Functional Expression of Endogenous Ligands of TLR4 and Associated Inflammatory Markers in Monocytes and Endothelial Cells During Normotensive Pregnancy, and Pregnancy Complicated by Pre-eclampsia

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Dedication

- My father, Aziz Al-ofi (1951-2003) and my mother Fatmah Salamah.

- My husband, Abdullah Al-amri, and my children Faisal and Abdulrahman.

- My brothers and sisters Azzah, Tareq, Waleed, Walaa and Wedyan.

Thanks to all who have always supported me with love, understanding and who “push me to the front”.

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Publications

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APCs	antigen-presenting cells
AT1	angiotensin type 1
ATP	adenosine 5`-triphoshate
Angs	angiopoietins
BrdU	bromodeoxyuridine
CBA	cytometric bead array
CCR	chemokine receptor
CK	cytokines
COX-2	cyclooxygenase-2
CRX-526	AGPs: Aminoalkyl glucosaminide 4-phosphates
DAMPs	damaged associated molecular patterns
DCs	dendritic cells
DIC	disseminated intravascular coagulation
DMC	decidual macrophages
dNK	decidual natural killer
ECs	endothelial cells
ECM	extracellular matrix
EDA	extra domain A
EDTA	ethylenediaminetetraacetic acid
FDP	fibrin degradation products
FGF	fibroblast growth factor
FGFR-1	fibroblast growth factor receptore-1
FGFR-2	fibroblast growth factor receptore-2
GM-CSF	granulocyte-macrophage colony-stimulating factor
HA	hyaluronan
HELLP	haemolysis, elevated liver enzymes and low platelet count
HLA-DR	human leukocyte class II DR antigens
HMGB1	high mobility group box protein 1
HSPs	heat shock proteins
HSPG	heparan sulfate proteoglycan
HTN	hypertension
HUVECs	human umbilical vein endothelial cells

ICAM-1	intercellular adhesion molecule 1
IDO	indoleamine 2,3-dioxygenase
IkB	I-kappa B
IL	interleukin
IRF	interferon regulatory factor
IUGR	intrauterine growth restriction
ISSHP	International Society of the Study of Hypertension in Pregnancy
LFA-1	leukocyte function-associated antigen 1
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein 1
M-CSF	macrophage colony-stimulating factor
MHC	major histocompability complex
MIP-1	macrophage inflammatory protein 1
MOs	monocytes
MQ	macrophages
MD2	myeloid differentiation factor 2
MMPs	matrix metalloproteinases
MyD88	myeloid differentiation factor 88
NF-kB	nuclear factor-kappa B pathway
NK	natural killer
NLRs	NOD-like receptors
NP	normal pregnancy
NO	nitric oxide
PA	plasminogen activator
PAI-1	plasminogen activator inhibitor type 1
PAMPs	pathogen associated molecular patterns
PCR	polymerase chain reaction
PDG	peptidoglycan
PE	PE
PGI_2	prostaglandin
PS	phosphatidylserine
PI	propidium iodide
PIGF	placental growth factor
RAGE	receptors for advanced glycation end product

RLRs	RIG-like receptors
ROS	reactive oxygen species
sFlt-1	soluble fms-like tyrosine kinase 1
STBMs	syncytiotrophoblast basement membrane fragments
TAT	thrombin-antithrombin complex
TGF-β	transforming growth factor-β
TLRs	toll like receptors
TIE2	tyrosine kinase receptor
TNF-α	tumour necrosis factor-α
tPA	tissue plasminogen activator
VCAM-1	vascular endothelial adhesion molecule 1
VEGF	vascular endothelial growth factor
VEGFR-1	vascular endothelial growth factor receptore-1
VEGFR-2	vascular endothelial growth factor receptore-2
VLA-4	very-late antigen 4

Abstract

A leading cause of pregnancy-related mortality and morbidity is PE (PE). The innate immune system recognises pathogens through transmembrane proteins called toll-like receptors (TLRs). Lipopolysaccharide (LPS) is recognised by TLR4 and peptidoglycan (PDG) is recognised by TLR2. However, other endogenous ligands such as heparan sulfate, hyaluronan, fibrinogen, fibronectin, and HMGB1 can stimulate TLR4 and trigger innate immunology. Thus, in Chapter 1 the pathophysiology of PE women is described with special emphasis on immune cells (monocytes/macrophages), inflammatory responses, and endothelial cell inflammation. Also, the expression of TLR in the foeto-maternal interface and PE was explained.

To further characterise the systemic effects of PE on circulating cells, monocyte subpopulations in normal pregnant (NP) and PE patients were analysed using flow cytometry (Chapter 3). We demonstrated for the first time that non-classical $CD14^{low}CD16^{+}$ monocytes were significantly increased in women with PE, and these women also displayed irregular expression of several chemokine receptors and antigen presentation molecules. The most striking phenotypic difference in cell surface molecules was the marked upregulation of TLR4 expression, where both $CD14^{high}CD16^{+}$ and $CD14^{low}CD16^{+}$ monocytes demonstrated higher levels in PE patients than their NP counterparts. In Chapter 4, we reported that stimulation of PE monocytes with bacterial TLR4 ligands resulted in profound secretion of various cytokines, in comparison to NP controls. In addition to LPS and PDG, fibrinogen was the only endogenous ligand that stimulated PE monocytes, producing inflammatory cytokines.

The interactions between PE/NP monocytes and endothelial cells were examined. The inflammatory responses following stimulation with TLR2 and 4 ligands were investigated in monocyte-HUVEC co-cultures by CBA (Chapter 5). Co-culture of PE monocytes with endothelial cells demonstrated profound secretion of inflammatory cytokines (IL-6) and chemokines (IL-8 and MCP-1), in comparison to NP controls. However, production of the anti-inflammatory cytokine (IL-10) was lower in PE co-cultures. Additionally, we showed that stimulation of PE co-cultures with TLR ligands led to an abnormal disturbance in cytokines and chemokines as compared to NP co-cultures.

In Chapter 6, the effects of TLR ligands on the expression levels of angiogenic (vascular endothelial growth factor: VEGF) and anti-angiogenic factors (soluble fms-like tyrosine kinase 1: sFlt-1), and adhesion molecules (vascular endothelial adhesion molecule 1: VCAM-1) were examined with CBA and ELISA in PE/NP co-cultures. VEGF production was significantly lower for PE monocyte-HUVEC co-cultures compared to NP controls. The expression level of sFlt-1 from HUVECs was down-regulated when co-cultured with monocytes from NP women; down-regulation of sFlt-1 by NP monocytes was significantly higher than for PE monocytes. Stimulation of monocyte-HUVEC co-cultures by bacterial ligands, induced sFlt-1 production in NP and PE cultures. Interestingly, exposure of PE monocytes co-cultured to fibrinogen resulted in down-regulation of sFlt-1 production, whilst this was unchanged for NP controls. Compared to NP, PE monocytes induced higher expression levels of VCAM-1 released by endothelial cells. Furthermore, exposure of PE monocytes and HUVEC co-cultures to TLR ligands, led to an exaggerated VCAM-1 response compared to NP controls.

In Chapter 7, plasma levels of endogenous ligands were measured with ELISA; and fibrinogen was investigated in placentae of PE and NP women, using immunohistochemistry. Fibrinogen, fibronectin, and HMGB1 levels were higher in the plasma of PE women compared to NP women, whilst heparan sulfate levels did not differ. Also, placental expression of fibrinogen was lower in PE women than in NP.

In summary, this thesis examined the physiology and innate immunology of human monocytes, and their interaction with vascular endothelial cells in the pathogenesis of hypertensive pregnancy disorders. It investigated the possible role of the endogenous ligands of TLR2 and TLR4 in this common pregnancy disorder that still contributes considerable maternal and foetal deaths and disease worldwide.

Chapter 1: Introduction

Literature review

Pre-eclampsia (PE) is a pregnancy disorder characterised by the new onset of hypertension and proteinuria after 20 weeks gestation (Brown *et al.*, 2001). It is a major cause of foeto-maternal morbidity and mortality worldwide. PE results in varied complications for the mother and the fetus. For the mother, PE may cause renal failure, liver failure, haemolytic syndrome, elevated liver enzymes and low platelet counts (HELLP syndrome), seizures, stroke, or death. Furthermore, PE can result in foetal growth restriction, preterm birth, hypoxic encephalopathy, or death of the neonate (Baumwell and Karumanchi, 2007, Sibai *et al.*, 2005). Although information regarding the aetiology of this disease is scant, its pathophysiology is characterised by abnormal placentation, endothelial dysfunction, and an exaggerated inflammatory response. To understand the pathophysiology of PE, we must first understand the normal physiology of pregnancy: implantation, placental development, and cardiovascular adaptation in pregnancy.

1.1 Normal physiology of human pregnancy

1.1.1 Implantation

Implantation is a critical and complex process that allows the embryo to adhere to and penetrate into the endometrium (Johnson and Everitt, 2007). Both the embryo and endometrium undergo several physiological changes and events to support the implantation process. These include: embryo development, uterine receptive changes (window of implantation), formation of blood vessels and endometrial stroma (decidualization), and inflammatory responses at the implantation site. The synchronization of embryo development and the changing receptivity of the uterus is an essential step for successful implantation and pregnancy (Johnson and Everitt, 2007).

Embryo development

Fertilisation of the ovulated oocyte with spermatozoa occurs in the ampulla part of the fallopian tube, within 24 to 48 hours of ovulation. After fertilization, the zygote (fertilised oocyte) undergoes a series of divisions (called cleavages), in which the total size of the zygote remains unchanged and enclosed within the zona pellucida (Johnson and Everitt, 2007). The zygote cleaves into its initial stages and then into a 32-cell stage (morula) where it is transferred to the uterine cavity by movement of cilia in the oviduct; this occurs within 3.5 days of fertilization. Then, the morula undergoes morphological changes (compaction) leading to fluid accumulation between the outer layer (trophoblasts) and the inner cell mass, to form the blastocyst. The trophoblast layer of the blastocyst will become the placenta, the blastocystic cavity will become the amniotic cavity, and the inner cell mass of the blastocyst will become the embryo (Johnson and Everitt, 2007, Red-Horse *et al.*, 2004).

Window of implantation

During the mid-luteal phase of the menstrual cycle, morphological changes occur in the endometrium so that it becomes receptive to embryo implantation (window of implantation) (Johnson and Everitt, 2007). These morphological changes include: thinning of the mucin coating, loss of surface negative charge, shortening of the microvilli, and the appearance of protrusions from the apical part of the luminal epithelial cells of the endometrium called pinopodes. The pinopodes may help the blastocyst attach to the endometrium and absorb fluids in the uterine cavity, leading to its occlusion and swelling of the stroma. The steroid hormones regulate these receptive changes during the window of implantation. However, hormonal regulation during implantation varies between species. Both oestrogen and progesterone are required in mice and rats for the establishment of the implantation window, but in pigs and rabbits oestrogen is not needed. In humans, although progesterone is critical for uterine receptive changes, the endometrium seems to be primed with oestrogen (Johnson and Everitt, 2007).

Implantation process

The implantation process has three stages: apposition, adhesion, and penetration (Chard and Lilford, 1998, Dominguez *et al.*, 2005, Johnson and Everitt, 2007). The trophoblast cells of the blastocyst come into close contact with the lumenal epithelium of the

endometrium (apposition). First of all, to ensure the attachment, proteolytic enzymes produced by either the trophoblast or the uterine endometrium digest the zona pellucida that encases the blastocyst. This loose apposition is followed by the strong adherence of the blastocyst to the endometrium (adhesion), which is supported by microvilli of the trophoblast cells interdigitating with those on the endometrial surface. Within a few hours of attachment, the blastocyst penetrates the lining of the luminal epithelium and invades the stroma up to the uterine vessels, forming a haemochorial placenta in order to create a low-pressure circulation to ensure adequate nutrition to the embryo (invasion) (Johnson and Everitt, 2007). The molecular factors that regulate the invasion process include: cytokines such as the leukaemia inhibitory factor, growth factors such as the vascular endothelial growth factor (VEGF), and extracellular matrix proteins such as heparan sulfate proteoglycan (HSPG), and metalloproteinases such as MMP9 (Dominguez *et al.*, 2005, van Mourik *et al.*, 2009).

1.1.2 Placental development

The placenta is the interface between the mother and the fetus that provides the nutrient interchange and metabolic exchange. Within a few hours of embryo invasion into the maternal endometrium, the trophoblastic cells of the embryonic pole of the blastocyst undergo a differentiation process (Johnson and Everitt, 2007). The trophoblasts differentiate into mononucleated cytotrophoblasts (acting as stem cells and rapidly proliferative cells that fuse together, resulting in the syncytiotrophoblast), the syncytiotrophoblast (the outer layer that is in direct contact with the maternal blood space), and the extravillous trophoblastic cells that invade the endometrium, which are non-proliferative cells (Red-Horse *et al.*, 2004). The trophoblast continues with proliferative activities, branching to form a villous tree (Benirschke *et al.*, 2006). The structure of a villous tree consists of stem villi connecting the chorionic plate to the basal plate of the cytotrophoblast (they act as mechanical supports for the villous tree, but they do not have a role in the metabolic functions), and terminal villi which make up the smaller filamentous network of the villous tree (they act as the principal functional units of the placenta). The intervillous space is the place where the maternal blood flows smoothly between the chorionic villi, consequently allowing metabolic exchange (Johnson and Everitt, 2007).

Additionally, the extravillous trophoblast invasion and infiltration reaches the walls of the maternal spiral arteries, leading to smooth muscle remodelling (Burrows *et al.*, 1996). As a result, the muscular walls of the spiral arteries are transformed from high-resistance to lower-resistance vessels with larger diameter; maintaining the blood supply to the placenta and growing embryo (Lyall *et al.*, 2001). The early stages of placenta development occur in a hypoxic field, but oxygenation is increased through placental angiogenesis (Salomon *et al.*, 2013).

1.1.3 Haemostasis in pregnancy

To understand haemostasis in pregnancy, we need an overview of the coagulation and haemostasis systems. Coagulation is a complex cascade of procedures that leads to blood clot formation. The main aim of coagulation is to stop the bleeding tendency and maintain physiological haemostasis (Hoffbrand *et al.*, 2005). Normal haemostasis is required to prevent blood loss from damaged blood vessels into extra-vascular spaces, and this is achieved through the formation of the fibrin mesh. Several factors are involved in this process, such as tissue factor, platelets, and injured endothelial cells (Hoffbrand *et al.*, 2005). The coagulation process is categorised into intrinsic and extrinsic pathways, which overlap to activate factor Xa, which mediates the cleavage of prothrombin into thrombin. Thrombin then cleaves the fibrinogen into fibrin that is implicated in polymerization to form a fibrin clot (De Caterina *et al.*, 2013). This haemostatic network is controlled by anticoagulant proteins such as protein C, which is produced by the liver and functions within the negative or positive feedback loops. Additionally, fibrinolysis is important to regulate the dissolution of the fibrin clot, and to ensure that fibrin deposition does not exceed the required amount, which can lead to thromboembolic complications (Mosesson, 2005). Plasmin is a major factor of fibrinolysis, which hydrolyses the fibrinogen and fibrin involved in the blood clot into fibrin degradation products (FDP). Tissue plasminogen activator (tPA) that is produced by endothelial cells converts the plasminogen into plasmin. Also, endothelial cells and platelets produce plasminogen activator inhibitor type 1 (PAI-1) to inactivate the plasmin and tPA. Thus, the haemostatic network starts and ends in a controlled fashion to ensure the formation of blood clots in the necessary places, and to prevent extensive propagation of blood clots (Hoffbrand *et al.*, 2005).

Normal pregnancy features a hypercoagulability state in order to maintain and prepare the coagulation and haemostasis systems for any anticipated haemorrhage at delivery (Brenner, 2004). Compared to pre-pregnancy, pregnant plasma levels of several clotting factors are increased, such as V, VII, VIII, X, von Willebrand factor, and most predominantly, fibrinogen (Thornton and Douglas, 2010). Fibrinogen is increased to promote the coagulation state, and fibrinolysis activity is suppressed (Thornton and Douglas, 2010). The fibrinolytic system in a healthy pregnancy can be controlled by regulatory factors that compensate the procoagulant state of the endothelium, including tPA, and PAI-1 (Sorensen *et al.*, 1995).

1.1.4 Circulating angiogenic factors

Vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) belong to a growing angiogenic family including VEGF-A, -B, -C, and -D. VEGF is secreted by a number of cells like endothelial cells, fibroblasts, and neutrophils; and PIGF is mainly produced by the placenta, lungs, and heart (Tammela *et al.*, 2005, Yano *et al.*, 2006). They act through two endothelial tyrosine kinase receptors VEGFR-1 (flt-1) and VEGFR-2 (flk-1) that are expressed by endothelial cells and some inflammatory cells. The functions of VEGF include: the induction of endothelial cell proliferation, stimulation of vasodilatation, and increased vascular permeability. It also recruits inflammatory cells and stimulates expression of proteases which are involved in angiogenesis. Thus, it is an important regulatory factor in normal and abnormal angiogenesis (Arcondeguy *et al.*, 2013). Hypoxia, cytokines, nitric oxide, and bacterial endotoxins are potent stimuli for VEGF production (Tammela *et al.*, 2005). It has been demonstrated that VEGF has vasodilator effects on the coronary arteries of dogs and humans; an infusion of VEGF results in nitric oxide-dependent vasodilation mainly via upregulation of nitric oxide and prostacyclin in the endothelial vessels (He *et al.*, 1999, Ku *et al.*, 1993). Additionally, patients undergoing inhibition of VEGF during anti-angiogenic cancer therapy develop hypertension and proteinuria (Yang *et al.*, 2003). Immunohistochemical studies have demonstrated that VEGF and its receptor VEGFR-1 are expressed on human villous invasive cytotrophoblasts, endothelium, and macrophages (Kaufmann *et al.*, 2004). Therefore, it has been suggested that they play

an important regulatory function in trophoblast invasion and placental angiogenesis. In the early stages of pregnancy, VEGF and VEGFR-2 are upregulated, in order to stimulate the branching angiogenesis, and their levels decrease as the pregnancy progresses. PIGF level is increased at the beginning of the second trimester of pregnancy, reaching its peak between 29 to 32 weeks, and then declines (Powe *et al.*, 2011). Conversely, soluble VEGFR-1 expression is increased as the pregnancy advances, when branching angiogenesis mechanisms are shifted to non-branching ones (Kaufmann *et al.*, 2004). Moreover, the angiopoietins (Ang1 and Ang2) mediating signalling via tyrosine kinase receptors (mainly TIE2, and to a lesser extent TIE1), are essential for normal placental angiogenesis development. Ang1, Ang2, and TIE2 are expressed in early human pregnancy placenta, and thereafter, Ang2 and TIE2 decrease while Ang1 increases towards the term placenta (Leinonen *et al.*, 2010).

1.2 Pre-eclampsia (PE)

1.2.1 Epidemiology of PE

Incidence of PE varies between 2% and 7% in healthy primigravida women (Sibai *et al.*, 2005). Epidemiological studies have identified genetic predispositions and other maternal and paternal factors associated with PE. Diabetes mellitus, chronic hypertension, and thrombophilia increase the risk of PE. Insulin resistance and obesity are also known to be associated with an increased risk of PE (Baumwell and Karumanchi, 2007). Interestingly, cigarette smoking is associated with a lower risk of PE (Bakker and Faas, 2007). Several epidemiological factors suggest that immunological factors predispose women to preeclampsia. PE is typically a disease of first pregnancies. The risk of PE increases in women who conceive following intercourse with men to whom have a lower frequency of sperm exposure (Einarsson *et al.*, 2003). The more frequent the sperm exposure from the same partner, the lower the risk of developing PE. This may explain the increased risk of PE in teenage mothers. Furthermore, a previous abortion or a previous normal pregnancy from the same spouse reduces the risk of PE, and this protection is lost with a change of spouse. Women have a higher risk in their second pregnancy if they were impregnated by men who fathered a

previous pre-eclamptic pregnancy with a different female (Lie *et al.*, 1998). There is also an increased risk of PE in infertile females who conceive through assisted reproductive technology during their first pregnancy with donated gametes (Sibai *et al.*, 2005). These observations suggest that understanding the immunology of PE remains pivotal to any future preventive and therapeutic measures.

1.2.2 Genetics in PE

The definitive genetic mechanisms in the pathogenesis of PE are still not clear. However, PE is speculated to be a multigenic syndrome. A series of studies show that PE has a familial tendency. These studies demonstrate that first-degree relatives of PE women have a two- to five-fold higher risk of developing the disease (Mutze *et al.*, 2008). Polymorphisms affecting two genes, angiotensinogen and endothelial nitric oxide synthase (eNOS) genes, have been shown to influence predisposition to PE (Bashford *et al.*, 2001, Medica *et al.*, 2007). M235T polymorphism is the most common one affecting the angiotensinogen gene. This polymorphism is associated with abnormal remodelling of maternal spiral vessels (Morgan *et al.*, 1997). Although E298D polymorphism of eNOS is not related to the pathogenesis of PE, it has been reported to be associated with an increased risk of PE complications. However, reports of these gene polymorphisms are conflicting and have not been confirmed by some studies (Cross, 2003, Medica *et al.*, 2007). It is also possible that genes inherited by a baby from the male parent could contribute to the development of the disease. Factors involved in normal placentation and trophoblast migration may be encoded in foetal genes (Mutze *et al.*, 2008). The involvement of foetal genes is also suggested by studies that demonstrate that men who father a pregnancy complicated by PE confer a higher risk of PE on other women in a future pregnancy (Lie *et al.*, 1998). Furthermore, there is an increased risk of PE in women impregnated by men who are themselves born to a pre-eclamptic mother (Esplin *et al.*, 2001). Thus, susceptibility to PE is suggested to come from complex interactions between paternal and maternal genotypes, maternal predisposing factors, and environmental factors.

1.2.3 Diagnosis and classification of PE

The classification of hypertensive disorders of pregnancy includes chronic hypertension, PE/eclampsia, PE superimposed on chronic hypertension, and gestational hypertension (Brown *et al.*, 2001). In the last four decades, multiple definitions and classifications of pregnancy-induced hypertension and PE have been endorsed by different international organizations or groups of experts (Lyall and Belfort, 2007). Nowadays, the most accepted diagnostic criteria for PE, established by the International Society of the Study of Hypertension in Pregnancy (ISSHP) in 2000, are the new onset of raised blood pressure (systolic BP ≥ 140 mmHg and/or diastolic BP ≥ 90) and proteinuria (≥ 300 mg/24hours) after 20 weeks of gestation.

Other manifestations of severe PE include headaches or visual disturbances, oliguria, renal failure, and hepatic failure **(Table 1.1)**. More considerable blood pressure elevation and a higher level of proteinuria are characteristics of severe PE. Eclampsia is a convulsion state that occurs in women with PE without any other attributable factors. Furthermore, PE has been sub-classified according to its onset: early onset PE (less than 34 weeks gestation) and late onset PE (more than 34 weeks of gestation) (Lisonkova and Joseph, 2013, von Dadelszen *et al.*, 2003). It has been speculated that the aetiology and pathogenesis of the early- and late-onset PE are different. Also, the clinical presentations of both types are different (Huppertz, 2008). However, this classification is still under research.

Table 1.1 Diagnostic criteria of PE according to ISSHP (2004)

Criteria	Mild to moderate PE	Severe PE
Blood pressure	Systolic blood pressure ≥ 140mmHg or, diastolic pressure ≥ 90 mmHg after 20 weeks of gestation in women with previous normal blood pressure	Systolic blood pressure ≥ 160 mmHg or, diastolic pressure ≥ 110 mmHg on two different occasions at least 6 hours apart during bed rest
Proteinuria	Protein ≥ 0.3g in 24 hours urine collection or ≥ +1 in urine dipstick test	Protein ≥ 5g in 24 hours urine collection or ≥ +3 in urine dipstick test at least 4hours apart of tow random urine samples
Other manifestations		• Oliguria (≤ 500 mL of urine in 24 hours) • Headache or visual disturbances • Epigastric or right upper quadrant pain • Hepatic failure • Thrombocytopenia • Pulmonary edema or cyanosis • Intrauterine growth restriction

1.2.4 Pathogenesis of PE

During the last few decades, many theories have been put forth regarding the aetiology of PE. These include: abnormal placentation, vascular endothelial dysfunction, exaggerated systemic inflammatory response, and foetal-maternal immunological factors (Lyall and Belfort, 2007). A unifying hypothesis for the pathophysiology of PE and the role of these factors in the development of the disease is summarised in **Figure 1.1.**

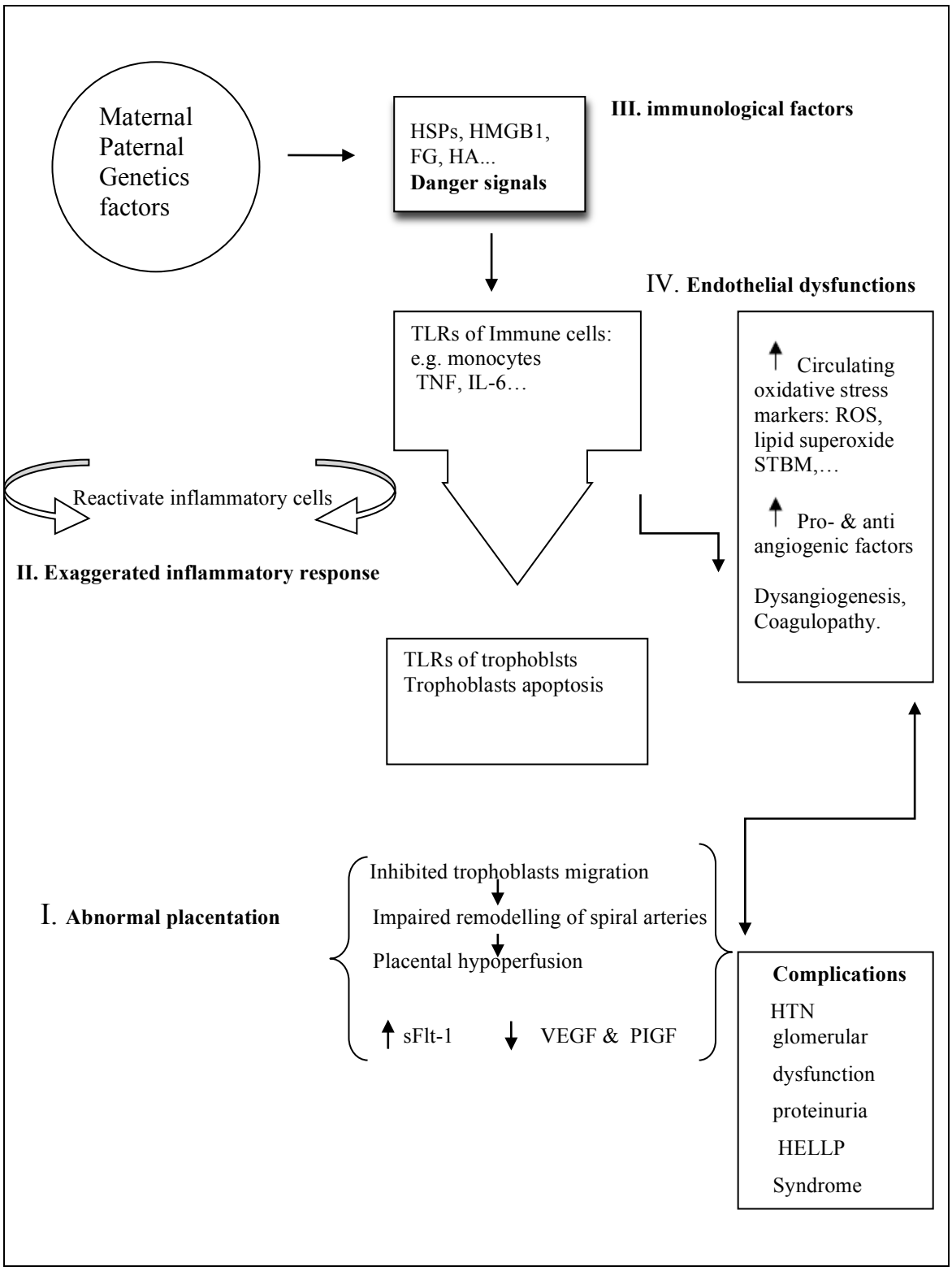

Figure 1.1 Potential mechanisms of PE aetiology

Abbreviations: FG; fibrinogen, HA; hyaluronan, HMGB1; high mobility group box protein 1, HSPs; heat shock proteins, HTN; hypertension, HELLP; haemolysis, elevated liver enzymes and low platelet count, ROS; reactive oxygen species, sFlt-1; soluble fms-like tyrosine kinase 1, STBMs; syncytiotrophoblast basement membrane fragments, PIGF; placental growth factor, VEGF; vascular endothelial growth factor.

1.2.4.1 Abnormal placentation

Abnormal placentation is an important finding in women with PE (Furuya *et al.*, 2008, Huppertz, 2008). Delivery of the placenta leads to the resolution of the hypertension associated with PE. Women who develop the gestational trophoblastic disease, in which there is no foetus, develop a severe early-onset type of PE (Baumwell and Karumanchi, 2007). Although abnormal placental development is necessary for PE, the initial triggers for this problem are still unclear. During normal pregnancy, extravillous trophoblasts invade maternal spiral arteries and differentiate to an endothelial phenotype (Burrows *et al.*, 1996, Kaufmann *et al.*, 2004). The high-resistance maternal spiral arteries are then transformed to high-capacitance vessels in order to provide the increased blood flow required for nutrient exchange at the foeto-placental interface. In PE, these trophoblasts are described as being unable to invade the myometrium adequately (Khong *et al.*, 1986). Consequently, the remodelling of the maternal spiral arteries is limited, resulting in uteroplacental hypoperfusion and hypoxia of the foeto-placental unit. Placental ischemia leads to the release of pro- and anti-angiogenic circulating mediators, which diffuse systemically to cause endothelial dysfunction, coagulopathy, and multi-organ damage (Leanos-Miranda *et al.*, 2013, Levine *et al.*, 2004).

The invasive trophoblasts in normal pregnancy express markers of the endothelial phenotypes after a pseudo-vascularization process, such as vascular endothelial adhesion molecule 1 (VCAM-1) and extracellular matrix metalloproteinases (Lyall, 2006). However, in PE this process of cellular phenotype transition fails; for example, invasive trophoblasts of PE do not express matrix metalloproteinase MMP9. Additionally, hypoxia has been suggested as contributing to this abnormal transition process as the in-vitro cultured cytotrophoblasts fail to invade and switch on their integrin receptors under hypoxic environments (Lyall, 2006).

1.2.4.2 Oxidative stress

It has been shown that PE is associated with both placental and maternal circulatory oxidative stress (Hubel, 1999, Siddiqui *et al.*, 2010). The PE placenta expresses higher levels of superoxide and lower levels of antioxidants as compared to normal placenta (Siddiqui *et al.*, 2010). Additionally, the serum of PE women demonstrates oxidative disturbance of lipids and proteins products, while it shows a decrease in antioxidants levels. It has been suggested that the ischemic placenta produces a shedding of placenta

microparticles into maternal circulation, which becomes involved in the oxidative stress and endothelial dysfunction. The circulating mediators released by abnormal ischemic placenta include: deported syncytiotrophoblast basement membrane fragments (STBMs) (Goswami *et al.*, 2006, Guller *et al.*, 2008), syncytiotrophoblast microvesicles (Gardiner *et al.*, 2011), oxygen free radicals (Hubel, 1999), and perhaps other as yet unidentified factors (Guller *et al.*, 2008).

1.2.4.3 Vascular endothelial dysfunction

The endothelium acts as a haemostasis barrier that regulates vascular tone and permeability, and coagulation and thrombosis cascade. Vascular endothelial dysfunction, which is a common hallmark for PE, is associated with disturbances in these functions (Lyall and Belfort, 2007, Powe *et al.*, 2011). Circulating factors of injured endothelium have been found in women with PE. These factors include: fibronectin, endothethlin-1, von Willebrand factor VIII, adhesion molecules (VCAM-1 and intercellular adhesion molecule 1 (ICAM-1), inflammatory cytokines, and oxidative stress markers. Additionally, morphological studies of endothelium obtained from the kidneys or uteroplacental arteries of PE patients reveal glomerular capillary endotheliosis or acute atherosis, respectively (Roberts and Post, 2008, Stillman and Karumanchi, 2007). For further support, the sera of PE women induce endothelial injuries and cytotoxicity in vitro indicating the presence of circulating factors leading to endothelial dysfunction (Rodgers *et al.*, 1988). The sections below will describe the circulating pro- and anti-angiogenic factors involved in PE pathogenesis, the renin-angiotensin system, and coagulopathy in PE.

(1) Circulating pro- and anti-angiogenic factors

The soluble fms-like tyrosine kinase 1 (sFlt-1; also known as sVEGFR-1) is an unbound circulating splice variant of membrane-bound Flt-1, a receptor for VEGF and PIGF. The molecule sFlt-1 acts as a potent antagonist for vascular VEGF and PIGF. *In vitro* studies have demonstrated inhibition of VEGF and PIGF vasodilatory functions and endothelial tube formation by recombinant sFlt-1 (Kendall and Thomas, 1993). Gene expression studies have shown that there is an upregulation of placental sFlt-1 in PE (Maynard *et al.*, 2003). The placenta produces a high level of circulating sFlt-1 in pregnancies complicated by PE, which falls after delivery. Additionally, its elevation

was found to start five weeks before the onset of clinical syndrome (Chaiworapongsa *et al.*, 2005, Levine *et al.*, 2004). The increased amounts of sFlt-1 in PE are associated with decreased circulating levels of free VEGF and PIGF. Although both are decreased, PIGF is more related to the clinical severity than VEGF (Chappell *et al.*, 2013). In early-onset PE and diseases associated with severe IUGR, the alteration of sFlt-1 and PIGF is more dramatic (Levine *et al.*, 2004, Powe *et al.*, 2011). Subsequently, the sFlt-1/PIGF ratio is a better indicator of PE than each alone. It has been suggested that inhibition of VEGF/PIGF by sFlt-1 may lead to impaired angiogenesis and abnormal endothelial function, hypertension, and proteinuria in females who develop PE. To lend further support to the role of sFlt-1 in the pathogenesis of PE, it has been shown that administration of exogenous sFlt-1 to pregnant and non-pregnant rats stimulates hypertension, proteinuria, and the histological finding of glomerular endotheliosis features which represent one of the final common pathways in pathogenesis of PE in humans (Maynard *et al.*, 2003).

Another important anti-angiogenic protein is soluble endoglin, which acts by blocking transforming growth factor-β (TGF-β) induced vasodilatation, introduced by the ischemic placenta into maternal circulation (Venkatesha *et al.*, 2006). The level of 65-kDa soluble endoglin monomer is four-fold higher in PE placenta as compared to NP placenta. Also, it has a synergetic action with sFlt-1when both are injected into pregnant rats by inducing haemolysis, hepatic injuries, and placental necrosis (HELLP-syndrome) (Powe *et al.*, 2011, Venkatesha *et al.*, 2006). Moreover, it has been observed that high levels of circulating soluble endoglin and sFlt-1/PIGF ratio appear prior to the onset of PE (Levine *et al.*, 2006, Levine *et al.*, 2004). Therefore, the combination of soluble endoglin and sFlt-1/PIGF ratio is a valid predictor for the onset of the disease. **Figure 1.2** illustrates the hypothetical pathophysiologic mechanism of regulation of angiogenesis in the placenta by sFlt-1, VEGF, and PIGF.

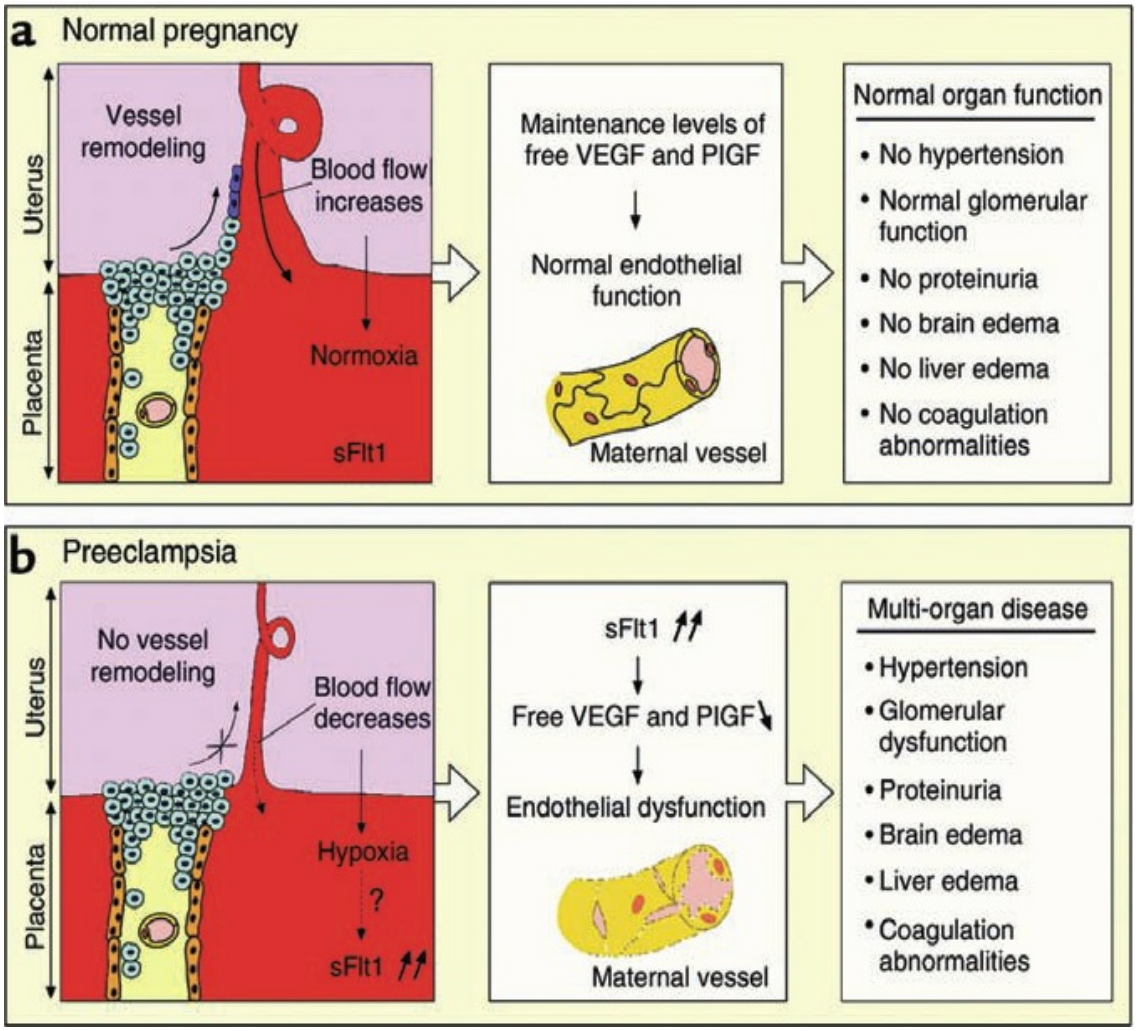

Figure 1.2 sFlt-1, VEGF and PLGF in normal and hypoxic placentas

Adapted from reference (Luttun and Carmeliet, 2003) with permission from the publisher (American Society for Clinical Investigation).

(2) The renin-angiotensin system

Normal pregnancy is characterised by a decrease in vascular resistance, vasodilation, and resistance to the vasoconstrictor effects of angiotensin II (Coad and Dunstall, 2011). However, this resistance is absent and hypersensitivity to angiotensin II is increased in pregnancy-induced hypertension (Irani and Xia, 2008). Angiotensin II binds to its receptor angiotensin type 1 (AT1), which mediates vasoconstriction, aldosterone release, sympathetic activity, and increase of blood pressure. The heterodimerisation of AT1 with bradykinin receptors could explain the hypersensitivity to angiotensin II in PE. In PE, angiotensin II and aldosterone levels are reduced, but recently a novel oxidised angiotensinogen, which increases angiotensin synthesis, has been discovered in the maternal circulation of women with PE (Zhou *et al.*, 2010).

(3) Coagulopathy in PE

Despite normal pregnancies showing alterations in haemostasis because of increased clotting factors, and suppression of anticoagulants and the fibrinolytic system (as shown in Section 1.1.3 haemostasis in pregnancy), pregnancy-induced hypertension shows profound hypercoagulability and haemostasis changes. The imbalances of haemostasis appear to be excessive in PE because of increases in tissue factor, thrombin-antithrombin complex (TAT), and PAI-1 (Schjetlein *et al.*, 1997). Additionally, morphological studies have demonstrated multi-organ (placental spiral arteries and glomerular endothelium of kidney and liver sinusoids) fibrin deposits in women with PE and HELLP syndrome (Arias and Mancilla-Jimenez, 1976, Bonnar *et al.*, 1971, Petrucco *et al.*, 1974, Roberts and Post, 2008, Stillman and Karumanchi, 2007). The fibrin deposition in the placental vessels could explain the placental hypoperfusion in these women. Also, its deposition in the glomerular endothelium of the kidneys may lead to renal damage and proteinuria, and renal ischemic-induced hypertension (Bonnar *et al.*, 1971).

To understand the inflammatory response of the immune system at the foeto-maternal interface, and its participation in the pathophysiology of PE, we first need to introduce the immune system.

1.3 The immune system

1.3.1 Innate and adaptive immunity

Human host defence against microbiotic pathogens is established by the immune system. Non-microbiotic substances can provoke the immune system as well. The immune system consists of two types: innate immunity and adaptive immunity (Abbas *et al.*, 2012).

Innate immunity is the first line of defence against microbes, and it also has the ability to distinguish between "pathogen non-self" and "host self". It is composed of barriers (physical or chemical); for example, epithelium surfaces, phagocytic cells (macrophages and neutrophils), natural killer cells, dendritic cells, complement factors, and cytokines.

Adaptive immunity is characterised by specificity and memory developed after repeated exposure to the same pathogen, with a vigorous response. It is composed of two types referred to as cellular immunity and humoral immunity (Abbas *et al.*, 2012). Cellular immunity is mediated by T lymphocytes, which recognise the antigens on microbes and then either destroy the infected cells directly or help phagocytic cells to kill the phagocytosed microbes. Thus, T lymphocytes are divided functionally into helper T lymphocytes, which release cytokines (to act as messenger molecules between immune cells) to activate other immune cells such as macrophages and B lymphocytes, and induce inflammation; and cytotoxic T lymphocytes that kill infected cells with viruses or intracellular microbes. Humoral immunity is mediated by antibodies, which are produced by B lymphocytes. Antibodies recognise the extracellular microbial antigens and then eliminate them using different effector mechanisms.

Innate and adaptive immunity are complementary to each other – working together to protect and defend the host body (Abbas *et al.*, 2012). The molecular and cellular

mechanisms of both types act cooperatively. The innate immune system provides effective mechanisms against the pathogens. However, some of these pathogens escape from innate immunity by resisting the mechanisms, and this is when the more powerful mechanisms of adaptive immunity come into effect. At the same time, adaptive immunity enhances the phagocytic functions of innate immunity.

1.3.2 Cellular components of the immune system

Lymphocytes

Lymphocytes are particular cells of the adaptive immune system, which are expressly cloned for distribution to antigenic receptors; each lymphocyte clone is specific to a single antigen determinate. They are divided into B lymphocytes, which are differentiated and matured in the bone marrow (thus called B cells), and T lymphocytes, which are differentiated and matured in the thymus (thus called T cells). B and T cells consist of subsets that are phenotypically and functionally distinct. The main B lymphocyte subsets are: marginal zone B cells, follicular B cells, and B-1 B cells, and each subset is located in a different organ's lymphoid tissues. The main two subsets of T lymphocytes, which express antigen receptor, are: $CD4^+$ helper T cells and $CD8^+$ cytotoxic cells. Also, there is another population of T cells that express γδ antigen receptor (Abbas *et al.*, 2012).

Natural killer cells (NKs)

NKs are lymphocytes that are different from T and B cells, and they have a major role in the innate immune system. They function against intracellular viruses, bacteria, and damaged cells. They are called killer cells because they accomplish killing functions without the other clonal and differentiation expansion requirements of cytotoxic T cells. NKs compose 5–15 % of the total mononuclear cells in the blood and spleen. They are produced from bone marrow precursors as large lymphocytes with abundant cytoplasmic granules. They are demonstrated in the blood by positive expression of CD56 and negative expression of CD3 (Abbas *et al.*, 2012).

Monocytes/Macrophages

Mononuclear cells originate from a common precursor of the myeloid linage in the bone marrow. The differentiated monocytes enter blood circulation from the bone marrow. Their diameter ranges from 10 to 15 μm, and they have large, bean-shaped nuclei. Monocytes have been defined as a heterogeneous population of cells with different phenotypes and functions (Abbas *et al.*, 2012). Monocytes in human peripheral blood can be divided into three distinct populations based on expression of the lipopolysaccharide (LPS) receptor CD14, and the Fcγ-III receptor, CD16. The nomenclature and classification of these monocyte subsets has recently been updated into three populations: classical monocytes ($CD14^{high}CD16^{-}$), intermediate monocytes ($CD14^{high}CD16^{+}$), and non-classical monocytes ($CD14^{low}CD16^{+}$) (Ziegler-Heitbrock *et al.*, 2010, Ziegler-Heitbrock and Hofer, 2013). The classical $CD14^{high}CD16^{-}$ phenotype strongly expresses CD14, but is negative for CD16 and is the most common phenotype in the peripheral blood. The non-classical $CD14^{low}CD16^{+}$ phenotype was previously referred to as pro-inflammatory monocytes because of higher intracellular expression of TNF-α and low-to-absent IL-10 after LPS stimulation (Belge *et al.*, 2002, Ziegler-Heitbrock, 1996). This phenotype also shows higher levels of MHCII expression, and this suggests a higher ability of antigen presentation for $CD4^{+}$ T cells. Moreover, an increased proportion of $CD14^{low}CD16^{+}$ monocytes have been reported in human sufferers of inflammatory and infectious diseases such as rheumatoid arthritis, haemodialysis, atherosclerosis, Kawasaki disease, septic shock, and human immunodeficiency virus (HIV) (Blumenstein *et al.*, 1997, Fingerle *et al.*, 1993, Kawanaka *et al.*, 2002a, Kawanaka *et al.*, 2002b, Nockher and Scherberich, 1998, Schlitt *et al.*, 2004, Ziegler-Heitbrock, 2007).

The massive investigation efforts that have recently been conducted by several researchers have demonstrated that these monocytes subpopulations have distinct functions and phenotypes (Cros *et al.*, 2010, Wong *et al.*, 2011, Wong *et al.*, 2012, Zawada *et al.*, 2011, Zawada *et al.*, 2012, Ziegler-Heitbrock and Hofer, 2013). The newest methodological techniques, such as gene microarray, flow cytometry and cytokine secretion studies, were used to identify the differences between these monocytes subsets. Table 1.2 summarises the main immunological features from these recent studies.

Differentiation and maturation of the monocytes subsets

Zeigler-Heitbrock and his colleagues have suggested that human monocyte subpopulations represent different stages of monocyte maturation. They suggested that $CD14^{low}CD16^{+}$ monocytes are the more mature phenotype because they exhibit features that are characteristic with tissue macrophages (Ziegler-Heitbrock, 1996, Ziegler-Heitbrock *et al.*, 1993). Additionally, *in-vitro* maturation studies have shown that $CD14^{low}CD16^{+}$ monocytes can also be differentiated from the more common $CD14^{high}CD16^{-}$ monocytes (Frankenberger *et al.*, 2008, Ziegler-Heitbrock *et al.*, 1993). Moreover, recent gene array studies have demonstrated that the intermediate and non-classical monocytes subtypes are more closely related (Wong *et al.*, 2011, Wong *et al.*, 2012). This close relationship suggests a direct development between these two subtypes. However, there remains considerable debate regarding the implications of the finding of these subtypes. A common feature can also be found between the $CD14^{high}CD16^{-}$ and $CD14^{low}CD16^{+}$ monocytes and type 1 macrophages or type 2 macrophages, respectively (Geissmann *et al.*, 2010, Ghattas *et al.*, 2013). Therefore, the particular monocyte subsets may differentiate into specific types of tissue macrophages. However, this cellular differentiation depends upon a certain microenvironment and interactions with other cell types, such as lymphocytes.

The vascular role of the intermediate and non-classical monocytes subsets

Zawada *et al*. (2011) have described that the intermediate $CD14^{high}CD16^{+}$ monocytes are characterised by pro-angiogenic behaviour. The intermediate subset expressed multiple surface makers of pro-angiogenesis, such as tyrosine kinase (TIE2, CD202b), endoglin (ENG) and VEGFR2 (Zawada *et al.*, 2011). Monocyte recruitment into the vascular wall is an important stage for vascular repair and angiogenesis. The $CD14^{low}CD16^{+}$ monocytes express a lower level of VEGFR-1 and these cells showed impaired migration toward VEGF and PIGF (Czepluch *et al.*, 2011). On the other hand, Cros *et al*. (2010) reported that the non-classical $CD14^{low}CD16^{+}$ monocytes exhibited a crawling "patrolling" behaviour on the endothelium after being transferred intravenously into mice. This behaviour indicates that the non-classical subset is often surveying the endothelium for signs of inflammation and might be transmigrated rapidly. Additionally, recent gene profiles have shown that the non-classical subset expressed higher levels of genes associated with cytoskeleton mobility, which might be responsible for the highly motile behaviour of this subset (Wong *et al.*, 2011).

Phagocytic function of the classical monocytes subset

Gene array studies have shown that the classical $CD14^{high}CD16^{-}$ subpopulation expresses higher levels of scavenger receptors, a proinflammatory S100 gene family and intracellular bacterial killing factors than non-classical monocytes (Wong *et al.*, 2011). Accordingly, researchers have suggested that $CD14^{high}CD16^{-}$ monocytes have superior phagocytic function (Wong *et al.*, 2012). This hypothesis is in agreement with previous functional studies that showed that $CD14^{low}CD16^{+}$ monocytes have lower phagocytic activity with less capacity to uptake a latex bead as compared to $CD14^{high}CD16^{-}$ monocytes (Cros *et al.*, 2010). On the other hand, the non-classical $CD14^{low}CD16^{+}$ monocytes may indirectly activate the immune system with the production of higher levels of inflammatory cytokines, such as TNFα (Belge *et al.*, 2002, Wong *et al.*, 2012).

Once monocytes enter into tissues, they mature and differentiate into macrophages. Macrophages are referred to using different names according to their specific location, such as Kupffer cells in the liver, microglia in the central nervous system, osteoclasts in bones, and decidual macrophages in the uterine decidua. They have several functions in innate and adaptive immunity, which include: 1) killing microbes through proteolytic digestion with lysosomal enzymes in phagolysosomes, and enzymatic conversion of reactive oxygen and nitrogen species that destroy microbes; 2) stimulated macrophages release cytokines for activating signalling in other cells to enhance immune response; 3) cleaning up the apoptotic cells that are released during physiological conditions such as growth, development, and tissue repairs; 4) repairing damaged tissues by enhancing angiogenesis and fibrosis; 5) and finally, acting as antigen-presenting cells for T lymphocytes (Abbas *et al.*, 2012).

Table 1.2 Comparison of different monocyte subpopulation phenotypes and functions (Wong *et al.*, 2012).

Features	**Classical monocytes** $CD14^{high}CD16^{-}$	**Intermediate monocytes** $CD14^{high}CD16^{+}$	**Non-classical monocytes** $CD14^{low}CD16^{+}$
Proportions to total monocytes	85%	5%	10%
Surface markers expressed	CD62L, CCR2, CLEC4D, CLEC5A, IL-13Rα1, CXCR1 CXCR2	CD74, HLA-DR, TIE2 (CD202B), ENG (CD105)	Siglec10, CD43, SLAN (subpopulation)
Surface markers not expressed	CX3CR1, CD123, P2RX1, Siglec10	CD62L, CXCR1, CXCR2, CLEC4D, IL-13Rα1	CCR5, CD62L, CXCR1, CXCR2, CD163, CLEC4D, IL-13Rα1
Cytokine responses to LPS	IL-10, G-CSF, CCL2, RANTES, IL-6, IL-8	IL-6, IL-8	TNFα, IL-1β, IL-6, IL-8
Described functions	Superior phagocytosis	T-cell proliferation and stimulation, superior ROS production, angiogenesis ($TIE2^{+}$ subpopulation)	T-cell proliferation and stimulation ($SLAN^{+}$ subpopulation), "patrolling" behaviour *in vivo*
Defined gene signature	Wound healing and coagulation, S-100 proteins, scavenger receptors, C-type lectin receptors, anti-apoptosis, responses to stimuli	MHC class II presentation and processing	Cytoskeletal rearrangement, complement components, pro-apoptosis, negative regulation of transcription

Neutrophils

Neutrophils are named polymorphonuclear leukocytes because their nuclei are segmented into three-to-five interconnected lobules. They are the most abundant leukocyte population in the peripheral blood and mediate the initial stages of the inflammatory response. Neutrophils arise from a common myeloid lineage of the bone marrow with mononuclear cells. Within a few hours of infection with microbes, neutrophils migrate toward the infected location. If they are not recruited to the inflammatory sites, they undergo apoptosis within six hours and are phagocytosed by macrophages (Abbas *et al.*, 2012).

Antigen-presenting cells (APCs); Dendritic cells

APCs are populations of cells that capture microbes and other antigens, and then present them to the lymphocytes. Also, they are involved in signalling for lymphocyte proliferation and differentiation. Dendritic cells (DCs) are a major type of APC that initiate T lymphocyte response. Also, macrophages and B lymphocytes display antigens to T lymphocytes. APCs connect the innate immune system and adaptive immune system responses. DCs arise from a myeloid precursor of hematopoietic cells that can be differentiated to monocytes but not to granulocytes. They are commonly distributed in the lymphoid tissues, organ parenchyma, and mucosal epithelium. DCs bind to antigens of microbes and respond by releasing cytokines and initiating phagocytic activity. Also, they respond by migrating to lymph nodes and presenting the microbial antigens to T lymphocytes. Therefore, they act as cells for both innate and adaptive immunity, and link both types together (Abbas *et al.*, 2012).

1.3.3 Inflammatory responses

The innate immune system responds to infection or products of damaged cells (dead cells) through acute inflammation processes. Inflammation is a process where leukocytes and plasma proteins are recruited from the blood, and accumulate at the sites of infection or damaged cells in order to eliminate them. The most abundant leukocytes, which are recruited during the initial phases of acute inflammatory response, are neutrophils. However, monocytes/macrophages become the more dominant populations as inflammation progresses. Plasma proteins, which are involved in the inflammation

process, include antibodies, complements, and acute phase reactants. The interactions between cells of the innate immune system and other host cells during inflammation are mediated by cytokines. Cytokines are large heterogeneous proteins that are released by numerous cell types. Synthesis of cytokines is initiated by new gene transcription due to cellular stimulations. Inflammatory cytokines are either produced locally (autocrine or paracrine action) or systemically to enter circulation (endocrine action). Cytokines either have the same synergetic effects with stimulation of each other, or some of them antagonise the action of others and inhibit their production. Leukocytes are recruited and migrate from blood to the inflamed tissues by the action of chemotactic cytokines called chemokines, which are a large lineage of structurally homologous cytokines (Abbas *et al.*, 2012). Some of the most important cytokines and chemokines involved in the inflammatory responses of the innate immune system are shown in **Table 1.3.**

Table 1.3 Cytokines of innate immunity (Abbas *et al.*, 2012)

Inflammatory markers		**Principal cell source**	**Principal cellular targets and biologic effects**
Pro-inflammatory cytokines	**TNFα**	Macrophages, T cells	Endothelial cells: activation (inflammation & coagulation) Hypothalamic: fever Neutrophil: activation Liver: synthesis of acute phase proteins Many cell types: apoptosis
	IL-1	Macrophages, endothelial cells, some epithelial cells	Endothelial cells: activation (inflammation & coagulation) Hypothalamic: fever Liver: synthesis of acute phase proteins
	IL-6	Macrophages, endothelial cells, T cells	Liver: synthesis of acute phase proteins B cells: proliferation of antibody-producing cells
Anti-inflammatory cytokine IL-10		Macrophages, T cells (mainly regulatory T cells)	Macrophages, dendritic cells: inhibition of IL-12 production and expression of costimulators and class II MHC molecules
Chemokines	**CC** **CCL2 (MCP-1)** **CCL3 (MIPα)** **CCL5 (RANTES)**	Macrophages, endothelial cells, T cells, fibroblast, platelets	Leukocytes: chemotaxis, activation & migration into tissues
	CXC **CXCL8 (IL-8)**		

1.3.4 Leukocyte-endothelial cells interactions

Leukocytes transmigrate from peripheral blood into tissues through the endothelium of blood vessels. This migration process requires the adhesion of leukocytes to vascular endothelial cells (ECs), and this is facilitated by the actions of two molecular groups: selectins and integrins, with support from chemokines (Abbas *et al.*, 2012, Beekhuizen and van Furth, 1993). Selectins are plasma membrane carbohydrate-binding adhesion molecules that initiate the first steps of leukocytes' low-affinity adhesion to ECs of the vasculature (Abbas *et al.*, 2012, Beekhuizen and van Furth, 1993). Endothelial cells express two types of selectin, called E-selectin and P-selectin. P-selectin is expressed by platelets and assist adhesion between leukocytes and ECs. E-selectin is upregulated and produced by endothelial cells as a result of IL-1 and TNF cytokine stimulation and lipopolysaccharide products of pathogen (Abbas *et al.*, 2012, Beekhuizen and van Furth, 1993, Mantovani *et al.*, 1997, Sun *et al.*, 2013). L-selectin is expressed on the white blood cells but not on endothelial cells.

Integrins are heterodimer cell surface proteins that enable cell-cell or cell-extracellular matrix adhesion via binding to their ligands (Abbas *et al.*, 2012, Beekhuizen and van Furth, 1993). The two principal integrins of the innate immune system are leukocyte function-associated antigen 1 (LFA-1; also known as CD11aCD18), and very-late antigen 4 (VLA-4 also known as CD49aCD29) (Abbas *et al.*, 2012, Beekhuizen and van Furth, 1993). Intercellular adhesion molecule 1 (ICAM-1, CD54) is a well-known ligand for LFA-1 (Abbas *et al.*, 2012, Beekhuizen and van Furth, 1993). ICAM-1 is a membrane-bound glycoprotein that is upregulated by cytokine-stimulated endothelial cells and many other cells like macrophages, lymphocytes, and fibroblasts. The binding of ICAM-1 to LFA-1 is an important step in leukocyte-endothelial interaction. Vascular endothelial adhesion molecule 1 (VCAM-1, CD106) binds to VLA-4 integrin (Abbas *et al.*, 2012, Beekhuizen and van Furth, 1993). Also, it is highly upregulated by the cytokine-stimulated endothelial cells (Mantovani *et al.*, 1997), and binding of VCAM-1 to VLA-4 is important for leukocyte migration through endothelium, transferring to inflamed tissues (Abbas *et al.*, 2012, Beekhuizen and van Furth, 1993). Mac-1 (CD11bCD18) is another important integrin, which is expressed by circulatory monocytes and binds to ICAM-1 to mediate the attachment to endothelium (Diamond *et al.*, 1990, Fink *et al.*, 2013).

Leukocyte-endothelial interaction is a multi-step process that mediates leukocyte transferral into tissues. At the infection or tissue injury site, macrophages release pro-inflammatory cytokines that include IL-1 and TNF (Abbas *et al.*, 2012, Beekhuizen and van Furth, 1993). These cytokines induce endothelial cells in the nearby small blood vessels to express selectin, chemokines, and ligands of integrins (ICAM-1 and VCAM-1). Selectin enables rolling of circulatory leukocytes on the surface of endothelium by weak interactions between leukocytes and endothelial cells. The sheer force of the blood flow allows leukocytes to roll throughout the endothelium of the blood vessels through repetitive detaching and re-binding. Chemokines are produced by different cells (such as macrophages and endothelial cells) in the adjacent infected tissues, and bind to their chemokine receptors on the rolling leukocytes, and then influence signalling to convert the leukocyte integrins from low- to high-affinity integrins. The high-affinity integrins are now ready to bind their ligands on the endothelial cells and enhance the firm attachment of the leukocytes. The leukocytes then crawl through endothelial junctions by reversible and transient protein disruption, mainly VE-cadherin complex (a process called paracellular transmigration). It has been suggested that binding of leukocyte integrins to VCAM-1 or ICAM-1 stimulates kinases that phosphorylate the cytoplasmic tail of VE-cadherin, causing reversible disruption of the junction complex. Monocytes, neutrophils, and lymphocytes use principally similar mechanisms to extravasate from blood circulation (Abbas *et al.*, 2012).

1.3.5 Pattern recognition receptors (PRRs)

The innate immune system recognises invading microorganisms by using pattern recognition receptors (PRRs) (Abbas *et al.*, 2012, Akira *et al.*, 2001, Takeda and Akira, 2005). The PRRs are able to recognise the conserved molecular patterns shared by large groups of microorganisms called pathogen associated molecular patterns (PAMPs). Also, they can recognise the endogenous molecules that are released from the damaged cells called damaged associated molecular patterns (DAMPs). The PRRs are expressed on the plasma membrane, endosomal membrane, and cytoplasm of different types of immune and non-immune cells such as neutrophils, macrophages, DCs, and epithelial cells. Toll-like receptors (TLRs) are an evolutionary-preserved family of PRRs that can be located either on the cell's plasma membrane (TLRs 1, 2, 4, 5 and 6) and recognise

the extracellular PAMSs, or intracellular on the endosomal membrane (TLRs 3, 7, 8 and 9) and recognise the nucleic acids of phagocytosed microbes. Also, the cells respond to cytoplasmic PAMPs and DAMPs through cytosolic PRR receptors called NOD-like receptors (NLRs) and RIG-like receptors (RLRs). When these receptors bind to PAMPs and DAMPs, they activate signalling transduction pathways that mediate the pro-inflammatory and anti-pathogenic functions. The innate immune system distinguishes the healthy normal cells from pathogens and damaged cells with the help of these PRRs, which are present everywhere in the human body to ensure the effective function of the innate immune system and elimination of harmful substances.

1.3.6 Toll-like receptors (TLRs)

At the end of the 90s, a protein named Toll was recognised in *Drosophila* as an essential receptor for host defence against fungal infection. In 1997, a mammalian Toll-like receptor (TLR) homologue of *Drosophila* was discovered (Medzhitov *et al.*, 1997). A few years later, more proteins structurally and functionally related to *Drosophila* were discovered and grouped into a family called the TLRs. TLRs are type I transmembrane proteins with an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic carboxy-terminal toll-interleukin 1 receptor domain. The cytoplasmic portion of TLRs shows high similarity to the IL-1 receptor family and is termed the Toll/IL-1 receptor (TIR) domain (Sandor and Buc, 2005). There are at least 10 different TLRs in humans that are specialised for the recognition of PAMPs and DAMPs. Lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, is recognised by TLR4. TLR2 in association with TLR1 or TLR6 recognises Gram-positive peptidoglycan (PDG), lipoproteins, and fungal zymosan. TLR3, TLR7, and TLR8 are specific to viruses (Akira *et al.*, 2001, Sandor and Buc, 2005) (**Table 1.4**).

1.3.6.1 Toll-like receptor 4 (TLR4)

TLR4 was the first human TLR homologue of *Drosophila* discovered in 1997 (Medzhitov *et al.*, 1997). One year later, LPS signalling was described and the mammalian TLR4 protein was reported to recognise LPS and transduce its signal across the plasma membrane (Hoshino *et al.*, 1999, Poltorak *et al.*, 1998). An additional

molecule is required for TLR4-mediated LPS signalling, this is myeloid differentiation factor 2 (MD2). Following ligation of LPS with the receptor complex, composed of CD14, MD2, and TLR4, there is the recruitment of an intracellular signalling adaptor protein, myeloid differentiation factor 88 (MyD88), which activates the nuclear factor-kappa B (NF-κB) pathway. NF-kB is a family of transcription factors that govern the expression of many pro-inflammatory cytokines such as TNFα, IL-1, and IL-6, chemokines such as CCL2 and CXCL8, co-stimulatory molecules such as CD80, CD86, and CD40, and endothelial adhesion molecules such as E-selectin. There are 11 known physiological NF-κB dimers that are composed of five distinct subunits (p100/p52, p105/p50, RelA (p65), RelB, and c-Rel) (Cookson and Chapman, 2010). The most commonly studied dimer consists of p50 and p65 subunits. During un-stimulated, resting conditions the p50-p65 heterodimer is coupled to I-kappa Ba (IkBa). After stimulation with ligands of TLR2 and TLR4, the IkBa is phosphorylated causing dissociation of IkBa-p50-p65 complex. This results in nuclear translocation of NF-κB subunits, which induces expression of inflammatory cytokines. Another pathway of TLR4 signalling involves the activation of interferon response factor 3 (IRF-3), as well as the late phase of NF-κB activation in a MyD88-independent manner (Takeda and Akira, 2005). TLR signalling pathways are summarised in **Figure 1.3**.

Table 1.4 Human TLRs and their common ligands

TLRs	Ligands	Study
TLR1	**Bacteria/ Mycobacteria** Lipoproteins (triacylated lipopeptides)	(Shimizu *et al.*, 2007)
TLR2	**Bacteria** Lipoproteins (di- & tri-acylated lipopeptides) Peptidoglycans Lipotechoic acid Atypical LPS **Fungi** Zymosan	(Sumikawa *et al.*, 2006) (Asong *et al.*, 2009) (Han *et al.*, 2003) (Werts *et al.*, 2001) (Roeder *et al.*, 2004, Sato *et al.*, 2003)
TLR3	**Viruses** Double-stranded RNA (dsRNA)	(Kalali *et al.*, 2008)
TLR4	**Gram negative bacteria** Lipopolysaccharides (LPS) Lipid A **Endogenous ligands** Heat shock proteins: HSP60, HSP70, HSP90 and GP96 High mobility group box 1 protein (HMGB1) Fibrinogen Extra domain A (EDA) of fibronectin Hyaluronan Heparan sulfate proteoglycan Lung surfactant A	(Hoshino *et al.*, 1999, Poltorak *et al.*, 1998) (MacArthur *et al.*, 2007, Rallabhandi *et al.*, 2008) (Chen *et al.*, 2009, Tsan and Gao, 2004a, Tsan and Gao, 2009) (Yu *et al.*, 2006) (Hodgkinson *et al.*, 2008, Smiley *et al.*, 2001) (Okamura *et al.*, 2001) (Taylor *et al.*, 2007) (Johnson *et al.*, 2002) (Guillot *et al.*, 2002)
TLR5	**Gram negative bacteria** Flagellin	(Hayashi *et al.*, 2001)
TLR6	**Bacteria** Lipoproteins (diacylated lipopeptides) **Fungi** Zymosan	(Nakao *et al.*, 2005) (Roeder *et al.*, 2004)
TLR7/8	**Viruses** Singal-stranded RNA (ssRNA)	(Larange *et al.*, 2009)
TLR9	**Bacteria/ viruses** CpG DNA **Protozoa (Malaria)** Hemozoin	(Kline and Krieg, 2008) (Engwerda and Good, 2005)

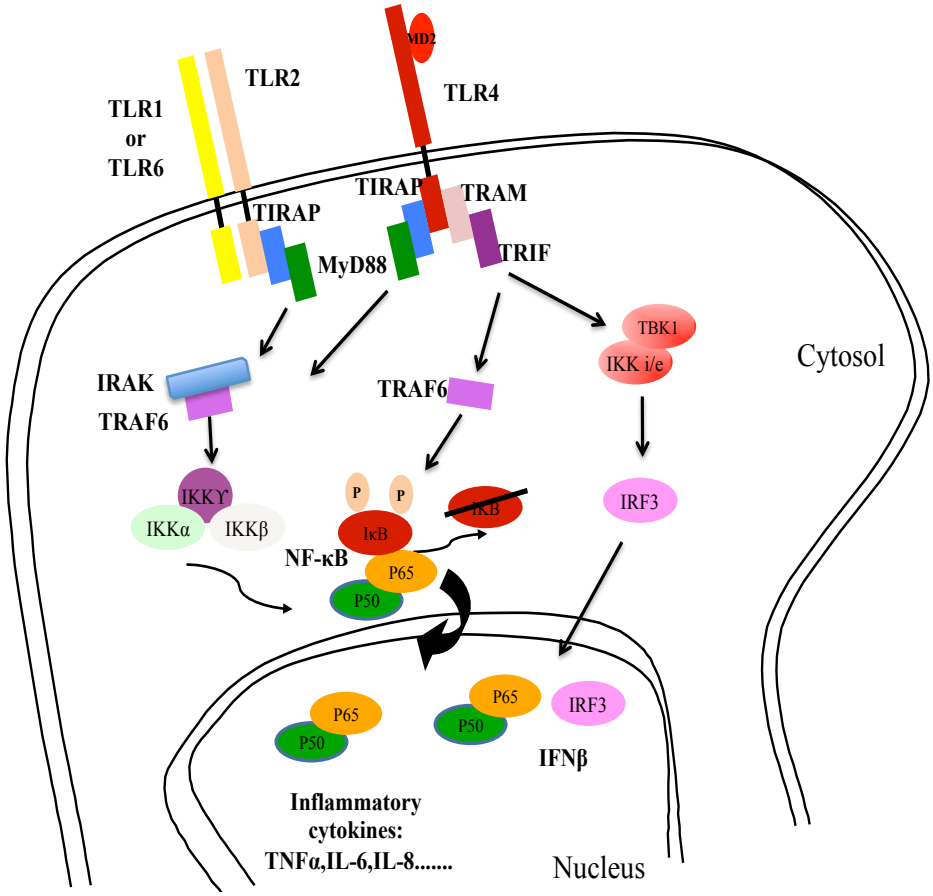

Figure 1.3 TLR 2 and TLR 4 signalling pathway

TIRAP, a TIR domain-containing adaptor, is involved in the MyD88-dependent signalling pathway via TLR2 and TLR4. A TIRAP and MyD88 associates with the cytoplasmic TIR domain of TLRs, and recruits IRAK to the receptor upon ligand binding. IRAK then triggers TRAF6, leading to the stimulation of the IκB kinase (IKK) complex comprising of IKKα, IKKß and IKK$^{\gamma}$. The IKK complex phosphorylates IκB, resulting in nuclear translocation of NF-κB that produces the inflammatory cytokines. In TLR4-mediated signalling pathways, stimulation of IRF-3 and production of IFN-ß are detected in a MyD88-independent pathway. A TRIF, another TIR domain-containing adaptor, is an important for the MyD88-independent pathway. A TRAM, TIR domain-containing adaptor, is exclusive to the TLR4-mediated MyD88-independent/TRIF-dependent pathway. Non-typical TBK1 and IKKs, IKKi/IKKe, lead to triggering of IRF-3 downstream of TRIF. Abbreviations: MD-2, a secreted glycoprotein; MyD88, myeloid differentiation primary response protein 88; IKK, IκB kinase; IRAK, interleukin-1 receptor-associated kinase; IRF3, interferon response factor 3; IFN, interferon; NF-κB, Nuclear factor κB; TRAF6, TNF receptor-associated factor 6; TRIF, TIR domain–containing adaptor protein inducing interferon-β; TRAM, TRIF-related adaptor molecule. Redrawn from references (Cookson and Chapman, 2010) and (Takeda and Akira, 2005).

1.3.6.2 Endogenous ligands for TLR4

In addition to LPS, TLR4 interacts with a host of endogenous molecules and triggers inflammatory and immune responses in the absence of infection (Tsan and Gao, 2004b). These endogenous ligands include: proteins released from damaged cells during stress, such as heat shock proteins (HSPs: HSP60, HSP70, HSP90, and GP96), high-mobility group box 1 protein (HMGB1), and lung surfactant A. Other endogenous ligands include: fibrinogen, fibronectin, extracellular matrix hyaluronan, and heparan sulfate proteoglycan (**Table 1.3**). Specifically, TLR2 and TLR4 were shown to mediate expression of inflammatory genes and trigger dendritic cell maturation by these endogenous agents (Beg, 2002). Whether the endogenous ligands of TLR4 activate cytokine production as result of contamination with the LPS and LPS-associated molecular patterns is not yet clear, but there is increasing evidence that such endogenous substances do exist and may mediate some autoimmune and other diseases (Erridge, 2010).

A. Heat shock proteins (HSPs)

In the past decade, expanded work has speculated that heat shock proteins (HSPs) could be strong stimulators of innate immunity. Stressful environmental, physiological, or pathological conditions stimulate production of HSPs in excessive amounts. The main function of HSPs is to act as molecular chaperones involved in the folding and assembly of proteins. Additionally, some HSPs (HSP60, HSP70, and GP96) from microbial and mammalian sources have been demonstrated to stimulate the release of inflammatory cytokines; for example, IL-1, IL-6, and TNF-α, and produce C-C chemokines and NO by immune cells (Tsan and Gao, 2004a, Tsan and Gao, 2009). Moreover, they stimulate the maturation of dendritic cells (DCs) recognised by the upregulation of MHCI, MHCII, and co-stimulatory molecules like CD80. HSPs are now called chaperonkine, a new term that expresses their double function. Moreover, their cytokine functions are delivered by the CD14/TLR4 and TLR2 complex signal transduction pathways contributing to the stimulation of NF-κB (Tsan and Gao, 2004a). Thus, it has been proposed that HSPs act as endogenous ligands for TLR4 and TLR2. However, there is still controversy regarding whether HSPs themselves activate TLR2/TLR4, or whether any such activity is due to contaminated bacterial recombinant protein being used in such studies.

B. High-mobility group box 1 protein (HMGB1)

HMGB1 is a DNA-binding nuclear protein of 215 amino acid lengths that is composed of two domains (HMG box A and B) (Huang *et al.*, 2010). It is highly distributed in most cells of mammalian bodies, especially in the lymphoid tissue, thymus, and neonatal liver. HMGB1 has several physiological functions, which include its role as a DNA-binding protein by conserving the structure of the nucleosome and regulating gene transcription, promoting cell proliferation, being involved in the regeneration of damaged tissues, and stimulating embryogenic stem cell migration and proliferation. Additionally, it acts as pro-inflammatory cytokine, stimulating neutrophils to release pro-inflammatory cytokines such as IL-1β, IL-8, IL-6, and TNFα, maturation of DCs with expressing co-stimulatory molecules such as CD54 and CD80, and acts as a strong chemoattractant and bactericide. It binds to receptors for advanced glycation end product (RAGE), TLR2, and TLR4 inducing NF-κB signalling pathway. During inflammation, extranuclear HMGB1 is released by activated macrophages and injured-necrotic cells, and acts as pro-inflammatory cytokine. Therefore, it is involved in different pathological conditions including sepsis, rheumatoid arthritis, atherosclerosis, and disseminated intravascular coagulation (DIC) (Huang *et al.*, 2010, Yu *et al.*, 2006).

C. Heparan sulfate proteoglycan (HSPG)

HSPG is an acidic copolymer on cell surfaces and extracellular matrices. Heparan sulphate promotes the integrity of the endothelial barrier, and protects against complements and oxidants. It has been demonstrated that neutrophils, T cells, and ischemic injury cause HSPG to be shed from endothelial cells. Accordingly, this loss of HSPG might explain the abnormal structure and function of blood vessels in inflammation and immune reactions (Tang *et al.*, 2007). In one previous study, it was shown that HSPG induces the presentation of antigen and the expression of co-stimulatory molecules by DCs as seen with LPS. This study also determined whether the responses of murine DCs to HSPG depended on TLR4, as does the response to LPS. They showed that HSPG induced expression of CD40, CD80, CD86, and activation of NF-κB in a fashion similar to LPS that was dependent on TLR4, but that neither HSPG nor LPS induced these changes in DC without the presence of TLR4 or non-functional TLR4. Thus, responses to HSPG are mediated by TLR4. Consequently, HSPG is

referred to as an endogenous agonist for TLR4 (Johnson *et al.*, 2002, Rosenberg, 2007, Tang *et al.*, 2007).

D. Hyaluronan (HA)

HA is one of the major extracellular matrix components. It consists of repeated disaccharide polymer composed of N-acetylglucosamine and glucuronic acid. Normally it is present as high-molecular-weight molecules in the epithelial and the connective tissues (Fraser *et al.*, 1997). However, it is degraded to intermediate- and low-molecular-weight molecules by a specific hyalurondiase enzyme during inflammatory conditions such as arthritis and wound repair. CD44, ICAM-1 (intracellular adhesion molecule-1), RHAMM (receptor for HA-mediated motility), TLR2, and TLR4 act as surface receptors for hyaluronan (Goueffic *et al.*, 2006, Taylor *et al.*, 2007). Hyaluronan has several functions and is implicated in cell proliferation, migration, tissue repair, embryogenesis, angiogenesis, and inflammatory processes (Nangia-Makker *et al.*, 2000, Taylor *et al.*, 2004, Termeer *et al.*, 2000). The inflammatory effect of HA is related to its size. Whilst high-molecular-weight hyaluronan has an anti-inflammatory effect, low-molecular-weight HA has a pro-inflammatory effect. Recently, a small hyaluronan (degraded-HA) was shown to stimulate inflammatory cells to release cytokines such as TNF-α, IL-6, and IL-1ß. Moreover, Yamawakie *et al.* showed in a recent study that small HA induces the release of IL-6 and monocyte chemoattractant protein (MCP-1) from monocytes via TLR4 (Yamawaki *et al.*, 2009).

E. Fibrinogen

Fibrinogen is a 45-nm-long glycoprotein consisting of two sets of three polypeptide chains called Aα, Bβ, and γ, which are symmetrically interconnected through five disulphide bridges (Mosesson, 2005). It is produced by hepatocytes in the liver and forms the major plasma coagulation factor that is converted by thrombin to fibrin. The plasma levels of fibrinogen range between 2-4 g/L and it has a half-life of approximately 3-4 days (Tennent *et al.*, 2007). But it is increased during pathological disorders and considered as a classical positive protein of acute phase reactant. Fibrinogen has several binding sites for platelets, endothelial cells, and leukocytes, for promoting its functions. The vascular endothelial (VE) cadherin receptor expressed on ECs binds to fibrinogen at $B\beta^{15-42}$ sequence, which stimulates capillary tube formation

and angiogenesis (Bach *et al.*, 1998). Also, the $B\beta^{15-42}$ sequence binds to heparin and leads to platelet spreading, fibroblast proliferation, and participation in cell-matrix interaction (Mosesson, 2005). The leukocyte integrin receptor CD11b/CD18 or $\alpha_M\beta_2$ (Mac-1) is highly expressed in monocytes with high affinity to fibrinogen that activates signalling pathways including NF-κB to release inflammatory cytokines such as IL-1β and TNF-α (Fan and Edgington, 1993, Lishko *et al.*, 2004, Perez *et al.*, 1999, Perez and Roman, 1995). Recent research has indicated the involvement of fibrin(ogen) and its degradation products in inflammatory diseases such as atherosclerosis, rheumatoid arthritis, stroke, multiple sclerosis, bacterial infection, and some cancers (Davalos and Akassoglou, 2012). Fibrinogen is able to bind and activate signalling of immune cells via CD11b/CD18 integrin receptors, adhesion molecules, and most recently has been found to act as an endogenous ligand for TLR4 (Hodgkinson *et al.*, 2008, Smiley *et al.*, 2001, Ugarova and Yakubenko, 2001). Fibrinogen stimulates TLR4 of macrophages to release inflammatory cytokines and chemokines such as IL-6, IL-8, TNF-α, monocyte chemoattractant protein 1(MCP-1), macrophage inflammatory protein 1 (MIP-1) α & β, matrix metalloproteinase (MMP) 1, and MMP9 (Hodgkinson *et al.*, 2008, Smiley *et al.*, 2001). Also, Fibrin(ogen) induces peripheral blood mononuclear cells to produce IL-1 β, TNF-α, and IL-6 (Jensen *et al.*, 2007). Fibrinogen acts as a bridge for the adhesion of monocytes to the endothelium by attaching to ICAM-1 on endothelial cells, which then interfere with monocyte-endothelial cells' adhesion and transmigration (Languino *et al.*, 1995, Languino *et al.*, 1993). Fibrinogen binds the VEGF and fibroblast growth factor 2 that are able to promote endothelial cell proliferation after binding (Sahni and Francis, 2000, Sahni *et al.*, 1999). However, it is well known as an independent factor for predicting cardiovascular dysfunction like hypertension and atherosclerosis (Lominadze *et al.*, 2010). Fibrinogen causes microvascular dysfunction by activating signalling mechanisms that trigger a decrease in endothelial cell-layer integrity, and increases in vascular reactivity and blood viscosity (Lominadze *et al.*, 2010). IL-6 and IL-1 participate in fibrinogen synthesis, which is also involved in the elevation of blood pressure (Chae *et al.*, 2001, Vasse *et al.*, 1996). Therefore, overproduction of fibrinogen might be a cause of high blood pressure, or be involved in its progression. Overall, fibrinogen has overlapping balanced functions of coagulation and haemostasis, inflammatory response, and vascular angiogenesis; and any excesses in one of these functions could affect the others and lead to pathological disorders.

F. Fibronectin

Fibronectin is a dimer composed of two identical subunits (~250 kDa) that are connected covalently adjacent to their C- terminals by a pair of disulfide links. Fibronectin is produced by hepatocytes of the liver and is widely expressed by various cell types. It is very important in the development of vertebrates. It is a soluble component of plasma and other bodily fluids. Also, it exists in the extracellular matrix that is involved in cell attachment, migration, growth, and tissue repair. Fibronectin acts as a ligand to dozens of cellular integrin receptors that link the extracellular matrix to the intracellular cytoskeleton. Also, it can bind to multiple biologically fundamental molecules including heparin, HSPG, collagen, and fibrin. The binding of fibronectin with fibrin is suggested to be important for cell adhesion and migration into fibrin clots. Also, their interaction may be involved with macrophage clearance of fibrin clots from peripheral blood after tissue injury or inflammation. It has been demonstrated that the recombinant extra domain A of fibronectin induces TLR4 activation and expression of MMP9 in THP-1 cells (Okamura *et al.*, 2001, Pankov and Yamada, 2002).

1.3.6.3 TLR4 activation stimulates VEGF production and enhances angiogenesis

Park and his colleagues demonstrated that the binding of TLR4 with LPS stimulates cyclooxygenase2 (COX2) production, which leads to prostaglandin I_2 (PGI_2) synthesis by macrophages. The released PGI_2 promotes macrophages to secrete VEGF. Moreover, they demonstrated that TLR4 stimulated by LPS results in the release of PGI_2 via the Akt signalling pathway. Furthermore, they suggest that LPS-induced VEGF expression is dependent on the synthesis of PGI_2 (Park *et al.*, 2007). Since macrophages and other leukocytes have an important role in the stimulation of angiogenesis via the secretion of VEGF mediated by TLR4, their activation may prove a key observation in the study of the pathogenesis of vascular disorders such as PE. It is plausible that genetic polymorphisms, or indeed epigenetic modifications of the TLR4 gene, may contribute to the development or protection of pregnant women against PE (Choudhury and Friedman, 2012, Molvarec *et al.*, 2008). Whether any such variations and observations will prove the cause or the consequence of the process of PE remains unclear. Alterations in the functional expression of TLR4 in placental and maternal vascular tissue in PE may reflect compensatory mechanisms to preserve VEGF-modulated

angiogenesis against endothelial dysfunction from dysregulated placental-derived sFlt-1 and PIGF.

1.4 The role of the immune system in normal pregnancy and PE

As to reasons why the foetus (a semi-allograft) is not rejected by the maternal immune system, a theory was generated 50 years ago (Mor *et al.*, 2011) based on the supposition that the placenta is an allograft organ acquiring paternal antigens, which should be rejected under normal immunological circumstances. Therefore, it was hypothesised that different mechanisms could allow the foetus to escape from the mother's immune system. These include the lack of polymorphic major histocompatibility complex (MHC) antigen expression on the surface of foetal-derived trophoblast cells, T-cell apoptosis and tryptophan catabolism by the indoleatimine, 2,3-dioxygenase (IDO) (Davies, 2007, Guleria and Sayegh, 2007, Spencer *et al.*, 2012).

The MHC is a large complex of genes organised into three regions that encode class I and class II human leukocyte antigens (HLA) (Davies, 2007, Loke and King, 2000). While the HLA class 1 and class II proteins are structurally similar, they are assembled from different subunits and the main function of these proteins is to present a peptide antigen to the T lymphocytes (Davies, 2007). There are two types of MHC class I genes: classical and non-classical (Davies, 2007, Loke and King, 2000). The classical HLA class I molecules (HLA-A, HLA-B and HLA-C) are highly polymorphic and they are present on the surface of nearly all somatic cells (Davies, 2007). The non-classical HLA class I molecules (HLA-E, HLA-F and HLA-G) are monomorphic and they have limited cellular expression (Davies, 2007, Loke and King, 2000). The HLA class II molecules are expressed only by professional cells, known as antigen presenting cells (APC), such as dendritic cells and B lymphocytes (Davies, 2007). Multiple studies have examined the expression of HLA molecules at implantation sites (Guleria and Sayegh, 2007). In humans and mice most of the polymorphic MHC class I antigens are lacking on the foetal-originated trophoblast cells (Guleria and Sayegh, 2007). The MHC class II antigens are also never expressed on trophoblast cells (Davies, 2007, Guleria and

Sayegh, 2007). The lack of MHC expression at the foeto-maternal interface may be critical for preventing harmful maternal immune responses against the foetus. The invasive extravillous cytotrophoblast expresses class I non-classical monomorphic HLA-E, HLA-F and HLA-G molecules and the classical class I polymorphic HLA-C molecule (Davies, 2007). The expression of non-classical HLA class I molecules by extravillous cytotrophoblast is believed to modulate immune responses at the foeto-maternal interface by the inhibition of uterine leukocyte activity and foetal protection against immune-mediated rejection (Davies, 2007). In contrast, the extravillous cytotrophoblast does not express HLA-A, HLA-B, HLA-DR, HLA-DP and HLA-DQ molecules that are the key targets for alloreactive T lymphocytes during transplantation (Davies, 2007, Doridot *et al.*, 2013).

It has been suggested that the existence of an immune system at the implantation site is the result of clonal deletion of anti-foetal effector cells that recognise the paternal antigens (Guleria and Sayegh, 2007, Spencer *et al.*, 2012). However, the T-cell activation is persistent and it can recognise the semi-allograft foetus (Spencer *et al.*, 2012). Therefore, to avoid allograft rejection, several mechanisms of peripheral T-cells tolerance allow the paternal alloantigens to exist without affecting the anti-infectious immune responses (Spencer *et al.*, 2012). For example, the Fas ligand expressed by trophoblasts cells may lead to apoptosis of the activated Fas-expressing maternal T lymphocytes (Spencer *et al.*, 2012). The role of Fas-FasL interaction upon foetal-maternal tolerance was shown to promote foetal rejection (Guleria and Sayegh, 2007). Moreover, T-cell foetal-maternal tolerance is regulated by a specialised subset of $CD4^+$ T cells expressing the IL-2 receptor α-chain (CD25), known as T regulatory cells (Guleria and Sayegh, 2007, Spencer *et al.*, 2012). T regulatory cells are pivotal in the regulation of immune responses with the maintenance of natural self-tolerance and they are involved in preventing autoimmunity. IDO is an enzyme that catabolizes the essential amino acid tryptophan and reduces its metabolites (Guleria and Sayegh, 2007). It is expressed on extravillous trophoblasts and villous trophoblasts in humans and giant trophoblasts in mice. Thus, it has been believed to inhibit the activation of maternal T-cells by depriving T-cells of tryptophan (Guleria and Sayegh, 2007).

The self-versus-non-self theory requires that the immune response be triggered by all foreign constituents (non-self), not by the host's own constituents (self) (Burnet, 1969).

In the 1990s, Matzinger established the danger theory, which claims that immune responses are triggered by danger signals released by one's own body's cells (Matzinger, 1994, Matzinger, 2012). As opposed to the self-versus-non-self theory, the danger theory suggests that the self components can trigger the immune responses if they are dangerous and the non-self components can be tolerated if they are not dangerous. The danger theory may help explain immune tolerance for organ transplantations and cancers (Pradeu and Cooper, 2012). Accordingly, maternal immune system might consider the foetal allograft to be dangerous or it might not; however, applying the self-or non-self theory, the semi-allograft foetus might be rejected and not tolerated.

The maternal immune system has been recognised at the maternal-foetal interface. Up-to-date insights on maternal-foetal immune cross-talk have been acknowledged, including the interactive pathways that link invasive trophoblasts, various maternal immune cells and decidual stromal cells (decidualization) (Arck and Hecher, 2013). Any failure of the immune system, either complete or partial, will lead to miscarriage or complications, such as PE (Redman and Sargent, 2010). The challenge is to identify the exact immunological mechanisms involved in protecting normal pregnancies as well as the mechanisms that may lead to complications, such as PE, IUGR, pre-term labour and recurrent miscarriages (Arck and Hecher, 2013, Hamilton *et al.*, 2013).

1.4.1 Immune cells at the maternal-foetal interface

There are two maternal-foetal interfaces as defined by Sargent: interface 1 between invasive trophoblasts and the maternal leukocytes in the endometrial mucosa (decidua); and interface 2 between syncytiotrophoblasts and leukocytes in maternal blood (Sargent *et al.*, 2006a, Sargent *et al.*, 2006b). The transformation of the endometrial tissue to decidua is referred as decidualization, which occurs in the pre-implantation stage (secretory phase of the menstrual cycle) in order to prepare and support the endometrium for implantation and placental development (Gellersen *et al.*, 2007). Decidua is the outer endometrial lining that is shed at parturition. It protects the foetus from intrauterine infection and the maternal immune system during pregnancy. The cellular composition of the endometrial decidua includes stromal cells, fibroblasts, and leukocytes. The leukocyte population comprises of the decidual natural killer cells

(~70%), the decidual macrophages (~25%), and T cells (< 20%) (Erlebacher, 2013a). There is evidence that decidual leukocytes play a major role in the development of the foeto-placental unit, and in successful implantation (Erlebacher, 2013a, Gomez-Lopez *et al.*, 2010, Mor *et al.*, 2011).

Natural killer cells (NKs)

During the first trimester, uterine decidual NKs (dNKs) ($CD56^{bright}CD16^{-}$) are the dominant phenotype in the decidual leukocytes that are quite distinct from the majority of peripheral blood NKs ($CD56^{dim}CD16^{+}$), because they express higher levels of cytokines, chemokines, and angiogenic factors (Arck and Hecher, 2013, Gomez-Lopez *et al.*, 2010, Sargent *et al.*, 2006b). Therefore, there are two theories that attempt to explain their origin; either dNKs arise from a specific lineage of NKs from hematopoietic precursor with distinct pregnancy functions, or they are recruited and result from maturation of $CD56^{bright}CD16^{-}$ peripheral blood NKs (which compose 10% of peripheral blood) (Arck and Hecher, 2013, Erlebacher, 2013a, Gomez-Lopez *et al.*, 2010). Although it has been suggested that they play a fundamental critical role in successful pregnancies due to their abundant composition in human decidua, these roles have not been clarified yet. Their possible functions include: regulation of extravillous trophoblast invasion, decidual vascular remodelling, and local antiviral functions. Additionally, it has been suggested that dNKs interact with major histocompatibility complex (MHC) antigens expressed on invading extravillous trophoblasts (Arck and Hecher, 2013, Sargent *et al.*, 2006b). The MHC expression on the invasive extravillous trophoblasts, which differs from the adult or foetal cells, lacks the polymorphic highly classical HLA class I molecules (HLA-A and HLA-B), which are the main target of graft rejection by T cell response. However, these cells express the polymorphic HLA-C molecule and the monomorphic non-classical HLA class I molecules (HLA-E, HLA-G, and HLA-F). In contrast, syncytiotrophoblast does not express MHC class I antigens, and both the invasive extravillous trophoblasts and syncytiotrophoblast do not express MHC class II antigens. The MHC antigens interact with dNKs via numerous receptors. The CD94/NKG2A receptor is believed to interact with HLA-E antigen on extravillous trophoblasts, and inhibit their killing. The killer immunoglobulin-like (KIR) 2DL4 receptors on the dNKs are thought to interact with HLA-G and stimulate production of cytokines, chemokines, and angiogenic factors that influence trophoblast invasion and vascular remodelling during implantation. Most interestingly, it is the interaction

between the KIR family on the dNKs and HLA-C on trophoblasts, which is polymorphic. Certain combinations of KIRs and HLA-C promote vascular remodelling, while others could lead to abnormal placentation (most importantly PE) (Kopcow and Karumanchi, 2007, Sargent *et al.*, 2007). There are two groups of HLA-C antigens; C1 that are less inhibitory for NKs, and C2 that are strongly inhibitory for NKs. Also, there are two important haplotypes: A, which has inhibitory receptors, and B, which has from one to five activating receptors. The risk of developing PE is increased when pregnancies are a product of a combination between the maternal KIR-AA genotype and the foetal HLA-C2 allele. This could explain the paternal inheritance of PE, due to inheritance of the foetal HLA-C2 allele that is associated with the strong inhibitory effects on dNKs.

Monocytes/ macrophages (DCM)

The second most abundant immune cells in the human decidua are decidual macrophages (DCM), which express CD14, CD68, and MHC class II surface molecules (Bulmer *et al.*, 1988, Vince *et al.*, 1990). Some immunological functions of DCM have been defined recently. DCM mainly functions in pathogenic recognition and phagocytosis to protect the foetus from intrauterine infection (Singh *et al.*, 2005). These cells are believed to have an important role in cell remodelling during implantation of the embryo, as they may regulate apoptotic debris clearance in normal pregnancy (Abrahams *et al.*, 2004b). Also, in early pregnancy, they are suggested to have immunosuppressive properties and may act as an inhibitory pattern of APCs, because these cells are demonstrated to produce high levels of spontaneous IL-10 and indoleamine 2,3-dioxygenase (IDO; known to prevent T cell activation in allogeneic foetuses) (Heikkinen *et al.*, 2003). The IL-10 anti-inflammatory cytokines are highly expressed in human decidua; their main cellular sources are dNKs, stromal cells, and type 2 macrophages, but their role is not very well established. Monocytes migrate to the maternal-foetal interface, and may cause either local cytokines production or upregulation of cell adhesion molecules. Graham *et al.* showed that human first-trimester trophoblast HTR-8/SV neo cells are able to release monocyte-associated chemokines (CCL2 and CCL5) in response of TNF stimulation (Renaud *et al.*, 2009). Moreover, it has been demonstrated that human trophoblasts are able to recruit lymphocytes and monocytes by releasing CXCL16 chemokine and interacting with CXCR6 receptors of first trimester decidual leukocytes (Arck and Hecher, 2013, Huang

et al., 2008). Recently, it has been shown that IL-10, combined with macrophage colony-stimulating factor (M-CSF), induced human peripheral blood monocyte maturation and differentiation into macrophages with features of DCM (Svensson *et al.*, 2011). Therefore, it is believed that dNKs, which produce more IL-10 (Thaxton and Sharma, 2010), could help macrophages differentiate and then maintain them in an immunosuppressive state.

Controversially, it has been shown that DCM are decreased (Burk *et al.*, 2001), increased (Schonkeren *et al.*, 2011), or not changed (Kim *et al.*, 2007b) in PE compared to normal pregnancy controls. A previous study demonstrated that tissue macrophages are severely reduced in pre-eclamptic placenta compared to normal deciduas (Burk *et al.*, 2001). Another study showed no significant difference of DCM between PE and controls (Kim *et al.*, 2007b). A more recent study examined the phenotypic characteristics of DCM in PE (Schonkeren *et al.*, 2011). They found that the total number of macrophage $CD14^+$ cells was significantly increased in the deciduas of PE women compared to controls. However, the number of type 2 macrophages (phenotypes characterised with anti-inflammatory, apoptotic cell clearance, and tissue remodelling functions (Anderson and Mosser, 2002)) was significantly reduced in the PE decidua's. Additionally, their data were confirmed by gene expression studies of human DCM phenotypes that showed a predominance of type 2 macrophages in normal pregnancies (Gustafsson *et al.*, 2008). Also, the abnormal distribution of DCM at PE maternal-fetal interface act as barrier between the invasive trophoblasts and spiral arteries lead to failure of vascular remodelling (Abrahams *et al.*, 2004b). The production of IL-10 was decreased in the placental tissue of PE patients (Hennessy *et al.*, 1999). Therefore, macrophages are thought to play an important immunoinhibitory function at the foeto-maternal side to maintain successful pregnancy. Any disturbance in these cells, either due to defects of migration and differentiation of circulatory monocyte/macrophages, or an increase in DCM consumption by enhanced phagocytosis, may lead to abnormal pregnancies like IUGR and PE. However, very little is known about the DCM functions, and most research concentrates on their distribution and expression, and work in the DCM-rodent model is still not well developed.

T cells

The proportion of T cells is lower relative to other decidual leukocytes during early pregnancy (5–20%). Decidual T cells include approximately 30–45% $CD4^+$ helper T cells and 45–75% $CD8^+$cytotoxic T cells, but their functions in pregnancy are largely unknown (Erlebacher, 2013a, Erlebacher, 2013b). The ratios of T cell subtypes are different from peripheral blood T cells. Type 1 $CD4^+$ T cells (Th1) release IL-2, IFN-γ, and TNF-α inflammatory cytokines that are the principle effectors of phagocytosis, and protect against intracellular microbes. Type 2 $CD4^+$ T cells (Th2) release IL-4, IL-5, IL-6, IL-10, and IL-13 cytokines (Sykes *et al.*, 2012). Wegmann was the first to postulate the predominance of Th2 in pregnancy, and the suppression of Th1 at the maternal-foetal interface, which is maintained in a successful pregnancy by anti-inflammatory cytokines such as IL-10 and IL-4, and by suppressing harmful cytokines such as IFN-γ and TNF-α (Wegmann *et al.*, 1993). Then, measurement of cytokine levels in murine and human subjects enforced this hypothesis (Sykes *et al.*, 2012). In PE, it was proposed that the Th1/Th2 ratio would deviate toward Th 1 rather than Th2 as in normal pregnancy due to an excessive inflammatory immune responses. However, the Th1/Th2 ratio does not change in PE, and the proportions of decidual T cell subsets change little compared to normal pregnancy (Borzychowski *et al.*, 2005, Erlebacher, 2013a). A recent study demonstrated elevation of granulysin-producing cytotoxic T cells from peripheral blood of PE women which could be contribute to predominance of Th1 with a pro-inflammatory immune responses (Molvarec *et al.*, 2011).

1.4.2 Inflammatory response

Normal pregnancy is associated with a mild inflammatory systemic response that strengthens as the pregnancy advances. These inflammatory responses are characterised by an increase of acute phase reactants such as plasma fibrinogen and caeruloplasmin, leukocyte activation including neutrophils, monocytes, and granulocytes, and an increase of circulatory inflammatory cytokines such as IL-6 and TNF-α. In PE, similar systemic inflammatory responses occur but with superior intensity (Borzychowski *et al.*, 2006, Redman and Sargent, 2003).

There is strong evidence that PE is an exaggerated inflammatory syndrome characterised by an increased cytokine production and an abnormal activation of

inflammatory and endothelial cells (Redman and Sargent, 2003, Redman and Sargent, 2005, Sibai, 2004). Previously, Fass and his colleagues developed an animal model of PE. They infused very low doses of endotoxin into pregnant rats and demonstrated pathological changes resembling those observed in PE (hypertension, proteinuria, glomerular lesions, and thrompocytopenia), but which were not seen in non-pregnant control rats (Faas *et al.*, 1994). The endotoxin-treated rats also demonstrated increased white blood cell and granulocyte counts, and decreased monocyte TNF-α production compared to saline-treated rats (Faas *et al.*, 2004). However, extrapolating observations from experimental animal models of PE may not reflect the pathology of PE, which is a unique disorder of human primates. Furthermore, the advanced renal damage noted in the experimental animals infused with endotoxin is not a usual feature of human PE.

A. Leukocyte exaggeration in PE

White cell activation appears to be a hallmark feature of PE. Sacks et al. showed in their study that leukocytes are activated in normal pregnancy compared to non-pregnancy, and are overactivated in PE (Sacks *et al.*, 1998). Upregulation of the activation markers CD11b, CD14, and CD64 has been demonstrated in monocytes and granulocytes from women with PE as compared to normotensive pregnant women. Furthermore, both monocytes and granulocytes produce increased amounts of intracellular reactive oxygen species (ROS) in women with PE compared to normal pregnant women. Also, they demonstrated that leukocytes from both normal and PE pregnancies are activated with similar changes as in sepsis (Sacks *et al.*, 1998). PE neutrophil produces more superoxide and ROS than in normal pregnant women (Lee *et al.*, 2003, Tsukimori *et al.*, 1993).

Another study showed that CD11b is upregulated in monocytes of PE women, and there are increased levels of ROS in these cells (Gervasi *et al.*, 2001). Monocytes are shown to express adhesion molecules (i.e., CD11a and CD54, both binding to ICAM-1), and progressively are upregulated throughout normal pregnancy (Luppi *et al.*, 2002). A study demonstrated that plasma from both normal pregnant women and those with PE activated monocytes leading to the expression of ICAMs and increased production of reactive oxygen species (Faas *et al.*, 2008). A previous study showed that the placental microparticles (namely STBMs) can activate peripheral blood monocytes expressing

CD54 and releasing IL-6, 1L-8, and IL-1β cytokines (Messerli *et al.*, 2010). More recently, Holder and colleagues demonstrated that placental-derived microvesicles of PE women activate peripheral blood mononuclear cells to produce cytokines and chemokines, as compared to term placental-derived microvesicles of NP women (Holder *et al.*, 2012).

B. Cytokines and chemokines in PE

Numerous studies have shown that TNF-α and IL-6 are elevated in the sera of pre-eclamptic patients compared to normotensive pregnant women (Conrad *et al.*, 1998, Freeman *et al.*, 2004, Greer *et al.*, 1994, Jonsson *et al.*, 2006, Kupferminc *et al.*, 1996, Vince *et al.*, 1995, Xiao *et al.*, 2012). They are multifunctional acute pro-inflammatory cytokines that mediate acute immune and inflammatory responses, stimulate endothelial cell inflammation and procoagulant states, and induce synthesis of acute phase reactants by the liver; see Table 1.2 (Abbas *et al.*, 2012). Altogether, these functions suggest that TNF-α and IL-6 could play a critical role in the endothelial inflammation and impairment in PE. Furthermore, several studies have indicated that IL-6 is involved in vascular functions. A study showed that IL-6 induces endothelial dysfunctions (Desai *et al.*, 2002), and another one showed that IL-6 induces aberrant angiogenesis (Nilsson *et al.*, 2005), while another showed that angiogenesis can be inhibited by IL-6 (Hatzi *et al.*, 2002). All these studies indicate that IL-6 could be a key modulatory factor in the regulation of physiological or pathological angiogenesis in normal pregnancy, or one with pregnancy-induced hypertension.

Several studies have reported that IL-10, an anti-inflammatory cytokine, is suppressed in PE plasma and placenta as compared to normotensive women's plasma and placenta (Bowen *et al.*, 2005, Hennessy *et al.*, 1999, Makris *et al.*, 2006). Kalkunte and colleagues, while studying rat maternal-foetal interfaces, discovered that IL-10 has immuno-modulatory activities and plays a protective role in high blood pressure and inflammation-mediated vascular dysfunction (Kalkunte *et al.*, 2011, Lai *et al.*, 2011, Tinsley *et al.*, 2010). They demonstrated that the administration of exogenous IL-10 to mice with pregnancy-induced hypertension resulted in an improvement of the hypertension and endothelial dysfunction (Tinsley *et al.*, 2010).

Additionally, it has been demonstrated that plasma of severely pre-eclamptic women expresses higher levels of chemokines, i.e., IL-8 and MCP-1, and plasma from PE women induces HUVEC to release IL-8 and MCP-1 (Kauma *et al.*, 2002). These chemokines are known to trigger firm adhesion of monocytes to the E-selectin expressed by monolayers of vascular endothelium (Gerszten *et al.*, 1999, Lukacs *et al.*, 1995), which might enhance monocyte adherence to vascular endothelium and be involved in its damage in PE. Moreover, a recent study proved PE to be a pro-inflammatory systemic condition characterised by vascular dysfunction by detecting increased circulatory levels of pro-inflammatory cytokines IL-6 and TNF-α, chemokines MCP-1 and IL-8, and adhesion molecules VCAM-1 and ICAM-1 in PE women as compared to NP women (Szarka *et al.*, 2010). Overall, the production of pro-inflammatory cytokines in PE IL-6 and TNF-α could induce endothelial cell activation and expression of their adhesion molecules that will recruit more leukocytes by chemokines MCP-1 and IL-8, which are known to be produced in response to acute inflammatory cytokines, i,e., TNF-α and IL-1.

1.4.3 Endothelial cell inflammation in PE

As mentioned above in Section 1.3.4, endothelial cells are known to regulate leukocyte adhesion and migration from the vasculature to the inflammatory sites through expression of endothelial surface adhesion molecules, i.e., E-selectin, VCAM-1, and ICAM-1 (Barleon *et al.*, 1996, Faruqi and DiCorleto, 1993, Takahashi *et al.*, 1996a). During inflammation, IL-1, IFN-γ, and TNF-α pro-inflammatory cytokines induce leukocyte and endothelial cell activation and shed off these adhesion molecules into circulation, which indicates vascular inflammation and endothelial cell activation (Blake and Ridker, 2001). Endothelial cell activation is a hallmark in the pathogenesis of PE. Whether this vascular cell activation results from an excessive inflammation of leukocytes due to the release of pro-inflammatory cytokines; or contradictious the activation of endothelial cells as a result of placental circulating factors or intrinsic vascular dysfunction, this will participate in exaggeration of the inflammatory response in those women; research in this area remains unclear. However, in PE, many studies demonstrate the elevation of soluble adhesion molecules such as VCAM-1, ICAM-1, and E-selectin, indicating activation of both cell types (Austgulen *et al.*, 1997, Budak *et al.*, 1998, Heimrath *et al.*, 2004, Higgins *et al.*, 1998, Kim *et al.*, 2004, Krauss *et al.*,

1997). And a few studies have demonstrated the interactions between the two cellular types.

Neutrophils are known to be markedly activated in PE and express higher levels of adhesion markers (Clark *et al.*, 1998b). Wang *et al*. studied the interaction between neutrophils, isolated from non-pregnant women, and human umbilical vein endothelial cells (HUVECs), isolated from normal pregnant and PE women. They found that neutrophils are strongly adhesive to HUVECs of PE women, and they express significantly higher levels of P-selectin, lower levels of ICAM, and largely unchanged levels of VCAM-1 and E-selectin. Additionally, they blocked the effects of these adhesion molecules by specific antibodies, which completely or partially blocked the adhesion of neutrophils to PE HUVECs (Wang *et al.*, 1998). Another study demonstrated that PE neutrophils express higher levels of CD11b and are more likely to adhere to endothelial cells compared to neutrophils from non-pregnant and normal pregnant women, and this is most likely mediated by neutrophil-derived reactive oxygen species (Tsukimori *et al.*, 2008).

A recent study investigated the correlation between the circulating anti-angiogenic factors (sFlt-1 and soluble endoglin) and the markers of neutrophil activation (α-defensins, calprotectin) in severe PE and systemic inflammatory conditions (chorioamnionitis, pyelonephritis and appendicitis). They found that the elevation of systemic inflammation in PE and systemic inflammatory condition does not correlate with elevation of anti-angiogenic factors, which are exclusively increased in PE (Ramma *et al.*, 2012).

Interestingly, plasma from severe PE women, associated with increased lipid peroxides levels, leads to upregulation of HUVECs NF-κB activity and ICAM-1; and this activation is suppressed by anti-oxidant treatments including vitamin E and N-acetyl-cysteine (Takacs *et al.*, 2001). Previously, Hey and colleagues demonstrated that the sera of PE women activate the cultured HUVECs by expression of VCAM-1 and ICAM-1 (Heyl *et al.*, 1999). Another group has recently shown that plasma of PE, but not NP women, leads to activation of endothelial cells, which express significantly higher levels of ICAM-1 and ROS, and this occurs only in the presence of the monocyte cell line (Faas *et al.*, 2010). Also, in another study they demonstrated that PE

microparticles with monocytes lead to activation of endothelial cells similar to the plasma from PE women in combination with monocytes (Lok *et al.*, 2012). This suggests that monocytes activated by PE sera may induce microparticle shedding from endothelial cells, and then induce their activation. To support this notion, it is better to study the effect of direct contact of activated PE monocytes on endothelial cell activation rather than the monocyte cell line with the sera of PE women.

1.4.4 TLR expression in the maternal-foetal interface

Innate immune cells present at the maternal-foetal interface may be necessary for successful pregnancy. Expression of all ten TLRs has been demonstrated in the human placenta (Koga and Mor, 2008). Various cell types in the term and preterm placenta, including Hofbauer cells (macrophages in placenta), express TLR2, and TLR4 (Patni *et al.*, 2009). TLRs are expressed not only in immune cells but also in non-immune cells such as trophoblasts, decidual cells, and endothelial cells. It has been shown that cultured cytotrophoblast and syncytiotrophoblasts, isolated from human term placenta, express TLRs 2, 6, and 9 (Koga and Mor, 2010). Also, expression of TLRs seems not to be constant throughout pregnancy, but is adjusted in a temporal way (Koga and Mor, 2010). For example, TLR4 is more highly expressed in human term placenta than in the first trimester (Beijar *et al.*, 2006); and despite TLR6 being expressed at third trimester trophoblast (Mitsunari *et al.*, 2006), it is not expressed in the first trimester (Abrahams *et al.*, 2004a). In pregnant mice, Gonzalez demonstrated upregulated gene expression of TLR2, 3, 4 and 9 in the uterus and cervix, but TLR4 was significantly downregulated in the placenta when the pregnancy advanced, indicating the immunosuppressive characteristics of placenta, which properly serve as protective mechanisms, or due to the higher likelihood of responding to infections (Gonzalez *et al.*, 2007). These findings suggest potential roles for TLRs in normal placentation, but these mechanisms are not well understood.

The functional roles of TLR4 and TLR2 during the first and third trimesters have been described. The tissue responses to TLRs at the maternal-foetal interface differ according to the specific TLR that is activated, and the type of stimuli. For example, TLR2 binding with PDG in trophoblasts causes apoptosis, whereas binding of TLR4 with LPS

in first trimester trophoblasts promotes cytokine production (Abrahams *et al.*, 2004a). Although LPS does not induce apoptosis in first trimester trophoblasts, chlamydial HSP60 has been shown to induce apoptosis in trophoblasts through TLR4 (Equils *et al.*, 2006). On the other hand, the *Mycoplasma fermentans* induce signalling of TLR2 and increased expression of cyclooxygenase 2 and prostaglandin during the third trimester (Mitsunari *et al.*, 2006). Additionally, Abrahams demonstrated that first trimester trophoblasts release chemokine profiles in response to bacterial LPS, through TLR4, and viral poly (I:C), through TLR3, which results in an increase of monocytes and neutrophil migration toward trophoblast cells. Although the innate immune cells at the maternal-foetal interface are necessary for successful pregnancy, elevated and altered white blood cell infiltration may contribute to complications of pregnancy such as preterm birth, IUGR, and PE (Abrahams *et al.*, 2005). As placental tissue is most easily available for studies postpartum, little is known about receptor changes during human pregnancy across all pregnancy trimesters. TLR2 and TLR4 expression in maternal neutrophils has been described throughout pregnancy, and therefore it is possible to compare TLR4 expression during normal pregnancies to that during complicated pregnancies such as preterm labour, infection, or PE (Nitsche *et al.*, 2010).

1.4.5 TLR expression in PE

Gene polymorphism studies have investigated whether allelic variants of TLR4 are related to early-onset PE and its severe variant, HELLP syndrome. They found that the allelic variants T399I and D299G of maternal TLR4 were more common in women with a history of early-onset PE than in controls, and the higher frequencies for these TLR4 variants were observed in women who developed HELLP syndrome, a severe form of PE. Taken together, these early findings suggest the involvement of the maternal innate immune system in severe hypertensive disorders of pregnancy such as PE (van Rijn *et al.*, 2008). The TLR4 receptor activity in women with PE may obtund the TLR4-mediated synthesis of VEGF, leading to, or contributing to, dysregulated angiogenesis and endothelial dysfunction. It is, however, likely that the relationship will prove far more complex than this simplistic model, as several other endothelial, placental, and white cell-derived molecules are likely to play regulatory roles. Another study evaluated the production of TNF-α, pro-inflammatory cytokine, and IL-10, an anti-inflammatory cytokine, by monocytes from pre-eclamptic patients induced with TLR ligands. The

responses to TLR2 and TLR4 ligands did not differ between non-pregnant and normal pregnant females. However, the release of TNF-α via monocytes induced by TLR2/4 ligands was severely reduced in PE, whereas IL-10 synthesis was not changed (Mazouni *et al.*, 2008). The unexpected anti-inflammatory role of monocytes in PE is speculated to be caused by the imbalance between the pro- and anti-inflammatory cytokines.

As PE is associated with an inflammatory process without strong evidence of an infective origin, any ligands of TLR4 which may modulate the dysregulated angiogenesis and inflammation seen in PE are likely to be endogenous, rather than exogenous, PAMPS on microbes. Holmlund *et al.* demonstrated that HMGB1, a ligand for TLR4, is highly expressed in decidua from pre-eclamptic patients (Holmlund *et al.*, 2007). HMGB-1 induces NF-κB activation and the release of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8. Indeed, recently, Rose *et al.* demonstrated that TLR2 bacterial ligand, but not TLR4 ligand, induces necrosis in murine trophoblastic stem cells and is associated with the release of HMGB1 (Rose *et al.*, 2011). These data suggest that placental necrotic cells might release HMGB1, which is involved in the activation of TLR4 as an endogenous ligand, resulting in the exacerbation of innate immunology at the maternal-foetal interface.

Kim and colleagues evaluated a potential link between "danger signals" and PE via TLR4 (Kim *et al.*, 2005). The expression pattern of TLR4 and TLR2 in the trophoblasts at the placental bed was analysed by double immunohistochemistry. They demonstrated that TLR4 expression in trophoblasts was significantly higher in women with PE than in women with preterm labour with or without chorioamnionitis. Furthermore, TLR4 expression was co-localised with activated NF-κB, TNF, and M30 (an apoptosis factor specific for trophoblast cells) in interstitial trophoblasts inside the placental bed of PE patients. This proposes that inflammatory cytokines could stimulate TLR4 expression and encourage more trophoblast response to "danger signals". They have also shown that TLR4 expression was induced with LPS and TNF-α, but not with IL-10 in vitro, and LPS-induced TLR4 blocked the migration of trophoblast cells, and this effect may explain the inadequate invasion of trophoblasts into the spiral vessels in the myometrium as seen in PE patients. The strong point in this study is the description of TLR2 and TLR4 expression within the human placental tissue in vivo. However, the limitation is that a cause-and-effect relationship of observations with PE could not be

deduced because a temporal link between the observations and the subsequent development of PE could not be established. A recent study examined neutrophil TLR2, TLR4, and intracellular cryopyrin (a caspase-activating recruitment domain) in PE, and women with normotensive IUGR and normal pregnancy (Xie *et al.*, 2009). They demonstrated higher levels of neutrophil TLR2, TLR4, and cryopyrin expression in early-onset PE compared to normal pregnant and normotensive IUGR women.

Although most research is concentrated on the placental expression of TLR2 and 4 in the pathogenesis of PE, a few studies concern other TLRs. Recently, Pineda *et al.* analysed placental expression of TLR2, 3, 4, and 9, using confocal microscopy (Pineda *et al.*, 2011). Interestingly, in addition to higher expression of these TLRs in all parts of PE placenta, the trophoblastic co-localization of TLR2 with TLR4, and TLR3 with TLR9, was demonstrated. This indicates that novel mechanisms of TLR2 and TLR4 co-operation to activate the innate immune system in PE may exist. A more recent study examined the role of double-strand RNA or single-strand RNA play in placental TLR3 or TLR7/8 activation, respectively, which are mainly produced either from apoptotic/necrotic cells or viral infections, in humans and mice (Chatterjee *et al.*, 2012). They demonstrated that TLR3, 7, and 8 agonists (poly I:C, R-837 and CLO97, respectively) are likely to induce more significant expression of these receptors in PE than in NP trophoblasts. Additionally, they found that treatment of pregnant mice with these agonists caused hypertension, placental inflammation, and endothelial dysfunction. Therefore, the excessive apoptosis or necrosis at the maternal-foetal interface during abnormal placentation and vascular remodelling might lead to amplification of the innate immune systems, which will lead to exuberance of the pathology of PE including vascular dysfunction.

Research hypothesis and objectives

We hypothesise that TLR4 activity may play a key role in the pathogenesis of PE through one or more of its endogenous ligands (e.g., heparan sulphate, fibrinogen, fibronectin, hyaluronan and HMGB1), produced by the ischaemic placenta. Additionally, these endogenous ligands may play some role in the activation of leukocytes and endothelial dysfunction, resulting in the exaggerated systemic inflammatory response seen in this condition. On the other hand, endogenous ligand activity may represent a protective adaptive mechanism of the human host to combat the dysregulation of angiogenesis that occurs in PE from reduced VEGF expression, itself secondary to sFlt-1 release as a consequence of placental dysfunction.

Objective 1: In Chapter 3, as monocytes and macrophages are critical for successful pregnancy, we set out to further characterise the monocyte phenotype in PE patients.

1. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood from PE, normal pregnant, and non-pregnant women (Non-P) and analysed using flow cytometry for identifying total PBMCs, total monocytes, and monocyte subpopulations amongst study groups.
2. Expression levels of TLR -2 and -4, CCR2 (a receptor of the monocyte chemo-attractant protein 1), and CCR5 (a receptor of the macrophage inflammatory protein), HLA-DR (a MHC class II cell surface receptor), and TIE2 (a receptor of angiopoietin), were investigated on monocytes and their subpopulations from PE, NP, and Non-P.

Objective 2: In Chapter 4, we aim to identify which of the endogenous ligands of TLR4 (namely: fibrinogen, fibronectin, heparan sulphate, and hyaluronan) may play a functional role in the pathogenesis of PE.

1. Monocytes were cultured from PE, NP, and Non-P and treated with LPS (TLR4 ligand), PDG (TLR2 ligand), and endogenous ligands.
2. The inflammatory cytokine profiles were measured in culture mediums of all three groups by using a cytometric bead array.

Objective 3: In Chapters 5 and 6, we seek to identify whether the inflammatory PE monocytes lead to endothelial cell activation and inflammation. We aim to investigate the inflammatory responses from monocytes and HUVEC co-culture models from PE women, and compare those responses to NP women. Also, we determine whether TLR4 endogenous and exogenous ligands contribute to the stimulation and elaboration by monocytes of pro-inflammatory cytokines, which may then promote expression of cell adhesion molecule (VCAM-1) by endothelial cells and disturb their release of pro-angiogenic (VEGF) and anti-angiogenic (sFlt-1), as mechanisms of the endothelial damage that epitomises PE. Finally, we seek to determine the role of PE/NP monocytes on endothelial cell viability and proliferation.

1. HUVECs were co-cultured with PE or NP monocytes, and then treated with LPS, PDG, fibrinogen, and HMGB1.
2. The inflammatory cytokine and chemokine profiles were measured in culture mediums of mono- and co-cultures of normal and PE-complicated pregnancies using a cytometric bead array.
3. VEGF and VCAM-1 were measured in culture mediums of mono- and co-cultures of normal and PE-complicated pregnancies using a cytometric bead array.
4. sFlt-1was measured in culture mediums of mono- and co-cultures of normal and PE-complicated pregnancies using Enzyme-Linked Immunosorbent Assay (ELISA).
5. Annexin V detection assay and BrdU labelling were carried out to study endothelial cell apoptosis and proliferation, respectively, using flow cytometry.

Objective 4: In Chapter 7, this study also attempts to determine the plasma levels of endogenous ligands, namely fibrinogen, fibronectin, heparan sulphate, HMGB1, and hyaluronan, in PE women; these levels are compared with the levels in both NP and Non-P women.

1. Plasma levels of endogenous ligands from three study groups were measured using ELISA.
2. Placental expression of fibrinogen was detected in the placentae of PE and NP women using immunohistochemistry.

Chapter 2: Methodology

2.1 Materials

2.1.1 Ethical approval

Ethical approval for this study was received from the South Yorkshire Research Ethics Committee (09/H1310/12). A detailed information sheet was given to all study participants and written informed consent was obtained from them.

2.1.2 Health and safety

Before starting the laboratory work, the requirements and precautions requested by the Control of Substances Hazardous to Health (COSHH) regulations were applied. Blood, placenta, and umbilical cord tissues were handled according to the health and safety guidelines of the University of Sheffield. Laboratory coats and gloves were worn during laboratory work.

2.1.3 Subjects

In this study, three groups of women were recruited.

Women with PE (PE)

Women with established PE at < 34 weeks gestation, diagnosed by the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP) (2004) were recruited to the study. The patients were approached in the Day Assessment Unit of Jessop Wing Hospital when the diagnosis was confirmed.

Inclusion criteria

1. Maternal age between 18-40 years.
2. Systolic blood pressure equal to or higher than 140mmHg, or diastolic blood pressure equal to or higher than 90mmHg after twenty weeks of gestation in women with previously normal blood pressure.

3. Proteinuria equal to or more than 0.3g protein in 24 hour urine collection which usually corresponds to +1 or greater in a urine dipstick test.

Exclusion criteria

1. Women aged under 18 or over 40 years.
2. Women with high systolic or diastolic blood pressure but without proteinuria.
3. Women with previous history of chronic hypertension.
4. Women with a urinary tract infection.
5. Women with a history of systemic infection, inflammatory, renal, or cardiovascular disorders such as rheumatoid arthritis and atherosclerosis.
6. Any other pregnancy complications, for example, gestational diabetes, antiphospholipid syndrome, severe IUGR, or recurrent miscarriage.

Normotensive pregnant women (NP)

Healthy normotensive pregnant controls were recruited from women attending the routine antenatal clinics at the Jessop Wing.

Inclusion criteria

1. Maternal age between 18-40 years
2. Normal blood pressure with systolic blood pressure less than 140mmHg and diastolic blood pressure less than 90mmHg.
3. No proteinuria on urine dipstick test.

Exclusion criteria

1. Women aged under 18 or over 40 years.
2. Women with a history of PE in previous pregnancies.
3. Women with a urinary tract infection.
4. Women with a history of systemic infection, inflammatory, renal, or cardiovascular disorders such as rheumatoid arthritis and atherosclerosis.
5. Any other pregnancy complications, for example, gestational diabetes, antiphospholipid syndrome, severe IUGR, and recurrent miscarriage.

Non-pregnant women (Non-P)

Healthy non-pregnant female volunteers were also studied to determine baseline non-gestational levels of the study proteins of interest.

Inclusion criteria

1. Women aged between 18-40 years
2. Normal blood pressure with systolic blood pressure less than 120mmHg and diastolic blood pressure less than 80mmHg.
3. Normal regular menstrual cycles.

Exclusion criteria

1. Women aged under 18 or over 40 years.
2. Women on hormonal contraception.
3. Women with a urinary tract infection.
4. Women with a history of systemic infection, inflammatory, renal, or cardiovascular disorders such as rheumatoid arthritis and atherosclerosis.

2.1.4 Samples

Blood

Following the consent obtained from the study groups, an additional 12ml of fresh blood was collected from each subject at the time of a scheduled blood. Each sample was collected into a tube containing EDTA which prevents clotting of the blood. Blood so obtained was then immediately taken to the research laboratories at the Academic Unit of Obstetrics and Gynaecology at the Jessop Wing for isolation of monocytes, storage of aliquotted plasma samples, and further laboratory work.

Placenta

The placenta was taken from the patients who have previously given consent immediately after normal vaginal delivery or caesarean section. Two or three pieces were cut around the umbilical cord from the foetal part down to the maternal part. These pieces were immediately soaked and fixed for 24-48 hours in 10% formalin; and automatically wax embedded for immunohistochemistry staining.

Human umbilical cords

Consent was obtained from ten normal pregnant women prior to delivery in the labour ward at Jessop Wing Hospital. Then the umbilical cord was collected in a sterile container with 100ml of cord collection medium [(5ml of penicillin-streptomycin solution, 5ml of amphotericin, and 6ml of sodium bicarbonate (7.5%) were added to 500ml of 1X MEM] (Cheung, 2007). Note: fresh medium was prepared for each cord collection). This was then stored at 4°C until used.

2.1.5 Sample size calculation

For the sample size calculation, we used the differences in the expression levels of TLR4 and TLR2 on monocytes as the main outcome in this study. Using the preliminary flow cytometry data arising from the present case control study to calculate an appropriate sample size, GraphPad StatMate software, was used. The mean percentage difference in TLR4 on monocytes between PE and normal pregnancy was 21.17% with a mean standard deviation (SD) of 11.53. The sample size of 12 in each group has a 99% power to detect a difference between means of 21.17 with a significant level α of 0.05 (two-tailed). The mean percentage difference in TLR4 on monocytes between normal pregnancy and non-pregnancy was 26.12% with a mean SD of 10.45. The sample size of 7 in each group has a 99% power to detect a difference between means of 26.12 with a significant level α of 0.05 (two-tailed). The mean percentage difference in TLR2 on monocytes between pre-e-clampsia and normal pregnancy was 14.36% with a mean SD of 7.81. The sample size of 12 in each group has a 99% power to detect a difference between means of 14.36 with a significant level α of 0.05 (two-tailed). The mean percentage difference in TLR2 on monocytes between normal pregnancy and non-pregnancy was 11.36% with a mean SD of 5.59. The sample size of 10 in each group has a 99% power to detect a difference between means of 11.36 with a significant level α of 0.05 (two-tailed). We anticipated that this sample size would also permit a similar investigation of CCR2, CCR5, TIE2, HLA-DR, endogenous ligands, chemokines and cytokines (although different analytical approaches were taken for individual experiments [see relevant chapters]).

2.1.6 Laboratory equipment

Measurement equipment

Different sizes of pipettes (200µl-1000µl, 50µl-200µl, and 2µl-20µl) were used. 5ml, 10ml and 20ml pipettes were used for media preparation and to change the media from tissue culture flasks. 15ml and 50ml Falcon tubes were used in cell isolation. Ported IV cannula 14G-orange (SP services, catalogue number: IN/223P), injection bungs (Vygon, catalogue number: 880.5) and plastic tubing clamps (VWR, catalogue number: 229-0332) were used in HUVECs isolation.

Centrifuges

A centrifuge (MSE Mistral 2000, Calserve Ltd) was used in the cell separation process. Microcentrifuge for Eppendorf tubes (Sigma, Type 1-14, Germany) was used in antibody staining.

Tissue culture laboratory

A well-cleaned and sterilized tissue culture laboratory on the fourth floor of the Jessop Wing Hospital was fully equipped with two cell culture hoods, a light microscope, refrigerator, water bath and two incubators. Cell culture hoods (Walker Safety Cabinets Ltd, Derbyshire, UK) were used to prevent cell contamination during the isolation of peripheral blood monocytes and human vascular endothelial cells (HUVEC) and co-culture experiments. Incubator (Thermo Scientific, UK) was used to incubate cells at 37°C with 5% CO_2. A syringe filter unit (0.02mm; filtrospur) (Sarstedt, catalogue number: 83.1826.001) was used to filter the culture media.

Microscopes

A light microscope was used for cell counting (VWR International Belgium Microscope) with ×10 magnification power. For microscopic observation and photographing of immunostaining slides, an Olympus CKX41SF (Olympus Corporation, Tokyo, Japan) microscope connected to an Olympus digital camera was used.

Storage equipment

A laboratory refrigerator at 2-4°C was used for the storage of antibodies and ELISA kits. Freezers at -20°C or -80°C were used to store plasma samples, supernatants and aliquots of TLR-2 and -4 ligands.

2.1.7 Reagent and media

Reagents and media, which were of the highest quality available, were used for peripheral blood mononuclear blood cells (PBMCs) and human vascular endothelial cells (HUVECs) isolation and cultures. Hank's balanced solution (HBSS) without Phenol red, Ca^{2+} or Mg^{2+} and foetal bovine serum (FBS) were both purchased from Lonza with catalogue numbers: BE10-547F and DE14-80 respectively. Ficoll-Paque supplied by GE Healthcare (catalogue number: 17-1440-02) was used in the monocytes isolation experiment. The following were also used:

- Phosphate buffered saline (PBS), supplied by Sigma (catalogue number: P4417)
- Stain buffer purchased from BD Bioscience (catalogue number: 554656)
- Penicillin-streptomycin and L-Glutamine, supplied by Sigma (catalogue numbers: P4333 and G3126 respectively)
- Amphotericin solution obtained from VWR (catalogue number: AMREK721-20)
- MEM 500ml and HEPES buffer 1M 100ml, both purchased from Lonza (catalogue numbers: BE12-611F and BE17-737 respectively)
- M199 10X solution, 500ml supplied by Fisher (catalogue number: VX21180021)
- Gelatin from porcine skin, 100g, heparin Sodium Salt, collagenase Type IV, new-born calf serum and trypsin-EDTA, all supplied by Sigma (catalogue numbers: G1890, H3149, C5138, N4637, T4299 respectively)
- Endothelial cell growth supplement, 50mg purchased from Tebu Bio (catalogue number: 1003)

Bacterial and endogenous ligands of TLR -2 and -4 used for the stimulations of PBMCs and HUVECs (mono- and co-cultures)

Lipopolysaccharides (rough strains) from *Escherichia coli EH 100*

Supplier: Sigma-Aldrich, UK

Catalogue number: L9641

Size: 5mg

Dilution and storage: 5ml of sterile balanced salt solution was added to whole powder in the vial, then aliquotted in small tubes and stored at -20°C.

Working dilution: 100ng/ml of culture media

Peptidoglycans from *staphylococcus*

Supplier: Sigma-Aldrich, UK

Catalogue number: 77140

Size: 10mg

Dilution and storage: 10ml of sterile balanced salt solution was added to whole powder, then aliquotted in small tubes and stored at -20°C.

Working dilution: 5µg/ml in culture media

Fibrinogen from human plasma

Supplier: Sigma-Aldrich

Catalogue number: F3879

Size: 250mg

Dilution: The fibrinogen was layered on top of pre-warmed 0.9% saline, and then fibrinogen-saline solution was gently agitated until dissolved and filtered with a 0.2µm filter. After sterile filtration, fibrinogen was aliquotted and stored at -20°C.

Working dilution: 2mg/ml in culture media

Fibronectin from human serum

Supplier: BD Biosciences, UK

Catalogue number: 354008

Size: 1mg

Dilution and storage: 1ml of sterile distilled water was added to whole powder in the vial, then aliquotted in small tubes and stored at -20°C.

Working dilution: 5µg/ml in culture media.

Heparan Sulfate Proteoglycan basement membrane

Supplier: Sigma-Aldrich, UK

Catalogue number: H4777

Size: 1mg

Dilution and storage: 1ml of sterile balanced salt solution was added to whole powder, then aliquotted in small tubes and stored at -20°C.

Working dilution: 10µg/ml in culture media

Select-Hyaluronan from molecular mass: 125-175kDa

Supplier: Sigma-Aldrich, UK

Catalogue number: S0201

Size: 1mg

Dilution and storage: 1ml of sterile balanced salt solution was added to whole powder, then aliquotted in small tubes and stored at -20°C.

Working dilution: 100µg/ml in culture media

Recombinant human HMGB1

Supplier: R and D Systems, UK

Catalogue number: 1690-HM-025

Size: 25µg

Dilution and storage: 950µl of sterile PBS was added to 50µl stock solution of HMGB1 then aliquotted in small tubes and stored at -80°C.

Working dilution: 0.7µg/ml in culture media

Monoclonal antibodies used for Flow Cytometry

Anti-human CD14 conjugated with Alexa Flour 700

Supplier: BioLegend, UK

Catalogue number: 325614

Clone number: HCD14

Format: Alexa Flour 700

Size: 100µg

Isotype: Mouse IgG1, κ

Storage: 2-8°C in the dark

Anti-human CD16 conjugated with e Fluor 450

Supplier: eBioscience, UK

Catalogue number: 48-0168

Clone number: CB16

Format: e Fluor 450

Size: 100 tests

Isotype: Mouse IgG1, kappa

Storage: 2-8°C in the dark

Anti-human CD146 conjugated with Phycoerythrin / Cy7

Supplier: BioLegend, UK

Catalogue number: 342010

Clone number: SHM-57

Format: Phycoerythrin / Cy7

Size: 100 tests

Isotype: Mouse IgG2a, κ

Storage: 2-8°C in the dark

Anti-human TLR4 (CD284) conjugated with Phycoerythrin

Supplier: eBioscience, UK

Catalogue number: 12-9917-42

Clone number: HTA125

Format: Phycoerythrin

Size: 100 tests

Isotype: Mouse IgG2a, kappa

Storage: 2-8°C in the dark

Anti-human TLR2 (CD282) conjugated with FITC

Supplier: eBioscience, UK

Catalogue number: 11-9922

Clone number: TL2.1

Format: FITC

Size: 100 tests

Isotype: Mouse IgG2a, kappa

Storage: 2-8°C in the dark

Anti-human HLA-DR (MHCII) conjugated with PerCP-Cy 5.5

Supplier: BD Bioscience

Catalogue number: 552764

Clone number: G46-6

Format: PerCP-Cy5.5

Size: 50 Tests

Isotype: Mouse IgG2a, κ

Storage: 2-8°C in the dark

Anti-human TIE2 conjugated with Allophycocyanin (APC)

Supplier: R & D Systems

Catalogue number: FAB3131A

Clone number: 83715

Format: APC

Size: 100 tests

Isotype: Mouse IgG1

Storage: 2-8°C in the dark

Anti-human CCR2 conjugated with Phycoerythrin

Supplier: R & D Systems

Catalogue number: FAB151P

Clone number: 48607

Format: Phycoerythrin

Size: 100 tests

Isotype: mouse IgG2B

Storage: 2-8°C in the dark

Anti-human CCR5 (CD195) conjugated with FITC

Supplier: BioLegend, UK

Catalogue number: 313706

Clone number: HEK/1/85a

Format: FITC

Size: 100 tests

Isotype: Rat IgG2a, κ

Storage: 2-8°C in the dark

Mouse IgG2a K isotype control conjugated with Phycoerythrin

Supplier: eBioscience, UK

Catalogue number: 12-4724

Clone number: eBM2a

Format: Phycoerythrin

Size: 100 tests

Isotype: Mouse IgG2a, kappa

Storage: 2-8°C in the dark

Mouse IgG2a K isotype control conjugated with FITC

Supplier: eBioscience, UK

Catalogue number: 11-4724

Clone number: eBM2a

Format: FITC

Size: 100 tests

Isotype: Mouse IgG2a, kappa

Storage: 2-8°C in the dark

Mouse IgG2a, k isotype control conjugated with PerCP-Cy 5.5

Supplier: BD Bioscience

Catalogue number: 558020

Clone number: MOPC-173

Format: PerCP-Cy 5.5

Size: 50 tests

Isotype: Mouse IgG_{2a}, κ

Storage: 2-8°C in the dark

Mouse IgG1, κ isotype control conjugated with Allophycocyanin (APC)

Supplier: BD Bioscience

Catalogue number: 555751

Clone number: MOPC-21

Format: APC

Size: 100 tests

Isotype: Mouse IgG1, κ

Storage: 2-8°C in the dark

Mouse IgG2B isotype control conjugated with Phycoerythrin

Supplier: R & D systems

Catalogue number: IC0041P

Clone number: 133303

Format: Phycoerythrin

Size: 100 tests

Isotype: Mouse IgG2B

Storage: 2-8°C in the dark

Antibodies used in the immunohistochemistry

Rabbit anti-human fibrinogen polyclonal antibody

Supplier: Dako UK Ltd

Code number: A0080

Volume: 2ml

Specificity: The antibody reacts with human fibrinogen as well as with the fibrinogen fragments D and E.

Storage: at 2-8°C

Rabbit Immunoglobulin Fraction (Solid-Phase Absorbed)

Supplier: Dako UK Ltd

Code number: X0936

Volume: 2ml

Storage: at 2-8°C

2.2 Methods

2.2.1 Cells

2.2.1.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh venous blood on the top of Ficoll-Paque and centrifugation using a protocol previously described (de Almeida *et al.*, 2000, Graziani-Bowering *et al.*, 1997, Murdoch *et al.*, 2007). Ficoll-Paque separation is based on density gradient centrifugation to separate distinct blood cells. It is produced by GE Healthcare company (Amersham Biosciences).

The blood was mixed with Hank's balanced solution, HBSS (without phenol red, Ca^{2+} or Mg^{2+}) enriched with 2% (v/v) foetal bovine serum (FBS) at the rate of 1:1, into 50ml Falcon tube. Then the mixture of blood and HBSS was overlaid slowly onto the top of 20ml Ficoll-Paque solution in the 50ml Falcon tube. The Falcon tube was centrifuged for 20 minutes at 800xg under 4°C. After centrifugation, three layers were visible in the conical tube, from top to bottom: plasma, Ficoll-Paque, and erythrocytes with granulocytes (**Figure 2.1**). The PBMCs layer (seen as a distinct white layer of cells between the upper plasma and lower Ficoll layers) was removed and transferred to a fresh vessel. Then the cells were washed three times by re-suspending the pelleted cells with 15ml of HBSS (without phenol red, Ca^{2+} or Mg^{2+}) enriched with 2% FBS and centrifuged for ten minutes.

The PBMCs were counted using a haemocytometer method by placing a 10µl drop of cell suspension on each side of cover slips which were then positioned on the haemocytometer and the cells were counted in four squares. The mean cell count for each patient approximated to 26 X 10^6cells/ml. These cells were divided into two parts. The first part was processed immediately for flow cytometry to determine monocyte phenotypes, TLR expression, migration, and angiogenic factors. The second part was used for monocyte culturing and stimulation with different ligands of TLR-2 and -4.

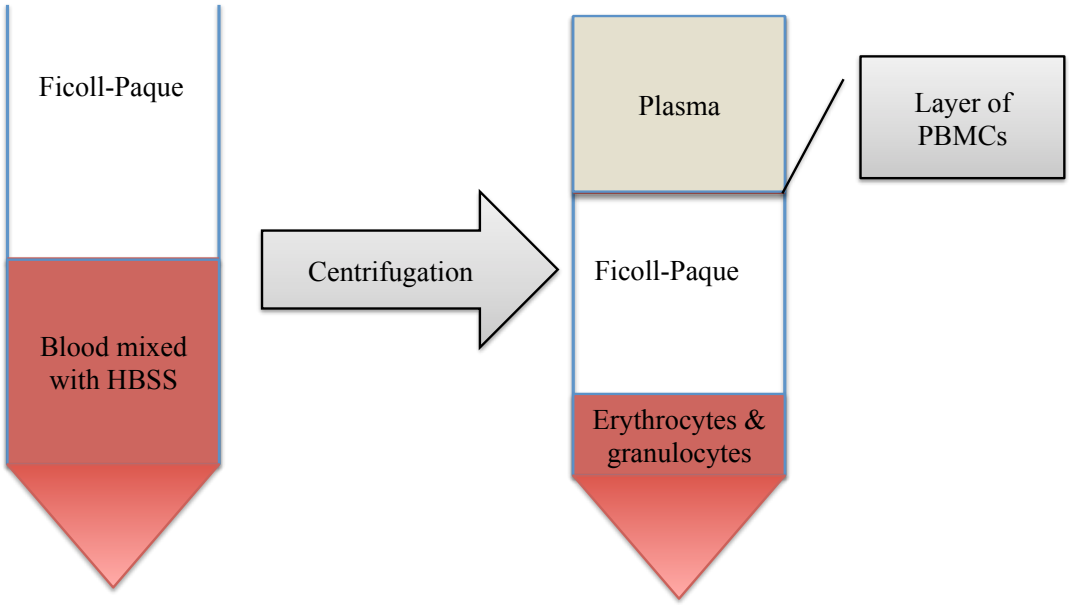

Figure 2.1 Isolation of peripheral blood mononuclear cells (PBMCs)

2.2.1.2 Isolation of monocytes from peripheral blood mononuclear cells

Monocytes from pregnant women (NP or PE) were isolated from PBMCs to be used in co-culture experiments of primary human monocytes and vascular endothelial cells.

The pan monocyte isolation kit (Miltenyi Biotic, Bergisch Gladbach, Germany catalogue number: 130-096-537) was used to isolate monocytes from PBMCs. They were isolated by density gradient centrifugation on top of Ficoll-Paque (as discussed previously page number 66). The pan monocyte isolation kit uses indirect magnetic labelling of non-monocyte cells such as basophils, NK cells, B cells, T cells, and dendritic cells. Thus it depletes the non-monocytic cells and isolates highly pure (recovery > 90%) unlabelled monocytes from human PBMCs (negative selection).

This pan monocyte kit has improved biotin-cocktail antibodies for the enrichment of classical ($CD14^{high}$ $CD16^{-}$), intermediate ($CD14^{high}$ $CD16^{+}$) and non-classical ($CD14^{low}$ $CD16^{+}$) monocytes (Ziegler-Heitbrock *et al.*, 2010). The kit includes a cocktail of biotin-conjugated monoclonal antibodies against antigens which are not expressed by human monocytes (CD3, CD7, CD19, CD56, CD123, and Glycophorin A) and microbeads conjugated to monoclonal anti-biotin antibody.

Purification of monocytes was performed using the pan monocyte kit and enriched by negative selection using the MidiMACS separator system and LS columns (Miltenyi Biotec Bergisch Gladbach, Germany, catalogue numbers: 130-042-302 and 130-042-401 respectively), according to the manufacturer's instructions. In summary, the PBMCs number was determined with haemocytometer and an average of 22 X 10^{6}cells/ml was obtained from 10ml of anti-coagulated fresh peripheral blood from the donors. The cell suspension was centrifuged at 300xg for 10 minutes and the supernatant was aspirated completely. The cell pellet was re-suspended in 60µl of buffer (a solution containing PBS, 0.5% of bovine serum albumin and 2mM EDTA). FCR blocking reagent (20µl) and Biotin antibodies cocktail (20µl) were added in the cell suspension, mixed and incubated for 5 minutes in the refrigerator (2-8°C). 60µl of buffer and then 40µl of microbeads conjugated to monoclonal anti-Biotin antibody were added in cell suspension, mixed and incubated for an additional 10 minutes in the refrigerator (2-8°C). After that, the cells were re-suspended in 500µl of buffer, and magnetic separation was then undertaken. The LS column was placed in the magnetic

field of the MidiMACS separator and rinsed with 3ml of buffer. The cell suspension was then applied onto the column, and the flow-through containing the unlabelled cells was collected. This represented the enriched monocytes. The column was washed with 3ml of buffer three times to collect all unlabelled enriched monocyte cells. The cell suspension was centrifuged at 300xg for 10 minutes and the supernatant was discarded. The cell pellet was re-suspended in 1ml of culture media (DMEM enriched with 5% FBS) and a haemocytometer cell count was carried out. The mean of monocytes isolated from PBMCs was adjusted to 3 X 10^6cells/ml.

2.2.1.3 Isolation of human umbilical vein endothelial cells (HUVECs)

Endothelial cells were isolated from freshly collected umbilical cords using standard published techniques used regularly in the Department of Cardiovascular Science of the Medical School in the University of Sheffield (Baudin *et al.*, 2007, Bonifacino, 1998, Cheung, 2007).

Buffer preparation

M199 Medium

50ml of 10X M199 was diluted in 430ml of distilled water, and 5ml of penicillin-streptomycin (10,000 IU/ml), 5ml L-glutamine and 10ml of sodium bicarbonate, was added.

Complete growth medium

M199 was supplemented with 10% new-born calf serum, 10% foetal bovine serum, 20µg/ml of endothelial growth supplement, and 90µg/ml of heparin.

0.1% (w/v) Collagenase solution

For each umbilical cord 0.02g of collagenase from *Clostridium histolyticum* was dissolved in 20ml of M199. The solution was passed through a syringe filter (gauge is 0.2µm) into a sterile tube. Collagenase solution was prepared fresh or used within 2 days if stored at 4°C.

Gelatin coated flasks

0.2% (w/v) gelatin solution in distilled water was used to coat tissue culture flasks.

Sufficient volume was added to cover the surface and left to coat the flasks for a minimum of 30 minutes before use.

Procedure

The work was done under a Class II hood, all surfaces were wiped with alcohol wipes, and a clean Pyrex dish was used for umbilical cord manipulation. Any damaged umbilical cords with holes/tears or those less than 10cm in length were discarded. The clamped ends and traumatised regions of cord were cut off.

Excess blood was removed by gently massaging the umbilical cord along its length. The umbilical vein was cannulated with a 14G plastic cannula and tightly secured with string. Then the cord was flushed through by perfusing with 30-40ml of medium M199 using a 50ml syringe. This was continued until the vein was free of remaining blood. The free end of the umbilical cord was secured with a surgical clamp and an injection bung was placed over the cannula. 0.1% collagenase solution was injected into the vein using a 20ml syringe and then the cord was incubated at room temperature for 10 minutes (**Figure 2.2)**. The digested layer of endothelial cells was collected into a sterile 50ml tube. The vein was flushed with 10-15ml of medium M199 to collect any partially dislodged cells. The cell suspension was centrifuged at 1000 RPM for 5 minutes at room temperature and the supernatant was carefully removed. Pelleted cells were re-suspended in 5ml of complete growth media and transferred to gelatin-coated 25mm^2 tissue culture flask (Greiner bio-one, catalogue number: 690175). Endothelial cells were then incubated at 37°C, 5% CO_2.

Twenty-four hours after isolation, the medium was removed and the adherent endothelial cell layer was washed once with PBS, to remove any non-adherent cells. Endothelial cells were re-fed with complete growth media and thereafter, fed every two days until they reached confluence. Typically endothelial cells reach confluence within 8-10 days.

At confluence, cells were once washed with PBS and incubated with a small volume (1-2ml) of 0.1% trypsin/0.02% EDTA in PBS, until the cells rounded up and detached. Endothelial cells were collected in a sterile 50ml tube containing 5-10ml of complete growth medium. The flask was washed with an additional 1-2ml of trypsin/EDTA and

was added to the cell suspension. Cells were centrifuged for 5 minutes at 1000 RPM and the supernatant was carefully removed. Endothelial cells were re-suspended in complete growth medium and were split in a 1:3 or 1:4 ratio onto gelatin–coated 75mm^2 tissue culture flasks (Greiner bio-one, catalogue number: 658175) containing 10ml of complete growth media.

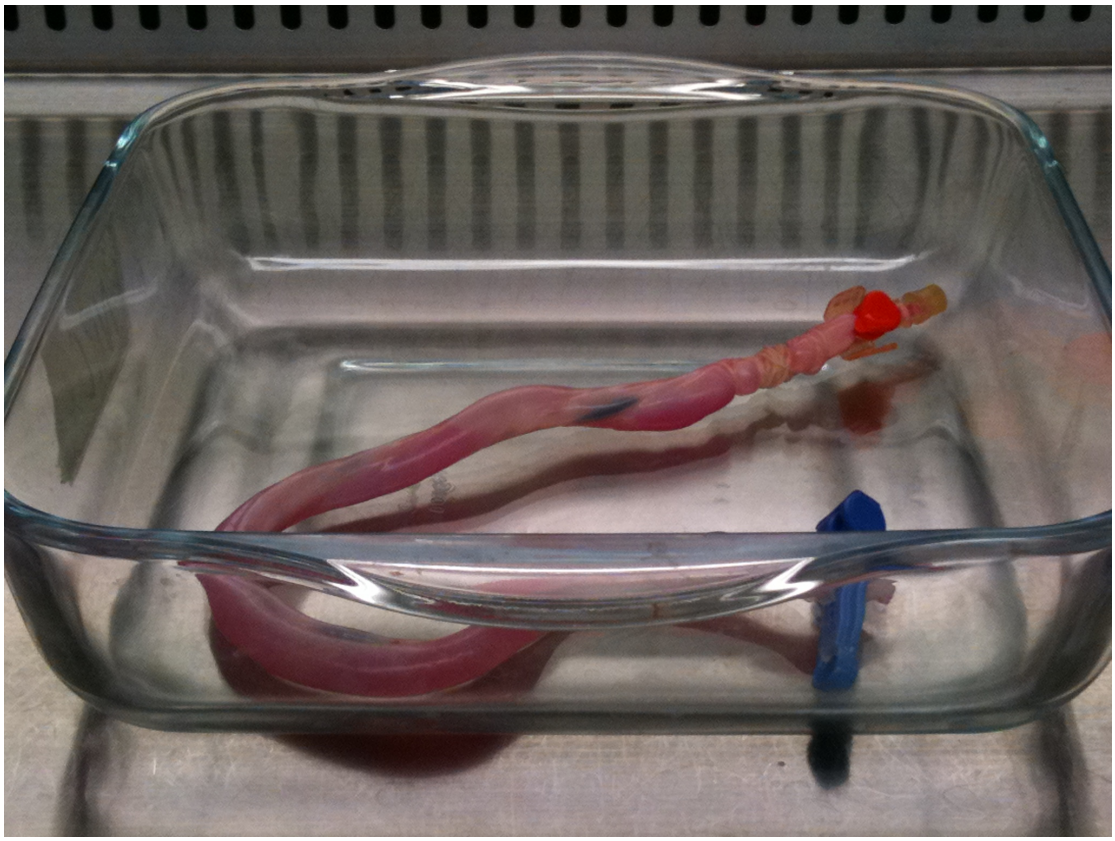

Figure 2.2 Human umbilical cord incubated with 0.1 % collagenase for isolating endothelial cells

2.2.2 Tissue cultures

2.2.2.1 Monocyte culturing (plate-attached method)

The peripheral blood mononuclear cells were isolated (see previously page number 66) and seeded in 12-well tissue culture flat bottom plate (Greiner bio-one, catalogue number: 665180) at a density of 1.8 X 10^6 cells/well. The cells were incubated with medium (IMDM supplemented with 2% FBS, 2mM L-Glutamine and penicillin-streptomycin) at 37°C with 5% CO_2. After two hours of incubation the medium with non-monocytic cells was removed and the monocytes, which adhere to the surface of the plates, were washed twice with cold PBS. The medium was then replaced (IMDM supplemented with 2% FBS, 2mM L-Glutamine and penicillin-streptomycin) for monocyte cells in the tissue culture plate.

The purified monocytes were then treated with 100ng/ml Lipopolysaccharides (rough strains) from *Escherichia coli*, 5µg/ml Peptidoglycan from *Staphylococcus aureus*, 10µg/ml Heparan Sulfate Proteoglycan, 5µg/ml Fibronectin, 2mg/ml Fibrinogen and 100µg/ml select-Hyaluronan of molecular mass: 125-175kDa as described in previous studies (Johnson *et al.*, 2002, Smiley *et al.*, 2001, Yamawaki *et al.*, 2009). The stimulated monocytes were incubated for 24 hours at 37°C with 5% CO_2 and the conditioned medium was collected after this time. The medium was aliquotted into small volumes and frozen at -20°C until analysis was undertaken.

2.2.2.2 Co-culture experimental model of primary human monocytes and HUVECs

Co-culture experiments of primary human monocytes (either isolated from normotensive pregnant or pre-eclamptic women) and endothelial cells (isolated from umbilical cord of end product of healthy pregnant women) were optimized according to previously published studies (Schubert *et al.*, 2008, Ward *et al.*, 2009). Several attempts were made to avoid cell culture contamination: with regular observation of the cells growing in culture, and by changing the media every other day. The precaution was taken of wiping all surfaces with alcohol, along with the regular filtration of media.

The human vascular endothelial cells (HUVECs) were isolated, passaged, and used at 2–5 passages for all experiments as described in page numbers 69-71. Endothelial cells were trypsinised from sub-confluent 75mm^2 tissue culture flask (Greiner bio-one, catalogue number: 658175), centrifuged for 5 minutes at 1000 RPM and supernatants were removed. The pellet was re-suspended in 1ml of culture medium and haemocytometer counting was carried out. HUVECs concentration was adjusted to 4 X 10^6cells/ml. Endothelial cells were seeded onto 0.2% gelatin-coated 12-well tissue culture flat bottom plate (Greiner bio-one, catalogue number: 665180) at a density of 10^5 cells per well. The cells were incubated with 1ml of medium (DMEM supplemented with 10% heat inactivated FBS, 2mM L-Glutamine and penicillin-streptomycin) at 37°C with 5% CO_2 until they reached 70-90% of confluence (usually within 2 - 3 days). The day before the experiment, the cells were washed with 1ml of pre-warmed PBS and cultured with low serum media (DMEM supplemented with 5% heat inactivated FBS without antibiotics) for serum starvation.

The monocytes were isolated from the fresh blood of donors collected in EDTA tubes and separated, using the negative magnetic selection method as described in page number 68, immediately after the blood was drawn. Following 24 hours of serum starvation, HUVECs were washed with 1ml of pre-warmed PBS and low serum media were replaced (DMEM supplemented with 5% heat inactivated FBS without antibiotics), and then monocytes were plated on top of HUVECs at the approximate proportion of 1 monocyte to 5 HUVECs. Co-culture assays were performed in DMEM with 5% heat inactivated FBS without antibiotics media. At the same time, monocytes or HUVECs cells were cultured separately with the equivalent density (mono-cultures).

Thereafter, mono- and co-cultures were treated with 100ng/ml Lipopolysaccharides (rough strains) from *Escherichia coli*, 5µg/ml Peptidoglycan from *Staphylococcus aureus*, 2mg/ml Fibrinogen and 0.7µg/ml HMGB1 as described in previous studies (Smiley *et al.*, 2001, Yu *et al.*, 2006). Then cells were incubated for 24 hours at 37°C with 5% CO_2 and the conditioned medium was collected after this time. The medium was aliquotted into small volumes and frozen at -80°C until analysis was undertaken.

2.2.3 Flow Cytometry

Flow Cytometry was used to identify and quantify peripheral blood monocytes and the subpopulations from different study groups, and to identify expression levels of toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4), human leukocyte class II DR antigens (HLA-DR), CCR2 (a receptor of the monocytes chemo-attractant protein 1), CCR5 (a receptor of the macrophage inflammatory protein), and TIE2 (a receptor of angiopoietin). It was also used to confirm presence of monocytes and endothelial cells in our co-culture experiments model.

2.2.3.1 Basic principles of flow cytometry

Flow Cytometry is a technique used for analysing cells and sub-cellular particles by passing them through a fluid system and laser beam. The absorbed lights and fluorescent intensities are measured and used as an indicator for cell size and granularity. Additionally, if these particles have been labelled with fluorochromes, specific surface proteins are measured by laser excitation of these dyes. Flow cytometry is a combination of fluidics, optics, and software to allow measurements of tiny particles at thousands of events per second. The fluidics system is responsible for the transfer of particles within the sample in solutions from three-dimensional space into a stream of single ordered particles. Optics are fundamental to flow cytometry for revealing light scattering signals and the fluorescent emission of labelled and unlabelled particles. Modern flow cytometers employ many laser light sources for cell excitation. Light is gathered by two lenses known as the forward (FSC) and side (SSC) scatter channels. The FSC provides information about the particle size, while the SSC can be used to gain information about the granular content. A Recent Flow Cytometer utilizes software for data acquisition and analysis. Data produced for analysis is usually displayed with histograms or dot plots. Fluorochromes are dyes that absorb one colour of light at a specified wavelength and re-emit a different colour of light with a longer wavelength. These two procedures are referred as excitation and emission. The aim of a fluorescent probe is to attach to an epitope of interest and then enable its biochemical structures to be measured by flow cytometry. Fluorescent probes are essential to different applications that include recognition of distinct cell populations, cell surface proteins, intracellular organelles and study of apoptosis. At the same time, the

fluorescence needs a negative control to detect where the positivity begins. Modern flow cytometers, including LSRII machines, use multi-colour fluorescence to analyse numerous factors of sample at the same time. However, multi-colour fluorescence brings with it the danger of spectral overlap, which can be avoided by fluorescence compensation (Melamed *et al.*, 1990, Ormerod, 2000, Shapiro, 2003). The basic principles of flow cytometry are depicted in Figure 2.3.

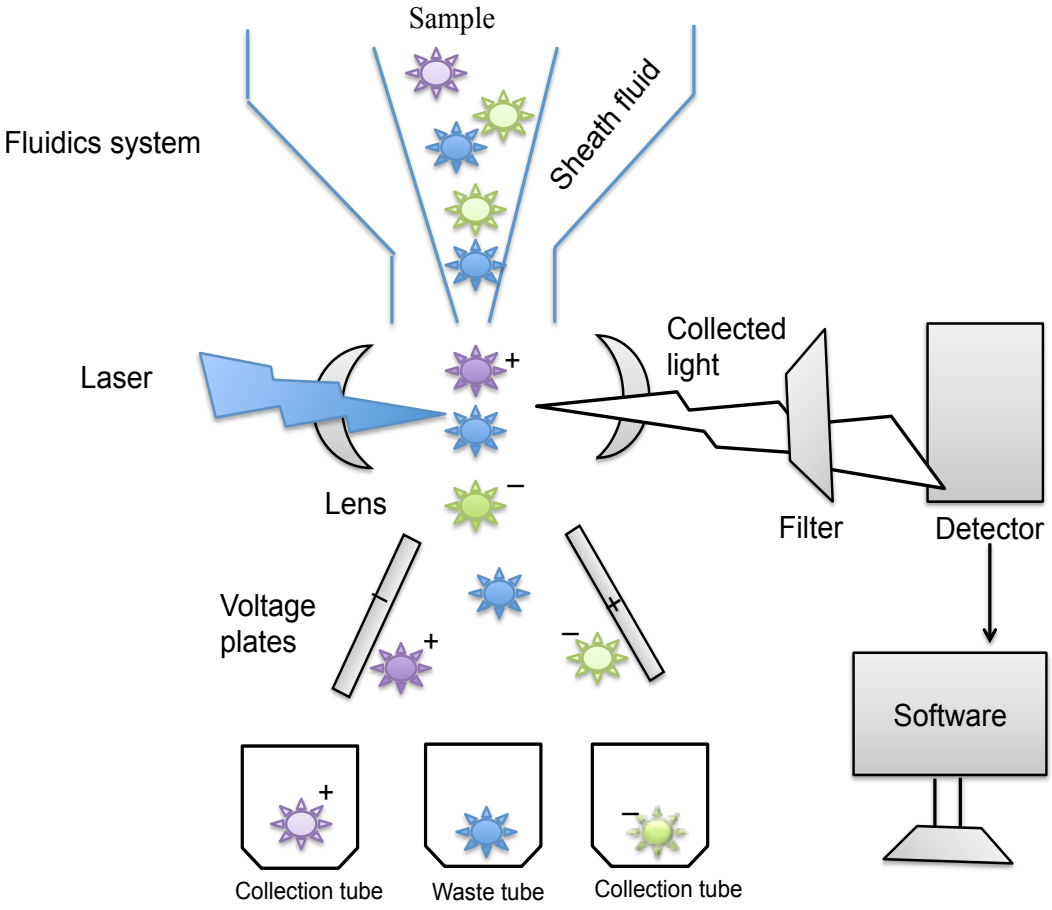

Figure 2.3 Diagram depicting electrostatic cell sorting in the Flow Cytometry operation

2.2.3.2 Peripheral blood mononuclear cells stained with monoclonal antibodies

The first part of the isolated PBMCs (1.3 X 10^7 cells/ml) was used for flow cytometric staining and analysis. The cells were further subdivided into two parts to be employed in separate experiments. One part was used for the determination of TLR2, TLR4, and HLA-DR expression levels. The second part was utilized for identifying CCR2, CCR5, and TIE2 expression levels.

In the first experiment, the cell suspension was equally distributed into four microcentrifuge Eppendorf tubes and blocked with 5µl of anti-human Fc receptor blocking reagent (MACS-Miltenyi Biotic, catalogue number: 1 30-059-901). It was refrigerated for 10 minutes. The cells were then labelled as follows: The first tube had unstained cells only. The second one had isotype negative controls matched for monoclonal antibodies (mouse IgG2a-κ conjugated to FITC, IgG2a conjugated to PE, and IgG2a-k conjugated to PerCP-Cy 5.5) to prevent non-specific bindings. The third Eppendorf tube had PBMCs stained with all monoclonal antibodies (5µl of CD14 conjugated to Alexa Fluor 700 antibody, 5µl of CD16 conjugated to e Flour450 antibody, 20µl of TLR2 conjugated to FITC antibody, 5µl of TLR4 conjugated to PE antibody, and 5µl of HLA-DR conjugated to PerCP-Cy 5.5 antibody) as mentioned in the company's sheets. And the last Eppendorf tube had cells mixed with 5µl of CD14 conjugated to Alexa Fluor 700 antibody, 5µl of CD16 conjugated to e Flour450 antibody and isotype negative controls matched for TLR2, TLR4, and HLA-DR monoclonal antibodies (the fluorescence minus one method).

In the second experiment, four microcentrifuge Eppendorf tubes were prepared and the cell suspension was equally disturbed and blocked with 5µl of anti-human Fc receptor blocking reagent as mentioned above. Afterwards the tubes were stained as follows: The first Eppendorf tube had unstained cells only. The second tube had isotype negative controls matched for monoclonal antibodies (mouse IgG2a-κ conjugated to FITC, IgG2B isotype conjugated to PE, and IgG1, κ conjugated to APC) to exclude non-specific bindings. The third Eppendorf tube with PBMCs stained by all monoclonal antibodies (5µl of CD14 conjugated to, Alexa Fluor 700 antibody, 5µl of CD16 conjugated to e Flour450 antibody, 20µl of CCR5 conjugated to FITC antibody, 20µl of CCR2 conjugated to PE antibody, and 20µl of TIE2 conjugated to APC antibody) as

mentioned in company's sheets. And fourth tube had cells mixed with 5µl of CD14 conjugated to Alexa Fluor 700 antibody, 5µl of CD16 conjugated to e Flour 450 antibody and isotype negative controls matched for CCR2, CCR5, and TIE2 monoclonal antibodies (the fluorescence minus one method).

All Eppendorf tubes were incubated in a refrigerator for 30 minutes. Cells were then washed twice by 1ml of cold BD flow cytometry stain buffer (FSB, catalogue no: 554656) and centrifuged at 5000 RPM for 2 minutes each time. The supernatants were removed completely and cells were re-suspended in 500µl of FBS. They were then sent immediately for FACS analysis.

2.2.3.3 Flow cytometry was used to confirm presence of monocytes and endothelial cells in co-culture experiments

The Flow Cytometry staining and analysis was repeated three times from HUVECs (isolated from three different umbilical cords of healthy pregnant women) and monocytes (isolated from peripheral blood of three different healthy pregnant women). The CD14 surface marker is expressed mainly by 90% of peripheral monocytes (Ziegler-Heitbrock and Ulevitch, 1993). Therefore it was used as a specific marker to identify monocytes in our co-culture experiments. And the CD146 surface marker was used particularly to label endothelial cells as mentioned in literature (Bardin *et al.*, 2001).

HUVECs, with or without monocytes, (incubated for 24 hours at 37°C with 5% CO_2) were harvested from 12 tissue culture plates by using cell dissociation solution obtained from Sigma (catalogue number: C5914) as directed by the company sheet. Briefly the cells were washed two times with warmed PBS without calcium and magnesium, gently rocked for 30 seconds and the buffer was removed. Then 750µl/well of cell dissociation solution was added and incubated for 15 minutes at 37°C with 5% CO_2. Complete growth media was then added to the cells and pipetted repeatedly to dissociate clumps. The cell suspension was centrifuged at 1000 RBM for 10 minutes at 4°C, and the supernatants were discarded.

Each of the cell pellets (HUVECs, monocytes, or monocytes with HUVECs) was then re-suspended in the 1ml HBSS and distributed equally in four microcentrifuge Eppendorf tubes for monoclonal antibodies staining. Initially cells were blocked with 5µl of anti-human Fc receptor blocking reagent and refrigerated for 10 minutes. Then the cells were mixed with monoclonal antibodies as follows: The first Eppendorf tube had unstained cells only, the second one had cells stained with 5µl Alexa Fluor 700 conjugated antibody against CD14, the third tube had cells stained with 20µl PE-cy7 conjugated antibody against CD146, and the last tube had cells stained with both monoclonal antibodies. All Eppendorf tubes were incubated in a refrigerator for 30 minutes. Cells were then washed twice by 1ml of cold BD flow cytometry stain buffer (FSB) and centrifuged at 5000 RPM for 2 minutes each time. Cells were aliquotted in 500µl of FBS and sent immediately for flow cytometry.

2.2.3.4 Flow cytometric analysis

Analysis was done using a multi-laser and detector analyser (LSR II Flow Cytometer from BD Bioscience, UK) at the Flow Cytometry Department in the core facility of the University of Sheffield Medical School (**Figure 2.4**). A general description of the analysis procedure is provided below.

The LSR II machine was switched on and the connected computer started up. The BD FACS Diva Software 6.0 was selected and a new folder was created every month and two experiments were added per patient. One was for the determination of TLR2, TLR4, and HLA-DR, and another experiment was for identifying CCR2, CCR5, and TIE2 expression levels in total monocytes and its sub-populations. In each experiment four tubes were created as follows: unstained tube, isotype tube, all monoclonal antibodies, and minus-one tube.

Auto compensation was calculated using anti-mouse compensation bead sets obtained from BD Bioscience (catalogue number: 552843), and was performed as directed by the manufacturer. In summary, the BD flow cytometry stain buffer was aliquotted into 100µl in the microcentrifuge Eppendorf tubes and one drop from each compensation bead was added. Then they were stained with monoclonal antibodies separately, incubated for 30 minutes in a refrigerator and washed twice by 1ml of cold FBS and

centrifuged at 5000 RBM for 2 minutes. Then they were re-suspended in 500µl of FBS and processed for FACS analysis. In every experiment before the cells were acquired, the compensation tube was created and calculated according to the required fluorochromes.

After compensation had been applied, the cells in the unstained tube were loaded and the different blood cell populations were identified in the forward scatter (FSC) and side scatter (SSC) dot plots. Fluorescent voltages were adjusted appropriately for all the participants in this study. A gate-specific for the monocytes population was created and monocyte sub-populations were identified according to CD14 and CD16 surface markers as defined previously by Ziegler-Heitbrock (Ziegler-Heitbrock, 1996, Ziegler-Heitbrock, 2007) **(Figure 2.5)**. A total of 10,000 events for monocyte gates were acquired in the acquisition dashboard. All the samples in the tubes then were loaded and data acquired and recorded accordingly. In monocytes-HUVEC co-culture experiments, the cell populations were identified in the forward scatter (FSC) and side scatter (SSC) dot plots. A gate-specific for the monocytes and HUVEC populations was created and a total of 10,000 events were acquired. After loading and recording all tubes, the machine was cleaned with FACS Clean, FACS Rinse and dH_2O.

Several analytical methods can be used to quantify the samples by BD Bioscience FACS Diva software 6.0. However, Median Fluorescence Intensity (MFI) is the more robust and reliable than the mean (Huber and Ronchetti, 2009). Thus, the MFI was preferred to identify the expression of TLR2, TLR4, HLA-DR, CCR2, CCR5, and TIE2 in monocytes subpopulations. MFI was calculated by the following formula: MFI = median of certain fluorochrome from all antibody tube/median of equivalent fluorochrome from minus-one tube.

Figure 2.4 LSR II Flow Cytometer from BD Bioscience, UK

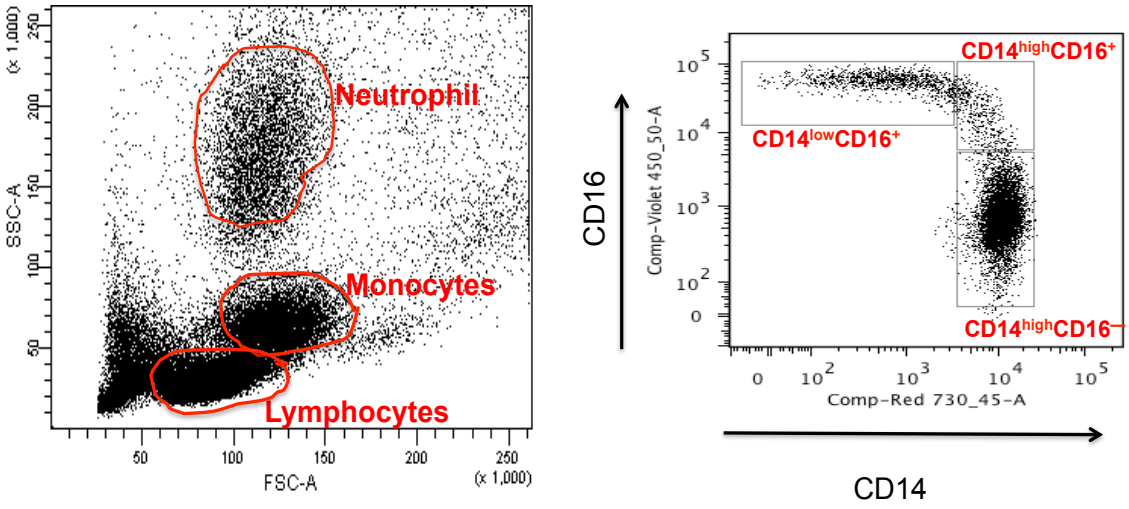

Figure 2.5 Peripheral blood mononuclear cells from non-pregnant women

A gate-specific for the monocytes population was created and monocyte sub-populations were identified according to CD14 and CD16 surface markers as defined previously by Ziegler-Heitbrock.

2.2.4 Cytometric bead array

The cytometric bead array technique was used to measure the inflammatory cytokines: Interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) and the anti-inflammatory cytokines; Interleukin-10 (IL-10) within monocyte conditioned medium (plate-attached method). It was also used to measure the inflammatory cytokines: Interleukin-1β (IL-1β) and 1L-6, the anti-inflammatory cytokines: IL-10, the chemokines: Interleukin-8 (IL-8) and monocyte chemo-attractant protein (MCP-1), the adhesion molecule: soluble vascular adhesion molecule (VCAM) and the angiogenic factor: vascular endothelial growth factor (VEGF) in supernatant medium collected from monocytes-HUVECs mono- or co-cultures.

BD Cytometric bead array (CBA) is a method used to measure multiple soluble proteins such as cytokines in small volumes (<50µl). BD-CBA kits contain suspended beads bound with antibodies that capture soluble protein of interest in the sample (**Figure 2.6**). These beads are incubated with the sample and then incubated further in the presence of a fluorescent antibody conjugated with the phycoeryithrin (PE) fluorochrome. Additionally, a set of standards is prepared that enables protein measuring between 10-2500 pg/ml. Thereafter, soluble analytes are analysed by detection of a fluorescence-based, Flow Cytometric technique (Morgan *et al.*, 2004, Varro *et al.*, 2007).

Supernatants collected from mono- and co-culture experiments of primary human monocytes with HUVECs were run undiluted for IL-1B, IL-10, VCAM-1, and VEGF. However, the IL-6, IL-8 and MCP-1 cytokines were above standard levels for the undiluted aliquot. Subsequently, serial dilutions (1:10 up to 1:200) were attempted for optimising the desired dilution. Then either 1:50 or 1:100 dilutions were applied for the samples and each sample was multiplied according to its dilution factor before analysis was taken.

This assay was performed following the instructions of the master buffer kit manual (BD Bioscience, UK, catalogue number: 558683) at the Flow Cytometry Core Facility at the Sheffield University Medical School with the help of Susan Newton (chief technician in the Flow Cytometry lab). The BD FACS Array flow cytometry machine and FCAP Array software were used for analysis.

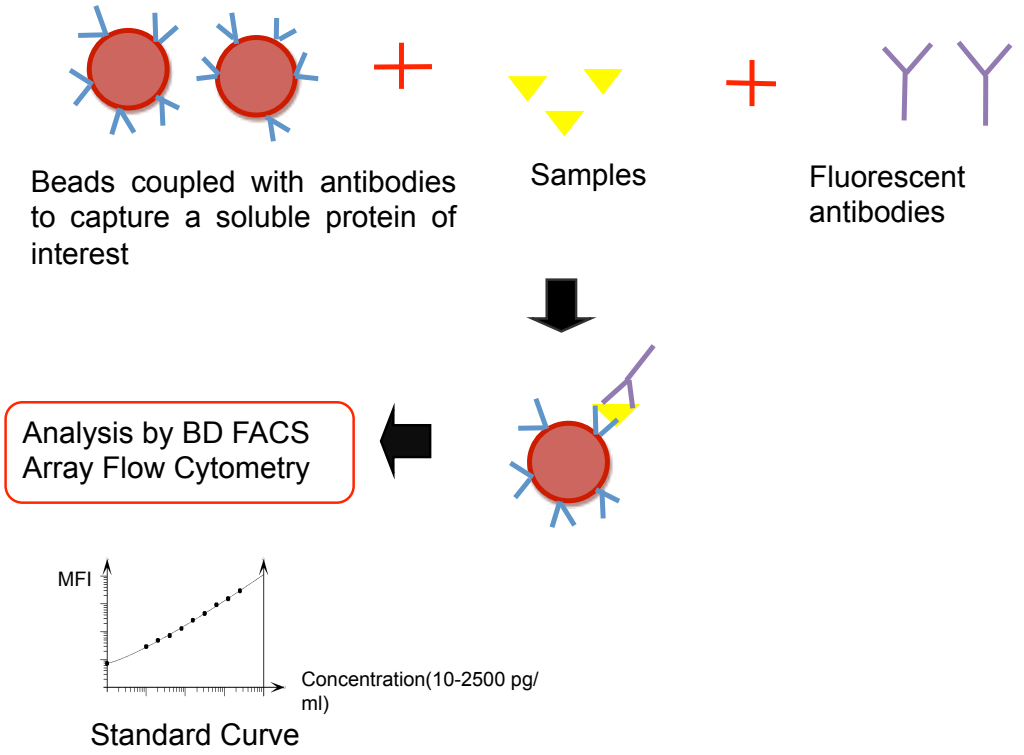

Figure 2.6 Overview of the Cytometric Bead Array procedure

The CBA beads are incubated with the sample and fluorescent antibodies, a set of standards is prepared that enables protein measuring between 10 and 2500 pg/ml. Soluble analytes are analysed by FACS Array Flow Cytometry. MFI: median fluorescence intensity.

2.2.5 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was used to measure the plasma levels of endogenous ligands of TLR2 and TLR4 in the three groups of subjects we studied. Plasma was first collected from the top layer of the blood centrifuged on top of Ficoll-Paque, as seen in **Figure 2.1**. The plasma from these subjects was then frozen at −80°C (the subjects were pre-eclamptic pregnant patients, pregnant women with no issues, and non-pregnant women). Additionally, ELISA was used to measure the sFlt-1 levels in the supernatant collected from monocyte-HUVEC co-culture experiments.

Enzyme-linked immunosorbent assay, also known as enzyme immune assay (EIA), is one of a number of biochemical techniques of immunoassay used to identify the existence of a substance in fluids like urine, plasma, and culture supernatants. The main function of an ELISA is to involve an enzyme to determine the attachment of an antigen-antibody complex. In ELISA, the multiple liquid reactions are transmitted to colour signals that quantify the substance by reading optical signals. The main advantage of the ELISA assay is that it is a quantitative and a very specific method (Lequin, 2005).

In our study, a sandwich ELISA was used as follows: First, it was used to measure the amount of antigen (either endogenous ligands—fibrinogen, fibronectin, heparan sulfate, and HMGB1—in plasma, or sFlt-1 in a monocyte-HUVEC medium) between two specified antibody layers; then it was used to capture the antibody absorbed onto micro-wells, and then the biotin-streptavidin detection antibody. The proteins in standards and samples are first bound to a pre-coated antibody absorbed onto the micro-wells. A biotin-conjugated antibody is then added, and binds to the protein captured by the first antibody. Following incubation, the unbound biotin-conjugated antibody is removed during a wash step. A streptavidin HRP is added, and binds to the biotin-conjugated antibody. All unbound material is then washed away, and a peroxidase enzyme substrate is added. The colour development is stopped and the intensity of the colour is measured.

A competitive ELISA assay was used to measure the hyaluronan (HA). In this assay, the colourimetric signal was inversely proportional to the amount of HA present in the

sample. The samples to be assayed were first mixed with the HA detector, and then added to the HA ELISA plate for competitive binding. An enzyme-linked antibody and colourimetric detection were used to detect the HA detector bound to the plate.

Fibrinogen assay

(Please see assay description in appendix 3)

The human fibrinogen ELISA kit provided by Abnova, Germany (catalogue number: KA0475) was used to detect fibrinogen in the plasma. The plasma samples were diluted (1:2000) in mixed diluent, and the assay was carried out according to the manufacturer's sheet. Intra-assay and inter-assay co-efficients of variation were 5.1% and 7.5% respectively, both according to the manufacturer's information. The minimum detectable concentration of fibrinogen is typically 0.16µg/ml.

Fibronectin assay

(Please see assay description in appendix 3)

An enzyme-linked immunosorbent assay for quantitative detection of human fibronectin provided by Bender MedSystems, Vienna, Austria (catalogue number: BMS2028) was used to measure plasma fibronectin. The plasma samples were diluted (1:10,000) with assay buffer, and the assay was carried out as mentioned in the protocol sheet. The inter- and intra-assays were evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 serum samples, each containing different concentrations of human fibronectin. The overall calculated intra-assay coefficient of variation was 5.3%, and the inter-assay coefficient of variation was 6.7%, according to the manufacturer information. The human fibronectin level detected in human plasma (EDTA) is 117-338µg/ml.

Heparan sulfate (HS) assay

(Please see assay description in appendix 3)

The ELISA kit for human heparan sulfate provided by Uscn Life Science Inc. Wuhan, China (catalogue number: E0161Hu) was used for the in-vitro quantitative measurement of human HS in plasma. The plasma samples had a 1000-fold dilution with PBS

(pH=7.2), and the assay was performed as in the instruction manual. The minimum detectable dose of human HS is typically less than 1.17 ng/ml. This assay has high sensitivity and excellent specificity for the detection of human HS. No significant cross-reactivity or interference was observed.

High mobility group box 1 (HMGB1) assay

(Please see assay description in appendix 3)

The HMGB1 ELISA kit was developed by Shino-Test Corporation, Japan, in cooperation with Professor Dr Ikuro Maruyama, Kagoshima University. IBL International Hamburg in Germany supplied the product (catalogue number: ST51011). This product is a two-step, sandwich enzyme-linked immunosorbent assay, and is used for quantitative determination of HMGB1 in human plasma. The samples were diluted 1:5 in a sample diluent solution, and the assay was carried out as described in the manufacturer's sheet. Cross-reactivity with HMGB2 is less than 2%. The sensitivity of this HMGB1 kit is 0.12ng/ml. Intra-assay precision was assessed in 11 samples, and coefficients of variation (CVs) were 3.2%-13.7%, whereas inter-assay precision was tested in 9 samples, and CVs were 1.3%-13.7%, in accordance with the manufacturer's information.

Hyaluronan (HA) assay

(Please see assay description in appendix 3)

The HA ELISA kit provided by Echelon Biosciences Inc. Salt Lake City, UT (catalogue number: K-1200) offers a simple, effective method for determining HA levels in human biological fluids. The plasma samples were analysed with no dilution, as recommended, and the assay was performed as shown in the manual. The HA ELISA kit detects HA molecules as small as 6.4kDA. The sensitivity of this HA kit is 25ng/ml.

Human sFlt-1 assay

(Please see assay description in appendix 3)

Human sVEGFR-1 / sFlt-1 Immunoassay (Quantikine ELISA kit catalogue number: DVR100B) was provided by R&D Systems, Europe, Ltd., and was used for the

quantitative determination of human sFlt-1 concentrations in cell culture supernatants. All measurements were made in triplicate on 1:5 dilutions of the culture media. The procedure was carried out as described in the company's sheet. No significant cross-reactivity or interference was observed. Intra-assay precision was tested in three samples, and the coefficients of variation were 2.6%, 3.8%, and 3.2%, whereas the inter-assay precision coefficients of variation were 9.8%, 7% and 5.5%. The minimum detectable dose is 3.5pg/ml, as mentioned in the instruction sheet.

Calculation of results

After the assay was performed according to the manufacturer's instructions for each ELISA kit, an ELISA microplate reader (Multiskan EX from Labsystems and Life Science International, UK LTD) was set at the wavelength indicated (usually 450nm) in order to measure the optical density of the wells, and the absorbance was read immediately. The average absorbance values for each set of triplicate standards and samples were calculated using Ascent software version 2.6 (Labsystems UK LTD), as described in the individual manual sheets. A standard curve was created by plotting the mean absorbance for each standard concentration on the y-axis against the concentration on the x-axis. The best-fit line was then determined using the four-parameter, logistic curve-fit.

2.2.6 Immunohistochemical staining from paraffin-embedded placental sections

Immunohistochemistry was used to identify fibrinogen expression in sections taken from wax-embedded placental tissues. The protocol used in this thesis was based on the study that was carried out previously (Karehed *et al.*, 2010). This method was used in the Histology Core Facility of Sheffield University Medical School. The technique was optimised before all experimental staining was carried out. All the placental samples from pregnant and pre-eclamptic women were then immunostained at the same time. The general staining protocol that was performed is described below.

Paraffin-embedded placental sections (5μm thickness) were de-waxed in two dishes of xylene for five minutes each, and then rehydrated using ethanol. The slides were placed

in two dishes containing 99% alcohol for five minutes each before being transferred directly to H_2O_2/methanol.

Endogenous peroxidase activity was blocked by incubating the slides in 3% of hydrogen peroxide (H_2O_2) (Fisher, catalogue number: H/1800/15, UK) for 30 minutes. 30ml of stock 30% H_2O_2 was added to 270ml of methanol to make a 3% concentration. The slides were subsequently washed three times in distilled water for one minute each, and then two times in PBS pH 7.3 baths for five minutes each.

A proteolytic enzyme digestion method was used for antigen retrieval. The slides were covered by proteinase K (Dako catalogue number: S3020, UK) for 10 minutes at room temperature. They were then washed twice in a PBS bath for five minutes each. Non-specific binding of the antigen and the antibody were blocked by incubating the sections in normal horse serum (Vector Universal Impress anti-mouse/rabbit Ig kit, catalogue number: MP-7500) for 20 minutes. The serum was then removed from the sections by tapping the slide's edge with tissue paper.

The 1:300 dilution of the primary antibody or the rabbit immunoglobulin in PBS pH 7.3 fraction was prepared for all sections directly before use. 200µl was added to each slide, and the slides were then incubated in a sealed, humidified container at room temperature for 30 minutes. The rabbit immunoglobulin fraction (Dako, catalogue number: X0936) and omission of the primary antibody were used as negative controls. The dilution of the primary antibody or the rabbit immunoglobulin fraction had been optimised in a previous experiment in order to find the best concentration. Serial dilutions of the primary antibody or the negative rabbit immunoglobulin control were prepared in PBS (from 1:200 up to 1:400). In the case of the slides on which rabbit immunoglobulin fraction and omission of primary antibody were used, non-specific background staining was not detected in all concentrations.

The slides were then washed twice in PBS for five minutes each. After washing, the slides were incubated with a peroxidase-labelled universal secondary antibody (Vector Impress anti-mouse/rabbit Ig kit, catalogue number: MP-7500) in a sealed container for 30 minutes at room temperature. The sections were then washed twice in PBS for five minutes each.

Nova Red Chromogen solution (Vector kit, catalogue number: SK-4800) was prepared immediately before use by measuring 5ml of distilled water, adding three drops of reagent 1 and mixing well, then adding two drops of reagent 2 and mixing well, and then adding two drops of reagent 3 and mixing well. Lastly 2 drops of hydrogen peroxide solution were added and mixed well. 200µl of Nova Red Chromogen solution was added to slides and incubated for ten minutes at room temperature. After incubation, the slides were rinsed in distilled water and washed in tap water for five minutes.

Nuclei were counterstained with Gill's II haematoxylin (Leica catalogue number: 3801521E) for twenty seconds The slides were washed in running tap water for 3-5 minutes until the rinse water was colourless and the tissue on the slides had turned blue.

Slides were dehydrated in gradual ethanol 70%, 95% and 99% for one minute each. Sections were cleared in two xylene baths for two minutes each.

The last step was covering the stained sections with glass cover slips using permanent Consul Mount (Thermo Scientific Shandon, catalogue number: 9990441) to improve viewing quality under the microscope, and to protect the tissue sections from scratching.

2.2.7 Statistical analysis

All statistics were performed using GraphPad Prism software version 5.0. D'Agostino-Pearson normality test was applied to all column data before selecting the powerful comparative tests amongst variance study groups or stimulators. Most of the column data from non-pregnant, normal pregnant and pre-eclamptic women are abnormally distributed. Therefore, Non-parametric test (Mann-Whitney U-test) was applied for most variable data (Monocytes subpopulations; TLR2, TLR4, HLA-DR, TIE2, CCR2 and CCR5 expressions on monocytes; inflammatory cytokines and chemokines released from cell cultures after TLR ligands stimulations and plasma levels of endogenous ligands). Also, Bonferroni test was applied for multiple comparisons. Appropriate comparative test was undertaken for individual experiments [see relevant chapters]. Results are illustrated as mean ± standard error of the mean (SEM). Differences between samples were deemed significant if $P < 0.05$.

Chapter 3: Characterisation of monocyte subpopulations in women with PE and normotensive pregnant women.

3.1 Introduction

Monocytes in human peripheral blood can be divided into three distinct populations based on expression of the lipopolysaccharide (LPS) receptor, CD14, and the Fcγ-III receptor, CD16. The nomenclature and classification of these monocyte subsets has recently been updated into three populations: classical monocytes ($CD14^{high}CD16^{-}$), intermediate monocytes ($CD14^{high}CD16^{+}$), and non-classical monocytes ($CD14^{low}CD16^{+}$) (Ziegler-Heitbrock *et al.*, 2010, Ziegler-Heitbrock and Hofer, 2013).

Emerging evidence indicates that each subpopulation has a unique gene signature and may also have differential functions in inflammation and immunity (Cros *et al.*, 2010, Zawada *et al.*, 2011); although, the hierarchical relationship and differentiation lineage between the subpopulations is still unclear. The recent reports on functional and phenotypic differences amongst the monocytes subpopulations has demonstrated that the non–classical $CD14^{low}CD16^{+}$ monocytes express a greater production of TNFα and a lower production of IL-10 after LPS stimulation; the intermediate $CD14^{high}CD16^{+}$ monocytes express greater HLA-DR and TIE2; and the classical $CD14^{high}CD16^{-}$ monocytes express greater CCR2 and CD62L and lower CX3CR1 (Cros *et al.*, 2010, Wong *et al.*, 2011, Wong *et al.*, 2012, Zawada *et al.*, 2011, Ziegler-Heitbrock and Hofer, 2013). More features of different monocyte subpopulations are summarised in the table 1.2.

Based on gene array data, the non-classical subpopulation most highly expressed multiple genes associated with cytoskeleton mobility, such as Rho GTPases (Wong *et al.*, 2011). This may represented the highly motile "patrolling" behaviour of non-classical monocytes on endothelium that has been observed after being transferred into mice (Cros *et al.*, 2010). The non-classical $CD14^{low}CD16^{+}$ monocytes are believed to be more mature than the classical $CD14^{high}CD16^{-}$ monocytes (Frankenberger *et al.*, 2008, Ziegler-Heitbrock *et al.*, 1993). Additionally, the previous hypothesis of the maturation of the non-classical from the classical subpopulations (Ziegler-Heitbrock, 1996) has been supported by recent microarray studies (Wong *et al.*, 2011).

Monocytes from women with PE are activated and display increased expression of CD14 and CD11b and produce more interleukin (IL)-1β, IL-6 and IL-8 than healthy

pregnancy controls (Gervasi *et al.*, 2001, Holthe *et al.*, 2004, Luppi and Deloia, 2006, Sacks *et al.*, 1998). These phenotypic changes are thought to affect monocytes in several ways, including their adherence to the vasculature as well as a down-regulation in their immunosuppressive functions once localized at the feto-maternal interface (Nagamatsu and Schust, 2010). In support of these notions, several studies have reported abnormal numbers of decidual macrophages (Burk *et al.*, 2001, Kim *et al.*, 2007b, Schonkeren *et al.*, 2011) and decreased expression of the immunosuppressive cytokine, IL-10, in PE maternal blood serum compared to normal pregnancy controls (Hennessy *et al.*, 1999, Sharma *et al.*, 2007).

As monocytes and macrophages are critical for successful pregnancy, we set out to further characterize the monocyte phenotype in PE patients. We hypothesised that this will differ from that of normotensive pregnant women, akin to observations in other disease states characterised by dysregulated inflammation such as upregulation of the activation markers (CD11b, CD14, and CD64) and reactive oxygen species (ROS) by monocytes, granulocytes and neutrophils from PE women (Gervasi *et al.*, 2001, Lee *et al.*, 2003, Luppi *et al.*, 2002, Sacks *et al.*, 1998, Tsukimori *et al.*, 1993).

3.2 Materials and methods

(For more details, please refer to chapter 2)

Subjects

Women with established PE were diagnosed by the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP) (2004), and were recruited from the antenatal clinics and obstetric day care unit of the Jessop Wing, University of Sheffield. Healthy non-pregnant (Non-P) female volunteers were also studied to determine baseline non-gestational levels. The Non-P women had normal menstrual cycles and were not on hormonal contraception. No study participants had any systemic infection or urinary tract infection. Experiments were performed on blood samples from 17 cases of PE, 11 NP women and 11 Non-P women. Our normal pregnancy (NP) cohort included 10 Caucasian women and 1 black woman, the PE cohort included 15 Caucasian women and 2 black women, and the non-pregnant cohort included 11 Caucasian women. Subject details are summarised in Table 3.1. All participants gave informed written consent for 12ml of fresh venous blood to be collected into a tube containing EDTA anticoagulant.

Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh venous blood layered over Ficoll-Pague Plus. The cells were blocked with 5µl of anti-human Fc receptor blocking reagent (Miltenyi Biotic, Surrey) for 10 minutes at 4°C. PBMCs were labelled with the following monoclonal antibodies for 30 minutes at 4°C: anti-CD14-AlexaFluor700 (clone HCD14; Biolegend), anti-CD16-eFlour450 (clone CB16; eBioscience), anti-CCR2-PE (clone FAB151P; R&D Systems), anti-CCR5-FITC (clone HEK/1/85a; Biolegend), anti-HLA-DR-PerCP-Cy5.5 (clone LN3; eBioscience), anti-TIE2-APC (clone FAB3131A; R&D Systems), anti-TLR2-FITC (clone TL2.1; eBioscience), and anti-TLR4-PE (clone HTA125; eBioscience). PBMCs were also labelled with isotype negative controls. Cells were washed twice by cold BD flow cytometry stain buffer, before acquisition was done on a LSRII Flow Cytometer (BD Bioscience). DIVA software (BD Bioscience) was used for analysis. Compensation was calculated using anti-mouse compensation bead sets (BD Bioscience). A gate specific for the monocyte population was created on the forward scatter (FSC) and side scatter

(SSC) dot plot and a total of 10,000 events were acquired. Monocyte sub-populations were identified according to CD14 and CD16 surface markers as defined previously (Ziegler-Heitbrock, 1996, Ziegler-Heitbrock, 2007, Ziegler-Heitbrock *et al.*, 2010).

Statistical analysis

Results are illustrated as mean ± standard error of the mean (SEM). Mann-Whitney *U*-test was used to compare differences between Non-P and NP or NP and PE using GraphPad Prism software version 5.0. *P* values < 0.05 were considered significantly different.

3.3 Results

3.3.1 Clinical characteristics

Patient characteristics are shown in table below.

Table 3.1 Patient characteristics

	Non-pregnant (n = 11)	Normal pregnant (n = 11)	Pre-eclampsia (n = 17)	P value (NP vs PE)
Age (years)	31.0 ± 5.4	29.3 ± 4.9	31.4 ± 5.6	0.26
Gestational age (weeks)		32.0 ± 3.7	32.9 ± 3.7	0.38
Gravidity		1-4	1-4	
SBP (mm Hg)	110.0 ± 10.0	114.0 ± 11.0	150.4 ± 8.6	0.0001
DBP (mm Hg)	70.0 ± 10.0	70.0 ± 10.0	98.3 ± 4.2	0.0001
24 hour urine collection (g/24hrs)	0	0	0.84 ± 1.3	
Urine Dipstick Protein Test	0	0	(1+ - 3+)	

3.3.2 Total peripheral blood mononuclear cells and total monocytes amongst groups were studied

Blood samples were collected from non-pregnant (Non-P), normal pregnant (NP), and pregnant women with PE (PE). Peripheral blood mononuclear cells (PBMCs) were isolated from these samples and analysed by flow cytometry. Cell counting was carried out in unstained tube. Cell debris was removed by increasing the signal threshold.

The mean percentage of gated PBMCs did not differ from NP women compared to non-pregnant women, or PE women compared to NP women. Also, there was no significant difference in the total number of monocytes studied between the three groups (**Table 3.2**).

Table 3.2. Mean percentage of gated PBMCs and monocytes

PBMCs from non-pregnant (Non-P), normal pregnant (NP), and PE (PE) patients were isolated and acquired by LSRII flow cytometry. PBMCs were plotted on FSC/SSC and then monocytes were gated. Statistical significance was determined by Mann-Whitney *U* test.

	Non-p	NP	PE	*P* value NP vs Non-p	*P* value PE vs NP
Mean % of gated PBMCs	69.9%	68.1%	68.6%	0.89	0.95
Mean % of gated monocytes	12.9%	12.1%	11.9%	0.82	0.75

3.3.3 Monocyte subpopulation frequency is skewed in pregnant women with PE

The isolated PBMCs were stained with anti-CD14 and anti-CD16 antibodies and analyzed by flow cytometry. Gates were first placed around monocytes on FSC/SSC plots. And the three monocytes subpopulations were identified as defined previously by Ziegler-Heitbrock (Ziegler-Heitbrock, 1996, Ziegler-Heitbrock, 2007).

Significant differences in monocyte subpopulations were prevalent between the three groups (**Figure 3.1**). NP patients exhibited a greater proportion of $CD14^{high}CD16^{-}$ monocytes and a lower proportion of $CD14^{low}CD16^{+}$ monocytes when compared with Non-P patients. Interestingly, this pattern was reversed in women with PE, where the non-classical $CD14^{low}CD16^{+}$ monocytes were significantly increased over NP controls. No differences were observed in the intermediate $CD14^{high}CD16^{+}$ monocyte population between any of the patients.

3.3.4 PE is associated with changes in monocyte phenotype

Next, we investigated the phenotype of monocytes in Non-P, NP, and PE women.

In relation to Non-P patients, the monocytes of NP patients expressed lower levels of CCR2 (a receptor of the monocytes chemo-attractant protein 1) and CCR5 (a receptor of the macrophage inflammatory protein), but higher levels of HLA-DR (a MHC class II cell surface receptor), TIE2 (a receptor of angiopoietin), and TLR2 (**Figure 3.2**). The number of $TLR4^{+}$ monocytes was equivalent between the two groups.

When comparing NP and PE monocytes, PE patients displayed a reduced number of $HLA\text{-}DR^{+}$ and $TIE2^{+}$ monocytes, whereas $CCR5^{+}$ and $TLR4^{+}$ monocytes were dramatically increased (**Figure 3.2**). A similar expression profile for CCR2 and TLR2 was observed between the two groups. These data indicate that monocytes from PE patients are phenotypically different from NP patients.

To determine whether the differences we observed were due to changes in expression by specific monocyte subpopulations, we examined the phenotypic markers on classical, intermediate, and non-classical subpopulations. Interestingly, classical monocytes did not exhibit any differences between the three patient cohorts. All the observed changes could be attributed to intermediate or non-classical monocytes.

Expression levels of CCR2, HLA-DR, and TLR2 were not different among monocytic subpopulations of NP and PE patients (**Figure 3.3**). However, the intermediate $CD14^{high}CD16^{+}$ monocyte population of PE patients displayed higher expression of CCR5 and TLR4 than NP patients. TLR4 was also significantly increased on non-classical $CD14^{low}CD16^{+}$ monocytes from PE when compared with NP controls. Moreover, TIE2 expression was significantly reduced on the $CD14^{low}CD16^{+}$ monocytes subset in PE. These data suggest that phenotypic differences between NP and PE patients can be attributed to specific monocyte subpopulations.

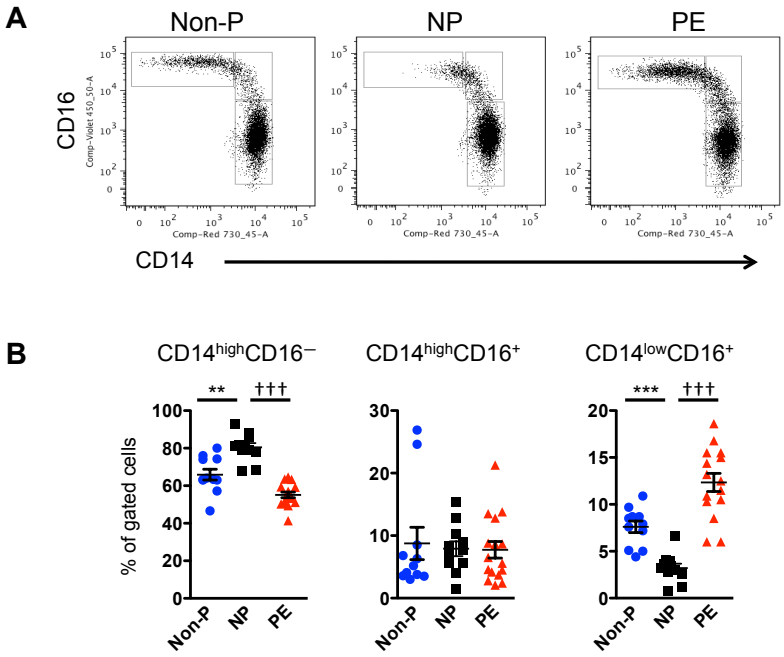

Figure 3.1 The proportion of monocyte subpopulations is skewed in patients with PE

PBMCs from non-pregnant (Non-P), normal pregnant (NP), and pre-eclampsia (PE) patients were stained with anti-CD14-AlexaFluor700 and anti-CD16-eFluor450 antibodies then analysed by flow cytometry. (**A**) Dot plots showing CD14 and CD16 expression on gated monocytes. (**B**) Graphic representation of percentage of gated cells (mean ± SEM). Statistical significance was determined by Mann-Whitney *U* test (n = 11-17). $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with Non-P; $^{\dagger\dagger\dagger}p < 0.001$ as compared with NP.

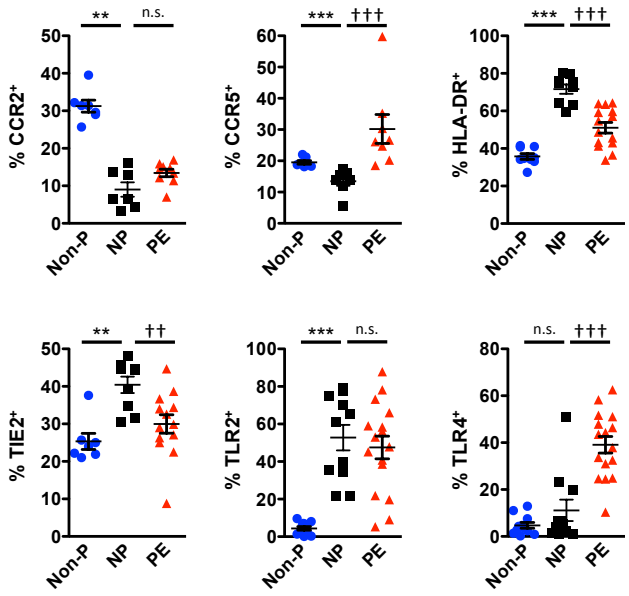

Figure 3.2 NP and PE monocytes display marked phenotypic differences

PBMCs from non-pregnant (Non-P), normal pregnant (NP), and PE (PE) patients were stained with anti-CD14-AlexaFluor700, anti-CD16-eFluor450, anti-CCR2-PE, anti-CCR5-FITC, anti-HLA-DR-PerCP-Cy5.5, anti-TIE2-APC, anti-TLR2-FITC, and anti-TLR4-PE antibodies then analysed by flow cytometry. Gates were placed around monocytes on FSC/SSC plots and the percentage of positive cells was calculated based off isotype controls. Values are illustrated as mean ± SEM. Statistical significance was determined by Mann-Whitney *U* test (n = 5-16). $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with Non-P; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ as compared with NP.

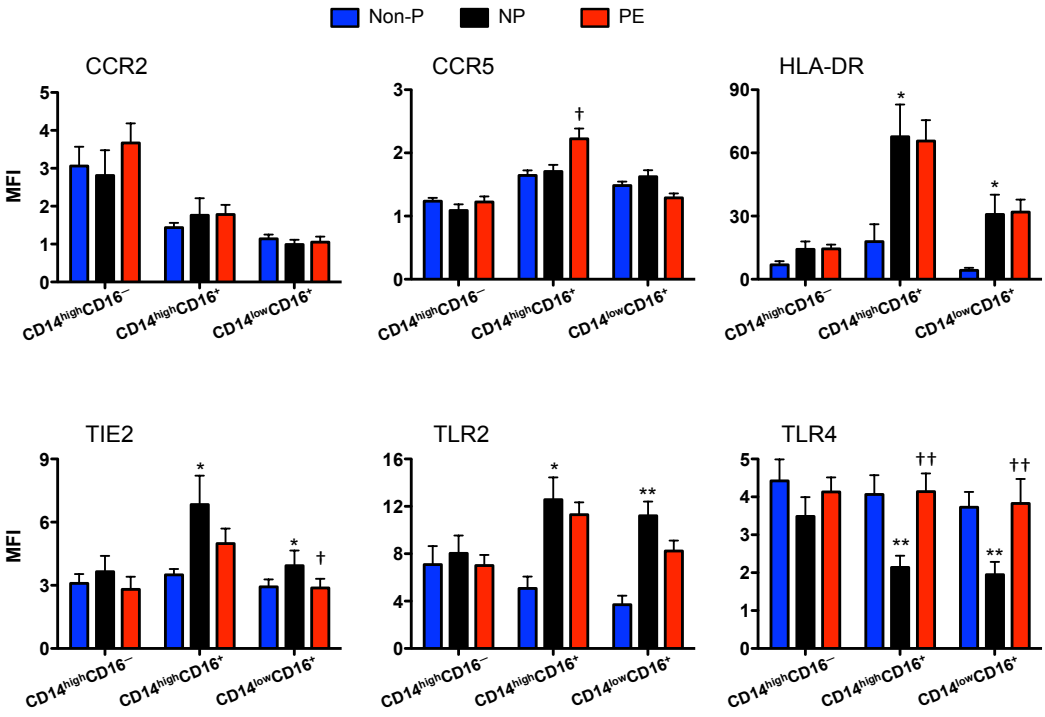

Figure 3.3 Expression of phenotypic markers on monocyte subpopulations

PBMCs from non-pregnant (Non-P), normal pregnant (NP), and PE (PE) patients were stained with anti-CD14-AlexaFluor700, anti-CD16-eFluor450, anti-CCR2-PE, anti-CCR5-FITC, anti-HLA-DR-PerCP-Cy5.5, anti-TIE2-APC, anti-TLR2-FITC, and anti-TLR4-PE antibodies then analysed by flow cytometry. Gates were placed around CD14/CD16 monocyte subpopulations and median fluorescence intensity (MFI) was calculated based off isotype controls. Values are illustrated as mean ± SEM. Statistical significance was determine by Mann-Whitney *U* test (n = 7-17). $*p < 0.05$, $**p < 0.01$ as compared with Non-P; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ as compared with NP.

3.4 Discussion

In this study, we report that the proportion of non-classical $CD14^{low}CD16^{+}$ monocytes in PE patients is increased, while classical $CD14^{high}CD16^{-}$ monocytes are decreased in comparison with normal pregnant controls. The phenotype of PE monocytes was also altered, exhibiting aberrant expression of chemokine receptors, antigen presentation molecules, and Toll-like receptors. These results provide further evidence that the phenotype of monocytes in PE patients is abnormal and may be associated with the pathogenesis of PE.

Evidence from PE patients indicates that their circulating monocytes display an aberrant activation status, with altered expression levels of surface antigens, cytokines and reactive oxygen species when compared with healthy pregnancy controls (Gervasi *et al.*, 2001, Holthe *et al.*, 2004, Luppi and Deloia, 2006, Sacks *et al.*, 1998). This report supports previously published findings and provides additional data describing the proportional and phenotypic differences between monocyte subpopulations of PE and NP women. We found that the non-classical, $CD14^{low}CD16^{+}$ monocyte subpopulation is increased in PE patients where they exhibit lower expression levels of HLA-DR and TIE2, and higher levels of CCR5 and TLR4.

We have observed that normal pregnant women exhibited a greater proportion of $CD14^{high}CD16^{-}$ monocytes and a lower proportion of $CD14^{low}CD16^{+}$ monocytes when compared with the non-P controls. Interestingly, this pattern was reversed in women with PE, in whom the non-classical $CD14^{low}CD16^{+}$ monocytes significantly increased over the NP controls. Therefore, it is plausible that the increased immature $CD14^{high}CD16^{-}$ monocytes from NP women may differentiate into the apparently more mature phenotype $CD14^{low}CD16^{+}$ cells in the end stages of pregnancy. This may contribute to the mild inflammatory response present in NP women. It is plausible that the maturation process of these monocytes could occur at earlier stages in PE women, resulting in the exaggerated systemic inflammatory response seen in this condition. Furthermore, a trigger factor that is expressed in PE might be inducing early monocyte maturation. This assumption may be supported by the findings from a recent study that demonstrated that monocyte subsets from third trimester normal pregnant women are shifted toward the pre-pregnancy stage (Bardou *et al.*, 2014). That study also found that

MCP-1 expressing monocytes (classical and intermediate) are reduced in NP women and in preterm premature rupture of membranes (PROM) women as compared to non-P women. However, spontaneous labour was associated with the return of MCP-1-expressing monocytes to pre-pregnancy levels in both the NP women and the PROM women. Thus, this suggests that monocyte activation contributes to the onset of labour. However, a larger study is required that examines monocyte subpopulations and their phenotypes, which includes pre-pregnancy, first trimester, second trimester, third trimester and post-delivery stages.

The elevation of $CD14^{low}CD16^{+}$ monocytes has been reported in other diseases such as rheumatoid arthritis, atherosclerosis, Kawasaki disease, septic shock and human immunodeficiency virus (HIV) infection (Blumenstein *et al.*, 1997, Fingerle *et al.*, 1993, Kawanaka *et al.*, 2002a, Kawanaka *et al.*, 2002b, Nockher and Scherberich, 1998, Schlitt *et al.*, 2004, Ziegler-Heitbrock, 2007). As such, expansion of non-classical monocytes may be a general phenomenon of inflammatory and infectious diseases. However, their role in the manifestation or propagation of these diseases is unclear, as very little is known about $CD14^{low}CD16^{+}$ monocyte function. After adoptive transfer into immunocompromised mice, these cells attach to the endothelium, crawl along vessels and exhibit a 'patrolling' behaviour whereas classical and intermediate monocytes do not (Cros *et al.*, 2010). $CD14^{low}CD16^{+}$ monocytes from healthy non-pregnant patients are weakly phagocytic of latex beads and give little response to LPS stimulation (Cros *et al.*, 2010).

The proportion of $CD16^{+}$ monocytes has been shown to be selectively increased in the peripheral blood of atherosclerosis patients (Rogacev *et al.*, 2012, Schlitt *et al.*, 2004, Zawada *et al.*, 2012). Moreover, the presence of $CD14^{high}CD16^{+}$ monocytes is considered to be an independent factor for predicting cardiovascular disease (Rogacev *et al.*, 2012). The potential significance of $CD14^{low}CD16^{+}$ monocytes in atherosclerosis is determined by their ability to bind to vascular endothelium and by their accumulation in atherosclerotic plaque via CX3CR1 and CCR5 (Ancuta *et al.*, 2003, Ghattas *et al.*, 2013). On the other hand, $CD14^{high}CD16^{-}$ monocytes represent the predominant subpopulation recognised in atherosclerotic plaque in response to activated endothelium

(Ghattas *et al.*, 2013). Therefore, all the monocyte subpopulations might be involved in atherogenesis initiation and progression.

The disturbance of monocyte subsets and their functions has been reported in autoimmune diseases, such as systemic lupus erythematous (SLE) (Cairns *et al.*, 2002, Henriques *et al.*, 2012). At parturition, pregnant women with SLE were found to be similarly affected as women with a healthy pregnancy, in terms of reduced percentages of chemokine receptors (CCR2, CCR5 and CXCR3) of both the classical and non-classical monocyte subsets, in comparison with the non-pregnancy controls (Bjorkander *et al.*, 2013). However, pregnant women with SLE had increased expression of CCR5 in the non-classical monocytes compared to the healthy pregnant women.

Our data indicate that TLR4 is upregulated in $CD14^{low}CD16^{+}$ monocytes of PE women. Increased TLR4 levels have also been observed on neutrophils of PE women (Xie *et al.*, 2009), suggesting that induction of TLR4 expression on circulating myeloid cells is a specific consequence of PE pathophysiology. It is tempting to speculate that a trigger factor(s) released by an abnormally ischemic placenta may be inducing early monocyte maturation – such as $CD14^{high}CD16^{-}$ monocyte differentiation into $CD14^{low}CD16^{+}$ monocytes – and/or upregulation of TLR4. In support of this notion, placental microparticles, namely syncytiotrophoblast basement membrane fragments derived from human term placentas, activate cytokine release from peripheral blood monocytes (Messerli *et al.*, 2010) and plasma from PE patients can induce ICAM-1, an adhesion receptor, in a monocytic cell line (Faas *et al.*, 2008). It will be interesting to determine whether TLR4 expression on newly recruited decidual macrophages correlates with TLR4 expression on circulating monocytes and whether signalling through this receptor exacerbates PE in murine models.

In contrast to our findings and others (Xie *et al.*, 2009), one study has also reported reduced expression of *TLR4* mRNA in neutrophils of PE women (Nitsche *et al.*, 2011). The results from these studies may be difficult to compare as gestational age, severity of the disease (i.e. proteinuria), gravidity, and genetic background of each patient population is dramatically different. These dissimilarities strongly suggest caution is warranted when interpreting this type of data.

In addition to TLR4, we observed differences in CCR5, HLA-DR, and TIE2 between PE and NP patients; although, the ramifications of their altered expression is also unclear. CCR5 binds the chemokine CCL5/RANTES which activates signalling pathways leading to migration. In our study, CCR5 upregulation was specific to the intermediate $CD14^{high}CD16^{+}$ monocytes, indicating that this subpopulation may be recruited by CCL5 expression in the placenta. HLA-DR was found at reduced levels in PE and may affect T cell activation in these patients, as this molecule is important for antigen presentation. Of note, TIE2-expressing monocytes have been shown to promote angiogenesis in mouse tumor models (Murdoch *et al.*, 2007, Venneri *et al.*, 2007), so their decrease in PE could have major implications for the reduced oxygenation observed in PE. All of these hypotheses remain to be tested.

In conclusion, the alterations of monocyte subpopulations seen in PE with predominance of $CD14^{low}CD16^{+}$ monocytes may result in the disturbance of decidual leukocyte distribution and function. Also, the upregulation of TLR4 suggests a role for this pattern recognition receptor in the exaggerated systemic inflammatory response seen in PE but it is unclear whether this occurs as a cause or consequence of PE.

Chapter 4: The effect of bacterial and endogenous ligands of TLR2 and TLR4 on inflammatory cytokine responses of human monocytes.

4.1 Introduction

Prior studies have shown that PE (PE) is characterised by an exaggerated systemic inflammatory response (Faas and Schuiling, 2001, Gervasi *et al.*, 2001, Redman *et al.*, 1999). In the circulation of women with PE, both monocytes and granulocytes display aberrant expression levels of surface antigens, cytokines and reactive oxygen species (Gervasi *et al.*, 2001, Holthe *et al.*, 2004, Luppi and Deloia, 2006, Sacks *et al.*, 1998). For example, monocytes display increased expression of CD14 and CD11b and produce more interleukin (IL)-1β, IL-6 and IL-8 than healthy pregnancy controls (Gervasi *et al.*, 2001, Holthe *et al.*, 2004, Luppi and Deloia, 2006, Sacks *et al.*, 1998).

We demonstrated in other experiments (Chapter 3) that PE monocytes have distinct phenotypes, with a predominance of the non-classical ($CD14^{low}CD16^{+}$) monocyte subpopulation as compared to normotensive and non-pregnant women. Furthermore, monocytes from women with PE express higher levels of Toll-like receptor 4 (TLR4).

In the past two decades, mammalian TLRs have been discovered, and their interaction with pathogen-associated molecular patterns (PAMPs), leads to activation of MyD88-dependent or independent signalling pathways and nuclear translocation of the NF-κB, inducing the inflammatory responses resulting in the secretion of cytokines, chemokines and adhesion molecules (Akira *et al.*, 2001, Sandor and Buc, 2005). Researchers have suggested that host-derived molecules will act as endogenous ligands for TLR2 and TLR4, leading to activation of signalling pathways in the absence of infection (Beg, 2002, Tsan and Gao, 2004b). These molecules may be produced endogenously as a result of tissue injury and cell necrosis. Several endogenous ligands have been proposed including heat shock proteins (HSPs), fibrinogen, fibronectin, heparan sulphate, hyaluronan and HMGB1. Recently, Erridge in his article reviewed more than 20 proposed endogenous ligands and summarised the evidence of their intrinsic capacity to stimulate TLR2 or TLR4, excluding risk of bacterial contamination (Erridge, 2010). He found that these endogenous molecules fall into one of the following categories: extracellular matrix components, intracellular proteins, oxidative modified lipids and other soluble markers. The risk of endotoxin contamination lipopolysaccharides (LPS) exists in the preparation methods of these molecules, as a result of using recombinant proteins prepared in *E. coli.* Also, Erridge emphasised that in subsequent studies

approximately one third of these endogenous molecules have been identified to have no intrinsic TLR4-stimulating properties, when highly purified reagents were used. For example, HSP 60 and 70 failed to stimulate TLR4 signalling when these reagents were prepared with free LPS methods (Bausinger *et al.*, 2002, Gao and Tsan, 2003). Also, Erridge proposed that extracellular matrix molecules produced during tissue damage could act as binding pathogen molecules to facilitate presentation of the PAMPs to the cells, rather than serve as endogenous ligands to TLR4. For this reason, we used highly purified commercially produced proteins isolated from human blood (fibrinogen and fibronectin) or extracellular matrix proteins (heparan sulphate and hyaluronan) in this study.

Subsequently, we hypothesised that TLR4 up-regulation in PE monocytes plays an important role in the systemic inflammation and the pathogenesis of vascular dysfunction in PE, and that alterations in endogenous ligand activity in PE may modulate these processes. Experiments were therefore designed to assess the effect of LPS (TLR4 ligand), peptidoglycan (PDG) (TLR2 ligand) and endogenous ligands of TLR4 (fibrinogen, fibronectin, heparan sulfate and hyaluronan) on monocyte cultures, and to compare the inflammatory cytokine expression profiles of monocytes from women with PE to those from normal pregnant (NP) women, in order to identify which of these endogenous ligands of TLR4 may play a role in the pathogenesis of PE for further study.

4.2 Materials and methods

(General description of the methods employed are detailed in chapter 2)

Subjects

Please see Chapter 3 Section 3.2 for further details about subjects and samples used.

Monocyte stimulation

The isolated PBMCs were seeded in 12-well tissue culture flat bottom plates, at a density of 1.8×10^6 cells/well. The cells were incubated with medium (IMDM supplemented with 2% FBS, 2 mM L-Glutamine and penicillin-streptomycin) at 37°C with 5% CO_2. After two hours of incubation the medium was removed and the adherent monocytes were washed twice with cold PBS. The medium was then replaced. Cells were treated with 100ng/ml Lipopolysaccharides (rough strains) from *Escherichia coli* (Sigma-Aldrich), 5µg/ml Peptidoglycan from *Staphylococcus aureus* (Sigma-Aldrich), 5µg/ml Fibronectin (BD Bioscience), 2mg/ml Fibrinogen (Sigma-Aldrich), 10µg/ml Heparan Sulfate Proteoglycan (Sigma-Aldrich), and 100µg/ml select-Hyaluronan of molecular weight: 125-175 kDa (Sigma-Aldrich) or left untreated for 24 hours. Conditioned medium was collected after this time, and frozen at -20°C.

Cytometric bead array

Cytometric bead array technique, as described in Chapter 2, was used to measure interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) cytokines, within monocyte conditioned medium.

Statistical analysis

Results are illustrated as mean ± standard error of the mean (SEM). Mann-Whitney *U*-test was used to compare differences between Non-P and NP, or NP and PE using GraphPad Prism software version 5.0. One Way ANOVA (Bonferroni multiple comparison test) was used to compare differences between the un-stimulated cells to different stimulators (bacterial and endogenous ligands of TLRs). *P* values < 0.05 were considered significantly different. To calculate fold change, the median fluorescence intensity (MFI) of LPS-, PDG-, fibrinogen-, fibronectin-, heparan sulphate- and hyaluronan- treated was divided over the un-stimulated MFI.

4.3 Results

4.3.1 Clinical characteristics

Participant characteristics are shown in table 3.1, please see chapter 3.

4.3.2 Monocytes from PE patients exhibit an amplified response to TLR bacterial ligands

Since TLR4 expression was increased on PE monocytes, we asked whether the observed phenotypic changes have any consequence on monocyte activity. We treated monocytes from Non-P, NP, and PE patients with TLR4 ligands and monitored cytokine expression.

Without stimulation, basal IL-6, IL-10 and TNF-α release by PE monocytes was lower in relation to NP; although, only IL-10 secretion was significantly less (**Figure 4.1**).

LPS and PDG significantly increased the release of all three cytokines by monocytes from each patient cohort, compared to their respective un-stimulated monocytes. However, TLR ligand-treated PE monocytes exhibited a marked and significant increase over that seen in TLR ligand-treated NP monocytes. Taken together, these data indicate that increased activity of TLR4 on monocytes from PE patients results in an exaggerated response to TLR4 ligands.

4.3.3 Fibrinogen induces inflammatory cytokine release from PE monocytes

In addition to LPS and PDG, the monocyte cultures from Non-P, NP, and PE patients were treated with different endogenous ligands of TLR-4 (fibrinogen, fibronectin,

heparan sulfate and hyaluronan), and then cytokine expression levels were measured in frozen supernatants.

We found that fibrinogen induced inflammatory cytokine release (IL-6, TNF-α) and anti-inflammatory cytokines (IL-10) from PE monocytes, to an extent comparable to bacterial ligands **(Figure 4.2)**; statistical significance was achieved only with IL-6. In contrast, the other endogenous ligands did not induce any cytokine release from cultured PE monocytes.

In comparison with normotensive pregnant women, the IL-6, TNF-α and IL-10 cytokines were significantly increased from PE monocytes stimulated by fibrinogen (P value < 0.05), but not from those stimulated with other endogenous ligands **(Figure 4.3)**.

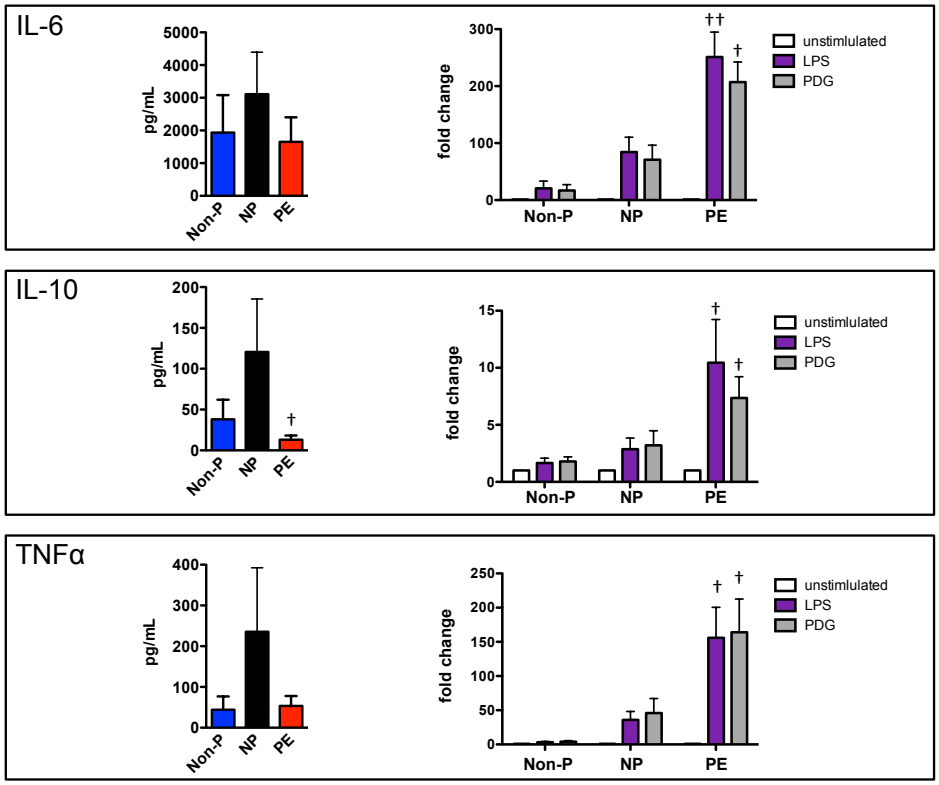

Figure 4.1 Monocytes from PE patients exhibit an amplified response to bacterial TLR ligands

Left panels: Basal levels of cytokine production by monocytes. ***Right panels****:* Graphic representation of the response of monocytes to LPS and PDG. Fold change was calculated by dividing MFI values over untreated control MFI values and illustrated as mean ± SEM. Statistical significance was determined by Mann-Whitney *U* test (Non-P n = 5-7; NP n = 11; and PE n = 15-17). $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ as compared with NP.

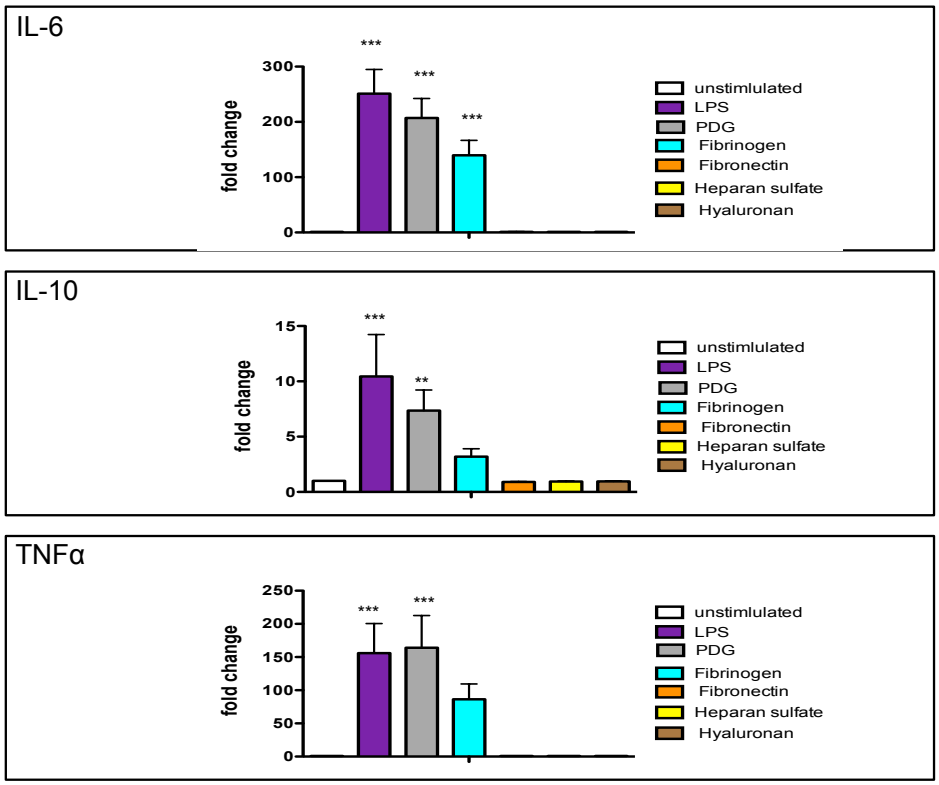

Figure 4.2 Fibrinogen induces cytokine release from PE monocytes

Graphic representation of the response of PE monocytes to different ligands. The data are presented as fold change, calculated by dividing MFI values of fibrinogen, fibronectin, or heparan sulphate groups over unstimulated control. Values are illustrated as mean ± SEM. Statistical significance was determined by One Way Anova to compare differences between the un-stimulated cells to different stimulators (bacterial and endogenous ligands of TLRs) (n= 15-17). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with un-stimulated cells.

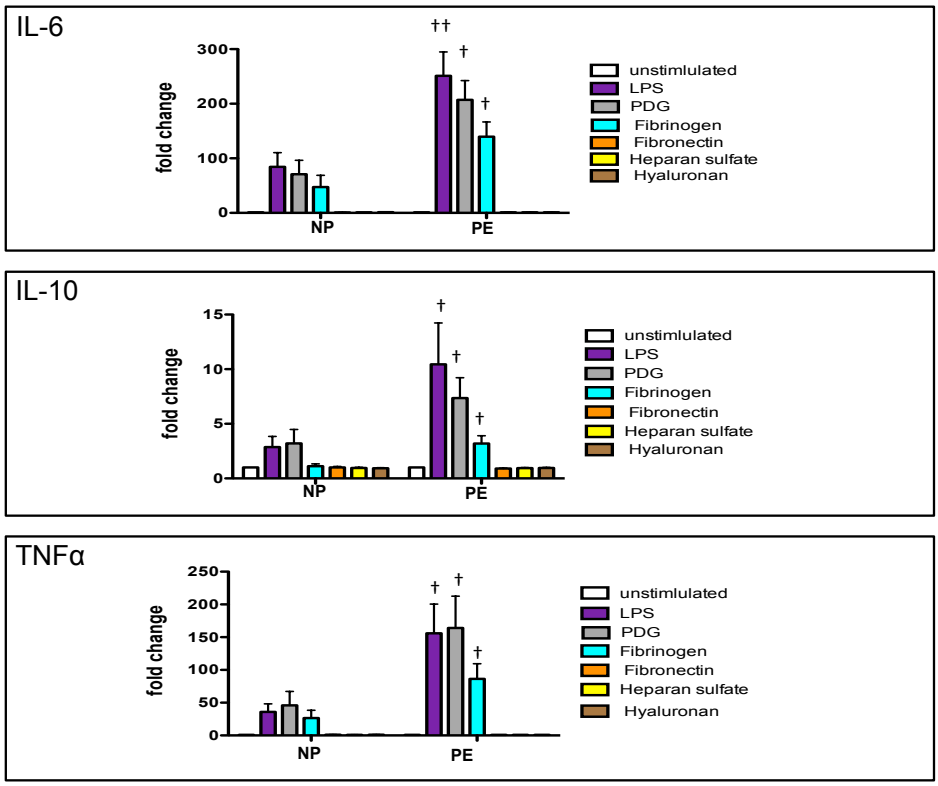

Figure 4.3 Fibrinogen induces more cytokine release from PE monocytes versus NP

Graphic representation of the response of PE and NP monocytes to different ligands. The data are presented as fold change, calculated by dividing MFI values of fibrinogen, fibronectin, or heparan sulphate groups over unstimulated control. Values are illustrated as mean ± SEM. Statistical significance was determined by the Mann-Whitney *U* test (NP n = 11; and PE n = 15-17). $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ as compared with NP.

4.4 Discussion

The cytometric bead array method was applied to investigate whether endogenous ligands of TLR4, namely fibrinogen, fibronectin, heparan sulfate and hyaluronan, would induce the release of inflammatory cytokines from monocytes.

E. coli-derived LPS and *Staphylococcus* PDG, were associated with an exaggerated release of TNF-α, IL-6 and IL-10 cytokines by monocytes from PE patients, consistent with the role for monocytes in the exaggerated systemic inflammatory response. This increase in cytokine production from PE monocytes treated with *Staphylococcus* PDG and *E. coli*-derived LPS, suggests increased functional activity of these receptors (TLR -2 and -4, respectively) in this tissue. This is consistent with data in Chapter 3 showing that TLR4 is particularly upregulated in the non-classical $CD14^{low}CD16^{+}$ monocytes of women with PE. These data suggest that PE monocytes appear hyper-responsive to TLR ligands, and this may contribute to exacerbation of the disease, by producing more inflammatory cytokines in the circulation of PE women that may be secondary to stimulation of these receptors with any pathogenic or endogenous molecules. It is well known that PE is characterised by excessive circulating inflammatory cytokines, especially IL-6 and TNF-α (Conrad *et al.*, 1998, Freeman *et al.*, 2004, Greer *et al.*, 1994, Jonsson *et al.*, 2006, Kupferminc *et al.*, 1996, Vince *et al.*, 1995, Xiao *et al.*, 2012). Also, the TLR4 expression is increased in the tissues from PE women such as monocytes, neutrophil and placental trophoblasts (Al-ofi *et al.*, 2012, Kim *et al.*, 2005, Xie *et al.*); any infectious or non-infectious factors could exacerbate the disease. Rustveld and colleagues investigated the relation between maternal infection and PE by systematic review of pervious epidemiologic studies (Rustveld *et al.*, 2008). They have demonstrated a significant increased risk of PE with any infectious agent (viral or bacterial) and that could explain their association with inflammation in PE. However, no study has identified the exact mechanisms by which these infectious agents may trigger inflammation in PE.

In contrast to our findings, Mazouni and colleagues have reported impaired secretion of TNF-α after LPS and PDG treatment of PE monocytes (Mazouni *et al.*, 2008). Results of this study may be difficult to compare, as gestational age, severity of the disease (i.e. proteinuria), gravidity, and genetic background of each patient population is

dramatically different from our study. Moreover, the concentration of TLR4 ligands, as well as the bacterial source of the ligands differs widely among various studies. Mazouni's study was performed on monocytes which were isolated from NP women with gestational age [GA] = 29.1 ± 4.3 weeks, n=10, PE (GA =30.8 ± 4.8 weeks, n=20, proteinuria (g/24 h) = 2.5 ± 1.1), and non-pregnant women (n=10); the isolated monocytes were treated with 1μg/ml of LPS obtained from Alexis-Biochemicals. On the other hand, the current study was done on monocytes that were isolated from NP women (GA = 32.0 ± 3.7 weeks, n=11), PE (GA =32.9 ± 3.7 weeks, n=17, proteinuria (g/24 h) = 0.84 ± 1.3), and non-pregnant women (n=11); the isolated monocytes were treated with 100 ng/ml of LPS obtained from Sigma-Aldrich. These dissimilarities may explain the differences in findings between the two studies, and strongly suggest that caution is warranted when interpreting this type of data.

Our results show reduction of IL-10 anti-inflammatory cytokine release from PE monocytes at basal levels, but significantly increased after LPS and PDG treatment. This contradicts a previous study (Mazouni *et al.*, 2008), which demonstrated that IL-10 was unchanged with LPS and PDG stimulation. This could be explained by a study that showed that IL-6 acts as an anti-inflammatory cytokine by suppressing IL-1β and TNF-α and increasing production of IL-10, and IL-1RA (Petersen and Pedersen, 2006). Thus, the up-regulation of IL-10 from PE monocytes observed by us may be secondary to an exaggerated release of IL-6 from PE monocyte cultures.

We hypothesised that one or more endogenous ligands of TLR4, perhaps released from the deranged placenta in PE, may be implicated in the pathogenesis of abnormal vascular reactivity and dysfunction in PE. However, stimulating PE monocytes with some of the well-described endogenous TLR4 ligands did not demonstrate significant inflammatory cytokine expression, except for fibrinogen. This finding is in agreement with previous works that have demonstrated fibrinogen to serve as an endogenous ligand for TLR4. It was shown to stimulate mice and human TLR4 of macrophages to release inflammatory cytokines and chemokines such as IL-6, IL-8, TNF-α, monocyte chemoattractant protein 1(MCP-1), macrophage inflammatory protein 1 (MIP-1) α & β, matrix metalloproteinase (MMP) 1, and MMP9 (Hodgkinson *et al.*, 2008, Smiley *et al.*, 2001).

Our findings show that intact fibronectin did not stimulate cytokine release from PE monocytes. Interestingly, Okamura *et al.* demonstrated that the recombinant extra domain A (EDA) of fibronectin, induces TLR4 signalling in THP-1 (monocytes/macrophages) and HEK 293 (human embryonic kidney cells, normally lacking TLR4, transfected with TLR4 and MD2 cDNA) to express MMP9, and to activate NF-κB signaling pathway (Okamura *et al.*, 2001). Additionally, they revealed that the intact fibronectin and its other recombinant domains did not stimulate MMP9 expression. They proposed that EDA-containing fibronectin fragments could stimulate TLR4 and serve as an endogenously produced molecule. However, as no cytokine release was investigated, one could interpret that this finding by Okamura was based on an assumption. In order to confirm such a proposition, further studies are required to investigate cytokine release and its mechanism involved in the binding of TLR4. Moreover, Gondokaryono demonstrated in his work that recombinant EDA of fibronectin stimulates the release of cytokines such as IL6, IL-1β and TNF-α from murine mast cells which are mediated by TLR4 (Gondokaryono *et al.*, 2007). However, this stimulation was not observed in response to other domains of fibronectin fragments. Further research confirmed Okamura's suggestion that there was specific physical binding between recombinant EDA of fibronecton and TLR4-expressing HEK 293 cells, stimulating the NF-κB pathway (Lasarte *et al.*, 2007). Furthermore, it was shown that cytokine release (TNF-α and IL-12) and dendritic cells (DC) maturation was in response to DC induction by the EDA of fibronectin via TLR4 (Lasarte *et al.*, 2007). Collectively, the EDA of fibronectin is considered an endogenous ligand for TLR4, not the intact protein.

No cytokine release was observed in response to hyaluronan from PE monocyte culture, in this study. On the contrary it has been shown that hyaluronan fragments do stimulate TLR2 and TLR4 from a variety of cells, including dendritic cells, endothelial cells and cumulus oocyte, releasing cytokines (Shimada *et al.*, 2008, Taylor *et al.*, 2004, Termeer *et al.*, 2000). However, a more recent study is in agreement with the results presented in this study where research shows that highly purified hyaluronan, without LPS contamination, does not stimulate NF-κB and cytokine production in murine macrophages (Krejcova *et al.*, 2009). This could explain that the low-molecular-weight hyaluronan was used in the current study, commercially produced with high

pharmacological purification, did not stimulate cytokines secretion from each patient cohort compared to un-stimulated monocytes.

In this study, heparan sulphate basement membrane fragments were not able to produce inflammatory cytokines from PE monocytes cultures. Furthermore, in chapter 7, it has been shown that HSPG did not differ in PE and NP women. This mainly suggested that HSPG may not be involved in the pathogenesis of PE.

Overall, the full-length fibrinogen stimulates TLR4; but other proteins such as fibronectin, heparan sulfate and hyaluronan stimulate TLR4 only when they are degraded to their fragments (Gondokaryono *et al.*, 2007, Hodgkinson *et al.*, 2008, Johnson *et al.*, 2002, Lasarte *et al.*, 2007, Okamura *et al.*, 2001, Shimada *et al.*, 2008, Smiley *et al.*, 2001, Taylor *et al.*, 2004, Termeer *et al.*, 2000). In the current experiments full-length of fibrinogen and fibronectin and fragments of heparan sulfate and hyaluronan, were used. Our finding of increased inflammatory cytokine expression levels in PE monocytes in response to fibrinogen suggests an important role for this endogenous ligand of TLR4 in the pathogenesis of PE. On the other hand, the fibronectin, heparan sulfate and hyaluronan did not demonstrate significant inflammatory cytokine expression from women with PE. If these endogenous ligands contribute in the way we postulated, this is likely to be partial, and may well involve other endogenous ligands that we did not study in these experiments. One of the limitations of this study is that it fails to prove whether the fibrinogen-induced PE inflammatory cytokines is mediated with TLR4 or other well established leukocytes integrin receptors expressed by monocytes, details of which are described elsewhere in this thesis Chapter 1 Section 1.3.6.2.E, (i.e. CD11b which is highly expressed in PE monocytes (Gervasi *et al.*, 2001)). This could be achieved by using a specific TLR4 antagonist such as Eritoran (Leon *et al.*, 2008). The current study (initially) aimed to identify which of the endogenous molecule stimulates inflammatory response from PE monocytes. However, recruitment of participants in the study groups was a time consuming process, as was analysing the differences between non-stimulated versus stimulated cultures. Thus, further work is suggested to use the same methodology of PE monocyte stimulation with *E. coli*-derived LPS and fibrinogen, with or without TLR4 antagonists. Another problem with this approach is that we used intact fibronectin to stimulate monocyte cultures instead of EDA-containing fibronectin fragment.

Numerous studies revealed an elevation of fibronectin from women with pregnancy-induced hypertension, and it was believed that it was shed off from vascular injuries in those women, but the exact mechanism is still ill-understood (Brubaker *et al.*, 1992, Chen *et al.*, 1994, Thurnau *et al.*, 1987). Also, our results from chapter 7 are consistent with increased fibronectin levels in the circulation of PE women as compared to NP women. Therefore, it will be more interesting if we isolate EDA-containing fibronectin fragments from PE, NP placentae, or vascular endothelium to demonstrate their potential role in stimulating TLR4 signalling; and inhibiting their effects by one of the well-recognised TLR4 antagonists.

Chapter 5: Exploring interactions between vascular endothelial cells and peripheral blood monocytes in normotensive pregnant women and women with PE, using a co-culture experimental model.

5.1 Introduction

As detailed in Chapter 1 Section 4, PE is characterised by an exaggerated systemic inflammatory response (Ahn *et al.*, 2011, Gervasi *et al.*, 2001, Holthe *et al.*, 2004, Luppi and Deloia, 2006, Redman *et al.*, 1999, Sacks *et al.*, 1998, Sibai, 2004) and severe generalised endothelial dysfunction. Numerous circulating markers mediating endothelial cell damage are elevated in PE, such as endothelin, plasminogen activator inhibitor-1 (PAI-1), cellular fibronectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Austgulen *et al.*, 1997, Budak *et al.*, 1998, Heimrath *et al.*, 2004, Higgins *et al.*, 1998, Kim *et al.*, 2004, Krauss *et al.*, 1997, Roberts *et al.*, 1989, Veas *et al.*, 2011, Yinon *et al.*, 2010).

In Chapters 3 and 4, we demonstrated that monocyte subpopulations from women with PE are abnormally skewed and exhibit exaggerated responses to toll-like receptor (TLR) 2 and 4. We also showed that PE monocytes demonstrate an abnormal phenotype as compared to normotensive pregnant (NP) women. PE patients displayed a reduced number of HLA-DR^+ (a MHC class II cell surface receptor) and $TIE2^+$ (a receptor of angiopoietin) monocytes, whereas the number of $CCR5^+$(a receptor of the macrophage inflammatory protein) and $TLR4^+$ monocytes was dramatically increased.

Endothelial cells (ECs) act as a physiological barrier between tissues and blood; and also play a role in the innate immune system by producing and responding to cytokines (Mantovani *et al.*, 1997). It has been shown that human ECs from different sources express TLR4 predominantly, and TLR2 (Faure *et al.*, 2000) weakly. Moreover, TLR4 ligand (lipopolysaccharides; LPS) induces activation of NF-κB by EC (Faure *et al.*, 2000), production of pro-inflammatory cytokines such as IL-6, IL-8, and monocyte chemo-attractant protein-1 (MCP-1), and also up-regulation of adhesion molecules such as VCAM-1 and ICAM-1 (Zeuke *et al.*, 2002, Zhao *et al.*, 2001). Previously, it has been shown that ECs are stimulated by LPS endotoxin to induce tissue factor expression whilst suppress thrombomodulin (Moore *et al.*, 1987). Additionally, LPS stimulates release of PAI-1 by ECs that leads to the inhibition of fibrinolysis (van den Berg *et al.*, 1988). Therefore, LPS alters the vascular endothelium from an anti-thrombotic state to a vigorous pro-thrombotic state. Also, the pro-inflammatory cytokines IL-1 and TNFα have been shown to induce ECs, like LPS, to up-regulate adhesion molecules and

induce the release of inflammatory cytokines and chemokines (Beekhuizen *et al.*, 1991, Mantovani *et al.*, 1992, Swerlick *et al.*, 1992).

During inflammation and injury; the attachment of monocytes to the vascular endothelium is amplified in order to maintain adequate number of monocytes/macrophages in the inflamed tissues. In addition, the monocyte-ECs interaction is influenced by various inflammatory mediators (Issekutz *et al.*, 1981). Many studies have reported that co-culture of monocytes with endothelial cells produces more inflammatory mediators, including IL-6, IL-1β, IL-1α, IL-8, MCP-1, IFN γ-inducible protein 10, and granulocyte-macrophage colony-stimulating factor (GM-CSF)(Kasama *et al.*, 2002, Lukacs *et al.*, 1995, Takahashi *et al.*, 1996b, Ward *et al.*, 2009), and adhesion molecules (Takahashi *et al.*, 1996a) than monoculture of either cell type individually. The Cardiovascular Group at the University of Sheffield Medical School have established primary human monocytes and human vascular endothelial cell (HUVECs) co-culture system, and have examined the inflammatory mechanisms of this system after stimulation with TLR ligands (Ward *et al.*, 2009). They showed that LPS-treated monocyte-HUVEC co-cultures released higher levels of IL-6, IL-1β and CXCL8 cytokines than LPS-treated monocyte or HUVECs monocultures. Moreover, there is evidence to show that monocytes play an important role in the regulation of endothelial proliferation and angiogenesis (Schubert *et al.*, 2008), and that they enhance ECs survival (Noble *et al.*, 1999, Schubert *et al.*, 2011).

Therefore, we postulated that activated "inflammatory" monocytes in PE may impair vascular endothelial cell function, leading to altered chemo-attractant, angiogenic and inflammatory responses. We aimed to compare the inflammatory responses from HUVECs co-incubated with PE monocytes to those co-incubated with NP monocytes. This chapter therefore details our study of the effect of activated monocytes from PE women on endothelial cell function, using a monocyte-HUVEC co-culture model.

5.2 Materials and methods

(For more details, please refer to chapter 2.)

Subjects and samples

Consent for use of umbilical cords was obtained from NP women in early labour in the delivery suite of the Jessop Wing Hospital (n=12). The harvested umbilical cords were used for the isolation of HUVECs.

Women with established PE were diagnosed and recruited as per criteria detailed in Chapter 2 Section 2.1.3, as were healthy non-pregnant controls. Monocyte and HUVEC co-culture experiments were performed using blood samples from nine PE and nine NP women. During a scheduled blood collection, an additional 10ml of fresh blood was collected from each subject with consent, into a tube containing EDTA to prevent clotting. Blood obtained was then immediately taken to the research laboratories at the Academic Unit of Obstetrics and Gynaecology at Jessop Wing for isolation of monocytes, for the co-culture experiment.

Stimulation of monocytes and HUVEC cultures

Human vascular endothelial cells were isolated from umbilical cords, cultured and passaged and used on the second through fifth passages as described elsewhere (Chapter 2 Section 2.2.1.3). HUVECs were seeded onto gelatin-coated, 12-well tissue-culture plates at a density of 10^5 cells per well and incubated with one ml of medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-Glutamine and penicillin-streptomycin) at 37°C with 5% CO_2 until they reached 70-90% of confluence, usually within two to three days. Then monocytes, freshly isolated from peripheral blood of PE or NP women by using the negative magnetic selection method (Chapter 2 Section 2.2.1.2), were plated on top of the HUVEC monolayer at the fractional rate of 1 monocyte to 5 HUVECs. Co-culture assays were performed in DMEM with 5% heat-inactivated FBS without antibiotics media.

Mono- and co-cultures were stimulated with 100ng/ml lipopolysaccharides (TLR4 ligand), 5μg/ml peptidoglycan (TLR2 ligand), 2mg/ml fibrinogen (an endogenous

TLR4 ligand) and 0.7 μg/ml high mobility group box protein (HMGB1) (an endogenous TLR4 ligand), for 24 hours of incubation at 37°C with 5% CO_2. After 24 hours, the conditioned medium was collected and stored at -80°C.

Flow cytometry

Flow cytometry was used to confirm the presence of monocyte and endothelial cell populations in the co-culture experimental models (Chapter 2 Section 2.2.3.3).

Cytometric bead array

The cytometric bead array technique was used to measure the inflammatory cytokines (IL-1B and IL-6), the anti-inflammatory cytokine (IL-0), and chemokines (IL-8 and MCP-1) within conditioned medium collected from monocytes and the HUVECs co-culture. The IL-1B and IL-10 cytokines were measured in the undiluted aliquot (30μl). However, IL-6, IL-8 and MCP-1 cytokines were above standard levels for undiluted aliquots. Subsequently, serial dilutions (1:10 up to 1:200) were attempted to optimise the desired dilution. Thereafter, according to the optimum concentration within the sample, either 1:50 or 1:100 dilutions were applied to the samples, and each sample was multiplied according to its dilution factor before analysis was initiated. For more details, please refer to Chapter 2 Section 2.2.4.

Statistical analysis

The results are illustrated as mean ± standard error of the mean (SEM). The Mann-Whitney *U*-test was used to compare differences between NP monocyte HUVECs and PE monocyte HUVECs co-cultures, using GraphPad Prism software version 5.0. The one way anova (Bonferroni multiple comparison test) was used to compare differences between the un-stimulated cells to different stimulators (bacterial and endogenous ligands of TLRs). P values < 0.05 were considered significantly different. To calculate the fold change of the median fluorescence intensity (MFI), the MFI of LPS-, PDG-, fibrinogen-, or HMGB1- treated was divided by the un-stimulated MFI. To calculate the fold change of concentration (pg/ml), the pg/ml of LPS-, PDG-, fibrinogen-, or HMGB1- treated was divided by the un-stimulated pg/ml.

5.3 Results

5.3.1 Clinical characteristics

Patient characteristics are shown in the table below.

Table 5.1 Patient characteristics

	Normal pregnant (n = 9)	Pre-eclampsia (n = 9)	*P* value
Age (years)	31.6 ± 6.4	31.5 ± 3.6	0.91
Gestational age (weeks)	33.15 ± 4.0	33.18 ± 5.8	0.67
Gravidity	1-4	1-3	
SBP (mm Hg)	114.0 ± 11.0	154 ± 5.8	0.0001
DBP (mm Hg)	70.0 ± 10.0	90.8 ± 2.4	0.0001
24 hour urine collection (g/24hrs)	0	1.6 ± 1.7	
Urine Dipstick Protein Test	0	(1+ - 4+)	

5.3.2 Monocyte and endothelial cell populations exist in the co-culture model

Mono- and co-cultures of HUVEC-monocyte were harvested from the 12-well tissue-culture plate, stained with anti-CD14 and anti-CD146 antibodies and analysed by flow cytometry.

Two populations of monocytes and endothelial cells were recognised according to their sizes, granularity and quantity on the forward scatter (FSC), and side scatter (SSC) plot. As seen in the graph below **(Figure 5.1),** the monocyte population is smaller in size and less granular than the endothelial cell population. It can also be observed that the monocyte population is present in the proportion of approximately one monocyte to five HUVECs.

Additionally, the two populations of cells were gated (G1 is gated monocytes and G2 is gated endothelial cells) and identified according to CD14 and CD146 surface markers, as depicted in the graph below. In the monoculture tube, HUVECs were strongly stained for CD146 and weakly stained for CD14, while the majority of monocytes were strongly positive to CD14 and were weakly stained for CD146. In the co-culture tube, HUVECs were strongly positive to CD146, but monocytes were strongly positive to CD14.

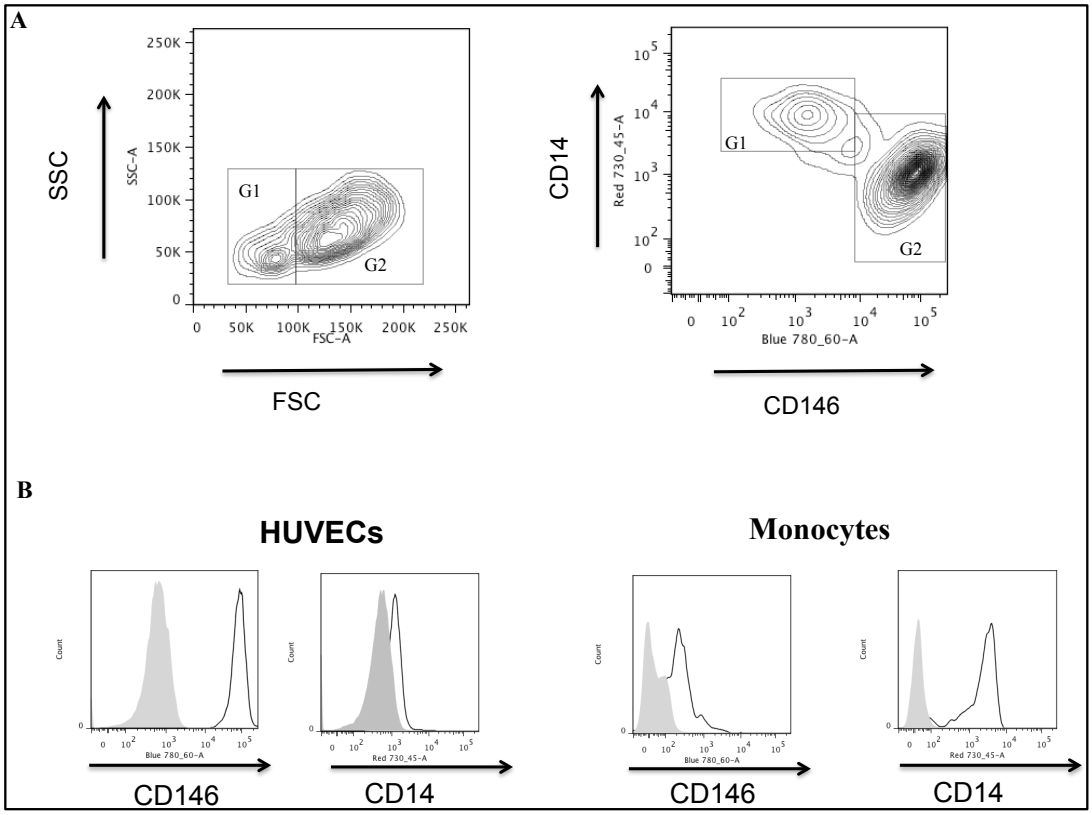

Figure 5.1 Flow cytometry graphs representing monocyte and HUVECs co-culture

A. Flow cytometry pseudo-popular graph of monocyte-HUVECs co-culture (***left panel***-FSC/SSC plot and ***right panel***-CD14/CD146 plot); G1 is gated monocytes and G2 is gated endothelial cells.
B. CD14 or CD146 flow cytometry histogram of gated HUVECs or NP monocytes

5.3.3 Monocytes of PE women alter inflammatory responses of vascular endothelial cells in co-culture experimental model

In comparison with NP, co-cultures of PE monocytes with endothelial cells without any stimulation demonstrated statistically significant, profound secretion of inflammatory cytokine (IL-6) and chemokines (IL-8 and MCP-1) ($P<0.05$), along with declining production of the anti-inflammatory cytokine IL-10 (**Figures 5.2-6**).

Because of higher production of inflammatory cytokines and chemokines at basal levels from HUVEC-PE monocyte co-cultures, demonstration of reponses after culture treatments varied depending on ligand stimulant, and method of representing data (fold-change or as mean concentration). There was no such difference between data obtained and analysed either from pg/ml concentration, or median fluorescence intensity (MFI) for all inflammatory cytokines and chemokines. For example, IL-6 production from LPS- or PDG-treated HUVEC-PE monocyte co-cultures reached maximum levels and was significantly higher compared to LPS- or PDG-treated HUVEC-NP monocyte co-cultures by mean concentrations. However, it did not achieve statistical significance between the two co-culture groups when compared with the fold change owing to differences in basal secretion levels.

Consequently, in the following sections, analysis has been performed for each cytokine or chemokine produced from co-culture experiments with mean concentration, MFI and both fold change.

IL-6

At the basal level, IL-6 was not released by HUVECs monocultures, however after LPS stimulation, endothelial cells significantly produced IL-6 ($P<0.01$). Additionally, non-stimulated monocytes-HUVECs co-cultures from pregnancy groups were significantly released, higher IL-6 levels than monocytes monocultures; particularly with more than10-fold increase from HUVEC-PE monocyte co-culture as compared to PE monocytes monoculture ($P<0.01$). After stimulation with LPS and PDG, IL-6 significantly increased in both NP and PE co-culture models ($P<0.05$) (**Figure 5.2**).

Compared with NP (mean concentration or MFI), TLR bacterial ligands-treated HUVEC-PE monocytes exhibited a marked and significant increase of IL-6, over that seen in TLR bacterial ligands-treated HUVEC-NP monocytes ($P<0.05$). TLR endogenous ligands-treated co-cultures showed no significant difference.

Expressed as a fold-change, TLR bacterial ligands-treated HUVEC-PE monocytes did not appear to differ significantly from TLR bacterial ligands-treated HUVEC-NP monocytes. However, interestingly, fibrinogen treatment produced lower levels of IL-6 from HUVEC-PE monocyte co-cultures, in comparison to HUVEC-NP monocyte co-cultures when using the fold change comparison.

IL-1β

The IL-1β basal level was slightly higher from HUVEC-NP monocyte co-culture than from NP monocytes monoculture. However, in PE basal levels of IL-1β did not differ between mono- and co-cultures. Nevertheless, HUVECs monocultures did not release IL-1β, even after stimulation with TLR ligands (**Figure 5.2**).

LPS, PDG, fibrinogen and HMGB1 significantly induced IL-1β in PE monocytes with or without HUVECs when compared to the non-stimulated control ($P<0.05$). Additionally, LPS, PDG and fibrinogen but not HMGB1, significantly induced IL-1β in HUVEC-NP monocytes when compared to the non-stimulated control ($P<0.05$).

Using all analysis methods (pg/ml concentration, MFI and fold change), fibrinogen-treated HUVEC-PE monocytes exhibited a marked increase of IL-1β over that seen in fibrinogen-treated HUVEC-NP monocytes. However, TLR bacterial ligands-treated HUVEC-PE monocytes did not appear to differ significantly from TLR bacterial ligands-treated HUVEC-NP monocytes.

IL-10

The IL-10 basal level was slightly higher from HUVEC-NP monocyte co-culture than from NP monocytes monoculture. However, in PE basal levels of IL-10 did not differ between mono- and co-cultures. Compared to non-stimulated controls, LPS significantly induced IL- 10 in NP or PE mono- and co-culture models. However,

endothelial monoculture did not release IL-10, either with or without stimulation with TLR ligands (**Figure 5.4**).

In contrast to the above results from IL-6 and IL- β, the anti-inflammatory cytokine IL-10 did not change between TLR ligands-treated HUVEC-PE monocytes and TLR ligands-treated HUVEC-NP monocytes, using any analysis method.

IL-8

The IL-8 basal level was slightly higher from HUVEC-NP monocyte co-culture than from NP monocytes monoculture. Furthermore, HUVEC-PE monocyte co-culture produced extremely higher basal levels of IL-8 than PE monocyte monoculture ($P<0.05$).

Compared to non-stimulated-HUVEC-PE monocyte co-cultures, IL-8 secretion was significantly induced by LPS and PDG, was suppressed by fibrinogen, but it did not change with HMGB1. Yet, in HUVEC-NP monocyte treated co-cultures, IL-8 was significantly induced by all TLR ligands, compared to un-stimulated co-cultures ($P<0.05$). LPS significantly induced IL-8 in HUVECs monoculture compared to untreated endothelial monoculture ($P<0.001$) (**Figure 5.5**).

Compared with treated HUVEC-NP monocytes (mean concentration or MFI analysis methods), LPS *E. coli* and PDG-treated HUVEC-PE monocytes exhibited a marked and significant increase of IL-8 ($P<0.05$). In contrast, fibrinogen and HMGB1-treated co-cultures showed no significant difference between the two co-culture groups. On the other hand, there appeared to be significant reduction in IL-8 secretion when analysis was performed as fold-change for all four TLR ligands-treated HUVEC-PE monocytes, as compared to using fold change analysis on all four TLR ligands-treated HUVEC-NP monocytes ($P<0.05$).

MCP-1

HUVECs and NP/PE monocytes in monocultures did not produce MCP-1. Conversely, when both cell types interacted during co-cultures experiments, there was a significant increase of MCP-1 production, especially from HUVEC-PE monocyte co-cultures ($P<0.001$).

Compared to non-stimulated controls, MCP-1 was released in huge amounts from PDG-treated HUVEC-PE monocytes; it was not produced by LPS-treated HUVEC-PE monocytes; and was suppressed in fibrinogen- and HMGB1-treated HUVEC-PE monocyte co-cultures. However, MCP-1 was induced by TLR bacterial ligands-treated HUVEC-NP monocyte co-cultures compared to non-stimulated controls. Additionally, MCP-1 was significantly exaggerated by LPS from the endothelial monoculture ($P<0.001$) (**Figure 5.6**).

Compared with NP, MCP-1 secretion is highly increased with LPS and PDG stimulation of a HUVEC-PE monocyte co-culture, when analysed as mean concentrations or as MFI ($P<0.05$). However, it did not change after stimulation with fibrinogen and HMGB1. On the other hand, when analysed as fold-change there was a significant reduction of MCP-1 production by TLR ligands-treated HUVEC-PE monocytes (except for PDG), compared to ligands-treated HUVEC-NP monocytes ($P<0.05$).