

Vitamin D₃ deficiency enhances allergen-induced lymphocyte responses in a mouse model of allergic airway disease

Shelley Gorman, Daryl H. W. Tan, Misty J. M. Lambert, Naomi M. Scott, Melinda A. Judge & Prue H. Hart

Telethon Institute for Child Health Research, Centre for Child Health Research, University of Western Australia, Perth, Western Australia, Australia

To cite this article: Gorman S, Tan DHW, Lambert MJM, Scott NM, Judge MA, Hart PH. Vitamin D₃ deficiency enhances allergen-induced lymphocyte responses in a mouse model of allergic airway disease. *Pediatr Allergy Immunol* 2012; **23**: 83–87.

Keywords

vitamin D; asthma; mice; lymphocytes.

Correspondence

Dr. Shelley Gorman, Telethon Institute for Child Health Research, Centre for Child Health Research, University of Western Australia, PO Box 855, West Perth, Western Australia 6872, Australia.

Tel.: +61 8 9489 7884

Fax: +61 8 9489 7700

E-mail: shelleyg@icmr.uwa.edu.au

Accepted for publication 22 December 2010

DOI:10.1111/j.1399-3038.2011.01146.x

Abstract

There is debate as to whether vitamin D deficiency contributes towards the extent of the asthma epidemic. In this study, using a mouse model, we determined whether vitamin D deficiency *in utero* and during early life modulated the severity of asthma. Using dietary restriction, vitamin D₃-replete and vitamin D₃-deficient colonies of BALB/c mice were established. Utilizing the allergic airway disease model of asthma with the experimental allergen ovalbumin (OVA), we examined asthma-like responses 24 h after airway challenge with OVA in adult offspring born to vitamin D₃-replete and vitamin D₃-deficient mothers. The ability of airway-draining lymph node cells to proliferate and secrete cytokines in response to OVA *ex vivo* was significantly enhanced by vitamin D₃ deficiency. However, other aspects of allergic disease, including the numbers and proportions of inflammatory cells and cytokines in the lungs and the quantity of OVA-specific IgE in serum, were not modified. These results suggest that vitamin D₃ deficiency modulates the capacity of lymphocytes to respond to allergens.

Asthma is a chronic illness of the airways characterized by wheeze, shortness of breath, chest tightness and cough. The prevalence of asthma is increasing in westernized communities; vitamin D deficiency might contribute to this increase as many people have less sun exposure and spend more time indoors than previously (1). The production of the active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25[OH]₂D₃), begins in the skin following the conversion of 7-dehydrocholesterol into pre-vitamin D₃ by ultraviolet B (UVB, 280–315 nm) radiation present in sunlight. The concentration of the metabolite 25-hydroxyvitamin D₃ (25[OH]D₃) in serum is used to determine the vitamin D status of individuals.

Recent investigations have painted a confusing picture of the role that vitamin D may play in modulating allergic disease. In two human studies, high maternal intake of vitamin D during pregnancy was associated with a reduced prevalence of wheeze in 3- and 5-yr-olds (2, 3). In contrast, maternal levels of 25(OH)D₃ in serum were positively associated with asthma in 9-yr-old children (4). Similarly, the incidence of asthma in adults (31-yr-olds) was higher in those who had

received vitamin D supplementation regularly during the first year of life (5). However, serum 25(OH)D₃ levels were not assessed in any of these studies. Serum 25(OH)D₃ levels were inversely correlated with outcomes such as hospitalization and airway hyperresponsiveness in asthmatic children (6). To explore this issue further, we have investigated the effects of vitamin D₃ deficiency throughout life (from conception until adulthood) upon asthma outcomes, using a classical murine model of acute asthma induced by the experimental allergen, ovalbumin (OVA).

Materials and methods

Mice and diet

Ethical guidelines of the National Health and Medical Research Council of Australia and the Telethon Institute for Child Health Research Animal Ethics Committee were followed. For each experiment, six female 3-wk-old BALB/c mice were placed on vitamin D₃-deficient (with 25 g/kg

Ca; SF05-033, Specialty Feeds, Western Australia, Australia) or control diets containing 2280 IU vitamin D₃/kg (with 15 g/kg Ca; SF05-34, Specialty Feeds). At 8 wks of age, female mice were mated with adult male vitamin D₃-replete BALB/c mice. Offspring were maintained on the deficient or control diets. There was no difference in the numbers of offspring produced per dam (5 ± 1 [mean $\pm$ SEM] for both vitamin D₃-replete and vitamin D₃-deficient dams), and the ratio of male to female offspring was also equivalent (1:1 for both vitamin D₃-replete and vitamin D₃-deficient dams). Mice were housed under fluorescent lighting, which emitted no detectable UVB radiation as measured using a UV radiometer (UVX Digital Radiometer; Ultraviolet Products Inc., Upland, California, USA). Serum 25(OH)D₃ levels were measured using an IDS OCEIA ELISA kit (Immunodiagnostic Systems Ltd, Boston Business Park, UK). To determine bone mineral content, dual-energy X-ray absorptiometry scans were performed using a GE Lunar Prodigy scanner (GE Lunar Corporation, Madison, WI, USA).

Sensitization and challenge of mice with ovalbumin

When offspring reached 8 wks of age, OVA (Sigma Chemical Company, St Louis, MO, USA) in an aluminium hydroxide suspension (Alum; Serva, Heidelberg, Germany) was delivered i.p. on day 0 (20 μ g OVA in 4 mg aluminium hydroxide per mouse; 200 μ l volume) and again on day 14. Mice were then challenged on day 21 with a 1% OVA-in-saline aerosol delivered using an ultrasonic nebulizer (UltraNeb, DeVilbiss, Somerset, PA, USA) for 30 min. Twenty-four hours after OVA challenge, the posterior mediastinal, tracheobronchial and parathymic lymph nodes (airway-draining lymph nodes [ADLN]) were removed, pooled within experimental groups, physically disaggregated and cultured at 10^5 cells/200 μ l per well (six replicates per treatment) with OVA (10 μ g/ml) as described previously (7). ³[H]-thymidine incorporation was used as a measure of cellular proliferation when added for the final 24 h of a 96-h culture. Culture supernatants were also obtained at 96 h. Using flow cytometry, the phenotypes of ADLN cells were examined as described previously (8). Twenty-four hours after the aerosol challenge with OVA, bronchoalveolar lavage fluid (BALF) was collected as described previously (7). IL-4, IL-5, IL-10 and IFN- γ were detected in tissue culture supernatants and BALF samples as described previously (7). At day 20 (24 h before OVA challenge) of the OVA-induced allergic airway disease model, sera were tested for OVA-specific IgE using an *in vivo* passive cutaneous anaphylaxis assay described previously (7). IgE titres were expressed as log₂.

Results

Dietary restriction induces vitamin D deficiency in mice

Mice fed the vitamin D₃-containing diet exhibited significantly greater ($p < 0.05$, Student's *t*-test) serum levels of 25(OH)D₃ than mice fed the vitamin D₃-deficient diet, which had very low serum levels of 25(OH)D₃ (< 20 nmol/l; Fig. 1a). There was no difference in the serum calcium levels, bone

mineral densities or weights of mice fed either diet (data not shown).

Responses by cells of airway-draining lymph nodes to OVA *ex vivo* are enhanced by vitamin D₃ deficiency

For three independent experiments, the capacity of ADLN cells to proliferate in response to OVA stimulation *ex vivo* was significantly enhanced for those cells from either male or female vitamin D₃-deficient mice (Fig. 1b). In addition, after 96 h, there were significant increases in the quantities of IL-4 (data not shown), IL-5 (Fig. 1c), IL-10 (data not shown) and IFN- γ (Fig. 1d) in the culture supernatants of ADLN cells from vitamin D₃-deficient mice. The proportions of various ADLN cells, including CD4⁺ T cells, CD8⁺ T cells, CD19⁺ B cells, CD11c⁺ MHC classII⁺ dendritic cells and CD4⁺ Foxp3⁺ regulatory T cells, were not modified by vitamin D₃ deficiency, when examined 24 h after OVA aerosol (Fig. 1e). These results indicate that the responsiveness of lymphocytes to OVA was significantly enhanced by vitamin D₃ deficiency.

Inflammatory cells and cytokines in the bronchoalveolar lavage fluid and IgE levels in serum are not affected by vitamin D₃ deficiency

Vitamin D₃ deficiency did not modify the number (Fig. 2a) or type (Fig. 2b) of inflammatory cells present in BALF collected 24 h after OVA challenge. Similarly, the concentration of IL-4, IL-5, IL-10 and IFN- γ in the BALF was also not altered by vitamin D₃ deficiency (data not shown). With multiple aerosols (three OVA aerosols at 24-h intervals), the proportion of eosinophils increased from $\sim 10\%$ to 30% of the total number of inflammatory cells present; however, vitamin D₃ deficiency did not affect the proportion of eosinophils or other cells in the BALF (data not shown). Further inspection of H&E- or periodic acid Schiff-stained lung sections revealed no difference in the extent of inflammation or numbers of mucus-secreting cells in the lungs of vitamin D₃-deficient or vitamin D₃-replete mice (data not shown). Vitamin D₃ deficiency did not modify the quantities of anti-OVA-specific IgE antibody in the serum of either male or female offspring (Fig. 2c). Anti-OVA IgG1 antibody levels were also not altered by vitamin D₃ deficiency (data not shown).

Discussion

Here, we show for the first time that vitamin D₃ deficiency enhanced allergen-induced lymphocyte responses in a mouse model of allergic airway disease. ADLN cells from vitamin D₃-deficient mice had an augmented ability to proliferate and secrete cytokines *ex vivo*. Vitamin D₃ deficiency affected the responses of ADLN cells from male and female mice to a similar degree. However, other asthma parameters (such as lung inflammation and allergen-specific antibody induction) that were suppressed in vitamin D₃-replete mice acutely or chronically treated with 1,25(OH)₂D₃ (reviewed in 1) were not affected by vitamin D₃ deficiency. In a previous study by

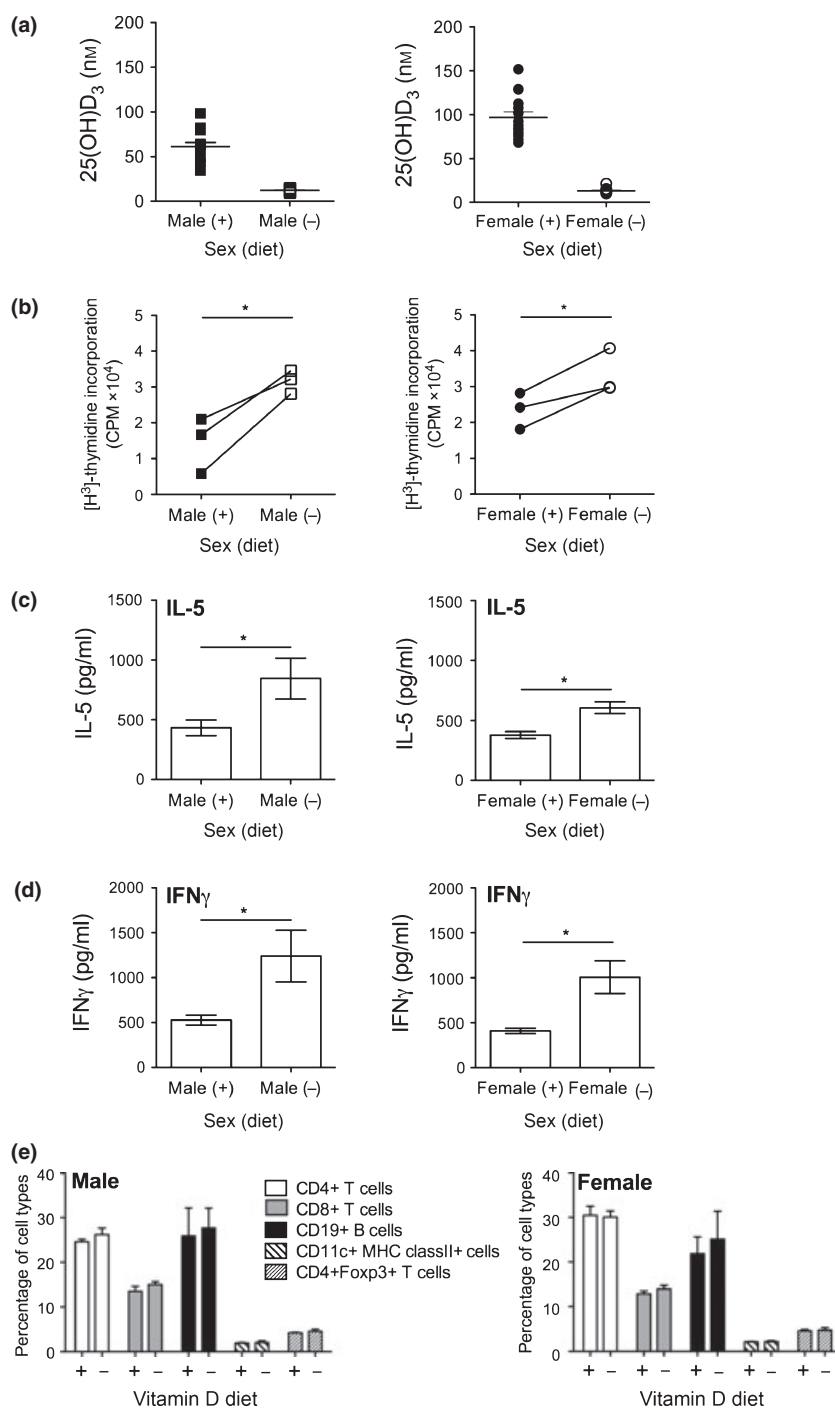


Figure 1 Vitamin D₃ deficiency enhances ADLN responsiveness in both male and female mice. (a) At 8 wks of age, serum from offspring ($n = 15$ mice/gp) fed the vitamin D₃-replete (+) and vitamin D₃-deficient (-) diets was collected and tested for 25(OH)D₃ with results for individual mice shown, and the horizontal line indicating mean 25(OH)D₃ levels in serum. At this time, mice were also intraperitoneally sensitized with ovalbumin (OVA) (20 μ g) and alum (4 mg), boosted with the same regimen 2 wks later and challenged with an aerosol containing 1% OVA after a further 7 days. Twenty-four hours after the OVA challenge, ADLN cells were pooled from

mice within treatment groups (five mice per treatment) and cultured with 10 μ g/ml OVA for 96 h. In (b), mean levels of ³[H]-thymidine incorporation by proliferating ADLN cells are shown for the last 24 h of a 96-h culture for three independent experiments. In (c) and (d), IL-5 and IFN- γ levels in supernatants are depicted, which were collected after 96-h culture. In (e), the proportions of various ADLN cell types were determined for cells collected 24 h after OVA challenge. Results were pooled from three independent experiments (with cultures tested in triplicate) and are shown as mean $\pm$ SEM (* $p < 0.05$, Student's t -test).

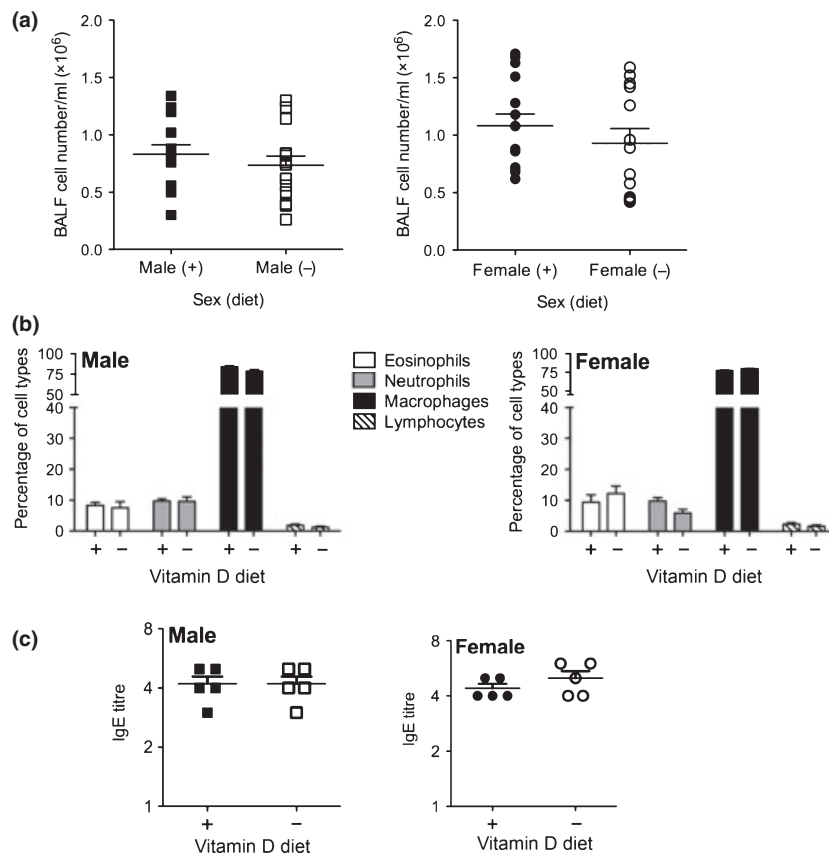


Figure 2 Vitamin D₃ deficiency does not modify inflammatory cell numbers in the bronchoalveolar lavage fluid (BALF) or serum ovalbumin (OVA)-specific IgE levels. At 8 wks of age, offspring mice were intraperitoneally sensitized with OVA (20 μ g) and alum (4 mg), boosted with the same regimen 2 wks later, and challenged with an aerosol containing 1% OVA after a further 7 days. Twenty-four hours after the OVA challenge, (a) the total number of cells and (b) the proportions of eosinophils, neutrophils, macrophages and lymphocytes were determined in BALF for male (left column) and female (right

column) vitamin D₃-replete (+) and vitamin D₃-deficient (-) offspring. Results are shown as mean $\pm$ SEM ($n = 15$ mice/gp with data pooled from three independent experiments), with results for individual mice shown in (a), where the horizontal line indicates mean BALF cell numbers/ml. In (c), a passive cutaneous anaphylaxis assay was used to determine OVA-specific IgE levels in the serum of male and female offspring 24 h prior to OVA challenge. Results are representative of one of three independent experiments ($n = 5$ mice per treatment per experiment) and are shown as mean $\pm$ SEM.

Wittke et al. (9), lung inflammation and allergen-specific IgE levels were also not modified by feeding vitamin D₃-deficient mice diets containing 1,25(OH)₂D₃.

As well as the type of vitamin D used to supplement the diets of the deficient mice (1,25[OH]₂D₃ vs. vitamin D₃), a major difference in the experimental protocols of Wittke et al. (9) and our own was the strain of mice in which allergic airway disease was induced (C57Bl/6 (9) vs. BALB/c). Serum calcium levels increased in mice fed the 1,25(OH)₂D₃-containing diet (9) whereas calcium levels in mice fed the vitamin D₃-replete or vitamin D₃-deficient diets were not modified in the current studies. Finally, while Wittke et al. (9) observed no difference in the responsiveness of spleen cells to OVA *ex vivo* during deficiency, local responses in the ADLN were not examined.

Interactions of dendritic cells, Th2 effector cells and regulatory T cells in the ADLN control the capacity of these cells

to proliferate *ex vivo* (reviewed in 8). In preliminary investigations, we find that CD11c⁺ cells (putative dendritic cells) purified from the lymph nodes of vitamin D₃-deficient mice have an enhanced capacity to prime immune responses *in vivo* (data not shown). Our studies have previously shown that topically applied 1,25-dihydroxyvitamin D₃ subverts the *in vivo* priming ability of skin-derived dendritic cells by increasing the suppressive capacity of CD4⁺CD25⁺ regulatory T cells in the skin-draining lymph nodes, where skin-derived dendritic cells from mice treated with 1,25-dihydroxyvitamin D₃ expressed increased mRNA levels of the tolerogenic enzyme indoleamine 2,3-dioxygenase (IDO, 10). In further preliminary investigations, increased IDO mRNA levels were also detected in ADLN cells from vitamin D₃-replete mice 24 h after OVA challenge (data not shown), suggesting that vitamin D₃ may act through IDO-dependent mechanisms to affect allergen-induced lymphocyte prolifera-

tion and cytokine production. In other studies, UVB irradiation of shaved skin reduced the responsiveness to OVA of ADLN cells (7, 8) by affecting the number of CD4+CD25+ Th2 effector cells and did not alter dendritic cell or regulatory T-cell number or function (8). Further investigations are required to determine which ADLN cells are affected by vitamin D₃ deficiency.

Preliminary data suggest that in the current model, we may be administering allergen to mice at doses far greater than that required to induce allergic airway disease and that more extensive immunomodulation may be difficult to achieve. Another limitation of this model is that mice differ from humans in that they are nocturnal animals with fur. The single-OVA aerosol asthma model has been used previously by our selves (7, 8) and many others to investigate asthmatic phenotypes in mice. The advantages of this model have been previously discussed and in particular enable for airway hyperresponsiveness to be measured using a well-

respected modified low-frequency forced oscillation technique (7). Airway hyperresponsiveness was not investigated in this study as the differences observed during vitamin D₃ deficiency seemed to be limited to the ADLN, in the absence of changes in lung inflammation and also allergen-specific IgE responses. In conclusion, vitamin D₃ deficiency modified the ability of ADLN cells to respond to allergen, but had no further effects on other asthma outcomes. Further investigations are required to determine whether vitamin D₃ deficiency may affect allergic sensitization in humans.

Acknowledgments

We thank Ms Claire Elliot for her assistance with the passive cutaneous anaphylaxis assays. This study was funded by grants from the Raine Foundation (SG), the Asthma Foundation of Western Australia (SG and PHH) and the University of Western Australia (SG).

References

1. Litonjua AA, Weiss ST. Is vitamin D deficiency to blame for the asthma epidemic? *J Allergy Clin Immunol* 2007; **120**: 1031–5.
2. Devereux G, Litonjua AA, Turner SW, et al. Maternal vitamin D intake during pregnancy and early childhood wheezing. *Am J Clin Nutr* 2007; **85**: 853–9.
3. Carmargo CA Jr, Rifas-Shiman SL, Litonjua AA, et al. Maternal intake of vitamin D during pregnancy and risk of recurrent wheeze in children at 3 y of age. *Am J Clin Nutr* 2007; **85**: 788–95.
4. Gale CR, Robinson SM, Harvey NC, et al. Princess Anne Hospital Study Group. Maternal vitamin D status during pregnancy and child outcomes. *Eur J Clin Nutr* 2008; **62**: 68–77.
5. Hyponen E, Sovio U, Wjst M, et al. Infant vitamin D supplementation and allergic conditions in adulthood: northern Finland birth cohort 1966. *Ann N Y Acad Sci* 2004; **1037**: 84–95.
6. Brehm JM, Celedon JC, Soto-Quiros ME, et al. Serum vitamin D levels and markers of severity of childhood asthma in Costa Rica. *Am J Respir Crit Care Med* 2009; **179**: 765–71.
7. McGlade JP, Gorman S, Zosky GR, et al. Suppression of the asthmatic phenotype by UVB-induced, antigen-specific regulatory cells. *Clin Exp Allergy* 2007; **37**: 1267–76.
8. McGlade JP, Strickland DH, Lambert MJ, et al. UV inhibits allergic airways disease in mice by reducing effector CD4+ T cells. *Clin Exp Allergy* 2010; **40**: 772–85.
9. Wittke A, Chang A, Froicu M, et al. Vitamin D receptor expression by the lung micro-environment is required for maximal induction of lung inflammation. *Arch Biochem Biophys* 2007; **460**: 306–13.
10. Gorman S, Judge MA, Hart PH. Topical 1,25-dihydroxyvitamin D₃ subverts the priming ability of draining lymph node dendritic cells. *Immunology* 2010; **131**: 415–25.