

Cite this: *Photochem. Photobiol. Sci.*, 2011, **10**, 1894

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PAPER

Differences in control by UV radiation of inflammatory airways disease in naïve and allergen pre-sensitised mice

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Received 29th June 2011, Accepted 5th September 2011

DOI: 10.1039/c1pp05206c

Exposure of skin to UV radiation (UVR) prior to allergen exposure can inhibit inflammatory airways disease in mice by reducing effector CD4⁺ T cells in both the trachea and the airway draining lymph nodes. This study analysed the immunomodulatory properties of UVR delivered to naïve *versus* allergen pre-sensitised mice. In a model of inflammatory airways disease, BALB/c mice were sensitised by peritoneal injection of the allergen, ovalbumin (OVA) (20 µg/mouse), in the adjuvant, alum (4 mg/mouse), on days 0 and 14. On day 21, the mice were exposed to aerosolised OVA and 24 h later, proliferative responses by the cells in the airway draining lymph nodes were examined. UVR (8 kJ m⁻²) was administered 3 days prior to first OVA sensitisation (day -3), or OVA aerosol challenge (day 18). UVR before sensitisation reduced immune responses associated with expression of allergic airways disease; seven days after first OVA sensitisation, regulation of OVA-induced proliferation *in vitro* but not *in vivo* by CD4⁺CD25⁺ cells from UV-irradiated mice was detected. UVR administered to pre-sensitised mice regulated allergen responsiveness by cells from the airway draining lymph nodes only with a sensitisation protocol involving allergen and adjuvant at 5% strength of the original dose (1 µg OVA in 0.2 mg alum/mouse). These results suggest that UVR may modulate allergic airways disease by two mechanisms. The first, and more potent, is by reducing effector cells in respiratory tissues and requires UV delivery prior to sensitisation. The second, associated with administration to pre-sensitised mice, is weaker and is detected when the mice are sensitised with lower levels of allergen and adjuvant.

Introduction

UV irradiation of skin prior to allergen sensitisation in mice can reduce many of the subsequent hallmark signs of asthma including reduced responses upon allergen challenge by the lungs, the trachea and cells in the lymph nodes draining the airways.^{1,2} There are human correlates with a positive latitude gradient described for some allergic diseases,^{3,4} and debated for others.^{5,6} In the murine models in which UVR was regulatory, allergen sensitisation occurred either by intraperitoneal administration of OVA plus the adjuvant, alum¹ or by daily administration of intranasal papain.² We had previously hypothesised^{1,2} that at the time of the inflammatory airways disease, UVR-induced regulatory cells controlled the immune responses associated with the effector phase of the allergic airways response. This hypothesis reflected previous demonstrations in rodent models of allergic airways disease that regulatory CD4⁺CD25⁺ cells can accumulate at the airway wall and reduce expression of allergen reactivity.^{7–10} However, an intense search of cells in the large airways and in the airway draining lymph nodes in UV-irradiated mice failed to

find any regulatory cells.¹¹ It was concluded that after exposure to aerosolised allergens and disease expression, there were reduced numbers of effector Th2 cells in the airways of UV-irradiated mice. The mechanisms of regulation of systemic immune responses by UV irradiation of skin remained unclear, particularly as previous studies of UVR-immune suppression have focused on UV-regulation of Th1 immune models.^{12–14}

The literature suggests UVR may be immunomodulatory by induction of CD4⁺CD25⁺ T regulatory cells,¹⁵ or other types of suppressive CD25-Foxp3- T cells (Tr1)^{16,17} or regulatory B cells.¹⁸ UV irradiation of mouse skin can also increase the regulatory ability of naturally occurring 'innate' CD4⁺CD25⁺ cells.¹⁹ In our studies of allergic airways disease, we proposed that UV irradiation of skin allowed the induction and/or activation of regulatory cells which in turn controlled the level of sensitisation of allergen-specific effector T cells.

To better understand the mechanisms of UVR control of allergen sensitisation, we sought UVR-induced regulatory cells in the airway draining lymph nodes at a time closer to UVR exposure, namely 7 days after allergen sensitisation and 10 days after UV irradiation. This contrasted with our previous unsuccessful search for UVR-associated regulatory cells in the airway draining lymph nodes 24 days after UVR.¹¹ Further, to consider therapeutic approaches using controlled UVR for asthmatics who would be

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already sensitised to allergens, the ability of UVR to modulate responses to aeroallergens by allergen pre-sensitised mice was investigated. In this study, the concentration of the allergen and adjuvant used was important with UVR consistently regulating the responses of mice pre-sensitised to lower levels of allergen and adjuvant. This study suggests that there may be two mechanisms of UVR control of experimental allergic airways disease. Firstly, when administered prior to allergen sensitisation, UVR can regulate the outcome of strong sensitisation protocols. Secondly, UVR administered to allergen pre-sensitised mice can regulate a subsequent allergen challenge, but only when pre-sensitised to lower doses of allergen and adjuvant.

Materials and methods

Animals

BALB/c female mice were obtained from the Animal Resources Centre (Murdoch, Western Australia) and used when 8 weeks old. 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. All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia and with approval from the Animal Ethics Committee of the Telethon Institute for Child Health Research.

UV irradiation

The UVR source was a bank of six FS40 UVB lamps (Westinghouse Corporation, Pittsburgh, PA) emitting a broad band of UVR, 250–360 nm, with 65% of the output in the UVB range (290–320 nm).²⁰ The radiation output for each experiment was measured using a UVX radiometer with a UVX-31 sensor (Ultraviolet Products Inc., San Gabriel, CA). Mice were irradiated with 8 kJ m⁻² (approximately 3–4 minimal edematous doses)²⁰ on a clean-shaven 2 × 4 cm dorsal patch of skin either before or post OVA sensitisation. A polyvinylchloride plastic sheet removed wavelengths less than 290 nm. Control mice were shaved but were not exposed to UVR.

Sensitisation and challenge of mice to ovalbumin

Unless otherwise stated, mice were injected intraperitoneally with OVA (Sigma Chemical Company, St. Louis, MO) in alum (aluminium hydroxide, Serva, Heidelberg, Germany) on day 0 and day 14 (20 µg OVA in 4 mg alum/mouse; 200 µl volume, 100% strength).^{1,11} On day 21, the mice were challenged with with a 1% OVA-in-saline aerosol delivered using an ultrasonic nebulizer (UltraNeb®, DeVilbiss, Somerset, PA) for 30 min. In some experiments, the lymph nodes draining the peritoneal cavity were harvested on day 7. In other experiments, mice were sensitised and boosted intraperitoneally with a 5% dose (1 µg OVA, 0.2 mg alum/mouse), a 1% dose (0.2 µg OVA, 0.04 mg alum/mouse) or a 0.1% dose (0.02 µg OVA, 0.004 mg alum/mouse). The OVA in alum suspension was diluted with 0.9% saline so that the injection volume (200 µl) remained constant.

Culture of single cell suspensions from peritoneal- and airway draining lymph nodes

The posterior mediastinal, tracheobrachial and parathymic lymph nodes (which drain both the peritoneal cavity and the airways)

were removed from mice at the indicated times and physically disaggregated in glucose-containing buffer supplemented with 10% FCS. The resultant cell suspensions were filtered through 100 µm nylon mesh and washed. Cells were incubated at 10⁵ cells/200 µl/well in RPMI medium (Gibco, Auckland, NZ) with 10% FCS, 2 mM L-glutamine, 50 µM 2-ME, 5 µg ml⁻¹ gentamicin, 10 mM non-essential amino acids and 1 mM sodium pyruvate (RPMI-10) at 37 °C with 5% CO₂, with or without OVA (100 µg ml⁻¹). Methyl-³H-thymidine (Amersham Pharmacia Biotech, NJ; 50 µCi/ml, 10 µl/well) was added at 72 h and cells harvested at 96 h with ³H-thymidine incorporation used as a measure of cellular proliferation. Cytokine levels in culture supernatants were measured as previously described.^{1,11}

CD4+CD25+ cell enrichment

CD4+CD25+ cells were purified from lymph node single cell suspensions by the AutoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany).¹¹ Briefly, a negative selection method was used to isolate CD4+ cells by labelling cells expressing CD8 α , CD11b, CD45R, CD49b and Ter-119 with biotin-conjugated monoclonal anti-mouse antibodies and then using anti-biotin microbeads (Miltenyi Biotec) to select for these cells. Purified CD4+ cells were then labelled with anti-CD25-PE and a positive selection method was used to purify CD25+ cells to >95% purity (determined by flow cytometry) using anti-PE microbeads (Miltenyi Biotec). For intracellular Foxp3 staining, a Foxp3 staining kit was used according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). The flow cytometry data were acquired on an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and analysed using FlowJo software (version 8.7.3).

Co-cultures using responder cells from OVA-sensitised BALB/c mice

Purified cell types from lymph nodes draining the peritoneal cavity of non- and UV-irradiated, OVA-sensitised mice in RPMI-10 were gamma-irradiated at 830 rad using a GammaCell 3000 Elan Irradiator (MDS Nordion, Ontario, Canada) before co-culture with unfractionated lymph node cells from non-irradiated, OVA-sensitised, -boosted and -challenged mice at various ratios (potential regulatory cell: responder cell ratios of 1:1, 1:2, 1:4, 1:8). The number of unfractionated responder cells was kept constant over all ratios (10⁵ cells per well). OVA protein was added at 100 µg ml⁻¹ and cells cultured in 200 µl final volume RPMI-10 at 37 °C for 96 h. Methyl-³H-thymidine was added after 72 h and cells harvested at 96 h (see above).

Tracking of CFSE+CD4+ cells from DO11.10 mice adoptively transferred into UV-irradiated, OVA-sensitised BALB/c mice

CD4+ cells from DO11.10 mice were labelled with 5 µM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Oregon, USA) as previously described.¹¹ Cells were resuspended in saline and 5 × 10⁶ cells/200 µl/mouse were adoptively transferred into non- or UV-irradiated, BALB/c recipient mice that had been sensitised to OVA in alum (20 µg OVA in 4 mg alum/mouse) 3 or 6 days previously. Twenty-four hours later, the recipient mice were given an OVA aerosol challenge. The lymph nodes draining the airways were removed from recipient mice three

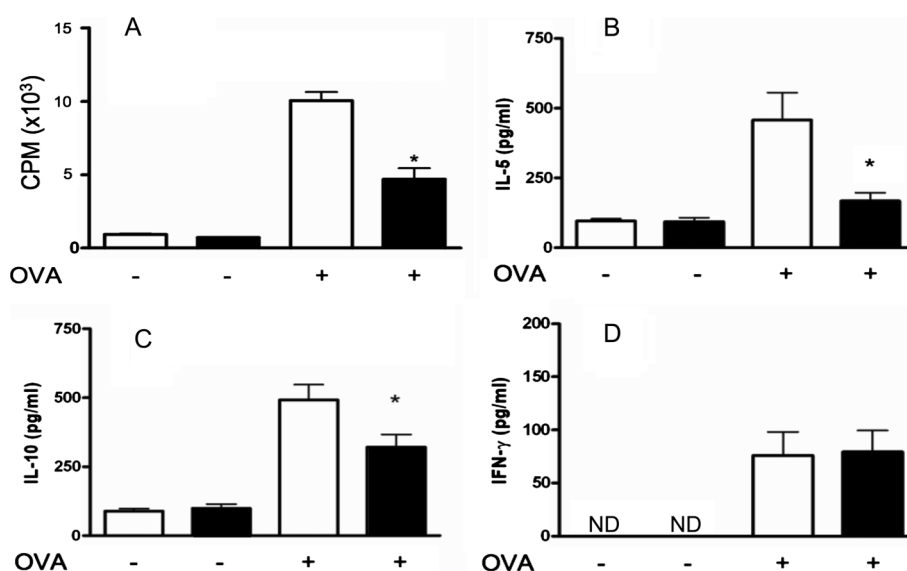


Fig. 1 OVA-induced proliferation (A), and cytokine production (B–D) by cells from the lymph nodes draining the peritoneal cavity and harvested 7 days after OVA in alum sensitisation. Cells were incubated without (–) or with (+) OVA ($1 \mu\text{g ml}^{-1}$) for 96 h. UV irradiation (solid bars) was administered 3 days prior to OVA sensitisation. Cells from non-irradiated mice are shown by open bars. (A) ^3H -thymidine incorporation during the last 24 h (CPM/well). (B) IL-5, (C) IL-10, (D) IFN γ levels in the culture supernatants. Mean $\pm$ SEM, $n = 3$ replicate cultures. An asterisk represents a significantly reduced level for cells from UV-irradiated mice, $p < 0.05$. ND, not detectable.

days after challenge and physically disaggregated into single cell suspensions. Cells were then labelled with [OVA-TCR]-PE-Cy5 (Caltag Laboratories, California, USA) and anti-CD4-APC. In flow cytometric analysis, CFSE levels in CD4+[OVA-TCR]+ cells were assessed. Reduced CFSE content indicated cellular division; the percentage of cells per division was also evaluated.

Measurement of serum OVA-specific IgG1

Using a 96-well ELISA format, wells were coated with OVA ($1 \mu\text{g}$) overnight at 4°C in carbonate buffer ($100 \text{ mM Na}_2\text{CO}_3$, 100 mM NaHCO_3 , pH 9.6), washed, incubated with blocking buffer (0.5% BSA in Tris-HCl, pH 7.4) before incubation for 2 h at room temperature with serum samples either undiluted or diluted $1:10$, $1:10^2$, $1:10^3$, $1:10^4$ and $1:10^5$ in a Tris-based assay buffer. Biotinylated mouse anti-IgG1 (Southern Biotechnology Associates Inc, Alabama, USA) was added before IgG1 measurement in a dissociation-enhanced time-resolved fluorescence immunoassay with europium as the label.

Statistical analyses

When results from multiple experiments were considered, levels of significance were calculated using raw data and a 2-way ANOVA using [Experiment Number] as one variable and [+UV] treatment as the other variable. Student's t test was used for replicates in a single experiment. Each experimental group consisted of 5 mice unless otherwise indicated.

Results

Regulation by UVR of OVA-driven proliferation of cells in the airway draining lymph nodes 7 days after sensitisation

The shaved backs of mice were given a single exposure to UVR (8 kJ m^{-2}) three days before intraperitoneal injection with $20 \mu\text{g}$

OVA in 4 mg alum/mouse . After a further 7 days, the lymph nodes draining the peritoneal cavity were harvested, and single cell suspensions incubated with OVA protein ($100 \mu\text{g ml}^{-1}$) for 96 h. The OVA-induced proliferation by cells from the UV-irradiated mice was significantly less than that by cells from non-irradiated mice (Fig. 1A). The levels of the Th2 cytokines (IL-5, IL-10) (Fig. 1B,C), but not the Th1 cytokine (IFN γ) (Fig. 1D), were significantly lower in the supernatants of cells from the UV-irradiated mice. Less production of all cytokines, including IL-10, suggested reduced sensitisation to OVA. However, it remained possible that UVR-induced regulatory cells in the lymph node cell suspensions contributed to the reduced responses.

Regulatory activity of CD4+CD25+ cells in a proliferation assay using non-fractionated lymph node cells from OVA-sensitised mice as responders

At this same time point (7 days after first intraperitoneal OVA in alum injection), CD4+ cells that were CD25+ or CD25- were purified from the lymph nodes draining the peritoneal cavity. Yields of CD4+CD25+ cells were low with approximately 5×10^4 cells obtained from the peritoneal lymph nodes/mouse, and were very similar from UV- and non-irradiated mice (data not shown). Yields of CD4+CD25- cells were approximately 10-fold higher than those of CD4+CD25+ cells. Of the CD4+CD25+ cells (–UV, $4.9 \pm 0.1\%$ of lymph node cells; +UV, $5.1 \pm 0.2\%$ of lymph node cells, mean $\pm$ SEM, $n = 3$), $88 \pm 0.5\%$ from non-irradiated mice and $89 \pm 0.8\%$ from UV-irradiated mice expressed Foxp3. Of the CD4+CD25- cells, $1.6 \pm 0.1\%$ from non-irradiated mice and $1.7 \pm 0.3\%$ from UV-irradiated mice expressed Foxp3.

The regulatory ability of the CD4+CD25+ and CD4+CD25- cells was examined using a proliferation assay with responder cells from OVA-sensitised mice. The CD4+CD25+ and CD4+CD25- cells (γ -irradiated to prevent their proliferation)

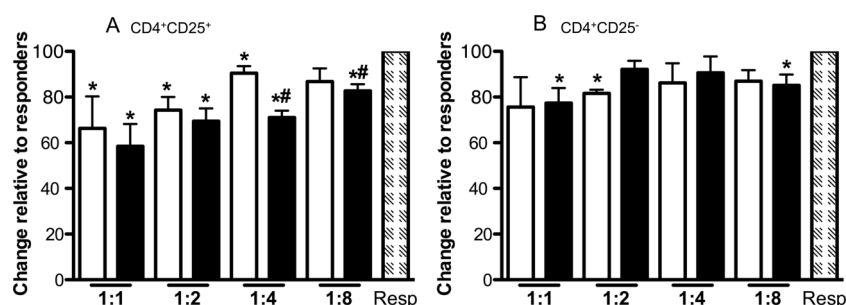


Fig. 2 Effect of UV irradiation of mice on the ability of lymph node CD4+CD25+ and CD4+CD25- cells to suppress proliferation *in vitro* by responder cells (unfractionated lymph node cells from OVA-sensitised mice). The lymph nodes draining the peritoneal cavity were harvested 7 days after OVA in alum sensitisation and CD4+CD25+ and CD4+CD25- cells isolated by microbead enrichment. In (A), CD4+CD25+ cells, and (B), CD4+CD25- cells were γ -irradiated before incubation with unfractionated cells from OVA-sensitised mice, together with OVA. ^3H -thymidine was added for the last 24 h of the 96 h culture. For each of 4 independent experiments, the level of ^3H -thymidine incorporated by responder cells alone (Resp, hatched bars) was normalized to 100% and reductions due to added CD4+CD25+ or CD4+CD25- cells calculated. Solid bars indicate the result for cells from UV-irradiated mice; open bars indicate the result for cells from non-irradiated mice. Mean + SEM. An asterisk represents significantly reduced ^3H -thymidine incorporation by cultures with added CD4+CD25+ or CD4+CD25- cells compared with that measured in cultures of responder cells alone (hatched bars), $p < 0.05$. A hash demonstrates a significant difference in ^3H -thymidine incorporation for cultures containing CD4+CD25+ cells from UV-irradiated mice compared with those from non-irradiated mice at the same dilution, $p < 0.05$.

were incubated at 1:1, and decreasing ratios, with responder cells and OVA. To validate the assay, we have previously shown that CD4+CD25+ cells from DO11.10 mice (OVA-TCR+) can significantly regulate OVA-induced proliferation by cells from OVA-sensitised mice, even at a ratio of 1:16, *i.e.* 1 CD4+CD25+ cell incubated with every 16 responder cells.¹¹ The cumulative results for 4 independent experiments are shown in Fig. 2A and B. CD4+CD25+ cells for all groups but one (CD4+CD25+ cells from non-irradiated mice diluted 1:8, *i.e.* one CD4+CD25+ cell to 8 responder cells) suppressed the proliferation by responder cells from OVA-sensitised mice (Resp, hatched bar, Fig. 2). In Fig. 2A, at a ratio of 1:4 or 1:8, the proliferation by responder cells was significantly lower if incubated with CD4+CD25+ cells from the UV-irradiated mice than from non-irradiated mice suggesting greater regulatory activity for CD4+CD25+ cells from UVR-exposed mice (Fig. 2A, 2-way ANOVA). Whether these cells contributed to the differences in proliferation by cells from UV- and non-irradiated mice (Fig. 1A) was not determined, particularly as an anti-CD25 antibody depletion may also remove activated cells. CD4+CD25- cells from UV-irradiated mice suppressed the proliferation of the responder cells at a ratio of 1:1, and from non-irradiated mice at a ratio of 1:2 (Fig. 2B). However, CD4+CD25- cells from the UV-irradiated mice were not more regulatory than CD4+CD25- cells from non-irradiated mice at any of the ratios tested.

Search for UVR-induced regulatory cells *in vivo*. Adoptive transfer of CFSE-labelled CD4+ cells from DO11.10 mice into UV-irradiated and non-irradiated, OVA-sensitised mice

To investigate the potential involvement of UVR-induced regulatory cells of any type^{15–19} and not only CD4+CD25+ and CD4+CD25- cells, an *in vivo* assay was established. In this assay, CFSE-labelled CD4+ responder cells were transferred into UV-irradiated mice and their proliferation monitored. The shaved backs of mice were given a single exposure to UVR (8 kJ m⁻²) three days before intraperitoneal sensitisation with 20 μg OVA in 4 mg alum/mouse. Six days later, the mice were given *via* their tail vein 5×10^6 CFSE-labelled CD4+ cells from DO11.10 mice. Twenty-four h later, they were given a 1% OVA-in-saline aerosol. A further 3 days later (4 days since injection of the cells), the lymph nodes draining the airways from individual mice were isolated and single cell suspensions prepared ($n = 4$ mice/group of UV- and non-irradiated mice). CFSE-dilution in the CD4+[OVA-TCR]+ cells was analysed. In response to the OVA aerosol, the transferred [OVA-TCR]+ cells proliferated with approximately 15% of cells proliferating 4 times (reaching 4 generations). However, there was no significant difference in proliferative responses by cells transferred into UV- or non-irradiated, OVA-sensitised mice (Table 1). A repeat experiment in which CFSE-labelled CD4+ cells from DO11.10 mice were transferred 3 not 6 days after OVA

Table 1 Proliferation of CFSE-labelled CD4+ cells from naïve DO11.10 mice adoptively transferred into non-irradiated or UV-irradiated, OVA-sensitised mice. Transfer occurred 6 days after OVA in alum sensitisation, and 24 h before OVA aerosol challenge. CFSE expression by cells in the airway draining lymph nodes was measured 72 h after OVA challenge

Generation ^a	0	1	2	3	4
–UV ^b	14.9 $\pm$ 4.0%	12.4 $\pm$ 1.4%	19.7 $\pm$ 0.9%	22.9 $\pm$ 2.2%	15.3 $\pm$ 1.6%
+UV	19.1 $\pm$ 5.4%	12.5 $\pm$ 0.9%	16.6 $\pm$ 1.4%	19.7 $\pm$ 1.9%	15.6 $\pm$ 1.5%

^a Generation 0 = undivided cells, Generations 1, 2, 3, 4 = 1, 2, 3, 4 divisions, respectively, measured by dilution of CFSE in [OVA-TCR]+CD4+ cells.

^b Recipient mice were irradiated with UV (8 kJ m⁻²), or not (–UV), 3 days before OVA sensitisation on day 0. 5×10^6 cells were transferred into 4 mice per group; mean $\pm$ SD.

in alum sensitisation gave very similar results (data not shown). Thus, the immunosuppressive properties of the CD4+CD25+ cells shown *in vitro* using cell ratios of 1 : 4 and 1 : 8 (Fig. 2A) could not be confirmed *in vivo*. Further, the activity of other putative UVR-induced regulatory cells was not detected (Table 1).

Regulation of allergen responses by UV irradiation of skin of pre-sensitised mice

To demonstrate greater potential therapeutic benefit, UVR should be modulatory in allergen pre-sensitised mice. Mice were sensitised to 20 µg OVA in 4 mg alum/mouse (designated as 100% strength) on day 0 and boosted on day 14. On day 18 (3 days before OVA aerosol challenge), they were given 8 kJ m⁻² UVR to their shaved back. Twenty-four hours after OVA aerosol challenge (day 22), the proliferation *in vitro* by airway draining lymph nodes was investigated. OVA-induced proliferation by cells from UVR-treated mice was not significantly different to that by cells from non-irradiated mice (Fig. 3A for cumulative data from 3 independent experiments). Due to inter-experimental variability in cytokine levels in the culture supernatants, the OVA-induced levels for IL-5 and IL-10 for cells from non-irradiated mice were normalised to 100% and changes due to UVR calculated

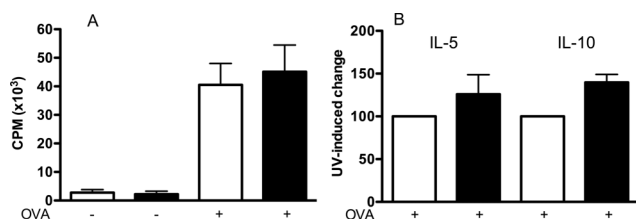


Fig. 3 Effect of UVR delivered to mice pre-sensitised to OVA in alum at 100% strength on OVA-induced proliferation by airway draining lymph node cells harvested 24 h after OVA aerosol challenge. Cells were incubated with (+) or without (-) OVA (1 µg ml⁻¹) for 96 h. UVR (solid bars) was administered 3 days prior to OVA aerosol challenge. Cells from non-irradiated mice are shown by open bars. (A) ³H-thymidine incorporation during the last 24 h (CPM/well). Mean + SEM for 3 independent experiments. (B) IL-5 and IL-10 in the culture supernatants of cells incubated with OVA. Levels for cells from non-irradiated mice were designated as 100%.

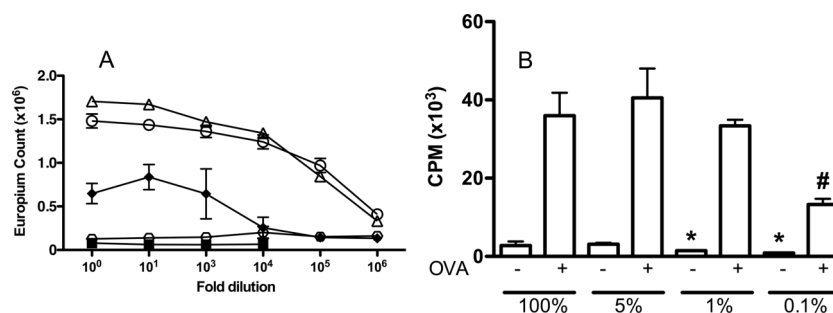


Fig. 4 Effect of reducing the concentration of the OVA in alum mixture for sensitisation. Mice were sensitised and boosted intraperitoneally on days 1 and 14 with OVA in alum undiluted (100% strength, open circles) or diluted with 0.9% saline to 5% (open triangles), 1% (closed diamonds), 0.1% (open hexagon) or left non-sensitised (closed squares). (A) Serum OVA-specific IgG1 levels on day 18. Mean ± SEM, *n* = 3. (B) Proliferation by airway draining lymph node cells. Airway draining lymph nodes were harvested 24 h after OVA aerosol challenge and incubated for 96 h with (+) or without (-) OVA. ³H-thymidine incorporation during the last 24 h (CPM/well). Mean + SEM for 6 replicate wells. ³H-thymidine incorporation by cells from mice sensitised to 100%, 1% or 0.1% strength OVA in alum was compared with that by cells from mice sensitised to 5% strength OVA in alum. An asterisk is shown for significantly different ³H-thymidine incorporation by cells incubated without OVA; a hash is shown for cells incubated with OVA, *p* < 0.05.

(Fig. 3B). There was no significant change detected for cells from UV-irradiated mice.

To investigate whether UVR can regulate responses developed in response to lower concentrations of allergen and/or adjuvant, the intensity of the sensitisation protocol was investigated. Mice were injected intraperitoneally on days 0 and 14 with OVA in alum at 100%, 5%, 1% and 0.1% relative strength (both components proportionally diluted). On day 18, the mice were tail bled for measurement of OVA-specific serum IgG1. As shown in Fig. 4A, serum IgG1 levels were not significantly different between mice sensitised with 100% and 5% OVA in alum. Mice sensitised to 1% OVA in alum had significantly reduced IgG1 levels whilst 0.1% OVA in alum did not induce detectable IgG1 levels. Twenty-four hours after an OVA-aerosol challenge on day 21, the airway draining lymph nodes were harvested for assessment of OVA reactivity *in vitro*. OVA-induced proliferation by cells from the airway draining lymph nodes was similar in response to sensitisation with 100%, 5% and 1% strength OVA in alum (Fig. 4B). In the absence of OVA addition to the culture, reduced proliferation was measured by cells from mice sensitised with OVA in alum at 1% and 0.1% strength (Fig. 4B).

In response to the results of Fig. 4A and B, mice were sensitised and boosted with 5% strength OVA in alum, *i.e.* the lowest dose giving similar sensitisation measures (both cellular and humoral responses) as 100% strength OVA in alum. Their shaved backs were exposed to 8 kJ m⁻² UVR 3 days before OVA aerosol challenge on day 21. For 3 independent experiments, the proliferation by cells from the airway draining lymph nodes of UV-irradiated mice harvested 24 h after OVA challenge was significantly reduced (Fig. 5A). Cytokine levels in the culture supernatants were also reduced (Fig. 5B). Thus, when a less concentrated suspension of OVA in alum was used for sensitisation, UV irradiation of skin of pre-sensitised mice suppressed subsequent OVA-induced responses by airway draining lymph node cells.

Discussion

UV irradiation of skin can suppress murine models of allergic asthma. In previous studies when UVR was administered prior to allergen sensitisation, the asthma-like responses in the large

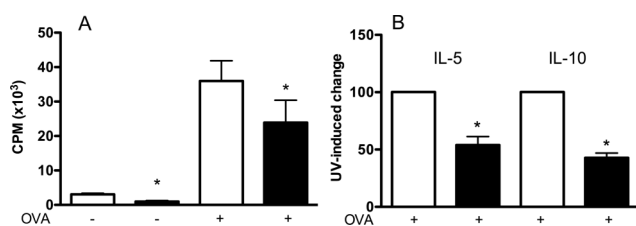


Fig. 5 Effect of UVR delivered to mice pre-sensitised to OVA in alum at 5% strength on OVA-induced proliferation by airway draining lymph node cells harvested 24 h after OVA aerosol challenge. Cells were incubated with (+) or without (–) OVA ($1 \mu\text{g ml}^{-1}$) for 96 h. UVR (solid bars) was administered 3 days prior to OVA aerosol challenge. Cells from non-irradiated mice are shown by open bars. (A) ^3H -thymidine incorporation during the last 24 h (CPM/well). Mean $\pm$ SEM for 3 independent experiments. (B) IL-5 and IL-10 in the culture supernatants of cells incubated with OVA. Levels for cells from non-irradiated mice were designated as 100%. An asterisk represents a significant change due to UVR, $p < 0.05$.

airways, lung and draining lymph nodes were reduced consistently by approximately 50%.^{1,11} In the present study, we show that UVR administered to pre-sensitised mice can be suppressive; however, a less concentrated allergen and adjuvant suspension was required for sensitisation. For 3 independent experiments in which UV was delivered to OVA-pre-sensitised mice, proliferation *in vitro* by single cell suspensions from the airway draining lymph nodes was reduced by a mean of 37%. Thus, we propose that UV may regulate allergic airways disease by at least two different pathways that associate with exposure to UVR before and after allergen sensitisation.

At the time of expression of allergic airways disease (*i.e.* after aerosolised allergen challenge), the reduced responses to allergen in the UV-irradiated mice are due to fewer effector Th2 cells.¹¹ We now report that 7 days after the initial OVA in alum sensitisation when OVA-driven proliferation of airway draining lymph node cells was reduced (Fig. 1), CD4+CD25+ cells from UV-irradiated mice had greater regulatory properties in proliferative assays *in vitro* than CD4+CD25+ cells from non-irradiated mice. These CD4+CD25+ cells in the nodes of UV-irradiated mice may help to control the development and number of effector cells. However, the regulatory properties of these CD4+CD25+ cells from UV-irradiated mice were not robust as these cells were unable to reduce OVA-aerosol-induced proliferation of CFSE-labelled CD4+ cells from OVA-TCR transgenic mice adoptively transferred into the UV-irradiated recipient mice. This *in vivo* assay had been designed to detect all types of UVR-induced regulatory cells (including regulatory B cells) but instead helped to determine the relative down-regulatory potential *in vivo* of CD4+CD25+ cells from UV-irradiated mice. It remains possible that UV-induced regulatory cells affected the differentiation of CD4+ responder cells that was not detected in the assay measuring dilution of CFSE in proliferating OVA-TCR+ cells in the lymph nodes. In this model of allergic airways disease, UVR may also control allergen sensitisation and effector cell development directly, without a major role for UVR-activated/induced regulatory cells. The ability of UVR to suppress immune responses by reduced effector and memory T cells, and without induction of regulatory cells, has been reported previously by at least one other laboratory.²¹

In the studies of UVR delivered to pre-sensitised mice, UV irradiation of skin was not initially immunomodulatory (*i.e.* when using 100% strength OVA in alum suspension). However, with a sensitisation protocol using 5% of the initial allergen concentration, and 5% of the adjuvant alum, UVR was immunosuppressive. In this 5% strength model, the mice may have been sensitised by a different immune process. UVR may also have been down-regulatory by a different mechanism, or at least one that may have been operative but was not the dominant mechanism when UVR was administered prior to sensitisation. It is possible that UVR activated pre-existing regulatory cells, either OVA-induced or naturally occurring T regulatory cells. In previous studies of naïve mice, UV irradiation of skin,¹⁹ and topical 1,25-dihydroxyvitamin D₃²² can increase the activity of CD4+CD25+ cells in the draining nodes, and further, upon transfer into OVA-pre-sensitised mice can down-regulate the responses associated with allergic airways disease.²³

Regulation by UVR of immune responses in pre-sensitised mice has been previously reported. In one laboratory, post-immune UVR induced Tr1-like regulatory T cells (IL-10-producing, CD25-Foxp3- cells) that were able to suppress B cell antibody production.¹⁶ Very high doses of UVR were administered (23 kJ m^{-2}).¹⁶ UVR control of allergic airway inflammation was also examined;¹⁷ however, the UVR was delivered one week after the first intraperitoneal sensitisation but on the very same day that the mice were given a second OVA sensitisation, followed by a third sensitisation one week later.¹⁷ Thus, the effects of the UV irradiation of skin were present during, and potentially controlled the outcome of, the second and third intraperitoneal sensitisations.¹⁷ In our study, the mice were twice sensitised intraperitoneally (2 weeks apart) and the UVR was delivered 4 days after the second sensitisation, and 3 days prior to aerosol challenge. In a further study of UVR regulation of immune responses in pre-sensitised mice, a Th1 model of delayed-type hypersensitivity to *Candida albicans* was studied and antigen-specific suppressor T cells were implicated.²⁴ The authors reported significant suppression if the UVR (80 kJ m^{-2} solar simulated UV) was given 7 to 9 days post immunisation and 3 to 1 days before challenge on day 10.²⁴

UV-irradiation of skin induces vitamin D₃ production, and for humans, most of our vitamin D₃ is obtained by irradiation of skin with UVB. Whether UVR-induced vitamin D₃ is responsible for the UVR regulation of allergic airways disease in our studies is a very important question particularly as it would support vitamin D₃ supplementation for treatment of inflammatory airways disease. It is possible that UVR-induced vitamin D₃ may regulate one but not the other mechanism of control proposed in this study. Chronic feeding of mice a vitamin D₃-deficient diet enhanced allergen sensitisation in the OVA-alum model of allergic airways disease.²⁵ A recent study of mice with experimental autoimmune encephalomyelitis concluded that UVR could regulate the disease by mechanisms unrelated to vitamin D₃.²⁶ Thus, further novel approaches are required to determine whether vitamin D₃ produced in response to acute UVR contributes to UVR control of sensitisation in our model of allergic airways disease.

This study cautions the interpretations of findings obtained using one of the most commonly used rodent models of allergic airways disease. We have observed that the doses of OVA and

alum used in most studies^{27–30} are such that a 20-fold dilution of both components allows for comparative induction of OVA-specific IgG1 levels, and similar proliferation to OVA *in vitro* by airway draining lymph node cells. We propose that in humans, the sensitisation to allergens is more subtle, does not occur in the context of powerful adjuvants, and such an acute high dose of allergen, with adjuvant, is not required. From another perspective, modulation of sensitisation to the higher doses of OVA in alum can show the potency of regulatory agents like UVR. Alternatively, the ability of some agents to regulate *in vivo* may not be appreciated with the high doses of OVA and alum frequently used.^{27–30} Perhaps, more importantly, the inflammatory airways disease induced by the higher concentrations of OVA in alum may be different to that induced by lower concentrations of OVA in alum.

In summary, this study investigated UVR control of inflammatory airways disease. We propose two mechanisms of suppression, the first and most potent occurs when UVR is delivered prior to allergen sensitisation. The second, detected when UV was administered to pre-sensitised mice, is less potent in this murine model but in humans in the absence of strong adjuvants may be significant. Studies of the effect of delivery of controlled UVR and/or vitamin D₃ to patients with allergic airways disease are required.

Abbreviations

CFSE	Carboxyfluorescein succinimidyl ester
OVA	Ovalbumin
UVR	UV radiation.

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.

Acknowledgements

This work was supported by grants from the National Health and Medical Research Council of Australia (#458612 to P.H.H.), the Cancer Council, Western Australia (to P.H.H. and S.G.).

References

- 1 J. P. McGlade, S. Gorman, G. R. Zosky, A. N. Larcombe, P. D. Sly, J. J. Finlay-Jones, D. J. Turner and P. H. Hart PH, Suppression of the asthmatic phenotype by ultraviolet B-induced, antigen-specific regulatory cells, *Clin. Exp. Allergy*, 2007, **37**, 1267–1276.
- 2 J. P. McGlade, S. Gorman, J. C. Lenzo, T. W. Tan, T. Watanabe, J. J. Finlay-Jones, W. R. Thomas and P. H. Hart PH, Effect of both UVB irradiation and histamine receptor function on allergic responses to an inhaled antigen, *J. Immunol.*, 2007, **178**, 2794–2802.
- 3 S. K. Weiland, A. Husing, D. P. Strachan, P. Rzehak and N. Pearce, the ISAAC phase One Study Group, Climate and the prevalence of symptoms of asthma, allergic rhinitis, and atopic eczema in children, *Occup. Environ. Med.*, 2004, **61**, 609–615.
- 4 G. Krstic, Asthma prevalence associated with geographical latitude and regional insolation in the United States and Australia, *PLoS One*, 2011, **6**, e18492.
- 5 A. A. Litonjua and S. T. Weiss, Is vitamin D deficiency to blame for the asthma epidemic?, *J. Allergy Clin. Immunol.*, 2007, **120**, 1031–1035.
- 6 P. N. Black and R. Scragg, Relationship between serum 25-hydroxyvitamin D and pulmonary function in the third national health and nutrition examination survey, *Chest*, 2005, **128**, 3792–3798.
- 7 P. G. Holt, D. H. Strickland, M. E. Wikstrom and J. L. Jahnsen, Regulation of immunological homeostasis in the respiratory tract, *Nat. Rev. Immunol.*, 2008, **8**, 142–152.
- 8 D. H. Strickland, P. A. Stumbles, G. R. Zosky, L. S. Subrata, J. A. Thomas, D. J. Turner, P. D. Sly and P. G. Holt, Reversal of airways hyperresponsiveness by induction of CD4+CD25+ regulatory T cells, *J. Exp. Med.*, 2006, **203**, 2649–2660.
- 9 J. Kearley, D. S. Robinson and C. M. Lloyd, CD4+CD25+ regulatory T cells reverse established allergic airway inflammation and prevent airway remodelling, *J. Allergy Clin. Immunol.*, 2008, **122**, 617–624.
- 10 I. P. Lewkowich, N. S. Herman, K. W. Schleifer, M. P. Dance, B. L. Chen, K. M. Dienger, A. A. Sproles, J. S. Shah, J. Kohl, Y. Belkaid and M. Wills-Karp, CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function, *J. Exp. Med.*, 2005, **202**, 1549–1561.
- 11 J. P. McGlade, D. H. Strickland, M. J. M. Lambert, S. Gorman, J. A. Thomas, M. A. Judge, J. T. Burchell, G. R. Zosky and P. H. Hart, UV inhibits allergic airways disease in mice by reducing effector CD4+ T cells, *Clin. Exp. Allergy*, 2010, **40**, 772–785.
- 12 S. E. Ullrich SE, Mechanisms underlying UV-induced immune suppression, *Mutat. Res., Fundam. Mol. Mech. Mutagen.*, 2005, **571**, 185–205.
- 13 M. Norval, The mechanisms and consequences of ultraviolet-induced immunosuppression, *Prog. Biophys. Mol. Biol.*, 2006, **92**, 108–118.
- 14 S. Beissert, A. Schwarz and T. Schwarz, Regulatory T cells, *J. Invest. Dermatol.*, 2006, **126**, 15–24.
- 15 T. Schwarz, 25 years UV-induced immunosuppression mediated by T cells- From disregarded T suppressor cells to highly respected regulatory T cells, *Photochem. Photobiol.*, 2008, **84**, 10–18.
- 16 L. Wang, M. Toda, K. Saito, T. Hori, H. Shiku, K. Kuribayashi and T. Kato, Post-immune UV irradiation induces Tr1-like regulatory T cells that suppress humoral immune responses, *Int. Immunol.*, 2007, **20**, 57–70.
- 17 L. Wang, K. Saito, M. Toda, T. Hori, M. Torii, N. Ma, N. Katayama, H. Shiku, K. Kuribayashi and T. Kato, UV irradiation after immunization induces type 1 regulatory T cells that suppress Th2-type immune responses via secretion of IL-10, *Immunobiology*, 2010, **215**, 124–132.
- 18 S. N. Byrne and G. M. Halliday, B cells activated in lymph nodes in response to ultraviolet irradiation or by interleukin-10 inhibit dendritic cell induction of immunity, *J. Invest. Dermatol.*, 2005, **124**, 570–578.
- 19 S. Gorman, J. W-T. Tan, S. T. Yerkovich, J. J. Finlay-Jones and P. H. Hart, 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.*, 2006, **127**, 915–924.
- 20 P. H. Hart, M. A. Grimaldeston, G. J. Swift, A. Jaksic, F. P. Noonan and J. J. Finlay-Jones, Dermal mast cells determine susceptibility to ultraviolet B-induced systemic suppression of contact hypersensitivity responses in mice, *J. Exp. Med.*, 1998, **187**, 2045–2053.
- 21 S. Rana, S. N. Byrne, L. J. MacDonald, C. Y-Y. Chan and G. M. Halliday, Ultraviolet B suppresses immunity by inhibiting effector and memory T cells, *Am. J. Pathol.*, 2008, **172**, 993–1004.
- 22 S. Gorman, L. A. Kuritzky, M. A. Judge, K. M. Dixon, J. P. McGlade, R. S. Mason, J. J. Finlay-Jones and P. H. Hart, Topically applied 1,25-dihydroxyvitamin D₃ enhances the suppressive activity of CD4+CD25+ cells in the draining lymph nodes, *J. Immunol.*, 2007, **179**, 6273–6283.
- 23 S. Gorman, M. A. Judge, J. T. Burchell, D. J. Turner and P. H. Hart, 1,25-dihydroxyvitamin D₃ enhances the ability of transferred CD4+CD25+ cells to modulate T helper type 2-driven asthmatic responses, *Immunology*, 2010, **130**, 181–192.
- 24 D. X. Nghiem, N. Kazimi, D. L. Mitchell, A. A. Vink, H. N. Ananthaswamy, M. L. Kripke and S. E. Ullrich, Mechanisms underlying the suppression of established immune responses by ultraviolet radiation, *J. Invest. Dermatol.*, 2002, **119**, 600–608.
- 25 S. Gorman, D. H. W. Tan, M. J. M. Lambert, N. M. Scott, M. A. Judge and P. H. Hart, Vitamin D₃ deficiency enhances allergen-induced lymphocyte responses in a mouse model of allergic airway disease, *Pediatr. Allergy Immunol.*, 2011, in press, DOI: 10.1111/j.1399-3038.2011.01146.x.
- 26 B. R. Becklund, K. S. Severson, S. V. Vang and H. F. De Luca, UV radiation suppresses experimental autoimmune encephalomyelitis independent of vitamin D production, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 6418–6423.

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- 27 A. J. Coyle, S. Tsuyuki, C. Bertrand, S. Huang, M. Aguet, S. S. Alkan and G. P. Anderson, Mice lacking the IFN- γ receptor have an impaired ability to resolve a lung eosinophilic inflammatory response associated with a prolonged capacity to T cells to exhibit a Th2 cytokine profile, *J. Immunol.*, 1996, **156**, 2680–2685.
- 28 C. L. Hardy, L. Kenins, A. C. Drew, J. M. Rolland and R. E. O’Hehir, Characterization of a mouse model of allergy to a major occupational latex glove allergen Hev b 5, *Am. J. Respir. Crit. Care Med.*, 2003, **167**, 1393–1399.
- 29 M. Ohshima, A. Yokoyama, H. Ohnishi, H. Hamada, J. Higaki and T. Naka, Overexpression of suppressor of cytokine signalling-5 augments eosinophilic airway inflammation in mice, *Clin. Exp. Allergy*, 2007, **37**, 735–742.
- 30 C. Lee, T. B. Kolesnik, I. Caminschi, A. Chakravorty, W. Carter, W. S. Alexander, J. Jones, G. P. Anderson and S. E. Nicholson, Suppressor of cytokine signalling 1 (SOCS1) is a physiological regulator of the asthma response, *Clin. Exp. Allergy*, 2009, **39**, 897–907.