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Functional Toll-like receptors in primary first trimester trophoblasts

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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.

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 the primary trophoblasts. For endosomal TLRs, TLR3 and TLR9 ligand exposure increased receptor specific production of IL-8 ($P < 0.01$) and IP-10 ($P < 0.001$) or 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 a role for trophoblast TLR2, TLR3, TLR4, TLR5 and TLR9 in early placental inflammation.

KEY WORDS: Toll-like receptors; trophoblasts; first trimester; pregnancy; inflammation

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 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, Ma et al., 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 related to 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, Anton et al., 2012, Wang et al., 2011, Abrahams et al., 2006). 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 between the TLRs warrants a combined study of these receptors for an improved 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 of 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 consents, and gestational age at collection was the only available information from these pregnancies.

Trophoblasts were isolated from first trimester placental tissue (on average $4.6x10^6$ 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 $1.4x10^8$). 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, Ohio, USA) and centrifuged at 400xg 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, Hampshire, United Kingdom) supplemented 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 $4x10^5$ 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 addition of TLR ligands at 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, USA), flagellin (TLR5, 1 µg/ml, #tlrl-stfla, InvivoGen), R848 (TLR7/TLR8, 1 µg/ml, #tlr-r848-5, InvivoGen, San Diego, USA) and oligodeoxynucleotide (ODN) 2006 CpG (TLR9, 20 µM, TIBMolBiol, Berlin, Germany). LPS was sonicated for 5 minutes 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 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 450xg for 5 min at 4°C, and analyzed undiluted.

2.4. Quantitative RT-PCR (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 twelve 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 triplicates on a Chromo4 detector using the 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 in the range of 17 to 32 were considered positive gene expression.

2.5. Statistical analysis

The qPCR data was analyzed using a generalized version of the comparative C_T or Livak/$2^{-\Delta\Delta CT}$ 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 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 main fixed effect, and cell population as a main random effect (Steibel et al., 2009). Log fold change (logFC) in *TLR* gene expression (using *TBP* as reference gene) was estimated as linear contrasts of the coefficients in the ANOVA model. Hypotheses tests were performed with ANOVA t-tests on the logFC 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).

Differences in trophoblast baseline and TLR ligand-induced cytokine production were tested for significance using two-tailed paired t-tests on log2 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 expressed (Fig. 1A). Among endosomal *TLR*s, *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 to the primary trophoblasts (significantly lower for 32 of 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 (Fig. 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 between 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, B, C and E). *TLR1* and *TLR2* gene expression levels varied the most between the six primary trophoblast populations, while *TLR6* mRNA was more consistently expressed (Fig. 2A, B and E). *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 absence or presence of TLR ligands

Cultured primary first trimester trophoblasts released considerable baseline amounts of IL-6, IL-8, IP-10 and 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 included pregnancies (Table 2). The 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 to 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).

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 (Figure 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 (Fig. 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 that of *TLR1* and *TLR2* mRNA (Fig. 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 a central role for early gestational trophoblasts 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., 2006, Wang et al., 2011, Abrahams et al., 2005, 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 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 and equivalent response, reflecting the complexity of the TLR2 signaling system. In trophoblast cell lines, a role for TLR6 in regulating the TLR2/1-response towards cytokine release has been suggested (Abrahams et al., 2008). The findings in this study indicate that TLR2 and TLR4-mediated trophoblast activation in early gestation may impact placentation, and the existence of TLR2 and TLR4-activating endogenous danger signals supports an inflammatory role for 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 increased cytokine production. This may suggest *TLR5* gene expression at levels below the detection limit of the qPCR assay, but above the threshold required for production of functional TLR5 protein, or that flagellin exposure may 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 NLRC4 involvement.

Enhanced placental expression of endosomal TLRs in PTB and PE in humans have 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-analogue 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 might have harmful consequences. Trophoblast TLR9 activation may be induced by microbial or placenta-derived DNA (Tabeta et al., 2004, Goulopoulou et al., 2012), and a role for TLR9 activation in development of PE has been suggested (Goulopoulou et al., 2012). The present study supports a role for TLR9 in trophoblasts 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 immunologic variation was observed between 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 regards to both TLR gene expression and function, and this unresponsiveness of BeWo cells to TLR activation is 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 immunologic role for primary first trimester trophoblasts than what is currently 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 syncytiotrophoblast 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 an active immunological role for 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.

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Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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Figure legends

Fig.1 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 (logFC) 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 RT-qPCR 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 (logFC) 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. Gestational age, GA; ND, not detected.

Fig.3 RT-qPCR 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 (logFC) 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. Gestational age, GA; ND, not detected.

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 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 two-tailed paired t-test on log2 transformed data. ND, not detected. **$P < 0.001$; *$P < 0.01$.

Table 1

TABLE 1: Specifications for qPCR.

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

TBP, TATA box binding protein; TLR, Toll-like receptor; bp, base pairs.

[a]Primers sequences for *TLR2* , *TLR3* and *TLR4* have been published (Grimstad *et al*. 2011).

Table 2

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	12912	13779	25308	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.

Supplementary Table 1

SUPPLEMENTARY TABLE 1: Cytokine production (pg/ml) in primary first trimester trophoblasts

Stimuli	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)
No stimuli	IL-6	688	2269	1332	724	871	977
	IL-8	4029	4101	1031	6979	1505	551
	IP-10	41	114	14	39	19	21
	VEGFA	5198	5717	9122	12912	13779	25308
P3CSK4	IL-6	835	3485	1189	1260	1053	1353
	IL-8	5685	6257	1092	9223	2138	1183
	IP-10	54	70	13	51	26	20
	VEGFA	4313	6770	9868	13621	15253	25256
FSL-1	IL-6	810	3876	1338	1346	967	1002
	IL-8	3343	4602	1162	9263	1382	514
	IP-10	41	70	16	49	21	17
	VEGFA	5018	6409	10609	15497	12852	24802
Poly(I:C)	IL-6	1298	4544	1888	3000	6900	4983
	IL-8	4410	5590	2591	8678	7584	4111
	IP-10	352	972	128	1130	645	2450
	VEGFA	5828	6160	8604	15254	19572	24338
LPS	IL-6	1212	3402	2127	1711	1460	923
	IL-8	10957	8985	2277	18366	2297	848
	IP-10	52	95	16	61	25	19
	VEGFA	5858	5608	8602	13445	12809	23826
Flagellin	IL-6	1140	5733	1711	2167	1414	1378
	IL-8	5960	12787	1330	11328	2874	980
	IP-10	57	317	19	91	31	20
	VEGFA	5951	8896	9105	14281	13870	21575
R848	IL-6	948	4365	1423	1960	1065	915
	IL-8	24448	102470	1183	14620	2229	507
	IP-10	62	116	11	83	21	19
	VEGFA	6113	8909	11322	14567	12487	22514
CpG ODN	IL-6	657	2168	1659	773	1217	606
	IL-8	2414	12387	1192	6804	1293	280
	IP-10	115	247	22	104	39	19
	VEGFA	7959	9820	10995	16919	19983	31376

Trb, trophoblast; GA, gestational age; IP-10, IFN-γ-induced protein 10; VEGFA, vascular endothelial growth factor A. Primary trophoblasts (n = 6) were cultured in absence or presence of indiated 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 hours, and the cytokine release to the supernatant was quantified by multiplex immunoassay analysis. Data represents the mean of biological triplicates.

Figure 1
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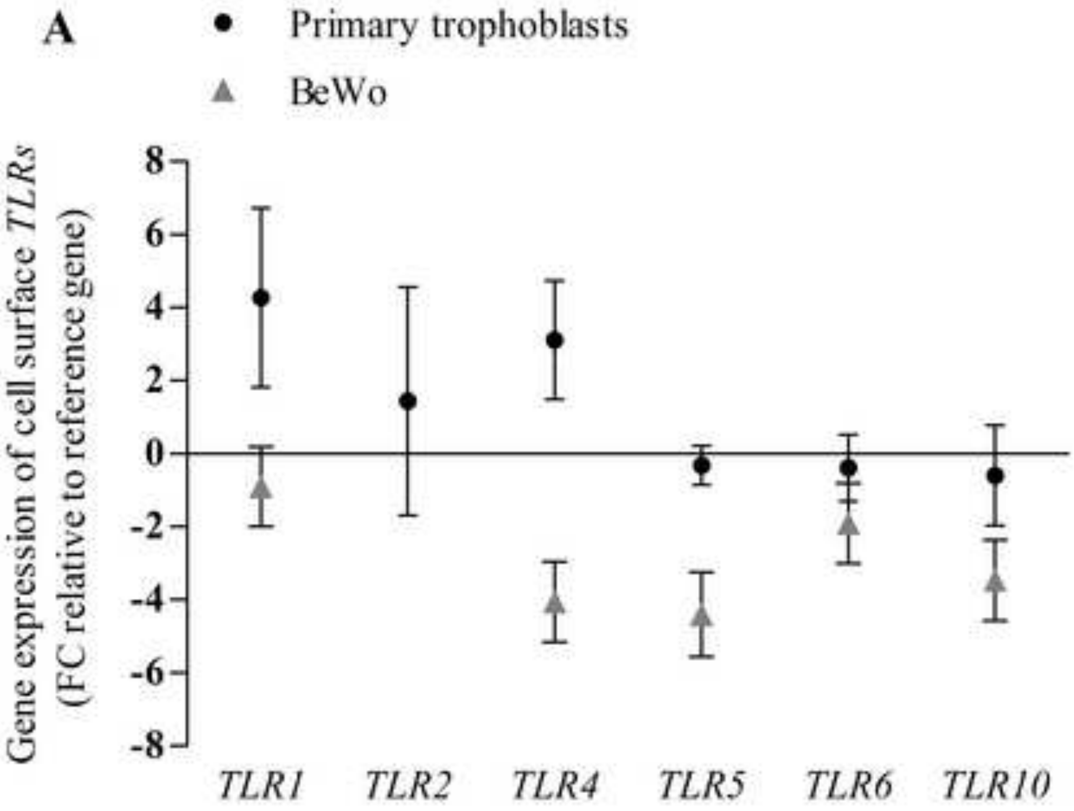

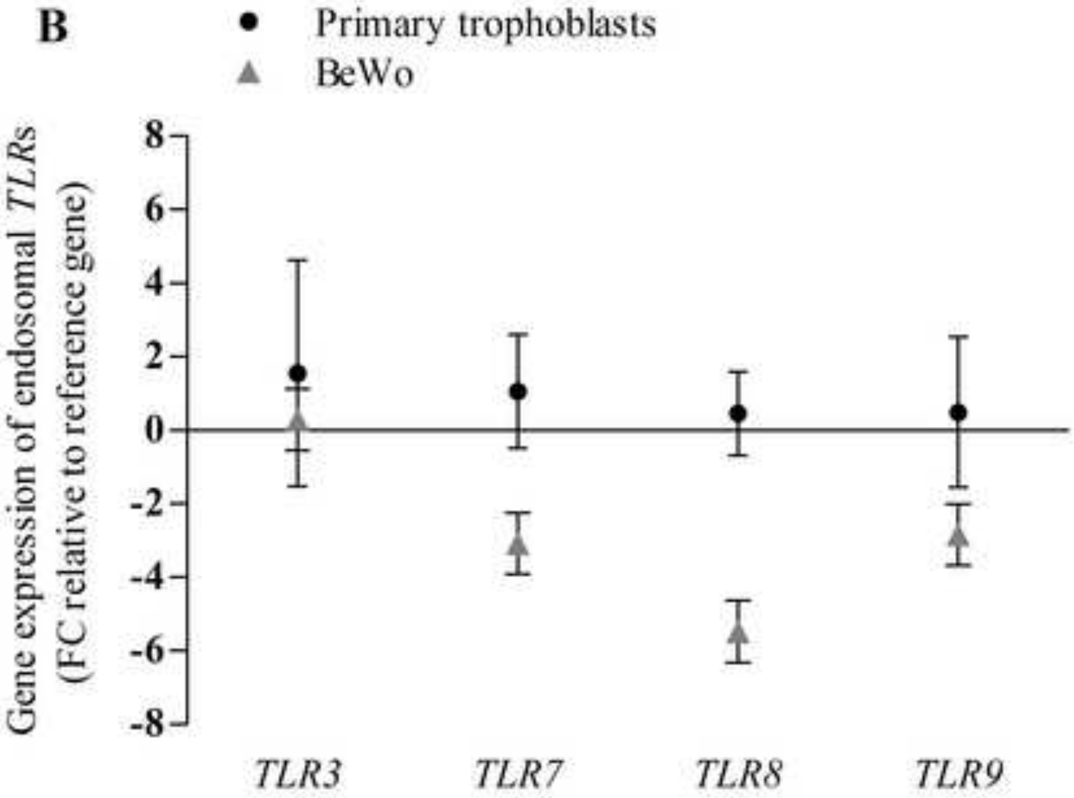

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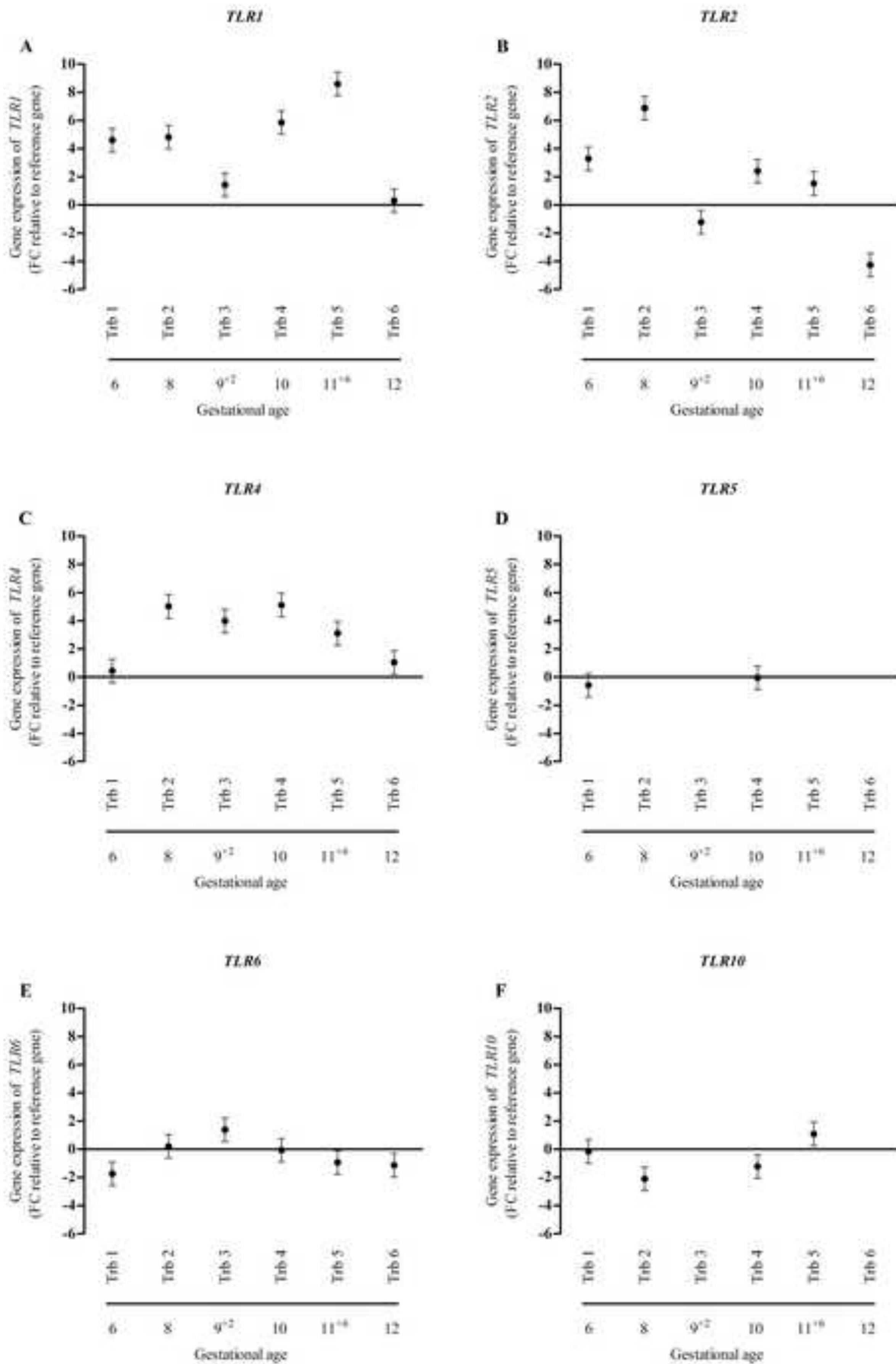

Figure 3

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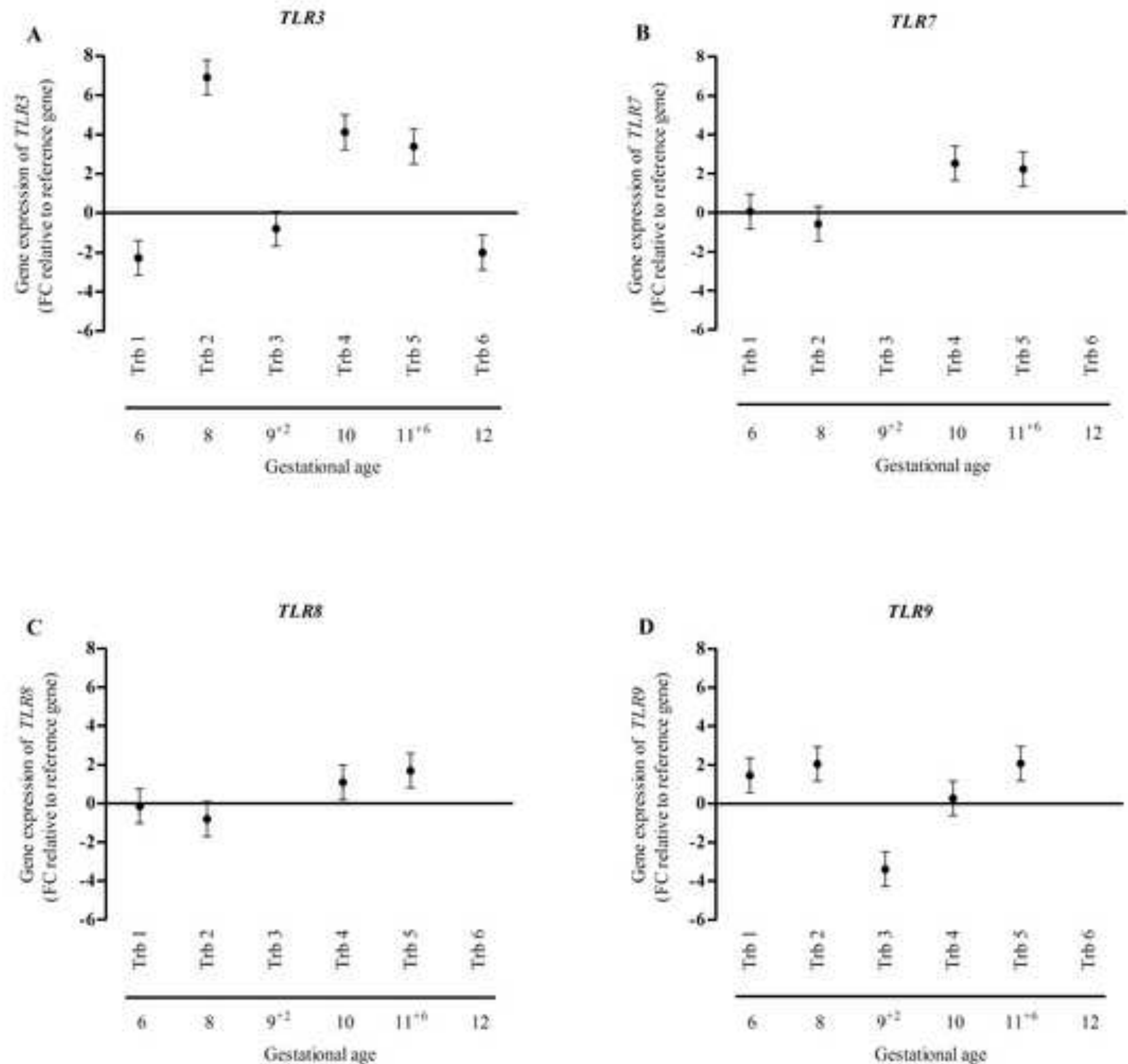

Figure 4
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