ASSOCIATION OF PLACENTAL SYNCYTIOTROPHOBLAST MEMBRANE MICRO-PARTICLES (STBM) WITH MATERNAL IMMUNE RESPONSE IN ADVERSE PREGNANCY OUTCOMES.

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Summary

Placental syncytiotrophoblast microvesicles (STBM) are biologically active, membrane-bound, subcellular vesicles shed into maternal circulation during pregnancy as a normal turnover of the placental surface. Quantitative and possibly qualitative STBM changes take place in adverse pregnancy outcomes such as pre-eclampsia (PE). However, the role of STBM in recurrent miscarriage (RM) remains insufficiently explored.

The objective of this study is to evaluate the immunogenicity of STBM in women with RM. Women with PE were studied as a comparison group. Study hypotheses are: i) STBM stimulate antigen presenting cells (APCs), such as dendritic cells (DCs) & macrophages, more in disease compared to health; ii) STBM bioactive contents, e.g. proteins and lipids, differ in disease and health; and iii) decidual DCs at maternal-foetal interface differ in disease and health.

Placentas from patients with a history of PE (n=13) or RM (>2 consecutive losses, <20 weeks) (n=22) and gestational age-matched normal controls were collected at the time of delivery or loss. STBM were generated using placental explant culture and then used in DC and macrophage stimulation assays as well as in mixed leukocyte reactions (MLR). Immune response was characterized by detecting cytokine secretion and changes in surface phenotype. Microvesicle bioactive contents were investigated by mass-spectrometry (ESI LC-MS/MS) of proteins and lipids. Decidual dendritic cell subsets were studied by a 13-colour flow cytometry.

The results show that STBM from RM and PE patients were more immunogenic as evidenced by significantly increased production of pro-inflammatory cytokines such as

IL-6 and TNF-α from stimulated DCs and macrophages, compared to the normal controls. STBM proteins include alarmins (heat shock proteins and fibronectin), stress and immune-regulatory molecules (annexins, integrins, and peroxiredoxins), complement and complement regulatory molecules (C1q, C3, C4, CD46, CD55, CD59 and vitronectin), lipid metabolism regulatory molecules (apolipoproteins A, and E) and transcriptional-regulatory molecules (histones). Among these, 14 proteins were found to be differentially expressed in adverse pregnancies compared to healthy pregnant controls.

Lipidomic analysis revealed that the major lipid classes in STBM are sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidic acid (PA) and phosphatidylinositol (PI). SM/PC ratio showed a unique reversal (3:1) compared to that normally found in human cells. PS was upregulated and PI, PA and GM3 was downregulated in adverse pregnancies compared to healthy pregnant controls.

Three myeloid DC subsets could be identified in human decidua: i) CD14+ DCs; ii) CD11c+ DCs; and iii) $CD141^{hi}$ DCs. One particular patient with 5 previous miscarriages demonstrated a markedly higher frequency of $CD141^{hi}$ DCs (1.48% of $CD45^{+}$ cells), compared to the other RM (0.2%) and the normal controls (0.2%).

Increased immunogenicity of STBM, as well as differential expressions of STBM proteins and lipids that are implicated in inflammation, coagulation, oxidative stress, apoptosis and immune response, suggest their pathophysiologic relevance in RM and PE. Presence of the most efficient antigen presenting cell $CD141^{hi}$ DCs in decidua may have implications in the immune dysregulation of RM.

Collectively, the findings of this study lend support to the emerging concept of immune-dysregulation in adverse pregnancy outcomes.

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List of Abbreviations

Antigen presenting cells	APC
Antiphospholipid syndrome	APS
Apoptotic bodies	AB
Ceramide	Cer
Decidual natural killer	dNK
Dendritic cells	DC
Dithiothreitol	DTT
Endoplasmic reticulum	ER
Enzyme-linked Immunosorbent assay	ELISA
Extracellular matrix	ECM
Fas legand	FasL
Fluorescence activated cell sorting	FACS
Ganglioside mannoside 3	GM3
Gene ontology	GO
Glucocyl-ceramide	GluCer
Granulocyte macrophage colony-stimulating factor	GM-CSF
Heat shock protein	HSP
High-performance thin-layer chromatography	HPTLC
Human umbilical vein endothelial cells	HUVEC
Human leukocyte antigen	HLA
Indoleamine 2,3 dioxygenase	IDO
Immunohistochemistry	IHC
Intercellular adhesion molecule	ICAM
Interleukin	IL
Intravenous immunoglobulin	IVIG
Iodoacetamide	IAA
Lymphokine activated killer	LAK
Multivesicular bodies	MVB
Magnetic activated cell sorting	MACS
Major histocompatibility complex	MHC
Nanoparticle tracking analysis	NTA
National Medical Research Council	NMRC
Natural killer	NK
Peripheral blood mononuclear cell	PBMC
Peripheral natural killer	pNK
Phosphatidylethanolamine	PE
Phosphotidylserine	PS
Phosphatidylinositol	PI
Phosphatidylglycerol	PG
Phosphatidylserine	PS
Phosphatidic acid	PA
Plasmacytoid DC	pDC
Placental alkaline phosphatase	PLAP
Phosphate buffered saline	PBS
Phorbol-12-myristate-13-acetate	PMA
Pre-eclampsia	PE

Red blood cells	RBC
Recurrent miscarriage	RM
Recurrent pregnancy loss	RPL
Recurrent spontaneous abortions	RSA
Sodium dodecyl sulphate	SDS
Sphingomyelin	SM
Standard error of mean	SEM
Syncytiotrophoblast microvesicle	STBM
Systemic lupus erythematosus	SLE
Regulatory T cells	Treg cells
T helper cells	Th Cells
Tissue factor	TF
TNF-related apoptosis-inducing ligand	TRAIL
Tris-buffered saline	TBS
Tumor necrosis factor	TNF
Triacylglycerides	TAG
uracil-N-glycosylase	UNG
Vascular endothelial growth factor	VEGF
White blood cells	WBC
World Health Organization	WHO

Chapter 1: Introduction

1.1 Microvesicles

1.1.1 Definition

Microvesicles are biologically active membrane-bound, sub-cellular elements produced during apoptosis, cellular activation or by vesicle secretion. These have been long viewed as artifacts or inert cellular debris. However, recent intense research interest in the pathophysiological relevance of microvesicles in autoimmune, cardiovascular, thromboembolic and inflammatory disorders has revealed the contrary. Current evidence recognizes their novel role as modulators of intercellular communication and immune function (Morelli etal., 2006, Schiller et al., 2008).

Microvesicles are nano-sized (<1μm) membrane fragments released into extracellular matrix by various healthy and diseased cells (Debski et al., 1991, Thery et al., 2002, Redmanet al., 2008, Lok et al., 2008, Cocucci et al., 2009, Redman et al., 2011). They are sealed by cell membrane lipid bilayer and may contain bioactive materials such as diverse membrane and cytosolic proteins, lipids, signaling molecules, DNA, messenger and microRNAs (Southcombe et al., 2011). There are three main types of sub-cellular vesicles: i) vesicles that bud directly from the cell membrane, ii) exosomes that were generated from multivesicular bodies within the cell, and iii) apoptotic bodies (ABs) that are small sealed membrane vesicles generated by cells undergoing cell death by apoptosis.

1.1.2 Biogenesis and shedding of microvesicles

Microvesicles are found commonly in cell cultures *in vitro* and in body fluids *in vivo*. The shedding process of microvesicles is stimulated by various events including inflammatory stimuli or calcium influx. The process is calpain-dependent (Yano et al, 1993). Examples of inflammatory stimuli facilitating shedding include cytokines such as TNF-α or IL-1β, or laboratory agents such as phorbol ester, ionomycin, Phorbol-12-myristate-13-acetate (PMA), concanavalin A, or activated complement C5a (Distler et al., 2005). Calcium chelation inhibits microvesicle generation. Increased cytosolic calcium activates various cytosolic enzymes relevant to microvesicle formation including calpain, facilitating breakdown of cytoskeleton, activation of apoptosis, and shedding of membrane blebs. The nature of stimulus may determine the type of vesicles generated. Microvesicles are released from viable cells during activation or dying cells during apoptosis by shedding or direct blebbing of the plasma membrane in a regulated way. They range in size between 0.1 to 1 μm (Figure 1.1). Exosomes or nanovesicles are, however, secreted via exocytosis from intracellular multivesicular bodies of the endosome and are much smaller in size (30-100 nm). Apoptotic bodies (500 nm-3 μm in diameter) are released by cells undergoing apoptosis by separation of surface blebs from the cellular remnant (Schiller et al., 2008, Dragovic et al., 2011).

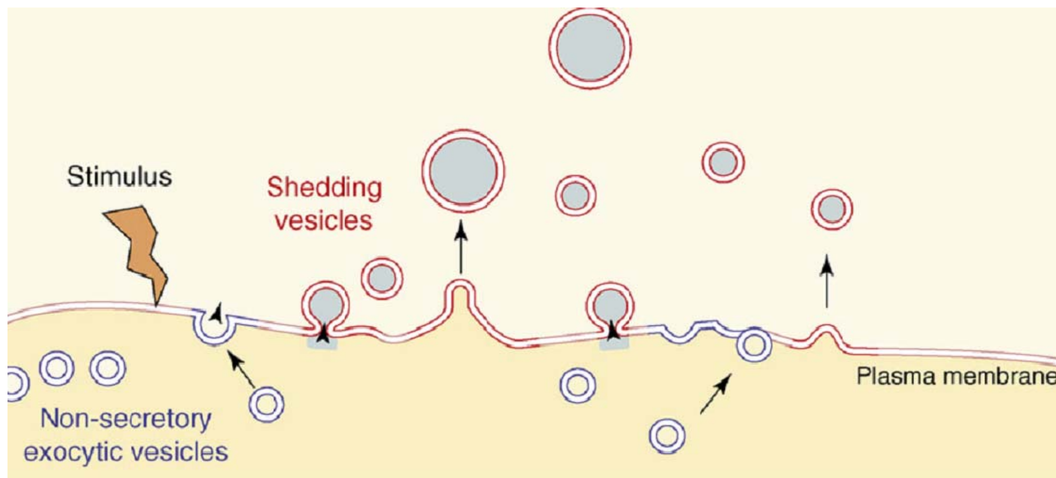

Figure 1.1: Generation of shedding vesicles. Reproduced from Coccucci et al., 2009.

Endosomes produced at plasma membrane fuse to form endocytic cisternae and subsequently multivesicular bodies (MVB) containing numerous 30-100 nm vesicles. There are two types of MVBs; the degradative MVBs that evolve into lysosomes and the exocytic MVBs that fuse their membrane with plasma membrane upon cell stimulation or activation leading to secretion and exposition of their internal vesicles known as exosomes (Murk et al., 2002) (Figure 1.2).

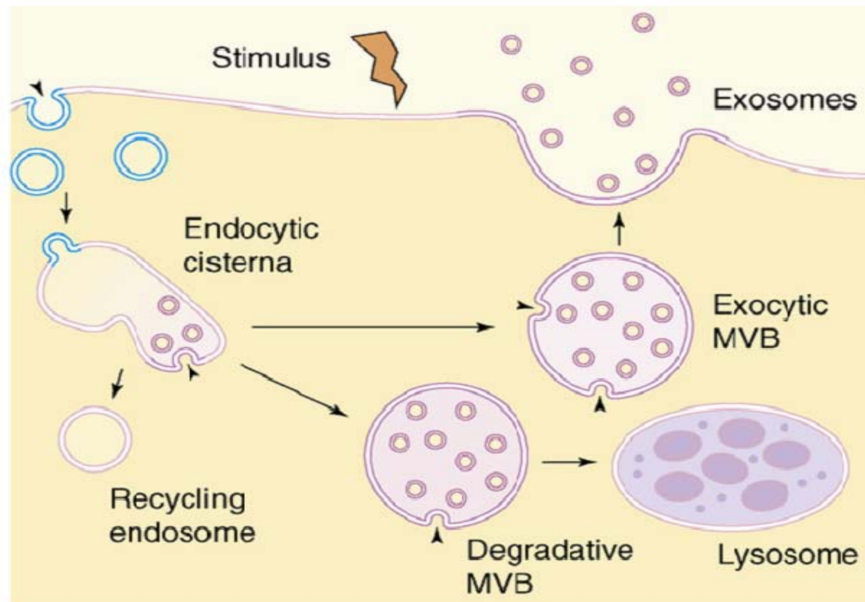

Figure 1.2: Generation of exosomes. Reproduced from Coccucci et al., 2009.

Apoptosis is a highly regulated and energy-dependent process culminating in formation of sealed sub-cellular fragments termed apoptotic bodies (AB) (Cotter et al., 1990) (Figure 1.3). Their formation and rapid engulfment by environmental phagocytes ensure a regulated, anti-inflammatory clearance of apoptotic debris under physiological conditions. If energy depletion occurs during the cascade of apoptosis, the process of cell death deviates to the energy-independent cellular breakdown termed secondary necrosis (Nicotera et al., 2000). An incomplete execution of the apoptosis program followed by degeneration and necrosis has been termed aponecrosis (Formigli et al., 2000). Necrotic debris may lead to a pro-inflammatory response in pathological conditions.

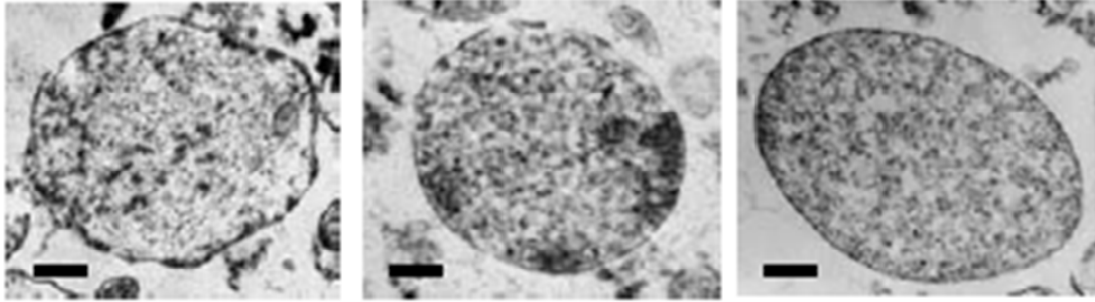

Figure 1.3: Transmission Electron Microscopy of ABs showing membrane-coated vesicles, 500 nm in size. Bar represents 100 nm. Reproduced from Schiller et al., 2008.

1.1.3 Bioactive contents of microvesicles

During apoptosis or activation, cellular components are packed within membrane vesicles or blebs and antigens are relocalised towards the surface of the cells (Radic et al., 2004, Frisoni et al., 2005). Thus, microvesicles seem to be mediators of intercellular communication by virtue of their bioactive contents such as diverse membrane and cytosolic proteins, lipids, nuclear proteins, nucleic acids including messenger and microRNAs, and signaling molecules from the cells of origin.

Protein composition

Proteomes of microvesicles or exosomes isolated from human platelets, plasma, malignant lymphocytes, B cells, dendritic cells, mast cells, and intestinal epithelial cells have been described (Garcia et al., 2005, Jin et al., 2005, Smalley et al., 2007, Little et al., 2010, Miguet et al, 2006, Wubblots et al. 2003, Thery et al., 1999, Thery et al., 2001, Skokos et al., 2001, Van Niel et al., 2001). These proteins are broadly divided into two categories: i) ubiquitous proteins which are produced by almost all cells, and ii) cell-specific proteins which are unique to the cells of origin. Exosomal proteins include

cytosolic, endosomal and membrane proteins. About 80% of exosomal proteins are conserved among species and characteristic protein families include tetraspannins (CD9, CD63, CD81 and CD82), heat shock proteins (HSP70, HSP90), structural/cytoskeletal proteins (tubulin, actin, actin binding proteins), metabolic enzymes, membrane transport and fusion proteins (annexins and RAB proteins), signal transduction proteins (protein kinases, 14-3-3, G proteins), integrins and MHC class I and II molecules (Thery et al., 2002). These ubiquitous proteins are most likely involved in exosome biogenesis (Figure 1.4).

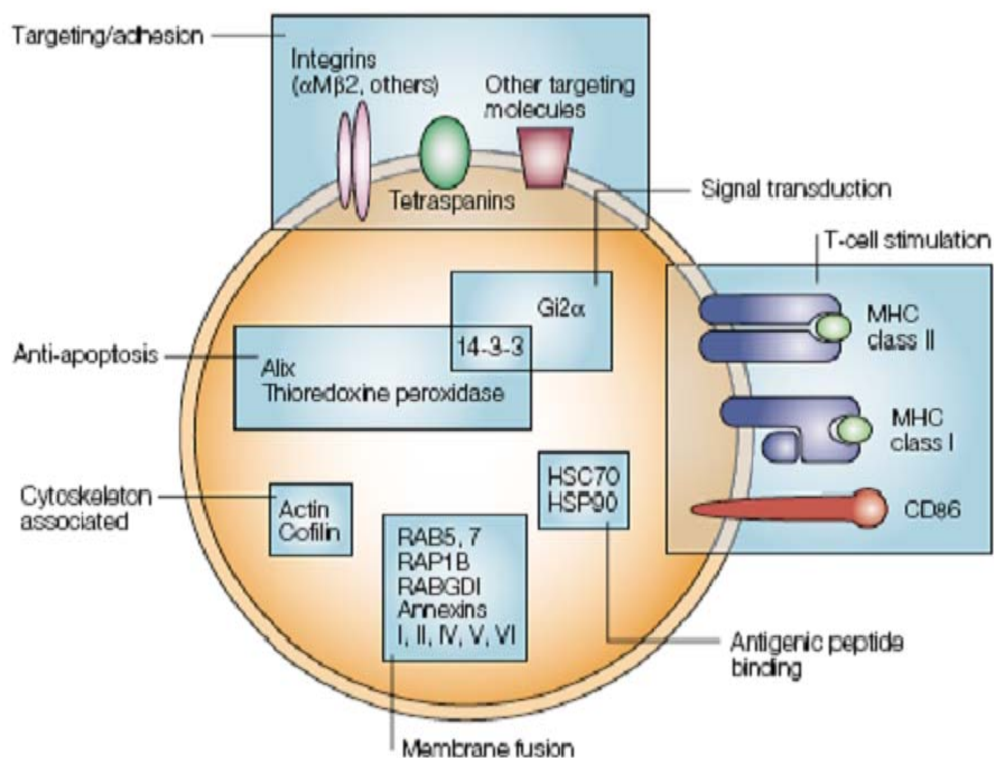

Figure 1.4: Schematic representation of exosomes produced by dendritic cells. Reproduced from Thery et al., 2002.

Examples of cell-specific proteins include MHC class II in exosomes from all cells expressing MHC class II, CD 86 from DC-derived exosomes which is a co-stimulatory molecule for T cells, T-cell receptors for T-cell derived exosomes, immunoglobulin family

members (CD54 on B cells, P-selectin on platelets), etc.. In contrast to exosomes, microvesicles/apoptotic bodies released by apoptotic cells contain nuclear and organeller proteins, DNA, messenger and microRNA. Both exosomes and microvesicles are now considered to provide unconventional routes of protein secretion (Nickel et al., 2005).

Lipid composition

Current evidence on lipid composition of microvesicle population is limited so far. They are encapsulated by a lipid bilayer. A key early event in their mode of formation is reorganization of the lipid asymmetry of the plasma membrane with externalization of phosphatidylserine (PS) which is normally present on the cytosolic side of plasma membrane. Lipid rafts, which are the plasma-membrane microdomains, and their accompanying cholesterol and proteins are concentrated where the membrane buds. Therefore, microvesicles are enriched in raft associated lipids.

Lipids have important roles as signaling molecules in various physiologic processes including immune response. There is increasing evidence suggesting that lipids in microvesicles can elicit cellular responses. For example, platelet-derived microvesicles treated with secretory phospholipase A_2 have been shown to activate endothelial cells and cause platelet aggregation with arachidonic acid being the active principle (Barry, et al., 1997, 1998). Platelet or RBC-derived microvesicles treated with a combination of phospholipase A_2 and sphingomyelinase cause platelet aggregation with lysophosphatidic acid being the active principle (Fourcade, et al., 1995). Endothelial cells exposed to peroxides, release microvesicles containing oxidized phospholipids and activate neutrophils (Patel et al., 1992). Apoptotic blebs and oxidized microvesicles have been shown to contain proinflammatory oxidized phospholipids that stimulate endothelial

cells to specifically bind with monocytes, initiating atherogenesis (Huber et al., 2002). Collectively this evidence on the bioactive content of microvesicles lends support to their emerging role as mediators of intercellular communication.

1.1.4 Functions of microvesicles

Microvesicles can influence physiology of their target cells by inducing intracellular signaling following binding to receptors or conferring new properties by transfer of bioactive materials such as lipids, cytokines and growth factors after fusion or endocytosis (Orozcoet al., 2010, Coccucci et al., 2009, Chaput & Thery, 2010, Thery 2009). It has now become established that apoptotic blebbing and microvesicle release plays a significant role for the interaction of the parent cell and immune system resulting in immunomodulation (Thery et al., 2002). These vesicles may interact with surrounding cells or are engulfed by phagocytes or antigen presenting cells as macrophages, dendritic cells, and B cells which may lead to immunogenic presentation of antigen in an inflammatory context. Facilitation of antigen presentation may lead either to a pro-inflammatory or anti-inflammatory response, such as by exposure of FasL (CD95) and induction of apoptosis in Fas-bearing cells (Figure 1.5).

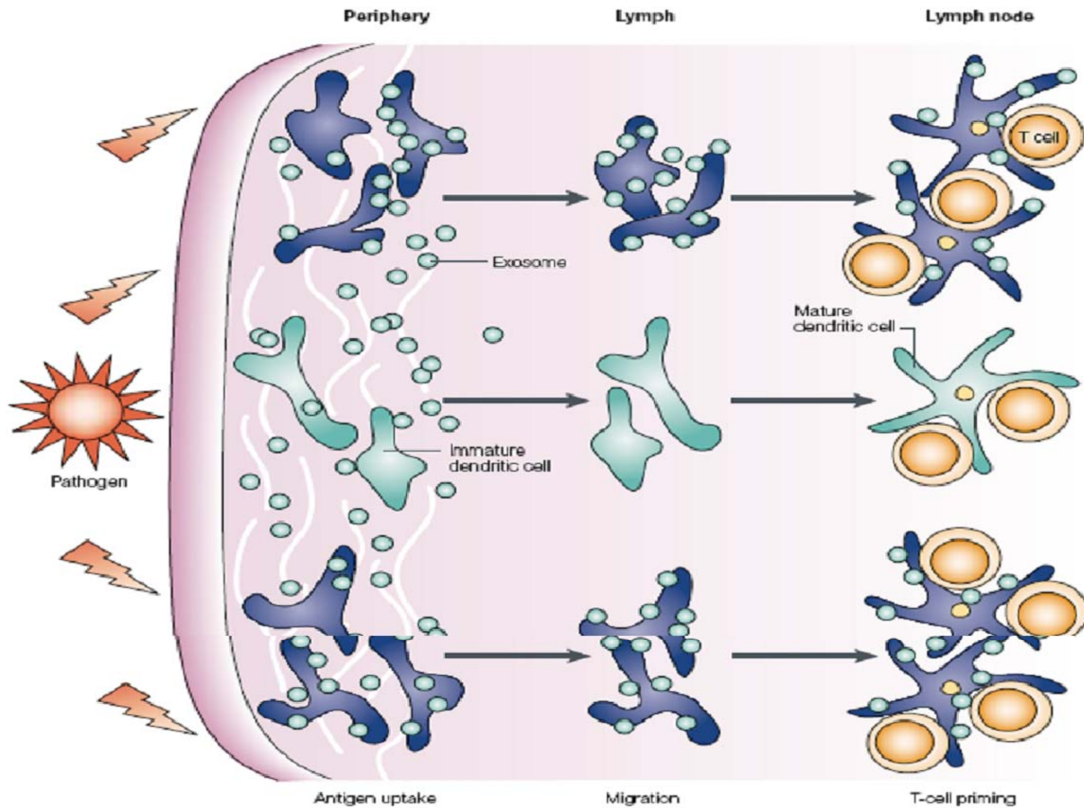

Figure 1.5: Role of exosomes in immune response. Uptake of pathogens in the periphery leads to generation of peptide-MHC complexes by the immature or maturing dendritic cells (DCs) in the tissues (green). Some of these complexes could be secreted in exosomes. This would lead to sensitization of other DCs (blue) which are not exposed to the pathogen directly. Next, all these DCs migrate out of the tissue towards the draining lymph nodes. An exchange of exosomes between the newly arrived DCs and the resident ones could occur inside the lymph nodes. Thus exosome production would lead to increased number of DCs that bear the relevant peptide-MHC complex and subsequent amplification of the magnitude of immune response. In the absence of inflammation, spontaneous migration of exosome-bearing DCs could lead to tolerance induction. Reproduced from Thery et al., 2002.

Microvesicles also participate in physiological processes including hemostasis and thrombosis by exposing binding sites for activated coagulation factors and tissue factor, the initiator of coagulation *in vivo* (Nieuwland et al., 2000, Biro et al., 2003), inflammation (Berckmans et al., 2005), angiogenesis (Kim et al., 2004), vasoconstriction (Boulanger et al., 2001), immune modulation (Peche et al., 2003, Taylor et al., 2006) and endothelial dysfunction (Vanwijk et al., 2002). Their circulating levels, cellular origin, composition

and function have been shown to be altered in various pathological conditions including atherosclerosis and coronary artery disease, pre-eclampsia, hematological and inflammatory diseases, diabetes and malignancies. However, it is unclear whether these changes cause disease development or are a consequence of the disease process itself.

1.2 Pregnancy-Associated Microvesicles

1.2.1 Microvesicles in normal pregnancy

Microvesicles present in plasma of pregnant women originate from blood cells, endothelial cells and the placenta (Figure 1.6).

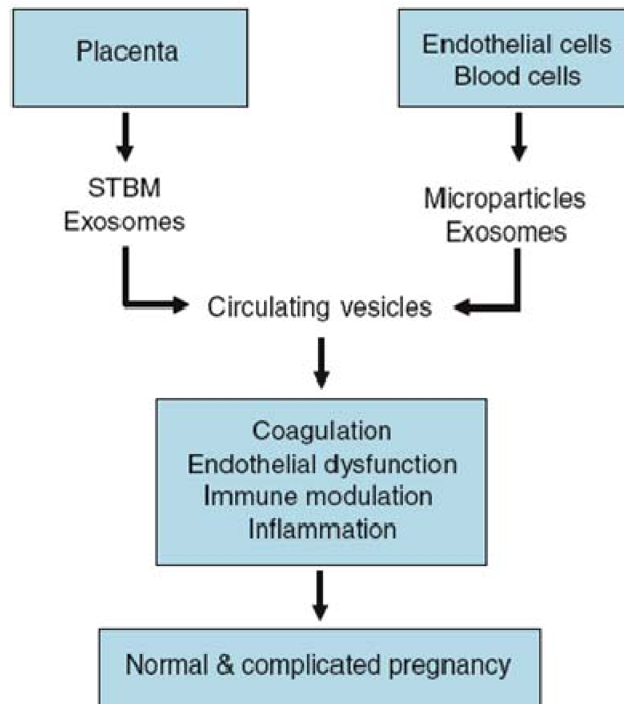

Figure 1.6: Microvesicles in pregnancy and the processes thought to be affected by these vesicles in health and disease. Reproduced from Toth et al., 2007.

The most abundant microvesicles in pregnancy are platelet-derived. Compared to non-pregnant women, increased levels of microvesicles occur in pregnant women (Knight et al., 1998). It is recognized that normal pregnancy is a pro-inflammatory and pro-coagulable condition. Hence the activations of leukocytes and platelets are expected to contribute to this increase in microvesicle population. The presence of placenta-derived syncytiotrophoblast microvesicle (STBM) is unique to pregnancy (Figure 1.7a)

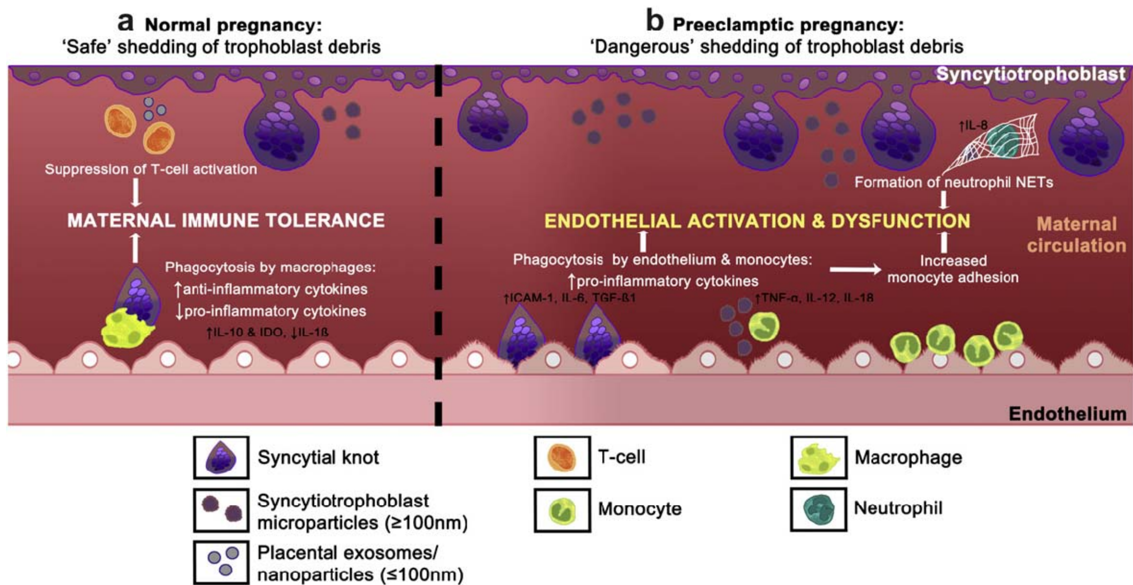

Figure 1.7: (a). Deficient placentation, placental hypoxia & generation of STBM in pre-eclampsia. Reproduced from van der Post et al., 2011. (b). Schematic presentation of known immune & vascular effects of trophoblast debris. Reproduced from Askelund et al., 2011.

STBM are shed from placenta into maternal circulation as a result of the normal turnover and renewal of placental surface and thus they interact with circulating maternal immune cells and endothelial cells (Figure 1.7b) (Askelund et al., 2011). STBM constitutes 1.5-3% of the total microvesicle population in a healthy pregnancy and 2.2-6% in pre-eclamptic pregnancy (Lok et al., 2008). During normal pregnancy, STBM are taken up and cleared by monocytes and dendritic cells, resulting in the production of proinflammatory cytokines such as TNF-α, IL-12 as well as marginal levels of IL-18 and IFN-γ. STBM are also considered physiologically relevant in establishing tolerance at feto-maternal interphase by transporting immunomodulatory bio-molecules from parent syncytiotrophoblast cells such as HLA-G, FasL, TRAIL, IDO, and complement inhibitors (CD46, CD55, CD59). This contributes to the suppression of Th1 responses observed in successful pregnancy. These mechanisms are employed to protect the fetal allograft and allow it to evade detection by paternally-inherited fetal antigen recognizing maternal T

cells. STBM concentration increases with advancement in gestation as the placenta size increases to support the growing fetus.

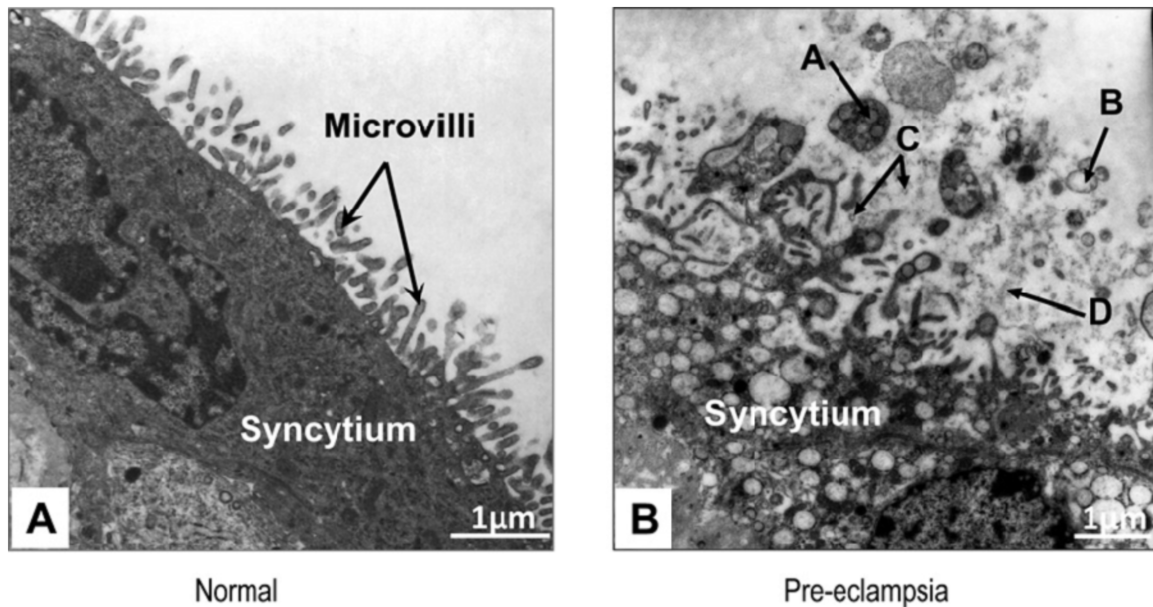

Figure 1.8: Ultra structure of normal and preeclamptic syncytial surface (tissue fixed immediately after delivery). (A) Normal syncytium showing normal microvilli on apical surface (B) Syncytium in severe preeclampsia showing distorted microvilli and shedding of debris, such as, A. apoptotic bodies (1μm), B. microvesicles (100nm-1μm), C. exosomes (70nm-120nm), Reproduced from Redman et al., 2011.

1.2.2 Microvesicles in adverse pregnancy outcomes

In adverse pregnancy outcomes such as pre-eclampsia, recurrent miscarriages with or without association with autoimmune antibodies, premature labour, there is dysregulation of immunomodulation, inflammation, endothelial dysfunction and thrombosis. STBM shedding may undergo changes in these pregnancy complications (Figures 1.8A & 1.8B). The numbers of total and platelet-derived microvesicles are decreased in preeclampsia (PE), however the number of P-selectin exposing platelet-derived microvesicle is increased (Lok et al., 2008, Brettle et al., 2003) reflecting platelet activation (Lok et al., 2007, Maccy et al., 2010). Elevated level of erythrocyte-derived microvesicles has been found in PE as a result of haemolysis and/or

haemoconcentration (Lok at al., 2008). In PE, elevated numbers of microvesicles derived from T cells, monocytes and granulocytes have also been reported reflecting granulocyte activation (Meziani et al., 2006, van Wijk et al., 2002, Lok et al., 2009). Some authors have reported elevated numbers of endothelial cell-derived microvesicles (Gonzalez-Quintero et al., 2003, 2004). Plasma samples from PE patients have shown increased levels of STBM compared to normal pregnancy controls and this is thought to result from placental hypoperfusion and increased trophoblast apoptosis and shedding. Paternal genes are expressed preferentially in the syncytiotrophoblast and immune tolerance towards the fetus requires specific and sufficient suppression and adaptation of maternal immune system. Maternal maladaption to the fetal allograft may lead to increased trophoblast apoptosis and shallow trophoblast invasion resulting in incomplete substitution of maternal spiral arterial endothelium. Resulting deficient spiral artery remodeling, insufficient placentation and placental hypoperfusion leads to ischaemia-perfusion injury and oxidative stress. This probably culminates into increased trophoblast shedding and raised STBM level in maternal plasma (2-6% in PE vs 1-3% in normal healthy pregnancy) (Knight et al., 1998, Redman et al., 2007, Redman et al., 2008). Lok et al showed a decrease in total plasma microparticles (MP) and a statistically insignificant increase in STBM level in PE (Lok et al., 2008) (Table 1). However, the authors reported significant increases in STBM level in some of the preeclamptic patients who did not distinguish themselves clinically from the other patients. Interestingly, in a separate study, statistically significant increase in STBM level was found only in the case of early-onset PE, <34 weeks of gestational age (Figure 1.9) (Goswami et al., 2006).

Circulating MP	Gestational Age						
	12	20	24	28	32	36	pp
Total MP (10^9/L)	–	–	–	2.3* (↓) (1.1–4.5)	3.3 (1.9–6.6)	2.3* (↓) (1.2–5.6)	7.2 (2.6–24.8)
% Platelet-derived MP	–	–	–	95.2 (↓) (90.0–98.8)	95.0*(↓) (92.4–98.4)	93.2 (82.4–98.9)	98.0 (87.1–99.2)
% Erythrocyte-derived MP	–	–	–	5.3* (↑) (1.5–12.3)	2.9 (↑) (0.6–5.8)	4.8 (↑) (0.7–12.6)	1.1 (0.3–12.6)
% T_{helper}-cell-derived MP	–	–	–	0.6 (0–5.3)	0.4 (0–4.3)	< 0.1 (ND)	< 0.1 (ND)
% Monocyte-derived MP	–	–	–	2.1* (↑) (0.9–3.5)	1.3* (↑) (0–3.7)	1.5* (↑) (0.2–3.6)	< 0.1 (ND)
% B-cell-derived MP	–	–	–	< 0.1 (ND)	< 0.1 (ND)	0.3* (↑) (0–0.8)	< 0.1 (ND)
% Endothelial-cell-derived MP	–	–	–	0.6 (0–0.8)	0.8 (0–3.0)	1.2 (0.4–4.9)	0.5 (0–1.1)
% Placental-derived MP	–	–	–	2.2 (1.3–3.3)	3.9 (1.3–10.0)	5.6 (1.8–10.3)	0.9 (0.1–6.2)

MP numbers are presented as median (range (min-max)). The subpopulations are expressed as median percentage of the total number of MP. The subpopulations are expressed as median percentage of the total number of MP. Percentages were calculated for every patient independently. Therefore, the sum of the median percentages of all subgroups approaches 100% and is not exactly 100%. pp = postpartum, ND = not detectable. Differences in the fraction of MP between preeclamptic patients and the normotensive women with a probability value of <0.05 were considered statistically significant (*),↑: elevated or ↓: decreased (total number or fraction of MP) compared with normotensive pregnant women. Granulocyte-derived MP were < 0.1% for all gestational ages. The percentage of $T_{suppressor}$-cell-derived MP was only > 0.1% at 28 weeks (0.3%, range 0% to 0.8%) but this was not significantly different compared with normotensive pregnant women.

Table 1.1. Cellular origin of circulating microparticles (MP) in pre-eclampsia .Reproduced from Lok et al., 2008.

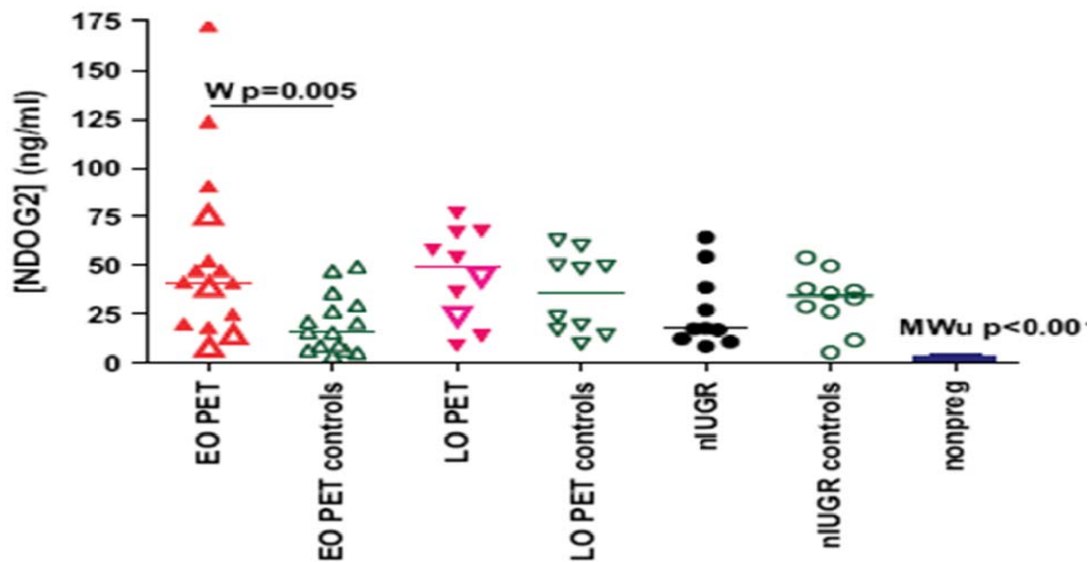

Figure 1.9: Peripheral venous blood concentrations of STBM (NDOG2) in women with early-onset pre-eclampsia (EOPET; open triangles- birth weight <5th percentile for gestational age), late-onset pre-eclampsia (LOPET; open triangles- birth weight <5th percentile for gestational age), normotensive intrauterine growth restriction (nIUGR), normal pregnancy, and non-pregnancy (non-preg). Horizontal bars represent median values Non-pregnancy values significantly lower than all other pregnancy groups. MWu- Mann Whitney *U* test*,* W- Wilcoxon test*.* Reproduced from Goswami et al., 2006.

While STBM in PE is a vibrant area of research, the role of STBM in RM remains yet unclear.

1.2.3 Functions of microvesicles in adverse pregnancy outcomes

Circulating placental microvesicles have the potential to interact with various maternal immune cells. The cargo they carry may provide them with the capabilities to participate in various physiologic ((Figure 1.10) and pathogenic processes (Figure 1.11). Redman et al in their recent review on STBM have emphasized this based on their unpublished observations on STBM proteome (Redman et al., 2011, Tannetta et al., unpublished observations). Proinflammatory effects of STBM may be due to their expression of danger molecules such as Hsp 70 and HMGB1. Procoagulant activity may be due to their expression of tissue factor. Sflt-1 and endoglin expressed by STBM may contribute to endothelial dysfunction while FasL and MICA/B may be responsible for their immunosuppressive or immunoregulatory function regulating maternal NK cell or T cell responses. The expressions of all these proteins may alter in adverse pregnancies compared to normal pregnancies.

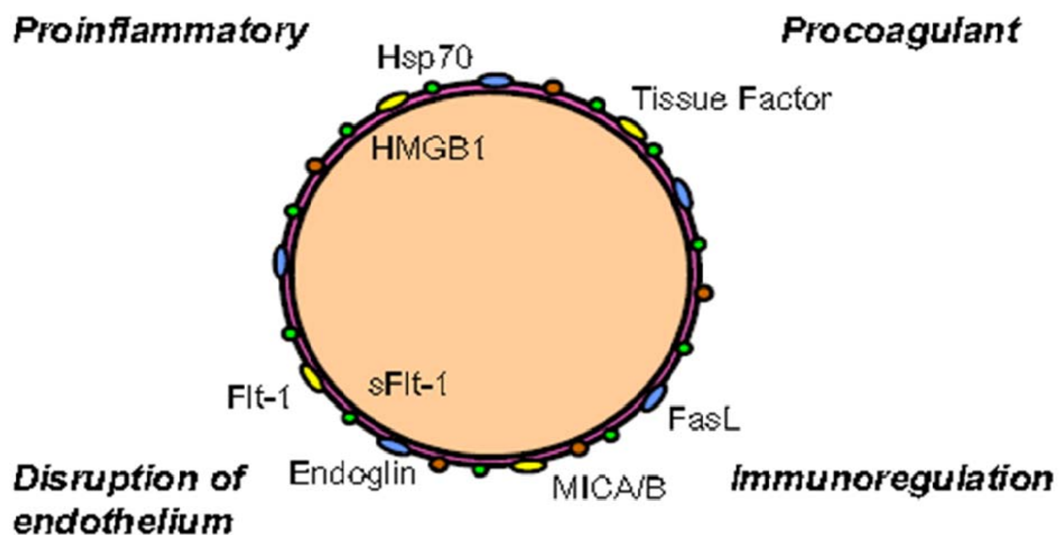

Figure 1.10: Functional activities of STBM and the relevant cargo they carry. Reproduced from Redman et al., 2011.

In a recent report, Rajakumar et al have shown that syncytial knots, shed from placental explants and loaded with sFlt1 protein and mRNA, are metabolically active and capable of *de novo* gene transcription and translation (Rajakumar et al., 2012). The authors speculated that these shedded particles represent an autonomous source of sFlt1 delivery into maternal circulation. This process of microvesicle formation, shedding and appearance in maternal circulation may be a novel mean by which sFlt1 and other toxic proteins such as soluble endoglin may be delivered systemically into maternal circulation. This may subsequently mediate the major events of adverse pregnancy outcomes such as PE. Thus, STBM may contribute to the pathogenesis of adverse pregnancy outcomes such as PE and RM.

Vascular response

Cell-derived microvesicles modulate the several cellular responses *in vitro* as well as *in vivo* (Figure 1.10 & 1.11). Plasma microvesicle samples from PE patients, but not from normal pregnant controls, impair vascular dilatation of myometrial arteries *in vitro* (Vanwijk et al., 2002). Also microvesicles from PE patients induced increased nitric oxide production associated-vascular hyporeactivity of human omental arteries and mouse aortas (Meziani et al., 2006). The proliferation of endothelial cells is inhibited and endothelial cell monolayer is disrupted in the presence of STBM (Gupta et al., 2005, Kertesz et al., 2000, Smarason et al., 1993). However, expression of inflammation-related genes in endothelial cells was found not to be affected by pre-eclamptic microvesicles or STBM (Lok et al., 2006). Nevertheless, supernatants from co-cultured endothelial cells and STBM activated peripheral blood leukocytes such as granulocytes, monocytes and lymphocytes, in vitro (von Dadelszen et al., 1999). This evidence suggests that factors released from endothelial cells in presence of diseased STBM can

indeed affect various cellular responses and thus may play a substantial role in pathogenesis of PE.

A subgroup of women with RM with increased number of procoagulant microvesicles even in non-pregnant state are thought to be in permanent prothrombotic state. These micorvesicles, originated from endothelial cells (Carp et al., 2004), as well as blood cells (Laude et al., 2001), expose phosphatidylserine (PS) and are believed to induce coagulation (Vanwijk et al., 2003). Microparticles also lead to increased expression of adhesion molecules (Barry et al., 1997; Mesri and Altieri, 1998), thus amplifying the procoagulant and/or inflammatory response on the endothelial cell surface. The presence of endothelial and blood cellular microparticles in the interval between pregnancies may be a chronic state of blood vessel and blood cellular activation which only becomes apparent in pregnancy.

The evidence suggests that pregnancy loss may be related to vascular endothelial cell activation.

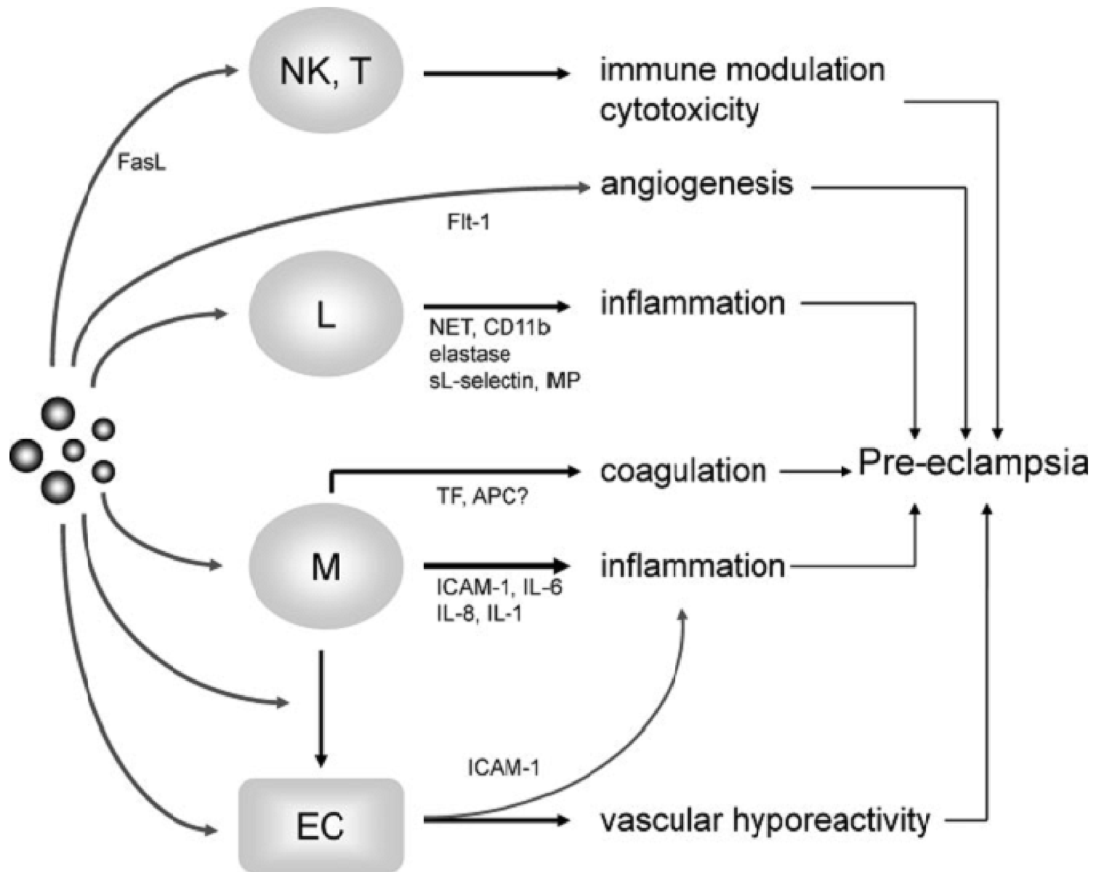

Figure 1.11: Functions of circulating microvesicles in pre-eclampsia . The microvesicles interact with leukocytes (L), monocytes (M) and endothelial cells (EC), thus affecting inflammation, coagulation and vascular reactivity.Reproduced from van der Post et al., 2011.

Haemostasis

There is an exaggeration of the procoagulant and proinflammatory state of normal pregnancy in PE. Microvesicles may contribute to coagulation by providing a membrane surface for activated coagulation factors to bind and by exposing tissue factor (TF), the initiator of blood coagulation. The increased level of plasma coagulation activation markers in pregnancy is further increased in PE and so is the TF activity expressed by STBM shed from preeclamptic placenta (Vanwijk et al., 2002, Gardiner et al., 2011). In RM, increased fibrin deposits are present in intervillus spaces of the placenta suggesting RM may be secondary to an exaggerated haemostatic response of pregnancy (Dolitzky et al., 2006, Rai et al., 2003). This hypercoagulability may be attributed to other procoagulant factors including microvesicles and exosomes (Vanwijk et al., 2003). Coagulation-promoting capacity of circulating microvesicles, based on a prothrombinase assay, in women with RM were found to be increased compared to the non-pregnant controls, and was more pronounced among patients with early losses (gestational age<10 weeks) (Laude et al., 2001). In pregnant mice, injection of artificially prepared procoagulant phospholipid vesicles containing phosphatidylserine induced thrombosis in the placental bed and led to reduced birth weight (Sugimura et al., 1999). Thus, the presence of circulating procoagulant microvesicles in women with RM may be an acquired thrombophilia that becomes manifest during pregnancy when additional factors come into play promoting coagulation. However their precise role in the pathogenesis of adverse pregnancies remains unclear.

25% of both coagulant and anticoagulant activities in blood are associated with platelet-derived microvesicles. Platelet activation not only leads to an increase in its procoagulant phenotype but also its ability to inactivate factor Va by activating protein C

(Tans et al., 1991). PE it is known to be associated with a decrease in the total and platelet-derived microvesicle number (Lok et al., 2008). Resulting insufficient activation of protein C may lead to reduced inhibition of coagulation factors and a net increase in coagulation activation, i.e. both a quantitative and qualitative change in plasma microvesicles may be an inciting factor here.

Inflammatory response

STBM derived from normal placenta are immunogenic and induce secretion of proinflammatory cytokines such as TNF-α, IL-12p70 and IL-18 by PBMCs from healthy non-pregnant women, suggesting their potential contribution in the proinflammatory state of normal pregnancy and possibly PE (Germain et al., 2007). STBM from normal placenta have been shown to stimulate monocytes, upregulate their surface expression of ICAM-1 and induce them to release proinflammatory cytokines such as IL-8, IL-6 and IL-1β (Messerli et al., 2010). Leukocyte activation in PET has been evidenced by increased plasma level of leukocyte activation markers (L-selectin, elastase) as well as increased number of various leukocyte-derived microvesicles and upregulation of NF-ќB (Lok et al., 2009). STBM activate neutrophils, as evident by exposure of CD11b and formation of neutrophil extracellular traps (NETs), present in large numbers in intervillous spaces of preeclamptic placentas (Gupta et al., 2005, Gupta et al., 2006). In contrast, release of cytokines and gene expression by human endothelial cells remained unaffected by PE plasma microvesicles (Donker et al., 2005). Nevertheless, in co-culture with monocytes, increased expression of ICAM-1 could be observed suggesting a complex interaction between microvesicles including STBM, monocytes to which they are mostly bound, and endothelial cells contributing to the proinflammatory state of PE (van der Post et al., 2011).

Complement inhibition

It could be speculated that complement regulatory proteins such as CD46 (membrane cofactor protein), CD55 (decay accelerating factor) and CD59 (membrane inhibitor of reactive lysis) are expressed on STBM since trophoblasts express high levels of these immunosuppressive proteins (Hsi et al., 1991, Holmes et al., 1992). These proteins are known to contribute towards prevention of complement-mediated lysis and protection of the extra embryonic tissues from maternal anti-paternal cytotoxic antibodies. Thus one could speculate that adverse pregnancy outcomes associated with immune-dysregulation could involve reduced expression and altered function of these complement regulatory proteins. Complement activation constitutes an integral part of increased systemic inflammatory response of PE. Increased C-reactive protein exposing microvesicles have been reported in PE compared to normal controls (Biro et al., 2007). The physiologic role of this protein is to bind to phosphotidylcholine on surface of dead or dying cells or bacteria to activate the complement system via C1Q complex. Thus, C-reactive protein exposing microvesicles could contribute to the proinflammatory state of PE.

Angiogenesis

STBM as well as platelet-derived microvesicles in PE, expose transmembrane vascular endothelial growth factor (VEGF) receptor Flt-1 (Lok et al,.2008) It is possible that Flt-1 can be transported to target cells, which then bind VEGF and thus play a role in angiogenesis.

Tolerance and Immunoregulation

The modulation of immune response is among the most important functions of microvesicles in pregnancy and is crucial in maintenance of tolerance. Failure to modulate maternal immune response can lead to placentation failure which in turn may lead to partial or total rejection of the fetus. Circulating placental microvesicles at term express more FasL and are more efficient in suppression of genes involved in T cell apoptosis, compared to those derived from women at preterm labour (Taylor et al., 2006). T cell signaling components such as CD3-zeta, JAK3 and STAT5 are suppressed by placental microvesicles isolated from maternal blood in normal pregnancies (Sabapatha et al., 2006). It is proposed that the inability to suppress such signaling molecules results in a failure to impair maternal T cells as well as NK cells sufficiently leading to pregnancy losses (Sabapatha et al., 2006). Human placental microvesicles express various NKG2D ligands, binding of which to NKG2D receptor bearing cells such as CD8+T cells, NK cells, etc. reduced their *in vitro* cytotoxicity (Hedlundet al., 2009), suggesting a role of STBMs in tolerance of pregnancy. Specific suppression by apoptosis and signaling of maternal cytotoxic T cells and NK cells may be achieved partially by Fas L and HLA-G expression by circulating STBM. There is evidence to suggest that this mechanism may be dysregulated in adverse pregnancy outcomes such as PET and RM. Preeclamptic women's sera induce insufficient suppression of T cell signaling (TCR-CD3 zeta) and their decreased apoptosis compared to that from the normotensive controls, suggesting a deficiency in a serum factor in preeclampsia that may be involved in T cell suppression in normal healthy pregnancies (Whitecar et al., 2001). In contrast to normal uncomplicated pregnancies, sera from women experiencing RM induce increased T cell proliferation, production of Th1 cytokines and decreased T cell apoptosis (Taylor et al., 2004). The extent to which immune-dysregulation and

maternal immune maladaptation to the semi-allogeneic feto-placental graft contributes to the pathogenesis of adverse pregnancy outcomes is still unclear and further investigation into the roles of placental microvesicles in disease etiology and pathogenesis is required.

1.3 Generation, Isolation, and Quantification of STBM

1.3.1 Current limitations

Current methods of measurement of microvesicles are beset with limitations. Microvesicles and exosomes in ultracentrifuged pellets of biological fluids such as cell culture medium, plasma or urine can be demonstrated by electron microscopy. However, this method is not quantitative and requires extensive sample preparation. Western Blotting can determine the expression of known proteins in pelleted materials; however, vesicle numbers or sizes cannot be determined. ELISA assays cannot capture all vesicles and only detect soluble antigens. Conventional flow cytometry cannot detect microvesicles below the 300 nm in size and therefore excludes exosomes (Perez-pojol et al., 2007). Atomic force microscopy has been used recently for studying size distribution and number of $CD41^+$ microvesicles in plasma but this method is highly labor-intensive (Yuana et al., 2010). Dynamic light scattering method determines the size by light scattered from particles under Brownian motion; however, it is biased towards measuring larger particles (Lawrie et al., 2009).

1.3.2 Newer methodologies

Recently, a new method, Nanoparticle Tracking Analysis (NTA), has been described for real-time visualization, determination of size, size distribution, number as well as phenotype (by using fluorescent antibodies) of biological vesicles in suspension. NTA

has distinct advantages over the conventional methods (Dragovic et al., 2011). In NTA, nano-sized particles in Brownian motion suspended in liquid are visualized by light scattering using a light microscope. A video is taken and the NTA software then determines the size and total concentration of particles by relating their Brownian motion to size. The authors evaluated NTA to analyze human placenta-derived microvesicles and human plasma-derived microvesicles. They demonstrated various advantages of using NTA; such as analysis of vesicles by NTA is rapid, and vesicles as small as 30 nm can be measured. Also, surface marker expression to determine their cells of origin and function can be done by using fluorescent antibodies. Thus, NTA extends the power of flow cytometry in the field of research of microvesicles and nanovesicles.

1.4 The immune System

The immune system defends an organism against exogenous microbial infection as well as responds to endogenous danger signals such as tissue necrosis, apoptosis and malignancy. For optimal function, the immune system must recognize a wide variety of pathogens, distinguish them from the self and neutralize them. Disorders of immune system can result in autoimmune diseases, inflammatory diseases, malignancies or even immunodeficiency. The vertebrate immune system can be described into two intimately linked arms, the innate and the adaptive immunity (Figure 1.12).

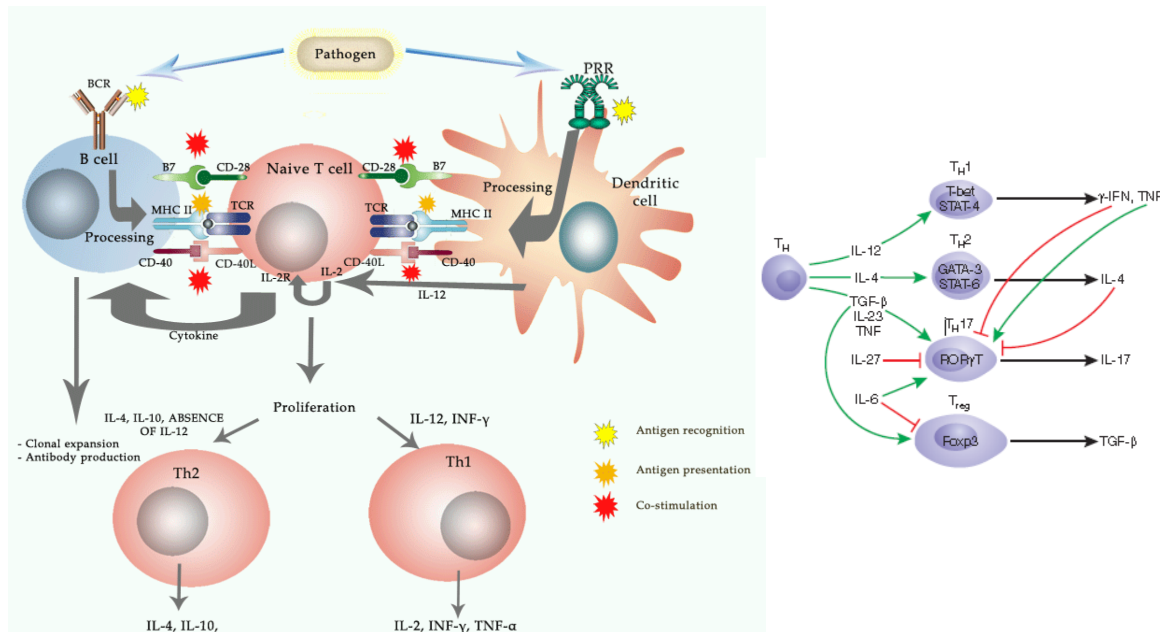

Figure 1.12: Innate and adaptive immunity. Reproduced from igem 2008 & Steinman 2007.

1.4.1 Innate and adaptive Immunity

The innate immune system constitutes the first line of defense against pathogens, reacts rapidly to danger signals, and works in a non-specific manner. The cells of innate immune system include phagocytes (macrophages, dendritic cells, neutrophils), basophils, eosinophils, mast cells and NK cells. They recognize and respond to pathogens in a generic way, but do not confer long lasting or protective immunity to the host.

The major functions of vertebrate innate immune system include:

a. Recruitment of immune cells to sites of infection, through production of chemical mediators known as cytokines
b. Activation of complement cascade to identify bacteria, activate cells, and promote clearance of apoptotic debris
c. Identification and removal of foreign substances by leukocytes
d. Activation of adaptive immune system by the process of antigen presentation
e. Constitution of a physical and chemical barrier against pathogens

The adaptive or specific immune response takes longer time, about 4-7 days, to develop, and is activated by the innate immune system. It provides the organism with immunological memory or the ability to recognize and remember specific pathogens and lasting protection against re-encounters with it. Re-encounters with the specific antigen can result in stronger immune response. The adaptive immune response comprises T-cell mediated cellular immunity and B-cell mediated, humoral or antibody immunity.

The major functions of adaptive immune system include:

a. Recognition of specific non-self antigens in the presence of self, during the process of antigen presentation
b. Generation of responses tailored to maximally eliminate specific pathogens
c. Development of immunological memory whereby each pathogen is remembered by a signature antibody. These memory cells can be called upon quickly to eliminate a pathogen in case of subsequent re-encounters.
d. Mounting stronger immune response against specific antigen in case of subsequent re-encounters.

1.4.2 Cells of innate immune response

The cells of innate immune system include phagocytes (macrophages, dendritic cells, and neutrophils), basophils, eosinophils, mast cells and NK cells. The phagocytic cells of the immune system include macrophages, dendritic cells and neutrophils that engulf or phagocytose pathogens or particles. Once inside the cell, the invading pathogen is contained inside an endosome which merges with a lysosome resulting in death and digestion of the particle. Phagocytes patrol the organism searching for pathogens and are also capable of secretion and reacting to a group of highly specialized molecular signals called cytokines which helps recruitment of other cells to the site of inflammation. Phagocytosis of host’s own cells is part of normal physiological turnover and growth of tissue. Following death of host cells by apoptosis or necrosis, phagocytes are responsible for their clearance prior to growth and development of new healthy cells and thus promote healing following tissue injury.

Macrophages

These are large phagocytic leukocytes capable of moving out of vascular system and entering intercellular spaces in search of invading agents. In tissues, organ-specific macrophages are differentiated from phagocytic cells present in blood such as the monocytes. Macrophages are the most efficient phagocytes. They are capable of generating and releasing reactive oxygen species to destroy pathogen.

Neutrophils

Neutrophils along with basophils and eosinophils are granulocytes due to presence of toxic substance containing granules in their cytoplasm designed to destroy pathogens. Neutrophils are the most abundant phagocytes representing 50-60% of total circulating leukocytes.

Dendritic cells (DCs)

Debdritic cells are phagocytic cells present in tissues that are in contact with external environment such as skin and inner mucosal lining of respiratory system (nose, lungs), gastro-intestinal system and reproductive system (uterus). They are of utmost importance in the process of antigen presentation to T cells and serve as the most efficient antigen-presenting cell (APC), between the innate and adaptive immune system (Banchereau and Steinman, 1998).

In their immature state, the DCs act as sentinels and through effective antigen sampling via macropinocytosis and endocytosis, they sense their macroenviroment for danger signals from pathogens and endogenous sources. On sensing a danger signal, they undergo maturation, mount an immune response leading to inflammation and

subsequent priming of adaptive immunity. The process of maturation is coupled with increased antigen processing and antigen presentation by upregulation of MHC I and MHC II components, upregulation of co-stimulatory molecules such as CD40, CD80 and CD86 and increased cytokine production such as IL-12, IL-6, TNF-α and IL-10.

DCs that have undergone functional maturation migrate to the T cell region of secondary lymphoid organs (Randolph et al., 2005) and are highly efficient at stimulating T cells via 3 distinct signals. These are: a) antigen specific TCR stimulation, b) costimulatory surface signals such as CD80/CD86 stimulation of CD28 receptor on T cells and c) cytokines such as IL-12. DC internalized antigens are degraded, loaded onto MHCII complexes and presented to CD4+ T helper cells that express antigen-specific TCR. Endogenous antigens are processed and loaded onto MHC I complex for priming of cytotoxic CD8+ T cells that express the specific TCR.

DCs can polarize adaptive immunity by inducing specific CD4+ T helper cell subsets (Guermonprez et al., 2002). The three main subsets of T helper responses are Th1, Th2 and Th17 responses. Differentiation of naïve CD4+T cells into Th1 requires IL-12p70 production by DCs (Trinchieri, 2003). Th1 cells produce IFN-γ and TNF-β and Th1 immunity protects against intracellular pathogens and tumors. Th2 cells produce IL-4, IL-5 and IL-13 and are responsible for humoral immunity against parasites. Th2 polarisation requires IL-4 and OX40L (Ito et al., 2005).

Th17 cells have been described more recently. They produce cytokines such as IL-17A, IL-17F and IL-22 and its differentiation is promoted by TGF-β, IL-6, IL-21 and IL-23 (Korn et al., 2009). They are implicated in defense against extracellular pathogens and development of autoimmune and inflammatory diseases.

Dysregulated immune cell activation is detrimental to systemic homeostasis and can lead to disorders of immunity. Regulatory T cells (Tregs) are specialized for immune suppression and they are important in maintenance of peripheral immunological self-tolerance. They suppress effector T cell proliferation and thus can actively downregulate activation and proliferation of self-reactive T cells (Sakaguchi et al., 2008). Naturally occurring Tregs arise in the thymus. T cell activation usually induces a population of Tregs and they are $CD4^{+}CD25^{+}Foxp3^{+}$. In addition to Tregs, DCs with tolerogenic properties exist that are crucial regulators of immunity (Morelli and Thomson, 2007). Immature DCs have been known to be tolerogenic and mature tolerogenic DCs do not express strong stimulatory signals. These tolerogenic DCs can present antigen to antigen-specific T cells but provide inadequate co-stimulatory signals or deliver net co-inhibitory signals for effector T cell activation and proliferation. This may result in T cell anergy, T cell death or Treg generation or expansion. Thus tolerogenic DCs have been shown to suppress autoimmunity (Menges et al., 2002, Verginis et al., 2005).

DC subsets

In the periphery, DCs act as sentinels for pathogens or peripheral self-antigen, then undergo maturation and migrate via lymphatics toward draining lymphoid organs and are categorized as migratory DCs (Wilson et al., 2003). Lymphoid tissue resident DCs are immature in steady state, $CD11c^{hi}CD45^{lo}MHC\ II^{int}$, which can be further described into 2 subsets: CD8+ conventional DC (cDC) and CD8- cDC (Naik, 2008). On maturation they become migratory and are MHC^{hi}.

Plasmacytoid DCs (pDC) resemble plasma B cells at resting state (Liu, 2005), are $CD11c^{int}$, express low levels of MHC I, MHC II and co-stimulatory molecules all of which

are upregulated upon activation. Once activated they produce high amounts of IFN-α and concomitantly acquire DC morphology and function.

In the human blood, DCs are a heterogenous cell population originating from bone marrow precursors and they make up about 1% of circulating PBMCs (Kassianos et al., 2010). Based on CD11c expression, $lin^{-}HLADR^{+}$ blood DC can be described in the $CD11c^{-}$ plasmacytoid (pDC) and $CD11c^{+}$ myeloid DC subsets. pDCs represent about 18% of blood DCs and express CD123, CD303 and CD304. mDCs comprise over 70% of blood DCs and are of 3 types: $CD1c^{+}$ subset (19%), $CD16^{+}$ subset (50%) and $CD141^{+}$ subset (3%) which is the rarest and least studied

Due to rarity of DCs, *in vitro* experiments have mostly relied on DCs generated from human blood monocytes cultured with GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994). A disadvantage of this method is that DCs generated this way are only found *in vivo* following an inflammation (Shortman and Naik, 2007).

1.4.3 Cells of adaptive immune response

T cells and B cells are the major cells of adaptive immune system involved in cell-mediated and humoral or antibody-mediated immune response respectively. T cells recognize a non-self target only after antigens have been processed and presented in combination with a self-receptor called MHC molecule. The major subtypes of T cells are cytotoxic T cell (CD8+) that recognize antigen coupled to MHC I molecule and helper T cell (CD4+) that recognize antigen coupled to MHC II molecule. In contrast, B cell antigen-specific receptor is an antibody molecule on B cell surface and recognizes whole pathogens without any need for antigen processing.

1.4.4 Cytokines

Cytokines are small cell signaling protein molecules secreted by various immune cells used extensively in intercellular communication. Major cytokines of immune system, their sources and functions are summarized in Figure 1.13.

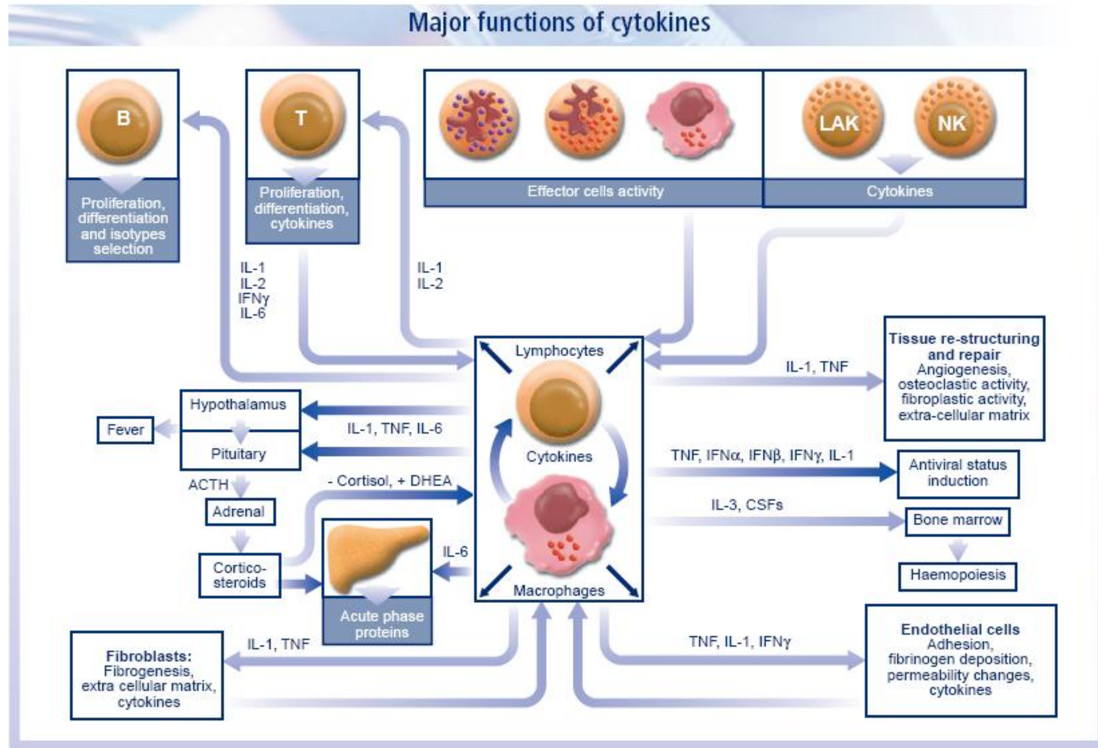

Figure 1.13: Cytokines of immune system. Reproduced from Calier, 2008.

Immunological cytokines can be described into those that enhance proinflammatory cellular responses, type 1 such as IL-6, IL-8,IL-12, IL-1β, and IFN-γ and type 2 (IL-2, IL-4, IL-10, TGF-β, IL-13) which favour an anti-inflammatory humoral response. IL-8, secreted by APCs and epithelial cells is proinflammatory, and serves as a chemical signal that attracts neutrophils to the site of inflammation and promotes angiogenesis. IL-6 acts as a pro-inflammatory cytokine, secreted by DCs, macrophages and T cells. It is among the most important mediators of acute phase inflammatory response and known

to stimulate acute phase protein synthesis, neutrophils and B cells. It is antagonistic to Tregs. However, it is also anti-inflammatory and this effect is mediated through its inhibitory effects on TNF-α and IL-1 and activation of IL-10. IL-12 is secreted by activated B cells, macrophages, and other antigen-presenting cells (APCs), but its production is inhibited by IL-4 and IL-10. Its biological activities include enhancement of cytotoxic T cells and lymphokine activated killer (LAK) cell generation and activation, increased natural killer (NK) cell cytotoxicity, induction of activated T cell and NK cell proliferation, induction of IFN-g production by NK cells and T cells. IFN-γ is known to enhance MHC class I and II expression on nucleated cells and stimulate many of the effector functions of mononuclear phagocytes. TNF-α, a potent endogenous pyrogen, secreted by activated macrophages and other cells, stimulate acute phase reaction such as fever, apoptosis, and sepsis. Recently described, IL-17 is a product of activated T lymphocytes and its biologic activities include stimulation of IL-6 and IL-8 production and enhanced ICAM-1 expression on human foreskin fibroblasts.

1.5 The Immunology Maternal-Fetal Interface in Health and Disease

The immunology of maternal-fetal interface has long been an area of intense research interest. Immunomodulation and establishment of tolerance at this dynamic interface are pivotal to successful coexistence between mother and the feto-placental semi-allograft expressing both maternal (self) and paternal (non-self) genes (Hunt et al., 2006, Mor et al., 2009, Robertson et al., 2010). During normal healthy pregnancy cytotoxic adaptive immune response is diminished, regulatory adaptive immunity is enhanced (Guerin et al., 2009, Lebar et al., 2010), while innate immunity remains intact to continue to provide host defense against infection and facilitate interaction with fetal tissue for optimal placentation (Barrientos et al., 2009, Dekel et al., 2010, Nagamatsu et al., 2010). Perturbations in this complex physiology could lead to adverse outcomes such as PE or pregnancy losses involving partial or complete rejection of the feto-placental graft (Figure 1.14).

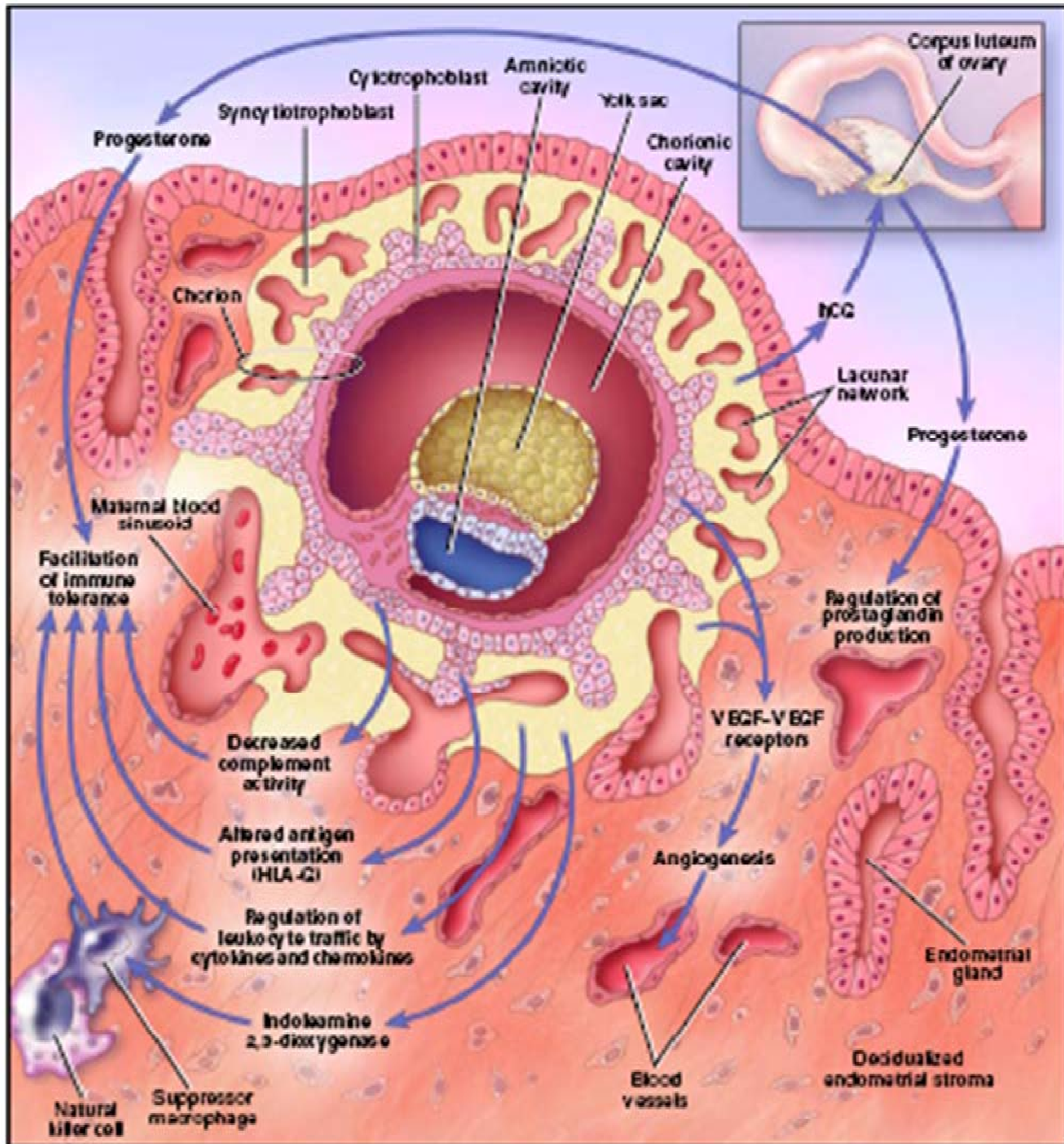

Figure 1.14: Maintenance of early pregnancy. An implanted embryo (approximately 14 days after conception) and the processes necessary for maintenance of an early pregnancy. Reproduced from Norwitz et al., 2001.

Maternal-fetal interface can be described as interface 1 and 2. Interface 1 involves local interactions between maternal immune cells and fetal trophoblasts in the decidua (specialized endometrium of pregnant uterus) at the implantation site that is dominant during early pregnancy. Interface 2 involves interactions between circulating maternal

immune cells and the syncytiotrophoblasts that form the outermost villus surface of the haemochorial placenta. This surface expands with the growth of the placenta for the support of the growing fetus and becomes the dominant interface towards the end of pregnancy engaging systemic instead of local immune response (Figure 1.15)

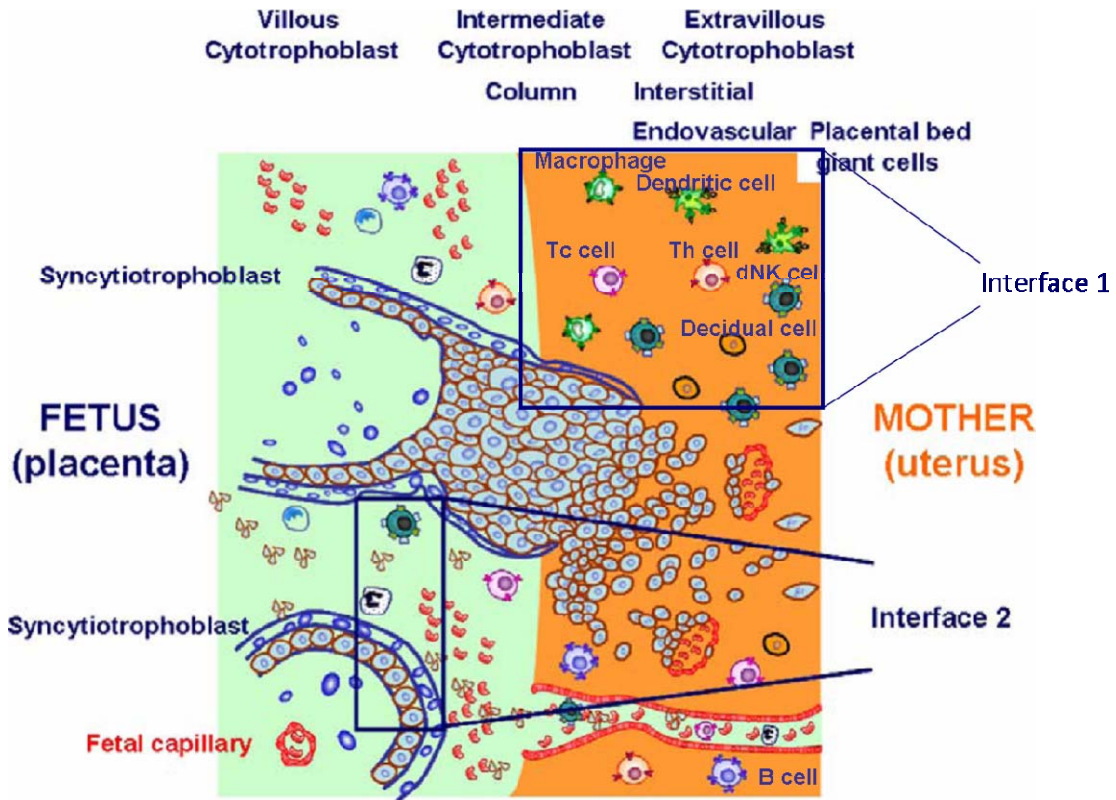

Figure 1.15: Schematic representation of the maternal-fetal interface. When trophoblast invades the deciduas, the immune cells constitute 40% of total decidual cell population and are dominated by mediators' innate immunity, such as decidual NK cells (70%), macrophages (25%) and dendritic cells (1-2%) while T cells (10%) are more sparsely distributed, Reproduced from Weiss et al., 2009.

1.5.1 The defense mechanisms of placenta

The placental trophoblasts are directly exposed to maternal blood and tissues. Uniquely, these fetal trophoblasts evade detection and destruction by maternal immune system programmed to attack non-self foreign antigen bearing cells or tissues. Fetal trophoblasts are derived from external trophectoderm layer of the blastocyst and choose

one of the three developmental pathways: a) remain quiescent as villus cytotrophoblasts (stem cells), b) proliferate and migrate into the deciduas as extravillus or invasive cytotrophoblast cells (forming chorion membrane), or c) they may merge into syncytiotrophoblast cell layer (a thin rim of multinucleated cytoplasm with no cell boundaries) to facilitate diffusion of gases and nutrients from mother to fetus.

Specific protective mechanisms for the trophoblasts include strict regulation of expression of HLA class I antigen (primary stimulators of graft rejection) and complete repression of HLA class II antigen (encode paternally derived foreign HLA class II HLA D). The extravillus cytotrophoblasts do not express HLA class Ia antigen (HLA -A or -B) and instead they express HLA-C and HLA class Ib antigen (HLA E, -F or –G) which may confer them the ability to dampen immune response and cytotoxicity exerted by NK cells, T cells and macrophages (Shakhawat et al., 2010). The syncytiotrophoblasts at the outer most layer of the placental villi, subjected to maximum exposure to maternal cells, express no membrane-bound HLA class 1a antigens. However, there is evidence of presence of HLA class 1b antigens in syncytiotrophoblast (Chu et al., 1998). Neither class of trophoblasts express HLA class II antigen.

Members of B7 family of costimulatory molecules such as B7H1 are uniquely expressed on syncytiotrophoblasts. This protein has lymphocyte inhibitory properties and therefore positioned to interfere with maternal circulatory lymphocyte activation (Petroff et al., 2003). Similarly, apoptosis inducing members of TNF supergene family such as TNF-α, FasL, TRAIL are also expressed on trophoblasts (Phillips et al., 2001) and are pivotal components of a successful pregnancy in order to diminish cytotoxic adaptive immunity. Also trophoblasts contain Indoleamine 2,3 dioxygenase (IDO), an inhibitor of tryptophan metabolism which is essential for T cells. It is therefore believed that IDO may contribute towards T cell inhibition by trophoblasts. The trophoblast expresses high levels of

complement regulatory proteins such as CD46, CD 55 and CD59 which are critically important for its protection against maternal anti-paternal cytotoxic antibodies since complement activation leads to opsonization and destruction of immunologic target (the fetal cells). Immunosuppressive molecules that permeate the pregnant uterine environment such as progesterone from placenta, prostaglandin E2 from resident macrophages and decidual stromal cells and the balance between Th1 and Th2 cytokines driven by progesterone contribute in maintenance of the unique immunomodulation of uterus.

There is evidence to show that the trophoblast secretes active FasL via exosomes and microvesicles (Abrahams et al., 2004, Frangsmyr et al., 2005). Thus, placenta-derived subcellular microvesicles containing an array of above mentioned protective proteins and RNA may play a significant role in immunomodulation critical for normal pregnancy. Their release is increased and composition is altered in pregnancy complications such as PE. Their role in RM remains yet unclear. Investigation into the pathophysiological relevance of placental microvesicles in PE and RM would be interesting and beneficial to the field of reproductive immunology.

1.5.2 Role of the pregnant uterus in local immune adaptation

Maternal immunological adjustment to accommodate the semi-allergenic feto-placental graft during pregnancy includes a significant change in the relative proportions of leukocyte subpopulations of the deciduas. The adaptive immune cells such as the T cells and B cells become scarce. The uterine NK cells population shifts from cytotoxic phenotype of peripheral NK cells (pNK) to cytokine producing benign phenotype decidual NK cells (dNK). Invasive trophoblasts become admixed with dNK cells,

macrophages and dendritic cells reflecting extensive maternal-fetal immunological cross-talk (King at al., 1989, Bulmer et al., 1988, Gardner et al., 2003).

Decidual NK cells have major roles in pregnancy such as facilitation of trophoblast invasion, decidual and placental angiogenesis and uterine vascular modifications. Alterations in decidual NK cell optimum number and activation status may play a role in RM. Decidual macrophages produce immunosuppressive factors which can limit inflammatory responses at maternal-fetal interface and may facilitate vascular remodeling in normal placentation.

The role and phenotype of decidual dendritic cells as the most potent antigen presenting cells is less clear and deserve further investigation. Animal studies have shown that they are critical for successful implantation and remodeling of maternal vasculature. Decidual DCs processing and presenting fetal antigens to the uterine T cells could be tolerogenic; insufficiently stimulating or inhibiting the T cells and thus contribute towards maternal tolerance of the fetal graft. Treg cells population reside and expand in deciduas. In adverse pregnancies such as PE, there are decreased numbers of peripheral and decidual Tregs suggesting their important role in maintenance of tolerance of pregnancy.

1.6 Adverse Pregnancy Outcomes Associated with Immune-Dysregulation (PE and RM)

Maternal immune maladaptation, excessive trophoblast apoptosis, dysregulation of the placental vasculature (anti-angiogenesis), inflammatory and hemostatic imbalance leading to pathological placentation are thought to be the common pathophysiologic mechanisms underlying PE and RM (Alijotas et al., 2009, Li et al., 2009, Matthiesen et al.,2012). PE involves partial rejection of the feto-placental graft with late manifestation (after 20 weeks), while RM represents complete rejection earlier in gestation (prior to 20 weeks) (Taylor et al., 2004) and these clinical outcomes are determined by time of onset as well as magnitude of the initial insult. Hence we were interested to study these two patient groups with adverse pregnancies.

Recurrent miscarriage (RM) is a vexing problem that affects up to 5% of couples trying to establish a family [Roman, 1984]. Recognized risk factors for RM include genetic disorders, structural pathologies, thrombophilias, endocrine and autoimmune diseases as well as environmental factors.

Over the last two decades, there has been increasing evidence that immunologic factors play an important role in RM [Gleicher, 1985; Xu, 1990; Stagnaro-Green, 1990; Maier, 1989; Silver, 1994; Coulam, 1995] and up to 70% of RM cases are associated with immunologic factors. Maternal adaptation of immunological responses to the implanting embryo is crucial for establishment of feto-placental unit. Inappropriate humoral or cellular responses have been demonstrated in patients with RM [Toth, 2010; De Carolis, 2010]. Various humoral abnormalities in RM include presence of anti-phospholipid

antibodies, anti-thyroid antibodies, anti-nuclear antibodies, anti-ovarian antibodies, and increased immunoglobulin M levels.

Increased /inappropriate cellular immune responses in RM (Table 1.2), include increased number and cytoxicity of natural killer (NK) cells in maternal peripheral blood (Kwak-Kim et al., 1995) and endometrium (Clifford et al., 1999), raised Th1 cytokine levels in endometrium (Lim et al., 2000), elevated Th1/Th2 cell ratios in peripheral blood (Kwak-Kim et al., 2003), increased number of IL-17^{+} T cells, reduced numbers and function of Foxp3^{+} regulatory T cells (Treg) in peripheral blood (Lee et al., 2011) and increased proportion of Th17 cells in peripheral blood and deciduas (Wang et al., 2010). Raised systemic and local inflammatory immune responses observed in RM may be induced by, among other factors, a currently emerging subject of much interest, the fetal cell debris including STBM. Defective trophoblast invasion resulting in abnormal uteroplacental perfusion and oxidative stress in PE and RM, may result in qualitative and subsequent functional changes in STBM. The role of STBM in immune-dysregulation of RM remains insufficiently explored and deserves further investigation.

	Normal pregnancy		Abortion		
	Peripheral blood	Uterus	Peripheral blood	Uterus	Depletion of Th1, Th2,Th17 or Treg cells
Th1 cells	↘	↓	↗ →	↑ →	Abortion is not observed.
Th2 cells	↗	↑	→	↓ → ↑(conflict data)	Abortion is not observed.
Th17 cells	→ ↘	↗	→ ↗	→ (missed abortion) ↑ (inevitable abortion) ↑ (recurrent abortion: inevitable abortion)	There is no data, but IL-17 null mice are fertile.
Treg cells	↑	↑↑	→	→	Abortion and implantation failure are observed in allogeneic pregnancy.

→ : no change, ↗: slightly elevate, ↑: elevate, ↑↑: markedly elevate, ↘: slightly decrease, ↓: decrease.

Table 1.2. Th1/Th2/Th17 and Treg cells in normal pregnancy and miscarriage. .Reproduced from Saito et al., 2010.

RM is defined as three or more consecutive miscarriages and generally, evaluation is performed after the third miscarriage. However, the physician may initiate work-up following two miscarriages, particularly in the setting of prior euploid miscarriages, advancing maternal age and concomitant infertility (Stephenson et al., 2007). This is why women with history of 2 or more consecutive miscarriages with all or some of the aforementioned factors were also recruited in our study.

Several features suggest that RM is a distinct clinical entity: (1) a woman's risk of miscarriage is directly related to the outcomes of previous pregnancies, (2) the observed incidence of RM (1%) is much higher than that expected by chance alone (0.34%), (3) unlike sporadic miscarriage, RM tends to occur even if the foetus is euploid, (4) RM seems to affect women with specific reproductive characteristics (Table 1.3).

	Number of women	
	(n=500)	(%)
>3 miscarriages	500	100
Conception delays/fertility investigations	160	32
Late miscarriage (second trimester)	109	22
Stillbirth or neonatal death	31	6
Ectopic pregnancy	23	5
Abortion for fetal abnormality	8	2
Previous livebirth	223	45
Pregnancy complicated by prematurity or fetal growth restriction	98	20

Table 1: Reproductive characteristics associated with poor pregnancy prognosis and recurrent miscarriage[15]

Table 1.3. Reproductive characteristics associated with poor pregnancy prognosis and RM. .Reproduced from Rai et al., 2006.

In early 2012, Beaman and colleagues introduced the term RSAI (recurrent spontaneous abortion of immunological origin) and defined it as the loss of two or more consecutive pregnancy losses with same partner prior to 20-28 weeks of gestation where endocrine,

anatomic, genetic and infectious causes have been ruled out (Beaman et al., 2012). This group of patients are the focus of our research interest in this study.

Current and future treatment options in RM associated with immunologic factors are summarized in Table 1.4, which are primarily antithrombotic, anti-inflammatory and immunosuppressive. Immune-regulators have been proposed recently which may have protective effects on trophoblasts, thus facilitating appropriate trophoblast differentiation and invasion and finally optimal placentation. To date the treatment options in RM of immunologic origin remain at best, limited and empiric. None of the treatment through randomized control trial has proven effective yet. It has been argued that when patients are selected for poor prognosis, namely those with positive immune tests, elevated NK cells or a greater number of miscarriages, the benefit of immunotherapy could be significant.

Treatment options
Tender-Loving-Care
Anticoagulants
Low molecular weight heparin
Acetylsalicylic acid
Hormones
Levothyroxine
Progesterone
Corticosteroids
'Immune-regulators'
Granulocyte–macrophage colony-stimulating factor
Human chorionic gonadotropin
M-CSF
Intravenous immunoglobulin
Lymphocyte Immunotherapy
Anti-TNF-α

Table 1.4. Present and possible future treatment options in RM. Reproduced from Matthiesen et al., 2012.

1.7 Summary of Introduction

Placental syncytiotrophoblast microvesicles (STBM) are membrane-bound, biologically active, subcellular vesicles shed into maternal circulation during pregnancy as normal turnover of the placental surface. They have emerged as novel vehicles of intercellular communications at maternal-fetal interface. Quantitative and possibly qualitative STBM changes take place in adverse pregnancy outcomes such as pre-eclampsia (PE). However, the role of STBM in recurrent miscarriage (RM) remains unclear. There has been increasing evidence in recent years that immunologic factors, both humoral and cellular, play an important role in RM. Raised systemic and local inflammatory immune responses observed in RM may be induced by the fetal cell debris including STBM. The role of STBM in immune-dysregulation of RM remains insufficiently explored. Investigation into the pathophysiological relevance of placental microvesicles in RM would be interesting and beneficial to the field of reproductive immunology. In the context of RM, interactions between STBM and antigen presenting cells, DCs and macrophages have not been investigated. Knowledge regarding the effect of STBM stimulation of DCs and macrophages bridging innate and adaptive immunity would provide an insight in to the immunopathogenesis of RM. Similarly, composition and differential expression of STBM protein and lipid are not known to date and determination of these biologically active contents of STBM would be helpful to unravel pathways involved in pathogenesis of RM. Finally, the roles decidual DCs in health and disease have not been sufficiently explored yet. An elaborate investigation of phenotype and functions of different decidual DC subsets in RM would elucidate *in vivo* alterations of this most efficient antigen presenting cell population and contribute towards a better understanding of RM.

1.8 Aims of the Study

Biologically active, placental STBM are considered novel vehicles of intercellular signaling in normal as well as adverse pregnancies. At the highly dynamic maternal-foetal interface, STBM are in constant contact with maternal immune cells, including the DCs and macrophages bridging innate and adaptive immunity.

The aim of this study is to explore immunogenicity of STBM in the context of RM where it may have pathophysiological relevance similar to PE. Women with PE were studied as a comparison group. Specific aims of this study are to:

i) Investigate whether STBM stimulate DCs & macrophages more in disease compared to health,
ii) Identify STBM bioactive contents, e.g. proteins and lipids, and determine whether they differ in disease and health, and
iii) Understand the distribution and role of decidual DC subsets at maternal-fetal interface in disease and health.

1.8.1 Investigate whether STBM stimulate DCs & macrophages more in disease compared to health

STBM are known to be proinflammatory. To date, most studies from this field have focused on peripheral blood mononuclear cells (PBMC) or monocytes stimulation. As antigen presenting cells (APC) uptake, process and present the foetal antigen to T cells, we decided to investigate STBM (normal and pathogenic) stimulation of DCs and macrophages. Cytokine production and changes in surface phenotype were studied.

1.8.2 Identify STBM bioactive contents, e.g. proteins and lipids, and determine whether they differ in disease and health

STBM are biologically active by virtue of presence of proteins, lipids, DNA and RNAs. However, the basis of their proinflammatory, procoagulant and anti-endothelial function is not known. We investigated STBM (normal and pathogenic) protein and lipid composition by mass spectrometric approach.

1.8.3 Understand the distribution and role of decidual DC subsets at maternal-fetal interface in disease and health

Optimum maternal immune adaptation to the developing semi-allogeneic feto-placental graft is pivotal to normal pregnancy outcome. However, the role of decidual DCs in immunedysregulation in RM remains insufficiently explored. We investigated the role of decidual DC subsets in women with RM. We used 13-colour flow cytometry to characterize decidual DC subsets.

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Chapter 2: Materials and Methods

2.1 Subjects and Sample Collection

The cases were patients with PE or history of RM. RM was defined as 2 or more consecutive pregnancy losses prior to 20 weeks of gestation where anatomic, infectious, chromosomal and endocrine causes were ruled out. Preeclampsia was defined as increased blood pressure (≥140/90 mm Hg) that occurred after 20 wks of gestation in previously normotensive women, accompanied with proteinuria ≥0.3g/day. The control group was gestational age-matched subjects (within two gestational weeks, similar mode of delivery) with no apparent related pregnancy complications.

Placental samples and peripheral blood samples were collected at the time of delivery or loss. Decidual tissues (decidua basalis) from central placental bed were collected following Caesarean delivery. All samples were processed within 2 hours. All samples were collected from the National University Hospital affiliated to the National University of Singapore. The research protocol was approved by the Domain Specific Review Board.

2.2 Cell Biology Techniques

2.2.1 Placental microvesicle (STBM) preparation

2.2.1.1 In vitro villous explant culture

Placental basal plate/decidua was carefully removed to access placental villous tissue. Three areas/cotyledons of 50 cents coin size were randomly sampled and rinsed in ~1L ice cold sterile PBS until blood-less, further cut into smaller pieces weighing ~200 mg, rinsed in PBS again. One such piece was distributed to each 100 mm culture dishes and then finally cut into explants/fragments of 5 mm. Completed medium (DMEM-F12 supplemented with 1% antimycotic/antibiotics, 10% heat-inactivated fetal bovine serum) was placed in hypoxic incubator to equilibrate to 8% O_2 for at least 3 hrs or preferably overnight. After equilibration, Flasks were parafilmed to cover filtered cap and kept in fridge to become ice-cold prior to adding to explants. Explants were cultured in 100 mm culture dishes in DMEM-F12 supplemented with 1% antimycotic/antibiotics, 10% heat-inactivated fetal bovine serum for 72 hrs for generation of STBM at 37^{0}C in 8% oxygen in the hypoxic incubator (Galaxy 48R CO_2 incubator with O_2 control 0.1 to 19% range). 40-50 explants in 15ml complete medium were set per culture dish and 6 culture dishes were set per sampling region per sample. At the end of culture, the supernatant was collected and frozen. The villous explant samples were snap-frozen for additional analysis and formalin-fixed (4% formalin in 1XPBS) for paraffin sectioning. All frozen samples were stored at -80^{0}C until further analysis. Fresh tissue was also preserved similarly for comparison purposes.

2.2.1.2 Isolation and characterization of STBM

STBM was isolated by differential centrifugation of explant culture supernatant (Gupta et al., 2005). Briefly, 30 ml culture supernatant was subjected to centrifugation at 1000 g for 10 min (for separation of whole cells), 10,000 g for 10 min (for separation of large cellular organelles, such as mitochondria, golgi, ER, etc.) and finally, 100 000 g for 60 min at 4^0C using Beckman polyallomer centrifuge tubes and SW 28 rotor, Beckman (for collecting subcellular membrane microparticles). The final pellet was collected, washed in 1.5 ml PBS at 7000 g for 10 min and resuspended in 0.5 ml sterile PBS and stored at -20^0C until use. The protein content in each STBM preparation was quantified by Bradford assay (Pierce, Rockford, IL) after lysis in RIPA lysis buffer with protease inhibitor cocktail for 30 min on ice. Typical STBM preparations yielded 0.2-0.4 mg/ml vesicles. STBM size distribution and concentration was determined by Nanoparticle Tracking Analysis (NTA) method (Dragovic et al., 2011). STBM samples were subjected to further evaluation by flow cytometry using trophoblast-specific marker placental alkaline phosphatase (Abcam, Cambridge, UK), apoptosis marker Annexin V (Invitrogen, Camarillo, CA), quantitative real-time PCR for detection of fetal gene SRY and finally scanning electron microscopy (SEM), described in details under sections 2.3 and 2.4.

2.2.2 Cell culture

2.2.2.1 Isolation of monocytes from human buffy coats

Peripheral blood leukocytes from healthy donors, in the form of buffy coat, were obtained from the National University Hospital Blood Donation Centre, Singapore with Institutional ethics approval. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. The cells

were plated for 2 hr at 37^0C followed by removal of non-adherent cells, mainly lymphocytes. The adherent fraction, mainly monocytes, was harvested. Monocytes isolated by this method were >90% pure, based on the expression of CD14 as judged by flow cytometry.

2.2.2.2 In vitro culture of monocyte-derived DC and macrophages

The isolated monocytes were cultured at a density of 1.5 x 10^6/ml in RPMI containing 10% (v/v) bovine calf serum (BCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1mM sodium pyruvate and 0.0012% (v/v) β-mercaptoethanol. The cells were cultured for 6 days in the presence of 20 ng/ml of GM-CSF (R & D Systems Inc, Minneapolis, MN) and 40 ng/ml of IL-4 (R & D Systems Inc) to differentiate the cells to DCs. Differentiation of monocytes to macrophages was performed by adding 20 ng/ml of M-CSF (R & D Systems Inc).

2.2.2.3 DC and macrophage stimulation

Differentiated cells were harvested. Cell stimulations were performed in triplicates at a density of 0.7X10^6 cells/ml. For flow cytometry, 1X10^6 cells/ml were seeded per stimulation. 100 μg/ml of STBM were used to stimulate cells for 24 hrs at 37^0C in 5% CO_2. LPS/IFN-γ stimulation was used as positive control, while unstimulated cells, as negative control. At the end of incubation period, cultured supernatants were collected and frozen at -20^0C until assayed.

2.2.2.4 Total, naïve and memory $CD4^+$ T cell isolation

Non-adherent PBMCs following monocyte adhesion, were either processed fresh for T cell isolation, or stored at 4^0C overnight in RPMI supplemented with 20% BCS for T cell

isolation the next day ($CD4^+$ T cell isolation kit II, naïve $CD4^+$ T cell isolation kit and memory $CD4^+$ T cell isolation kit, Miltenyi Biotec, Germany), following manufacturer's instructions.

2.2.2.5 Mixed Leukocyte Reaction (MLR)

For MLR, 0.1 ml T cells ($2x10^6$/ml) were co-cultured for 7 days with 0.1 ml DCs or macrophages ($2x10^5$/ml) in activation medium, in the presence or absence of STBM. The culture supernatant was collected and IFN-γ, IL-10 and IL-17 were measured by ELISA.

2.3 Molecular Biology Techniques

2.3.1 DNA extraction and qrt-PCR of STBM

350 µl STBM suspension (0.2-0.4 µg/µl) were used for automated DNA extraction by EZ1 Bio Robot (Qiagen) using the DNA Blood protocol, EZ1 DNA Blood Mini Kit reagent cartridges with elution volume 50 µl. Real-time PCR reactions were performed in triplicates for each sample in total reaction volume of 25 µl with the following components: TaqMan PCR Master Mix (Applied Biosystems)-12.5 µl, forward & reverse primer- human beta globin gene HBB (100 µM each)-0.06 µl, forward & reverse primer- human male gene SRY (100 µM each)-0.11 µl, TaqMan probe (50 µM) FAM – 0.05 or 0.11 µl, DNA template- 5 µl, nuclease-free water- to 25 µl final reaction volume. The reaction mixtures were run on the ABI Systems 7000 Real-Time PCR machine using the *absolute quantitation method.* The reaction conditions were as follows: carryover decontamination via uracil-N-glycosylase (UNG) for 2 min at 50^0C, AmpliTaq Gold pre-activation for 10 min at 95^0C, 35 cycles of melting step for 15 sec at 95^0C and combined annealing and extension step for 1 min at 60^0C. Corresponding calibration curve/standard curve for house keeping gene and target gene were run in parallel with each analysis. Absolute concentrations of the target gene DNA were expressed as copies/mg of STBM.

2.4 Imaging, Protein and Lipid Chemistry Techniques

2.4.1 Nanoparticle Tracking Analysis (NTA)

NanoSight LM10 system (NanoSight Ltd., Amesbury, UK) was used for real-time visualization of STBM and determination of their size distribution as well as concentration as previously described (Dragovic 2011). Briefly, 300 µl of diluted STBM suspension in double filtered PBS (1:200) was loaded onto the sample chamber and a video of 30 seconds duration was recorded, with a frame rate of 30 frame per second. Brownian movement of each particle was tracked and measured. In NTA method, the velocity of particle movement is used to calculate particle size. Particle movement as captured in the video was then analysed by NTA software to give a distribution of vesicle size and concentration at each vesicle size.

2.4.2 Immunofluorescence labeling and flow cytometry of STBM

Annexin V (AxV)-PI Assay- 500 ul STBM suspension isolated from 30 ml culture SN was washed in PBS (X2), resuspended in 1XAxV binding buffer (0.2-0.4 µg/µl), and aliquoted 100ul per tube. 5ul AxV and 10ul PI buffer were added in, followed by incubation at room temperature for 15 min in the dark. 400ul AxV binding buffer was finally added, and subjected to flow cytometry within an hour. 10 000 events were read.

Placental Alkaline Phosphatase (PLAP) Assay- 500 ul STBM (0.2-0.4 µg/µl), was washed in PBS (X2), resuspended in 1XPBS, aliquoted 100ul per tube. Labeling was performed with primary antibody (PLAP Mo anti-human) 100ul, 1:100 dilution (10ug/ml), incubated at 37^0C, for 30 min in the dark. 200ul PBS was added. STBMs were washed

at 13000 rpm for 10 min, followed by incubation with secondary antibody (Alexa-Fluor 488 FITC Conj Goat Anti-Mo), 100 ul, 1:100 dilution for 30 min at 4^0C. STBM were then washed in PBS, resuspended in 250-500ul PBS and finally subjected to flow cytometry within an hour. 10 000 events were read.

2.4.3 H/E & immunohistochemical staining of placental syncytiotrophoblast layer

4% buffered formalin-fixed placental villi were embedded in paraffin and 5 µm thick sections were prepared. After deparaffinization with xylene, rehydration with decreasing concentrations of alcohol and fixation with additive methanol, slides were stained in eosin for 1.5 minutes and in thiazine or hematoxylin for 30 seconds.

Slides were destained with increasing concentrations of alcohol and then subjected to antigen retrieval by boiling in Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) for 2.5 minutes using a pressure cooker. Immunohistochemical staining was initiated by permeabilization first, in case of intracellular antigens, using methanol-acetone or CHAPS. For surface antigens of syncytiotrophoblast, eg., PLAP, blocking step was initiated first, followed by primary and secondary antibody incubation steps. Slides were washed in between steps 3X, for 5 min with PBST, finally air-dried in the dark, stained with DAPI, covered with cover slip and finally sealed with nail varnish.

2.4.4 Western blotting of STBM

STBM proteins (40 µg) resolved by 1D SDS-PAGE were electroblotted to nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences) and blocked for an hr in 5%BSA in TBST before an overnight incubation at 4^0C in primary antibody (rabbit anti-human PLAP or FasL and beta-actin, Abcam). Primary and secondary incubations were done in

blocking buffer. The membrane was washed 3X for 10 mins with TBST and finally visualized using SuperSignal® West Pico Chemiluminescent Substrate (Pierce).

2.4.5 Scanning electron microscopy of STBM

For SEM examination, 40 µg STBM was diluted in 1 ml PBS. Primary fixation was done by 2.5% Glutaraldehyde in 1xPBS overnight followed by distilled water wash at RT for 10-20 min, X3-5. Secondary fixation was performed with 1.4% Osmium tetroxide in distilled water at RT for 1-2 hrs followed by washing. Dehydration with increasing concentration of alcohol was then instituted with incubation at RT for 10 min at each concentration (25%, 50%, 70-75%, 90-95%, and 100% ethanol). Dehydration with 100% ethanol was performed twice. Finally, the specimens were subjected to critical point drying, mounted on specimen stub by silver or graphite paste, coated with gold and analyzed by Phillips JSM 5600LV scanning electron microscope.

2.4.6 ELISA of stimulation assay supernatant

ELISA on supernatant of DC/macrophage stimulation assay was performed using commercially available human cytokine detection kits. The Duoset® ELISA Development System (R & D Systems Inc, Minneapolis, MN) was used for TNF-α and IL-17, while the BD OptEIA™ ELISA set (BD Biosciences, San Dieago, CA) was obtained to quantitate IL-6, IL-8, IL-10, IL-12p40, IL-12p70 and IFN-γ and the Ready-SET-Go! Kit (eBioscience, San Dieago, CA) was used for IL-23 detection.

2.4.7 Flow cytometry of stimulated DCs

Following stimulation by STBM, DCs were harvested and incubated for 30 minutes on ice with fluorochrome-conjugated antibodies, e.g., CD80, CD83 (BD Biosciences), CCR7

(R & D), CD86, MHC I, MHC II (Ancell, Bayport, MN), isotypes IgG1 (Ancell). For non-conjugated antibodies, e.g., CD40 (eBioscience), a second step of incubation was performed using a PE-conjugated goat-anti-mouse IgG. Data acquisition and analysis were performed on a Dako Cyan flow cytometer using the Summit 4.3 software (Dako-cytomation).

2.4.8 Confocal microscopy of stimulated DCs

DCs were cultured on Lab-Tek Chambered Coverglass (Nunc) with STBM stimulation or left untreated for 24 hrs and then stained with PE-conjugated CD11c antibody. To detect STBM internalized by DC, non-conjugated FasL antibody (Abcam) and secondary antibody (FITC-conjugated goat-anti mouse antibody) were used. Confocal images were taken using the FLUVIEW FV 1000 Confocal Microscope System, version 1.6.2.5 and images were analysed using the Zeiss Image Browser 4.0. Various antibodies used in this study are shown in Table 2.1 and 2.2.

Table 2.1

Antibodies used in this study to characterize STBM

Antibody	Host Species	Dilution	Specificity	Source
Placental Alkaline Phosphatase (PLAP)	Mouse	1:100	Human	Abcam
FasL	Mouse	1:100	Human	Abcam

Table 2.2

Antibodies used in this study to characterize DCs

Antibody	Conjugate	Specificity	Dilution	Source
CD40	Unconjugated	Human (Costimulatory molecule expressed by APCs)	1:100	eBioscience
CD80	PE	Human (Costimulatory molecule expressed by APCs)	1:20	BD
CD83	PE	Human (Costimulatory molecule expressed by APCs)	1:20	BD
CD86	PE	Human (Costimulatory molecule expressed by APCs)	1:100	Ancell
CCR7	PE	Human (Marker for cell migration)	1:25	R & D
MHC I	FITC	Human (Marker for antigen presentation)	1:100	Ancell
MHC II	FITC	Human (Marker for antigen presentation)	1:100	Ancell
IgG1 Isotype PE	PE		1:100	Ancell
IgG1 Isotype FITC	FITC		1:100	Ancell
Goat anti-mouse PE mix for CD40	PE		1:50	

2.4.9 Protein separation, in-gel digestion and LC-MS/MS analysis of STBM proteins

STBM protein lysate from ~200µl STBM suspension was subjected to acetone precipitation overnight, reduced with 10mM dithiothreitol (DTT) and alkylated with 20mM iodoacetamide (IAA). Proteins (~50µg) were separated on a 10.5-14% Tris-HCL SDS-gel (BIO-RAD) and stained with PageBlue^{TM} protein staining solution (Fermentas) containing Coomassie Brilliant Blue G-250 dye. Each lane in gel was divided into 13 slices and in-gel digestion was carried out with trypsin. Samples were lyophilized and reconstituted with 10 µl of 0.1% formic acid.

For LC-MS/MS analysis, each digestion product was separated by a 60 minute gradient elution at a flow rate 0.30µl/min with Agilent 1200 HPLC system interfaced with 6520 QTOF MS system. The analytical column was Zorbax 300SB (75µm ID, 150 mm length) packed with C-18 resin (300 A, particle size 5µm). Mobile phase A consisted of 100% H_2O with 0.1% formic Acid, and mobile phase B consisted of 100% acetonitrile with 0.1% formic acid. The mass spectrometer was operated in scanning mode and recorded a range of 120 m/z to 2200 m/z. Ions were selected based on charge state and then by abundance.

The data were searched against human IPI database using Mascot program (Matrix Science) and validated using X! Tandem in Scaffold program (Proteome Software). The following search parameters were used in all of the Mascot searches: maximum of 3 missed trypsin cleavages, maximum error tolerance 300 ppm for MS and 0.8 Da for MS/MS. Proteins were designated as hits only when there were at least 2 unique peptides matches and Protein Identification Probability value >95%.

The quantitation of protein expressions by spectral counts for each identified proteins (defined as total spectral counts for all the peptides of a given protein) was carried out using Scaffold program (Proteome Software). Only proteins with spectral counts ≥10 and a ratio of spectral counts between two groups (patients and normal controls) of > 2 or < 0.5 were tested further by t-test and validation. We performed t-test to examine significant difference in protein spectral counts between STBM proteins from patients and normal controls, p value of <0.05 was used to determine differentially expressed proteins which would then be candidates for validation by immunohistochemistry and/or western blotting.

2.4.10 Immunohistochemistry of villous explants for LC-MS data validation

Villous explants were fixed and paraffin-embedded. Tissue microarrays were prepared. IHC was performed according to manufacturer's instructions (Leica Microsystems). Briefly, after deparaffinization and heat-induced epitope retrieval, endogenous peroxidase blocking was performed. Slides were incubated with primary antibodies for 45 minutes. Antibodies used are listed in Table 2.3. After washing, the sections were exposed to anti-rabbit poly-HRP-IgG in 10% animal serum for 10 minutes, followed by DAB reagents for 3 minutes. DAB reaction was stopped and counterstaining with hematoxylin was performed for 5 minutes. After washing, sections were dehydrated and finally mounted in synthetic mounting media (Electron Microscopy Services, Hatfield, USA). Controls included omission of primary antibodies. After IHC, tissue microarray slides were scanned, analyzed and scored (Leica Microsystems slide scanner and Slidepath image analysis software).

Table 2.3
Antibodies used for Immunohistochemistry

Antibody	Source	Cat#	Host	Conditions
S100-A11	Abcam	ab97329	Rabbit	1:5000 pH9
Histone H2A	Abcam	ab18255	Rabbit	1:2500 pH6
Histone H4	Abcam	ab10158	Rabbit	1:10000 pH6
Fibronectin	Abcam	ab2413	Rabbit	1:250 pH6
Integrin Beta 3	Abcam	ab75872	Rabbit	1:100 pH6
Annexin IV	Abcam	ab33009	Rabbit	1:250 pH9
Peroxiredoxin 6	Abcam	ab59543	Rabbit	1:2000 pH9
Annexin A1	Abcam	ab33061	Rabbit	1:100 pH9
Clathrin Heavy Chain	Abcam	ab21679	Rabbit	1:5000 pH6

2.4.11 Lipid extraction from STBM

Lipids were extracted using the modified Bligh and Dyer's method (Bligh and Dyer 1959). Briefly, 750µl of ice cold chloroform: methanol (1:2, v/v) was added to microparticles resuspended in 100 ul of PBS. The mixture was vortexed vigorously for 1 minute. After shaking at 1200rpm at 4°C for 1 hour, 250 µl ice cold chloroform and 350 µl of ice cold water was added to the samples. The samples were then subjected to another 1 min vortexing. The phases were separated by centrifugation at 9000 rpm and the lower organic phase containing the lipids was transferred to a fresh tube. Lipids were re-extracted from the remaining aqueous phase with 500 µl of ice cold chloroform. The two extracts were pooled and vacuum-dried. Dried lipid film was then reconstituted in chloroform: methanol 1:1 (v/v) prior to analysis by mass spectrometry. The lipid samples were spiked with a mixture of internal standards prior to mass spectrometry. The standard cocktail contained -PA 17:0/17:0, PE 17/17:0, PG 14:0/14:0, d31-PI 18:1/16:0,

PS 14:0/14:0, PC 14:0/14:0, C17 ceramide, C8 glucosylceramide, d6-cholesterol, d6-cholesterol ester and SM 18:1/12:0.

2.4.12 ESI-LC MS MRM of STBM lipids

Quantification of individual lipid molecular species was performed using an Agilent 1200 HPLC system coupled with an Applied Biosystem Triple Quadrupole/Ion Trap mass spectrometer 3200Qtrap (Applied Biosystems, Foster City, CA) as described previously (Shui et al., 2011, PMID:21573191). In multiple reaction monitoring (MRM) based experiments, the first quadrupole, Q1, is set to pass the precursor lipid ion of interest to the collision cell, Q2, where it undergoes collision induced dissociation. The third quadruple, Q3, was set to pass the structure-specific product ion characteristic of the precursor lipid of interest. Lipids were infused using an Agilent 1200 system and separated on Silica based column chromatography (Luna 3u Silica 100A, 150 X 2mm, Phenomenex, USA) before being introduced into the mass spectrometer. The separation was carried out using a gradient as described previously (Shui et al., 2011, PMID: 21573191). The mobile phase A had chloroform: methanol: 28% NH_4OH (895:100:5) and B had Chloroform : methanol : water : 28% NH_4OH (550:390:55:5). Mass spectrometry was recorded under both positive and negative modes, respectively. Under the negative mode, phosphatidylglycerol (PG), PI, phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylethanolamine (PE) and ganglioside (GM3) were eluted and separated by a step gradient elution, lasting a total of 48 min per sample. Under the positive mode, phosphatidylcholine (PC), ceramide, glucoceramide and sphingomyelin (SM) were separated by another 50-min step gradient elution. The flow rate was maintained at 0.3ml/min throughout the run with the injection load of 10μl for both negative and positive mode. Free cholesterol (chol) and its congeners, e.g. cholesteryl esters (CE), 24-hydroxycholesterol (24-OHC), 7β-hydroxycholesterol (7-OHC), 7-

ketocholesterols (7-KC), and squalene were analyzed using HPLC/APCI/MS/MS as previously described (Shui et al., 2011, PMID:21621788).

2.4.13 Flow Cytometry of decidual DCs

Pieces of decidual tissue were rinsed, chopped finely, and digested overnight with 0.8 mg/ml collagenase (Type IV, Worthington-Biochemical) in RPMI (PAA) with 10% FCS (AutogenBioclear) for 2 hr. Viability was >90% by DAPI exclusion (Sigma). RBC lysis was performed followed by staining for flow cytometry. Peripheral blood mononuclear cells were isolated by density centrifugation (Ficoll-Paque; GE Healthcare).

Flow cytometry was performed on a BDLSRII and FACSCanto and data analyzed with FlowJo (Treestar). Antibodies used are listed in Table 2.4.

Decidua samples were analyzed using the same flow strategy to map known blood DC subsets to peripheral tissue populations as previously described, Figure 1 (Haniffa et al., 2012). Cells were first gated by FSC-A and SSC-A and dead cells were excluded by DAPI staining. Leukocytes were identified using CD45 followed by exclusion of doublets using the SSC-A and SSC-W. Antigen Presenting Cells (APC) were identified within the HLA-DR+ and Lineage- (CD3, CD19, CD20 and CD56 all in FITC) fraction. Having the Lineage cocktail in the FITC channel also allowed us to exclude autofluorescent macrophages in peripheral tissues.

Table 2.4
Antibodies used for Flow Cytometry of DC Subsets in human blood & decidua

Antibody	Fluorochrome	Source	Cat#
CD45	V500	Invitrogen	HI30
CD3	FITC	BioLegend	SK7; UCHT1
CD7	FITC	eBioscience	4H9
CD19	FITC	BioLegend	HIB-19
CD20	FITC	BioLegend	2H7
CD56	FITC	BioLegend	MEM188
HLA-DR	Qdot605	Invitrogen	Tu36
CD14	ECD	Beckman Coulter	RM052
CD16	APC-H7	BioLegend	3G8
CD123	PerCPCy5.5	BioLegend	7G3; 6H6
CD11c	V450	BioLegend	B-ly6
CD1c	PECy7	BioLegend	L161
CD141	PE or APC	Miltenyi Biotec	AD5-14H12
CD34	PE	R&D	563

2.5 Experimental Repeats and Statistical Analysis

Results presented are representative of at least two independent experiments. Data were expressed as mean values of experimental triplicates ± SEM. Cytokine data had a non-normal distribution while the mass spectrometry data were normally distributed (D'Agostino & Pearson omnibus normality test). Analysis of statistical significance of differences in cytokine production by DCs in response to STBM stimulation were sought using Mann Whitney *U* test, while differences in STBM protein spectral counts, STBM protein staining intensities, STBM lipid molar fractions and frequency of DC subsets between patient group and normal healthy pregnant group were sought using Student's two-tailed unpaired t-test. Analyses were performed using GraphPad Prism 5 software. Results were considered to be statistically significant if $P<0.05$.

2.6 Buffers

1x PBS p^H 7.4 (diluted from 10x stock from Invitrogen, Singapore)

- KH_2PO_4 1.76 mM
- Na_2HPO_4 10.4 mM
- NaCl 137 mM
- KCl 2.7mM

FACSwash buffer

- 1xPBS
- Heat-inactivated bovine calf serum 2.5% (v/v) (Invitrogen, Singapore)
- Sodium azide 0.05% (w/v)

MACS buffer

- 1xPBS
- 0.5% bovine serum albumin (BioRad, Singapore)
- 2 mM EDTA

Tris buffered saline

- Tris-HCl, p^H 7.5, 50 mM
- NaCl 150 mM

10 x SDS-PAGE electrophoresis buffer

- Tris base 250 mM
- Glycine 2.5 M

- SDS 1% (w/v)

5 x reducing laemmli buffer (BioRad, Singapore)

- Tris-HCl, p^H 6.8, 250 mM
- Glycerol 50% (v/v)
- SDS 10% (w/v)
- Bromophenol Blue 0.05% (w/v)
- Dithiothreitol (DTT) 0.5 M (added fresh before use)

10 x Western blot transfer buffer

- Tris base 250 mM
- Glycine 1.92 M
- To prepare 1 x western blot transfer buffer- 10 x buffer diluted1 part to 2 parts 100% methanol & 7 parts deionized water

PBS-T or TBS-T buffer

- 1 x PBS or 1 x TBS with Tween-20, 0.05% (v/v)

Western blot blocking and antibody incubation buffer

- 5% bovine serum albumin in TBST

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Chapter 3: Immunomodulation by Placental STBM in Adverse Pregnancy Outcomes

3.1 Introduction

Placental syncytiotrophoblast microvesicles (STBM) are shed into the maternal circulation during pregnancy as normal turnover of the placental surface. STBM are membrane vesicle populations (0.1 to 1 µm), produced as a result of syncytiotrophoblast apoptosis (Huppertz et al., 2006, Redman et al., 2008, Pantham et al., 2011). STBM also include placenta-derived exosomes (10-100 nm), secreted from endosomal multivesicular bodies (MVB) upon fusion with the plasma membrane (Redman et al., 2007, Dragovic et al., 2011). Membrane vesicles are composed of lipid bilayer with transmembrane proteins and enclose part of cytosol of the parent cell. They are metabolically active by virtue of the presence of lipids, proteins and nucleic acids (Gupta et al., 2008, Cocucci et al., 2009, Redman et al., 2012, Rajakumar et al., 2012). They have the ability to directly activate T cells or transfer antigens to dendritic cells for subsequent presentation to T cells and can be inhibitory by induction of T cell death, inhibition of dendritic cell maturation or inhibition of T cell cytotoxic activity (Thery et al., 2009). STBM may be novel vehicles for intercellular communication, especially with maternal immune cells at maternal-fetal interface (Southcombe et al., 2011).

STBM are known to be proinflammatory (Germain et al., 2007, Messerli et al., 2010, Southcombe et al., 2011), procoagulant (Gardiner et al., 2011) immune-regulatory (Arkwright et al., 1994) and anti-angiogenic (Smarason et al., 1993, Gupta et al., 2005). They circulate in significantly increased amounts in adverse pregnancy outcomes such as preeclampsia (PE), which is characterized by exaggerated systemic inflammatory response, coagulation and endothelial dysfunction (Lok et al., 2008, Redman et al.,

2009, Van Der Post et al., 2011). Thus, circulating STBM may play a role in the pathogenesis of PE and possibly other pregnancy complications such as RM, which involves pathological placentation. In the present study, RM was defined as 2 or more consecutive pregnancy losses prior to 20 weeks of gestation where infectious, chromosomal and hormonal causes were ruled out.

Up to 70% of RM is associated with immunologic factors as evidenced by increased inflammatory immune responses in RM including increased number and cytoxicity of natural killer (NK) cells in peripheral blood of non-pregnant and pregnant women with history of RM regardless of outcome of the index pregnancy, i.e. live births or non-live births (Kwak-Kim et al., 1995) and endometrium (Clifford et al., 1999), raised Th1 cytokine levels in endometrium regardless of outcome of subsequent pregnancy (Lim et al., 2000), elevated Th1/Th2 cell ratios in peripheral blood in non-pregnant women with RM (Kwak-Kim et al., 2003), increased number of IL-17^{+} T cells , reduced numbers and function of Foxp3^{+} regulatory T cells (Treg) in peripheral blood in non-pregnant women with RM (Lee et al., 2011) and increased proportion of Th17 cells in peripheral blood and deciduas of RM patients with ongoing loss (Wang et al., 2010).

As the role of STBM in immune-dysregulation of RM remains yet unclear, we sought to determine if STBM from women with history of RM, regardless of outcome of index pregnancy, were more immuno-stimulatory compared to normal pregnant women by using explant STBM to evaluate the in vitro responses of antigen presenting cells such as monocyte-derived dendritic cells (DC), macrophages as well as T cells.

3.2 Subject Characteristics

We studied 22 pregnant women with RM, 13 pregnant women with PE, and gestational age-matched healthy normal pregnant women (Table 3.1 and Table 3.2). Of the RM cases, 6 had definite autoimmune disease such as systemic lupus erythematosus (SLE) and/or antiphospholipid syndrome (APS) that fulfilled American College of Rheumatology classification criteria (Tan et al., 1982, Hochberg et al., 1997, Wilson et al., 1999), 10 had positive antinuclear antibody or antithyroid antibody, 14 had elevated NK cells (>12%) and 10 had more than one poor prognostic factor. All RM patients received either passive immunotherapy in the form of IVIG and/or antithrombotic therapy (aspirin and/or heparin), while those with PE received conventional anti-hypertensive therapy.

Table 3.1

Maternal and foetal outcomes of STBM immunomodulation study- RM

	Pregnant women with RM		Normal Pregnant Controls	Normal Pregnant Controls
	Live birth (n=20)	Non-live birth (n=2)	Live birth(n=20)	Non-live birth (n=4)
Maternal age (y)	36.22[5.19]	35 [1.41]	35.27 [5.27]	33.17 [3.46]
Gestational age (w)	34.81 [7.70]	14 [5.66]	36.83 [2.61]	16 [4.54]
Number of miscarriages	2.68 [1.05]	3.5 [0.71]	-	-
Positive Immune Test	10	0	-	-
Definite Autoimmune Disease	6	0	-	-
NK cells >12%	14	0	-	-
IVIG	12	0	-	-
LMWH	11	1	-	-

Results are mean [SD]

Table 3.2

Maternal and foetal outcomes of STBM immunomodulation study- PE

	Pregnant women with PE		Normal Pregnant Controls	Normal Pregnant Controls
	Live birth (n=11)	Non-live birth (n=2)	Live birth (n=13)	Non-live birth (n=4)
Maternal age (y)	37.17[3.82][a]	33.33 [3.06]	35.17[5.38]	33.17 [3.46]
Gestational age (w)	31.82[3.24]*	16.97 [6.51]	35.35[3.09]	16 [4.54]
SBP (mm Hg)	161.50[18.95]***		120.67[15.31]	-
DBP (mm Hg)	107[6.06]***		73[6.08]	
Proteinuria (g/24h)	1.14[0.57]		-	
Infant birth wt (g)	1687.95[888.96]*		2650[642.25]	
Placental wt (g)	395.50[121.93]**		578.75[101.21]	

3.3 STBM Characterization

Results of STBM characterization are presented in Figure 3.1. H/E and immunofluorescent staining of placental villous explants confirmed the integrity of the syncytiotrophoblast layer, shed as STBM. Scanning electron microscopy demonstrated expected morphology. Conventional flow cytometry and nanoparticle tracking analysis method showed lowest forward and variable side scatters, placental syncytiotrophoblast specific protein PLAP expression and STBM size distribution to be around 250 nm. Quantitative Real time (TaqMan) PCR analysis of STBM DNA showed amplification of male fetal DNA (SRY gene).

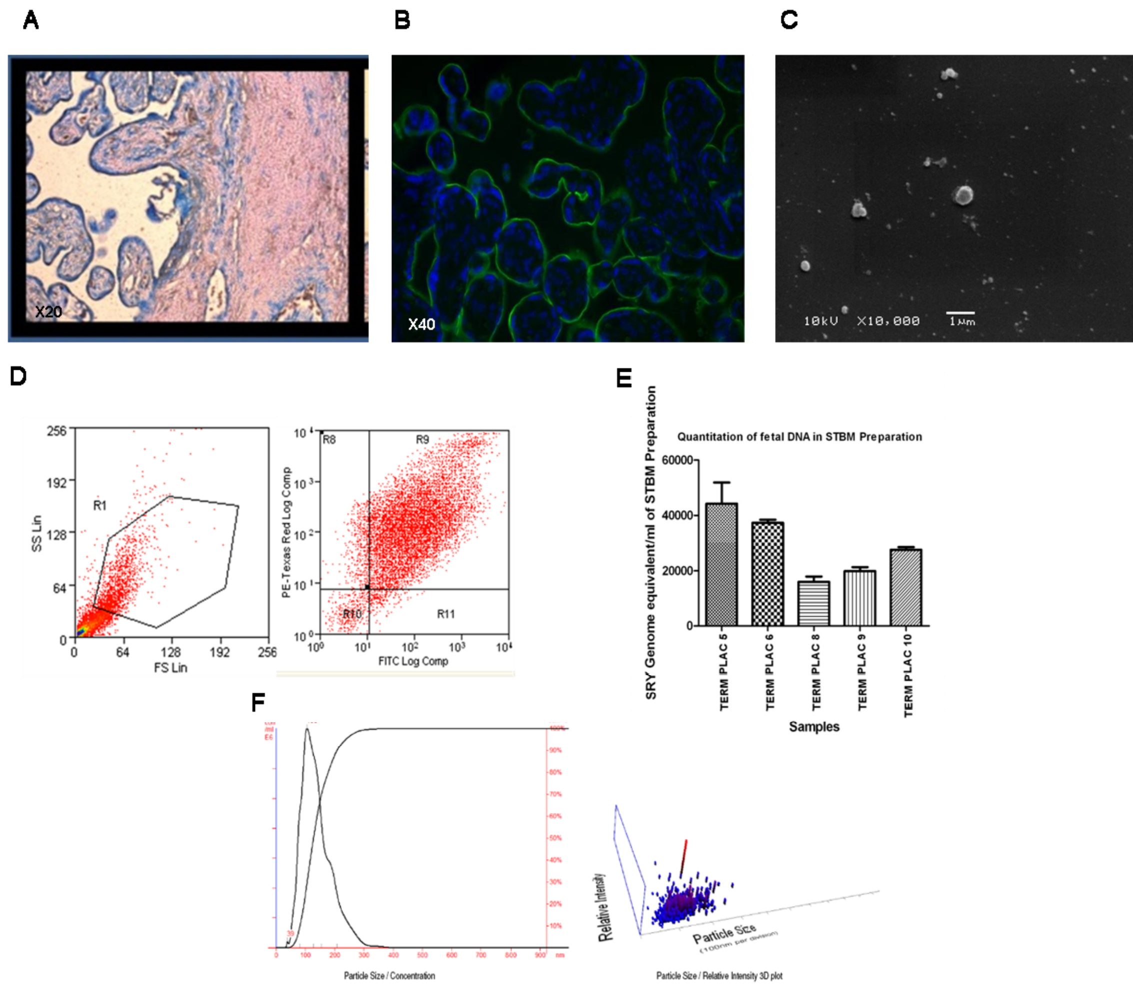

Figure 3.1: Representative images of STBM basic characterization. A. H/E staining of villous explants showing deep blue syncytiotrophoblast (STB) layer at periphery (X20); B. Immunofluorescence (IF) image of villous explants demonstrating the bright green layer of STB stained for Placental Alkaline Phosphatase, PLAP (X40); C. Morphology of STBM by scanning electron microscopy (X10.000); D. PLAP expression of STBM by flow cytometry; E. Quantitative Real time (TaqMan) PCR analysis of STBM DNA for detection of male fetal DNA (SRY gene). 100 µl of STBM preparation was used for extraction of DNA by EZ1 DNA Blood Mini Kit, elution 50 µl, 3 µl/reaction used as template for PCR; F. Nanoparticle tracking analysis of STBM, showing STBM size to be between 200-300 nm.

3.4 Human Monocyte-Derived DCs Internalize STBM and STBM Do not Affect the Viability of DCs

Immunofluorescent staining and confocal microscopy confirmed uptake of STBM by DCs, while DCs that were not exposed to STBM did not show fluorescence signal (Figure 3.2). STBM stimulation showed insignificant impact on DC viability based on the phenotype in flow cytometry (Figure 3.3).

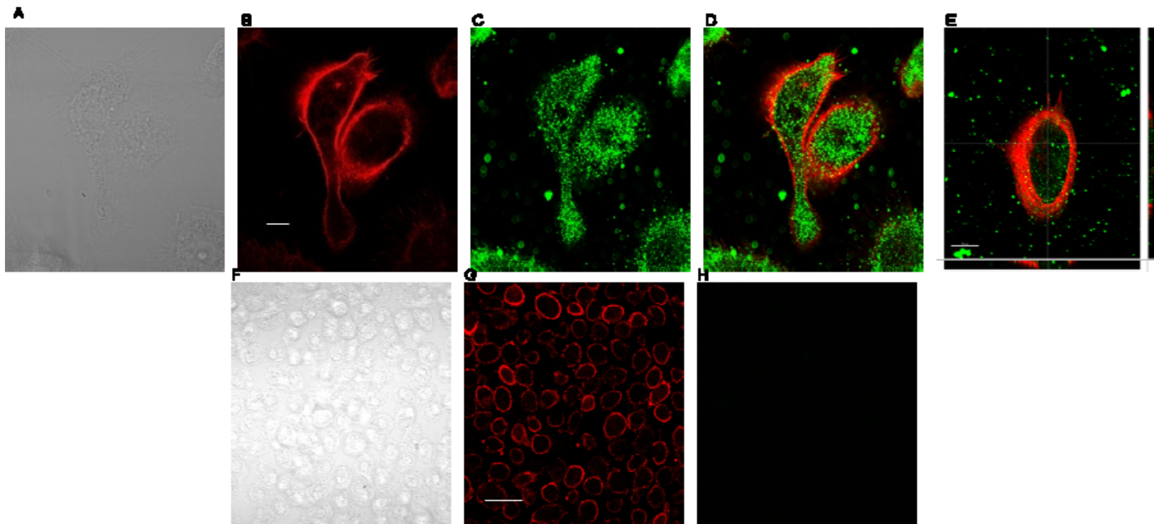

Figure 3.2: Representative confocal images of dendritic cells after being incubated with (top panel) or without (bottom panel) 100 µg/ml STBM for 24 hr. Monocyte-derived dendritic cells showing, (A) brightfield image; (B) CD11c (red); (C) STBM (green); (D) overlay; (E) single monocyte-derived dendritic cell with 3-D image; DC left unstimulated showing (F) brightfield image; (G) CD11c (red); (H) no green fluorescence in green filter. Bar represents 20 µm.

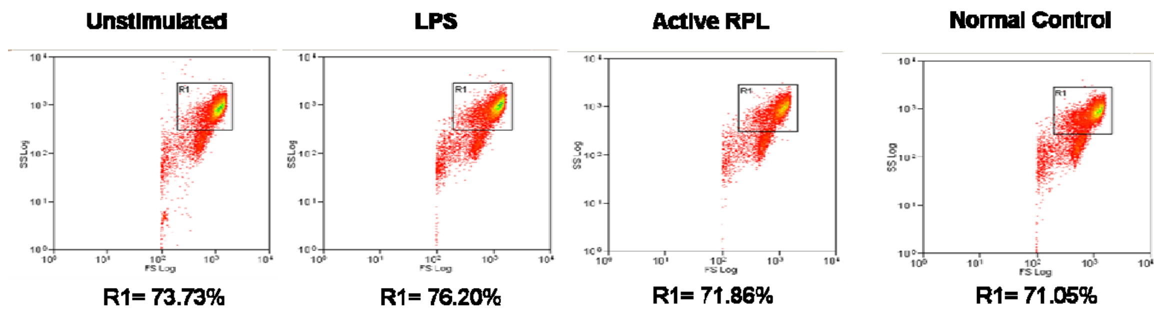

Figure 3.3. Representative forward scatter/side scatter (FSC/SSC) dot plots of DC, left unstimulated or incubated with indicated preparation of 100 µg/ml STBM or 500 ng/ml LPS for 24 hr.

3.5 STBM Induce Marginal Expression of Maturation Markers on DCs

DCs are known to express basal levels of co-stimulatory molecules and relatively higher levels of MHC molecules. However, no differential expression profile of these markers across the three study groups relative to untreated cells was observed (Figures 3.4, 3.5, 3.6).

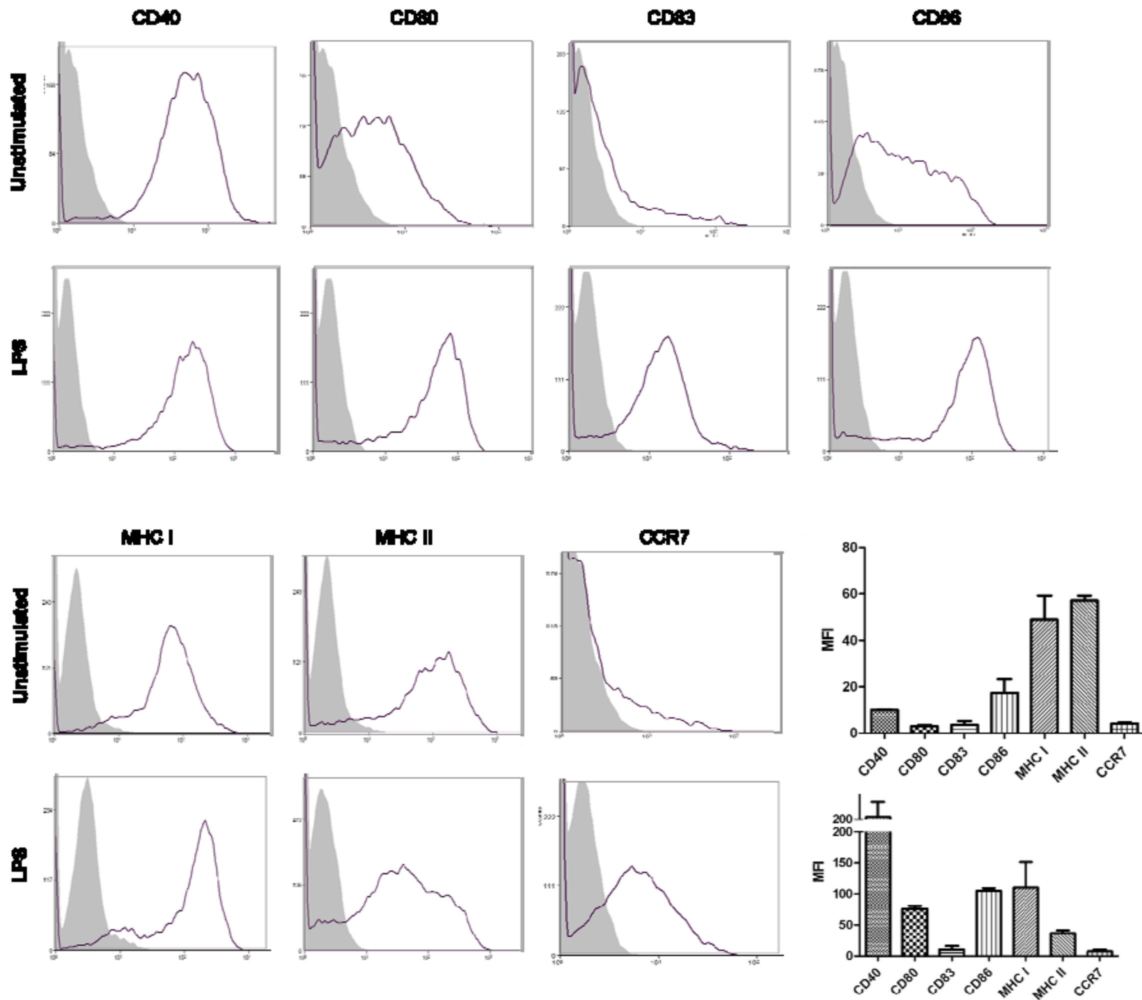

Figure 3.4. Surface phenotype of unstimulated (immature) and LPS-stimulated (mature) DC (n=3). DCs differentiated from monocytes after 6 days of culture were considered immature DC. Harvested cells were left unstimulated or stimulated with LPS (500 ng/ml) for 24 hrs to allow maturation. CD40, CD80, CD83, CD86, MHC I, MHC II and CCR7 antibodies were used to stain the DC. Filled histograms are signals obtained with isotype IgG. Open histograms are staining with specific antibodies

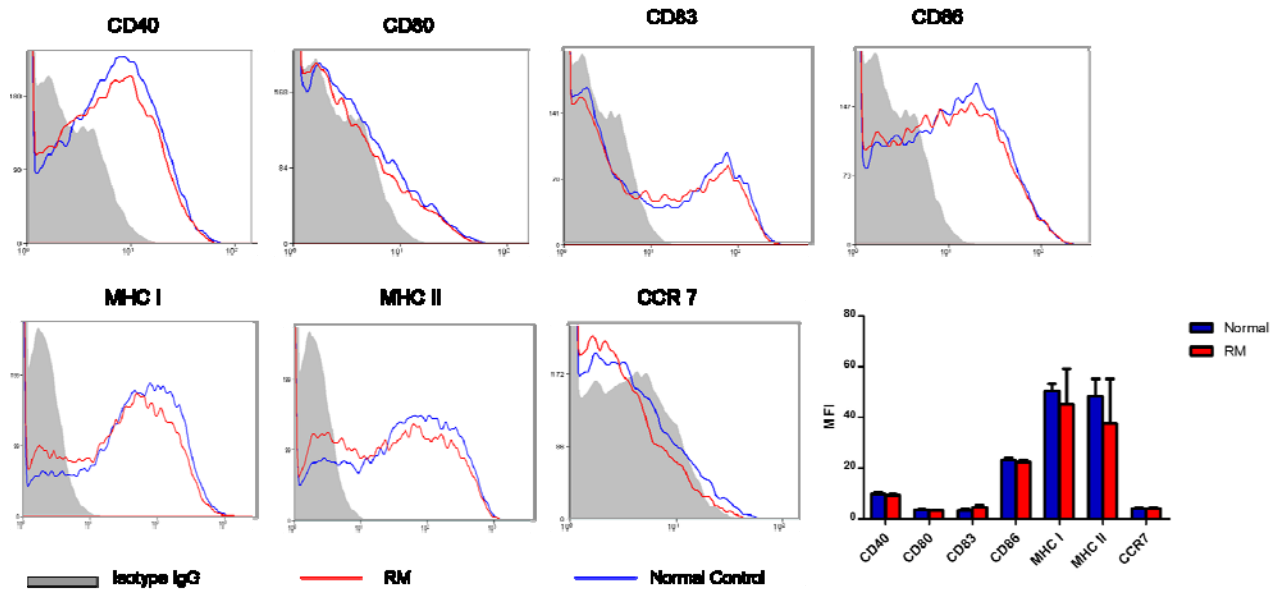

Figure 3.5. Surface phenotype of DC stimulated by STBM from woman with RM and gestational age matched normal control (n=2). DCs were stimulated with STBM for 24 hrs. Filled histograms are signals obtained with isotype IgG. Open histograms are staining with specific antibodies, red histograms, RM STBM; blue histograms, normal control STBM.

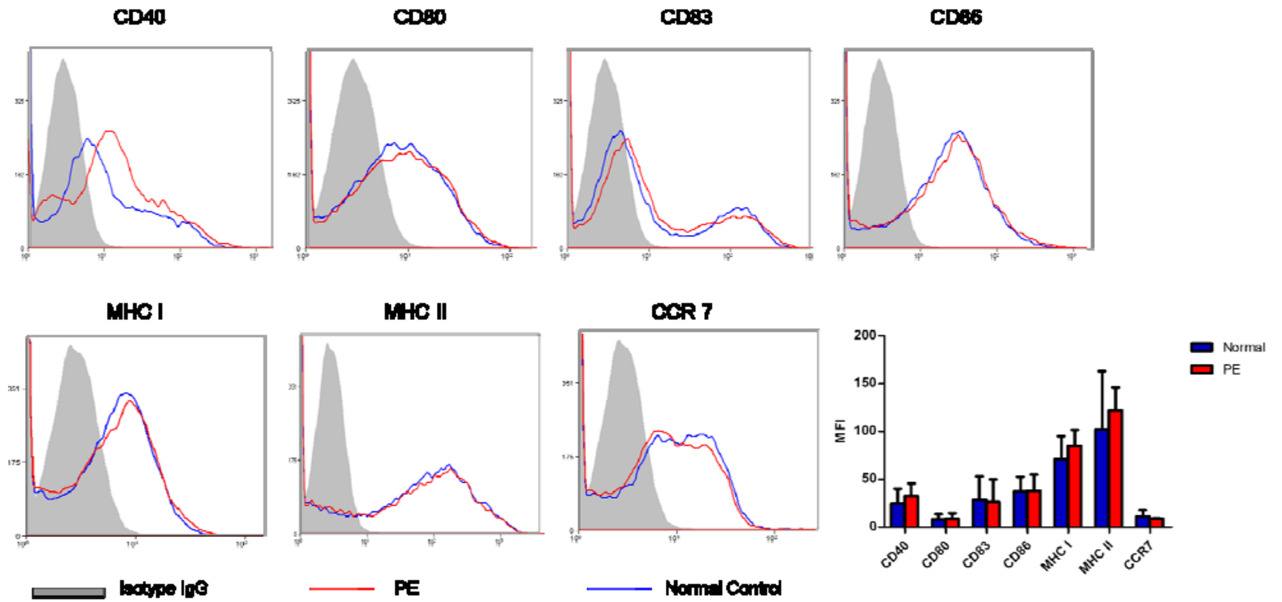

Figure 3.6. Surface phenotype of DCs stimulated by STBM from woman with PE and gestational age matched normal control (n=2). DCs were stimulated with STBM for 24 hrs. Filled histograms are signals obtained with isotype IgG. Open histograms are staining with specific antibodies, red histograms, PE STBM; blue histograms, normal control STBM

3.6 STBM Induce Dose-Dependent Production of Pro-Inflammatory Cytokines Following Uptake by DCs

STBM stimulated dose-dependent release of pro-inflammatory cytokines IL-8, IL-6 and anti-inflammatory cytokine IL-10 from DCs (Figure 3.7). Importantly, additional 48 hr culture of aliquots of explant culture supernatants prior to STBM isolation showed no evidence of bacterial growth and endotoxin contamination (data not shown).

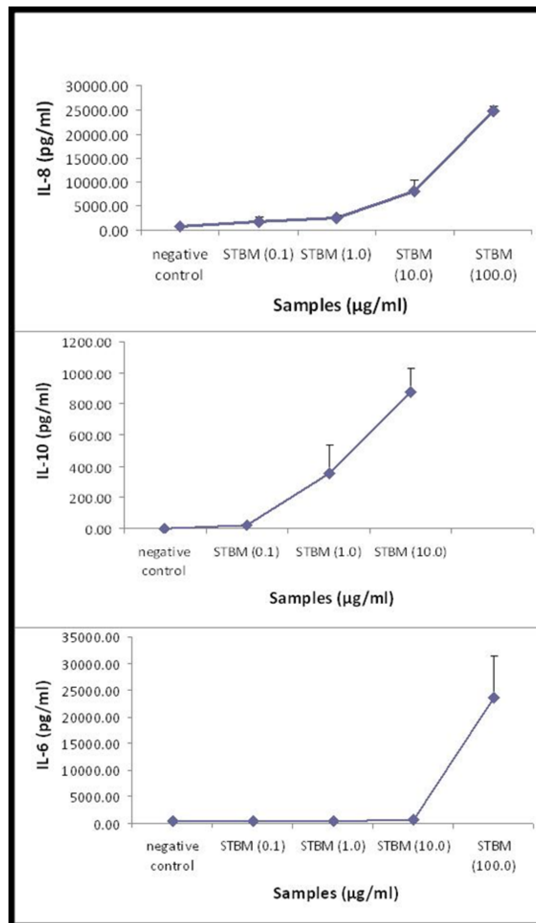

Figure 3.7: Normal STBM-mediated dose-dependent secretion of cytokines by monocyte-derived dendritic cells (n=2). Cells were left untreated (negative control) or co-incubated for 24 h with different doses of STBM. Data is representative of at least 2 independent experiments.

3.7 STBM from RM Patients with non-live birth or Severe PE Requiring Termination are Immuno-stimulatory

STBM from the RM patient with non-live birth or severe PE patients requiring pregnancy termination were immuno-stimulatory. This was demonstrated by significantly increased or a trend to increased production of pro-inflammatory cytokines in PE and RM group, respectively, such as IL-8 ($P=0.0574$ in PE), IL-6 ($P<0.01$ in PE), and TNF-α ($P<0.01$ in PE) by the stimulated DCs, (Figures 3.8 and 3.9, Tables 3.4 and 3.6). Placental histopathology was supportive, as evident by massive perivillous fibrin deposition, inflammation, thrombosis in RM and severe villous ischaemia in severe PE. Cytokines showing marginal changes in the initial stage of the study, namely IL-10, IL-12p70, IL-23, and IL-17, were not included in further analysis. STBM stimulated macrophages showed similar results (data not shown). T cell data are not available for these patients.

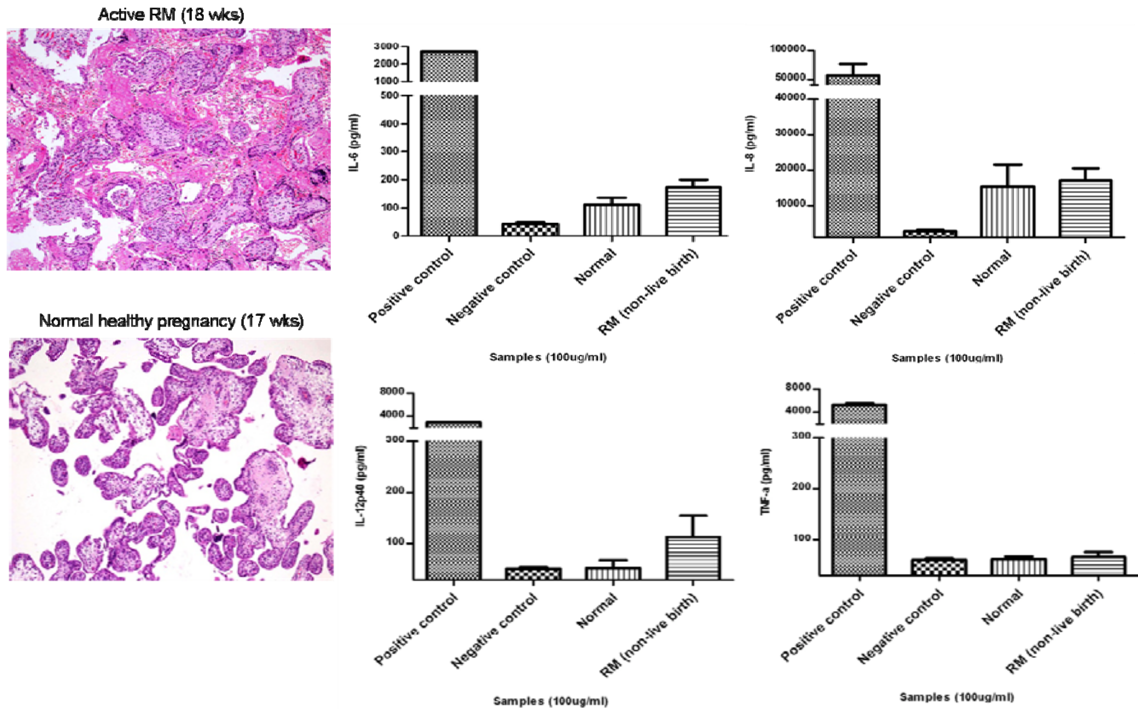

Figure 3.8. RM (non-live birth)- a trend to increased proinflammatory cytokine release by DCs. DCs (7 X 10^4 per well) were stimulated for 24 hr with placental STBM from patient & gestational age matched normal healthy control (n=2), LPS (positive control) or left unstimulated (negative control). Cytokine production was determined by ELISA. All experiments were performed in triplicates with at least 2 independent experiments carried with the same batch of samples, Results are expressed as mean values of experimental triplicates ±SEM. Histopathology of placenta shows massive perivillous fibrinoid deposition.

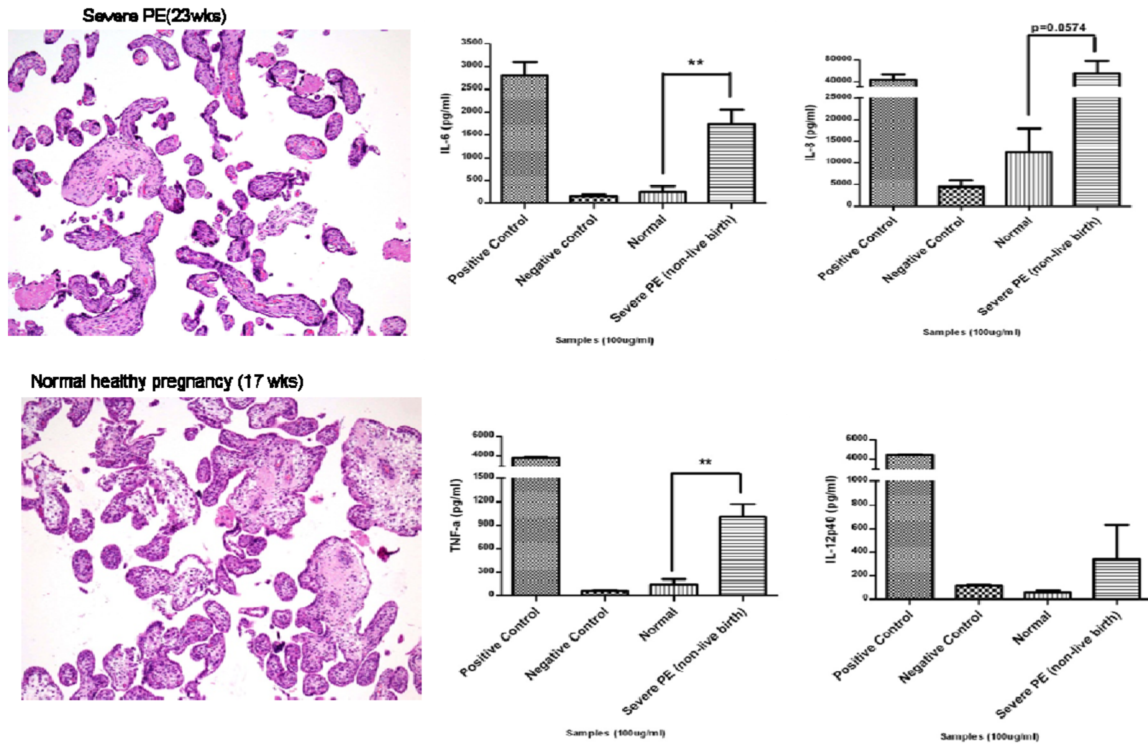

Figure 3.9. Severe pre-eclampsia requiring termination- significant proinflammatory cytokine release by DCs. DCs were stimulated with placental STBM from patient & gestational age matched normal healthy control (n=2), LPS (positive control) or left unstimulated (negative control). Results are expressed as mean values of experimental triplicates ±SEM. Histopathology of placenta shows marked villous ischaemia, villi are thin and slender with widening of intervillous space.

3.8 STBM from RM or PE Patients with live birth have increased immunogenicity

STBM from RM patients with live birth had significantly increased immunogenicity. This was demonstrated by production of significantly greater levels of pro-inflammatory cytokines, e.g. IL-6 and TNF-α ($P<0.05$) (Table 3.3). PE patients showed similar results (Table 3.5). Placental villous histology showed scattered fibrin deposition in intervillus space and minimal inflammatory infiltrate in decidua (Figures 3.10 and 3.11).

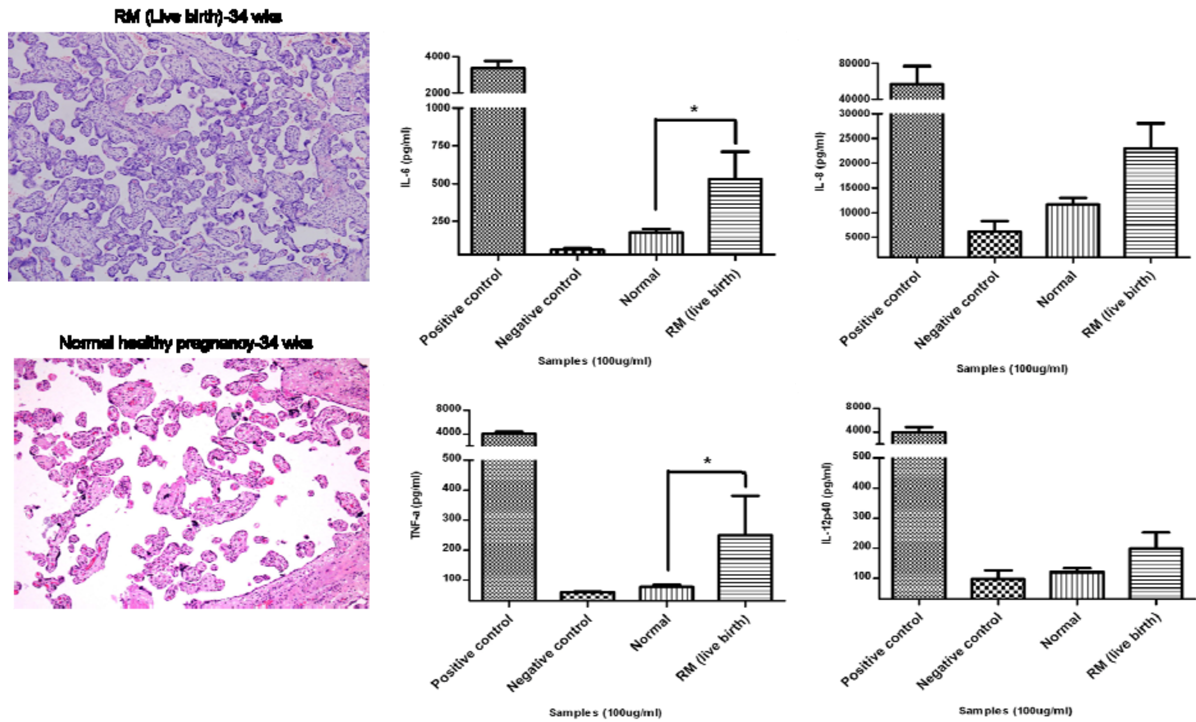

Figure 3.10. RM (live birth) – significantly increased proinflammatory cytokine release by Dendritic cells. DCs were stimulated with placental STBM from patients & gestational age matched normal healthy controls (n=20), LPS (positive control) or left unstimulated (negative control). Results are expressed as mean values of experimental triplicates ±SEM. Section show placental tissue with focal congestion, prominent is tissue knobs and scattered intervillus fibrin deposition. No infarct is noted. No fibrin thrombi identified. A few inflammatory cells composed of lymphocytes and a few neutrophils are seen in the decidua.

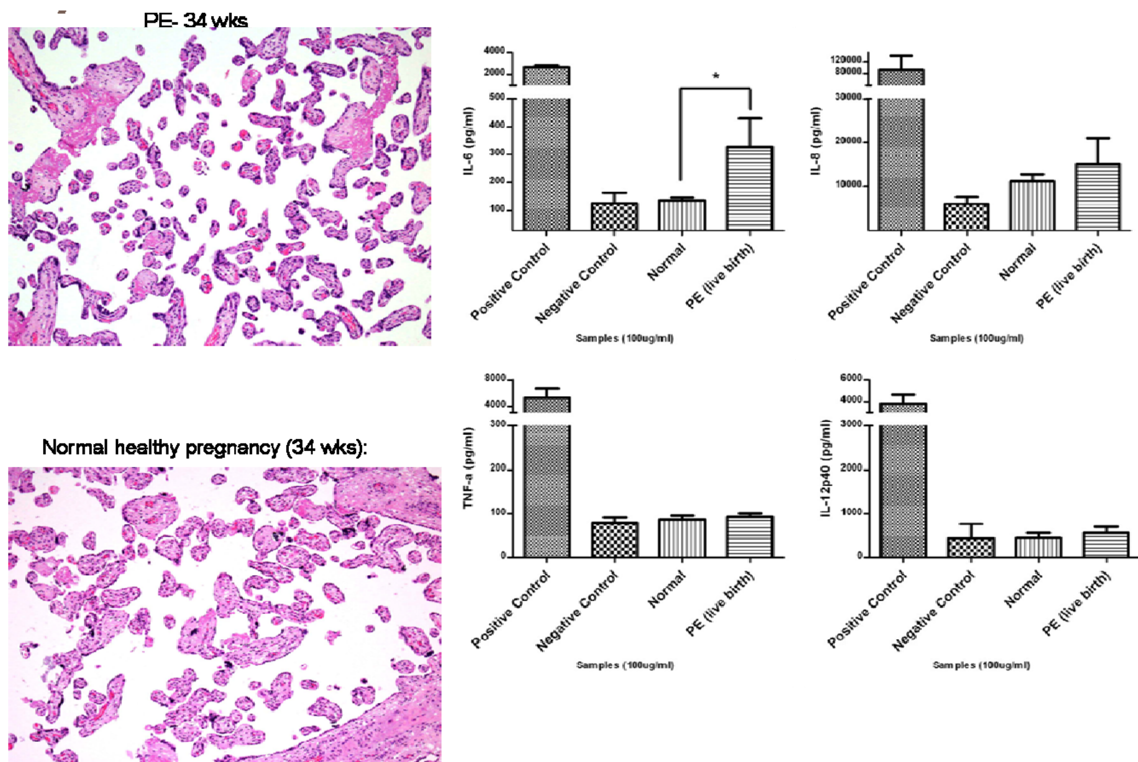

Figure 3.11. PE (live birth) – significantly increased proinflammatory cytokine release by Dendritic cells. DCs were stimulated with placental STBM from patients & gestational age matched normal healthy controls (n=11), LPS (positive control) or left unstimulated (negative control). Results are expressed as mean values of experimental triplicates ±SEM. Histopathology of placenta show evidence of villus ischaemia.

Table 3.3
Cytokine profile of STBM-stimulated DC (RM live birth)

	RM			Normal			
	Mean (pg/ml)	SEM	N	Mean (pg/ml)	SEM	N	P value
IL-6	529.70	181.80	20	173.20	21.52	20	0.0133
IL-8	23000	5043	20	11670	1354	20	0.1636
IL-12p40	199.50	53.84	20	120.50	11.50	20	0.2393
TNF-α	250.40	130.80	20	73.44	6.61	20	0.0066
IFN-γ	468.90	208.00	6	421.10	123.70	5	0.8559
IL-10	125.70	24.38	6	107.10	28.65	5	0.6304

Table 3.4
Cytokine profile of STBM-stimulated DC (RM non-live birth)

	RM			Normal			
	Mean (pg/ml)	SEM	N	Mean (pg/ml)	SEM	N	P value
IL-6	176.00	48.37	2	110.30	17.97	4	0.5333
IL-8	13670	954	2	15210	6247	4	1
IL-12p40	151.60	32.89	2	53.03	10.71	4	0.1333
TNF-α	64.24	17.70	2	61.83	5.00	4	1

Table 3.5
Cytokine profile of STBM-stimulated DC (PE live birth)

	PE			Normal			
	Mean (pg/ml)	SEM	N	Mean (pg/ml)	SEM	N	P value
IL-6	326.40	102.10	11	137.10	10.71	13	0.0321
IL-8	15240.00	5832.00	11	11120.00	1694.00	13	0.3247
IL-12p40	561.80	139.70	11	450.90	110.20	13	0.643
TNF-α	92.95	7.71	11	86.95	8.87	13	0.3848
IFN-γ	1115.00	137.90	6	951.40	216.90	4	0.5196
IL-10	153.10	8.28	6	124.50	5.25	4	0.0332

Table 3.6
Cytokine profile of STBM-stimulated DC (PE non-live birth)

	PE			Normal			
	Mean (pg/ml)	SEM	N	Mean (pg/ml)	SEM	N	P value
IL-6	1727.00	329.00	2	254.20	114.10	4	0.0051
IL-8	55320	22840	2	12560	5420	4	0.0574
IL-12p40	336.20	297.80	2	60.72	16.65	4	0.2089
TNF-α	1006.00	164.90	2	144.90	71.30	4	0.0042

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Chapter 4: Proteomic Analysis of Placental Syncytiotrophoblast Microvesicles in Adverse Pregnancy outcomes

4.1 Introduction

Proteomes of microvesicles derived from platelets, plasma, malignant lymphocytes, endothelial cells, dendritic cells, mast cells, and intestinal epithelial cells have been published (Garcia et al., 2005, Jin et al., 2005, Miguet et al, 2006, Wubblots et al. 2003, Thery et al., 1999, Thery et al., 2001, Van Niel et al., 2001). Broadly, microvesicle proteins are either ubiquitous or specific to cells of origin. Ubiquitous proteins are most likely involved in microvesicle biogenesis. Examples include tetraspannins (CD9, CD63, CD81 and CD82), heat shock proteins (HSP70, HSP90), cytoskeletal proteins (tubulin, actin, actin binding proteins), metabolic enzymes, membrane transport and fusion proteins (annexins and RAB proteins), signal transduction proteins (protein kinases, 14-3-3, G proteins), integrins and MHC class I and II molecules (Thery et al., 2002). Examples of cell-specific proteins include MHC class II in exosomes from all cells expressing MHC class II, CD 86 from DC-derived exosomes (a co-stimulatory molecule for T cells), T-cell receptors for T-cell derived exosomes, and immunoglobulin family members (CD54 on B cells, P-selectin on platelets). In contrast to exosomes, microvesicles released by apoptotic cells do contain nuclear and organeller proteins, DNA, messenger and microRNA. Both exosomes and microvesicles may provide unconventional routes of protein secretion (Nickel et al., 2005).

Multiple pathophysiologic factors are implicated in PE including maternal immune maladaptation to the feto-placental unit, excessive fetal trophoblast apoptosis, and

increased trophoblast debris shedding. These result in increased systemic inflammatory response, haemostatic activation, endothelial dysfunction, and metabolic changes (Roberts, 2002). Primary reduction in placental perfusion leading to tissue hypoxia and oxidative stress may result in qualitative and subsequent functional changes in STBM. Serdar et al. showed quantitative changes in serum, placental and decidual lipid and protein oxidation products and anti-oxidant concentrations to be significantly associated with preeclampsia (Serdar et al., 2003). Proteomic analyses of normal and preeclampsia placentas (Gharesi-Fard et al., 2010), syncytiotrophoblasts (Okamura et al., 1981), cytotrophoblasts (Hoang et al., 2001, Hu et al., 2007, Hong et al., 2008, Johnstone et al., 2011) and plasmas (Blumenstein et al., 2009, Rasanen et al., 2010, Liu et al., 2011) have been previously reported. While the role of STBM in PE is a vibrant area of research, the role of STBM in RM remains unclear.

We hypothesized that STBM proteins are differentially expressed in PE and RM compared to normal pregnancies. Our objectives were to determine protein and peptide components of STBM that can incite pathogenic responses in pregnancy complications and explore whether these STBM protein expressions differ in pathologic and normal pregnancies. As opposed to earlier studies which employed 2D-LC-MS/MS (Rasanen et al., 2010, Johnstone et al., 2011), 2D PAGE and MALDI TOF/TOF (Gharesi et al., 2010, Shin et al., 2011), and 2D DIGE (Blumenstein et al., 2009), we used 1D Gel-LC-MS/MS approach to improve sensitivity.

4.2 Subject Characteristics

In a series of three ESI LC-MS/MS experiments, we studied 3 pregnant women complicated with preeclampsia, 2 pregnant women with recurrent miscarriages, and 2 healthy normal pregnant women. The clinical characteristics of the women and maternal and neonatal outcomes are summarized in Table 4.1. The patients underwent caesarean section at gestational age >34 weeks with otherwise healthy maternal and neonatal outcomes.

Table 4.1
Maternal and foetal outcomes of STBM proteomics study

	PE (n=3)	RM (n=2)	Normal controls (n=2)
Maternal age (y)	31[2.16][a]	30[5.66]	37.6[4.88]
Gestational age (w)	36.09[0.95]	36.35[2.93]	35.86[3.03]
SBP (mm Hg)	173.25[31]***	103.5 [19.09]	116.25[15.31]
DBP (mm Hg)	109.5[13.2]***	67[9.9]]	76.5[5.58]
Proteinuria (g/24h)	1.14[0.57]	-	-
Infant birth wt (g)	2658[792.02]	2845[827.31]	2302.5[38.89]
Placental wt (g)	508.33[79.74]	760[84.85]	500[70.71]

[a]Results are mean [SD], comparisons using two-sample t test (patient group, ie, PE or RM, versus normal pregnant controls), $P<0.05$ *, $P<0.0001$ ***

4.3 Identification of STBM proteins by 1D Gel-LC-MS/MS

Three gels from PE group and 2 gels from RM group including gestational age-matched healthy controls were analyzed and representative gels are presented in Figures 4.1 (a) and 4.1 (b). When lanes of STBM protein extracts from pathologic pregnancies are compared to that from normal healthy pregnancies, staining intensities show definite differences in several gel bands shown in Figure 4.1. After in-gel digestion, the tryptic peptides of each gel slice were analysed by LC-MS/MS. Over 300 STBM proteins were identified based on previously mentioned criteria in section 2.4. Spectral counts were used to quantify expression level of proteins in the whole lane.

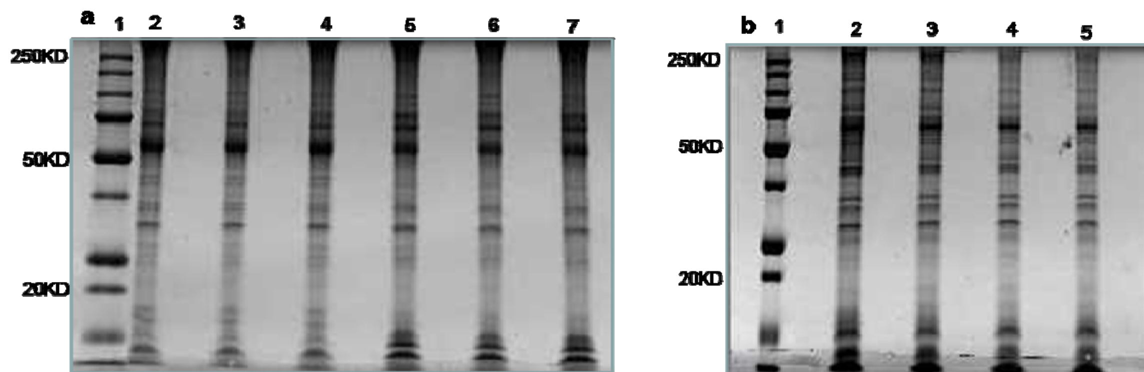


Figure 4.1: Representative 1-D SDS PAGE gel images of protein extracts from STBM.

(a) Lane 1: molecular weight marker. Lanes 2, 3 & 4: Normal STBM. Lanes 5, 6 & 7: PE STBM. Loading volume: 20 uL per lane (~50 ug of proteins).

(b) Representative 1-D SDS PAGE gel images of protein extracts from STBM. Lane 1: molecular weight marker. Lanes 2 & 3: RM STBM. Lanes 4 & 5: Normal STBMs. Loading volume: 20 uL per lane (~50 ug of proteins).

4.4 Global Overview

The STBM proteins were subjected to GO (gene ontology) analysis to identify major biological processes, cellular components and molecular functions. The major cellular components include membrane, cytoskeleton, cytoplasm as well as nucleus suggesting these microvesicles population indeed resulted from apoptotic membrane blebbing (Figure 4.2). The major molecular functions of STBM proteins include binding (proteins, nucleotides, receptors, transcription factors), enzyme regulation, transcriptional regulation, and molecular transduction (Figure 4.3). Finally, the major biological processes associated with STBM proteins include biological regulation, metabolic processes, immune response, apoptosis, adhesion and localization (Figure 4.4).

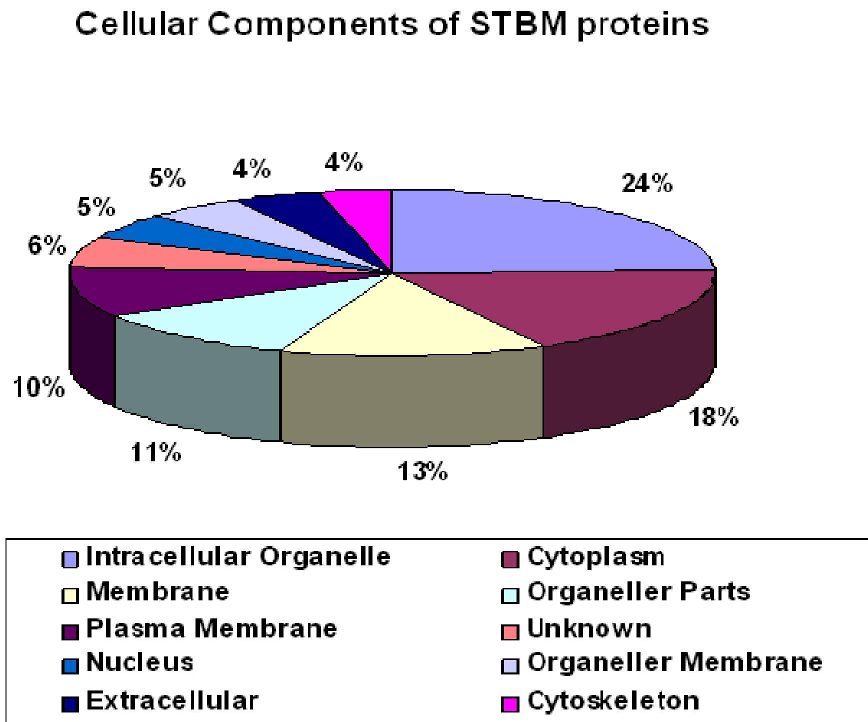
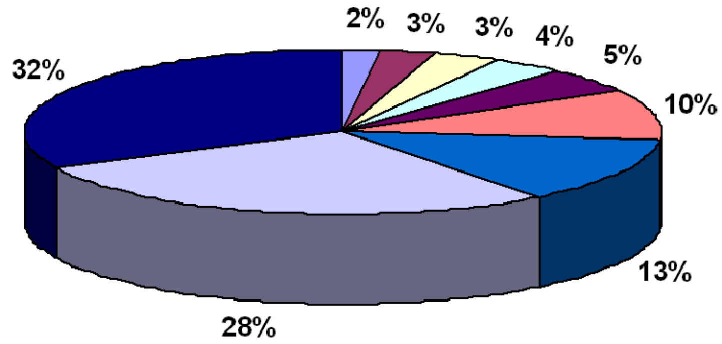


Figure 4.2. GO analysis of cellular components of STBM proteins

Molecular Functions of STBM proteins



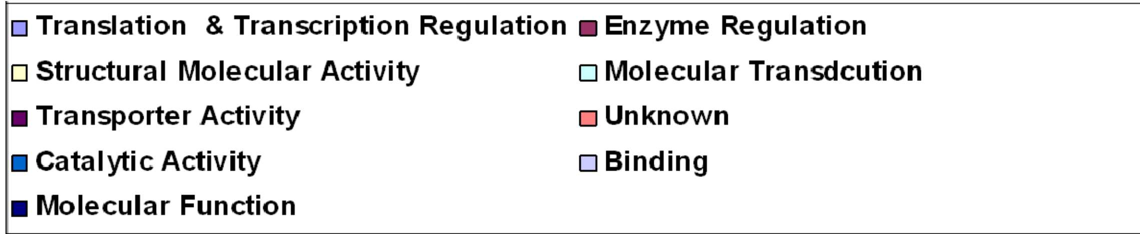


Figure 4.3. GO analysis of molecular functions of STBM proteins

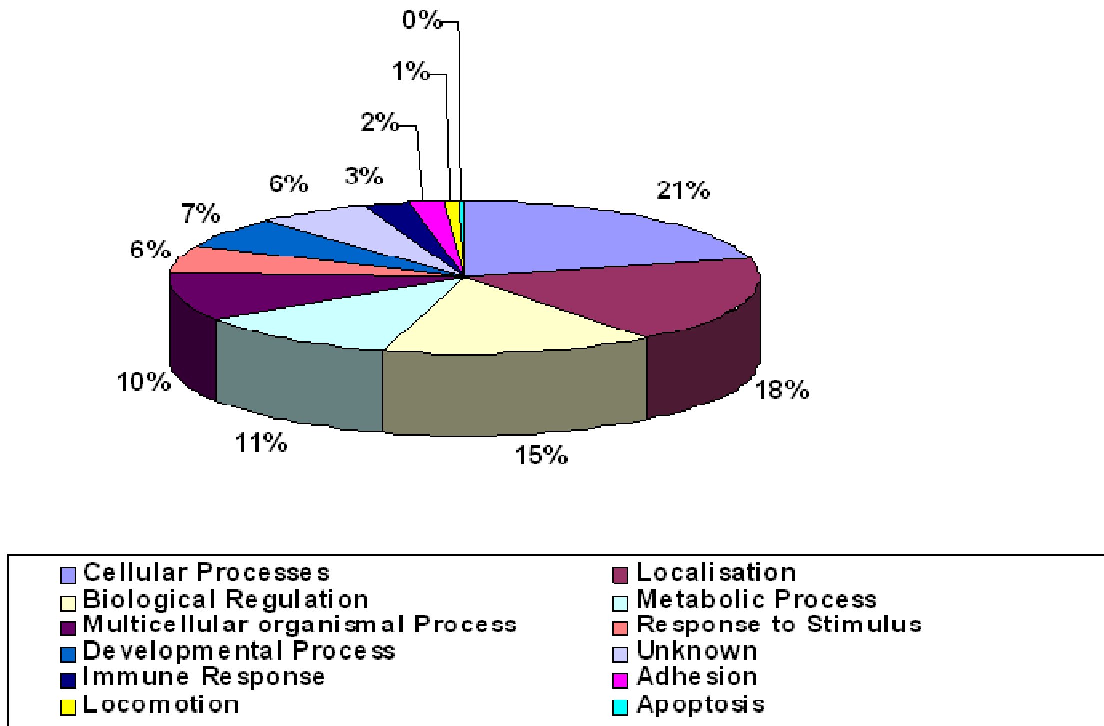


Figure 4.4. GO analysis of biological functions of STBM proteins

4.5 Identification of Differentially Expressed STBM Proteins in Adverse Pregnancies and Healthy Controls

We identified over 14 STBM proteins differentially expressed between pathologic and normal pregnancies, listed in Tables 4.2 and 4.3, notable among them are annexins, histones, integrins, fibrinogens, fibronectin, peroxiredoxins, clathrin and protein S-100 A11. All of these, except histones, were upregulated in adverse pregnancies. Their major functions are summarized in Table 4.4.

Table 4.2
Protein expression in STBM from patients with PE versus that from normal pregnant controls

protein name	accession numbers	molecular weight	Mean PE	SEM PE	Mean NP	SEM NP	Fold (PE/NP)	p- value
Annexin A1[a]	IPI00218918	39 kDa	133.69[b]	33.99	44.75	9.75	2.99	0.0412
Isoform 2 of Annexin A2	IPI00418169 (+1)	40 kDa	94.32	13.51	31.25	12.75	3.02	0.0137
Annexin A4	IPI00793199	36 kDa	109.76	24.56	24.25	8.75	4.53	0.0201
Histone H2B type 2-E	IPI00003935 (+3)	14 kDa	15.61	4.83	46.00	6.00	0.34	0.0079
Histone H4	IPI00453473	11 kDa	5.70	2.48	29.50	3.50	0.19	0.0028
Isoform Beta-3A of Integrin beta-3	IPI00303283	87 kDa	7.94	2.43	19.25	2.75	0.41	0.0165
Protein S100-A11	IPI00013895	12 kDa	13.85	2.52	4.75	2.75	2.92	0.0312
Peroxiredoxin-6	IPI00220301	25 kDa	10.20	0.57	1.50	1.50	6.80	0.0166

[a]Only proteins with a total spectral count of 10 or more, fold change >2 or <0.5 and p-value<0.05 (two-sample t-test) are listed.
[b]Numbers represent total spectral counts from all the peptides of a given protein.

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Table 4.3
Protein expressions in STBM from patients with RM versus that from normal pregnant controls

protein name	accession numbers	molecular weight	Mean RM	SEM RM	Mean NP	SEM NP	Fold (RM/NP)	p- value
Histone H2A type 1-H[a]	IPI00081836 (+4)	14 kDa	9[b]	2	19.25	1.25	0.47	0.0255
Isoform 1 of Clathrin heavy chain 1	IPI00024067 (+1)	192 kDa	17.25	0.75	5.5	2	3.14	0.0161
Isoform 1 of Fibronectin	IPI00022418 (+10)	263 kDa	12	0				
Isoform 1 of Fibrinogen alpha chain	IPI00021885 (+1)	95 kDa	10	0				
Fibrinogen beta chain	IPI00298497	56 kDa	24	0				
Isoform Gamma-B of Fibrinogen gamma chain	IPI00021891 (+3)	52 kDa	16.5	0				

[a]Only proteins with a total spectral count of 10 or more, fold change >2 or <0.5 and p-value<0.05 (two-sample t-test) are listed.
[b]Numbers represent total spectral counts from all the peptides of a given protein.

Table 4.4
Functional association of proteins differently expressed in STBM from patients with PE and RM

protein name	Functional Association
Annexin A1 Isoform 2 of Annexin A2 Annexin A4	Calcium-dependent phospholipid binding, anti-apoptosis, signal transduction
Histone H2A type 1-H Histone H2B type 2-E Histone H4	DNA binding, nucleosome assembly, DNA repair, transcriptional regulation
Isoform Beta-3A of Integrin beta-3	Cell adhesion, trophoblast invasion, vasculature remodeling
Isoform 1 of Fibronectin Isoform 1 of Fibrinogen alpha chain Fibrinogen beta chain Isoform Gamma-B of Fibrinogen gamma chain	Extracellular matrix remodeling, trophoblast differentiation, tissue repair, acute phase respo
Peroxiredoxin-6	Cell redox homeostasis, antioxidant activities, cell proliferation
Isoform 1 of Clathrin heavy chain 1	Intracellular protein transport, receptor internalisation
Protein S100-A11	Signal transduction, negative regulation of cell proliferation

4.6 Validation of Proteomics Results by Immuno-histochemistry (IHC) analysis

IHC analysis of placenta for validation of LC-MS/MS-based STBM-proteomics data confirmed presence of all the selected proteins in the syncytiotrophoblast layer at periphery, which is shed as STBM, shown in Figure 4.5, 4.6, and 4.7. Negative controls showed no signals, Figure 4.8. There was a trend towards downregulation of all proteins, namely, histones, annexins, integrins, peroxiredoxins, fibronectin, protein S100, and clathrin (Figure 4.9) and Tables 4.5 and 4.6.

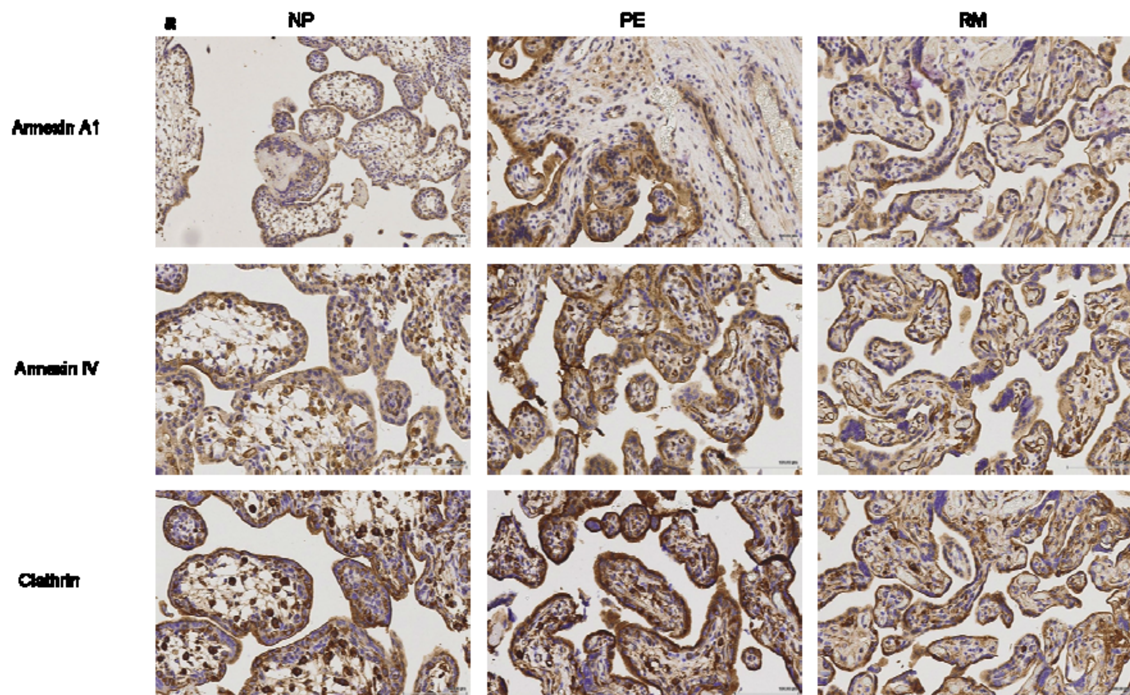

Figure 4.5: Representative images of Immunohistochemical validation of mass spectrometry-based STBM proteiomic data in placental villous tissues. There was a trend towards decrease in the studied protein expressions in PE and RM placental villi. Proteins are localized in the trophoblasts. Bars represent 100 µm (X20).

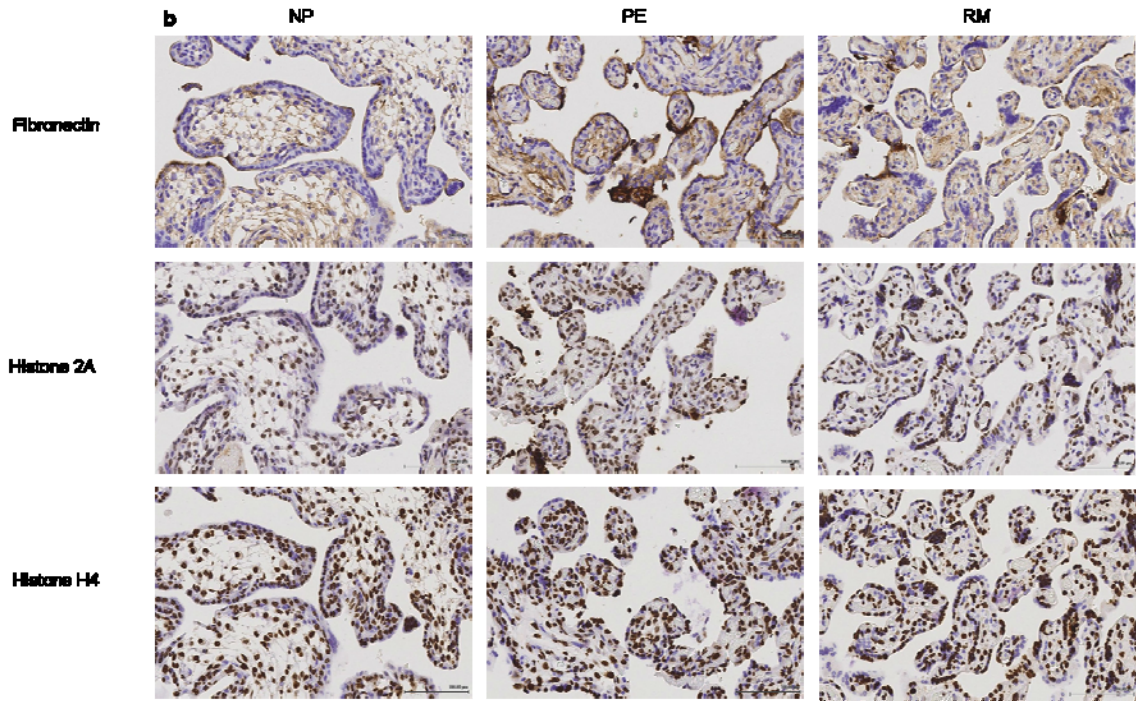

Figure 4.6: Representative images of Immunohistochemical validation of mass spectrometry-based STBM proteiomic data in placental villous tissues. There was a trend towards decrease in the studied protein expressions in PE and RM placental villi. Proteins are localized in the trophoblasts. Bars represent 100 μm (X20).

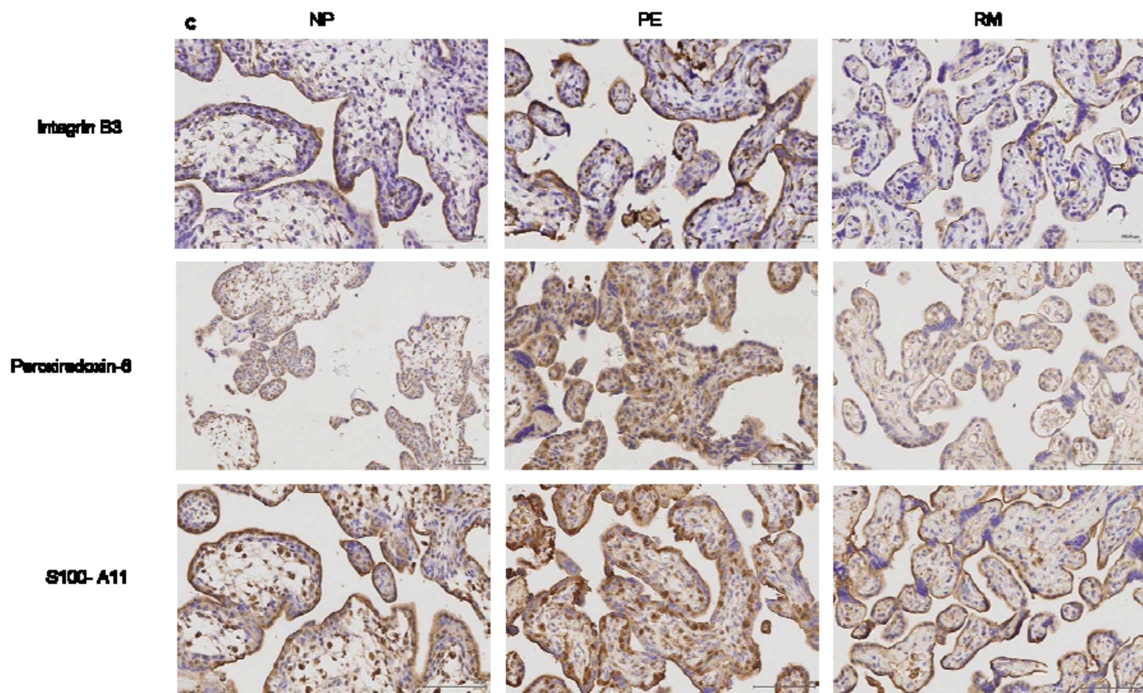

Figure 4.7: Representative images of Immunohistochemical validation of mass spectrometry-based STBM proteiomic data in placental villous tissues. There was a trend towards decrease in the studied protein expressions in preeclampsia (PE) and recurrent miscarriages (RM) placental villi. Proteins are localized in the trophoblasts. Bars represent 100 µm (X20).

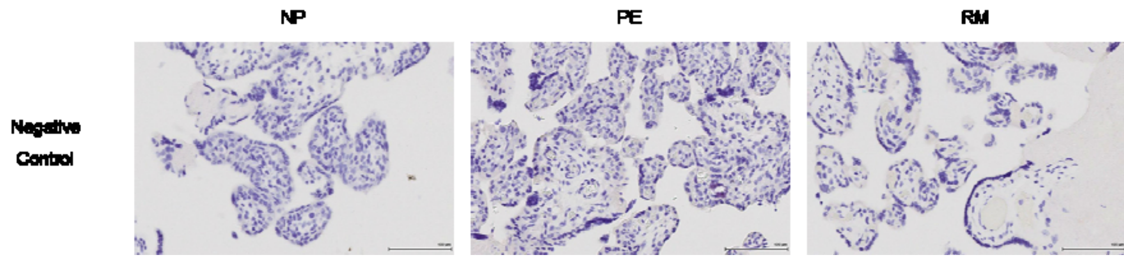

Figure 4.8: Representative images of negative controls of placentas of PE and RM patients. Negative controls showed no signals. Bars represent 100 µm (X20).

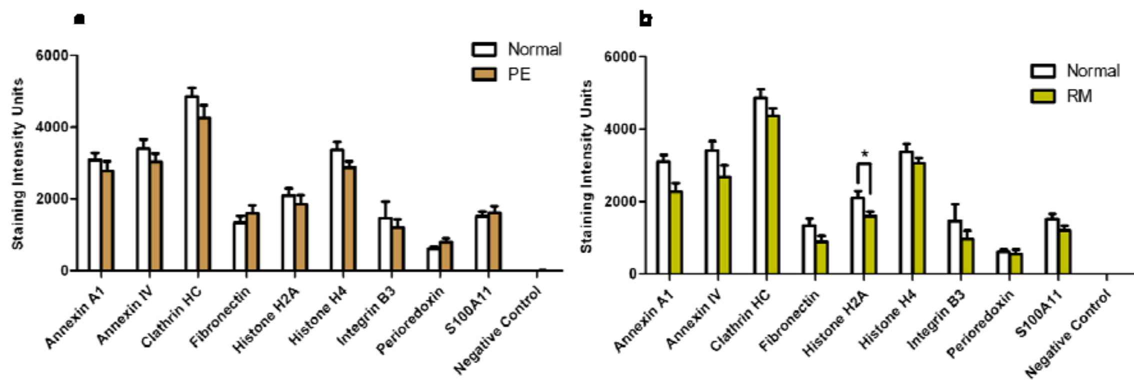

Figure 4.9: Summary of Immunohistochemistry analysis of placenta of PE and RM patients (a) PE-STBM protein expressions; (b) RM-STBM protein expressions.

Table 4.5
Immunohistochemical validation of RM STBM protein expression -- based on staining intensity

	RM			Normal			
	Mean	SEM	N	Mean	SEM	N	P value
Annexin A1	2266.69	243.34	13	3091	193.901	8	0.0406
Annexin IV	2680.15	317.35	13	3406.25	255.125	8	0.1259
Clathrin HC	4368.23	206.903	13	4859.25	242.786	8	0.1481
Fibronectin	883.615	161.633	13	1337.63	187.724	8	0.0893
Histone H2A	1592.85	132.047	13	2091.63	203.172	8	0.0440
Histone H4	3059.31	144.681	13	3369.63	221.248	8	0.2344
Integrin B3	962.077	224.9	13	1465.88	463.424	8	0.2872
Perioredoxin	543.385	130.234	13	609.25	60.348	8	0.7093
S100A11	1185.69	148.802	13	1512.13	139.868	8	0.1541
Negative Control	1.69231	0.44049	13	2.5	0.88641	8	0.3747

Table 4.6
Immunohistochemical validation of PE STBM protein expression -- based on staining intensity

	PE			Normal			
	Mean	SEM	N	Mean	SEM	N	P value
Annexin A1	2777.17	267.953	12	3091	193.901	8	0.4026
Annexin IV	3034.25	234.685	12	3406.25	255.125	8	0.3091
Clathrin HC	4258.17	360.172	12	4859.25	242.786	8	0.2322
Fibronectin	1590.58	234.659	12	1337.63	187.724	8	0.4493
Histone H2A	1850.25	249.996	12	2091.63	203.172	8	0.4988
Histone H4	2882.25	162.992	12	3369.63	221.248	8	0.0866
Integrin B3	1199.08	235.909	12	1465.88	463.424	8	0.5801
Perioredoxin	790	115.85	12	609.25	60.348	8	0.1121
S100A11	1611.67	184.414	12	1512.13	139.868	8	0.4903
Negative Control	4.75	2.50492	12	2.5	0.88641	8	0.4879

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Chapter 5: Lipidomic Analysis of Placental Syncytiotrophoblast Microvesicles in Adverse Pregnancy outcomes

5.1 Introduction

Defective trophoblast invasion resulting in abnormal uteroplacental perfusion and oxidative stress in pre-eclampsia and RM may result in qualitative and subsequent functional changes in STBM. Besides bioenergetics, lipids are also known to play important roles in cellular signaling, membrane structure and function. Recent evidence shows that alterations in the lipidome mediates physiologic cellular adaptation during health and pathologic alterations during disease (Gross et al., 2011).

Pre-eclamptic plasma analysis of lipid peroxides, antioxidants, triglycerides, and cholesterol profiles have been previously reported (Bayhan et al., 2000, Barden et al., 2001, Adiga et al., 2007, Lima et al., 2011, Ahmedi et al, 2012, Gohil et al, 2012). Oxidative stress and dyslipidemia in the form of increased malondialdehyde (marker of lipid peroxidation), decreased total anti-oxidant capacity, decreased high-density lipoprotein cholesterol concentration and increased total cholesterol, low-density lipoprotein cholesterol, very low -density lipoprotein cholesterol and triglycerides concentration have been shown to be significantly evident in pre-eclampsia. In a recent report, Oliveira et al characterized the plasma lipid profiles of plasma samples from women with pre-eclampsia using a MALDI-MS approach (Oliveira et al., 2012). The main classes identified were glycerophosphocholines, glycerophosphoserines, glycerophosphoglycerols, glycosyldiradylglycerols, and glycerophosphates.

Weerheim et al., first reported the phospholipid composition of total microvesicles isolated from plasma of healthy individuals using one-dimensional high-performance thin-layer chromatography (HPTLC). Microvesicles contained PC (59%), SM (20.6%), and PE (9.4%), with relatively minor (<5%) quantities of other phospholipids (Weerheim et al., 2002). Subsequently, lipid composition of human B cell-derived microvesicles applying MALDI-MS was reported and found to be enriched in cholesterol, SM, and GM3 (Wubblots et al., 2003). Oxidized microvesicles and apoptotic blebs stimulated endothelial cells to specifically bind monocytes, with oxidized phospholipids being the active principle responsible for their biological activity (Huber et al., 2002). Serdar and coworkers found changes not only in serum, but also in placental and decidual lipid and protein oxidation products and anti-oxidant concentrations to be significantly associated with PE (Serdar et al., 2003). STBM from PE placentas differ in quality with regard to lipid (fatty acid) composition from those from normal placentas (Cester et al., 1994). Reduction in lipid levels in STBM reduced their potential to inhibit human umbilical vein endothelial cells (HUVEC) proliferation and blocked their potential to induce apoptosis (Gupta et al., 2008). Collectively these data suggest that STBM lipid content may play a role in the endothelial dysfunction and immune-deregulation of PE.

The role of STBM lipid profiles in RM patients has not been explored yet. Furthermore, other than sterol lipids, knowledge on polar lipids, e.g. sphingolipids and glycerophospholipids is lacking. These major membrane lipids form precursors for second messengers during cellular signalling (Fernandis et al., 2007)

Our research interest is to study STBM lipid composition in preeclampsia and RM. Our objectives are to determine lipid components of STBM using lipidomics approaches and explore whether STBM lipid metabolites differ between normal pregnancies and pregnancies associated with preeclampsia and RM. Changes in various polar lipids

(Figure 5.1, Table 5.1) in STBM would provide an insight into the pathogenic changes that occur at the maternal-foetal interface during adverse pregnancies and which lipid components could possibly incite such changes.

Table 5.1
Functional association of major polar lipid classes

Lipid class	Functional Association
Phosphatidylinositol (PI) Phosphatidic Acid (PA)	Regulatory signaling molecules involved in migration, proliferation, senescence, apoptosis
Sphingomyelin (SM), Ganglioside (GM3), Ceramide (Cer) Lyso phosphatidylcholine (Lyso PC) Phosphoglyceride (PG)	Inflammation, immune response, oxidative stress, apoptosis
Phosphatidylserine (PS)	Regulatory signaling molecules involved in apoptosis & coagulation
Phosphatidylcholine (PC) Phosphatidylethanolamine (PE)	Binding sites for inter & intracellular proteins
Oxidised Phospholipids	Hypoxia, redox cell signaling, activation of signaling pathways
Cholesterol (Chol) Cholesteryl esters (CE) 7-ketocholesterol (7-KC) 24-OH cholesterol (24-OHC)	Membrane structure maintenance, regulation of signal transduction Form lipid-soluble core of lipoprotein, associated with atherosclerosis Apoptosis, oxidation, inflammation Cholesterol elimination & homeostasis

5.2 Subject Characteristics

We investigated lipid profiles of microvesicles from 6 pregnant women complicated with preeclampsia, 6 gestational age-matched healthy normal pregnant women as preeclampsia controls, 9 pregnant women with history of RM, and 9 gestational age-matched healthy normal pregnant women as RM controls. The clinical characteristics of the women and maternal and neonatal outcomes are summarized in Table 5.2. The patients underwent Caesarean or vaginal delivery at gestational age >28 weeks with live births. All RM patients received either passive immunotherapy in the form of intravenous immunoglobulin (IVIG) and/or antithrombotic therapy (aspirin and/or heparin), while those with pre-eclampsia received conventional anti-hypertensive therapy.

Table 5.2
Maternal and foetal outcomes of STBM lipidomics study

	PE (n=6)	PE-Normal controls (n=6)	RM (n=9)	RM-Normal controls (n=9)
Maternal age (y)	37.17[3.82][a]	35.17[5.38]	32.67[3.94]	36.22[5.19]
Gestational age (w)	31.82[3.24]*	35.35[3.09]	36.9[1.72]	36.36[2.88]
SBP (mm Hg)	161.50[18.95]***	120.67[15.31]	103.5 [19.09]	116.25[15.31]
DBP (mm Hg)	107[6.06]***	73[6.08]	67[9.9]	76.5[5.58]
Proteinuria (g/24h)	1.14[0.57]	-	-	-
Infant birth wt (g)	1687.95[888.96]*	2650[642.25]	2823.25[533.62]	2725.56[571.65]
Placental wt (g)	395.50[121.93]**	578.75[101.21]	589.75[203.75]	565.86[73.54]

[a]Results are mean [SD], comparisons using unpaired t test (patient group, ie, PE or RM, versus normal pregnant controls), P <0.05*, P <0.001**, P <0.0001 ***

5.3 Identification of STBM Lipids by ESI-LC MS MRM

We used LC gradient elution to separate various classes of lipids (Figure 5.1) in STBM. The various MRM transitions for corresponding lipid species were monitored, their intensities measured and compared to spiked internal standards. Each lipid species in each sample was then normalized to the total lipid levels in the corresponding sample. Analyses of total lipid fraction of individual classes of lipids are shown in Figure 5.2 and 5.3 and 5.4. Surprisingly, contrary to the normal lipid composition observed in human cells or plasma, SM was the most abundant phospholipid species in STBM (normal and pathologic) instead of PC, followed by cholesterol, PS, PC, PI, and PA. PS was significantly higher in the STBM from preeclampsia patients compared to that from normal controls ($p<0.005$), while SM, Cer, and LPC, showed a similar but non-significant trend. PI ($p<0.0005$), PA ($p<0.005$), and GM3 ($p<0.05$) were significantly lower in the STBM from preeclampsia patients relative to that from normal controls, while PC, PE, PG, LPI and GluCer showed a similar but non-significant trend, Figure 5.5 (a). STBM from RM patients showed similar trends in total levels of these lipid classes with PI ($p<0.005$), significantly lower than that from normal subjects, Figure 5.5 (b). When women who had 3 or more losses (n=4) were separately analyzed, the increase in PS compared to the control group ($p<0.05$), were found to be more marked as opposed to the women who had 2 losses (non-significant increase). While RM patients treated with heparin (n=4) showed no significant STBM lipid changes relative to the control group, RM patients with no heparin treatment showed significantly increased PS ($p<0.005$) and significantly decreased PI ($p<0.005$).

Among the sterol lipids, cholesterol and CE showed a trend to increase while 24-OHC and 7-OHC, a trend to decrease in adverse pregnancies.

The levels of individual lipid species in STBM were quantified and are illustrated in

Figure 5.6 and 5.7.

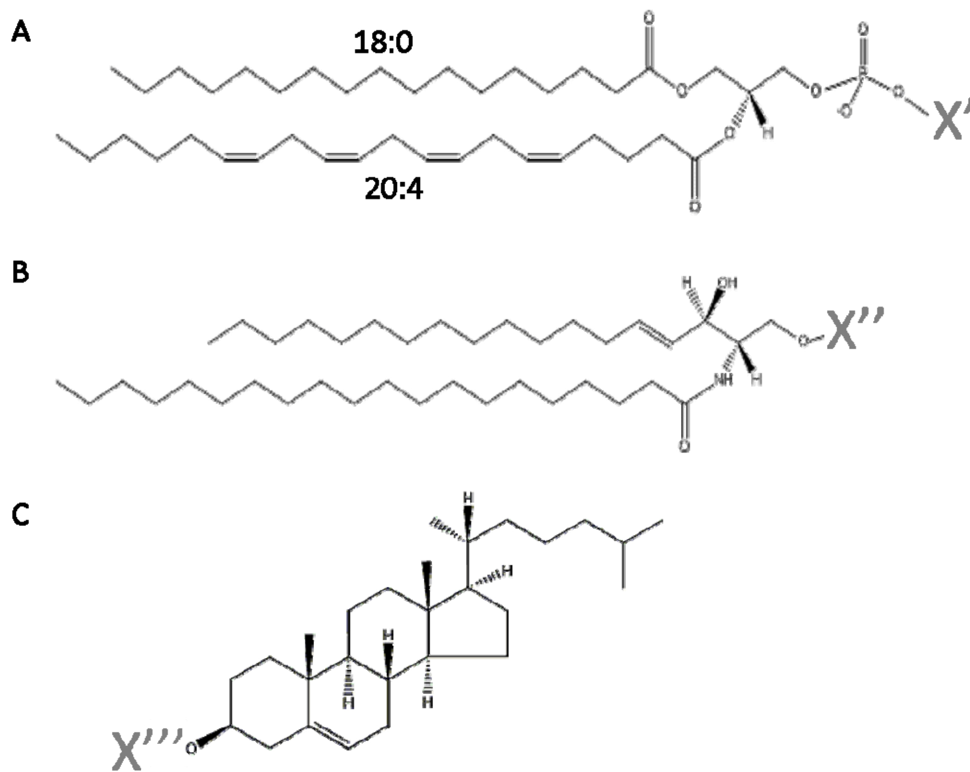


Figure 5.1: Examples of polar lipid species and explanation of the lipid nomenclature. (A) Structure of phospholipid, where x is the headgroup. X'= choline (PC), ethanolamine (PE), serine (PS), inositol (PI), glycerol (PG), phosphate (PA). Each of the phospholipid species has two fatty acyl chains. These may vary in length and degree of unsaturation. ESI MRM lipid profiling is able to identify each lipid species by their headgroup (X'), total carbon number in the two fatty acid chains, and degree of unsaturation. In this case, there are fatty acids 18:0 and 20:4. If X'= choline, the molecule would be PC38:4. The same nomenclature applies to both (B) and (C). (B) Structure of sphingolipid. X''= hydrogen (Cer), p-choline (SM), aNeu5Ac(2-3)bDGalp(1-4)bDGlcp(1-1) (GM3). (C) Structure of sterol. X'''= hydrogen (cholesterol), fatty acid (cholesteryl ester).

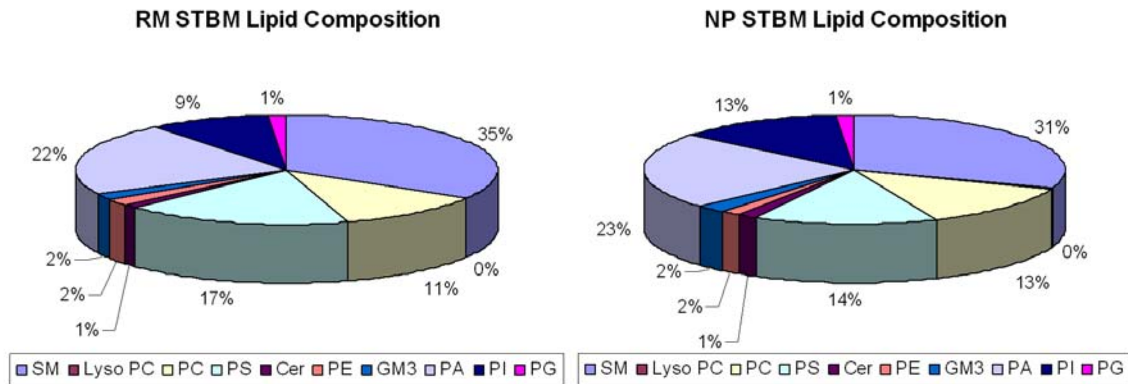


Figure 5.2: Lipid composition of RM STBM. Comparison of mean molar fractions of phospholipids and sphingolipids classes in STBM from RM patients (n=9) and normal pregnant women (n=9).

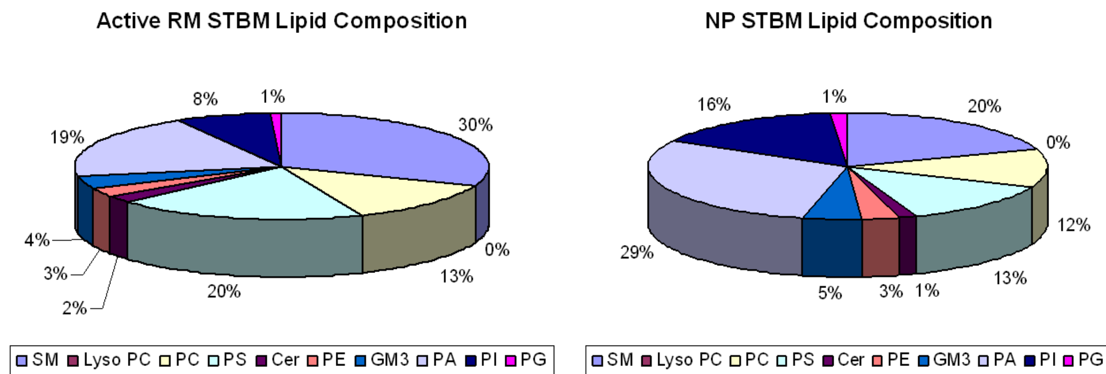


Figure 5.3: Lipid composition of active RM STBM. Comparison of mean molar fractions of phospholipids and sphingolipids classes in STBM from RM patient with ongoing loss (n=1) and normal pregnant women (n=1).

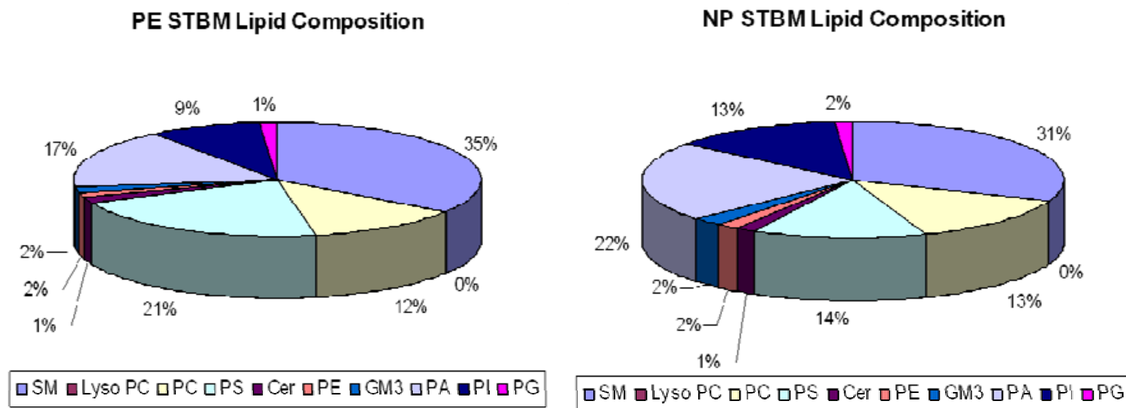
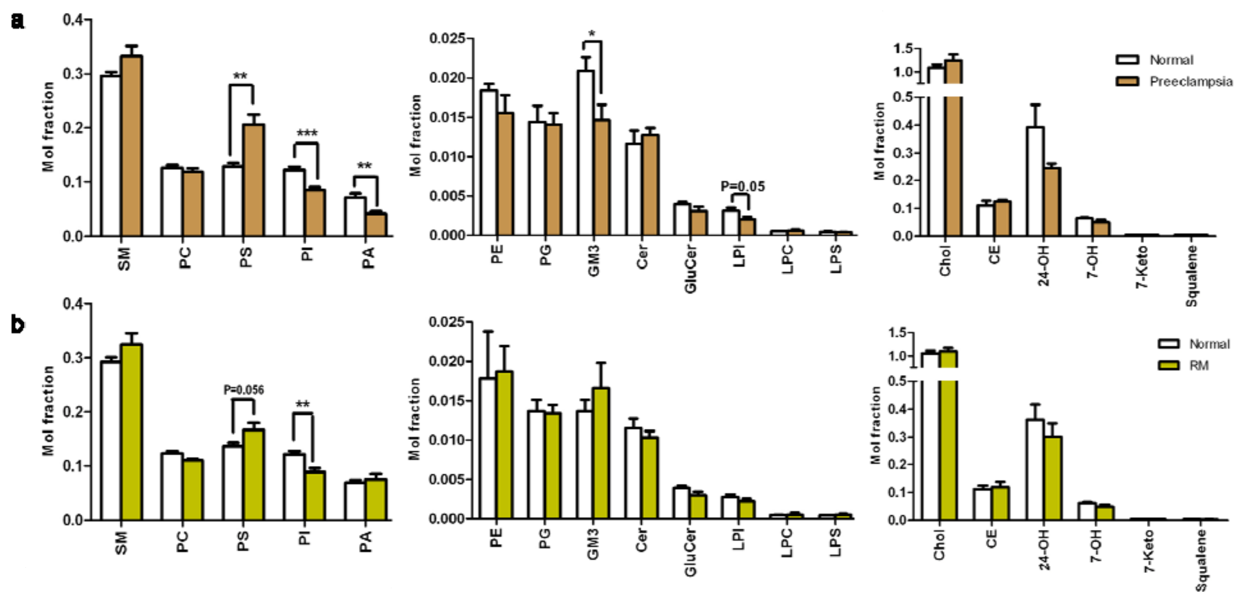


Figure 5.4: Lipid composition of pre-eclampsia STBM. Comparison of mean molar fractions of phospholipids and sphingolipids classes in STBM from pre-eclampsia patients (n=6) and normal pregnant women (n=6).



Figures 5.5 (a) & (b). (a). Major Lipid Classes in PE STBM. Comparison of total fractions of phospholipid, sphingolipid and sterol lipid classes in STBM from preeclampsia patients (n=6) and normal pregnant women (n=6). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. Figure (b). Major Lipid Classes in RM STBM. Comparison of total molar fractions of phospholipid, sphingolipid, and sterol lipid classes in STBM from RM patients (n=9), and normal pregnant women (n=9). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

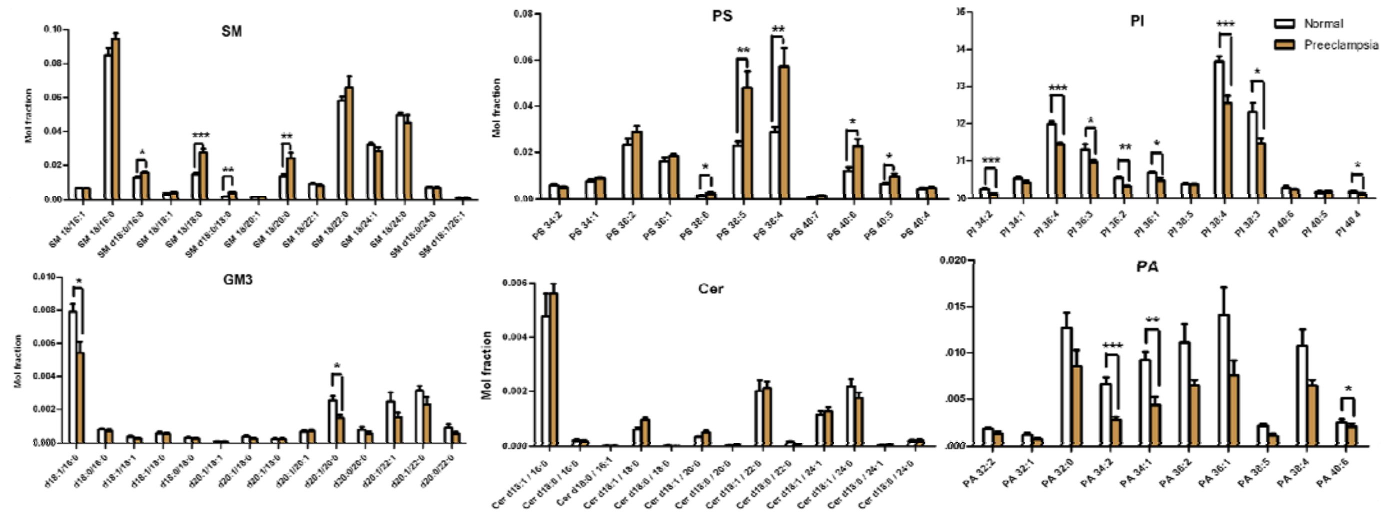


Figure 5.6: Major lipid species within each class in pre-eclampsia STBM. Comparison of mean molar fractions of individual phospholipids and sphingolipids species in STBM from pre-eclampsia patients (n=6) and normal pregnant women (n=6). * p <0.05.

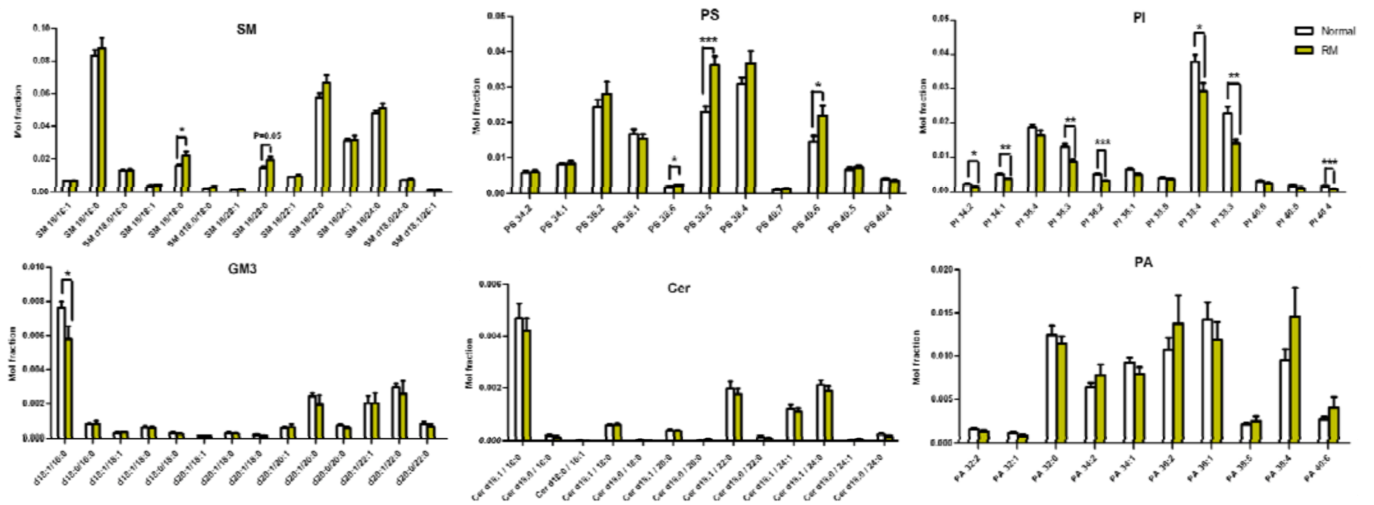


Figure 5.7: Major lipid species within each class in RM STBM. Comparison of mean molar fractions of individual phospholipids and sphingolipids species in STBM from RM patients (n=9) and normal pregnant women (n=9). * $p < 0.05$

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Chapter 6 Dendritic Cells Subset Analysis in Human Decidua Shows Presence of CD141^{hi} DCs

6.1 Introduction

Immunomodulation and establishment of tolerance at the dynamic maternal-foetal interface are pivotal to successful coexistence between mother and the feto-placental semi-allograft expressing both maternal (self) and paternal (non-self) genes (Hunt et al., 2006, Mor et al., 2010, Mor et al., 2011). During normal healthy pregnancy cytotoxic adaptive immune response is diminished, regulatory adaptive immunity is enhanced (Guerin et al., 2009), while innate immunity remains intact to continue to provide host defense against infection and facilitate interaction with foetal tissue for optimal placentation (Barrientos et al., 2009, Dekel et al., 2010, Nagamatsu et al., 2010). Immune privilege at maternal-foetal interface occurs when maternal immune cells encounter immunomodulatory molecules, e.g. progesterone, IDO, HLA-G and FasL from foetal trophoblasts, and GM-CSF from uterine epithelial cells. Perturbations in this complex physiology could lead to adverse outcomes such as recurrent miscarriages (RM) involving rejection of the feto-placental graft.

A significant portion of unexplained RM is associated with immunologic factors as evident by increased inflammatory immune responses in RM including increased number and cytoxicity of natural killer (NK) cells in maternal peripheral blood (Kwak-Kim et al., 1995) and endometrium (Clifford et al., 1999), raised Th1 cytokine levels in endometrium (Lim et al., 2000), elevated Th1/Th2 cell ratios in peripheral blood (Kwak-Kim et al., 2003), increased number of IL-17^{+} T cells , reduced numbers and function of Foxp3^{+} regulatory T cells (Treg) in peripheral blood (Lee et al., 2011), and increased

proportion of Th17 cells in peripheral blood and deciduas (Wang et al., 2010). Collectively this evidence suggests that enhanced pro-inflammatory immune responses with suppressed immune regulation may be an important immune mechanism involved in RM.

During pregnancy, invasive foetal trophoblasts become admixed with decidual immune cells including dendritic cells (DCs) reflecting extensive maternal-foetal immunological cross-talk (Bulmer et al., 1988a, Bulmer et al., 1988b, Gardner et al., 2003). Gardner and colleagues first identified and described the phenotype and distribution of human decidual DCs, as an HLA-DR+, CD11c+ lin- (CD3-, CD19-, CD56-, CD14-) population using three-color flow cytometry. The cell isolates were prepared from first-trimester decidual tissue. The decidual DCs comprised approximately 1.7% of CD45+ cells in the isolates and had the phenotype of immature myeloid DCs. Decidual DCs are critical for activating the T cell response mediating maternal immune tolerance of the semi-allogeneic foetus.

Human DC subsets in peripheral tissues, e.g. skin, lung and liver, have recently been reported, Figure 6.1 (A-F) (Haniffa et al., 2012). The functions of different DC subsets have been summarized in Table 6.1. Of the major DC subsets, $CD141^{hi}$ DCs is found to be the most efficient antigen presenting cells. DC subsets in decidual tissue from women with normal pregnancy or RM have not been reported yet.

As the role of decidual DCs in immune-dysregulation of RM remains unclear, we sought to determine if known DC subsets, $CD141^{hi}$ DCs in particular, were present in decidua and also whether RM patients had a differential distribution of DC subsets compared to normal healthy pregnant women, applying 13-colour flow cytometry approach.

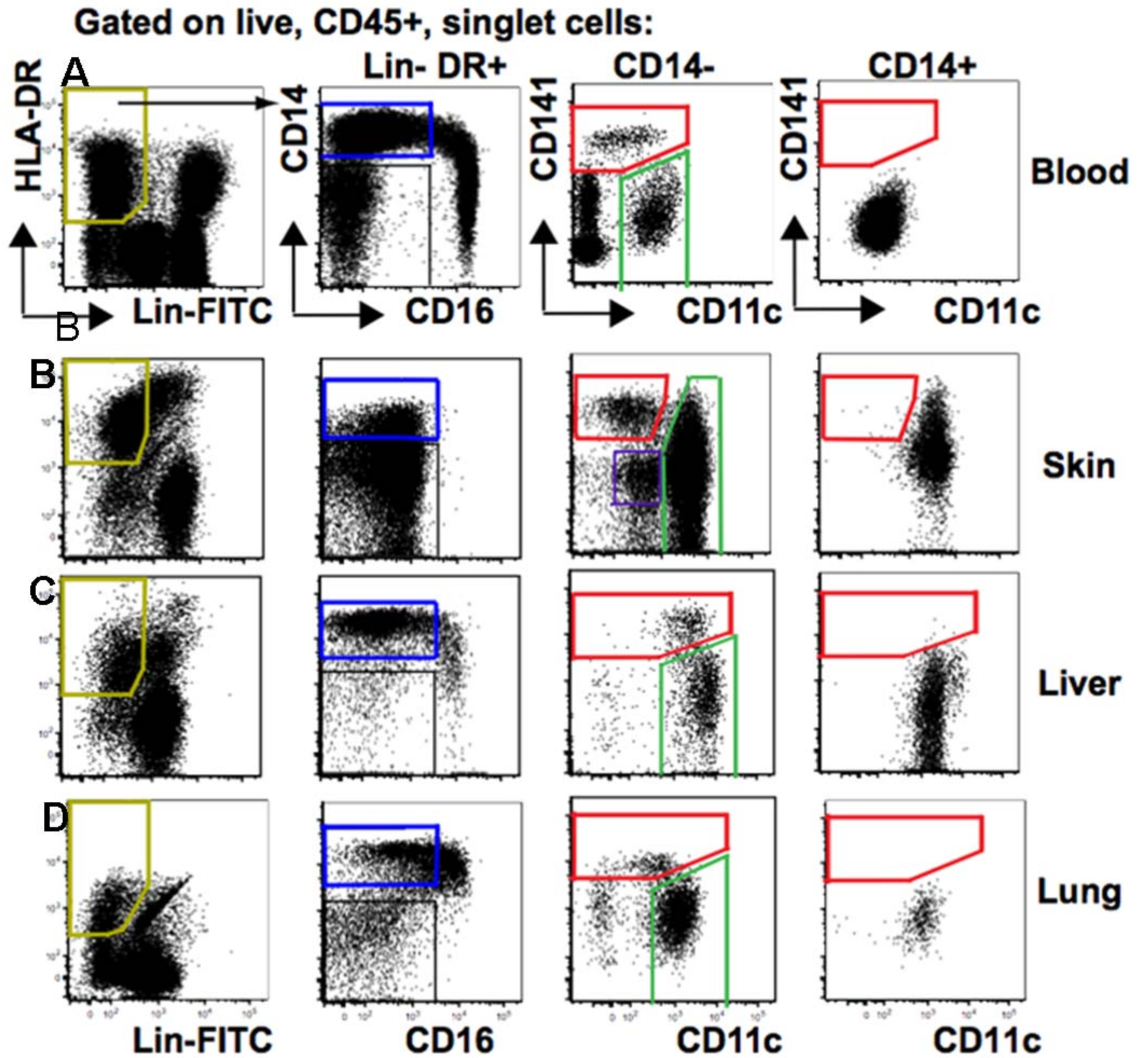


Figure 6.1 (A-D): Identification of CD141hi DCs in Human Tissues. Flow cytometry of peripheral blood, collagenase-treated whole skin, liver, and mechanically dispersed lung. Gating strategy used to identify three myeloid DC subsets within LinHLA-DR+ fraction (yellow gate) in tissues: (1) CD14+ DCs (blue gate), (2) CD14CD11c+ DCs (green gate), and (3) CD14CD11cloCD141hi DCs (red gate). Reproduced from Haniffa et al., 2012

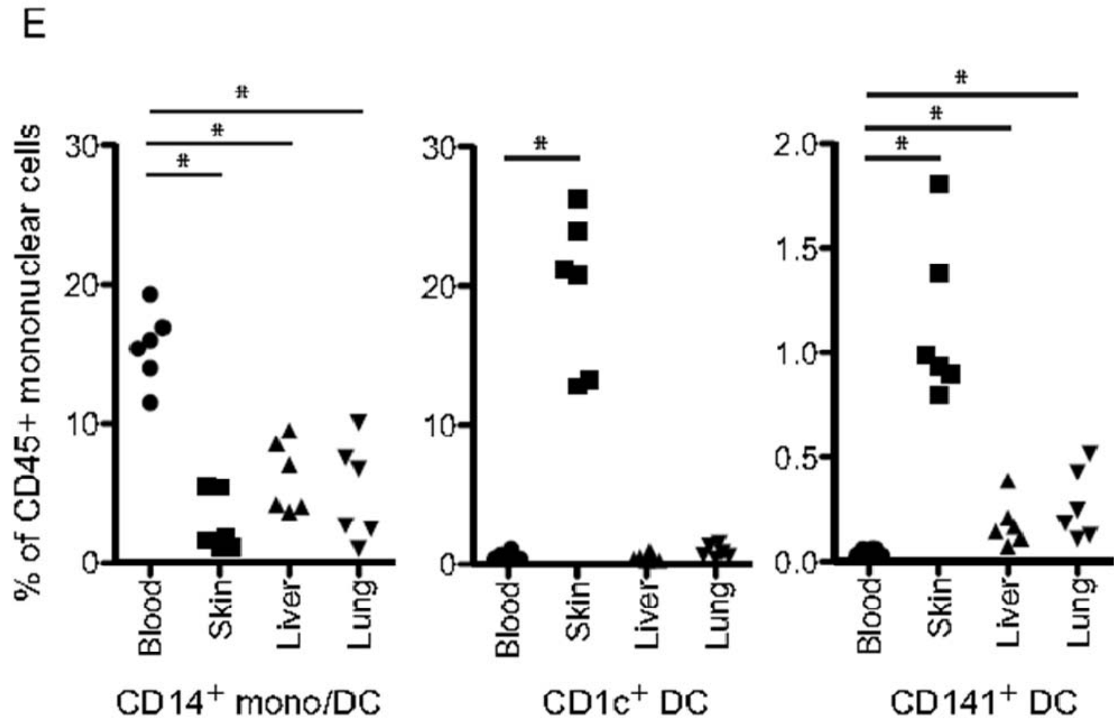


Figure 6.1 (E) Frequency of CD14 DC, CD1c DC and CD141 DC in blood, liver and skin as a % of CD45+ mononuclear cells. Scatterplot from 6 donors are shown. *p < 0.05, Mann-Whitney U test. Reproduced from Haniffa et al., 2012

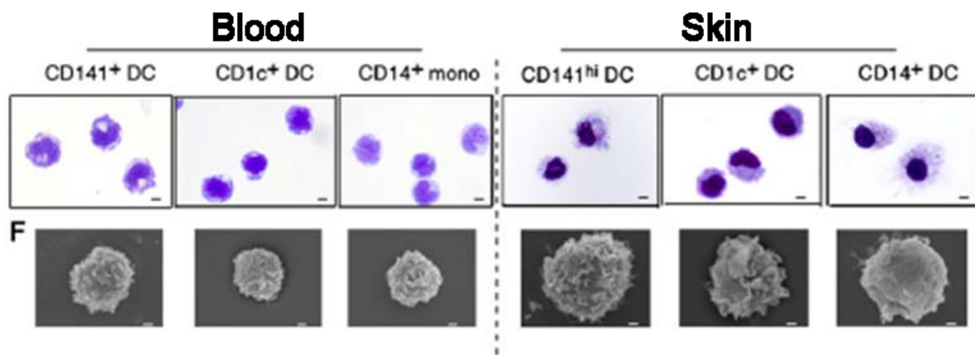


Figure 6.1 (F): Morphology of FACS-sorted blood and skin CD141+, CD1c+, and CD14+ DCs and monocytes. (upper panel) visualized by GIEMSA staining of cytospin preparations (X100) and (lower panel) by SEM. Scale bars represent 10 mm in (upper panel) and 1 mm in (lower panel). Reproduced from Haniffa et al., 2012.

Table 6.1
Functional association DC subsets

DC Subsets	Functional Association
$CD14^+$	Less immunogenic, less potent antigen presenting cells and stimulators of allogeneic MLRs
$CD14^+CD16^+$	Higher phagocytosis capacity and expression of IL-1 & MHC (HLA DR)
$CD16^+$	Induce IL-4 and TGF-β production by T lymphocytes (Th2 responses) during acute & chron
pDC	Produce IFN-α which upregulates antigen presentation to T cells, activates macrophages &
$CD11c^+$ DC	Potent inducers of allogeneic $CD4^+$ and $CD8^-$ T cell proliferation
CD141+ DC	Most efficient in cross-presentation of soluble antigens and induction of CTL responses produce CXCL10 and TNF-a on stimulation but not IL-12 and IL-23 (Haniffa et al., 2012) immunoregulatory, produce IL-10, and induce regulatory T cells (Chu et al., 2012) .

6.2 Subject Characteristics

We studied 2 pregnant women with RM and 7 gestational age-matched healthy normal pregnant women (Table 6.2). The patients and normal control subjects underwent caesarean delivery at gestational age >34 weeks. Of the RM patients, 1 had 5 previous unexplained RM. All RM patients received either passive immunotherapy in the form of IVIG and/or antithrombotic therapy (aspirin and/or heparin).

Table 6.2
Maternal and foetal outcomes in decidual DC subset study

	RM (n=2)	Normal controls (n=7)
Maternal age (y)	36[1.41][a]	31.43[2.94]
Gestational age (w)	36.65[2.90]	38.56[0.52]
Infant birth wt (g)	3200[176.78]	2706.2[159.63]
Placental wt (g)	824[0]	537.75[17.11]

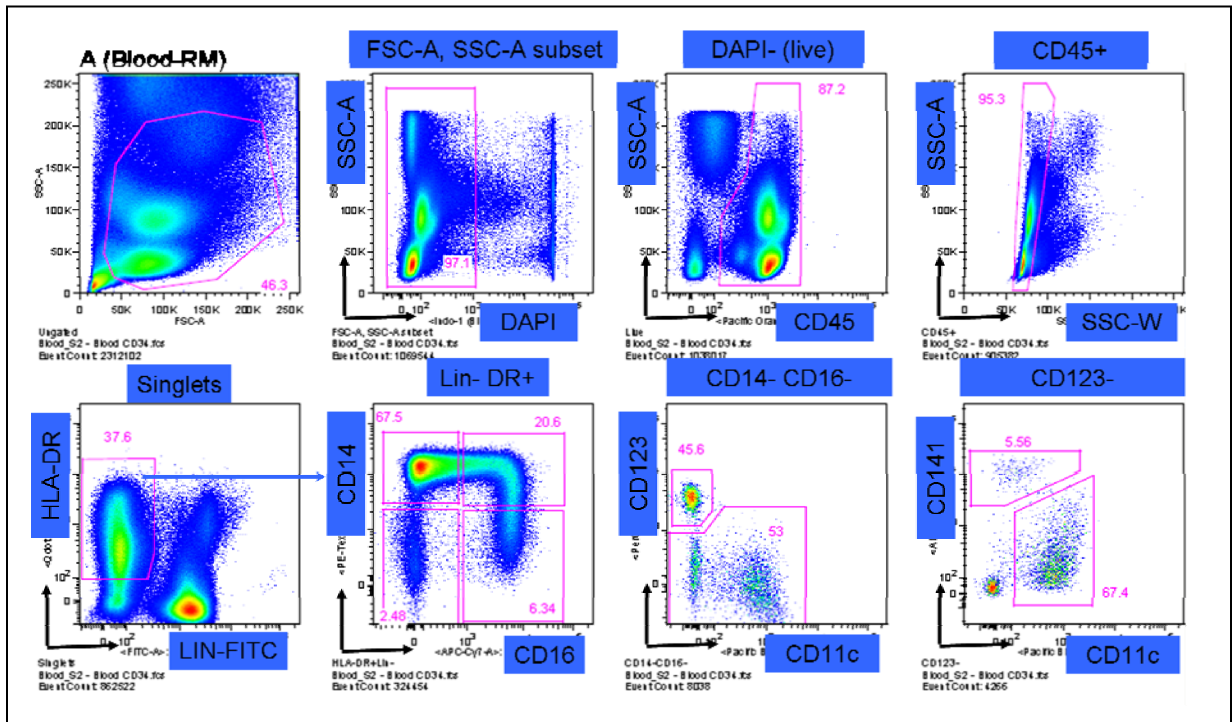
[a]Results are mean [SD]

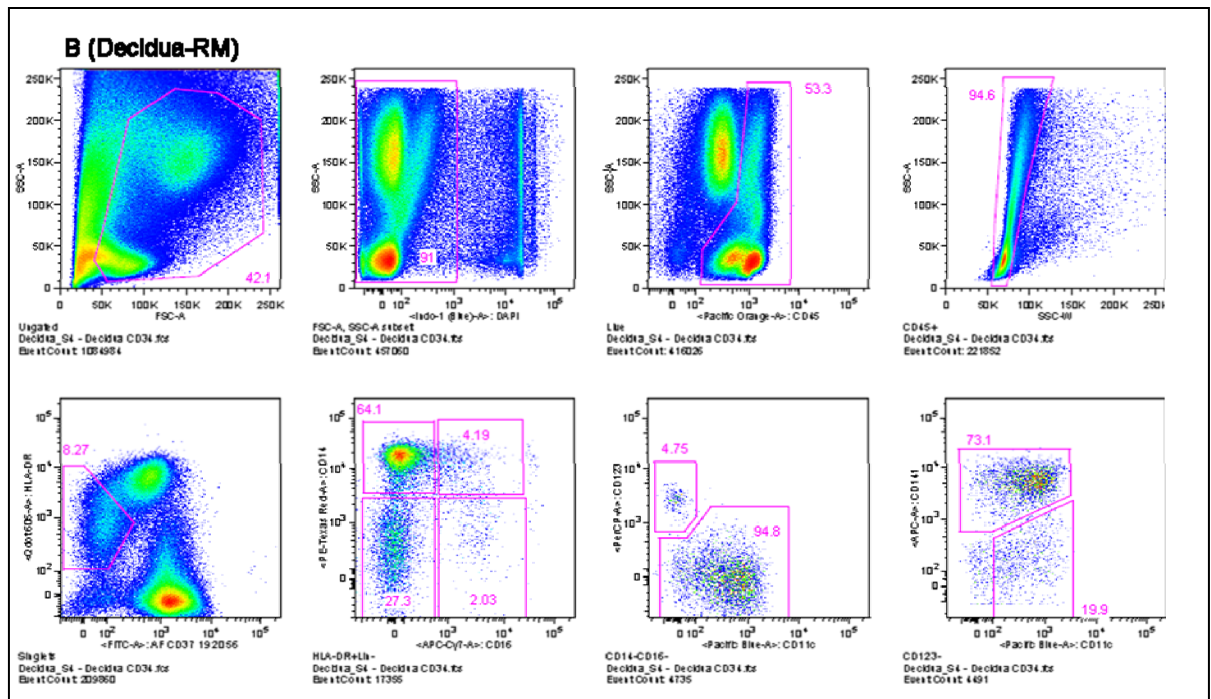
6.3 Identification DC subsets in Human Decidual Tissues

Using the gating strategy described in *section 2.3*, three myeloid DC subsets could be identified in human decidua: i). CD14+ DCs corresponding to blood CD14+ monocytes; ii) CD11c+ DCs corresponding to blood CD11c+ DCs; and iii) CD141hi DCs corresponding to CD141+ blood DCs. CD11c-CD141- cells comprised of CD123+ plasmacytoid DCs (pDCs) and $CD34^{+}$ haematopoietic progenitor cells. Full gating strategy and percentage of DC populations in blood and decidual tissue from patients and normal controls are shown in Figures 6.2 (A-D) and 6.3.

HLA-DR^{+} $lineage^{-}$ cells in blood and decidua comprised a $CD14^{-}$ and $CD14^{+}$ fraction (Figure 1A). Blood also contain $CD16^{+}$ monocytes or equivalent cells. The $CD14^{-}$ fraction was further separated by CD141 and CD11c expression. In blood, typical $CD141^{+}$ DCs are a distinct population with lower CD11c expression. This population is mirrored in decidua as CD141hi cells with low-to-intermediate CD11c expression.

Of the RM cases, 1 had suffered 5 unexplained miscarriages previously. This particular patient demonstrated a markedly higher frequency of CD141hi DCs (1.48% of $CD45^{+}$ cells), compared to the other RM (0.2%) and the normal controls (0.2%) (Figure 6.3).





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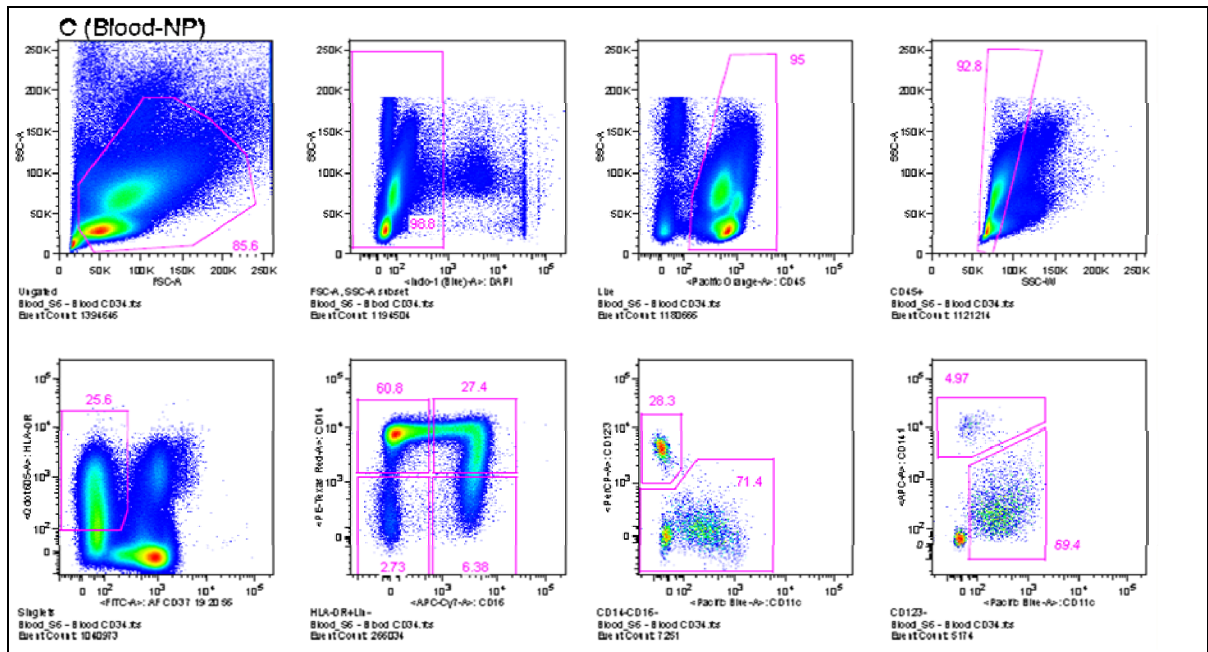

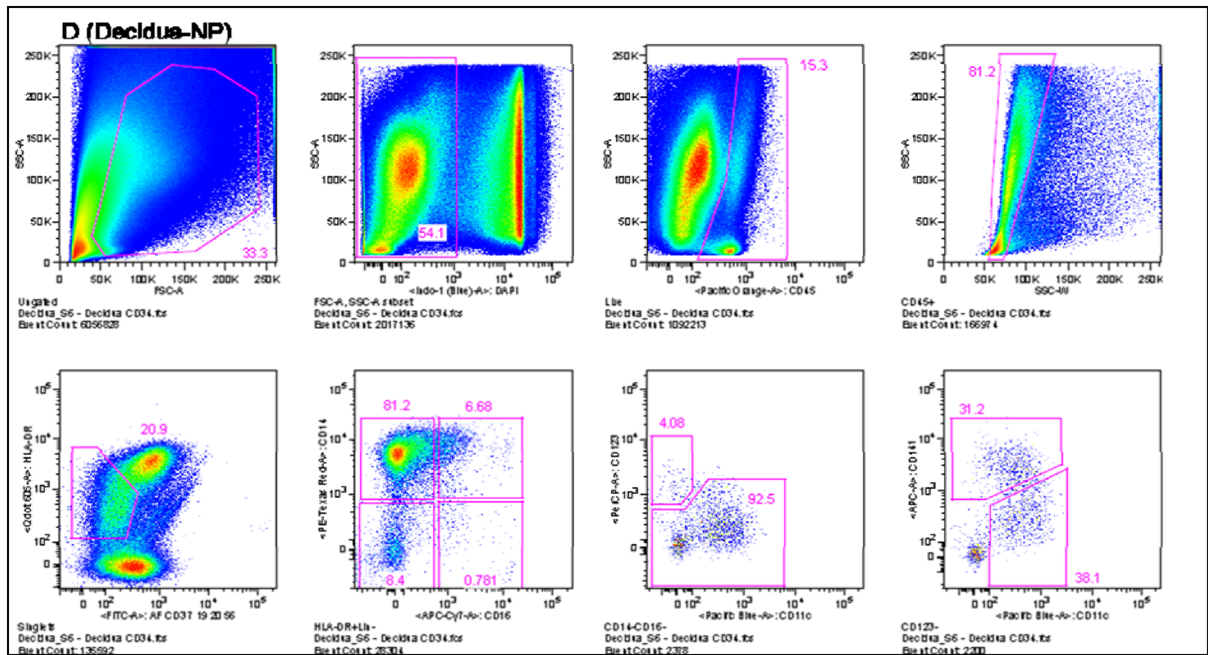

Figure 6.2 (A-D): Flow-cytometry gating strategy for (A) blood from RM patients, (B) collagenase-digested decidua from RM patients, (C) blood from normal pregnant controls (D) collagenase-digested decidua from normal pregnant controls. Decidua samples were analyzed using the same flow strategy to map known blood DC subsets to peripheral tissue populations (Haniffa et al., 2012). Cells were first gated by FSC-A and SSC-A and dead cells were excluded by DAPI staining. Leukocytes were identified using

CD45 followed by exclusion of doublets using the SSC-A and SSC-W. Antigen Presenting Cells (APC) were identified within the HLA-DR+ and Lineage- (CD3, CD19, CD20 and CD56 all in FITC) fraction. Having the Lineage cocktail in the FITC channel also allowed us to exclude autofluorescent macrophages in peripheral tissues. Three myeloid DC subsets can be identified in human decidua:1. CD14+ DCs corresponding to blood CD14+ monocytes; 2. CD11c+ DCs corresponding to blood CD11c+ DCs; and 3. CD141hi DCs corresponding to CD141+ blood DCs. CD11c-CD141- cells are comprised of CD123+ plasmacytoid DCs (pDCs) and CD34+ haematopoietic progenitor cells in the blood.

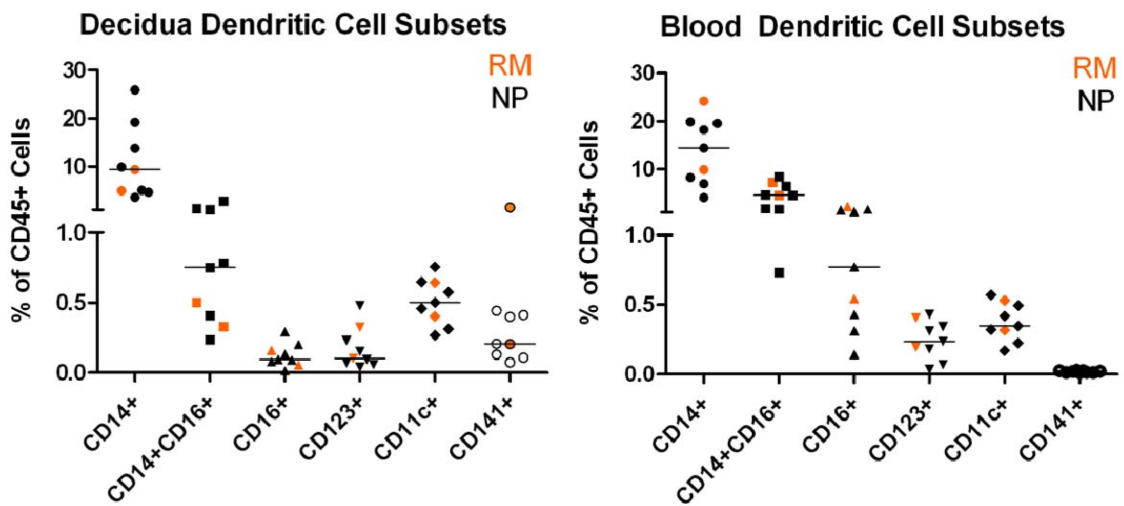

Figure 6.3. Frequency of DC subsets in decidua & blood as a percentage of CD45+ mononuclear cells. Scatterplot from 2 recurrent miscarriages (RM) patients & 7 normal pregnant controls (NP) (with median) are shown. Lines represent the median. Results are combined data from analysis of consecutive samples.

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Chapter 7 Discussions

7.1 Immunomodulation by Placental Syncytiotrophoblast

7.1.1 Microvesicles in adverse pregnancy outcomes

Our study investigated the effects of RM and PE associated STBM prepared in vitro from placental villous explants on antigen presenting cells. Explant STBM were produced under physiological conditions (Redman et al., 2005, Jauniaux et al., 2006, Heazell et al., 2008, Hung et al., 2008). Furthermore, explant STBM have been shown to be pro-inflammatory in previous reports of monocyte or PBMC stimulation assay (Messerli et al., 2010, Southcombe et al., 2011). This validates the application of these shedding STBM as a sound and acceptable approach to study the role of placental microvesicles in women with adverse pregnancy outcomes.

Microvesicles can transfer antigens to dendritic cells for subsequent presentation to T cells for indirect immune cell stimulation, or act independently of antigens by exposing immune cells to stimulatory factors, such as heat shock protein-70 or HMGB1(Thery et al., 2009, Redman & Sargent, 2012). Micorvesicles have been shown to affect the physiology of neighbouring recipient cells in various ways, from inducing intracellular signaling following binding to receptors to conferring new properties after the acquisition of new receptors, enzymes or even genetic material from the vesicles.

Southcombe & co-workers have shown that STBM bind monocytes, and to a lesser extent B cells. STBM binding to and/or internalization by dendritic cells are likely to be

similar to their parent cells, i.e. monocytes. Monocytes are able to rapidly phagocytose STBM, up to ~60%, at the end of an hour of incubation. This suggests that the receptor for STBM on monocytes may be involved with phagocytosis as well as binding. Possible receptors include Toll-like Receptors, Receptor for Advanced Glycation End Products (RAGE) and Integrins. Toll-like receptors (TLR) form the major family of pattern recognition receptors (PRR) that are involved in innate immunity. At the maternal–fetal interface, TLRs are expressed not only in the immune cells but also in non-immune cells including trophoblasts; moreover, their expression patterns vary according to the stage of pregnancy. They interact with endogenous danger molecules known to be expressed on STBM (e.g. HSP, HMGB1, and fibronectin). Binding of TLRs results in release of cytokines via intracellular signaling pathway. It is known that trophoblast is able to 'educate' immune cells, where constitutively expressed cytokines/chemokines, e.g. IL-8, MCP-1 and other signals originated from trophoblast could determine the subsequent immune cell behavior. This proper trophoblast–immune cell cross-talk may be essential for a normal pregnancy, and changes or defects in this interaction may lead to pregnancy complications as evidenced by animal models and clinical observations. TLR stimulation is known to induce fetal resorption when it occurs in early pregnancy (Li et al, 2010). Anti-phospholipid antibodies, which is known to be involved in the pathology of recurrent miscarriage, pre-eclampsia and preterm labor, was also shown to induce a pro-inflammatory response in first-trimester trophoblast via TLR4 pathway (Mulla et al., 2009).

Furthermore, among PBMCs, STBM bind preferentially to monocytes (~80%) and B cells (~40%) and over a 20-hr period of incubation time, up to 90% of STBM undergo internalization by the monocytes. Among PBMCs, the monocytes are responsible for the production of the majority of the most abundantly produced pro-inflammatory cytokines

(TNFa, IL-6, IL-8 and IL-1b). Koppler and colleagues showed that transfer of microvesicles to monocytes was largely complement independent, but dependent on calcium and annexin V, and was found to change the cytokine profile of monocytes (Koppler et al., 2006).

We found that although the STBM are pro-inflammatory to DCs and macrophages, they only induce a basal to modest expression of co-stimulatory molecules such as CD40, CD80, CD83 and CD86 as well as MHC I and MHC II molecules. Besides, they do not activate T cells. Our findings (Figure 3.5 and 3.6) are consistent with a recent report where the maternal monocytes were induced to produce pro-inflammatory cytokines by STBM but no upregulation of HLA-DR (MHC class II surface receptor) expression was observed (Southcombe et al., 2011). The authors argued that as T cells are not activated in normal pregnancy, a reduced expression of these markers of maturation/antigen presentation by APCs would reduce the potential for antigen presentation and prevent subsequent activation of $CD4^+$ T helper cells. In addition, DCs with tolerogenic properties are known to exist, which are crucial regulators of immunity (Morelli and Thomson, 2007). These tolerogenic DCs can present antigen to antigen-specific T cells but provide inadequate co-stimulatory signals or deliver net co-inhibitory signals for effector T cell activation and proliferation. We speculate that fetal-derived STBM incur similar tolerogenic properties on DCs at maternal-fetal interface. Besides, cytokines may be the primary signal via which STBM-mediated stimulation of DCs and T cells are carried out.

Our study showed that STBM induce release of pro-inflammatory cytokines such as IL-6, IL-8, TNF- α and IL-12p40 from monocyte-derived DCs. These cytokines have been reported to be increased locally at placenta or in maternal peripheral blood in normal pregnancy (Montagnana et al., 2008, Kraus et al., 2010). The pro-inflammatory effects of

cytokines of maternal origin are considered relevant in early pregnancy-interaction with fetal tissues to promote embryo implantation, angiogenesis and placenta formation. Thus, in the process of reproduction, cytokines are actively involved in the inflammatory process which results in either tissue damage or repair.

However, IL-6 is also anti-inflammatory and this effect is mediated through its inhibitory effects on TNF-α and IL-1 and activation of IL-10. As IL-6 has both pro-inflammatory and anti-inflammatory role, its presence in pregnancy may counter balance highly pro-inflammatory cytokines. IL-12 drives Th1 differentiation of T helper cell. Bioactive IL-12 (IL-12p70) is a p35/p40 heterodimer. IL-12p40 dimerises with IL-12p35 to produce bioactive IL-12. We could only observe upregulation of IL-12p40 in our study, possibly due to the physiologic and relatively mild nature of explant STBM stimulation that were used in the DC stimulation assay. STBM prepared from perfusion of placental cotyledon has been previously shown to upregulate IL-12p70 in PBMCs (Germain et al., 2007) and are also markedly more pro-inflammatory than explant STBM (Messerli et al., 2010, Southcombe et al., 2011). We found significant difference in pro-inflammatory cytokine production in STBM-induced DCs from women with RM or PE compared to normal pregnancies. These findings, together with histopathological evidence of inflammation and thrombosis, support the notion that excessive upregulation of these pro-inflammatory cytokines contributed to an exaggerated immune response that led to adverse pregnancy outcomes.

As expected in a clinical setting, almost all of our patients received passive immunotherapy in the form of IVIG and/or anti-thrombotic therapy (Low molecular weight heparin, LMWH) /low dose aspirin, ASA). IVIG may act possibly by correction of the immune dysregulation, a generally accepted phenomenon in play in immunologic RM (Dwyer et al., 1992; Mettheisen & Sharma, 2012). Anti-idiotypic antibodies in IVIG

preparations possibly bind to harmful autoantibodies associated with RM, and clear those. This would prevent binding of an autoantibody to its antigen, e.g. APA to trophoblast, prevent excessive apoptosis of trophoblast, and correct dysregulation of placental vasculature underlying RM. Also, anti-idiotypic antibodies may bind to and downregulate receptors for antigen, on APCs such as B-cell, and possibly dendritic cells and macrophages and modulate exaggerated immune response. Finally, it is postulated that anti-idiotypic antibodies may be recognized by regulatory T-cells leading to their activation and release of immunosuppresive lymphokines.

ASA and LMWH administration in RM may have a wide array of interactions with immune cells, e.g.dendritic cells and macrophages. They have anti-inflammatory effects by blocking, (i) production of pro-inflammatory cytokines, e.g. IL-6 and TNF-α, (ii) antigen-antibody binding, (iii) adhesion and chemotaxis of pro-inflammatory cells, (iv) and inhibiting complement activation. Besides, anti-coagulant effects; anti-apoptotic effects, thus conferring protection against trophoblast apoptosis; and finally pro-angiogenic effects have also been described.

The described drug effects suggest that the results of our experiments involving DC/ macrophage/T cell immune response using placentae from patients with history of RM could have been tempered by response to treatment administered to them. In our in vitro experiments, immune cells were derived from PBMCs from unrelated healthy donors, thus possible modulatory effects of drugs on immune cells were possibly excluded. Significant increase in level of pro-inflammatory cytokines by RM STBM could be demonstrated. Similar effects on trophoblasts, however, cannot be ruled out and could have influenced our findings, as demonstrated by mass-spectrometry analysis of STBM lipids. Eleven out of 20 women in RM group had received heparin (low molecular weight

heparin, LMWH). While RM patients treated with LMWH showed no significant STBM lipid changes relative to the control group, RM patients not treated with LMWH showed significantly increased PS ($p<0.005$) and significantly decreased PI ($p<0.005$). Of note, the lipid component of STBM has been shown to be associated with their pro-inflammatory potential (Huber et al., 2002, Gupta et al., 2008). In the present study, no differences in DC immune responses to STBM derived from between various treatment groups (IVIG vs. no IVIG or LMWH vs. no LMWH) could be observed.

In order to exclude these drug effects on experiments, larger number of RM patients with or without various treatment regimens could be studied. More importantly, study on RM patients in a randomized control trial on efficacy of immunotherapy needs to be performed to demonstrate drug effects.

The placenta is known to exert immunomodulatory effects in pregnancy (Chaouat et al., 1987) and shedding STBM which carry regulatory lipids, proteins, and microRNAs (Baig et al., in preparation, Tannetta et al., unpublished observations) possibly extend this immunomodulatory effect systemically. Specific suppression by apoptosis and inhibition of signaling of maternal cytotoxic T cells and NK cells may be achieved partially by FasL, HLA-G, and syncytiotrophoblast membrane glycoproteins expression by circulating STBMs (Gercel-Taylor et al., 2002, Taylor et al., 2006, Sabapatha et al., 2006, Arkwright et al., 1994). In another recent report, trophoblast debris generated in vitro from placental explants of normal placenta, were exposed to peripheral blood-derived macrophages (Abumaree et al., 2012). The macrophages acquired an immunosuppressive phenotype and functions. It was concluded that phagocytosis of trophoblast debris from normal placentae alters the phenotype of macrophages towards tolerance and away from inflammation. There is evidence to suggest that this

mechanism may be dysregulated in adverse pregnancy outcomes such as PE and RM (Whitecar et al., 2001, Taylor et al., 2004) and this may explain higher immunogenicity of pathogenic STBM.

We found both normal and pathologic pregnancy- associated STBM to be pro-inflammatory with a trend towards higher proinflammatory cytokine signals from DC as well as macrophages stimulated by pathogenic STBM. The discrepancy of these results may be attributed to different methodology of preparation of STBM including 8% O_2 used in villous explant culture in our study, similar to Southcombe et al. as opposed to 20% O_2 used by the Abumaree et al., which may have effected the immunogenicity of STBM. Oxygen is known to influence cell turnover and expression of regulators of apoptosis such as p53, p21 and Mdm2 in human placental trophoblast (Heazell et al., 2008). Also, STBM shed by explants from normal placenta cultured in air (20% O_2) has previously been shown be pro-inflammatory as demonstrated by up-regulation of monocyte cell surface expression of the adhesion molecule CD54 and production of interleukin IL-8, IL-6 and IL-1β (Messerli et al., 2010), supporting the results of our study.

Maternal immune dysregulation to the fetal allograft may lead to increased trophoblast apoptosis and dysfunction. In miscarriage, development of placento-decidual interface is severely impaired leading to premature, widespread onset of maternal blood flow with major oxidative insult by oxygen free radicals and subsequent pregnancy loss (Jauniaux et al., 2000). In PE the trophoblast invasion is sufficient to allow early phases of placentation. However, shallow trophoblast invasion results in incomplete substitution of maternal spiral arterial endothelium leading to impaired placentation, repetitive ischaemia-reperfusion phenomenon and chronic oxidative stress. This probably culminates into increased trophoblast shedding and raised STBM level (Knight et al., 1998, Redman et al., 2007, Redman et al., 2008, Lok et al., 2008). Besides this

quantitative change, STBM possibly undergo qualitative changes due to oxidative damage to its content explaining their higher immunogenicity (Pantham et al., 2011). Investigation of STBM lipid and protein contents in pregnancy pathologies is currently in progress in our laboratory.

In conclusion, we have shown STBM in health and disease (RM and PE) to be pro-inflammatory in the context of stimulation of antigen presenting cells that bridge innate and adaptive immunity. Our study provides new insight into the pathophysiologic relevance of STBM in adverse pregnancy outcomes.

7.2 Proteomic Analysis of Human Placental STBM in Adverse Pregnancy Outcomes

We sought to determine the protein composition of STBM using 1D gel-LC-MS/MS approach to gain an insight into the pathophysiologic changes of PE and RM. To our knowledge this study is the first report of STBM proteome in PE and RM in comparison to that of normal healthy pregnancies.

14 proteins were found to be differentially expressed between STBM derived from women with PE and RM compared to healthy controls; notably annexins, histones, integrins, fibrinogens, fibronectin, peroxiredoxins, clathrin and protein S-100 A11 which were upregulated with the interesting exception of histones. The annexins are calcium dependent-phospholipid binding proteins involved in various functions including signal transduction, stress response, coagulation, anti-apoptosis and lipid metabolism. Annexin A1 is anti-inflammatory and has been shown to be associated with inhibition of acute inflammation (Ahmad M Kamal, 2005). The higher expression of annexin A1(3-fold change, $p<0.05$) in PE STBM than in control STBM may suggest an attempt by homeostatic mechanisms to limit increased systemic inflammatory response in PE. The up-regulation of anti-apoptosis protein in PE STBM, such as annexin A4 (5-fold change, $p<0.05$) (Kebir et al., 2008), can be explained by increased apoptotic activity of the placental syncytiotrophoblasts in PE which would trigger the generation of anti-apoptotic proteins in an attempt to counteract pro-inflammatory molecules such that homeostasis is achieved.

Peroxiredoxins are a family of proteins involved in the metabolism of reactive oxygen species. Peroxiredoxins function as a scavenger for H_2O_2 and contribute to cellular protection against H_2O_2-induced cell damage and apoptosis (Berggren et al., 2001). In

our study, we found upregulation of Peroxiredoxin-6 in PE STBM (7-fold change, $p<0.05$). An increased expression of this group of proteins may be explained as a response to counteract the existing oxidative stress in PE and RM.

Clathrin mediates sorting of cell membrane cargo and recruitment of lipid modifiers (Brodsky et al., 2012). We found upregulation of clathrin in RM STBM (3-fold change, $p<0.05$). Components of glycolytic pathways such as glyceraldehyde-3-phosphate dehydrogenase was expressed, having important functional relevance in anaerobic glycolysis in trophoblasts enriched in glycogen. Weak expressions of various procoagulant complement components as well as complement regulatory proteins such as C1q, C3, C4, CD46, CD55 and CD59 were also found, suggestive of possible role of STBM in hemostatic activation as well as regulation. Two recently described proteins in PE sera, chorionic somatomammotropin hormone (CSH) secreted by placental syncytiotrophoblast and fibulin were also found in our study (Liu et al., 2011). CSH have a role in lypolysis and may contribute towards dyslipidemia of PE (Muraj et al., 1997). Fibulin as well as protein S100-A11 are involved in ECM remodeling. We found upregulation of protein S100-A11 in PE STBM (3-fold change, $p<0.05$). This expression in STBM may be suggestive of tissue injury, endothelial damage, ischaemia and hypoxia in adverse pregnancies (Redfern et al., 2000). Collectively all these protein expressions possibly depict the syncytiotrophoblast response at maternal-fetal interphase to underlying pregnancy pathology in PE and RM.

Integrin signaling (including ligands for fibronectin) has important role in trophoblast invasion and vasculature remodeling (Huppertz et al., 2012). Its downregulation in PE STBM (0.04-fold change, $p<0.05$) may be associated with shallow trophoblast invasion and defective placental vasculature in PE and RM. The fibrinogens are involved in coagulation and their upregulation may be associated with the pro-coagulant state of PE

and RM. Fibrinogen, in association with fibronectin, is known to have an important role in extracellular matrix (ECM) remodeling (Mosesson et al., 2005). Fibronectin is also known to have an important role in trophoblast differentiation (Benoit et al., 2007). Increased expression of these proteins, as found in RM STBM in the present study, may represent a protective response to tissue injury in pathologic pregnancies. Fibronectin is already considered a promising biomarker to predict PE (Leeflang et al., 2007) and it would be interesting to investigate further its biomarker potential in prediction of RM.

Major functions of histones include DNA binding, DNA repair and transcriptional regulation (Liang et al., 2012). The unique finding of downregulation of histones in both PE and RM STBM in this study (0.3-and 0.4-fold change, respectively, $p<0.05$) may be suggestive of defective DNA repair, increase in damaged DNA and raised inflammatory response in adverse pregnancies.

Interestingly, we found that STBM proteins include endogenous danger molecules or alarmins which may render them intensely pro-inflammatory such as extracellular free actin, S100, fibronectin, transthyretin, besides heat shock proteins (Redman et al., 2012). This supports the currently emerging role of STBM as contributors to the pro-inflammatory state of PE and RM.

We acknowledge the small number of observations as a significant weakness of this study and that our findings are preliminary. Due to constraints of resources, sample number could not be increased. However, this number gives us sufficient statistical power. Future biomarker discovery work will include STBM proteomic analysis of higher number of placentas from more severe phenotypes of RM and PE and validation of biomarker potentials of differentially expressed proteins in patients' plasma. We also acknowledge the limitations of using label-free method and spectral counts in

demonstrating the true representation of differential expression of low abundance proteins. Confirmation of some of the differentially expressed proteins needs to be carried out with isotope-encoded peptides corresponding to the tryptic peptides of the selected proteins. This will help determine absolute concentration of targeted proteins.

Immunohistochemistry was performed on placenta instead of Western analysis of STBM proteins, due to sample constraints. Furthermore, there are limitations of using label-free method and spectral counts in demonstrating the true representation of differential expression of low abundance proteins. Confirmation of some of the differentially expressed proteins needs to be carried out with isotope-encoded peptides corresponding to the tryptic peptides of the selected proteins. This will help determine absolute concentration of targeted proteins.

Immunohistochemistry was performed on placenta instead of STBM. The protein effects observed in STBM analyzed by LC-MS/MS diluted out as immunohistochemical staining intensities were measured in syncytiotrophoblast layer of placental sections. Compared to the parent cell syncytiotrophoblast, STBM is likely to be enriched in a variety of signaling proteins, similar to other microvesicle populations. This may explain the upregulation of certain proteins relevant to pathologic pregnancies observed in STBM but not in placenta.

In conclusion, the present study shows the main biological pathways associated with PE and RM include inflammation, coagulation, endothelial dysfunction and lipid metabolism. While previous evidence for increased activation of hemostasis and endothelial dysfunction exists, the concept and proposed roles of immune-dysregulation and lipid metabolism in pathophysiology of adverse pregnancies are novel and yet to be established. Our findings are consistent with recent reports on PE pregnancy sera

(Blumenstein et al., 2009, Liu et al., 2011), demonstrating pro-inflammatory and lipid-regulatory protein expression, besides those involved in hemostasis and endothelial dysfunction. Targeting these novel pathways may offer newer strategies of management of adverse pregnancies.

7.3 Lipidomic Analysis of Human Placental STBM in Adverse Pregnancy Outcomes

Dysregulated lipid metabolism is currently emerging among the key pathogenetic pathways implicated in adverse pregnancy outcomes such as PE and RM. Recent reports on proteomic analysis of plasma from pre-eclampsia patients show presence and differential expression of lipid metabolism-related proteins which may lead to protein-lipid interactions including annexins and clotting proteins, e.g. fibrinogens related to PS, apolipoproteins related to cholesterols and triacylglycerides (TAG), and serum amyloid proteins A2 and A4 related to cholesterols (Blumenstein et al., 2009, Liu et al., 2011). It is also known that TNF-α and other proinflammatory cytokines which are upregulated in PE and RM, mediate lipid dysregulation in adverse pregnancies by inducing insulin resistance, inhibiting lipogenesis, stimulating lypolysis and releasing FFA in maternal circulation.

To date most published reports on dyslipidemia of PE have focused on plasma level of lipid peroxides, cholesterols and TAG (Bayhan et al., 2000, Barden et al., 2001, Adiga et al., 2007, Lima et al., 2011, Ahmedi et al, 2012, Gohil et al, 2012). However, knowledge on other polar lipids, e.g. sphingolipids and glycerophospholipids is lacking. These major membrane lipids form precursors for second messengers and functional assembly matrices on membrane domains during cellular stimulation and signaling (Fernandis et al., 2007). This present study investigated lipid profiles of placental microvcesicle STBM shed from syncytiotrophoblast membrane, with a focus on phospholipids and sphingolipids. We separated and quantified various polar lipids in placental STBM using ESI LC MS MRM approach. To our knowledge this study is the first report of STBM lipidome in PE and RM in comparison to that of normal healthy pregnancies.

Our results showed that in STBM, the most abundant polar lipid is SM (up to 35%), followed by PS, PA, PC and PI. A previous report on lipidomic analysis of microvesicles derived from human B cells similarly showed an enrichment of SM, beside GM3 and cholesterol (Wubblots et al., 2003). We also observed a unique reversal of SM/PC ratio (3:1) contrary to that in normal human cells. Although similar trend of SM/PC ratio was observed between patients with successful pregnancy outcomes and normal control groups, the ratio showed marked difference between an RM patient with ongoing pregnancy loss (SM:PC, 30%:13%) and her gestational age-matched normal control (SM:PC, 20%:12%). SM is known to be involved in various biological pathways such as inflammation, immune response, and oxidative stress (Lahiri et al., 2007), which are likely to be upregulated in active RM with an ongoing pregnancy loss. This may explain the observed upregulation of SM in active RM compared to the control. Besides, selective incorporation of biologically active components including lipids are known phenomenon in microvesicle biogenesis and their role as vehicles of intercellular signaling may have necessitated an enrichment of SM.

Recently aberrant expressions of sphingolipids including gangliosides resulting in disorganizations of membrane microdomains have been implicated in pathogenesis of metabolic disorders, e.g. type 2 diabetes, which also involves oxidative stress (Inokuchi et al., 2009). Thus significantly elevated levels of few SM species and decreased levels of some GM3 species in STBM from adverse pregnancies, as observed in the present study, may also be relevant in pathogenesis of complicated pregnancies.

We also observed significant upregulation of total levels of PS and downregulation of PI, PA and GM3 in preeclampsia and similar trends in RM, compared to healthy pregnant controls. This might have implications in pathogenesis of preeclampsia. Also, STBM lipids may be relevant in a subset of idiopathic RM with increased number of circulating

procoagulant microvesicles and this may be of interest to investigate further in the future. Laude and coworkers reported this population to be 59% in patients with early losses (<10 gestational weeks) and 48% in patients with late losses (> 10 weeks) (Laude et al., 2001). PS is among important signaling molecules involved in coagulation and apoptosis (Leventis et al., 2010) and these pathways are known to be exaggerated in preeclampsia and RM (Li et al., 2009, Cindrova-Davies et al., 2009), possibly mediated by placenta-derived factors such as reactive oxygen species (ROS). ROS activate various stress pathways, including proapoptotic p38 and SAPK/JNK MAPK pathways and inflammatory NF-kB pathway These pathways promote increased shedding of microvesicles, anti-angionenic factors and inflammatory cytokines, as well as initiates the blood coagulation cascade.

Thus an elevated expression of PS in STBM could be reflective of activation of apoptotic pathways in placental trophoblast in adverse pregnancies. PI and PA are involved in regulation of apoptosis, proliferation and differentiation (Kabayama et al., 2007) and their downregulation may represent a protective response against placenta-derived pro-apoptotic stressors such that homeostasis is achieved. Interestingly, in the RM group, levels of PS and PI changed significantly with number of losses and heparin treatment. It is suggested that heparin may be beneficial in RM by exerting protection against trophoblast apoptosis, pro-angiogenic, anti-inflammatory, and anti-coagulant effects (Matthiesen et al., 2012). The possible role of heparin in correction of dyslipidemia in RM may be of interest to investigate further in the future. Sphingolipids including ganglioside GM3 are important signaling molecules in immune response, inflammation and oxidative stress pathways (Corda et al., 2002, Kabayama et al., 2007, Paccalet et al., 2010). GM3 downregulation as noted in the present study could be a response to the state of increased inflammatory and oxidative stress known to exist in adverse pregnancies.

Cholesterol is an essential mammalian cell membrane component (Simons et al., 2002). Aberrant levels of sterol lipids are known to be associated with coronary heart disease, stroke, and diabetes (Simons et al., 2002, Kingsbury et al, 1969, Gross et al., 2007). 7β-OHC and 7-KC are formed as a result of cholesterol oxidation and serve as biomarkers for oxidative stress in atherosclerosis and other diseases (Witztum et al., 1994, Larsson et al., 2007, Lee et al., 2009)). In the present study, cholesterol and CE, showed a trend towards increase in STBM from adverse pregnancies compared to that from healthy pregnancies, while 24-OHC and 7-OHC showed a trend towards decrease in preeclampsia and RM. 24-OHC and 7-OHC have regulatory roles in cholesterol homeostasis and elimination of cholesterol. Their decreased expression as observed in diseased STBM in our study could be associated with the lipid dysregulation, elevated free cholesterols as well as cholesteryl esters, in these immune-dysregulated pregnancies.

We observed substantial changes in the polyunsaturated species in PI and PS, with those in the former class being significantly decreased and those in the latter being significantly increased in both preeclampsia and RM patients compared to controls. This may imply that there is a preference for PS over PI synthesis in patients. Both PS and PI synthesis involve the same substrate (CDP-diacylglycerol or CDP-DAG) (KEGG pathway database for glycerophospholipid metabolism). There is probably an upregulation of PS synthesizing enzyme, PS synthase, and a downregulation of PI synthesizing enzyme (CDP-DAG-inositol-3-phosphatidyltransferase) in patients that led to the observed lipid metabolite differences. Hence, future work can be carried out to establish the enzyme activities in these patients.

In conclusion the present study suggests that the biological pathways associated with preeclampsia and RM may include lipid metabolism. Further studies are required to investigate the roles of differentially expressed lipids in STBM from preeclampsia and

RM patients as potential biomarkers in peripheral blood plasma for detection of onset of these adverse pregnancies. While previous evidence for increased activation of immune-response and haemostasis as well as endothelial dysfunction exists, the concept and proposed roles of lipid metabolism in pathophysiology of adverse pregnancies is novel and yet to be established. Targeting these novel pathways may offer newer strategies of management of adverse pregnancies.

7.4 Dendritic Cell Subsets Analysis in Human Decidua Shows Presence of CD141^{hi} DCs

This study is the first report of the DC subsets in decidua from normal pregnant subjects and RM patients. Within the HLA-DR^{+}lineage^{-}CD14^{-} fraction of leukocytes we found a discrete human DC subset identified by high CD141 and low-to intermediate CD11c expression, recently reported to be isolated from skin, lung, and liver (Haniffa et al, 2012). The authors found that skin CD141hi DCs are proliferating and include a subset of immature cells related to but distinct from blood CD141^{+} DCs, suggesting that they are potentially derived from CD141^{+} blood DCs. *In vitro* CD141hi DCs were the most efficient antigen presenting cells compared to other interstitial DCs. These cells produce very little IL-12 and IL-23 when stimulated but produce CXCL10 and TNF-α. CXCL10 and XCL1 form a chemokine circuit between DCs expressing XCR1 and activated NK cells or Th1 cells (Dorner et al., 2009; Crozat et al., 2010; Contreras et al., 2010). In addition to CD141hi DCs, human decidua contains CD11c^{+} DCs and CD14^{+} DCs, as previously described, notably in the skin (Lenz et al., 1993; Nestle et al., 1993; Haniffa et al., 2009). A recent study isolated CD 141^{+} DCs from human skin which also produce IL-10, and induce regulatory T cells (Chu et al., 2012). Haniffa et al argued this population of CD 141^{+} DCs isolated by Chu and colleagues corresponds to CD14^{+} DCs which the former found to have similar phenotype and function.

CD141+ dermal DCs were found to possess lymph node migratory capacity, induce T cell hyporesponsiveness, cross-present self-antigens to autoreactive T cells, and induce potent regulatory T cells that inhibit skin inflammation. These CD141+ dermal DC-like cells can be generated *in vitro* and, once transferred *in vivo*, have the capacity to inhibit xeno-graft versus host disease and tumor alloimmunity. These findings suggest that

CD141+ dermal DCs play an essential role in the maintenance of skin homeostasis and in the regulation of both systemic and tumor alloimmunity.

Likewise, $CD14^+CD141^+$ DCs could be relevant at maternal-fetal interface and may play an important role in maintenance of tolerance leading to a normal healthy pregnancy outcome. It would be interesting to study distribution of these cells in adverse pregnancies associated with immunedysregulation e.g. RM which has been shown to be associated with increased number of IL-17^+ T cells as well as reduced numbers and function of $Foxp3^+$ regulatory T cells (Treg) in peripheral blood (Lee et al., 2011) and increased proportion of Th17 cells in peripheral blood and deciduas (Wang et al., 2010). Various factors including GM-CSF regulates the efficiency with which uterine DCs and macrophages activate T cells, and it is essential for optimal MHC class II- and class I-mediated indirect presentation of reproductive antigens. Insufficient GM-CSF may impair generation of T cell-mediated immune tolerance at the outset of pregnancy and may contribute to the altered DC profile and dysregulated T cell tolerance evident in RM (Moldenhauer et al., 2010).

Enhanced pro-inflammatory immune responses with suppressed immune regulation is considered an important immune mechanism involved in RM and a reduced number of $CD14^+CD141^+$ DCs could reflect maternal immune maladaptation to the foetus in RM.

We found a considerably high frequency of CD141hi DCs in the decidua of a treated RM patient with a history of 5 recurrent miscarriages and subsequent successful pregnancy outcome. We speculate CD141hi DCs could be the population capable of most efficient cross-presentation of soluble fetal antigens to maternal T cells, production of TNF-α and induction of CTL responses. Intervention or treatment may have dampened the

stimulatory function of these cells and this immunosuppression could have facilitated a healthy pregnancy outcome.

This component of the PhD is an ongoing study and our findings on decidual DC subsets would need further validation in a larger group of RM patients. Our findings may aid development of novel strategies for diagnosis and treatment of immune-dysregulation in adverse pregnancies.

7.5 Conclusions

Placental syncytiotrophoblast microvesicles (STBM) are biologically active, membrane-bound, subcellular vesicles shed into maternal circulation during pregnancy as a normal turnover of the placental surface. They interact with maternal immune cells and vascular endothelium in intervillous space and uterine decidua. A successful feto-maternal cross-talk is considered pivotal in immunomodulation of normal pregnancy leading to the acceptance of the feto-placental semi-allograft. Quantitative and possibly qualitative STBM changes take place in adverse pregnancy outcomes such as pre-eclampsia (PE). However, the role of STBM in recurrent miscarriage (RM) remains insufficiently explored.

The study objectives were to evaluate the immunogenicity of STBM in women with RM by investigating whether i) STBM stimulate antigen presenting cells (APCs), e.g. dendritic cells (DCs) & macrophages more in disease compared to health; ii) STBM bioactive contents, e.g. proteins and lipids, differ in disease and health; and iii) decidual DCs involved in fetal antigen presentation to T cells at maternal-fetal interface differ in disease and health.

STBM from RM and PE patients demonstrated significantly increased immunogenicity, causing significantly increased production of proinflammatory cytokines, e.g. IL-6 and TNF-α from DCs, compared to STBM from gestational age-matched healthy pregnant women.

Next, to understand the factors attributing to the immunogenicity of STBM, we investigated STBM protein and lipid composition. Among the STBM proteins, 14 were found to be differentially expressed in STBM derived from women with PE and RM

compared to healthy controls. Notable among these proteins were annexins, histones, integrins, fibrinogens, fibronectin, peroxiredoxins, clathrin and protein S-100 A11, all of which were upregulated with the interesting exception of histones.

Lipidomic analysis revealed that the major lipid classes in STBM are sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidylinositol (PI). SM/PC ratio showed a unique reversal (3:1) compared to that normally found in human cells. PS was upregulated and PI, PA and GM3 was downregulated in adverse pregnancies compared to healthy pregnant controls.

Collectively, differential expressions of STBM proteins and lipids in RM and PE could be observed. These proteins and lipids have been implicated in inflammation, coagulation, oxidative stress, apoptosis and immune response, suggesting a pathophysiologic relevance of STBM in adverse pregnancy outcomes. The observed qualitative changes in STBM possibly represent homeostatic responses by the syncytiotroblast positioned at maternal-fetal interface.

Finally, to investigate the *in vivo* decidual DC population, we analyzed dendritic cell subsets. Significant findings include the presence of $CD141^{hi}$ DCs which has been recently reported to be the most efficient antigen presenting cell in human peripheral tissues (Haniffa et al., 2012). One particular patient with 5 previous miscarriages demonstrated a markedly higher frequency of $CD141^{hi}$ DCs (1.48% of $CD45^{+}$ cells), compared to the other RM (0.2%) and the normal controls (0.2%). This study is ongoing and validation of present findings would be required in a larger group of patients. Nevertheless, I would like to speculate that $CD141^{hi}$ DCs may contribute locally to maternal-foetal immune maladaptation in PE and RM.

In summary, the findings of this study lend support to the emerging concept of immune-dysregulation in adverse pregnancy outcomes. This in turn has implications in development of novel approaches of diagnosis and therapy of these significant yet perplexing, unresolved clinical problems.

7.6 Limitations of current study and future works

In the present study, the number of study subjects in RM with non-live birth group is small. We acknowledge that data from pregnant patients with history of RM who subsequently miscarry would be of great interest to the scientific community and contribute towards better understanding of pathogenesis of RM. Recruitment of patients to obtain higher number of cases in this group and analysis is ongoing and we hope to publish this data in future.

Peripheral blood leukocytes from healthy non-pregnant donors, in the form of buffy coat, were used in this study to generate antigen presenting cells (APCs), DCs and macrophages. Autologous source of peripheral blood leukocytes (matched maternal blood) would have probably increased the cytokine signals elicited in stimulation assays; as during pregnancy, monocytes are known to be primed to produce pro-inflammatory cytokines (Germain et al., 2007). Currently matched maternal monocytes are being stored and will be tested in DC stimulation assays in future.

Placental explant culture was used in this study to generate STBM (eSTBM) as it remains the simplest method to generate STBM in large number. However, STBM generated by perfusion of placental cotyledon (pSTBM) is known to be superior to eSTBM with regard to immunogenicity (Messerli et al., 2010). In addition, the use of a cell culture system to generate STBM will also introduce artefacts from cell types other than trophoblasts (such as fibroblasts, endothelial cells, blood cells from foetal blood vessels in placental villi) into the system. Using pSTBM probably would have resulted in better stimulation of APCs. In future, pSTBM generation system will be established and used for further experiments.

The data on DC stimulation by STBM were derived *in vitro*. A better model could have been *in vivo* animal model such as abortion-prone mouse model or humanized mouse model.

For STBM proteomic analysis, label-free method and spectral counts were applied, which have limitations in demonstrating the true representation of differential expression of low abundance proteins. Confirmation of some of the differentially expressed proteins needs to be carried out with isotope-encoded peptides corresponding to the tryptic peptides of the selected proteins. This will help determine absolute concentration of targeted proteins.

Immunohistochemistry was performed on placenta instead of on STBM due to sample constraints. The protein effects observed in STBM analyzed by LC-MS/MS diluted out as immunohistochemical staining intensities were measured in syncytiotrophoblast layer of placental sections, instead of on STBM. A labelled method to determine absolute concentration of targeted proteins in STBM will be carried out in future. Also, synthetic microvesicles could be used as vectors for specific proteins or lipids to test their immunogenic potential in an animal model.

Decidual DC subset analysis is an ongoing study and our findings on decidual DC subsets would need further validation in a larger group of RM patients.

Notwithstanding these limitations, our study offers an insight into the pathophysiologic relevance of STBM in RM and provides a foundation for further elaborate research into the pathogenesis of RM.

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Appendix A: List of Abstracts and Presentations

Sonia Baig[1], Sherry Sze Yee Ho[1], Narasimhan Kothandaraman[1], Boon King Teh[3], Sheila Vasoo[2], Jin Hua Lu[3], Markus Wenk[4], Lin Lin Su[1], Mahesh Choolani[1]. [1]Obstetrics and Gynaecology, [2]Medicine, [3]Microbiology, [4]Biochemistry, National University of Singapore, Singapore. Association of placentally-derived biologically active microparticles with, immune-dysregulation in adverse pregnancy outcomes (preeclampsia and recurrent pregnancy loss). Poster presentation, X1 ICRI 2010 (International Congress of Reproductive Immunology), Cairns, Australia, Aug 15-19, 2010. Abstract of the paper published in Journal of Reproductive Immunology, Aug 2010; vol 86: page 65 (Poster Abstract P76).

Sonia Baig, Sherry Sze Yee Ho, Bee Lian Ng, Lily Chiu, Evelyn SC Koay, Gek Har Leow, Leena Gole, Jenny Chan, Xiaoli Sun, Narasimhan Kothandaraman, Yiong Huak Chan, Mary Rauff, Lin Lin Su, Arijit Biswas, Mahesh Choolani. Development of Quantitative Fluorescence polymerase chain reaction for rapid prenatal diagnosis of common chromosomal aneuploidies in 1,000 samples in Singapore. Original article, Singapore Medical Journal April 2010; 51(4):343-48.

Sonia Baig[1], Sheila Vasoo[2], Boon King Teh[3], Sherry Sze Yee Ho[1], Narasimhan Kothandaraman[1], , Jin Hua Lu[3], Markus Wenk[4], Lin Lin Su[1], Mahesh Choolani[1]. [1]Obstetrics and Gynaecology, [2]Medicine, [3]Microbiology, [4]Biochemistry, National University of Singapore, Singapore. Association of placentally-derived biologically active microparticles with, immune-dysregulation in adverse pregnancy outcomes (preeclampsia and recurrent pregnancy loss). Oral Presentation, Exosomes & Microvesicles 2011 Conference, Orlando, Florida, USA, Oct 15-17, 2011.

Sonia Baig[1], Sheila Vasoo[2], Boon King Teh[3], Sherry Sze Yee Ho[1], Narasimhan Kothandaraman[1], , Jin Hua Lu[3], Markus Wenk[4] , Lin Lin Su[1] , Mahesh Choolani[1]. [1]Obstetrics and Gynaecology, [2]Medicine, [3]Microbiology, [4]Biochemistry, National University of Singapore, Singapore. Association of placentally-derived biologically active microparticles with, immune-dysregulation in adverse pregnancy outcomes (preeclampsia and recurrent pregnancy loss). Poster Presentation, 5th Autoimmunity Congress Asia 2011 (ACA), Singapore, Nov 17-19, 2011.

Sonia Baig[1], Sheila Vasoo[2], Boon King Teh[3], Sherry Sze Yee Ho[1], Narasimhan Kothandaraman[1], Jin Hua Lu[3], Markus Wenk[4] , C Arquio[1], A Kale[1], LL Su [1], A Biswas [1], Mahesh Choolani[1]. [1]Obstetrics and Gynaecology, [2]Medicine, [3]Microbiology, [4]Biochemistry, National University of Singapore, Singapore. Immunomodulation by placental microvesicles in adverse pregnancy outcomes (preeclampsia and recurrent pregnancy loss). Oral presentation, University Obstetrics & Gynaecology Congress (an International Congress), Singapore, May 25-27, 2012.

Sonia Baig[1], Sheila Vasoo[2], Boon King Teh[3], Sherry Sze Yee Ho[1], Narasimhan Kothandaraman[1], Jin Hua Lu[3], Markus Wenk[4] , C Arquio[1], A Kale[1], LL Su [1], A Biswas [1], Mahesh Choolani[1]. [1]Obstetrics and Gynaecology, [2]Medicine, [3]Microbiology, [4]Biochemistry, National University of Singapore, Singapore. Immunomodulation by placental microvesicles in adverse pregnancy outcomes (preeclampsia and recurrent pregnancy loss). Oral Presentation as top three finalists. Joint International Congress of American & European Society for Reproductive Immunology, Hamburg, Germany, May 31-June 02, 2012. Abstract of the paper published in Journal of Reproductive Immunology, May 2012; vol 94 (1): page 65 (Oral Presentation Abstract P17).

Appendix B: Manuscripts Submitted and in Preparation

<u>Sonia Baig</u>[1], Boon King Teh[3], Jing Yao Leong[3], Sherry SY Ho[1], Jinhua Lu[3], Kenneth TE Chang[4], Anita Kale[1], Lin Lin Su[1], Arijit Biswas[1], Sheila Vasoo[2] , Mahesh Choolani[1]. [1]Division of Maternal-Fetal Medicine, Department of Obstetrics & Gynaecology; [2]Division of Rheumatology, Department of Medicine; [3]Immunology Programme, Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore; [4]Department of Pathology and Laboratory Medicine, KK Women's and Children's Hospital, Singapore. Immunomodulation by Placental Syncytiotrophoblast Microvesicles in Adverse Pregnancy Outcomes. ***Manuscript (original article) submitted, Journal of Reproductive Immunology, revision in progress.***

<u>Sonia Baig</u>[1], Narasimhan Kothandaraman[1], Chin Wee Lai [3], Wan Yu Tan[3], Felicia Yeoh[3], Anita Kale[1], Lin Lin Su[1], Arijit Biswas[1], Sheila Vasoo[2] , Mahesh Choolani[1].[1]Division of Maternal-Fetal Medicine, Department of Obstetrics & Gynaecology; [2]Division of Rheumatology, Department of Medicine; [3] NUS High School of Mathematics and Science, Singapore. Proteomic Analysis of Human Placental Syncytiotrophoblast Microvesicles in Adverse Pregnancy outcomes***. Manuscript (original article) submitted, Placenta Journal, revision in progress.***

<u>Sonia Baig</u>[1], Jing Yan Lim[3], Aaron Fernandis[3], Markus Wenk[3], Anita Kale[1], Lin Lin Su[1], Arijit Biswas[1], Sheila Vasoo[2] , Guanghou Shui [4], Mahesh Choolani[1]. [1]Division of Maternal-Fetal Medicine, Department of Obstetrics & Gynaecology; [2]Division of Rheumatology, Department of Medicine; [3]Department of Biochemistry, Yong Loo Lin School of Medicine, [4]Life Science Institute, National University of Singapore & National University Health System. Lipidomic Analysis of Human Placental Syncytiotrophoblast Microvesicles in Adverse Pregnancy Outcomes. ***Manuscript (original article) accepted by Placenta Journal, IF 3.7, revision in progress***.

Sonia Baig[1], Amanda Shin[3], Kenneth TE Chang[4], Anita Kale[1], Lin Lin Su[1], Arijit Biswas[1], Sheila Vasoo[2] , Florent Ginoux[3] , Mahesh Choolani[1]. [1]Division of Maternal-Fetal Medicine, Department of Obstetrics & Gynaecology; [2]Division of Rheumatology, Department of Medicine; [3]Singapore Immunology Network, Agency for Science, Research & Technology, Singapore; [4]Department of Pathology and Laboratory Medicine, KK Women's and Children's Hospital, Singapore. Dendritic cells subset analysis in human decidua shows presence of CD141^{hi} DCs. ***Manuscript (original article) in preparation.***

Appendix C: Awards

Travel Award, International Society for Immunology of Reproduction, XI International Congress of Reproductive Immunology (ICRI). Cairns, Australia, Aug 15-19, 2010.

S$200,000 NMRC Grant, May 2010, study title- "Association of placental syncytiotrophoblast membrane micro-particles (STBMs) with maternal immune response in recurrent pregnancy losses", NMRC/NIG/1022/2010.

Best Research Paper Award 2010 (First Prize), Singapore Medical association, Title of paper- "Development of Quantitative Fluorescence polymerase chain reaction for rapid prenatal diagnosis of common chromosomal aneuploidies in 1,000 samples in Singapore. Singapore Medical Journal April 2010; 51(4):343-48.'

Second Prize, New Investigator Award Competition of the American Society for Reproductive Immunology, Clinical Science Category, Joint International Congress of American & European Society for Reproductive Immunology, Hamburg, Germany, May 31-June 02, 2012.

Best Graduate Oral presentation Award for presentations in international conferences, Annual Graduate Scientific Congress, January 30, 2013, YLL School of Medicine, National University of Singapore.

Best Poster Presentation Award, Annual Graduate Scientific Congress, January 30, 2013, YLL School of Medicine, National University of Singapore.