

Prenatal vitamin D₃ supplementation suppresses LL-37 peptide expression in *ex vivo* activated neonatal macrophages but not their killing capacity

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Abstract

Vitamin D has regulatory effects on innate immunity. In the present study, we aimed to assess the effect of prenatal vitamin D₃ (vitD₃) supplementation on neonatal innate immunity in a randomised, placebo-controlled trial by evaluating cathelicidin (LL-37) expression and the killing capacity of macrophages. Healthy pregnant women (*n* 129) attending a clinic in Dhaka were randomised to receive either a weekly oral dose of 0.875 mg vitD₃ or placebo starting from 26 weeks of gestation up to delivery. Serum, plasma and monocyte-derived macrophages (MDM) were obtained from the cord blood. 25-Hydroxyvitamin D (25(OH)D) concentration was measured in serum. MDM were stimulated with or without Toll-like-receptor 4 ligand (TLR4L). Innate immune function was assessed by measuring LL-37 peptide levels in the culture supernatant of MDM by ELISA, LL-37 transcript levels by quantitative PCR, and *ex vivo* bactericidal capacity of MDM. vitD₃ supplementation did not increase LL-37 peptide levels in plasma or in the extracellular fluid of macrophages with or without TLR4L induction. However, stimulated intracellular LL-37 expression (ratio of stimulated:unstimulated MDM) was significantly reduced in the vitamin D group *v.* placebo (*P*=0.02). Multivariate-adjusted analyses showed that intracellular LL-37 peptide concentration from stimulated MDM was inversely associated with 25(OH)D concentration in serum (*P*=0.03). TLR4L stimulation increased the bactericidal capacity of MDM compared with the unstimulated ones (*P*=0.01); however, there was no difference in killing capacity between the two groups. A weekly dose of 0.875 mg vitD₃ to healthy pregnant women suppressed the intracellular LL-37 peptide stores of activated macrophages, but did not significantly affect the *ex vivo* bactericidal capacity of cord blood MDM.

Key words: Vitamin D₃; Antimicrobial peptides; Macrophages; Innate immunity; Neonates

Globally, vitamin D deficiency is common during pregnancy. However, epidemiological studies determining the associations between vitamin D status and adverse pregnancy outcomes such as pre-eclampsia, gestational diabetes, low birth weight, preterm labour and infectious diseases have shown conflicting results⁽¹⁾. Therefore, the benefits of routine vitamin D supplementation for improving pregnancy outcomes remain to be established⁽²⁾.

Vitamin D plays an important role in defence against infectious diseases by modulating both innate and adaptive immune responses. All major immune cells, including T cells, B cells,

neutrophils and antigen-presenting cells (monocytes, macrophages and dendritic cells), express the vitamin D receptor. Macrophages, the front-line responders to microbial infection, sense pathogen-associated molecular patterns by utilising pattern recognition receptors such as Toll-like receptors (TLR). TLR triggering induces Cytochrome p450 27B1 (CYP27B1), a vitamin D-activating enzyme that converts 25-hydroxyvitamin D (25(OH)D) into the active form (1,25-dihydroxyvitamin D; 1,25(OH)₂D), and vitamin D receptor expression⁽³⁾. The 1,25(OH)₂D–vitamin D receptor complex directly induces cathelicidin antimicrobial peptide (*CAMP*; gene encoding

Abbreviations: 1,25(OH)₂D, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; AViDD, Antenatal Vitamin D in Dhaka; CBMC, cord blood mononuclear cell; CFU, colony-forming unit; ICDDR,B, International Centre for Diarrheal Disease Research, Bangladesh; ICF, intracellular fluid; LPS, lipopolysaccharide; MDM, monocyte-derived macrophages; TLR, Toll-like receptor; TLR4L, Toll-like receptor 4 ligand; vitD₃, vitamin D₃.

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LL-37) transcription and corresponding increases in the expression of the antimicrobial peptide, LL-37⁽⁴⁾.

Antimicrobial peptides function as front-line host defence effector molecules, acting as both endogenous antibiotics and paracrine immunomodulators⁽⁵⁾. Human cathelicidin (the cleaved active component of which is LL-37) is synthesised by several immune cell types (neutrophils, macrophages, B cells, T cells, natural killer cells and mast cells) as well as by barrier/mucosal epithelial cells^(6–8). In response to some infections (diarrhoeal diseases and gonorrhoea), LL-37 has been found to be down-regulated^(9–12), while in some other diseases, it is up-regulated (psoriasis)⁽¹³⁾. The localisation of LL-37 to skin⁽¹⁴⁾, vernix caseosa⁽¹⁵⁾ and breast milk⁽¹⁶⁾ suggests a potential role for the antimicrobial peptide in response to infection in young infants. Antimicrobial peptides, including LL-37, exhibited bactericidal activity against organisms responsible for bacterial infections in newborns⁽¹⁷⁾. They are up-regulated in tracheal secretions during neonatal lower respiratory tract infections⁽¹⁸⁾, suggesting that clinical or nutritional interventions to enhance the production of antimicrobial peptides may reduce the risk of neonatal infections. Little is known regarding the effect of vitamin D supplementation during pregnancy on the synthesis of cathelicidin/LL-37 or other aspects of neonatal innate immune defences.

Therefore, in the present study, we aimed to evaluate the effect of prenatal vitamin D₃ (vitD₃) supplementation on neonatal innate immune function. In a previous double-blind, placebo-controlled trial of vitD₃ supplementation (0.875 mg/week) during the third trimester of pregnancy among Bangladeshi women, vitD₃ has been shown to significantly increase the mean 25(OH)D concentration in cord blood by approximately 2.5-fold compared with the placebo group (103 *v.* 39 nmol/l)⁽¹⁹⁾. In the present trial cohort, we studied the effect of this substantial rise in vitamin D status on the expression of cord blood LL-37 peptide and mRNA transcript levels in stimulated macrophages and the killing capacity of neonatal macrophages *ex vivo*.

Methods

Study design and participants

The Antenatal Vitamin D in Dhaka (AViDD) study was a randomised, double-blind, placebo-controlled trial of vitamin D supplementation of pregnant women in the third trimester conducted in Dhaka, Bangladesh. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human participants were approved by the Ethics Committees of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B) and Johns Hopkins Bloomberg School of Public Health, Hospital for Sick Children (protocol no. PR-09058). Written informed consent was obtained from all eligible participants. Details of the trial design and primary biochemical outcomes have been published previously⁽¹⁹⁾. Briefly, 160 pregnant women were enrolled at the Shimantik Urban Primary Health Care Project Maternity Center, a non-governmental facility that provides basic antenatal and

obstetric services in a low-income community. Pregnant women were included if they were aged between 18 and <35 years, at a gestational age of 26–29 weeks, currently residing in Dhaka with plans to stay there throughout pregnancy and for at least 1 month after delivery, and had plans to deliver at the maternity centre. Study participants were allocated to receive a weekly dose of either 0.875 mg vitD₃ (VitD group: cholecalciferol, Vigantol oil; Merck KGaA) or placebo oil (placebo group: Miglyol oil; Merck) until delivery. Of the 160 women enrolled in the AViDD trial, there were 129 mother–infant pairs (81%), sixty-four from the placebo group and sixty-five from the VitD group, with adequate volume of cord blood for immune function assays.

Cord blood collection, plasma and mononuclear cell isolation

Venous cord blood was collected immediately after delivery and transferred to a central laboratory in Dhaka for processing on the same day (within 2–18 h). Maternal blood was also collected during the time of delivery. Cord blood mononuclear cells (CBMC) and cord blood plasma were separated from the whole blood by Ficoll-Paque (Amersham Pharmacia Biotech, Inc.) density gradient centrifugation. The isolated CBMC were resuspended in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco; Invitrogen) containing autologous plasma, and cultured in two parallel sets of tissue culture plates (NUNC; Thermo Fisher Scientific) for 3 d to develop macrophages. A dose–response assessment was performed to determine the optimum concentration of lipopolysaccharide (LPS; 2.5, 5.0 and 10 µg/ml) and the incubation time needed for monocyte-derived macrophages (MDM) to produce maximum levels of LL-37. We found that stimulation with 5.0 and 10 µg LPS/ml produced similar levels of LL-37 peptide by MDM, and the concentration of the peptide was comparable or slightly higher in 48 h culture as opposed to 24 h incubation. Thus, macrophages derived from CBMC were cultured with or without Toll-like receptor 4 ligand (TLR4L) for a further 48 h. The extracellular fluid was collected from one set of MDM cultures, and the remaining macrophages were treated with 0.1% saponin in RPMI-1640 medium to release and collect the intracellular fluid (ICF). After removal of the ICF, RNAlater (Qiagen GmbH) was added to the MDM for further extraction of LL-37 transcripts. The LL-37 peptide was measured in the ICF, extracellular fluid and cord plasma. The second set of MDM cultures was used for the assay of MDM-mediated killing capacity. In all assays, LPS (from *Escherichia coli* serotype 0111:B4, 5 µg/ml; Sigma) was used as TLR4L.

Assessment of vitamin D status

Details of serum 25(OH)D measurements have been described elsewhere⁽¹⁹⁾. To assess vitamin D status, serum 25(OH)D was measured by HPLC–tandem MS; only 25(OH)D₃ was detected in serum, not 25(OH)D₂. As no standard classification of 25(OH)D concentrations in cord blood exists, for the purpose of the present study, we followed the classification of

vitamin D sufficiency or deficiency based on previous publications^(20–23). All participants were grouped into four categories of 25(OH)D concentrations: (1) ≥ 76 nmol/l (high); (2) 50–75 nmol/l (moderate); (3) 30–49 nmol/l (low); (4) < 30 nmol/l (very low).

Measurement of LL-37 peptide concentration

LL-37 peptide concentration was measured by ELISA (Hycult Biotechnology), according to the manufacturer's recommendations. The lower limit of detection of the kit was 0.14 ng/ml, and the intra- and inter-assay CV were 4.96 and 7.89%, respectively. The concentration of LL-37 peptide measured in the supernatant was then normalised to per million macrophages.

Quantitative real-time RT-PCR amplification of LL-37 mRNA

RNA was extracted from macrophages using the RNeasy Mini Kit (Qiagen GmbH), according to the manufacturer's instructions, and corresponding complementary DNA was synthesised using the SuperScript III First-Strand Synthesis System (Invitrogen). The *CAMP* gene encoding LL-37 transcripts relative to the housekeeping 18S rRNA was measured in triplicate from the complementary DNA samples by quantitative real-time RT-PCR using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) and the 18S rRNA-housekeeping gene kit (Applied Biosystems). The sequences of forward and reverse primers for LL-37 transcripts were 5'-TCACCAGAGGATTGTGACTTCAA-3' and 5'-TGA GGGTCACTGTCCCCATAC-3', respectively (Primer Express; Applied Biosystems). Results were analysed using the relative standard method⁽⁹⁾.

Assay of monocyte-derived macrophage-mediated killing capacity

The capacity of MDM to kill ingested bacteria was determined by quantifying the number of viable *E. coli* K-12 colonies (avirulent) in agar plates after a period of phagocytosis and removal of extracellular bacteria. MDM were infected with *E. coli* K-12 at a multiplicity of infection of 100 (100 bacteria per one macrophage) and incubated for 2 h. Extracellular bacteria were removed by washing, and infected macrophages were cultured in media with 10% autologous plasma overnight. The infected macrophages were lysed with 0.3% saponin to release intracellular bacteria. Cell lysates were cultured on MacConkey agar plates (Becton Dickinson) overnight and viability of the bacteria was determined by counting the colony-forming units (CFU). A 'relative CFU count' was calculated for each participant as the ratio of CFU in LPS-stimulated to unstimulated cells, to account for inter-subject variations in the baseline (unstimulated) killing activity of macrophages.

Statistical analyses

Primary outcomes were fold difference in intracellular LL-37 peptide concentration (indicator of the effect of prenatal

vitD₃ supplementation on LPS-induced LL-37 synthesis in cord blood cells) and the relative CFU count (an indicator of the effect of vitD₃ supplementation on LPS-induced neonatal macrophage killing activity). Secondary outcomes were unstimulated and LPS-stimulated LL-37 concentrations in the intracellular and extracellular fluid, LL-37 mRNA transcript copy numbers in MDM, and LL-37 concentration in plasma.

The distributions of LL-37 and bacterial CFU counts are expressed as ranges, means and standard deviations and 95% CI, or medians and interquartile ranges for non-normally distributed variables. The fold difference in the intracellular concentration of LL-37 peptide was calculated for each participant as the ratio of LL-37 concentration in LPS-stimulated to unstimulated cells. Unstimulated and LPS-stimulated intracellular LL-37 peptide concentrations and fold difference in intracellular LL-37 peptide concentration did not follow normal distributions even after transformation; therefore, they were analysed by non-parametric statistics (Mann–Whitney *U* test). Bootstrap linear regression was used to verify inferences from non-parametric tests. Factors that have been previously reported in the literature to be associated with cord blood LL-37 concentration including cord serum 25(OH)D concentration, gestational age (categorised by preterm ≤ 37 weeks of gestation or term > 37 weeks of gestation), infant sex and delivery mode were assessed for associations with LL-37 concentration using the bootstrap procedure for unadjusted bivariate and adjusted multivariate regression analyses. Cord plasma LL-37 was log-transformed as data were not normally distributed, and between-group comparisons were analysed by Student's *t* test. For the aforementioned LL-37 outcomes, sensitivity analyses that excluded LL-37 observations below the minimum detectable limit of the ELISA assay (< 0.14 ng/ml) were also conducted. Participants were categorised into four groups based on 25(OH)D concentrations, as described previously; comparisons between groups with respect to the LL-37 peptide and transcript levels were evaluated by one-way ANOVA.

To calculate the relative CFU count, one CFU was added to all values due to multiple observations with bacterial colony counts of zero. Relative CFU counts did not follow a normal distribution and were therefore log-transformed before the analysis. The number of bacterial colonies that remained after treatment with or without LPS was considered as count data; however, because the Poisson distribution was a poor fit for the over-dispersed count data, comparisons were based on negative binomial regression models, using generalised estimating equations to account for the correlation between colony counts of LPS-stimulated and unstimulated cells within the subjects. The primary parameter of interest in the negative binomial generalised estimating equation model was the interaction between vitamin D supplementation and LPS stimulation on CBMC-derived macrophage killing activity. Statistical analyses were performed using Stata/IC 12.1 for Mac (StataCorp), and $P \leq 0.05$ was considered significant.

Results

Of the 160 women enrolled in the AViDD trial, there were 129 mother–infant pairs (81%), sixty-four from the placebo group



and sixty-five from the VitD group, with adequate volume of cord blood for immune function assays. The mean age (22.4 (SD 3.5) *v.* 22.4 (SD 3.5) years) and gestational age (27.73 (SD 1.1) *v.* 28.0 (SD 1.1) weeks) of the pregnant women in the VitD group were similar to those in the placebo group.

Vitamin D and LL-37 levels in cord blood

25(OH)D concentration ranged from 20 to 192 nmol/l in the study cohort, which was similar to the entire cohort as reported previously⁽¹⁹⁾. Maternal 25(OH)D concentration in the VitD group (mean 101.52 (SD 30.67) nmol/l) was significantly increased at delivery compared with the placebo group (mean 40.30 (SD 17.83) nmol/l) ($P < 0.001$) (Table S1, available online). The concentration of LL-37 in cord plasma ranged from 16.6 to 101.7 ng/ml. There was no significant difference in plasma LL-37 concentrations between the two groups (Table 1). Cord serum 25(OH)D was not significantly associated with LL-37 concentration in cord plasma (Table 2).

Vitamin D₃ supplementation suppressed intracellular LL-37 peptide expression in Toll-like receptor 4 ligand-stimulated macrophages

Concentrations of LL-37 ranged from 0.05 to 4.93 ng/ml in the ICF of unstimulated macrophages. No significant differences were observed between the VitD and placebo groups (Table 1). The bootstrap linear regression analysis did not show an effect of vitD₃ supplementation on unstimulated intracellular LL-37 concentration (mean between-group difference 0.12 ng/ml, 95% CI -0.16, 0.39; $P = 0.41$).

In TLR4L-stimulated macrophages, LL-37 concentration in the ICF ranged from 0.07 to 7.72 ng/ml. The distribution of TLR4L-induced intracellular LL-37 concentrations was slightly

lower in the VitD group than in the placebo group, although the difference did not reach statistical significance as determined by the non-parametric Mann-Whitney *U* test ($P = 0.09$; Table 1). Similarly, the results of the bootstrap linear regression analysis revealed that the mean decrease in TLR4L-induced intracellular LL-37 concentrations in the VitD *v.* placebo group did not reach statistical significance (mean 0.31 ng/ml, bootstrap 95% CI -0.64, 0.023; $P = 0.068$).

Overall, individual fold differences in intracellular LL-37 concentration ranged from 0.12 to 3.64. Fold differences in the VitD group were lower, on average, than those in the placebo group, and the difference between the distributions was significant as determined by the non-parametric Mann-Whitney *U* test (Table 1). However, when group means were compared using bootstrap regression, the effect of vitD₃ supplementation on the fold difference in intracellular LL-37 concentration did not reach statistical significance ($P = 0.15$). Moreover, there was no significant interaction between vitD₃ supplementation and TLR4L stimulation in terms of their effects on mean intracellular LL-37 concentration (data not shown).

Cord serum 25(OH)D was not significantly associated with LL-37 concentration in the unstimulated ICF, or fold difference in intracellular LL-37 concentration (Table 2). However, 25(OH)D in cord serum was inversely related to TLR4L-induced intracellular LL-37 in both unadjusted bivariate and adjusted multivariate analyses (Table 2). For every 10 nmol/l increase in cord serum 25(OH)D concentration, TLR4L-induced intracellular LL-37 concentration decreased by 0.05 ng/ml ($P = 0.03$). No significant associations were observed between LL-37 concentration and preterm birth, delivery mode or infant sex (Table 2). Excluding low LL-37 values in the sensitivity analysis did not change the inferences from any of the above-mentioned analyses (data not shown). Upon stratifying the participants into four groups based on serum 25(OH)D concentrations, LL-37 peptide

Table 1. Antimicrobial peptide LL-37 concentration in umbilical cord plasma and cord blood mononuclear cell culture, overall and by treatment group (Medians and interquartile ranges (IQR); geometric means and 95% confidence intervals)

Tissue	Treatment group*						P†
	Overall* (n 129)		Vitamin D (n 63)		Placebo (n 66)		
	Median	IQR	Median	IQR	Median	IQR	
Cord blood plasma‡							0.91
Geometric mean	41.2		41.1		41.4		
95% CI	38.9, 43.7		37.7, 44.8		38.1, 44.8		
Intracellular fluid							
Unstimulated cells	0.5	0.4–0.9	0.5	0.4–0.8	0.6	0.4–0.9	0.43
Stimulated cells	0.5	0.4–0.9	0.5	0.4–0.7	0.6	0.4–1.1	0.09
Fold difference§	1.02	0.8–1.3	0.96	0.8–1.2	1.1	0.9–1.4	0.02
Extracellular fluid							
Unstimulated cells	1.92	1.4–3.4	1.9	1.46–3.5	1.84	1.4–3.1	0.52
Stimulated cells	1.96	1.4–3.5	2.2	1.35–3.6	1.90	1.40–3.6	0.73
mRNA transcripts							
Unstimulated cells	124.86	63.7–232.9	141.3	85.5–217.8	99.8	55.6–249.9	0.54
Stimulated cells	171	104.5–321.6	176.94	108.6–369.9	171.279	102.9–318.1	0.91

* LL-37 peptide concentrations are expressed as ng/ml and mRNA as copy numbers.
 † *P* values were based on the non-parametric Mann-Whitney *U* test, unless otherwise indicated.
 ‡ Vitamin D group *n* 64 and placebo group *n* 65; *P* value was based on the *t* test.
 § Fold difference was calculated as the ratio of LL-37 concentration in stimulated to unstimulated cells.
 || Vitamin D group *n* 42 and placebo group *n* 37.

Table 2. Unadjusted and adjusted multivariate associations between LL-37 concentration (in cord plasma and intracellular fluid) and cord serum 25-hydroxyvitamin D (25(OH)D), gestational age, infant sex and delivery mode (β -Coefficients and 95% confidence intervals)

	Unadjusted models			Multivariate models		
	β	Bootstrap 95% CI	<i>P</i>	β	Bootstrap 95% CI	<i>P</i>
Cord plasma LL-37 (ng/ml)						
Cord serum 25(OH)D*	-0.204	-0.773, 0.365	0.48	-0.221	-0.804, 0.362	0.46
Gestational age†	-2.308	-9.533, 4.916	0.53	0.504	-5.022, 6.030	0.86
Infant sex‡	3.290	-1.569, 8.146	0.18	4.472	-0.159, 9.102	0.06
Delivery mode	-3.925	-9.249, 1.399	0.15	-2.778	-7.826, 2.271	0.28
Unstimulated intracellular LL-37 (ng/ml)						
Cord serum 25(OH)D*	-0.016	-0.058, 0.025	0.44	-0.016	-0.059, 0.028	0.48
Gestational age†	-0.245	-0.796, 0.306	0.38	-0.205	-0.788, 0.378	0.49
Infant sex‡	0.049	-0.229, 0.327	0.73	0.075	-0.219, 0.368	0.62
Delivery mode	-0.134	0.444, 0.177	0.40	-0.065	-0.396, 0.266	0.70
Stimulated intracellular LL-37 (ng/ml)						
Cord serum 25(OH)D*	-0.049	-0.090, -0.008	0.02	-0.047	-0.090, -0.005	0.03
Gestational age†	-0.240	-0.772, 0.293	0.38	-0.214	-0.771, 0.342	0.45
Infant sex‡	0.122	-0.202, 0.446	0.46	0.135	-0.209, 0.480	0.44
Delivery mode	-0.264	-0.371, 0.318	0.88	0.033	-0.336, 0.401	0.86
Intracellular fold difference in LL-37§						
Cord serum 25(OH)D*	-0.015	-0.046, 0.015	0.33	-0.015	-0.047, 0.017	0.34
Gestational age†	-0.050	-0.260, 0.160	0.64	-0.038	-0.273, 0.197	0.75
Infant sex‡	-0.028	-0.224, 0.168	0.78	-0.047	-0.246, 0.152	0.65
Delivery mode	-0.006	-0.211, 0.198	0.95	-0.027	-0.248, 0.195	0.81

* Cord serum 25(OH) was scaled by 10 nmol/l.

† Gestational age was categorised as preterm (≤ 37 weeks of gestation) or term (> 37 weeks of gestation).

‡ Infant sex is female v. male.

§ Fold difference was calculated as the ratio of stimulated:unstimulated cells.

concentrations in the ICF of TLR4L-stimulated MDM were significantly reduced in the high (≥ 76 nmol/l), moderate (50–75 nmol/l) and low (30–49 nmol/l) groups ($P=0.005$, $P=0.035$ and $P=0.029$, respectively) compared with the very low (< 30 nmol/l) group (Fig. 1(a)).

Effect of prenatal vitamin D₃ supplementation on LL-37 mRNA transcription

There was no significant effect of vitamin D supplementation on LL-37 transcript copy number in unstimulated or TLR4L-stimulated BMC (Table 1), nor was there an interaction between vitamin D supplementation and TLR4L stimulation (data not shown). TLR4L stimulation increased LL-37 transcript levels in both groups (Table 1), but no significant differences were observed between the groups.

Among the four groups stratified based on the categories of vitamin D status, we found that TLR4L-induced LL-37 transcript copy number in MDM was higher in the moderate group compared with the low ($P=0.07$) and high ($P=0.047$) groups (Fig. 1(b)).

Effect of prenatal vitamin D₃ supplementation on bacterial killing capacity of activated macrophages

The macrophage killing assay was performed on 112 cord blood cell cultures (fifty-six from the placebo group and fifty-six from the VitD group) since adequate cells were not available from the other seventeen participants. Overall, the range of viable bacterial counts after the MDM-mediated *E. coli* killing was 0 to 2.50×10^4 CFU for unstimulated cells

and 0 to 2.25×10^4 CFU for TLR4L-stimulated cells, and the relative CFU counts ranged from 0 to 1.25×10^3 . The regression analysis did not show any significant effect of vitD₃ supplementation on the relative CFU count, or on the relative CFU counts in unstimulated and TLR4L-stimulated macrophages (Table 3). However, TLR4L stimulation augmented bacterial killing in both groups. Stratifying the participants based on serum 25(OH)D concentrations did not show any difference among the four groups (data not shown).

Discussion

Despite the potent effect of vitD₃ supplementation (0.875 mg/week) during the third trimester of pregnancy on maternal–infant vitamin D status⁽¹⁹⁾, the present study showed that prenatal vitD₃ supplementation did not yield significant benefits in terms of indicators of innate immune function. In fact, we observed that vitD₃ supplementation may have slightly suppressed intracellular LL-37 peptide synthesis in activated cord blood macrophages, suggesting a potential immunosuppressive effect. Furthermore, we did not find any significant influence of vitD₃ supplementation on the bactericidal capacity of macrophages with or without TLR4L activation.

In vitro studies using primary monocytes/macrophages cultured in 25(OH)D-sufficient sera or culture media containing the active form of vitamin D (1,25(OH)₂D₃) have shown that vitamin D mediates the induction of cathelicidin (LL-37) and β -defensin 4 transcripts in macrophages^(3,24,25). *In vitro* addition of 1,25(OH)₂D₃ or 25(OH)D₃ in cord blood cultures also showed increased TLR-independent or -dependent cathelicidin mRNA expression⁽²³⁾. In the present study, *in vivo* vitD₃

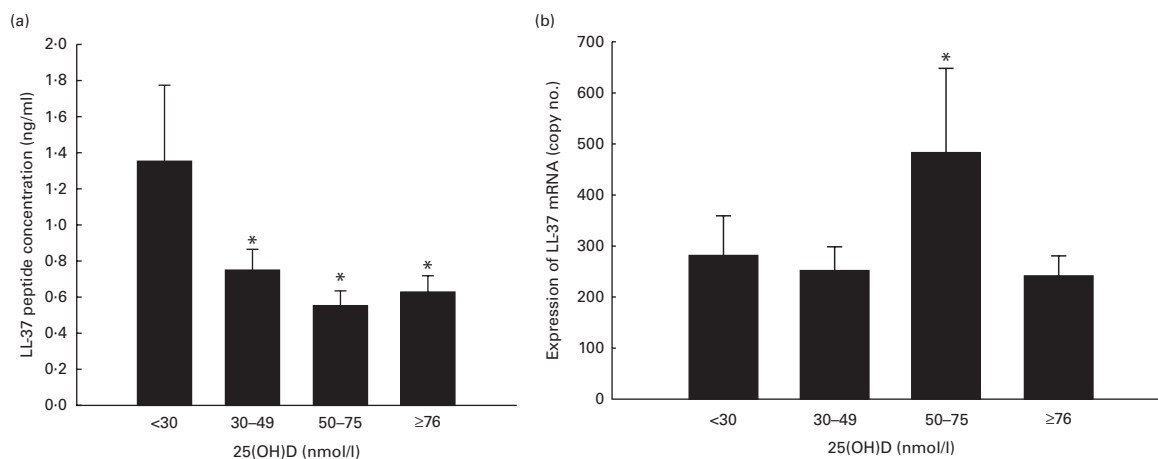


Fig. 1. Participants were stratified into four groups based on serum 25-hydroxyvitamin D (25(OH)D) concentrations: (1) ≥ 76 nmol/l (high, n 57); (2) 50–75 nmol/l (moderate, n 10); (3) 30–49 nmol/l (low, n 37); (4) < 30 nmol/l (very low, n 25). Values are means, with their standard errors represented by vertical bars. (a) * Mean value was significantly different from that of the very low group ($P < 0.05$). (b) * Mean value was significantly different from that of the high group ($P < 0.05$).

supplementation did not affect LL-37 mRNA levels in neonatal macrophages with or without activation. The main difference between the other reports and the present study is that here vitD₃ was given orally to pregnant women and autologous plasma was used instead of adding 1,25(OH)₂D₃ or 25(OH)D₃ exogenously in the macrophage culture. However, it is noteworthy that TLR4L activation was associated with increased LL-37 transcript levels in macrophages cultured in the presence of serum 25(OH)D of the moderate group (50–75 nmol/l) compared with those with poorer vitamin D status (30–49 or < 30 nmol/l). This finding was substantiated by Walker *et al.*⁽²³⁾ who reported that TLR4L-stimulated cord blood monocytes showed higher LL-37 mRNA in the presence of 25(OH)D > 50 nmol/l compared with 25(OH)D < 30 nmol/l. They further showed that 25(OH)D concentration < 50 nmol/l was linked with diminished TLR4L-mediated induction of CYP27B1 enzyme, the key enzyme responsible for the conversion of 25(OH)D to the active form in macrophages, and diminished TLR4L-mediated induction of cathelicidin. However, they also showed reduced cathelicidin mRNA levels in the presence of 25(OH)D > 75 nmol/l compared with 25(OH)D > 50 nmol/l⁽²³⁾. Similarly, in the present study, LL-37 transcript levels declined in the high (≥ 76 nmol/l) group compared with the moderate (50–75 nmol/l) group. These findings suggest that 25(OH)D

concentration > 75 nmol/l suppresses the induction of LL-37 transcript levels.

Although most *in vitro* studies^(3,23,25–29) have shown an increase in cathelicidin mRNA expression after exposure to vitD₃, none of these studies measured the cathelicidin/LL-37 peptide concentration in the intracellular or extracellular compartment of macrophages. Liu *et al.*⁽³⁾ demonstrated the presence of cathelicidin peptide in macrophages by flow cytometry, immunofluorescence staining and the surface-enhanced laser desorption ionisation time-of-flight method, but LL-37 peptide concentration in macrophages was not measured. Only one study in Canada reported the *in vivo* effects of vitD₃ supplementation⁽³⁰⁾ that corroborated our findings, showing that 8 months of supplementation with vitD₃ significantly decreased serum LL-37 levels compared with pre-supplementation levels in healthy participants. They did not find any significant difference in TLR2/1L-induced LL-37 peptide concentration (assessed by Western blot and densitometry) in MDM between pre- and post-vitD₃ supplementation. The decrease in peptide concentration in the ICF of macrophages following TLR4L activation in the VitD group may have been due to a post-transcriptional regulatory block. It is important to acknowledge that this effect was quantitatively small, not consistent across multiple statistical approaches, and not mirrored by

Table 3. Effect of prenatal vitamin D supplementation *v.* placebo on the number of viable *Escherichia coli* colony-forming units (CFU) after culture with cord blood macrophages, with and without Toll-like receptor 4 ligand stimulation (Medians and interquartile ranges (IQR); ratios of CFU counts and 95% confidence intervals)

Specimens	CFU count						Ratio of CFU counts*	95% CI	P
	Overall (n 112)		Vitamin D (n 56)		Placebo (n 56)				
	Median	IQR	Median	IQR	Median	IQR			
Unstimulated cells	1775	378–4385	1685	445–4113	1858	282–4385	0.98	0.68, 1.42	0.91
LPS-stimulated cells	505	80–2063	440	43–1825	1008	128–2508	0.79	0.54, 1.14	0.21
Relative CFU count†	0.55	0.2–1.03	0.47	0.13–1.1	0.68	0.36–1.03	–	–	0.10‡

LPS, lipopolysaccharide.

* Based on the negative binomial regression model for comparison between the vitamin D and placebo groups.

† Relative CFU count for each participant was calculated as the ratio of CFU in LPS-stimulated to unstimulated cells.

‡ Based on the *t* test for comparison between the vitamin D and placebo groups, using log-transformed data.

a decline in LL-37 mRNA transcript levels. Furthermore, evaluation of the extracellular fluid did not show any increased release of LL-37 from the intracellular compartment of macrophages, which might have been expected in the context of an important increase in intracellular LL-37 peptide synthesis. In our recent report on the oral supplementation of healthy adult volunteers with 0.125 mg vitD₃ for 4 d, no significant changes in the concentration of either LL-37 peptide or mRNA were found in macrophages or lymphocytes⁽³¹⁾. The present results thus collectively suggest that *in vitro* findings related to the effect of vitamin D metabolites on LL-37 expression may not be consistent and may not predict the *in vivo* effects of vitD₃ supplementation.

We did not find any significant effect of oral vitD₃ supplementation on the killing activity of macrophages, as determined by the number of viable bacterial colonies remaining after the killing assay in TLR4L-stimulated and unstimulated macrophages. In both groups, TLR4L activation enhanced bacterial killing compared with unstimulated macrophages by reducing the number of viable bacterial colonies by approximately 40%, which is consistent with previous evidence that the antimicrobial activity of macrophages is dependent upon activation⁽³⁾. Notably, the antibacterial killing capacity of macrophages remained unaffected by 25(OH)D in the context of TLR4L activation, even though intracellular LL-37 peptide expression was slightly reduced by 25(OH)D. In the above-mentioned previous study involving healthy adult volunteers, we found that vitD₃ supplementation significantly increased the antibacterial killing activity of macrophages in the absence of any increase in the levels of LL-37 peptide or mRNA expression⁽³¹⁾. However, there are some key differences between our two studies. In our previous study⁽³¹⁾, vitD₃ supplementation was administered for only 4 d (even though the daily dose was the same), while in the present study, supplementation was given for about 3 months⁽¹⁹⁾. The previous study involved healthy adults, while the participants of the present study were healthy pregnant women, and immune function was studied in cord blood, not in maternal specimens. Macrophages have many arsenals; thus, it is possible that other vitamin D-dependent, but cathelicidin-independent, immune responses come into play, contributing to the innate killing by macrophages⁽³²⁾. It is likely that vitamin D plays a complex dual role in immunity; on the one hand, it may contribute to direct antimicrobial activities (e.g. by enhancing increased macrophage-mediated antimicrobial activity), on the other hand, it modulates immune cell functions that tend to reduce local inflammation (e.g. by controlling the release of antimicrobial peptides).

One area of particular interest regarding the effect of vitamin D on the immune response is in the treatment of tuberculosis infection, for which several randomised clinical trials of vitamin D supplementation have been reported^(33–36) or are ongoing⁽³⁷⁾ (NCT01130311, NCT00366470, NCT00507000, NCT00677339, NCT00918086 and NCT01580007). The reports published so far have conflicting results. An Indonesian study showed favourable outcome in tuberculosis patients with earlier sputum conversion⁽³⁴⁾. However, studies by Wejse *et al.*⁽³⁶⁾ and Martineau *et al.*⁽³³⁾ did not find any overall effect of vitD₃ supplementation on disease outcome; however,

the dose and duration of vitD₃ supplementation as well as genetic polymorphisms may modify treatment responses⁽³³⁾.

There are several studies reporting the effects of vitamin D supplementation on infectious diseases, particularly in lung diseases in children^(38–40). However, there is little conclusive evidence from controlled trials about the effect of vitamin D supplementation on the risk of infectious diseases in early infancy.

In conclusion, our findings showed that prenatal vitD₃ supplementation with a weekly dose of 0.875 mg in the last trimester may have slightly reduced intracellular LL-37 peptide concentration, but did not adversely affect the antibacterial activity of macrophages in neonates. We did not find mechanistic evidence of a promising beneficial effect of prenatal vitD₃ supplementation on newborn innate immunity. However, vitamin D has complex immune-regulatory roles, indicating the need for further short- and long-term studies to evaluate the effects of prenatal vitamin D exposures on infant and child immune responses.

Supplementary material

To view supplementary material for this article, please visit <http://dx.doi.org/10.1017/S0007114514001512>

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There is no conflict of interest.

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