BOR Papers in Press. Published on November 12, 2008 as DOI:10.1095/biolreprod.108.073577

Vitamin D Induces Innate Antibacterial Responses in Human Trophoblasts via an Intracrine Pathway

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Abbreviated title: Vitamin D and trophoblast innate immunity

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This work was supported by NIH grant RO1AR050626 to M.H.

Abstract

The active form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)$_2$D) is a potent inducer of the antimicrobial protein cathelicidin, CAMP (LL37). In macrophages this response is dependent on intracrine synthesis of 1,25(OH)$_2$D from precursor 25-hydroxyvitamin D (25OHD), catalyzed by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1). In view of the fact that trophoblastic cells also express abundant CYP27B1, we postulated a similar intracrine pathway for induction of CAMP in the placenta. Analysis of placenta explants, primary cultures of human trophoblast, and the 3A trophoblastic cell line treated with 1,25(OH)$_2$D (1-100 nM) revealed dose-dependent induction of *CAMP* similar to that observed with primary cultures of human macrophages. Also consistent with macrophages, induction of trophoblastic *CAMP* was enhanced via intracrine conversion of 25OHD to 1,25(OH)$_2$D. However, in contrast to macrophages, induction of *CAMP* by vitamin D in trophoblasts was not enhanced by co-stimulation with toll-like receptor ligands such as lipopolysaccharide. Despite this, exposure to vitamin D metabolites significantly enhanced antibacterial responses in trophoblastic cells: 3A cells infected with *Escherichia coli* (*E. coli*) showed decreased numbers of bacterial colony-forming units compared to vehicle-treated controls when treated with 25OHD (49.6 ± 10.9 %) or 1,25(OH)$_2$D (45.4 ± 9.2 %), both $p<0.001$. Treatment with 25OHD (1-100 nM) or 1,25(OH)$_2$D (0.1-10 nM) also protected 3A cells against cell death following infection with *E. coli* (13.6-26.9 and 22.3-40.2% protection respectively). These observations indicate that 1,25(OH)$_2$D can function as an intracrine regulator of CAMP in trophoblasts, and may thus provide a novel mechanism for activation of innate immune responses in the placenta.

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Introduction

The active form of vitamin D, 1,25-dihydroxyvitamin D (1,25$(OH)_2$D), is a potent modulator of human immune responses [1-3]. In particular, recent studies have shown that 1,25$(OH)_2$D promotes innate immunity by stimulating synthesis of the antimicrobial protein cathelicidin (CAMP) [1-5]. This response is due to direct transcriptional regulation of the gene for CAMP [6, 7], and 1,25$(OH)_2$D-induced CAMP expression has been demonstrated in a variety of cell types expressing the intracellular vitamin D receptor (VDR) [7-10]. Although this mechanism appears to play a central role in the combating infection by *Mycobacterium tuberculosis* (M. tb) [5, 8, 11], CAMP is known to be an effective inhibitor of other pathogens such as *Staphylococcus aureus* [12-14], as well as exhibiting antiviral properties [15-17]. In a similar fashion, although vitamin D-induced CAMP-mediated bacterial killing is a pivotal feature of macrophages [8, 11], an equivalent mechanism has also been described for keratinocytes following epidermal injury [9]. Thus, CAMP expression has been linked not only to tuberculosis but other diseases such as sepsis [18], inflammatory bowel disease [19], and inflammatory lung disease [20]. Collectively, these observations support the hypothesis that a key non-classical function of 1,25$(OH)_2$D is to enhance innate immunity through localized production of CAMP.

As indicated above, the ability of vitamin D to promote antibacterial activity is dependent on the expression of VDR by cells such as macrophages. The availability of ligand for the VDR may be due to endocrine production of 1,25$(OH)_2$D in the kidneys, catalyzed by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) which is expressed in the proximal tubules [21, 22]. However, current data suggest that innate immune responses to vitamin D are more likely to involve an intracrine mechanism, with cells such as macrophages acting as an extra-renal site for expression of CYP27B1 and synthesis of 1,25$(OH)_2$D [23, 24]. Consistent with this, several studies have demonstrated induction of CAMP following treatment with the inactive precursor metabolite 25OHD, as well as active 1,25$(OH)_2$D [8, 9, 25].

Another key extra-renal site for expression of CYP27B1 is the placenta, with both fetal trophoblast and maternal decidua demonstrating synthesis of 1,25$(OH)_2$D [26, 27], Expression of placental CYP27B1 is induced early in pregnancy [28, 29], and we have postulated that a key function of the enzyme in this tissue is to support both innate and adaptive immunity in utero [29]. In previous studies we have described intracrine induction of CAMP by 25OHD in maternal decidual cells from first trimester human placentas [25]. In data presented here we have explored this function of vitamin D in trophoblasts, a cell type closely linked to innate immune responses within the placenta [30]. Consistent with previous studies of decidual cells, trophoblasts potently expressed CAMP in response to treatment with either 25OHD or 1,25$(OH)_2$D, confirming an intracrine mode of action. This innate immune response was similar to that observed with macrophages. In common with macrophages, the induction of CAMP by 1,25$(OH)_2$D enhanced antibacterial responses in trophoblasts and also abrogated the cell death that occurs following infection of these cells. However, in contrast to macrophages in which the vitamin D system is activated via toll-like receptors (TLR), trophoblast responses to vitamin D metabolites appears to be due to constitutive

expression of VDR and CYP27B1. Collectively these data suggest that intracrine induction of placental innate immunity may be an important function for vitamin D during pregnancy.

Materials and Methods

Cell lines and reagents

3A and JEG3, human trophoblastic cell lines were obtained from American Type Tissue Culture Collection (ATCC). Cells were cultured as adherent monolayers in Modified Eagles Medium (MEM) (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal-calf serum (FCS), 10 mM HEPES, 1 mM sodium pyruvate, and 100 nM penicillin/streptomycin (Invitrogen Life Technologies). Macrophages, 3A trophoblastic cells, JEG-3 cells, placenta explants and primary trophoblasts were treated with 1,25(OH)$_2$D (1-100 nM), 25OHD (1-100 nM) or vehicle (0.1% ethanol) for 24-48 hrs. 3A trophoblastic cells were treated with reagents from a toll-like receptor (TLR) ligand kit (Invivogen, San Diego, CA, USA). These reagents included: Pam3CSK4, TLR1/2 agonist; HKLM, TLR2 agonist; Poly(I:C), TLR3 agonist; Lipopolysaccharide (LPS) (*Escherichia coli* K12), TLR4 agonist; Flagellin *Salmonella typhimurium*, TLR5 agonist; FSL1, TLR6/2 agonist; Imiquimod, TLR7 agonist; ssRNA40/LyoVec, TLR8 agonist; ODN2006, TLR9 agonist. All TLR agonists were added to culture medium at the manufacturer's recommended dose (Invivogen).

Isolation and culture of placental tissue and primary trophoblasts

Tissue was isolated from third-trimester placentas from healthy full-term pregnant women undergoing elective caesarian section. All samples were obtained in accordance with Cedars-Sinai Medical Center Institute Review Board protocols. Unpurified placental tissue was separated from membranes, minced using forceps and scalpel and then washed Hank's Balanced Salt Solution (HBSS) (Invitrogen Life Technologies) and centrifuged three times to remove excess blood and cell debris. The resulting placental explants were then resuspended in Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen Life Technologies) supplemented with 10% normal human serum (Gemini Bio-Products, West Sacremento, CA). Trophoblastic cells were isolated as described previously [31]. Briefly, placental tissue specimens were washed with cold HBSS to remove excess blood. Cells were scraped from the membranes, transferred to trypsin-EDTA (Invitrogen Life Technologies) digestion buffer and incubated at 37°C for 10 min with shaking. An equal volume of DMEM containing 10% FCS was added to inactivate the trypsin. This mixture was vortexed for 20 s and allowed to sediment, and the supernatant was collected. This was repeated twice and the collected supernatant was centrifuged at 1500 rpm for 10 min. Contaminating red blood cells were removed by resuspending the cellular pellet with HBSS, layering this over the same volume of Lymphocyte Separation Media (ICN Biomedicals, Irvine, CA), and centrifuging at 2000 rpm for 25 min. The cellular interface containing the trophoblast cells was collected and resuspended in DMEM supplemented with 10% normal human serum and cultured at 37°C/5% CO_2 for three passages. Purity of the trophoblast cells was >98% as determined by immunostaining for cytokeratin-7 (Sigma-Aldrich, St Louis, USA) [31].

Isolation and culture of human macrophages
Peripheral blood mononuclear cells were isolated from buffy coat preparations of human blood obtained commercially from the Virology Core Unit at UCLA. Monocytic cells were adhered to 24 well cultured in serum-free RPMI 1640 (RPMI) culture medium for 2 hrs, after which non-adherent cells were removed and the remaining adherent monocytes washed with further serum-free RPMI. The monocytes were then cultured for 5 days in RPMI supplemented with 10% fetal calf-serum (FCS) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Immunex, Seattle, WA). The resulting cells showed increased expression of macrophage makers such as CD14 [23].

Extraction of RNA and reverse transcription
RNA was extracted from mouse tissues using the RNeasy Total RNA extraction kit as detailed by the manufacturer (Qiagen, Valencia, CA). RNA was eluted in RNase-free elution solution and aliquots (1.5 µg) were reverse-transcribed using Powerscript MMLV reverse transcriptase as described by the manufacturer (ABI, Foster City, CA).

Quantitative real time RT-PCR amplification of cDNAs
Expression of mRNAs for *VDR*, *CYP27B1*, 24-hydroxylase (*CYP24*), *CAMP*, β-defensin-4 (*DEFB4*) and other specified genes was quantified using an ABI 7700 sequence detection system (ABI) as described previously [32]. Approximately 50 ng of cDNA was used per reaction. All reactions were multiplexed with the housekeeping *18S* rRNA gene (Assays-on-Demand (ABI) primer and probe mix Hs99999901_s1), enabling data to be expressed in relation to an internal reference to allow for differences in sampling. Data were obtained as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line), and used to determine ΔCt values (Ct of target gene–Ct of housekeeping *18S* rRNA gene). PCR amplification of target gene cDNA was carried out using the following Taqman human gene expression assays: 1α-hydroxylase (*CYP27B1*), forward primer 5’-TTGGCAAGCGCAGCTGTAT-3’, reverse primer 5’-TGTGTTAGGATCTGGGCCAAA-3’,TaqMan probe 5’-TTGCAATTCAAGCTCTGCCAGGCG-3’; vitamin D receptor (*VDR*), forward primer 5’-CTTCAGGCGAAGCATGAAGC-3’, reverse primer 5’-CCTTCATCATGCCGATGTCC-3’, Taqman probe 5’-AAGGCACTATTCACCTGCCCCTTCAA-3’; 24-hydroxylase (*CYP24A1*), Forward primer, 5’-CAAACCGTGGAAGGCCTATC-3’; reverse primer 5’-AGTCTTCCCCTTCCAGGATCA-3’;TaqMan probe 5’-ACTACCGCAAAGAAGGCTACGGGCTG-3’; *CD14*, Assays-on-Demand (ABI) primer and probe mix Hs00169122_g1; toll-like receptor 2 (*TLR2*) Hs00610101_m1 ; *TLR4*, Hs00152939_m1; cathelicidin (*CAMP*), Hs00189038_m1; inducible nitric oxide synthase (*NOS*2) Hs00167257_m1. All cDNAs were amplified under the following conditions: 50 °C for 2 min; 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. All reactions were performed in triplicate and initially expressed as mean ± SD ΔCt values which were employed in statistical comparisons. Visual representation of data was carried out by converting ΔCt values to fold-change data relative to ΔCt values for control (vehicle-treated) cells using the equation $2^{-\Delta\Delta Ct}$.

*Measurement of $1,25(OH)_2D$ synthesis by macrophages and trophoblasts***.**
Activity of CYP27B1 in 3A trophoblasts and macrophages was assessed by quantifying

the conversion of ^{3}[H]-25OHD (180 Ci/mmol, Amersham Biosciences, Piscataway, NJ) to ^{3}[H]-1,25$(OH)_2$D. For each assay, 10 nM ^{3}[H]-25OHD suspended in 0.2 ml serum-free RPMI was added to 3A cells or macrophages and incubated at 37°C for 5 hrs, with the reaction being terminated by freezing at –20°C. Protein from these samples was initially precipitated with added acetonitrile (1:1). Vitamin D metabolites were then extracted from the reaction mixtures by elution on C18-OH columns according to manufacturer's instructions (Diasorin, Stillwater, MN). The resulting eluent was resuspended in 25 μl of elution solvent hexane:methanol:isopropanol (90:5:5), vortexed for 15 seconds, and individual metabolites separated by HPLC using a Beckman Gold system with an Agilent Technologies Zobax Sil normal phase column (Agilent Technologies, Palo Alta, CA) eluted at a rate of 1.5ml/min for 20 minutes. Elution profiles for standard vitamin D metabolites (25OHD, 24,25$(OH)_2$D, 1,25$(OH)_2$D) were determined by UV absorbance at 264nm. Elution of metabolites of ^{3}[H]-25OHD was assessed using a β-Ram Model 4 in-flow detector (IN/US, Tampa, Fl) in conjunction with Ultima-Flo M scintillation fluid (Perkin-Elmer, Boston, MA) at a 2:1 ratio with a 5 second dwell time to designate the increments for data collection. Lauralite 3 software (LabLogic, Sheffield, UK) was used to quantitate peaks of radioactivity corresponding to 25OHD, 24,25$(OH)_2$D or 1,25$(OH)_2$D. Data were reported as mean fmoles metabolite synthesized/hr/10^6 cells ± S.D. following n=3 separate experiments.

Analysis of 3A trophoblastic cell infection with Escherichia coli
3A cells were seeded at $3x10^4$cells/well in a 24-well plate. Cells were cultured with MEM + 10% FBS at 37°C, 5% CO_2, for 72 hrs and then treated with either 10nM 1,25$(OH)_2$D or 100nM 25OHD for 48 hrs. E. *coli* were cultured in 5ml LB broth overnight with shaking at 250rpm and 37°C. From these cultures, an aliquot (100 μl) of bacteria were added to 10ml fresh LB medium, and culture continued at 37°C with shaking at 250rpm for 2 ~ 3 hours until the optical density (OD) at 600 nm reached 0.4. The resulting bacteria were then centrifuged at 3000 rpm for 10 min and the pellet washed twice with PBS and then resuspended in MEM + 10% FBS. From this suspension, E. *coli* were added into each well of cultured 3A cells at a dose of 50 bacteria/cell in a total volume of 200 μl/well of 3A cells. The cell culture plates were then centrifuged at 500 rpm for 5 min and then incubated at 37°C with 5% CO_2 for 30min. Extracellular E. *coli* were then removed by aspiration and the 3A cells washed once with PBS. Any residual extracellular bacteria were then killed by the addition of 5 μg/ml gentamicin in MEM + 10% FBS. At 24 hours after the addition of gentamicin, the antibiotic removed by aspiration and cells washed 3 times with PBS. 3A cells were then lysed by the addition of 1 ml distilled water to each well. The resulting lysates were then removed from each well, transferred to an eppendorf tube and then vortexed to ensure complete disruption of the cells. Serial dilutions of each lysate tube were added to LB plates and incubated at 37°C over night, after which the resulting colonies were counted.

Analysis of 3A trophoblastic cell death following infection with E. coli
Assays for *E. coli* infection were performed essentially as described before [33]. Briefly, E coli (strain DH5α) (Invitrogen) were grown in TH Broth to mid-log phase (~ 10^8 CFU/ml; OD_{600}=0.4), washed in PBS, resuspended in RPMI with 10% FBS, and used to infect the 3A trophoblastic cells. The resulting cell cultures were centrifuged at 700g for

5 min to settle the bacteria on the monolayer surface then incubated at 37°C in 5% CO_2 for 1 hour. To kill extracellular bacteria, cells were washed 3x with sterile PBS and incubated with media containing gentamicin (100 μg/ml) and penicillin (5 μg/ml). The concentration and purity of each E. coli inoculum was confirmed by quantitative culture on THB agar plates. 3A cell death was assessed by measuring cell culture supernatant lactate dehydrogenase levels by performing colorimetric lactate dehydrogenase (LDH) assay (Roche Diagnostics) according to the manufacturer's instructions. Data were reported as the LDH released upon Triton X-100 treatment of cells relative to control infected cells.

Statistical analyses
Data were expressed as mean ±SD unless otherwise stated. Statistical analysis of data was carried out using a student's t-test applied to raw data, including ΔCt values from real time RT-PCR assays. All statistical analyses were carried out using Sigmastat 3.1 software (Systat Inc., San Jose, CA, USA).

Results
1,25(OH)$_2$D induces expression of CAMP in human trophoblasts
To determine whether vitamin D stimulates innate immune response in placental cells, we assessed the effects of 1,25(OH)$_2$D on expression of *CAMP* in various in vitro trophoblastic cell models relative to human macrophages. Results in Figure 1 showed a dose-dependent induction of *CAMP* in placental explant tissue cultures, primary human trophoblasts and 3A transformed human trophoblasts in response to 24 hr treatment with 1,25(OH)$_2$D (1-100 nM). The sensitivity and magnitude of this response was similar to that observed with primary cultures of human macrophages, the principal target cell for 1,25(OH)$_2$D-induction of *CAMP*. Similar studies were also carried using the choriocarcinoma trophoblastic cell line JEG-3 but this showed no response to treatment with 1,25(OH)$_2$D (results not shown).

Expression and activity of CY27B1 in human trophoblasts
Further RT-PCR analyses indicated that vitamin D-activating enzyme CYP2B1 was expressed by human trophoblasts and by the 3A trophoblastic cell line (Figure 2). Levels of mRNA for *CYP27B1* were relatively low in the trophoblastic cultures compared to human macrophages. However, measurement of actual CYP27B1 activity (conversion of 25OHD to 1,25(OH)$_2$D utilizing radiolabeled 25OHD as substrate) revealed comparable levels of 1,25(OH)$_2$D synthesis in 3A trophoblasts (13.2 ± 3.6 fmoles/hr/10^6 cells) relative to macrophages (16.7 ± 5.1 fmoles/hr/10^6 cells). Furthermore, trophoblasts and 3A cells showed levels of mRNA for the VDR that were 2-3 times higher than macrophages, underlining the potential for autocrine/intracrine response to endogenously synthesized 1,25(OH)$_2$D in these cells (Figure 2). The trophoblastic cells and macrophages showed similar levels of mRNA for the feedback control enzyme 24-hydroxylase (CYP24), which is potently induced in response to exogenous or endogenous 1,25(OH)$_2$D. However, enzyme activity analyses showed enhanced production of the CYP24 metabolite 24,25(OH)$_2$D in 3A trophoblasts (19.0 ± 4.6 fmoles/hr/10^6 cells) compared to macrophages (4.3 ± 2.7 fmoles/hr/10^6 cells).

Intracrine vitamin D-induction of innate immune responses in 3A trophoblasts is specific for CAMP

Having demonstrated expression and activity of CYP27B1 in trophoblastic cells, we then sought to determine whether this was sufficient to induce expression of *CAMP* in an intracrine fashion. Results in Figure 3 showed that incubation of 3A cells with 10 or 100 nM 25OHD for 24 hrs stimulated expression of *CAMP* to a similar degree as 1 and 10 nM 1,25$(OH)_2$D. Furthermore this response was specific to *CAMP* as neither 25OHD nor 1,25$(OH)_2$D affected expression of other putative antibacterial gene products such as β-defensin 4 (*DEFB4*) or inducible nitric oxide synthase (*NOS2*).

Intracrine induction of trophoblast CAMP by vitamin D is independent of TLR responses

Results in Figure 4 showed that the TLR4 ligand LPS had no effect on *CAMP* induction by either 25OHD or 1,25$(OH)_2$D by either cell type. Interestingly, in primary trophoblasts LPS added alone suppressed expression of *CAMP* but this effect was abrogated by co-incubation with 25OHD. Results in Figure 5 showed that, in macrophages, LPS induced expression of *CYP27B1*, *VDR*, *TLR2* or *TLR4* and this was unaffected by co-treatment with 25OHD. By contrast, in 3A cells, LPS had no effect on expression of *CYP27B1*, *VDR*, *TLR2* or *TLR4*, despite the fact that *TLR4* was detectable in 3A cells. Further studies using a panel of ligands to TLR1-TLR9 also showed no effect on *VDR* or *CYP27B1* expression in 3A cells (see Supplemental Figure 1, available online at www.biolreprod.org). In primary trophoblast cultures, LPS stimulated expression of *TLR2* in a similar fashion to that observed with macrophages but, paradoxically suppressed levels of mRNA for *TLR4*. In both cases these changes were unaffected by co-treatment with 25OHD. Also unlike macrophages, treatment of primary trophoblastic cells with either 25OHD or 1,25$(OH)_2$D acted to suppress expression of *CYP27B1*.

Vitamin D-induced expression of CAMP in 3A trophoblasts suppresses bacterial infection and counteracts infection-associated cell death

To assess the functional significance of vitamin D-induced expression of *CAMP*, further studies were carried out using 3A trophoblastic cells infected with *E. coli* . Analysis of *E. coli* colony forming units (cfu) in 3A cells showed that pre-treatment with 25OHD (100 nM) or 1,25$(OH)_2$D (10 nM) significantly suppressed levels of bacterial infection (49.6 ± 10.9 % and 45.4 ± 9.2 % relative to vehicle-treated controls, both $p<0.001$) (Figure 6A). We have shown previously that infection of trophoblasts with GBS leads to the induction of cell death [33]. Therefore, we carried out similar studies using 3A cells treated with vehicle, 25OHD (1-100 nM) or 1,25$(OH)_2$D (0.1-10 nM). Results in Figure 6B showed that infection of 3A cells with *E. coli* stimulated the release of lactate dehydrogenase (LDH), a marker of cell death [33]. However, 3A cells pre-treated with 25OHD or 1,25$(OH)_2$D for 48 hrs showed significantly lower levels of LDH, indicating that these cells were protected against *E. coli*-induced cell death. Similar studies were also carried out using 3A cells infected with *Group B streptococcus* (GBS). In this case 25OHD (100 nM, 24hrs) and 1,25$(OH)_2$D (10 nM, 24 hrs) suppressed LDH release by 12.5 ± 3.1% and 8.3% ± 3.1% respectively

Discussion

The placenta is one of the principal extra-renal sites for synthesis of the active vitamin D metabolite 1,25$(OH)_2$D [26-28, 34, 35]. In keeping with the classical endocrine function of 1,25$(OH)_2$D, expression of CYP27B1 by the placenta has been linked to the regulation of maternal and fetal calcium homeostasis during pregnancy [36]. However, studies using knockout (-/-) mice for the VDR have cast some doubt on this by showing that VDR -/- or +/- offspring from either VDR -/- or +/- mothers have normal mineral calcium and phosphate levels, and skeletal development [37]. In addition, we have shown that the placental CYP27B1 and VDR are induced early in gestation and prior to normal skeletal development [29]. This has raised the question as to whether vitamin D fulfills an alternative function in the placenta. In particular, the accumulating evidence linking vitamin D with the regulation of both innate and adaptive immunity [1-4] has prompted suggestions that the main role of CYP27B1 in the placenta is immunomodulatory [25, 29, 38]. In previous studies using maternal decidual cells we have shown that intracrine synthesis of 1,25$(OH)_2$D can act to both enhance antimicrobial proteins whilst suppressing inflammatory cytokine production [25]. The fact that trophoblastic cells also express CYP27B1 [28], suggests that the fetal component of placenta may also be subject to vitamin D-mediated immunoregulation. In the current study we have investigated the effects of active and inactive metabolites of vitamin D on trophoblast innate antibacterial responses.

Recent studies have highlighted the importance of placental innate immunity as a mechanism for counteracting uterine infection during pregnancy [39, 40]. Although this appears to be mediated in part through pathogen sensing and antibacterial responses in the decidua [39, 41, 42], it is now evident that fetal trophoblast may also be involved [40, 43]. Human trophoblastic cells have been shown to express various naturally-occurring antimicrobial proteins including β-defensins 1-3 [40], secretory leuokocyte protease inhibitor [42] and β-defensin 5 [44]. Moreover, proteomic analysis of bovine conceptus fluid has reported the presence of antibacterial proteins such as bovine cathelicidin-1 [45]. However, to the best of our knowledge, results presented in this study are the first evidence for expression of the cathelicidin CAMP by trophoblastic cells. Cathelicidins are a family of antimicrobial proteins with a conserved n-terminal and homology to the cysteine protease inhibitor cathelin [46]. In humans there is a single cathelicidin, for which the full-length pro-form protein is known as hCAP-18. However, the n-terminal of this protein is cleaved to form an active c-terminal mature peptide of CAMP, LL37, which is incorporated into phagocytic vacuoles [47]. CAMP achieves its antibacterial effects in neutrophils and macrophages by permeabilizing the bacterial cell wall and membrane [47]. In view of the fact that previous studies have highlighted a potential phagocytic function for human trophoblastic cells [43], we hypothesize that concomitant bacterial killing by trophoblasts may be promoted by intracrine activation of vitamin D.

In macrophages the ability of vitamin D to counteract bacterial infection of these cells is dependent on upregulation of both VDR and CYP27B1 to provide an effective intracrine mechanism for induction of CAMP [8]. This appears to be a pivotal facet of macrophage responses to gram positive bacteria such as *Mycobacterium tuberculosis*,

which enhances vitamin D metabolism and function via TLR2 [5]. Data for trophoblastic cells suggest that basal expression of VDR and CYP27B1 is sufficient to support intracrine synthesis and action of 1,25$(OH)_2$D, in the absence of any toll-like receptor (TLR) stimulus. Using a panel of TLR ligands we were unable to demonstrate any significant effects on VDR or CYP27B1 expression in 3A cells and the TLR4 ligand LPS did not modulate these genes in primary cultures of trophoblastic cells, despite being sensitive to LPS with respect to expression of TLR2 and 4 themselves. The regulation of trophoblast TLR2 expression by LPS was similar to that observed with human macrophages and was also consistent with previous studies from our group demonstrating LPS induction of TLR2 in human endothelial cells [48]. In both macrophages and endothelial cells LPS also induced its own receptor TLR4, whereas trophoblastic cells showed LPS auto-inhibition of TLR4 (see Figure 5). The latter effect has been reported previously for mouse macrophages where LPS can induce transient suppression of TLR4 expression [49]. Thus it is possible that the LPS suppression of *TLR4* mRNA in trophoblastic cells simply represents a temporal variation relative to macrophages.

It is also possible that low TLR expression renders trophoblasts relatively insensitive to TLR ligands such as LPS at least with respect to regulation of VDR and CYP27B1 expression. Similar observations have been made for keratinocytes which have low baseline expression of TLR2 and 4, and are therefore unable to induce CYP27B1 or VDR in response to their ligands [9]. In this latter instance CYP27B1 was induced by transforming growth factor beta (TGFB), a factor commonly associated with epidermal wounding. The resulting increase in 1,25$(OH)_2$D production stimulated TLR expression and this in turn increased sensitivity to TLR ligands to enable vitamin D-induced intracrine induction of CAMP similar to that observed with macrophages [9]. We were unable to demonstrate a similar mechanism in trophoblasts, in that treatment with 1,25$(OH)_2$D or 25OHD had no effect on expression of TLR2 or TLR4. Indeed it was notable that these treatments inhibited expression of CYP27B1 consistent with the feedback control of this enzyme previously described for renal cells [50]. Thus, it would appear that the induction of placental CYP27B1 and VDR that occurs early in gestation is sufficient to support intracrine induction of innate immunity independent of TLR involvement. However, we cannot exclude the possibility that other factors such as inflammatory cytokines may potentiate synthesis of 1,25$(OH)_2$D or expression of VDR. In future studies it will be important to identify these factors.

Trophoblast and macrophage vitamin D metabolism also differed at the level of catabolism by the enzyme CYP24. Both cell types expressed similar amounts of *CYP24* mRNA but enzyme activity analyses showed enhanced production of the metabolite 24,25$(OH)_2$D in 3A trophoblasts compared to macrophages. One possible explanation for this is that macrophages express a splice variant form of *CYP24* encoding a metabolically inactive truncated protein that is nevertheless still able to bind 25OHD or 1,25$(OH)_2$D [51]. Unlike the wild type CYP24 which functions by catalyzing conversion of 1,25$(OH)_2$D to less active 24-hydroxylated metabolites, the splice variant appears to sequester 25OHD and may therefore act to attenuate synthesis of 1,25$(OH)_2$D [51].

Induction of CAMP expression is a key feature of innate immune response to infection by a diverse array of pathogens including organisms such as *Mycobacterium tuberculosis* (*M. tb*) [5, 8, 11] and Gram-positive *Staphylococcus aureus* [12-14], and Group A streptococcus [52]. CAMP can also target Gram-negative bacteria [53], and in the current study we demonstrated that the induction of this CAMP in trophoblasts is associated with killing of *E. coli*. Gram-positive group B streptococcus (GBS) is an important pathogen during and immediately after pregnancy [54, 55]. However, sensitivity of GBS to antimicrobial peptides is variable and several strains of this bacterium appear to be resistant to CAMP [56]. In data presented here we have shown that induction of CAMP in trophoblasts is not only linked to the anti-bacterial effects of vitamin D but may also be involved in protection against infection-associated cell death (see Figure 6B).

In previous studies we have shown that infection with GBS induces cell death in trophoblast and fibroblasts from human placentas [33]. Other groups have shown that in addition to its antibacterial effects CAMP is a potent modulator of apoptosis, suppressing programmed cell death in neutrophils whilst having the opposite effect in lung epithelial cells [57]. Here we have demonstrated that vitamin D–induced CAMP may also help to protect against trophoblast death following bacterial infection. This novel facet of vitamin D function may help to maximize bacterial killing following induction of CAMP. Recent studies have demonstrated how pathogens such as *M. Tb* can avoid innate immune surveillance by blocking macrophage apoptosis [58]. Thus, it is possible to speculate that local production of 1,25$(OH)_2$D not only enhances mechanisms for bacterial killing but also sustains this bacterial killing by promoting host cell viability. Moreover, it is important to recognize that CAMP has also been implicated in diverse biological responses that extend beyond its antibacterial actions [59, 60]. These include effects on angiogenesis [61] and heparin sulfate-binding [62] which may also impact on placental function. In future studies it will be interesting to determine whether these factors are also modulated by vitamin D.

In summary, data presented here show for the first time that induction of trophoblastic CAMP may be a key facet of placental innate immunity. Importantly, we also demonstrate that expression of CAMP by trophoblastic cells is potently enhanced following intracrine activation of vitamin D. Given that circulating levels of the pro-hormone form of vitamin D (25OHD) vary significantly in pregnant women [63], we hypothesize that placental capacity to synthesize CAMP and mount effective innate immune responses may also be subject to substantial variation. Although the precise function of vitamin D within the placenta remains to be defined, data from this study suggest that local synthesis of active 1,25$(OH)_2$D may play a key role in placental innate immunity. We further postulate that improvement of maternal vitamin D status through dietary supplementation may act to potentiate placental innate immune responses during pregnancy.

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Figure Legends

Figure 1. Induction of cathelicidin (*CAMP*) in macrophages, placenta and trophoblastic cells by 1,25-dihydroxyvitamin D (1,25$(OH)_2$D). Varying doses of 1,25$(OH)_2$D (1-100 nM) were used to treat: 1) primary cultures of human peripheral blood-derived macrophages (macrophages); 2) placental tissue explants from 3rd trimester human pregnancy (placenta); 3) primary trophoblasts isolated from 3rd trimester human placenta (trophoblast); 4) 3A human trophoblastic cells (3A cells). Each set of tissue/cell cultures was incubated with 1,25$(OH)_2$D for 24 hrs after which total RNA was isolated. Subsequent real-time RT-PCR was used to assess the expression of mRNA for the antibacterial protein *CAMP*. Data are shown as the mean (± SD) fold-induction of *CAMP* mRNA relative to vehicle (0.1% ethanol)-treated controls for n=3 tissue/cell cultures. * = statistically different to vehicle samples, $p<0.05$. ** = statistically different to vehicle samples, $p<0.01$. *** = statistically different to vehicle samples, $p<0.001$.

Figure 2. Trophoblastic cells express vitamin D receptors and vitamin D metabolic enzymes. Expression of mRNAs for the vitamin D receptor (*VDR*), 25-hydroxyvitamin D-1α-hydroxylase (*CYP27B1*), and vitamin D-24-hydroxylase (*CYP24*) by: 1) human macrophages; 2) primary human trophoblasts; 3) 3A trophoblastic cells. Data are shown as the mean (± SD) fold-induction of mRNA relative to vehicle (0.1% ethanol)-treated controls for n=3 tissue/cell cultures.

Figure 3. Regulation of antibacterial gene expression by vitamin D metabolites in 3A trophoblastic cells. Expression of mRNAs for *CAMP*, β-defensin 4 (*DEFB4*) and inducible nitric oxide synthase (*NOS2*) was analysed in 3A trophoblastic cells treated for 24 hrs with either 25-hydroxyvitamin D (25OHD) or 1,25-dihydroxyvitamin D (1,25$(OH)_2$D) (1-100 nM for both treatments). Data are shown as the mean (± SD) fold-induction of *CAMP* mRNA relative to vehicle (0.1% ethanol)-treated controls for n=3 tissue/cell cultures. * = statistically different to vehicle samples, $p<0.05$. ** = statistically different to vehicle samples, $p<0.01$. *** = statistically different to vehicle samples, $p<0.001$.

Figure 4. Effect of toll-like receptor activation on 25OHD or 1,25$(OH)_2$D-induced regulation of *CAMP* in 3A trophoblastic cells and primary (1°) cultures of human trophoblasts. Cells were treated for 24 hrs with the toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS) with or without 100 nM 25OHD (25) or 10 nM 1,25$(OH)_2$D (1,25). The resulting cells were then used to assess expression of mRNA for *CAMP*.

Data are shown as the mean (± SD) fold-induction of *CAMP* mRNA relative to vehicle (0.1% ethanol)-treated controls for n=3 tissue/cell cultures. * = statistically different to vehicle samples, $p<0.05$. ** = statistically different to vehicle samples, $p<0.01$. *** = statistically different to vehicle samples, $p<0.001$. ### = statistically different to LPS only-treated samples, $p<0.001$. nd = not done.

Figure 5. Effect of toll-like receptor activation on 25OHD or 1,25(OH)$_2$D-induced regulation of *CYP27B1*, *VDR*, *TLR2* and *TLR4* in human macrophages, primary (1°) cultures of human trophoblasts, 3A trophoblastic cells and. Cells were treated for 24 hrs with the toll-like receptor 4 (TLR4) ligand lipopolysaccharide (L) with or without 100 nM 25OHD (25) or 10 nM 1,25(OH)$_2$D (1,25). The resulting cells were then used to assess expression of mRNA for *CYP27B1*, *VDR*, *TLR2* and *TLR4*. Data are shown as the mean (± SD) fold-induction of mRNA relative to vehicle (0.1% ethanol)-treated controls for n=3 cell preparations. * = statistically different to vehicle samples, $p<0.05$. ** = statistically different to vehicle samples, $p<0.01$. *** = statistically different to vehicle samples, $p<0.001$.

Figure 6. Effect of 25OHD and 1,25(OH)$_2$D on bacterial infection and infection-associated cell death in trophoblastic cells. A. 3A cells were pre-treated with 25OHD (100 nM), 1,25(OH)$_2$D (10 nM) or vehicle (0.1% ethanol) for 48 hrs and then infected with *E. coli* for 24 hrs. After removal of exogenous bacteria, monolayers of 3A cells were lysed and assessed for surviving intracellular E. coli (colony-forming units, CFU). B. 3A cells were pre-treated with 25OHD (1-100 nM), 1,25(OH)$_2$D (0.1-10 nM), or vehicle (0.1% ethanol) for 48 hrs and then infected with *E. coli* for 24 hrs After removal of exogenous bacteria, cell death was assessed by analysis of the release of lactate dehydrogenase (LDH) into cell culture supernatants. Data are shown as the level of LDH in supernatants from infected 3A cells relative to infected vehicle-treated cells. * = statistically different to vehicle samples, $p<0.05$, ** = statistically different to vehicle samples, $p<0.01$ *** = statistically different to vehicle samples, $p<0.001$.

Figure 1

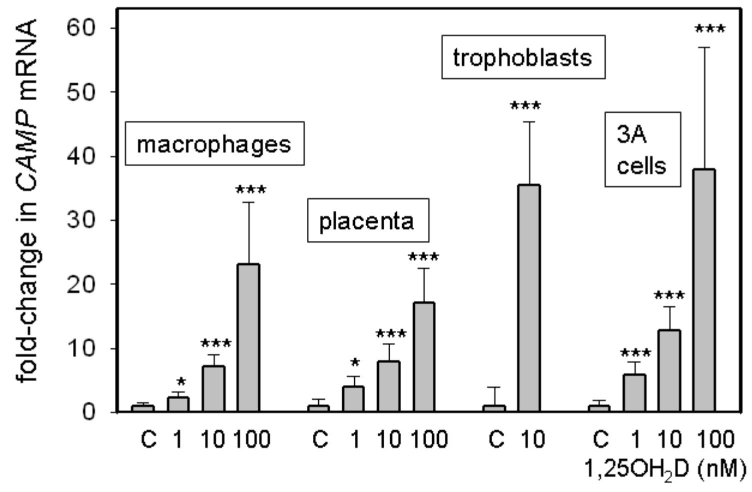
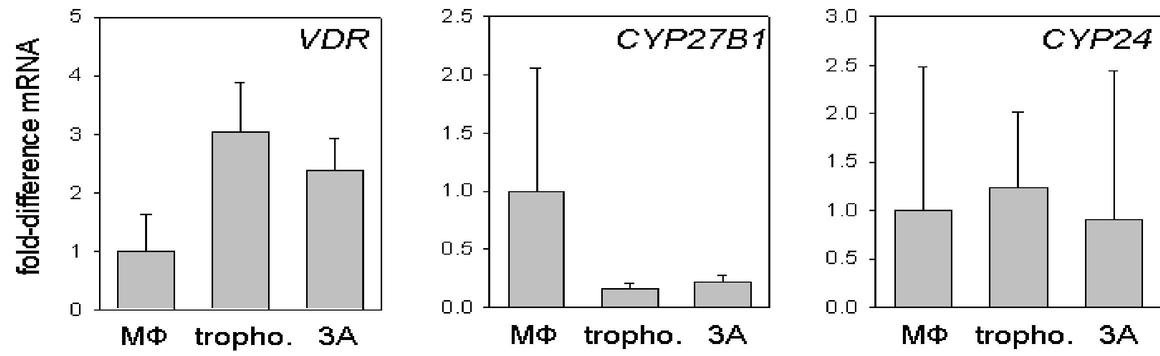


Figure 2



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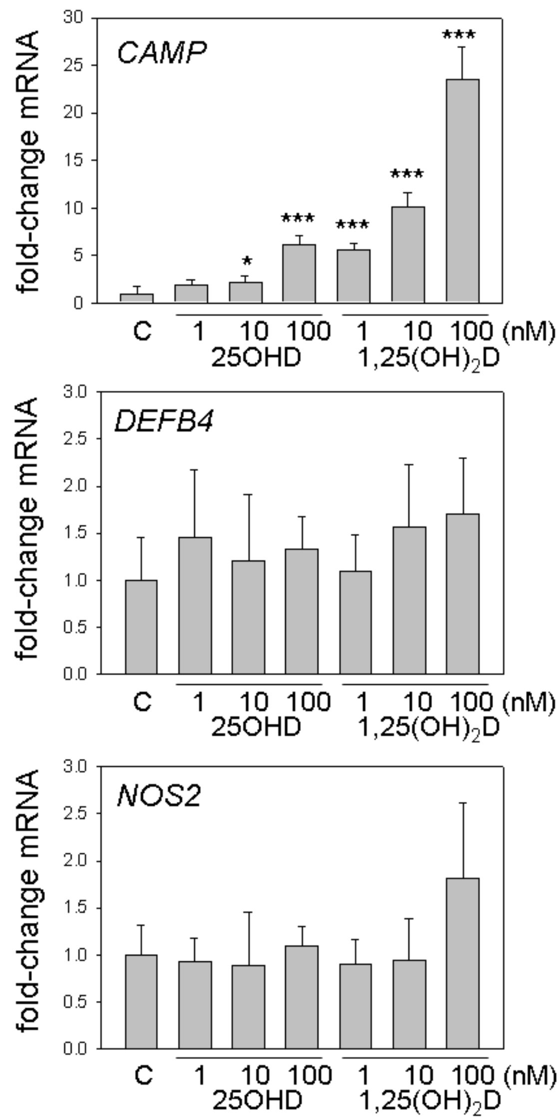

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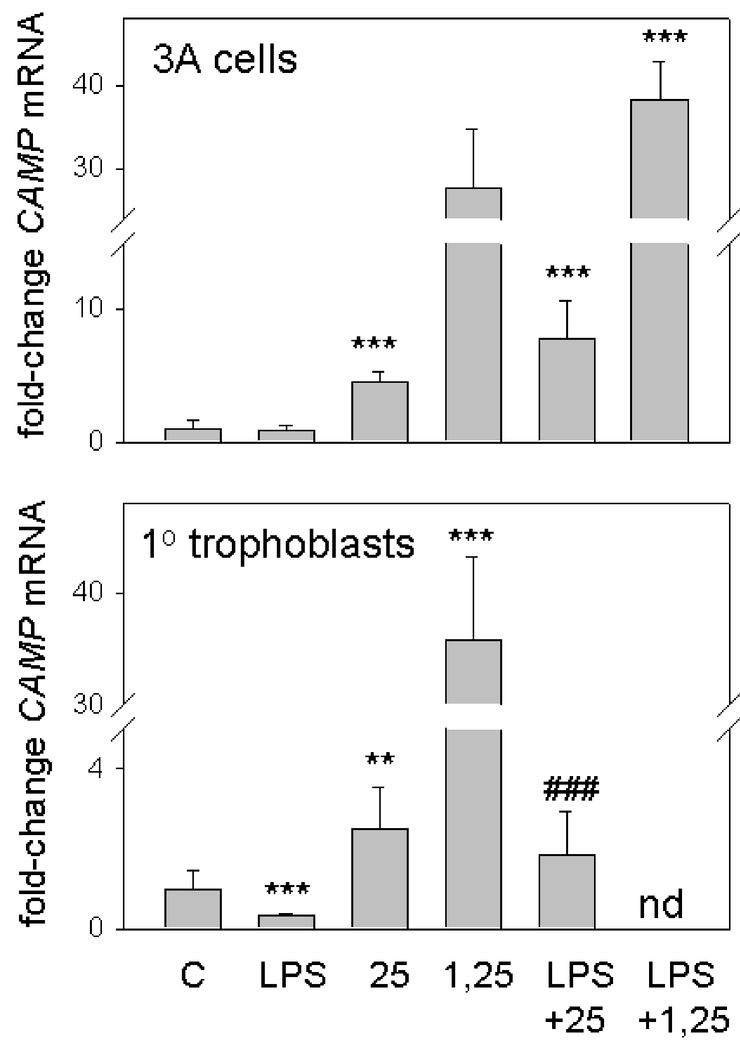

Figure 5

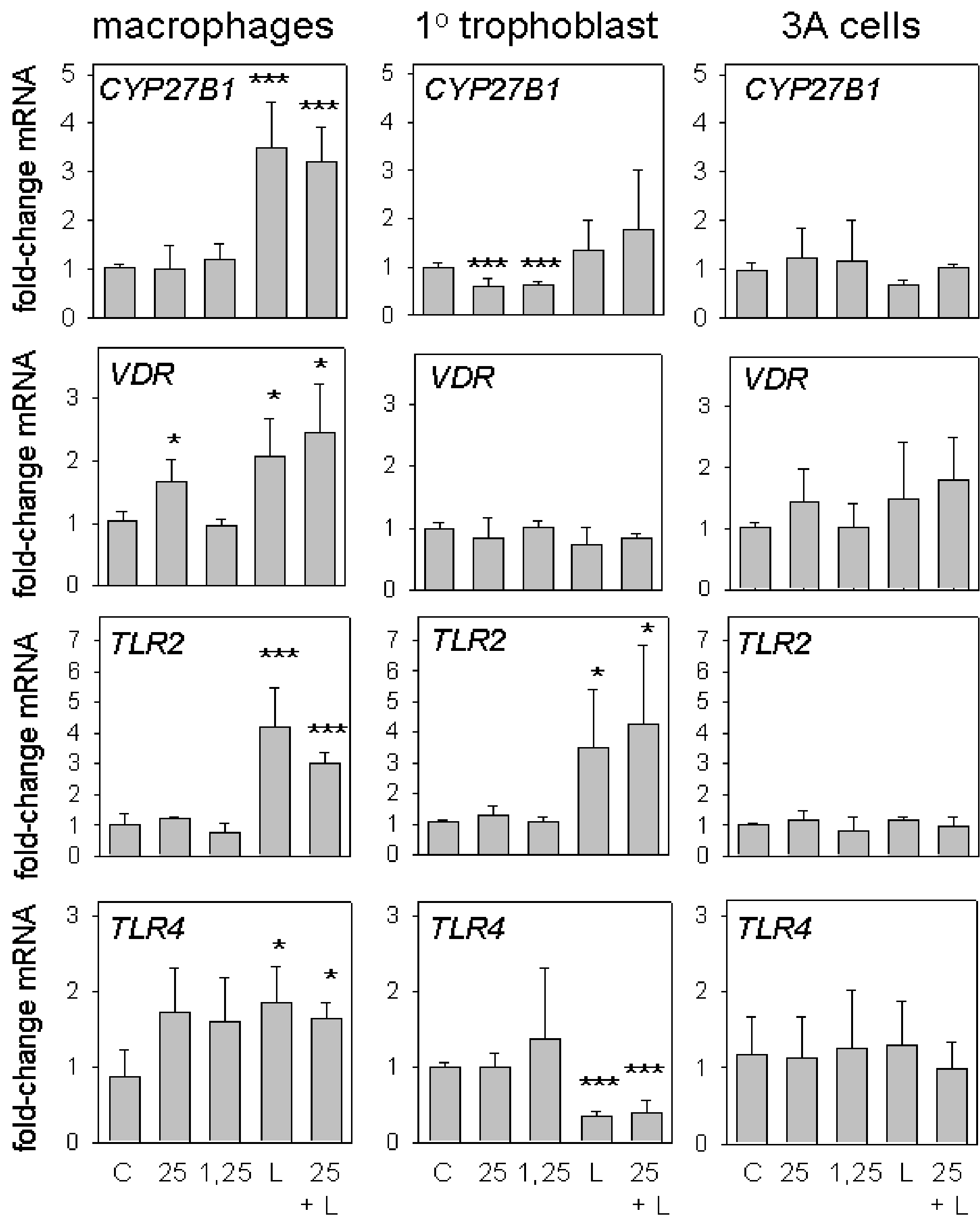

Figure 6