Examination of the inflammatory nature of different placental syncytiotrophoblast microparticles (STBM) preparations on human monocytes

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ABBRIVIETATIONS

APC	Antigen-presenting cells
AP-1	Activator protein 1
6AQ	6-Amino-4-(4-phenoxyphenylethylamino)quinazoline
BCL3	B-cell CCL/lymphoma 3
CCL2	Chemokine (C-C-motif) ligand 2
CD	Cluster of differentiation
CRP	C-reactive protein
CSF	Colony stimulating factor
DAF	Decay accelerating factor
DMSO	Dimethyl sulfoxide
dNK cell	Decidual natural killer cell
EGR1	Epidermal growth factor 1
Eng	Endoglin
eS_3	Villous explant STBM prepared at 3% O_2
eS_{20}	Villous explant STBM prepared at 20% O_2
EVT	Extravillous trophoblast
FADD	Fas-Associated protein with Death Domain
FasL	Fas ligand
Fc	Fragment, crystallizable
FcR	Fc receptor
FFA	Free fatty acids
Flt-1	Fms-like tyrosine kinase-1 receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HELLP	Hemolysis, elevated liver enzymes, and low platelet counts
HLA	Human leukocyte antigen
IDO	Indoleamine-2,3-dioxygenase
IFN	Interferon
IL	Interleukin
IL-1R1	IL-1 receptor type 1
IP-10	Interferon-inducible-protein-10
IUGR	Intrauterine growth restriction
KIR	killer cell immunoglobulin receptor
LDL	Low-density lipoproteins
LFA-1	Lymphocyte function-associated antigen 1
LIR	leukocyte Ig-like receptor

LPS	Lipopolysaccharide
LTA	Lymphotoxin α
MAC	Membrane attack complex
MCP	Membrane co-factor protein
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
mS	STBM prepared by mechanical dissection
MyD88	Myeloid differentiation primary response gene 88
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nIUGR	Normotensive intrauterine growth restriction
NK cell	Natural killer cell
NOD-1	Nucleotide-binding oligomerization domain 1
OFR	Oxygen free radical
PA	Perillyl alcohol
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed death 1
PHA	Phytohemagglutinin
PLAP	Placental alkaline phosphatase
PlGF	Placental growth factor
PMA	Phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocytes
pS	STBM prepared by dual perfusion of a placental cotyledon
RT	Room temperature
sEng	Soluble endoglin
sFlt-1	Soluble fms-like tyrosine kinase-1 receptor
STBM	Syncytiotrophoblast microparticle
TCR	T-cell receptor
TGF	Transformation growth factor
Th	T helper
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
T_{reg}	Regulatory T-cells
TREM-1	Triggering receptor expressed on myeloid cells 1
VEGF	Vascular endothelial growth factor

SUMMARY

Background: Healthy human pregnancies are accompanied by a mild systemic maternal inflammatory response, which includes activation of peripheral blood monocytes. This generalized inflammation is exaggerated in preeclampsia, a placenta-dependent disorder specific to human pregnancies. It has been proposed that placental syncytiotrophoblast membrane microparticles (STBM), which are released into the peripheral blood, might contribute to the maternal response in normal pregnancy and preeclampsia.

Aim: The aim of this work was to assess the inflammatory properties of STBM generated *in vitro* from human term placentas by four different approaches which should mimic physiologic or patho-physiologic conditions, and their mode of action on human monocytes *in vitro*.

Methods: STBM were prepared by: (1) placental explant cultures of villous tissue incubated at 20% O_2/5% CO_2 (air) at 37°C for 72 hours (eS_{20}); (2) perfusion of the maternal side of a placental cotyledon *(pS)*; (3) placental explant cultures of villous tissue incubated at 3% O_2/5% CO_2 (hypoxia) at 37°C for 72 hours (eS_3); and (4) mechanical dissection of villous tissue *(mS)*. In all approaches, STBM were isolated by serial high-speed centrifugation. STBM were co-incubated with either the human monocytic cell line Mono Mac 6 or human peripheral blood monocytes. In some cases, agents which inhibit cellular functions or signalling pathways were used. Analysis of viability, phenotype and function were performed by real-time PCR, flow cytometry, ELISA and fluorescence microscopy.

Results: Viability of Mono Mac 6 cells was not impaired following treatment with STBM. However, STBM only induced a marginal response in Mono Mac 6. None of the STBM population affected the viability of primary monocytes. eS_3 and *mS* decreased the expression of CD54 on peripheral blood monocytes, but did not induce the secretion of IL-1β, IL-6 and IL-8. However, *pS* and eS_{20} up-regulated the cell surface expression of CD54 on primary monocytes and stimulated the secretion of IL-1β, IL-6 and IL-8 in a dose- and time-dependent

manner. Interestingly, eS_{20} derived from normal and preeclamptic placentas stimulated monocyte activation to similar degrees.

eS_{20} induced the transcription of several NF-κB responsive genes, including IL-6 and IL-8, and the secretion of IL-6 and IL-8 was reduced upon treatment with NF-κB inhibitors.

eS_{20} was located at the monocytic cell surface and a phagocytosis inhibitor did not reverse the eS_{20} induced production of IL-6 and IL-8.

Primary monocytes and the non-responding Mono Mac 6 cells expressed toll like receptors (TLRs) differently. Pre-incubation of primary monocytes with an inhibitor of intracellular TLR signalling reduced the inflammatory response triggered by eS_{20}.

Conclusions: STBM populations evoked neither a proinflammatory nor an anti-inflammatory phenotype in Mono Mac 6 cells. However, STBM prepared at conditions which are believed to mimic the physiologic situation in human pregnancy (eS_{20} and *pS*) triggered the secretion of IL-1β, IL-6 and IL-8 and up-regulated the expression of the adhesion molecule CD54 on peripheral blood monocytes. These findings indicate that Mono Mac 6 cells are not the appropriate cells to study the interaction between monocytes and STBM.

STBM prepared at non-physiologic *(mS)* and hypoxic (eS_3) conditions, which are thought to mimic the patho-physiologic situation in preeclampsia, did not induce an inflammatory response in peripheral blood monocytes. In addition, the observation that eS_{20} derived from normal as well as from preeclamptic placentas triggered an equally strong and dose-dependent inflammatory response in primary monocytes, suggests that there are no or only minor qualitative differences between the microparticles. These findings presume that the overt maternal inflammation associated with preeclampsia may be due to the higher concentration of circulating STBM, rather than to a qualitative difference between microparticles released from normal and patho-physiologic placentas.

The results also suggest that the inflammatory reaction in monocytes may be initiated by the attachment of STBM to the cell surface and the activation of TLRs. In turn, NF-κB mediates

transcription of proinflammatory genes, including IL-1β, IL-6, IL-8 and CD54. The altered expression of CD54 may modulate the adhesion properties of monocytes, whereas the secretion of IL-1β, IL-6 and IL-8 could recruit further immune cells, leading to generalized inflammation.

INTRODUCTION

1. Human placentation

Although reproduction is a fundamental feature of life, in humans it is paradoxically a quite inefficient process. The probability of conception per menstrual cycle just accounts for 30% and only 60% of human pregnancies are progressing beyond week 20 of gestation [1, 2].

Successful human pregnancy relies on well coordinated complex processes, including implantation of the fertilized egg into the hormonally primed uterus, followed by placentation to ensure fetal supply with oxygen and nutrients derived from the maternal circulation. Furthermore, the fetal and placental oxygen demand and the interactions between maternal and feto-placental cells are changing throughout gestation. Any disturbance in this well controlled development, can lead to pregnancy disorders or even pregnancy loss.

1.1. Implantation

During the passage of the fallopian tube, the fertilized ovum (zygote) divides to form the morula (12 to 16 cells). Six to seven days following conception, the 128 cell stage mass (blastocyst) starts to implant into the uterine wall (Figure 1). Human implantation can be divided in three steps. The first unstable adhesion of the blastocyst is called *apposition* and is characterized by the contact between microvilli of the trophoblast cells and the large and smooth projections (pinopodes) of the uterine epithelium. During the *stable adhesion* these interactions are increasing, leading to the final step of implantation, namely the *invasion* of the blastocyst into the uterus. As soon as the blastocyst had penetrated the uterine wall, the trophoblast cells, which form the extra-embryonic tissue covering the inner cell mass, which

is preset to develop into the embryo, migrate deeper into the uterine stroma to form the placenta (Figure 1).

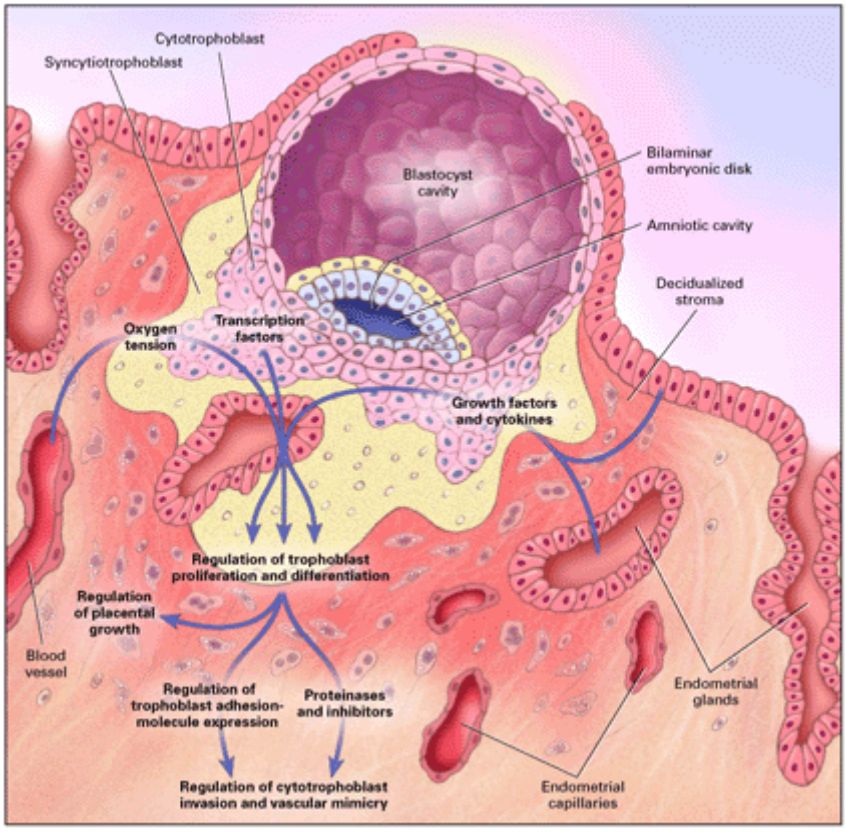

Figure 1: Implantation. Successful invasion of the blastocyst into the maternal uterus involves several highly regulated processes. Oxygen tension, transcription factors and growth factors/cytokines control trophoblast proliferation and differentiation [3].

1.2. Haemochorial placentation

Placentation is the process of the development of the placenta during pregnancy and is the key event to ensure the appropriate transfer of nutrients and oxygen from the mother to the growing fetus.

Among mammalian pregnancies, there exist several types of placentation, which highly vary in respect of the invasive potential of the placental cells into the uterus (Figure 2) [4]. *Epitheliochorial* placentation involves no invasion at all. In *endotheliochorial* placentas the trophoblast cells are in direct contact with the endothelium of maternal vessels.

Several mammals, including rodents, monkeys and humans, have the invasive *haemochorial* placentation. However, rodents and monkeys are not complete models for human placentation, since the intermixture of fetal and maternal cells is highest in humans.

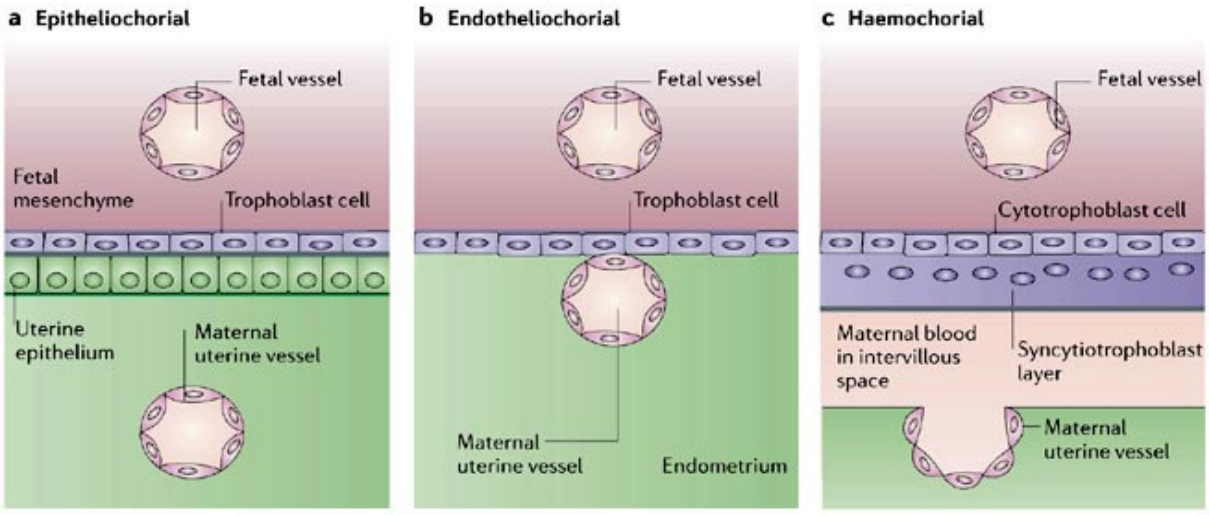

Figure 2: The three major types of placentation. Depending on the mode of placentation, the relationship between maternal blood cells and fetal trophoblast cells is different. (a) Epitheliochorial placentation is characterised by the apposition of the uterine epithelium and the trophoblast cell layer, in the absence of trophoblast invasion into the maternal tissue and vessels. (b) In endotheliochorial placentation the uterine epithelial cell layer is ruptured and trophoblasts are in direct tangency with the maternal endothelium. (c) The most invasive type of placentation is the haemochorial one, where trophobalst cells are penetrating maternal vessels and the syncytiotophoblast is in direct contact with maternal blood in the intervillous space [4].

During human placentation, there are three main changes in the uterus. First, the uterine mucosa (endometrium) differentiates into a dense cellular matrix (decidua) by a process called decidualization [5]. Second, trophoblast cells are invading the decidua and the underlying myometrium [5]. Third, the uterine spiral arteries are transformed into widened, low resistance vessels, to direct an increased maternal blood flow into the placenta [5]. This enlargement is mainly done by *extravillous trophoblast cells (EVTs)*, comprising interstitial and endovascular EVTs, which penetrate maternal vessels, disrupt and replace the

endothelium and some parts of the muscle coat, resulting in a pseudo-endothelium being half fetal and half maternal [6].

Beside EVT cells, there exist the villous trophoblast cells, which form the cellular shell of the villous tree. The core fetal stroma, containing fetal vessels, which merge into the umbilical vein or the two umbilical arteries, is covered by germinative and proliferative *villous cytothrophoblasts* (Figure 3). The fusion of the mononuclear cytotrophoblasts ensures the formation and renewal of the external multinucleated and non-dividing *syncytiotrophoblast* layer (Figure 3). The syncytiotrophoblast is in constant contact with the maternal blood in the intervillous space and as a counterbalance to the syncytial fusion, old cellular material of the syncytiotophoblast is steadily released into the maternal blood (Figure 3). In healthy human pregnancies this release most likely involves apoptotic mechanisms, characterized by well controlled, consecutive morphological changes. Syncytiotophoblast debris, known as syncytial knots, morphologically resemble apoptotic bodies, except that they contain several nuclei [7-9].

Towards term, the syncytiotrophoblast reaches a surface area of 12-14m^2 and several grams of syncytial knots are released daily into the maternal blood [10, 11].

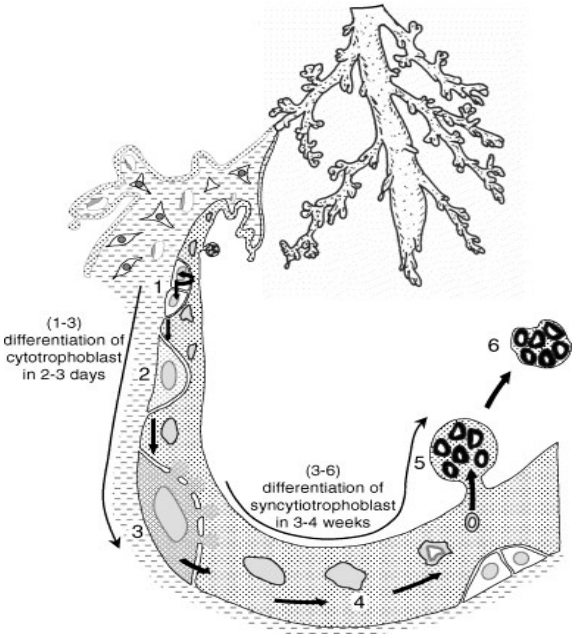

Figure 3: Syncytiotrophoblast turnover. The syncytiotophoblast is steadily renewed by the underlying germinative and proliferative cytotrophoblast cells. Syncytial fusion of differentiated mononuclear cytotrophoblasts leads to the formation of the non-proliferative multinucleated syncytiotrophoblast. Due to normal cell turnover, cellular and subcellular syncytiotrophoblast material is released into the intervillous space [12].

1.3. Physiological hypoxia in early placentation

Early human placentation is not truly haemochorial. In fact, when EVT start to invade the uterine tissue, they also obstruct the tips of the spiral arteries by forming cellular plugs (Figure 4) [13]. On the one hand, this cell shell firmly connects placenta and maternal tissue. On the other hand, these plugs filter maternal blood and strongly limit blood flow into the intervillous space. In this stage, placental villi only contain few capillary vessels, leading to a low feto-placental blood flow. Only few spiral arteries in the periphery are never plugged by EVT and allow a limited maternal blood flow into the placenta [13]. Additionally, the fetus in the amniotic cavity and the placenta are spatially separated by the exocoelomic cavity (Figure 4). All these anatomical changes mediate a hypoxic environment, which is essential for the development of the fetus. Fetal organogenesis is very susceptible to teratogenic damage by reactive oxygen free radicals (OFRs) and hypoxia is needed to keep the full pluripotency of stem cells [14].

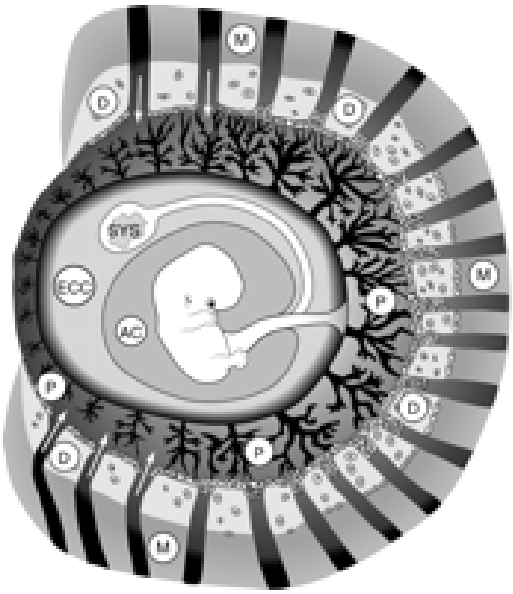

Figure 4: Early human pregnancy (End of 2nd month). Obstruction of the tips of the spiral arteries by extravillous trophoblast cells prevents the flow of maternal blood into the intervillous space of the placenta, resulting in a hypoxic environment for the placenta and the growing fetus**.** Only few spiral arteries in the periphery are never plugged allowing a minimal maternal blood flow into the intervillous space (white arrows). M = myometrium, D = decidua, P = placenta, ECC = exo-coelomic cavity, AC = amniotic cavity, SYS = secondary yolk sac [13].

1.4. Physiological placental oxidative burst at the end of the first trimester

After week 12 of gestation the decidual partial pressure of O_2 (PO_2) is two to three times higher than between week 8 and 10 [15, 16]. This rise in the intraplacental PO_2 at the end of the first trimester triggers an oxidative burst in the periphery of the early placenta [16]. This higher local O_2 concentration damages the trophoblasts and leads to villous degeneration, inducing the formation of fetal membranes [17].

The increased placental oxygenation triggers trophoblast growth and differentiation, and invasive EVT extensively transform the spiral arteries, including the myometrial segments [13]. Spiral arterial rearrangement is complete by week 20 and allows an increased maternal blood supply of the placenta.

2. Immunology of human pregnancy

2.1. Immunological interfaces

The invasive nature of haemochorial placentation implicates direct contact of maternal and fetal cells. There are two immunological interfaces between mother and child in human pregnancy (Figure 5). *Interface 1* involves the local interactions between maternal cells and placental trophoblasts in the decidua and is the main feto-maternal interface during early pregnancy (Figure 5A) [18]. *Interface 2* is set up towards the end of the first trimester with the onset of maternal blood flow into the placental intervillous space and takes place between the syncytiotrophoblast, the outer lining of the placenta, and circulating maternal blood cells (Figure 5B) [16, 18, 19]. Due to the large size of the placenta, the second interface becomes the main interface towards term pregnancy [18]. The syncytiotrophoblast constantly releases placentally-produced factors, including hormones, cytokines, angiogenic factors and trophoblast debris, into the intervillous space, from where they are carried with the maternal blood flow into the entire circulation *(extended interface 2)* (Figure 5C) [18].

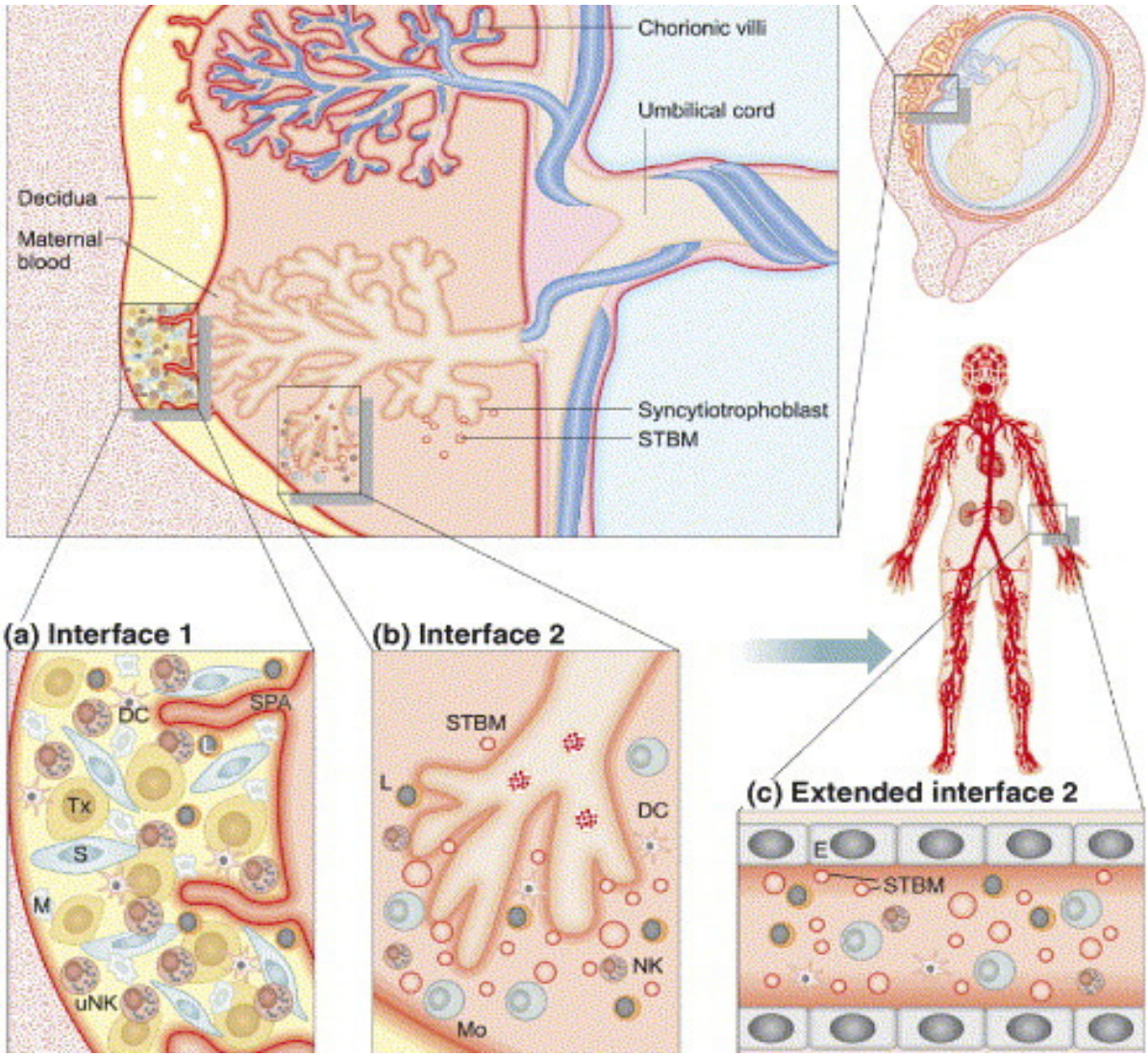

Figure 5: Immunological feto-maternal interfaces in human pregnancy. (a) Interface 1 takes place between decidual immune cells and fetal trophobalst cells and is the main feto-maternal interface in early human pregnancy. (b) Towards end of pregnancy, interface 2, implying interactions between the syncytiotrophoblast and circulating maternal blood cells, becomes the predominant feto-maternal interface. (c) The syncytiotrophobalst constantly releases cellular and subcellular trophoblast debris, including STBM, into the maternal blood. Thus, STBM continuously encounter maternal blood cells throughout the circulation, described as extended interface 2. NK = NK cells, uNK = uterine NK cells, L = lymphocytes, M= macrophages, Mo = monocytes, DC = dendritic cells, Tx = invasive extravillous trophoblasts, S = stromal cells, SPA = spiral arteries, E = endothelial cells, STBM = syncytiotophoblast microparticles [18].

2.2. Immunological interactions

In immunological term, the growing fetus in the uterus is considered as a semi-allograft, having half of the genes from the mother (maternal) and the other half from the father (paternal). Interestingly, in healthy human pregnancies the fetal and maternal cells are coexisting, without leading to the rejection of the developing fetus. This feto-maternal tolerance is believed to be carried out by several mechanisms used by the maternal immune system and placental trophoblast cells locally at interface 1, but also systemically in the maternal circulation.

2.2.1. Predominant Th2 immunity

In 1993 Wegmann and co-workers suggested, that the human fetus is not rejected by the maternal immune system thanks to a prevalent production of T helper cell 2 (Th2) cytokines, both systemically and in the placental bed [20-22]. Th2 mediators, such as interleukin (IL)-4, IL-5, IL-6, IL-10 and IL-13, attenuate the production of proinflammatory Th1 cytokines, such as IL-2 and interferon (IFN)-γ. Thereby, the Th2 response interferes with the stimulation of $CD8^+$ cytotoxic T-cells and natural killer (NK)-cells, which may be harmful for the fetal cells [20].

The Th2 cytokine IL-4 is continuously expressed by the syncytiotrophoblast, cytotrophoblast cells and decidual macrophages [23, 24]. In addition, the spontaneous secretion of the Th1 mediator IL-12 by peripheral blood mononuclear cells (PBMC) from pregnant women is significantly decreased than in non-pregnant controls [25]. However, few years ago, the question raised, if this bias towards Th2 cytokine secretion is important for successful pregnancy, or if it is just a secondary effect [26]. Although, in humans, a propensity to Th1 immune reactions has been reported in pregnancy complications, such as recurrent spontaneous abortions, so far, a T-cell mediated attack of the fetus, triggered by a shift towards Th1 immunity has only been shown in an abortion-prone mouse model [27, 28].

Thereby, the abortions in the matings of CBA/J females with DBA/2 males were mediated by the production of the Th1 cytokine TNF-α in the decidua and could be hindered by the Th2 cytokine IL-10 [28, 29].

2.2.2. Absent trophoblast expression of MHC class II and classical polymorphic MHC class I molecules

Major histocompatibility complex (MHC) class I and class II molecules present peptides, derived from pathogens or foreign cells, on the cell surface of antigen presenting cells (APCs) to cytotoxic $CD8^+$ T-cells and helper $CD4^+$ T-cells, respectively [30]. MHC class I and II themselves are recognized as non-self antigens by the immune cells of a recipient of an allogeneic organ graft [31, 32]. Thus, an analogy could be drawn between the fetus and an allograft. However, all human trophoblast cell subsets completely lack MHC class II expression and display an unconventional pattern of MHC class I molecules [4]. The classical polymorphic MHC class I molecules human leukocyte antigen (HLA)–A and HLA-B, which are driving the T-cell-dependent transplant rejection, are not expressed by any trophoblast cell subset [18]. Intriguingly, EVT cells display the classical polymorphic HLA-C and the non-classical, less polymorphic HLA-G, HLA-E and HLA-F [33-37].

Originally it was believed that the syncytiotrophoblast cells are deficient of MHC class I and II expression, but lately it has been proposed that they might display non-classical HLA-E and produce soluble HLA-G [4, 38-40].

2.2.3. Decidual NK (dNK) cells and their interaction with trophoblast HLA-E, -G, -C

70% of all leukocytes at the implantation site are made up by a particular $CD56^{hi}CD16^-$ decidual natural killer (dNK) cell subset, which are recruited in great numbers in early pregnancy and remain the most abundant maternal immune cells in the decidua until mid-gestation, before their quantity is decreasing again towards term [41, 42].

Despite their expression of perforin, granzyme A and B, and some NK-activating receptors, and the close contact with infiltrating interstitial trophoblasts, dNK cells do not mediate cytotoxicity against the allogeneic trophoblast cells [43, 44]. This is believed to be due to the interaction between the non-classical, largely monomorphic MHC class I molecules HLA-G and HLA-E displayed by EVT and its receptors expressed by dNK cells. HLA-E is suggested to bind to the inhibitory receptor CD94/NKG2A, which is highly expressed by dNK cells [45]. A number of inhibitory NK cell receptors, including the killer cell immunoglobulin receptor (KIR) KIR2DL4 (CD158d) and the leukocyte Ig-like receptor (LIR-1), have been suggested to bind to HLA-G and, thus, prevent NK-mediated killing [46, 47].

In addition to tolerance induction, the great abundance of dNK cells also indicates an essential role in placentation. Indeed, dNK cells are suggested to actively support vascular remodelling by secreting interferon (IFN)-γ, vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) [48, 49]. Furthermore, dNK cells produce the chemokines IL-8 and interferon-inducible-protein-10 (IP-10), which trigger migration of invading trophoblasts [49]. It has also been proposed that specific dNK cell – EVT interactions might be more beneficial for the transformation of the spiral arteries [50]. EVT express the classical, polymorphic MHC class I molecule HLA-C, which interacts with specific members of the KIR family of NK cell receptors, namely KIR2D (contain two immunoglobulin-like domains) [51]. Depending on the combination of the haplotypes, KIR2D may be more activating or more inhibiting [51]. Particular (more activating) KIR2D-HLA-C pairs may be more favourable for the enlargement of the spiral arteries, suggesting that activated dNK cells might be essential for an efficient vascular remodelling [4, 50].

2.2.4. Decidual macrophages

20-30% of the maternal cells at the implantation site account for decidual macrophages and their numbers remain high throughout pregnancy [52]. Decidual macrophages display CD14,

the MHC class II molecule HLA-DR and low levels of the T-cell co-stimulatory cell surface markers CD80 and CD86 [52-54]. *In vitro,* decidual macrophages spontaneously produce high levels of the anti-inflammatory cytokine IL-10 and express the tryptophan catabolising enzyme indoleamine dioxygenase (IDO), suggesting that decidual macrophages exhibit immunosuppressive functions, which may be important to maintain feto-maternal tolerance [54, 55].

Since decidual macrophages are localized in the proximity of apoptotic trophoblast cells in the placental bed, it has been speculated that the engulfment of these dying cells by decidual macrophages might result in a decreased synthesis of proinflammatory cytokines and an enhanced secretion of anti-inflammatory Th2 mediators [52]. The uptake of apoptotic trophoblast cells may prevent secondary necrosis, which is characterized by the release of potentially proinflammatory and immunogenic intracellular material from the dying cells [52].

2.2.5. Regulatory T-cells (T_{reg})

The human $CD4^+CD25^{high}$ regulatory T-cells (T_{reg}) are involved in tolerance induction against self-antigens and allogeneic organ grafts [56, 57].

Levels of peripheral blood T_{reg} almost double during pregnancy, compared to non-pregnant controls, and drop again postpartum [58]. T_{reg} are also localized in the decidua and during early pregnancy they account for 22% of $CD4^+$ cells. Spontaneous abortion is associated with a reduction of circulating T_{reg} to non-pregnant levels and a decrease of the $CD4^+CD25^{high}/CD4^+$ ratio to 7% in the decidua [59, 60].

These observations suggest that T_{reg} are essential for the maintenance of the maternal tolerance towards the growing fetus and, thus, may contribute to the successful progression of human pregnancy.

2.2.6. B7 family:

Activation of naive T-cells requires the engagement of the T cell receptor (TCR) by binding to an antigen peptide – MHC protein complex on APCs. To complete T-cell stimulation, a costimulatory signal is needed, which is mediated by the interaction between T-cell expressed CD28 and members of the B7 family, displayed by the partner cell [61]. However, B7 molecules are able to trigger both, activating and inhibitory signals in lymphocytes.

The expression of at least five of the seven known B7 family members has been reported in the human placenta [62, 63]. From all placentally expressed B7 family members, only B7-H1 has been documented to play a key role in maternal tolerance toward the fetus, since inhibition or genetic deletion of B7-H1 resulted in an elevated rejection of the allogeneic fetus in animal experiments [64]. In humans, placental expression of B7-H1 is found on syncytiotrophoblasts, villous and extravillous cytotrophoblasts, and is increasing throughout pregnancy [62]. B7-H1 interacts with the immunoinhibitory molecule programmed death (PD)-1, which is present on activated T-cells, B-cells and monocytes [65]. The *in vitro* ligation of human PD-1 blocks T-cell proliferation and cytokine production [66]. As human decidual T-cells express PD-1, the induction of the PD-1-B7-H1 pathway may provide a mechanism of immunological tolerance towards the fetal semi-allograft [67].

2.2.7. Indoleamine-2,3-dioxygenase (IDO):

Indoleamine-2,3-dioxygenase (IDO) is a tryptophan-degrading enzyme, which is expressed at the feto-maternal interfaces by trophoblasts, syncytiotrophoblasts and glandular epithelial cells [60]. By depleting tryptophan from the local microenvironment, IDO can inhibit the activation of T-cells, which are particularly sensitive to the loss of this essential amino acid [68, 69]. IDO may mediate T-cell suppression either by limiting the availability of tryptophan, or by indirect effects on the biology of IDO-expressing cells [69].

2.2.8. Fas-Fas ligand system:

Expression of the death-triggering receptor-ligand pair Fas (CD95) – Fas ligand (FasL/CD95L) is found at the feto-maternal interface. FasL is expressed by EVT, villous cytotrophoblasts, syncytiotrophoblasts and maternal decidual cells, and eliminates activated Fas-positive maternal immune cells invading the uterus [70-72]. *In vitro,* human trophoblast cells induce Fas/FasL dependent apoptosis in T-cells [73, 74]. Recently, Abrahams et al. demonstrated that trophoblast cells from the first trimester placenta do not express cell-surface FasL [75]. However, the first trimester trophoblast cells are able to secrete FasL *in vitro*, which might protect them from the recognition by the maternal immune system [75].

2.2.9. Complement regulatory proteins:

The complement system is an essential component of the immune defence, assembled by a set of proteins, and mediates clearance of pathogens, apoptotic cells and immune complexes [76, 77]. The net result of the complement cascade is the formation of the membrane attack complex (MAC), leading to cellular lysis [76]. The syncytiotrophoblast, villous cytotrophoblasts and EVT all express the three complement regulatory proteins decay accelerating factor (DAF/CD55), membrane co-factor protein (MCP/CD46) and CD59, which protect healthy cells from cell lysis [78-82].

2.2.10. Deportation of syncytiotrophoblast debris into maternal blood

As mentioned earlier, the old cellular material of the syncytiotrophoblast, such as syncytial knots and microparticles, is constantly released into the intervillous space (Figure 5). Syncytial knots are detectable in the maternal uterine veins, but not in the peripheral blood of pregnant women, which might be due to their uptake by alveolar macrophages in the lungs [83-85]. Engulfment of syncytial knots by a macrophage cell line induces an anti-inflammatory response *in vitro*, suggesting that the shedding of apoptotic debris is not only a

mechanism to dispose aged syncytiotrophoblast cells, but also a mechanism to provide tolerogenic parental antigens to the maternal immune system [85].

2.3. Systemic maternal inflammation

Healthy third-trimester pregnant women display a generalized inflammatory response, which is in some regards as extensive as in sepsis [86]. However, this controlled systemic inflammatory reaction does not appear to be harmful for the mother.

Markers of overall maternal inflammation include an elevated total white blood cell count (leukocytosis) and increased levels of the proinflammatory cytokines IL-1, IL-6, TNF-α and the acute phase protein C-reactive protein (CRP) in the serum of pregnant women compared to non-pregnant subjects [87-90]. CRP levels are moderately raised as early as week 4 of gestation, providing evidence that there is a low-grade maternal inflammation already during the implantation of the conceptus [90]. Furthermore, there is an activation of peripheral blood leukocytes, which is characterized by the significantly elevated cell surface expression of the adhesion molecules CD11b, CD11c, CD64 and the pattern recognition receptor CD14 on granulocytes and monocytes compared to non-pregnant controls [18, 86, 91]. Moreover, monocytes, granulocytes and lymphocytes display significantly increased values of basal intracellular reactive oxygen species [86].

Granulocytes from pregnant donors spontaneously produce the proinflammatory cytokine IL-6 and the chemokine IL-8 [92]. Phorbol 12-myristate 13-acetate (PMA)/ionomycin-stimulated third trimester granulocytes synthesise significantly more IL-8 than PMA/ionomycin-stimulated cells from non-pregnant women, suggesting that circulating maternal granulocytes are primed to produce inflammatory mediators [92].

Monocytes from pregnant women display an enhanced phagocytic rate with peak levels in the third trimester and exhibit a progressive up-regulation of the cell surface adhesion molecule CD54 [92-94]. In addition, PMA/ionomycin triggers a higher production of intracellular IL-1β in second- and third-trimester monocytes than in the cells from pregnant women in the first trimester and non-pregnant controls [92]. These findings suggest a continuous activation of maternal monocytes throughout pregnancy.

It has also been shown, that during pregnancy, the platelets, the complement - and the clotting system are activated [18].

Until now the cause of this maternal inflammation is unknown. However, it is generally accepted, that it consists in a sterile inflammatory reaction, which is stimulated by the pregnancy itself and not by an infection. Candidate triggers include placental factors released into maternal blood, such as cytokines and angiogenic factors [95, 96].

2.3.1. Placental cytokines

Pro-inflammatory cytokines and chemokines, including IL-6, IL-1β, IL-8 and TNF-α, are produced by the placenta, as shown by *in vitro* cultures of placental villous tissue [97-100]. Beside their local action, they are also secreted into the maternal circulation, where they may contribute to the mild systemic inflammation by attracting and stimulating maternal immune and endothelial cells [101]. The placental expression of the different cytokines varies in function of the gestational age, most likely reflecting a specific function at particular stages of pregnancy [102, 103].

2.3.2. Placental angiogenic factors

Placental angiogenesis is of great importance in pregnancy to ensure appropriate blood supply to the growing fetus. Angiogenesis is supported by placentally-produced angiogenic factors, such as PlGF and VEGF, which interact with receptors expressed by the vascular endothelium, including the VEGF receptor (VEGFR / Flt-1) and endoglin (Eng) [104-110]. In addition, soluble forms of Flt-1 (sFlt-1) and Eng (sEng) with anti-angiogenic properties are produced by the placenta [96, 111]. These factors are released into the maternal peripheral blood, where they could disturb the maternal endothelium and explain the vascular reactivity and mild glomerular endotheliosis found towards term [112, 113]. The expression of these markers varies with the gestational age [96, 114].

A potential role in the maternal inflammatory response is also ascribed to membrane microparticles released from the syncytiotrophoblast into the maternal circulation, which are discussed later, in chapter 3 of the introduction [115].

3. Preeclampsia

3.1. Epidemiology

Preeclampsia is a heterogeneous placenta-dependent disorder specific to human pregnancies and is characterized by new onset hypertension (systolic and diastolic pressure of ≥140 and 90mm Hg) and proteinuria (protein excretion of ≥300 mg in a 24 h urine collection, or a dipstick of ≥2+) after week 20 of gestation [116, 117]. The maternal symptoms become apparent in the second half of pregnancy and may be either early (<34 weeks of gestation) or late (>34 weeks of gestation), and mild or severe, according to the degree of hypertension and/or protenuria. In addition, patients may exhibit edema, reduced blood supply to several organs, including the placenta, and can end up in organ (mainly renal or liver) failures. In severe cases preeclamptic women may develop the HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet counts) or the disorder may evolve into eclampsia, which is characterized by convulsions and seizures, pre- or postnatally. Preeclampsia may manifest as a maternal syndrome only, or it can affect the fetus, through in utero growth restriction (IUGR) or sudden fetal distress.

The reported incidence of preeclampsia is 2 to 7% among human pregnancies world wide [117]. However, there are large differences in the frequency of incidence among populations, which may be due to racial, geographic, social and economic distinctions. Other predisposing factors, which make women more prone to become preeclamptic during pregnancy, include obesity, chronic hypertension, diabetes or insulin resistance, multiple gestations and a history of previous preeclampsia [118].

Owing to the excellent medical care in western countries, preeclampsia is less and less fatal [119]. However, in underdeveloped countries preeclampsia remains the major cause of maternal death. In Latin America and Caribbean over 25% of maternal deaths are attributed to

pregnancy associated hypertensive disorders [120]. In Columbia the number of maternal deaths caused by preeclampsia (42%) is ten times higher than in the United States [121].

Pregnancy may be lengthened by giving antihypertensive drugs to treat the symptoms of the disorder [122]. To prevent imminent eclampsia magnesium sulphate, a promising anticonvulsant drug, is applied [123-126]. However, none of these drugs prevents the onset of preeclampsia and the only definite cure of preeclampsia is the removal of the placenta by elective preterm delivery, either by induction or caesarean section. Though, preterm delivery (<37 weeks of gestation) accounts for 75% of perinatal mortality and preterm babies are at increased risk for long-term medical problems affecting the neurological, respiratory, cardiovascular and gastrointestinal systems [127].

Although the acute maternal symptoms of preeclampsia can be cured by delivering the baby, women have an increased risk to develop cardiovascular diseases in later life [128].

Since preeclampsia is a life-threatening disorder, it is very demanding to recognize the disease early enough. In the past few years, research focussed in the finding of biophysical and biochemical markers, which are differently expressed in preeclamptic women or in women, who will develop the disease, compared to healthy pregnant controls, and could be used for detection and prediction [129].

Several clinical studies were/are evaluating the efficiency of prophylactic agents, such as the anti-oxidant vitamins C and E, low-dose aspirin, folic acid and calcium [117, 130-136]. However, the results of different clinical trials are either contradictory or not efficacious.

3.2. Pathophysiology

Preeclampsia may be divided into two main stages. The first stage is related to the asymptomatic preclinical phase in early pregnancy. The clinical stage appears in the second half of pregnancy and is characterized by the maternal syndrome.

3.2.1. Preclinical stage: shallow trophoblast invasion and incomplete vascular remodelling

Preeclampsia may develop in pregnancies without a viable fetus (hydatidiform moles), but its onset always depends on the presence of a placenta [118]. A long lasting hypothesis has been that the onset of preeclampsia relies on a deficient development of the early placenta and an incomplete remodelling of the maternal spiral arteries (Figure 6). This poor placentation takes place before week 20 of gestation [137]. The invasion of the decidua by EVT is reduced and the transformation of spiral arteries into dilated tubes is shallow or absent.

However, reduced placental perfusion cannot be the only trigger of preeclampsia since normotensive intrauterine growth restriction (nIUGR) is associated with an incomplete placentation in the absence of the maternal syndrome [138].

Furthermore, the current hypothesis on the placental origin of preeclampsia has been challenged [139]. It has been suggested that the failure of the development and the differentiation of the trophoblast lineage at various time-points may result in preeclampsia with late-onset appearance and mild symptoms, or IUGR.

However, women suffering from arterial diseases, hypertension, obesity and diabetes before conception are predisposed to preeclampsia, and in this so-called maternal preeclampsia the challenge rather lies in an inappropriate maternal response than in an abnormal placentation [140, 141].

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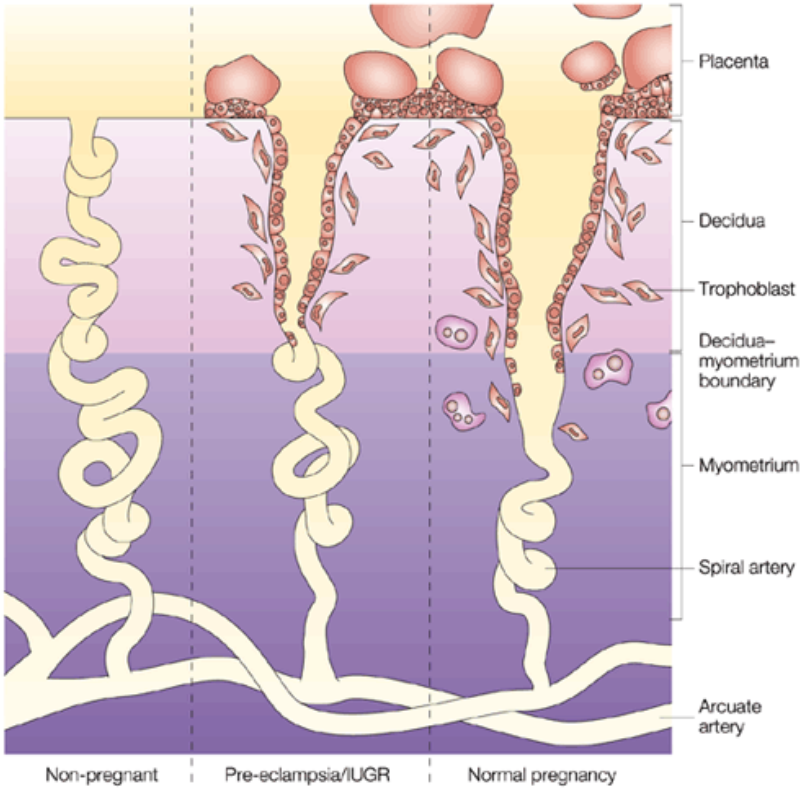

Figure 6: Incomplete remodelling of spiral arteries in preeclampsia. During normal pregnancy uterine spiral arteries are extensively remodelled. In order to increase the placental and fetal blood supply, the spiral arteries are enlarged by replacing the vascular endothelium by infiltrating trophoblast cells. In preeclampsia and IUGR vascular transformation is shallow resulting in a reduced blood flow in the intervillous space [41].

3.2.1.1. Placental oxidative stress

The shallow remodelling of spiral arteries implicates maintenance of smooth muscle cells in the placental vascular walls and persistence of up to 50% of the vascular contractibility [13]. This might result in an intermittent perfusion of the intervillous space mediating transient hypoxic conditions [142]. In *in vitro* experiments, Hung and collaborators demonstrated that oxidative stress, which is characterized by an imbalance favouring oxidant over antioxidant forces, occurs after reoxygenation of hypoxic placental tissue [143]. Enhanced placental oxidative stress is associated with increased tissue damage mediated by reactive oxygen free radicals (OFRs) [13].

Lipid peroxidation, induced by OFRs, enhances the incorporation of cholesterol, oxidized free fatty acids (FFAs) and low-density lipoproteins (LDLs) into cell membranes [144]. Furthermore, in regions of spiral and myometrial arteries, where the physiologic remodelling is missing, pathologic lesions known as acute atherosis can be found [143]. Acute atherosis shares a lot of clinical features with atherosclerosis, namely clusters of macrophages loaded with lipids (foam cells), fibrinoid necrosis of vascular walls, dysfunctional endothelium and accumulations of platelets [145-148]. Markers of oxidative stress have been found in the peripheral blood of preeclamptic women [149, 150]. Hence, it has been proposed that oxidatively stressed placentas of preeclamptic patients release soluble factors into the maternal bloodstream, where they might affect the maternal vascular endothelium [151].

3.2.1.2. Maternal-paternal immune maladaptation

There are multiple lines of evidence that imply a crucial role of the maternal immune system in the onset of preeclampsia.

Preeclampsia is a disorder of first pregnancies (primigravidity) and the risk to develop preeclampsia decreases upon an earlier healthy pregnancy [152, 153]. However, the change of the partner reverses this natural protection generated by multiple pregnancies (multigravidity). Thus, Robillard and co-workers described preeclampsia as a “disease of new couples” (primipaternity), according to the observation that the length of sexual cohabitation before conception inversely correlates with the risk of preeclampsia [154]. Though, the use of barrier contraceptives, such as condoms, does not reduce the risk of developing preeclampsia, leading to the hypothesis that preeclampsia might develop due to missing seminal priming [155].

Other mechanisms of tolerance induction are altered or missing in preeclampsia as well. A reduced trophoblast FasL expression and an elevated Fas expression have been observed and this correlates with increased apoptosis of EVTs and villous trophoblast cells [156-160]. Furthermore HLA-G expression on EVTs is reduced or even missing, and, thus, the resulting

deficient interactions with dNK cells might lead to dNK-cell-mediated cytolysis of EVTs [161, 162]. Santoso and co-workers reported a decreased expression of the T-cell inhibitor IDO in preeclamptic placentas [163]. In addition, in pregnancy pathologies, such as preeclampsia and recurrent pregnancy loss, an increased activation of the complement system has been found [128, 164-166]. However, studies gave controversial results about the frequency of peripheral blood T_{reg} cells in women suffering from preeclampsia [167-169].

In addition, preeclampsia is associated with a predominant Th1 response in the peripheral maternal blood, as well as in the placenta, in contrast to the Th2 bias in normal pregnancy [170]. The spontaneous and phytohemagglutinin (PHA)-stimulated production of the Th1 cytokines TNFα, IL-2 and IFNγ by PBMC from preeclamptic patients is higher than those from normal pregnant controls [171, 172]. Furthermore, the *in vitro* stimulation of PBMC from preeclamptic women with the classical Th1 cytokine IFNγ results in an increased production of IL-12 and IL-18 [173]. In the presence of IL-12, IL-18 does not act as a Th2 mediator anymore, but supports the IL-12 driven Th1 response [174]. Th1 cytokines are known to trigger chronic inflammation, and IFNγ, together with the proinflammatory factors IL-1 and TNFα, has been shown to amplify this chronic inflammatory response [170].

3.2.2. Clinical stage: excessive maternal systemic inflammation and placental factors

An excessive, generalized maternal inflammatory response including a dysfunctional maternal endothelium is thought to be at the basis of the maternal clinical manifestations [175, 176].

This overt inflammatory response is believed to be the extreme end of the mild inflammation found in healthy pregnancy [177]. There is a significantly increased leukocytosis and a significantly higher concentration of the proinflammatory mediators IL-6 and IL-8 in the peripheral blood of preeclamptic women relative to normal pregnant women [178-185].

Enhanced activation of peripheral blood leukocytes is marked by the higher basal as well as PMA-induced production of intracellular reactive oxygen species and a significant increased

cell surface expression of the integrin CD11b, in monocytes and granulocytes from preeclamptic women compared to the monocytes and granulocytes from healthy pregnant women [86, 91, 186].

Additionally, expression of the activation marker HLA-DR is significantly enhanced on monocytes derived from preeclamptic women relative to the cells of normotensive pregnant women [25]. However, the findings on the level of CD14 expression on monocytes are contradictory [25, 91]. Furthermore, spontaneous intra-monocytic synthesis of IL-1β, IL-6 and IL-8 is higher in cells from preeclamptic women than in monocytes derived from normal pregnant and non-pregnant subjects [187]. Monocyte-derived microparticles were also more elevated in preeclamptic patients compared with pregnant controls, reflecting activation of their parental cells in preeclampsia [188].

A dysfunctional endothelium is marked by the increased plasma concentrations of the vasoconstrictive mediators' asymmetric dimethylarginine and endothelin, and the released integrin fibronectin in women with adjacent preeclampsia relative to women with uncomplicated pregnancies [189-192].

Furthermore, there is an elevated activation of the complement cascade, the clotting system and the platelets in the peripheral blood of preeclamptic women compared to normal pregnant women [193-195].

As it is the case in normal pregnancy, candidate triggers are believed to be derived from the placenta [196]. The expression of placental cytokines and placenta-derived angiogenic factors is altered compared to normal pregnancy. Placental tissue from preeclamptic women produce increased levels of the proinflammatory cytokines TNFα, IL-1, and IFN-γ relative to the placenta of normal pregnant subjects [95]. The anti-angiogenic factors sFlt-1 and sEng, which are secreted by the placenta, are elevated in the peripheral blood of preeclamptic women for a long time before the onset of the disease [96, 197-200]. On the other hand, the levels of the pro-angiogenic factor PlGF are reduced before and during onset of preeclampsia [129, 201].

The rates of syncytial apoptosis and shedding of debris are significantly increased in preeclampsia compared to normotensive pregnancy (Figure 7), consistent with the increased placental damage and dysfunction observed in this pathological condition [160]. Other markers for apoptosis, such as cytokeratin and cell free fetal DNA of placental origin are also elevated in preeclampsia [202, 203]. It was suggested that the shedding of placental debris might be exacerbated by apo-necrosis or even necrosis in this pathologic condition (Figure 7) [204]. Among this syncytial debris, there are small membrane microparticles released from the syncytiotrophoblast [115].

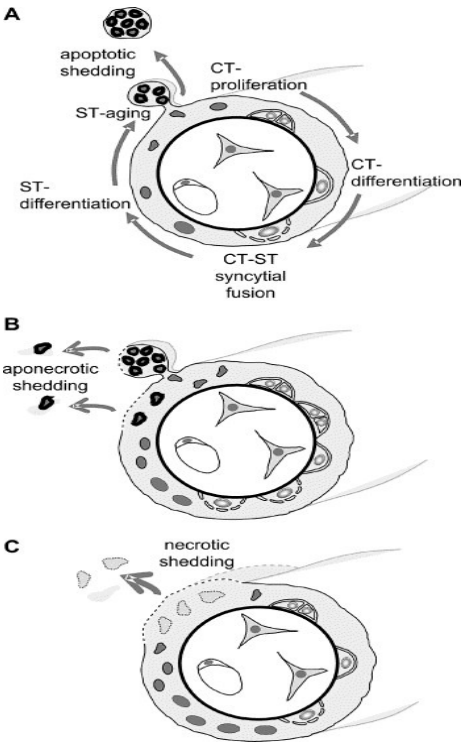

Figure 7: Release of Syncytial knots and STBM by apoptosis, apo-necrosis and necrosis. (A) Controlled apoptotic shedding of syncytial knots and STBM due to normal cell turnover. (B) If the final steps of apoptosis fail and the membranes of the apoptotic bodies break (apo-necrotic shedding), released intracellular material may trigger inflammation. (C) Necrotic rupture of the syncytiotrophoblast layer leads to uncontrolled disposal of the cellular content, inducing a maternal inflammatory response. CT = cytotrophoblast; ST = syncytiotrophoblast [205].

3.3. Microparticles

3.3.1. Microparticles in general

Microparticles are subcellular membrane-sealed fragments and exhibit a mean diameter of 100 nm. They are shed from the cell surface in both physiologic and patho-physiologic conditions, and are generated during cell death and cellular activation [206]. The properties of microparticles may differ according to the characteristics of the parental cell, including membrane components, content of proteins, lipids and messenger RNA, size and antigenicity [206].

Microparticles are components of normal peripheral blood (5-50µg microparticles/ml blood) and are released from leukocytes, endothelium, erythrocytes and platelets [206]. Platelet-derived microparticles are the most abundant ones in normal serum (80%), whereas microparticles released from endothelial cells and leukocytes only account for 10% each [206]. However, their numbers are increasing during inflammation, cell injury, infection, thrombosis, cardiovascular diseases and platelet activation [206]. Furthermore, microparticles are released from tumour cells in cancer patients [207-209].

Due to their interactions with cells, microparticles are essential modulators of cell to cell communication. Hence, microparticles may affect the function or nature of the target cell by the following means [206, 210]:

- Stimulation of the target cell by a ligand/receptor interaction
- Transfer of membrane molecules
- Transfer of cytoplasmic proteins, mRNA, lipids
- Delivery of pathogens, such as HIV, prions

Indeed, it has been assumed, that lots of cell-free receptors and molecules detected in body fluids are in truth microparticle-associated [211-213].

3.3.2. Microparticles in inflammation

During inflammation the numbers of circulating microparticles in peripheral blood are increased, exerting various effects on cells of the immune system and the endothelium. Microparticles shed from endothelial cells have been shown to mediate procoagulant activity in monocytes [214]. Platelet-derived microparticles display IL-1β and have been reported to change the expression of adhesion molecules and to trigger the production of proinflammatory mediators in monocytes and endothelial cells [206, 215]. Microparticles, also named ectosomes, released from monocytes display procoagulant properties [216]. Upon activation, monocytes secrete bioactive IL-1β, which is associated with microparticles [217]. IL-1β is then released from the microparticles. On the contrary, ectosomes derived from activated polymorphonuclear leukocytes (PMN) and erythrocytes possess immunosuppressive activities. Gasser et al. described a PMN-ectosome dependent increase of transformation growth factor (TGF)-β1 production by macrophages, whereas the secretion of the proinflammatory mediators IL-8 and TNF-α was not induced [218]. Furthermore, ectosomes derived from PMN and erythrocytes inhibited the zymosan A and LPS induced activation of macrophages [218, 219].

3.3.3. Syncytiotrophoblast microparticles (STBM)

Next to microparticles derived from platelets, leukocytes and endothelium, in pregnant women, unique circulating microparticles originated from placental syncytiotrophoblasts can be found [84].

Syncytiotrophoblast microparticles (STBM) are 100-200 nm in diameter and are free of nuclei [115, 220]. Unlike the bigger syncytial knots, STBM are not trapped in the maternal lungs and get into the maternal peripheral circulation, where they encounter maternal immune and endothelial cells, and, thus, may affect their function and phenotype [84].

In the peripheral blood of preeclamptic women there are significantly increased concentrations of STBM compared to normotensive subjects [84, 221].

3.3.4. STBM and the maternal inflammation

Circulating STBM have been proposed to be involved in the systemic feto-maternal tolerance, as well as in the generalized maternal inflammation. But, as placental microparticles represent less than 6% of the total amount of microparticles in the maternal peripheral blood in normal pregnant women, it is very difficult to obtain appropriate numbers of pure STBM from the maternal blood [222]. Divers *ex vivo* and *in vitro* approaches for the preparation of STBM from term placentas have been published [220]. Some approaches may better mimic the physiologic situation in human pregnancy, whereas others could reflect more closely non-physiologic or the patho-physiologic conditions found in preeclampsia. Thus, depending on the mode of preparation, STBM induce different responses in target cells.

On the one hand, STBM have been shown to affect the adaptive immune system. Both, STBM generated by mechanical dissection of villous tissue from human term placentas as well as STBM prepared by *in vitro* explant cultures of villous tissues incubated in air significantly inhibited the proliferation of phorbol ester and Ca^{2+} ionophore stimulated peripheral blood T-cells, whereas STBM isolated from the wash of the maternal side of a dually-perfused placental lobe enhanced proliferation [223]. Gercel-Taylor and co-workers isolated shed placental membrane fragments from the serum of pregnant women [224]. These "naturally occurring" placental microvesicles expressed FasL and triggered Fas/FasL-mediated apoptosis and the down-regulation of CD3-ζ on a T-cell line, suggesting a role of placental membrane fragments in the systemic maternal immune tolerance [224]. However, this study cannot be directly compared with the one from Gupta and co-workers, as Gercel-Taylor et al. isolated membrane fragments, which were smaller in size (exosomes) than the *in*

vitro prepared STBM. Furthermore, they used a T-cell line and not peripheral blood T-cells to evaluate the effects of the exosmes [224, 225].

On the other hand, artificially generated STBM have also shown to activate innate immune cells. STBM prepared by *in vitro* villous explant cultures incubated in air and STBM collected from the maternal side of an *ex vivo* dually perfused placental cotyledon induced partial inhibition of endothelial cell proliferation, but no apoptosis [220, 226]. However, in the same experiment STBM prepared by mechanical dissection of villous tissue triggered endothelial cell detachment from the collagen matrix, and apoptosis, supporting the proposal that STBM prepared by the physical disruption of the villous tissue integrity are released during cellular necrosis [220]. In addition, these STBM triggered the production of superoxide radicals in neutrophils. This production was higher upon co-culture with STBM prepared from preeclamptic placentas [227]. This observation correlated with the N-formyl-methionyl-leucyl-phenylalanine (FMLP)-induced synthesis of superoxide radicals in neutrophils from normal pregnant and preeclamptic women, suggesting that STBM might be a trigger of the production of superoxide radicals in maternal neutrophils [227].

Furthermore, STBM generated by *in vitro* culture of villous tissue incubated in air significantly increased the expression of the activation marker CD11b on peripheral blood neutrophils [228]. The same STBM preparations mediated the formation of fibrous extracellular lattices containing DNA, called neutrophil extracellular traps (NETs), which are known to be generated upon an inflammatory signal, such as gram-negative and gram-positive bacteria, IL-8 and PMA, in neutrophils [228-230]. As large numbers of NETs have been observed in the intervillous space of preeclamptic placentas, it has been suggested that STBM might be a key activator of maternal neutrophils and, thus, mediate the formation of NETs [228].

Less is known about the inflammatory effects of STBM on human peripheral blood monocytes. Monocytes belong to the mononuclear leukocytes and develop from monoblasts

in the bone morrow [231]. Once entered the blood, human monocytes circulate for few days, before they invade tissues, where they may differentiate into macrophages or dendritic cells [232]. Two main monocyte subsets may be distinguished according to their expression of CD14, which is part of the lipopolysaccharide receptor, and CD16 (FcγRIII): the "classical" $CD14^{+}CD16^{-}$ monocytes, which account for 95% of all blood monocytes, and the "non-classical" $CD14^{lo}CD16^{+}$ monocytes [232]. The key functions of monocytes during inflammation are the processing of antigens, the release of cytokines and thereby stimulating other immune cells. A previous study revealed that STBM isolated *ex vivo* from the maternal perfusion of a placental cotyledon induced the intra-monocytic production of the Th1 cytokines TNF-α and IL-12p70, thereby strongly suggesting that placental microparticles may play a role in the development of the inflammatory state in pregnancy [173].

RESEARCH OBJECTIVE

Since maternal monocytes are progressively activated during normal pregnancy and are further activated in preeclampsia, the aim of this PhD project was to evaluate STBM as candidate trigger for this activation [86, 91, 92, 101, 176, 187]. The inflammatory nature of STBM was investigated by their co-incubation with monocytes *in vitro*.

STBM were artificially generated from healthy or preeclamptic human term placentas. Several conditions, either mimicking the physiological situation of normal pregnancy or the patho-physiological conditions found in preeclampsia, were used.

Functional analysis was performed by assessing the following items in STBM-treated monocytes:

- cell viability
- gene expression
- cytokine secretion
- cell surface expression
- interaction/communication
- intracellular signalling

MATERIALS AND METHODS

1. Preparation of syncytiotrophoblast microparticles (STBM)

This study was approved by the Cantonal Institutional Review Board of Basel, Switzerland, and the Ethical Committee Review Board for studies in human subjects of Lund, Sweden, and written informed consent was received in all cases. Human placentas were obtained from the University Women's Hospital Basel, or the Department of Obstetrics and Gynecology, Lund University Hospital, immediately after elective caesarean section or vaginal delivery from uncomplicated pregnancies or cases of preeclampsia. STBM were generated by four different approaches, indifferently of the mode of delivery.

Preparations of STBM by *in vitro* cultures of placental villous fragments and by mechanical dissection of villous tissue were performed in the laboratory of Prenatal Medicine in Basel, Switzerland.

1.1. *In vitro* explant cultures (eS_{20} and eS_3)

After the removal of the decidua, the villous tissue was cut into small pieces and washed 3 times in phosphate buffered saline (PBS) to eliminate clotted blood. The villous fragments were dissected into smaller pieces (2-4 mm^3) free of any visible vessels. Villous explants were cultured in 10 cm-diameter culture dishes (Corning, NY, USA) in Dulbecco Modified Eagle's Medium (DMEM):F12 Nutrient Mixture (1:1) (Gibco, Grand Island, NY, USA) supplemented with 10% FCS (Amimed, Allschwil, Switzerland), 1 x antibiotic/antimycotic (Gibco, Grand Island, NY, USA), 25 IU/ml heparin (B. Braun Medical AG, Sempach, Switzerland) and 50 U/ml aprotinin (Fluka, Buchs, Switzerland) for 72 h at 37 °C. Incubation was performed in 20% O_2 / 5% CO_2 (air) for healthy as well as for preeclamptic placentas, or in 3% O_2 / 5%

CO_2 (hypoxia) for normal term placentas. 25 µg/ml of vitamin C (Sigma, Saint Louis, MO, USA) was added to the cultures incubated in 20% O_2 / 5% CO_2, to preserve villous tissue from elevated programmed cell death or necrosis [233].

1.2. Mechanical dissection (*mS*)

The villous tissue from healthy term placentas was separately dissected using the protocol described before in [220]. In brief, small fragments of villous tissue were extensively washed three times in ice cold 100 mM $CaCl_2$ to remove blood cells and stirred in 0.15 M NaCl supplemented with 400 U/ml penicillin/streptomycin (Gibco, Grand Island, NY, USA) overnight at 4 °C.

1.3. Placental dual perfusion (*pS*)

Placental dual perfusions of healthy term placentas were carried out in the laboratory of Prof. Dr. S. Hansson at the Department of Obstetrics and Gynecology, University Hospital Lund, Lund, Sweden.

A suitable cotyledon was set up for separate dual perfusion of the maternal (intervillous space) and the fetal compartment (villous vasculature), without recirculation at flow rates of approximately 12 and 6 ml/min respectively, using a well-described perfusion system [234]. Particulate matter was collected with a 30 minute wash of the maternal side of the placental cotyledon.

In all approaches STBM were isolated from supernatants by a three step centrifugation at 4 °C: 1000 × g for 10 min, 10’000 × g for 10 min, and 60’000 × g for 90 min. The final pellets contained STBM, were washed with PBS and resuspended in PBS containing 5% sucrose. STBM and STBM-free supernatants were stored at -20 °C until use.

1.4. STBM quantification

A surrogate assay was used to quantify STBM. Thereby, the protein content of the different STBM preparations was measured with the Advanced protein assay reagent from Cytoskeleton Inc. (Denver, CO, USA), according to the manufacturer's recommendations. The optical density was read at 595 nm with the Spectramax 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

1.5. Caspases activity assay

Whole protein extracts were obtained by homogenizing the explants in 50 mM Tris pH 7.6, 150 mM NaCl, 1 x protease inhibitor cocktail, 1% Triton X-100 and 0.5mM PMSF, with a Polytron PT 1200 E tissue homogenizer (Kinematica AG, Littau-Lucerne, Switzerland). The protein content was quantified by the Bradford assay. An adaptation of the fluorimetric homogeneous caspases assay (Roche Diagnostics GmbH; Mannheim, Germany) was used to assess activated caspases 2, 3, 6, 7, 8, 9 and 10. In brief, protein extracts were diluted 1/10 in the incubation buffer, 1 volume of the substrate working solution was added and the reaction was incubated at 37 °C for 24 h. The cleavage of the substrate by active caspases released was measured with an excitation filter set at 485 nm, and an emission filter set at 538 nm. The cut off value was set at 530 nm. Relative fluorescence units (RFU) were measured on a Spectramax Gemini spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA), using the Softmax Pro software (Molecular Devices, Sunnyvale, CA, USA). The results are presented as RFU / µg of proteins

1.6. Lactate dehydrogenase (LDH) cytotoxicity assay

The release of LDH into the culture medium was assessed relative to a positive control, made from a villous explant lysed in 1% Triton X-100, using an adaptation of the colorimetric lactate dehydrogenase based toxicity assay from Sigma (Saint Louis, MO, USA). In brief,

culture supernatants were diluted 1/3 in the assay mixture consisting of equal amounts of substrate, enzyme and dye solutions. The reaction was stopped with 1 N HCl and the optical density (OD) was measured at 490 nm in a Spectramax 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Percent necrotic cell death was calculated as (OD of sample / OD of Triton X-100-lysed explant) × 100%.

2. Cell culture

2.1. Cell line: Mono Mac 6

The monocytic cell line Mono Mac 6 (kindly provided by Prof. R. Landmann, Department of Biomedicine, University of Basel, Switzerland) was established in 1985 from the peripheral blood of a 64-year-old man with relapsed acute monocytic leukemia (AML FAB M5) upon myeloid metaplasia [235]. Mono Mac 6 cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA), containing 10% FCS (Amimed, Allschwil, Switzerland), 4 mM glutamine (Gibco, Grand Island, NY, USA), 100 U/ml penicillin/streptomycin (Gibco, Grand Island, NY, USA), 1 x non-essential amino acids (Gibco, Grand Island, NY, USA), 1 mM sodium pyruvate (Gibco, Grand Island, NY, USA) and 9 µg/ml human insulin (Gibco, Grand Island, NY, USA).

2.2. Human blood monocytes

40 ml of venous blood from male donors, collected in EDTA-containing tubes, were diluted in PBS supplemented with 2 mM EDTA and peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation on Histopaque (Sigma, Saint Louis, MO, USA). PBMC were washed twice with PBS / 2 mM EDTA. The remaining erythrocytes were lysed with the red blood cell (RBC) lysis solution (Qiagen, Valencia, CA, USA). Monocytes were isolated by negative selection using the human Monocyte Isolation Kit II and magnetic cell separation (MACS) (Miltenyi Biotec Inc., Auburn, CA, USA), according to the manufacturer's protocol. Purified populations of monocytes generally contained >94% $CD14^+$ cells, as confirmed by flow cytometry, and were resuspended in RPMI-1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% FCS (Amimed, Allschwil, Switzerland), 4 mM glutamine (Gibco, Grand Island, NY, USA) and 100 U/ml penicillin/streptomycin (Gibco, Grand Island, NY, USA).

2.3. Co-culture of monocytes and STBM

Peripheral blood monocytes or Mono Mac 6 cells were cultured at 5×10^5 cells / ml in complete RPMI-1640 medium (Gibco, Grand Island, NY, USA). STBM were added in different concentrations, ranging from 10 µg/ml up to 300 µg/ml, as indicated in the figure legends. STBM preparations were tested individually on at least 2 different monocyte populations to exclude a donor-specific response. As a positive control of the monocyte response, cells were treated with lipopolysaccharide (LPS) from gram-negative bacteria (Sigma, Saint Louis, MO, USA). In some experiments, STBM were pre-treated with 0.4 µg/ml or 1 µg/ml of a mouse anti-human IL-8 blocking antibody (R&D Systems Inc., Minneapolis, MN, USA) or 10 µg/ml of a mouse anti-human CD54 antagonistic antibody (R&D Systems Inc., Minneapolis, MN, USA) for 15 min before co-culture with monocytes. Alternatively, the cells were cultured with 1 ng/ml, 10 ng/ml and 100 ng/ml of recombinant human IL-8 (Sigma, Saint Louis, MO, USA). In some cultures monocytes were pre-incubated for 15 min with 1 µM and 10 µM of cytochalasin B (Sigma, Saint Louis, MO, USA), 10 µM of 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline (6AQ) (Calbiochem, San Diego, CA, USA) and 10 µM of Perillyl alcohol (PA) (Sigma, Saint Louis, MO, USA), and for 24 h with 1 µM of the MyD88 homodimerization peptide (Imgenex, San Diego, CA, USA), followed by the addition of the STBM. Cells and culture supernatant were separately harvested after 4 h or 16 h incubation at 37 °C in air / 5% CO_2.

3. Functional Analysis

3.1. Cell viability (WST-1 assay)

The colorimetric WST-1 assay (Roche Diagnostics GmbH; Mannheim, Germany) was used to assess cell viability and is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. Following co-culture, monocytes were harvested, washed, resuspended in 100 µl complete RPMI-1640 medium and transferred into 96-well plates. 10 µl of WST-1 reagent was added to each well. As a negative control, the same volume of culture medium and WST-1 reagent were used. The plate was incubated for 2 h at 37 °C, in air / 5% CO_2 and analyzed on the Spectramax 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 450 nm, corrected by the reference wavelength of 600 nm.

3.2. Flow cytometry

To block unspecific binding through Fc receptors (FcRs), monocytes were pre-incubated with purified human IgG (Sigma, Saint Louis, MO, USA), diluted to a working concentration of 200 µg/ml in PBS / 2 mM EDTA / 1% FCS for 5 min at 4 °C. FACS stainings were performed for 15 min at 4 °C with ready to use concentrations of FITC-conjugated antibodies against CD14 (BD Pharmingen, San Jose, CA, USA) and TLR2 (eBioscience, San Diego, CA, USA), PE-conjugated antibodies against CD54, CD11b (BD Pharmingen, San Jose, CA, USA), TLR1 and TLR4 (eBioscience, San Diego, CA, USA), and APC-conjugated antibody against CD11a (BD Pharmingen, San Jose, CA, USA). For the detection of TREM-1, the supernatant of the human TREM-1-specific hybridoma 21C7 (kindly provided by Prof. Dr. Christoph Müller, University of Berne, Switzerland) - diluted 1/10 in PBS / 2 mM EDTA / 1% FCS - and, as a second step, PE-conjugated goat anti-mouse Ig (SouthernBiotech, Birmingham, AL, USA) - diluted 1/1000 in PBS / 2 mM EDTA / 1% FCS - were used. After

washing, the stained cells were resuspended in PBS / 2 mM EDTA / 1% FCS and 10'000 events were acquired on a Dako Cyan flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analyzed with the Summit software.

3.3. Enzyme-linked immunosorbent assay (ELISA)

3.3.1. IL-1β, IL-6, IL-8 and CD54 ELISA

IL-1ß, IL-6, IL-8 and CD54 were quantified with commercial DuoSet® ELISA Development Kits (R&D Systems Inc., Minneapolis, MN, USA) following the manufacturer's instructions. Precisely, 96-well maxisorb immunoplates (Nunc, part of Thermo Fisher Scientific, Rochester, NY, USA) were coated with 50 µl/well capture antibody, diluted 1/180 in PBS, sealed and kept at RT overnight. Wells were rinsed once with H_2O and blocked at RT with 250 µl of PBS / 0.5% BSA / 1 mM EDTA. After 1 h, plates were rinsed with H_2O and 50 µl/well of samples and standard, diluted in 1x PBS / 0.5% BSA / 1 mM EDTA, were added. Plates were kept at RT for 2 h and washed 4 times with PBS / 0.05% Tween. 50 µl of the biotin-conjugated detection antibody, diluted in 1x PBS / 1% BSA to the requested working concentration, were pipetted into each well and incubated at RT. After 2 h the plates were washed 4 times with PBS / 0.05% Tween and 50 µl of streptavidin-conjugated horseradish-peroxidase (HRP), diluted 1/200 in PBS / 1% BSA, were added into each well. Plates were incubated for 30 min at RT and washed 4 times with PBS / 0.05% Tween. Into each well 100 µl of TMB substrate solution (Sigma, St. Louis, MO, USA) were added and kept in the dark at RT until colour development. The colour reaction was stopped with 50 µl of 2 N H_2SO_4. Optical density was read on the Spectramax 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 450 nm, adjusted by the wavelength correction of 562 nm, using Softmax Pro software (Molecular Devices, Sunnyvale, CA, USA).

Samples and standard were measured in duplicates and mean values were calculated in each experiment.

3.3.2. IL-10 ELISA

IL-10 production was measured using the human IL-10 ELISA Ready-SET-Go kit from eBioscience (San Diego, CA, USA), according to the manufacturer's protocol. Plates were coated with 100 µl/well capture antibody, diluted 1/250 in coating buffer, sealed and incubated overnight at 4 °C. The next day, plates were washed 4 times with PBS / 0.05% Tween and blocked with 200 µl/well of 1 x assay diluent for 1 h at RT. Wells were washed 4 times with 1x PBS / 0.05% Tween and 100 µl of the samples and the recombinant IL-10 standard, diluted in 1 x assay diluent, were added. After 2 h at RT, plates were washed 5 times with PBS / 0.05% Tween and 100 µl of detection antibody, diluted 1/250 in 1 x assay diluent, were pipetted into each well. Following incubation at RT for 1 h, plates were washed 5 times with PBS / 0.05% Tween and 100 µl/well of avidin-HRP, diluted 1/250, were added and incubated for 30 min at RT. The last washing step was repeated 7 times with PBS / 0.05% Tween. 100 µl/well of TMB substrate solution were added and kept in the dark at RT until colour development. The reaction was stopped with 50 µl 2 N H_2SO_4. Optical density was measured on the Spectramax 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 450 nm, corrected with the reference wavelength of 562 nm.

Samples and standard were measured in duplicates and mean values were calculated in each experiment.

3.3.3. Placental alkaline phosphatase (PLAP) ELISA

96-well maxisorb immunoplates (Nunc, part of Thermo Fisher Scientific, Rochester, NY, USA) were coated overnight at RT with 1 µg/ml of the monoclonal mouse anti-human placental alkaline phosphatase (PLAP) Ab-5 antibody (Lab Vision Corporation, Fremont, CA,

USA). After rinsing with H_2O, plates were blocked for 1 h at RT with 250 µl/well PBS / 0.5% BSA / 1 mM EDTA. Samples and PLAP standard (Sigma, St. Louis, MO, USA) were diluted in PBS / 0.5% BSA / 1 mM EDTA, distributed 50 µl/well and incubated at RT for 3 h. Plates were washed 4 times with PBS / 0.05% Tween and 100 µl/well pre-warmed pNPP substrate solution (Sigma, St. Louis, MO, USA) were added and incubated for 2-3 h at RT till the colour reaction was completed. Optical density was measured on the Spectramax 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 405 nm.

Samples and standard were measured in duplicates and mean values were calculated in each experiment.

3.4. RNA extraction

Using the RNAeasy Mini kit from Qiagen (Valencia, CA, USA), RNA was isolated from monocytes co-cultured for 4 h with 300 µg/ml eS_{20} derived from three different STBM preparations. Cells were washed with ice cold PBS, centrifuged at 400 × g for 5 min at 4 °C and resuspended in 500 µl Trizol reagent (Gibco, Grand Island, NY, USA). Tubes were vortexed vigorously and incubated at RT for 5 min. 100 µl of RF chloroform were added, mixed well, kept at RT for 3 min and centrifuged at 16'000 × g for 15 min at RT. 500 µl of the aqueous phase were transferred into new eppendorf tubes, supplemented with 250 µl of 100% ethanol, mixed well and applied onto columns. After centrifugation at 8'000 × g for 15 seconds at RT, the flow-through was discarded, 350 µl of solution RW1 were pipetted onto the columns and centrifuged again at 8'000 × g for 15 seconds at RT. In turn, the flow-through was discarded, 10 µl of DNAse 1 and 70 µl of the solution RDD were mixed and directly pipetted onto the matrix of the column and incubated for 15 min at RT. 350 µl of solution RW1 were added and centrifuged at 8'000 × g for 15 seconds at RT. The flow-through was discarded and the columns were washed twice with 500 µl of buffer RPE and centrifuged at 8'000 × g for 2 min at RT. Columns were put into new eppendorf tubes and

RNA was eluted from the matrix with 50 µl of RNAse free H_2O by centrifugation at 8'000 × g for 15 seconds at RT.

RNA was quantified using nanodrop (Wilmington, DE, USA).

3.5. GAPDH real-time PCR with mRNA

To control for contamination of mRNA with genomic DNA, real-time PCR for GAPDH mRNA was done, in the presence or absence of the reverse transcription enzyme. For each sample, the reaction consisted of 1 x one-step RT-PCR Master Mix (Applied Biosystems, Foster City, CA, USA) containing 0.2 µM of forward primer (5'-GAAGGTGAAGGTCGGAGT-3') (Microsynth AG, Balgach, CH), 0.2 µM of reverse primer (5'-GAAGATGGTGATGGGATTTC-3') (Microsynth AG, Balgach, CH) and 0.2 µM of 6-carboxyfluorescein-labeled probe (Applied Biosystems, Foster City, CA, USA) for the amplification of the GAPDH mRNA 5' end. All samples were performed in duplicates, with and without the addition of 1 x reverse transcriptase mix, to exclude the presence of GAPDH DNA, and run under the following conditions: 30 min at 48 °C, 10 min at 95 °C and 45 cycles (15 seconds at 95 °C, 1 min at 60 °C).

3.6. Reverse transcription reaction

For the reverse transcription reaction (TRIO-Thermoblock; Biometra, Goettingen, Germany) 167 ng RNA were added to the reaction mix containing 4 µl $MgCl_2$, 2 µl of RT 10 x buffer, 2 µl of 10 mM dNTP mix, 0.5 µl rec RNAsin, 1 µl of 0.5 mg/µl random primers and 0.6 µl of 25 U/µl AMV RT transcriptase (Promega, Madison, WI, USA) and filled up with H_2O to a total volume of 20 µl. The reaction was performed using the following conditions: 10 min at 37 °C, 60 min at 45 °C, 5 min at 95 °C and 15 min at 4 °C. The cDNA products of all eS_{20} treated and untreated monocytes were pooled separately.

3.7. Human NF-κB Signalling Pathway RT^2 Profiler PCR Array

The real-time PCR based array from SABiosciences (Frederick, MD, USA) profiles expression of 84 key genes involved in the NF-κB signalling transduction. Two 96-well real-time PCR plates were delivered from SABiosciences, pre-coated with the forward and reverse primers of the respective genes. The pipetting, described next, was performed at 4 °C. For one 96-well plate 102 µl of cDNA were mixed with 1275 µl of 2x SuperArray RT^2 qPCR master mix and filled up with H_2O to the final volume of 2550 µl. 25 µl of the experimental cocktail were pipetted into each well of the PCR array. Plates were sealed with the optical thin-wall 8-cap strips, shortly spun down to remove bubbles, and run at the following PCR cycling program for ABI 7000 (Applied Biosystems Inc., Forster City, USA): 10 min at 95 °C, 40 cycles (15 seconds at 95 °C, 1 min at 60 °C).

For the quantification test mRNA transcripts (Ct) were normalized to the reference gene actin. Results were presented as the transcript fold change of the respective gene normalized to actin (ΔCt) and relative to untreated controls (ΔΔCt), using the $2^{-\Delta\Delta Ct}$ method.

3.8. Fluorescence microscopy

STBM were stained with 5 µM of the red fluorescent membrane dye PKH26 (Sigma, St. Louis, MO, USA) and incubated for 5 min at RT. The staining was stopped with 2 ml of heat inactivated FCS and kept at RT for 2 min. 4 ml of PBS were added and centrifuged for 30 min at 60'000 × g. The PKH-26 stained STBM were washed twice in PBS and centrifuged for 1 h at 60'000 × g. The STBM-pellet was resuspended in PBS supplemented with 5% sucrose and stored at -20 °C until use.

Monocytes were isolated from peripheral blood as described above and resuspended in PBS. 1 × 10^6 cells were labelled with 1 µM of the green fluorescent cytoplasmic dye carboxyfluorescein succinimidyl ester (CFSE) (kindly provided by Prof. Dr. G. Spagnoli, Department of Biomedicine, University of Basel, Switzerland) for 10 min in the dark at 37

°C. The staining reaction was stopped with 2 ml of RPMI medium containing 10% FCS for 5 min at RT and centrifuged for 5 min at 400 × g. The cell pellet was washed 3 times in PBS and resuspended in complete RPMI medium to a concentration of 5×10^5 cells/ml.

PKH26-stained STBM were co-cultured with CFSE-labelled monocytes for 16 h as described above. Cells were harvested and washed once in PBS. 100'000 cells were transferred on one microscope slide using cytospin centrifugation (Shandon cytospin 3, Histocom AG, Zug, CH) at 400 rpm for 5 min. Slides were dried at RT in the dark and cells were fixed with 4% formaldehyde for 30 min. DNA was stained with DAPI and slides were immediately covered with coverslides. Analysis was done using a Zeiss Axioplan 2 imaging fluorescent microscope (Carl Zeiss AG, Feldbach, CH).

3.9. Statistical analysis

To calculate the statistical significance of differences between experimental groups, the Mann-Whitney test was performed using the statistical analysis software SPSS (Statistical Package for the Social Sciences; Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

1. STBM preparations

STBM were prepared by four different approaches, which are believed to mimic physiological and patho-physiological conditions [204]. STBM generated by *in vitro* cultures of explants from villous tissue incubated at 20% O_2 (eS_{20}), or collected from the maternal side of *ex vivo* dually perfused placental lobe (*pS*) are believed to represent the most physiological conditions with apoptotic shedding as part of normal placental turnover. Generation of STBM by mechanical dissection of villous tissue (*mS*) is, in essence, a necrotic process of release, whereas STBM released from villous tissues cultured at 3% O_2 (eS_3) could reflect the hypoxic conditions seen in preeclampsia. To assess the degree of apoptosis and necrosis in placental explant cultures incubated in 20% or 3% oxygen, tissue caspases activity and the release of LDH were measured (Figure 8). Villous tissue explants cultured at 3% O_2 showed lower caspases activity but higher LDH release relative to the explants incubated at 20% O_2 (Figure 8a and b), strongly suggesting higher rate of apoptotic tissue turnover in 20% O_2, and conversely, increased necrosis in tissue cultured under hypoxia.

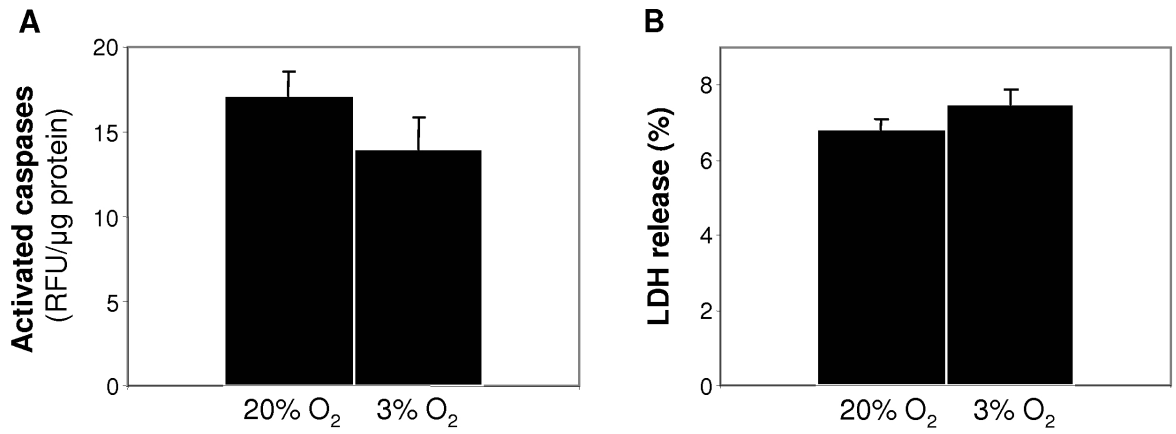

Figure 8. Expression of activated caspases and release of LDH by villous tissue explants.
Villous explants were cultured for 72 h at 37 °C in either 20% O_2 or 3% O_2. The same placentas were used for cultures under both oxygen conditions. (A) The levels of active caspases in protein extracts of villous explants are expressed as relative fluorescence units (RFU) / µg of tissue protein. (B) The percentage of LDH released in the culture medium is indicated relative to the LDH activity from an explant that was lysed in detergent. Bars are presented as mean ± SEM of 3 explant cultures. Statistics were not performed as the experiment included only 3 explant cultures.

1.1. All microparticle preparations contain the syncytiotrophoblast specific placental alkaline phosphatase (PLAP)

The placental origin of the *in vitro* prepared STBM was confirmed by quantification of their content of placental alkaline phosphatase (PLAP) by ELISA (Table 1). All STBM populations tested contained PLAP. However, the levels of *mS*-associated PLAP were up to 4 times higher than the ones enclosed in eS_{20} and eS_3 (Table 1). As previously shown by my colleagues, the STBM prepared by dual perfusion of a cotyledon contained similar concentrations of PLAP as the STBM generated by villous tissue cultures incubated in 20% O_2 [220].

Table 1. Content of PLAP in the STBM populations was measured by ELISA. All results are shown by mean ± SEM of STBM generated from 3-4 different placentas for each condition.

	PLAP (µg/mg STBM)
eS_{20}	38.4 ± 10.4
eS_3	40.5 ± 14.7
mS	155.6 ± 55.6

1.2. STBM associated cytokines

The placental syncytiotrophoblast produces many proinflammatory cytokines, which might be associated with the microvesicles when the latter shed off from the syncytiotrophoblast membrane. In particular, it has been previously shown by my colleagues that STBM prepared from villous explant cultures incubated in air, contained detectable amounts of IL-8 [228]. As cytokines influence many aspects of monocyte activation, we analysed the different STBM preparations for the presence of proinflammatory factors.

None of the STBM preparations contained detectable levels of IL-1ß or TNF-α (data not shown). IL-6 was either undetectable or present at very low concentrations (Table 2). IL-8 was similarly absent or low in *pS*, eS_3 and *mS*. However, high levels of IL-8 were reproducibly associated with preparations of eS_{20} (Table 2).

Table 2. Presence of STBM-associated cytokines was measured by ELISA. All results are shown as mean ± SEM of STBM generated from 3-5 different placentas for each condition

	IL-8 (ng/mg STBM)	IL-6 (ng/mg STBM)
eS_{20}	44.0 ± 3.2	0.13 ± 0.08
pS	0.0	0.0
eS_3	0.7 ± 0.3	0.07 ± 0.06
mS	0.07 ± 0.03	0.0

1.3. Conclusion

Placental explant cultures incubated in 20% O_2 exhibited an increased apoptotic cell turnover, whereas necrosis was higher in placental villous tissues cultured at hypoxic conditions.

All STBM preparations contained the syncytiotrophoblast-specific PLAP and high levels of the chemokine IL-8 were associated with eS_{20}.

2. STBM prepared from normal term placentas do not activate the monocytic cell line Mono Mac 6

To investigate the effects of STBM on human monocytes the monoblastic cell line Mono Mac 6, which is derived from the peripheral blood of a man with relapsed acute monocytic leukemia upon myeloid metaplasia, was chosen [235].

Faas and co-workers previously used Mono Mac 6 to evaluate the monocytic cell surface expression of the adhesion molecule CD54 and the synthesis of reactive oxygen species following treatment with plasma from normal pregnant and preeclamptic women [236].

2.2. STBM only marginally alter the expression and median fluorescent intensity of cell surface markers on Mono Mac 6 cells

STBM did not induce apoptosis in Mono Mac 6 cells, as assessed by visual inspectation and the distribution of the cells in the forward scatter (FSC) / side scatter (SSC) dot plots of flow cytometric analysis (data not shown).

CD14, CD54 (intercellular adhesion molecule 1/ICAM-1) and the MHC class II molecule HLA-DR have been shown to be differently expressed by peripheral blood monocytes in normal pregnant and preeclamptic women compared to non-pregnant controls [25, 91, 92].

10% of Mono Mac 6 cells displayed CD14 and its median fluorescent intensity (MFI) was low (Figure 9a). Both, the number of cells expressing CD14 as well as its MFI were increased upon treatment with LPS for 16h. However, none of the three STBM preparations (eS_{20}, eS_3 and *mS*) altered the frequency of expressing cells and MFI of CD14 (Figure 9a).

Mono Mac 6 cells were highly positive for CD54, which was further enhanced following stimulation with LPS (Figure 9b). While eS_{20} and eS_3 did not trigger any change, *mS* decreased the frequency of CD54 expressing cells in a dose-dependent manner (Figure 9b and d). Furthermore, MFI of CD54 was 3.5 fold increased after LPS treatment and reduced upon

incubation with *mS* (17.9 ± 1.9), compared to basal MFI (25.9 ± 2.1) (Figure 9b and d). The percentage of HLA-DR expressing cells remained equal in untreated cells and cells incubated with LPS or STBM (Figure 9c). However, MFI of HLA-DR was dose-dependently declined by *mS* (Figure 9c and e).

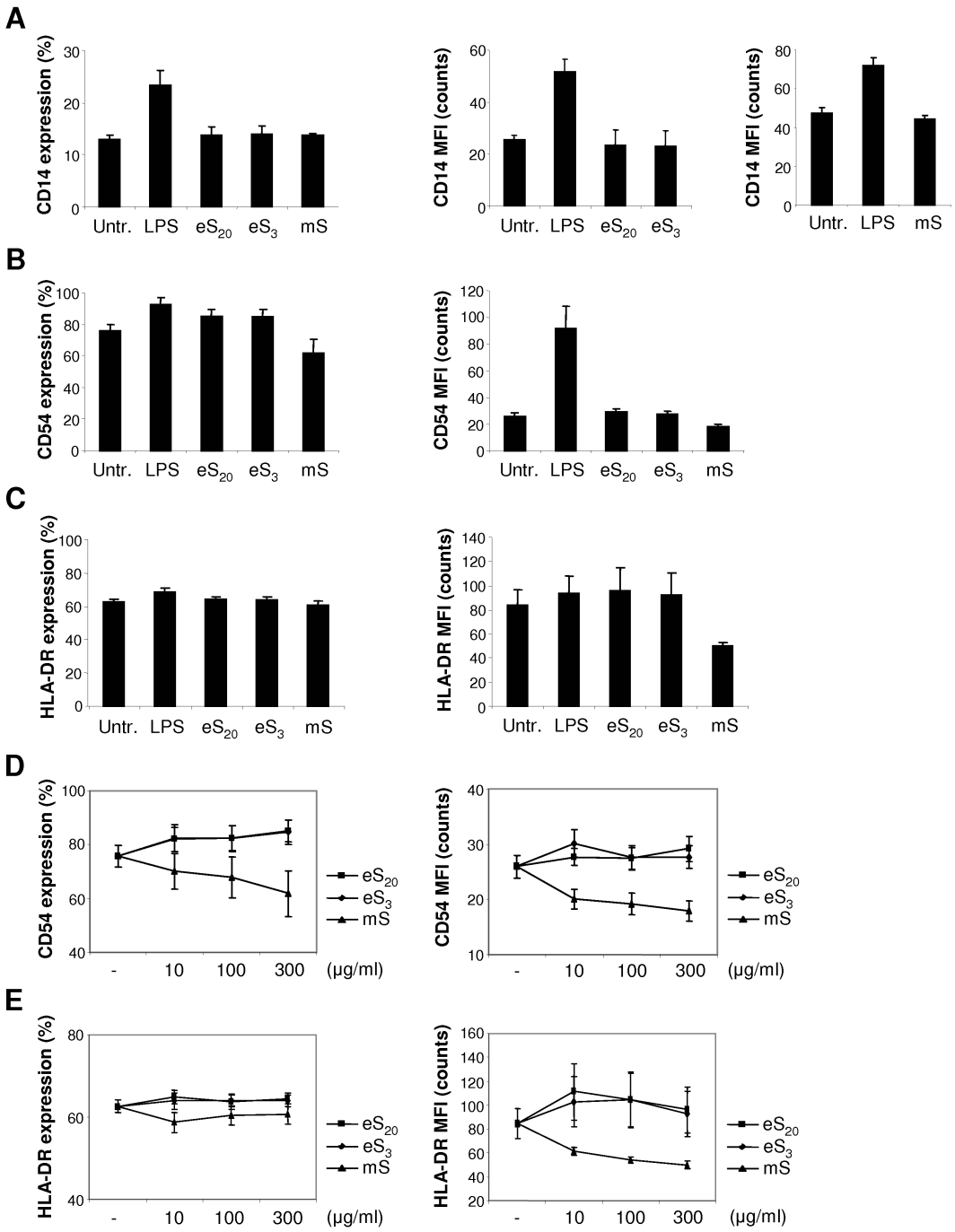

Figure 9. Expression of CD14, CD54 and HLA-DR on Mono Mac 6 cells co-incubated with STBM prepared from normal term placentas. Percentage of expressing cells and median fluorescent intensity (MFI) of CD14 (A), CD54 (B) and HLA-DR (C) of Mono Mac 6 cells co-cultured with 300 µg/ml STBM or 1 µg/ml LPS for 16 h. (D, E) Percentage of expressing cells and MFI of CD54 and HLA-DR on Mono Mac 6 cells following treatment with three different concentrations of STBM. Data are presented as mean ± SEM of 2 independent co-culture experiments with STBM prepared from 3 different placentas for each condition.

2.3. STBM do not trigger the secretion of pro-inflammatory cytokines in Mono Mac 6 cells

As another approach to evaluate the activation status of Mono Mac 6 cells upon STBM treatment, secretion of the proinflammatory cytokines interleukin (IL)-6 and IL-1ß were measured (Table 3).

Mono Mac 6 cells produced trace amounts of IL-6 and IL-1β in the absence of any trigger (Table 3). Stimulation with LPS induced a 76.6 fold and a 7.9 fold increase in IL-6 (826.8 ± 78.1pg/ml) and IL-1β (35.6 ± 18.1pg/ml) secretion, respectively (Table 3). However, there was no induction of IL-6 and IL- 1β production after co-culture with any of the three STBM population (Table 3).

Table 3. Production of IL-6, IL-1β and IL-10 by Mono Mac 6 cells cultured for 16 h in the absence of any treatment and following stimulation with 1 µg/ml LPS or co-incubation with STBM. All results are shown as mean ± SEM of 2 co-culture experiments with STBM generated from 3-4 different placentas for each condition.

	IL-6 (pg/ml)	IL-1β (pg/ml)	IL-10 (pg/ml)
Untreated	10.8 ± 9.7	4.5 ± 7.8	4.7 ± 0.9
1 µg/ml LPS	826.8 ± 78.1	35.6 ± 18.1	66.6 ± 23.4
10 µg/ml eS_{20}	25.0 ± 18.0	3.8 ± 0.8	14.6 ± 9.2
100 µg/ml eS_{20}	21.0 ± 14.8	3.0 ± 0.8	2.7 ± 2.7
300 µg/ml eS_{20}	29.0 ± 11.4	2.3 ± 0.8	3.1 ± 3.1
10 µg/ml eS_3	15.0 ±11.0	0.0	1.6 ± 1.6
100 µg/ml eS_3	18.0 ± 14.7	0.0	2.9 ± 2.9
300 µg/ml eS_3	26.0 ± 16.6	0.0	10.2 ± 4.4
10 µg/ml mS	0.8 ± 0.7	0.0	6.3 ± 3.4
100 µg/ml mS	0.8 ± 0.7	0.0	6.1 ± 3.6
300 µg/ml mS	0.8 ± 0.7	0.0	5.8 ± 2.9

2.4. STBM do not induce an anti-inflammatory response in Mono Mac 6 cells

Analysis of activation markers, including cell surface molecules and proinflammatory mediators, showed that none of the STBM preparations stimulated an inflammatory response in Mono Mac 6 cells. To investigate a potential STBM-mediated anti-inflammatory effect on Mono Mac 6 cells, the secretion of IL-10 was assessed (Table 3). Untreated Mono Mac 6 cells produced trace amounts of IL-10 (4.7 ± 0.9pg/ml), which was enhanced by LPS (66.6 ± 23.4pg/ml), but not altered by any of the STBM preparations (Table 3).

2.5. Conclusion

Although Mono Mac 6 responded to LPS, STBM did not trigger an inflammatory response in the cell line. Co-cultures of STBM and Mono Mac 6 might not be the appropriate model to study the effects of placental membrane fragments on monocytes.

Thus, next the inflammatory nature of STBM was analyzed on peripheral blood monocytes.

3. STBM prepared from normal term placentas activate human peripheral blood monocytes

3.1. STBM do not affect the viability of human monocytes

The human peripheral blood monocytes only represent 5-10% of blood leukocytes and to avoid activation of the cells during isolation, monocytes were enriched by a negative selection method using the magnetic cell separation approach [232]. This resulted in purified populations of monocytes, which generally contained >94% $CD14^+$ cells, as confirmed by flow cytometry (data not shown). These cells were incubated with the same STBM preparations used in the experiments with Mono Mac 6 cells, namely eS_{20}, eS_3 and *mS*, as well as with STBM, which were collected from the maternal side of *ex vivo* dually perfused placental lobe (*pS*). Next to eS_{20}, *pS* are believed to represent the physiologic situation found in human pregnancy. None of the STBM preparations substantially altered monocytic viability as observed in the flow cytometer analysis (Figure 10a). Furthermore, cell viability was also routinely quantified by a colorimetric assay, which measures mitochondrial dehydrogenase activity, and was always >80% relative to the untreated monocytes that were cultured in parallel to the STBM-treated cells (data not shown).

3.2. STBM differently alter the expression of cell surface markers on human primary monocytes

In order to investigate the effect of STBM on the phenotypic activation status of monocytes, the cell surface expression of CD14, CD54 and CD11a (alpha L integrin) was measured (Figure 10). Like CD14 and CD54, CD11a is differently expressed on monocytes of pregnant and preeclamptic women compared to non-pregnant controls [92].

Neither the well known monocyte activator LPS nor the different STBM populations altered CD14 expression relative to the untreated cells following a 16 hour co-incubation (Figure 10b

and c). In contrast, stimulation of monocytes with LPS led to an increased median fluorescent intensity (MFI) of CD54 and a decrease in the expression of CD11a (Figure 10b and c). Incubation with eS_{20} and *pS* also enhanced monocytic expression of CD54, whereas eS_3 and *mS* led to decreased CD54 MFI (Figure 10b and c). Cell surface expression of CD11a was reduced, albeit to differing extents, by all microparticle populations except eS_{20} (Figure 10b and c).

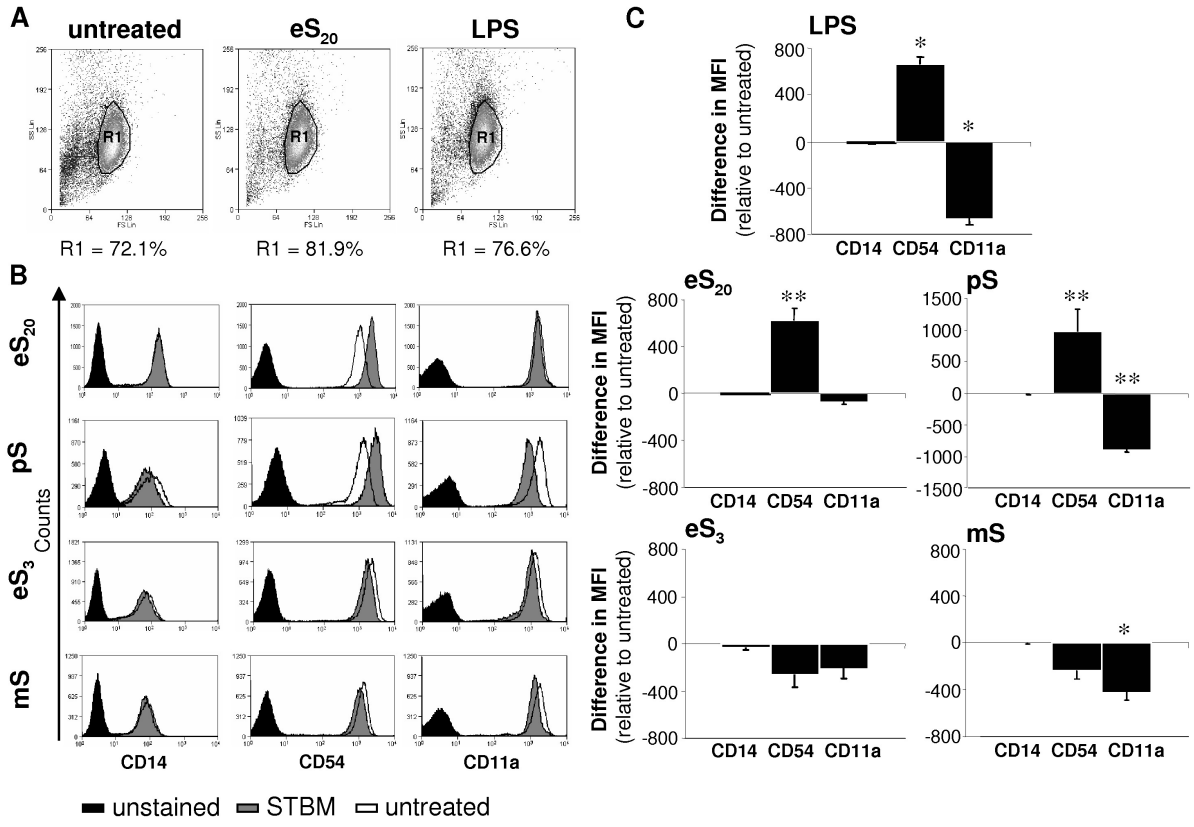

Figure 10. Expression of CD14, CD54 and CD11a on monocytes co-incubated with different STBM populations.

(A) Representative forward scatter (FSC) / side scatter (SSC) dot plots of monocytes, left untreated, incubated with 300 µg/ml STBM or 1 µg/ml LPS for 16 h. (B) Representative histograms of CD14, CD54 and CD11a on R1 gated cells co-cultured with indicated STBM preparations. (C) Differences in median fluorescent intensity (MFI) of CD14, CD54 and CD11a triggered by LPS and the different STBM populations. Bars represent mean ± SEM of 2 independent monocyte co-culture experiments with STBM generated from 3-5 different placentas for each condition (* P value < 0.05; ** P value < 0.01).

3.3. *eS$_{20}$* and *pS* induce the secretion of proinflammatory cytokines by human monocytes

Since the changes in cell surface expression of adhesion molecules upon incubation with STBM may indicate monocytic activation, we next investigated whether STBM could stimulate the secretion of the chemokine IL-8 and the proinflammatory cytokines IL-1ß and IL-6 (Figure 11).

Monocytes cultured for 16 h in the absence of treatment produced basal levels of IL-8, but did not secrete detectable levels of proinflammatory cytokines (Figure 11a). As expected, stimulation with LPS enhanced IL-8 secretion and induced the production of IL-1β and IL-6 compared to the untreated cells. Incubation with *eS$_{20}$* led to a comparable increase in IL-8 release, while at the same dose of STBM protein, *pS* triggered a 3-fold higher secretion of IL-8 than *eS$_{20}$* (Figure 11a upper panel). Both, *eS$_{20}$* and *pS* also stimulated IL-6 and IL-1ß production, although to varying degrees. Whereas the cellular response to *eS$_{20}$* remained modest and was always lower than that induced by LPS, the stimulatory effect of *pS* on cytokine expression was extreme. IL-6 and IL-1β secretion were, respectively, 3.5-fold and 2.6-fold higher upon incubation with *pS* than following stimulation with LPS (Figure 11a, medium and lower panels). In contrast, *eS$_3$* and *mS* did not induce monocytic release of IL-6, IL-1ß or IL-8.

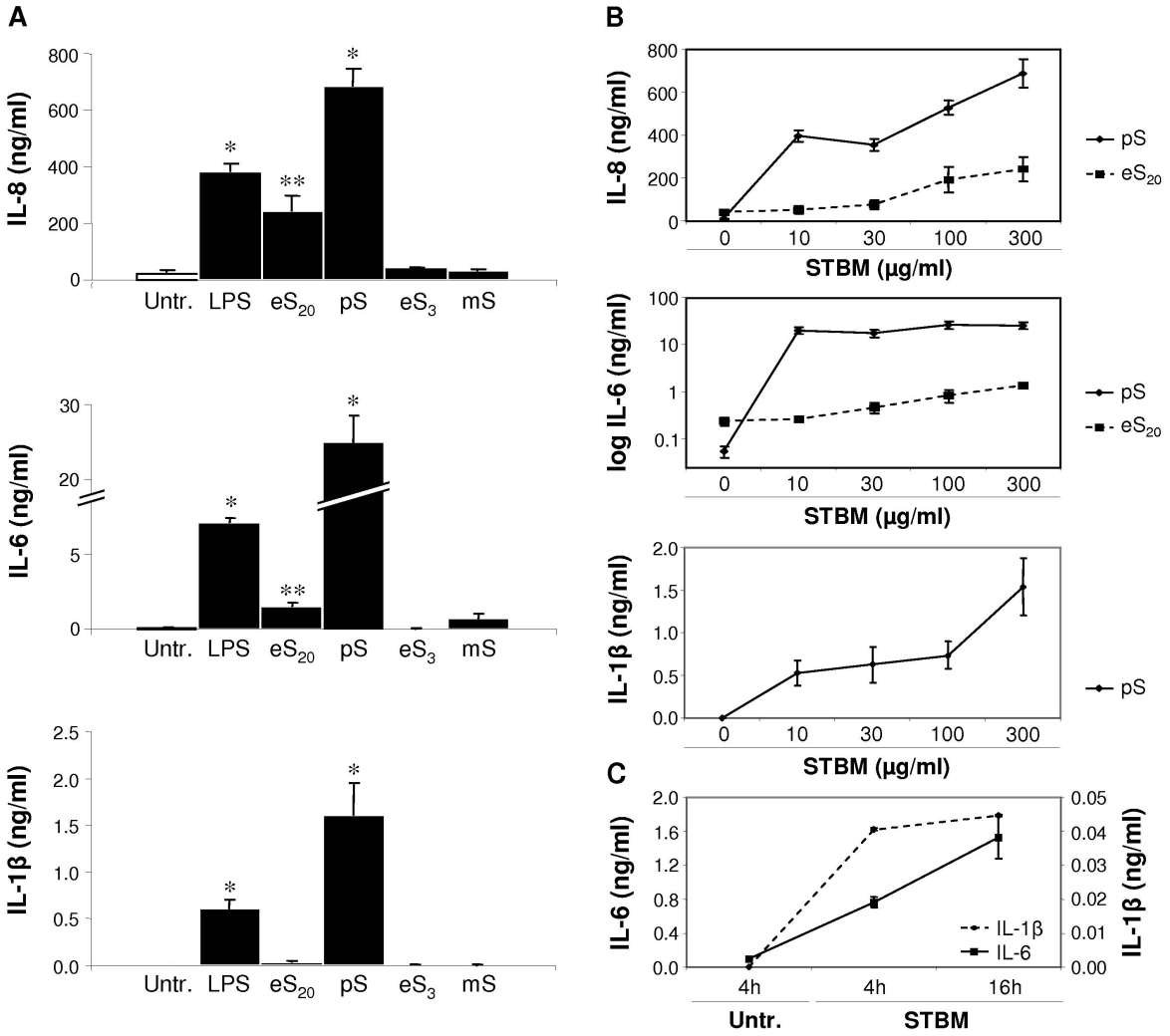

Figure 11. STBM-mediated secretion of cytokines by monocytes. (A) Quantification of IL-8, IL-6 and IL-1ß secreted by monocytes by ELISA. Cells were left untreated, co-incubated for 16 h with 300 µg/ml STBM or 100 pg/ml LPS. (B) Dose-dependent release of IL-8 and IL-6 by *pS* and *eS*$_{20}$. (C) Time-dependent secretion of IL-6 and IL-1ß by monocytes upon treatment with *eS*$_{20}$. Data are presented as mean ± SEM of 2 monocyte co-culture experiments with STBM generated from 3-5 different placentas for each condition (* P value < 0.05; ** P value < 0.01). *eS*$_{20}$-associated IL-8 was deduced from total levels of IL-8 measured in the co-cultures (in order to evaluate monocytic contribution).

3.4. STBM-induced cytokine secretion is dose- and time-dependent

The secretion of proinflammatory factors stimulated by *eS*$_{20}$ and *pS* was dependent on the concentration of microparticles, which was evaluated by their protein content. Increasing amounts of *eS*$_{20}$ and *pS* mediated a steady rise in IL-8 production (Figure 12b, upper panel). IL-6 secretion in response to *eS*$_{20}$ was also dose-dependent (Figure 12b, medium panel). In

contrast, the levels of IL-6 already reached a plateau at the lowest dose of *pS* and did not increase further. Moreover, *pS* induced a higher cytokine response than eS_{20} at all doses of STBM tested. pS also stimulated IL-1β secretion in a dose-dependent manner (Figure 12b, lower panel).

Cytokine production in response to STBM was also time-dependent. While IL-6 secretion increased steadily from 4 h to 16 h following stimulation, IL-1ß release reached maximum levels already at 4 h and was sustained with longer incubation (Figure 12c).

3.5. IL-8 is not responsible for eS_{20} mediated cytokine secretion

As shown in Table 2, eS_{20} contain detectable levels of IL-8. Although IL-8 has never been described as a stimulus for monocytes, we confirmed that it was not responsible for the eS_{20}-mediated activation of the cells. First, monocytes, which were stimulated with three different concentrations of human recombinant IL-8 for 16 h did not produce IL-6 (data not shown). Furthermore, the IL-6 secretion in response to eS_{20} was not affected by the presence of an anti-human IL-8 blocking antibody (Figure 13).

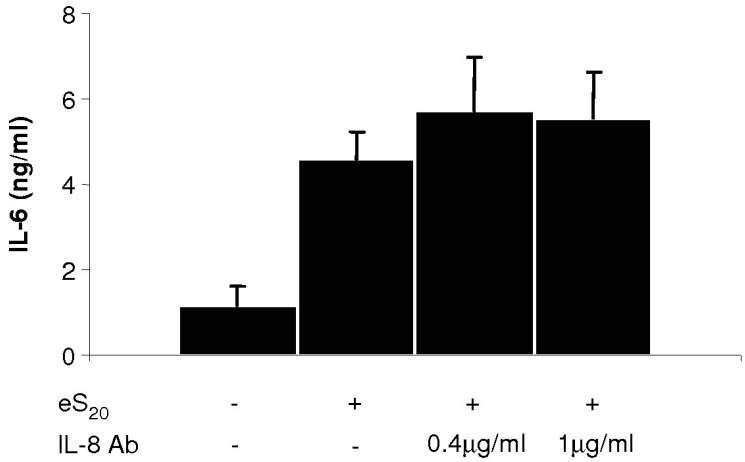

Figure 13. STBM-induced IL-6 secretion in presence of IL-8 blocking antibody. 300 µg/ml eS_{20} were pre-treated with mouse anti-human IL-8 antibody with the indicated concentrations, before co-culture with monocytes for 16 h. All data are presented as mean ± SEM of 2 monocyte co-culture experiments with eS_{20} prepared from 3 different placentas.

3.6. Conclusion

pS and eS_{20} activated peripheral blood monocytes in a dose-dependent manner and it was not due to STBM-associated IL-8.

4. STBM prepared from preeclamptic placentas equally activate human peripheral blood monocytes as STBM from healthy placentas

4.1. eS_{20} prepared from term preeclamptic placentas modify the expression of cell surface molecules

So far, all co-culture experiments were done using STBM from healthy term placentas. The next step was to evaluate the effects of STBM derived from preeclamptic placentas on monocytes and to compare them with the ones of STBM form healthy term placentas. Thus, the eS_{20} preparation approach was used to generate eS_{20} from preeclamptic placentas. To investigate if eS_{20} generated from preeclamptic placentas induce changes in the expression profile of cell surface markers as well, the expression of CD14, CD54 and CD11a on primary human monocytes treated with eS_{20} from preeclamptic placentas was compared with that on untreated monocytes. Monocytic CD14 expression remained unchanged. However, MFI of CD54 was highly increased (641.2 ± 122.5) and MFI of CD11a decreased (-583.4 ± 77.2) on monocytes co-cultured with eS_{20} from preeclamptic placentas (Figure 13b and c). The changes in cell surface expression were similar to these induced by eS_{20} from normal term placentas (see Figure 10).

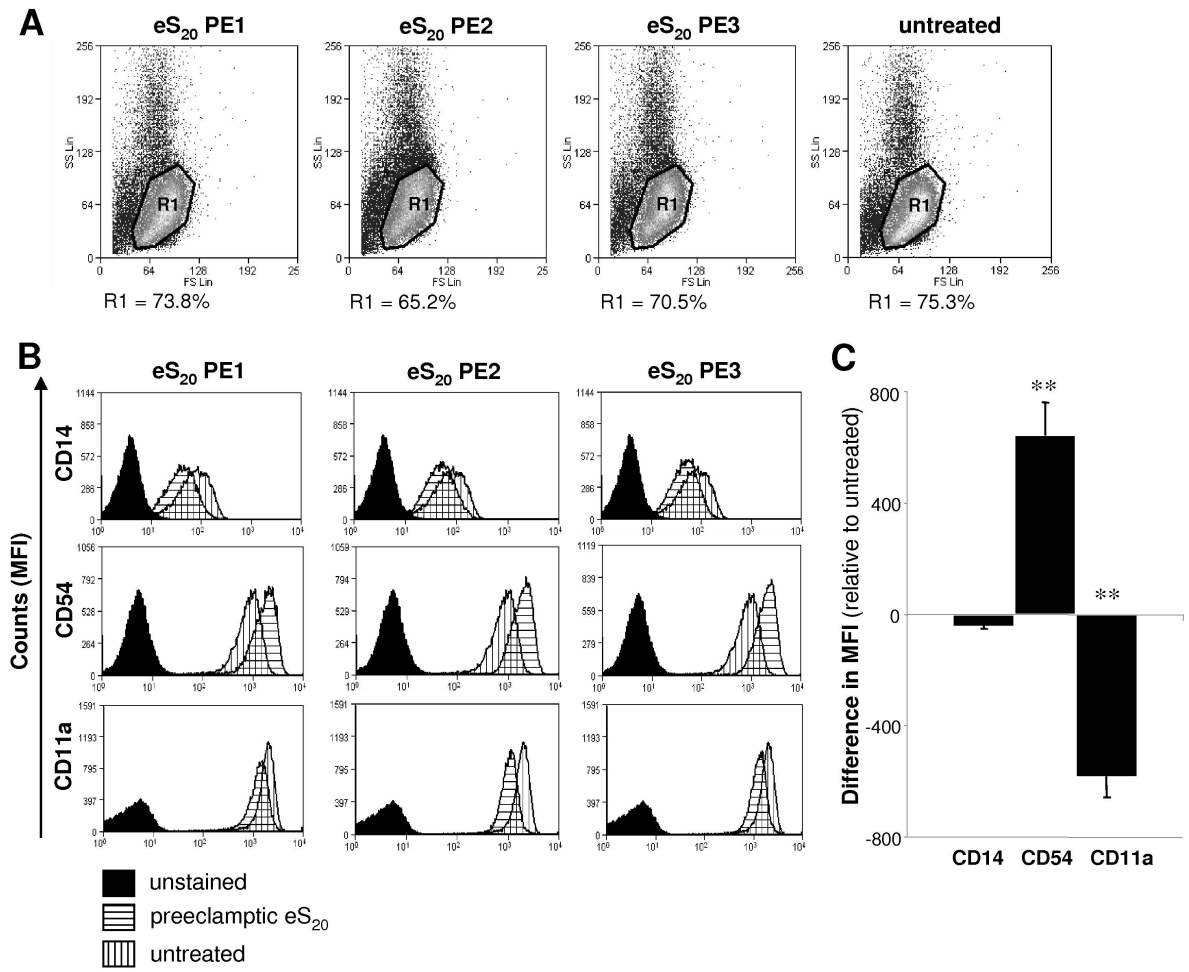

Figure 13. Expression of CD14, CD54 and CD11a on monocytes co-incubated with *eS*$_{20}$ prepared from preeclamptic placentas. (A) Representative forward scatter / side scatter dot plots of monocytes treated with 300 µg/ml of three different preparations of *eS*$_{20}$ isolated from preeclamptic placentas (*eS*$_{20}$ PE1, *eS*$_{20}$ PE2, *eS*$_{20}$ PE3) and untreated monocytes. (B) Representative histograms of CD14, CD54 and CD11a on R1 gated cells co-cultured with the three STBM preparations. (C) Differences in median fluorescent intensity (MFI) of CD14, CD54 and CD11a triggered by *eS*$_{20}$ generated from 3 different preeclamptic placentas. Bars represent mean ± SEM of 2 independent monocyte co-culture experiments with *eS*$_{20}$ prepared from 3 different preeclamptic placentas (* P value < 0.05; ** P value < 0.01).

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4.2. *eS20* generated from normal and preeclamptic placentas similarly trigger the secretion of proinflammatory cytokines

Knowing that *eS20* from healthy term placentas induced a dose-dependent production of proinflammatory mediators by primary monocytes, the effect of *eS20* generated from preeclamptic placentas on the monocytic secretion of IL-6 and IL-8 was analysed (Figure 14). *eS20* derived from preeclamptic placentas stimulated IL-6 and IL-8 secretion by monocytes in a dose-dependent manner and to a similar extent as *eS20* isolated from normal placentas (Figure 14a and b).

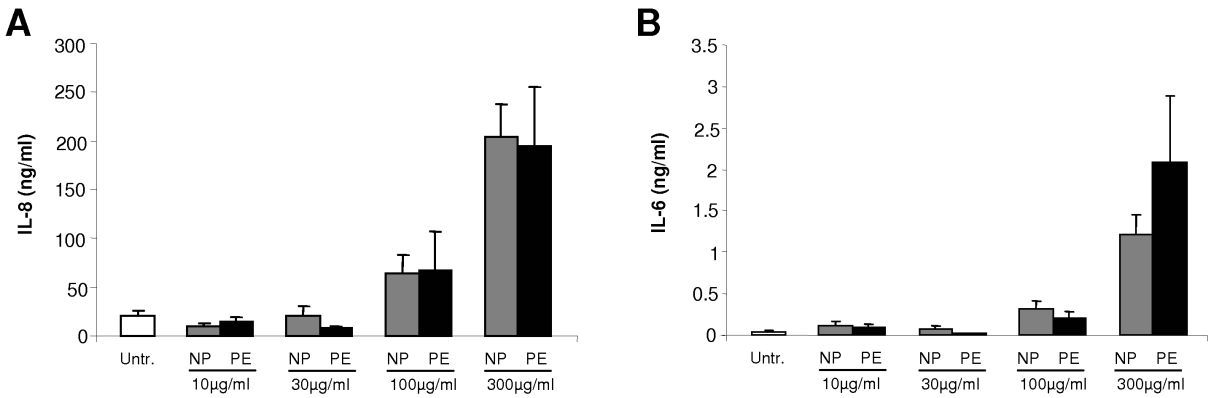

Figure 14. *eS20* prepared from normal and preeclamptic placentas induce dose-dependent release of IL-6 and IL-8 in a similar range. Measurement of IL-6 (A) and IL-8 (B) secretion by monocytes by ELISA. Cells were left untreated or incubated with 10, 30 ,100 and 300µg/ml *eS20* derived from normal (NP) or preeclamptic (PE) placentas for 16 h. All results are defined as mean ± SEM of 2 different monocyte co-culture experiments with *eS20* generated from 3 preeclamptic placentas and form 5 healthy placentas.

4.3. Conclusion

eS20 from preeclamptic placentas showed a similar proinflammatory action on monocyte activation as *eS20* from normal term placentas.

5. STBM induce secretion of proinflammatory mediators by primary human monocytes in a NF-κB-dependent manner

So far, this work showed that *pS* isolated from normal term placentas and eS_{20} prepared from normal as well as from preeclamptic placentas triggered monocytic cell surface expression of CD54 and secretion of proinflammatory mediators by primary human monocytes. Gene expression of CD54, IL-1β, IL-6 and IL-8 is known to be induced by the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [237].

Hence, the potential role of NF-κB in the activated phenotype of STBM-treated monocytes was analysed.

Since there was a higher abundance of normal term placentas than preeclamptic ones, the following experiments were performed using eS_{20} prepared from normal term placentas only.

5.1. The transcription of proinflammatory mediators is induced by eS_{20}

Treatment of primary monocytes with eS_{20} for 4 h induced the transcription of multiple NF-κB responsive genes relative to their expression in untreated cells (Figure 15a).

IL-6 transcription was highly induced in monocytes co-cultured with eS_{20}, demonstrated by a $2^{-\Delta\Delta Ct}$ value of 120.26 relative to untreated controls (Figure 15a). Genes with a transcript fold change between 10 and 21 were the chemokine CCL2 (chemokine (C-C motif) ligand 2) ($2^{-\Delta\Delta Ct}$ = 18.77), which is also known as monocyte chemotactic protein (MCP)-1, the cytokines colony-stimulating factor (CSF)-2 ($2^{-\Delta\Delta Ct}$ = 17.03) and CSF-3 ($2^{-\Delta\Delta Ct}$ = 20.97), TNF ($2^{-\Delta\Delta Ct}$ = 11.16) and IL-1α ($2^{-\Delta\Delta Ct}$ = 15.24) and the transcription factor EGR1 (early growth response 1) ($2^{-\Delta\Delta Ct}$ = 16.34). Furthermore, a fold increase of gene transcription ranging between 2 and 10 was measured for the cytokines IL-1β ($2^{-\Delta\Delta Ct}$ = 6.02), lymphotoxin (LT)-α ($2^{-\Delta\Delta Ct}$ = 3.53), the chemokine IL-8 ($2^{-\Delta\Delta Ct}$ = 3.78), the IL-1 receptor type 1 (IL-1R1) ($2^{-\Delta\Delta Ct}$ = 2.35), and Jun ($2^{-\Delta\Delta Ct}$ = 3.78), which forms, either as homodimer or heterodimer, the functional transcription

factor AP-1 (activator protein-1). However, in addition to genes encoding for proinflammatory mediators, monocytic gene transcription of the anti-inflammatory cytokine IL-10 ($2^{-\Delta\Delta Ct} = 5.82$) was increased following co-culture with eS_{20} (Figure 15a).

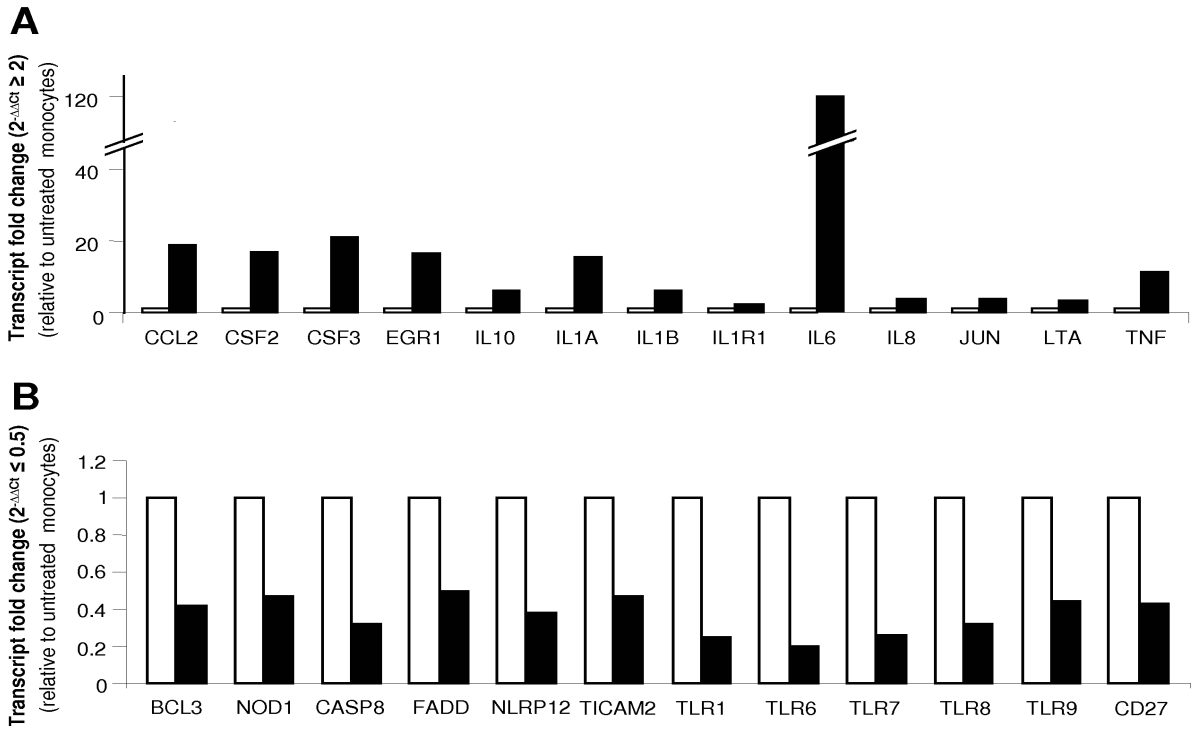

Figure 15. Differential expression of genes involved in NF-κB signalling following treatment with eS_{20}. Monocytes were co-cultured for 4 h with 300 µg/ml of three different eS_{20} preparations and the pooled cDNA was analysed for the transcription of several genes. (A) eS_{20} induced a transcript fold change ($2^{-\Delta\Delta Ct}$) $\geq$ 2 in the following genes: chemokine (C-C motif) ligand 2 (CCL2), Colony-stimulating factor 2 (CSF2), CSF3, Epidermal growth factor 1 (EGR1), interleukin (IL)-10, IL-1α (IL1A), IL-1β (IL1B), IL-6, IL-8, JUN, lymphotoxin-α (LTA) and tumour necrosis factor (TNF). (B) eS_{20} mediated a transcript fold change $\leq$ 2 in the following genes: B-cell CCL/lymphoma 3 (BCL3), nucleotide-binding oligomerization domain containing (NOD)-1, caspase 8 (CASP8), Fas-Associated protein with Death Domain (FADD), NLR family, pyrin domain containing 12 (NLRP12), TICAM2, Toll-like receptor (TLR)-1, -6, -7, -8, -9 and CD27.

5.2. *eS20* decrease the gene expression of pro-apoptotic mediators, pattern recognition receptors and their adaptor molecules

Beside induction, *eS20* mediated down-regulation of several NF-κB responsive genes, which were defined by a $2^{-\Delta\Delta Ct}$ value ≤ 0.5 (Figure 15b).

On the one hand, the gene expression of the pro-apoptotic molecules caspase-8 ($2^{-\Delta\Delta Ct} = 0.32$), CD27 ($2^{-\Delta\Delta Ct} = 0.43$), a member of the TNFR superfamily, and FADD (Fas-Associated protein with Death Domain) ($2^{-\Delta\Delta Ct} = 0.5$) was decreased (Figure 15b). On the other hand, *eS20* reduced the transcription of the pattern recognition receptors toll-like receptor (TLR)1 ($2^{-\Delta\Delta Ct} = 0.25$), TLR6 ($2^{-\Delta\Delta Ct} = 0.2$), TLR7 ($2^{-\Delta\Delta Ct} = 0.26$), TLR8 ($2^{-\Delta\Delta Ct} = 0.32$), TLR9 ($2^{-\Delta\Delta Ct} = 0.44$) and nucleotide-binding oligomerization domain containing (NOD)-1 ($2^{-\Delta\Delta Ct} = 0.47$), the intracellular TLR4 adaptor molecule TICAM2 ($2^{-\Delta\Delta Ct} = 0.47$) and NLRP12 (NLR family, pyrin domain containing 12) ($2^{-\Delta\Delta Ct} = 0.38$), which encodes the NOD-like receptor monarch-1. Furthermore, gene expression of the transcriptional NF-κB co-activator BCL3 (B-cell CCL/lymphoma 3) ($2^{-\Delta\Delta Ct} = 0.42$) was halved in response to *eS20* (Figure 15b).

5.3. Inhibitors of NF- κB reduce cytokine secretion mediated by *eS20*

To confirm the involvement of NF-κB in the activation of peripheral blood monocytes by *eS20*, cells were treated with agents, which are known to block NF-κB activation (Figure 16). 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline (6AQ) is a cell-permeable quinazoline compound, which functions as inhibitor of NF-κB transcriptional activation [238]. Perillyl alcohol (PA) is suggested to block calcium-dependent NF-κB signalling [239].

Both inhibitors reduced LPS-triggered IL-6 synthesis, whereas the effect on IL-8 secretion was marginal (Figure 16a and b). 6AQ and PA similarly decreased IL-6 and IL-8 production in *eS20*-treated monocytes compared to cells co-cultured with *eS20* in the absence of 6AQ and PA (Figure 16a and b).

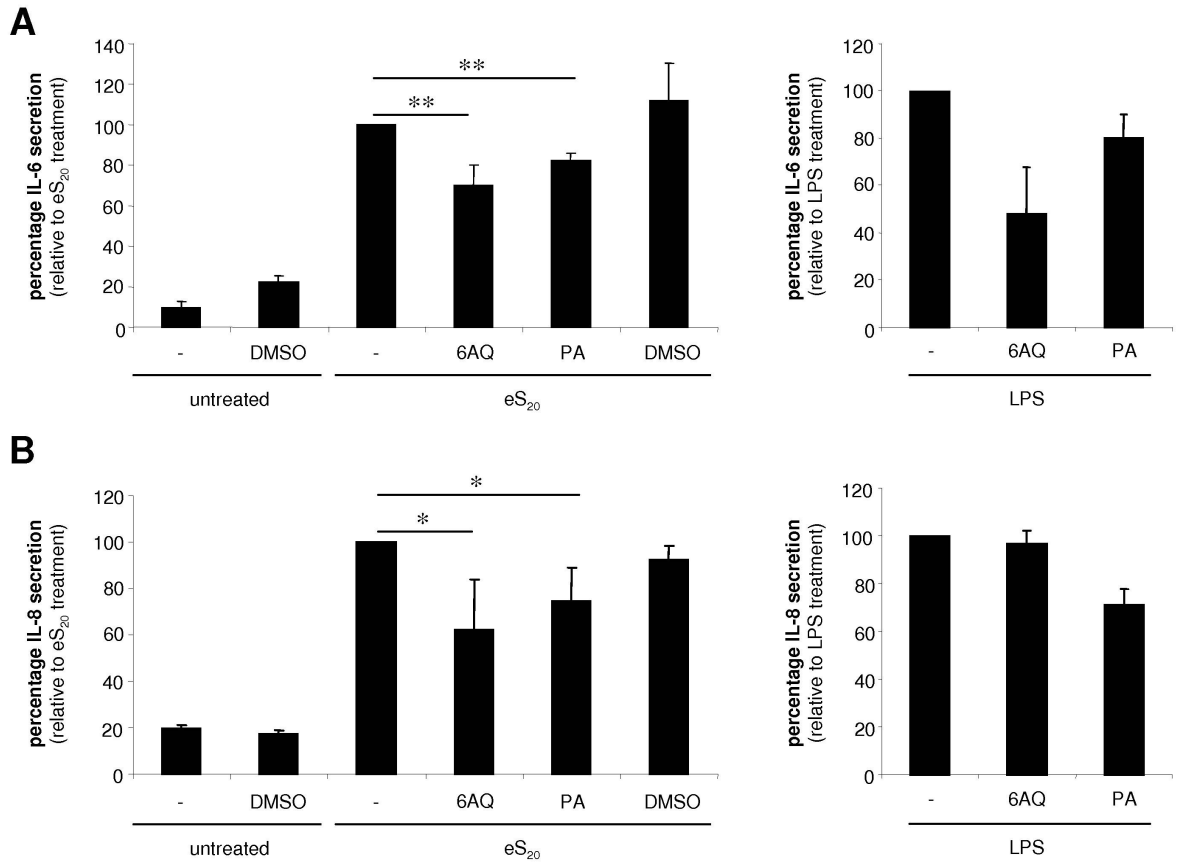

Figure 16. NF-κB inhibitors reduce the *eS$_{20}$*-induced IL-6 and IL-8 secretion in monocytes. Monocytes were pre-incubated with 10 µM of 6-amino-4-(4-phenoxyphenylethylamino) quinazoline (6AQ), perillyl alcohol (PA) or DMSO, the solvent of 6AQ, before co-culture with 300 µg/ml *eS$_{20}$* or 100 pg/ml LPS for 16 h. IL-6 (A) and IL-8 (B) secretion was quantified by ELISA. All data are presented as mean ± SEM of 3 different monocyte co-culture experiments with *eS$_{20}$* generated form 4 placentas (* *P* value < 0.05; ** *P* value < 0.01). Statistics were not performed for LPS, as the experiment has only been done three times.

5.4. Conclusion

Incubation of STBM with monocytes triggered changes in the transcription of several genes, which was partially mediated by NF-κB.

6. Interaction of STBM and primary human monocytes

6.1. *eS$_{20}$* attach to the monocytic cell surface

To assess if STBM directly interact with human monocytes, *eS$_{20}$* were stained with the red fluorescent membrane dye PKH-26 before co-culture with monocytes. Flow cytometric analysis revealed that all monocytes were highly positive for PKH-26, suggesting a binding of STBM to the monocytic cell surface (Figure 17a). This finding was confirmed by fluorescent microscopy (Figure 17b). Therefore monocytes were stained with the green fluorescent cytoplasmic dye carboxyfluorescein succinimidyl ester (CFSE) and treated with PKH26-labelled *eS$_{20}$*. Single STBM or aggregates of STBM were localized at the boundary of the CFSE-stained monocytes (Figure 17b). The nuclei were stained with the DNA-binding dye 4',6-Diamidin-2'-phenylindol- dihydrochlorid) (DAPI).

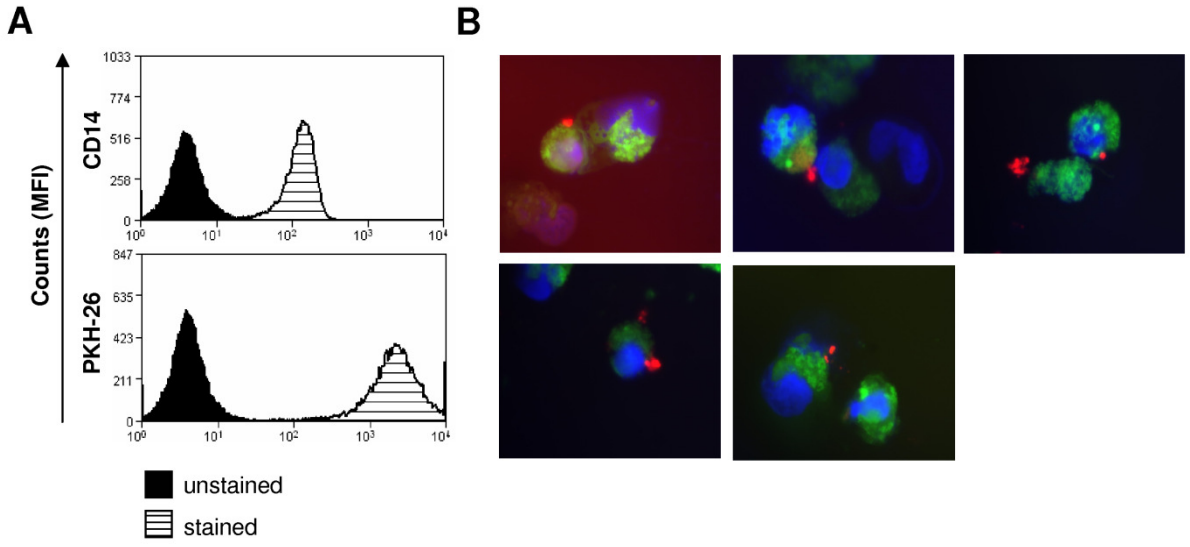

Figure 17. STBM bind to the monocyte surface. (A) Representative histograms of flow cytometry analysis of monocytes co-cultured with PKH-26-labelled *eS$_{20}$* for 16 h. (B) Fluorescence microscopy of CFSE-stained monocytes (green) incubated with PKH-26-labelled *eS$_{20}$* (red) for 16 h. DNA was stained with DAPI (blue). 2 independent monocyte co-cultures were performed with *eS$_{20}$* prepared from 3 different placentas.

6.2. *eS20* mediated secretion of proinflammatory mediators is independent of phagocytosis by human monocytes

Although Figure 17b does not suggest an engulfment of STBM, next, it was tested if phagocytosis was important for the *eS20*-mediated cytokine secretion. To block phagocytosis, monocytes were pre-treated with cytochalasin B, a cell-permeable mycotoxin that shortens actin filaments by inhibiting monomer addition, before stimulation with *eS20* (Figure 18). Cytochalasin B did not reverse the *eS20*-induced secretion of IL-8 and IL-6 (Figure 18a and b), showing that phagocytosis is not required for the induction of the inflammatory response.

However, the localization of *eS20* at the monocytic cell surface suggested that monocytes express cell surface molecules, which interact with *eS20*-associated markers and convey the activation signal into the cell.

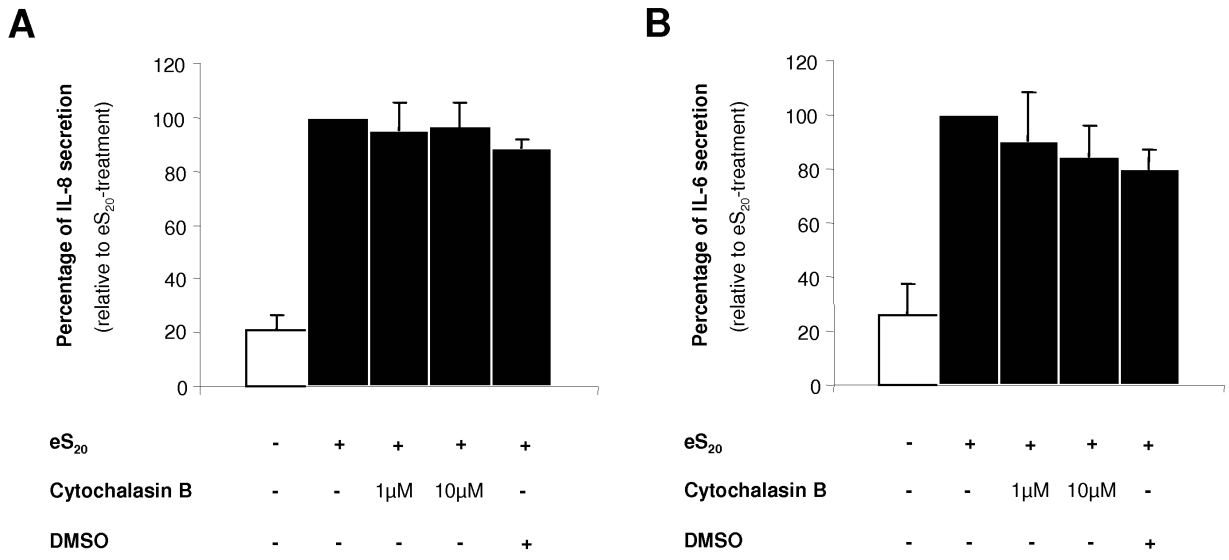

Figure 18. Inhibition of phagocytosis does not reverse STBM-mediated secretion of proinflammatory mediators by monocytes. Monocytes were pre-treated with 1 or 10 µM of the phagocytosis inhibitor cytochalasin B or with DMSO, the solvent of cytochalasin B, before addition of 300 µg/ml *eS20*. Release of IL-8 (A) and IL-6 (B) was quantified by ELISA. All data are presented as mean ± SEM of 3 different monocyte co-culture experiments with *eS20* prepared from 3 placentas.

6.3. STBM contain CD54

It has been previously proposed that monocytes strongly adhere via LFA-1 to CD54 displayed by cultured syncytiotrophoblasts [240]. As it is likely that STBM express the same membrane molecules as their parental cells, it was speculated that STBM-associated CD54 could bind LFA-1 expressed by monocytes. Thus, in a next step, the potential existence of STBM-associated CD54 was analyzed.

All STBM populations that activated monocytes, namely eS_{20} and *pS* prepared from healthy term placentas and eS_{20} generated from preeclamptic placentas, contained CD54 (Table 4). However, there was less microparticle-associated CD54 in *pS*, which always triggered the highest STBM-mediated inflammatory response in monocytes, than in eS_{20}, suggesting that CD54 may not be the major molecule mediating the interaction.

Table 4. Presence of STBM-associated CD54 was measured by ELISA. All results are shown as mean ± SEM of STBM prepared from 3 different placentas for each condition. NP = normal pregnancy; PE = preeclampsia.

	CD54 (ng/mg STBM)
eS_{20} NP	2.2 ± 0.8
eS_{20} PE	2.6 ± 0.5
pS	0.2 ± 0.1

6.4. STBM-associated CD54 is not responsible for eS_{20} mediated cytokine secretion

Although the concentration of STBM-associated CD54 was lower in *pS* than eS_{20}, its possible involvement in monocyte activation was evaluated. Therefore, the potential CD54-mediated adhesion of eS_{20} to primary monocytes was blocked with an anti-human CD54 antagonistic antibody. However, the secretion of IL-6 and IL-8 was not affected by the presence of the blocking antibody (Figure 19a and b), showing that CD54 is not required for the eS_{20}-mediated inflammatory response.

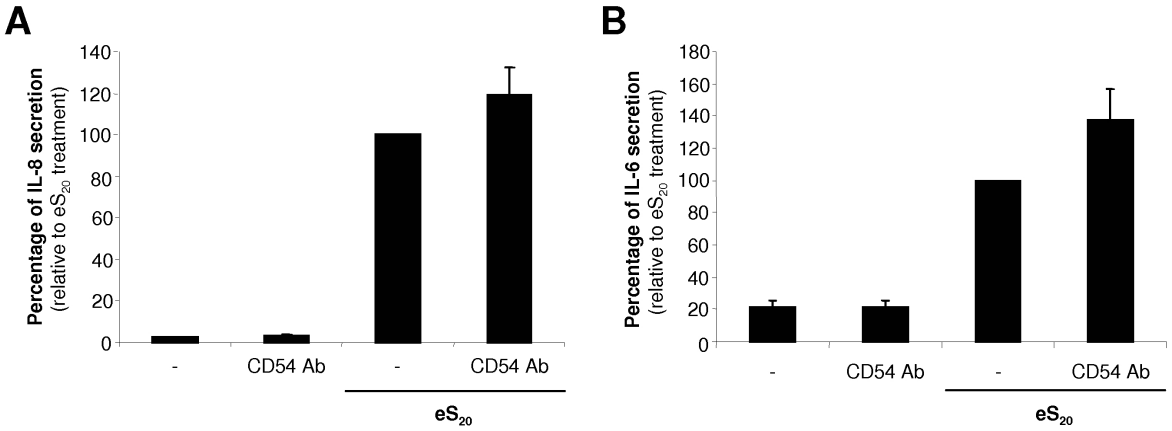

Figure 19. STBM-associated CD54 is not responsible for the proinflammatory response of monocytes. 100 µg/ml eS_{20} were pre-incubated with 10 µg/ml of anti-human CD54 adhesion antibody before adding to monocytes. After 16 h of co-culture, IL-8 (A) and IL-6 (B) secretion by monocytes was measured by ELISA. Bars represent mean ± SEM of 2 monocyte co-culture experiments with eS_{20} prepared from 3 different placentas.

6.5. Basal expression of cell surface marker is different in Mono Mac 6 cells and peripheral blood monocytes

Previous results from this work showed that eS_{20} triggered an inflammatory response in primary human blood monocytes, but not in the monocytic cell line Mono Mac 6. The lack of the cell surface expression of a specific molecule could be responsible for the unresponsiveness of Mono Mac 6 cells towards STBM treatment. Thus, basal expression of

monocytic markers, which might be involved in the binding of eS_{20} to monocytes and the subsequent intracellular signalling, was assessed on Mono Mac 6 cells and peripheral blood monocytes (Figure 20a and b). Comparison of CD14 expression confirmed previous results. Monocytes were highly positive for CD14, whereas Mono Mac 6 cells expressed low levels of CD14 (17.1 ± 5.1%). Both cell types were positive for CD54 and CD11a (Figure 20a and b). Peripheral blood monocytes and Mono Mac 6 highly expressed toll like receptor (TLR) 2 (98.9 ± 0.2% and 86.6 ± 2.1%, respectively), whereas they were negative for TLR4 (Figure 20a and b). However, there was a huge difference in the expression profile of TLR1: almost all primary cells (86.2 ± 7.8%) were positive for TLR1, in contrast to the Mono Mac 6, which did not express TLR1 (Figure 20a and b). In addition, primary monocytes (14.9 ± 3.9%) displayed triggering receptor expressed on myeloid cells (TREM)-1, in contrast to Mono Mac 6 (Figure 20a and b).

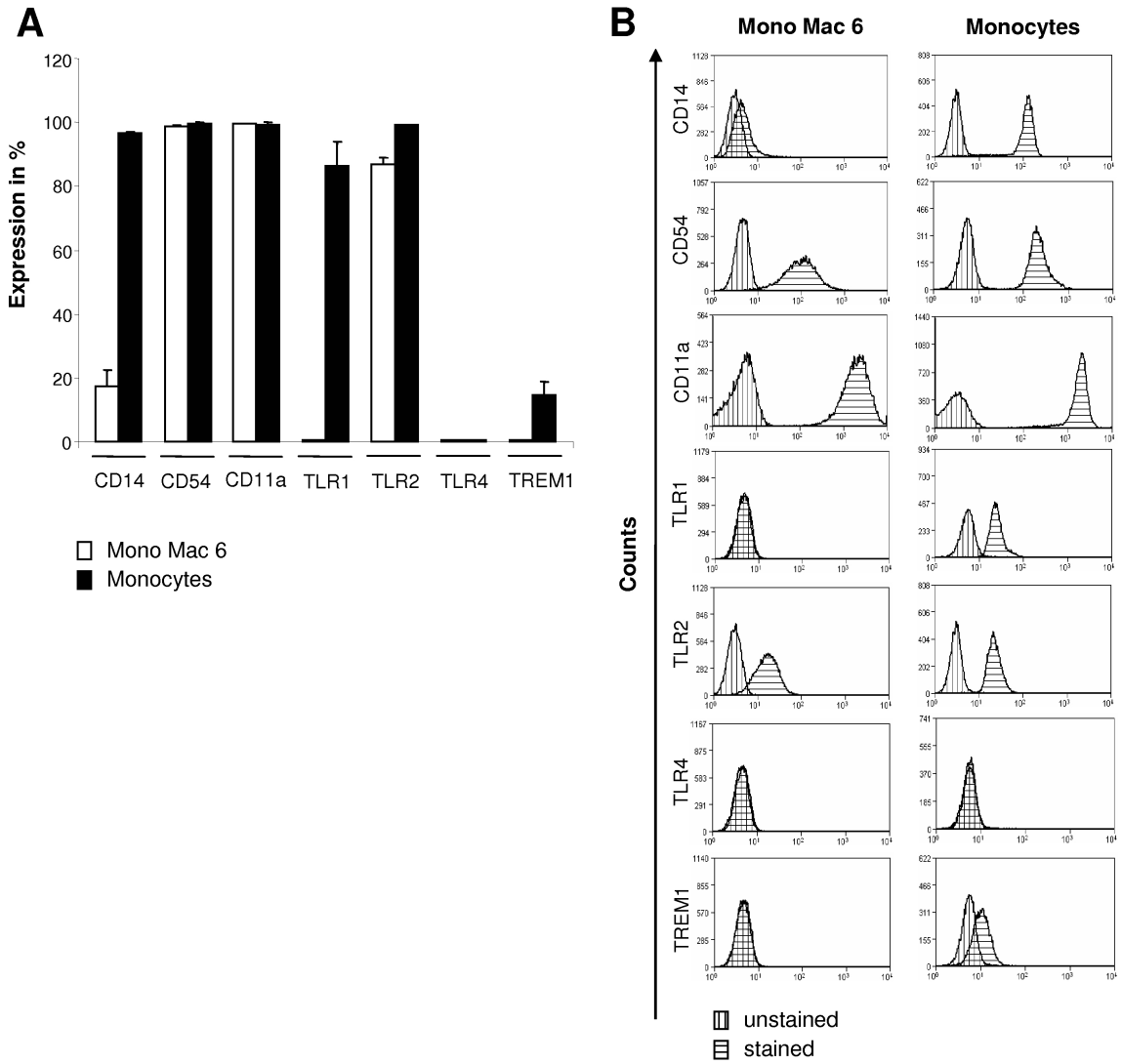

Figure 20. Different basal expression of cell surface molecules on Mono Mac 6 and primary monocytes. (A) Percentage of Mono Mac 6 cells and peripheral blood monocytes expressing CD14, CD54, CD11a, TLR1, TLR2, TLR4 and TREM-1. Bars represent mean ± SEM of 2 experiments. (B) Representative histograms of all cell surface molecules analysed.

6.6. MyD88 inhibitor peptide reduces *eS*$_{20}$ mediated secretion of proinflammatory mediators

TLR are potential receptors for STBM, as STBM are assembled of characteristic lipids [241]. In addition, the finding that TLR1 was differently expressed on primary monocytes and Mono Mac 6, suggested that the *eS*$_{20}$-induced monocyte activation may be mediated through TLR signalling.

Myeloid differentiation primary response gene (MyD) 88 is an intracellular adaptor protein, which is used by TLRs to induce the transcription factor NF-κB [242]. To investigate the involvement of TLR activation in *eS*$_{20}$-mediated secretion of proinflammatory mediators by monocytes, TLR signalling was blocked by a MyD88 homodimerization inhibitory peptide (Figure 21). Pre-treatment of monocytes with the inhibitory peptide reduced LPS-triggered IL-6 and IL-8 secretion (Figure 21a and b). The highest inhibition was achieved when the cells were treated with the lowest dose of LPS.

Treatment of monocytes with *eS*$_{20}$ in the presence of the MyD88 inhibitory peptide impaired monocyte activation, as shown by the reduced secretion of IL-6 (78.6 ± 16.5% and 87.1 ± 4.9%, respectively) and IL-8 (80.8 ± 3.2% and 72.7 ± 5.4%, respectively) compared to cells cultured with *eS*$_{20}$ alone (Figure 21a and b).

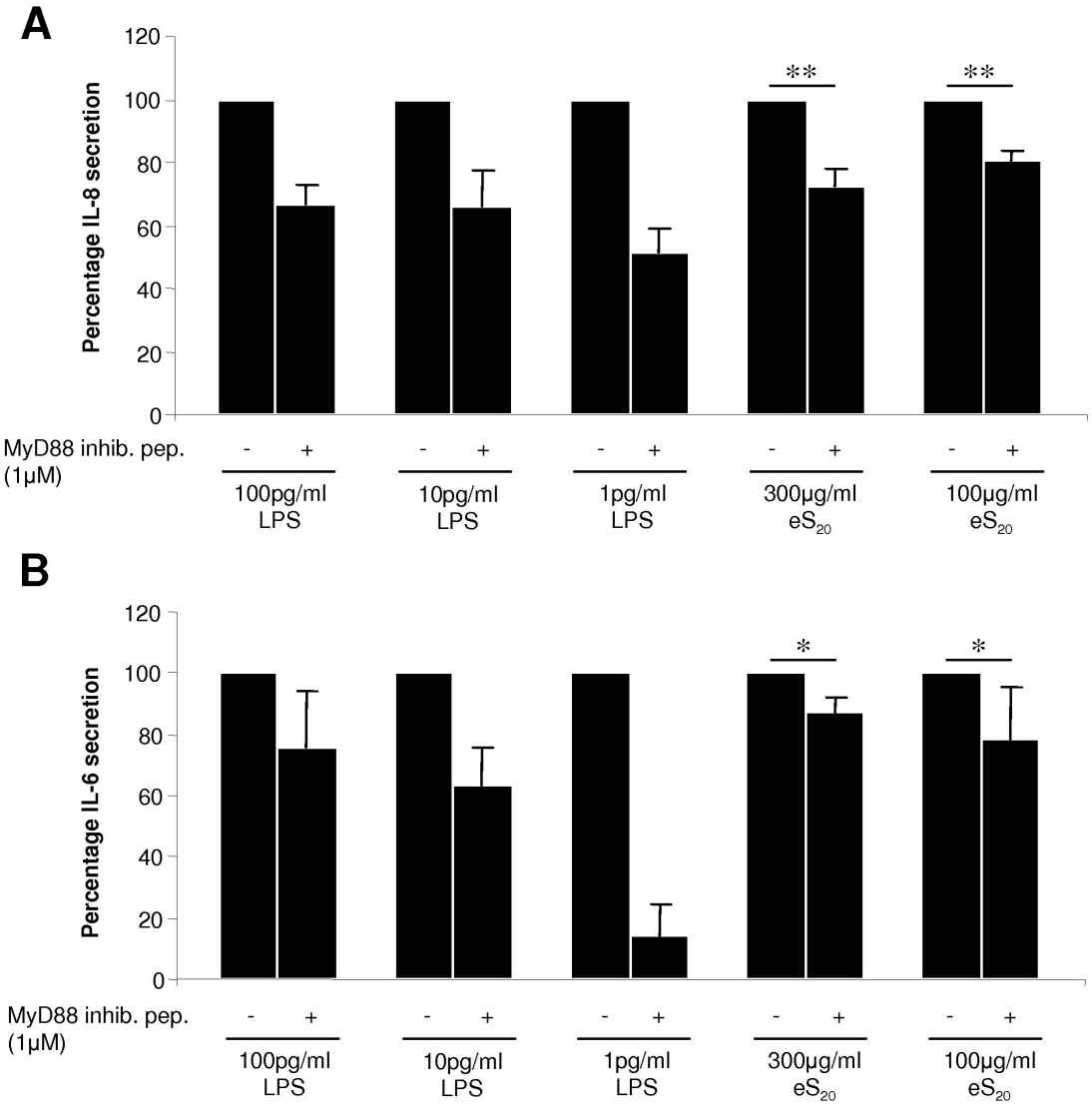

Figure 21. MyD88 homodimerization peptide reduces the STBM mediated proinflammatory response. Primary monocytes were treated with 1 µM of the MyD88 inhibitor peptide for 24 h before culturing with *eS*$_{20}$ or LPS for 12 h. Quantification of IL-8 (A) and IL-6 (B) released by monocytes was performed by ELISA and presented as percentage secretion relative to monocytes treated with *eS*$_{20}$ or LPS without pre-incubation with the MyD88 homodimerization peptide. Data are presented as mean ± SEM of 3 monocyte co-culture experiments with *eS*$_{20}$ prepared from 4 different placentas (* *P* value < 0.05; ** *P* value < 0.01). Statistics were not performed for LPS, as the experiment has only been done three times.

6.7. Conclusion

eS$_{20}$ bound to the monocyte cell surface and *eS*$_{20}$ partially mediated activation of monocytes through the TLR-signalling pathway.

DISCUSSION

Syncytiotrophoblast microparticles (STBM) have been attributed potential functions in the systemic maternal inflammatory response during normal human pregnancy and in the exaggerated generalized inflammation found in preeclampsia [115]. My colleagues and others showed, that STBM prepared from term placentas directly activate human cells of the inflammatory network, comprising endothelial cells and neutrophils, *in vitro* [220, 226-228, 243].

In the current work, the monocytic cell line Mono Mac 6 and primary human monocytes were used to investigate the potential contribution of STBM on the cellular viability, phenotype and function of monocytes. Thereby, STBM were prepared from healthy term placentas by four different *in vitro* approaches at conditions, which are believed to reflect either the physiologic situation in normal pregnancy (STBM washed from the maternal side of a dually perfused placental cotyledon and STBM shed by explants cultured in air) or the patho-physiologic one found in preeclampsia (STBM isolated by mechanical dissection and STBM generated from villous explant cultures incubated in hypoxic conditions). The dual perfusions of placental cotyledons and the cultures of villous tissue explants may represent best the *in vivo* situation, as the tissue structure and integrity are at least partially retained. In addition, Di Santo et al. has previously shown that the trophoblast viability und functionality was even better maintained during dual perfusions than in villous tissue explant cultures incubated in air, by directly comparing tissues from the same placenta for 7 hours [244]. Although the conditions of the present study are not exactly as those in Di Santo et al., one may assume that the same would be true when comparing a dual perfusion of 30 minutes and a villous explant culture incubated in air of 72 hours. In order to mimic the placental hypoxic conditions observed in preeclampsia, villous tissue explants were cultured in 3% O_2. Indeed, the current results suggest that there is a shift from apoptotic to necrotic cell death in villous tissue cultured in

3% O_2 in contrast to the villous tissue incubated in 20% O_2. Furthermore, physical disruption of tissue integrity by mechanical dissection of villous tissue is, in essence, a necrotic process.

None of the STBM populations triggered an inflammatory response in Mono Mac 6, although an activated phenotype was inducible by LPS. However, decreased CD54 expression on Mono Mac 6 cells incubated with *mS* could indicate changes in adhesion properties. Faas and co-workers recently observed a similar decreased CD54 expression on Mono Mac 6 cells following treatment with plasma derived from normal pregnant and preeclamptic women for 24 h, suggesting that STBM in the maternal plasma could mediate this reduced expression [236].

The absence of responsiveness of Mono Mac 6 cells upon co-incubation with STBM, may be attributed to its immature phenotype [245]. This is confirmed by the present results, which revealed low frequency of Mono Mac 6 cells expressing CD14, in contrast to the mature peripheral blood monocytes, which are highly positive for CD14. Thus, the inflammatory potential of *in vitro* prepared STBM was assessed on primary human peripheral blood monocytes. The data show, that different STBM populations triggered various responses in primary monocytes without affecting cell viability, which confirms that Mono Mac 6 cells and primary monocytes are not identical, concerning function and phenotype.

While the most patho-physiologic STBM populations (eS_3 and *mS*) had no or only minor influences on the monocytic phenotype and function, STBM generated in conditions mimicking more closely the physiologic situation (eS_{20} and *pS*) induced a proinflammatory response.

That the monocytic response varied according to the mode of preparation of the STBM is not surprising in the light of previous findings with other cell types. For instance, it was shown that *mS* triggered endothelial cell detachment from the collagen matrix and apoptosis, whereas eS_{20} and *pS* induced a partial inhibition of endothelial cell proliferation, but no apoptosis [220,

226]. Furthermore, *eS*$_{20}$ and *mS* significantly inhibited, whereas *pS* enhanced, the proliferation of T lymphocytes induced with phorbol ester and Ca^{2+} ionophore [223].

The current data show that primary monocytes treated with *pS* and *eS*$_{20}$ acquired an activation-like phenotype and secreted proinflammatory immune mediators. Up-regulation of the adhesion molecule CD54 is of interest in regard of a previous report documenting a significant increase in CD54 expression on peripheral blood monocytes from third trimester pregnancies [92]. The results suggest that placental microparticles could be the mediators of this observation.

pS and *eS*$_{20}$ also stimulated the production of the proinflammatory molecules IL-8, IL-6 and IL-1β. These factors have been analysed because of their enhanced intracellular expression in peripheral blood monocytes of preeclamptic women [187]. Furthermore, they are also present in elevated concentrations in the serum of preeclamptic patients [183-185]. The current results are also in line with a recent report, which showed that STBM prepared by dual perfusion of a placental cotyledon, but not STBM generated by mechanical dissection of villous tissue, induced a functional response in primary monocytes *in vitro*, as measured by the increased intra-cellular production of TNF-α and IL-12p70 [173]. However, in the study of Germain and co-workers, STBM were incubated with the whole peripheral blood mononuclear cell (PBMC) fraction, leaving open the possibility that the up-regulation of cytokine expression in monocytes could be indirect, for instance by cell-cell contact between monocytes and STBM-activated third-party cells, or alternatively, through stimulation by a soluble mediator produced by the latter. Our findings allow us to conclude that STBM are capable of directly inducing monocytic cytokine production.

The monocytic response was time-dependent. As expected, IL-6 secretion increased steadily with incubation time, whereas the secretion of IL-1β was rapid and sustained. This observation is in agreement with the knowledge that IL-1β is a mediator of acute inflammation, whereas IL-6 is a secondary pleiotropic regulator of inflammatory responses.

IL-1β is stored as an inactive precursor molecule in the cytoplasm, which is cleaved and released as a proactive form in the extra-cellular compartment immediately after stimulation [246].

The results also show that monocyte activation in response to STBM was dose-dependent. Moreover, at identical protein concentration, the response stimulated by *pS* was much higher than that induced by eS_{20}. This could indicate that these two preparations of microparticles are functionally different. On the other hand, this might also reflect a technical limitation of this work. To conform to previous studies, the STBM-associated protein concentration was used to quantify the STBM populations [173, 220]. However, this is a surrogate measurement for microparticle numbers as the protein content of differentially prepared STBM may vary.

Another aspect that needs to be considered when interpreting the current results is that, although the presence, in our STBM preparation, of syncytial-membrane microparticles could be confirmed by the detection of syncytiotrophoblast-specific PLAP, a possible occurrence of microparticles derived from non-trophoblastic elements of the villous tissue or from residual maternal blood cell debris cannot be excluded.

eS_{20} prepared from preeclamptic placentas induced a proinflammatory reaction in peripheral blood monocytes, as well. eS_{20} from normal as well as from preeclamptic placentas triggered an equally strong and dose-dependent inflammatory response in primary monocytes, suggesting that there are no or minor qualitative differences between the microparticles. From these observations one may speculate that the overt maternal inflammation associated with preeclampsia might be due to the higher concentrations of circulating STBM, rather than to qualitative differences between the microparticles shed from the healthy and patho-physiologic placentas.

eS_{20} partially induced the inflammatory response through NF-κB. NF-κB is a master transcription factor involved in inflammatory pathways, which regulates the expression of several genes, including CD54, IL-8, IL-6 and IL-1β [237]. eS_{20} triggered the transcription of

a number of NF-κB-regulated genes, which contribute to the amplification of an inflammatory reaction, by either attracting or inducing production and differentiation of other immune cells. Beside a tremendous increase in the transcription rate of IL-6, there was a high induction of the small proinflammatory chemokine CCL2, which chemotactically attracts monocytes to sites of inflammation [247]. Furthermore, *eS*$_{20}$ induced the transcription of the cytokines CSF-2 and -3, which control production, differentiation and function of macrophages and granulocytes, TNF, which is a major mediator of acute inflammation, and the TNF family member LT-α, which mediate various inflammatory and immunologic responses [248-250]. Although, *eS*$_{20}$ triggered a huge release of IL-8 by monocytes, its transcription was only marginally enhanced. This observation could point to the presence of an intracellular reservoir of pre-stored IL-8, as it is the case in Weibel-Palade bodies in microvascular endothelial cells [251]. However, it has to be considered that gene expression and secreted protein levels of IL-8 were measured at different time points following *eS*$_{20}$ treatment (after 4 and 16 hours, respectively).

eS$_{20}$ not only stimulated the secretion of IL-1β, but also its gene transcription. However, the transcription of the other isoform of IL-1, namely IL-1α, was induced more by *eS*$_{20}$. IL-1β and IL-1α both stimulate chemokine production, when they bind IL-1 receptor 1 (IL-1R1).

eS$_{20}$ also induced gene expression of the transcription factors EGR1 and JUN. EGR1 belongs to a family of immediate early genes, which exhibit a critical role in cellular growth, development and differentiation [252]. JUN either forms homodimers (JUN/JUN) or heterodimers (JUN/FOS and JUN/ATF) to generate the functional AP-1 transcription factor, which regulates gene expression of crucial molecules of the immune system, including cytokines [253].

However, *eS*$_{20}$ also triggered transcription of the anti-inflammatory cytokine IL-10, representing a typical negative feedback regulator, which is usually produced by activated cells to maintain homeostatic control and to prevent potential injury mediated by an overt

inflammatory reaction [254]. The eS_{20}-mediated decrease in gene expression of TLR1, TLR6, TLR7, TLR8, TLR9 and TICAM2, an intracellular adaptor molecule restricted to TLR4 signalling, may contribute to the resolution of the inflammatory response as well [255, 256]. In addition, the reduced transcription of the transcriptional co-activator of NF-κB BCL3 may limit the transcriptional activity of NF-κB and, thus, support the termination of the inflammatory reaction [257, 258].

On the one hand, down-regulated transcription of the cytoplasmic pattern recognition receptor NOD1 (CARD4) could also account for the restriction of the inflammation, because NOD1 functions as activator of NF-κB [259]. On the other hand, reduced NOD1 transcription prevents apoptosis, as NOD1-mediated activation of the pro-apoptotic molecule caspase 9 is inhibited [259].

NF-κB is also known as a cell survival factor, and the eS_{20}-mediated decrease in gene expression of the apoptosis-inducing molecules FADD, caspase 8 and the TNF receptor CD27, could explain the high viability of monocytes following STBM treatment, compared to monocytes cultured in absence of any trigger [237, 260-262].

The requirement of NF-κB for the regulation of IL-6 and IL-8 production could be demonstrated by the finding that the two NF-κB inhibitors 6AQ and PA partially prevented the secretion of the proinflammatory mediators upon stimulation with eS_{20}. eS_{20}-triggered release of proinflammatory factors was more reduced following treatment with 6AQ than with PA. This is not surprising, as these two compounds interfere with different steps of NF-κB activation. PA decreases intracellular calcium levels and thereby blocks the calcium-dependent NF-κB pathway, only [239]. However, 6AQ is a low molecular weight inhibitor of NF-κB transcriptional activation and might not be restricted to one of the pathways activating NF-κB [238]. Activation of the NF-κB signal transduction pathway in PBMC of preeclamptic women remains controversial [263, 264]. Luppi et al. showed an increased activation of NF-κB compared to normal pregnant controls [263]. In contrast, McCracken and co-workers

published a suppression of the NF-κB activation pathway relative to normal pregnant and non-pregnant subjects [264]. However, this suppression might rather be attributed to the T-cell subset than the monocytes, as McCracken et al. recently documented the down-regulation of NF-κB in peripheral blood T-cells of pregnant women [265].

The finding that there is a response in eS_{20}-treated monocytes, raised the question of a direct interaction between monocytes and STBM. Results obtained by flow cytometry and fluorescence microscopy revealed, that eS_{20} attach to the monocytic surface. These results are in line with the work of Germain and collaborators, who recently gave evidence for binding of placental-derived microparticles on circulating monocytes in the peripheral blood of normal pregnant and preeclamptic women [173].

The observed localization of eS_{20} at the boundary of monocytes, suggests that STBM interact with monocytes via one or several receptor-ligand-pair(s).

pS and eS_{20} preparations led to decreased CD11a expression on monocytes. CD11a forms together with CD18 the heterodimeric integrin lymphocyte function-associated antigen-1 (LFA-1). It has been suggested that monocytes strongly adhere via LFA-1 to CD54 displayed by cultured syncytiotrophoblasts [240]. Since STBM are likely to encompass the same membrane molecules as the parent cell from which they are derived, it has been speculated that the observed changes in CD11a fluorescence intensity might be due to the interaction between the STBM and the monocytes through these partner molecules, resulting in some form of masking of the CD11a epitope to the antibody used for the present flow cytometry analysis. The results obtained during this work confirmed the presence of CD54 on STBM. Although binding was not analyzed, the adhesion blockade of CD54 did not affect the production of IL-6 and IL-8 in primary monocytes induced by eS_{20}.

The STBM used in this study also contained proinflammatory mediators. According to the data, a role for IL-8 that is associated with eS_{20}, or the proinflammatory cytokines IL-6, IL-1β and TNF-α, which are present only in trace concentrations in all STBM populations, is

unlikely. However, it has been previously shown that STBM isolated by placental perfusion of the maternal circuit contained the biologically active isoform of IL-1β [234]. In contrast to the present work, where *pS* have been retrieved from placental washes lasting for 30 minutes, Di Santo and co-workers prepared STBM after seven hours of perfusion of the maternal compartment.

STBM are potential ligands for TLRs, as STBM are constituted of characteristic lipids and contain DNA molecules [241, 266]. According to the data, TLR1 is a candidate receptor for STBM, as Mono Mac 6 cells, which did not respond to STBM, were lacking TLR1 expression, whereas nearly all primary monocytes were positive for TLR1. TLR1 forms heterodimers with TLR2 and binds preferentially triacylated lipopeptides [267]. Blocking the TLR signalling by the cell permeable MyD88 homodimerization peptide reduced IL-6 and IL-8 secretion, which gave further evidence for the involvement of TLRs. TLR signalling via the adaptor molecule MyD88 results in NF-κB activation [268]. However, some TLRs, such as TLR3 and TLR4, may trigger inflammation in a MyD88-independent way as well [268]. In the case of TLR4 it is known, that MyD88-dependent signalling is already induced at the plasma membrane, whereas activation of the MyD88-independent pathway needs endocytosis of the receptor/ligand complex [269]. Provided that this observation of Kagan et al. is true for other TLRs, which signal through MyD88, and knowing that engulfment of STBM is not required for the proinflammatory response in monocytes, one could speculate, that STBM are recognized on the monocyte membrane by one or several TLRs, which then trigger the MyD88-dependent cascade and induce the inflammatory response in absence of TLR internalisation.

However, the MyD88 inhibitory peptide did not completely block the inflammatory response in eS_{20}-treated monocytes. This could point out the involvement of further membrane molecules, which signal through other pathways. A candidate molecule is TREM-1, which was not expressed on Mono Mac 6, but present at low levels on primary monocytes. TREM-1

is known to transduce the extracellular signals through the immunoreceptor tyrosine-based activation motif (ITAM) into the nucleus, inducing the production of proinflammatory mediators, such as IL-8 and TNFα [270, 271]. However, the ligand of TREM-1 is still illusive.

In conclusion, the present analysis suggests that STBM prepared by *in vitro* approaches, which probably mimic best the physiologic conditions of human pregnancy, bear dose-dependent proinflammatory properties for primary monocytes. This is interesting in regard to the *in vivo* situation in normal pregnancy. The progressive monocytic activation in the maternal peripheral blood may be caused by the steady increase in the load of placental microparticles with gestational age. In addition, the present study suggests, that there are minor qualitative differences between STBM prepared from healthy and preeclamptic placentas. This could further indicate that the overt monocyte activation associated with preeclampsia may be attributed to the enhanced circulatory concentrations of STBM, rather than to a differential nature of the microparticles, compared to normal pregnancy.

The results also suggest that STBM induce the MyD88-dependent TLR signalling at the cell surface, leading to NF-κB activation. In turn, NF-κB mediates the transcription of proinflammatory mediators, including IL-1β, IL-6 and IL-8, and adhesion molecules, such as CD54, resulting in a systemic inflammation.

In future, the following questions should be solved in order to understand the inflammation-inducing components of STBM: which molecules displayed by STBM are recognized by which TLR? Are there other receptors expressed by monocytes binding to respective ligands on STBM? Although it is very demanding to get decent and pure amounts of naturally occurring STBM from maternal blood, it would be interesting to compare their molecular expression profile with the one of *in vitro* prepared placental microparticles.

To extend the knowledge of potential *in vivo* effects of the STBM populations, analyzed in the present study, the next steps include their examination in pregnant mice. Possible xeno-reactions should be ruled out by the administration of unspecific microparticles, for example human red blood cell microparticles (RBC ghost).

However, preeclampsia is a multifactorial disorder and it is unlikely, that STBM alone are responsible for the maternal syndrome. Thus, data from all lines of ongoing research in preeclampsia should be considered, to get a more complete picture of the factors mediating the disease, which hopefully can be translated into new diagnostic and screening tools.

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