

Dietary Vitamin D Supplementation Is Ineffective in Preventing Murine Cow's Milk Allergy, Irrespective of the Presence of Nondigestible Oligosaccharides

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Keywords

Cow's milk allergy · Nondigestible oligosaccharides · Vitamin D · Regulatory T cells · Dendritic cells

Abstract

Introduction: Cow's milk allergy (CMA) is one of the most common food allergies especially early in life. A mixture of nondigestible short-chain galacto-oligosaccharides, long-chain fructo-oligosaccharides, and pectin-derived acidic-oligosaccharides (GFA) may reduce allergy development and allergic symptoms in murine CMA. Recently, vitamin D (VitD) has been suggested to have beneficial effects in reducing allergy as well. **Objective:** In this study, the immune modulatory effect on allergy prevention using the combination of GFA and VitD was investigated. **Methods:** Female C3H/HeOuJ mice were fed a control or GFA-containing diet with depleted, standard (1,000 IU/kg), or supplemented (5,000 IU/kg) VitD content for 2 weeks before and during whey sensitization ($n = 10\text{--}15$). Mice were sensitized 5 times intragastrically with PBS as a control, whey as cow's milk allergen, and/or cholera toxin as adjuvant on a weekly interval. One week after the last sensitization, mice were intradermally challenged in both ear pinnae and orally with whey, subsequently the acute allergic skin response and shock symptoms were

measured. After 18 h, terminal blood samples, mesenteric lymph nodes, and spleens were collected. Whey-specific immunoglobulin (Ig) E and IgG1 levels were measured by means of ELISA. T cell subsets and dendritic cells (DCs) were studied using flow cytometry. **Results:** Additional VitD supplementation did not lower the allergic symptoms compared to the standard VitD diet. CMA mice fed the GFA diet supplemented with VitD (GFA VitD⁺) significantly decreased the acute allergic skin response of whey sensitized mice when compared to the CMA mice fed VitD (VitD⁺) group ($p < 0.05$). The effect of GFA was not improved by extra VitD supplementation even though the CMA mice fed the GFA VitD⁺ diet had a significantly increased percentage of CD103⁺ DCs compared to the VitD⁺ group ($p < 0.05$). The VitD-deprived mice showed a high percentage of severe shock and many reached the humane endpoint; therefore, these groups were not further analyzed. **Conclusions:** High-dose VitD supplementation in mice does not protect against CMA development in the presence or absence of GFA.

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Published by S. Karger AG, Basel

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Edited by: H.-U. Simon, Bern.

Introduction

Food allergy occurs when the immune system incorrectly identifies a food protein as a threat and triggers an immune response, causing sensitization and upon re-exposure, allergic symptoms. Cow's milk allergy (CMA) is one of the most common food allergies in children (3–5%), and symptoms can range from gastrointestinal to dermatological to respiratory manifestations and in the worst case might cause life-threatening anaphylactic shock reactions [1, 2].

Nondigestible oligosaccharides resembling structural and functional aspects of human milk oligosaccharides have been developed, such as short-chain galacto-oligosaccharides (scGOS), long-chain fructo-oligosaccharides (lcFOS), and pectin-derived acidic-oligosaccharides (pAOS) (GFA). A human study has shown positive effects of GFA in allergic disease prevention, particularly in the prevention of atopic dermatitis in infants [3]. However, a recent publication indicated no preventive effect of atopic dermatitis when high-risk children were supplied with a partially hydrolyzed whey formula in which whey proteins are enzymatically processed into smaller fragments when supplemented with the oligosaccharides GFA [4]. In a CMA mouse model, supplementation of GFA to the diet was found to decrease allergic symptoms [3, 5–7]. In the latter study, the dietary intervention was started 2 weeks prior to and continued during allergic sensitization and challenge with cow's milk protein whey, and in this setting, the possible preventive effect of GFA was assessed. These studies revealed that the allergy protective effect of GFA was associated with improved regulatory T cell (Treg) function [8, 9]. Hence, even though lower symptom severity was observed in the latter study, this was the result of the preventive action of the dietary intervention, which was provided as a prophylactic measure.

Vitamin D (VitD) is obtained via the diet or synthesized in the skin upon UV light exposure. VitD is known for its importance in bone mineralization. However, recently VitD has gained attention in terms of its possible role in allergic diseases as well. VitD activating enzymes were found, for example, in epithelial cells, T cells, dendritic cells (DC), and activated macrophages [10, 11]. According to multiple studies, a substantial portion of the world population has low serum VitD levels. Major risk factors for the low or too low VitD status are aging, female gender, living at higher latitudes, winter season, darker skin pigmentation, obesity, and/or low dietary intake [10, 12, 13]. The low VitD levels found in people living further

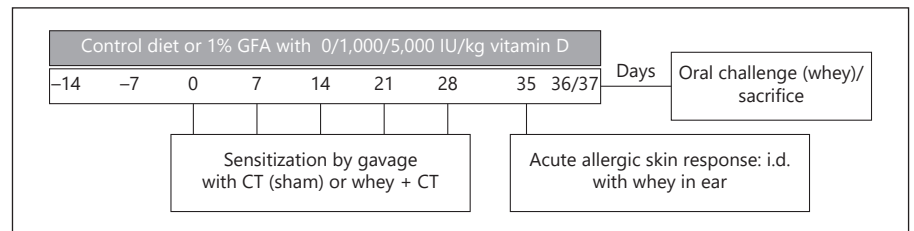
from the equator and the coinciding increase in food allergy prevalence suggest an implication of the influence of VitD on food allergy development [10–12, 14, 15].

Activation of VitD occurs in 2 steps: first in the liver, it is modified to a prohormone, followed by conversion to the biological active hormone in the kidneys [16]. As mentioned above, VitD activation also occurs in structural cells and cells of the immune system [10, 11]. After VitD is enzymatically activated, it will bind to the nuclear vitamin D receptor (VDR); the VDR will form a homodimer or a heterodimer with retinoic x receptor bound to retinoic acid to activate specific genes in the nucleus. The VDR/retinoic x receptor complex can bind with high affinity to specific genes to up- or downregulate transcription. Detailed information concerning VitD receptor functions, regulation, and binding possibilities can be found in reviews by Christakos et al. [17] and Martens et al. [18].

VitD signaling leads to tolerogenic immature interleukin (IL)-10 producing DC, which favors Treg upregulation and reduces T cell activation [11, 15, 19, 20]. Simultaneously, DCs interacting with VitD have a better migratory pattern toward lymph nodes and Peyer's patches [20]. Not only is T cell activation reduced by VitD exposed DC but also VitD has a direct influence on T cells. VitD can directly inhibit IL-2, interferon γ (IFN- γ), and IL-17 production for T helper (Th) 1 and Th17 cells, and upregulates IL-10 [11, 21, 22]. On the other hand, when VitD and transforming growth factor β were added to human CD4⁺ T cells in a cell proliferation assay, more CD25⁺ Foxp3⁺ cell expansion and more IL-2 production were measured [23]. For the regulation of Th2 cytokines IL-4 and IL-13 by VitD, data remain inconclusive [10, 17, 21–24]. VitD can also enhance antimicrobial mechanisms via increasing various antimicrobial peptides, strengthening beneficial microbiota, as well as improving the intestinal epithelial barrier function [19, 24–27]. VitD can, for example, upregulate tight junction proteins in the intestinal epithelium to maintain adequate barrier integrity [19, 25]. In a colitis mouse model, it was shown that providing extra VitD can still positively regulate beneficial bacteria even when the VitD receptor is knocked out [28].

Due to differences in design and the small number of studies with well-defined clinical parameters to determine VitD effects, the relationship between VitD and food allergy is unclear [22, 24, 29]. There are studies suggesting a role for VitD not only in the DC-T cell interaction, but also a regulatory role in B cell-mast cell interaction [11, 30]. VitD can stabilize mast cells in vitro by bind-

Fig. 1. Design of experiment. GFA, short-chain galacto-oligosaccharides (scGOS), long-chain fructo-oligosaccharides (lcFOS), and pectin-derived acidic-oligosaccharides (pAOS); CT, cholera toxin.



ing to intracellular signaling protein Lyn, which inhibits mast cell degranulation [31]. Upon dietary VitD supplementation in vivo, immunoglobulin (Ig)-E mediated mast cell activation of ovalbumin (OVA)-treated mice is also inhibited [31]. In another study using OVA allergic mice, an association between VitD deficiency and exacerbation of OVA-induced allergy was shown. OVA-specific IgE and IgG1, *Il4* mRNA levels in mesenteric lymph nodes (MLN), and diarrhea score as indicators for allergic sensitization or symptoms were found to be enhanced [11, 14]. In OVA allergic children who developed natural tolerance to egg, increased serum VitD levels were associated with changes in innate immune profiles [32].

Even though VitD may affect the infant's immune function, its role in food allergy development remains unclear. A recent study in a small cohort of egg allergic children suggested that VitD insufficiency did not correlate with food allergy [33]. However, VitD insufficiency in Asian children was found to correlate with a risk for allergic sensitization to cow's milk at the age of 2 [34]. To further study the contribution of VitD depletion or supplementation in allergy development, the current study investigates the role of VitD in the prevention of CMA in the presence or absence of dietary GFA supplementation in mice prior to and during sensitization and challenge with whey protein.

Materials and Methods

Diets

The control diet, supplied by Research Diet Services, Wijk bij Duurstede, the Netherlands, is a semisynthetic cow's milk free AIN93G chow, in which casein and whey are replaced by soy proteins [35]. The diet contains no VitD and "standard" 1,000 IU VitD/kg, while the VitD-supplemented diet contains 5,000 IU VitD/kg. The deprivation dose was started upon arrival when mice were 4 weeks of age. Before that, the mice received normal VitD amounts from their mothers and standard chow without casein and whey. For the supplementation dose, 5 times supplementation of the amount compared to control was chosen, because no direct adverse effects have been found in mice using this approach as shown by Agrawal et al. [36]. The prebiotic diet is a control diet with standard or high VitD and additional isocaloric supplementa-

tion of 1% GFA in a 9:1:2 ratio (scGOS [Vivinal, GOS; FrieslandCampina Domo, Zwolle, The Netherlands], lcFOS [Raftiline HP; Orafit, Wijchen, the Netherlands], pAOS [Südzucker, Mannheim, Germany]).

Animals

Upon arrival, 4-week-old (>11 g bodyweight), specific pathogen-free female C3H/HeOJ mice (Charles River, Sulzfeld, Germany) were fed control diet, diet supplemented with GFA, diet supplemented with VitD, or diet supplemented with VitD and GFA throughout the study (Fig. 1). Food and tap water were available ad libitum. Mice ($n = 10\text{--}15$ per group) were randomly allocated to a cage and group housed (5/cage) in Makrolon type III cages per treatment, with 9 kGy irradiated sawdust bedding (Lignocel 9 s; J. Rettenmaier & Söhne GmbH, Rosenberg, Germany), 2 red-transparent polycarbonate cages as environmental enrichment, and no nesting material due to the potential effect on allergy parameters [37] at the animal facility (Intravacc, Bilthoven, the Netherlands). Mice were on light/dark cycle of 12 h/12 h and 65–70% relative humidity. Animal procedures, housing, and care were performed in accordance with the EU guidelines (2010/63/EU) and approved by a licensed Animal Ethics Committee (DEC-Consult, Soest, the Netherlands).

Oral Sensitization and Challenge of Mice

After 2 weeks, mice were sensitized weekly for 5 times with 10 μg cholera toxin (List Biological Laboratories, Campbell, CA, USA) or 20 mg whey (sweet whey protein concentrate 60 [sWPC60]; Milei, Leutkirch im Allgäu, Germany) + 10 μg cholera toxin per 500 μL DPBS (Life Technologies, Inc., Invitrogen, Carlsbad, CA, USA) per mouse per oral gavage using a blunt needle. After the third sensitization, temperature transponders (IPTT-300; BMDS, Seaford, DE, USA) were injected subcutaneously into the mice under isoflurane. At day 35 following initial sensitization, whey protein was intradermally (i.d.) injected (10 μg whey product/20 μL PBS/ear) into the ear pinnae and the corresponding delta (Δ) ear swelling at 1 h was measured as readout for the local activation of mast cells. One day after the ear challenge, 18 h before termination, the mice were challenged intragastrically (i.g.) with 50 mg whey product/500 μL DPBS to determine the mast cell degranulation. Finally, at day 37, blood and MLN were isolated under terminal anesthesia and stored for further analysis (Fig. 1).

Evaluation of the Skin Allergic Response

To measure the acute allergic skin response, mice were challenged intradermally in the ear pinnae with 10 μg whey protein per ear. Ear thickness was recorded before and 1 h after the intradermal challenge using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands), and the acute skin response was calculated as

Table 1. Decision tree on the monitoring of well-being during the development of anaphylactic shock following intradermal challenge adapted from van Esch et al. [5]

Anaphylactic shock score	Body temperature >32°C	Body temperature ≤32°C
0 no subjective symptoms	No action	Heating pad Unlikely to occur
	Mouse is likely to recover within 60 min	Mouse is likely to recover within 60 min Repositioning to home cage when body temperature ≥35°C
1 scratching around nose and/or mouth	No action	Heating pad Unlikely to occur
	Mouse is likely to recover within 60 min	Mouse is likely to recover within 60 min Repositioning to home cage when body temperature ≥35°C
2 swollen eyes and/or mouth, piloerection, reduced mobility, increased breath frequency	No action	Heating pad
	Mouse is likely to recover within 60 min	Mouse is likely to recover within 60 min Repositioning to home cage when body temperature ≥35°C
3 shortness of breath and/or increased breath frequency, bluish color around mouth and tail, further reduced/painful mobility	No action	Heating pad
	Unlikely to occur	Mouse is likely to recover within 60 min
	Mouse is likely to recover within 60 min	Repositioning to home cage when body temperature ≥35°C
4 no mobility following stimulation, convulsions	Humane endpoint	Humane endpoint
	Unlikely to occur	
	Mouse not likely to recover and is actively euthanized	Mouse not likely to recover and is actively euthanized

The severity of the anaphylactic shock as well as the body temperature were monitored at level of behavior and mobility every 15 min following intradermal challenge.

Δ = ear thickness at 1 h – basal ear thickness and is expressed as delta micrometer. The body temperature and the anaphylactic shock symptoms were scored according to the decision table as shown (Table 1), which is adapted from the method previously described by van Esch et al. [5].

Whey-Specific Igs and Mouse Mast Cell Protease-1 in Serum

Serum whey-specific Igs were quantified by means of an ELISA as previously described with few modifications [38]. Briefly, high-binding 96-well plates (Costar® Assay Plate, Corning, NY, USA) were coated with whey (20 mg/L) overnight, washed, and blocked with 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA). Three different dilutions of the samples and standards were added and incubated for 2 h and washed. This was followed by application of biotin-labelled rat anti-mouse IgE, IgG1, or IgG2a (1 mg/L; Becton Dickinson, Temse, Belgium) and incubation for 1 h. The plates were washed, incubated for 1 h

with streptavidin peroxide (Sanquin, Amsterdam, The Netherlands) in the dark, washed, and incubated with 3,3',5,5'-tetramethylbenzidine 1-step substrate (Thermo Scientific) for up to 15 min. The reaction was stopped with 10% sulfuric acid (Sigma-Aldrich, Steinheim, Germany), followed by measurement at 450 nm with microplate reader (BioTek, PowerWave HT, Vermont, USA). The mouse mast cell 1 (mMCP1) concentrations were determined using a Mouse MCPT-1 ELISA Ready-SET-Go!® kit (eBioscience, San Diego, CA, USA), using a protocol obtained from the manufacturer.

Isolation of Lymphocytes

To remove RBCs, spleens were lysed according to Kostadinova et al. [39]. Single cell suspensions of MLNs and spleens were made with a 70-µm cell strainer (Thermo Fisher Scientific, Amsterdam, the Netherlands) and then resuspended in RPMI 1640, 10% fetal bovine serum, and penicillin (100 U/mL)/streptomycin (100 µg/

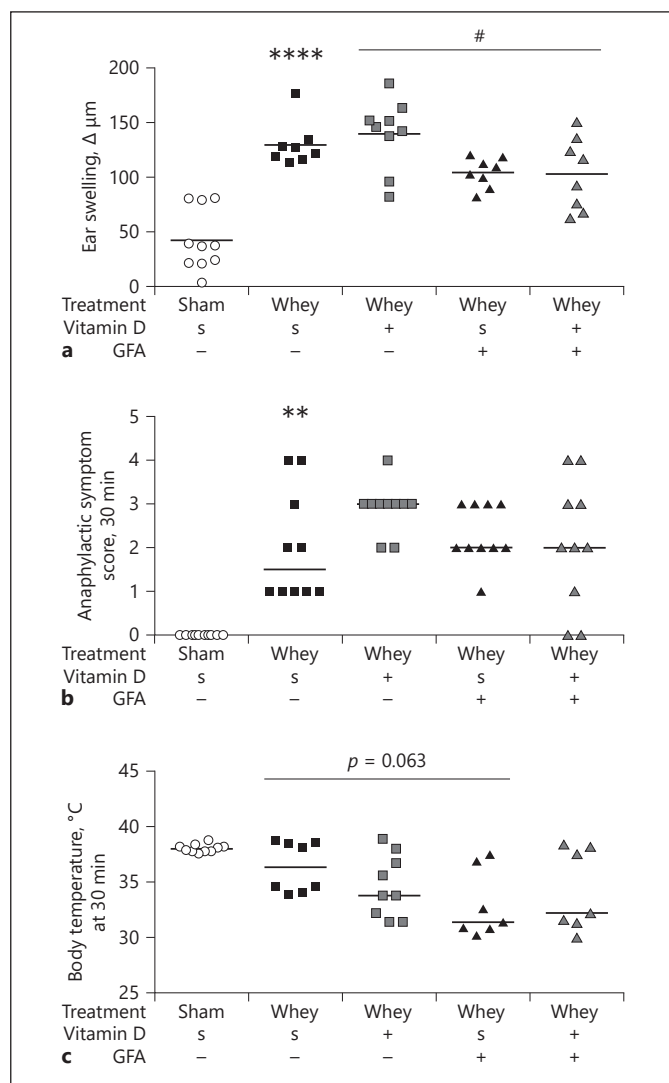


Fig. 2. Clinical symptoms in sham- and whey-sensitized mice fed a control or GFA diet with standard (s) or extra (+) vitamin D. The acute allergic skin response (**a**) was measured 1 h after i.d. injection. Values are means \pm SEM, $n = 8-10$, a one-way ANOVA and Bonferroni post hoc test were used. Δ indicates ear thickness before i.d. injection deducted from thickness after i.d. injection. The anaphylactic symptom scores (**b**) of sham-sensitized mice fed control diet and whey-sensitized mice fed control or GFA diet with standard (s) or extra (+) vitamin D, $n = 8-10$. Data were calculated with the Kruskal-Wallis and Dunn's post hoc tests with preselected pairs. The body temperature (**c**) was measured 30 min after i.d. injection via transponder readout of sham-sensitized mice fed control diet and whey-sensitized mice fed control or GFA diet with standard (s) or extra (+) vitamin D. Values are means \pm SEM, $n = 8-10$, a one-way ANOVA and Bonferroni post hoc test were used to calculate the dataset. Asterisks indicate a significant difference compared to sham-sensitized mice, **** $p < 0.0001$, ** $p < 0.01$. Hash tag indicates significance compared to whey-sensitized mice on a control diet, # $p < 0.05$. GFA, short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic-oligosaccharides; s, standard 1,000 IU/kg vitamin D; +, 5,000 IU/kg vitamin D.

mL). The cells were quantified using a Coulter Z1 particle counter (Beckmann Coulter, Brea, CA, USA).

Flow Cytometry of Immune Cells

One million cells per well were added to polypropylene V-bottom 96-well plates (BD Biosciences, Heidelberg, Germany) and blocked with rat anti-mouse CD16/32 (BD Biosciences, Heidelberg, Germany) for 20 min at 4°C to block nonspecific binding sites. For surface staining, the cells were incubated with the different antibodies of the staining panels for 30 min at 4°C . After the antibody incubation, the cells were fixed using BD cell fix (Becton Dickinson, Temse, Belgium). The Th1/Th2 and Th17/Treg staining contained intracellular markers (Tbet, Gata3, Ror γ t, and Foxp3); therefore, directly after the staining procedure for extracellular markers, these cells were permeabilized using a fixation/permeabilization buffer (eBioscience, San Diego, CA, USA) overnight. The next day, the cells were blocked again and incubated with the intracellular marker antibodies for 30 min at 4°C . Then, the cells were fixed with the BD cell fix. Fluorescence minus ones and unstained cells were used as controls. In addition, compensation staining with UltraComp beads (eBioscience, San Diego, CA, USA) was performed to correct for the spectral overlap, which occurs in multicolor staining. Antibodies used were CD8a-APC-Cy7, CD11c-PerCP-Cy5.5, and CD25-Pe-Cy7 from BD Biosciences (San Jose, CA, USA) and CD4-PerCP-Cy5.5, CD69-Pe-Cy7, CD11b-PE, CD103-APC, CX3CR1-FITC, B220-APC, IL-10-PE, IFN- γ -APC, IL-4-Pe-Cy7, IL-17 α -FITC, Ror γ -Pe, Gata3-PE, Tbet-APC, and Foxp3-APC from eBioscience (San Diego, CA, USA). The analysis of the stained cells was performed using FACS Canto II cytometer (BD Biosciences, San Jose, CA, USA) and FACSDiva software (BD Biosciences, Heidelberg, Germany).

Statistical Analysis

The data are represented as the mean, mean \pm SEM, or Tukey box-and-whisker plots. Not normally distributed data were log transformed. Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). One-way ANOVA and Bonferroni multiple comparison post-test comparing selected groups were used to analyze the data. Selected groups are sham versus allergic control mice, allergic control mice versus allergic control mice fed VitD⁺, allergic control mice versus allergic control mice fed GFA, allergic control mice fed VitD⁺ versus allergic control mice fed GFA VitD⁺, and allergic control mice fed GFA versus allergic control mice fed GFA VitD⁺. The Kruskal-Wallis test was used to analyze the anaphylactic shock score, followed by Dunn's multiple comparison post-test, because the shock score data are not normally distributed. p value < 0.05 was considered significant.

Results

Allergic Clinical Symptoms

To measure clinical symptoms, the acute allergic skin response, anaphylaxis (Table 1), and body temperature were measured. The acute allergic skin response, measured as Δ ear swelling, and anaphylactic symptom score were significantly increased in whey-sensitized mice (al-

Table 2. Total number of mice per group suffering from anaphylactic shock symptoms during 1 h after the intradermal ear challenge

Vitamin D	Amount in chow; intake	GFA	% anaphylactic shock (mice/ total)	% humane endpoint (mice/mice with shock)
Deprivation	0 IU/kg; 0 IU/day	–	100 (15/15)	40 (6/15)
		+	89 (8/9)	38 (3/8)
Normal	1,000 IU/kg [35]; ≈5 IU/day	–	60 (6/10)	30 (2/6)
		+	90 (9/10)	22 (2/9)
Supplementation	5,000 IU/kg [36]; ≈25 IU/day	–	100 (10/10)	10 (1/10)
		+	80 (8/10)	25 (2/8)

lergic control mice) compared to sham-sensitized mice fed control diet (sham) ($p < 0.01$) (Fig. 2a, b). VitD supplementation in whey-sensitized mice fed the GFA diet (allergic control mice fed GFA VitD⁺) significantly decreased the allergic skin response compared to the VitD supplemented mice fed the control diet (allergic control mice fed VitD⁺) ($p < 0.05$). Nine mice, fed food deprived of VitD, reached the humane endpoint during the 60 min establishment of the clinical allergic symptoms, which was calculated as a loss of 38–40% in the VitD-deprived group versus 22–30% in the CMA mice fed the standard VitD levels (Table 2). These VitD-deprived mice were not included in the rest of the data.

The anaphylactic shock scores, measured at 30 min, of the groups fed the GFA diet with or without VitD were not significantly different from sham (Fig. 2b). Furthermore, the body temperature of the mice was monitored during the acute allergic skin response to determine systemic shock symptoms (Fig. 2c). No significant differences were observed between groups with regard to body temperature; however, in the GFA group, the body temperature tended to be lower than the allergic controls fed standard diet. No significant differences were measured in bodyweight (data not shown).

Mouse Mast Cell Protease and Whey-Specific Igs

To investigate whether the Th2- and Th1-type humoral responses are related to the differences in acute allergic skin responses of the different groups, whey-specific IgE, IgG1, and IgG2a were measured. The whey-specific IgE and IgG1 levels were significantly increased in the allergic control mice when compared to sham ($p < 0.01$) (Fig. 3a, b). However, the whey-specific IgE and IgG1 levels were not affected by the GFA diet with or without VitD supplementation. The concentration of mMCP-1, which is a

mediator released by mucosal mast cells upon degranulation, and whey-specific IgG2a levels were not increased in the allergic versus sham control mice and not affected by the diet (data not shown).

T Cells and DCs

The effect of the diets on Th1 (CD4⁺ Tbet⁺), Th2 (CD4⁺ Gata3⁺), Treg (CD4⁺ CD25⁺ Foxp3⁺), CD11c⁺ CD103⁺ DC and plasmacytoid DCs (pDC) (CD11c^{low} B220⁺) was determined in spleen and MLN. No significant differences were observed in the percentages of Th1 and Th2 in the spleen (Fig. 4a) and MLN (Fig. 4b), and no significant differences were observed in pDC and Treg cell frequency for both the spleen (Fig. 5a) and MLN (Fig. 5b). There was a significantly higher percentage of CD11c⁺ CD103⁺ DC in the allergic mice fed GFA VitD⁺ group compared to the allergic mice fed VitD⁺ ($p < 0.05$) (Fig. 5b).

Discussion

VitD is known to modulate the function of the innate and adaptive immune system. In the current study, it was investigated whether VitD deprivation or supplementation could affect CMA development in the presence or absence of dietary nondigestible oligosaccharide mixture GFA.

Normal VitD levels are for 90% acquired via UV-B radiation-mediated conversion of cholesterol in the skin and further activation in the liver and kidney to form the biologically active 1,25-dihydroxy VitD, while 10% is acquired via dietary intake in humans. The advised human VitD levels are optimized for bone health, and it is unknown if these levels are also adequate for optimizing im-

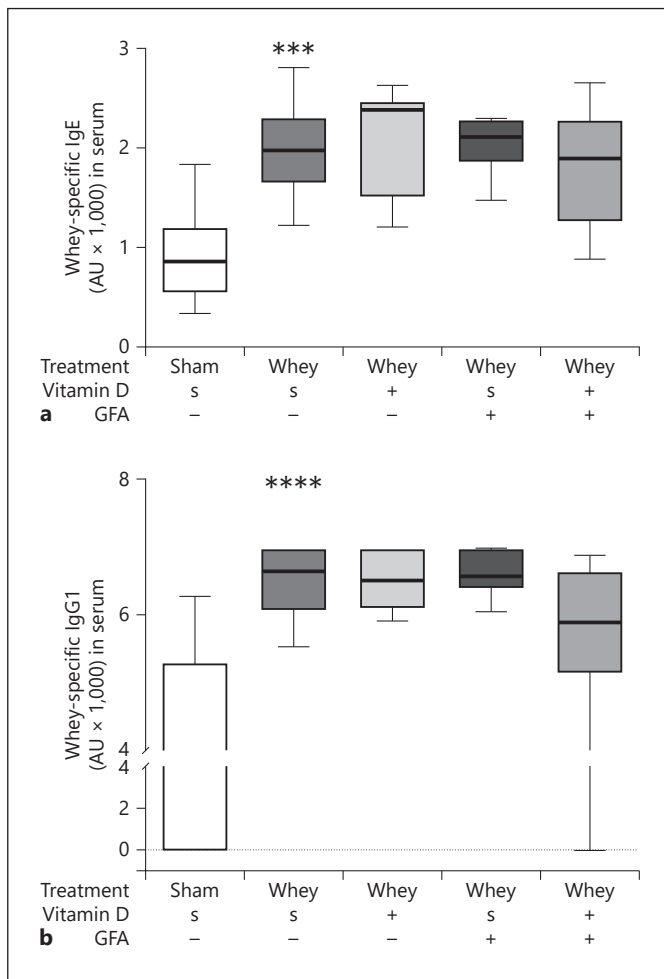


Fig. 3. Whey-specific IgE and IgG1 measured in serum of mice. Whey-specific IgE (AU \times 1,000) (**a**) and whey-specific IgG1 (AU \times 1,000) (**b**) were measured in serum from sham-sensitized mice fed control diet and whey-sensitized mice fed control or GFA diet with standard (s) or extra (+) vitamin D. Values are means \pm SEM, $n = 8$ –10. Data were calculated with a one-way ANOVA and Bonferroni post hoc test, if required, log transformation was used to normalize data distribution. Asterisks indicate a significant difference compared to sham-sensitized mice, **** $p < 0.0001$, *** $p < 0.001$. GFA, short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic-oligosaccharides; IgE, immunoglobulin E; IgG1, immunoglobulin G1; AU, arbitrary units; s, standard 1,000 IU/kg vitamin D; +, 5,000 IU/kg vitamin D.

immune properties. Furthermore, VitD has a “U”-shaped effectiveness curve, too little or too much is not desirable [10, 15]. In mice not much is known regarding the acquisition and processing of VitD. VitD dosages applied in standard mouse chow are extrapolated from studies in rats [40]. The dosage used in this study, five times higher than control, was previously shown not to cause adverse

effects in mice [36]. For mice, just as for humans, VitD is an essential vitamin for many body functions. The VitD mice in the current study were supplied with VitD-depleted chow starting one week after weaning; hence, in the first three weeks of life, they had access to VitD supplies via their mother and chow. VitD is a fat-soluble vitamin, which can be stored in fatty tissues to form a depot.

Previously, an allergy preventive effect of GFA was shown; however, in the current study, GFA alone did not significantly suppress the allergic symptoms in CMA mice [5, 8, 41, 42], although it lowered the acute allergic skin response in the GFA VitD + group than in the VitD + group. In addition, VitD supplementation did not beneficially affect the clinical symptoms such as the acute allergic skin response and anaphylactic shock. However, the percentage of mice that were actively euthanized due to severe shock upon intradermal whey injection was highest in the CMA mice deprived from VitD fed with or without GFA. This indicates that VitD amounts below the standard levels in chow may negatively affect the immune status of the mice and promote the development of severe allergic symptoms. This is in line with study of Matsui et al. [14], where the VitD-deprived mice showed worse OVA allergy via increased OVA-specific Ig levels. In a C57BL/6 VitD-deficient mouse model, it was shown that eosinophilic cells activate spontaneously and subsequently release inflammatory mediators, which also cause intestinal epithelial barrier dysfunction [43]. Furthermore, Liu et al. [31] showed positive inhibitory effects of VitD supplementation on allergic symptoms in an OVA Balbc mouse model; however, in these studies, histamine and tumor necrosis factor- α were measured in serum as a reflection of mast cell activation. In the current study, no significant difference in whey-specific IgE was observed (data not shown).

VitD supplementation in the presence or absence of GFA did not have a significant effect on activated Th2 or Th1 cells in this model. Also no difference in Th cells between the control and CMA mice was observed. In a previous study, it was shown that CMA mice fed GFA had a higher increase of Th1 *Tbet* mRNA compared to Th2 *Gata3* mRNA [41]. The diets did not affect the percentage of CD25⁺ Foxp3⁺ Treg cells and pDC in this study. However, the percentage of CD11c⁺ CD11b⁺ CD103⁺ DC was significantly increased in the allergic GFA VitD⁺ group when compared to the allergic mice fed VitD⁺. This subtype of DC may have tolerogenic properties and support the generation of functional Treg, which may be capable of suppressing mast cell degranulation and subsequently decrease the acute allergic skin response [44–46]. VitD

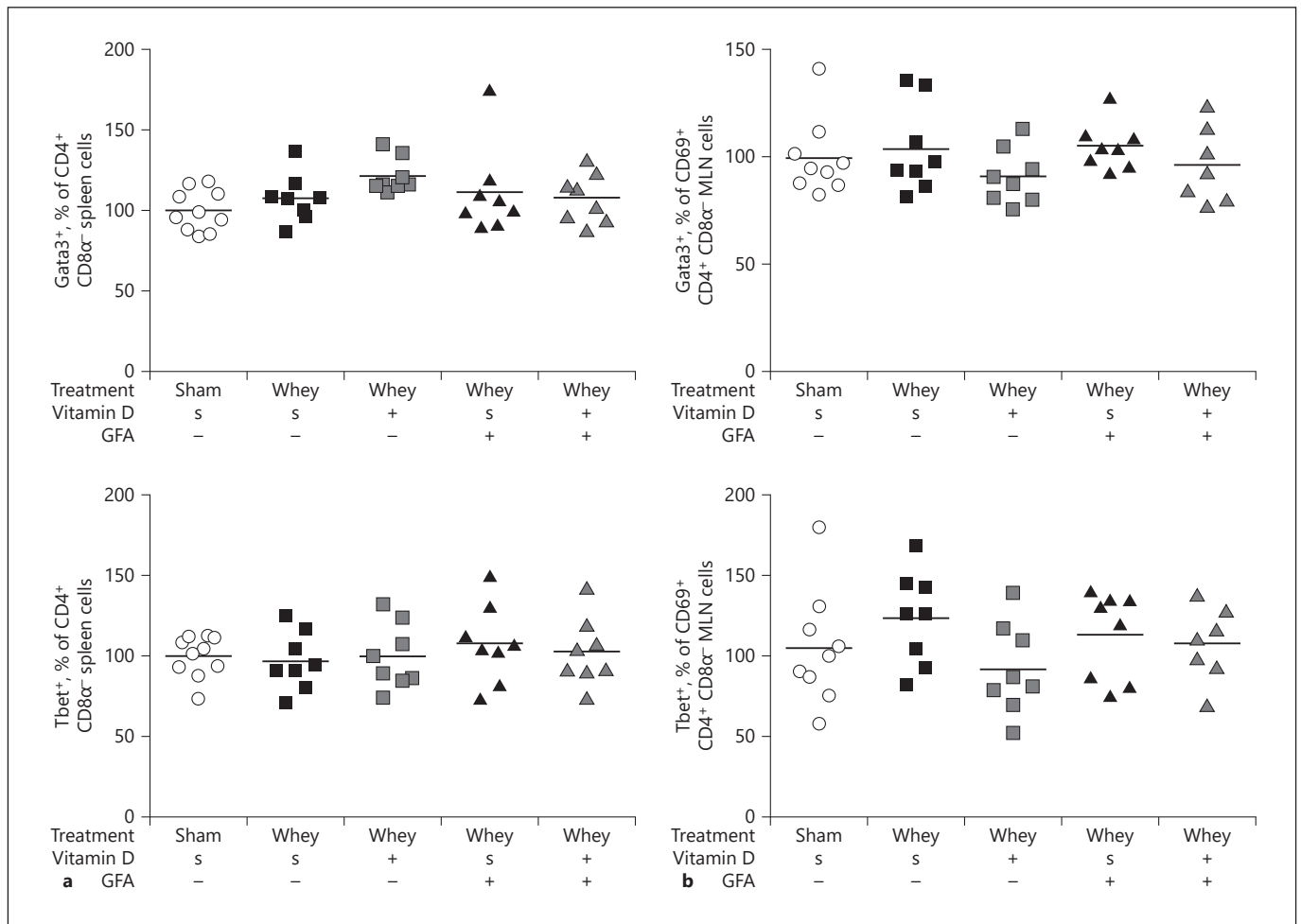


Fig. 4. T helper (Th) cell population, Th1 and Th2, measured in the spleen (**a**) and MLN (**b**). Spleens (**a**) were removed after sacrifice of mice and stained for CD4-PERCP-Cy5.5⁺ CD8α-APC-Cy7⁻, and then 2 subpopulations were gated, one for Th1, the intracellular Tbet-APC⁺, and the other for Th2, the intracellular Gata3-PE⁺. MLNs (**b**) were removed after sacrifice of mice and stained for CD4-PERCP-Cy5.5⁺ CD8α-APC-Cy7⁻, then for CD69-Pe-Cy7⁺ and subsequently for 2 subpopulations, one for Th1, the intracellular Tbet-APC⁺, and the other for Th2, the intracellular Gata3-PE⁺. Representative flow cytometric plots for spleen and

MLN are shown in online suppl. Fig. 2 and 3; for all online suppl. material, see www.karger.com/doi/10.1159/000509750. Results are shown in percentage increase or decrease compared to the mean of sham-sensitized mice fed control diet. Values are means ± SEM, *n* = 8–10, and were calculated with a one-way ANOVA and Bonferroni post hoc test. CD, cluster of differentiation; GFA, short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic-oligosaccharides; s, standard 1,000 IU/kg vitamin D; +, 5,000 IU/kg vitamin D.

indeed has been shown to regulate different dermal and epidermal DC subsets and even to induce transforming growth factor β-dependent Foxp3 Treg or IL-10-dependent IL-10⁺ Treg through the modulation of DC function [23, 47]. However, in the current study, intracellular IL-10 levels in DC in the MLN or spleen remained below detection (data not shown). Although we did not observe any significant differences in pDC, another study suggests that VitD can regulate pDC function [48]. In the latter study, bone marrow-derived DCs were used and a

tumor mouse model was used to multiply pDC in lymphoid organs, which is quite different compared to the food allergy model [48].

Still, very little is known about the effect of VitD status on food allergy development. In this study, we evaluated the effect of dietary VitD supplementation or deprivation in a murine model of CMA. VitD deprivation may enhance allergic symptoms, since 38–40% of CMA mice fed the VitD-deprived diet had very severe anaphylactic shock manifestations, while this was 22–30% in CMA

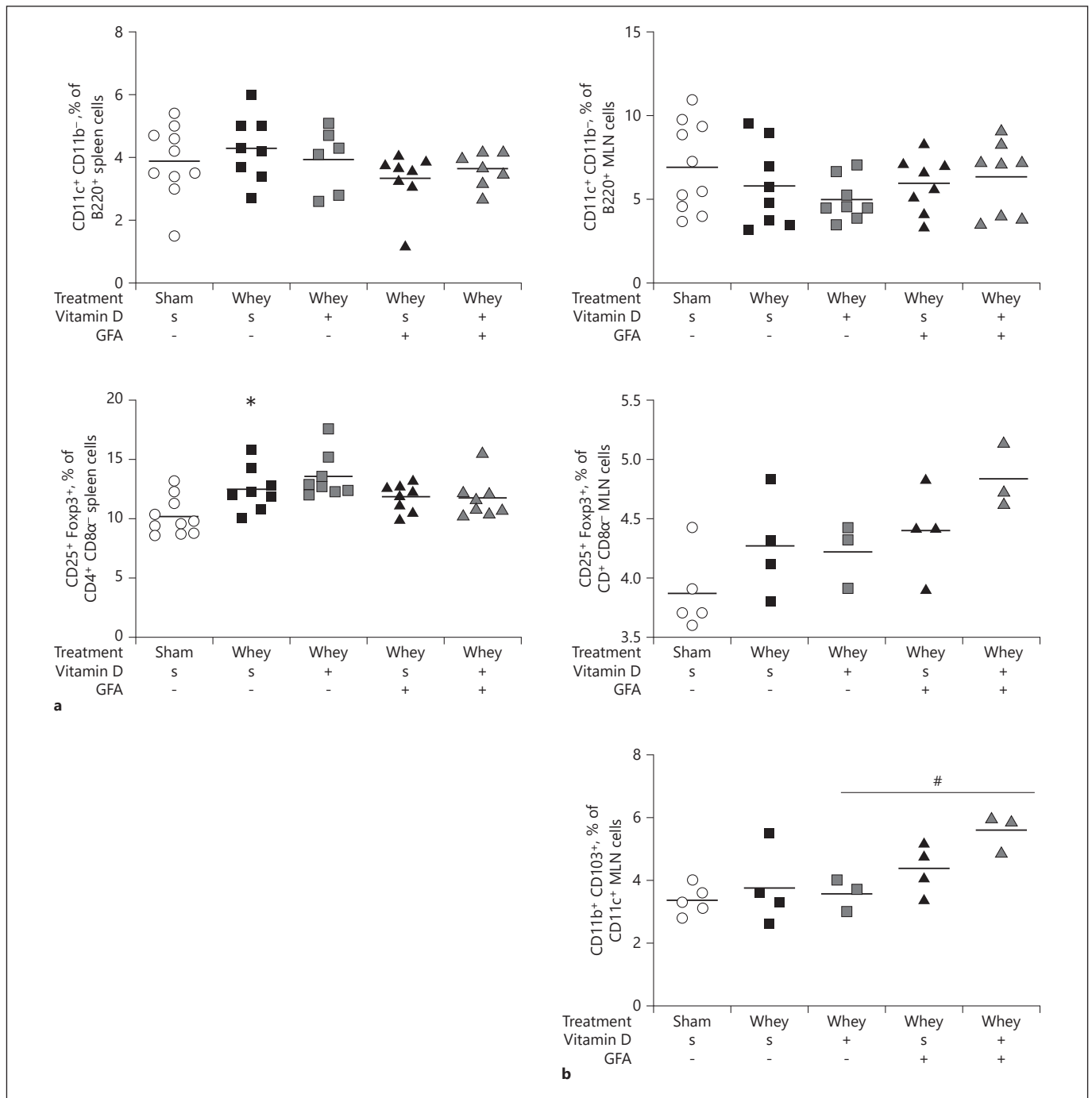


Fig. 5. Plasmacytoid and CD103⁺ dendritic cell population and Foxp3⁺ CD25⁺ regulatory T cell population measured in the spleen (**a**) and MLN (**b**). Spleen (**a**) was stained for plasmacytoid DC (pDC), B220-APC-Cy7⁺, CD11c-PERCP-Cy5.5⁺ CD11b-PE⁻, and for Treg cells, CD4-PERCP-Cy5.5⁺ CD8α-APC-Cy7⁻, Foxp3-APC⁺ CD25-PE-Cy7⁺. MLNs (**b**) were removed after sacrifice of mice and stained for pDC, B220-APC-Cy7⁺, CD11c-PERCP-Cy5.5⁺ CD11b-PE⁻; for migratory DC, CD11c-PERCP-Cy5.5⁺, CD103-APC⁺, CX-3CR1-FITC⁻ and CD11b-PE⁺, CD8α-APC-Cy7⁻; and for Treg cells, CD4-PERCP-Cy5.5⁺ CD8α-APC-Cy7⁻, Foxp3-APC⁺ CD25-

PE-Cy7⁺. Values are means ± SEM, *n* = 3–10, and were calculated with a one-way ANOVA and Bonferroni post hoc test. For the MLN for the CD103 as well as the Treg population, only data of 1 cohort were available. Representative flow cytometric plots are shown in online suppl. Fig. 4. Asterisk indicates a significant difference compared to sham-sensitized mice, #*p* < 0.05. CD, cluster of differentiation; GFA, short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic-oligosaccharides; s, standard 1,000 IU/kg vitamin D; +, 5,000 IU/kg vitamin D.

mice fed standard chow with normal VitD levels. However, extra supplementation of VitD did not protect against CMA development. As previously shown, dietary supplementation with GFA lowered the development of allergic symptoms in the CMA mice. Extra VitD supplementation did not further enhance the allergy protective effect of GFA-containing diet in CMA.

Statement of Ethics

Animal procedures, housing, and care were performed in accordance with the EU guidelines (2010/63/EU) and approved by a licensed Animal Ethics Committee (DEC-Consult, Soest, the Netherlands). This is also described in the manuscript.

Conflict of Interest Statement

J.K.: No conflict of interest. D.V.-G. was employed by Nutricia Research and reports personal fees and nonfinancial support from Nutricia Research during the conduct of the study, and provided help conducting the study and with the writing of the manuscript. T.W. is employed by Nutricia Research and reports personal fees and nonfinancial support from Nutricia Research during the conduct of the study, and provided help conducting the study and with the writing of the manuscript. A.O.: No conflict of interest. P.V.J. was employed by Nutricia Research and reports personal fees and nonfinancial support from Nutricia Research during the conduct of the study, and provided help conducting the study and with the writing of the manuscript. J.G. is partly employed by Nutricia Research and reports personal fees and nonfinancial support from Nutricia Research during the conduct of the study, and

provided help conducting the study and with the writing of the manuscript. In addition, the author has a patent nondigestible oligosaccharides for oral induction of tolerance against dietary proteins pending to Nutricia Research, the Netherlands. L.M.J.K. is employed by Nutricia Research and reports personal fees and nonfinancial support from Nutricia Research during the conduct of the study, and provided help conducting the study and with the writing of the manuscript. In addition, the author has a patent nondigestible oligosaccharides for oral induction of tolerance against dietary proteins pending to Nutricia Research, the Netherlands. L.E.M.W.: No conflict of interest. The work was conducted within the framework of a strategic alliance between the Division of Pharmacology of the Utrecht University and Nutricia Research B.V., and the author is employed at the Utrecht University within this context.

Funding Sources

These studies were financed by the Utrecht University and are performed within the framework of a strategic alliance between the Division of Pharmacology of the Utrecht University and Nutricia Research B.V.

Author Contributions

J.K., D.V.-G., P.V.J., L.M.J.K., and L.E.M.W. designed research; J.K., D.V.-G., T.W., and A.O. conducted research; J.K., D.V.-G., A.O., and L.E.M.W. analyzed data and/or performed statistical analysis; J.K., D.V.-G., P.V.J., J.G., L.M.J.K., and L.E.M.W. wrote the paper or critically read the paper; and J.K. and L.E.M.W. had primary responsibility for final content. All authors have read and approved the final manuscript.

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