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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).