Research Article

Dietary Vitamin D Increases Percentages and Function of Regulatory T Cells in the Skin-Draining Lymph Nodes and Suppresses Dermal Inflammation

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Skin inflammatory responses in individuals with allergic dermatitis may be suppressed by dietary vitamin D through induction and upregulation of the suppressive activity of regulatory T (T_{Reg}) cells. Vitamin D may also promote T_{Reg} cell tropism to dermal sites. In the current study, we examined the capacity of dietary vitamin D_3 to modulate skin inflammation and the numbers and activity of T_{Reg} cells in skin and other sites including lungs, spleen, and blood. In female BALB/c mice, dietary vitamin D_3 suppressed the effector phase of a biphasic ear swelling response induced by dinitrofluorobenzene in comparison vitamin D_3-deficient female BALB/c mice. Vitamin D_3 increased the percentage of T_{Reg} (CD3+CD4+CD25+Foxp3+) cells in the skin-draining lymph nodes (SDLN). The suppressive activity of T_{Reg} cells in the SDLN, mesenteric lymph nodes, spleen, and blood was upregulated by vitamin D_3. However, there was no difference in the expression of the naturally occurring T_{Reg} cell marker, neuropilin, nor the expression of CCR4 or CCR10 (skin-tropic chemokine receptors) on T_{Reg} cells in skin, SDLN, lungs, and airway-draining lymph nodes. These data suggest that dietary vitamin D_3 increased the percentages and suppressive activity of T_{Reg} cells in the SDLN, which are poised to suppress dermal inflammation.

1. Introduction

Vitamin D plays an intrinsic role in shaping innate and adaptive immune responses [1, 2]. Vitamin D is produced following skin exposure to ultraviolet B photons of sunlight, resulting in the conversion of the precursor 7-dehydro-cholesterol into vitamin D_3, which can also be acquired through dietary supplementation. The vitamin D-binding protein (VDBP) transports much of this vitamin D_3 into the liver, where a hydroxylation reaction converts vitamin D_3 into 25-hydroxyvitamin D_3 (25(OH)D_3). This form of vitamin D_3 is found at nanomolar levels in blood, and because of its relative stability and longer half-life, it is used as a measure of vitamin D sufficiency, with 50 nM currently considered the tipping point for insufficiency by the National Institute of Health [3] (although this remains controversial [4]). In renal proximal tubule epithelial cells, and other cells including disease-activated macrophages (reviewed in [5]), 25(OH)D_3 is converted into the most active vitamin D metabolite, 1,25-dihydroxyvitamin D_3 (1,25(OH)$_2D_3$). It is this form of vitamin D_3 which has the most potent effects on regulating immune responses, with circulating levels in the picomolar range [1, 6].

Central to the ability of 1,25(OH)$_2D_3$ to modulate immune responses are changes to regulatory T cells (T_{Reg} cells) and dendritic cells (DCs) [7]. Topical (skin) application of 1,25(OH)$_2D_3$ enhanced the suppressive capacity [8, 9] and proliferative activity [10] of CD4+CD25+Foxp3+ T_{Reg} cells. Stimulation of DCs with bacterial products like lipopolysaccharide or cytokines like transforming growth factor-ß may result in the synthesis of 1,25(OH)$_2D_3$ from circulating 25(OH)D_3, promoting T_{Reg} cell activity (reviewed in [1, 2]). The VDBP may also play an important role in this process, whereby high affinity VDBP can prevent conversion of 25(OH)D_3 to 1,25(OH)$_2D_3$ by DCs and thus their ability to modulate T_{Reg} cell activity [11]. With the right costimulators,

including interleukin-2, $1,25(OH)_2D_3$ can modulate the suppressive functions of T_{Reg} cells independently of DCs [12].

While the capacity for $1,25(OH)_2D_3$ to regulate adaptive immunity through its effects on T_{Reg} cells and DCs is clear, most studies have used supraphysiological levels of $1,25(OH)_2D_3$ (≥ 10 nM). During monocyte differentiation into macrophages, increased concentrations of $1,25(OH)_2D_3$ (up to 1 nM) were detected in cell culture media, but this was not observed during monocyte differentiation to DCs [13]. This increased production of $1,25(OH)_2D_3$ could have paracrine effects on colocated DCs [13] and T cells [14]. However, most *in vitro* studies have used substantially more $1,25(OH)_2D_3$ (≥ 10 nM) to modulate DC and T cell phenotype and function. T_{Reg} cell numbers and/or their suppressive activity correlate with circulating $25(OH)D_3$ levels. This has been observed in patients with pancreatitis [6], multiple sclerosis [15], and asthma [16, 17] or those chronically infected with the hepatitis C virus [18]. Supplementation with vitamin D_3 or an analogue increased T_{Reg} cell numbers in healthy individuals (140,000 IU oral vitamin D_3/month) [19] or patients with undifferentiated connective tissue disease (0.5 μg oral alfacalcidol/day) [20]. Other studies report a positive correlation between serum $1,25(OH)_2D_3$ levels (but not $25(OH)D_3$ levels) and circulating T_{Reg} cell numbers in patients with multiple sclerosis [21]. A negative correlation between $25(OH)D_3$ and T_{Reg} cell numbers has been reported in cord blood [22]. Most studies support a positive relationship between circulating $25(OH)D_3$ levels and T_{Reg} cell activity; however, this has not always been associated with improved disease-related outcomes [15, 19].

Another intriguing aspect of vitamin D biology includes its ability to modulate the tropism of cells for certain tissues. Tropism for skin has been suggested in some studies, where $1,25(OH)_2D_3$ or an analogue (nM range) increased the expression of the skin-tropic chemokine receptor CCR10 on cultured T cells [14, 23]. The $1,25(OH)_2D_3$ analogue TX527 significantly upregulated other skin-homing molecules like CCR4 (but not CLA) on T cells, as well as inflammation-homing molecules (e.g., CCR5, CXCR3, and CXCR6) but downregulated expression of lymph node-homing molecules (CD62L, CCR7, and CXCR4) [23]. Serum $25(OH)D_3$ levels are associated with increased expression of the skin-tropic chemokine receptors CCR4 and CLA on circulating T_{Reg} cells from healthy male volunteers [24]. Other studies in HIV-infected participants suggested that serum $25(OH)D_3$ was negatively associated with CCR4 expression on circulating T_{Reg} cells. Vitamin D_3 supplementation (25,000 IU/week) of these participants increased CCR4 and CCR10 expression on blood T_{Reg} cells [25]. Collectively, these studies suggest that $1,25(OH)_2D_3$ promotes homing of T_{Reg} cells towards skin or sites of inflammation.

The results of a recent meta-analysis of clinical trials suggest that dietary vitamin D_3 supplementation may reduce symptoms of atopic dermatitis [26], an inflammatory skin disease. In this study, we investigated how the tissue distribution and suppressive function of T_{Reg} cells are regulated by dietary vitamin D_3. We used a murine model of dietary-induced vitamin D_3 restriction to induce vitamin D_3 deficiency and compared the effects of dietary vitamin D_3 on T_{Reg} cell function and numbers in various tissues and skin inflammation induced by a hapten.

2. Materials and Methods

2.1. Mice and Diet. All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia and with approval from the Telethon Kids Institute Animal Ethics Committee (AEC#229). BALB/c mice were purchased from the Animal Resources Centre, Western Australia. Mice transgenic for the $OVA_{323-339}$-specific (ISQAVHAAHAEINEAGR) T cell receptor (DO11.10) on a BALB/c background were originally purchased from the Jackson Laboratory and bred in-house. Expression of $OVA_{323-339}$-specific T cell receptor on T cells from DO11.10 mice was confirmed as previously described [9]. Female 3-week-old BALB/c or DO11.10 transgenic mice were placed on semipure diets, which were supplemented with vitamin D_3 (2280 IU vitamin D_3/kg with 1% calcium, SF05-34, Specialty Feeds, Perth, Western Australia) or were not supplemented with vitamin D_3 (0 IU vitamin D_3/kg with 2% calcium, SF05-033, Specialty Feeds) as previously described [27, 28]. At 8 weeks of age, female mice were mated with adult male mice maintained on standard mouse chow (Specialty Feeds, containing 2000 IU vitamin D_3/kg). Female or male offspring were maintained on the same vitamin D_3-replete or vitamin D_3-deficient diets (as their mothers) for the rest of the experiment. All results shown are for female offspring, unless otherwise stated.

2.2. Measurement of Serum 25-Hydroxyvitamin D_3 ($25(OH)D_3$). Serum $25(OH)D_3$ levels were measured in BALB/c mice using IDS EIA ELISA kits (Immunodiagnostic Systems Ltd., Fountain Hills, AZ) as described by the manufacturer (limit of detection was 7 nmol·L^{-1}). We have previously shown that results from this assay correlate highly ($r = 0.99$) [29] with a liquid chromatography-tandem mass spectrometry method, which has been certified to a reference measurement procedure developed by the National Institute of Standards and Technology and Ghent University [30, 31].

2.3. Biphasic Ear Swelling Assay. A biphasic ear swelling response [32, 33] was induced by painting both sides of each ear pinnae with 10 μL of 0.05–0.2% 2,4-dinitrofluorobenzene (DNFB, Sigma, St Louis, MO) in acetone using a micrometer to measure ear thickness in a blinded fashion at the indicated times. Results are shown as the change in ear thickness, with baseline measures subtracted from those measured at each time point.

2.4. Identification of T_{Reg} Cells by Flow Cytometry. Single cell suspensions were generated from minced ear skin or whole lung digested for 90 min with collagenase IV (3 mg/mL, Worthington) at 37°C with frequent vortexing. To isolate peripheral blood mononuclear cells (PBMC), heparinized blood was diluted 1 : 2 in 0.9% saline (Baxter, Old Toongabbie, NSW, Australia) and layered over 1 mL Lymphoprep (Axis-Shield, Oslo, Norway) for every 2 mL of diluted blood. Samples were then centrifuged at 800 ×g for 20 min at room temperature

with PBMCs collected from the resulting interface. Skin-draining lymph nodes (SDLN; brachial, inguinal, and axillary), airway-draining lymph node cells (ADLN; posterior mediastinal, tracheobrachial, and parathymic), mesenteric lymph nodes (MLN), or spleens were removed from mice and physically disaggregated to generate single cell suspensions as previously described [9]. Staining of surface (CD3, CD4, CD25, CCR4, CCR10, neuropilin, MHC class II, and CD11c) and intracellular (Foxp3) antigens was performed as previously described [9]. At least 10,000 cells of interest were collected using the FACS LSRII (BD Biosciences) flow cytometer. Data were analyzed using FlowJo software (v9.5.2, TreeStar Inc., Ashland, OR, USA).

2.5. Assessing the Suppressive Capacity of T_{Reg} Cells.

We isolated T_{Reg} cells from vitamin D_3-replete or vitamin D_3-deficient DO11.10 mice to test the capacity of dietary vitamin D_3 to modify the suppressive activity of cells located in a number of different immune sites. As the majority of T_{Reg} cells express the $OVA_{323-339}$ receptor [9], they will suppress the IL-2-secreting capacity of cocultured $OVA_{323-339}$ receptor-specific CD4+ T cells in the presence of antigen-presenting cells and $OVA_{323-339}$ peptide, as we have demonstrated previously [9]. CD4+CD25+ cells (≥95% pure, as determined by flow cytometry) were isolated from specified tissues of DO11.10 mice using a CD4+CD25+ regulatory T cell isolation kit (Miltenyi Biotec) or by cell sorting as previously described [9, 34]. Greater than 90% of the purified CD4+CD25+ cells expressed Foxp3 (confirmed by flow cytometry). Peripheral lymph node cells (including SDLN, ADLN, MLN, auricular-draining lymph nodes, and para-aortic lymph nodes) of naïve DO11.10 mice were used as responder cells. These were resuspended in RPMI with 10% FCS and 2 μM 2-ME and aliquot into round-bottomed 96-well plates at 10^5 cells/0.1 mL/well. CD4+CD25+ cells were added to responder cells at ratios of 1:8, 1:16, or 1:32. $OVA_{323-339}$ peptide was added at a final concentration of 1 μg/mL. After incubation for 92 h at 37°C in 5% CO_2, supernatants were harvested and the concentration of interleukin-2 (IL-2) was determined by ELISA as previously described [9].

2.6. Assessing the Ability of Dendritic Cells to Induce T_{Reg} Cells.

A single cell suspension of ADLN cells was prepared by physically disaggregating lymph nodes and digesting samples with collagenase IV (1 mg/mL, Worthington) and DNase I (0.1 mg/mL, Sigma) for 30 min at 37°C. Conventional DCs were enriched from the ADLN cells by removal of CD3+, Thy1.1+ CD19+, GR-1+, and TER-119+ cells using magnetic bead separation as previously described [35]. The remaining cells were then labelled with antibodies specific for CD11c and MHC class II [9] and MHC class II^{hi}CD11c^{med} cells sorted by FACS using the FACSAria (BD Biosciences). MHC class II^{hi}CD11c^{med} cells were incubated with peripheral lymph nodes from naïve DO11.10 (vitamin D-replete) mice (see [9]) at a ratio of 1:40 with 1 μg/mL $OVA_{323-339}$ peptide. DCs were not added to some cultures as controls. Cells were incubated for 62 h at 37°C and 5% CO_2, and then CD3+CD4+CD25+Foxp3± cells were identified by flow

cytometry using the FACS LSRII, where at least 5,000 Foxp3+ cells were collected. Data were analyzed using FlowJo software.

2.7. Statistical Analyses.

Data were compared using an unpaired two-way Student's t-test using Prism 5 statistical analysis program for Mac OS X. Differences were considered significant with a p value < 0.05. Data are shown throughout as mean ± SEM.

3. Results and Discussion

3.1. Vitamin D Deficiency Promoted Allergic Dermatitis Responses Measured during a Biphasic Ear Swelling Response.

We investigated the effects of dietary vitamin D on allergic dermatitis responses mimicked by inducing a biphasic ear swelling response. We tested adult female offspring of vitamin D_3-replete or vitamin D_3-deficient BALB/c dams, which were maintained on the same diet as their mothers. Serum levels of $25(OH)D_3$ were <20 nmol·L^{-1} for offspring fed the vitamin D_3-deficient diet and >50 nmol·L^{-1} for offspring fed the vitamin D_3-replete diet (Figure 1(a)). These diets did not significantly alter serum calcium [27, 28]. The contact sensitizer DNFB was then used to initiate a biphasic ear swelling response [32, 33]. The ears of vitamin D_3-deficient or vitamin D_3-replete mice were sensitized with 0.05–0.2% DNFB (in acetone), and ear swelling was recorded over a 3-week period. The ability of dietary vitamin D_3 to suppress ear swelling responses depended on the sensitizing dose of DNFB, where responses to ≤0.1% DNFB were suppressed at 144 h after sensitization, corresponding to the second peak of ear swelling (Figure 1(b)). As expected, the ear swelling response was biphasic, with an initial peak at 3 h and later peak at 168 h after DNFB treatment (Figure 1(c)). Previous studies have shown that this first peak represents an early innate influx of neutrophils and inflammatory cells into ear skin, which may depend on histamine release by mast cells [32], while the second peak is an antigen-specific (DNFB) effector response driven by CD8+ T cells expressing interferon-γ [33]. Dietary vitamin D_3 significantly suppressed (by 61%) this second "efferent" ear swelling response, which peaked at 168 h after DNFB application in vitamin D_3-sufficient mice as compared to responses observed in deficient mice (Figure 1(c)).

3.2. Increased Percentages of T_{Reg} Cells Were Observed in the Skin-Draining Lymph Nodes of Vitamin D_3-Replete Mice.

We have previously published that topically applied $1,25(OH)_2D_3$ increased the capacity of T_{Reg} cells to suppress contact hypersensitivity responses initiated by DNFB [9]. To examine the effects of dietary vitamin D_3 on T_{Reg} cells, their percentages in various tissues were measured in naïve female mice prior to sensitization with DNFB. The percentages of CD3+CD4+CD25+Foxp3+ T_{Reg} cells in the skin, SDLN, lung, ADLN, MLN, spleen, and blood were determined by flow cytometry (Figure 2(a), a representative plot for a MLN sample is shown). CD4+ T_{Reg} cell percentages were increased in the SDLN (from 5.0 ± 0.2 (vitD−) to 5.7 ± 0.1 (vitD+); 14% increase; n = 6/treatment) and reduced in the ADLN (from 4.8 ± 0.3 (vitD−) to 3.3 ± 0.2 (vitD+); 31% reduction;
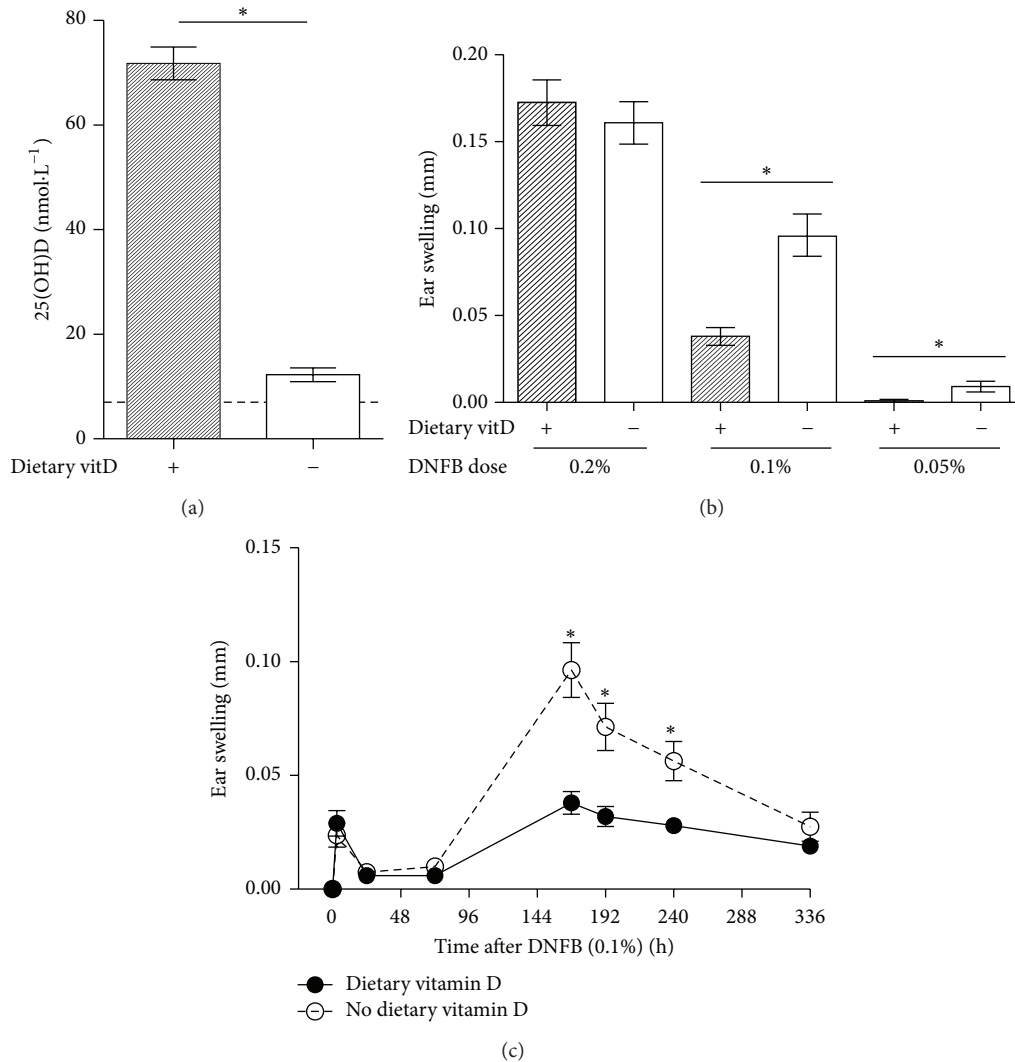
FIGURE 1: Dietary vitamin D suppressed the biphasic ear swelling response induced by the hapten DNFB. Female offspring born to vitamin D_3- (vitD-) replete (+) or vitamin D_3-deficient (−) BALB/c mothers were maintained on vitamin D_3-replete or vitamin D_3-deficient diets (resp.). (a) Serum $25(OH)D_3$ levels of female offspring at 8 weeks of age (mean ± SEM for ≥5 mice per group). The broken line indicates the level of detection for $25(OH)D_3$ (7 pg/mL). In (b), the ear pinnae of mice were sensitized with 0.2, 0.1, or 0.05% DNFB and the ear swelling response was measured at the second peak of the biphasic response (144 h). In (c), the ear pinnae of mice were sensitized with 0.1% DNFB and the resulting biphasic ear swelling response was measured over 305 h. Data are shown as mean ± SEM in (b) and (c) for 8–12 ear pinnae of 4–6 mice per treatment ($^*p < 0.05$). In (b), data are from a single experiment and in (c) they are representative of 2 experiments.

$n = 6$/treatment) of vitamin D_3-replete mice in comparison to vitamin D_3-deficient mice, but there was no difference in the percentages of these cells in the skin, lungs, MLN, spleen, or blood (Figure 2(b)). There was also a trend ($p = 0.08$, Student's t-test) for increased Foxp3 expression (by 16%) on CD3+CD4+CD25+Foxp3+ cells from the SDLN of vitamin D_3-replete mice, when geometric mean fluorescence intensity was compared (1053 ± 43 (vitD+); 910 ± 63 (vitD−); $n = 6$/treatment, data from cells collected in Figure 2(b)). There was no difference in the expression of Foxp3 on CD3+CD4+CD25+Foxp3+ cells from the other tissues (data not shown). With the exception of blood, CD3+CD4+CD25+Foxp3− T "effector" cell (T_{Eff}) (Figure 2(c)) percentages were unaffected by vitamin D_3

deficiency. In male mice, CD3+CD4+CD25+Foxp3+ T_{Reg} cell percentages were affected in a similar way by dietary vitamin D_3 as those observed in female mice and were increased in the SDLN (by 21%) (from 4.1 ± 0.3 (vitD−) to 5.0 ± 0.2 (vitD+); $n = 6$/treatment) and reduced in ADLN (by 23%) (from 4.3 ± 0.3 (vitD−) to 3.3 ± 0.1 (vitD+); $n = 6$/treatment). There was also an increase (42%) in the percentage of CD3+CD4+CD25+Foxp3− T_{Eff} cells in the SDLN of male mice fed a vitamin D_3-containing diet (from to 0.31 ± 0.04 (vitD−) to 0.44 ± 0.04 (vitD+); 42% increase; $n = 6$/treatment).

The number of cells isolated from the SDLN was altered by vitamin D_3 supplementation of female mice. Significantly fewer SDLN cells (2.8 ± 0.4 × 10^7/mouse (vitD−);
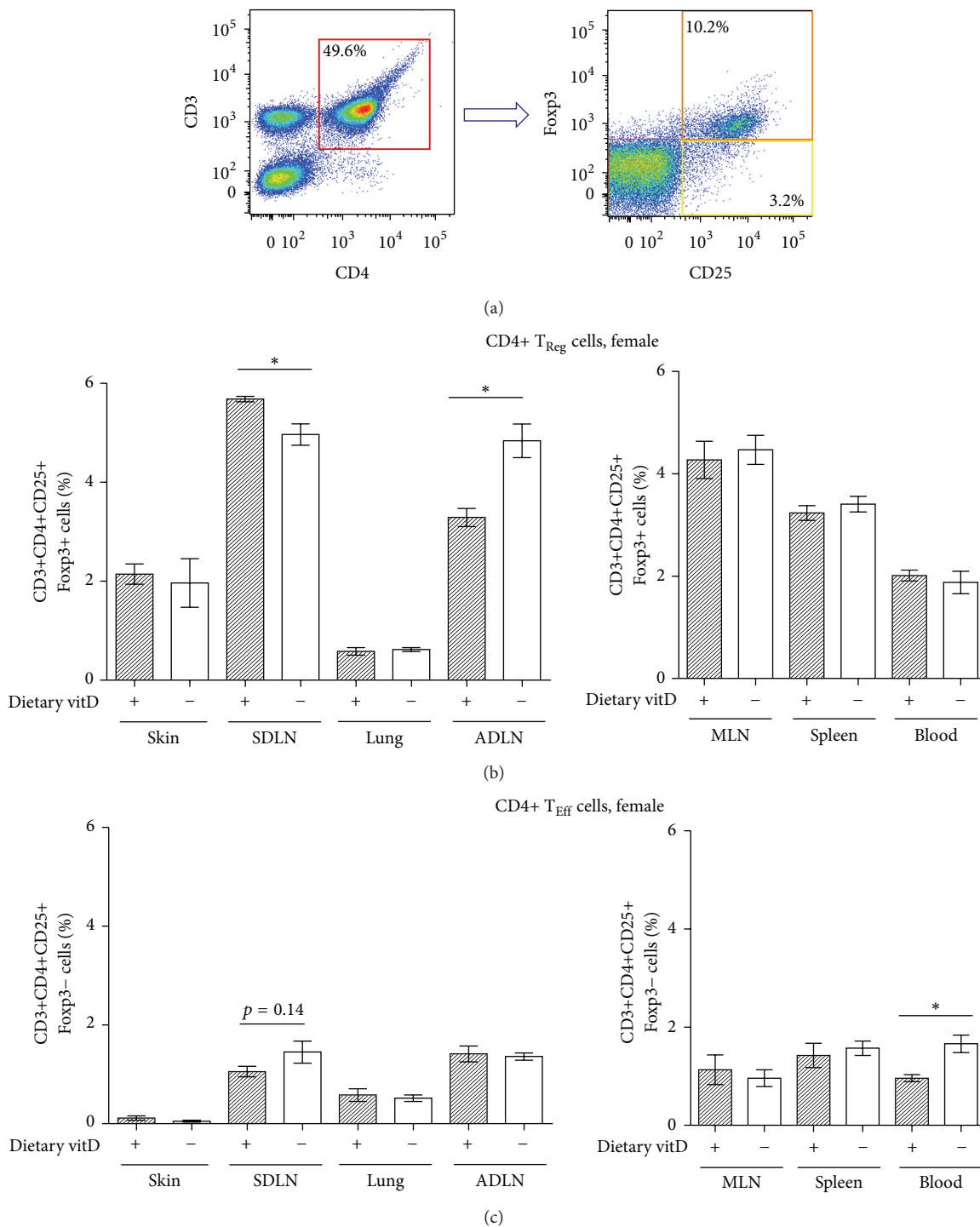
(a)

(b)

(c)

FIGURE 2: Dietary vitamin D_3 increased the percentage of Foxp3+ T_{Reg} cells in the SDLN. Female offspring born to vitamin D_3- (vitD-) replete (+) or vitamin D_3-deficient (−) BALB/c mothers were maintained on the vitamin D_3-replete or vitamin D_3-deficient diets (resp.). (a) The FACS gating strategy for determining the percentage of CD3+CD4+CD25+Foxp3+ (T_{Reg} cells, orange) or CD3+CD4+CD25+Foxp3− (T_{Eff} cells, yellow) cells in skin, SDLN, lung, ADLN, MLN, spleen, and blood. Representative plots from a MLN sample are shown. For all tissues, a gate was used to exclude red blood cells using forward and side scattering properties of cells prior to selection of the various T cell populations. (b) and (c) The percentage of T_{Reg} cells and T_{Eff} cells (resp.) in various tissues. Data are shown as mean ± SEM for 6 mice/treatment with results combined from two experiments; $^*p < 0.05$.
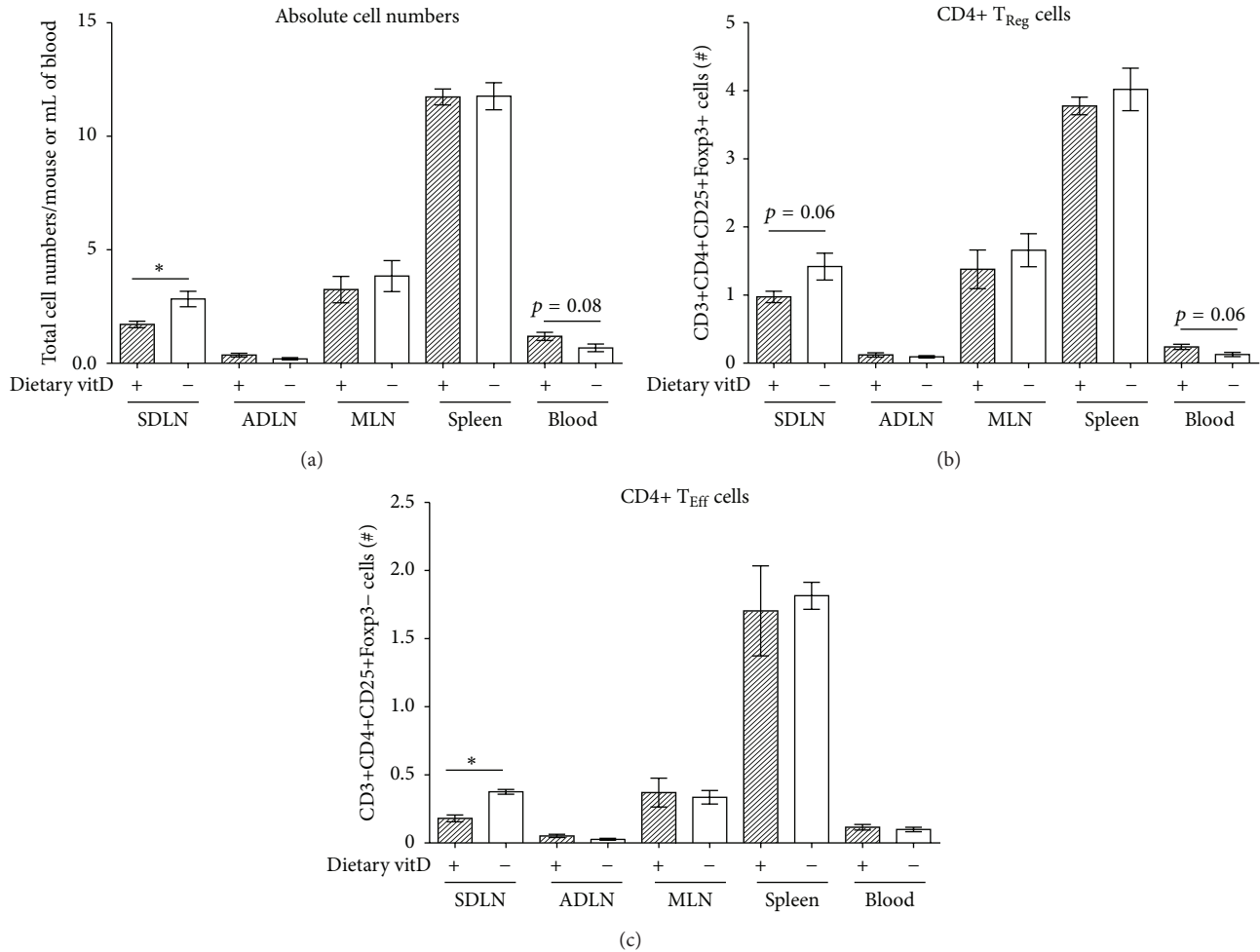
(a)

(b)

(c)

FIGURE 3: Dietary vitamin D_3 reduced the absolute number of cells in the SDLN. Female offspring born to vitamin D_3- (vitD-) replete (+) or vitamin D_3-deficient (−) BALB/c mothers were maintained on the vitamin D_3-replete or vitamin D_3-deficient diets (resp.). In (a), the number of cells ($\times 10^7$)/mouse or mL of blood isolated from the SDLN, ADLN, MLN, spleen, or blood is shown. The numbers of CD3+CD4+CD25+Foxp3+ (T_{Reg} cells) or CD3+CD4+CD25+Foxp3− (T_{Eff} cells) cells in these tissues were calculated using the percentages depicted in Figure 2. In (b) and (c), the number of CD4+ T_{Reg} and T_{Eff} cells ($\times 10^6$)/mouse or mL of blood is shown. Data are shown as mean $\pm$ SEM for 6 mice/treatment with results combined from two experiments; $^*p < 0.05$.

$1.7 \pm 0.1 \times 10^7$/mouse (vitD+); 39% reduction; $n = 6$/treatment) were isolated from vitamin D_3-supplemented mice (Figure 3(a)). These effects were in the opposite direction to those of dietary vitamin D_3 on T_{Reg} cell percentages in the SDLN. There was no difference in the numbers of cells isolated from other tissues (Figure 3(a); data not shown for skin and lung). There was a trend for fewer CD4+ T_{Reg} cell numbers in the SDLN ($1.5 \pm 0.2 \times 10^6$/mouse (vitD−); $0.9 \pm 0.1 \times 10^6$/mouse (vitD+)); 40% reduction; $n = 6$/treatment) of vitamin D_3-supplemented mice in comparison to vitamin D_3-deficient mice (Figure 3(b)). Similarly, numbers of CD3+CD4+CD25+ Foxp3− T_{Eff} cells were significantly reduced in the SDLN of mice fed a vitamin D_3-supplemented diet ($3.8 \pm 0.2 \times 10^5$/mouse (vitD−); $1.8 \pm 0.2 \times 10^5$/mouse (vitD+); 53% reduction; $n = 6$/treatment) (Figure 3(c)). There was no effect of dietary vitamin D_3 on the total cell numbers or numbers of T_{Reg} or T_{Eff} cells identified in the SDLN, ADLN, or blood of male mice (data not shown). These

data suggest that while the proportions of CD4+ T_{Reg} cells increased in the SDLN with dietary vitamin D_3, this significant increase did not persist when cell numbers were considered, as significantly fewer SDLN cells were isolated from mice fed a diet containing vitamin D_3.

3.3. The Suppressive Activity of T_{Reg} Cells Was Enhanced by Dietary Vitamin D_3 in Most Immune Tissues but Not the Airway-Draining Lymph Nodes. An in vitro assay was used to test if dietary vitamin D_3 altered the suppressive function of T_{Reg} cells in comparison to those from vitamin D_3-deficient mice. Purified CD4+CD25+(Foxp3+) cells were cocultured with responder lymph node cells from DO11.10 mice and OVA$_{323-339}$ peptide for 92 h. Responder CD4+ T cells expressing the OVA$_{323-339}$ TCR proliferate and produce cytokines like IL-2 in response to presentation of the OVA$_{323-339}$ peptide by antigen-presenting cells. We assessed IL-2 levels as a measure of the proliferative capacity of responder cells, where

cocultured T_{Reg} cells significantly suppressed supernatant levels of IL-2 in a dose-dependent manner (Figure 4). T_{Reg} cells produce very low levels of IL-2 when stimulated *in vitro*. These levels are up to 10 times less than CD4+ T_{Eff} responder cells [12]. T_{Reg} cells therefore do not significantly contribute towards the pool of IL-2 in coculture assays. CD4+CD25+(Foxp3+) cells from the SDLN (Figure 4(a)), MLN (Figure 4(c)), spleen (Figure 4(d)), and blood (Figure 4(e)) of vitamin D_3-replete mice had increased capacity to suppress IL-2 production by cocultured responder cells. There was no significant difference in suppressive function observed for CD4+CD25+(Foxp3+) cells from the ADLN (Figure 4(b)) of vitamin D_3-replete or vitamin D_3-deficient mice. The results reported in Figure 4 were for suppressive activities of CD4+CD25+(Foxp3+) cells from female mice; however, similar results were obtained for cells from male mice (data not shown). We were not technically able to assess the suppressive activity of T_{Reg} cells in the skin or lungs as their numbers were too infrequent for efficient isolation. These data suggest that dietary vitamin D_3 is required for the optimal activity of T_{Reg} cells at various immune sites throughout the body, with the exception of the ADLN.

3.4. Vitamin D_3 Did Not Induce T_{Reg} Cells in the Periphery.
Surface expression of neuropilin can be used to identify naturally occurring T_{Reg} cells [36]. We examined the expression of neuropilin on T_{Reg} cells from the skin, SDLN, lungs, or ADLN of vitamin D_3-replete or vitamin D_3-deficient mice and observed no difference in the expression of this molecule (Figure 5(a)). These results suggest that dietary vitamin D_3 did not favour the induction of new T_{Reg} cells in the SDLN. The observed reduction in the percentage of T_{Reg} cells in the ADLN of mice fed a vitamin D_3-containing diet was a surprising result. A lack of difference in neuropilin expression on T_{Reg} cell from the ADLN of vitamin D_3-replete and vitamin D_3-deficient mice suggested that dietary vitamin D_3 did not prevent the induction of new T_{Reg} cells (Figure 5(a)). However, to confirm this observation, we then tested whether there was a functional difference between DCs from vitamin D_3-deficient and vitamin D_3-replete mice, as DCs are required for the induction of new T_{Reg} cells in the periphery [37]. Conventional MHC class II^{hi}CD11c^{med} DCs were sorted (Figure 5(b)) from the ADLN of vitamin D_3-replete and vitamin D_3-deficient mice and cocultured with lymph node cells from naïve DO11.10 mice and OVA peptide. The percentage of CD4+CD25+Foxp3± cells was determined after 62 h of coculture (Figure 5(c)). CD4+CD25+Foxp3− T_{Eff} cell and CD4+CD25+Foxp3+ T_{Reg} cell percentages were increased (>2-fold) by the presence of DCs in the cocultures (Figures 5(c) and 5(d)). However, there was no effect of dietary vitamin D_3 on the ability of ADLN DCs to regulate T_{Reg} cell percentages (Figure 5(d)). These results suggest that dietary vitamin D_3 did not impair the induction of new T_{Reg} cells in the ADLN.

3.5. Dietary Vitamin D_3 Did Not Affect the Expression of CCR4 or CCR10 on T_{Reg} Cells in the SDLN or ADLN.
Dietary vitamin D_3 could induce the migration of T_{Reg} cells to augment the percentages of these cells in the SDLN, facilitated by the expression of chemokine receptors. There was no difference in the expression of the skin-homing receptors CCR4 or CCR10 on T_{Reg} cells in the skin, SDLN, lungs, or ADLN of vitamin D-replete or vitamin D-deficient mice (Figures 6(a) and 6(b)). Significant levels of CCR4 (Figure 6(a)) were detected on T_{Reg} cells in skin and SDLN with less expression on cells from the lung and ADLN, while similar levels of CCR10 (Figure 6(b)) were identified on T_{Reg} cells from these tissues. While dietary vitamin D_3 promoted T_{Reg} cell accumulation in the SDLN, there was no difference in the expression of CCR4 and CCR10 skin-homing receptors once cells entered the SDLN.

3.6. Dietary Vitamin D_3 May Promote Dermal Tolerance to Reduce Skin Inflammation.
Our findings of the immunosuppressive effects of dietary vitamin D_3 in controlling DNFB-induced skin inflammation reiterate emerging data from clinical trials, which suggest that vitamin D_3 supplementation reduces symptoms of allergic dermatitis [26]. Similar suppressive effects of dietary vitamin D_3 have been observed in other animal models that used haptens to induce skin inflammation [38]. In other studies, hypocalcaemia induced by vitamin D_3 deficiency may have impaired hapten-induced ear swelling responses [39]. The vitamin D_3-containing diets used in our studies and those of others [38] were enriched with calcium to prevent hypocalcemia [27, 28]. While clinical trials suggest that maintaining optimal serum levels of 25(OH)D through dietary vitamin D_3 supplementation (or safe sun exposure) reduces symptoms of atopic dermatitis [26], we are still uncertain of the optimal levels of 25(OH)D needed to limit atopic dermatitis. It is also important to note that some studies have observed no significant effect of vitamin D_3 supplementation [40], with suggestions that genetic or other population-based factors (e.g., initial circulating 25(OH)D levels) or the supplementation regimen (e.g., dose, schedule of treatment, and type of vitamin D) may have limited the efficacy of the vitamin D treatment.

3.7. Dietary Vitamin D_3 Increased the Percentages and Activity of T_{Reg} Cells in the SDLN.
We observed a greater suppressive activity of CD4+CD25+(Foxp3+) cells isolated from the SDLN, MLN, spleen, and blood of mice fed the vitamin D-containing diet, suggesting a systemic effect of dietary vitamin D on T_{Reg} cell activity. It is important to note that we examined T_{Reg} cell numbers and function prior to sensitization with DNFB, and so these findings are independent of skin inflammation induced by the irritant. These observations were accompanied by a lack of effect of dietary vitamin D on the suppressive activity of cells from the ADLN. This curious observation is difficult to explain but may represent a site from which T_{Reg} cells actively migrate (to the SDLN). We did not observe increased expression of skin-tropic chemokine receptors CCR4 or CCR10 on T_{Reg} cells from any of the tested sites (skin, SDLN, lung, and ADLN) of vitamin D_3-fed mice, but it may be that these molecules are upregulated during transition (in blood) between locations, which could be a focus of future studies. Crosstalk between the immune reactions initiated in the skin and
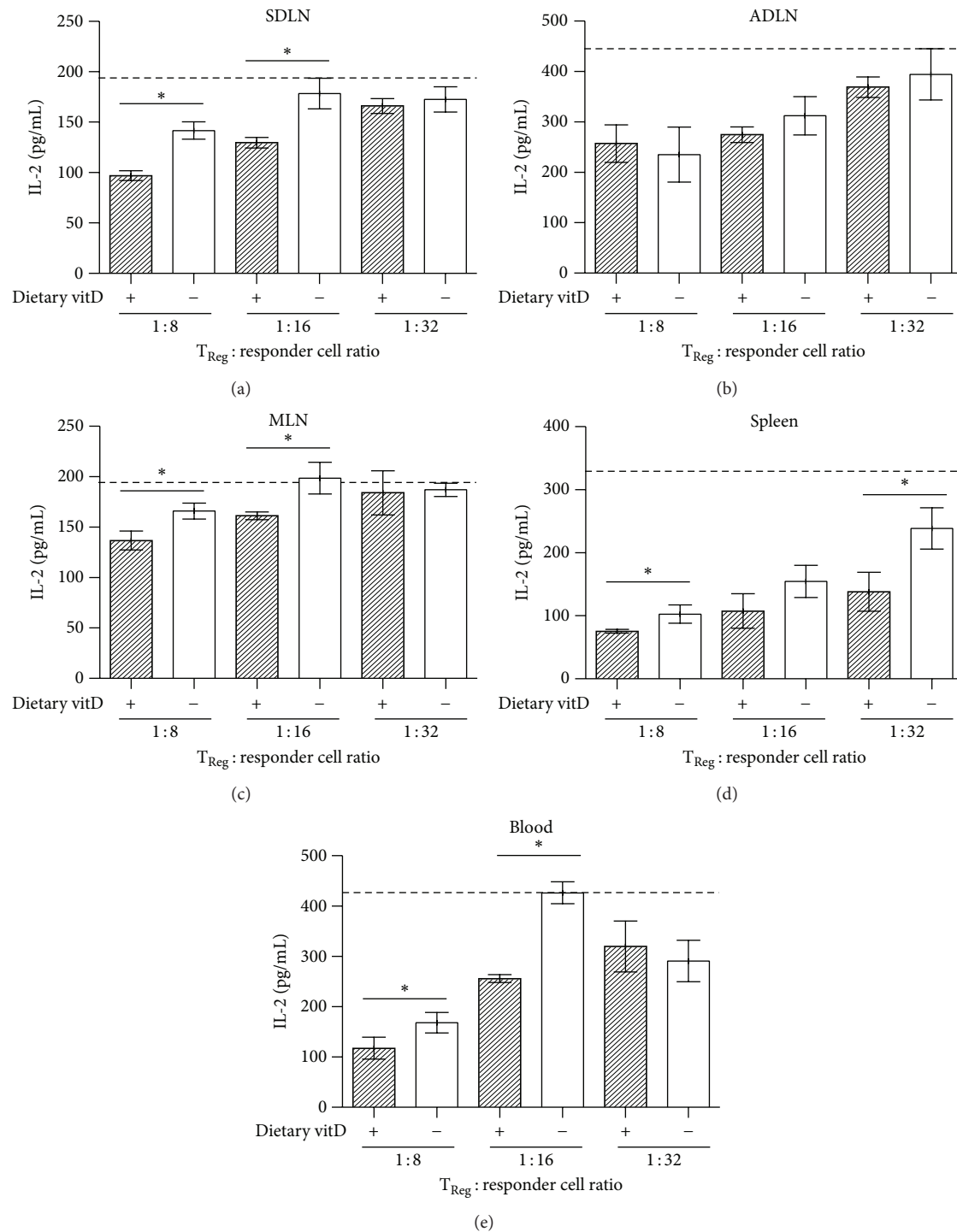
FIGURE 4: Dietary vitamin D enhanced the activity of Foxp3+ T_{Reg} cells at multiple immune sites. Female offspring born to vitamin D_3-(vitD-) replete (+) or vitamin D_3-deficient (−) DO11.10 mothers were maintained on vitamin D-replete or vitamin D-deficient diets (resp.) until 8 weeks of age. CD4+CD25+(Foxp3+) cells were purified from (a) SDLN, (b) ADLN, (c) MLN, (d) spleens, or (e) blood of mice and cocultured with lymph node responder cells from DO11.10 mice at ratios of 1:8, 1:16, or 1:32 and OVA peptide. IL-2 levels in the coculture supernatants were measured after 96 h. The broken lines indicate the levels of IL-2 measured in supernatant of responder cells cultured with OVA peptide alone. Data are shown as mean ± SEM for 6 wells/treatment, representative of two experiments; $^*p < 0.05$.
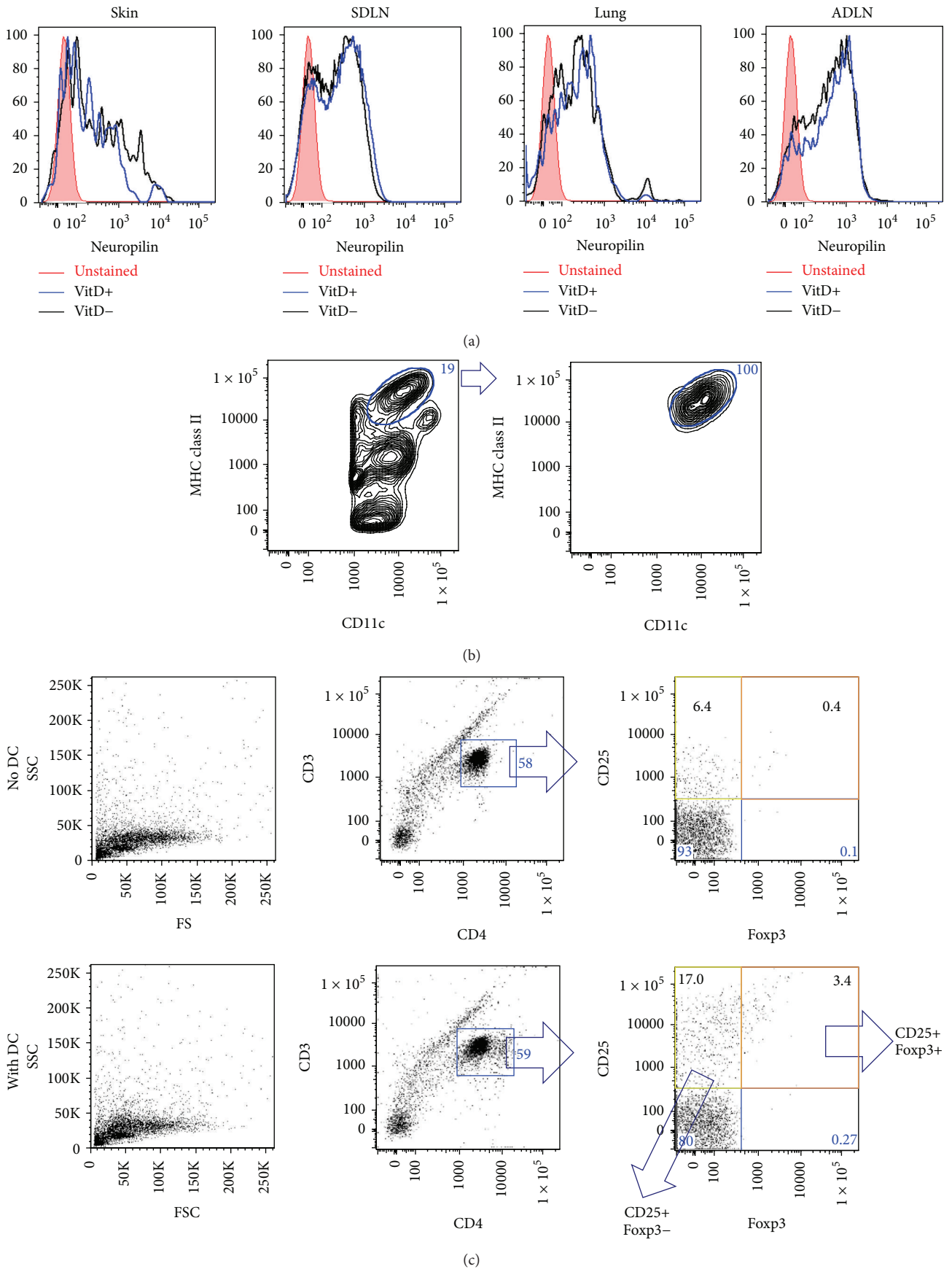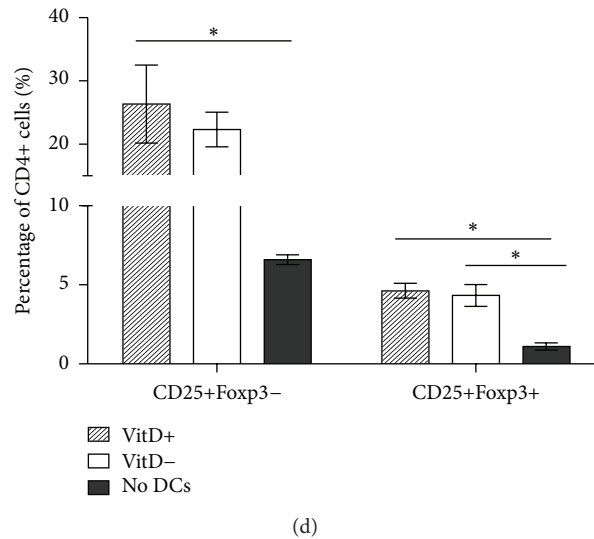
(a)

(b)

(c)

Figure 5: Continued.

(d)

FIGURE 5: Dietary vitamin D_3 did not modify neuropilin expression on Foxp3+ T_{Reg} cells from the skin, SDLN, lung, or ADLN or alter the capacity of DCs from the ADLN to induce new Foxp3+ T_{Reg} cells. Female offspring born to vitamin D_3-replete or vitamin D_3-deficient BALB/c mothers were maintained on the vitamin D_3-replete (vitD+) or vitamin D_3-deficient (vitD−) diets (resp.) until 8 weeks of age. In (a), the expression of neuropilin in the skin, SDLN, lungs, and ADLN is shown, with cells from vitamin D_3-replete and vitamin D_3-deficient mice shown in blue and black, respectively (unstained = red shaded). Data are representative of two experiments. In (b), MHC class II^{hi}CD11c^{med} cells from the ADLN were sorted with the prepurity (left) and postpurity (right) shown, as determined by flow cytometry. These cells were cocultured with lymph node cells from OVA-TCR transgenic mice (DO11.10 mice) at a ratio of 0 : 1 (no DC) or 1 : 40 (with DC) for 62 h with OVA peptide. (c) The gating strategy for determining the percentage of CD25+Foxp3− and CD25+Foxp3+ cells after 62 h of coculture. In (d), the percentages of CD25+Foxp3− and CD25+Foxp3+ cells of CD4+CD3+ cells are shown as mean ± SEM for 3 wells/treatment. $^{*}p < 0.05$.

airways is illustrated by the "atopic march" concept, where allergic responses in the skin affect immunity in the airways. Indeed, hapten-induced skin inflammation can worsen signs of allergic airway disease in mice [41]. We suggest that dietary vitamin D_3 may prevent the "atopic march" by promoting T_{Reg} cell accumulation and activity in the SDLNs. The lack of difference in neuropilin levels suggests that vitamin D_3 may increase SDLN T_{Reg} cell accumulation through migration.

3.8. Inconsistencies between These Observations and Other Published Data. Urry et al. (2012) observed a positive correlation between circulating 25(OH)D and the percentages of T_{Reg} cells in the airways (lavage fluid) of children with severe asthma [42]. We did not see any difference in the percentages of T_{Reg} cells in the lungs of mice fed a vitamin D_3-replete or vitamin D_3-deficient diet and did not assess the percentages of these cells in the trachea or bronchoalveolar lavage fluid. Furthermore, reduced percentages of T_{Reg} cells were observed in the ADLN of mice fed a vitamin D_3-replete diet. In addition, it is possible that, upon respiratory stimulation with allergen, T_{Reg} cell percentages could increase in the lungs of vitamin D_3-replete mice. In other studies, Mann et al. (2015) found that more CD4+ cells stimulated with 1,25(OH)$_2$D (100 nM) *in vitro* expressed neuropilin compared to control cells [43]. It is therefore possible that new T_{Reg} cells may be generated under conditions of highly concentrated 1,25(OH)$_2$D$_3$.

3.9. Modelling Skin Inflammation. We induced a biphasic cutaneous skin reaction using the contact sensitizer DNFB

to examine the effects of dietary vitamin D_3 on skin inflammation. A humanized mouse model would have been an interesting alternative means of comparing these treatments through xenotransplantation of human skin [44] or bioengineered human skin equivalents [45] onto immunodeficient mice. These models can induce some (but not all) clinical manifestations of atopic dermatitis, particularly acute lesions [45]. While such models would improve our understanding of the effects of dietary vitamin D_3 in humanized settings, we were more interested in the capacity of dietary vitamin D_3 to modulate T_{Reg} cell proportions and function in certain tissues (e.g., skin or lungs) prior to the onset of inflammation.

4. Conclusion

Our studies suggest that dietary vitamin D_3 increased the percentages and suppressive function of T_{Reg} cells in the SDLN and that these cells are poised to suppress dermal inflammation. These studies support the notion that maintaining adequate serum 25(OH)D through dietary vitamin D_3 supplementation or safe sun exposure is important to reduce the severity of allergic dermatitis and other inflammatory skin conditions.

Abbreviations

1,25(OH)$_2$D$_3$: 1,25-Dihydroxyvitamin D_3
25(OH)D$_3$:   25-Hydroxyvitamin D_3
ADLN: Airway-draining lymph nodes
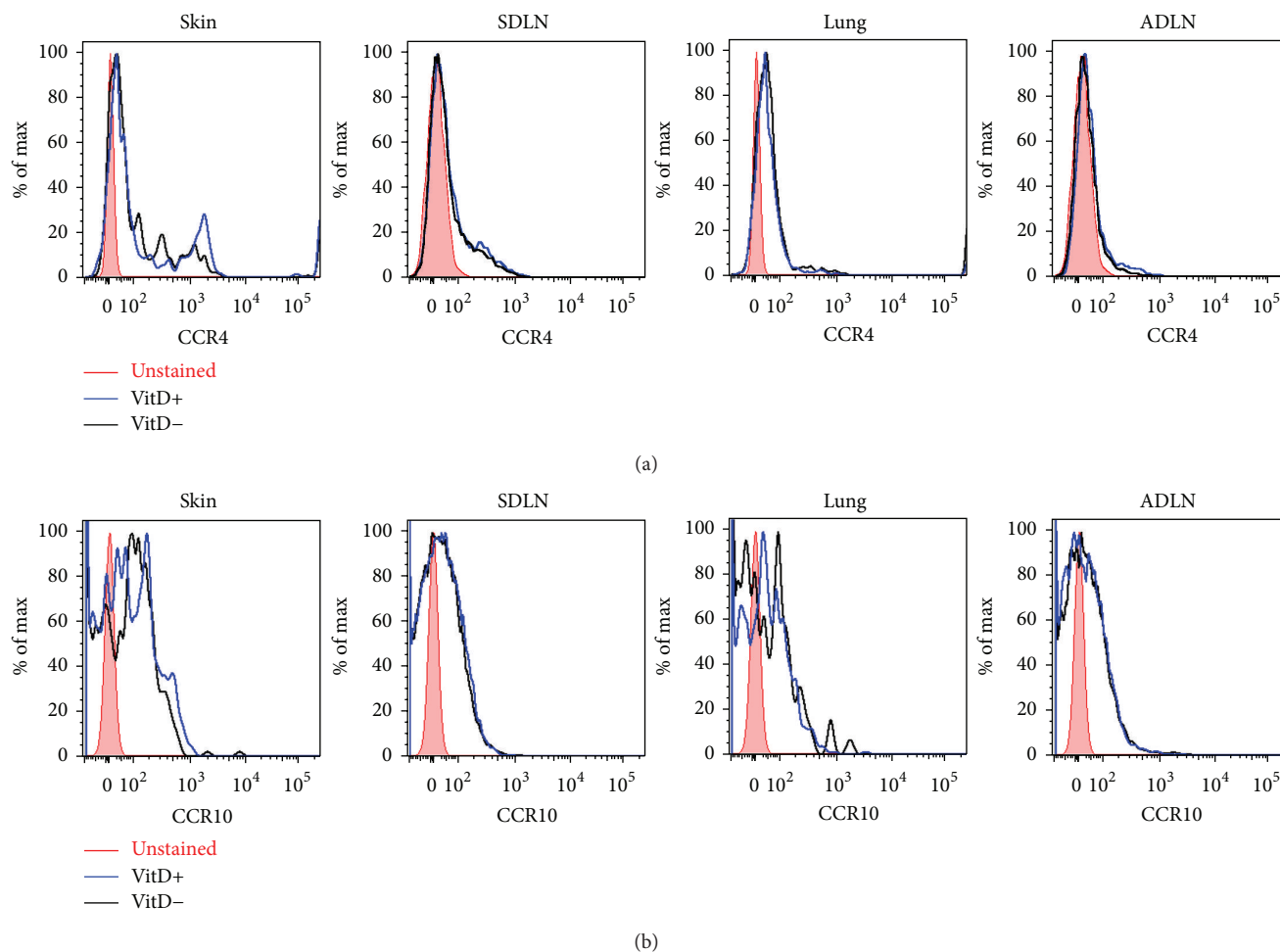DNFB: 2,4-Dinitrofluorobenzene

(a)

(b)

FIGURE 6: Dietary vitamin D_3 did not modify CCR4 or CCR10 expression on Foxp3+ T_{Reg} cells from the skin, SDLN, lung, or ADLN. Female offspring born to vitamin D_3-replete or vitamin D_3-deficient BALB/c mothers were maintained on the vitamin D_3-replete (VitD+) or vitamin D_3-deficient diets (VitD−) (resp.) until 8 weeks of age. The expression of (a) CCR4 and (b) CCR10 was measured on CD3+CD4+CD25+Foxp3+ cells in the skin, SDLN, lungs, and ADLN, with cells from vitamin D_3-replete and vitamin D_3-deficient mice shown in blue and black, respectively (unstained = red shaded). Data are representative of two experiments.

T_{Eff} cells:	Effector T cells
MLN:	Mesenteric lymph nodes
T_{Reg} cells:	Regulatory T cells
SDLN:	Skin-draining lymph nodes
VDBP:	Vitamin D-binding protein.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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