Calcitriol regulates immune genes *CD14* and *CD180* to modulate LPS responses in human trophoblasts

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Abstract

We assessed the response of primary cultures of placental villous mononucleated trophoblasts and multinucleated syncytiotrophoblast to calcitriol, the most biologically active form of vitamin D. Whole-genome microarray data showed that calcitriol modulates the expression of many genes in trophoblasts within 6 hours of exposure and RT-qPCR revealed similar responses in cytotrophoblasts, syncytiotrophoblasts and villous explants. Both cytotrophoblasts and syncytiotrophoblasts expressed genes for the vitamin D receptor, for LRP2 and CUBN that mediate internalization of calcidiol, for *CYP27B1* that encodes the enzyme that converts calcidiol into active calcitriol, and for *CYP24A1* that encodes the enzyme that modifies calcitriol and calcidiol to inactive calcitriol. Notably, we found an inverse effect of calcitriol on expression of *CD14* and *CD180/CD180*, proteins that differentially regulate toll-like receptor 4-mediated immune responses. Supported by gene ontology analysis, we tested the hypothesis that *CD14* and *CD180* modulate the inflammatory response of syncytiotrophoblast to bacterial lipopolysaccharide (LPS). These cells showed a robust response to a wide range of LPS concentrations, with induction of active NF- κ B and increased secretion of IL-6 and IL-8. siRNA-mediated knockdown of *CD14* reduced the secretion of IL-6 and IL-8 in response to LPS. Collectively, our data showed that calcitriol has a rapid and widespread effect on villous trophoblast gene expression in general, and a specific effect on the innate immune response by syncytiotrophoblast.

Reproduction (2017) **154** 735–744

Introduction

Vitamin D refers to a group of important nutrients whose recommended trivial names (IUPAC-IUB 1982, Colvin *et al.* 2015) include ergocalciferol, which is vitamin D₂, and cholecalciferol, which is vitamin D₃. Both isoforms are converted to calcidiol (25-hydroxycholecalciferol) in the liver, and in some cell types, notably the kidney, it has been shown that the heterodimeric receptor pair, megalin (LRP2) and cubilin (CUBN), mediate internalization of calcidiol (Christensen & Birn 2002, Nielsen *et al.* 2016), the most stable vitamin D molecule. Much of the circulating calcidiol is converted in the kidney by activity of the CYP27B1 P450 cytochrome 1- α -hydroxylase to calcitriol, (1 α ,25-dihydroxycholecalciferol), which is the most bioactive vitamin D isoform. Circulating calcidiol can also be converted to calcitriol in other cell types and tissues, including the placenta (Shin *et al.* 2010). Inactivation of vitamin D molecules results from the CYP24A1 hydroxylase, which converts both calcidiol and calcitriol to the biologically inactive calcitriol (1 α ,24R,25-trihydroxycholecalciferol). Notably, placental

villous trophoblasts express both CYP24A1 and CYP27B1 (Shin *et al.* 2010).

Studies of different ethnic groups suggest that vitamin D deficiency or inadequacy affects 20–80% of pregnant women, based on measured calcidiol blood levels (Hollis & Wagner 2006), also see Institute of Medicine report at <http://www.iom.edu/Reports/2010/Dietary-Reference-Intakes-for-Calcium-and-Vitamin-D.aspx>. Moreover, dysregulation of calcidiol activation, catabolism, uptake, or signaling in pregnancy associates with preeclampsia (Díaz *et al.* 2002, Ma *et al.* 2012, Tamblyn *et al.* 2017), gestational diabetes mellitus (Burris *et al.* 2012), intrauterine growth restriction (Nguyen *et al.* 2015), preterm birth (Bodnar *et al.* 2014, Bodnar *et al.* 2015), bacterial vaginosis (Aghajafari *et al.* 2013, Bodnar *et al.* 2015), neonatal skeletal health (Hollis & Wagner 2013), and the placental response to pathogens (Liu *et al.* 2009), highlighting the importance of the vitamin D pathway in pregnant women.

Calcitriol mediates transcriptional effects through binding to the intracellular vitamin D nuclear hormone receptor (VDR) to modulate the expression of hundreds of

target genes over hours or days (DeLuca 2004, Shin *et al.* 2010, Pike 2011). Bioactive vitamin D isoforms are well known to regulate calcium and phosphate homeostasis, but calcitriol also regulates non-classical pathways in epithelial cells (Bikle *et al.* 1986, Ogunkolade *et al.* 2002, Ardesia *et al.* 2015), and in cells of the innate immune response (Chun *et al.* 2014). Notably, calcitriol binding to the VDR in human trophoblast influences placental functions, such as trophoblast secretion of the hormones hCG, hPL, and estradiol (Barrera *et al.* 2012, Noyola-Martínez *et al.* 2014), the secretion of the antimicrobials, cathelicidin (CAMP) and β -defensin 4 (Liu *et al.* 2009, Olmos-Ortiz *et al.* 2015), and, in cases of preeclampsia, secretion of the cytokines IL-6 and IL-8 by trophoblasts (Noyola-Martínez *et al.* 2014).

We assessed the response of primary cultures of human trophoblasts to calcitriol and used whole-genome microarrays to identify hypothesis-generating changes in gene expression. Notably, the microarray identified a marked inverse effect of calcitriol on gene expression for two immunomodulators, CD14 and CD180/RP105 (hereafter designated CD180), both of which regulate toll-like receptor 4 (TLR4)-mediated responses to bacterial ligands (Kelley *et al.* 2013, Gay *et al.* 2014, Ortiz-Suarez & Bond 2016). We then tested the hypothesis that calcitriol-upregulation of CD14 enhances the response of villous syncytiotrophoblasts to bacterial lipopolysaccharide (LPS).

Materials and methods

Study approval and tissue acquisition

The study was approved by the Institutional Review Board of Washington University School of Medicine in St Louis, MO that allowed verbal consent as no patient data was obtained other than the knowledge that the placenta was from a normal, term pregnancy. Primary human villous cytotrophoblasts and placental villous explants were obtained from uncomplicated pregnancies delivered by repeat C-section without labor at 39 weeks' gestation under epidural anesthesia.

Reagents

Catalog numbers of reagents are indicated within parentheses. Calcidiol (D1530, Sigma) and calcitriol (D7938, Sigma) were dissolved in ethanol and stored at -70°C prior to use. Ultrapure LPS EK (tlrl-pekllps, InvivoGen, San Diego, CA, USA) was dissolved in water and stored at -70°C prior to use. Fetal bovine serum (FBS, 2140079, Life Technologies) was treated with dextran-coated charcoal (C6241, Sigma) according to the manufacturer's instructions to generate charcoal stripped (cs) FBS that was depleted for vitamin D and related molecules (Yang *et al.* 1994, Dang & Lowik 2005, Cao *et al.* 2009).

Isolation and culture of primary trophoblasts and explants

Primary human villous cytotrophoblasts were isolated as described (Kliman *et al.* 1986, Chen *et al.* 2010, 2015), plated

at 300,000 cells/cm², washed thoroughly 4 h after plating to eliminate villous fragments and nonviable cells, and the medium was then replaced with DMEM containing 2% or 10% csFBS. Four hours after plating was designated time zero for all experiments. Medium was refreshed every 24 h, and supernatants for assay or cells for RNA or protein were isolated at times indicated in the tables or figure legends. All cell and explant culture was done in ambient (20%) oxygen in a humidified chamber with 5% CO₂. After plating, primary cytotrophoblasts spontaneously fuse and differentiate into multinucleated syncytiotrophoblast from 24 h to 72 h, as described (Chen *et al.* 2010, 2015). Staining for cytokeratin 7 expression and nuclear DNA in cultured cells was done as described previously (Longtine *et al.* 2012a,b).

We used medium with heat inactivated 2% csFBS to limit effects from bovine soluble CD14 in experiments using LPS or siRNA (Yang *et al.* 1994). ELISA indicated that medium with 2% or 10% csFBS both contained undetectable levels of CD14 (data not shown). Cell death, as determined by lactate dehydrogenase (LDH) release and MTS assays, revealed no difference in the viability of trophoblasts cultured in medium with 2% csFBS compared to cells cultured in 10% csFBS (data not shown). All cytotrophoblast cultures were harvested for media supernatant, RNA, or protein after 24 h of culture and all syncytiotrophoblasts after 72 h of culture.

Explants were isolated from regions of villous tissue free of obvious infarct or calcification, washed extensively in medium, and allowed to adapt to culture conditions for 4 h (Chen *et al.* 2010, 2015). Medium was then replaced, and explant culture was continued for 24 h, when supernatants were collected and tissue was harvested.

Calcitriol and LPS treatment

Calcitriol and LPS were added to cultures for the times and concentrations indicated in the table and figure legends.

Microarray analysis

Microarray analysis was conducted as described previously (Cvitic *et al.* 2013) using primary cultures ($n=6$) of human cytotrophoblasts from term placentas, with phenotype verified as previously described. Ethanol as control or 100 nM calcitriol was added for the final 6 h of culture. RNA was harvested, extracted, labeled, and hybridized to GeneChip Human 1.0 ST arrays (Affymetrix, Cleveland, OH, USA), as described (Cvitic *et al.* 2013).

Hybridization and primary analysis were completed at the Division Core Facility for Molecular Biology at the Centre of Medical Research at the Medical University of Graz, Austria. Microarray data were analyzed with Partek Genomic Suite v6.4 software (Partek Inc, St Louis, MO, USA) (Downey 2006). The import process of the .CEL files contained RA normalization (robust multi-chip average) including background correction, quantile normalization across all arrays and median polished summarization based on log transformed expression values. Data are available online at <https://www.ncbi.nlm.nih.gov/geo/> under GEO database accession number GSE95497.

The change in expression of each gene was calculated by determining the fold-change (FC) of the mean intensity for treated vs control samples. ANOVA was performed to reveal significant changes by calcitriol treatment. Genes with a $P < 0.05$ were considered significant. Pathway analysis was performed using Ingenuity Pathway Analysis Software (IPA: Ingenuity Systems, Redwood City, CA, USA).

Quantitative reverse-transcriptase PCR (RT-qPCR) analyses

Nucleic acid isolation from cultured cells and villous tissue, DNA removal by enzymatic digestion, and cDNA synthesis, were done as described (Chen *et al.* 2015). Samples were normalized to parallel reactions with *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta) (Meller *et al.* 2005, Drewlo *et al.* 2012) and the fold expression determined by using the $2^{-\Delta\Delta C_t}$ method (Schmittgen & Livak 2008). We confirmed that *YWHAZ* was unaffected by calcitriol as revealed by microarray analysis and by *YWHAZ* having very similar C_t values in multiple RT-qPCR reactions from samples incubated in the presence or absence of calcitriol. Melting temperatures (T_m) of the PCR products were determined by sequential 0.5°C increases in temperature to 95°C. RT-qPCR primer sequences were designed to span an intron–exon boundary and are shown in Supplementary Table 1 (see section on supplementary data given at the end of this article). All RT-qPCR reactions yielded a product with a single T_m and showed a single band of the expected size upon agarose gel electrophoresis.

Protein extraction and immunoblotting

Protein extraction, separation by SDS-PAGE, transfer to PVDF membrane and detection by chemiluminescence (34076, Thermo Fisher Scientific) were done as described previously (Chen *et al.* 2010). Primary antibodies used included goat anti-actin (SC-1616, 1:3000, Santa Cruz Biotechnology), mouse monoclonal anti-CD14 (SC-58591, 1:1000, SCBT), and rabbit monoclonal anti-CD180 (AB18495, 1:1000, Abcam). HRP-conjugated secondary antibodies were from Cell Signaling Technology and were used at a ratio of 1:5000.

siRNA treatment

siCTRL (si control; 1027310, Qiagen), siVDR (S100008211, Qiagen) and siCD14 (S1000012383, Qiagen) RNAs were resuspended in water at 20 µM and stored at –70°C until use. Primary human trophoblasts were cultured for 24 h and medium exchanged for Opti-MEM medium (31985, Invitrogen) with 2% csFBS. siRNAs were complexed with lipid, using DharmaFECT 1 transfection reagent (T-2001-03, Dharmacon, Lafayette, CO, USA), and 50 nM siRNAs were added to the Opti-Mem with 2% csFBS. After 16 h incubation, the medium was replaced with DMEM with 2% csFBS. Calcitriol or LPS were added during the final 24 h of culture, at concentrations and times noted in figure legends, with cell harvest at 72 h.

ELISA

ELISA of cell extracts for active, DNA-binding NF-κB (1007889, Cayman Chemical), and of culture supernatants for secreted CD14 (DY382, R&D Systems), secreted IL-6 (DY206, R&D Systems), secreted IL-8 (DY208, R&D Systems) and secreted TNF-α (DY201, R&D Systems) were performed according to the manufacturer's instructions. hCG ELISA were performed as described previously (Chen *et al.* 2016).

Data analysis and statistics

Each experiment was reproduced with the numbers of placentas indicated in the table and figure legends. Statistical analyses were performed with the KaleidaGraph software (Synergy Software, Reading, PA, USA) using Student's *t*-test for two comparisons, and ANOVA with Bonferroni *post-hoc* analysis for more than two comparisons, as noted in the table and figure legends, with $P < 0.05$ considered significant.

Results

Differentiation of primary cultures of trophoblasts

Primary cultures of trophoblasts in these experiments replicated our previously published description of differentiation of human cytotrophoblasts into syncytiotrophoblasts (Chen *et al.* 2015, Wang *et al.* 2016). Briefly, more than 95% of the cells isolated from term placental villi were cytokeratin 7 positive mononucleated cells as determined by immunofluorescence staining, indicative of being villous cytotrophoblasts. These cells remained as the mononucleated cytotrophoblast phenotype through 24 h and then progressively formed syncytiotrophoblasts by cell fusion between 24 h and 72 h of culture. By 72 h, ~85% of the nuclei were in syncytia with a greater than five-fold increase of hCG levels in the media, as indicated by ELISA analysis, indicating morphological and hormonal differentiation. For ease of reference, we hereafter denote cells cultured for 24 h as cytotrophoblasts and those cultured for 72 h as syncytiotrophoblasts.

Calcitriol modulates gene expression in trophoblasts and villous explants

We validated our models by examination of expression of genes involved with vitamin D responses and metabolism by RT-qPCR using gene-specific primers under baseline conditions of culture with no added vitamin D (Supplementary Fig. 1). As expected (Ma *et al.* 2012), we found that both villous trophoblast phenotypes and villous explants expressed *CYP24A1* and *CYP27B1* and the vitamin D receptor (*VDR*). Moreover, all three models expressed *megalyn* (*LRP2*) and *cubilin* (*CUBN*), suggesting the encoded proteins could participate in

internalization of calcidiol into trophoblasts and the subsequent conversion to calcitriol by CYP27B1. Under baseline conditions, explants showed higher expression of all vitamin D pathway genes than either trophoblast phenotype, consistent with previous findings that non-trophoblast components of the villous core also express vitamin D-related genes (Ma *et al.* 2012, Knabl *et al.* 2015).

We next assessed the functional responses of vitamin D pathway genes in our three models after exposure to exogenous calcitriol (Table 1). VDR expression was not changed in any system, while CYP27B1 levels showed a modest, yet significant, decrease in cultured trophoblasts. Notably, CYP24A1 expression was significantly induced within 6 h of exposure of cytotrophoblasts and syncytiotrophoblasts to calcitriol, with expression further increased after 24 h of exposure. The response was concentration dependent, as 24 h exposure to 10 nM of calcitriol yielded increased CYP24A1 expression compared to 2 nM. Likewise, calcitriol increased CYP24A1 expression in explants. The expression of the CAMP gene, which encodes the antimicrobial peptide cathelicidin, known to be induced by calcitriol in human trophoblasts (Liu *et al.* 2009), was also rapidly induced by calcitriol in all three models. As expected, trophoblasts are able to convert calcidiol to calcitriol intracellularly, as indicated by changes in gene expression after 24 h exposure to calcidiol. Together, these data indicate that cytotrophoblasts, syncytiotrophoblasts and villous explants express genes involved in vitamin D metabolism and undergo transcriptional responses to calcitriol in a time- and concentration-dependent manner.

Microarray analysis of cytotrophoblasts exposed to calcitriol

Next, we conducted whole-genome microarray analysis of RNA from cytotrophoblasts exposed to calcitriol for 6 h. We found 1590 genes showing significantly altered expression. Restricting the analysis to genes with a fold-change of ≥ 1.25 , we identified 114 genes with altered expression after calcitriol treatment (Supplementary Table 2): 62 with increased and 52 with decreased expression. Twenty-three genes showed a 1.4-fold or greater change in expression. These highly regulated genes did not include *IL-6*, *IL-8*, *TNF- α* or any genes encoding toll-like receptor proteins. The most highly induced gene identified by the microarray was CYP24A1, while other highly induced genes included *HBEGF*, *GOS2*, *CD14* and *IL1B*, with the most highly repressed genes being *RPL23P8* and *CD180*.

We then validated the response of selected genes identified by microarray analysis and compared their expression in cytotrophoblasts, syncytiotrophoblasts and explants. RT-qPCR confirmed calcitriol induction of *CD14*, *GOS2*, *HBEGF* and *IL1B* and reduced expression of *CD180* (Table 2). RT-qPCR analyses showed no effect on *IL-6* expression after 24 h exposure of cytotrophoblasts or syncytiotrophoblasts to 100 nM calcitriol (Supplementary Table 3). We then investigated the expression of six other genes (*CCL5*, *CYR61*, *HSPA4L*, *INHBA*, *TGFB2* and *VEGFA*) whose expression was unchanged in the microarray. As expected, calcitriol did not affect the expression of any of these genes in cytotrophoblasts, syncytiotrophoblasts or explants (Supplementary Table 3). Collectively, these data validate the general findings of the microarray experiments and show that calcitriol induces similar,

Table 1 RT-qPCR of baseline conditions of vitamin D pathway genes.

	Time (h)	Vit D	nM	Cytotrophoblast	Syncytiotrophoblast	Explant
VDR	0	None	0	1.02 $\pm$ 0.01 (4)	1.02 $\pm$ 0.01 (4)	1.02 $\pm$ 0.02 (4)
	24	Calcitriol	100	1.05 $\pm$ 0.31, <i>P</i> =0.75 (4)	0.91 $\pm$ 0.19, <i>P</i> =0.34 (4)	1.21 $\pm$ 0.42, <i>P</i> =0.43 (4)
CYP27B1	0	None	0	1.03 $\pm$ 0.03 (6)	1.0 $\pm$ 0.01 (5)	1.00 $\pm$ 0.01 (4)
	24	Calcitriol	100	0.71 $\pm$ 0.33, <i>P</i> =0.04 (6)	0.74 $\pm$ 0.01, <i>P</i> <0.01 (5)	1.14 $\pm$ 0.27, <i>P</i> =0.15 (6)
CYP24A1	0	None	0	1.02 $\pm$ 0.04 (14)	1.01 $\pm$ 0.01 (13)	1.01 $\pm$ 0.02 (6)
	24	Calcitriol	2	1.58 $\pm$ 1.74, <i>P</i> =0.62 (5)	2.74 $\pm$ 1.26, <i>P</i> =0.02 (5)	nd
	24	Calcitriol	10	7.35 $\pm$ 4.47, <i>P</i> =0.05 (5)	8.66 $\pm$ 4.64, <i>P</i> =0.05 (5)	nd
	6	Calcitriol	100	5.88 $\pm$ 4.26, <i>P</i> <0.01 (10)	37.19 $\pm$ 24.16, <i>P</i> <0.01 (13)	nd
	24	Calcitriol	100	10.10 $\pm$ 11.30, <i>P</i> <0.01 (14)	53.14 $\pm$ 26.19, <i>P</i> <0.01 (13)	17.33 $\pm$ 9.46, <i>P</i> <0.01 (6)
	24	Calcidiol	100	12.23 $\pm$ 6.51, <i>P</i> =0.04 (5)	9.45 $\pm$ 4.1, <i>P</i> <0.01 (5)	nd
	24	Calcitriol	100	12.23 $\pm$ 6.51, <i>P</i> =0.04 (5)	9.45 $\pm$ 4.1, <i>P</i> <0.01 (5)	nd
CAMP	0	None	0	1.03 $\pm$ 0.02 (14)	1.02 $\pm$ 0.06 (13)	1.01 $\pm$ 0.02 (6)
	24	Calcitriol	2	1.73 $\pm$ 1.22, <i>P</i> =0.42 (6)	2.04 $\pm$ 2.24, <i>P</i> =0.39 (6)	nd
	24	Calcitriol	10	9.04 $\pm$ 4.14, <i>P</i> <0.01 (5)	10.34 $\pm$ 5.17, <i>P</i> <0.01 (5)	nd
	6	Calcitriol	100	1.95 $\pm$ 0.43, <i>P</i> <0.05 (4)	4.50 $\pm$ 5.17, <i>P</i> <0.01 (4)	nd
	24	Calcitriol	100	19.65 $\pm$ 19.60, <i>P</i> <0.01 (14)	16.45 $\pm$ 11.00, <i>P</i> <0.01 (13)	2.06 $\pm$ 0.94, <i>P</i> =0.02 (6)
	24	Calcidiol	100	9.05 $\pm$ 9.40, <i>P</i> <0.05 (5)	9.45 $\pm$ 4.10, <i>P</i> <0.01 (5)	nd

Cells or explants were treated with control (EtOH), calcitriol or calcidiol as indicated for the time shown and gene expression assayed by RT-qPCR. Mean $\pm$ s.d., with the number within parentheses indicating the number of primary human trophoblast cultures or explants from different placentas assayed. Bold font denotes significance (*P*<0.05 by Student's *t*-test compared to control) with *P* values indicated. nd, not done.

Table 2 RT-qPCR verification of selected genes responsive to calcitriol in microarray analysis.

	Time (h)	Vit D	nM	Cytotrophoblast	Syncytiotrophoblast	Explant
<i>CD14</i>	0	None	0	1.0±0.01 (11)	1.00±0.00 (11)	1.02±0.02 (6)
	6	Calcitriol	100	2.16±0.87 , <i>P</i> <0.01 (4)	2.45±1.06 , <i>P</i> <0.01 (13)	nd
	24	Calcitriol	100	3.07±0.55 , <i>P</i> <0.01 (11)	3.52±2.20 , <i>P</i> <0.01 (10)	3.19±1.34 , <i>P</i> <0.01 (6)
<i>HBEGF</i>	0	None	0	1.03±0.03 (14)	1.00±0.01 (9)	1.01±0.02 (6)
	6	Calcitriol	100	1.53±1.23, <i>P</i> =0.09 (3)	3.15±1.90 , <i>P</i> <0.01 (7)	nd
	24	Calcitriol	100	2.19±1.11 , <i>P</i> <0.01 (14)	3.25±1.93 , <i>P</i> <0.01 (9)	1.11±0.50, <i>P</i> =0.77 (6)
<i>GOS2</i>	0	None	0	1.02±0.04 (10)	1.01±0.03 (10)	1.01±0.02 (6)
	6	Calcitriol	100	7.27±4.41 , <i>P</i> <0.01 (8)	13.35±1.75 , <i>P</i> <0.01 (8)	nd
	24	Calcitriol	100	7.22±4.12 , <i>P</i> <0.01 (10)	10.86±3.71 , <i>P</i> <0.01 (10)	2.34±0.47 , <i>P</i> <0.01 (6)
<i>IL1B</i>	0	None	0	1.01±0.01 (11)	1.01±0.02 (9)	1.01±0.02 (6)
	6	Calcitriol	100	1.51±1.36, <i>P</i> =0.10 (4)	1.38±0.63, <i>P</i> =0.09 (7)	nd
	24	Calcitriol	100	1.28±0.27 , <i>P</i> =0.02 (11)	1.45±0.57 , <i>P</i> =0.03 (9)	1.38±0.22 , <i>P</i> <0.01 (6)
<i>CD180</i>	0	None	0	1.02±0.03 (10)	1.01±0.00 (10)	1.01±0.02 (6)
	6	Calcitriol	100	0.48±0.31 , <i>P</i> <0.01 (5)	0.69±0.27 , <i>P</i> <0.01 (5)	nd
	24	Calcitriol	100	0.45±0.46 , <i>P</i> <0.01 (10)	0.29±0.19 , <i>P</i> <0.01 (10)	0.49±0.34 , <i>P</i> <0.01 (6)

Cells or explants were treated with control (EtOH) or calcitriol as indicated for the time shown and gene expression assayed by RT-qPCR. Mean±s.d., with the number within parentheses indicating the number of primary human trophoblast cultures or explants from different placentas assayed. Bold font denotes significance (*P*<0.05 by Student's *t*-test compared to control) with *P* values indicated. nd, not done.

but not identical, gene responses in cytotrophoblasts, syncytiotrophoblasts and villous explants.

We performed ontology analysis of the genes in cytotrophoblasts that were responsive to calcitriol treatment by ≥1.25 fold. Ingenuity Pathway Analysis (IPA) identified a network for immune cell trafficking (Supplementary Fig. 2) as most significantly affected. The network shows potential interactions of highly calcitriol-regulated genes (*CD14*, *GOS2* and *CD180*) with the NF-κB complex. Furthermore, IPA identified the immune and inflammatory-related diseases and biofunctions as highly affected and comprising 8 of 10 most significantly regulated disease and function categories (Supplementary Table 4) with participation of *CD14*, *CD180*, *CCL3* and *IL1B*.

Response of *CD14* and *CD180* to calcitriol

Our microarray data showed a substantial upregulation of *CD14* and downregulation of *CD180* in trophoblasts exposed to calcitriol. Notably, these proteins have opposite effects on the TLR4 response (Miyake 2003, Kelley et al. 2013, Ortiz-Suarez & Bond 2016). *CD14* is a TLR4 co-receptor (Jersmann 2005) and is expressed in two forms, one as a GPI-linked membrane protein and one as a soluble form (sCD14) present in serum. Soluble and membrane-associated *CD14* can bind to both TLR4 and to LPS, enhancing the sensitivity of the TLR4-mediated response to LPS. In contrast, *CD180* is a TLR4 accessory protein that acts as a decoy receptor and inhibits the activation of the TLR4 response in the presence of TLR4 ligands.

We thus pursued this pathway to dissect the effects of calcitriol on *CD14* and *CD180* expression in trophoblasts using immunoblot and ELISA analyses. In concordance with the RNA analysis, calcitriol increased

CD14 and decreased *CD180* protein levels in both cytotrophoblasts and syncytiotrophoblasts (Fig. 1A), with the magnitude of the response being greater in syncytiotrophoblasts than that in cytotrophoblasts. Calcitriol exposure also increased sCD14 expression from both primary cell phenotypes (Fig. 1B), with again a larger response in syncytiotrophoblasts, as well as increased sCD14 expression by villous explants (Fig. 1C). Collectively, these data indicate that calcitriol differentially modulates *CD14* and *CD180* gene and protein expression in both trophoblast phenotypes, with a more robust response in syncytiotrophoblast.

VDR mediates the calcitriol-induced changes in *CD14* expression in syncytiotrophoblasts

The multinucleated syncytiotrophoblast forms the outer layer of placental villi and thus is in direct contact with the maternal circulation and likely to be exposed to pathogens present in the maternal blood. Thus, we pursued the response of *CD14* and *CD180* in syncytiotrophoblasts exposed to calcitriol.

Based on other systems, we suspected that the effects of calcitriol on gene expression were mediated by the VDR. To test this idea, we treated cultured syncytiotrophoblasts with control or VDR siRNAs, exposed the cells to calcitriol and assayed the effects of VDR-knockdown on gene expression. As expected, reduction of VDR levels significantly reduced the ability of calcitriol to induce expression of the vitamin D responsive genes, *CAMP*, *CYP24A1* and *GOS2* RNAs (Fig. 2A). Similarly, VDR knockdown inhibited calcitriol-mediated induction of *CD14* mRNA (Fig. 2A) and of *CD14* protein (Fig. 2B). In contrast, VDR knockdown yielded increased levels of *CD180* mRNA (Fig. 2A) in calcitriol-exposed cells. These results indicate that

Figure 1 Calcitriol regulates CD14 and CD180 protein levels in trophoblasts. Cultured primary cytotrophoblasts (Cyto) and syncytiotrophoblasts (Syn) were exposed to vehicle control or 100 nM calcitriol (Calc) for their final 24 h of culture: (A) expression of cellular CD14 and CD180 as determined by immunoblotting normalized to actin as a loading control, and by ELISA analysis of soluble CD14 (sCD14) in (B) cytotrophoblasts and syncytiotrophoblasts as well as (C) explants (Exp). Mean $\pm$ S.D., $n \geq 4$ primary trophoblast cultures or explants from different placentas. (A and B) Conditions without a common italicized letter differ as analyzed by ANOVA with Bonferroni *post-hoc* test ($P < 0.05$). (C) Student's *t*-test ($*P < 0.05$).

calcitriol enhanced CD14 expression and repressed CD180 expression via the VDR in syncytiotrophoblasts.

The above data suggested that in syncytiotrophoblasts CD14 is a downstream target of the VDR pathway in response to calcitriol. Thus, we assayed the effects of CD14 knockdown. Efficient knockdown of CD14 was obtained, with siCD14 resulting in a $\sim 70\%$ reduction of CD14 mRNA (Fig. 2A) with commensurate reductions in membrane bound (Fig. 2B) and secreted (Fig. 2C)

CD14 protein. As expected, CD14 knockdown did not affect the expression of VDR, CYP24A1, CAMP, GOS2 or CD180 mRNA (Fig. 2A), confirming CD14 expression is downstream of the VDR and is not upstream of CYP24A1, CAMP, GOS2 or CD180.

Syncytiotrophoblast response to LPS is enhanced by CD14

The expression of CD14 and CD180 by villous trophoblasts suggested that these cells respond to TLR4 ligands via activation of the TLR4 signaling pathway. To investigate this possibility, calcitriol-exposed syncytiotrophoblasts were treated with increasing concentrations of LPS and we assayed the effects on TLR4 signaling and response. As shown in Fig. 3A, levels of LPS of 2 nM or greater administered ≥ 4 h yielded a significant increase of active (DNA binding) NF- κ B. Similarly, 24 h exposure of syncytiotrophoblasts to 2 nM or greater LPS resulted in a significant increase in expression of the pro-inflammatory cytokines, IL-6 (Fig. 3B) and IL-8 (Fig. 3C). We hypothesized that elevated CD14 enhances LPS binding to TLR4 and amplifies the response of trophoblasts to this ligand. Indeed, in calcitriol-exposed syncytiotrophoblasts subjected to as low as 2 ng/mL of LPS, siCD14 knockdown significantly reduced the TLR4 mediated induction of both IL-6 (Fig. 3D) and IL-8 (Fig. 3E). Collectively, these data indicate that syncytiotrophoblasts undergo a rapid TLR4-mediated pro-inflammatory response to LPS and that this response is increased by elevated levels of CD14.

In some systems, including monocytes (Dickie *et al.* 2010) and in trophoblasts from pre-eclamptic women, calcitriol has been shown to reduce expression of IL-6 and IL-8 (Noyola-Martínez *et al.* 2013). TNF α also has been suggested to be decreased in response to calcitriol in trophoblasts isolated from pre-eclamptic women (Noyola-Martínez *et al.* 2013). As noted above, we did not detect an effect of calcitriol on IL-6 mRNA expression by microarray analysis or by qRT-PCR, or on TNF- α or IL-8 by microarray. Using ELISA, we were unable to quantify TNF α as the levels were below the level of detection in both control and calcitriol-exposed trophoblasts. Moreover, ELISA analysis indicated that exposure of syncytiotrophoblasts to 100 nM calcitriol for 24 h did not affect the level of secreted IL-6 (1.08 ± 0.19 -fold calcitriol vs control, $P = 0.99$, $n = 8$, Student's *t*-test) or secreted IL-8 (0.92 ± 0.83 -fold calcitriol vs control, $P = 0.99$, $n = 8$, Student's *t*-test).

Discussion

The data showed that calcitriol modulates the expression of multiple genes in human placental villous cytotrophoblasts and syncytiotrophoblast within 6 hours of exposure to ligand. Both trophoblast phenotypes

Figure 2 Effect of knockdown of VDR and CD14 on calcitriol-regulated gene expression. Syncytiotrophoblasts were exposed to 100 nM calcitriol (Calc) after siCTRL, siVDR or siCD14 treatment and expression was assayed by (A) RT-qPCR, (B) immunoblotting normalized to actin as loading control or (C) ELISA. Mean $\pm$ s.d., $n \geq 4$ primary trophoblast cultures from different placentas. (A) Student's *t*-test (* $P < 0.05$) vs siCTRL-treated cells (value normalized to 1.0, as shown by the dotted line). (B) Conditions without a common italicized letter differ as analyzed by ANOVA with Bonferroni *post-hoc* test ($P < 0.05$). (C) Student's *t*-test (* $P < 0.05$).

express genes that encode proteins for vitamin D metabolism and signaling. Notably, cytotrophoblasts and syncytiotrophoblasts respond to calcitriol with a marked increase in expression of both the soluble and the membrane-associated isoforms of CD14, a TLR4 co-receptor that enhances the sensitivity of TLR4 signaling in response to LPS, while showing a concomitant reduction in CD180 expression, which inhibits TLR4 activity. Moreover, syncytiotrophoblasts show a robust response to a wide range of LPS concentrations, with induction of active NF- κ B and increased secretion of IL-6 and IL-8. Collectively, the data indicate that calcitriol has widespread effects on villous trophoblast gene expression in general, and a specific effect on the innate immune response.

We found that the calcitriol-mediated induction of CD14 occurs via a VDR-dependent mechanism and siRNA knockdown of CD14 strongly suggests that the elevated CD14 contributes to the increased secretion of IL-6 and IL-8 in response to LPS. This calcitriol-

mediated induction of CD14 and strengthening of the innate immune response to TLR4 ligands should be considered during the inflammatory response of the fetal-placental unit.

An important part of the innate immune response to pathogens involves the TLR proteins (Beutler 2009, Koga *et al.* 2014), which are directly involved in recognition of pathogen. In monocytes, calcitriol has been shown to reduce the expression of TLR2 and TLR4 (Sadeghi *et al.* 2006, Dickie *et al.* 2010) and of TLR9 (Dickie *et al.* 2010), with a variable response time (~ 12 to >24 h), with this downregulation yielding hyporesponsiveness to TLR ligands. Unlike for monocytes, in primary trophoblasts (from pre-eclamptic placentas), calcitriol did not alter TLR2 or TLR4 mRNA levels, although mRNA for TLR2 increased and mRNA for TLR4 decreased after LPS exposure (Liu *et al.* 2009). Using trophoblasts from normal, term placentas we did not detect any effects of calcitriol on TLR expression by microarray analysis, or, by microarray, qRT-PCR and ELISA, of IL-6 or IL-8.

Figure 3 CD14 is involved in the syncytiotrophoblast inflammatory response to LPS. (A, B and C) Syncytiotrophoblasts were exposed to 100 nM calcitriol for 24 h and then to the indicated concentrations of LPS for (A) 4 h or (B and C) 24 h and the levels of active NF- κ B, IL-6 and IL-8 were assayed by ELISA. (D and E) Syncytiotrophoblasts were exposed to siCTRL or siCD14 for 24 h in the absence or presence of 100 nM calcitriol and 10 ng/mL LPS, as indicated, and the levels of IL-6 and IL-8 were assayed by ELISA. Mean $\pm$ s.d., $n \geq 4$ primary trophoblast cultures from different placentas. Calc, calcitriol. (A, B and C) Conditions without a common italicized letter differ as analyzed by ANOVA with Bonferroni *post-hoc* test ($P < 0.05$). (D and E) Student's *t*-test (* $P < 0.05$).

However, further investigation of the role of TLR proteins, and the effects of calcitriol and toll-like receptor ligands on the innate immune response of placental trophoblasts is clearly warranted.

We studied the gene expression profile of primary human placental trophoblast by calcitriol after a relatively short time of 6 hours of ligand exposure. We found multiple responsive genes, but with modest levels of induction, compared to control. A longer exposure to calcitriol would likely have yielded more robust responses and more genes, at the expense of detection of non-primary calcitriol and VDR target genes. The short exposure to calcitriol also allowed us to minimize the confounding effects that could occur over longer exposure due to the *in vitro* differentiation of human cytotrophoblasts to syncytiotrophoblasts, which begins after 24 h of culture. It is worth pointing out, however, that long-term exposure of trophoblasts to different levels of calcitriol (as would occur *in vivo* during pregnancy of pregnancies with insufficient or adequate vitamin D levels) could have large-scale effects on the expression of many genes and proteins in the placenta.

We confirmed our expectation that not only villous tissues (Ma *et al.* 2012, Knabl *et al.* 2015), but also both villous trophoblast phenotypes express vitamin D pathway components. The strength of our study is that multiple genes emerging as vitamin D responsive from our microarray study were confirmed by RT-qPCR. We recognize a weakness in our study is the lack of assessment of primary trophoblast cells from placentas of male vs female offspring, which would allow a direct comparison of the independent variable of gender. Although trophoblastic choriocarcinoma cell lines show some responses to vitamin D (Knabl *et al.* 2015), our study targeted primary cells and villous explants that more likely reflect biological responses that occur in the human placenta.

Notably, in response to calcitriol, villous trophoblasts show a robust increase in the antimicrobial cathelicidin, an increase in *CYP24A1* and a decrease in *CYP27B1*. Villous core expression of several genes was substantially higher in villi compared to either villous trophoblast phenotype, suggesting that villous core endothelium or connective tissue cells are sources for a paracrine interaction between trophoblast and endothelium, as suggested previously (Ma *et al.* 2012, Knabl *et al.* 2015). Indeed, disruption in the vitamin D signaling in villi from pre-eclamptic women has been suggested by this group (Ma *et al.* 2012).

Vitamin D enhances the antibacterial responses of human trophoblast, in part by a robust induction of cathelicidin (CAMP) (Liu *et al.* 2009). Our data extend the understanding of the vitamin D-induced innate immune responses of human trophoblast by identifying a likely role for the upregulation of CD14, and downregulation of CD180, in response to calcitriol binding to the VDR in trophoblast. These two CD proteins influence the

cellular TLR4 response in opposite directions (Ortiz-Suarez & Bond 2016). TLRs are expressed in many epithelia (Gay *et al.* 2014) including human trophoblasts, and multiple bacterial products, including LPS, activate TLR4. Notably, membrane-bound CD14 enhances the sensitivity of TLR4 when LPS concentrations are low (Jersmann 2005) while sCD14 may bind LPS in plasma and present this ligand to TLR4 on trophoblasts in women exposed to systemic infections (Gay *et al.* 2014). Clearly, determination of the dosage for dietary supplementation that yields optimal vitamin D effects on placental villi require further basic research into end organ transport, binding and metabolism of all the vitamin D metabolites.

The calcitriol-mediated induction of CD14 in trophoblasts, and the increased expression of IL-6 and IL-8 in response to TLR4 ligands, should be considered during inflammatory responses of the fetal-placental unit.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-17-0183>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Department of Obstetrics and Gynecology at Washington University School of Medicine, St Louis, MO, USA, and by funds of the Oesterreichische Nationalbank (Anniversary Fund, project number: 16442) Graz, Austria.

Acknowledgements

The authors would like to thank M Laura Costa for helpful discussions.

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Received 27 March 2017

First decision 10 May 2017

Revised manuscript received 17 August 2017

Accepted 4 September 2017