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Vitamin D insufficiency is associated with reduced regulatory T cell frequency in food-allergic infants

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To the Editor,

The influence of vitamin D on human health is strongly associated with tolerogenic immune function, skewing the immune response toward a regulatory phenotype. Ecological and epidemiological studies have resulted in a proposed link between reduced levels of the most abundant circulating form of vitamin D, 25-hydroxyvitamin D (25(OH)D), and the development of food allergy in children.[1] We have also shown that infants with vitamin D insufficiency (≤ 50nmol/L) were 11 times more likely to have a peanut allergy and 3 times more likely to have an egg allergy relative to infants with sufficient vitamin D levels.[2] Interestingly, in the same cohort, 25(OH)D levels positively correlated with tolerogenic immune responses in 4-year-old children who had naturally outgrown their food allergy.[3] There is no single accepted mechanism for the proposed association between vitamin D insufficiency and food allergy.[4] Rather, a combination of several mechanisms is hypothesized

to be involved, including the potential to modulate immune cell proportions or function, including that of regulatory T cells.[4]

In this study, we explored the relationship between early life serum 25(OH)D concentration and the circulating immune profile of infants with challenge-confirmed IgE-mediated peanut and/or egg allergies. Immune profiling was performed by mass cytometric analysis of peripheral blood mononuclear cells (PBMCs) obtained from (n = 18) 1-year-old food-allergic infants in the HealthNuts cohort (Table 1).[5,6] Serum 25(OH)D levels were measured by liquid chromatography tandem mass spectrometry as described.[2] Half (n = 9) of the infants had 25(OH)D levels (51.7 - 110.69 nmol/L) above the sufficiency threshold of 50 nmol/L, whereas the other half had insufficient levels (16.84 - 49.79 nmol/L). Allergies were either to peanuts (n = 2), eggs (n = 6), or both (n = 10).

Mass cytometry data were interpreted with unsupervised analysis, primarily using the FlowSOM algorithm and confirmed with the RPhenograph algorithm. Manual gating was used as further confirmation of the computational results. Cluster frequencies were compared between the infants with a linear mixed model using both discrete and continuous 25(OH)D levels. See supplemental data for the detailed methodology.

FlowSOM clustering analysis identified 11 cell clusters (Figure 1A). Based on the expression profiles, 10 clusters were classified as distinct cell types (Figure 1B). One cluster was negative for all markers and was denoted as undefined. Frequencies of the clusters for the 18 individual infants are presented as a stacked bar graph (Figure S1). Amongst all identified cell types, only the circulating regulatory CD4 T cells (Tregs) showed a statistically significant difference in frequency between infants with sufficient (median = 2.55% of live cells, range = 1.84-3.32%) and insufficient (median = 1.86%, range = 0.79-2.81%) vitamin D (Figure 1C, p = 0.0013, FDR-adjusted p = 0.0138). This increased proportion of Tregs in infants with higher levels of 25(OH)D compared to those with lower 25(OH)D measurements was further established when examining 25(OH)D levels as a continuous variable (Figure 1D, p = 0.0016, FDR-adjusted p = 0.0181). To validate these results, we utilized RPhenograph as a second unsupervised analysis approach (Figure S2). Among the clusters identified by RPhenograph, the cluster corresponding to Tregs was the only one showing a significant difference in frequency between infants with sufficient and insufficient 25(OH)D (Figure S3A, p = 0.0023, FDR-adjusted p = 0.0570) as well as a significant association between frequencies and serum 25(OH)D concentrations (Figure S3B, p = 0.0038, FDR-adjusted p =

0.0957). Finally, utilizing manual gating, the trends of Treg frequency differences between vitamin D sufficient and insufficient infants (Figure 2A, p = 0.0109) as well as when examining 25(OH)D as a continuous variable (Figure 2B, p = 0.0255) were confirmed.

This analysis provides important new evidence for the relationship between 25(OH)D levels and Tregs in food-allergic infants. Vitamin D was previously shown to be associated with increased Tregs in both healthy patients and those with autoimmune diseases.[7] However, most studies making this connection have focused on older children and adults, while food challenge tests indicate food allergies are also common in younger children, as 3% of Australian infants are peanut allergic and 9% are egg allergic.[5,7,8] Furthermore, while 25(OH)D levels have been associated with allergic status, the correlation between Treg and 25(OH)D levels in patients with allergies is less studied. A study on asthmatic children (aged 6-16 years) similarly to our results found a correlation between proportion of Treg cells and 25(OH)D levels.[9] Another study found a positive correlation between 25(OH)D levels and the absolute count of Tregs in a pool of infants less than 9 months old with recently confirmed cow's milk allergy and non-allergic controls.[10] As far as we know, this relationship had never previously been investigated in peanut and/or egg allergic infants.

Follow-up food challenges for egg and peanut were performed at age 4 years for a subset of the participants. All 8 infants with confirmed peanut allergy at age 1 remained peanut allergic at age 4. Eight infants with egg allergy were retested at age 4, revealing that 3 were persistently egg allergic and 5 had naturally resolved their egg allergy. No clear association between early life vitamin D levels, Tregs and food allergy outcomes in childhood were observed in this study, warranting further investigation in a larger cohort.

This study has several limitations. We utilized available data from prior work thus resulting in a limited sample size when stratifying by vitamin D status.[6] Additionally, the available data did not allow the inclusion of non-food allergic infants with sufficient and insufficient vitamin D levels as comparison group. Of the 12 non-allergic infants from the original study, only one showed insufficient serum 25(OH)D levels. As expected, this was significantly less (p = 0.024, Fisher's Exact Test) than the 50% (9/18) with insufficient serum 25(OH)D levels of the food-allergic infants that we included in our analysis.[2,3] Comparing immune profiles of food-allergic and non-allergic infants with vitamin D insufficiency, potentially stratified by allergies to specific foods, should be done in a larger cohort to validate our findings. Additionally, information such as ethnicity and history of vitamin D supplementation should

be included, as there is some evidence to suggest this may play a role in the correlation between 25(OH)D levels and food allergy.[2] When interpreting our findings, it should also be considered that blood collection was performed within 2 hours following an oral food challenge.[6] We have previously reported no differences in cellular activation when blood samples were taken on a non-food challenge day vs. an active oral food challenge day in food-allergic infants.[3]

In summary, our findings suggest that vitamin D enhances circulating levels of Tregs in food-allergic infants. This confirms previous studies emphasizing a relationship between vitamin D and Tregs, while providing new evidence correlating 25(OH)D and circulating Treg levels in peanut and egg allergic 12-month-old infants.[7] This observation is consistent with the hypothesis that increased sun exposure or vitamin D supplements during the first 12 months of life could increase the proportion of Tregs in infancy, thereby promoting immune tolerance. Whether this could play a preventative or protective role in food allergies needs to be further examined. A follow-up study that includes non-allergic infants, while also considering the severity of allergies from infants that are included, as well as maternal vitamin D levels during pregnancy, would be beneficial.

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Conflict of Interest:

Dr. Nadeau reports grants from National Institute of Allergy and Infectious Diseases (NIAID), National Heart, Lung, and Blood Institute (NHLBI), and National Institute of Environmental Health Sciences (NIEHS); Food Allergy Research & Education (FARE), Director of World Allergy Organization (WAO) Center of Excellence at Stanford; Advisor at Cour Pharma; Co-founder of Before Brands, Alladapt, Latitude, and IgGenix; National Scientific Committee member at Immune Tolerance Network (ITN) and National Institutes of Health (NIH) clinical research centers; DSMB member for NHLBI, US patents for basophil testing, multifood immunotherapy and prevention, monoclonal antibody from plasmoblasts, and device for diagnostics. The remaining authors have nothing to disclose.

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Table 1: Allergy and 25(OH)D levels of the 18 participants (ID) included in this study. Table is organized by serum 25(OH)D level, from highest to lowest concentrations.

ID	Sex	Peanut allergy	Peanut sIgE (kuA/L)	Peanut SPT (mm)	Egg allergy	Egg sIgE (kuA/L)	Egg SPT (mm)	Vitamin D sufficiency	25(OH)D level (nmol/L)
P13	M	N	0.93	2.5	Y	34.6	6.5	Y	110.69
P30	M	Y	2.02	8	N	0.19	3	Y	106.7
P18	M	N	1.66	4	Y	21.3	10	Y	90.9
P31	F	Y	1.26	12.5	Y	2.03	3	Y	78.68
P29	M	Y	NA	11.5	Y	NA	4	Y	72.3
P32	M	Y	77	10	Y	2.54	4	Y	67.3
P28	M	Y	NA	10	Y	NA	3	Y	63.57
P26	M	Y	11.8	10	Y	21.6	7.5	Y	54.7
P27	F	Y	16.8	8	N	0.15	0	Y	51.7
P35	F	Y	0.81	9	Y	0.32	2	N	49.79
P25	F	Y	NA	7	Y	2.58	4	N	47.8
P36	M	Y	6.5	8	Y	0.93	2.5	N	41.04
P20	M	N	1.17	8	Y	3.46	5	N	39
P34	M	Y	0.31	5	Y	0.41	4.5	N	28.91
P22	F	N	0.8	3.5	Y	0.6	3	N	27.97
P19	M	N	9.31	4	Y	31.8	1	N	25.2
P23	F	N	2.66	2.5	Y	43	3	N	22.32

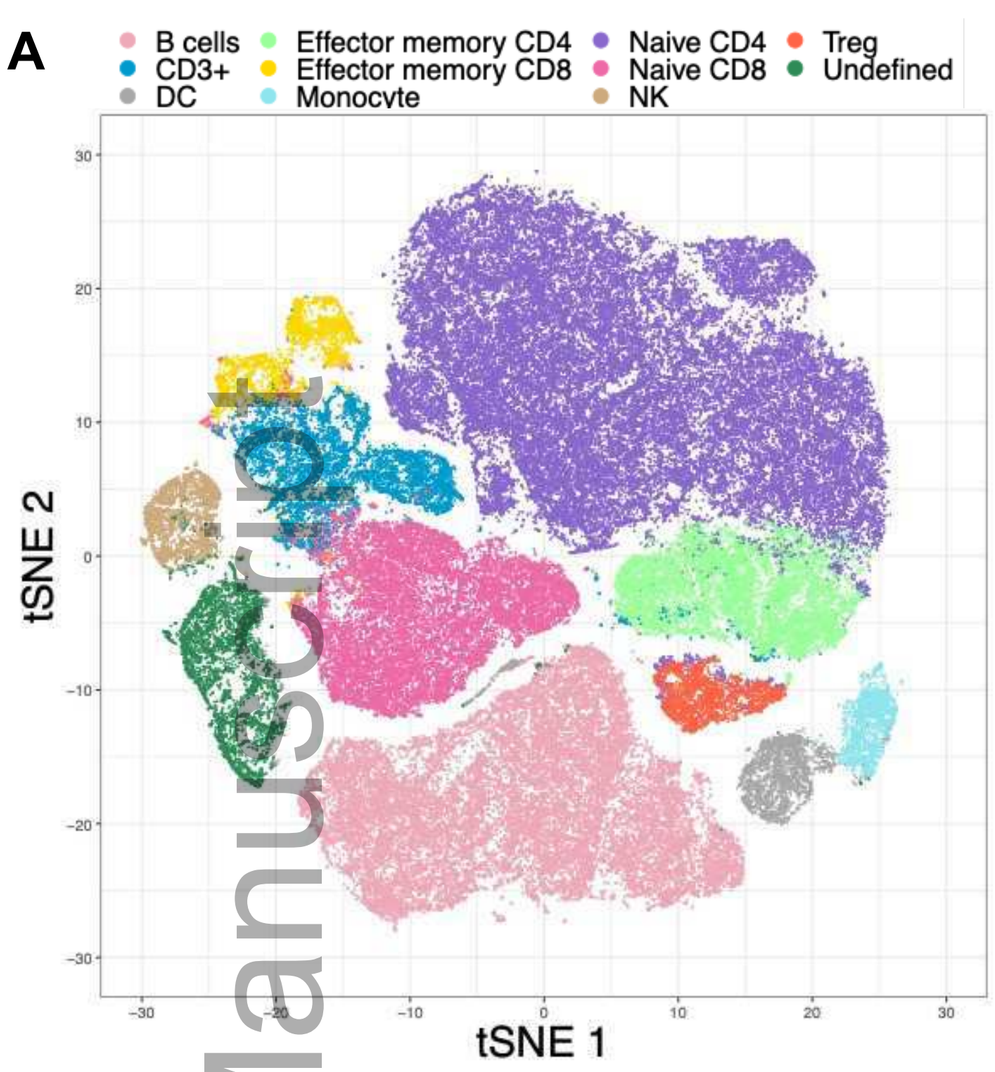
P33	M	Y	4.24	9	Y	0.4	4	N	16.84

Figure legends:

Figure 1: (A) tSNE plot of 5600 cells per sample, colored according to their FlowSOM cluster. **(B)** A heatmap showing the arcsinh transformed median expression for each cluster for each lineage marker. The number in parentheses at the row names is the percent live cells. **(C)** and **(D)** show the frequency of Tregs compared with serum 25(OH)D levels in the participants. The p-values were calculated from a linear mixed model with **(C)** 25(OH)D level as a discrete or **(D)** with 25(OH)D levels as a continuous variable. For each analysis the p-values of all clusters were FDR-adjusted.

Figure 2: The frequency of regulatory T cells (Tregs) found by manual gating compared with serum 25(OH)D levels in the participants. The p-values were calculated from a linear mixed model with **(A)** 25(OH)D level as a discrete variable (p=0.0109) or **(B)** with vitamin D sufficiency as a continuous variable (p=0.0255).

A



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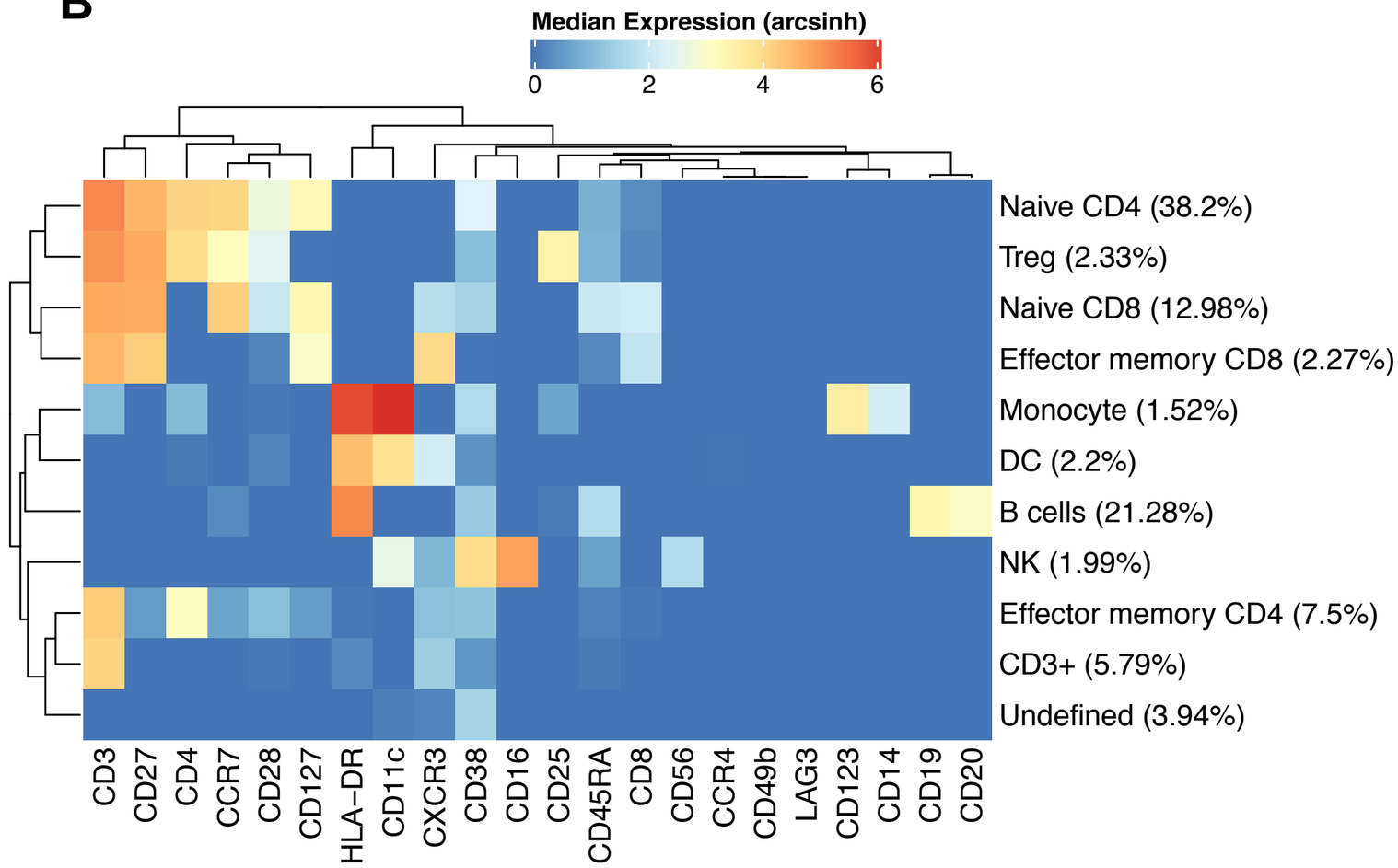

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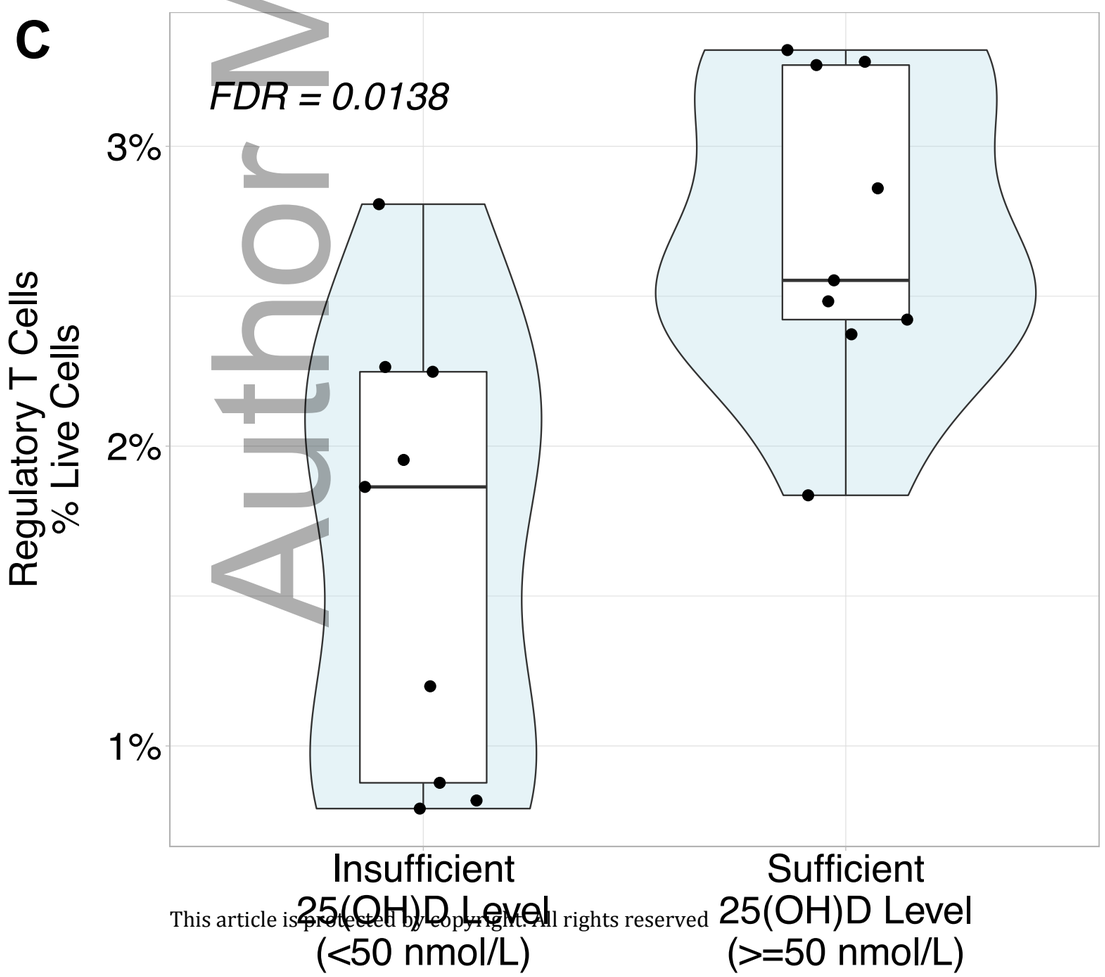

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