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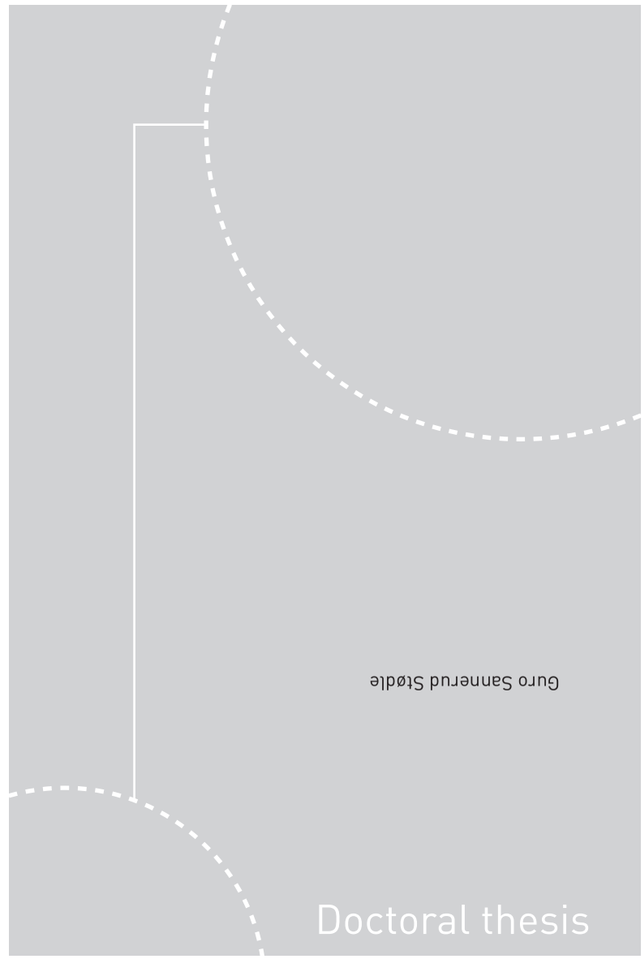

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Guro Sannerud Stødle

Pattern recognition receptor mediated inflammation in placental trophoblasts

Doctoral thesis

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NTNU
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Thesis for the Degree of
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Studie av mekanismer for betennelse i morkaken

Betennelse (inflammasjon) er en immunrespons som kroppen setter i gang for å beskytte seg mot infeksjoner og vevsskade. Alle mennesker er født med "mønster-gjenkjennende" pattern recognition reseptorer (PRRer) som en del av immunforsvaret. Disse reseptorene er avgjørende for igangsettelse av betennelsesreaksjoner, blant annet gjennom produksjon av signalmolekyler kalt cytokiner. En betennelsesreaksjon er som regel både nødvendig og gunstig for kroppen, men hvis responsen blir for kraftig eller langvarig kan den føre til stor skade og sykdom. Et normalt svangerskap er assosiert med en mild betennelsestilstand, mens hos kvinner som utvikler svangerskapsforgiftning (preeklampsi) vil nivået av betennelsesmarkører, som cytokiner og C-reaktivt protein (CRP), være forhøyet. Man tror svangerskapsforgiftning begynner tidlig i svangerskapet med en ufullstendig utvikling av morkaken. En mangelfull utvikling av morkaken vil gi redusert blodtilførsel fra mor til morkake, og kan resultere i «stressede» celler og betennelse i morkaken. En slik stresset morkake produserer faresignaler som kan aktivere PRRer på f.eks trofoblaster - fosterceller som utgjør den største delen av morkaken. Aktivering av PRRer på trofoblaster kan igangsette overdreven immunrespons i morkaken, som igjen vil kunne bidra til betennelsen man observerer ved svangerskapsforgiftning, men kunnskapen om de underliggende sykdomsmekanismene er mangelfull. Formålet med denne avhandlingen var å bidra til økt kunnskap om betennelse igangsatt av PRRer i morkaken ved friske svangerskap og svangerskapsforgiftning.

Vi studerte PRR-aktivering av trofoblaster isolert fra morkaker tidlig i svangerskapet, og sammenliknet resultatene med syv trofoblast-cellelinjer (modifiserte celler med forlenget levetid). De isolerte trofoblastene uttrykte et bredt repertoar av fungerende PRRer, men kun én av syv cellelinjer viste seg å ha et liknende uttrykk av funksjonelle PRRer. Det brede uttrykket av PRRer vi påviste i de isolerte trofoblastene tyder på at disse cellene spiller en viktig rolle ved betennelse i morkaken. Videre viser våre resultater at det er viktig å velge riktig trofoblast-cellelinje når man skal studere disse betennelsesreaksjonene og tilgangen til isolerte trofoblaster er begrenset.

For å avklare om kjente PRR-aktiverende faresignaler som kolesterol, urinsyre og HMGB1 spiller en rolle ved utvikling av svangerskapsforgiftning, ble morkakevev og blodprøver fra kvinner med og uten svangerskapsforgiftning undersøkt. Vi avdekket høyere nivåer av kolesterol og urinsyre, men ikke HMGB1, i blodprøver fra kvinner med svangerskapsforgiftning sammenlignet med friske gravide kvinner. I morkaken fant vi at HMGB1 og PRRen som gjenkjenner HMGB1, TLR4, var høyest uttrykt i morkaker fra kvinner med svangerskapsforgiftning. HMGB1 aktivering av TLR4 økte produksjonen av cytokinet interleukin (IL)-8, og vi målte forhøyet IL-8 nivå både i morkakevev og blodprøver fra kvinner med svangerskapsforgiftning. PRRen NLRP3, som aktiveres av kolesterol og urinsyre, var spesielt høyt uttrykt av trofoblaster i morkaken, og vi målte et høyere uttrykk av det NLRP3 responsive cytokinet IL-1β i trofoblaster ved svangerskapsforgiftning. Videre viste vi at de aktuelle PRR mekanismene lar seg aktivere av kolesterol og HMGB1 i morkakevev og en trofoblast-cellelinje. Studiene av PRR mekanismene TLR4 og NLRP3 påviste forhøyede nivåer av kolesterol, urinsyre og/eller HMGB1 som mulige underliggende årsaker til betennelse i morkaken og utvikling av svangerskapsforgiftning.

Resultatene i denne avhandlingen indikerer at trofoblaster i morkaken og bestemte PRR mekanismer er involvert i betennelsen som observeres i både normale svangerskap og ved svangerskapsforgiftning. Det langsiktige målet med arbeidet er å kunne identifisere kvinner med økt risiko for svangerskapsforgiftning, samt kartlegge nye muligheter for behandling av sykdommen.

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LIST OF PAPERS

I. Tangerås LH* / Stødle GS*, Olsen GD, Leknes AH, Gundersen AS, Skei B, Vikdal AJ, Ryan L, Steinkjer B, Myklebost MF, Langaas M, Austgulen R, Iversen AC. **Functional Toll-like receptors in primary first-trimester trophoblasts.** Journal of Reproductive Immunology 2014; 106:89-99. *Both authors contributed equally.

II. Gierman LM, Stødle GS* / Tangerås LH*, Austdal M, Olsen GD, Follestad T, Skei B, Rian K, Gundersen AS, Austgulen R, Iversen AC. **Toll-like receptor profiling of seven trophoblast cell lines warrants caution for translation to primary trophoblasts.** Placenta 2015; 36(11):1246-53. *Both authors contributed equally.

III. Tangerås LH, Stødle GS, Silva GB, Gierman LM, Skei B, Collett K, Beversmark AL, Skråstad RB, Thomsen LC, Bjørge L, Iversen AC. **The role of high mobility group box 1 (HMGB1) in placental inflammation and preeclampsia.** Submitted to Clinical Science July 2017.

IV. Stødle GS, Silva GB, Tangerås LH, Gierman LM, Nervik I, Dahlberg UE, Sun C, Aune MH, Thomsen LC, Bjørge L, Iversen AC. **Inflammation in preeclampsia by nod-like receptor protein (NLRP)3 inflammasome activation in trophoblasts.** Submitted to American Journal of Obstetrics and Gynecology July 2017.

LIST OF ABBREVIATIONS

ABCA	ATP-binding cassette transporter
AIM	Absent-in-melanoma
ALR	AIM2-like receptor
ATP	Adenosine triphosphate
CARD	Caspase recruitment domain
CD	Cluster of differentiation
CK	Cytokeratin
CT	Cytotrophoblast
CXCL	C-X-C motif ligand
CXCR	C-X-C chemokine receptor
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
ds	Double-stranded
ER	Endoplasmic reticulum
ET	Endovascular trophoblasts
EVT	Extravillous trophoblast
FGR	Fetal growth restriction
GC	Giant cells
HIN	Hematopoietic interferon-inducible nuclear
HMGB1	High mobility group box 1
hsCRP	High-sensitivity C-reactive protein
HSP	Heat shock protein
IFN	Interferon
IL	Interleukin
IP-10	Interferon-γ-inducible protein 10
IRF	IFN-regulatory factor
IT	Interstitial trophoblast
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAL	MyD88-adaptor like
MAPKs	Mitogen-activated protein kinases
MD-2	Myeloid differentiation factor 2
mRNA	Messenger ribonucleic acid
MSU	Monosodium urate
MyD88	Myeloid differentiation primary-response gene 88
NEK7	NIMA related kinase 7
NF-κB	Nuclear factor-κB
NK	Natural killer
NLR	NOD-like receptor
NLRP	NOD-like receptor protein
NLRC4	NLR family CARD domain containing 4
NOD	Nucleotide binding and oligomerization domain
PAMP	Pathogen-associated molecular pattern
Pam3Cys	Pam3CysSerLys4

Poly(I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
PTD	Preterm delivery
PYD	Pyrin domain
RAGE	Receptor for advanced glycation end products
REC	Regional Committee for Medical and Health Research Ethics
RIG-I	Retinoic acid-inducible gene-I
RLR	Retinoic acid-inducible gene-I-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxidative species
SCT	Syncytiotrophoblast
sFlt-1	Soluble fms-related tyrosine kinase 1
SGHPL-5	Saint George's Hospital cell line 5
ss	Single-stranded
TCC	Terminal complement complex
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFN-β
VEGF	Vascular endothelial growth factor

1. INTRODUCTION

1.1. INFLAMMATION

Inflammation is a response induced by the immune system to protect the body from harmful stimuli or conditions, such as infections and tissue damage [1]. The inflammatory response is designed to remove the source of harm, repair damaged tissue and restore homeostasis. Normally, inflammation is necessary and beneficial, but it also has the potential to cause substantial damage if its extent or duration is dysregulated [1]. Thus, many physiological processes involve inflammation, including the menstrual cycle, establishment of pregnancy and birth, as well as pathological conditions such as sepsis and atherosclerosis [1, 2].

The initiation of inflammation happens through the activation of specialized receptors in the immune system, which sets off a signalling cascade resulting in the production of inflammatory mediators such as cytokines [1]. The release of cytokines will activate cells located in the surrounding tissue and recruit immune cells from the circulatory system. A successful inflammatory response will eliminate the harmful stimuli and culminate in tissue repair before completed [1]. However, failure to remove the causative stimuli or continuance of the inflammatory response may result in progression to chronic inflammation [3]. When inflammation is not limited to a local tissue, but additionally involves blood vessels and other organs, it can be considered a systemic inflammatory response [3].

1.2. INNATE IMMUNITY AND PATTERN RECOGNITION RECEPTORS

Two major subdivisions constitute the immune system; the innate and the adaptive immune system. The immune system protects the human body against microbial invasions and endogenous signal molecules released by damaged or altered host cells. The innate immune system is ready to be mobilized immediately upon infection or cell damage, whereas the adaptive immune response requires days for full activation [4]. Natural killer (NK) cells, neutrophils, macrophages and dendritic cells are important cells of the innate immune system, and recognize microorganisms and danger molecules through a limited number of germline-encoded receptors called pattern recognition receptors (PRRs) [5, 6]. The adaptive immune system depends on clonal expansion of antigen specific receptors produced by B and T lymphocytes, and mediates immunologic memory by producing T and B memory cells [4].

Despite these differences, the components of the innate and adaptive immune system are closely entwined and cooperate to mount a solid and optimal immune response.

The PRRs are continuously expressed on both professional innate immune cells and non-immune cells like epithelial cells, endothelial cells and fibroblasts [5, 7]. Microorganisms express various classes of signature molecules, including proteins, lipids, carbohydrates and nucleic acids that are sensed by PRRs [5]. These molecules are known as pathogen-associated molecular patterns (PAMPs). PRRs are also responsible for the recognition of damage-associated molecular patterns (DAMPs) on endogenous molecules released from stressed or damaged cells [7].

Today, several families of PRRs are known. These include toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), DNA receptors, C-type lectin receptors, absent-in-melanoma (AIM)-like receptors (ALRs), and hematopoietic interferon-inducible nuclear (HIN)-200 proteins [5, 7-10]. TLRs and C-type lectin receptors sense PAMPs and/or DAMPs both at the cell surface and in endosomes, whereas RLRs, NLRs, DNA receptors, ALRs and HIN-200 proteins detect target molecules only in intracellular compartments [7, 10, 11].

Recognition of PAMPs and DAMPs by PRRs induce inflammation through the production of pro-inflammatory cytokines, chemokines and type I interferons (IFNs) via complex signal transduction pathways [5, 7]. The production of these pleiotropic molecules regulate cell death, modify vascular endothelial permeability, recruit blood cells to inflamed tissues and induce production of acute-phase proteins [7]. This culminates in elimination of invading microbes and damaged host cells through recruitment and activation of immune cells, phagocytosis and autophagy [11]. Each PAMP or DAMP may activate several PRRs [9]. Hence, the defense mechanisms induced depend on which PRRs are activated and on the origin and amount of the PAMP/DAMP [7]. Defect sensing or dysregulated signalling by PRRs may predispose the host to recurrent infections or cell damage and development of immune related diseases such as preeclampsia, inflammatory bowel disease and cancer [12-15].

1.2.1. Toll-like receptors

The TLRs are the most widely studied PRRs. TLRs are expressed by various cell types, including immune cells such as dendritic cells, macrophages and B cells, and non-immune cells like epithelial cells and fibroblasts [5, 7]. TLRs are type I membrane glycoproteins with unique horseshoe shaped structures consisting of an extracellular leucine rich repeat (LRR) domain, a transmembrane region and a cytoplasmic Toll interleukin (IL)-1 receptor (TIR) domain [5]. The LRR domain recognizes and binds ligands, while the TIR domain is responsible for the recruitment of different TIR adaptor molecules and further activation of intracellular signalling pathways. Upon ligand binding TLRs typically form dimers, and by operating in both heterodimers and homodimers they display a unique ability to recognize a wide range of PAMPs and DAMPs [7, 9, 11]. So far, ten human TLR family members have been identified, and mice have been shown to express twelve TLRs [5]. TLR12 and TLR13 have been detected in mice only, while TLR10 is only expressed in humans [5]. Primarily, TLR1, TLR2, TLR4, TLR5 and TLR6 are localized at the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are expressed intracellularly within endocytic compartments [5]. TLR2 and TLR4 are also expressed on endosomes, appearing to be important for IFN responses [16, 17]. The function and ligand for TLR10 is not yet determined.

1.2.2. Toll-like receptor ligands

TLR activation induced by microbes may result from direct binding of PAMPs, or indirectly through the detection of changes in concentration or sub-cellular localization of DAMPs released from damaged tissue elicited by invading microorganisms [11]. Importantly, immune responses causing inflammation is not always induced by exogenous pathogens. In diseases such as atherosclerosis, gout and diabetes 2, damaged cells secrete DAMPs that interact with TLRs and induce responses to maintain the homeostatic state in health and disease [18].

Microorganisms such as mycobacteria, bacteria, viruses, fungi and parasites display PAMPs recognized by different TLRs (Figure 1) [5]. These include lipoproteins (sensed by TLR1, TLR2, and TLR6), zymosan and peptidoglycan (TLR2 agonists), double-stranded (ds) RNA (recognized by TLR3), lipopolysaccharide (LPS) (TLR4 ligand), flagellin (TLR5), single-stranded (ss) RNA (TLR7 and TLR8), and DNA (TLR9) (Figure 1) [9]. Examples of DAMPs that act as TLR ligands are endogenously produced lipoproteins (binding to TLR2/1 and

TLR2/6), self RNA (recognized by TLR3), self DNA (TLR7 and TLR9) (Figure 1), and numerous molecules recognized by TLR4; the nuclear protein high mobility group box 1 (HMGB1), heat shock proteins (HSPs), fibronectin, oxidized phospholipids, oxidized low-density lipoprotein (LDL) and heparan sulfate [18]. In addition to these natural TLR ligands, synthetic TLR ligands have been developed for the imitative activation of TLRs in research studies concerning TLR signalling and function. Examples of such synthetic TLR ligands are Pam3CysSerLys4 (Pam3Cys) crystals (sensed by TLR2/1), follistatin-like 1 (FSL1) (recognized by TLR2/6), polyinosinic-polycytidylic acid (poly(I:C)) (TLR3), R848 (TLR7 and 8) and cytosine-guanine pairs (CpG oligodeoxynucleotides) (TLR9) [5, 19, 20].

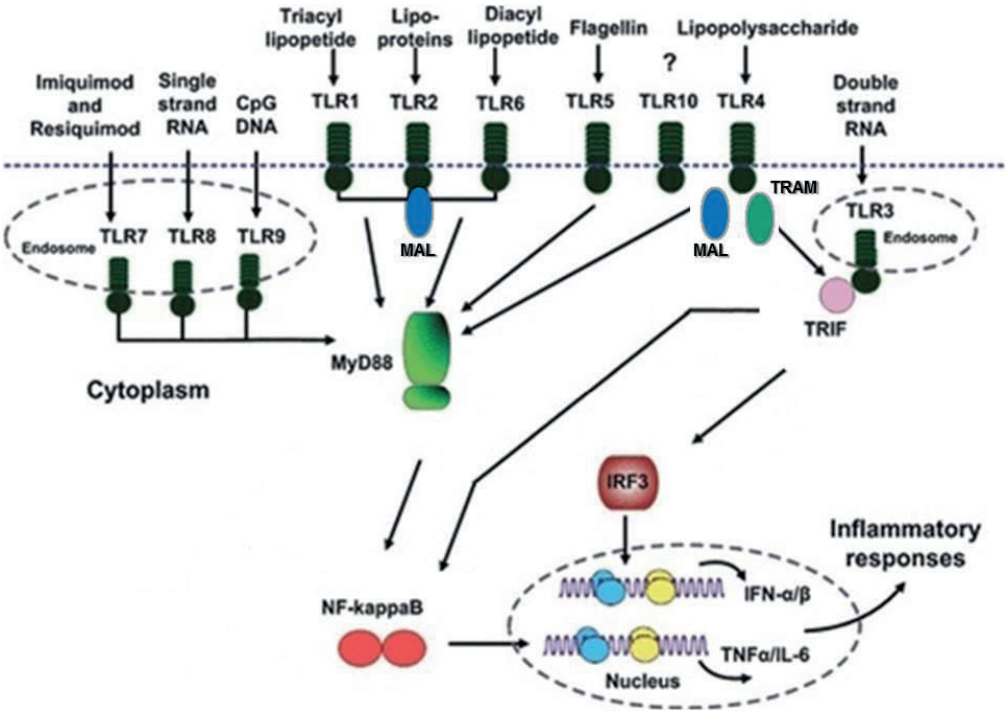

Figure 1: TLR1 - TLR10, their ligands, cellular localization and the signalling pathways they activate. *Adapted from Xie et al. [21], modified and reproduced with permission.*

1.2.3. Toll-like receptor signalling

Upon ligand binding, tighter interactions between the two TLR molecules forming a dimer are induced. This promotes a conformational change where the two cytosolic TIR domains are brought closer together, and adaptor molecules containing TIR domains are recruited to the TIR domains of the different TLRs [5, 9]. The primary adaptor molecules utilized by TLRs are called myeloid differentiation factor 88 (MyD88) and TIR-domain containing adaptor inducing protein inducing IFN-β (TRIF), and these are linked to certain TLRs by one of two bridging adaptors; myeloid adaptor-like protein (MAL) or TRIF-related adaptor molecule (TRAM) (Figure 1) [22]. MyD88 and TRIF activate downstream transcription factors such as nuclear factor (NF)-κB, IFN regulatory factor-3 (IRF-3) and various mitogen-activated protein kinases (MAPKs), inducing the production of inflammatory mediators (Figure 1) [5, 23]. The numerous TLR signalling pathways can therefore be divided into two groups: MyD88- and TRIF- dependent signalling pathways [9]. All TLRs, except TLR3, utilize MyD88 in their activation of NF-κB through a cascade of kinases, leading to the induction of inflammatory cytokines such as IL-6, IL-8 and interferon-γ-inducible protein (IP)-10 (Figure 1) [9, 23, 24]. TLR2 and TLR4 depend on the additional adaptor molecule MAL for the recruitment of MyD88 (Figure 1) [9]. TLR4 and TLR3 activate both NF-κB and IRF-3 by using TRIF as adaptor molecule, and this induces the production of both type I IFNs and cytokines (Figure 1) [9]. TRAM is essential for the activation of TRIF by TLR4 (Figure 1) [9]. It has also been shown that the TRAM/TRIF pathway is important for TLR2 signalling [25].

HMGB1 - The archetypical DAMP

HMGB1 is one of the first identified DAMP molecules [26]. The functions of HMGB1 depend on location, posttranslational modifications and binding partners of the molecule [27, 28]. HMGB1 organizes DNA and normally functions as a transcription factor in the nucleus. Upon cell activation or injury, nuclear HMGB1 can translocate to the cytoplasm, where it is involved in regulation of autophagy and apoptosis. HMGB1 is actively secreted from cells when they are infected by pathogens or stimulated by pro-inflammatory cytokines (Figure 2). HMGB1 is also passively released from necrotic or damaged cells (Figure 2). When secreted or released into the extracellular milieu, HMGB1 acts as a danger signal and binds to TLR2,

TLR4, receptor for advanced glycation end products (RAGE) and C-X-C chemokine receptor type 4 (CXCR4) (Figure 2) [28, 29]. TLR ligation triggers MyD88-mediated NF-κB activation and induces production of pro-inflammatory cytokines, and maturation and migration of immune cells (Figure 2). The association of HMGB1 with RAGE has been implicated in cell migration, cell growth, differentiation and autophagy (Figure 2). HMGB1 forms a complex with C-X-C motif ligand (CXCL)12 that is recognized by CXCR4, exerting cell migration (Figure 2).

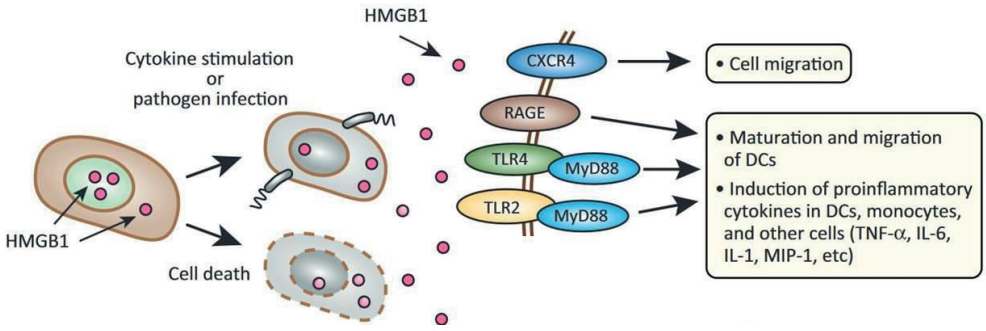

Figure 2: Receptors for extracellular HMGB1. *Adapted from Yanai et al. [29], modified and reproduced with permission.* DC, dendritic cells.

Receptor usage and subsequent biological activities of HMGB1 are determined by the redox state of three cysteines in the HMGB1 molecule (C23, C45 and C106) (Figure 3) [30]. The pro-inflammatory activity of HMGB1 requires a disulfide link and one thiol group (Figure 3). The disulfide-HMGB1 binds and signals via the TLR4/myeloid differentiation factor-2 (MD-2) complex and CD14 co-receptor to induce cytokine production (Figure 2) [31-33]. Acting as a cytokine, disulfide-HMGB1 initiates and perpetuates immune responses during infectious and sterile inflammation, and has been associated with divergent clinical conditions such as sepsis, rheumatoid arthritis and atherosclerosis [34-36]. HMGB1 expressing three cysteine thiol residues (all-thiol-HMGB1) exerts chemoattractant activity by forming a complex with C-X-C motif chemokine ligand 12 (CXCL12), which binds to the receptor CXCR4 and initiates chemotaxis (Figure 2 and 3) [37]. By this mechanism inflammatory cells are recruited to the site of HMGB1 release. The “chemokine-HMGB1” and the “cytokine-HMGB1” cannot activate each other’s signaling pathways [38]. Oxidized-HMGB1, with any

of the cysteines oxidized, has no identified pro-inflammatory activity but accumulates during resolution of inflammation (Figure 3) [28].

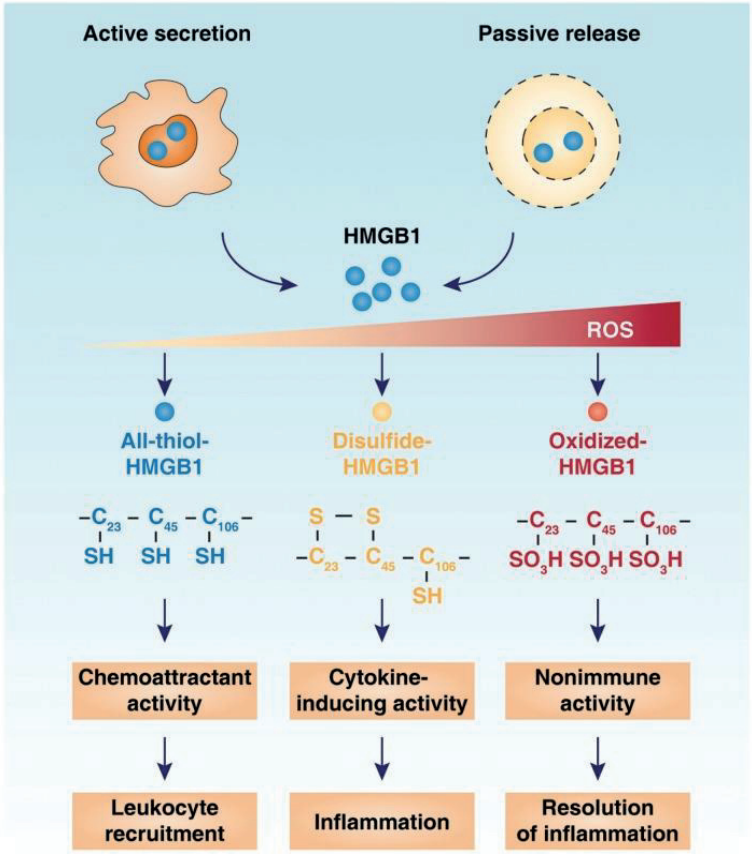

Figure 3: HMGB1 redox states. *Adapted from Tang et al. [39] and reproduced with permission.* ROS, reactive oxygen species.

1.2.4. Nod-like receptors and inflammasomes

NLRs are cytoplasmic PRRs that recognize microbial products and DAMPs, exerting downstream signalling that culminate in transcriptional activity and inflammatory responses [10]. In humans, 22 members of the NLR family have been identified [10]. These receptors are characterized by a C-terminal LRR domain, a central NOD domain (also known as

NACHT domain) and a N-terminal protein-protein interaction domain [10]. The LRR domain is thought to be involved in ligand interaction, whereas the NOD domain has ATPase activity and is thought to play a role in the oligomerization of proteins [40]. Although NLRs share common LRR and NOD domains, the N-terminal regions differ between NLRs.

Members of the NLR family are central components in the formation of so-called inflammasomes, which are multiprotein signalling platforms assembling upon the detection of various PAMPs and DAMPs [41]. Following detection of a ligand, the inflammasomes activate caspases to produce cytokines and induce pyroptotic cell death [41]. Five distinct inflammasomes have been identified, with the assembly of each being dictated by a unique PRR [41]. Three of the five inflammasomes are regulated by NLR members; NOD-like receptor protein (NLRP)1, NLRP3, and NLR family CARD domain containing 4 (NLRC4) [41]. The NLRP3 inflammasome is the most studied inflammasome, and the only inflammasome known to sense a wide variety of DAMPs [42, 43].

1.2.5. NLRP3 inflammasome ligands

NLRP3 (also known as cryopyrin) responds to a surprisingly various set of stimuli, including several bacterial, fungal and protozoan pathogens, and structurally diverse DAMPs, such as adenosine triphosphate (ATP), asbestos, silica, glucose, alum and amyloid-β [41, 42]. Cholesterol and uric acid crystals are also known agonists of the NLRP3 inflammasome and inducers of inflammatory disease [44, 45]. However, the exact mechanisms by which NLRP3 is activated by these diverse ligands are still unclear. There is no evidence that NLRP3 binds directly to any of its diverse activators, and given the wide variety of stimuli, it is likely that NLRP3 responds to a common cellular event that is triggered by these activators [41, 46]. Many different mechanisms have been proposed (Figure 4), including 1) ATP-induced potassium efflux and formation of large membrane pores allowing extracellular NLRP3 agonists to enter the cytosol and directly engage NLRP3, 2) engulfment of crystalline or particulate NLRP3 agonists followed by lysosomal destabilization and sensing of lysosomal content by NLRP3, and 3) PAMP/DAMP-induced production of reactive oxygen species (ROS) [41, 42].

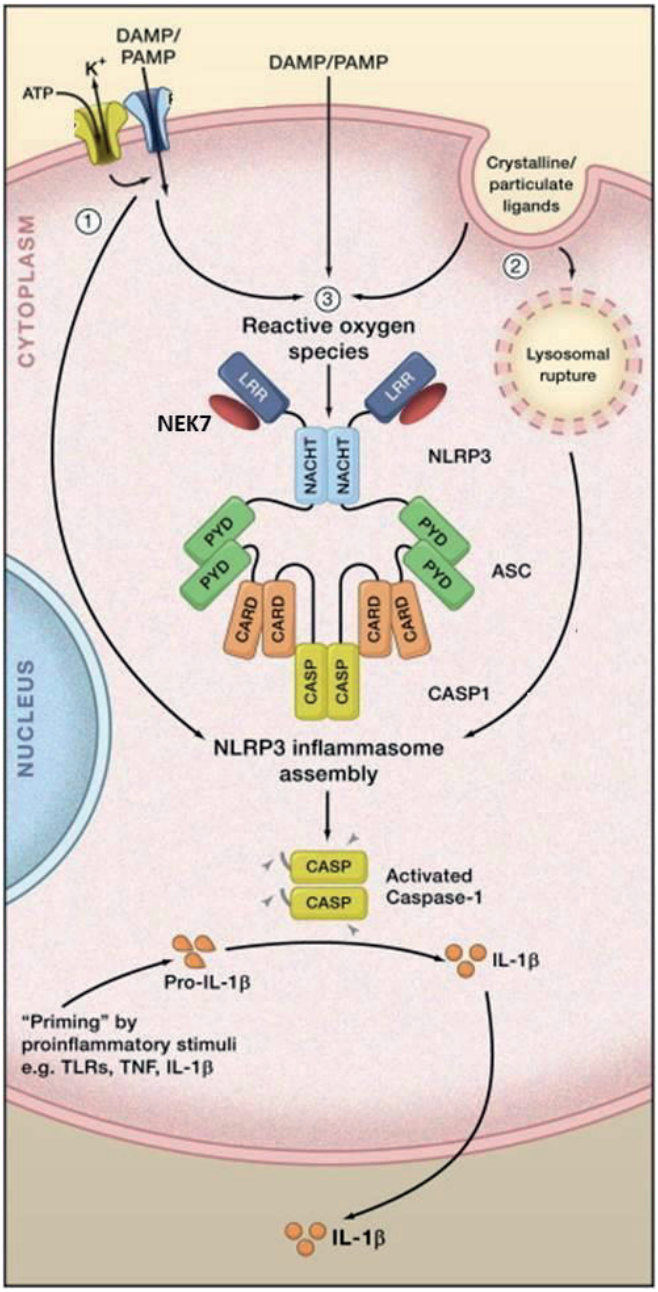

Figure 4: NLRP3 inflammasome activation, assembly and downstream signalling.
Adapted from Schroder and Tschopp [42], modified and reproduced with permission.

1.2.6. Signalling through the NLRP3 inflammasome

After direct or indirect activation by the ligand, NIMA-related kinase 7 (NEK7) directly binds NLRP3 inducing NLRP3 oligomerization, and each NLRP3 of the oligomer binds to apoptosis-associated speck-like protein containing a CARD (ASC) via the homotypic interaction of their pyrin domains (PYDs) (Figure 4). ASC in turn binds to the inactive precursor form of the caspase-1 cysteine protease via its caspase recruitment domains (CARDs), which becomes active and cleaves pro-IL-1β (p35) to its biologically active form (p17) (Figure 4) [41]. Mature IL-1β is a potent pro-inflammatory cytokine that activates immune cells and initiate the acute phase response, and its production is associated with the severity of several pathologies, including atherosclerosis and diabetes [46, 47].

It is well described that a "priming" signal is necessary prior to NLRP3 inflammasome assembly and activation by its ligand [41]. The release of mature IL-1β is tightly controlled by induction of pro-IL1β and upregulation of NLRP3 transcription [48]. NF-κB activation is required for this priming step, and is typically accomplished *in vitro* using a microbial TLR ligand (Figure 4) [43]. However, during the progression of sterile inflammatory diseases, inflammasome activation is primed by endogenous factors such as ROS, oxidized LDL, complement factors, amyloids and misfolded proteins [43].

Uric acid and cholesterol crystal activation of NLRP3

Cholesterol crystals and uric acid crystals trigger NLRP3 activation mainly through phagocytosis followed by lysosomal damage [49]. Thus, NLRP3 does not sense the crystals per se, but recognizes lysosome contents leaking into the cytosol (e.g. the lysosomal proteases cathepsin B and L) [45, 49, 50]. In addition, uric acid crystals can directly engage the cellular membrane without any known receptor being involved [49]. Recent studies have shown that the complement system controls several cellular processes involved in cholesterol and uric acid crystal-induced NLRP3 activation [51, 52]. The complement system is an integral component of the innate immunity and has been shown to contribute to the pathology of several inflammatory diseases [53]. Activation of complement lead to the generation of the potent anaphylatoxin C5a and the terminal complement complex (TCC). C5a can trigger inflammasome activation in the presence of crystalline material in human cells, and tumor necrosis factor (TNF) might help sustain this priming of NLRP3 over time [51, 52].

1.3. HUMAN PREGNANCY AND THE PLACENTA

The human pregnancy starts when a maternal egg cell is fertilized by a paternal sperm cell and attaches to the maternal uterine wall. The trophoblast is the first cell lineage to differentiate during fetal development. This happens four to five days after conception, when the fertilized egg is between the state of morula and blastocyst [54]. A blastocyst consists of the embryoblast surrounded by a single layer of mononucleated trophoblasts (Figure 5). The cell mass constituting the embryoblast will later in pregnancy become the embryo, umbilical cord and mesenchymal parts of the placenta, while most of the placenta will rise from the trophoblasts [54].

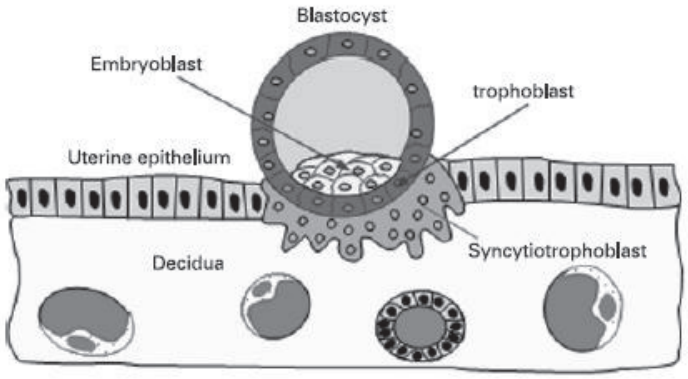

Figure 5: Blastocyst attachment to the uterine wall and formation of the syncytiotrophoblast. *Adapted from Huppertz [54], modified and reproduced with permission.*

1.3.1. The placenta

During pregnancy, the important interchange between the mother and her fetus is provided by the placenta - the fetal organ supporting normal growth and development of the fetus [54, 55]. The placenta has four main functions. The first is transport of oxygen, water and nutrients (carbohydrates, amino acids, lipids, vitamins and minerals) to the fetus, and removal of carbon dioxide and other waste products. Second, several substances are metabolized in the placenta before released into maternal and/or fetal circulation. Third, the placenta has an important endocrine function, providing both fetus and mother with hormones that affect pregnancy, fetal growth, metabolism, parturition and other functions. Fetal/maternal

communication through such blood-borne substances is essential because the placenta does not hold any nerves [55]. Last, but not least, the placenta protects the fetus against harmful molecules, infections and maternal disease [55].

The placentation starts when mononucleated cytotrophoblasts overlying the embryoblast undergoe fusion and generate an oligonucleated syncytiotrophoblast, also referred to as the syncytium (Figure 5) [54]. The syncytiotrophoblast displays an invasive phenotype, making the blastocyst able to penetrate the uterine epithelium. At this stage of development the syncytiotrophoblast is the only embryonic tissue coming into direct contact with maternal cells and fluids, while the mononucleated cytotrophoblasts remain in the second row without contacting maternal tissues. The cytotrophoblasts display stem cell properties, with rapid cell division and subsequent fusion with the syncytiotrophoblast, resulting in a expansion of the syncytium that will continue throughout pregnancy. The establishment of the syncytium is of great importance for the further development of a well-functioning placenta. At birth, an average placenta has the full diameter of about 22 cm and a thickness of 2,5 cm, weighing about 420 g [54]. The flat and circular organ has a fetal surface; the chorionic plate, and a maternal portion; the decidua basalis, and these parts of the placenta are separated by the intervillous space and chorionic villi (Figure 6) [54, 56].

The chorionic plate contains chorionic vessels that receive oxygen-poor fetal blood from the umbilical arteries and transports well oxygenated blood back through the single umbilical vein (Figure 6) [55]. Decidua basalis, from now on called decidua, is the area of endometrium lying between the placental villi trees and the maternal myometrium (Figure 6 and 7). It is a mixture of fetal trophoblast cells, different maternal cells (decidual stroma cells and immune cells) and components of the extracellular matrix (Figure 7) [6, 54, 56]. The intervillous space contains branched villous structures filled with fetal arteries and veins (blood cells, endothelial cells, smooth muscle cells) and villous stroma (fibroblasts and leukocytes) (Figure 6) [54, 57, 58]. These chorionic villi are bathed in maternal blood, which is released into the intervillous space from maternal circulation via spiral arteries located in the endometrium (Figure 6 and 7) [6, 54]. At the terminal branches of the villi trees, only three or four cell layers separate the fetal and maternal blood, and this is where the majority of exchange between mother and fetus occurs [55]. The most distal parts of the villous trees are left only with cytotrophoblasts and syncytiotrophoblast, serving the decidua with a third subtype of

trophoblasts; extravillous trophoblasts (Figure 7) [6, 54, 55]. Maternal blood drains back into the mother's circulation through endometrial veins after the fetal/maternal exchange (Figure 6) [54].

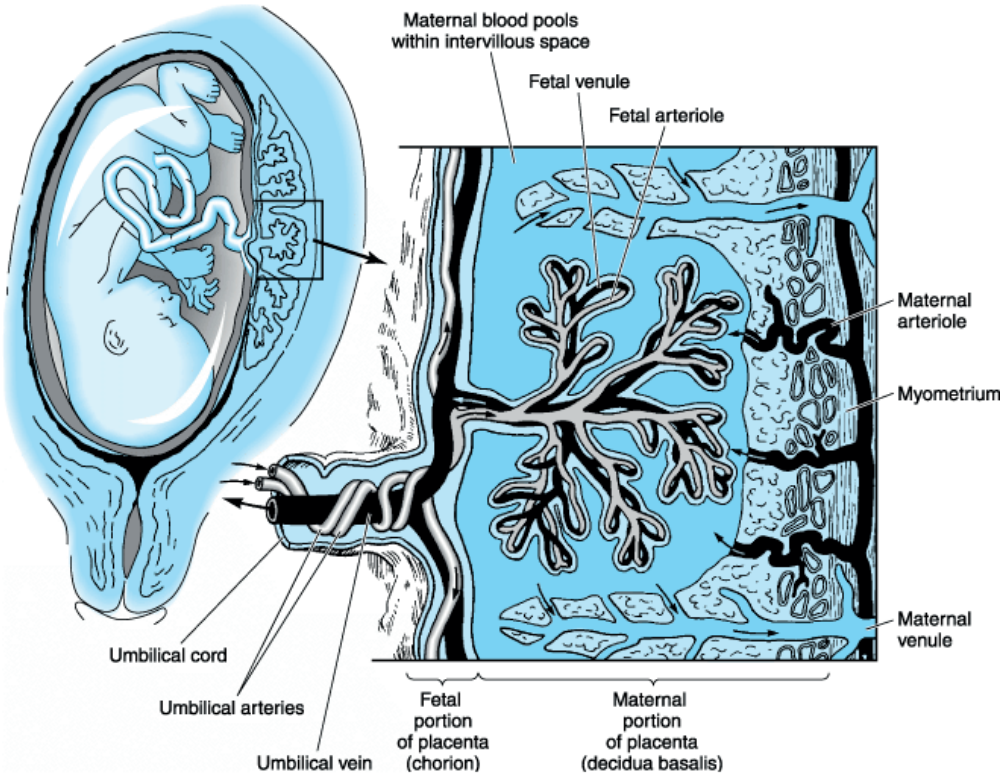

Figure 6: Illustration of fetal and placental localization inside the uterus, and the important components of a well-adapted placenta. The chorion and the decidua basalis are separated by the intervillous space and chorionic villi. *Adapted from Morgan et al. [56], and reproduced with permission.*

1.3.2. Remodelling of maternal spiral arteries

To assure adequate delivery of oxygen and nutrients to the placental intervillous space and further to the fetus, it is essential that the maternal spiral arteries are converted into dilated vessels (Figure 7 and 8) [6]. This provides the reduced contractility, pressure and rate of blood flow necessary for a constant influx of maternal blood to the placenta [59]. The transformation process depends on the extravillous trophoblasts invading the maternal endometrium early in pregnancy, destructing the arterial muscular wall and temporarily and partly replacing the arterial endothelium (Figure 7) [6, 54, 60, 61].

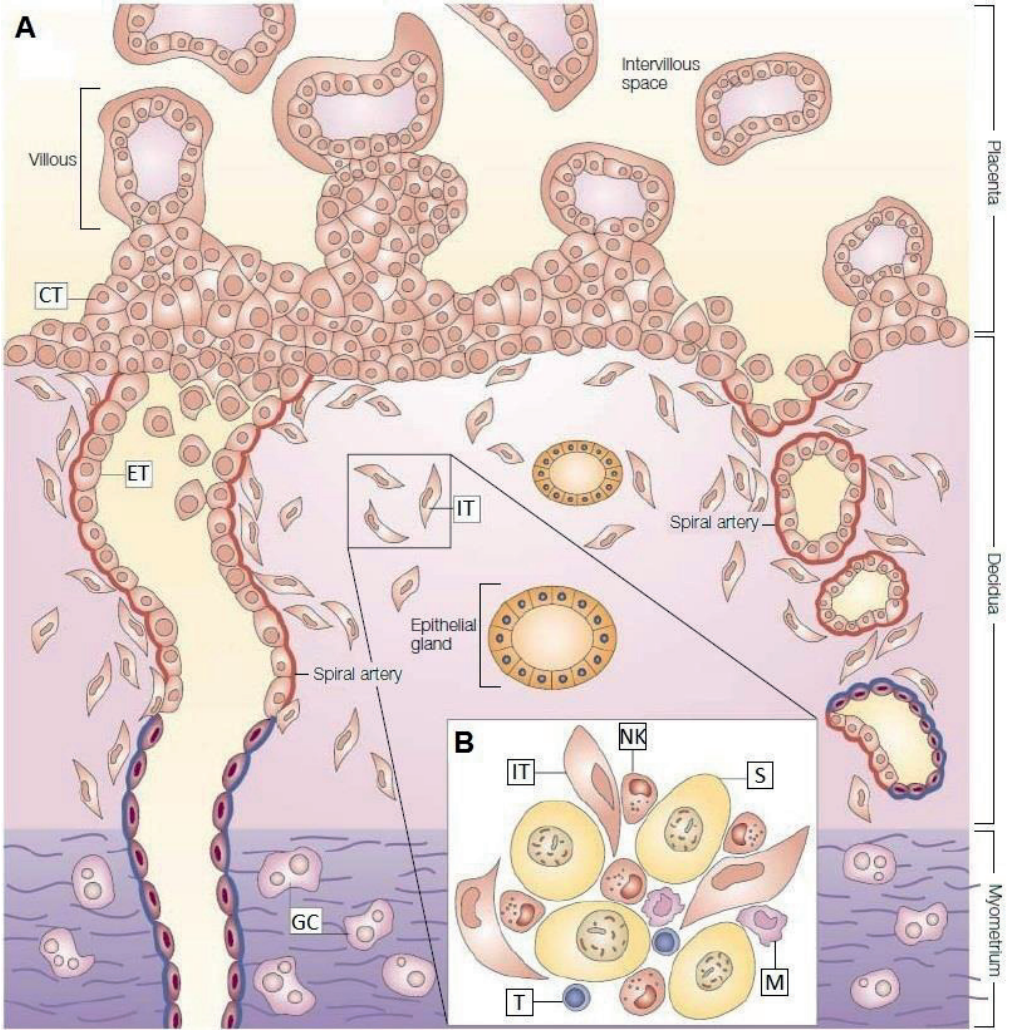

Figure 7: Trophoblast migration into maternal decidua, spiral arteries and myometrium (A) and cells of the decidual stroma (B). Trophoblast migration into the maternal portion of the placenta and dilation of maternal spiral arteries is essential for the formation of an optimal placenta. Cytotrophoblasts (CT) migrate from the chorion to the distal parts of the villous trees, where they differentiate and serve the decidua with two types of extravillous cytotrophoblasts; interstitial trophoblasts (IT) and endovascular trophoblasts (ET). The arterial muscular walls are destroyed by the ITs and replaced by fibrinoid material, which result in dilated arteries, and the arterial endothelial cells are partly replaced by ETs. Some trophoblasts migrate all the way into the maternal myometrium and form giant cells (GC). In the decidua, trophoblasts come in close contact with maternal stroma cells (S), NK-cells (NK), macrophages (M) and T cells (T), and the establishment of a state of immune tolerance, between the fetal and maternal cells, is considered crucial for successful trophoblast invasion. *Adapted from Moffett-King [6], and reproduced with permission.*

Throughout pregnancy, the amount of oxygen and nutrients demanded by the growing fetus is steadily increasing. If these demands are not matched by increased blood flow and placental growth, it may have dramatic effects on the intrauterine environment, and consequently, the fetal development [55]. When comparing the spiral arteries in a pregnant woman with a non-pregnant woman, the spiral arteries are invaded by trophoblasts, less curly and substantially diluted (Figure 8). However, if the trophoblast invasion is hampered, the important remodeling and dilation of maternal spiral arteries will fail, leading to formation of an insufficient placenta and potentially result in pregnancy complications such as preeclampsia and fetal growth restriction (FGR) (Figure 8) [6, 59].

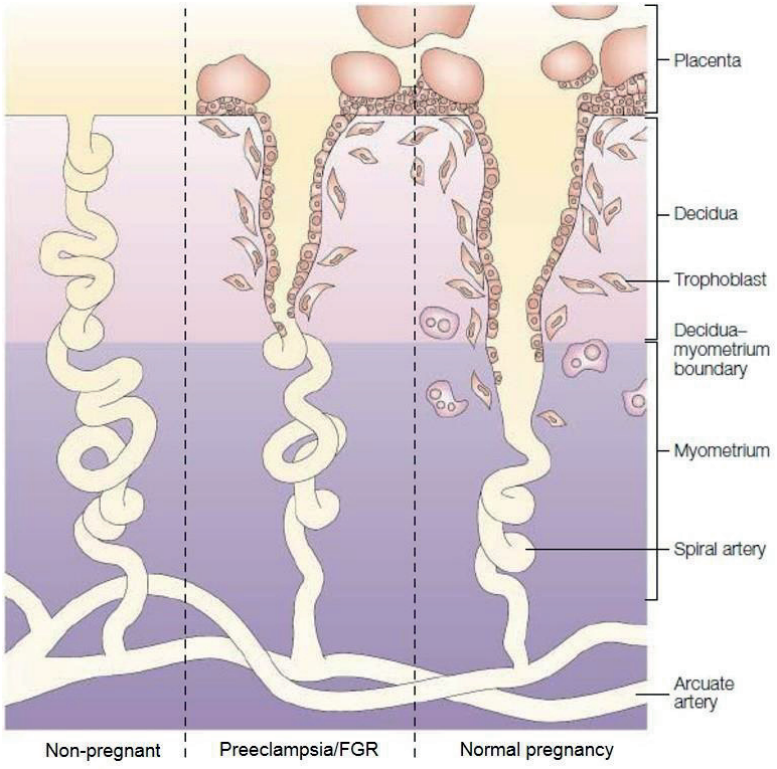

Figure 8: Trophoblast invasion and remodeling of maternal spiral arteries in normal and complicated pregnancies. In normal pregnancies, trophoblast migration into the decidua promotes remodeling and dilation of the maternal spiral arteries to assure adequate delivery of oxygen and nutrients to the placenta. Reduced trophoblast invasion early in pregnancy and less dilated spiral arteries can lead to the development of pregnancy complications such as preeclampsia and FGR later in pregnancy. *Adapted from Moffett-King [6], modified and reproduced with permission.*

1.4. PREECLAMPSIA

Preeclampsia is an inflammatory pregnancy disorder. Importantly, preeclampsia can occur in patients with hydatidiform moles [62], proving that a placenta, and not the fetus, is required for the development of this disease. Indeed, the only effective treatment for preeclampsia is delivery of the placenta. The pathophysiology of preeclampsia is highly complex, however the actual cause of this pregnancy complication remains largely unknown and many theories have been proposed [63]. Therefore, the disease is often called “the disease of theories”, reflecting the wide range of potential causes, clinical signs and complications that are associated with preeclampsia.

1.4.1. Prevalence, definition and diagnosis of preeclampsia

Worldwide, 2-8 % of all pregnancies are affected by preeclampsia, and a majority of maternal and perinatal mortality and morbidity is caused by preeclampsia [63]. In low- and middle-income countries 10-15 % of direct maternal deaths are associated with preeclampsia and eclampsia [64]. The rate of preeclampsia in the USA has been slightly increasing during the past 30 years [65]. This increase might be related to an increased prevalence of disorders predisposing towards preeclampsia, such as chronic hypertension, diabetes mellitus and maternal obesity [65]. In high-income countries, such as Norway, Sweden, Canada and Australia, there has been a consistent decline in the prevalence of preeclampsia since 2002, despite the increasing rates of predisposing disorders [66]. This may be due to elective delivery prior to due date for high-risk women and the use of interventions that reduce the risk of preeclampsia progression [67].

Preeclampsia is a pregnancy disorder principally diagnosed by increased blood pressure (hypertension) and increased urinary protein (proteinuria) in the mother, both occurring later than mid-gestation [68]. The diagnostic clinical criteria for preeclampsia in Norway are given by the Norwegian association for Obstetrics and Gynecology [69]. According to these guidelines, preeclampsia is defined as a pregnancy-specific syndrome that occurs after week 20 with 1) *de novo* appearance of hypertension of ≥140/90 mmHg and 2) proteinuria of ≥0.3 g/L in a 24-hour urine sample, and both hypertension and proteinuria should be measured twice.

The clinical presentation of preeclampsia is heterogeneous. Women are mostly asymptomatic, and the disease is often diagnosed during routine antenatal care [70]. However, different sub-phenotypes of preeclampsia have been defined according to severity of maternal outcomes, time of onset and presence of FGR [71]. Placental dysfunction may complicate pregnancy also for the fetus, and consequently, preeclampsia is often associated with FGR.

1.4.2. Aetiology and pathogenesis of preeclampsia

Multiple pathogenic mechanisms have been implicated in preeclampsia, however the leading hypotheses strongly rely on disturbed placental development and function in early pregnancy, with abnormal trophoblast invasion into the decidua and impaired remodelling of the maternal spiral arteries as a primary causative factor [63]. However, the failure of physiological transformation of the spiral arteries is neither specific to preeclampsia nor sufficient to cause it [72]. Therefore, preeclampsia should be considered a multisystem disorder that is associated with several pathological features including immune maladaptation, impaired placentation, intrauterine infections, angiogenic imbalance, placental stress (oxidative stress and endoplasmic reticulum (ER) stress), inflammation and endothelial dysfunction [72-74]. It has become increasingly clear that preeclampsia is not one disorder, but rather different aetiologies leading to a common phenotype (hypertension and proteinuria), with manifestation of one or more insults in the placenta [72]. The complex interplay between factors produced by the placenta and the maternal adaptions to these factors are crucial for the development of preeclampsia.

1.4.3. Inflammation in pregnancy and preeclampsia

A mild early intrauterine as well as a later systemic state of inflammation is established in all pregnant women, representing the body's adaption to pregnancy [75, 76]. It has been proposed that the early and local inflammation is due to a controlled production of pro-inflammatory cytokines by maternal immune cells upon interaction with trophoblasts invading the decidua during placental development [6, 75, 76]. The late and systemic inflammation is also associated with enhanced cytokine production, but by circulating immune cells in maternal blood or maternal endothelial cells upon their meeting with villous trophoblasts or activators released from the placenta [77]. Inflammation in pregnancy causes metabolic adaptions such as insulin resistance [78] and hyperlipidemia [79] to ensure fetal

growth and development, and parturition depends on inflammation in the final trimester [75, 76, 80].

Preeclampsia is characterized by an exaggerated inflammation, both locally in the placenta and systemically in the mother [74, 75]. The enhanced production of pro-inflammatory cytokines observed in preeclamptic placentas is believed to arise from the interplay between several processes. Shallow trophoblast invasion and poor spiral artery remodeling and, consequently, reduced placental perfusion, increased oxidative/ER stress and elevated levels of DAMPs released from apoptotic cells [77, 81-83]. There are close links between these processes, with each being able to induce the others. Intrauterine infections by PAMP-bearing microorganisms leading to induction of pro-inflammatory cytokines have also been suggested a role in preeclampsia [73]. It is further hypothesized that inflammatory mechanisms generate atherosclerotic-like lesions termed acute atherosis in the decidual spiral arteries of preeclamptic women [84]. These lesions are characterized by the accumulation of lipid-filled foam cells, fibrinoid necrosis and leukocyte infiltration, and likely impair intervillous blood flow contributing to placental oxidative stress and dysfunction [84].

The elevated systemic inflammation observed in preeclamptic women is thought to be triggered by activators released from an already stressed and inflamed placenta into the maternal circulation [85]. E.g. products of oxidative/ER stress, DAMPs, pro-inflammatory cytokines, syncytiotrophoblast microparticles and soluble fms-related tyrosine kinase 1 (sFlt-1). The levels of all these activators are significantly increased in preeclampsia [81, 83, 85]. As the burden on the placenta increases with fetal growth, the release of inflammatory factors escalates after mid-pregnancy, triggering the late stage of preeclampsia with maternal systemic inflammation and endothelial dysfunction. Endothelial dysfunction or inappropriate endothelial activation is a hallmark of the development of preeclampsia [86]. As important players in the inflammatory network, endothelial cells stimulate and are stimulated by immune cells. In preeclampsia, the interaction between immune cells and endothelial cells is increased, and through the induction of capillary permeability, platelet thrombosis and increased vascular resistance excessive endothelial cell damage leads to onset of symptomatic preeclampsia manifesting as hypertension and proteinuria [86]. Several pathophysiological features are shared between preeclampsia and cardiovascular diseases, including vascular

lesions, systemic inflammation and endothelial dysfunction, and women with a history of preeclampsia have increased risk for later life cardiovascular disease [87].

Some women developing preeclampsia do not feature abnormal placentation. Instead, the mother is predisposed to systemic inflammation (e.g. chronic hypertension, obesity and insulin resistance), with the maternal vessels reacting abnormally to the stress of even a healthy pregnancy. However, cases without or with placental involvement are not easy to separate clinically.

1.5. PRRs IN PREGNANCY

PRRs are crucial mediators of inflammation, and as discussed in the previous chapter, inflammation is essential to pregnancy and relevant for preeclampsia development. PRRs provide a mechanism by which both exogenous and endogenous danger signals can initiate, sustain and worsen inflammation in pregnancy.

The current knowledge of PRRs in pregnancy extends to PRR responses in maternal immune cells and fetal trophoblasts, animal models of pregnancy complications, and observations in clinical samples, but much remains to be elucidated. The main focus of PRR research in pregnancy has been on pathogen-mediated TLR activation and the association to adverse pregnancy outcomes such as preterm delivery (PTD) [88]. However, a growing body of evidence implicate DAMP-mediated PRR activation in pregnancy-related inflammation [83, 89, 90]. Evidence also points towards the placenta being an essential source of PRR-induced cytokines and apoptosis during pregnancy [88, 91].

1.5.1. PRR-induced inflammation in the placenta

In healthy pregnancies, PRR signalling in the placenta is important for regulation of a unique immune environment required for successful implantation, growth and parturition [76, 88]. The expression of PRRs enables maternal and fetal cells to communicate with each other, modulate their surroundings and respond to PAMPs and DAMPs [23]. In the placenta, both professional immune cells such as fetal macrophages (Hofbauer cells) and non-immune cells like endothelial cells, fibroblasts and trophoblasts have been shown to express various PRRs [92-94]. All members of the TLR family and some cytoplasmic-based NLRs (NOD1, NOD2, NLRP1, NLRP3 and NLRC4) have been detected in human placentas [90, 95-100], and the

placental expression of these receptors is dominated by trophoblasts (Table 1). In addition, a recent study showed expression of RLRs in human term trophoblasts [101].

Different trophoblast phenotypes express PRRs, including extravillous trophoblasts [92, 102], cytotrophoblasts [96, 103] and syncytiotrophoblasts [104-106], indicating the importance of these immune receptors in placental cells that display different biological functions such as migration, tissue preservation and immunity. PRR expression reported in first trimester trophoblasts is somewhat more restricted than at a later gestation (Table 1).

Table 1. Pattern recognition receptor expression and function in trophoblasts*.

	First trimester	Third trimester	References
PRR protein expression	TLR2, TLR4	TLR2, TLR4	[89, 92, 93, 96, 97, 102, 105, 107-111]
	TLR3	TLR3	[95, 96, 100, 101, 111]
		TLR6	[107]
		TLR7, TLR8	[100, 101]
		TLR9	[101, 111]
	TLR10	TLR10	[112]
	NOD1, NOD2	NOD1	[106, 113]
	NLRP1	NLRP1	[104]
PRR gene expression	TLR2, TLR4	TLR2, TLR4	[102, 107, 114]
		TLR3	[107]
		TLR5	[107]
		TLR6	[107]
	TLR8		[115]
	TLR10	TLR10	[112]
		NOD1	[106]
	NLRP1, NLRP3	NLRP1, NLRP3	[103, 104]
***In vitro* PRR activation**	TLR4	TLR2, TLR4	[93, 95, 107, 114, 116-119]
	TLR3		[95, 120, 121]
		TLR6	[107]
	NOD1, NOD2	NOD1	[106, 113, 122]
		NLRP3	[123]

* The table summarizes findings from trophoblasts located in human placental tissue samples or primary trophoblasts isolated from human placentas.

In vitro PRR activation experiments in trophoblasts isolated from human placentas have confirmed the functionality of some of these receptors (Table 1), and additional PRRs have been reported as functional in trophoblast cell lines [100, 115, 124, 125]. Ligation of TLR4 or NLRP3 typically initiates pro-inflammatory responses characterized by IL-6 and IL-8 or IL-1β production, respectively [114, 123]. Stimulation of TLR3 can induce the release of various chemokines and IFN-β [95, 121], while the TLR2 ligand peptidoglycan may cause apoptosis in trophoblasts [102, 112]. Collectively, these findings support the hypothesis implying that

trophoblasts are important immune competent cells of the placenta, protecting the fetus from invading pathogens and sterile damage, recruiting professional immune cells to the site of danger, and regulating cell death [88], but further studies concerning PRR activation of trophoblasts are needed to determine their role in the immune system.

1.5.2. PRRs in preeclampsia

It can be harmful for the pregnancy if the immune system, which is already activated because of the pregnancy itself, mounts an over-active inflammatory response towards PAMPs or DAMPs [73, 77]. Several studies have been performed to elucidate the role of PRRs in development of preeclampsia and other pregnancy complications [15]. Injection of PRR ligands in pregnant rodents has shown association between PRR activation and preeclampsia symptoms [100, 126, 127]. In humans, PRR gene polymorphisms have been suggested to affect the susceptibility to preeclampsia [128, 129]. Further, increased expression of PRRs and pro-inflammatory cytokines have been observed both systemically [130-138] and in the placenta [89, 100, 110, 111, 130, 137, 139, 140] of women with preeclampsia compared to healthy pregnant women.

In preeclamptic pregnancies, the harmful inflammation may be triggered by PRR-mediated pro-inflammatory cytokine production in response to PAMPs or DAMPs in a stressed or infected placenta [88, 91]. Both synthetic PRR ligands and microbes have been shown to activate PRRs in trophoblasts, promote secretion of pro-inflammatory cytokines and enhance migration of immune cells [15, 23, 73, 95, 118, 124]. In addition, PRR activation enhance placental apoptosis, a phenomena that is associated with preeclampsia and FGR [15]. It is believed that the induction of an exaggerated local inflammation during placental development may cause unsuccessful trophoblast invasion of the maternal endometrium and failed transformation of spiral arteries [73]. For example, pro-inflammatory cytokines such as IL-6, IFN-γ and TNF-α have been shown to be cytotoxic to trophoblasts and inhibit their migration into the decidua [73, 141-145]. An improper transformation of endometrial arteries may further lead to preeclampsia by inducing placental hypoxia, placental apoptosis and the release of cellular debris [73]. Hypoxia-induced reactive oxygen species, endogenous molecules released by apoptotic cells and cellular debris can all activate PRRs expressed locally in the placenta or by maternal immune cells circulating in the maternal blood,

subsequently leading to production of pro-inflammatory cytokines known to play a role in the inflamed placenta in preeclampsia. Therefore, PRRs have been assigned an important role in both the early placental inflammation and the late systemic inflammation observed in preeclamptic women.

PRR involvement in preeclampsia development has predominantly been examined in response to bacterial and viral ligands, even though infection is not directly related to preeclampsia [73]. However, PRR recognition of endogenous danger dignals most likely contributes significantly to preeclampsia development by mediating sterile inflammation [146]. In addition to dysregulated expression of the PRRs, enhanced concentrations of many DAMPs such as HMGB1 and cholesterol are present in the placenta and/or the maternal circulation of preeclamptic women [146].

HMGB1 in preeclampsia

Pathological processes such as necrosis, hypoxia and oxidative stress are all central to the development of a preeclamptic placenta [147], and these processes induce release of HMGB1 [26, 148, 149]. Preeclampsia has recently been associated with increased HMGB1 protein expression in placental tissue and localized to the cytoplasm of trophoblasts [150, 151]. Others have linked trophoblast-derived HMGB1 to the maternal endothelial dysfunction observed in preeclampsia [152, 153]. Supporting this, elevated concentrations of extracellular HMGB1 [150, 154, 155] and HMGB1-expressing placental debris [156, 157] have been detected in the circulation of preeclamptic women. Acting as a DAMP, extracellular HMGB1 located to the placenta or in the maternal blood has the ability to induce the release of pro-inflammatory cytokines from trophoblasts in a TLR4-dependent manner [158]. Although several studies indicate that HMGB1 may play a role as mediator of inflammation in preeclampsia, the findings are controversial [159, 160].

Cholesterol and uric acid in preeclampsia

Cholesterol and uric acid (monosodium urate, MSU) have been shown to be elevated in the maternal circulation of pregnancy pathologies such as preeclampsia and FGR [161-168]. An association between maternal early pregnancy hypercholesterolemia and the subsequent risk of preeclampsia is reported [161]. In addition, Baumann et al. reported accumulation of

cholesterol in preeclamptic placentas, and associated this with defect cholesterol transporter proteins [169]. Elevated cholesterol levels has also been observed in decidua in women with preeclampsia [170]. Accumulated cholesterol crystals are recognized as a hallmark of atherosclerotic lesions, activating inflammation via the NLRP3 inflammasome [45], suggesting an inflammatory role also for cholesterol activation of NLRP3 at the maternal fetal interface. Recently, convincing data on uric acid crystal activation of the NLRP3 inflammasome in human placentas was published [123]. This study also demonstrated that injection of uric acid crystals into pregnant rats led to FGR, which was characterized by a pro-inflammatory profile and immune cell infiltration of the placenta [123]. Furthermore, studies using trophoblast cells have shown that IL-1β is produced in response to uric acid crystals via activation of the NLRP3 inflammasome [104, 123, 171]. Thus, elevated uric acid associated with preeclampsia could be related to activation of the NLRP3 inflammasome in trophoblast, further increasing the IL-1β levels in the placenta and contributing to the pathogenesis of preeclmapsia and FGR.

2. AIMS

Inflammation is an immune response induced to protect the body from infections and tissue damage, and PRRs are crucial for initiating the inflammatory responses needed to eliminate pathogens and repair the damage. Although inflammation is usually both necessary and beneficial, it also has the potential to cause substantial damage if its extent or duration is dysregulated. Healthy pregnancy is characterized by a low-grade placental inflammation as well as a mildly increased maternal systemic inflammatory state, while an exaggerated inflammatory response in the woman and the placenta is associated with the development of preeclampsia. This pathogenic inflammation is thought to be initiated by insufficient placental development and mediated by activators released from a stressed and inflamed placenta. Fetal trophoblasts are the main cell type of the placenta and express PRRs. A role for trophoblast PRRs in placental inflammation is strongly implicated, and PRR-mediated placental inflammation may be linked to the exaggerated inflammation observed in preeclamptic women, but the knowledge is limited.

The main aim of this thesis was to characterise PRR-mediated placental inflammation in healthy and preeclamptic pregnancies. This aim was accomplished by addressing the following specific aims:

I. Identify trophoblast immune potential in early pregnancy by broadly examining TLR1-10 gene expression and activation in primary first trimester trophoblasts (paper I).

II. Assess the utility of trophoblast cell lines as models for primary trophoblasts by identifying their immunological role through characterization of TLR1-10 gene expression and activation (paper II).

III. Investigate if HMGB1 activation of TLR2 or TLR4 is involved in placental inflammation in healthy and preeclamptic pregnancies (paper III).

IV. Investigate whether the inflammatory pathway of cholesterol crystal-induced NLRP3 inflammasome activation is part of placental inflammation and a possible mediator of the harmful inflammation in preeclampsia (paper IV).

3. MAIN RESULTS

Paper I: Functional Toll-like receptors in primary first-trimester trophoblasts.

In this study, gene expression of all ten TLRs in primary trophoblasts isolated from first trimester placental tissue was reported. All included trophoblast populations expressed TLR1, TLR2, TLR3, TLR4, and TLR6 mRNA, while a more restricted number of trophoblast populations additionally expressed TLR5, TLR7, TLR8, TLR9 and TLR10 mRNA. Receptor specific ligand activation of TLR2/1, TLR4 and TLR5 increased IL-6 and/or IL-8 release from primary trophoblasts, and TLR3 and TLR9 ligand exposure increased the production of IL-8 and IP-10 or vascular endothelial growth factor A (VEGFA). The trophoblast cell line BeWo expressed lower TLR mRNA levels and did not respond to TLR activation compared to primary trophoblasts.

Paper II: Toll-like receptor profiling of seven trophoblast cell lines warrants caution for translation to primary trophoblasts.

TLR gene expression and receptor specific activation was analysed in seven trophoblast cell lines; five of choriocarcinoma origin and two SV40 large T antigen-transfected cell lines, and results were compared to primary trophoblasts. All choriocarcinoma cell lines demonstrated broad TLR gene expression, but *in vitro* TLR activation did not induce any functional cytokine responses. In contrast, the transfected cell lines showed a more restricted TLR gene expression pattern, but responded to TLR ligand exposure with cytokine responses; SGHPL-5 cells secreted increased levels of IL-6, IL-8, IL-12 and VEGFA after TLR3 and/or TLR4 activation, while after TLR2 activation increased IL-6 and IL-8 levels were seen. HTR8/SVneo cells responded to TLR3 activation by increased IL-6 and IFN-γ. The TLR gene expression profile and cytokine response of the SGHPL-5 cell line most closely resembled primary trophoblasts.

Paper III: The role of high mobility group box 1 (HMGB1) in placental inflammation and preeclampsia.

Protein expression of the DAMP HMGB1, its receptor TLR4, and the responsive inflammatory cytokine IL-8 was demonstrated in placental syncytiotrophoblasts by immunohistochemistry. The expression level of TLR4 was significantly increased in syncytiotrophoblasts from preeclamptic pregnancies compared to healthy pregnancies. *In vitro*, the cytokine-HMGB1 isoform induced a TLR4-dependent release of IL-8 from placental explants and cultured trophoblasts. Furthermore, maternal serum levels of IL-8 were significantly increased in preeclamptic compared to healthy pregnancies, and showed a significant positive correlation with syncytiotrophoblast expression of TLR4.

Paper IV: Inflammation in preeclampsia by nod-like receptor protein (NLRP)3 inflammasome activation in trophoblasts.

Cholesterol and uric acid were elevated in maternal serum from preeclamptic women compared to healthy pregnancies, and the levels correlated to the levels of high-sensitivity C-reactive protein (hsCRP) and the placental preeclampsia marker sFlt-1. Co-localization of NLRP3 inflammasome pathway components (NLRP3, caspase-1, IL-1β) and complement factors (C5a and TCC) was demonstrated in the placental syncytium. When comparing preeclamptic to healthy pregnancies, syncytiotrophoblast production of IL-1β and formation of TCC were significantly increased. Syncytium expression of C5a was mainly observed in preeclamptic placentas. NLRP3 pathway functionality was confirmed *in vitro* in primed placental explants and trophoblasts by crystal-induced NLRP3 dependent IL-1β production. These findings strongly support an important role for the NLRP3 inflammasome in placental inflammation and in the pathogenesis of preeclampsia.

4. DISCUSSION

4.1. METHOLOGICAL STRENGTHS AND LIMITATIONS

4.1.1. Clinical material and study design

The diagnostic criteria for preeclampsia are based on maternal signs and symptoms, and because of considerable diversity in the phenotypic representation among affected women it can be challenging for clinicians to set the diagnosis. In addition, the clinical guidelines diverge and occasionally change. As an example, the development of proteinuria is not a diagnostic requirement in all countries; the combination of hypertension and any maternal end-organ damage is sufficient for diagnosing preeclampsia in several guidelines [72]. It is therefore important to be mindful about the possibilities for inconsistencies in the clinical destription of preeclampsia when results from different studies are compared [172]. For the clinical material used in our studies (paper III and IV), preeclampsia was diagnosed following the prevailing guidelines at time of inclusion. Since the collection of preeclamptic samples extended from 2002 to 2014, the diagnoses were re-evaluated by obstetricians in 2014 as an important quality control. The 2014 version of the clinical guidelines from the Norwegian Association for Obstetrics and Gynecology [69] was applied, and consequently, the diagnostic criteria for preeclampsia and FGR are in agreement in paper III and IV.

Studies comparing placental samples from preeclamptic and healthy pregnancies are often biased by differences in gestational age at delivery, as patients with severe and early onset preeclampsia often deliver early. It is challenging to include placentas from gestational age-matched uncomplicated pregnancies for comparison (in the same way it is possible for blood samples). Control placentas from pregnancies with preterm labour may be used to overcome the potential confounding effect of gestational age [92, 110], but underlying placental pathology causing preterm labour may be shared with preeclampsia [173] and interfere with the interpretation of results. In paper III and IV, the mean gestational age of included preeclamptic placentas was significantly lower than for the healthy pregnancy placentas, and this might have influenced the measured protein expression that were compared between these groups. However, TLR4 expression is increased in preeclamptic placentas also when compared to gestational age-matched controls [110], supporting that the difference observed

in paper III is related to preeclampsia. To our knowledge, there are no reports of measured IL-1β expression in gestational age-matched controls, so this knowledge is lacking for IL-1β.

4.1.2. *In vitro* experiments: Primary trophoblasts and placental explants

For the examination of TLR expression and function in early pregnancy, primary trophoblasts were isolated from first trimester placentas (paper I and II). The cell preparations were characterized as trophoblasts based on morphology, positive expression of the trophoblast marker cytokeratin (CK) 7 and negative expression of the leukocyte marker CD45. The isolated cells also released the other common trophoblast markers human chorionic gonadotropin and human placental lactogen. However, the trophoblasts were not characterized as one specific trophoblast subtype due to limited cell yield. Based on the uniform mononuclear morphology of the cells, their ability to proliferate, no observation of syncytialization, and the fact that they were isolated from the placental villis, the cell population was most likely dominated by cytotrophoblasts [174]. Despite the quality controls performed, extravillous trophoblasts could also have been present.

For practical reasons, all trophoblast populations were frozen in liquid nitrogen prior to TLR stimulation experiments. This procedure has also been used by others [175, 176], and reported cell modifications are reduced ability to adhere, impaired syncytialization and increased cell death [177]. We experienced that freezing had minimal effect on cell viability as the cryopreserved primary trophoblasts were adherent after thawing. Their TLR functionality was also clearly preserved, as indicated by the potent cytokine responses observed in paper I and II.

In vitro cultivation of cells, including trophoblasts, is generally performed at ~20% O_2, which is equivalent to a partial pressure of ~150 mmHg O_2. Given that the partial pressure of O_2 in human arterial blood ranges between 75 and 100 mmHg, routine cell culture therefore subjects cells and tissues to substantially greater O_2 levels than in blood with maximum O_2 saturation. Consideration of the *in vivo* oxygen tension in human placenta suggests that 3% O_2 mimics placental conditions prior to 10 weeks gestation, while 8% O_2 reflects the *in vivo* condition from 12 weeks of pregnancy until term [178]. If the placental oxygen tension is increased too early in pregnancy, it can lead to a loss in placental mass or even spontaneous abortion [179]. In our *in vitro* experiments, primary first trimester trophoblasts were

cultivated at 21% O_2 (paper I and II), while 8% were used for third trimester placental explants (paper III and IV). Consequently, the primary trophoblast experiments were performed under hyperoxic conditions, and given the importance of oxygen for trophoblast differentiation and function [179], we cannot exclude the possibility that the high O_2 tension might have affected trophoblast physiology.

In paper III and IV, stimulation experiments were performed in third trimester placental explants to assess placental responses to HMGB1 and crystals. Such stimulation of whole tissue more closely resemble the *in vivo* cell composition and environment compared to isolated cells. The trophoblasts probably respond more optimally (with a wider production of cytokines) when kept in their natural environment with the possibility to adhere and interact with neighbouring cells. This might underlie the release of a broader spectrum of cytokines in response to HMGB1 in explants than in trophoblast cultures (paper III). However, it is difficult to attribute functional properties to a specific cell type when using explants [180]. Although trophoblasts constitute the main cell type in chorionic villi explants, the broader cytokine response in explants (paper III) might indicate additional HMGB1-responsive cell types in the placenta villi. These cell types may for example be endothelial cells and macrophages of the chorionic villi, as TLR4 expression has been detected in these cells [92, 93].

4.1.3. Immunohistochemistry

Immunohistochemistry is a complex staining method in which multiple parameters may introduce variations, including tissue fixative and fixation time, different practice among technicians and varying reagent conditions. In our studies (paper III and IV), all histology samples were fixed in the same fixative, but the fixation time varied. However, correlation analysis showed no correlation between fixation time and the measured protein expression levels, confirming that the measured differences were independent of varying fixation time. All immunohistochemical stainings in paper III and IV were performed by the same engineer at the Cellular and Molecular Imaging Core Facility, assuring consequent interpretation of protocol steps and conditions of the reagents applied. Further, the choice of primary antibody is crucial to obtain a reliable staining result. Although most manufacturers ensure antibody specificity using a range of immunochemical techniques, it is important to include tissue and

reagent controls for verification of staining results [181]. Immunohistochemical stainings of twelve different proteins in placental tissue are presented in this thesis (paper III and IV). The primary antibodies and staining procedures used were thoroughly evaluated; with a total of 21 different primary antibodies tested, using at least four different staining protocols. Specificity and optimal concentration of the primary antibodies were evaluated in positive control tissues, and isotype controls were performed to exclude unspecific staining. There are limitations regarding three of the antibodies used for immunohistochemistry in paper IV. The NLRP3 antibody reacts with an epitope in the C-terminal of the protein, while an epitope localized to the N-terminal would be preferable since this domain is more variable between NLRs. However, the manufacturer confirms that the antibody has been tested for cross-reactivity towards other NLRs. The caspase-1 antibody detects non-cleaved caspase-1, and is therefore less suitable for detecting the biologicaly active caspase-1 variant that more directly reflects NLRP3 inflammasome activation. The IL-1β antibody strongly detects all mature IL-1β, but weak detection of pro-IL-1β is also reported by the manufacturer. Ideally, we would have preferred to use an antibody that detects only the active form, cleaved IL-1β. It should be mentioned that the protein expression of ASC in placental tissue is not presented in paper IV, despite its importance for NLRP3 inflammasome activation. Although we tested several sources of commercial ASC antibodies, non of the tested antibodies passed our rigourous specificity evaluation. However, others have identified the formation of NLRP3-ASC complexes in human preeclamptic placentas [90].

Proteins detected by immunohistochemistry was quantified automatically in the syncytium of placentas from healthy and preeclamptic pregnancies (paper III and IV). The automated method for quantification in the syncytium was developed by our research group with the use of computer software that automatically measures the staining intensity in microscopy images. The intention of the new method was to compare cases and controls using a more objective method than what is traditionally used. Importantly, we ensured consistency in all steps of the sample preparation, immunohistochemical staining procedure and microscopy imaging prior to quantification to avoid discrepancies introduced by methodological variations. Similar digital quantification methods have been used in studies with markers for prostate cancer and breast cancer [182, 183]. These studies confirm that automated quantification of immunohistochemical staining is more reproducible than manual scoring by

pathologists, suggesting that digital methods are preferable and especially warranted for studies of large sample sizes. To our knowledge, we are the first to apply such automated quantification of immunohistochemistry staining in the comparison of healthy and preeclamptic placental samples.

4.1.4. Sample size and statistical analysis

Generally, larger sample sizes lead to increased precision when estimating unknown parameters. However, sample sizes may be determined in different ways for studies with different purpose and design. Factors such as cost and availability of study material often influence the number of included samples. In the four papers included in this thesis, different sample sizes were used according to the intended purpose and design of each experiment. In paper I six primary trophoblast populations were included. Since the purpose was to study the overall TLR expression and function in first trimester trophoblasts, and no quantified comparison between clinical groups were made, the inclusion of six pregnancies was likely sufficient and comparable to similar studies [102, 114]. Calculation of appropriate sample size (power calculation) is an important part of study design when the aim is to make inferences about a population from a sample. However, a power calculation is based on known sample data, which often are obtained from a smaller pilot study or a corresponding study, but if no such sample data are available it is impossible to perform a reliable power calculation. For the comparison of quantified protein expression in placental tissue from healthy and preeclamptic pregnancies without and with FGR the sample sizes were 13, 11 and 12 placentas, respectively (paper III and IV). Since we used a new method for the immunohistochemical quantitation, there were no sample data available, and we were not able to do a reliable power calculation. Instead, sample sizes were chosen with the intention of being comparable to previously published studies [92, 159]. Also, because of costly immunohistochemistry experiments and extensive labour-intensive image analysis (identifying the syncytium area) prior to automatic intensity measurements, the number of included samples was restricted. Retrospective calculation of appropriate sample size, using IL-1β sample data obtained in paper IV as estimate for population parameters, indicates that 40 placentas should be included in each group to provide 80% power at a significance level of 5% when comparing healthy and preeclamptic pregnancies. Still, our study represents the largest report of cell-specific IL-1β protein expression in preeclamptic compared to healthy placental tissue. For serum

analyses in paper III and IV, all available samples in the Preeclampsia Biobank were included, giving sample sizes of 34 preeclamptic and 43 healthy pregnancies, which are generally lower than comparable studies of cytokine and CRP levels [132, 184]. For cholesterol serum levels there are few studies including larger cohorts, possibly causing the discrepancies in results observed among these studies (summarized in [162]). However, the increased maternal serum cholesterol levels observed in preeclamptic women, which did not reach statistical significance in our and other cohorts, were confirmed to be statistical significant in a large meta-analysis [162]. In conclusion, the interesting clinical findings reported in paper III and IV warrant further investigation in larger cohorts including sub-phenotyping of preeclampsia with presence and absence of FGR.

4.2. DISCUSSION OF MAIN RESULTS

4.2.1. Trophoblasts as immune competent cells

Combined, the four papers presented in this thesis define an immunological role for trophoblasts in the placenta, supporting the representation of trophoblasts as pregnancy-specific actors of the immune system [76, 185]. Different trophoblasts express PRRs, including extravillous trophoblasts [92, 102], cytotrophoblasts (papers I-IV, [96, 103]) and syncytiotrophoblasts (paper III and IV, [104-106]), indicating the importance of these immune receptors in placental cells that display different biological functions such as migration, tissue preservation and immunity. This encourages an expanded view of the trophoblasts' contribution to placenta development, placental function, and fetal-maternal interaction.

The detection of broad and functional PRR expression in trophoblasts (papers I-IV), indicates that these cells can respond to both endogenous danger signals and invading pathogens at different stages of pregnancy. PRR expression reported in first trimester trophoblasts is somewhat more restricted than at a later gestation (Table 1). This might be explained by researcher's limited access to first trimester placenta samples (and simply fewer studies performed on this material), or it might reflect a broader biological necessity of PRRs later in pregnancy. During placentation, physiological tissue damage is substantial because trophoblasts disrupt the uterine lining before invading the maternal tissue, and an abundance of endogenous PRR ligands are likely to be generated in this process [80, 146]. Our findings

of broad TLR gene expression in trophoblasts isolated from first trimester placentas (paper I) and trophoblast expression of TLR2, TLR4 and NLRP3 proteins in first trimester tissue samples (paper III and IV) support a considerable potential for PRR signaling in early pregnancy. At the end of pregnancy, endogenous activation of PRRs initiates labour and is crucial for the continued progress of labour and delivery [76]. Our detection of trophoblast PRR expression in healthy third trimester placentas (paper III and IV) supports the involvement of these receptors in the process of parturition. In the event of an infection (early or late in pregnancy), a pathogen may enter the placenta through the reproductive tract or the maternal blood and activate the trophoblasts through their PRRs [185].

The expression of PRRs enables maternal and fetal cells to communicate with each other through the secretion of PRR-induced cytokines and chemokines [6, 95]. Upon exposure to PRR ligands *in vivo*, the trophoblasts will enhance their release of several cytokines, as we observed *in vitro* (papers I-IV). When released from activated trophoblasts, the various cytokines will influence the biological processes of placentation, labour and elimination of infectious agents [76]. Cytokine secretion by trophoblasts will recruit various maternal immune cells, which will take part in the transformation and repair of the uterus during placenta development and parturition, and contribute to successful elimination of dangerous pathogens [80, 185]. Through these processes, activation of trophoblast PRRs may be essential to the development, protection and termination of a healthy pregnancy. However, PRR mediated trophoblast activation can also have detrimental effects for the pregnancy [185], a topic that is discussed in section 4.2.2.

In paper I and II, we demonstrated major discrepancies in TLR functionality between primary trophoblasts and trophoblast cell lines. Others have also reported similar differences [186, 187]. These findings underline the importance of being cautious when extending results from cell line experiments to primary cells and further the *in vivo* situation. As a consequence, trophoblasts possibly play a broader immunological role than what has been interpreted from studies performed in cell line studies, and our findings in paper I and II support this. However, the restricted availability of especially first trimester placenta tissue and the limited lifespan of primary cells in culture cause a need for trophoblast cell lines as surrogates for primary trophoblasts. Since the SGHPL-5 trophoblast cell line was identified as most suitable

for studying TLR function (paper II), this cell line was included as an *in vitro* model in paper III and IV.

4.2.2. Placental inflammation and preeclampsia

The origin of preeclampsia pathogenesis has been linked to the placenta, but which cells and molecular mechanisms that contribute to disease development are not ascertained. Several studies have reported an association between dysregulated PRR mechanisms and the development of preeclampsia [146]. In paper III and IV, we have specifically studied the role of TLR4 and NLRP3 inflammasome activation in clinical samples from healthy and preeclamptic women by HMGB1 and crystals, respectively. By identifying trophoblast contribution and activation of these PRRs in the placenta, we have linked this cell type and specific inflammatory mechanisms to preeclampsia.

At early gestation enhanced PRR activation by either PAMPs or DAMPs in the placenta will hamper placentation and lead to reduced trophoblast invasion and restricted blood flow through the maternal spiral arteries [83, 88]. Restricted blood flow into the intervillous space may result in a hypoxic placenta, in which oxidative stress will occur due to oxygen reperfusion injury. Released ROS will change harmless endogenous molecules into well-known DAMPs by oxidation (e.g. oxidized LDL, cytokine-HMGB1, oxysterols) [146, 188]. Consequently, these DAMPs can enhance and elongate an inflammatory response through continued PRR activation both in the placenta and in the maternal circulation, leading to the persistent inflammation observed in preeclamptic women. The first trimester material included in our studies (paper I-IV) was collected from elective terminations of pregnancies that were classified as healthy based on the mother's health status. If continued, most of these pregnancies would remain healthy, but some could become complicated if allowed to progress. Therefore, we cannot associate our first trimester findings with the development of either healthy or complicated pregnancies. However, the detection of trophoblast PRRs in first trimester placental samples (paper III and IV), and the broad cytokine responses initiated by first trimester trophoblasts upon PRR activation (paper I and II), imply a potential role for enhanced activation of trophoblast PRRs at early gestation in preeclamptic pregnancies. *In vitro* stimulation of placental explants and trophoblasts with the partly oxidized cytokine-HMGB1 induced a TLR4-dependent release of IL-8 (paper III), suggesting this as a possible

mechanism occurring under oxidative stress conditions in preeclamptic placentas. Our findings of elevated TLR4 protein expression in preeclamptic placentas (paper III) further support this belief.

Improper placental PRR activation may also occur following normal placental development and still lead to harmful consequences for the pregnancy. Placental cholesterol levels are physiologically increased with gestational age, because the fetus gradually starts to produce its own cholesterol in addition to the cholesterol supplied by the mother and placenta. In preeclampsia, the transplacental lipid transport is dysregulated due to reduced expression of an ATP-binding cassette transporter (ABCA1), causing cholesterol to accumulate in the syncytium and endothelial cells of preeclamptic placentas [169]. Consequently, the probability of NLRP3 mediated IL-1β induction in syncytiotrophoblasts by accumulated cholesterol is higher towards term in these pathologic pregnancies. Combined with our findings of elevated maternal serum levels of total cholesterol and syncytial protein expression of IL-1β in preeclamptic women in third trimester (paper IV), we suggest that activation of the NLRP3 inflammasome by cholesterol may be involved in the placental inflammation observed in preeclampsia.

We have identified the syncytium as an essential site for PRR activation in the placenta (paper III and IV), and this cell layer is surrounded by maternal blood. The maternal blood may carry PRR-activating components, such as pathogens and danger signals [188]. In paper III and IV, we suggest that maternal serum HMGB1, cholesterol and uric acid could activate TLR4 and NLRP3 expressed by the syncytium. When activated by PRR ligands or injured by ischemia/reperfusion, the syncytiotrophoblasts will release high concentrations of pro-inflammatory cytokines [188], and most likely some of these cytokines will be released into the maternal circulation. Our results indicate that enhanced production of IL-1β (paper IV) in the syncytium of preeclamptic placentas might contribute to the maternal systemic response in preeclampsia. Maternal serum IL-8 concentrations were elevated in preeclamptic women (paper III), and others have shown the same for IL-1β [168, 189, 190]. The *in vitro* stimulation of placental explants with HMGB1 and crystallized cholesterol and uric acid induced release of IL-8 and IL-1β from the placenta tissue, but even though it is likely, we cannot prove that the syncytium is responsible for this cytokine release (paper III and IV). Both these cytokines can contribute to maternal endothelial dysfunction [191-193]. However,

our results cannot confirm the potential link between PRR-mediated inflammatory activation in the placenta and maternal disease. It would provide essential information to perform a study that confirms the cellular origin of circulating cytokines in preeclamptic women, as this would indicate the source of the harmful inflammation observed in these women.

4.2.3. The role of DAMPs in placental inflammation and preeclampsia

It has previously been a dominating theory that the maternal immune system must be restricted during pregnancy to tolerate the paternal antigen-carrying fetus and placenta [194]. However, according to the newer danger model [195], the maternal immune system would not be provoked by the semi-foreign fetus. Instead, pathological conditions can cause excessive production of endogenous danger signals that may induce harmful inflammation associated with poor pregnancy outcomes [146]. Preeclampsia primarily represents a sterile inflammatory disease, because its inflammatory response usually occurs in the absence of microbial infection [196].

The available information on the role of endogenous PRR-activating danger signals in pregnancy is limited [146, 196]. We detected elevated placental HMGB1 (paper III) and serum total cholesterol and uric acid (paper IV) in clinical samples from preeclamptic women. Combined with the syncytial detection of TLR4 and NLRP3 (paper III and IV); PRRs that are activated by HMGB1 and crystalline cholesterol and uric acid, we suggest these endogenously produced components to be important inflammatory mediators in preeclamptic placentas.

A dysfunctional placenta, harmed by hypoxia, tissue injury, cell death and/or inflammation, will release DAMPs within the placenta and likely to the maternal blood [196]. Examples of known DAMPs that occur in preeclamptic placentas are uric acid, cell-free fetal DNA (cffDNA), HMGB1, heat shock proteins and TNF [146, 196]. In addition, a mother with e.g. hypercholesterolemia, chronic hypertension, hyperuricemia, anemia or insulin resistance, will release DAMPs such as uric acid, cffDNA, HMGB1, hyaluronan, heat shock proteins, oxidized LDL and pentraxin-3 to the maternal circulation [146, 161, 196]. Regardless of the origin of the DAMPs, PRRs expressed by maternal immune cells and endothelium and placental cells, especially the syncytium, may be activated by these inflammatory mediators [196]. The TLR4 and NLRP3 expression (paper III and IV) was clearly strongest on the

syncytium surface facing maternal blood, emphasizing the probability for PRR involvement in response to increased danger signals present in maternal blood in this cell layer.

The "molecular symphony" of DAMPs and PRRs in placental and maternal inflammation presently discovered in preeclamptic pregnancies indicates that the involvement of danger signals in the pathogenesis of preeclampsia is highly complex. It is likely the sum of these endogenously produced inflammatory mediators that generate the placental dysfunction and maternal phenotype characteristic of preeclampsia. It is therefore a limitation to study the contribution of only one specific DAMP/PRR mechanism; however the unknown aetiology of this disease makes also fragments of the whole picture valuable.

4.2.4. Possible mechanisms for inflammasome priming in trophoblasts

Before a functional NLRP3 inflammasome can be formed, transcriptional upregulation of NLRP3 and pro-IL-1β is required to achieve sufficient levels of these inflammasome components within the cell. This particular transcriptional upregulation is known as inflammasome priming [43]**.** LPS is regularly used for priming by researchers studying NLRP3 activation *in vitro,* however the plausible cause of inflammation *in vivo* is not always infection [43], at least not in the case of pregnancy-related inflammation [196]. Endogenous factors may prime the inflammasome during the progression of sterile inflammation. Obesity and other chronic inflammatory states prime the NLRP3 inflammasome, constituting serious predisposing factors for inflammasome activation in sterile inflammatory disease [43], including preeclampsia [197]. Examples of endogenous factors that prime the NLRP3 inflammasome are ROS, oxidized LDL and components of the complement system [43], which are all reported to be associated with preeclampsia [198-201]. In paper IV we show that cholesterol crystals induce a NLRP3-dependent IL-1β response in human trophoblasts primed with the combination of C5a and TNF-α. The choice of priming factors was based on reports of elevated TNF-α and complement activation in preeclampsia [131, 137, 140, 168, 190, 198, 202, 203], and further supported by our discovery of C5a and TCC in the syncytium of preeclamptic placentas (paper IV). Priming with C5a or TNF-α alone could not trigger IL-1β secretion. Consequently, we suggest that the combination of active complement and elevated inflammation observed in preeclamptic pregnancies provide relevant and necessary priming of cholesterol crystal-induced NLRP3 activation in trophoblasts. Further, HMGB1 is

converted into its cytokine isoform when encountering ROS [39], and this HMGB1 isoform activates trophoblasts through TLR4 (paper III). Therefore, when combining our results in paper III and IV, we also speculate that ROS-mediated oxidation of placental HMGB1 contributes to NLRP3 inflammasome priming in preeclampsia through activation of TLR4 on trophoblasts and subsequent release of TNF-α. These findings emphasizes a combined role of complement activation, inflammation and cholesterol accumulation in the complex pathology of a preeclamptic placenta.

4.2.5. Inflammasome activation and placental cell death

There are several forms of cell death occurring at the syncytium. Apoptosis is a normal constituent of trophoblast turnover and the release of apoptotic material does not lead to an inflammatory response of the mother [204]. In preeclampsia, however, other more harmful mechanisms have been suggested to take over, such as necrosis and aponecrosis [204]. These mechanisms lead to loss of placental surface integrity, with distorted microvilli and shedding of dying cells and cell components to the maternal circulation and thereby probably evoking the harmful maternal response [157]. Through our results in paper IV, we indicate that the NLRP3 inflammasome may be activated in syncytiotrophoblasts. NLRP3 activation in macrophages culminates in cell death by pyroptosis, a highly inflammatory form of programmed cell death [46]. Consequently, we suggest that syncytiotrophoblast pyroptosis induced by NLRP3 inflammasome activation represents another possible explanation for syncytium cell death in preeclampsia, but specialized studies of cell death mechanisms are required to further understand these potentially devastating processes.

5. CONCLUDING REMARKS

The overall aim of this thesis was to characterize PRR-mediated placental inflammation in healthy and preeclamptic pregnancies. Through the presented studies we have defined trophoblasts as potential mediators of PRR-induced placental inflammation in both early and late gestation of normal and preeclamptic pregnancies.

We have identified broad expression of functional PRRs in trophoblasts from first trimester (paper I) and third trimester (paper III and IV), supporting an active immunological role of trophoblasts in placental inflammation at different stages of healthy pregnancy. The cytokine response to *in vitro* PRR activation of trophoblasts and placental explants involved IL-1β, IL-6, IL-8, IP-10 and VEGFA (papers I-IV), representing potent inflammatory and/or angiogenic mediators with known involvement in placental development and several pregnancy complications. The consequences of trophoblast PRR activation may range from successful placentation and parturition in normal pregnancy to an exaggerated inflammatory response with detrimental effects for pregnancy. More specifically, we suggest a contribution of HMGB1 activation of TLR4 and complement-primed crystal activation of the NLRP3 inflammasome in the harmful placental inflammation in preeclampsia, pointing to the syncytiotrophoblasts as central cellular players (paper III and IV). Further, our results indicate that the syncytium is a possible source for elevated maternal serum IL-8 and IL-1β taking part in endothelial dysfunction characteristic of preeclampsia (paper III and IV). Our results showing elevated levels of placental HMGB1 and serum cholesterol and uric acid in preeclamptic pregnancies, in addition to placental expression of PRRs responding to the presence of these molecules, highlights the importance of DAMPs and sterile inflammation in the pathogenesis of preeclampsia (paper III and IV).

Altogether, data in this thesis suggest a role for trophoblast PRR activation in placental inflammation (papers I-IV), and even substantiate a more potent immunological role for trophoblasts than what is interpreted from previous cell line studies (paper I and II). However, more research is needed to elucidate the consequence of trophoblast PRR activation in healthy and preeclamptic pregnancies, hopefully leading to better understanding of the pathogenesis for various preeclampsia subgroups and detection of potential biomarkers.

6. FUTURE PERSPECTIVES

The work presented in this thesis generated evidence for the involvement of trophoblast PRR activation in placental inflammation, but also underpins the immunological complexity of placental contribution to preeclampsia. Some of the results are hypothesis generating, and further studies are required to elucidate the role of specific PRR activators, PRR mechanism components, cytokine functions and cell contribution in pregnancy. Especially, improved understanding of PRR-mediated responses to endogenous danger signals is awaited in the context of pregnancy. Our work also revealed the potential for methodological developments.

This thesis identifies several specific points that will be important to address in future work:

- The extent and mechanism for cholesterol uptake and crystal formation should be further addressed in the placenta. In macrophages, CD36-facilitated endocytosis of lipoprotein-packed cholesterol results in intracellular formation of cholesterol crystals [205], and the discovery of a corresponding mechanism in trophoblasts would be of high interest. Not only crystalline uric acid, but also soluble uric acid can activate NLRP3 [49], but it is still unclear whether cholesterol must be in its crystal form to activate NLRP3, suggesting another study question to be investigated.

- Since the biological properties of HMGB1 are altered through specific redox modifications, it is important to consider which redox form of HMGB1 is involved in physiological and pathological processes [27]. The cytokine-HMGB1, but not the chemokine-HMGB1, induced release of inflammatory cytokines from placental explants and trophoblasts (paper III), but the clinical relevance of the specific cytokine-HMGB1 isoform in preeclampsia remains unknown. This could be addressed by mass spectrometry analysis of HMGB1 isoforms in clinical samples from preeclamptic women, an experiment that we plan to perform. This could potentially identify HMGB1 as a therapeutic target in preeclampsia.

- The prominent PRR and cytokine expression in the syncytium facing maternal blood (paper III and IV), emphasizes the probability for syncytial PRR involvement in response to activators present in maternal blood and cytokine release to the maternal circulation. Therefore, the interaction between trophoblast PRRs and potential ligands

in the maternal blood, and the extent of placental contribution to the elevated levels of cytokines present in the circulation of preeclamptic women should be examined, but such studies require highly complex study design.

- An automated method for quantification of immunohistochemical staining intensity was used in this work (paper III and IV). However, specific targeting of syncytiotrophoblast cytoplasm had to be performed manually, and was immensely laborious. In ongoing studies, our research group is now further developing an automated method for the localization of specific cells in histological images, which will reduce the time spent on such analysis.

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Functional Toll-like receptors in primary first-trimester trophoblasts

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ABSTRACT

Toll-like receptors (TLRs) are an important part of the body's danger response system and crucial for initiating inflammation in response to cellular stress, tissue damage, and infections. Proper placental development is sensitive to inflammatory activation, and a role for TLRs in trophoblast immune activation has been suggested, but no overall examination has been performed in primary trophoblasts of early pregnancy. This study aimed to broadly examine cell surface and endosomal *TLR* gene expression and activation in first-trimester trophoblasts. Gene expression of all ten TLRs was examined by quantitative RT-PCR (RT-qPCR) in primary first-trimester trophoblasts ($n = 6$) and the trophoblast cell line BeWo, and cytokine responses to TLR ligands were detected by quantitative multiplex immunoassay. Primary first-trimester trophoblasts broadly expressed all ten TLR mRNAs; *TLR1*, *TLR2*, *TLR3*, *TLR4*, and *TLR6* mRNA were expressed by all primary trophoblast populations, while *TLR5*, *TLR7*, *TLR8*, *TLR9*, and *TLR10* mRNA expression was more restricted. Functional response to ligand activation of cell surface TLR2/1, TLR4, and TLR5 increased IL-6 and/or IL-8 release ($P < 0.01$) from primary trophoblasts. For endosomal TLRs, TLR3 and TLR9 ligand exposure increased receptor-specific production of IL-8 ($P < 0.01$) and IFN-γ-induced protein 10 (IP-10; $P < 0.001$) or vascular endothelial growth factor A (VEGFA; $P < 0.01$). In contrast, BeWo cells expressed lower *TLR* mRNA levels and did not respond to TLR activation. In conclusion, primary first-trimester trophoblasts broadly express functional TLRs, with inter-individual variation, suggesting that trophoblast TLR2, TLR3, TLR4, TLR5, and TLR9 might play a role in early placental inflammation.

Abbreviations: C_T, threshold cycle; FC, fold change; IP-10/CXCL10, IFN-γ-induced protein 10; ODN, oligodeoxynucleotide; PE, preeclampsia; Poly(I:C), polyinosinic–polycytidylic acid; PTB, preterm birth; RT-qPCR, quantitative RT-PCR; TBP, TATA box binding protein; T, threshold values; TLR, Toll-like receptor; Trb, trophoblast; VEGFA, vascular endothelial growth factor A.

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1. Introduction

Pregnancy is a natural inflammatory state (Redman and Sargent, 2004). While moderate inflammation may be beneficial to pregnancy, excessive production of pro-inflammatory cytokines is harmful and contributes to adverse pregnancy outcomes, such as miscarriage, preterm birth (PTB), and preeclampsia (PE) (Redman and Sargent, 2004; Wei et al., 2010; Calleja-Agius et al., 2012). During placentation fetal trophoblasts form the growing placenta and invade the maternal uterine wall, interacting with maternal cells and modifying the uterine vasculature. Aberrant placental inflammation triggered by infection or cellular stress may disturb trophoblast function and lead to improper placental development (Gomez and Parry, 2009).

Toll-like receptors (TLRs) serve as sensors for danger signals from bacteria, viruses, and damaged tissue, and are crucial for initiating an inflammatory response (Takeuchi and Akira, 2010). TLR activation results in the rapid release of IFNs and potent pro-inflammatory cytokines and chemokines such as IL-6, IL-8, TNF-α, and IFN-γ-induced protein 10 (IP-10/CXCL10) (Takeuchi and Akira, 2010). TLRs are expressed by both professional immune cells and other cells like endothelial cells and fibroblasts (Takeuchi and Akira, 2010). The human TLR family consists of TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 found primarily on the cell surface, and TLR3, TLR7, TLR8, and TLR9 expressed in intracellular endosomes (Blasius and Beutler, 2010). The cellular localization of a TLR reflects its ligand specificity; the cell surface TLRs recognize structures in bacterial membranes and released danger signals, while the intracellular TLRs require cellular uptake of their ligands, such as viral nucleic acids and nucleic acids released from damaged tissue (Blasius and Beutler, 2010). TLR2 forms heterodimers with TLR1, TLR6 or TLR10, while TLR4 acts as a homodimer in concert with several co-receptors, and each receptor responds to a variety of danger signals, ranging from bacterial cell wall components to endogenous heat shock proteins (Takeuchi and Akira, 2010; Guan et al., 2010). TLR5 forms homodimers or heterodimers with TLR4, to recognize bacterial flagellin (Hayashi et al., 2001; Mizel et al., 2003). Endosomal TLR3 is activated by dsRNA (Alexopoulou et al., 2001), TLR7 and TLR8 by ssRNA (Heil et al., 2004), and TLR9 by unmethylated DNA fragments (Hemmi et al., 2000).

TLRs have been implicated in pregnancy complications such as PTB and PE (Elovitz et al., 2003; Pineda et al., 2011; Koga et al., 2009). Reports of trophoblast TLR expression suggest that these receptors might play a role in placentation and inflammatory responses during pregnancy, but the majority of existing studies have examined placental tissue (Holmlund et al., 2002; Klaffenbach et al., 2005; Pineda et al., 2011; Chatterjee et al., 2012) or term trophoblasts (Chan and Guilbert, 2006; Mitsunari et al., 2006; Aye et al., 2012; Lucchi and Moore, 2007; Ma et al., 2006, 2007). TLR2, TLR4, and TLR10 expression have been demonstrated in primary first-trimester trophoblasts (Abrahams et al., 2004; Mulla et al., 2013) and of the endosomal TLRs, only *TLR3* and *TLR8* transcripts have been detected in early gestational placentas (Abrahams et al., 2005; Aldo et al., 2010). Functional TLR studies relating to the first trimester have largely been conducted on trophoblast cell lines (Abrahams et al., 2004; Klaffenbach et al., 2005; Mulla et al., 2013; Komine-Aizawa et al., 2008; Nakada et al., 2009; Chatterjee et al., 2012), and in primary first-trimester trophoblasts, TLR3- and TLR4-activated release of the pro-inflammatory cytokines IL-6, IL-8, and IFN-β has been reported (Abrahams et al., 2005, 2006; Anton et al., 2012; Wang et al., 2011). Collectively, these findings indicate that TLR-mediated trophoblast activation is of importance in pregnancy, but the knowledge is limited and the functional role of TLRs in early gestational trophoblasts has yet to be established. The complex interaction among the TLRs warrants a combined study of these receptors to improve understanding of their role in trophoblasts. The aim of this study was to broadly examine cell surface and endosomal *TLR* gene expression and function in primary human trophoblasts isolated from first-trimester placentas.

2. Materials and methods

2.1. Tissue collection and trophoblast isolation and culture

Placental tissue was collected from six healthy Norwegian women undergoing surgically induced elective abortions at 6–12 weeks' gestation at St. Olavs Hospital, Trondheim University Hospital, from 2009 to 2011. The study was approved by the Regional Committee for Medical Research Ethics, the participants signed informed consent, and gestational age at collection was the only information available from these pregnancies.

Trophoblasts were isolated from first-trimester placental tissue (on average 4.6×10^6 cells/g placental tissue), using an established protocol (Kliman et al., 1986; Aboagye-Mathiesen et al., 1996), with some modifications (Vince et al., 1990; Abrahams et al., 2004). The tissue was washed and cleaned for membranes and blood clots, before three enzymatic digestions for 20 min at 37 °C with a mix of 150 U/ml collagenase, 451 U/ml hyaluronidase, and 36 KU/ml DNAse (Sigma–Aldrich, St. Louis, MO, USA). Supernatants were collected and centrifuged (average cell yield 1.4×10^8). The cells were resuspended in 5 ml EMEM (Caisson Laboratories, Logan, UT, USA), layered on top of 3 ml lymphocyte separation medium (MP Biomedicals, Solon, OH, USA) and centrifuged at $400 \times g$ for 20 min. Trophoblasts were collected, washed in PBS, and seeded at approximately 80% confluence in trophoblast medium containing EMEM, with 10% FBS (BioWhittaker, Verviers, Belgium), 0.75 mg/ml $NaHCO_3$ (BioWhittaker), 1 mM sodium pyruvate (PAA Laboratories GmbH, Pasching, Austria), 1 μM HEPES (Gibco, Carlsbad, CA, USA), and 100 mg/ml penicillin-streptomycin (Sigma–Aldrich) at 37 °C and 5% CO_2 on collagen type IV-coated Petri dishes (Becton Dickinson, Franklin Lakes, NJ, USA). The trophoblasts were cultivated overnight before isolation of total RNA (Section 2.4). Following freezing in liquid nitrogen in trophoblast culture medium containing 10% DMSO (Sigma–Aldrich), trophoblasts were thawed and further experiments performed (Section 2.2).

The isolated primary cells displayed trophoblast morphology, expressed cytokeratin 7, were negative for the leukocyte marker CD45, and released human chorionic gonadotropin (Handschuh et al., 2007) and human placental lactogen (data not shown).

The human choriocarcinoma trophoblast cell line BeWo was generously provided by Professor Berthold Huppertz (Medizinische Universität Graz, Austria). The cells were cultivated in equal amounts of DMEM (BioWhittaker) and Ham's nutrient mixture F12 (SAFC Biosciences, Andover, Hampshire, UK) supplemented with 10% FBS, 20 μM L-glutamine (Sigma–Aldrich), and 100 mg/ml penicillin-streptomycin, at 37 °C and 5% CO_2.

2.2. Cell treatments

Cells were cultured in trophoblast medium at 100 μl 4×10^5 cells/ml per well in flat-bottom 96-well plates. After 4–6 h of incubation, 40 μl of the culture medium was replaced with 60 μl fresh culture medium with or without the addition of TLR ligands at the indicated final concentrations: Pam3CysSerLys4 (P3CSK4; TLR2/1, 100 ng/ml, #L2000, EMCmicrocollection GmbH, Tübingen, Germany), Pam2CGDPKHPKSF (FSL-1; TLR2/6, 50 ng/ml, #L7000, EMCmicrocollection GmbH), polyinosinic–polycytidylic acid (poly(I:C); TLR3, 50 μg/ml, #27-4729-01, Amersham Pharmacia Biotech, Uppsala, Sweden), *E. coli* LPS (TLR4, 100 ng/ml, #tlrl-pelps, InvivoGen, San Diego, CA, USA), flagellin (TLR5, 1 μg/ml, #tlrl-stfla, InvivoGen), R848 (TLR7/TLR8, 1 μg/ml, #tlr-r848-5, InvivoGen), and oligodeoxynucleotide (ODN) 2006 CpG (TLR9, 20 μM, TIB-MolBiol, Berlin, Germany). LPS was sonicated for 5 min prior to use. The supernatants were harvested after 24 h, centrifuged and stored at −80 °C. Cell morphology was monitored by light microscopy and cell viability assayed by MTT analysis, confirming that the stimuli had no toxic effect (data not shown).

2.3. Quantitative multiplex immunoassay

For quantification of cytokines, collected trophoblast supernatants were analyzed with a multiplex cytokine assay from Bio-Rad Laboratories (for the detection of IL-1β, IL-6, IL-8, IL-9, IL-10, IL-12 (p70), IP-10, TNF-α, IFN-γ, and vascular endothelial growth factor A (VEGFA)) on a Bio-Plex 200 system (Bio-Rad Laboratories) powered by Luminex xMAP Technology. The trophoblast supernatants were thawed on ice, centrifuged at 450 × *g* for 5 min at 4 °C, and analyzed undiluted.

2.4. Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)

Total RNA was isolated from one 80% confluent 8.5 cm Petri dish of trophoblasts using the High Pure RNA Isolation Kit (Roche Applied Sciences, Mannheim, Germany). cDNA was synthesized from 1 μg of total RNA using the iScript/qScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, US/Quanta Biosciences, Gaithersburg, MD, USA). The kits were carefully compared and found to give equivalent qPCR results. The cDNA synthesis reaction was incubated at 25 °C/22 °C (Bio-Rad/Quanta) for 5 min, 42 °C for 30 min, and 85 °C for 5 min.

For qPCR, 1.5 μl cDNA was added to SYBR Green Supermix/FastMix (Bio-Rad/Quanta) together with 400 nM/300 nM of forward and reverse primers (Bio-Rad/Quanta). The qPCR reagent mixes were carefully compared and found to give equivalent qPCR results. For TATA box binding protein (*TBP*) analysis, cDNA was diluted 1:20, and for *TLR* analysis cDNA was used undiluted. As previously suggested (Meller et al., 2005), *TBP* was found to be a suitable reference gene for this study by comparison of 12 common reference genes (data not shown), using the Human Reference Gene Panel kit (TATAA Biocenter, Rødovre, Denmark). The RT-qPCR primer pairs (Table 1) were designed using Clone Manager (Sci-Ed, Cary, NC, USA) and purchased from Sigma–Aldrich. These primers have been used in our laboratory for *TLR* gene expression studies in other cell types (Grimstad et al., 2011, and unpublished observations). The samples were analyzed in triplicate on a Chromo4 detector using MJ Opticon Monitor software version 3.1 (Bio-Rad Laboratories) at 95 °C for 5 min, 40 cycles of 95 °C for 5 s, 60 °C to 62 °C for 10 s, and 72 °C for 8 s. Threshold cycle (C_T) values within the range 17–32 were considered positive gene expression.

2.5. Statistical analysis

The qPCR data were analyzed using a generalized version of the comparative C_T or Livak/$2^{-\Delta\Delta C_T}$ method for relative quantification with normalization to expression of the reference gene *TBP*, using the statistical software R. The generalized threshold cycle is defined as $gC_T = C_T - \log 2$ (threshold value), for each experiment. The gene expression data sets for the primary trophoblast populations consisted of 144 observations of gC_T for cell surface *TLR* expression and 111 observations of gC_T for endosomal *TLR* expression, modeled by two-way ANOVA with target gene and cell population as the main effects. The interaction between gene expression and cell population was included and found to be significant ($P < 2.2 \times 10^{-16}$). For BeWo cells, the 71 (cell surface *TLRs*) and 63 (endosomal *TLRs*) observed gC_T values were modeled in multiple linear mixed effects models with target gene as the main fixed effect, and cell population as a main random effect (Steibel et al., 2009). Log fold change (log FC) in *TLR* gene expression (using *TBP* as the reference gene) was estimated as linear contrasts of the coefficients in the ANOVA model. Hypotheses tests were performed with ANOVA *t*-tests on the log FC contrasts. Primary trophoblast populations and BeWo cells were compared for each *TLR* (corrected for *TBP*) using the estimated means and standard deviations from each of the previous analyses in *z*-tests (assuming asymptotic normality). Multiple testing was handled by controlling the family-wise error rate at level 0.05, separately for cell surface and endosomal TLRs, using the Bonferroni method. For cell surface *TLR* expression, a total of 91 hypotheses tests were performed, and a cut-off of 0.05/91 = 0.00055 gave 50 significant findings (data not shown). For endosomal *TLR* expression, a total of 56 hypotheses tests were performed, and a cut-off of 0.05/56 = 0.00089 gave 36 significant findings (data not shown).

Table 1
Specifications for quantitative polymerase chain reaction (qPCR).

cDNA	Forward primer (5′–3′)[a]	Reverse primer (5′–3′)[a]	Amplicon size (bp)	Annealing temperature (°C)	Accession number
TBP	5′-ttgctgcggtaatcatgagg-3′	5′-gccagtctggactgttcttc-3′	109	61	NM_003194
TLR1	5′-agctgccagaagatgaggtc-3′	5′-aatcaggccagccctctaac-3′	124	61	NM_003263
TLR2	5′-tgactcccaggagctcttag-3′	5′-cttccttggagaggctgatg-3′	169	60	NM_003264
TLR3	5′-gccttctgcacgaatttgac-3′	5′-tccagctgaacctgagttcc-3′	155	61	NM_003265
TLR4	5′-cctggacctgagctttaatc-3′	5′-aaaggctcccagggctaaac-3′	193	61	NM_138554
TLR5	5′-gtcccttctgctaggacaac-3′	5′-tcagcaggagcctctcagtg-3′	187	61	NM_003268
TLR6	5′-gcccaaacctgtggaatatc-3′	5′-acacggtgtacaaagctgtc-3′	161	62	NM_006068
TLR7	5′-gtttctgtgcacctgtgatg-3′	5′-tgtggccaggtaaggaatag-3′	79	61	NM_016562
TLR8	5′-gttggaactacacggaaacc-3′	5′-ggactggcacaaatgacatc-3′	120	61	NM_138636
TLR9	5′-tcctgatgctagactctgccag-3′	5′-cgtccatgaataggaagcgc-3′	63	66	NM_017442
TLR10	5′-catggccagaaactgtggtc-3′	5′-catccagggagatcagttag-3′	199	61	NM_030956

TBP, TATA box binding protein; TLR, Toll-like receptor; bp, base pairs.
[a] Primer sequences for *TLR2*, *TLR3*, and *TLR4* have been published (Grimstad et al., 2011).

Differences in trophoblast baseline and TLR ligand-induced cytokine production were tested for significance using two-tailed paired *t*-tests on log 2 transformed data ($P < 0.01$ to take into account multiple testing) using GraphPad Prism v5.03.

3. Results

3.1. TLR gene expression in primary first-trimester trophoblasts

RT-qPCR analysis revealed that all ten *TLR* mRNAs were detected in primary first-trimester trophoblasts (Fig. 1). When comparing cell surface *TLR* expression in trophoblasts displaying positive *TLR* gene expression, *TLR1* mRNA was most highly expressed and *TLR10* mRNA lowest (Fig. 1A). Among endosomal *TLRs*, *TLR3* mRNA was expressed at the highest level, and *TLR8* and *TLR9* at the lowest level (Fig. 1B). In comparison, BeWo cells did not express detectable levels of TLR2 mRNA and the other nine *TLR* mRNAs (Fig. 1) were expressed at lower levels compared with the primary trophoblasts (significantly lower for 32 of the 43 possible comparisons, $P < 0.05$, Bonferroni-adjusted (data not shown)).

3.2. Individual TLR gene expression in primary first-trimester trophoblasts

Although all ten *TLR* mRNAs were detected overall (Fig. 1), not all six primary trophoblast populations expressed all ten receptors (Figs. 2 and 3). The cell surface (Fig. 2) and endosomal (Fig. 3) *TLR* gene expression profile varied extensively between trophoblast populations isolated from different placentas, and this was reflected by significant differences in 54 of the 104 possible comparisons of *TLR* mRNA levels among primary trophoblasts ($P < 0.05$, Bonferroni-adjusted (data not shown)).

Analysis of cell surface *TLR* gene expression showed that *TLR1*, *TLR2*, *TLR4*, and *TLR6* mRNA were expressed by all six primary first-trimester trophoblast populations (Fig. 2A–E). *TLR1* and *TLR2* gene expression levels varied the most among the six primary trophoblast populations, while *TLR6* mRNA was more consistently expressed (Fig. 2A–E).

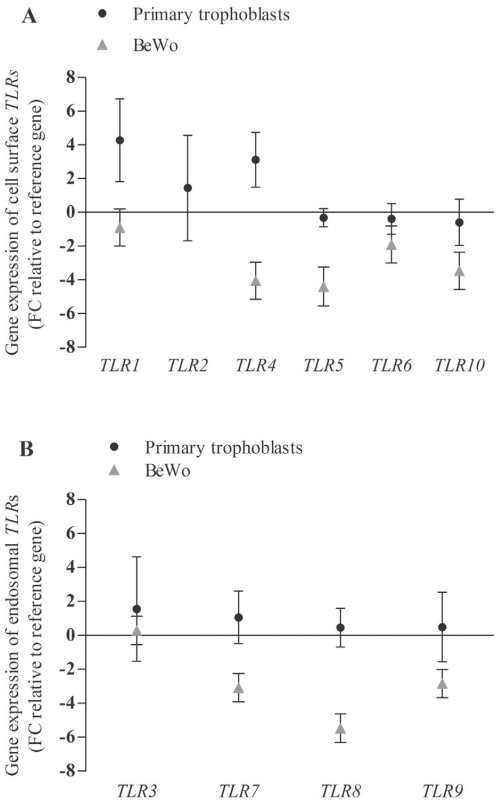

Fig. 1. Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR analysis of cell surface Toll-like receptor (*TLR*) (A) and endosomal *TLR* (B) gene expression in primary first trimester trophoblasts ($n = 6$) and the trophoblast cell line BeWo. The results are shown as log fold change (log FC) of *TLR* gene expression relative to expression of the reference gene TATA box binding protein (*TBP*), as calculated by the ANOVA method based on the generalized C_T-value. Data are shown as mean with 95% confidence interval of the positive *TLR* gene expression detected in six (*TLR1*, *TLR2*, *TLR3*, *TLR4*, and *TLR6*), five (TLR9), four (*TLR7*, *TLR8*, and *TLR10*) or two (*TLR5*) primary trophoblast populations (each run in three technical replicates), and three biological replicates for BeWo cells.

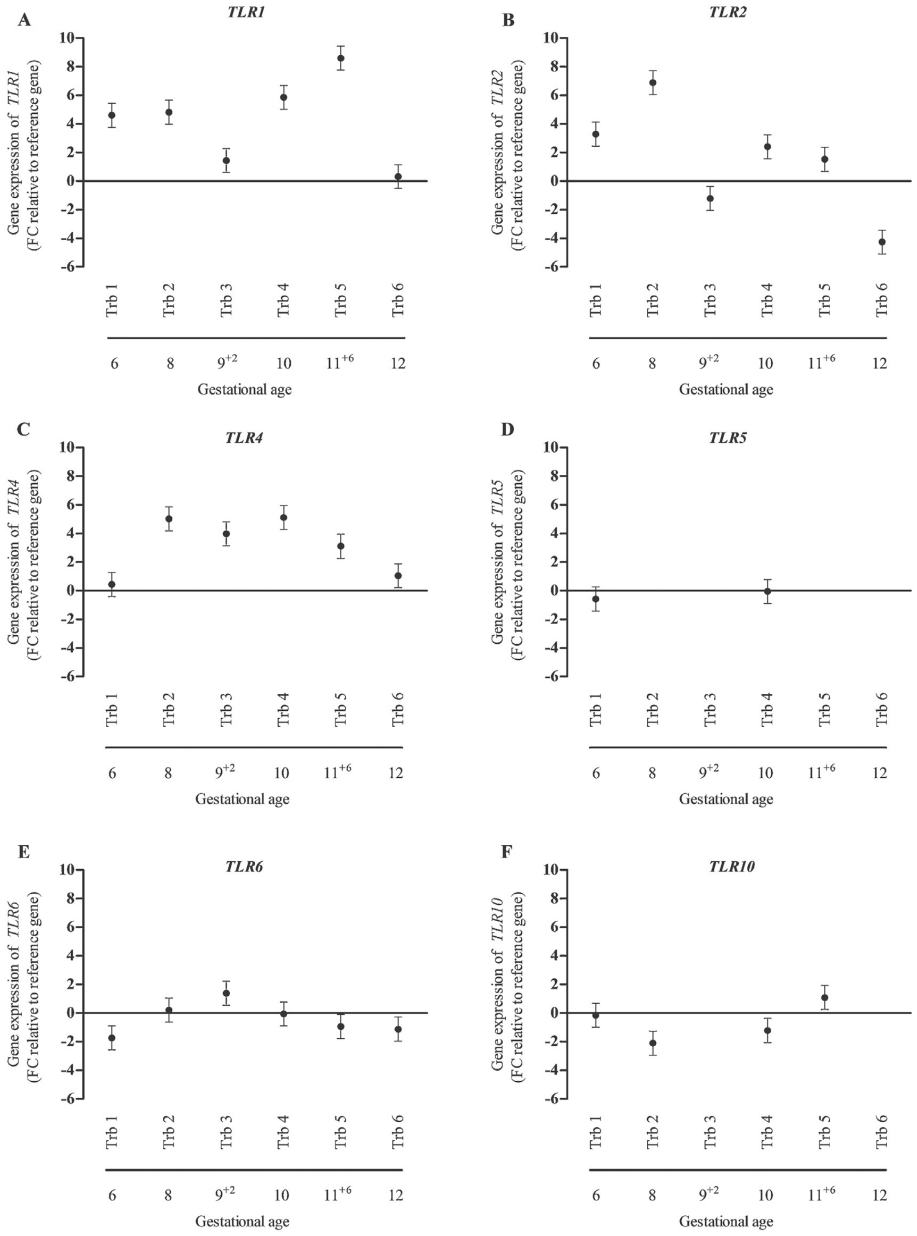

Fig. 2. Quantitative RT-PCR analysis of cell surface Toll-like receptor (*TLR*) gene expression (A–F) in primary first-trimester trophoblasts from six different placentas (Trb1–6). The results are shown as log fold change (log FC) of *TLR* gene expression relative to expression of the reference gene TATA box binding protein (*TBP*), as calculated by the ANOVA method based on the generalized C_T-value. Data are shown as mean with 95% confidence interval of triplicates.

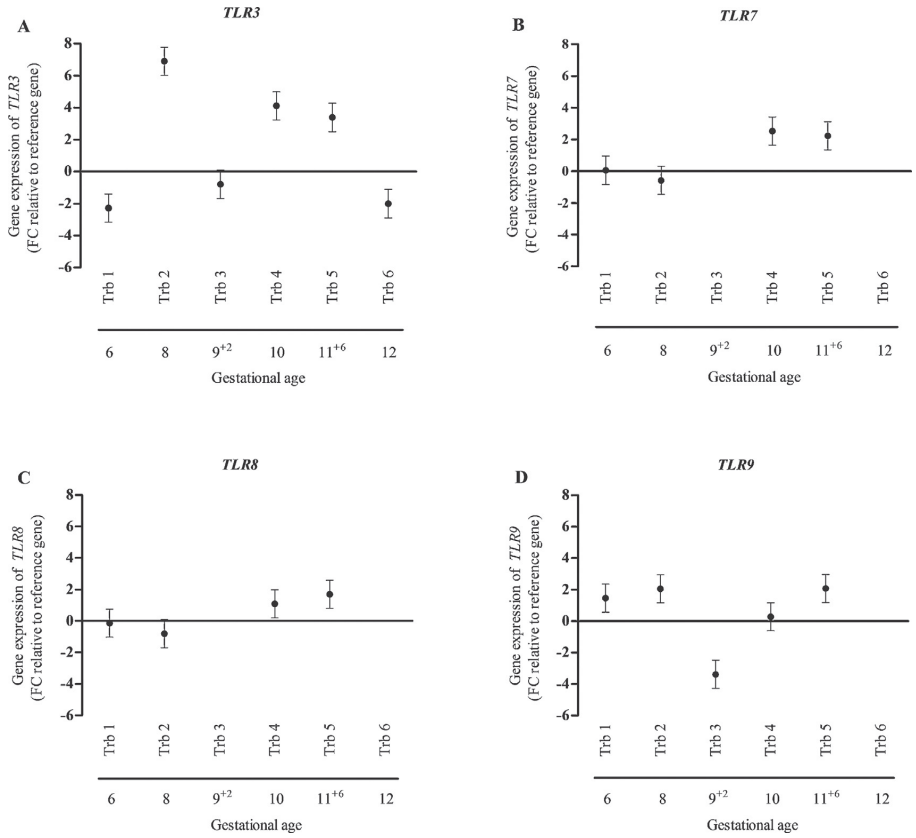

Fig. 3. Quantitative RT-PCR analysis of endosomal Toll-like receptor (*TLR*) gene expression (A–D) in primary first-trimester trophoblasts from six different placentas (Trb1–6). The results are shown as log fold change (log FC) of *TLR* gene expression relative to expression of the reference gene TATA box binding protein (*TBP*), as calculated by the ANOVA method based on the generalized C_T-value. Data are shown as mean with 95% confidence interval of triplicates.

TLR5 mRNA was detected in two and *TLR10* mRNA in four of the six primary trophoblast populations (Fig. 2D and F).

Gene expression of the endosomal receptor *TLR3* was detected in all six trophoblast populations, and with substantial variation in expression levels (Fig. 3A). Five trophoblast populations displayed *TLR3* as their most highly expressed endosomal *TLR* mRNA (Fig. 3), and for one of the trophoblasts *TLR3* was the only endosomal *TLR* mRNA detected (Fig. 3). Four trophoblast populations expressed both *TLR7* and *TLR8* mRNA (Fig. 3B and C), and five of the six trophoblast populations expressed *TLR9* mRNA (Fig. 3D).

3.3. Trophoblast cytokine production in the absence or presence of TLR ligands

Cultured primary first-trimester trophoblasts released considerable baseline amounts of IL-6, IL-8, IP-10, and

Table 2
Baseline cytokine production (pg/ml) in first-trimester trophoblasts.

Cytokines	Trb 1 (GA 6)	Trb 2 (GA 8)	Trb 3 (GA 9^{+2})	Trb 4 (GA 10)	Trb 5 (GA 11^{+6})	Trb 6 (GA 12)	BeWo
IL-6	688	2269	1332	724	871	977	29
IL-8	4029	4101	1031	6979	1505	551	ND
IP-10	41	114	14	39	19	21	17
VEGFA	5198	5717	9122	12,912	13,779	25,308	2202

Trb, trophoblast; GA, gestational age; IP-10, IFN-γ-induced protein 10; VEGFA, vascular endothelial growth factor A; ND, not detected. Data represents the mean of biological triplicates.

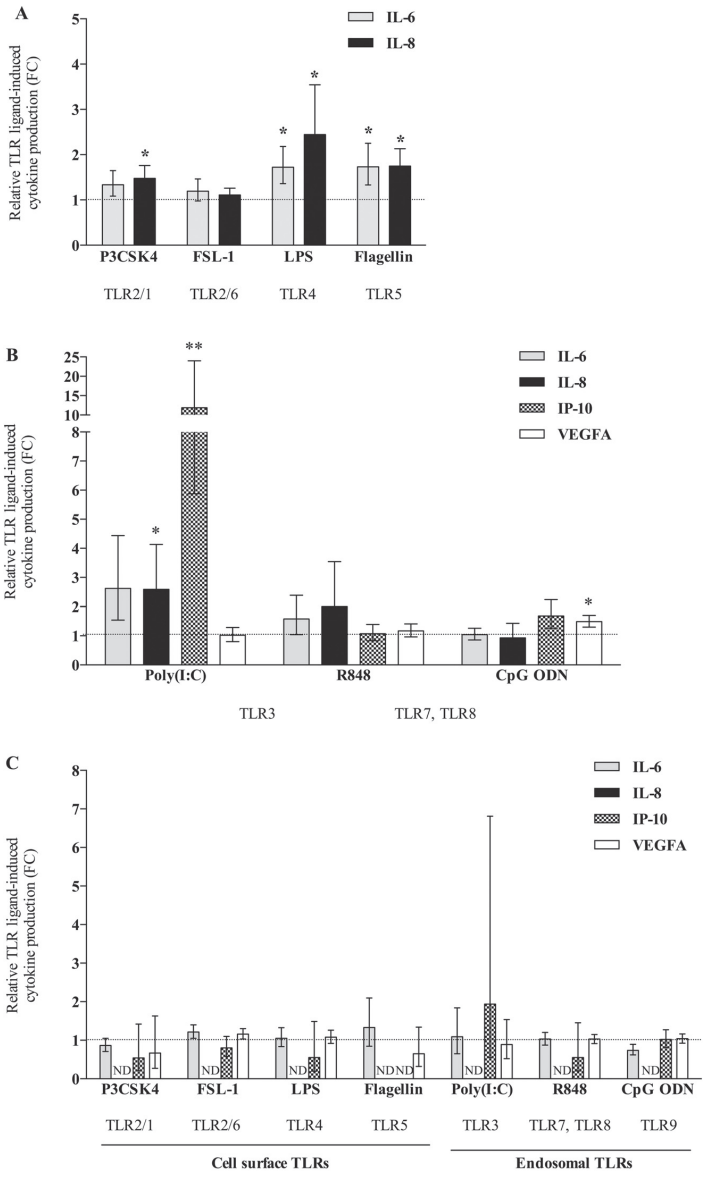

Fig. 4. Production of IL-6, IL-8, IP-10, and VEGFA in Toll-like receptor (*TLR*) ligand-activated first-trimester trophoblasts. Primary first-trimester trophoblasts ($n = 6$) (A, B) and the trophoblast cell line BeWo (C) were cultured in the absence or presence of the indicated TLR ligands (100 ng/ml P3CSK4, 50 ng/ml FSL-1, 50 μg/ml poly(I:C), 100 ng/ml LPS, 1 μg/ml flagellin, 1 μg/ml R848, 20 μM CpG ODN) for 24 h, and the cytokine release to the supernatant quantified by multiplex immunoassay analysis. The results are shown as fold change (FC) of TLR ligand-induced cytokine production relative to baseline cytokine production, as mean with 95% confidence interval of six primary trophoblasts (each run in three biological replicates) and three biological replicates of BeWo cells. Difference in baseline and TLR ligand-induced cytokine production were tested for significance using a two-tailed paired *t*-test on log 2 transformed data. ND, not detected. $^{**}P < 0.001$; $^{*}P < 0.01$.

VEGFA, but only low or no IL-1β, IL-9, IL-10, IL-12, TNF-α, and IFN-γ (Table 2 and data not shown). Of these, VEGFA was the most abundantly secreted cytokine and the levels corresponded with the gestational age of the six pregnancies included (Table 2). Cytokine production varied greatly among trophoblasts from different placentas; however, the highest level of cytokine release was not restricted to one single primary trophoblast population (Table 2). In BeWo cells, only VEGFA was produced in considerable amounts, but at five times lower levels compared with primary trophoblasts (Table 2 and data not shown).

Primary first-trimester trophoblasts responded to the cell surface TLR ligands P3CSK4, LPS, and flagellin by significantly increased production of IL-6 and/or IL-8 (Fig. 4A and Supplementary Table 1). The TLR2/6 ligand FSL-1 did not significantly induce IL-6 or IL-8 in primary first-trimester trophoblasts (Fig. 4A and Supplementary Table 1).

Supplementary Table 1 associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jri.2014.04.004.

The ligand for endosomal TLR3, poly(I:C), activated the primary first-trimester trophoblasts to a potent 23-fold increase in IP-10 production and a significant increase in IL-8 release (Fig. 4B and Supplementary Table 1). Exposure to the TLR9 ligand CpG ODN led to significantly increased VEGFA production in the primary trophoblasts, while the TLR7 and TLR8 ligand R848 did not significantly influence the cytokine production in primary trophoblasts (Fig. 4B).

Overall, these results confirmed functional TLR2/1, TLR3, TLR4, TLR5, and TLR9, but not TLR2/6, TLR7 or TLR8, in early gestational trophoblasts, and with distinct receptor-specific cytokine responses. Of all ten cytokines analyzed only IL-6, IL-8, IP-10, and VEGFA were significantly influenced by exposure to TLR ligands (Fig. 4, Supplementary Table 1, and data not shown). TLR ligand stimulation of BeWo cells did not significantly influence cytokine production (Fig. 4C), indicating non-functional TLRs in BeWo cells under the given conditions.

3.4. Comparison of TLR gene expression and ligand activation in primary first-trimester trophoblasts

TLR1, *TLR2*, and *TLR4* showed the highest mRNA levels when comparing cell surface TLR gene expression (Figs. 1A and 2) and ligand-induced activation of TLR2/1 and TLR4 led to enhanced IL-6 and/or IL-8 production by primary first-trimester trophoblasts (Fig. 4A). Furthermore, the *TLR6* mRNA levels were lower than those of *TLR1* and *TLR2* mRNA (Figs. 1A and 2A, B and E), supporting a significant IL-8 response to the TLR2/1 ligand P3CSK4 and not to the TLR2/6 ligand FSL-1 (Fig. 4A). Only for cell surface TLR5, the gene expression levels did not fully correspond with cytokine responses; *TLR5* mRNA was detected at low levels in only two of six primary trophoblast populations (Fig. 2D), and yet flagellin significantly induced IL-6 and IL-8 in most primary trophoblast populations (Fig. 4A and Supplementary Table 1).

For the endosomal TLRs, gene expression of TLR3 varied extensively between trophoblast populations from different placentas (Fig. 3A), and the overall IP-10 response to the TLR3 ligand poly(I:C) was substantial (Fig. 4B). However, the individual *TLR3* gene expression level did not directly correspond with the magnitude of individual cytokine response to stimulation with poly(I:C) (Fig. 3A and Supplementary Table 1). *TLR7* and *TLR8* gene expression levels also varied between primary trophoblast populations (Fig. 3B and C), but, except for one trophoblast population, the primary trophoblasts did not respond significantly to ligand-induced activation of TLR7 and TLR8 (Fig. 4B and Supplementary Table 1). *TLR9* gene expression in primary trophoblast populations from different placentas did not correspond with individual cytokine response to CpG ODN stimulation (Fig. 3D and Supplementary Table 1). However, the one primary trophoblast population lacking detectable *TLR9* mRNA was unresponsive to CpG ODN (Fig. 3D and Supplementary Table 1).

4. Discussion

This study is the first to collectively investigate all ten TLRs in primary first-trimester trophoblasts, and the broad functional cell surface and endosomal TLR expression shown here suggests that early gestational trophoblasts might play a central role in placental inflammation. To the authors' knowledge, this is the first report of gene expression of cell surface *TLR1*, *TLR5*, *TLR6* and all four endosomal *TLRs* in isolated primary first-trimester trophoblasts. Detection of *TLR2*, *TLR4*, and *TLR10* mRNA is supported (Abrahams et al., 2004; Mulla et al., 2013), but has not previously been collectively addressed, which is required to fully understand the functionality of these receptors. The gene expression findings indicate a potential for diverse TLR signaling in primary trophoblasts, and are supported functionally by cell surface TLR2/1, TLR4, and TLR5 ligand-induced activation of IL-6 and/or IL-8 release, and the distinct IL-8/IP-10 and VEGFA responses to endosomal TLR3 and TLR9 ligand activation. These findings, together with previous findings on TLR3 and TLR4 function (Abrahams et al., 2005, 2006; Wang et al., 2011; Anton et al., 2012), clearly demonstrate a broad potential for a diverse TLR-mediated trophoblast immune activation at the fetal–maternal interaction site. The consequences of both cell surface and endosomal TLR activation in first trimester may range from successful elimination of the danger to an exaggerated response with detrimental effects for pregnancy (Guleria and Pollard, 2000). The cytokine response to TLR activation of primary trophoblasts involved IL-6, IL-8, IP-10 and VEGFA, representing potent inflammatory and/or angiogenic mediators with known involvement in placental development and several pregnancy complications (Redman and Sargent, 2004; Szarka et al., 2010).

The LPS-induced cytokine response in primary first-trimester trophoblasts corresponds with findings in other studies (Abrahams et al., 2005; Anton et al., 2012), and TLR4-mediated inflammation is shown to be associated with PTB in humans (Tateishi et al., 2012) and fetal growth restriction and PE-like symptoms in rats (Cotechini et al., 2014). Whereas primary trophoblasts responded to the TLR2/1 ligand, stimulation with the TLR2/6 ligand failed to induce an equivalent response, reflecting the complexity of

the TLR2 signaling system. In trophoblast cell lines, it has been suggested that TLR6 might play a role in regulating the TLR2/1-response toward cytokine release (Abrahams et al., 2008). The findings in this study indicate that TLR2- and TLR4-mediated trophoblast activation in early gestation may have an impact on placentation, and the existence of TLR2- and TLR4-activating endogenous danger signals supports the inflammatory role of trophoblasts beyond an infectious response.

To the authors' knowledge, this is the first report of flagellin-mediated cytokine responses in primary first-trimester trophoblasts. However, primary trophoblasts lacking detectable *TLR5* mRNA still responded to the TLR5 ligand flagellin by increasing cytokine production. This may suggest *TLR5* gene expression at levels below the detection limit of the qPCR assay, but above the threshold required for the production of functional TLR5 protein, or that flagellin exposure might increase TLR5 expression to a functional level in first-trimester trophoblasts. Flagellin has also been shown to activate the receptor NLR family CARD domain-containing protein 4 (NLRC4) (Miao et al., 2006), but the lack of an IL-1β response to flagellin indicates no such NLRC4 involvement.

Enhanced placental expression of endosomal TLRs in PTB and PE in humans has been shown (Chatterjee et al., 2012; Pineda et al., 2011). Viruses such as cytomegalovirus may infect and replicate in the placenta, inducing local TLR3-mediated inflammation (Tabeta et al., 2004), and RNA released from damaged tissue or contained within endocytosed cells are endogenous TLR3 ligands (Kariko et al., 2004). In this study, dsRNA-analog activation of primary first-trimester trophoblasts led to increased IP-10 and IL-8, cytokines with known involvement in the excessive inflammation of PE (Szarka et al., 2010). It has also been shown that dsRNA activation of TLR3 induces murine miscarriage and PTB, and PE-like symptoms in rodents (Tinsley et al., 2009; Chatterjee et al., 2012; Koga et al., 2009). Combined with our findings, it is reasonable to assume that infectious or cell stress-related TLR3 activation in early gestational trophoblasts would have harmful consequences. Trophoblast TLR9 activation may be induced by microbial or placenta-derived DNA (Tabeta et al., 2004; Goulopoulou et al., 2012), and it has been suggested that TLR9 activation might play a role in the development of PE (Goulopoulou et al., 2012). The present study supports the role of TLR9 in trophoblast function during placentation by enhanced production of VEGFA and IP-10.

A clear correlation between *TLR* gene expression levels and the potency of response to TLR ligand activation for each individual primary trophoblast population was not apparent in this study. Nevertheless, considerable immunological variation was observed among the primary first-trimester trophoblast populations tested. Different individuals may respond differently to an infection or endogenous tissue damage signals during pregnancy and the accompanying TLR response may thus have different impacts on individual pregnancies. Hence, the idea of a scale of systemic inflammation with a gradual transition between normal pregnancy and conditions such as PE (Redman and Sargent, 2004) is supported by the findings of individual variation in *TLR* gene expression in this study.

Data from trophoblast cell lines, including BeWo cells, dominate previous reports on TLR function in early gestational trophoblasts (Komine-Aizawa et al., 2008; Klaffenbach et al., 2005; Aldo et al., 2010; Nakada et al., 2009). Our findings show substantial discrepancy between primary first-trimester trophoblasts and the trophoblast cell line BeWo, with regard to both TLR gene expression and function, and this unresponsiveness of BeWo cells to TLR activation has been supported by others (Fujisawa et al., 2000; Komine-Aizawa et al., 2008). This demonstrates the importance of including primary cells in functional studies, and substantiates a more potent immunological role for primary first-trimester trophoblasts than that interpreted from cell line studies.

This study demonstrates functional TLRs in primary first-trimester trophoblast preparations dominated by cytotrophoblasts, and therefore mostly reflects an *in vivo* potential for responding to active placental infections and placental inflammation with release of danger signals from neighboring cells. A small number of extravillous trophoblasts or syncytiotrophoblasts could also have been present in the trophoblast preparations, and *in vivo* these trophoblast types would be directly exposed to TLR-activating ligands in maternal blood, but to specifically examine these cell types, further studies are warranted.

In conclusion, the broad expression of functional TLRs in primary first-trimester trophoblasts supports the active immunological role of trophoblasts in placental inflammation and immune responses at the maternal–fetal interface. Furthermore, excessive or aberrant activation of trophoblast TLRs may contribute to pregnancy complications, by disturbing proper placentation and enhancing the normal pregnancy-associated inflammation to a harmful level.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Paper II

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Toll-like receptor profiling of seven trophoblast cell lines warrants caution for translation to primary trophoblasts

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ABSTRACT

Introduction: Excessive placental inflammation is associated with pregnancy complications. Toll-like receptors (TLRs) are sensors for danger signals from infections and damaged tissue and initiate inflammation. Trophoblasts in the placenta broadly express TLRs. Trophoblast cell lines are used as surrogates for primary trophoblasts for *in vitro* studies, but the inflammatory translatability of trophoblast cell lines warrants examination. We aimed to assess TLR1-10 gene expression and activation in seven trophoblast cell lines and compare this to primary trophoblasts.
Methods: The five choriocarcinoma trophoblast cell lines BeWo, JAR, JEG-3, AC1M-32 and ACH-3P, and the two SV40 transfected trophoblast cell lines HTR-8/SVneo and SGHPL-5 were included and compared to primary first trimester trophoblasts (n = 6). TLR1-10 gene expression was analyzed by RT-qPCR. Cells were stimulated by specific TLR1-9 ligands for 24 h and cytokine release was measured by a 10-plex immunoassay.
Results: All choriocarcinoma cell lines demonstrated broad TLR gene expression, but lacked functional cytokine response to TLR ligand activation. In contrast, SV40 transfected cell lines showed restricted TLR gene expression, but SGHPL-5 cells displayed significantly increased levels of interleukin (IL)-6, IL-8, IL-12 and vascular endothelial growth factor A after TLR3 and/or TLR4 activation ($P < 0.01$), while TLR2 activation increased IL-6 and IL-8 levels ($P < 0.05$). HTR8/SVneo cells responded to TLR3 activation by increased IL-6 and interferon (IFN)-γ ($P < 0.05$). The SGHPL-5 TLR profile most closely resembled primary trophoblast.
Discussion: The characterized trophoblast cell line TLR profiles serve as a reference and warrant caution when selecting trophoblast cell lines as *in vitro* models for immune responses in primary trophoblasts.

1. Introduction

Normal pregnancy is characterized by natural mild inflammation, while disturbances leading to excessive placental inflammation may have harmful consequences for mother and fetus [1,2]. Fetal trophoblasts are the main placental cell type and directly interact with maternal cells. In early pregnancy, trophoblasts invade the spiral arteries in the uterine wall to facilitate vessel adaptions required for optimal placental development. At later gestation, trophoblasts cover the fetal villous structures forming a

Abbreviations: CT, threshold cycle; FBS, fetal bovine serum; FC, fold change; IFN, interferon; IL, interleukin; IP, interferon-γ-inducible protein; P3CSK4, Pam3CysSerLys4; FSL-1, Pam2CGDPKHPKSF; poly(I:C), polyinosinic:polycytidylic acid; LPS, lipopolysaccharide; ODN, oligodeoxynucleotide; PCA, principal component analysis; TBP, TATA box binding protein; TLR, Toll-like receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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placental barrier that interacts with maternal blood [3]. The delicate interplay between trophoblasts and maternal cells is sensitive to danger signals from infections and tissue damage.

Toll-like receptors (TLRs), which belong to the family of pattern recognition receptors, serve as the body's immediate sensors of danger and are essential for initiating inflammation [4]. TLR activation induces an inflammatory repair process involving production of inflammatory cytokines and recruitment of immune cells to the site of injury. Ten different human TLRs, each responding to a specific set of ligands, have been identified [4]. TLR expression and activation in primary trophoblasts [5–10] may contribute substantially to development of inflammatory pregnancy complications such as preeclampsia [11–13]. In Tangerås/Stødle et al. [7] we demonstrated a broad functional TLR profile in primary first trimester trophoblasts, while the trophoblast cell line BeWo showed no TLR mediated cytokine response.

Trophoblast properties have been widely studied in normal and complicated pregnancies [14]. Primary trophoblasts are the ideal choice for such studies, but the availability of placental tissue is often restricted, isolation of trophoblasts is labor intensive, and the isolated cells have a restricted life span in culture. To overcome these limitations a variety of trophoblast cell lines are commonly used [15]. Among these are naturally immortalized cell lines obtained from choriocarcinoma tissue, such as BeWo [16], JAR [17] and JEG-3 [18]. The cell lines AC1M-32 [19] and ACH-3P [20] have been generated by fusion of the AC1-1 cell line (a JEG-3 mutant [21]) with primary term or first trimester trophoblasts, respectively. In addition, trophoblast cell lines such as HTR-8/SVneo [22] and SGHPL-5 [23] have been generated by SV40 large T antigen transfection. Trophoblast cell lines represent a valuable tool for studying placental function and it is essential that these models are thoroughly characterized and compared to primary trophoblasts. This study aimed to assess TLR1-10 gene expression and activation in seven trophoblast cell lines and compare this to primary first trimester trophoblasts.

2. Materials and methods

2.1. Cell lines

The choriocarcinoma trophoblast cell lines BeWo, JAR (#HTB-44, ATCC, Manassas, Virginia), JEG-3 (#HTB-36, ATCC), AC1M-32 and ACH-3P, and the SV40 transfected trophoblast cell lines HTR-8/SVneo and SGHPL-5 were included (Table 1). The BeWo, ACH-3P and AC1M-32 cell lines were generously provided by Professor Berthold Huppertz (Medizinische Universität, Graz, Austria), the HTR-8/SVneo cell line by Professor Charles H. Graham (Queens University, Kingston, Canada), and the SGHPL-5 cell line by Professor Guy Whitley (Saint George's Hospital, University of London, UK). All cell lines were cultured in specific medium (Table 1) with 100 mg/ml penicillin–streptomycin (Sigma–Aldrich, St. Louis, Missouri) at 37 °C and 5% CO_2 and tested negative for mycoplasma (Lonza, Basel, Switzerland).

2.2. TLR gene expression

TLR1-10 gene expression was analyzed by RT-qPCR as previously described [7]. In short, 1.5 μl cDNA (iScript/qScript cDNA synthesis kit, Bio-Rad, Hercules, CA/Quanta, Gaithersburg, Maryland) was added to SYBR Green Supermix/FastMix (Bio-Rad/Quanta) together with 400 nM/300 nM of forward and reverse primers for TLR1-10 or the reference gene TATA box binding protein (TBP) (Bio-Rad/Quanta) [7]. The samples were analyzed in triplicates on a Chromo4 detector using MJ Opticon Monitor software version 3.1 (Bio-Rad) at 95 °C for 5 min, 40 cycles of 95 °C for 5 s, 60 °C–66 °C for 10 s, and 72 °C for 8 s.

2.3. TLR ligand activation and quantitation of cytokine response

Cells were seeded in 96-well plates and stimulated at 80% confluence with or without specific TLR ligands in 100 μl culture medium (triplicates); Pam3CysSerLys4 (P3CSK4; TLR2/1 ligand, 100 ng/ml, #L2000, EMCmicrocollection GmbH, Tübingen, Germany), Pam2CGDPKHPKSF (FSL-1; TLR2/6 ligand, 50 ng/ml, #L7000, EMCmicrocollection GmbH), polyinosinic:polycytidylic acid (poly (I:C); TLR3 ligand, 50 μg/ml, #27-4729-01, Amersham Pharmacia Biotech, Uppsala, Sweden), *Escherichia coli* lipopolysaccharide (LPS) (TLR4 ligand, 100 ng/ml, #tlrl-pelps, InvivoGen, San Diego, CA), flagellin (TLR5 ligand, 1 μg/ml, #tlrl-stfla, InvivoGen), R848 (TLR7/TLR8 ligand, 1 μg/ml, #tlr-r848-5, InvivoGen), and CpG oligodeoxynucleotide (ODN) 2006 (TLR9 ligand, 20 μM, TIBMolBiol, Berlin, Germany). LPS was sonicated for 5 min prior to use. After 24 h supernatants were collected, centrifuged, and stored at −80 °C.

For quantification of cytokine responses, supernatants were thawed on ice and analyzed with a human 10-plex cytokine immunoassay (Bio-Rad) on a Bio-Plex 200 system (Bio-Rad) powered by Luminex xMAP Technology. The levels of IL-1β, IL-6, IL-8, IL-9, IL-10, IL-12 (p70), interferon-(IFN)-γ inducible protein (IP)-10, tumor necrosis factor (TNF)-α, IFN-γ, and vascular

Table 1
Characteristics and culture conditions of the seven trophoblast cell lines.

Cell line	Origin	Culture life span	Medium[b]	Supplement[b]	FBS[b]	Ref.
Choriocarcinoma cell lines						
BeWo	Choriocarcinoma	Unlimited	DMEM/Ham's F12	20 μM L-glutamine	10%	[16]
JAR	Choriocarcinoma	Unlimited	RPMI 1640	1 mM sodium pyruvate 1 μM HEPES D-glucose	10%	[17]
JEG-3	Choriocarcinoma	Unlimited	MEM	20 μM L-glutamine 1 mM sodium pyruvate 0.1 mM NEAA	10%	[18]
AC1M-32	AC1-1[a] fused with primary term trophoblast cells	Unlimited	Ham's F12		10%	[19]
ACH-3P	AC1-1[a] fused with first trimester cytotrophoblasts	Unlimited	Ham's F12		10%	[20]
SV40 transfected cell lines						
HTR-8/SVneo	Cells from tissue pieces of first trimester placental villi	Unlimited	RPMI 1640		5%	[22]
SGHPL-5	Primary first trimester extravillous trophoblasts	Passage 25	Ham's F10	20 μM L-glutamine	10%	[23]

[a] Hypoxanthine guanine phosphoribosyltransferase (HGPRT)-defective mutant of JEG-3.
[b] Fetal bovine serum (FBS) (BioWhittaker, Verviers, Belgium), DMEM (BioWhittaker), Ham's nutrient mixture F12 (SAFC Biosciences, Hampshire, UK), L-glutamine (Sigma–Aldrich, St. Louis, Missouri), RPMI 1640 (Sigma–Aldrich), sodium pyruvate (PAA Laboratories GmbH, Pasching, Austria), HEPES (Gibco), D-glucose (Sigma–Aldrich), MEM medium (Gibco, Carlsbad, CA), non-essential amino acid (NEAA) cell culture supplement (Lonza, Basel, Switzerland), Ham's nutrient mixture F10 (Gibco).

endothelial growth factor A (VEGF-A) were measured.

2.4. Primary first trimester trophoblasts

For comparison, data on TLR1-10 gene expression and activation in primary first trimester trophoblasts from our previous publication [7] was included. Trophoblasts were isolated from placental tissue collected from six healthy women undergoing surgically-induced elective abortions at 6–12 weeks gestation. The study was approved by the Regional Committee for Medical Research Ethics and all participants signed informed consent.

2.5. Statistical analysis

Statistical analysis was performed using SPSS 21.0 (SPSS Inc, Illinois), GraphPad Prism v.5.0 (GraphPad software, CA, USA) and Matlab 2013b (The Mathworks Inc., Massachusetts) with PLS Toolbox 7.3.1 (Eigenvector Research, Washington).

TLR gene expression data were analyzed using a generalized version of the comparative threshold cycle (C_T) method for relative quantification with normalization to expression of the reference gene TBP. The generalized C_T is defined as $gC_T = C_T - \log2(\text{threshold value})$ for each technical replicate and averaged for each biological replicate. The log-transformed gC_T values were modeled in a linear mixed effects model [24] with target gene and cell line, including their interaction, as fixed effects and biological replicate as random effect. Log fold change (FC) in TLR gene expression was estimated as linear contrast of the coefficients from the model. Each FC was tested for statistical significance by t-test for each contrast. A total of 109 tests were performed. To adjust for multiple testing a cut-off of $0.05/109 = 0.00046$ was used, controlling the family-wise error rate at level 0.05.

Cytokine responses in the trophoblast cell lines were tested for significance using two tailed paired t-tests on log2 transformed data. To take into account multiple testing $P < 0.01$ was considered statistically significant.

Principal component analyses (PCA) were performed to summarize the variation between cell types. For TLR gene expression, FCs as compared to TBP expression were included (TLR1-10, 10 variables) and lack of detectable gene expression was set to zero. For cytokine response, the absolute levels after stimulation corrected for basal levels were included (7 conditions × 10 cytokines, 70 variables), and negative values were set to zero. The datasets were autoscaled prior to PCA.

3. Results

3.1. TLR gene expression in seven trophoblast cell lines

RT-qPCR analysis of TLR1-10 mRNA demonstrated cell line specific TLR gene expression profiles (Table 2 and Supplementary Table 1). The choriocarcinoma trophoblast cell lines showed a broad TLR gene expression profile, while the TLR gene expression in the SV40 transfected trophoblast cell lines was more restricted (Table 2). TLR1 mRNA was the only receptor detected in all cell lines, while TLR2 mRNA was exclusively expressed by SGHPL-5 cells. TLR3 gene expression was found in all cell lines except JAR, and TLR5 was detected in all cell lines except SGHPL-5. BeWo expressed nine of the ten TLR mRNAs and mostly resembled the TLR gene expression profile of primary first trimester trophoblasts (Table 2, [7]).

Representative TLR gene expression profiles for four of the seven cell lines are presented (Fig. 1). JAR cells demonstrated TLR gene expression that was higher than the TBP expression (Fig. 1A), while JEG-3 cells expressed TLRs at lower levels compared to the expression of the reference gene TBP (Fig. 1B). The TLR gene expression profiles of the two SV40 transfected cell lines were more variable (Fig. 1C and D). The detected TLR mRNA levels did not differ significantly between the cell lines.

3.2. Cytokine response to TLR activation in seven trophoblast cell lines

To assess TLR functionality of the cell lines (Table 2), cytokine responses to TLR1-9 ligand activation were analyzed. None of the choriocarcinoma cell lines showed a significant alteration in cytokine release in response to TLR ligands (Table 3 and Supplementary Table 2). TLR3 activation of the SGHPL-5 cells induced a strong and broad cytokine response with significantly ($P < 0.01$) increased levels of IL-6, IL-8, IL-12 and VEGF-A (Fig. 2 and Supplementary Table 2), and also IFN-γ and IP-10 levels were higher ($P = 0.01$). TLR4 activation of the SGHPL-5 cells led to significantly ($P < 0.01$) increased IL-8 response (Fig. 2B). A similar tendency towards elevated IL-6 levels was found ($P = 0.02$, Fig. 2A). Moreover, TLR2/1 and TLR2/6 activation increased the production of IL-6 ($P = 0.02$ and $P = 0.04$, respectively) and IL-8 ($P = 0.01$ and $P = 0.04$, respectively) by SGHPL-5 cells (Fig. 2 and Supplementary Table 2). The TLR ligand responses of SGHPL-5 cells closely resembled the broad TLR responsiveness observed in primary first trimester trophoblasts (Table 3, Supplementary Table 2). No significant ($P < 0.01$) cytokine responses were found after TLR activation of the HTR-8/SVneo cell line (Table 3), but TLR3 activation led to increased levels of IL-6

Table 2
Toll-like receptor (TLR) 1–10 gene expression in trophoblast cell lines and primary first trimester trophoblasts.

	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10
Choriocarcinoma cell lines										
BeWo	x	–	x	x	x	x	x	x	x	x
JAR	x	–	–	–	x	x	x	x	x	x
JEG-3	x	–	x	–	x	x	x	x	x	x
AC1M-32	x	–	x	–	x	x	x	x	–	x
ACH-3P	x	–	x	–	x	x	x	–	–	x
SV40 transfected cell lines										
HTR-8/SVneo	x	–	x	x	x	–	–	–	–	–
SGHPL-5	x	x	x	x	–	–	x	–	–	–
Primary first trimester trophoblast*	x	x	x	x	x	x	x	x	x	x

x: Indicates positive gene expression as determined by RT-qPCR in at least three biological replicates (cell lines) or six different placentas (primary trophoblasts).
*: Indicates TLR1-10 gene expression data from primary trophoblasts isolated from six first trimester placentas as previously published in Tangerås/Stødle et al. [7].
–: Indicates lack of gene expression.
All values are shown in Supplementary Table 1.

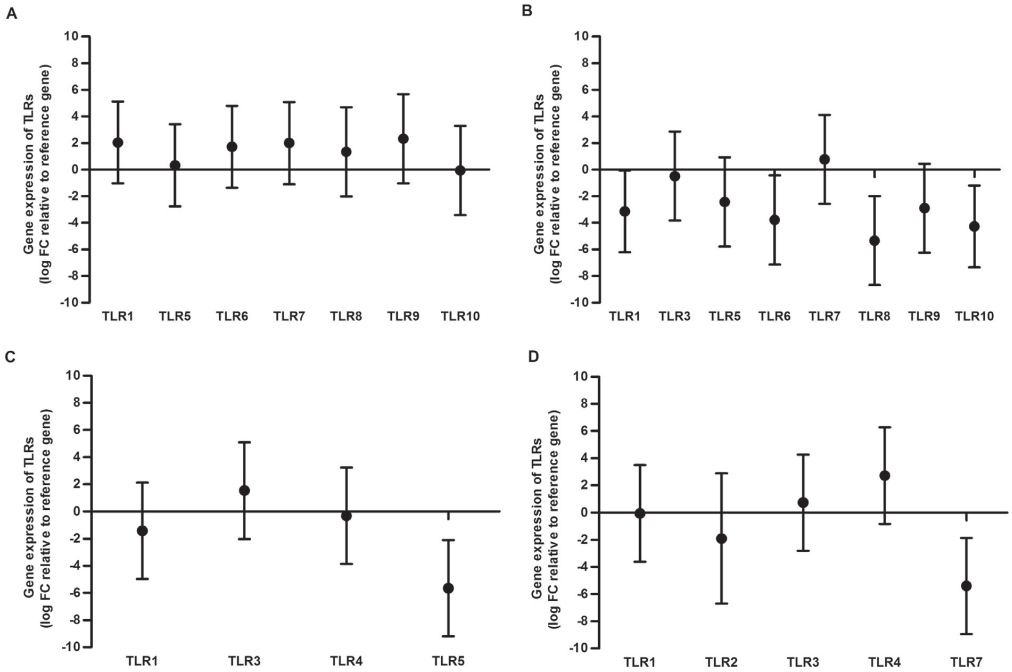

Fig. 1. Toll-like receptors (TLR) 1–10 gene expression levels in choriocarcinoma (JAR (A) and JEG-3 (B)) and SV40 transfected (HTR-8/SVneo (C) and SGHPL-5 (D)) trophoblast cell lines as analyzed by RT-qPCR. Gene expression levels are shown as log fold change (FC) of positive TLR gene expression relative to the reference gene TATA box binding protein (TBP) within each cell line, as estimated by the linear mixed model for the generalized CT value. Data are shown as mean with 95% confidence interval of the positive gene expression detected in three biological replicates of each cell line.

Table 3
Cytokine response in Toll-like receptor (TLR) ligand activated trophoblast cell lines and primary first trimester trophoblasts.

TLR ligand	P3CSK4 (TLR2/1)	FSL-1 (TLR2/6)	Poly(I:C) (TLR3)	LPS (TLR4)	Flagellin (TLR5)	R848 (TLR7/8)	CpG ODN (TLR9)
Choriocarcinoma cell lines							
BeWo	–	–	–	–	–	–	–
JAR	–	–	–	–	–	–	–
JEG-3	–	–	–	–	–	–	–
AC1M-32	–	–	–	–	–	–	–
ACH-3P	–	–	–	–	–	–	–
SV40 transfected cell lines							
HTR-8/SVneo	–	–	+	–	–	–	–
SGHPL-5	+	+	x	x	–	–	–
Primary first trimester trophoblasts							
	x	–	x	x	x	–	x

x: Indicates a significant cytokine increase ($P < 0.01$, two-tailed paired t-test) as determined in three biological replicates (cell lines) or six different placentas (primary trophoblasts), after 24 h TLR ligand stimulation compared to the unstimulated condition, for at least one of the cytokines interleukin (IL)-6, IL-8, IL-12 (p70), and/or vascular endothelial growth factor A (VEGF-A).
+: Indicates a cytokine increase at $P < 0.05$, but over the statistical significance threshold of $P < 0.01$, after TLR ligand stimulation for at least one of the cytokines IL-6, IL-8 and interferon (IFN)-γ.
–: Indicates no significant change in cytokine levels after TLR ligand stimulation.
All values are shown in Supplementary Table 2.

(Fig. 2C, $P = 0.04$) and IFN-γ ($P = 0.01$) (Table 3 and Supplementary Table 2).

3.3. Multivariate analysis of TLR gene expression and cytokine response profiles

PCAs of TLR1-10 gene expression data and cytokine responses were performed (Fig. 3). For TLR gene expression profiles (Fig. 3A and B), the principal component (PC) 1 separated JAR from the other cell lines and PC2 distinguished the primary trophoblasts (Fig. 3A). JAR cells, and to some degree AC1M-32 cells, could be identified by particularly high expression of TLR6 and the endosomal receptors TLR7, TLR8 and TLR9, while primary trophoblasts expressed the highest levels TLR1, TLR2 and TLR4 (Fig. 3B). The overall comparison of cytokine responses in the different cell lines showed a completely different pattern (Fig. 3C and D). PC1 clearly separated SGHPL-5 cells and primary trophoblasts from all other cell lines and PC2 described a difference between SGHPL-5 and

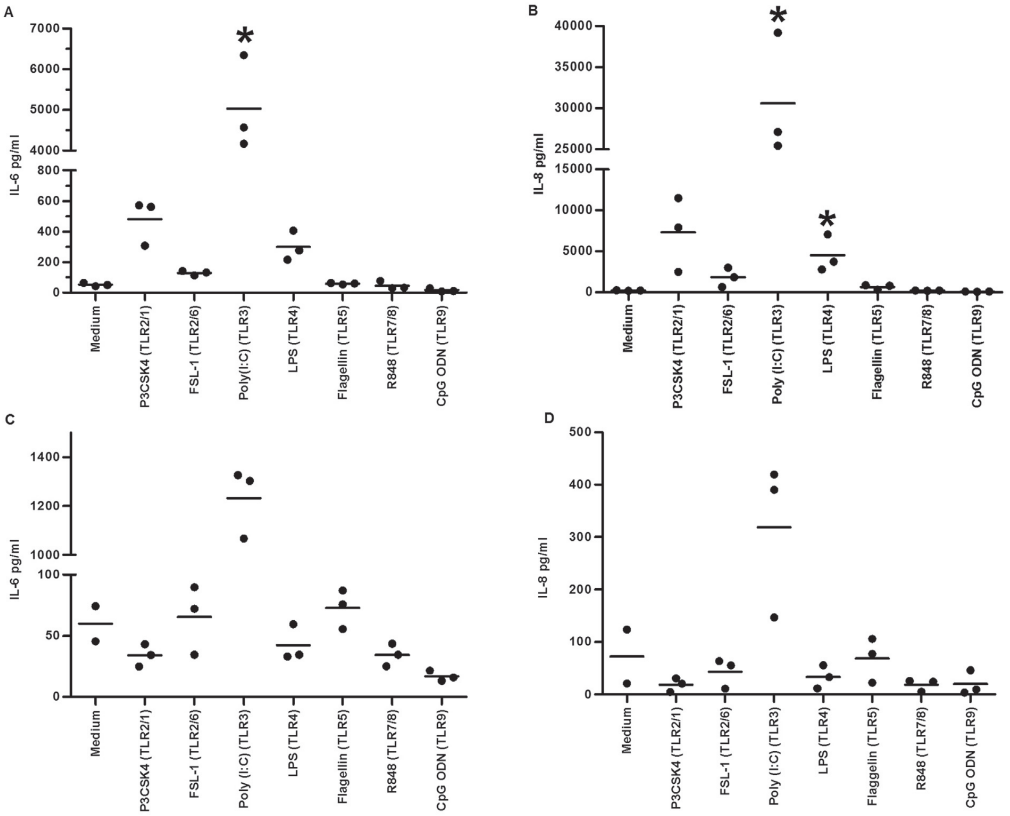

Fig. 2. Toll-like receptor (TLR) ligand activated interleukin (IL)-6 (A, C) and IL-8 (B, D) release (pg/ml) from SV40 transfected trophoblast cell lines SGHPL-5 (A, B) and HTR-8/SVneo (C, D) after 24 h stimulation, as quantified by 10-plex immunoassay. Differences between unstimulated and TLR ligand-induced cytokine production from three biological replicates were tested for significance using two-tailed paired t-test. Lines indicate the mean value. *$P < 0.01$.

primary trophoblasts (Fig. 3C). SGHPL-5 and primary trophoblasts showed higher levels of almost all cytokines compared to the other cell lines (Fig. 3D). IL-12, IP-10 and IL-9 levels were in general higher and TNFα and VEGF-A levels lower in the SGHPL-5 cells as compared to primary trophoblasts (Fig. 3D).

4. Discussion

Trophoblast cell lines are valuable tools for studying trophoblast function. In this study we demonstrated that the choriocarcinoma cell lines BeWo, JEG-3, JAR, AC1M-32 and ACH-3P broadly expressed TLR mRNA, but lacked functional cytokine responses to *in vitro* TLR ligand activation. The SV40 transfected trophoblast cell lines SGHPL-5 and HTR-8/SVneo showed a more restricted TLR mRNA profile, but SGHPL-5 responded to TLR ligand stimulation by increased production of several inflammatory cytokines. TLR gene expression varied extensively between the different cell lines and responses to TLR activation were, with the exception of SGHPL-5, far less prominent in cell lines compared to primary first trimester trophoblasts. This is the first study providing a broad characterization of TLR gene expression and function in seven different trophoblast cell lines in comparison to primary first trimester trophoblasts. The results show that most of these seven cell lines do not possess the TLR responsiveness assigned to primary trophoblasts.

None of the choriocarcinoma cell lines showed TLR responses despite expressing TLR mRNA. This could be due to lack of TLR protein expression. TLR2 and TLR4 protein expression has been reported for JEG-3 and JAR cells [25,26], but this is in contrast to the lack of TLR2 and TLR4 mRNA and cytokine responses described here. Another explanation might be that the TLR signaling pathways are non-functional or required adaptors such as MyD88 and CD14 are absent in the non-responsive cell lines. Gene expression of all ten TLRs and several adaptors have been detected in JAR and BeWo cells, but functionally only a very low IL-8 response after LPS activation in JAR cells has been reported [26]. The lack of TLR responsiveness in BeWo cells has been demonstrated previously [27,28], while this has not been investigated for ACH-3P and AC1M-32 cells. For the trophoblast cell lines expressing the endosomal receptors TLR3, TLR7, TLR8 and TLR9, the lack of cytokine response to TLR ligand exposure could be due to lack of TLR ligand uptake to endosomes [29].

The SV40 transfected cell lines showed a more restricted TLR gene expression profile compared to primary trophoblasts, which might be related to the SV40 vector transfection [30]. Interestingly, SGHPL-5 expressed functional TLRs essential for response to tissue damage and bacterial and viral infections, which are important trophoblast immune functions. IL-6 and IL-8 were the most

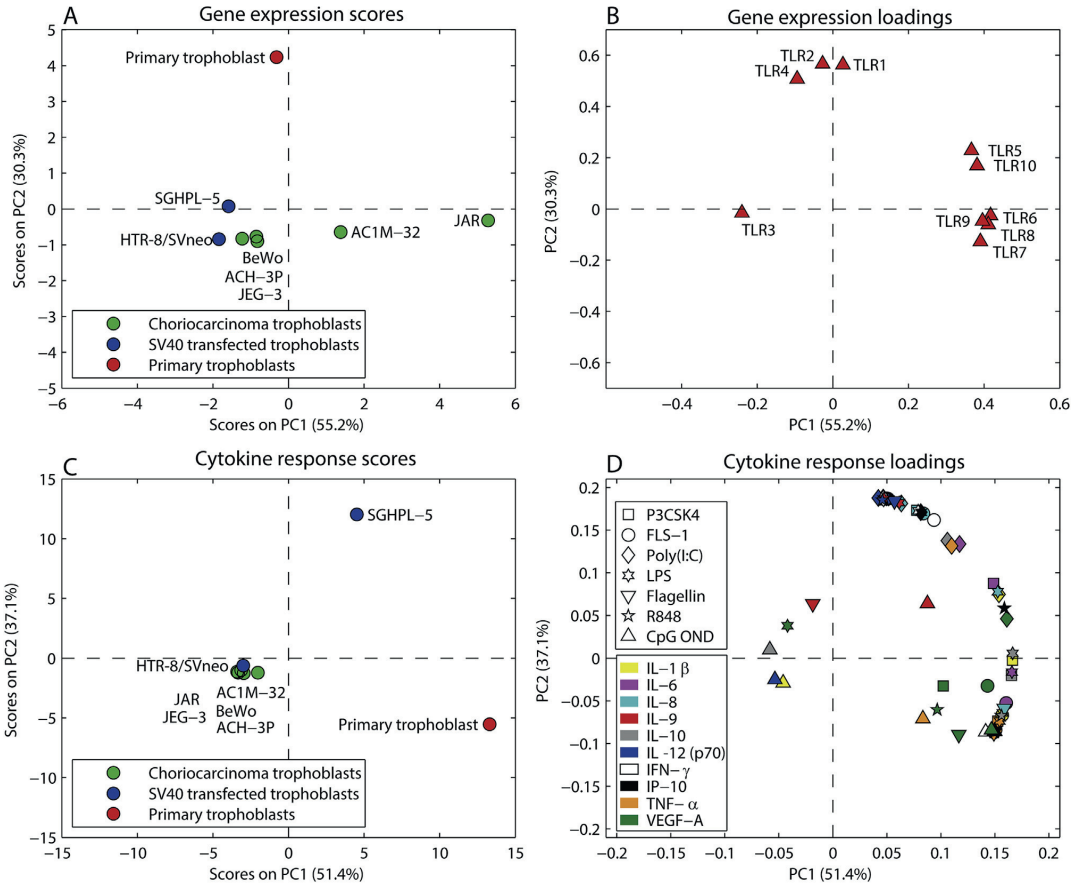

Fig. 3. Principal component analysis (PCA) of Toll-like receptor (TLR) gene expression (A, B) and cytokine responses (C, D) in choriocarcinoma trophoblast cell lines (BeWo, JEG-3, JAR, AC1M-32 and ACH-3P), SV40 transfected trophoblast cell lines (HTR-8/SVneo and SGHPL-5), and primary first trimester trophoblasts (n = 6). For TLR gene expression fold changes as compared to the reference gene TATA box binding protein (TBP) expression were included as input to the model, and for cytokine release the absolute levels measured in the supernatant after stimulation corrected for the unstimulated condition were included as input to the model. Score plots (A, C) and loading plots (B, D) are shown.

responsive cytokines to TLR activation in the SGHPL-5 cell line. This is in line with TLR responses observed in primary trophoblast [7,8] and these cytokines are implicated in several pregnancy complications [31,32]. TLR3 was the most responsive TLR in the SV40 transfected trophoblast cell lines, a finding supported by others [33,34]. LPS-induced IL-8 release from HTR-8 cells (the non-immortalized parental cell line of HTR-8/SVneo) has been reported, but only after very high doses of LPS [35]. In this study trophoblast cell lines have been compared to primary trophoblasts from first trimester, but the broad TLR expression profile and cytokine responsiveness has also been reported for primary trophoblasts at later gestations [8–10]. Our findings suggest the suitability of SGHPL-5 cells in studying trophoblast responses to infection and tissue damage.

The lack of TLR responses may also be a consequence of the nature of the TLR stimuli and selected responses, and does not exclude other functionalities of trophoblast TLRs. For instance, cytomegalovirus has been shown to interact with TLR2 in primary trophoblasts inducing TNF-α production [36]. Chlamydia heat shock protein 60 induced TLR4-dependent apoptosis in JEG-3 cells while this was not observed in response to LPS [25]. In BeWo cells, TLR3 and TLR9 activation has been shown to upregulate human choriongonadotrofine in presence of forskolin [28], and in JEG-3 cells LPS has been reported to increase corticotrophin-releasing hormone mRNA [37].

The PCA clearly showed that none of the cell lines directly reflected the TLR profile of primary first trimester trophoblasts. The placenta contains different trophoblast types with specialized functions [3], and trophoblast cell lines can only partly reflect this complexity. Functional and phenotypic differences between trophoblast cell lines and primary trophoblasts have been extensively studied [38–43]. Choriocarcinoma cell lines have a malignant origin which may be responsible for different functional characteristics, while in SV40 transfected cell lines the vector transfection may have affected the phenotype [30,43]. Gene expression profiling has demonstrated that choriocarcinoma and SV40 transfected trophoblasts differed considerably both from each other and from primary cytotrophoblast and extravillous trophoblast [41]. Additionally, miRNA fingerprints of trophoblast cell lines and primary first and term trophoblasts have been found dissimilar [42], supporting our results. However, there are many examples of functional agreement between primary trophoblasts and derivative cell

lines, supporting the use of these cell models [15,43]. Nevertheless, trophoblast cell lines need to be carefully selected and monitored for the process under investigation [15,44].

In this study, the SGHPL-5 trophoblast cell line most closely resembled primary first trimester trophoblasts with regard to TLR responses. Interestingly, this cell line was the only included trophoblast cell line with a limited culture life span, whereas the longer existence of the other cell lines might have influenced their phenotype [44]. This supports the use of the SGHPL-5 cell line in *in vitro* inflammation studies related to normal and pathological pregnancy conditions.

In summary, this is the first study examining TLR1-10 gene expression and activation in a panel of trophoblast cell lines of different origins. Trophoblast TLR activation has been implicated in pregnancy outcome and fetal development [13], but more research is needed to elucidate the consequence of placental TLR activation in normal and pathologic pregnancies. The use of trophoblast cell lines is crucial in achieving these goals. This study serves as a reference for selecting a trophoblast cell line for TLR studies. It designates that data should be corroborated with primary trophoblast experiments, and that caution must be used when interpreting immune responses from trophoblast cell line studies.

Conflict of interest statement

Toll-like receptor profiling of seven trophoblast cell lines warrants caution for translation to primary trophoblasts.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2015.09.004.

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