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

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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-1β, 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.
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 2017; [E pub].)

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

The majority of all asthma cases are diagnosed in early childhood, implying that the origin of the disease is in fetal or very early life. Recurrent wheeze, atopy, and lower respiratory tract infections 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 asthma 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.

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 virus and lower mononuclear cell proliferative responses to LPS. 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 responses and lower cord blood serum levels of IL-4, TNF-α, and IFN-γ.

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 neonatal immune cells with 1,25(OH)2D3 has been shown to express the expression of antimicrobial peptides, such as cathelicidin, and to influence innate immune signaling. 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 TH17 cytokine production in vitro while promoting regulatory T-cell phenotypes.

We have previously described upregulation of IL-10 gene expression in CD4 T cells and an increased response to dexamethasone in culture for IL-10 synthesis and cell phenotyping, 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 to 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.

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, 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 to 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 × 10⁶ cells/mL in supplemented RPMI medium either alone or with the addition of LPS (from Escherichia coli 0111:B4m, 0.01 μg/mL; InvivoGen, San Diego, Calif), PPG (peptidoglycan from Staphylococcus aureus, 1.25 μg/mL, InvivoGen), CpG (type C CpG oligonucleotide ODN 2395, 1 μg/mL, InvivoGen), or PHA (lectin from Phaseolus vulgaris, 15 μg/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 × 10⁶/mL in RPMI medium in the presence of plate-bound anti-CD3 (1 μg/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.

Abbreviations used

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>CBMC</td>
<td>Cord blood mononuclear cell</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>1,25(OH)2D3</td>
<td>1,25-Dihydroxyvitamin D3</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>Total 25-hydroxvitamin D (25-hydroxyvitamin D2 plus 25-hydroxyvitamin D3)</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<td>VDAART</td>
<td>Vitamin D Antenatal Asthma Reduction Trial</td>
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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 FACSDiva 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 μL 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−ΔΔCT 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),
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-1β ($P = .094$), 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).

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

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

$mDCs$: Myeloid dendritic cells; $pDCs$: plasmacytoid dendritic cells.

*Lineage = CD3, CD14, CD16, CD19, CD20, and CD56.
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.
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 ($P = 0.03$) 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 ($P = 0.018$, 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...
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.¹⁷,²⁰ 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 comorbidities 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-1β, 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, and 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 later in childhood. In addition, lower expression levels of TLRs on cord blood immune cells has been linked to maternal allergy.³⁵-³⁷ 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 Th17-associated cytokines play a crucial role in pulmonary immune defense to pathogens and in neonatal immunity 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.³⁸ 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 Th17 synthesis 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 asthma³³,³⁴ and could be relevant in the context of early life, when glucocorticoids and vitamin D have been proposed to influence lung development,³³ 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 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.³⁵

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.

We thank the Boston University clinical site of VDAART for their work on this project and in particular the women who participated in this trial. We thank the Antony Nolan Trust staff at King’s College Hospital for provision of cord blood samples for pilot studies. This research was also supported by the
National Institute for Health Research (NIHR) Clinical Research Facility at Guy’s & St Thomas’ NHS Foundation Trust and NIHR Biomedical Research Centre based at Guy’s and St Thomas’ NHS Foundation Trust and King’s College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

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.

REFERENCES


METHODS
VDAART clinical trial
VDAART 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 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 MHiPp9; 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; CD5 peridinin-chlorophyll-protein complex (BD Biosciences), clone SK7; CD14 phycoerythrin (PE; BD Biosciences), clone MHiPp9; 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 complex (BD Biosciences), clone L243; CD45 Pacific blue (AbD Serotec, Oxfordshire, United Kingdom), clone F10-89-4; CD4 eFluor 450 (eBioscience), clone BC96.

For stain combinations containing only cell-surface antibodies, 3 µL of each relevant antibody was added to an aliquot of 1 to 2 million fresh UCB, and 3 µL of 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 housekeeping 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, 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, 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).

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.

REFERENCES