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

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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; oligodeoxynucleotide (ODN); PCA, principal component analysis; TBP, TATA box binding protein; TLR, Toll-like receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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.

*Result*s: 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.

Keywords

Cell line, cytokine, inflammation, placenta, Toll-like receptor, trophoblast.

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 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 lifespan 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 to 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), E. 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 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 $gC_T = C_T\text{-log2(threshold value)}$ 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 x 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 (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 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).

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 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.

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Figures

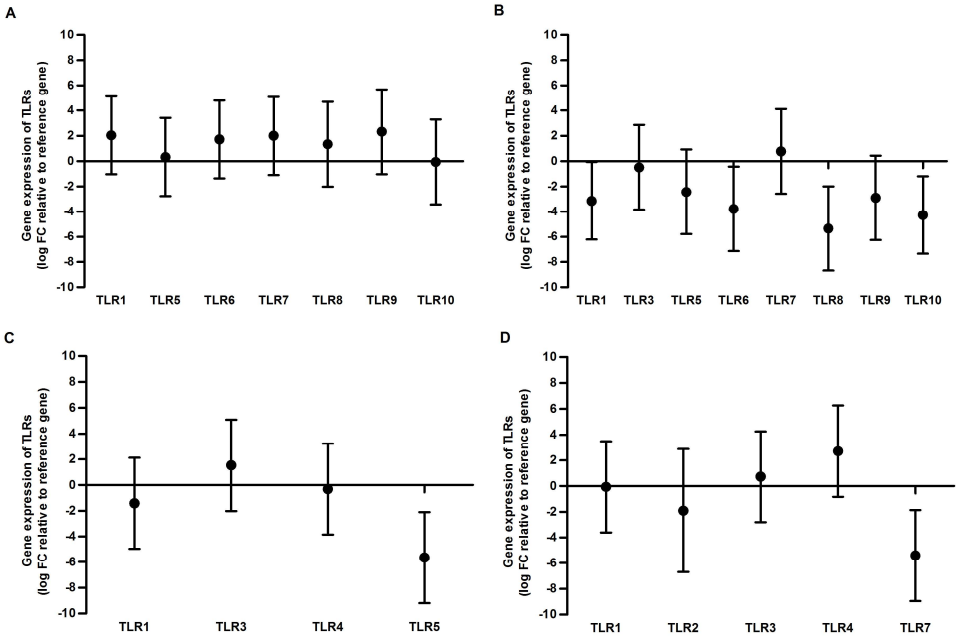

Fig. 1. Toll-like receptors (TLR) 1-10 gene expression levels in choriocarcinoma (JAR (A) and JEG-3 (B)) and SV40 transfected (HTR-8/SVneo (C) and SGHPL-5 (D)) trophoblast cell lines as analyzed by RT-qPCR. Gene expression levels are shown as log fold change (FC) of positive TLR gene expression relative 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.

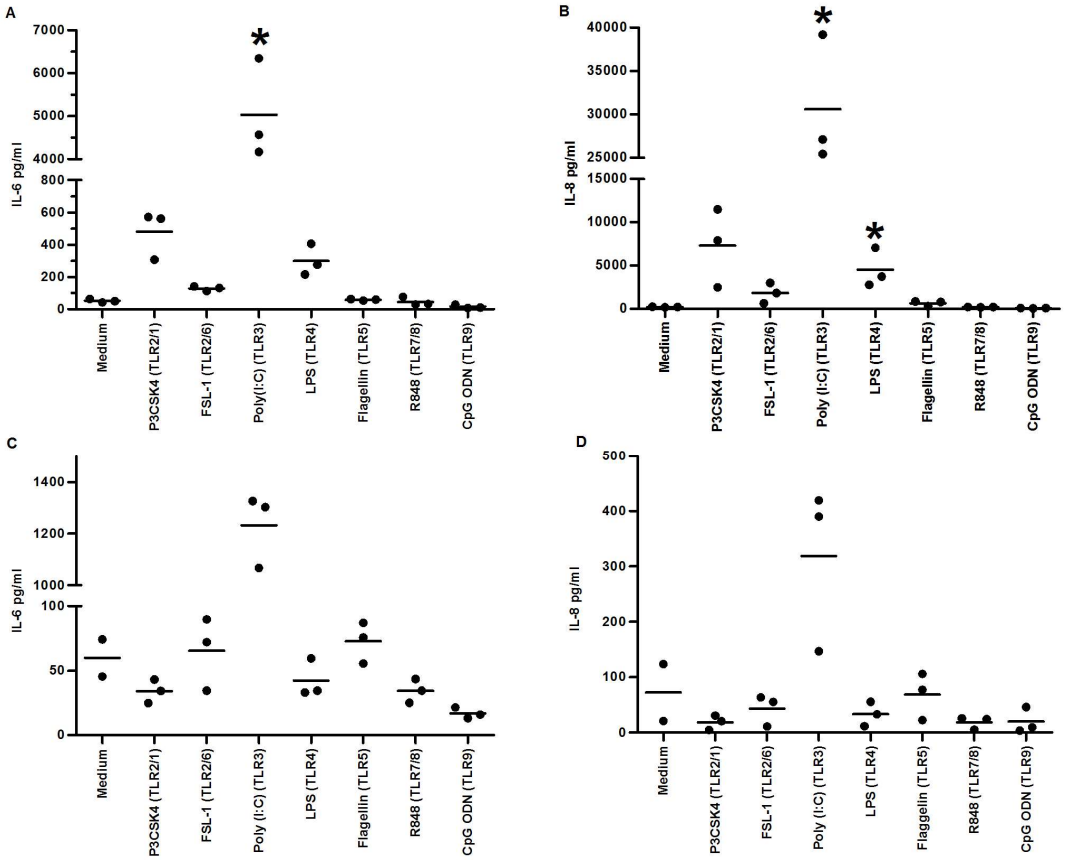

Fig. 2. Toll-like receptor (TLR) ligand activated interleukin (IL)-6 (A, C) and IL-8 (B, D) release (pg/ml) from SV40 transfected trophoblast cell lines SGHPL-5 (A, B) and HTR-8/SVneo (C, D) after 24 h stimulation, as quantified by 10-plex immunoassay. Differences between unstimulated and TLR ligand-induced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

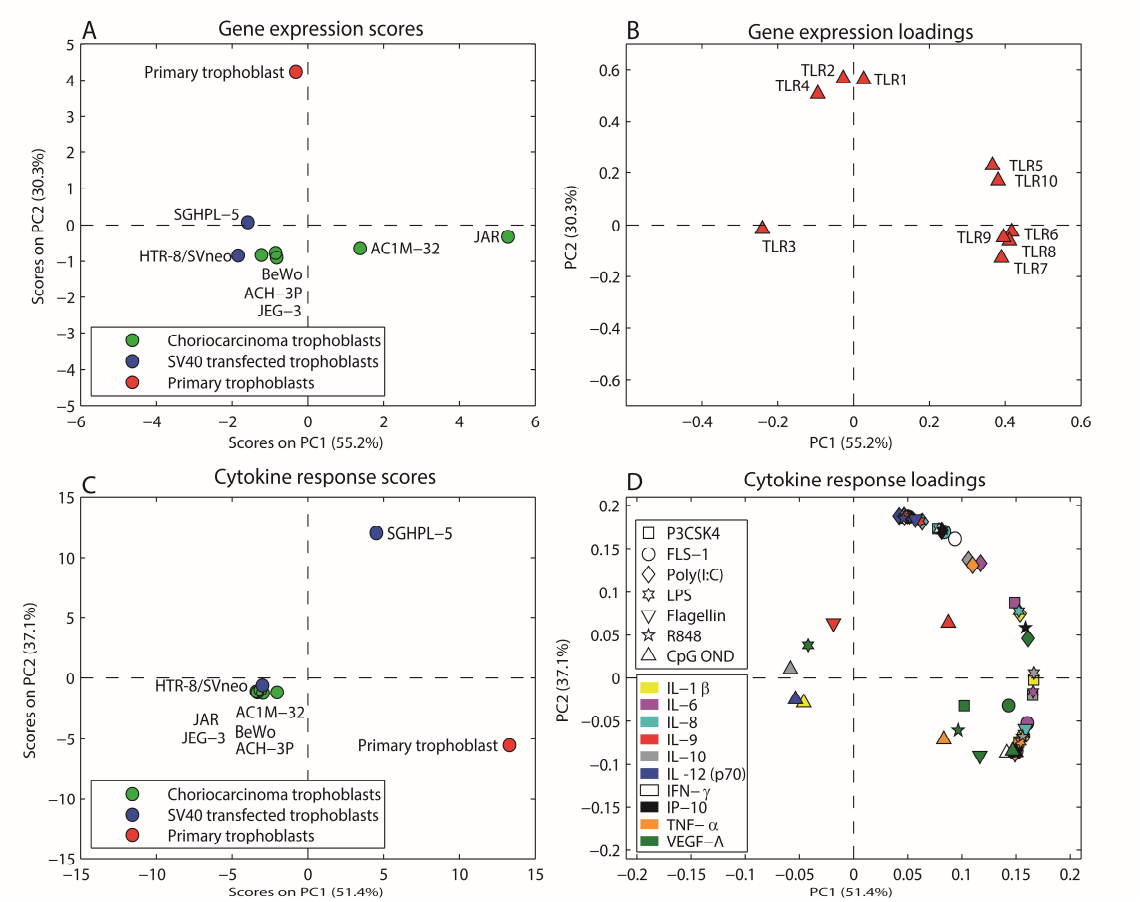

Fig. 3. Principal component analysis (PCA) of Toll-like receptor (TLR) gene expression (A, B) and cytokine responses (C, D) in choriocarcinoma trophoblast cell lines (BeWo, JEG- 3, JAR, AC1M-32 and ACH-3P), SV40 transfected trophoblast cell lines (HTR-8/SVneo and SGHPL-5), and primary first trimester 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.

Tables

Table 1

Characteristics and culture conditions of the seven trophoblast cell lines.

Cell line	Origin	Culture life span	Medium**	Supplement**	FBS**	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* fused with primary term trophoblast cells	Unlimited	Ham's F12		10%	[19]
ACH-3P	AC1-1* 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]

* hypoxanthine guanine phosphoribosyltransferase (HGPRT) -defective mutant of JEG-3

**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).

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.

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.

Supplementary Table 1

Generalized C_T values of Toll-like receptor (TLR) 1-10 gene expression in trophoblast cell lines and primary first trimester trophoblasts.

	TBP	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10
Choriocarcinoma cell lines											
BeWo	29.02	27.22	-	29.92	30.05	33.73	27.57	32.7	34.37	32.01	28.20
	29.15	30.75	-	29.15	34.31	33.70	31.73	33.32	36.88	34.03	33.26
	28.52	31.75	-	30.41	34.49	33.26	33.11	33.58	35.53	32.85	35.64
	32.69		-								
JAR	36.57	31.06	-	-	-	32.16	34.56	31.63	33.59	32.24	27.23
	35.41	27.91	-	-	-	30.29	25.37	28.72	28.76	28.43	29.16
	31.65	28.52	-	-	-	35.42	28.89	31.27	31.08	29.83	44.44
	31.38	39.33	-	-	-	35.82	39.31	35.37	-	-	-
JEG-3	30.68	33.53	-	31.62	-	32.83	31.94	28.10	34.72	33.70	32.64
	33.37	33.00	-	33.11	-	32.82	33.19	29.26	36.25	36.32	34.81
	31.29	34.97	-	32.19	-	35.71	40.29	34.41	38.05	34.96	36.63
	30.95	37.31	-	-	-	-	-	-	-	-	39.23
AC1M-32	32.31	31.79	-	32.38	-	34.51	31.84	30.65	34.16	34.10	33.65
	33.22	31.74	-	34.56	-	35.88	33.54	31.53	33.90	33.45	34.54
	32.98	29.06	-	33.74	-	34.54	29.76	31.06	31.05	32.12	31.22
ACH-3P	32.25	35.29	-	31.31	-	34.97	37.19	31.56	37.33	35.92	37.05
	28.88	28.57	-	31.55	-	34.61	29.77	34.33	39.02	34.65	29.53
	31.62	32.07	-	34.46	-	34.36	39.70	33.64	42.42	30.75	32.96
	31.16	36.48	-	36.94	-	-	-	31.93	37.13	34.96	
	31.24		-	38.27				30.53	30.77	-	
			-	37.75				33.56	34.59		
SV40 transfected cell lines											
HTR8/SVneo	31.59	32.51	-	30.90	31.03	37.65	-	-	-	-	-
	32.45	32.76	-	29.48	32.58	36.53	-	-	-	-	-
	31.05	34.06	-	30.08	32.38	37.82	-	-	-	-	-
SGHPL-5	32.29	32.28	34.53	31.48	29.01	-	-	34.29	-	-	-
	31.17	31.77	31.04	30.67	29.26	-	-	34.62	-	-	-
	31.76	31.33	-	30.87	28.79	-	-	42.51	-	-	-
Primary first trimester trophoblasts											
	33.72	28.71	30.41	35.98	33.26	34.28	35.44	33.64	33.84	32.24	33.85
	35.33	26.74	33.80	31.95	32.22	38.91	36.28	33.11	33.64	33.27	34.25
	34.92	30.10	28.03	28.02	29.90	38.16	35.14	35.50	35.49	32.87	37.01
	35.10	29.24	32.69	30.99	29.98	35.14	35.16	44.75	38.15	34.82	36.12
	31.15	30.83	35.40	33.15	30.10	38.70	32.27	33.75	33.79	-	43.69
	32.99	31.55	34.21	33.78	29.01	39.49	31.61	39.34	39.46	36.38	-

Data represents the mean of generalized C_T values from three technical replicates, with a mean value shown for each biological replicate. The generalized C_T is defined as $gC_T = C_T - \log2(\text{threshold value})$ for each technical replicate, calculated from C_T values representing positive gene expression within the range of 17-32. TATA box binding protein (TBP) was included as reference gene.

-: Indicates no detectable gene expression.

Supplementary Table 2

Cytokine levels (pg/ml) measured in supernatants from trophoblast cell lines and primary first trimester trophoblasts after 24 h of Toll-like receptor (TLR) ligand stimulation.

	Medium only	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								
IL-1β	0.01	0.23	0.15	0.14	0.03	0.19	0.15	0.03
IL-6	28.95	26.88	36.09	39.73	31.87	46.53	31.14	20.53
IL-8	-	-	-	-	-	-	0.17	-
IL-9	1.32	2.39	1.47	2.39	1.32	1.51	0.80	2.60
IL-10	0.27	0.29	0.46	0.66	0.43	0.32	0.35	0.66
IL-12	0.67	0.61	0.57	0.60	0.45	0.31	0.55	0.93
IFN-γ	4.15	7.28	4.15	4.15	-	-	-	14.51
IP-10	16.76	12.19	14.68	48.88	12.64	8.18	12.41	18.05
TNFα	1.74	3.25	2.16	4.06	1.24	3.71	2.58	4.24
VEGF-A	2202	1621	2514	2359	2483	1643	2320	2279
JAR								
IL-1β	0.05	0.04	0.04	0.08	0.04	0.05	0.05	0.04
IL-6	11.84	8.18	7.23	18.43	7.20	17.29	11.17	6.78
IL-8	-	-	0.04	0.05	0.01	0.33	0.09	0.09
IL-9	0.17	0.09	0.03	0.35	0.08	0.38	0.03	0.54
IL-10	1.56	1.39	1.17	1.96	1.49	0.94	0.85	2.63
IL-12	2.88	2.27	1.86	6.66	2.49	1.67	1.80	5.77
IFN-γ	-	-	-	-	-	-	-	-
IP-10	-	-	-	116.82	-	-	-	-
TNFα	-	-	-	0.03	-	0.13	-	-
VEGF-A	2509	1924	1486	2429	1953	1362	1594	2570
JEG-3								
IL-1β	-	-	-	-	-	0.01	-	0.02
IL-6	2.96	1.38	2.22	4.80	1.50	1.62	1.99	2.34
IL-8	0.38	0.30	0.33	0.53	0.23	0.21	0.32	0.40
IL-9	0.57	0.24	0.31	0.99	0.39	0.76	0.30	0.47
IL-10	1.93	1.21	1.46	2.89	1.72	1.13	1.55	1.68
IL-12	4.03	2.53	2.93	6.66	3.34	2.12	2.93	4.08
IFN-γ	-	-	-	-	-	-	-	-
IP-10	-	-	-	11.87	-	-	-	1.57
TNFα	0.01	-	-	-	-	-	0.01	0.01
VEGF-A	847	389	490	955	636	364	535	533
AC1M-32								
IL-1β	0.03	0.04	0.03	0.03	0.04	0.03	0.03	0.03
IL-6	18.00	15.34	13.33	16.28	17.26	16.94	18.72	10.55

IL-8	0.02	0.03	0.03	0.04	0.03	0.01	-	0.31
IL-9	0.41	0.25	0.41	0.47	0.39	1.24	0.51	0.64
IL-10	2.73	2.56	2.31	2.49	2.95	2.84	2.88	2.96
IL-12	7.24	6.56	6.05	5.78	7.33	6.93	8.45	7.18
IFN-γ	-	-	-	-	-	-	-	-
IP-10	-	-	-	-	-	-	-	-
TNFα	-	-	-	-	-	-	-	-
VEGF-A	4135	3957	3382	4027	4713	4307	4782	3351
ACH-3P								
IL-1β	-	-	-	-	-	-	-	-
IL-6	29.66	34.82	25.83	38.64	34.62	24.96	24.83	28.17
IL-8	0.42	0.50	0.45	0.42	0.60	0.28	0.38	0.90
IL-9	0.63	0.52	0.49	0.84	0.57	1.42	0.34	0.93
IL-10	1.91	2.05	1.69	3.58	2.00	1.26	1.44	3.35
IL-12	3.43	4.14	3.08	6.97	3.69	2.42	2.49	8.96
IFN-γ	-	-	-	-	-	-	-	-
IP-10	-	0.75	-	0.20	0.20	-	-	1.92
TNFα	-	-	-	0.01	0.01	0.01	-	-
VEGF-A	848	1356	880	962	1196	661	707	1654
SV40 transfected cell lines								
HTR-8/SVneo								
IL-1β	-	-	-	0.31	-	-	-	-
IL-6	59.93	34.16	65.43	***1232.52***	42.36	72.86	34.36	16.90
IL-8	72.23	18.65	43.32	318.81	33.24	68.37	18.34	19.69
IL-9	2.38	2.42	2.05	3.94	2.05	4.55	2.67	3.19
IL-10	0.88	0.92	0.97	0.77	0.98	0.88	0.82	0.58
IL-12	2.59	2.68	2.88	1.18	2.36	2.75	2.50	1.49
IFN-γ	-	-	-	***42.97***	-	-	-	-
IP-10	7.65	5.10	5.10	64.67	7.65	7.65	2.55	5.10
TNFα	-	-	-	0.60	-	-	-	-
VEGF-A	31.44	36.50	36.43	21.30	35.44	35.86	37.68	34.78
SGHPL-5								
IL-1β	0.05	0.77	0.18	3.14	0.70	-	-	0.05
IL-6	52.25	***480.44***	***129.23***	**5027.56**	***299.31***	59.08	46.03	16.16
IL-8	242	***7304***	***1845***	**30597**	**4535**	663	229	87
IL-9	6.14	12.39	10.53	26.94	13.14	7.29	9.35	7.31
IL-10	8.90	***10.75***	8.91	14.18	11.19	8.50	10.44	9.27
IL-12	40.66	52.57	***46.60***	**76.94**	58.50	44.23	47.97	39.29
IFN-γ	26.07	67.47	31.76	***361.29***	54.74	20.90	26.07	26.97
IP-10	31.67	47.94	48.37	***4420.45***	49.30	31.45	40.51	32.64
TNFα	0.09	0.78	0.21	5.78	0.76	0.25	0.42	0.14
VEGF-A	617	781	797	**1600**	861	608	734	783
Primary first trimester trophoblasts								

IL-1β	6.89	8.45	8.02	10.05	12.26	12.86	11.65	6.52
IL-6	1144	***1529***	1556	***3769***	**1806**	**2257**	1779	1180
IL-8	3033	**4263**	3378	**5494**	**7288**	**5877**	24243	4062
IL-9	4.58	3.79	3.55	4.54	5.05	4.95	4.10	5.57
IL-10	2.05	7.27	8.02	4.65	6.59	12.47	16.63	1.56
IL-12	1.51	1.53	1.19	1.12	1.67	1.68	1.41	1.35
IFN-γ	22.44	20.78	24.08	20.23	27.69	33.37	32.49	50.91
IP-10	41.14	39.12	35.59	**946.30**	44.72	***89.24***	51.68	***90.83***
TNF-α	4.57	11.89	9.70	7.42	14.33	23.01	74.43	6.59
VEGF-A	12006	12513	12531	13293	116927	12280	12652	**16175**

Data represents the mean of three biological replicates (cell lines) and six different placentas (primary trophoblasts).

-: Indicates no detectable cytokine levels in any of the biological replicates.

Numbers in plain bold are significantly ($P<0.01$, two-tailed paired t-tests) increased compared to the unstimulated culture condition. Numbers in italic bold indicate responses at $P<0.05$ but above the threshold of $P<0.01$. IL, interleukin; IFN, interferon; IP, IFN-γ inducible protein; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.