



Contents lists available at ScienceDirect

Allergy International

journal homepage: <http://www.elsevier.com/locate/alit>

Letter to the Editor

Vitamin D deficiency exacerbates sensitization and allergic diarrhea in a murine food allergy model

Dear Editor,

Food allergies (FA) are becoming increasingly common worldwide, and, while their causes remain unclear, season of birth, latitude, and sun exposure are all reportedly implicated in this increase in food sensitization.¹ Exposure to sunlight during early infancy has been inversely correlated with food sensitization,² and a notable increase in FA risk has been observed in winter; this risk is related to levels of vitamin D, which is produced following sunlight exposure. Serum levels of 25-hydroxyvitamin D (25(OH)D) are lowest during the winter months.¹ Moreover, vitamin D reportedly has multiple immunological effects, including the induction of tolerogenic dendritic cells and regulatory T (Treg) cells and the inhibition of B cell IgE production.¹

Vitamin D is also important for maintaining mucosal barrier function via the enhanced expression of genes encoding epithelial tight junction proteins, including ZO-1, claudin 1, claudin 2, and E-cadherin.¹ Vitamin D deficiency could therefore contribute to epithelial barrier dysregulation, causing excessive antigen exposure and thus accelerated sensitization to food antigens. Vitamin D deficiency caused by insufficient sun exposure has therefore been hypothesized to be involved in the development of FA.¹ However, the value of serum 25(OH)D level as an indicator of FA risk is debatable; some studies report an inverse relationship between serum 25(OH)D level and FA and sensitization, while others show that excess vitamin D as well as vitamin D supplementation increase FA risk.¹ While vitamin D deficiency reportedly exacerbates allergic airway inflammation and airway hyperresponsiveness,³ and enhances the number of eosinophils and neutrophils in bronchoalveolar lavage fluid in a murine model of allergic airway disease,⁴ the effect of vitamin D deficiency on FA development has thus far not been demonstrated in murine FA models. We therefore investigated this important question using a murine FA model to determine the effects of 25(OH)D deficiency (Supplementary Methods). All studies were approved by the Animal Experiment Committee at Gifu Pharmaceutical University, and were conducted according to the relevant guidelines.

Female BALB/c mice were fed either a vitamin D-deficient diet (Deficient) or a control vitamin D-sufficient diet (Control), and were intraperitoneally injected with an ovalbumin (OVA)/aluminum hydroxide gel (alum) mixture before receiving 9 oral doses of 30 mg OVA to boost sensitization to OVA and

induce FA (Fig. 1A). Plasma 25(OH)D levels were confirmed to be deficient in the Deficient mice, reaching a maximum of 20 ng/ml at day –1.⁴ Conversely, the Control mice maintained a significantly higher and stable plasma 25(OH)D concentration (Fig. 1B).

OVA-specific IgE and IgG1 were detectable in both Control and Deficient mice plasma after intraperitoneal OVA/alum and oral OVA administration, although the concentrations of both immunoglobulins were significantly higher in Deficient mice (Fig. 1C). Vitamin D deficiency therefore accelerated OVA sensitization induced by intraperitoneal then oral routes.

Allergic diarrhea following each 30 mg dose of OVA was used to assess FA, and was evaluated using previously described diarrhea scores⁵: 0, solid; 1, funicular; 2, slurry; and 3, watery. Scores of 2 or 3 were taken to indicate allergic diarrhea (Fig. 1D, left panel). The percent occurrence of allergic diarrhea following each OVA dose is also shown (Fig. 1D, right panel). Deficient mice displayed higher diarrhea scores and diarrhea occurrence than Control mice, indicating that vitamin D deficiency promoted sensitization to OVA and exacerbated the resulting FA symptoms.

Next, we investigated the mechanisms by which 25(OH)D deficiency promotes FA. Intestinal colonization with *Clostridium cocoides* and *Clostridium leptum* reportedly protects against FA development through the regulation of innate lymphoid cell function and intestinal epithelial permeability, as well as the induction of protective Treg cells.⁶ Furthermore, vitamin D receptor knockout mice reportedly display an altered commensal gut bacteria composition, including a lower proportion of *Clostridia*.⁷ We hypothesized that vitamin D deficiency impairs *Clostridia* colonization, and that these microbiome changes are related to the acceleration of sensitization and the exacerbation of food allergy symptoms. We found a non-significant reduction in *C. cocoides* prevalence in Deficient mice compared to Control mice ($p = 0.155$, Fig. 2A, left panel), and no differences in *C. leptum* prevalence (Fig. 2A, right panel). This suggests that changes to the intestinal flora with vitamin D deficiency were relatively minor in our model.

The cytokine mRNA levels in the mesenteric lymph nodes, the lymph nodes that drain from the small and large intestines, were then determined using quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) (Fig. 2B). Although levels of IFN- γ did not differ significantly between the groups, levels of TGF- β , a suppressive cytokine produced by Treg

Peer review under responsibility of Japanese Society of Allergy.

<http://dx.doi.org/10.1016/j.alit.2017.08.010>1323–8930/Copyright © 2017, Japanese Society of Allergy. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

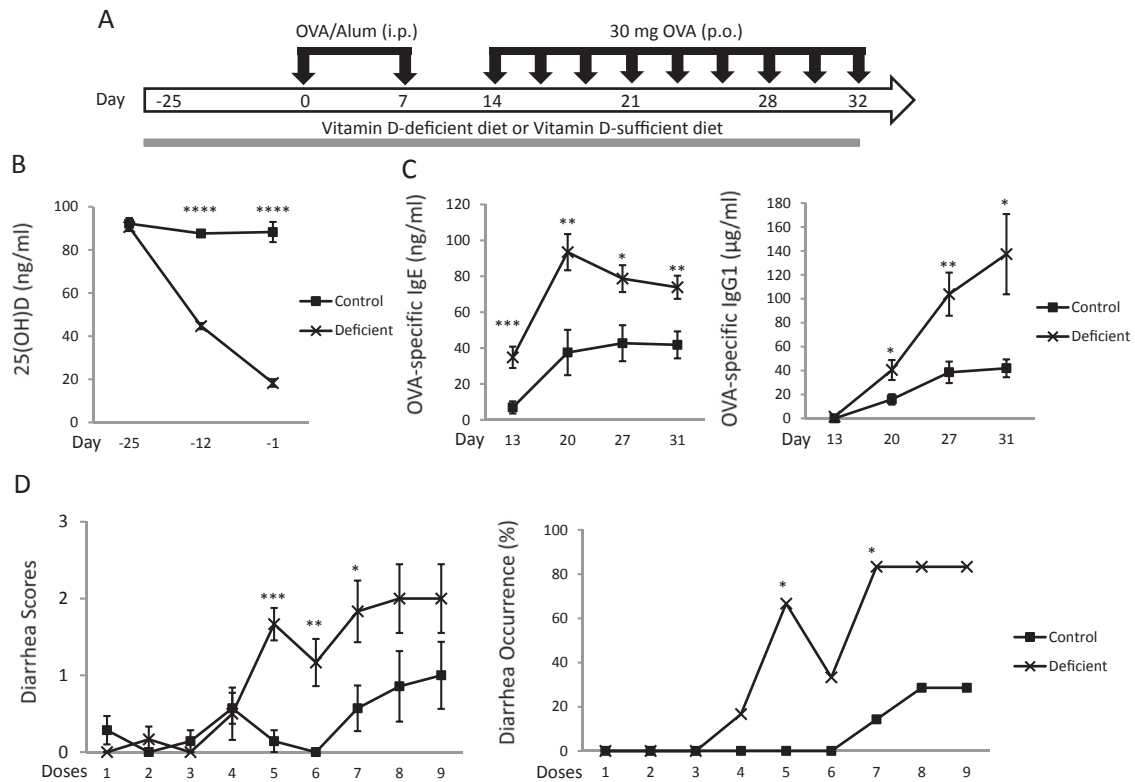


Fig. 1. 25(OH)D deficiency accelerates OVA sensitization and exacerbates allergic diarrhea. **(A)** BALB/c mice were fed either a vitamin D-deficient diet or a control vitamin D-sufficient diet for 25 days prior to intraperitoneal (i.p.) sensitization with a mixture of ovalbumin (OVA) and aluminum hydroxide gel (OVA/alum). Mice then received 9 oral doses of 30 mg OVA to boost sensitization and induce allergic diarrhea. **(B)** Plasma 25(OH)D levels in the mice fed the vitamin D-deficient diet (Deficient), and in the mice fed a vitamin D-sufficient control diet (Control), on days -25, -12, and -1. **(C)** Plasma concentrations of OVA-specific IgE (left panel) and OVA-specific IgG1 (right panel) in Deficient and Control mice on days 13, 20, 27, and 31. **(D)** Diarrhea scores (left panel) and diarrhea occurrence (%) (right panel) in Deficient and Control mice after each of the 9 doses of oral OVA (30 mg). Doses were administered between days 14 and 32. Diarrhea was defined as a score of 2 or 3. Values represent the mean $\pm$ standard error, and groups contained either six or seven mice. Data are representative of two independent experiments. p.o., *per os* (oral administration); i.p., intraperitoneally. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

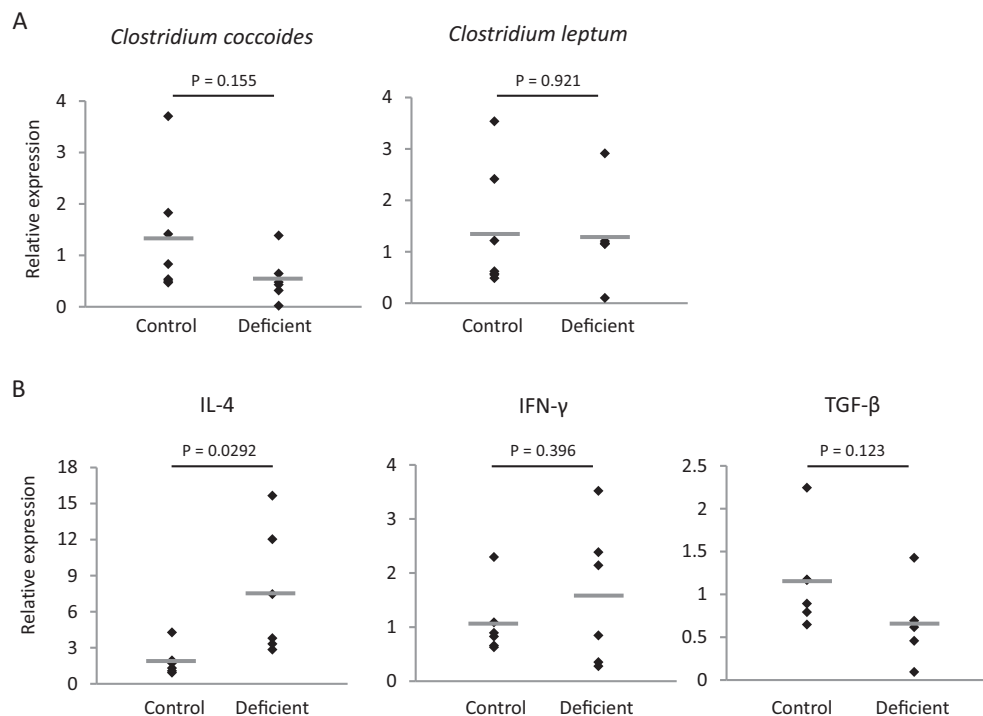


Fig. 2. 25(OH)D deficiency enhances IL-4 expression in the mesenteric lymph nodes and alters intestinal microbiota. **(A)** Expression of 16S rDNA from *Clostridium coccoides* and *Clostridium leptum* in fecal matter collected on day 31, as determined by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR). **(B)** Expression of the cytokines IL-4, IFN- γ , and TGF- β in mesenteric lymph nodes, collected on day 32 after the evaluation of allergic diarrhea. Expression was determined by qRT-PCR, and normalized to β -actin expression levels. Bars represent the mean. Either six or seven mice were used per group. Data were obtained from a single experiment. Deficient, mice fed the vitamin D-deficient diet; Control, mice fed a vitamin D-sufficient control diet.

cells that is also involved in Treg induction, were marginally lower in Deficient mice compared with Control mice ($p = 0.123$).

IL-4 is essential for the Th2 response, wherein it triggers B cell activation and interacts with antigen-specific B cells to give isotype switching and the generation of antigen-specific IgEs. IL-4 is also involved in Th2 cell development, mast cell activation, and eosinophil infiltration into the gut mucosa. Additionally, IL-4 deficiency reportedly impairs food allergy development.⁸ Conversely, gain-of-function mutations in the IL-4 receptor α -chain increase susceptibility to anaphylaxis.⁹ IL-4 is thus pivotal in the induction of FA symptoms. In our system, IL-4 expression was significantly elevated in the Deficient mice compared with the Control mice ($p = 0.0292$, Fig. 2B, left panel).

Although intraperitoneal sensitization is widely used in murine FA models, this is not considered to be a natural food sensitization route, and transcutaneous routes are thought to be more representative.¹⁰ In future studies, it will therefore be important to use a transcutaneous sensitization model to investigate the effect of vitamin D deficiency on FA development.

This is the first study to show conclusively that 25(OH)D deficiency promotes sensitization and exacerbates FA in a murine model, by mechanisms that could involve the increased expression of IL-4 in the mesenteric lymph nodes of vitamin D-deficient mice.

Acknowledgements

This study was supported by the Practical Research Project for Allergic Disease and Immunology, part of the Japan Agency for Medical Research and Development (grant number: 17ek0410019s0703), and by the Public Foundation of Elizabeth Arnold-Fuji.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.alit.2017.08.010>.

Conflict of interest

The authors have no conflict of interest to declare.

Teruaki Matsui^{a,b,c,*}, Hirotaka Yamashita^{a,d}, Ken-ichi Saneyasu^a, Hiroyuki Tanaka^{a,d}, Komei Ito^b, Naoki Inagaki^{a,d}

^a Laboratory of Pharmacology, Department of Bioactive Molecules, Gifu Pharmaceutical University, Gifu, Japan

^b Department of Allergy, Aichi Children's Health and Medical Center, Aichi, Japan

^c Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan

^d United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu, Japan

* Corresponding author. Laboratory of Pharmacology, Department of Bioactive Molecules, Gifu Pharmaceutical University, Daigaku-Nishi 1-25-4, Gifu 501-1196, Japan.

E-mail address: terumatsu1981@hotmail.com (T. Matsui).

References

- Vassallo MF, Camargo Jr CA. Potential mechanisms for the hypothesized link between sunshine, vitamin D, and food allergy in children. *J Allergy Clin Immunol* 2010;**126**:217–22.
- Matsui T, Tanaka K, Nakagawa T, Sasaki K, Nakata J, Sugiura S, et al. Sun exposure inversely related to food sensitization during infancy. *Pediatr Allergy Immunol* 2015;**26**:628–33.
- Agrawal T, Gupta GK, Agrawal DK. Vitamin D supplementation reduces airway hyperresponsiveness and allergic airway inflammation in a murine model. *Clin Exp Allergy* 2013;**43**:672–83.
- Gorman S, Weeden CE, Tan DH, Scott NM, Hart J, Foong RE, et al. Reversible control by vitamin D of granulocytes and bacteria in the lungs of mice: an ovalbumin-induced model of allergic airway disease. *PLoS One* 2013;**8**:e67823.
- Yamashita H, Takahashi K, Tanaka H, Nagai H, Inagaki N. Overcoming food allergy through acquired tolerance conferred by transfer of Tregs in a murine model. *Allergy* 2012;**67**:201–9.
- Stefka AT, Feehley T, Tripathi P, Qiu J, McCoy K, Mazmanian SK, et al. Commensal bacteria protect against food allergen sensitization. *Proc Natl Acad Sci U S A* 2014;**111**:13145–50.
- Ooi JH, Li Y, Rogers CJ, Cantorna. Vitamin D regulates the gut microbiome and protects mice from dextran sodium sulfate-induced colitis. *J Nutr* 2013;**143**:1679–86.
- Dourado LP, Saldanha JC, Gargiulo DL, Noviello Mde L, Brant CC, Reis ML, et al. Role of IL-4 in aversion induced by food allergy in mice. *Cell Immunol* 2010;**262**:62–8.
- Mathias CB, Hobson SA, Garcia-Lloret M, Lawson G, Poddighe D, Freyschmidt EJ, et al. IgE-mediated systemic anaphylaxis and impaired tolerance to food antigens in mice with enhanced IL-4 receptor signaling. *J Allergy Clin Immunol* 2011;**127**:795–805.
- du Toit G, Tsakok T, Lack S, Lack G. Prevention of food allergy. *J Allergy Clin Immunol* 2016;**137**:998–1010.

Received 22 November 2016

Received in revised form 25 August 2017

Accepted 28 August 2017

Available online xxx

Supplementary Methods

Animals

Female BALB/c mice (SLC, Inc., Hamamatsu, Japan) used in experiments were housed in an animal research facility at a temperature of $23 \pm 1^{\circ}\text{C}$ and a humidity of $50 \pm 10\%$.

Six or seven mice per group were used in all experiments.

Food allergy model

To create the food allergy model, we modified a previously reported protocol [E1]. Briefly, female BALB /c mice were intraperitoneally injected with a mixture of 1 μg ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO, USA) and aluminum hydroxide gel (Alum; Thermo Fisher Scientific, Waltham, MA, USA) on days 0 and 7 to induce sensitization. One week after the final sensitization with the OVA/alum mixture, sensitization to OVA was accelerated and food allergy was induced by the oral administration of 9 separate doses of 30 mg OVA dissolved in 0.2 ml PBS, at a rate of three doses per week.

Vitamin D deficiency

Mice were fed either a vitamin D-deficient diet (AIN93M; Oriental Yeast, Tokyo,

Japan) to induce vitamin D deficiency, or a vitamin D-sufficient regular chow diet containing 2100 IU/kg of vitamin D (CE-2; CLEA Japan, Tokyo, Japan) as controls, from 25 days prior to sensitization until the end of the experiment.

Evaluation of food allergy symptoms

Food allergy was defined as the occurrence of allergic diarrhea within 1 h of administration of 30 mg OVA, as described previously [E1]. The severity of diarrhea was assessed using the following scoring system: 0, solid; 1, funicular; 2, slurry; and 3, watery. Diarrhea scores of 2 or 3 were considered to be indicative of allergic diarrhea [E1].

Detection of OVA-specific IgE and IgG1 in plasma

Plasma samples were obtained on days 13, 20, 27, and 31. Plasma concentrations of OVA-specific IgE and IgG1 were measured using an enzyme-linked immunosorbent assay (ELISA) according to a previously reported protocol [E1]. Briefly, a high protein-binding capacity ELISA plate (Nunc, Roskilde, Denmark) was coated with either anti-mouse IgE antibody (MCA419, clone LO-ME-3; Serotec, Oxford, UK) or anti-mouse IgG1 antibody (STAR81, Serotec), to capture plasma OVA-specific IgE or

OVA-specific IgG1, respectively. Biotinylated OVA was then bound to the captured OVA-specific IgE and OVA-specific IgG1, before horseradish peroxidase-conjugated streptavidin (Dako Cytomation, Glostrup, Denmark) was bound to the biotin (Pierce, Rockford, USA). The complexes were detected by measuring the absorbance at 492nm (reference 690nm) with an automatic ELISA plate reader (Titertek Multiscan MS, version 8, Labsystems Oy, Helsinki, Finland).

Detection of *Clostridium* 16S rDNA in feces

Feces were collected on day 31 from all mice, and DNA was purified from all samples using a QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany). Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR), using SYBR Premix Ex Taq II master mix (Takara Bio) and a Takara Thermal Cycler Dice system, was performed as described previously to detect 16S rDNA from *Clostridium coccooides* and *Clostridium leptum* [E2]. The universal primers 926f and 1392r were used in control reactions. Primer sequences were as follows: 5'-AAACTYAAAKGAGACGG -3' for 926f; 5'-ACGGGCGGTGTGTRC-3' for 1392r; 5'-AAATGACGGTACCTGACTAA -3' and 5'-CTTGAGTTTCATTCTTGCGAA-3' for *Clostridium coccooides*; 5'-GCACAAGCAGTGGAGT-3' and 5'-CTTCCTCCGTTTTGTCAA-3' for *Clostridium*

leptum.

Detection of cytokine mRNA expression

Cytokine mRNA levels were measured using qRT-PCR as previously described [E1].

Briefly, after the evaluation of allergic symptoms on day 32, animals were euthanized and their mesenteric lymph nodes were collected. Total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), and complementary DNA was prepared using PrimeScript RT Master Mix (Takara Bio, Shiga, Japan). Cytokine mRNA levels in the mesenteric lymph nodes were measured by qRT-PCR, using SYBR Premix Ex Taq II master mix (Takara Bio) and a Takara Thermal Cycler Dice system.

β -actin (*Actb*) was used as an internal control. The following primer sets were used:

5'-CATCCGTAAAGACCTCTATGCCAAC-3' and 5'-ATGGAGCCACCGATCCACA-3' for

Actb; 5'-TCTCGAATGTACCAGGAGCCATATC-3' and

5'-AGCACCTTGGAAGCCCTACAGA-3' for *Il4*; 5'-CGGCACAGTCATTGAAAGCCTA-3'

and 5'-GTTGCTGATGGCCTGATTGTC-3' for interferon-gamma (*Infγ*); and

5'-GTGTGGAGCAACATGTGGAACCTCTA-3' and 5'-TTGGTTCAGCCACTGCCGTA-3'

for transforming growth factor-beta (*Tgfb*).

Statistical analysis

All statistical analyses were performed using the EZR software package (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [E3]. Data are expressed as the mean $\pm$ standard error. Differences in the levels of 25(OH)D, OVA- specific IgE, OVA- specific IgG1, mRNA, and 16S rDNA between groups were assessed using a Student's *t*-test. Differences in diarrhea score between groups were assessed using a Mann-Whitney *U* test, and differences in diarrhea occurrence were assessed using Fisher's exact test. P values <0.05 were considered to be statistically significant.

References

- E1. Yamashita H, Takahashi K, Tanaka H, Nagai H, Inagaki N. Overcoming food allergy through acquired tolerance conferred by transfer of Tregs in a murine model. *Allergy* 2012; **67**: 201-9.
- E2. Matsuki T, Watanabe K, Fujimoto J, Miyamono Y, Takada T, Matsumoto K, et al. Development of 16S rRNA-Gene-Targeted Group-Specific Primers for the Detection and Identification of Predominant Bacteria in Human Feces. *Appl Environ Microbiol* 2002; **68**: 5445-51.
- E3. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant* 2013; **48**: 452-8.