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In infants with sufficient vitamin D status at birth, vitamin D supplementation does not impact immune development

Jonatan Leffler¹ | Cristina Gamez^{1,2} | Anderson P. Jones^{1,2} | Kristina Rueter^{2,3} | James F. Read¹ | Aris Siafarikas^{1,2,4} | Ee-Mun Lim^{5,6} | Paul S. Noakes^{1,2,7} | Susan L. Prescott^{1,2,3} | Philip A. Stumbles^{1,8} | Debra J. Palmer^{1,2} | Deborah H. Strickland¹

¹Telethon Kids Institute, University of Western Australia, Nedlands, WA, Australia

²School of Medicine, The University of Western Australia, Crawley, WA, Australia

³Immunology Department, Perth Children's Hospital, Nedlands, WA, Australia

⁴Department of Endocrinology and Diabetes, Perth Children's Hospital, Nedlands, WA, Australia

⁵Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Nedlands, WA, Australia

⁶Department of Clinical Biochemistry, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands, WA, Australia

⁷School of Medicine, The University of Notre Dame, Fremantle, WA, Australia

⁸College of Science, Health, Engineering and Education, Murdoch University, Murdoch, WA, Australia

Correspondence

Deborah H. Strickland, Telethon Kids Institute, Perth Children's Hospital, 15 Hospital Avenue, Nedlands, WA 6009, Australia.
Email: deb.strickland@telethonkids.org.au

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Abstract

Background: Low vitamin D levels have been associated with allergic diseases. Vitamin D has potent immunomodulatory properties, but the mechanisms remain unclear. We have investigated the effect of oral vitamin D supplementation on circulating immune cell phenotypes in infants.

Method: A double-blinded randomised controlled trial was conducted to investigate the effect of oral vitamin D supplementation (400 IU/d) on eczema and immune development. A subset of 78 infants was included in this analysis. Phenotypic analysis of immune cell subsets was performed using flow cytometry.

Results: Vitamin D supplementation resulted in median 25(OH)D levels of 80.5 vs 59.5 nmol/L in the placebo group at 3 months of age ($P = .002$) and 87.5 vs 77 nmol/L at 6 months of age ($P = .08$). We observed significant changes in immune cell composition from birth (cord blood) to 6 months of age. Vitamin D supplementation did not impact these changes, nor did immune cell composition correlate with plasma 25(OH)D levels. Through exploratory analysis, we identified possible associations with eczema development and increased abundance of naïve CD4⁺ T cells at birth, as well as associations with basophils, iNKT and central memory CD4⁺ T cells, and altered expression patterns of IgE receptor (FcεR1) on monocytes and dendritic cells with eczema at 6 months.

Jonatan Leffler, Cristina Gamez, and Deborah H. Strickland have been Joint primary/senior authors.

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Conclusions: Vitamin D supplementation in infants who were vitamin D sufficient at birth did not affect developmental changes in immune cells during the first 6 months of life. However, immune cell profiles at birth and at 6 months of age were associated with early life eczema.

KEYWORDS

early life, eczema, immune cell profiles, randomised controlled trial, vitamin D supplementation

1 | INTRODUCTION

Vitamin D and serum levels of its metabolites 25(OH)D and 1,25(OH)₂D during early life have been proposed to be associated with the risk of developing allergic diseases, notably as the prevalence of allergic disease has been associated with increasing latitude.¹ Low vitamin D status during the first few years of life has also been reported to increase the risk of eczema,² food allergy,³ wheeze,⁴ allergic rhinitis⁵ and allergen sensitisation.³ The possible involvement of vitamin D in reducing allergic disease risk is further supported by experimental data, suggesting that vitamin D plays a regulatory role in the immune system.⁶ Vitamin D acts through the Vitamin D receptor which is expressed across a range of immune cells including dendritic cells (DC), T cells and B cells. Immune cells such as DC and macrophages also produce 1 α -25(OH) vitamin D3 hydroxylase enabling local production of the active 1,25(OH)₂D from 25(OH)D which may further assist with shaping immune responses.⁷ Experimental and in vitro studies have suggested that vitamin D influences a range of immune cells with relevance to allergic disease,⁶ including modulation of Th1/Th2 responses, IgE production in B cells, induction of regulatory T (Treg) cells and the ability of DC to induce tolerogenic immune responses and reduce expression of the IgE receptor (Fc ϵ RI)⁸; however, how these effects operate in humans remains unclear.

Eczema is one of the earliest manifestations of allergic disease and may affect up to 15%-20% of children.⁹ The pathogenesis of eczema is complex and appears as the result of both genetic and environmental factors leading to a defective skin barrier and a dysregulated immune system.⁹ Some infants with eczema become sensitised to common environmental and/or food allergens during the first year of life and are at a high risk for progressing to food allergy, allergic rhinitis and asthma.¹⁰ Notably, there is evidence of immune dysregulation already at birth in predisposed infants, with signs of attenuated immune responses following stimulation,¹¹ a decreased proportion of Treg cells in cord blood¹² and reduced IL-10 or IFN- γ . This is coupled with increased IL-4 production,¹³ increased proportion of memory (CD45RO⁺) T cells¹⁴ and alterations in circulating DCs with increased levels of Fc ϵ RI expression.¹⁵

To further examine the potential protective effect of 25(OH)D status on allergic disease development via immune modulation, we established a double-blind, randomised controlled trial (RCT) to investigate whether oral vitamin D supplementation over the first 6 months of life could modulate allergen-specific immune responses

Key Message

Low vitamin D levels are associated with development of allergic disease, possibly due to its immune modulatory properties. To assess the impact of vitamin D on immune cell development during infancy, we conducted a double blinded vitamin D supplementation RCT. Our analysis shows that in vitamin D sufficient infants, vitamin D supplementation did not impact the normal development of immune cells during the first 6 months of life, neither did it influence expression of functional immune cell markers. Given that these types of early-life intervention trials are rare, our findings provide important insights into the ongoing debate regarding vitamin D supplementation during infancy.

and reduce the incidence of eczema in infants with high atopic risk, but 25(OH)D-sufficient mothers.¹⁶ Here, we report the analysis of cellular immune profiles in the cord blood mononuclear cells and peripheral blood mononuclear cells at 6 months of age.

2 | METHODS

2.1 | Clinical study design

This analysis was conducted on a subset of 78 infants participating in the previously published infant oral vitamin D3 supplementation (400 IU/d) RCT (Australian New Zealand Clinical Trials Registry ACTRN12612000787886).¹⁶ The infant subset for this analysis was selected from the 195 infants included in the RCT based on the availability of cord blood and infant blood samples collected at 6 months of age (Figure 1). Infant participant characteristics are displayed in Table 1. Written informed consent was obtained before participation. Approval was granted by the Princess Margaret Hospital for Children Human Research Ethics Committee (HREC approval number 1959EP), and the study conforms to the standards of the Declaration of Helsinki.

Additional methods are available in Supplementary material.

FIGURE 1 Study flow of participants. CB, cord blood; CBMCs, cord blood mononuclear cells; PBMCs, peripheral blood mononuclear cells

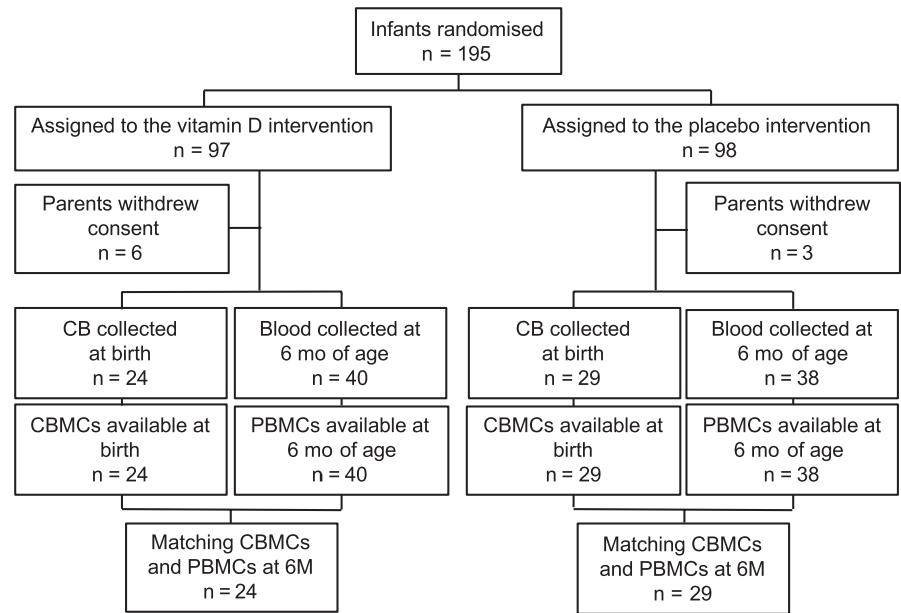


TABLE 1 Infant characteristics

	Vitamin D intervention group	Placebo group	P*
Infants (n)	n = 40	n = 38	
Maternal 25(OH)D level at late gestation, nmol/L, mean, (SD)	75.8 (14.1) ^a	73.6 (12.1)	.467
Birthweight, g, mean (SD)	3412.2 (376.3)	3462.5 (329.0)	.534
Gestational age at birth, weeks, median (IQR)	39.1 (38.4-40.0)	39.4 (38.5-40.2)	.114
Age at randomisation, days, median (IQR)	11.0 (8.0-15.0)	11.5 (9.7-13.0)	.943
Infant male sex, n (%)	20 (50%)	19 (50%)	1.0
Caesarean section, n (%)	12 (30.0%)	13 (34.2%)	.876
Caucasian infant, n (%)	33 (82.5%)	32 (84.2%)	1.0
Maternal history of allergic disease, n (%)	26 (65.0%)	25 (65.8%)	1.0
Paternal history of allergic disease, n (%)	29 (72.5%)	28 (73.7%)	1.0
Sibling history of allergic disease, n (%)	10 (25.0%)	8 (21.1%)	.874
Vitamin D supplementation in pregnancy, n (%)	13 (32.5%)	13 (33.3%)	1.0
Exclusively breastfed at randomisation, n (%)	25 (62.5%)	26 (68.4%)	.756
Currently breastfed at 6 mo, n (%)	28 (70.0%)	31 (81.6%)	.354
Any breastmilk within first 6 mo, n (%)	36 (90.0%)	37 (97.4%)	.387
Any formula within first 6 mo, n (%)	23 (57.5%)	20 (52.6%)	.838

^aData are displayed as mean (standard deviation; SD), median (interquartile range; IQR) or proportion depending on data distribution.

*P-values of statistical difference between experimental groups using either Student's *t* test, Mann-Whitney *U* test or Pearson's chi-square test depending on characteristics of the data.

3 | RESULTS

3.1 | Establishment of developmental trajectory

To assess the impact of vitamin D supplementation on immune development during the first 6 months of life, we established the

developmental trajectories of common immune cell subsets using flow cytometry, two custom-made antibody panels and a combination of FlowSOM and tSNE visualisation (Figure 2A,B). Using CATALYST¹⁷ and *diffcyt*¹⁸ R packages, the developmental changes from birth (cord) to 6 months were assessed using paired analysis. The proportion of cells in clusters deemed significantly different between the two time

points were displayed per major cell subset definition (Figure 2C). As expected, dramatic changes in cell abundances between birth and 6 months of age were observed with over 50% of cells in each cell definition changing abundance between the two time points. Defining immune cell subsets using conventional marker expression (Figure S1A,B), many of the changes occurring from birth to 6 months of age were confirmed. In particular, there were significant increases in B-cell and T-cell populations whereas abundance of natural killer (NK) cells, basophils, monocytes, innate lymphocyte cells (ILC) and invariant natural killer T (iNKT) cells significantly decreased (Figure 2D,E).

3.2 | No impact of vitamin D intervention

We then assessed whether vitamin D supplementation impacted the developmental trajectory of any of the identified immune cell subsets.

As this analysis was performed on a subset of the original RCT participants,¹⁶ we confirmed the impact of vitamin D supplementation on plasma 25(OH)D levels during the first 6 months of life. As maternal 25(OH)D levels of less than 50 nmol/L were exclusion criteria for infant participation in the RCT, the majority of infants were 25(OH)D sufficient at birth and no difference was observed between the groups before supplementation (Figure 3A). At 3 months of age, levels of 25(OH)D were significantly elevated in the intervention group, and at 6 months of age, a trend of increased levels remained compared to the placebo group (Figure 3A). Using the *diffcyt* package, we assessed if vitamin D supplementation significantly impacted cell subset abundance at 6 months of age. In clusters defined as NK cells, 1.5% of cells in this definition significantly differed in abundance between the groups, suggesting a limited impact by the intervention (Figure 3B). Minor effects in cells defined as T cells were also observed (Figure 3B). Using conventional gating, no significant difference in trajectories between

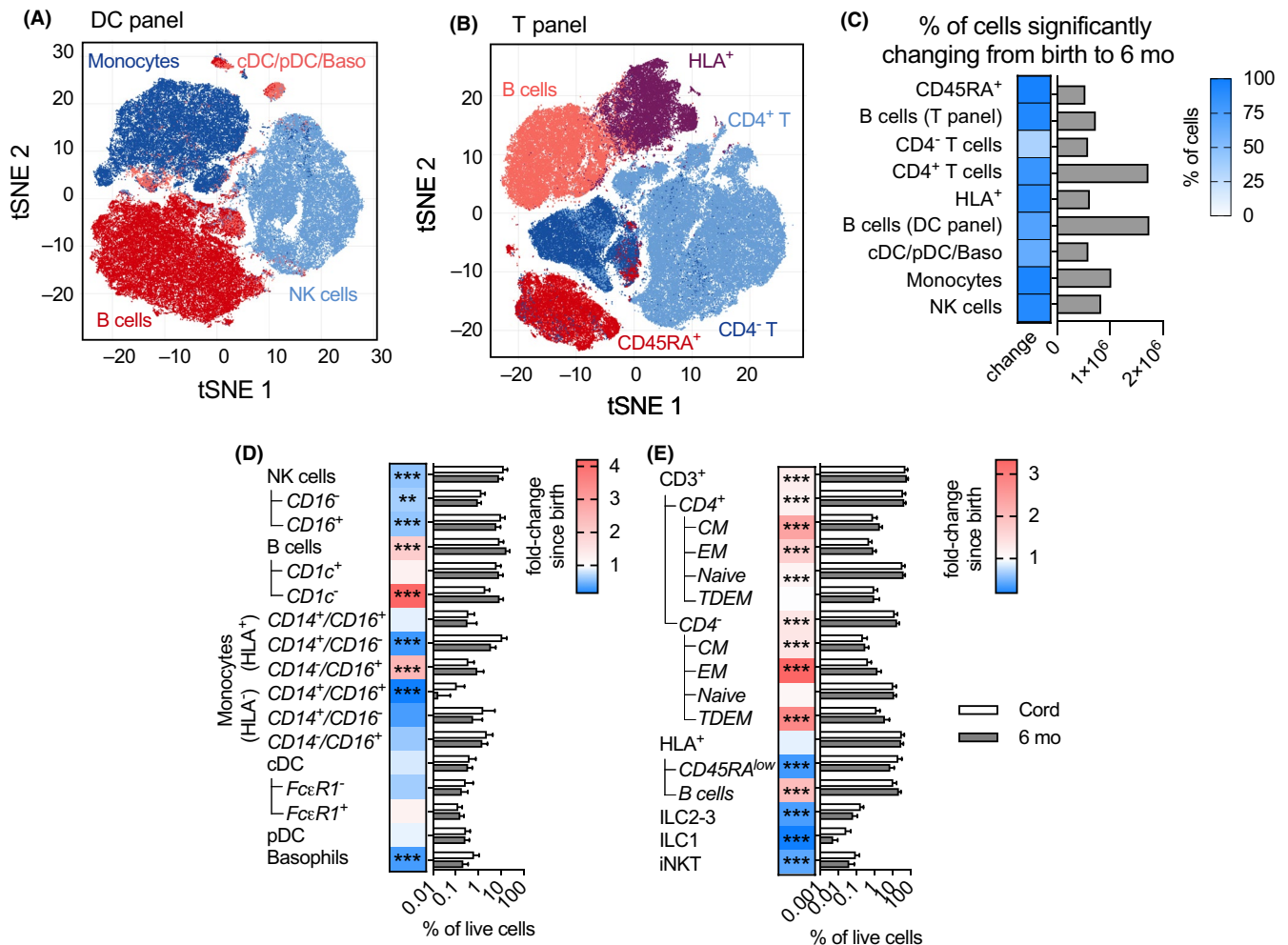


FIGURE 2 (A,B) Immune cell definitions were based on FlowSOM clustering and visualised using tSNE for the DC (A) and T-cell panel (B). (C) Paired comparison of cluster abundance at birth and 6 mo of age. The proportion of cells per each definition that are different between the two time points are displayed. (D,E) Using conventional gating and unpaired analysis, differences between cell subset abundance are compared between birth and 6 mo of age. In A,B, data are visualised from down-sampled files $n_{DC} = 89$, $n_T = 118$. In C, statistical significance is calculated using a paired generalised linear mixed model $n_{DC} = 35$, $n_T = 42$. In D,E, statistical significance of difference in cell subset abundance between the two time points is calculated using multiple *t* test with adjusted *P*-value and displayed as ****P* < .001, ***P* < .01. At birth, $n_{DC} = 46$, $n_T = 47$; at 6 mo, $n_{DC} = 42$, $n_T = 71$

well as a positive correlation with UV exposure at 3 months of age and abundance of effector memory (EM) and central memory (CM) CD4⁺ T cells (Table S4), suggesting that 25(OH)D and UV exposure only have limited impact on the abundance of specific immune cell subset during this period.

3.3 | Immune cell subsets associated with eczema in early infancy

The RCT did not detect a beneficial effect of vitamin D supplementation on eczema development during the first 6 months of life in 25(OH)D-sufficient infants.¹⁶ As an exploratory analysis, we assessed if abundance of specific immune cell subsets was associated with eczema development during the same period. At birth, *diffcyt* analysis revealed that cells defined as CD4⁺ T cells, followed by B cells, contained the largest proportion of cell subsets with significantly different abundance in infants who developed eczema compared to those who did not (Figure 4A). Indeed,

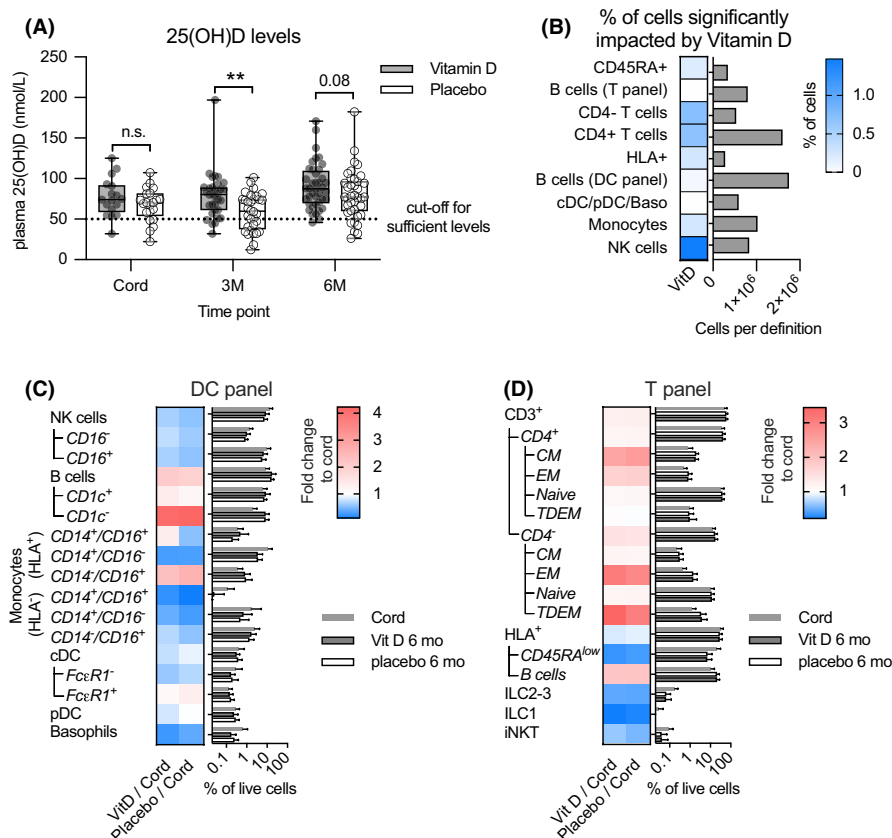


FIGURE 3 (A) 25(OH)D plasma levels at birth, 3 mo and 6 mo of age in the vitamin D intervention or placebo group. (B) Comparison of cluster abundance in the vitamin D intervention group or placebo at 6 mo of age. The proportion of cells that belong to clusters that are significantly different between the two groups are displayed for each cell definition in the two panels. (C,D) Using conventional gating, the ratio of cell subset abundance at 6 mo and at birth was compared between the vitamin D intervention and placebo group. In A, significance of difference was calculated using two-way ANOVA followed by Bonferroni's post hoc test, data are displayed as individual data points, and box/whiskers display median, 25%-75% percentile and range. In B, statistical significance is calculated using a generalised linear mixed model $n_{DC(vit D)} = 18$, $n_{DC(placebo)} = 24$, $n_{T(vit D)} = 38$, $n_{T(placebo)} = 33$. In C,D, statistical significance of difference in cell ratios between the two groups is calculated using multiple t test with adjusted P -value. At birth, $n_{DC} = 46$, $n_T = 47$; at 6 mo, $n_{DC(vit D)} = 18$, $n_{DC(placebo)} = 24$, $n_{T(vit D)} = 38$, $n_{T(placebo)} = 33$

using conventional gating, we observed elevated levels of naïve CD4⁺ T cells in infants who developed eczema compared to those who did not (Figure 4B). In a prospective analysis, there was also a trend ($P = .06$) that infants born in the top quartile of naïve CD4⁺ T cells were more likely to develop eczema compared to the rest of the population (Figure 4C). At 6 months of age, *diffcyt* analysis indicated that cells defined as CD4⁺ and CD4⁺ T cell contained the largest proportion of cell subsets with significantly different abundance in infants with eczema at 6 months of age (Figure 4D). However, conventional gating only confirmed that CM CD4⁺ T cells were significantly elevated in infants with eczema at 6 months of age (Figure 4E). In addition, manual gating also revealed that infants with eczema at 6 months of age displayed lower proportion of basophils (Figure 4F) and iNKT cells (Figure 4G) compared to those without eczema.

We recently reported a strong correlation between expression of FcεR1, IgE sensitisation and allergic asthma in children aged 6-14 years.¹⁹ To assess whether similar associations were observed during the first 6 months of life, we assessed expression of FcεR1 at 6 months of age across the relevant cell subsets (plasmacytoid DC (pDC), conventional DC (cDC), basophils and monocytes) in infants with and without diagnosed eczema. In infants with eczema at 6 months of age, an increased proportion of FcεR1⁺-expressing HLA⁺/CD14⁺/CD16⁺ monocytes (Figure 5A) as well as increased expression of FcεR1 on FcεR1⁺ cDC (Figure 5B) compared to those without eczema was observed, suggesting that the atopic trajectory begins early in life. The vitamin D intervention did not have an impact of FcεR1 expression (data not shown); however, the proportion of FcεR1⁺ cDC out of the total cDC population negatively correlated with 25(OH)D levels in plasma at 6 months (Figure 5C).

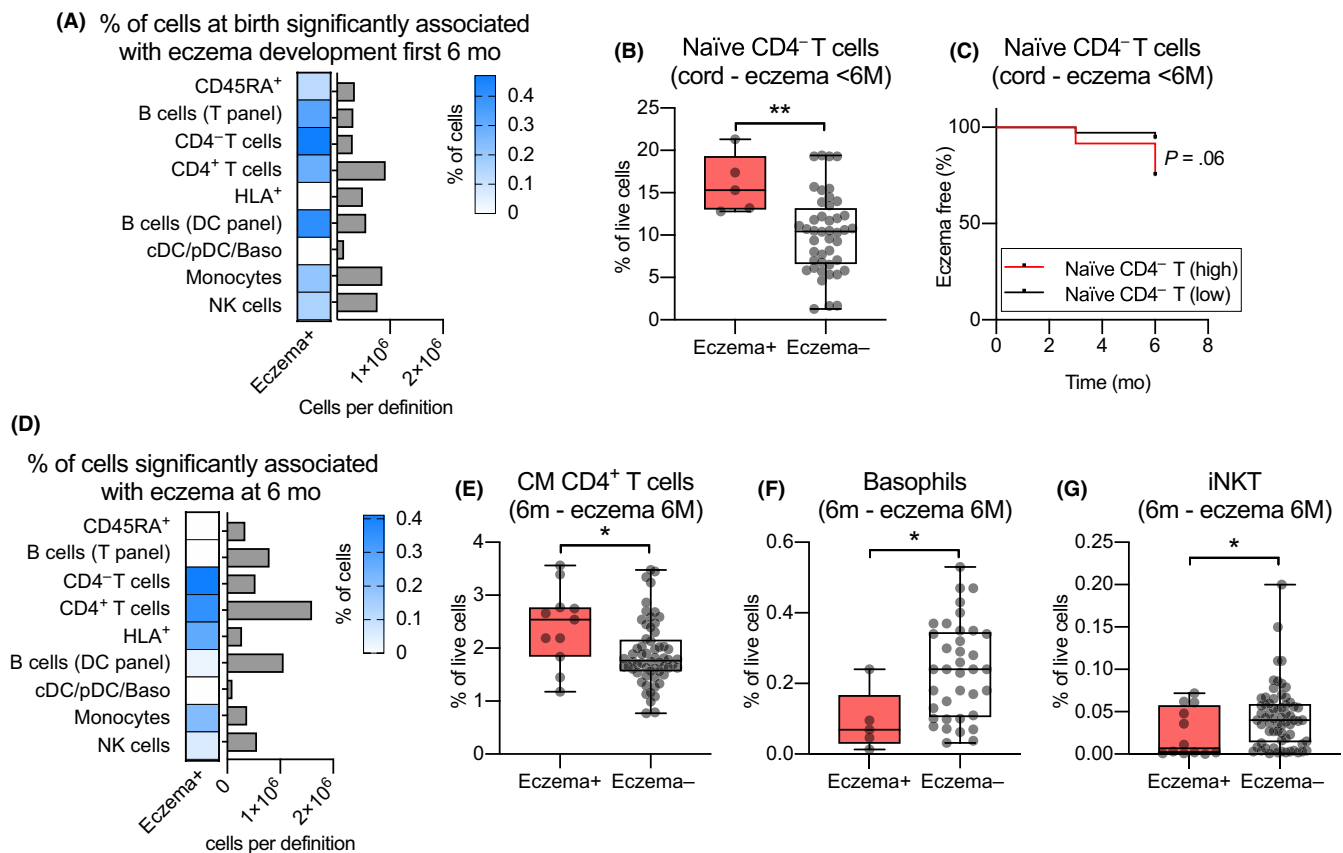


FIGURE 4 (A) Comparison at birth of cluster abundance in children who developed eczema within 6 mo of age compared to children without eczema. The proportion of cells that belong to clusters that are significantly different between the two groups are displayed for each cell definition in the two panels. (B) Using conventional gating, the abundance of naïve CD4⁺ T cells at birth in children who developed eczema within 6 mo of age compared to eczema-free children. (C) Eczema-free time in children born with the highest quartile of naïve CD4⁺ T cells compared to the remainder of the group. (D) Comparison at 6 mo of age of cluster abundance in children with eczema compared to children without eczema at 6 mo. The proportion of cells that belong to clusters that are significantly different between the two groups are displayed for each cell definition in the two panels. (E-G) Using conventional gating, the abundance of central memory CD4⁺ T cells (E), basophils (F) and iNKT cells (G) in children with or without eczema at 6 mo of age. In A and D, statistical significance is calculated using a generalised linear mixed model at birth $n_{DC(eczema+ <6M)} = 6$, $n_{T(eczema+ <6M)} = 5$, $n_{DC(eczema- <6M)} = 40$, $n_{T(eczema- <6M)} = 42$ and at 6 mo $n_{DC(eczema+ 6M)} = 5$, $n_{DC(eczema- 6M)} = 37$, $n_{T(eczema+ 6M)} = 11$, $n_{T(eczema- 6M)} = 60$. In B and E, F, significance of difference was calculated using Student's *t* test, and in G, significance of difference was calculated using Mann-Whitney *U* test. Data are displayed as individual data points, and box/whiskers display median, 25%-75% percentile and range. In C, statistical significance of difference between the two groups was calculated using log-rank test

4 | DISCUSSION

In the present study, we investigated the immunological impact of oral vitamin D supplementation from birth to 6 months of age. We observed dramatic changes in immune cell abundance during the first 6 months of infancy. By comparing the intervention versus placebo group, none of the changes in immune cell abundance or expression of functional markers appeared to be impacted by oral vitamin D supplementation, neither did the abundance of defined immune cell subsets correlate with 25(OH)D status at 6 months of age. We did, however, observe immune cell profiles that were associated with manifestations of eczema at 6 months of age such as increased CD4⁺ CM T cells and increased expression of FcεR1.

We used paired analyses where possible and a combination of algorithm-based and conventional gating strategies. Concordance between the two strategies was good in the majority of analysis; however, some discrepancies were noted. Detection and robust clustering of rare subsets due to intra assay variability and use of a subset from original clustering in the analysis may have contributed to this. Nevertheless, this type of analysis was a valuable tool that guided our gating strategy and analysis.

At birth, a high proportion of naïve CD4⁺ T cells (presumably CD8⁺ T cells, although we did not include CD8 in our panel) appeared to increase the risk of eczema development during the first 6 months of life. This is in contrast to previous studies that observed an increased proportion of CD4⁺/CD45RA⁺ T cells in cord blood of children who later developed atopic dermatitis.²⁰ We acknowledge that these findings are based on a limited number of samples and further studies are likely needed to clarify this association.

At 6 months of age, infants with eczema displayed increased proportion of CM CD4⁺ T cells which is in line with what has previously

been observed together with a skewing towards Th2-type immunity.²¹ In addition, infants with eczema displayed lower levels of basophils and iNKT cells, potentially suggesting decreased generation or increased tissue migration. During active disease, we have previously observed that high levels of circulating basophils are associated with recurrent asthma exacerbations in children.²² The role of iNKT cells in atopic disease is more controversial where low abundance previously has been observed in children with food allergy.²³ However, in adults with eczema, the abundance of iNKT cells may be higher compared to health controls.²⁴

Expression of FcεR1 is closely associated with manifestations of atopic disease in school-aged children and adults,^{15,19} possibly due to its immune modulatory function on antigen-presenting cells.²⁵ However, the impact of FcεR1 expression during the first year of life, particularly before 12 months of age, is less clear.²⁶ We observed that the proportion of FcεR1-expressing HLA⁺/CD14⁺/CD16⁺ monocytes was increased in children with eczema and, in addition, the level of expression was also increased on cDC. This suggests that FcεR1 expression already differs at 6 months of age in children on a trajectory towards atopic manifestations. However, given the variability in expression across individuals, larger sample sizes and longer follow-ups are likely needed to study these trajectories in further detail. Recently, vitamin D was shown to decrease FcεR1 expression on cDCs.⁸ Our results support this finding as we observed a negative association between 25(OH)D levels and proportion of FcεR1⁺ cells in the cDC population, although cDC constitute several subsets where only cDC2 express FcεR1.²⁷ Hence, our findings may relate to an association of 25(OH)D levels with the balance of cDC1/cDC2 rather than with expression of FcεR1 itself.

The impact of vitamin D on atopic outcomes, such as eczema, is currently debated. In this trial, vitamin D supplement was unable

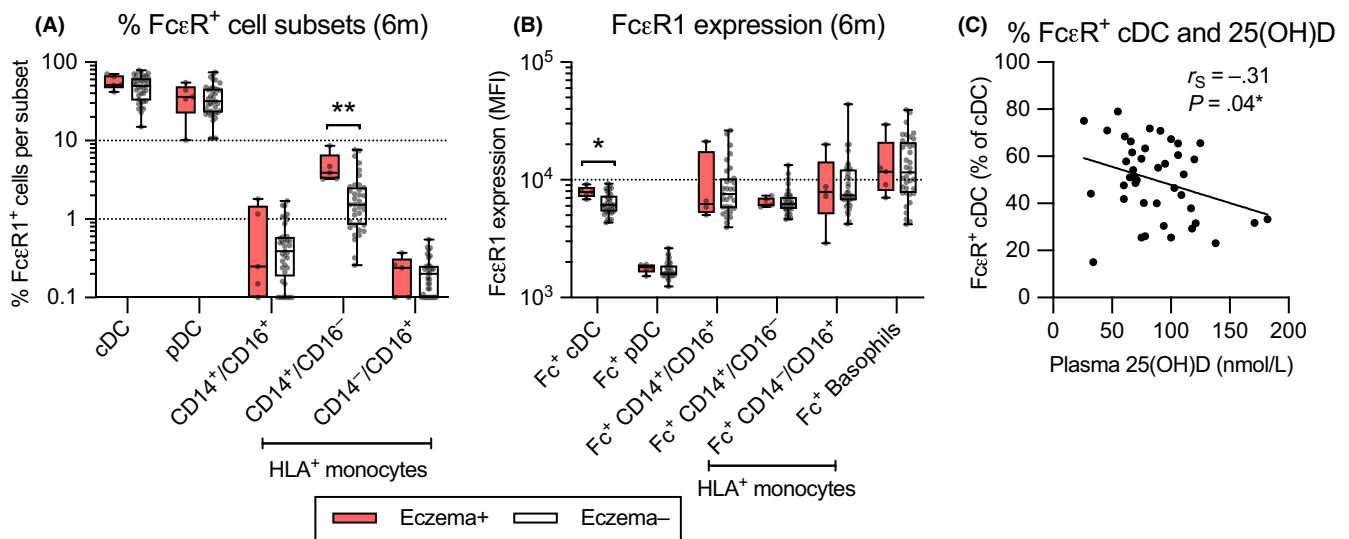


FIGURE 5 (A,B) Using conventional gating, the proportion of FcεR1-expressing DC and monocytes (A) and levels of FcεR1 expression on FcεR1⁺ cells (B) in children with or without eczema at 6 mo of age. (C) Correlation with plasma 25(OH)D levels at 6 mo of age and proportion of FcεR1-expressing cDC. In A,B, statistical significance is calculated using multiple t tests with adjusted *P*-values and displayed as individual data points, and box/whiskers display median, 25%-75% percentile and range, $n_{\text{Eczema}^+} = 5$, $n_{\text{Eczema}^-} = 37$. In C, statistical significance of correlation was assessed using Pearson's correlation test, $n = 42$

to prevent development of eczema during the first 6 months of life¹⁶ which is in accordance with previous attempts to prevent allergy development in infants through maternal vitamin D supplementation during pregnancy, although insufficient power may have been an issue.²⁸ It is important to note that based on maternal 25(OH)D levels in late pregnancy, no 25(OH)D-deficient mothers were included; thus, this study only focused on infants with sufficient 25(OH)D levels at birth. Aligned with the clinical outcomes of the study,¹⁶ we did not observe any impact of vitamin D supplementation on the abundance or development of cell subsets, or expression of their functional markers. Further, we did not observe any strong correlations between immune cell subset abundance and 25(OH)D plasma levels. A possible reason may be that the effect sizes were too small to be detected, the range of participant 25(OH)D levels were rather limited, local tissue conversion of vitamin D into 25(OH)D was not reflected in plasma 25(OH)D levels, or the duration/dose of the intervention was insufficient.

In summary, our data suggest that oral vitamin D supplementation to infants who were 25(OH)D sufficient at birth does not impact the developmental trajectory of the circulating cellular immune system. In addition, our exploratory analysis identifies possible associations with eczema development and increased levels of naïve CD4⁺ T cells at birth, as well as associations with basophils, iNKT and CM CD4⁺ T cells, and altered FcεR1 expression patterns with eczema at 6 months.

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CONFLICT OF INTEREST

The authors declare none.

AUTHOR CONTRIBUTION

Jonatan Leffler: Data curation (equal); Formal analysis (equal); Visualization (equal); Writing-original draft (equal). **Cristina Gamez:** Data curation (equal); Formal analysis (equal); Visualization (equal); Writing-original draft (equal). **Anderson P Jones:** Formal analysis (equal); Investigation (equal); Writing-review & editing (equal). **Kristina Rueter:** Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Investigation (equal); Project administration (equal); Writing-review & editing (equal). **James F Read:** Data curation (equal); Formal analysis (equal); Visualization (equal); Writing-review & editing (equal). **Aris Siafarikas:** Investigation (equal); Methodology (equal); Writing-review & editing (equal). **Ee-Mun Lim:** Investigation (equal); Methodology (equal); Writing-review & editing (equal). **Paul S Noakes:** Investigation (equal); Methodology (equal); Writing-review & editing (equal). **Susan L Prescott:** Investigation (equal); Methodology (equal); Writing-review & editing (equal). **Philip A Stumbles:** Investigation (equal); Methodology (equal); Writing-review & editing (equal). **Debra J**

Palmer: Conceptualization (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Supervision (equal); Writing-review & editing (equal).

Deborah H Strickland: Conceptualization (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Supervision (equal); Writing-review & editing (equal).

ORCID

Jonatan Leffler  <https://orcid.org/0000-0001-5674-8462>

REFERENCES

1. Krstic G. Asthma prevalence associated with geographical latitude and regional insolation in the United States of America and Australia. *PLoS ONE*. 2011;6(4):e18492.
2. Palmer DJ, Sullivan TR, Skeaff CM, Smithers LG, Makrides M, Team DOAF-U. Higher cord blood 25-hydroxyvitamin D concentrations reduce the risk of early childhood eczema: in children with a family history of allergic disease. *World Allergy Organ J*. 2015;8(1):28.
3. Allen KJ, Koplin JJ, Ponsonby AL, et al. Vitamin D insufficiency is associated with challenge-proven food allergy in infants. *J Allergy Clin Immunol*. 2013;131(4):1109-1116, 1116 e1101-1106.
4. Baiz N, Dargent-Molina P, Wark JD, Souberbielle JC, Annesi-Maesano I, Group EM-CCS. Cord serum 25-hydroxyvitamin D and risk of early childhood transient wheezing and atopic dermatitis. *J Allergy Clin Immunol*. 2014;133(1):147-153.
5. Dogru M, Suleyman A. Serum 25-hydroxyvitamin D3 levels in children with allergic or nonallergic rhinitis. *Int J Pediatr Otorhinolaryngol*. 2016;80:39-42.
6. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. *Nat Rev Immunol*. 2008;8(9):685-698.
7. Kreutz M, Andreesen R, Krause SW, Szabo A, Ritz E, Reichel H. 1,25-Dihydroxyvitamin D3 production and vitamin D3 receptor expression are developmentally regulated during differentiation of human monocytes into macrophages. *Blood*. 1993;82(4):1300-1307.
8. Cristi F, Perez-Mateluna G, Vera-Kellet C, et al. Vitamin D modulates the allergic phenotype of dendritic cells in children with atopic dermatitis. *Exp Dermatol*. 2019;28(3):308-311.
9. Weidinger S, Novak N. Atopic dermatitis. *Lancet*. 2016;387(10023):1109-1122.
10. Bantz SK, Zhu Z, Zheng T. The atopic march: progression from atopic dermatitis to allergic rhinitis and asthma. *J Clin Cell Immunol*. 2014;5(2). <https://doi.org/10.4172/2155-9899.1000202>
11. Macaubas C, de Klerk NH, Holt BJ, et al. Association between antenatal cytokine production and the development of atopy and asthma at age 6 years. *Lancet*. 2003;362(9391):1192-1197.
12. Schaub B, Liu J, Hoppler S, et al. Impairment of T-regulatory cells in cord blood of atopic mothers. *J Allergy Clin Immunol*. 2008;121(6):1491-1499, 1499 e1491-1413.
13. Biedermann T, Skabytska Y, Kaesler S, Volz T. Regulation of T cell immunity in atopic dermatitis by microbes: the yin and yang of cutaneous inflammation. *Front Immunol*. 2015;6:353.
14. Esaki H, Czarnowicki T, Gonzalez J, et al. Accelerated T-cell activation and differentiation of polar subsets characterizes early atopic dermatitis development. *J Allergy Clin Immunol*. 2016;138(5):1473-1477 e1475.
15. Stary G, Bangert C, Stingl G, Kopp T. Dendritic cells in atopic dermatitis: expression of FcεRI on two distinct inflammation-associated subsets. *Int Arch Allergy Immunol*. 2005;138(4):278-290.
16. Rueter K, Jones AP, Siafarikas A, et al. Direct infant UV light exposure is associated with eczema and immune development. *J Allergy Clin Immunol*. 2019;143(3):1012-1020 e1012.

17. Chevrier S, Crowell HL, Zanotelli VRT, Engler S, Robinson MD, Bodenmiller B. Compensation of signal spillover in suspension and imaging mass cytometry. *Cell Syst*. 2018;6(5):612-620 e615.
18. Weber LM, Nowicka M, Soneson C, Robinson MD. diffcyt: Differential discovery in high-dimensional cytometry via high-resolution clustering. *Commun Biol*. 2019;2(1):183.
19. Leffler J, Read JF, Jones AC, et al. Progressive increase of FcepsilonRI expression across several PBMC subsets is associated with atopy and atopic asthma within school-aged children. *Pediatr Allergy Immunol*. 2019;30(6):646-653.
20. Hagendorens MM, Ebo DG, Bridts CH, Van de Water L, De Clerck LS, Stevens WJ. Prenatal exposure to house dust mite allergen (Der p 1), cord blood T cell phenotype and cytokine production and atopic dermatitis during the first year of life. *Pediatr Allergy Immunol*. 2004;15(4):308-315.
21. Czarnowicki T, Esaki H, Gonzalez J, et al. pediatric atopic dermatitis shows only a cutaneous lymphocyte antigen (CLA)(+) TH2/TH1 cell imbalance, whereas adults acquire CLA(+) TH22/TC22 cell subsets. *J Allergy Clin Immunol*. 2015;136(4):941-951 e943.
22. Leffler J, Jones AC, Hollams EM, et al. Basophil counts in PBMC populations during childhood acute wheeze/asthma are associated with future exacerbations. *J Allergy Clin Immunol*. 2018;142(5):1639-1641 e1635.
23. Jyonouchi S, Abraham V, Orange JS, et al. Invariant natural killer T cells from children with versus without food allergy exhibit differential responsiveness to milk-derived sphingomyelin. *J Allergy Clin Immunol*. 2011;128(1):102-109 e113.
24. Wu WH, Park CO, Oh SH, et al. Thymic stromal lymphopoietin-activated invariant natural killer T cells trigger an innate allergic immune response in atopic dermatitis. *J Allergy Clin Immunol*. 2010;126(2):290-299, e291-294.
25. Shin JS, Greer AM. The role of FcepsilonRI expressed in dendritic cells and monocytes. *Cell Mol Life Sci*. 2015;72(12):2349-2360.
26. Vasudev M, Cheung DS, Pincsak H, et al. Expression of high-affinity IgE receptor on human peripheral blood dendritic cells in children. *PLoS ONE*. 2012;7(2):e32556.
27. Berings M, Gevaert P, De Ruyck N, et al. FcepsilonRI expression and IgE binding by dendritic cells and basophils in allergic rhinitis and upon allergen immunotherapy. *Clin Exp Allergy*. 2018;48(8):970-980.
28. Litonjua AA, Carey VJ, Laranjo N, et al. Effect of prenatal supplementation with vitamin D on asthma or recurrent wheezing in offspring by age 3 years: the VDAART randomized clinical trial. *JAMA*. 2016;315(4):362-370.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supplementary material

In infants with sufficient vitamin D status at birth, vitamin D supplementation does not impact immune development.

Jonatan Leffler, PhD^{1,*}, Cristina Gamez, PhD^{1,2,*}, Anderson P. Jones, PhD^{1,2}, Kristina Rueter, MD^{2,3}, James F. Read, BSc¹, Aris Siafarikas, MD^{1,2,4}, Ee-Mun Lim, MD, PhD^{5,6}, Paul S. Noakes, PhD^{1,2,7}, Susan L. Prescott, MD, PhD^{1,2,3}, Philip A. Stumbles, PhD^{1,8}, Debra J. Palmer, PhD^{1,2,*} and Deborah H. Strickland, PhD^{1,*}

* Joint primary/senior authors

¹ Telethon Kids Institute, University of Western Australia, 15 Hospital Ave, Nedlands, Western Australia, 6009, Australia.

² School of Medicine, The University of Western Australia, 35 Stirling Highway, Crawley, Western Australia, 6009, Australia.

³ Immunology Department, Perth Children's Hospital, 15 Hospital Ave, Nedlands, Western Australia, 6009, Australia.

⁴ Department of Endocrinology and Diabetes, Perth Children's Hospital, 15 Hospital Ave, Nedlands, Western Australia, 6009, Australia.

⁵ Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Nedlands, Western Australia, 6009, Australia.

⁶ Department of Clinical Biochemistry, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands, Western Australia, 6009, Australia.

⁷ School of Medicine, The University of Notre Dame, Fremantle, Western Australia, 6160, Australia

⁸ College of Science, Health, Engineering and Education, Murdoch University, Murdoch, Western Australia, 6150, Australia

METHODS

Blood collection

For processing of cord blood mononuclear cells (CBMC), blood was collected from the umbilical cord at birth by venepuncture of the umbilical vessels into Roswell Park Memorial Institute medium (RPMI-1640) (Life Technologies, Grand Island, NY, USA) supplemented with 10 IU/ml of heparin. Samples were transported at room temperature and processed within 12 hours of collection. Infant peripheral blood samples were collected at 6 months of age by venepuncture into lithium heparin tubes (Vacurette, Greiner Bio-One GmbH, Kremsmünster, Austria) and processed immediately after the clinic visit. Plasma and peripheral blood mononuclear cells (PBMC) and CBMC were isolated following separation of blood using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Plasma was stored at -20 °C and cells were cryogenically preserved in RPMI media supplemented with 43.5% heat-inactivated fetal calf serum (FCS) (Life Technologies, Grand Island, NY, USA) and 7.5% dimethylsulphate in liquid N₂.

Measurement of 25(OH)D levels

25(OH)D levels were measured in plasma by a competitive chemiluminescent immunoassay, automated on the Abbott Architectⁱ2000 (Abbott Laboratories, Abbott Park [operated by PathWest Laboratory Medicine, Nedlands, Western Australia]) using a 2-step incubation process with human serum calibrators. The Abbott Architectⁱ 2000 is accredited by the National Association of Testing Authorities for measurement of 25(OH)D levels. Here we have defined vitamin D deficiency as a 25(OH)D <50 nmol/L¹.

Flow cytometry

For phenotypic analysis of cell subsets, cryopreserved CBMC and PBMC samples were thawed as previously described². For staining, 2×10^6 CBMC/PBMC were incubated with viability dye, Zombie NIR (BioLegend, San Diego, CA, USA) for 15 minutes followed by incubation of 50 µg/ml human immunoglobulins (BD Biosciences, Australia) for 10 minutes to block nonspecific antibody binding by Fc receptors. After washing in PBS supplemented with 4% heat-inactivated FCS, (FACS buffer) cells were incubated for 30 minutes on ice with 2 separate multi-fluorochrome panels designed for analysis of either T lymphocyte or myeloid/DC subsets (Table S1 and S2). Following incubation, cells in the T cell panel, were washed, incubated with Streptavidin-AF700 (BD) for 20 minutes, washed and fix-permeabilised in the Transcription Factor Buffer Set (BD) according to manufacturer's instructions and stained for intracellular markers. For the DC panel, cells were washed in FACS buffer. Cells from both panels were subsequently resuspended in FACS buffer and analysed on a LSRFortessa (BD). Flow samples were analysed using FlowJo v10.1 software (BD). For analysis in R, compensated and down-sampled files, 50,000 events per sample, were exported from FlowJo and imported into R 3.6.1 using *FlowCore*³ and visualised using the *CATALYST*⁴. Differential abundances were assessed using the *diffcyt* package⁵.

Statistical Analysis

The study was powered to detect differences in allergen cytokine response in vitro. For flow cytometry data, we observed a mean SD of 60% allowing detection of a 35% difference in mean cell abundance between two experimental groups. The statistical significance of difference between two groups was calculated using Student's t-test or Mann-Whitney's test depending on distribution values; for multiple comparisons, Student's t-test was used and the p-value was adjusted using the Holm-Sidak method; for comparison of multiple experimental groups, Two-way ANOVA followed by Bonnferroni's post test was used; and for comparison of categorical data between groups, Pearson's χ^2 test was used; for survival analysis, log-rank test was used. For comparison of differential abundance of clusters a generalised linear mixed model was used through the *diffcyt* R-package. Statistical analysis was performed using R 3.6.1 (R-project.org) or Prism 8.02 (GraphPad Software, San Diego, CA).

FIGURES

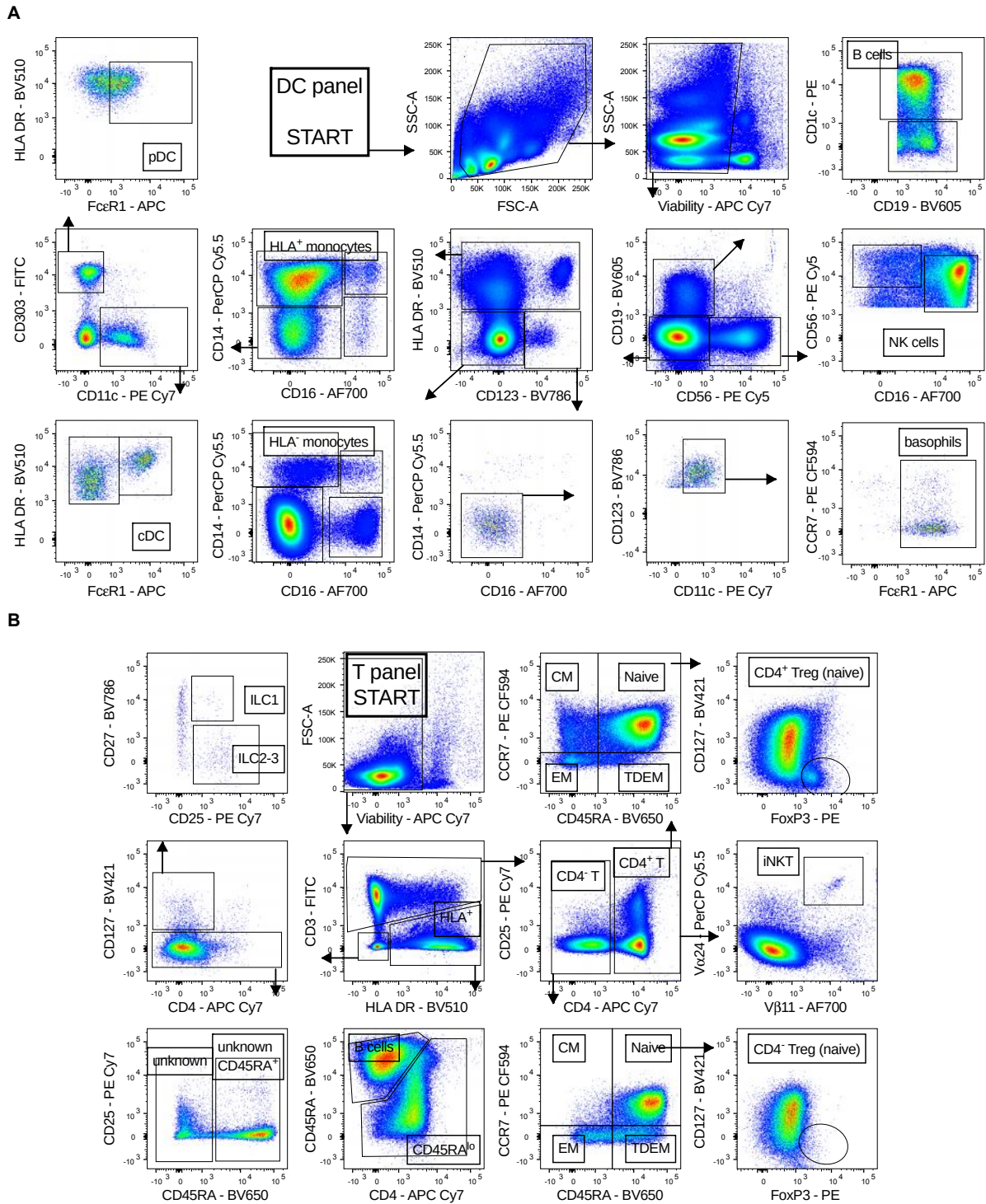


Figure S1

A-B Gating strategy for the DC (A) and T cell (B) flow cytometry panel. Data is displayed from a representative individual from $n_{\text{DC panel}} = 88$ and $n_{\text{T panel}} = 118$.

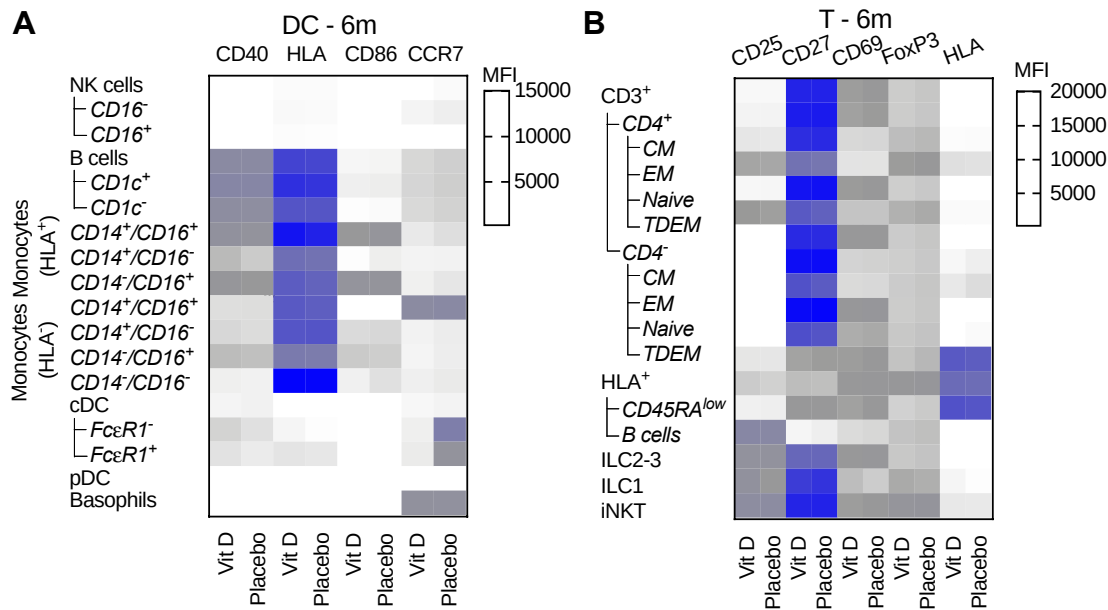


Figure S2

A-B Expression of surface markers in the DC (**A**) or T cell (**B**) flow cytometry panel that are associated with the function of immune cell subsets at 6 months of age in the vitamin D intervention and the placebo group. Statistical significance of difference in marker expression level between the two groups is calculated using multiple t-test with adjusted p-value; $n_{DC(vit\ D)} = 18$, $n_{DC(placebo)} = 24$, $n_{T(vit\ D)} = 38$, $n_{T(placebo)} = 33$. No significant difference were observed.

TABLES

Table S1

Antibody panel with focus on T cell lymphocytes (T panel)

Antigen	Fluorochrome	Clone	Company
Zombie NIR	APC-Cy7		BioLegend
CD3	FITC	UCHT1	BD
CD4	APC-H7	RPA-T4	BD
CD25	PE-Cy7	M-A251	BD
CD27	BV786	L128	BD
CD45RA	BV650	HI100	BD
CD69	BV605	FN50	BD
CD127	BV421	HIL-7R-M21	BD
CCR7	PE-CF594	150503	BD
HLA-DR	BV510	G46-6	BD
TCR-V α 24	PerCP-Cy5.5	C15	Beckman Coulter
TCR-V β 11	Biotin/Strep AF700	C21	Beckman Coulter
Intracellular			
FoxP3	PE	259D/C7	BD

Table S2

Antibody panel with focus on myeloid/DC (DC panel)

Antigen	Fluorochrome	Clone	Company
Zombie NIR	APC-Cy7		BioLegend
CD16	AF700	3G8	BD
CD40	BV421	5C3	BD
HLA-DR	BV510	G46-6	BD
CD19	BV605	SJ25C1	BD
CD86	BV650	2331	BD
CD141	BV711	1A4	BD
CD123	BV786	9F5	BD
CD303	FITC	AC144	Miltenyi Biotec
CD1c	PE	AD5-8E7	Miltenyi Biotec
CCR7	PE-CF594	150503	BD
CD56	PE-Cy5	B159	BD
CD11c	PE-Cy7	B-ly6	BD
CD14	PerCP Cy5.5	Møp9	BD
FcεR1	APC	AER-37	eBioscience

Table S3

Correlations with serum 25(OH)D levels at 3 and 6 months of age and abundance of cell subset at 6 months of age.

Cell type	25(OH)D levels at 3 months			25(OH)D levels at 6 months		
	r^2	p (ANOVA)	Direction (if $p < 0.2$)	r^2	p (ANOVA)	Direction (if $p < 0.2$)
DC panel						
NK cells	0.1	0.05*	Positive	0.002	0.77	
16^- NK	0.05	0.18		0.001	0.83	
16^+ NK	0.1	0.06	Positive	<0.001	0.99	
B cells	<0.001	0.88		<0.001	0.96	
$1c^+$ B	0.07	0.14	Positive	0.003	0.73	
$1c^-$ B	0.09	0.09	Negative	0.002	0.80	
HLA ⁺ Mono						
$14^+/16^+$	0.13	0.04*	Positive	0.006	0.64	
$14^+/16^-$	0.07	0.14	Positive	0.02	0.44	
$14/16^+$	0.07	0.13	Positive	0.001	0.81	
HLA ⁻ Mono						
$14^+/16^+$	0.11	0.05	Positive	<0.001	0.98	
$14^+/16^-$	0.05	0.22		0.02	0.37	
$14/16^+$	0.06	0.16	Positive	0.06	0.13	Positive
cDC	0.002	0.78		0.009	0.54	
Fc^- cDC	0.007	0.62		0.01	0.54	
Fc^+ cDC	0.007	0.63		0.004	0.7	
pDC	<0.001	0.88		0.05	0.16	Negative
Basophils	<0.001	0.92		0.002	0.76	
T cell panel						
CD3 ⁺	0.003	0.67		0.01	0.39	
CD4 ⁺ T	0.006	0.57		0.04	0.08	Negative
CM	0.02	0.33		0.003	0.68	
EM	0.05	0.11	Negative	0.005	0.57	
Naive	0.001	0.81		0.04	0.12	Negative
TDEM	0.07	0.05*	Negative	0.03	0.18	Negative
CD4 ⁻ T	<0.001	0.86		0.03	0.16	Positive
CM	0.002	0.72		0.002	0.68	
EM	0.003	0.66		0.03	0.17	Positive
Naive	0.02	0.28		0.008	0.46	
TDEM	0.01	0.39		0.02	0.25	
HLA ⁺	0.004	0.62		0.002	0.70	
CD45RA ^{low}	<0.001	0.99		0.01	0.39	
B cells (T)	0.01	0.45		<0.001	0.93	
ILC2-3	<0.001	0.9		0.04	0.09	Positive
ILC1	<0.001	0.86		0.02	0.24	
iNKT	0.02	0.26		0.01	0.32	

Table S4

Correlations with UV exposure at 3 months of age and abundance of cell subset at 6 months of age.

	UV exposure at 3 months		
Cell type	r^2	p (ANOVA)	Direction (if $p < 0.2$)
DC panel			
NK cells	0.007	0.7	
16^- NK	<0.001	0.96	
16^+ NK	<0.001	0.88	
B cells	0.003	0.77	
$1c^+$ B	0.05	0.3	
$1c^-$ B	0.01	0.63	
HLA ⁺ Mono			
$14^+/16^+$	<0.001	0.92	
$14^+/16^-$	0.04	0.32	
$14^-/16^+$	0.03	0.4	
HLA ⁻ Mono			
$14^+/16^+$	0.002	0.84	
$14^+/16^-$	0.005	0.75	
$14^-/16^+$	0.006	0.72	
cDC	0.03	0.38	
Fc^- cDC	0.09	0.16	Positive
Fc^+ cDC	0.07	0.19	Negative
pDC	0.1	0.13	Negative
Basophils	0.02	0.49	
T cell panel			
CD3 ⁺	0.02	0.4	
$CD4^+$ T	0.03	0.29	
CM	0.02	0.45	
EM	0.01	0.5	
Naive	0.04	0.25	
TDEM	0.03	0.34	
$CD4^-$ T	0.003	0.75	
CM	0.13	0.03*	Positive
EM	0.14	0.03*	Positive
Naive	0.01	0.55	
TDEM	0.01	0.55	
HLA ⁺	0.02	0.45	
$CD45RA^{low}$	0.04	0.24	
B cells (T)	<0.001	0.86	
ILC2-3	<0.001	0.98	
ILC1	<0.001	0.99	
iNKT	0.02	0.35	

REFERENCES

1. Holick MF, Binkley NC, Bischoff-Ferrari HA, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab.* 2011;96(7):1911-1930.
2. Leffler J, Read JF, Jones AC, et al. Progressive increase of FcepsilonRI expression across several PBMC subsets is associated with atopy and atopic asthma within school-aged children. *Pediatr Allergy Immunol.* 2019;30(6):646-653.
3. Hahne F, LeMeur N, Brinkman RR, et al. flowCore: a Bioconductor package for high throughput flow cytometry. *BMC Bioinformatics.* 2009;10:106.
4. Chevrier S, Crowell HL, Zanutelli VRT, Engler S, Robinson MD, Bodenmiller B. Compensation of Signal Spillover in Suspension and Imaging Mass Cytometry. *Cell Syst.* 2018;6(5):612-620 e615.
5. Weber LM, Nowicka M, Soneson C, Robinson MD. diffcyt: Differential discovery in high-dimensional cytometry via high-resolution clustering. *Communications Biology.* 2019;2(1).