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Topically Applied 1,25-Dihydroxyvitamin D_3 Enhances the Suppressive Activity of $CD4^+CD25^+$ Cells in the Draining Lymph Nodes[1]

Shelley Gorman,* L. Alexandra Kuritzky,* Melinda A. Judge,* Katie M. Dixon,† Jacqueline P. McGlade,* Rebecca S. Mason,† John J. Finlay-Jones,* and Prue H. Hart[2]*

The immunomodulatory effects of vitamin D have been described following chronic oral administration to mice or supplementation of cell cultures with 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active form of vitamin D. In this study, topically applied $1,25(OH)_2D_3$, enhanced the suppressive capacity of $CD4^+CD25^+$ cells from the draining lymph nodes. The effects of topical $1,25(OH)_2D_3$ were compared with those of UVB irradiation, which is the environmental factor required for $1,25(OH)_2D_3$ production in skin. $CD4^+$ cells from the skin-draining lymph nodes (SDLN) of either $1,25(OH)_2D_3$-treated or UVB-irradiated mice had reduced capacity to proliferate to Ags presented in vitro, and could suppress Ag-specific immune responses upon adoptive transfer into naive mice. This regulation was lost upon removal of $CD4^+CD25^+$ cells. Furthermore, purified $CD4^+CD25^+$ cells from the SDLN of $1,25(OH)_2D_3$-treated or UVB-irradiated mice compared with equal numbers of $CD4^+CD25^+$ cells from control mice had increased capacity to suppress immune responses in both in vitro and in vivo assay systems. Following the sensitization of recipient mice with OVA, the proportion of $CD4^+Foxp3^+$ cells of donor origin significantly increased in recipients of $CD4^+CD25^+$ cells from the SDLN of $1,25(OH)_2D_3$-treated mice, indicating that these regulatory T cells can expand in vivo with antigenic stimulation. These studies suggest that $1,25(OH)_2D_3$ may be an important mediator by which UVB-irradiation exerts some of its immunomodulatory effects. ***The Journal of Immunology,*** **2007, 179: 6273–6283.**

As well as being the main initiator of skin neoplasms, the UVB (290–320 nm) component of sunlight is responsible for initiation of the production of the active form of vitamin D (1,25-dihydroxyvitamin D_3; $1,25(OH)_2D_3$)[3]. This lipophilic molecule exerts its actions through the vitamin D receptor (VDR) (1). Upon irradiation with UVB, 7-dehydrocholesterol of skin converts to previtamin D_3, which then isomerises with body heat into vitamin D_3. The bulk of this binds to vitamin D binding protein and is transported to the liver for hydroxylation at the 25 position of vitamin D_3. This metabolite is further hydroxylated in the kidney at the C1α position to form $1,25(OH)_2D_3$. In addition, keratinocytes and other skin cells have an autonomous vitamin D_3 pathway and can produce substantial amounts of $1,25(OH)_2D_3$ (2). Based on studies in human skin equivalents and in human skin using microdialysis (3, 4), local concentrations of $1,25(OH)_2D_3$ in the order of 2–5 nM might be achieved.

The potential immunomodulating effects of vitamin D_3 on the immune system have been largely derived from in vitro observations following the supplementation of DC or T cell cultures with $1,25(OH)_2D_3$. This molecule blocks the maturation of myeloid DC by reducing the expression of costimulatory molecules, inhibiting secretion of IL-12 and increasing IL-10 production (5). $1,25(OH)_2D_3$ also enhances the secretion of CCL22 by DC in vitro, which is a chemokine that attracts T cells into the skin (5). $1,25(OH)_2D_3$ can inhibit the secretion of IFN-γ by T cells and stimulate Th2 cell development with increased production of IL-4, IL-5, and IL-10 (6). In addition, IL-10-producing regulatory T cells can be induced in vitro by $1,25(OH)_2D_3$ in the presence of dexamethasone (7).

Chronic feeding of mice with $1,25(OH)_2D_3$ suppressed autoimmune responses in a variety of different mouse models including type 1 diabetes (0.3 μg RI26–2198 (a $1,25(OH)_2D_3$ analog)/kg for 16 wk) (8), experimental autoimmune encephalitis (5 μg $1,25(OH)_2D_3$/kg thrice weekly for 30 days) (9), arthritis (20 ng $1,25(OH)_2D_3$/day, commencing one day before the induction of disease) (10) and asthma (1.6 IU $1,25(OH)_2D_3$/g diet) (11). In the model of type 1 diabetes, $CD4^+CD25^+$ regulatory T cell numbers were enhanced in NOD mice chronically fed five times a week for eight weeks with $1,25(OH)_2D_3$ (8). Together, these observations indicate that $1,25(OH)_2D_3$ induces regulatory-type environments both in vitro and in vivo.

Consequently, the effects of topical application of skin with $1,25(OH)_2D_3$ upon subsequent immune responses warrant closer inspection. It is not clear whether topical $1,25(OH)_2D_3$ can alter immune responses in models of contact hypersensitivity (CHS). When hapten was applied to the $1,25(OH)_2D_3$-treated sites in mice (using a local model of CHS), both no effect (12, 13) and also down-regulation (14) of CHS responses have been reported. In a recent study using human subjects, calcipotriene, a $1,25(OH)_2D_3$

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[3] Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; SDLN, skin-draining lymph nodes; CHS, contact hypersensitivity; PLN, peritoneal cavity-draining lymph nodes; DNFB, 2,4-dinitrofluorobenzene; VDR, vitamin D receptor.

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analog, significantly suppressed CHS responses by 64% (15). In this paper, increased regulatory activity of $CD4^+CD25^+Foxp3^+$ cells in the skin-draining lymph nodes (SDLN) following a single topical application of $1,25(OH)_2D_3$ is described. The effects of topical $1,25(OH)_2D_3$ were compared with those of UVB irradiation as this environmental factor can both induce and activate regulatory T cells in the SDLN of otherwise naive mice (16). These studies indicate that $1,25(OH)_2D_3$ may be an important mediator by which UVB-irradiation exerts some of its immunomodulatory effects.

Materials and Methods

Mice

Mice transgenic for the $OVA_{323–339}$ (ISQAVHAAHAEINEAGR)-specific TCR-$\alpha\beta$ (DO11.10) on a BALB/c background were originally purchased from The Jackson Laboratory and bred in-house. Female DO11.10 mice were used between the ages of 8–12 wks. Expression of $OVA_{323–339}$-specific TCR-$\alpha\beta$ on T cells was confirmed by staining lymph node cells with biotinylated-anti-DO11.10 TCR mAb (KJ1–26; Caltag Laboratories) and then PE-Cy5-streptavidin (BD Biosciences). Female 8-wk-old BALB/c mice were purchased from the Animal Resources Centre (Murdoch, Western Australia). 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 Institute for Child Health Research Animal Experimentation Ethics Committee.

Vitamin D application

A 100-μl aliquot containing 125 ng of 1,25-dihyroxyvitamin D_3 ($1,25(OH)_2D_3$; Sigma-Aldrich) diluted in ethanol, propylene glycol, and water mixed at a 2:1:1 ratio was painted onto a clean-shaven 8 cm^2 dorsal skin surface of mice. This was equivalent to 37 pmol $1,25(OH)_2D_3$ per cm^2 of skin, or 100 μl of 3 μM $1,25(OH)_2D_3$. Alternatively, the vehicle used to dilute $1,25(OH)_2D_3$ was applied in a similar manner. In studies of repair of UV-induced thymine dimers, the vehicle or $1,25(OH)_2D_3$ was applied immediately after UV irradiation. $1,25(OH)_2D_3$ was stored under argon gas at −80°C. The chemical integrity of $1,25(OH)_2D_3$ was routinely verified using a scanning spectrophotometer.

UV irradiation

A bank of FS40 sunlamps (Westinghouse) emitting a broad band of UV, 250–360 nm, with 65% of the output in the UVB range (290–320 nm), was used to irradiate mice to deliver a dose of 8 kJ/m^2 onto clean-shaven 8 cm^2 dorsal skin as previously described (17, 18). PVC plastic film (0.22 mm thick) was taped to the top of each perspex cage before irradiation to screen out wavelengths <290 nm (UVC radiation). The dose rate of UVB was monitored using a UVX radiometer with a UVX-31 sensor (UV Products) after using the PVC film to remove the UVC wavelengths. Mice were housed in individual compartments of perspex cages during irradiation. UV lamps were held 20 cm above the cages.

Quantification of cyclobutane pyrimidine dimer positive nuclei in UV-irradiated skin

Skin samples were taken from UV-irradiated dorsal skin at 0.5, 3 and 24 h post-UV, fixed in Histochoice (Amresco) and paraffin-embedded. Detection of cyclobutane pyrimidine dimers was by immunohistochemical analysis as previously described (19). Sections were deparaffinised through graded alcohol and endogenous peroxidase blocked by 3% H_2O_2 in H_2O, followed by Ag retrieval with citrate buffer. Nonspecific Ab staining was blocked using 10% normal horse serum in Tris-buffered saline. Sections were incubated with the primary mouse monoclonal IgG1 anti-thymine dimer Ab (Affitech) or isotype control (20 μg/ml) in combination with the Animal Research kit (DakoCytomation).

In vitro proliferation assay

$CD4^+$ cells were isolated from the SDLN (inguinal, axillary, and brachial) of treated or control DO11.10 mice. MHC class II^+ and $CD8\alpha^+$ cells were removed using supernatant from the TIB-120 hybridoma and rat anti-CD8α mAb (2.5 μg/ml; BD Biosciences) with M-450 sheep anti-rat IgG Dynabeads (Dynal Biotech). A $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec) was used to deplete $CD25^+$ cells from $CD4^+$ cells. $CD4^+$ cells (≥95% pure, as determined by flow cytometry) were labeled with 5 μM CFSE (Molecular Probes) for 10 min at room temperature. After washing three times, cells were resuspended in RPMI 1640 with 10% FCS and 2 μM 2-ME and aliquoted into round-bottom 96-well plates at 10^5 cells/0.1 ml/well. $OVA_{323–339}$ peptide (Proteomics International) was added at a final concentration of 1 μg/ml. For preparation of APC, lymph nodes of naive BALB/c mice were digested with collagenase type 4 (1 mg/ml; Worthington Biochemical) and DNase (0.1 mg/ml; Sigma-Aldrich) for 30 min at 37°C. CD11c microbeads (Miltenyi Biotec) were used with the autoMACS separator (Miltenyi Biotec) to positively select for $CD11c^+$ cells. Cells were washed with RPMI 1640 with 10% FCS and 2 μM 2-ME and added to $CD4^+$ cells at varying ratios (1:10 to 1:1000) in replicates of six. This APC population was composed of >90% $CD11c^+$ cells, confirmed by staining with PE-anti-CD11c mAb. After incubation for 92 h at 37°C in 5% CO_2, supernatants were harvested and cells washed and stained with PE-Cy5-anti-CD4 mAb. Data were analyzed using the proliferation algorithm of FlowJo software (version 4.6.1; TreeStar) on viable cells, which were gated according to forward and side scatter properties and CD4 expression; 50,000 events were collected on the flow cytometer. The percentage of total cells that had divided was determined by using measurements of the number of viable $CD4^+$ CFSE-lo cells. All results shown are of APC cultured at a 1:100 ratio with $CD4^+$ cells.

Cytokine detection in supernatants

IL-2, IL-4, IL-5, IL-10, IL-12, and IFN-γ were detected using rat anti-mouse IL-2, IL-4, IL-5, IL-10, IL-12, or IFN-γ ELISA capture and detection mAb (BD Biosciences) in a dissociation-enhanced time-resolved fluorescence immunoassay with Europium as the label (sensitivity 25 pg/ml). Recombinant mouse IL-2, IL-4, IL-5, IL-10, IL-12, or IFN-γ (BD Biosciences) were used as the standards.

Regulation of proliferation by $CD4^+CD25^+$ cells in vitro

$CD4^+CD25^+$ cells (≥95% pure, as determined by flow cytometry) were isolated from the SDLN of treated or control BALB/c mice using the $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec). $CD4^+CD25^-$ cells (≥95% pure) were purified from the lymph nodes of naive DO11.10 mice by also using the $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec) and then labeled with 5 μM CFSE (Molecular Probes). $CD4^+CD25^-$ cells were resuspended in RPMI 1640 with 10% FCS and 2 μM 2-ME and aliquoted into round-bottom 96-well plates at 10^5 cells/0.1 ml/well. The $CD4^+CD25^+$ cells were added to the $CD4^+CD25^-$ cells at ratios of 1:2, 1:5, or 1:10. $OVA_{323–339}$ peptide was added at a final concentration of 1 μg/ml. APC were prepared as detailed above, and were added to $CD4^+CD25^-$ cells at a ratio of 1:100. After incubation for 92 h at 37°C in 5% CO_2, supernatants were harvested and cells washed and stained with PE-Cy5-anti-CD4 mAb. The proliferation of the cells ($CD4^+CD25^-$) was determined as described above. Alternatively, methyl-[H^3]-thymidine (0.25 mCi; 10 μl/well; Amersham Biosciences) was added to cultures 24 h before the harvest of cells, with [H^3]thymidine incorporation used as a measure of cellular proliferation.

Contact hypersensitivity responses

Four days after treatment, 1.5×10^7 $CD4^+$ or $CD4^+CD25^-$ cells or 2.5×10^5 $CD4^+CD25^+$ cells were purified as described above from SDLN of untreated, $1,25(OH)_2D_3$-treated or UVB-irradiated BALB/c mice, and adoptively transferred via the tail vein into naive BALB/c mice (five mice per group). Flow cytometry was used to determine that these cells were routinely ≥95% pure. Twenty-four hours after adoptive transfer, mice were sensitized by painting 25 μl 0.5% 2,4-dinitrofluorobenzene (DNFB; Sigma-Aldrich) diluted in acetone onto the shaved ventral surface. After another 5 days, a CHS response was elicited by painting dorsal and ventral ear surfaces with 10 μl 0.2% DNFB (in acetone). After 24 h, the ear thickness was measured in a blinded manner as previously described (16–18). The mean ear swelling measured in mice that were challenged but not sensitized with DNFB (0.01 mm) was subtracted from ear measurements in each experiment.

FACS analysis and Abs

The following anti-mouse mAb were obtained from BD Biosciences: FITC- and biotinylated-anti-CD3; FITC-anti-CD62L, PE- and PE-Cy5-anti-CD4; PE-anti-CD44; PE-anti-CD69; biotinylated anti-CD45RB; biotinylated anti-CD54; biotinylated anti-CD103; allyphococyanin-anti-CD25; PE-Cy5-labeled streptavidin; PE-anti-CD11c; and anti-CD16/32. For staining of surface markers, lymph node cells were incubated with Abs and staining buffer (PBS, 0.1% BSA, and 0.01% sodium azide) for 30 min, washed and fixed in 0.3 ml 1% formaldehyde (in PBS). An anti-mouse/rat Foxp3-PE staining kit from eBioscience was used for intracellular staining as described by the manufacturers. A mouse anti-human ki67-FITC kit (BD

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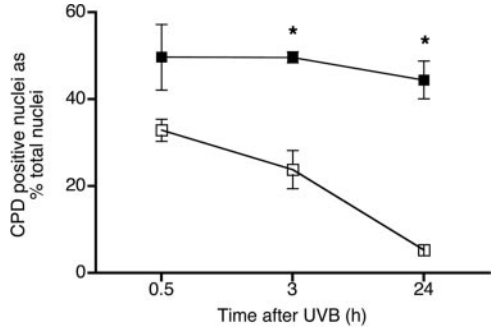

FIGURE 1. Topical $1,25(OH)_2D_3$ significantly reduced UVB-induced DNA damage in irradiated skin cells. The shaded dorsal skin of BALB/c mice was irradiated with 8 kJ/m^2 UVB and then either vehicle (■) or 125 ng $1,25(OH)_2D_3$ (□) applied immediately to the irradiated skin. After 0.5, 3, and 24 h, the numbers of cyclobutane pyrimidine dimers were calculated in skin sections as a percentage of total epidermal cell nuclei. Results are shown as mean ± SEM (n = 3 mice/group). *, A significant difference between vehicle and $1,25(OH)_2D_3$ treatments.

Biosciences) was used to detect intracellular expression of ki67 in conjunction with the anti-mouse/rat Foxp3-PE staining kit. Data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software.

Transfer of CD4$^+$CD25$^+$ cells and subsequent in vivo OVA sensitization of recipient mice

CD4$^+$CD25$^+$ cells were purified from the SDLN of $1,25(OH)_2D_3$-treated or control DO11.10 mice, as described above. Flow cytometry was used to determine surface expression of CD3, CD4, and CD25 so that CD4$^+$CD25$^+$ cells were routinely ≥95% pure. Twenty-four hours following the adoptive transfer of 1×10^5 CD4$^+$CD25$^+$ cells, naive BALB/c mice were sensitized by i.p. injection of 20 μg OVA protein (Grade V, 98% purity; Sigma-Aldrich) in a suspension with 4 mg of alumium hydroxide (Alu-Gel-S, 2% $Al(OH)_3$; Serva) in a volume of 200 μl. At 72 h after OVA-sensitization, the lymph nodes that drain the peritoneal cavity (parathymic) were removed from three mice per treatment.

Statistical analyses

Data were compared using the Student's t test or ANOVA with the Tukey's posthoc analysis using Microsoft Excel and Prism statistical analysis programs for Macintosh as appropriate.

Results

Physiological activity of 1,25(OH)$_2$D$_3$

Previous studies have shown that $1,25(OH)_2D_3$ can reduce DNA damage induced by UV irradiation (4, 19, 20). To confirm that the 125 ng dose of $1,25(OH)_2D_3$ was physiologically active, its capacity to reduce DNA damage was tested by removing skin samples from BALB/c mice at various times after UV-irradiation with either $1,25(OH)_2D_3$ or vehicle applied topically immediately after irradiation. $1,25(OH)_2D_3$ significantly reduced the number of nuclei positive for cyclobutane pyrimidine dimers as early as 3 h postirradiation (Fig. 1).

CD4$^+$ cells from 1,25(OH)$_2$D$_3$-treated mice had reduced capacity to proliferate in vitro

To determine whether CD4$^+$ cells from the SDLN of $1,25(OH)_2D_3$-treated mice had a diminished capacity to proliferate in vitro, these cells were purified from DO11.10 mice at 4 days posttreatment and cultured with OVA peptide and APC (CD11c$^+$ cells) derived from the lymph nodes of naive mice. The majority of CD4$^+$ cells in the DO11.10 mice express the $OVA_{323–339}$ TCR, and will thus proliferate in response to presentation of the $OVA_{323–339}$ peptide by APC either in vitro or in vivo. As

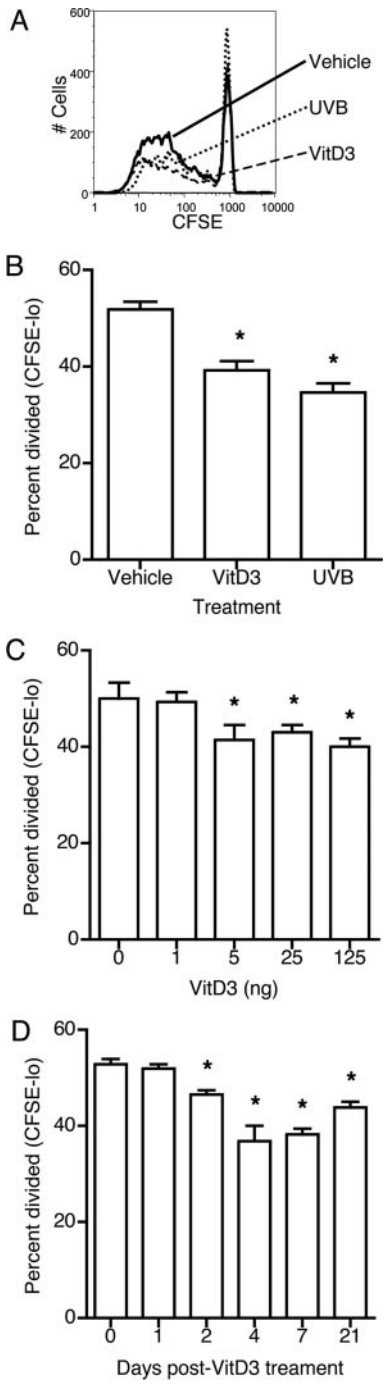

FIGURE 2. Topical $1,25(OH)_2D_3$ significantly reduced the capacity of CD4$^+$ cells from the SDLN of treated mice to proliferate in vitro. In *A* and *B* the shaved dorsal skin of DO11.10 mice was topically treated with vehicle, 125 ng $1,25(OH)_2D_3$ (VitD3) or 8 kJ/m^2 UVB. Results shown in *B* have been pooled from six independent experiments (mean + SEM). In *C*, the shaved dorsal surfaces of DO11.10 mice were topically treated with vehicle (0 ng of $1,25(OH)_2D_3$ or 1, 5, 25, or 125 ng $1,25(OH)_2D_3$. In *A–C*, four days after treatment, CD4$^+$ cells were purified from the SDLN and cultured with CD11c$^+$ cells purified from the lymph nodes of naive BALB/c mice at a 100:1 ratio, with 1 μg/ml $OVA_{323–339}$ peptide. In *D*, mice were administered vehicle (0 days) or 125 ng $1,25(OH)_2D_3$ and after 1, 2, 4, 7, and 21 days, CD4$^+$ cells were purified from the SDLN. In *A–D*, proliferation of the CD4$^+$ cells was determined by dilution of the intracellular dye CFSE after 92 h of coculture, which are shown as overlays in *A*. In *B*, *C* and *D*, the percentage of divided live CD4$^+$ (CFSE-lo) cells is shown. In *B*, *C* and *D*, an asterisk (*) denotes a significant suppression following $1,25(OH)_2D_3$ or UVB administration. Results show mean + SEM where cells were pooled from three mice per group and proliferation of cells determined from triplicate wells.

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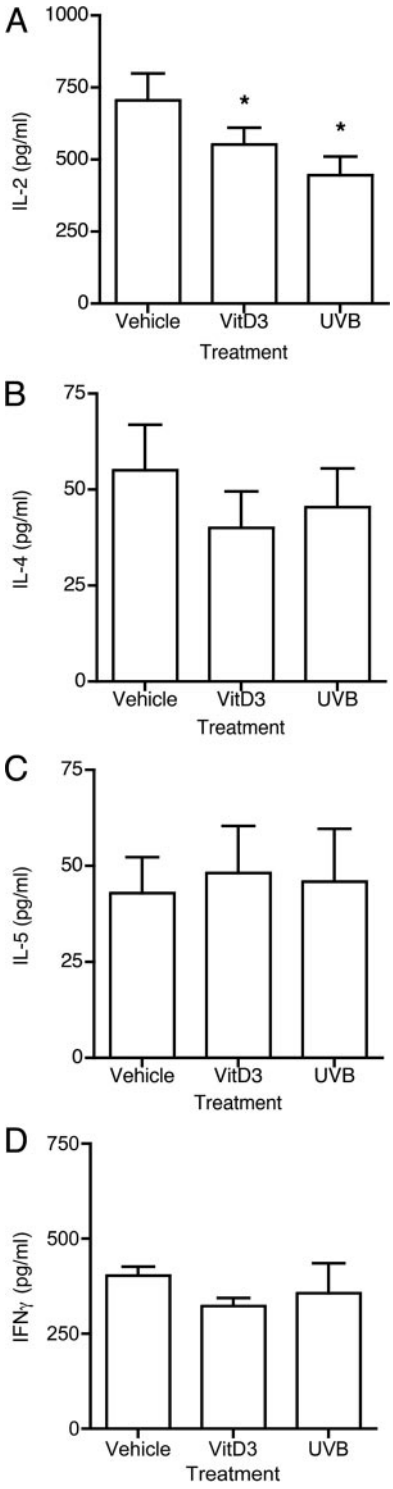

FIGURE 3. $CD4^+$ cells from the SDLN of $1,25(OH)_2D_3$-treated mice secreted less IL-2, but similar quantities of Th1- and Th2-type cytokines as $CD4^+$ cells from vehicle-treated mice. Supernatants were harvested from APC-T cell cocultures after 92 h and three samples were tested per treatment for each of six independent experiments. Levels of IL-2 (*A*), IL-4 (*B*), IL-5 (*C*), and IFN-γ (*D*) were detected using a modified ELISA technique. In *A*, an asterisk (∗) denotes a significant difference compared with vehicle treatment only. Results are shown as mean + SEM ($n = 18$).

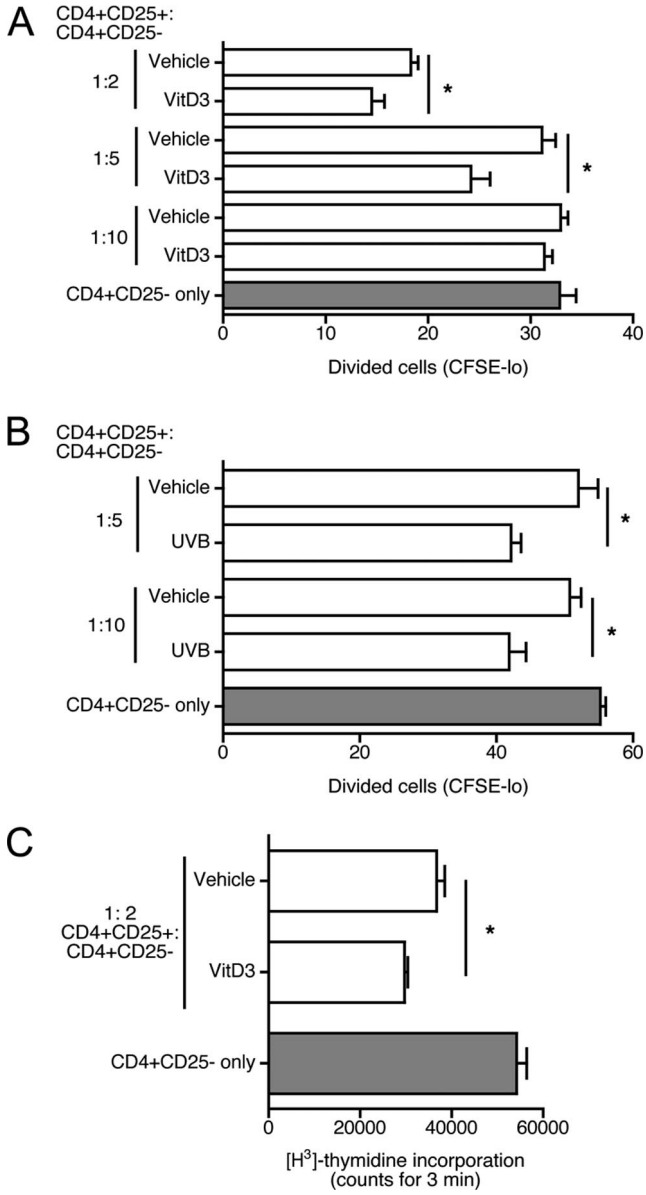

FIGURE 4. $CD4^+CD25^+$ cells from mice topically treated with $1,25(OH)_2D_3$ had enhanced capacity to suppress the proliferation of cocultured $CD4^+CD25^-$ cells. The shaved dorsal skin of BALB/c mice was topically treated with vehicle, 125 ng $1,25(OH)_2D_3$ (VitD3; *A* and *C*) or 8 kJ/m^2 UVB (*B*). $CD4^+CD25^+$ cells were purified from the SDLN at four days posttreatment, and then cocultured with CFSE-labeled $CD4^+CD25^-$ cells from lymph nodes of naive DO11.10 mice at ratio of 1:2, 1:5, or 1:10. $OVA_{323–339}$ peptide (1 μg/ml) and $CD11c^+$ cells were also added at a 1:100 ratio with the $CD4^+CD25^-$ cells. Proliferation of the $CD4^+CD25^-$ cells was determined by dilution of CFSE after 92 h of coculture, which are shown as the percentage of divided live $CD4^+$ cells (CFSE-lo) in *A* and *B*. In *C*, $CD4^+CD25^+$ cells were purified from the SDLN of BALB/c mice at four days post $1,25(OH)_2D_3$ treatment, and then cocultured with $CD4^+CD25^-$ cells from lymph nodes of naive DO11.10 mice at a ratio of 1:2 with $CD11c^+$ cells and OVA peptide. Proliferation of cells in these cultures was determined by the incorporation of [H^3]thymidine over the last 24 h of the 92 h culture period. ∗, A significant difference between treatments. Results show mean + SEM where cells were pooled from at least three mice per group and proliferation of cells determined from triplicate wells.

determined by dilution of the CFSE label on proliferating cells, there was a significant reduction in the ability of $CD4^+$ cells purified from the SDLN of $1,25(OH)_2D_3$-treated or UVB-irradiated mice to proliferate in vitro (Fig. 2*A*). For six experiments, the percentage of divided cells (CFSE-lo) was significantly reduced when $CD4^+$ cells were derived from the SDLN of $1,25(OH)_2D_3$-treated or UVB-irradiated mice (Fig. 2*B*). However, there was no difference

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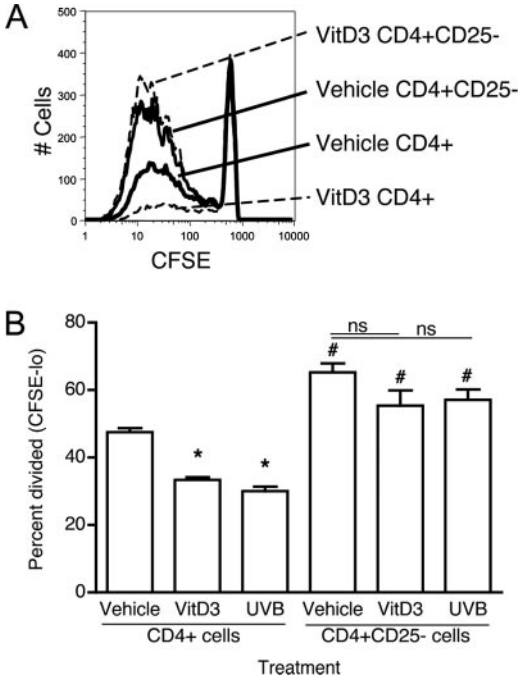

FIGURE 5. Depletion of $CD25^+$ cells from $CD4^+$ cells derived from mice treated with $1,25(OH)_2D_3$ ablated the suppression of proliferation observed in T cell cultures. The shaved dorsal skin of DO11.10 mice was topically treated with vehicle, 125 ng $1,25(OH)_2D_3$ (VitD3) or 8 kJ/m^2 UVB. Four days after treatment, $CD4^+$ or $CD4^+CD25^-$ cells were purified from the SDLN of these mice, labeled with CFSE and cultured with CD11c+ cells purified from the lymph nodes of naive BALB/c mice at a 100:1 ratio with 1 μg/ml $OVA_{323-339}$ peptide. Proliferation of cells was determined by dilution of CFSE after 92 h of coculture, which are shown as overlays in *A*, and as the percentage of divided live $CD4^+$ cells (CFSE-lo) in *B*. ∗, A significant reduction in the percentage of divided cells for $CD4^+$ cells from $1,25(OH)_2D_3$ -treated or UVB-irradiated mice. #, A significant increase in proliferation with the removal of $CD4^+CD25^+$ cells by comparing the extent of proliferation observed in cultures containing $CD4^+$ cells with those containing $CD4^+CD25^-$ cells within treatment groups. ns, Nonsignificant difference in the extent of proliferation was detected for $CD4^+CD25^-$ cells derived from vehicle and $1,25(OH)_2D_3$-treated or UVB-irradiated mice. Results show mean + SEM where cells were pooled from at least three mice per group and proliferation of cells determined from triplicate wells.

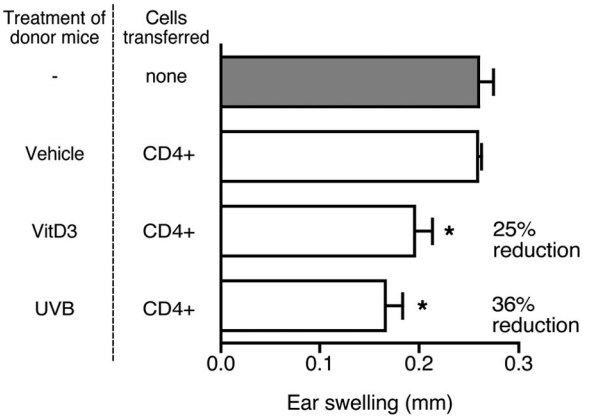

FIGURE 6. $CD4^+$ cells purified from the SDLN of $1,25(OH)_2D_3$-treated or UVB-irradiated mice suppressed contact hypersensitivity responses. The shaved dorsal skin of BALB/c mice was topically treated with vehicle, 125 ng $1,25(OH)_2D_3$ (VitD3) or 8 kJ/m^2 UVB. Four days after treatment, $CD4^+$ cells were purified from the SDLN and 1.5×10^7 cells adoptively transferred into recipient BALB/c mice (five mice per group). After 24 h, recipient mice were sensitized on shaved ventral skin by topical application of 25 μl 0.5% DNFB. Ear swelling responses were elicited after a further 5 days by applying 10 μl 0.2% DNFB to each ear pinna. Ear swelling was measured after a further 24 h. ∗, A significant difference between mice receiving $CD4^+$ cells from vehicle and $1,25(OH)_2D_3$-treated mice (25% reduction) or UVB treatments (36% reduction). Ear swelling responses for mice that did not receive $CD4^+$ cells are also shown. Results are shown as mean + SEM ($n = 5$).

in the extent of proliferation observed between $CD4^+$ cell cultures derived from $1,25(OH)_2D_3$-treated and UVB-irradiated mice (Fig. 2*B*).

To determine whether the suppressive effects of $1,25(OH)_2D_3$ were related to dose, DO11.10 mice were topically treated with vehicle or 1, 5, 25, or 125 ng $1,25(OH)_2D_3$. At 4 days posttreatment the SDLN were removed, $CD4^+$ cells purified and cells cultured with APC and OVA peptide. $CD4^+$ cell proliferation was lowest with a dose of 125 ng of $1,25(OH)_2D_3$ (Fig. 2*C*). However, significantly reduced proliferation was observed with a dose of 5 ng $1,25(OH)_2D_3$ (Fig. 2*C*). To establish the time post $1,25(OH)_2D_3$ treatment at which optimal reduction in proliferation occurs, the SDLN were removed at 1, 2, 4, 7, or 21 days after topical treatment with 125 ng $1,25(OH)_2D_3$. At these times, $CD4^+$ cells were purified from the SDLN and cells cultured with APC and OVA peptide. The greatest reductions in proliferation were observed at 4 and 7 days post $1,25(OH)_2D_3$ administration (Fig. 2*D*). Significant effects were also detected as early as 2 days and as late as 21 days post $1,25(OH)_2D_3$ treatment. Thus, application of 5–125 ng $1,25(OH)_2D_3$ caused reduced proliferation in vitro of $CD4^+$ cells from the SDLN of topically treated mice, and this effect on $CD4^+$ cell proliferation was observed for SDLN cells harvested up to 21 days posttreatment.

Topical $1,25(OH)_2D_3$ did not induce a Th2-bias within cultured $CD4^+$ cells

Previous analyses found that lymphocytes cultured with $1,25(OH)_2D_3$ added in vitro (21, 22) or derived from $1,25(OH)_2D_3$-treated mice (23, 24) secreted increased quantities of Th2-type cytokines such as IL-4 (21, 22), and reduced quantities of the Th1-type cytokine IFN-γ (22–24). To determine whether this occurred in our model, cytokine levels were examined in culture supernatants obtained 92 h after the coculture of APC with $CD4^+$ cells from the SDLN of treated mice. There was a significant reduction in IL-2 levels in cultures containing $CD4^+$ cells from the SDLN of $1,25(OH)_2D_3$-treated or UVB-irradiated mice (Fig. 3*A*). These reductions in IL-2 significantly correlated with the reduced proliferation observed in Fig. 2*B* ($r = 0.99$, $p < 0.01$). Levels of IL-4, IL-5, IL-10, IL-12, or IFN-γ present in cell culture supernatants of $CD4^+$ cells were not altered. IL-4, IL-5, and IFN-γ levels are shown in Fig. 3*B*, *C*, and *D*, respectively. Thus, with the in vitro conditions used in this study, $CD4^+$ cells derived from the SDLN of $1,25(OH)_2D_3$-treated mice did not secrete increased quantities of Th2-type cytokines or reduced quantities of Th1-type cytokines.

Enhanced capacity of $CD4^+CD25^+$ cells from $1,25(OH)_2D_3$-treated mice to suppress the proliferation of cocultured $CD4^+CD25^-$ cells

Reduced proliferation may reflect control by activated regulatory T cells. To determine whether $CD4^+CD25^+$ cells from the SDLN of $1,25(OH)_2D_3$-treated (125 ng) or UVB-irradiated (8 kJ/m^2) mice had an increased capacity to suppress the proliferation of cocultured $CD4^+CD25^-$ cells in vitro, $CD4^+CD25^+$ cells were purified

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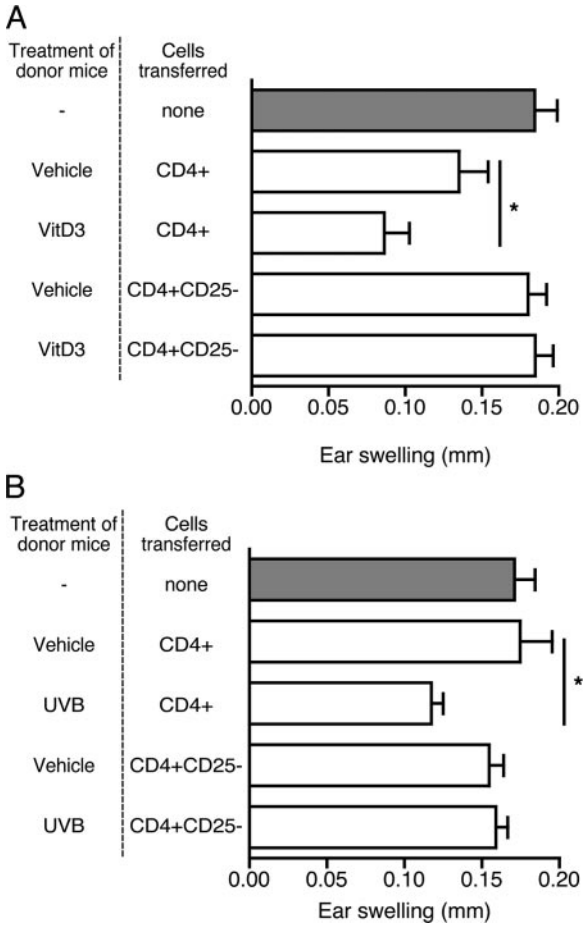

FIGURE 7. Depletion of $CD25^+$ cells from $CD4^+$ cells before adoptive transfer prevented the suppression of contact hypersensitivity responses in recipients of $CD4^+$ cells from $1,25(OH)_2D_3$-treated mice. The shaved dorsal skin of BALB/c mice was treated with vehicle, $1,25(OH)_2D_3$ (VitD3, *A*) or 8 kJ/m^2 UVB (*B*). Four days after treatment, $CD4^+$ or $CD4^+CD25^-$ cells were purified from the SDLN of these mice and 1.5×10^7 cells adoptively transferred into recipient BALB/c mice (five mice per group). After 24 h, recipient mice were sensitized by topical application of 25 μl 0.5% DNFB. Ear swelling responses were elicited after a further 5 days by applying 10 μl 0.2% DNFB to each ear pinna. Ear swelling was measured after a further 24 h. ∗, A significant difference between treatment pairs. Ear swelling responses for mice that did not receive $CD4^+$ or $CD4^+CD25^-$ cells are also shown. Results are shown as mean + SEM ($n = 5$).

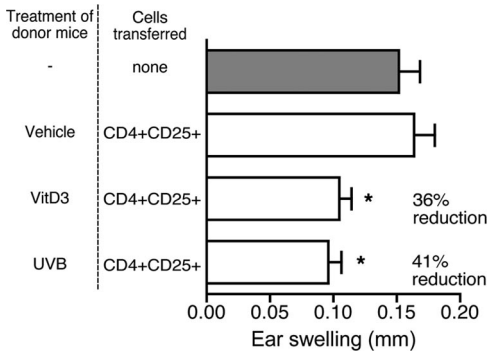

FIGURE 8. $CD4^+CD25^+$ cells purified from the SDLN of $1,25(OH)_2D_3$-treated or UVB-irradiated mice had increased capacity to suppress contact hypersensitivity responses. The shaved dorsal skin of BALB/c mice was topically treated with vehicle, 125 ng $1,25(OH)_2D_3$ (VitD3) or 8 kJ/m^2 UVB. Four days after treatment, $CD4^+CD25^+$ cells were purified from the SDLN and 2.5×10^5 adoptively transferred into recipient BALB/c mice (five mice per group). After 24 h, recipient mice were sensitized on shaved ventral skin by topical application of 25 μl 0.5% DNFB. Ear swelling responses were elicited after a further 5 days by applying 10 μl 0.2% DNFB to each ear pinna. Ear swelling was measured after a further 24 h. ∗, A significant difference between mice receiving $CD4^+CD25^+$ cells from vehicle and $1,25(OH)_2D_3$-treated (36% reduction) or UVB-irradiated mice (41% reduction). Ear swelling responses for mice that did not receive $CD4^+CD25^+$ cells are also shown. Results are shown as mean + SEM ($n = 5$).

from the SDLN of BALB/c mice at 4 days posttreatment. As controls, $CD4^+CD25^+$ cells were purified from vehicle-treated mice. These cells were cultured with $CD4^+CD25^-$ cells derived from the lymph nodes of DO11.10 mice at ratios of 1:2, 1:5, or 1:10 with APC from the lymph nodes of naive mice ($CD11c^+$ cells) and OVA peptide. As determined by dilution of the CFSE label upon proliferating $CD4^+CD25^-$ DO11.10 cells, $CD4^+CD25^+$ cells purified from the SDLN of $1,25(OH)_2D_3$-treated (Fig. 4*A*) or UVB-irradiated (Fig. 4*B*) mice had significantly increased capacity to suppress the proliferation of cocultured $CD4^+CD25^-$ DO11.10 cells than $CD4^+CD25^+$ cells from control mice. $CD4^+CD25^+$ cells from the SDLN of UVB-irradiated mice suppressed the proliferation of cells at both the 1:5 and 1:10 ratios, while those from $1,25(OH)_2D_3$-treated mice were more potent suppressors at only the 1:2 and 1:5 ratios. To confirm these observations with $1,25(OH)_2D_3$, a different measure of proliferation, [H^3]thymidine incorporation, was used. In this experiment, $CD4^+CD25^+$ cells from BALB/c mice were cultured at a 1:2 ratio with $CD4^+CD25^-$ DO11.10 cells with APC and OVA peptide. The incorporation of [H^3]thymidine was significantly reduced in cocultures containing $CD4^+CD25^+$ cells derived from the SDLN of $1,25(OH)_2D_3$-treated compared with those from vehicle-treated mice (Fig. 4*C*).

To confirm the increased suppressive capacity of $CD4^+CD25^+$ cells from the SDLN of $1,25(OH)_2D_3$-treated or UVB-irradiated mice, $CD4^+$ cells from the SDLN of DO11.10 mice were depleted of $CD25^+$ cells. The proliferation of these $CD4^+CD25^-$ cells was then compared with that of the $CD4^+$ cells by culturing them with $CD11c^+$ APC from the lymph nodes of naive mice, and OVA peptide for 92 h. Although the proliferation of $CD4^+$ cells from the SDLN of $1,25(OH)_2D_3$-treated (Fig. 5, *A* and *B*) or UVB-irradiated mice (Fig. 5*B*) was significantly reduced compared with $CD4^+$ cells from control mice, the removal of $CD25^+$ cells significantly enhanced proliferation of cells from all treatments (Fig. 5, *A* and *B*). Furthermore, depletion of the $CD25^+$ cells also removed the reduction in proliferation of cells from mice treated with vehicle and $1,25(OH)_2D_3$ or UVB (Fig. 5*B*). Together, these results indicate that $CD4^+CD25^+$ cells from the SDLN of $1,25(OH)_2D_3$-treated or UVB-irradiated mice had significantly enhanced capacity to suppress the proliferation of cocultured $CD4^+CD25^-$ cells compared with $CD4^+CD25^+$ cells from control mice.

Adoptive transfer of $CD4^+$ cells from $1,25(OH)_2D_3$-treated mice suppressed immune responses in vivo

To further confirm that topical $1,25(OH)_2D_3$ increases the suppressive activity of $CD4^+CD25^+$ cells in the SDLN, $CD4^+$ cells were purified from BALB/c mice treated with either the vehicle, $1,25(OH)_2D_3$ or UVB. In a previous study, we showed that transfer of 1.5×10^7 $CD4^+$ cells from UVB-irradiated mice was sufficient to suppress contact hypersensitivity (CHS) responses in naive recipients (16). Thus, 1.5×10^7 $CD4^+$ cells from the SDLN of either $1,25(OH)_2D_3$ or vehicle-treated mice were adoptively transferred into naive BALB/c recipients, which were then sensitized 24 h

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Table I. *Expression of various markers on $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from the SDLN of BALB/c mice treated four days earlier with vehicle or $1,25(OH)_2D_3$*

Marker (GMFI)[a]	Skin treatment	$CD4^+CD25^+$	$CD4^+CD25^-$
CD44	Vehicle	23.0 ± 2.6	6.7 ± 1.0
	$1,25(OH)_2D_3$	22.3 ± 3.2	5.7 ± 0.9
CD45RB	Vehicle	29.3 ± 2.0	144.7 ± 2.7
	$1,25(OH)_2D_3$	24.3 ± 4.9	154.7 ± 11.2
CD54	Vehicle	31.7 ± 2.0	7.3 ± 0.3
	$1,25(OH)_2D_3$	36.0 ± 4.0	9.0 ± 1.0
Marker (%)[a]	**Skin treatment**	**$CD4^+CD25^+$**	**$CD4^+CD25^-$**
$CD62L^{hi}$	Vehicle	57.8 ± 1.2	77.5 ± 1.7
	$1,25(OH)_2D_3$	59.3 ± 2.6	79.7 ± 1.1
$CD69^+$	Vehicle	34.7 ± 0.4	18.7 ± 0.4
	$1,25(OH)_2D_3$	35.8 ± 0.6	18.9 ± 1.0
$CD103^+$	Vehicle	26.1 ± 1.9	11.1 ± 0.6
	$1,25(OH)_2D_3$	24.5 ± 0.5	10.6 ± 1.1

[a] Data are shown as mean ± SEM for three experiments where the $CD3^+CD4^+$ T cells from the SDLN of three mice per treatment were tested for the expression of these markers by flow cytometry. GMFI, Geometric mean fluorescence index; %, percentage of cells that expressed high levels (hi) or were positive (+) for the particular marker.

later with 25 μl 0.5% DNFB. Ear swelling was elicited after another 5 days by applying 10 μl 0.2% DNFB to each ear pinna. Transfer of $CD4^+$ cells from the SDLN of mice treated with $1,25(OH)_2D_3$ or UVB significantly reduced the ear-swelling response in comparison with that observed in vehicle-treated control mice (Fig. 6).

$CD4^+CD25^+$ cells from the SDLN of $1,25(OH)_2D_3$-treated mice suppressed contact hypersensitivity responses in recipient mice

To determine whether $CD4^+CD25^+$ cells were responsible for reduced CHS responses following the adoptive transfer of $CD4^+$ cells from $1,25(OH)_2D_3$-treated mice, $CD4^+$ cells were depleted of $CD25^+$ cells before adoptive transfer. $CD4^+$ or $CD4^+CD25^-$ cells (1.5×10^7) from the SDLN of nonirradiated vehicle-treated, $1,25(OH)_2D_3$-treated, or UVB-irradiated BALB/c mice (isolated 4 days posttreatment) were adoptively transferred into naive recipients. $CD4^+$ cells from the SDLN of $1,25(OH)_2D_3$-treated (Fig. 7*A*) or UVB-irradiated (Fig. 7*B*) mice significantly reduced ear-swelling responses in recipient BALB/c mice to a similar extent, in comparison with responses observed in recipients of $CD4^+$ cells from vehicle-treated mice. Depletion of $CD25^+$ cells before adoptive transfer prevented the suppression observed in recipients of $CD4^+$ cells from either $1,25(OH)_2D_3$- (Fig. 7*A*) or UVB-treated (Fig. 7*B*) mice. In a further experiment, $CD4^+CD25^+$ cells (2.5×10^5 cells) purified from the SDLN of vehicle-treated, $1,25(OH)_2D_3$-treated, or UVB-irradiated BALB/c mice (isolated 4 days posttreatment) were adoptively transferred into naive recipients. $CD4^+CD25^+$ cells from vehicle-treated mice did not regulate CHS responses when compared with responses observed in mice that did not receive cells but were sensitized and challenged with DNFB (Fig. 8). $CD4^+CD25^+$ from both $1,25(OH)_2D_3$-treated and

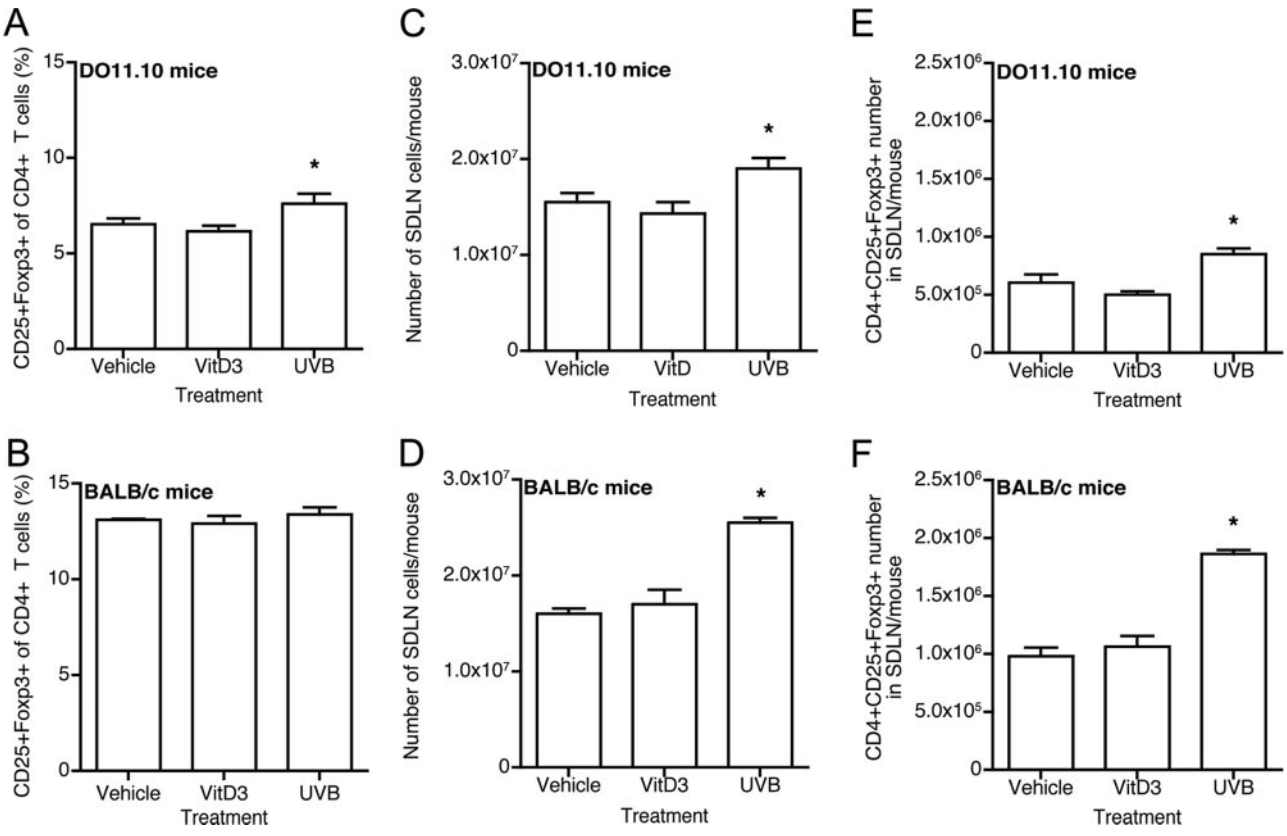

FIGURE 9. UVB irradiation but not topical $1,25(OH)_2D_3$ induced lymph node hypertrophy and increased the number of $CD4^+CD25^+Foxp3^+$ cells in the SDLN. The shaved dorsal skin of DO11.10 (*A*, *C*, and *E*) or BALB/c (*B*, *D*, and *F*) mice was topically treated with vehicle, 125 ng $1,25(OH)_2D_3$ (VitD3) or 8 kJ/m^2 UVB. After four days, the SDLN were removed and the number of cells counted (*C* and *D*). The proportions and numbers of $CD3^+CD4^+$ cells expressing CD25 and Foxp3 in the SDLN are shown in (*A* and *B*) and (*E* and *F*), respectively. Results were derived from six independent experiments where cells from three mice per group were pooled (mean + SEM). ∗, A significant difference between cells from vehicle and $1,25(OH)_2D_3$-treated or UVB-irradiated mice.

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UVB-irradiated mice significantly suppressed ear swelling responses in recipient mice (Fig. 8). Together these results indicate that the suppressive activity of $CD4^+CD25^+$ regulatory cells is enhanced following topical treatment with $1,25(OH)_2D_3$ or irradiation with UVB.

Topical $1,25(OH)_2D_3$ did not alter the phenotype or cellularity of the SDLN

UV-induced regulatory T cells express markers typical of the naturally occurring regulatory T cells, including CD25 (25) and Foxp3 (16, 26). To further examine the phenotype of regulatory T cells in the SDLN of mice topically treated with $1,25(OH)_2D_3$, a number of different T cell markers were examined on $CD4^+CD25^+$ or $CD4^+CD25^-$ cells from the SDLN of vehicle- or $1,25(OH)_2D_3$-treated BALB/c mice including the activation markers CD44 and CD69, the lymphocyte/memory markers CD45RB and CD62L, the costimulatory marker CD54, and the regulatory/mucosal cell marker CD103. The expression of these markers was not modified on either $CD4^+CD25^+$ or $CD4^+CD25^-$ cells from the SDLN of $1,25(OH)_2D_3$-treated mice (Table I). The majority of $CD4^+CD25^+$ cells from the SDLN also expressed Foxp3 (>90%). $1,25(OH)_2D_3$ had no effect upon the proportion (Fig. 9, *A* and *B*) or the number (Fig. 9, *E* and *F*) of $CD4^+CD25^+$ $Foxp3^+$ cells in the SDLN of either BALB/c or DO11.10 mice. The proportion of $CD4^+$ cells expressing Foxp3 and CD25 significantly increased following UVB irradiation in DO11.10 (Fig. 9*A*) but not BALB/c (Fig. 9*B*) mice, which have higher baseline levels of $Foxp3^+$ cells (Fig. 9*B*). UVB significantly increased the total number of SDLN cells, and therefore the number of $CD4^+CD25^+Foxp3^+$ cells in both DO11.10 (Fig. 9, *C* and *E*) and BALB/c (Fig. 9, *D* and *F*) mice.

The increased numbers of $CD4^+CD25^+$ cells in the SDLN of UVB irradiated mice was determined to be at least partially due to the proliferation of these cells. $CD4^+CD25^+$ cells were purified from the lymph nodes of naive DO11.10 mice, CFSE-labeled and 1.5×10^5 adoptively transferred into naive BALB/c recipients. After 18 h, recipient mice were treated with either vehicle, $1,25(OH)_2D_3$ or UVB. After four days, the SDLN were removed from these recipient mice, and the proportion of transferred cells ($OVA\text{-}TCR^+CD4^+$) that had subsequently divided (CFSE-lo) was determined. The proportions of dividing transferred cells were not altered by topical $1,25(OH)_2D_3$ (Fig. 10*A*). However, UVB irradiation significantly increased the number of dividing cells in the SDLN (Fig. 10*A*), where some cells had undergone up to two divisions (Fig. 10*B*). Together, these observations suggest that topical $1,25(OH)_2D_3$ increases the regulatory activity of $CD4^+CD25^+$ cells in the SDLN, whereas UVB irradiation has further proliferative effects upon these cells.

$CD4^+CD25^+$ cells from $1,25(OH)_2D_3$-treated mice can expand in recipient mice after Ag sensitization

As shown above, topical $1,25(OH)_2D_3$ alone did not cause the adoptively transferred $CD4^+CD25^+$ cells to proliferate. We then hypothesized that the presentation of Ag to these cells might induce their expansion in vivo. To test this, at four days post $1,25(OH)_2D_3$-treatment, $CD4^+CD25^+$ cells were purified from the SDLN of vehicle- or $1,25(OH)_2D_3$-treated DO11.10 mice, and 1×10^5 $CD4^+CD25^+$ cells adoptively transferred into naive BALB/c mice. After 24 h, recipient mice were sensitized i.p. with 20 μg OVA with Alum included as an adjuvant. Peritoneal cavity-draining lymph nodes (PLN) were removed at 72 h postsensitization and the expression of the regulatory T cell marker Foxp3 on the donor cells ($OVA\text{-}TCR^+$) was determined by flow cytometry. In addition, the expression of ki67, a

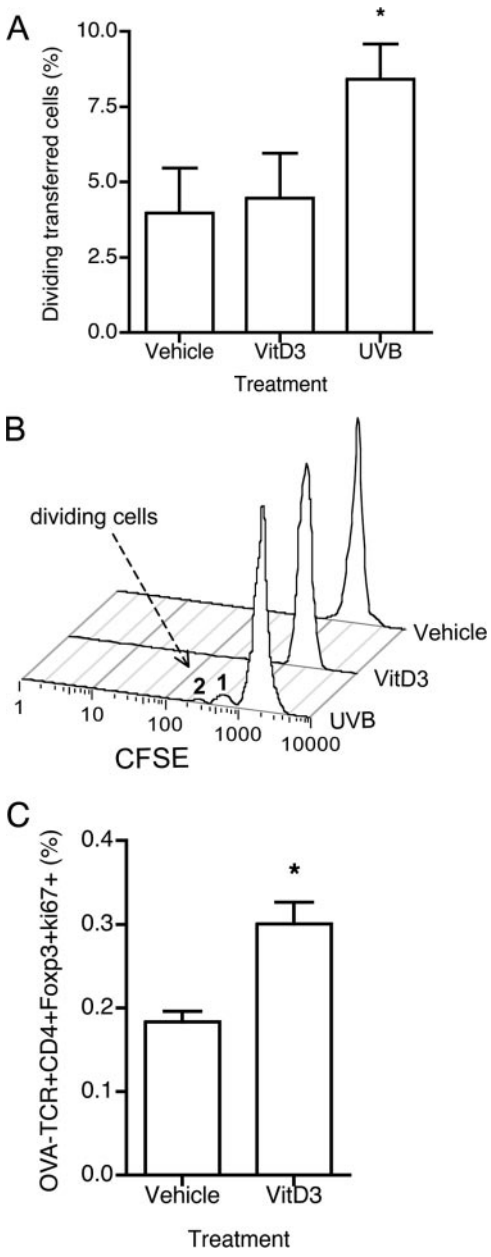

FIGURE 10. $CD4^+CD25^+$ cells from $1,25(OH)_2D_3$-treated mice proliferated after application of exogenous Ag. In *A* and *B*, $CD4^+CD25^+$ cells were purified from the lymph nodes of naive DO11.10 mice CFSE-labeled and 1.5×10^5 adoptively transferred into BALB/c recipients. After 18 h, the shaved dorsal skin of these recipient mice (n = 3/treatment) was topically treated with vehicle, 125 ng $1,25(OH)_2D_3$ (VitD3) or 8 kJ/m^2 UVB. Four days later, the SDLN were removed and CFSE levels in transferred cells ($OVA\text{-}TCR^+CD4^+CD25^+$) assessed by flow cytometry. Proliferation of the $CD4^+CD25^+$ cells was determined by dilution of CFSE, which are shown as the percentage of dividing cells (CFSE-lo) in *A* and as overlays in *B*. In *A*, an asterisk (*) denotes a significant difference in the percentage of dividing cells transferred from UVB-irradiated mice. Results are shown as mean + SEM (n = 3). In *B*, the numbers 1 and 2 refer to cells, which have undergone 1 or 2 divisions, respectively. In *C*, the shaved dorsal skin of DO11.10 mice was topically treated with vehicle or 125 ng $1,25(OH)_2D_3$ (VitD3). Four days after treatment, $CD4^+CD25^+$ cells were purified from the SDLN and 1×10^5 adoptively transferred into each of five recipient BALB/c mice. After 24 h, recipient mice were sensitized by intraperitoneal injection of 20 μg OVA with 4 mg alum. The PLN were removed 72 h after OVA sensitization and the proportion of $OVA\text{-}TCR^+CD4^+Foxp3^+ki67^+$'donor' cells in the PLN of recipients of $CD4^+CD25^+$ cells investigated. An asterisk (*) denotes a significant increase for cells transferred from $1,25(OH)_2D_3$-treated mice. Results are shown as mean + SEM (n = 3).

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nuclear cell proliferation-associated Ag expressed in all active stages of the cell cycle was determined. At 72 h, the proportion of $CD4^+Foxp3^+ki67^+$ cells that were derived from the donor mice (OVA-TCR^+) was significantly increased in the recipients of $CD4^+CD25^+$ cells from $1,25(OH)_2D_3$-treated mice (Fig. 10*C*). The expression of ki67 upon $CD4^+Foxp3^+$ cells indicated that these cells were cycling, and that the increased number in recipients of $CD4^+CD25^+$ cells from $1,25(OH)_2D_3$-treated mice was not due to enhanced survival of the transferred population. Instead, the increased proportion of $CD4^+Foxp3^+ki67^+$ cells of donor origin in recipients of $CD4^+CD25^+$ cells from $1,25(OH)_2D_3$-treated mice suggests that OVA sensitization induced expansion of the transferred cells.

Discussion

This study demonstrates for the first time that a single topical application of a physiologically relevant amount of $1,25(OH)_2D_3$ to the shaved dorsal skin of mice enhances the suppressive capacity of $CD4^+CD25^+$ regulatory T cells in the SDLN. This complements other in vitro studies where $1,25(OH)_2D_3$ stimulated regulatory DC and T cells (5–7), and in vivo models where autoimmunity was reduced by chronic oral administration of $1,25(OH)_2D_3$ (8–11). In this study, $CD4^+CD25^+$ cells activated by topical administration of $1,25(OH)_2D_3$ suppressed the proliferation in vitro of cocultured $CD4^+CD25^-$ cells to a greater extent than equal numbers of $CD4^+CD25^+$ cells from control, vehicle-treated mice. Even though the regulatory activity of the $CD4^+CD25^+$ cells was enhanced by topical $1,25(OH)_2D_3$, numbers of $CD4^+CD25^+Foxp3^+$ cells in the SDLN were not increased. In an in vivo model, when equal numbers of $CD4^+CD25^+$ cells from the SDLN of BALB/c mice topically treated with $1,25(OH)_2D_3$ or vehicle were adoptively transferred into BALB/c recipients subsequently sensitized and challenged with hapten, ear swelling responses were significantly reduced in those mice that received cells from $1,25(OH)_2D_3$-treated mice.

Transferred $CD4^+CD25^+$ cells were derived from the SDLN, and thus may have been enriched for TCR specific for skin-derived Ags. Upon sensitization with a new Ag, we hypothesize that the transferred cells facilitate proliferation or expansion of new regulatory T cells specific for that Ag. Indeed, following the sensitization of recipient mice with OVA, proportions of $CD4^+Foxp3^+$ cells of donor origin (OVA-TCR^+) were increased in recipients of $CD4^+CD25^+$ cells from DO11.10 mice treated with $1,25(OH)_2D_3$. These cells also expressed ki67, a marker of proliferation, which suggests that transferred $CD4^+CD25^+$ cells expand in recipient mice upon sensitization with Ag. Similar results have been observed in a mouse model of diabetes, where adoptive transfer of regulatory T cells increased the number of $Foxp3^+$ cells in pancreatic lymph nodes to block the development of diabetes in recipient NOD mice (27).

In the SDLN of DO11.10 mice, the proportion of cells expressing the OVA-TCR was lower for $CD4^+CD25^+$ (60%) in comparison with $CD4^+CD25^-$ cells (85%). The levels of expression of the OVA-TCR on $CD4^+CD25^+$ cells from DO11.10 mice were not modified by $1,25(OH)_2D_3$ or UVB. The expression of OVA-TCR on the majority of $CD4^+CD25^+$ cells may facilitate the capacity of regulatory T cells from $1,25(OH)_2D_3$-treated or UVB-irradiated mice to suppress OVA-specific immune responses. However, experiments using BALB/c mice for the donor cells (e.g., CHS adoptive transfer experiments) demonstrate that the activating effects of $1,25(OH)_2D_3$ on $CD4^+CD25^+$ cells could be detected in systems not dependent on the majority of $CD4^+CD25^+$ cells expressing TCR for the experimental Ag. Our experiments suggest that the polyclonal endogenous TCR α-chains on the $CD4^+CD25^+$ cells are essential for manifestation of the regulatory function of these cells in different experimental systems.

In the experiments described, topically applied $1,25(OH)_2D_3$ failed to modify the balance or quantity of Th1- (IFN-γ) or Th2-type (IL-4 or IL-5) cytokines secreted by $CD4^+$ cells derived from the SDLN when cultured with APC and Ag. This is contrary to previous observations in NOD mice with autoimmune prostatitis, where chronic administration of a VDR agonist (elocalcitol) at 100 μg/kg/day for 10–16 days reduced the capacity of lymph node-derived $CD4^+$ T cells to secrete IFN-γ upon further stimulation in vitro (24). Furthermore, reduced IFN-γ and increased IL-4 levels were detected in colon protein extracts from mice treated with 0.2 μg of calcitriol/kg/day for 3 days in a model of Th1-mediated colitis (23). In these previous studies, mice were chronically administered significantly greater doses of $1,25(OH)_2D_3$ or a VDR agonist than used in the current experiments. In addition, the chronic treatments were previously administered via oral (24) or intraperitoneal (23) routes instead of the single topical treatment with $1,25(OH)_2D_3$ given to mice in this study.

The production of $1,25(OH)_2D_3$ in the skin of UV-irradiated mice may act as a homeostatic mechanism for controlling some of the inflammatory effects of UVB. $1,25(OH)_2D_3$ repaired UVB-induced DNA damage in the epidermis of irradiated BALB/c mice in this study as well as in Skh:HR1 hairless mice (19). UV-induced DNA damage was also reduced by culturing irradiated human skin keratinocytes (4), fibroblasts, or melanocytes (19) with $1,25(OH)_2D_3$. In contrast, both $1,25(OH)_2D_3$ and UVB increased the regulatory capacity of $CD4^+CD25^+$ cells in the SDLN. Repair of UV-induced DNA damage by $1,25(OH)_2D_3$ occurs via a rapid-acting, nongenomic pathway (4, 19). Thus, $1,25(OH)_2D_3$ may act via a different pathway to activate regulatory T cells. This alternate pathway may be the genomic pathway, which uses the VDR.

The 125 ng dose of $1,25(OH)_2D_3$ applied to the skin of mice in these studies was equivalent to applying 37 pmol $1,25(OH)_2D_3$ per cm^2 of skin or 100 μl of 3 μM $1,25(OH)_2VitD_3$. This dose was used because based on the levels measured in human skin equivalents and in human skin using microdialysis (3, 4), local concentration of $1,25(OH)_2D_3$ in the order of 2–5 nM might be achieved. The 125 ng dose is similar to that used in other investigations of the immunomodulatory effects of topically applied $1,25(OH)_2D_3$ (4, 14, 19, 20). However, lower amounts of $1,25(OH)_2D_3$ were also found to be active in our studies. Concentrations of $1,25(OH)_2D_3$ of 10–100 nM have been used to treat DC and/or T cells in vitro (5–7).

UVB irradiation of skin and topical $1,25(OH)_2D_3$ were both able to enhance the capacity of $CD4^+CD25^+$ cells to suppress the proliferation of $CD4^+CD25^-$ cells when cocultured in vitro, and to diminish CHS responses following adoptive transfer in vivo. This study did not identify differences in the mode of activation of $CD4^+CD25^+$ cells after erythemal UVB irradiation or topical $1,25(OH)_2D_3$. The phenotype of $CD4^+CD25^+$ cells activated by $1,25(OH)_2D_3$ is the subject of further experimentation, as are studies of their mode of action.

The immunomodulatory effects of UVB may be explained by its capacity to promote the secretion of IL-10, prostaglandins, TNF-α, and other inflammatory by-products by irradiated keratinocytes (reviewed by Ref. 28). These molecules may further act to expand regulatory T cells in the draining lymph nodes. Indeed, UVB irradiation alone (in the absence of exogenous Ag) caused

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$CD4^+CD25^+$ cells to proliferate in recipient mice. UVB irradiation also caused hypertrophy of the SDLN, with an increase in the number of $CD4^+CD25^+$ cells as previously described by ourselves (16, 29) and others (30). In contrast, it was only after sensitization with Ag, that $CD4^+CD25^+$ from $1,25(OH)_2D_3$-treated mice expanded in vivo.

Although the effects of erythemal UVB are systemic (16), the ability of topical $1,25(OH)_2D_3$ to enhance the suppressive capacity of $CD4^+CD25^+$ regulatory cells may be limited to the skin and draining lymph nodes. Topical treatment with $1,25(OH)_2D_3$ had no effect upon the ability of $CD4^+$ cells from the spleens of DO11.10 mice to proliferate in response to presentation of the OVA peptide by cocultured APC (data not shown). Although the effects of topical $1,25(OH)_2D_3$ may not be systemic, suppression of $CD4^+$ cell proliferation ex vivo was observed for as long as 21 days after a single treatment, indicating that $1,25(OH)_2D_3$ may have long-lasting effects upon regulatory T cells. Together, these differences indicate that UVB irradiation has further proliferative effects upon $CD4^+CD25^+$ regulatory cells that do not occur after application of topical $1,25(OH)_2D_3$ and that $1,25(OH)_2D_3$ may be one of the molecules generated in skin which is responsible for the activation of the $CD4^+CD25^+$ cells.

In this study, we were not able to determine whether $1,25(OH)_2D_3$ applied to skin had direct or indirect effects on $CD4^+CD25^+$ cells. This is the subject of further studies, where we hypothesize that some of the effects of $1,25(OH)_2D_3$ and UVB may be due to modified Ag presentation by DC from the skin of treated mice. UVB irradiation also causes skin DC to migrate to the SDLN (31), and once there, the presentation of modified skin Ags by DNA-damaged DC may induce a tolerogenic environment for the activation of regulatory T cells (32). However, the effects of topical $1,25(OH)_2D_3$ on skin cells including DC, keratinocytes, and mast cells are not completely understood.

It has been reported that numbers of MHC $classII^+$ LC in $1,25(OH)_2D_3$-treated ear skin were significantly reduced following topical application of 200 ng $1,25(OH)_2D_3$ to the ear skin of mice for 7 days (14). $1,25(OH)_2VitD_3$ also inhibited the expression of costimulatory molecules upon LC in vitro, and impaired the ability of these cells to present Ag in a MLR (33). Furthermore, DC can metabolize $1,25(OH)_2D_3$ precursors to program T cells to express skin-homing molecules in vitro (34). Additional investigations are required to determine whether LC from $1,25(OH)_2D_3$-treated mice have altered capacity to present Ag and whether modified LC are responsible for the activation and increased suppressive capacity of $CD4^+CD25^+$ regulatory T cells in the SDLN. These may focus upon the role of RANK-RANKL (receptor activator of NF-κB ligand) interactions in $1,25(OH)_2D_3$-treated skin (35, 36), or the role of the inhibitory receptor ILT3 (37). In psoriatic lesions, expression of ILT3 upon DC increased after topical administration of the $1,25(OH)_2D_3$ analog calcipotriol, although in vitro studies showed that ILT3 expression was dispensable for the induction of regulatory T cells by $1,25(OH)_2D_3$-treated DC (37). Further investigations are required to better understand how $1,25(OH)_2D_3$ formed in response to the UVB wavelengths of sunlight may contribute toward curbing over-zealous immune responses.

Disclosures

The authors have no financial conflict of interest.

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