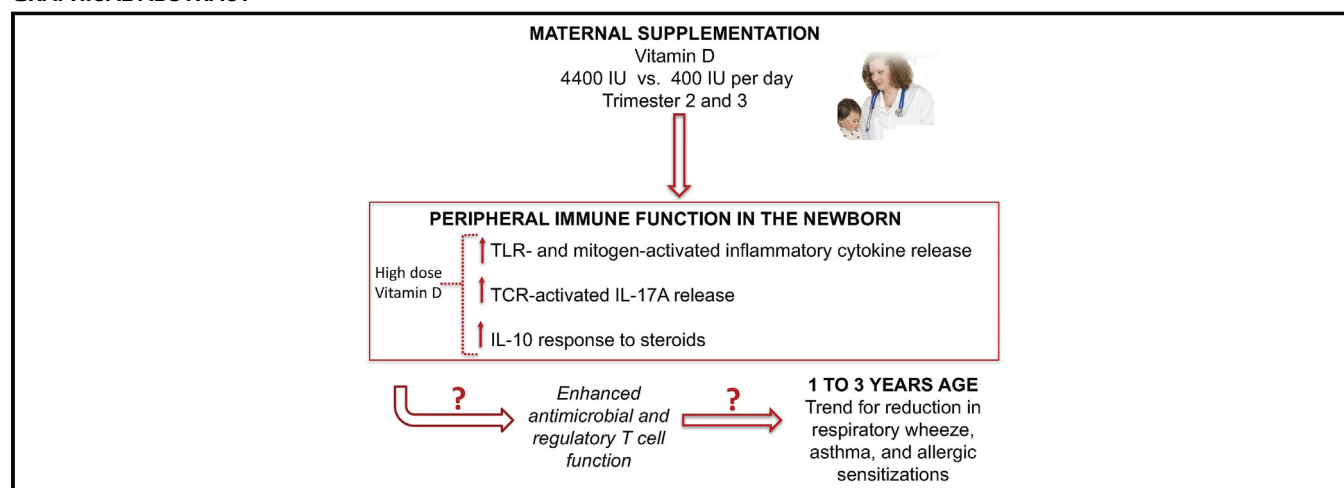


Vitamin D supplementation during pregnancy: Effect on the neonatal immune system in a randomized controlled trial



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GRAPHICAL ABSTRACT



Background: Programming of the immune system during fetal development can influence asthma-related risk factors and outcomes in later life. Vitamin D is a well-recognized immune modulator, and deficiency of this nutrient during pregnancy is hypothesized to influence disease development in offspring.

Objective: We sought to investigate the effect on neonatal immunity of maternal supplementation with 4400 IU/d vitamin D₃ during the second and third trimesters of pregnancy by using a subset of cord blood samples from a randomized, double-blind, placebo-controlled clinical trial (the Vitamin D Antenatal Asthma Reduction Trial).

Methods: Cord blood samples from neonates born to mothers supplemented with 4400 IU/d (n = 26) or 400 IU/d (n = 25) of vitamin D₃ were analyzed for immune cell composition by flow

cytometry, Toll-like receptor (*TLR*) expression by quantitative PCR, and cytokine secretion after stimulation with mitogenic, *TLR*, and T-cell stimuli by cytometric bead array. Responsiveness to the glucocorticoid dexamethasone was determined.

Results: Supplementation of mothers with 4400 IU of vitamin D₃ resulted in an enhanced broad-spectrum proinflammatory cytokine response of cord blood mononuclear cells to innate and mitogenic stimuli ($P = .0009$), with an average 1.7- to 2.1-fold increase in levels of several proinflammatory cytokines (GM-CSF, IFN- γ , IL-1 β , IL-6, and IL-8) across stimuli, a higher gene expression level of *TLR2* ($P = .02$) and *TLR9* ($P = .02$), a greater than 4-fold increase in IL-17A ($P = .03$) production after polyclonal T-cell stimulation, and an enhanced IL-10 response

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of cord blood mononuclear cells to dexamethasone treatment in culture ($P = .018$).

Conclusion: Vitamin D exposure during fetal development influences the immune system of the neonate, which can contribute to protection from asthma-related, including infectious, outcomes in early life. (J Allergy Clin Immunol 2018;141:269-78.)

Key words: Vitamin D, asthma, innate immunity, pregnancy

The majority of all asthma cases are diagnosed in early childhood,^{1,2} implying that the origin of the disease is in fetal or very early life. Recurrent wheeze,^{3,4} atopy,^{5,6} and lower respiratory tract infections⁷ in early childhood are considered risk factors for the development of asthma-related disease. Maternal vitamin D deficiency during pregnancy has also been proposed to be a risk factor for development of childhood asthma⁸ and the associated risk factors of allergy and infection; however, observational studies that have investigated a relationship between asthma-related outcomes in children and total 25-hydroxyvitamin D (25-hydroxyvitamin D₂ plus 25-hydroxyvitamin D₃; 25[OH]D) levels in maternal or cord blood have produced inconsistent results.^{9,10}

Immune measurements taken from the neonate at birth have been linked to the subsequent development of atopic and asthmatic disease, implying that early changes in the immune system can precede and underpin disease development. For example, neonates born to parents with a history of allergy or asthma, who are themselves at an increased risk of experiencing these diseases, have lower mononuclear cell cytokine responses to respiratory syncytial virus¹¹ and lower mononuclear cell proliferative responses to LPS.¹² Additionally, direct comparisons with disease outcomes have shown that neonates who go on to have atopic disease or asthma have lower LPS-induced mononuclear cell proliferative responses¹² and lower cord blood serum levels of IL-4, TNF- α , and IFN- γ .¹³

Cells of the innate and adaptive immune systems express the vitamin D receptor, and their function is modulated by exposure to 1,25-dihydroxyvitamin D₃ (1,25[OH]₂D₃) in culture. Direct *in vitro* treatment of innate immune cells with 1,25(OH)₂D₃ has been shown to enhance the expression of antimicrobial peptides, such as cathelicidin,^{14,15} and to influence innate immune signaling.¹⁶ Dysregulation of the adaptive immune system is also of prime importance in the pathology of asthma. 1,25(OH)₂D₃ has been described to directly inhibit T_H1^{17,18} and T_H17^{19,20} cytokine production *in vitro* while promoting regulatory T-cell phenotypes.^{19,21} We have previously described upregulation of IL-10 gene expression in CD4 T cells²² and an increased response to dexamethasone in culture for IL-10 synthesis²³ after supplementation of asthmatic patients with 1,25(OH)₂D₃. Notably, all these studies were performed in adults and might not reflect immunity in early life.

Vitamin D status during pregnancy has been linked to immune parameters in the neonate. For example, a positive correlation between cord blood 25(OH)D levels and IFN- γ release from cord blood mononuclear cells (CBMCs) on LPS stimulation²⁴ and serum IL-10 levels²⁵ has been shown.

To our knowledge, all studies to date that have investigated links between vitamin D status and immune outcomes in the neonate have been observational and therefore do not address directly whether vitamin D can actively alter the responsiveness of the neonatal immune system to innate and adaptive

Abbreviations used

CBMC:	Cord blood mononuclear cell
FITC:	Fluorescein isothiocyanate
1,25(OH) ₂ D ₃ :	1,25-Dihydroxyvitamin D ₃
25(OH)D:	Total 25-hydroxyvitamin D (25-hydroxyvitamin D ₂ plus 25-hydroxyvitamin D ₃)
PE:	Phycoerythrin
TLR:	Toll-like receptor
VDAART:	Vitamin D Antenatal Asthma Reduction Trial

challenge. Therefore in the current study we aimed to test the effect of daily vitamin D₃ supplementation during pregnancy on the immune system of the neonate using samples from a large, randomized, double-blind, placebo-controlled clinical trial, the Vitamin D Antenatal Asthma Reduction Trial (VDAART).^{9,26} First, we hypothesized that high-dose maternal vitamin D₃ supplementation would promote the innate immune system of the neonate, increasing the capacity of CBMCs to respond to Toll-like receptor (TLR) ligation. Second, we hypothesized a more nuanced effect of maternal vitamin D₃ supplementation on neonatal T-lymphocyte responses, with suppression of IFN- γ and IL-17A production but enhancement of IL-10. Additionally, we hypothesized that there would be greater dexamethasone-induced IL-10 production in neonates from mothers receiving high-dose supplementation.

METHODS

VDAART ancillary study

Umbilical cord blood was collected from 51 pregnant women recruited consecutively to the Boston Medical Center site of the parent VDAART study and who provided written informed consent to participate in this ancillary study. Sample size was based on power calculations performed by using data from human adult studies on 1,25(OH)₂D₃-induced IL-10 synthesis and cell phenotyping,^{21,22} in which studying 48 subjects would produce excellent power (>85%) to detect differences across dosing groups. We aimed to recruit up to an additional 12 subjects to allow for the risk of technical issues with investigative assays, such as insufficient blood volume. Participants were randomized at 10 to 18 weeks of pregnancy to high- or low-dose vitamin D supplementation; 26 of the ancillary study participants were from the study arm supplemented with 4400 IU/d vitamin D₃, and 25 were from the study arm supplemented with 400 IU/d.

Preparation and culture of CBMCs

CBMCs were isolated by means of standard density gradient centrifugation, and CBMCs from each donor were cultured at 2×10^6 cells/mL in supplemented RPMI medium either alone or with the addition of LPS (from *Escherichia coli* 0111:B4m, 0.01 μ g/mL; InvivoGen, San Diego, Calif), PPG (peptidoglycan from *Staphylococcus aureus*, 1.25 μ g/mL, InvivoGen), CpG (type C CpG oligonucleotide ODN 2395, 1 μ g/mL, InvivoGen), or PHA (lectin from *Phaseolus vulgaris*, 15 μ g/mL; Sigma, St Louis, Mo). Culture supernatants were harvested at 24 hours from each well and immediately stored, without pooling, at -80°C before cytokine analysis. CBMCs were cultured at 1×10^6 /mL in RPMI medium in the presence of plate-bound anti-CD3 (1 μ g/mL, OKT3) and soluble recombinant human IL-2 (50 U/mL; EuroCetus, Amsterdam, The Netherlands) to study adaptive T cell responses. Culture supernatants were harvested after 72 hours and stored at -80°C until analysis. All CBMC cultures were performed in duplicates, and cytokine levels from each well were measured independently.

TABLE I. Characteristics of VDAART participants contributing immunological data

Mother's characteristics	4400 IU		400 IU		P value
	n	Percent	n	Percent	
Race					
African American	10	38	14	56	
All other races	16	62	11	44	.21
History of eczema/atopic dermatitis	8	31	10	40	.49
History of asthma	10	38	9	36	.86
History of hayfever and allergic rhinitis	16	62	18	72	.43
Cesarean section delivery	8	31	11	44	.33
	n	Mean (SD)	n	Mean (SD)	
Age at childbirth	26	31.0 (4.9)	25	28.1 (6.2)	.06
BMI before pregnancy	22	29.4 (5.9)	20	29.4 (5.9)	.97
Plasma 25(OH)D ₃ (ng/mL) at enrollment	26	19.2 (6.7)	25	23.5 (8.6)	.05
Plasma 25(OH)D ₃ (ng/mL) at third-trimester visit	23	35.4 (13.8)	25	24.6 (9)	.002
	n	Percent	n	Percent	P value
Neonatal characteristics					
Sex					
Female	13	50	8	32	
Male	13	50	17	68	.19
	n	Mean (SD)	n	Mean (SD)	
Gestational age (wk) at delivery	26	39.4 (1.4)	25	39.4 (1.3)	.99
Birth weight (g)	26	3555.4 (513.3)	25	3247 (447.1)	.03
Birth length (cm)	26	51.4 (2.4)	25	49.9 (2.3)	.03
Cord blood plasma 25(OH)D ₃ (ng/mL)	26	25.4 (15.3)	23	16.2 (6.5)	.01

BMI, Body mass index.

Flow cytometry

For phenotypic characterization of the main immune cell populations in cord blood, samples were stained with appropriate antibodies (see the [Methods](#) section in this article's Online Repository at www.jacionline.org), and data were acquired on a BD LSR II flow cytometer by using BD FACS-Diva software (BD Biosciences, San Jose, Calif) and then analyzed with FlowJo software (version 9.6.4; TreeStar, Ashland, Ore).

Cytokine measurements

Cytokine concentrations in individual culture supernatants were determined by using a cytometric bead array assay (BD Biosciences), according to the manufacturer's instructions.

Quantitative PCR

CBMCs (2×10^6) were lysed in 750 μ L of Trizol Reagent (Ambion, Foster City, Calif) and stored at -80°C until RNA extraction and analysis. RNA was isolated according to the manufacturer's protocol and purified by using an RNeasy Mini column (Qiagen, Hilden, Germany), with additional DNase treatment (Ambion) performed per the manufacturer's instructions. RNA quantity was determined by using the Agilent RNA 6000 Pico kit (Agilent Technologies, Santa Clara, Calif). One hundred nanograms of RNA was reverse transcribed into cDNA. Quantitative RT-PCR was performed in triplicates with TaqMan gene expression technology and the ViiA7 system (Life Technologies, Grand Island, NY), and data were expressed by using the $2^{-\Delta\text{CT}}$ method.

Statistical analysis

All immune assays were performed in a blinded manner, and the data were subsequently unblinded and analyzed independently by a statistician. For

analysis of the effect of vitamin D supplementation on cytokine production by CBMCs in response to innate stimulation, a global null hypothesis of no effect of supplementation on mean cytokine production was tested by using fixed-effects multivariate ANOVA. Random-effects ANOVA models were used to test the effects of vitamin D supplementation on TLR expression in CBMCs and T-cell cytokine production after adaptive immune stimulation. All computations used R software (version 3.3; www.r-project.org). Two separate replicate CBMC cultures were conducted for each culture condition for each donor. The resulting biological replicates were appropriately analyzed as repeated measures to capture the effects of biological variation in the statistical analysis. In contrast, further technical replicates in PCR analyses were analyzed by using the mean average of replicate values per standard practice.

Full details of methods, reagents, and statistical analyses can be found in the [Methods](#) section in this article's Online Repository.

RESULTS

Characteristics of the trial population

Umbilical cord blood samples from 51 VDAART participants recruited consecutively into the Boston Medical Centre site of the trial were used for immunologic analyses: 26 from the study arm supplemented with 4400 IU/d vitamin D₃ and 25 from the study arm supplemented with 400 IU/d vitamin D₃. Characteristics of these participants are shown in [Table I](#). There were no significant differences in the mother's race, age, prepregnancy body mass index, or history of eczema, atopic dermatitis, asthma, hay fever, and allergic rhinitis between the 2 study arms of the trial. The proportion of cesarean section deliveries, sex of the neonate, and gestational age were also not significantly different between the 2 arms. At enrollment in the trial (10-18 weeks' gestation),

TABLE II. Effect of vitamin D supplementation in pregnancy on immune cell populations in cord blood

Immune population	4400 IU/d		400 IU/d		P value
	Mean (SE)	No.	Mean (SE)	No.	
CD3 ⁺ cells within CD45 ⁺ fraction (%)	19.18 (2.17)	15	24.744 (3.33)	16	.18
CD14 ⁺ cells within CD45 ⁺ fraction (%)	4.615 (0.36)	20	5.994 (0.82)	16	.11
Neutrophils within CD45 ⁺ fraction (%)	52.27 (3.66)	17	47.927 (3.44)	15	.40
CD19 ⁺ cells within CD45 ⁺ fraction (%)	5.329 (0.54)	14	5.143 (0.467)	14	.80
HLA-DR ⁺ Lin ⁻ cells within CD45 ⁺ fraction (%)	0.487 (0.05)	16	0.471 (0.06)	14	.84
CD11c ⁺ mDCs within HLA-DR ⁺ Lin ⁻ fraction (%)	48.86 (5.29)	14	34.25 (5.29)	11	.051
BDCA4 ⁺ pDCs within HLA-DR ⁺ Lin ⁻ fraction (%)	12.30 (2.19)	14	11.93 (1.68)	11	.90
CD4 ⁺ cells within CD3 ⁺ fraction	67.965 (1.64)	20	70.756 (1.66)	16	.24
CD8 ⁺ cells within CD3 ⁺ fraction (%)	24.88 (1.40)	20	23.144 (1.41)	16	.39
RA ⁺ RO ⁻ cells within CD3 ⁺ CD4 ⁺ fraction (%)	68.556 (2.87)	16	73.657 (4.06)	14	.30
RA ⁻ RO ⁺ cells within CD3 ⁺ CD4 ⁺ fraction (%)	8.389 (1.04)	18	5.864 (1.09)	14	.11
FoxP3 ⁺ CD25 ⁺ cells within CD3 ⁺ CD4 ⁺ fraction (%)	3.962 (0.57)	13	3.908 (0.4)	13	.94

mDCs, Myeloid dendritic cells; pDCs, plasmacytoid dendritic cells.

*Lineage = CD3, CD14, CD16, CD19, CD20, and CD56.

mean levels of circulating 25(OH)D in the mothers from both study arms were in the insufficient-deficient range (<30 ng/mL), although mean levels were lower ($P = .05$) in the group subsequently supplemented with 4400 IU/d vitamin D₃ versus the 400 IU/d group. After 22 to 30 weeks of supplementation (ie, in the third trimester of pregnancy), the pregnant women receiving 400 IU/d vitamin D₃ remained in the insufficient range (<30 ng/mL), with only 6 of 23 patients having a sufficient vitamin D level at this time point. The mean concentration in women receiving 4400 IU/d moved into the sufficient range (>30 ng/mL), achieving a mean 25(OH)D concentration of 35 ng/mL, with 17 of 25 participants demonstrating vitamin D sufficiency. Data for vitamin D status were missing for 3 women in the 400 IU arm and for 1 woman in the 4400 IU arm. Therefore 4400 IU/d was effective in increasing 25(OH)D levels to sufficiency within this subset of the larger trial patient population and resulted in a significant difference ($P = .002$) in maternal 25(OH)D₃ levels compared with the 400 IU/d group by the third trimester of pregnancy.

The effect of maternal supplementation in our patient population was also reflected in cord blood 25(OH)D levels, which were significantly ($P = .01$) higher in the group that received 4400 IU/d versus those who received 400 IU/d. Babies born in the 4400 IU/d group were significantly longer ($P = .03$) and heavier ($P = .03$) than in the 400 IU/d group.

Effect of vitamin D₃ supplementation in pregnancy on immune cell populations in cord blood

Supplementation with 4400 IU/d vitamin D₃ during pregnancy versus 400 IU/d vitamin D₃ had no significant effect on the relative proportions of the main immune cell types in cord blood, as measured by using flow cytometry (Table II). Myeloid, but not plasmacytoid, dendritic cells showed a trend ($P = .051$) toward an enhanced frequency in CBMCs from babies within the 4400 IU/d group in comparison with those of the 400 IU/d group.

Effect of vitamin D₃ supplementation in pregnancy on cytokine secretion from CBMCs after stimulation of innate immune responses

To address our first hypothesis that vitamin D₃ supplementation during pregnancy would promote the innate immune

system of the neonate, we cultured CBMCs from the 2 clinical trial study arms for 24 hours with LPS (TLR4 specific), peptidoglycan (TLR2 specific), the type C CpG oligonucleotide ODN 2395 (CpG, TLR9 specific), PHA (mitogenic), or medium alone. Each CBMC sample was cultured in 2 separate wells, and the resultant culture supernatants were then analyzed independently for concentrations of 7 innate cytokines. CBMCs cultured in medium alone released an undetectable or minimal amount of cytokine in the 24-hour period, with the exception of IL-8, levels of which ranged from 125 to 280,000 pg/mL (data not shown). Data are summarized in Fig 1, A, as a heat map in which the ratio of the geometric mean cytokine concentrations (adjusted for cytokine levels in medium control) in the 4400 IU/d supplementation group are calculated relative to those in the 400 IU/d supplementation group. Ratios of greater than 1 are indicative of a higher geometric mean cytokine concentration in the 4400 IU/d group. Geometric mean cytokine concentrations were greater in the 4400 IU/d vitamin D₃ supplementation arm than the 400 IU/d vitamin D₃ supplementation arm in 27 of the 28 cytokine-stimulation combinations ($P = .0009$, multivariate ANOVA-based summary test of treatment effect on 7-dimensional response; Fig 1, A; analysis for TLR stimuli alone, $P = .156$).

In further exploratory analyses the effect of vitamin D supplementation on production of individual innate cytokines was examined. For each cytokine measured, stimulation of CBMCs with each of the 3 TLR ligands and PHA significantly induced a range of cytokine concentrations in culture supernatants, and maternal vitamin D₃ supplementation dose exhibited significant or borderline significant effects on geometric mean cytokine levels of GM-CSF ($P = .010$), TNF- α ($P = .131$), IL-1 β ($P = .094$), and IFN- γ ($P = .077$); for IL-6, IL-8, and IL-10, P values for tests of supplementation effect exceeded 0.40 (Fig 1, B).

CBMCs from neonates of mothers supplemented with 4400 IU/d vitamin D₃ had higher expression levels of *TLR2* ($P = .02$) and *TLR9* ($P = .02$) gene expression compared with the 400 IU/d group, an observation that could in part explain increased sensitivities to peptidoglycan and CpG in this group. However, *TLR4* expression was not significantly greater in the 4400 IU/d group ($P = .14$, Fig 2).

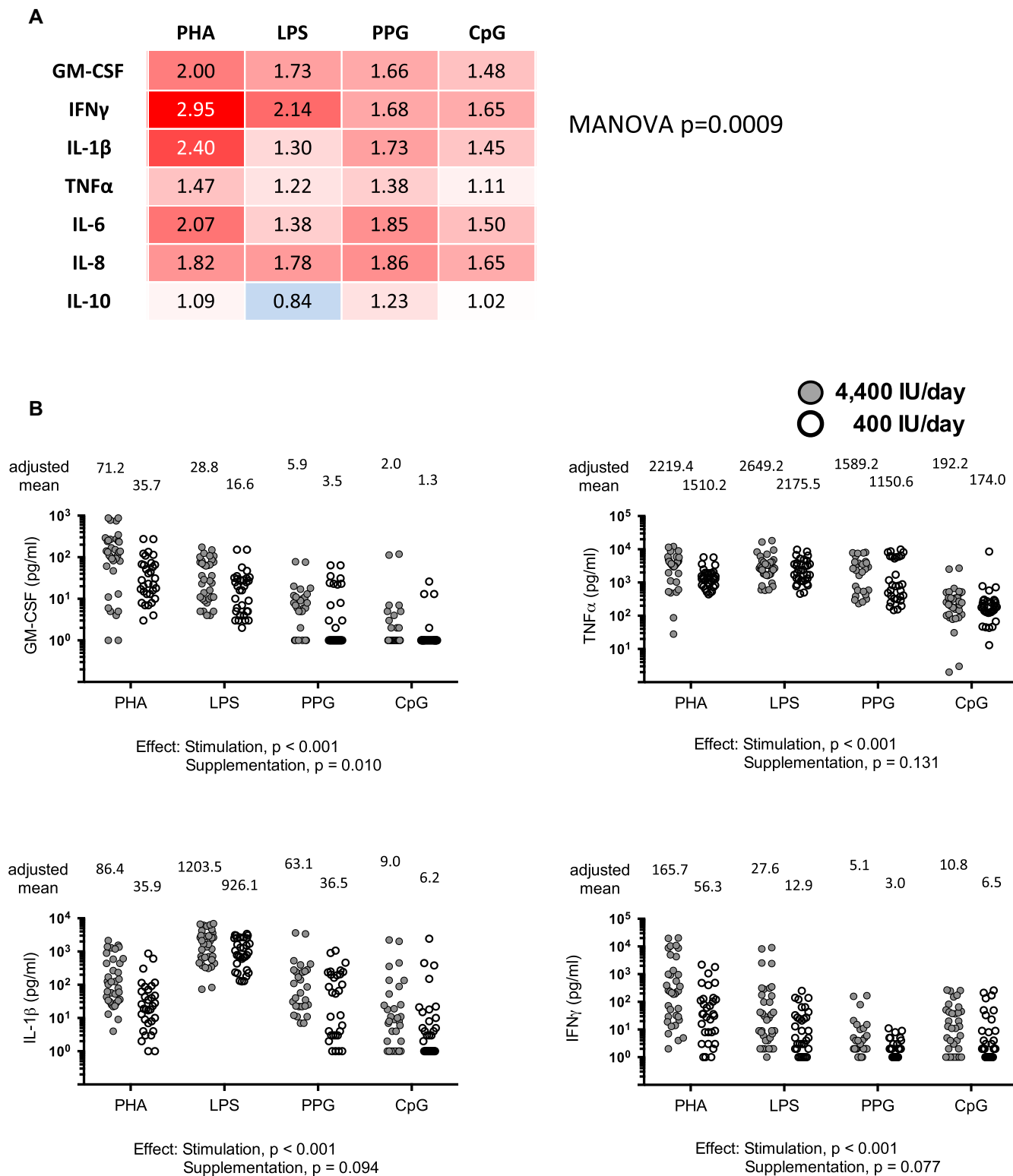


FIG 1. Effect of vitamin D₃ supplementation during pregnancy on innate and mitogenic cytokine responses. **A**, Heat map of ratios of geometric mean cytokine concentrations adjusted for medium control cytokine level. Ratios of greater than 1 (red scale) are indicative of a higher concentration in the 4400 IU/d group. **B**, Raw cytokine levels. Gray filled symbols, 4400 IU/d group (n = 14-18); open symbols, 400 IU/d group (n = 15-16). There were 2 biological replicates per CBMC sample.

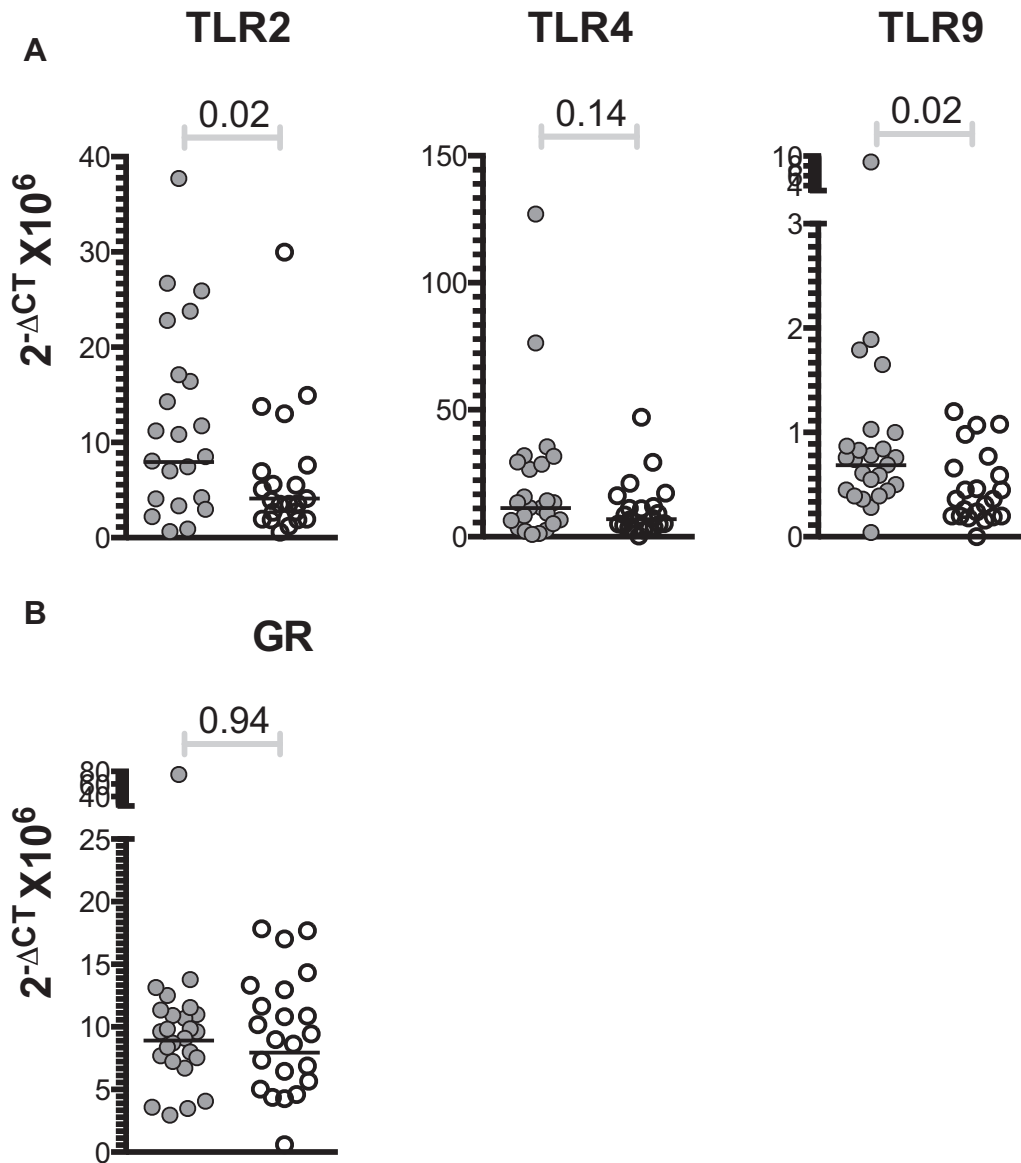


FIG 2. Effect of vitamin D supplementation in pregnancy on expression of *TLR2*, *TLR4*, and *TLR9* (A) and the glucocorticoid receptor (GR) in CBMCs (B). Expression levels were measured by using quantitative PCR and are expressed as $2^{-\Delta CT} \times 10^6$ relative to 18s. One biological replicate for each participant is shown ($n = 22$ –25 per group). Gray filled circles, 4400 IU/d; open circles, 400 IU/d.

Effect of vitamin D₃ supplementation in pregnancy on T-cell responses in the neonate

CBMCs from the 2 study arms were cultured with anti-CD3 and IL-2 to establish the effect of vitamin D₃ supplementation during pregnancy on adaptive T-lymphocyte responses in the neonate. Culture of CBMCs with this polyclonal T-cell stimulus resulted in detectable production of a range of T-cell cytokines (IFN- γ , IL-5, IL-10, IL-13, and IL-17A) after 72 hours (Fig 3). CBMCs from the group supplemented with 4400 IU/d of vitamin D₃ secreted greater geometric mean levels of IL-17A ($P = .03$) versus the 400 IU/d group, the opposite effect to that hypothesized. There were no significant differences in the levels of other cytokines measured between the 2 arms of the study; however, there was a trend for lower levels of IL-10 and higher levels of IL-13 in the 4400 IU/d group versus the 400 IU/d group.

Effect of vitamin D₃ supplementation in pregnancy on glucocorticoid responses in the neonate

We next assessed whether the higher dose of maternal vitamin D₃ supplementation would enhance dexamethasone-induced IL-10 production from CBMCs in anti-CD3- and IL-2-stimulated cultures. The change in IL-10 production in response to dexamethasone treatment was enhanced in CBMC cultures from the study arm supplemented with 4400 IU/d compared with the arm supplemented with 400 IU/d and was statistically significant between the 2 study arms ($P = .018$, Fig 4). However, no effect of vitamin D₃ supplementation during pregnancy on the expression level of glucocorticoid receptor in CBMCs was observed to explain this finding (Fig 2).

Production of IFN- γ , IL-13, IL-5, and IL-17A in response to dexamethasone was also studied and in both arms of the trial was

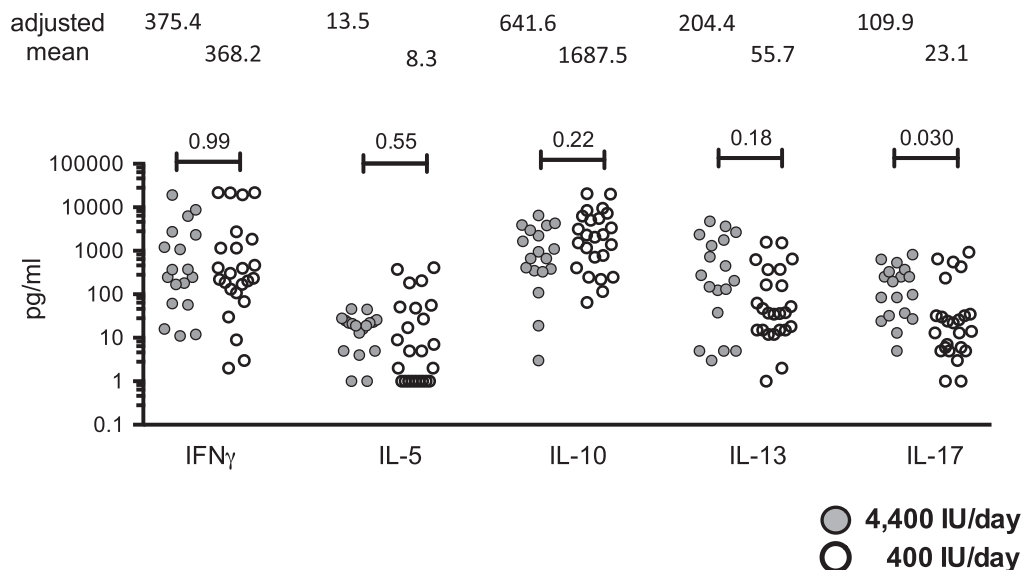


FIG 3. Effect of vitamin D supplementation in pregnancy on neonatal cytokine responses after T-cell stimulation. CBMCs were cultured with anti-CD3 and IL-2. Supernatants were harvested at 72 hours, and production of 5 T-cell cytokines was measured. Two biological replicates for each participant are shown. *Gray filled circles, 4400 IU/d; open circles, 400 IU/d.*

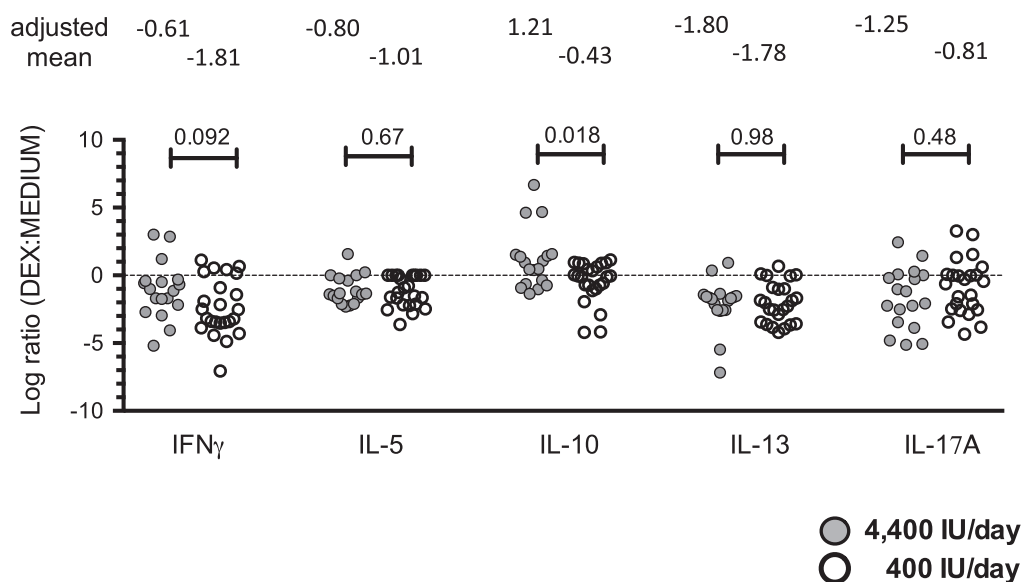


FIG 4. Effect of vitamin D supplementation on neonatal responses to dexamethasone. CBMCs were cultured with anti-CD3 and IL-2 for 72 hours in the presence or absence of 1×10^{-7} mol/L dexamethasone (DEX). Change in cytokine concentration in dexamethasone-treated versus untreated cultures is expressed as a log ratio. Two biological replicates per participant are plotted. *Gray filled circles, 4400 IU/d; open circles, 400 IU/d.*

reduced by addition of dexamethasone; there was no significant effect of vitamin D₃ supplementation on this response.

DISCUSSION

This study investigates the effect of supplementation with 4400 IU/d vitamin D₃ during the second and third trimesters of pregnancy on the immune responsiveness of the neonate by using a

subset of umbilical cord blood samples from babies born within VDAART.^{9,26} This regimen led to an increase in mean maternal circulating 25(OH)D levels into the sufficiency range (>30 ng/mL). In contrast, supplementation with the current recommended daily intake of 400 IU/d did not enhance 25(OH)D levels in the mother. Notably, even within the small cohort studied here, a significant increase in newborn weight and height was observed in babies born to mothers supplemented with 4400 IU/d.

Importantly, supplementation of pregnant women with 4000 IU/d vitamin D₃ appears to be safe and effective in reducing the morbidities of pregnancy.^{27,28}

Our first major finding was of significantly enhanced proinflammatory cytokine production in response to innate and mitogenic stimuli in CBMC cultures from babies born within the 4400 IU/d vitamin D₃ arm. This effect was not specific to any one particular cytokine or stimulation given that all cytokine stimulation combinations were on average higher in the 4400 IU/d group and resulted in mean fold increases across the 4 stimuli of 1.715 for GM-CSF, 2.105 for IFN- γ , 1.72 for IL-1 β , 1.7 for IL-6, and 1.778 for IL-8. In contrast, such an effect was not seen with the anti-inflammatory cytokine IL-10. The capacity of vitamin D₃ supplementation during pregnancy to enhance innate responses in the neonatal period is supported by observational studies in early life, showing a positive relationship between cytokine responses and 25(OH)D levels in cord blood.^{24,29}

Additionally, we observed higher gene expression levels for *TLR2* and *TLR9* in CBMCs from the 4400 IU/d group. Given that the strongest effect of vitamin D₃ supplementation was observed after PHA stimulation, the difference in expression of these receptors might not fully explain the difference in responsiveness between study arms. However, PHA will activate innate lymphocytes, such as natural killer cells, and has also been proposed to act as a TLR agonist.³⁰ Analysis of cell subset composition hinted at an increased frequency of myeloid, but not plasmacytoid, dendritic cells ($P = .051$) within CBMCs from the 4400 IU/d group. Although more detailed analyses are warranted based on these preliminary data, it is plausible that dendritic cells can contribute both directly to the enhanced proinflammatory cytokine levels and/or indirectly by enhancing T-cell activation in these short-term cultures. However, the mechanism by which high-dose maternal vitamin D₃ supplementation improves the fitness of innate immune cells to respond to stimulation remains uncertain.

Responsiveness of immune cells to infectious stimuli at birth is known to be highly variable between subjects.³¹ Stronger neonatal cytokine responses, in particular for IFN- γ , have been associated with reduced respiratory tract illness,³²⁻³⁴ as well as with the incidence of wheeze, allergy, and asthma^{11-13,35,36} later in childhood. In addition, lower expression levels of TLRs on cord blood immune cells has been linked to maternal allergy.^{37,38} Therefore the capacity of high-dose vitamin D₃ supplementation during pregnancy to enhance innate immune fitness in the neonate might limit the immune perturbations in early life that are linked to asthma development.

Our second major finding was that vitamin D₃ supplementation of 4400 IU/d led to enhanced IL-17A production in CBMC cultures in response to polyclonal T-cell stimulation. IL-17A and T_H17-associated cytokines play a crucial role in pulmonary immune defense to pathogens³⁹ and in neonatal immunity⁴⁰ but in excess can also have pathologic effects. Lack of IL-12/IL-23p40 in very premature babies is associated with an increased risk of sepsis, and this further suggests a role for IL-17 in neonatal defense to infection.⁴¹ We propose that upregulation in the present study would support the capacity of vitamin D to promote the protective antimicrobial actions of IL-17A in the neonate. These data were unexpected given previous reports demonstrating that 1,25(OH)₂D₃ inhibits IL-17A and T_H17 synthesis^{19,20,42} in addition to our own data showing that IL-17A is strongly inhibited in CBMC polyclonal T cell-stimulated cultures

in the presence of *in vitro* 1,25(OH)₂D₃ (data not shown). These conflicting findings highlight the importance of context and timing in the actions of vitamin D on the immune system.

Although no effect of vitamin D₃ supplementation in pregnancy on levels of IL-10 synthesized by CBMCs after innate or T-cell stimulation was observed, enhanced responsiveness of CBMCs from the 4400 IU/d vitamin D₃ group compared with the placebo group to *in vitro* dexamethasone treatment for the production of IL-10 was seen. This vitamin D phenomenon has been shown in adults with steroid-resistant asthma^{23,43} and could be relevant in the context of early life, when glucocorticoids and vitamin D have been proposed to influence lung development,⁴⁴ although the clinical effect of this is an area for further exploration.

Our study was underpowered to investigate links between immune parameters at birth and clinical outcomes in children; however, the main VDAART of 876 women reported a trend for a lower incidence of asthma/recurrent wheeze in the group whose mothers had been supplemented with 4400 IU/d compared with 400 IU/d vitamin D₃, a trend for reduced rates of lower respiratory tract infections, and significantly fewer allergens to which they were sensitized at age 3 years. Notably, an independent randomized controlled trial of vitamin D₃ supplementation in pregnancy, although with significant differences in study design, demonstrated a trend for similar clinical outcomes and in a secondary principle component analysis reported an upregulated neonatal airway immune profile in nasal lining fluid samples from the supplemented arm, which complements the present findings.⁴⁵

The strengths of this study are that participants were from a randomized controlled trial and that a comprehensive set of immunologic assays was conducted examining both innate and adaptive immune responses. The main limitation of this study is that it was underpowered for analysis of associations between immune parameters and subsequent clinical outcomes. Furthermore, additional stratified analyses based, for example, on sex were not pursued because no sex-specific effects were observed in the main VDAART and the size of the current study precluded meaningful analyses. Nevertheless, given the small size of this substudy of a randomized controlled trial, it is very encouraging how many immunologic parameters showed a significant effect from randomization to higher-dose vitamin D supplementation.

In summary, this is the first study to ascertain the effects of maternal vitamin D supplementation at levels that restored sufficiency in the majority of pregnant women on the responsiveness of the early-life immune system on innate and T-cell stimulation by using the rigorous approach of a randomized controlled trial. We report that neonates of mothers supplemented with 4400 IU/d vitamin D₃ had greater innate cytokine responses, greater IL-17A production in response to T-cell stimulation, and greater dexamethasone-induced IL-10 production. Given the evidence for strong neonatal immune responses in early life being associated with decreased development of asthma, this effect will likely lead to improved respiratory health in early life. Future studies should address the longitudinal effect of vitamin D₃ supplementation in pregnancy on clinical and immune outcomes in the infant.

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Key messages

- Vitamin D supplementation during pregnancy modifies the immune system of the neonate.
- This modified immune system might be better equipped to protect the host against pathogenic infections.

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METHODS

VDAART clinical trial

VDAART^{E1} is a multicenter, randomized, double-blind, placebo-controlled clinical trial designed to determine whether higher vitamin D intake in pregnant women will prevent asthma in offspring at age 3 years. Eight hundred seventy-six pregnant women were recruited from 3 clinical sites across the United States and randomized at 10 to 18 weeks of pregnancy into one of 2 arms: 4000 IU of vitamin D₃ plus a multivitamin containing 400 IU of vitamin D₃ to be taken daily or a placebo pill plus a multivitamin containing 400 IU of vitamin D₃ to be taken daily. Eligible participants were pregnant women between the ages of 18 and 39 years with a history of asthma, eczema, or allergic rhinitis (or the biological father had a history of asthma, eczema, or allergic rhinitis) and currently a nonsmoker. VDAART is registered at [ClinicalTrials.gov](https://clinicaltrials.gov) as NCT00920621.

Preparation of CBMCs

At the time of birth, cord blood samples were collected into tubes containing 10% sodium citrate and transported to the laboratory within 16 hours of delivery. CBMCs were isolated by using a Ficoll density gradient.

Flow cytometry

For phenotypic characterization of the main immune cell populations in cord blood samples, the following antibodies were used in various stain combinations: CD3 fluorescein isothiocyanate (FITC; BD Biosciences), clone SK7; CD14 FITC (BD Biosciences), clone MΦp9; CD16 FITC (BD Biosciences), clone 3G8; CD19 FITC (BD Biosciences), clone 4G7; CD20 FITC (BD Biosciences), clone 2H7; CD56 FITC (BD Biosciences), clone NCAM16.2; CD3 peridinin-chlorophyll-protein complex (BD Biosciences), clone SK7; CD14 phycoerythrin (PE; BD Biosciences), clone MΦp9; CD19 FITC (BD Biosciences), clone 4G7; HLA-DR peridinin-chlorophyll-protein complex (BD Biosciences), clone L243; CD45 Pacific blue (AbD Serotec, Oxfordshire, United Kingdom), clone F10-89-4; CD4 eFluor 450 (eBioscience, San Diego, Calif); CD8 allophycocyanin (BD Biosciences), clone OKT4; CD38 allophycocyanin (eBioscience), clone HIT2; CD45RA FITC (BD Biosciences), clone HI100; forkhead box P3 (FoxP3) PE (eBioscience), clone PCH101; CD45 RO 650NC (eBioscience), clone UCHL1; and CD25 PE-Cy7 (BD Biosciences), clone BC96.

For stain combinations containing only cell-surface antibodies, 3 μL of each relevant antibody was added to an aliquot of 125 μL of fresh UCB, and the sample was incubated on ice for 30 minutes. Fluorescence-activated cell sorting lysing solution (BD Biosciences) was subsequently added to lyse the majority of erythrocytes per the manufacturer's instructions. Cells were fixed with BD Cytofix. For stain combinations containing the intranuclear FoxP3 antibody, 3 μL of cell-surface antibodies were first added to 1 to 2 million CBMCs in a volume of 100 μL of PBS plus 2% FBS and incubated on ice for 30 minutes. FoxP3 staining was subsequently carried out by using a FoxP3 staining buffer set from eBioscience, according to the manufacturer's instructions. Gating of cell populations was carried out with FlowJo software (version 9.6.4). Doublets were excluded, and a CD45 gate was applied as a marker of leukocytes. Subsequent cell proportions were determined as a proportion of leukocytes or subpopulations of leukocytes. Neutrophils were identified based on forward- and side-scatter characteristics. Fluorescence minus one controls for individual samples were used to set the gates. Mean fluorescence intensity was reported as normalized to the fluorescence minus one sample.

Cytokine measurements

Cytokine concentrations in cell-culture supernatants were determined by using a cytometric bead array assay (BD Biosciences), according to the manufacturers' instructions. Samples from the assay were analyzed on a Fortessa flow cytometer (Becton Dickinson). Data were analyzed with FlowJo software (version 9.6.4) and GraphPad Prism software (version 5). The lower limit of detection for all cytokines in the assay was 1.5 pg/mL. Samples to be assayed for IL-6 and IL-8 were first diluted 1:10 because of the high concentration of these cytokines present in the samples. In these cases cytokine measurements were multiplied by 10 to produce the final measurements. In the minority of cases in which the measurement of the 1:10 sample was less than the standard curve of the assay, the assay was repeated with the undiluted sample. Results greater than the standard curve (despite dilution as above) were valued as 50,000 pg/mL (for GM-CSF, IFN-γ, IL-1β, TNF-α, and IL-10) or 500,000 pg/mL for IL-6 and IL-8. Results less than the standard curve were valued as 1 pg/mL.

Quantitative PCR primers

The following FAM-labeled primer and probe sets from Life Technologies were used: TLR2 (Hs00610101-m1), TLR4 (Hs00152939-m1), TLR9 (Hs00152973_m1), and NR3C1 (Hs00353740_m1). VIC-labeled eukaryotic 18s rRNA endogenous control (Life Technologies) was used as a house-keeping gene.

Statistical analysis

For analysis of the effect of vitamin D supplementation on cytokine production by CBMCs in response to innate stimulation, 2-way mixed-effects analyses were performed,^{E2} showing effects of stimulation and vitamin D₃ supplementation on *in vitro* production of different cytokines. The mean natural logarithm of each measured cytokine response plus one was analyzed with adjustment for response to sham, fixed effects of treatment arm and stimulus, and random effects of subject to accommodate correlation among replicates. Then for each different cytokine, separate random-effects ANOVAs were conducted to simultaneously test the effect of the different stimuli along with the effect of supplementation across the range of stimuli. Similar random-effects models were used to test the effects of vitamin D supplementation on TLR expression in CBMCs and T-cell cytokine production after polyclonal T-cell stimulation. The global null hypothesis of no effect of supplementation on mean cytokine production was tested by using fixed-effects multivariate ANOVA,^{E3} with the 7-dimensional response for each subject composed of log (1 plus measured cytokine concentration) minus log (1 plus mean replicated cytokine measure with sham stimulus), allowing effects of stimulus type, supplementation with vitamin D, and individual contributor. All computations used R software (version 3.3; www.r-project.org).

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