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1,25-dihydroxyvitamin D_3 enhances the ability of transferred $CD4^+$ $CD25^+$ cells to modulate T helper type 2-driven asthmatic responses

Shelley Gorman, Melinda A. Judge, Jennifer T. Burchell, Debra J. Turner and Prue H. Hart

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

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Correspondence: Dr S. Gorman, Telethon Institute for Child Health Research, Centre for Child Health Research, University of Western Australia, PO Box 855, West Perth, Western Australia, 6872, Australia.
Email: shelleyg@ichr.uwa.edu.au
Senior author: Prue H. Hart,
email: pruch@ich.uwa.edu.au

Summary

The severity of allergic diseases may be modified by vitamin D. However, the immune pathways modulated by the active form of vitamin D, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], are yet to be fully elucidated. In this study, naturally occurring $CD4^+$ $CD25^+$ cells from the skin-draining lymph nodes (SDLN) of mice treated with topical $1,25(OH)_2D_3$ had an increased ability to suppress T helper type 2 (Th2) -skewed immune responses. $CD4^+$ $CD25^+$ cells transferred from mice treated with topical $1,25(OH)_2D_3$ into ovalbumin (OVA) -sensitized mice challenged intranasally with OVA 18 hr later, significantly suppressed the capacity of airway-draining lymph node (ADLN) cells to proliferate and secrete cytokines in response to further OVA stimulation *ex vivo*. The $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated mice also reduced airway hyperresponsiveness and the proportions of neutrophils and eosinophils in bronchoalveolar lavage fluid (BALF). To test the effect of $1,25(OH)_2D_3$ on cells able to respond to a specific antigen, $CD4^+$ $CD25^+$ cells were purified from the SDLN of OVA-T-cell receptor (TCR) transgenic mice treated 4 days earlier with topical $1,25(OH)_2D_3$. $CD4^+$ $CD25^+$ cells from OVA-TCR mice treated with $1,25(OH)_2D_3$ were able to alter BALF cell content and suppress ADLN responses to a similar degree to those cells from non-transgenic mice, suggesting that the effect of $1,25(OH)_2D_3$ was not related to TCR signalling. In summary, topical $1,25(OH)_2D_3$ increased the regulatory capacity of $CD4^+$ $CD25^+$ cells from the SDLN to suppress Th2-mediated allergic airway disease. This work highlights how local $1,25(OH)_2D_3$ production by lung epithelial cells may modulate the suppressive activity of local regulatory T cells.

Keywords: asthma; $CD4^+$ $CD25^+$ regulatory T cells; lung; skin; vitamin D

Introduction

Currently, scientists in research laboratories around the world are working to optimize the conditions required to generate regulatory T-cell populations with increased capacity to modulate immune-mediated pathologies (reviewed in refs [1,2]), such as asthma. Asthma is a chronic illness of the airways characterized by wheeze, shortness of breath, chest tightness and cough. Dietary and environmental factors can modulate regulatory T-cell function. Examples of these include vitamin A (reviewed in ref.[2]), vitamin D[3] and ultraviolet B (UVB) irradiation.[4,5] One of the immune-driven diseases that can be modulated by environmental factors is asthma. Similarly, chronic treatment of mice with the active form of vitamin D [1,25-dihydroxyvitamin D_3; $1,25(OH)_2D_3$)] reduced airway hyperresponsiveness (AHR),[6] eosinophilia and interleukin-5 (IL-5) levels in bronchoalveolar lavage fluid (BALF).[7] Vitamin D deficiency may contribute towards the asthma epidemic;[8] however, the immune pathways modulated by vitamin D are yet to be fully elucidated.

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; ADLN, airway-draining lymph nodes; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; DC, dendritic cells; SDLN, skin-draining lymph nodes.

Vitamin D is synthesized from the precursor 7-dehydrocholesterol in the skin following exposure of skin to the UVB irradiation present in sunlight. Further enzymatic conversions in the kidneys and liver and other sites (including the skin) produce the active form of the hormone, $1,25(OH)_2D_3$. Many immune cells express the vitamin D receptor, including T cells, activated B cells and dendritic cells (DC; reviewed in ref.[8]), and so possess the potential to respond to $1,25(OH)_2D_3$ directly. However, most studies of $1,25(OH)_2D_3$ on immune cells have been performed *in vitro*, with concentrations of $1,25(OH)_2D_3$ greater than those observed in physiological settings. Vitamin D can regulate the *in vitro* function of various immune cells, including DC and T cells. Some DC become tolerogenic when cultured with $1,25(OH)_2D_3$. For example, co-stimulatory molecule expression on myeloid DC and Langerhans cells was reduced by $1,25(OH)_2D_3$.[9,10] The $1,25(OH)_2D_3$ also has tolerogenic effects on T cells; in particular, IL-10-secreting regulatory $CD4^+$ T cells were induced *in vitro* by $1,25(OH)_2D_3$ in the presence of dexamethasone.[11] In addition, $1,25(OH)_2D_3$ also up-regulates a skin-trophic chemokine receptor (CCR10) on T cells.[12] These *in vitro* observations point to an immunomodulatory role for $1,25(OH)_2D_3$ on DC and T cells.

We have examined the *in vivo* effects of topically applied $1,25(OH)_2D_3$ on immune cells as local skin concentrations in the order of 2–5 nM $1,25(OH)_2D_3$ may be achieved following UVB irradiation.[13,14] We[3] and others[15] propose that $1,25(OH)_2D_3$ produced in UVB-irradiated skin may initiate some of the immunomodulatory effects of UV irradiation. The $1,25(OH)_2D_3$ was applied to the skin of naive BALB/c mice and the phenotype and function of immune cells were examined in the draining lymph nodes.[3] $CD4^+$ T cells from the lymph nodes proliferated less in response to stimulation *ex vivo*;[3] this was the result of an increased suppressive activity of $CD4^+$ $CD25^+$ $Foxp3^+$ cells in the skin-draining lymph nodes (SDLN). Topical $1,25(OH)_2D_3$ augmented the ability of these cells to suppress the proliferation of co-cultured $CD4^+$ $CD25^-$ T cells *in vitro* and also their capacity to suppress T helper type 1 (Th1)/Th17-skewed contact hypersensitivity[16] responses upon adoptive transfer into naive recipient mice.[3] These results suggested that UVB-induced $1,25(OH)_2D_3$ production in skin affects downstream immune responses by increasing the suppressive activity of $CD4^+$ $CD25^+$ $Foxp3^+$ cells isolated from the SDLN.

As well as being produced by skin epithelial cells,[13,14] $1,25(OH)_2D_3$ is also synthesized by respiratory epithelial cells.[17] Therefore, $1,25(OH)_2D_3$ made in the respiratory environment could modify the activity of local $CD4^+$ $CD25^+$ cells. As a surrogate for $1,25(OH)_2D_3$ produced by airway epithelial cells, we investigated the potential of $CD4^+$ $CD25^+$ cells from the SDLN of $1,25(OH)_2D_3$-treated mice to modulate immune responses in the airways. In addition, we questioned whether the effects of topical $1,25(OH)_2D_3$ on $CD4^+$ $CD25^+$ cells were limited to modulating Th1/Th17-driven immune disease. As *in vitro* $1,25(OH)_2D_3$ up-regulates skin-homing chemokine receptors (CCR10) on T cells,[12] topical $1,25(OH)_2D_3$ treatment may promote the migration of these cells to skin sites and not the respiratory tract. We were uncertain as to whether $CD4^+$ $CD25^+$ cells could regulate Th2 immune responses in the respiratory tract even if transferred immediately before challenge, when signals that promote cell migration into the airways are generated by the lung challenge. Using a classical mouse model of acute asthma induced by the experimental allergen, ovalbumin (OVA), topical $1,25(OH)_2D_3$ augmented the ability of $CD4^+$ $CD25^+$ cells to suppress Th2-driven immune responses when cells were transferred immediately before airway challenge. Hence, topical $1,25(OH)_2D_3$ enhances the ability of $CD4^+$ $CD25^+$ cells to modify Th2-mediated (i.e. OVA-induced airway disease) immune pathologies.

Materials and methods

Mice

Female 8-week-old BALB/c mice were purchased from the Animal Resources Centre (Murdoch, Western Australia). Mice transgenic for the $OVA_{323–339}$ (ISQAVHAAHAEINEAGR)-specific T-cell receptor-$\alpha\beta$ (TCR-$\alpha\beta$; DO11.10) on a BALB/c background were originally purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in-house. Female DO11.10 mice were used between the ages of 8 and 12 weeks. 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 monoclonal antibody (KJ1-26; Caltag Laboratories, Burlingame, CA) and then phycoerythrin (PE) -Cy5-conjugated streptavidin (BD Biosciences, Heidelberg, Germany). All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia with approval from the Telethon Institute for Child Health Research Animal Ethics Committee.

Vitamin D application

A 100-μl aliquot containing 125 ng 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$; Sigma Chemical Company, St Louis, MO] 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. The $1,25(OH)_2D_3$ was stored under argon gas at − 80°; its

chemical integrity was routinely verified using a scanning spectrophotometer.

Purification of CD4+ CD25+ cells

Four days after topical skin treatment, $CD4^+$ $CD25^+$ cells ($\geq$ 95% pure, as determined by flow cytometry) were isolated from the SDLN (inguinal, axillary and brachial lymph nodes) of vehicle-treated or $1,25(OH)_2D_3$-treated mice using the $CD4^+$ $CD25^+$ regulatory T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and the AutoMACS cell separator (Miltenyi Biotec). In one experiment $CD4^+$ $CD25^+$ cells were purified from the SDLN of treated mice by fluorescence-activated cell-sorting (FACS) cells labelled with fluorescein isothiocyanate-conjugated (-FITC) CD3, allophycocyanin-conjugated (-APC) CD4, CD25-PE and propidium iodide (FACS Aria, see below for surface staining). More than 90% of $CD4^+$ $CD25^+$ cells from the lymph nodes of vehicle-treated or $1,25(OH)_2D_3$-treated BALB/c mice express the regulatory cell marker Foxp3 intracellularly ([3], Fig. 2).

FACS analysis and antibodies

The following anti-mouse monoclonal antibodies were obtained from BD Biosciences: FITC-anti-CD3; PE-anti-CD25; APC-anti-CD25; APC-CD4; and anti-CD16/32. For staining of surface antigens, lymph node cells were incubated with antibodies and staining buffer (phosphate-buffered saline, 0·1% bovine serum albumin, 0·01% sodium azide) for 30 min, washed and immediately acquired on a FACSCalibur flow cytometer (BD Biosciences). For some experiments cells were washed with 1 ml staining buffer with 1 μg/ml propidium iodide (Sigma). A Foxp3 intracellular staining kit (eBiosciences, San Diego, CA) was used to determine intracellular Foxp3 expression. Data were analysed using FlowJo software (v8.3.7) (Treestar Inc., Ashland, OR).

Adoptive transfer of CD4+ CD25+ cells into OVA-sensitized mice

Four days after topical skin treatment, $CD4^+$ $CD25^+$ cells were purified from the SDLN of treated mice and $2{\cdot}5 \times 10^5$ cells in 200 μl 0·9% saline were adoptively transferred through the tail vein of OVA-sensitized and boosted mice 18 hr before OVA aerosol challenge (see below, Fig. 1). In some studies, $CD4^+$ $CD25^+$ cells were labelled with 5 μM carboxyfluorescein succini,idyl ester (CFSE; Molecular Probes, Invitrogen Australia, Mulgrave, Australia) for 10 min at room temperature. After washing three times, cells were resuspended in 0·9% saline and $2{\cdot}5 \times 10^5$ cells were intravenously transferred into naive mice that were sensitized 18 hr later with OVA as described below.

Sensitization and challenge of mice with OVA

Ovalbumin (Sigma) in an aluminium hydroxide suspension (Alum; Serva, Heidelberg, Germany) was delivered intraperitoneally on day 0 (20 μg OVA in 4 mg aluminium hydroxide per mouse; 200 μl volume) and again on day 14 (Fig. 1). Mice were then challenged on day 21 with a 1% OVA-in-saline aerosol delivered using an ultrasonic nebulizer (UltraNebs; DeVilbiss, Somerset, PA) for 30 min (Fig. 1).

Preparation and culture of single-cell suspensions from airway-draining lymph nodes

Twenty-four hours after OVA challenge, the posterior mediastinal, tracheobrachial and parathymic lymph nodes (airway-draining lymph nodes; ADLN) were removed from mice, pooled within experimental groups, physically disaggregated and cultured at 10^5 cells/200 μl/well (six replicates per treatment) in RPMI-1640 medium (Gibco, Auckland, New Zealand) with 10% fetal calf serum, 2 mM L-glutamine, 50 mM 2-mercaptoethanol and 5 mg/ml

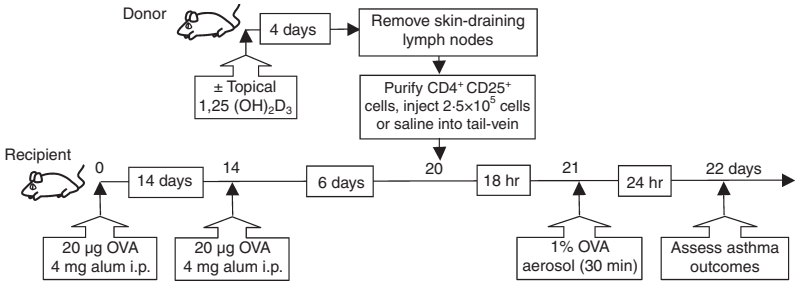

Figure 1. Experimental timeline for transfer of $CD4^+$ $CD25^+$ cells into ovalbumin (OVA) -sensitized mice. $CD4^+$ $CD25^+$ cells were purified from the skin-draining lymph nodes (SDLN) of 'donor' BALB/c or DO11.10 mice treated topically 4 days earlier with vehicle or 125 ng $1,25(OH)_2D_3$. Cells ($2{\cdot}5 \times 10^5$) or 0·9% saline (200 μl) were adoptively transferred into 'recipient' BALB/c mice intraperitoneally sensitized and boosted earlier with 20 μg OVA and 4 mg Alum adjuvant. These recipient mice were challenged with a 1% OVA in saline aerosol for 30 min, 18 hr after the adoptive transfer. Twenty-four hours later, various asthma outcomes were assessed in the recipient mice.

gentamicin. Single-cell suspensions were incubated at 37° with 5% CO_2, with OVA (10 μg/ml). Methyl-[^{3}H]thymidine [10 μl (0·25 mCi)/well; Amersham Pharmacia Biotech, Piscataway, NJ] was added at 72 hr and cells were harvested at 96 hr with [^{3}H]thymidine incorporation used as a measure of cellular proliferation. Culture supernatants were also obtained at 96 hr.

Cytokine detection in tissue culture supernatants and BALF

Interleukin-2, IL-4, IL-5, IL-10 and interferon-γ (IFN-γ) were detected using rat anti-mouse IL-2, IL-4, IL-5, IL-10 or IFN-γ enzyme-linked immunosorbent assay capture and detection of monoclonal antibodies (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 or IFN-γ (BD Biosciences) were used as the standards.

Measurement of airway hyperresponsiveness

Twenty-four hours after the aerosol challenge, a modified low-frequency forced oscillation technique was used to measure change in respiratory input impedance (Zrs) in AHR by using increasing doses of methacholine (0·1–30 mg/ml) as described previously.[18] Briefly, mice were anaesthetized with xylazine (2 mg/ml; Troy Laboratories, Wetherill Park, NSW, Australia) and ketamine (40 mg/ml; Troy Laboratories) delivered intraperitoneally at a dose of 0·01 ml/g. Mice were tracheostomized and ventilated (flexivent; Scireq, Montreal, Canada) at 450 breaths/min with a tidal volume of 8 ml/kg and a positive end expiratory pressure of 2 cmH_2O. Baseline values were obtained by measurement of Zrs five times at 1-min intervals. A 90-second aerosol was delivered with an ultrasonic nebulizer (Devilbiss UltraNeb® Large volume ultrasonic nebulizer, Sunrise Medical, Somerset, PA) and Zrs was measured five times at 1-min intervals. This was repeated with ½ $\log_{10}$ incremental doses of methacholine (0·1–30 mg/ml) and the peak response for each parameter was recorded for analysis. The constant phase model[19] was used to partition Zrs into components representing the conducting airway (airway resistance) and the lung parenchyma (tissue damping and tissue elastance).

Broncheoalveolar lung lavage

Twenty-four hours after the aerosol challenge with OVA, BALF was collected by flushing 1·2 ml 0·2% bovine serum albumin in phosphate-buffered saline into the lung. Lavage samples were centrifuged (300 ***g***, 4°, 10 min) and the supernatant was stored at − 20° until cytokine analysis. Cells were then centrifuged onto slides and stained using the DIFF-Quik Stain Set 64851 (Lab Aids, Narrabeen, NSW, Australia) as per the manufacturer's instructions.

Measuring messenger RNA in CD4$^+$ CD25$^+$ cells

The CD4$^+$ CD25$^+$ cells ($\geq 2{\cdot}5 \times 10^6$) were snap-frozen in 1 ml Trizol Reagent (Invitrogen Australia). RNA was extracted according to the manufacturer's instructions and DNAse-treated using the Turbo DNA-free kit (Ambion, Austin, TX). RNA was further purified using the RNA RT^2 qPCR-Grade RNA Isolation kit (SABiosciences, Frederick, MD) and then 100 ng was reversed transcribed into complementary DNA using the RT^2 First-Strand kit (SABiosciences). To examine CCR10 messenger RNA (mRNA) expression, a real-time polymerase chain reaction (PCR) was then performed according to the manufacturer's instructions, using 2 × SuperArray RT^2 qPCR Master Mix (SABiosciences) and the Th1-Th2-Th3 RT^2 profiler PCR array (PAM034A, 96-wells; SABiosciences). The PCR was performed using the standard two-step cycling conditions on the ABI 7000 SDS (Applied Biosystems, Foster City, CA). Alternatively, using primers designed in-house, the expression of IL-10 and transofrming growth factor-β (TGF-β) were determined in cDNA samples, quantified by real-time PCR using QuantiTect SYBR Green Master Mix (Qiagen, Doncaster, Vic, Australia) on the ABI PRISM 7900HT (Applied Biosystems). Melting-curve analysis was used to assess the specificity of the assay. Expression levels were determined by a standard curve created from serial dilutions of the PCR product and normalized to the reference genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH; as detected on the Th1-Th2-Th3 RT^2 profiler PCR array for CCR10) or eukaryotic translation elongation factor 1α.[20] In-house-designed primer pairs were: eukaryotic translation elongation factor 1α, 5′-CTGGAGCCAAGTGCTAATATGCC-3′ and 5′-GCCAGGCTTGAGAACACCAGTC-3′; IL-10, 5′-GGTTGCCAAGCCTTATCGGA-3′ and 5′-ACCTGCTCCACTGCCTTGCT-3′; TGF-β 5′-CACTGATACGCCTGAGTG-3′ and 5′-GTGAGCGCTGAATCGAAA-3′.

Statistical analyses

Data were compared using Student's *t*-test with the PRISM statistical analysis program for Macintosh (v4.0b) (GraphPad Prism, GraphPad Software Inc., La Jolla, CA).

Results

Do CD4$^+$ CD25$^+$ cells from 1,25(OH)$_2$D$_3$-treated mice suppress Th2-mediated immune pathologies?

Both naturally occurring[21–22] and antigen-induced[23] regulatory T cells have been implicated in controlling asthma-associated immune reactions in the airway mucosa, draining lymph nodes and lung parenchyma when transferred before the allergen challenge of pre-sensitized experimental animals. In the current study, CD4$^+$ CD25$^+$ cells were purified from the SDLN of mice topically treated 4 days

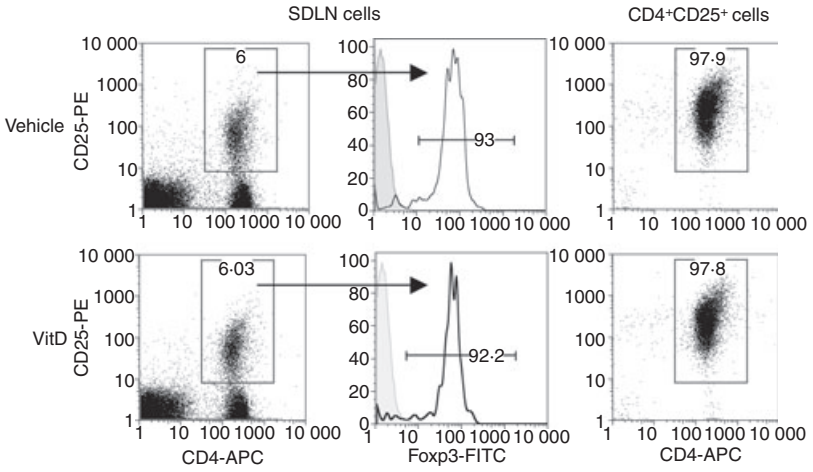

Figure 2. $CD4^+$ $CD25^+$ cells from the skin-draining lymph nodes (SDLN) of mice topically treated with vehicle or $1,25(OH)_2D_3$ have similar phenotypes. $CD4^+$ $CD25^+$ cells were purified from the SDLN of BALB/c mice treated topically 96 hr earlier with vehicle or 125 ng $1,25(OH)_2D_3$ (VitD). The proportion of live $CD4^+$ $CD25^+$ cells in the SDLN (left panels) and of isolated $CD4^+$ $CD25^+$ cells (right panels) are shown. The proportions of $Foxp3^+$ cells of the live $CD4^+$ $CD25^+$cells from the SDLN are shown in the middle panel with an isotype control also shown (shaded histogram). Results are representative of cells pooled from the SDLN of BALB/c mice from four individual experiments.

earlier with 125 ng $1,25(OH)_2D_3$ or vehicle (Fig. 1, donor mice). As shown previously,[3] there was no difference in the proportion of $CD4^+$ $CD25^+$ cells in the SDLN of vehicle or $1,25(OH)_2D_3$-treated mice (Fig. 2), such that $2{\cdot}4 \pm 0{\cdot}5 \times 10^5$ or $2{\cdot}1 \pm 0{\cdot}1 \times 10^5$ $CD4^+$ $CD25^+$ cells/mouse (mean ± SEM of $n = 4$ experiments, Fig. 2) were purified from the SDLN of vehicle-treated or $1,25(OH)_2D_3$-treated BALB/c mice, respectively. More than 90% of the $CD4^+$ $CD25^+$ cells expressed intracellular Foxp3 (Fig. 2) with no difference in the expression of CD25 (Fig. 2) or other T-cell activation/memory/mucosal cell markers as previously reported.[3] $CD4^+$ $CD25^+$ cells ($2{\cdot}5 \times 10^5$) were adoptively transferred into BALB/c mice, which had been sensitized and boosted earlier with 20 μg OVA and 4 mg Alum (Fig. 1, recipient mice). To control for the effects of cell transfer, a group of mice received 200 μl of 0·9% saline. Eighteen hours after cells were transferred, recipient mice were challenged through the airways with a 1% OVA in saline solution as an aerosol. After a further 24 hr, asthma-related parameters were examined including ADLN cell responses to OVA *ex vivo*, AHR and the effector cells and cytokines present in the BALF. For all of the tested parameters, there was no difference between the responses observed in the recipients of $CD4^+$ $CD25^+$ cells from vehicle-treated mice and those mice that received saline only (Figs. 3–5).

$CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated BALB/c mice reduce cytokine production by, and proliferation of, ADLN cells

The ADLN cells were removed 24 hr after OVA challenge and cultured for 96 hr with OVA. Results were pooled

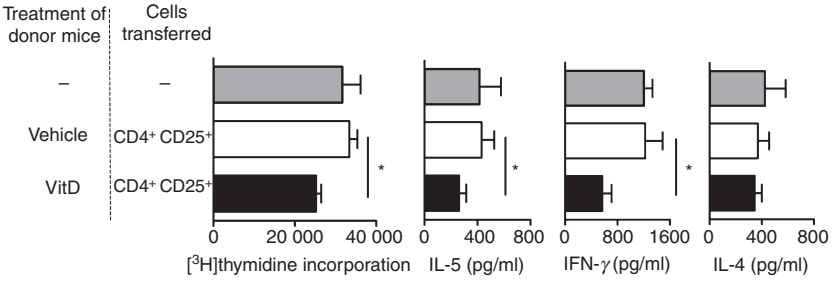

Figure 3. $CD4^+$ $CD25^+$ cells from the skin-draining lymph nodes (SDLN) of mice topically treated with $1,25(OH)_2D_3$ regulate *ex vivo* proliferation and cytokine production by airway-draining lymph nodes (ADLN) cells. $CD4^+$ $CD25^+$ cells were purified from the SDLN of BALB/c mice treated topically 4 days earlier with vehicle or 125 ng $1,25(OH)_2D_3$. Cells ($2{\cdot}5 \times 10^5$) or 0·9% saline (200 μl) were adoptively transferred into BALB/c mice sensitized and boosted earlier with ovalbumin (OVA). These recipient mice were challenged with an OVA aerosol 18 hr after the adoptive transfer. After a further 24 hr, ADLN cells were pooled from mice within treatment groups and cultured with 10 μg/ml OVA for 96 hr. [^{3}H]thymidine incorporation by ADLN cells is shown for the last 24 hr of a 96-hr culture with interleukin-5 (IL-5), interferon-γ (IFN-γ) or IL-4 levels in supernatants depicted, which were collected after a 96-hr culture. Results are pooled from three independent experiments ($n = 4$ mice/treatment/experiment) and are shown as mean + SEM (*$P < 0{\cdot}05$).

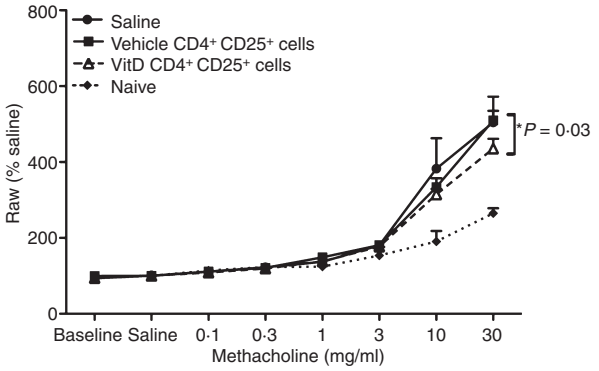

Figure 4. $CD4^+$ $CD25^+$ cells from the skin-draining lymph nodes (SDLN) of mice topically treated with $1,25(OH)_2D_3$ regulate airway hyperresponsiveness. $CD4^+$ $CD25^+$ cells were purified from the SDLN of BALB/c mice treated topically 4 days earlier with vehicle or 125 ng $1,25(OH)_2D_3$. Cells ($2{\cdot}5 \times 10^5$) or 0·9% saline (200 µl) were adoptively transferred into BALB/c mice sensitized and boosted earlier with ovalbumin (OVA). These recipient mice were challenged with an OVA aerosol 18 hr after the adoptive transfer. After a further 24 hr, airway resistance to increasing concentrations of the bronchoconstrictor methacholine was determined. Responses were also measured in age-matched naive BALB/c mice. Baseline = values obtained by measurement of Zrs five times at 1-min intervals after stabilization of the mouse on the ventilator; saline = values obtained by measurement of Zrs five times at 1-min intervals after a 90-second saline aerosol; % saline = % increase of saline values for increasing doses of methacholine. Results are shown as mean + SEM ($n = 8$ mice/treatment, $^*P = 0{\cdot}03$) relative to baseline levels (100%).

from three independent experiments ($n = 4$ mice/treatment/experiment). For mice receiving $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated donor mice, the capacity of ADLN cells to proliferate in response to OVA stimulation *ex vivo* was significantly reduced (Fig. 3). In the supernatants of these proliferating ADLN cells there were significant reductions in both Th1 (IFN-γ) and Th2 (IL-5) cytokine concentrations (Fig. 3). However, levels of IL-2 (data not shown) and IL-4 (Fig. 3) were not modified.

$CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated BALB/c mice suppress airway hyperresponsiveness

Twenty-four hours after the OVA-aerosol challenge, mice were tested for AHR by a forced oscillation method, using increasing concentrations of the bronchoconstrictor, methacholine.[18] Parameters of airway resistance, tissue damping and tissue elastance were measured using a constant-phase model.[19] $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated mice suppressed airway resistance by 30% ($P = 0{\cdot}03$, one-way *t*-test, $n = 8$ mice per treatment, 30 mg/ml methacholine) in comparison to responses observed in recipients of $CD4^+$ $CD25^+$ cells from vehicle-treated mice (Fig. 4). However, airway resistance was greater in recipients of $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated mice than in naive mice, suggesting that other factors control airway resistance. There was no difference in tissue damping or elastance in recipients of $CD4^+$ $CD25^+$ cells from either vehicle-treated or $1,25(OH)_2D_3$-treated mice (data not shown).

$CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated BALB/c mice reduce neutrophil and eosinophil numbers in the lungs

The BALF was collected immediately following measurement of AHR. Transfer of $CD4^+$ $CD25^+$ cells did not modify the total number of immune cells in the BALF

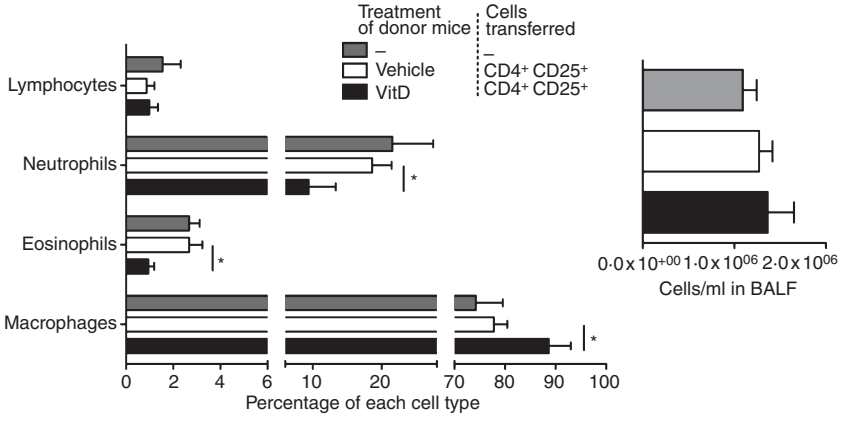

Figure 5. $CD4^+$ $CD25^+$ cells from the skin-draining lymph nodes (SDLN) of mice topically treated with $1,25(OH)_2D_3$ regulate cells in the bronchoalveolar lavage fluid (BALF). $CD4^+$ $CD25^+$ cells were purified from the SDLN of BALB/c mice treated topically 4 days earlier with vehicle or 125 ng $1,25(OH)_2D_3$. Cells ($2{\cdot}5 \times 10^5$) were adoptively transferred into BALB/c mice sensitized and boosted earlier with OVA. These recipient mice were challenged with an OVA aerosol 18 hr after the adoptive transfer. After a further 24 hr, the proportions of lymphocytes, neutrophils, eosinophils and macrophages and the total number of cells were determined in BALF. Results are shown as mean + SEM ($n = 8$ mice/treatment, $^*P < 0{\cdot}05$).

(Fig. 5) with the proportions of neutrophils and eosinophils in the BALF being similar to those observed in our previous investigations where mice were challenged with a single OVA aerosol.[24] However, there were significant reductions in the proportions of neutrophils and eosinophils (Fig. 5) in the BALF of mice that received $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated mice 24 hr before OVA challenge. Similar results were observed in three further experiments ($n = 4$ mice/treatment/experiment) where the average (± SEM) reductions in the proportion of neutrophils and eosinophils were 37% (± 14%) and 45% (± 16%), respectively, in mice that received $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated mice. Even though total BALF cell numbers did not change in recipients of $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated mice, the reduction of granulocyte populations indicates that inflammation was probably reduced in the lungs. The reduction in granulocyte numbers resulted in an increased proportion of macrophages in the BALF of recipients of $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated mice (Fig. 5). Cytokine levels in BALF, including IL-2, IL-4, IL-5 and IL-10, were not significantly altered by any treatment (data not shown). Together, these results indicate that topical $1,25(OH)_2D_3$ increased the ability of $CD4^+$ $CD25^+$ cells isolated from the SDLN to modulate Th2-driven allergic airway disease.

CCR10 mRNA levels in $CD4^+$ $CD25^+$ cells are not modified by topical $1,25(OH)_2D_3$

CCR10, a skin-homing chemokine receptor, is upregulated on T cells after *in vitro* treatment with $1,25(OH)_2D_3$.[12] The expression of CCR10 mRNA was examined for $CD4^+$ $CD25^+$ cells isolated 4 days after topical $1,25(OH)_2D_3$ treatment in three independent experiments. There was no difference in the expression of CCR10 mRNA with $1,25(OH)_2D_3$ treatment of skin (vehicle = 0·007 ± 0·002, $1,25(OH)_2D_3$ = 0·008 ± 0·002 mean ± SEM relative to the housekeeping gene GAPDH). This observation, along with the increased ability of $CD4^+$ $CD25^+$ cells to suppress respiratory immune responses, indicates that topical $1,25(OH)_2D_3$ did not modify or prevent their ability to migrate into the respiratory tract. Levels of mRNA for other immunoregulatory molecules including IL-10, TGF-β and Foxp3 were not modified in $CD4^+$ $CD25^+$ cells isolated from the SDLN 4 days after topical $1,25(OH)_2D_3$ (data not shown). The levels of CCR10 mRNA detected in $CD4^+$ $CD25^+$ cells were comparable with TGF-β and Foxp3 mRNA levels (C_t scores $\sim$ 30).

$CD4^+$ $CD25^+$ cell migration *in vivo* is not modified by topical $1,25(OH)_2D_3$

To determine if topical $1,25(OH)_2D_3$ altered the ability of $CD4^+$ $CD25^+$ cells to migrate to airway immune sites, the cells were purified from the SDLN of BALB/c mice 4 days after skin treatment with $1,25(OH)_2D_3$ or vehicle. Cells were labelled with CFSE, and then 10^5 cells were transferred intravenously into naive mice. Mice were sensitized 18 hr later with OVA and alum with the proportion of transferred cells ($CFSE^+$ $Foxp3^+$ $CD4^+$) in the ADLN and SDLN assessed 3 days later (Fig. 6a). All of the transferred cells expressed high levels of CFSE, indicating that none of them had begun to proliferate 3 days after OVA sensitization. The was no difference in the ability of cells from vehicle or $1,25(OH)_2D_3$-treated mice to migrate into and accumulate in the ADLN or SDLN (Fig. 6b). However, more of the transferred cells were identified in the SDLN than in the ADLN of recipient mice (Fig. 6b) such that about three times the number of transferred $CD4^+$ $CD25^+$ cells were detected in the SDLN than the ADLN of recipient mice (Fig. 6c).

$CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated OVA-TCR transgenic mice reduce cytokine production by, and proliferation of, ADLN cells as well as neutrophil and eosinophil numbers in the lungs

For the data described in Figs. 3–5, $CD4^+$ $CD25^+$ cells were derived from $1,25(OH)_2D_3$-treated but otherwise naive BALB/c mice. The effect on $CD4^+$ $CD25^+$ cells expressing the OVA-TCR was examined, where 'OVA-specific' $CD4^+$ $CD25^+$ cells were obtained from the SDLN of vehicle or $1,25(OH)_2D_3$-treated OVA-TCR transgenic DO11.10 mice. Approximately 60% of $CD4^+$ $CD25^+$ cells from the SDLN of DO11.10 mice express the OVA-TCR, and this is not modified by topical $1,25(OH)_2D_3$.[3] Cells ($2{\cdot}5 \times 10^5$) were transferred 18 hr before OVA challenge (Fig. 1). Results were pooled from two independent experiments ($n = 5$ mice/treatment/experiment) and are shown as mean + SEM. As observed following topical treatment of BALB/c mice, there was no difference in the proportion of $CD4^+$ $CD25^+$ cells in the SDLN of vehicle or $1,25(OH)_2D_3$-treated DO11.10 mice, such that $1{\cdot}1 \times 10^5$ or $1{\cdot}3 \times 10^5$ $CD4^+$ $CD25^+$ cells/mouse (mean of $n = 2$ experiments) were purified from the SDLN of vehicle or $1,25(OH)_2D_3$-treated mice, respectively. Similarly, the expression of CD25 and Foxp3 was not different for $CD4^+$ $CD25^+$ cells from the SDLN of vehicle or $1,25(OH)_2D_3$-treated DO11.10 mice (data not shown).

$CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated mice significantly reduced the proliferation and cytokine production (IL-4 and IL-5) by ADLN cells in response to OVA stimulation *ex vivo* (Fig. 7a) and also diminished the proportions of eosinophils in the BALF of OVA-challenged mice (Fig. 7b). In all treatments there was no change in total BALF cell numbers (data not shown). $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated DO11.10 mice suppressed responses by ADLN cells and in the BALF to a similar degree as those cells from BALB/c mice

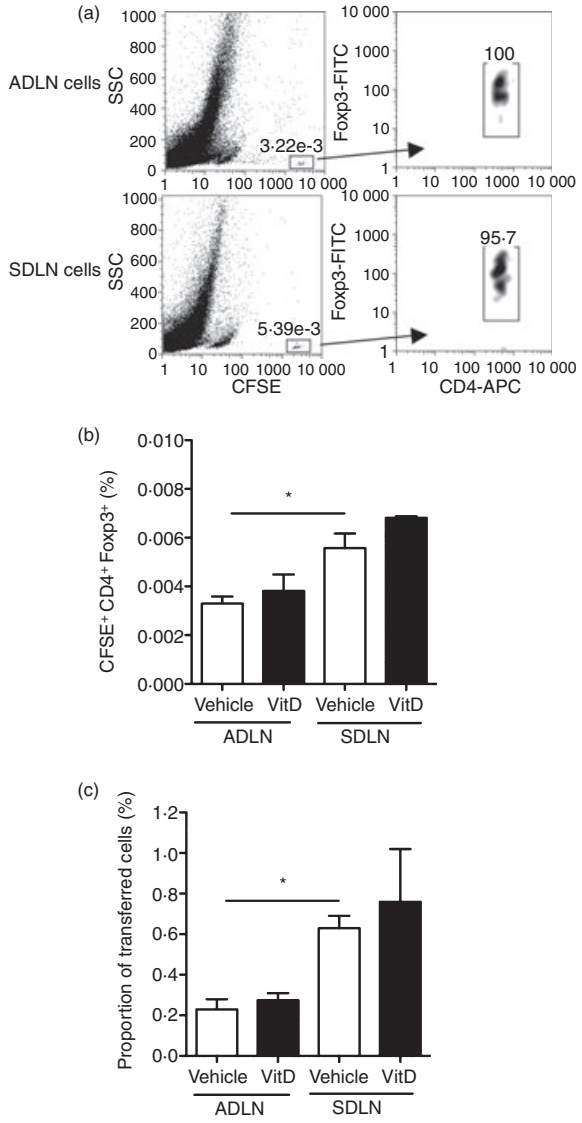

Figure 6. Topical $1,25(OH)_2D_3$ does not alter migration of $CD4^+$ $CD25^+$ cells to the airway-draining lymph nodes (ADLN). $CD4^+$ $CD25^+$ cells were purified from the skin-draining lymph nodes (SDLN) of BALB/c mice treated topically 4 days earlier with vehicle or 125 ng $1,25(OH)_2D_3$. Cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) and then 1×10^5 intravenously transferred into naive BALB/c mice, which were sensitized 18 hr later with ovalbumin (OVA). Three days after sensitization, (a) the proportions of transferred ($CFSE^+$ $Foxp3^+$ $CD4^+$) cells in the ADLN and SDLN were determined by first gating $CFSE^+$ SSC^{lo} cells and then cells that expressed both Foxp3 and CD4. (b) The percentage of $CFSE^+$ $Foxp3^+$ $CD4^+$ cells detected in the ADLN and SDLN and, (c) the proportion of total cells transferred cells. Results for (b) and (c) are depicted as mean + SEM ($n = 3$ mice/treatment, $^*P < 0{\cdot}05$).

(Figs 3, 5 and 7). However, the $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated DO11.10 mice were also able to reduce levels of IL-4 but not of IL-5 (Fig. 7c), IL-2 or IL-10 in the BALF (data not shown). These reductions in IL-4 production by ADLN cells and IL-4 accumulation in the BALF of recipients of $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated DO11.10 mice were not observed for recipients of cells from $1,25(OH)_2D_3$-treated BALB/c mice. Hence, $1,25(OH)_2D_3$ has similar but not identical immunoregulatory enhancing effects on $CD4^+$ $CD25^+$ cells from BALB/c and OVA-TCR transgenic mice to increase their ability to suppress Th2-driven immune responses in mouse models of allergic airway disease.

Discussion

In this study $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated mice had an increased capacity to suppress Th2-driven immune responses in a murine model of asthma. $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated mice reduced proliferation and cytokine production by ADLN cells, AHR and the numbers of granulocytes in the BALF of mice. Results were also comparable when $CD4^+$ $CD25^+$ cells were purified using magnetic bead separation or by cell-sorting techniques. It is of note that while the suppressive activity of these cells increased, their numbers did not change in the SDLN. $CD4^+$ $CD25^+$ cells from both BALB/c and OVA-TCR transgenic (DO11.10) mice had an increased ability to suppress aspects of Th2 immunity, indicating that the effects of topical $1,25(OH)_2D_3$ are largely non-antigen-specific and independent of OVA-TCR expression on the $CD4^+$ $CD25^+$ cells. $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated BALB/c or DO11.10 mice were able to suppress Th2 responses to a similar degree. However, $1,25(OH)_2D_3$ did not have identical effects as following topical $1,25(OH)_2D_3$, $CD4^+$ $CD25^+$ cells from OVA-TCR transgenic mice had an additional capacity to suppress IL-4 levels in the BALF, and IL-4 production by ADLN cells *ex vivo*, perhaps indicating a further effect of $1,25(OH)_2D_3$ on the Th2-regulatory capacity of $CD4^+$ $CD25^+$ regulatory T cells activated through the OVA-TCR.

'Naturally-occurring' $CD4^+$ $CD25^+$ regulatory T cells isolated from the lungs and spleens of naive mice can suppress airway inflammation, Th2 cytokine production, and AHR through IL-10 and TGF-β-dependent mechanisms.[21,22,25] However, $CD4^+$ $CD25^+$ cells from mice treated topically with $1,25(OH)_2D_3$ did not express increased levels of IL-10 or TGF-β mRNA. In addition, there was no change in the expression of Foxp3 mRNA nor in the intracellular levels of Foxp3 protein in SDLN $CD4^+$ $CD25^+$ cells with topical $1,25(OH)_2D_3$ treatment (Fig. 2 and data not shown). Furthermore, we considered it unlikely that the increased regulatory activity resulted from an increased ability of $CD4^+$ $CD25^+$ cells to proliferate following topical $1,25(OH)_2D_3$. In our previous studies, topical $1,25(OH)_2D_3$ increased the ability of $CD4^+$ $CD25^+$ cells to proliferate when subsequently exposed to OVA.[3] The OVA-specific $CD4^+$ $CD25^+$ cells transferred from mice topically treated with $1,25(OH)_2D_3$ proliferated after 3 days to a greater extent in recipient mice primed with

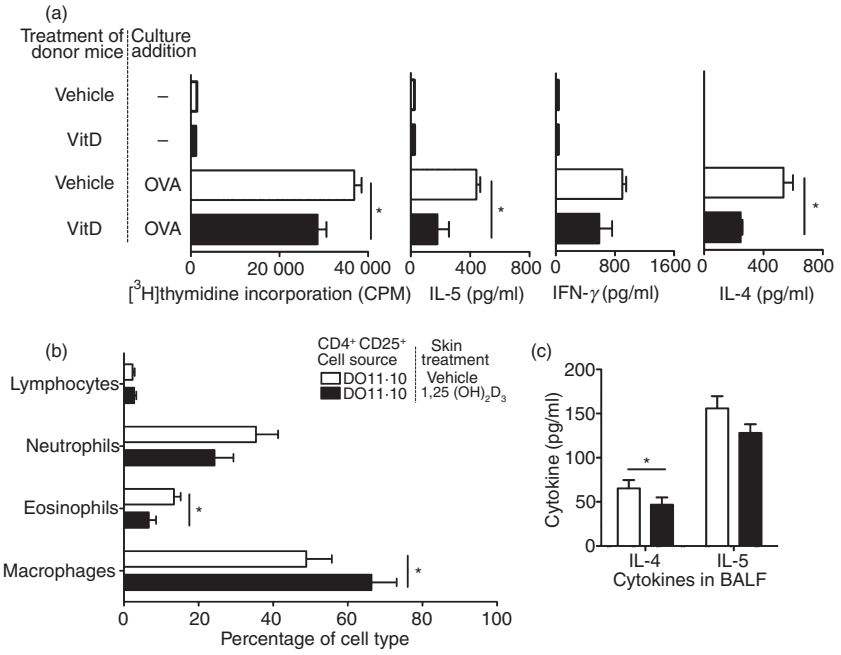

Figure 7. $CD4^+$ $CD25^+$ cells from the skin-draining lymph nodes (SDLN) of ovalbumin–T-cell receptor (OVA-TCR) transgenic mice topically treated with $1,25(OH)_2D_3$ regulate various asthma parameters. $CD4^+$ $CD25^+$ cells were purified from the SDLN of OVA-TCR transgenic (DO11.10) mice treated topically 4 days earlier with vehicle or 125 ng $1,25(OH)_2D_3$. Cells ($2{\cdot}5 \times 10^5$) or 0·9% saline (200 μl) were adoptively transferred into BALB/c mice sensitized and boosted earlier with OVA. These recipient mice were challenged with an OVA aerosol 18 hr after the adoptive transfer. After a further 24 hr, (a) airway-draining lymph node (ADLN) cells were pooled within treatments and cultured with and without (−) 10 μg/ml OVA for 96 hr. [^{3}H]thymidine was added to cultures for the last 24 hr of a 96 hr culture. Interleukin-5 (IL-5), interferon-γ (IFN-γ) or IL-4 concentrations in supernatants were determined after 96 hr of culture. At the same time, (b) the proportions of lymphocytes, neutrophils, eosinophils and macrophages and (c) concentrations of IL-4 and IL-5 were measured in bronchoalveolar lavage fluid (BALF). Results are pooled from two independent experiments (n = 5 mice/treatment/experiment) and are shown as mean + SEM (*$P < 0{\cdot}05$).

OVA.[3] However, in the current study using T cells from BALB/c or OVA-TCR transgenic mice, the $CD4^+$ $CD25^+$ cells in the recipient mice were exposed to OVA (administered as an aerosol) for only 24 hr. Furthermore, OVA-TCR^+ T cells begin to proliferate 48 hr after OVA priming (S. Gorman, P.H. Hart unpublished data). Using CFSE to label cells, similar numbers of transferred $CD4^+$ $CD25^+$ cells from mice topically treated with vehicle or $1,25(OH)_2D_3$ were identified in the ADLN of recipient mice 72 hr after transfer. These data suggest that topical $1,25(OH)_2D_3$ does not modify the capacity of $CD4^+$ $CD25^+$ cells to migrate to the ADLN. Studies are continuing to investigate the mechanisms by which $1,25(OH)_2D_3$ regulates $CD4^+$ $CD25^+$ cells.

It may be expected that $CD4^+$ $CD25^+$ cells from DO11.10 (that express the OVA-TCR) would inhibit respiratory responses to OVA challenge more effectively than those from BALB/c mice; however, this does not appear to be the case. In a recent study, OVA-TCR transgenic $CD4^+$ $CD25^+$ cells transferred intratracheally into recipient mice suppressed aspects of ragweed-induced allergic airway disease including airway hyperreponsiveness to a similar extent as that observed by these cells when transferred into OVA-sensitized and challenged mice.[26] These results suggest that antigen-specific TCR is not required for the capacity of $CD4^+$ $CD25^+$ regulatory T cells to suppress allergic airway disease and that non-antigen-specific cells may suppress as well as those that are antigen specific. The OVA-TCR^+ cells were closely associated with $CD8^+$ T cells and major histocompatibility complex class II^+ cells in the lungs of ragweed-sensitized mice, indicating that the transferred cells could indeed migrate to the lungs despite their antigen specificity.[26]

$CD4^+$ $CD25^+$ cells from both BALB/c and DO11.10 mice treated with $1,25(OH)_2D_3$ had an increased capacity to suppress the influx of both eosinophils and neutrophils into the lungs of the recipient mice. However, the mechanism by which this occurs is not known. In other studies depletion or transfer of $CD4^+$ $CD25^+$ cells affected eosinophil numbers in the BALF and lungs of mice sensitized and challenged with OVA.[27,28] $CD4^+$ $CD25^+$ cells also affected the recruitment of neutrophils into the kidneys and airways of mice with injury[29] or OVA-induced inflammation,[30] respectively. The effects on neutrophils may be dependent on IL-10 secreted by $CD4^+$ $CD25^+$ cells[29] but *in vitro* studies examining the effects of lipopolysaccharide-activated or TCR-activated $CD4^+$ $CD25^+$

cells on neutrophils have shown that TGF-β and contact-dependent mechanisms may also be important.[31]

$CD4^+$ $CD25^+$ ($Foxp3^+$) cells are generally considered regulatory cells; however, in this study $2{\cdot}5 \times 10^5$ $CD4^+$ $CD25^+$ cells from untreated mice were not sufficient to modify asthma responses in recipient mice after intravenous transfer. Topical treatment with $1,25(OH)_2D_3$ significantly enhanced the regulatory ability of $CD4^+$ $CD25^+$ cells such that the same number of $CD4^+$ $CD25^+$ cells could suppress the experimental asthma responses. Similarly, others have adoptively transferred (intravenously) 5×10^5 $CD4^+$ $CD25^+$ cells from non-transgenic mice (e.g. BALB/c) and found no effects on OVA-induced asthma responses.[21] These cells suppressed only when transferred through the trachea.[21] In contrast, in models of asthma using house dust mite (*Der p* I;[22]) or cockroach[25] antigens, intravenous transfer of 1×10^5 to 5×10^5 $CD4^+$ $CD25^+$ cells from naive mice modified lung and airway inflammation, AHR and Th2 cytokine production. It is possible that the inability of $\leq 5 \times 10^5$ $CD4^+$ $CD25^+$ cells from naive mice to suppress allergic airway disease following intravenous transfer may be specific to when OVA is used as an allergen.

In other studies,[32] $CD4^+$ $CD25^+$ cells from OVA-TCR transgenic mice modulated allergic airway disease following the intravenous transfer of 5×10^5 cells. Some of these cells were derived from the spleens of mice, as also performed for other studies using non-antigen-specific $CD4^+$ $CD25^+$ cells.[22,25] It is unlikely that splenic $CD4^+$ $CD25^+$ cells have an increased regulatory capacity in respiratory models. We did not isolate $CD4^+$ $CD25^+$ cells from the spleens of mice topically treated with $1,25(OH)_2D_3$ because results from our previous studies indicated that the suppressive capacity of $CD4^+$ $CD25^+$ cells from the spleen was not modified by topical $1,25(OH)_2D_3$.[3]

The aim of this study was to determine whether $1,25(OH)_2D_3$ applied *in vivo* could stimulate $CD4^+$ $CD25^+$ cells in lymph nodes to regulate allergic airway disease. To detect a difference in responses, low numbers ($2{\cdot}5 \times 10^5$) of $CD4^+$ $CD25^+$ cells were adoptively transferred. We were actually aiming for none or a limited amount of suppression by $CD4^+$ $CD25^+$ cells from control mice, so increasing the window for the detection of enhanced regulatory ability by $CD4^+$ $CD25^+$ cells from $1,25(OH)_2D_3$-treated mice. Low numbers of cells ($2{\cdot}5 \times 10^5$) were transferred because we had previously found that this number of cells from $1,25(OH)_2D_3$-treated mice was able to suppress contact hypersensitivity responses, even though $CD4^+$ $CD25^+$ cells from non-treated (vehicle) mice did not suppress.[3] In our experiments, in consideration of cell numbers and their potency, we have effectively 'concentrated' the effects of topical $1,25(OH)_2D_3$ by transferring $CD4^+$ $CD25^+$ cells from approximately two donor mice into one recipient mouse.

Cells were transferred 18 hr before OVA challenge (1% OVA aerosol, Fig. 1) because previous studies have indicated that $CD4^+$ $CD25^+$ cells from naive mice can suppress elements of allergic airway disease if transferred the day before the first respiratory challenge with allergen.[21,22] The timing of cell transfer is an important factor to consider as the transfer of $2{\cdot}5 \times 10^5$ $CD4^+$ $CD25^+$ cells from donor mice topically treated with $1,25(OH)_2D_3$ before the initial OVA sensitization of the recipient mice (18 hr before the first OVA/Alum injection, Fig. 1) had no effect on the asthma outcomes tested (data not shown). Transferring the cells immediately before the respiratory challenge with OVA may enable the migration of the cells to the challenge site, and their subsequent interactions with cells such as DC and Th2 effector cells, which mediate allergic airway disease and AHR in the trachea and respiratory tract.[23]

In other studies, a single topical application of $1,25(OH)_2D_3$ did not regulate OVA-induced allergic airway disease, such that it was necessary to transfer (intravenously) $CD4^+$ $CD25^+$ cells disaggregated from the SDLN. From this report, and highlighting the importance of this study, the ability of respiratory epithelial cells to synthesize $1,25(OH)_2D_3$[17] has implications for the ability of 'local' $CD4^+$ $CD25^+$ cells to modulate DC and Th2 cell interactions which mediate allergic airway disease in the respiratory tract. The production of $1,25(OH)_2D_3$ by respiratory epithelial cells highlights the dual role that $1,25(OH)_2D_3$ has to (i) modulate immune cells that regulate adaptive immune responses (such as regulatory T cells), and to (ii) promote antimicrobial activity through production of cathelicidin during infection.[17]

In vivo treatment of OVA-primed mice with $1,25(OH)_2D_3$ (chronic intraperitoneal injection) down-regulates the *in vivo* homing ability of $CD4^+$ T cells to ADLN.[33] In these studies, purified $CD4^+$ T cells were labelled with CFSE, and cells were injected intravenously into $1,25(OH)_2D_3$-treated or untreated control mice. After 3·5 hr, the accumulation of labelled cells in the spleen was unaffected by the various pre-treatments. In contrast, migration of cells to the lymph nodes of $1,25(OH)_2D_3$-treated mice was significantly decreased.[33] In our studies, topical $1,25(OH)_2D_3$ did not imprint the cells with chemokine receptor expression that prevented their migration to sites such as the airways. In contrast to findings with unfractionated T cells,[12] expression of mRNA for the skin-trophic chemokine receptor CCR10 was not modified in $CD4^+$ $CD25^+$ cells isolated from the SDLN of mice 4 days after topical $1,25(OH)_2D_3$. However, following intravenous transfer and subsequent OVA sensitization, more $CD4^+$ $CD25^+$ cells from the SDLN migrated to the SDLN than the ADLN, but importantly $1,25(OH)_2D_3$ did not alter the migratory patterns of these cells. Upon OVA aerosol challenge, sufficient $CD4^+$ $CD25^+$ cells migrated to the airway lymphatic tissue and presumably other respiratory

sites where those from $1,25(OH)_2D_3$-treated mice significantly regulated allergic airway disease.

The potential of topical $1,25(OH)_2D_3$ to augment the ability of $CD4^+$ $CD25^+$ regulatory T cells to suppress Th1/Th17 and Th2-driven immune responses may provide additional treatment options for the control of autoimmune and allergic diseases. Calcipotriene, a non-calcaemic analogue of $1,25(OH)_2D_3$, is already used topically to treat psoriasis.[34] In addition, with dexamethasone, vitamin D_3 induces IL-10-secreting regulatory T cells,[11,35] which potentially augment therapeutic responses in steroid-resistant asthma patients.[35] The apparent prevalence of vitamin D insufficiency around the world makes it important for us to better understand the immunomodulatory effects of vitamin D. In this study, in which $1,25(OH)_2D_3$ was applied topically to the experimental animal and not to cells in culture, $1,25(OH)_2D_3$ increased the immunoregulatory capacity of $CD4^+$ $CD25^+$ regulatory T cells, which in turn could modulate Th2-driven immune responses in a mouse model of asthma. It is proposed that sufficient levels of vitamin D are required for the optimal suppressive activity of $CD4^+$ $CD25^+$ regulatory T cells.

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Disclosures

None of the authors have any financial or other conflicts of interest or are associated with a company or institution that might benefit from the publication.

References

1 Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. *Immunity* 2009; **30**:656–5.

2 Miyara M, Wing K, Sakaguchi S. Therapeutic approaches to allergy and autoimmunity based on $Foxp3^+$ regulatory T-cell activation. *J Allergy Clin Immunol* 2009; **123**:749–55.

3 Gorman S, Kuritzky LA, Judge MA, Dixon KM, McGlade JP, Mason RS, Finlay-Jones JJ, Hart PH. Topically applied 1,25-dihydroxyvitamin D_3 enhances the suppressive activity of $CD4^+$ $CD25^+$ cells in the draining lymph nodes. *J Immunol* 2007; **179**: 6273–83.

4 Gorman S, Tan JW-Y, Yerkovich ST, Finlay-Jones JJ, Hart PH. $CD4^+$ T cells in lymph nodes of UVB-irradiated mice suppress immune responses to new antigens both *in vitro* and *in vivo*. *J Invest Dermatol* 2007; **127**:915–24.

5 Schwarz T. 25 years of UV-induced immunosuppression mediated by T cells – from disregarded T suppressor cells to highly respected regulatory T cells. *Photochem Photobiol* 2008; **84**:10–18.

6 Taher YA, van Esch BC, Hofman GA, Henricks PA, van Oosterhout AJ. 1α,25-dihydroxyvitamin D_3 potentiates the beneficial effects of allergen immunotherapy in a mouse model of allergic asthma: role for IL-10 and TGF-β. *J Immunol* 2008; **180**:5211–21.

7 Matheu V, Back O, Mondoc E, Issazadeh-Navikas S. Dual effects of vitamin D-induced alteration of TH1/TH2 cytokine expression: enhancing IgE production and decreasing airway eosinophilia in murine allergic airway disease. *J Allergy Clin Immunol* 2003; **112**:585–92.

8 Litonjua AA, Weiss ST. Is vitamin D deficiency to blame for the asthma epidemic? *J Allergy Clin Immunol* 2007; **120**:1031–5.

9 Penna G, Amuchastegui S, Giarratana N, Daniel KC, Vulcano M, Sozzani S, Adorini L. 1,25-dihydroxyvitamin D_3 selectively modulates tolerogenic properties in myeloid but not plasmacyoid dendritic cells. *J Immunol* 2007; **178**:145–53.

10 Fujita H, Asahina A, Komine M, Tamaki K. The direct action of $1\alpha,25(OH)_2$-vitamin D_3 on purified Langerhans cells. *Cell Immunol* 2007; **245**:70–9.

11 Barrat FJ, Cua DJ, Boonstra A *et al. In vitro* generation of interleukin-10-producing regulatory $CD4^+$ T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* 2002; **195**:603–16.

12 Sigmundsdottir H, Pan J, Debes GF, Alt C, Habtezion A, Soler D, Butcher EC. DCs metabolize sunlight-induced vitamin D3 to 'program' T cell attraction to the epidermal chemokine CCL27. *Nat Immunol* 2007; **8**:285–93.

13 Lehmann B, Sauter W, Knuschke P, Dressler S, Meurer M. Demonstration of UVB-induced synthesis of 1α,25-dihyroxyvitamin D_3 (calcitriol) in human skin by microdialysis. *Arch Dermatol Res* 2003; **295**:24–8.

14 Gupta R, Dixon KM, Deo SS, Holliday CJ, Slater M, Halliday GM, Reeve VE, Mason RS. Photoprotection by 1,25 dihydroxyvitamin D_3 is associated with an increase in p53 and a decrease in nitric oxide products. *J Invest Dermatol* 2007; **127**:707–15.

15 Ghoreishi M, Bach P, Obst J, Komba M, Fleet JC, Dutz JP. Expansion of antigen-specific regulatory T cells with the topical vitamin D analog calcipotriol. *J Immunol* 2009; **182**:6071–8.

16 He D, Wu L, Kim HK, Li H, Elmets CA, Xu H. IL-17 and IFN-γ mediate the elicitation of contact hypersensitivity responses by different mechanisms and both are required for optimal responses. *J Immunol* 2009; **183**:1463–70.

17 Hansdottir S, Monick MM, Hinde SL, Lovan N, Look DC, Hunninghake GW. Respiratory epithelial cells convert inactive vitamin D to its active form: potential effects on host defense. *J Immunol* 2008; **181**:7090–9.

18 Hantos Z, Collins RA, Turner DJ, Janosi TZ, Sly PD. Tracking of airway and tissue mechanics during TLC manoeuvers in mice. *J Appl Physiol* 2003; **95**:1695–705.

19 Hantos Z, Adamicza A, Govaerts E, Daroczy B. Mechanical impedances of lungs and chest wall in the cat. *J Appl Physiol* 1992; **73**:427–33.

20 Hamalainen HK, Tubman JC, Vikman S, Kyrola T, Ylikoski E, Warrington JA, Lahesmaa R. Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. *Anal Biochem* 2001; **299**:63–70.

21 Joetham A, Takada K, Taube C *et al.* Naturally occurring lung $CD4^+$ $CD25^+$ T cell regulation of airway allergic responses depends on IL-10 induction of TGF-β. *J Immunol* 2007; **178**:1433–42.

22 Leech MD, Benson RA, de Vries A, Fitch PM, Howie SEM. Resolution of *Der p* I-induced allergic airway inflammation is dependent on $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells. *J Immunol* 2007; **179**:7050–8.

23 Strickland DH, Stumbles PA, Zosky GR, Subrata LS, Thomas JA, Turner DJ, Sly PD, Holt PG. Reversal of airway hyperresponsiveness by induction of airway mucosal $CD4^+$ $CD25^+$ regulatory T cells. *J Exp Med* 2006; **203**:2649–60.

24 McGlade JP, Gorman S, Zosky GR, Larcombe AN, Sly PD, Finlay-Jones JJ, Turner DJ, Hart PH. Suppression of the asthmatic phenotype by ultraviolet B-induced antigen-specific regulatory cells. *Clin Exp Allergy* 2007; **37**:1261–3.

25 McGee HS, Agrawal DK. Naturally occurring and inducible T-regulatory cells modulating immune response in allergic asthma. *Am J Respir Crit Care Med* 2009; **180**:211–25.

26 Joetham A, Takeda K, Okamoto M, Taube C, Matsuda H, Dakhama A, Gelfand EW. Antigen specificity is not required for modulation of lung allergic responses by naturally occurring regulatory T cells. *J Immunol* 2009; **183**:1821–7.

27 Kearley J, Robinson DS, Lloyd CM. $CD4^+$ $CD25^+$ regulatory T cells reverse established allergic airway inflammation and prevent airway remodelling. *J Allergy Clin Immunol* 2008; **122**:617–24.

28 Boudousquie C, Pellaton C, Barbier N, Spertini F. $CD4^+$ $CD25^+$ T cell depletion impairs tolerance induction in a murine model of asthma. *Clin Exp Allergy* 2009; **39**:1415–26.

29 Kinsey GR, Sharm R, Huang L, Li L, Vergis AL, Ye H, Ju S-T, Okusa MD. Regulatory T cells suppress innate immunity in kidney ischemia–reperfusion injury. *J Am Soc Nephrol* 2009; **20**:1744–53.

30 Suto A, Nakajuma H, Kagami S-I, Suzuki K, Saito Y, Iwamoto I. Role of $CD4^+$ $CD25^+$ regulatory T cells in T helper 2 cell-mediated allergic inflammation in the airways. *Am J Respir Crit Care Med* 2001; **164**:680–7.

31 Lewkowicz P, Lewkowicz N, Sasiak A, Tchorzewski H. Lipopolysaccharide-activated $CD4^+$ $CD25^+$ T regulatory cells inhibit neutrophil function and promote their apoptosis and death. *J Immunol* 2006; **177**:7155–63.

32 Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperreactivity after *in vivo* transfer of $CD4^+$ $CD25^+$ regulatory T cells is interleukin 10 dependent. *J Exp Med* 2005; **202**:1538–47.

33 Topilski I, Flaishon L, Naveh Y, Harmelin A, Levo Y, Shachar I. The anti-inflammatory effects of 1,25-dihydroxyvitamin D3 on Th2 cells *in vivo* are due in part to the control of integrin-mediated T lymphocyte homing. *Eur J Immunol* 2004; **34**:1068–76.

34 Segaert S, Duvold LB. Calcipotriol cream: a review of its use in the management of psoriasis. *J Dermatol Treat* 2006; **17**:327–37.

35 Xystrakis E, Kusumakar S, Boswell S *et al.* Reversing the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients. *J Clin Invest* 2006; **116**:146–55.