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Vitamin D3 deficiency enhances contact hypersensitivity in male but not in female mice

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ABSTRACT

To ascertain the influence of vitamin D3 and its metabolites on the function of the skin immune system and the induction of the contact hypersensitivity (CHS) response, a population of vitamin D3-deficient BALB/c mice was established, through dietary vitamin D3 restriction and limitation of exposure to UVB irradiation. Vitamin D3 normal female mice had higher CHS responses than their male counterparts, and dietary vitamin D3 deficiency significantly increased the CHS responses in male, but not in female, mice. This change in the vitamin D3-deficient male mice was not due to an alteration in skin dendritic cell function including antigen carriage, migration or costimulatory molecule expression. In addition, 18 h after sensitisation, the lymph node populations in the vitamin D3-deficient and normal male mice showed similar proliferation and IFN- γ production. However, during the sensitisation phase of CHS, there was lower lymphocyte recruitment to the skin draining lymph nodes of the vitamin D3-deficient and normal male mice compared with their female counterparts which could account for the difference between the sexes in the extent of the CHS response. These results indicate the vitamin D system can influence cutaneous immune responses in male mice, but this did not occur through the modulation of the dendritic cell functions analysed.

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1. Introduction

The skin is the largest organ in the body and contains a specialised immune system. Ultraviolet (UV),¹ particularly UVB (280–315 nm), radiation suppresses the skin immune system via complex mechanisms [1,2]. There is production of various cytokines and mediators by keratinocytes, mast cells and other cutaneous cell types. These factors together with direct DNA damage alter antigen presentation by cutaneous dendritic cells (DCs), resulting in antigen-specific immune suppression mediated by suppressor/regulatory T cells [3].

UVB radiation of the skin also causes the conversion of 7-dehydrocholesterol to previtamin D3 within keratinocytes, which is then thermally isomerised to vitamin D3. Classically, vitamin D3 is transported to the liver where it is enzymatically hydroxylated to 25-hydroxyvitamin D3 (25(OH)D3). In the kidney a

second hydroxylation takes place to form the biologically active 1 α ,25-dihydroxyvitamin D3 (1 α ,25(OH)₂D3). 1 α ,25(OH)₂D3 exerts its actions via the vitamin D receptor (VDR), a member of the hormone nuclear receptor superfamily, and a second, yet to be identified, membrane receptor [4]. While the importance of vitamin D3 for bone health has been known for decades, only in more recent years has an immunomodulatory role for 1 α ,25(OH)₂D3 been identified. 1 α ,25(OH)₂D3 affects the differentiation and function of DCs, T cells [5] and B cells [6]. *In vitro* 1 α ,25(OH)₂D3 can alter the function of DCs by inhibiting their differentiation and maturation, which may lead to the induction of regulatory T cells or to the poor activation of antigen-specific T cells (reviewed in [7]).

There is some evidence to support a role for vitamin D3 in influencing the skin immune system. Keratinocytes and Langerhans cells (LCs), the major epidermal antigen presenting cells, have the capacity to convert vitamin D3 to 1 α ,25(OH)₂D3 following UVB radiation (reviewed in [8]) and they, dermal DCs, mast cells and T cells all express the VDR [9]. Culture of LCs *in vitro* with 1 α ,25(OH)₂D3 reduces their maturation and results in lowered allogeneic T cell proliferation [10,11]. The topical application of 1 α ,25(OH)₂D3 or an analogue of it to skin prior to the application of haptens reduces (although not totally) the contact hypersensitivity (CHS) response in humans [12] and in mice [13]. It has been proposed that 1 α ,25(OH)₂D3, produced by keratinocytes upon skin

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¹ Abbreviations used: UV, ultraviolet; CHS, contact hypersensitivity; D+, vitamin D3-replete; D–, vitamin D3-deficient; DC, dendritic cell; 1 α ,25(OH)₂D3, 1 α ,25-dihydroxyvitamin D3; FITC, fluorescein isothiocyanate; 25(OH)D3, 25-hydroxyvitamin D3; LC, Langerhans cell; VDR, vitamin D receptor; EAE, experimental allergic encephalitis.

exposure to UVB radiation, may contribute to UVB-induced immunosuppression [14].

To study the effects of the vitamin D3 endocrine system on skin DCs, we developed a mouse model of vitamin D3 deficiency. This was performed through dietary vitamin D3 restriction and limitation of exposure to UVB radiation, and was confirmed by low plasma or serum 25(OH)D3 levels. Using this model we investigated the CHS response in vitamin D3 deficiency, allowing the influences of the skin and skin draining lymph node micro-environment on DC function to be compared with those in vitamin D3-supplemented mice, considered vitamin D “replete”. We hypothesised that adequate circulating 25(OH)D3 would allow the local production of $1\alpha,25(\text{OH})_2\text{D}_3$ in the skin, thus inhibiting the migration of DCs from the skin and their maturation, and resulting in a reduction in the CHS response compared with the situation in the vitamin D3-deficient mice. The results show that dietary vitamin D3 significantly decreased the CHS response in male but not in female mice, and that the mechanism of this was not centred on perturbation of the function of the cutaneous DCs.

2. Methods

2.1. Mice

Inbred male and female BALB/c mice were obtained from the University of Tasmania Central Animal House. They were housed in rooms where plastic covers over fluorescent lighting shielded the mice from UVB exposure and/or under individual cage plastic covers. Mice were kept at 25°C with a 12 h light-dark cycle. To establish a 25(OH)D3-deficient mouse colony, BALB/c mice were weaned from mothers fed the standard wheat-based mouse diet at 3 weeks of age and placed on rice-based vitamin D3-deficient (SF05-33, Specialty Feeds, Western Australia) or control diet (Vitamin D3 2195 IU/kg)(SF05-034, Specialty Feeds, Western Australia). At 8 weeks of age, representative mice were bled from the lateral tail vein (samples were stored at –20°C until testing) and plasma or serum concentrations of 25(OH) D3 levels established (γ -B 25-hydroxy Vitamin D Radioimmunoassay IDS, USA). Vitamin D3 deficiency was classed as a plasma or serum concentration of <25 nmol/L and sufficiency >100 nmol/L. Once achieved, vitamin D3-deficient and replete breeding pairs were formed and the offspring (maintained on the same diet as their parents) used in subsequent experiments. Random sampling of experimental animals was undertaken to ensure vitamin D3 deficiency was being maintained. Mice were killed by carbon dioxide asphyxiation at the completion of experiments. All experimentation was conducted in accord with accepted standards, under the ethical approval of the University of Tasmania Animal Ethics Committee permit number A0007770/A0009250.

2.2. Contact sensitisation

Age, sex and diet matched groups (6–8 mice per group) were sensitised with 150 μ L of freshly prepared 1.5% solution (in a 4:1 acetone:olive oil) of oxazalone (Sigma Chemical, USA) on shaved dorsal skin. The CHS response was elicited 7 days later by challenged with 10 μ L of 0.25% oxazalone on each side of both ears. Prior to and at 24 h after challenge the ear thickness was measured using engineer's spring loaded micrometer (Mituyo, Japan). To measure non-specific inflammation, the animals were sensitised with 150 μ L of the solvent alone and were challenged with 0.25% oxazalone solution. The CHS was calculated by averaging the increase in ear thickness for each mouse and then taking the mean for the group. Statistical significance between mouse groups was obtained using the Student's unpaired, two tailed *t* test.

2.3. Epidermal sheet preparation and staining

Following carbon dioxide asphyxiation, epidermal sheets from dorsal skin were prepared as outlined previously [15]. To identify LCs the epidermal sheets were incubated overnight at 4°C in anti-MHCII rat antibody (TIB120 hybridoma supernatant, neat, ATCC, Manassas, VA, USA), followed by a 2 h incubation at room temperature with goat anti-rat Alexa 488 (A-11006 at 1:400, Molecular Probes, Victoria, Australia), after washing in PBS. Sheets were washed in PBS and mounted in fluorescent mounting medium (S3023; DakoCytomation, Carpinteria, CA, USA). Negative controls were prepared using the secondary antibody alone. Stained epidermal sheets were imaged using a DMBL2 fluorescence microscope (Leica, Nussloch, Germany) and images (six fields) were captured at 600 $\times$ using an Optronics Digital Camera (Chelmsford, MA, USA) with Magnafire software and analysed using Adobe Photoshop version 7.0 (Adobe, San Jose, CA, USA). The average density of LC depleted was calculated using the formula: average density of LC 18 h after vehicle application—density of LC remaining 18 h following contact sensitiser application.

2.4. Lymph node suspensions and culture

Groups of 3–4 mice were sensitised on their shaved backs with oxazalone or vehicle and the draining axillary, brachial and inguinal lymph nodes were collected 18 h later into RPMI supplemented with 10% foetal calf serum (FCS; CSL Biosciences, Melbourne, Australia), 2 mmol/L L-glutamine (JRH Biosciences, Lenexa, KS, USA), 30 μ g/mL gentamicin (David Bull Laboratories, Melbourne, Australia) and 5×10^{-5} mol/L 2- β -mercaptoethanol (Sigma, USA). The lymph nodes were pressed through a metal sieve, using a sterile syringe plunger, into a sterile petri dish. The suspension was centrifuged at 1500 rpm for 10 min at 4–7°C and the pellet resuspended in supplemented RPMI. Cells were resuspended at 2×10^6 cells/ml and 200 μ L of suspension placed in 96 well microplates (IWAKI, Funahashi, Japan), in replicates of three (for proliferation) and 24 for cell supernatant collection. Cells were incubated at 37°C in 5% CO₂ in a fully humidified atmosphere for 4 days. Each well was pulsed with 37 kBq tritiated thymidine (TRA310; Amersham Pharmacia Biotech, Tullamarine, Australia) for the last 18 h of culture before harvesting on a Combi cell Harvester (Skatron, Norway) and counted using a Rackbeta liquid scintillation counter (LKB-Wallac, Turku, Finland).

2.5. Cytokine detection in supernatants

Cell supernatants were harvested (2000 rpm, 10 min) from the lymph node cultures after 72 h. IFN- γ , IL-4, IL-10 and IL-12 levels were determined using rat anti-mouse ELISA capture and detection mAb using a standard streptavidin peroxidase protocol, StreptABC Complex/HRP (Dakocytomation, Denmark) with SureBlue TMB Microwell Peroxidase substrate (KPL, MO, USA) and capture and detection antibody for IFN- γ , IL-4, IL-10, IL-12 (BD Biosciences, New South Wales, Australia). Standard curves were prepared using known dilutions of IFN- γ (BD Biosciences). Plates were read using a SpectramaxM2 (Molecular Devices, Victoria, Australia).

2.6. FITC⁺ dendritic cell isolation, enrichment and costimulatory molecule analysis

150 μ L of 1% fluorescein isothiocyanate (FITC) dissolved in a vehicle solution of 4:1 acetone: di-*n*-butylphthalate (28073; BDH Limited, Victoria, Australia) was applied to shaved dorsal skin, and lymph node suspensions prepared as described above 18 h later. For FITC⁺ cell enrichment, dissected lymph nodes were incubated with 1:70 dilution of Liberase Blendzyme 2 (Roche Applied Sci-

Table 1

Weight, plasma/serum 25(OH)D3 and serum calcium levels in male and female BALB/c mice fed on vitamin D3-replete (D+) and deficient (D–) diets.

	Male		P value	Female		P value
	D+	D–		D+	D–	
Weight (g±SD) (age = 12 weeks)	31.0±3.4 (n=56)	30.6±3.2 (n=31)	NS	22.9±2.1 (n=55)	21.9±2.0 (n=49)	p<0.01
Plasma/serum 25(OH)D3 (nmol/L ^{−1} ±SD)	122.2±28.0 (n=8)	23.7±3.6 (n=6)	p<0.001	135.5±38.1 (n=17)	20.5±4.0 (n=18)	p<0.0001
Serum calcium (mmol/L ^{−1} ±SD)	2.59±0.3 (n=28)	2.57±0.3 (n=41)	NS	2.44±0.4 (n=66)	2.29±0.3 (n=54)	p<0.05

ences, Victoria, Australia) in RPMI for 40 min at 37 °C with slight agitation. Following washes, the cells were resuspended in MACS buffer (degassed PBS-0.1% FCS) and isolated according to the manufacturer's instructions using anti-FITC Microbeads (Miltenyi Biotec, Germany). Cells were washed and incubated with phycoerythrin-labelled anti-CD11c, or biotinylated hamster anti-mouse CD80, rat anti-mouse CD86 or CD40 or appropriate isotype controls followed by streptavidin–allophycocyanin secondary antibody (all from BD Biosciences) for 20 min at 4 °C in staining buffer (PBS-2% FCS). Labelled cells were analysed using a Coulter ELITE ESP flow cytometer (Beckman Coulter, Los Angeles, CA, USA) and CellQuest software version 3.3 (Becton Dickinson, San Jose, CA, USA). To identify the cell fraction that included DC carrying FITC to the skin draining lymph nodes the forward and side scatter characteristics of the CD11c⁺ cell populations were identified from within the FITC⁺ enriched cell fraction. The forward and side scatter gate was then used to identify the DC rich cell fraction within the whole LN suspensions.

3. Results

3.1. Physical and biochemical characteristics of vitamin D3-deficient (D–) and replete mice (D+)

Both the male and female D– mice, born from D– parents and fed on the D– diet, had greatly reduced plasma or serum 25(OH)D3 levels compared with the D+ male and female mice (Table 1). The D+ and D– male mice achieved the same body weight and serum calcium level. Although there was a small difference in body weight and serum calcium level when the D– female mice were compared with the D+ (Table 1), the D– females were physically normal and showed no evidence of bony deformity on radiological examination, and their calcium levels were within the normal range.

3.2. Vitamin D3 and sex influence the contact hypersensitivity response in BALB/c mice

The shaved backs of D– and D+ mice were treated with oxazalone and 1 week later the CHS response was elicited. Fig. 1 illustrates, first, that D+ male mice had a significantly reduced CHS response compared with D+ female mice, secondly that D– male mice had an increased CHS response compared with D+ male mice, and thirdly that D+ and D– female mice developed similar CHS responses. Thus, dietary vitamin D3 exhibits sexual dimorphism in its regulation of cutaneous immune system function. The increase in the CHS response found in some groups was not due to non-specific inflammation as no difference between groups was seen when the ears were treated with vehicle alone before challenge with oxazalone (Fig. 1).

3.3. Vitamin D3 does not alter Langerhans cell depletion in response to sensitisation

Epidermal LCs play an important role in carrying antigen to the regional skin draining lymph nodes to initiate an immune

response. D+ and D– female mice were found to have similar LC densities as were D+ and D– male mice but the D+ females had a significantly higher number than the D+ males as shown in Fig. 2a. To determine if the migration of LCs from the skin, induced by sensitisation, was altered by vitamin D3 status, the number of MHCII⁺ cells was counted in epidermal sheets 18 h after the application of oxazalone. There was no significant differences between any of the groups in the number of the LC that remained in the epidermis 18 h following contact sensitisation (Fig. 2b), however, there was a significantly greater reduction in the LC density of D+ female compared to D+ male mice (Fig. 2c). When taking into account the lower initial LC density within D+ male epidermis, vitamin D3 did not affect the relative LC depletion in either the male or the female D+ or D– mice (Fig. 2d). Thus the differences in CHS response were not secondary to differences in numbers of LC leaving the epidermis between D+ and D– male mice. However, increased LC leaving the epidermis of D+ female mice (compared to D+ male mice) may contribute to the sex differences in the CHS response in D+ mouse populations.

3.4. Vitamin D3 does not alter antigen carriage to the skin draining lymph nodes

The carriage and presentation of cutaneous antigens by DCs is a prerequisite for inducing the CHS response. To assess antigen carriage, lymph node cell suspensions were analysed 18 h after

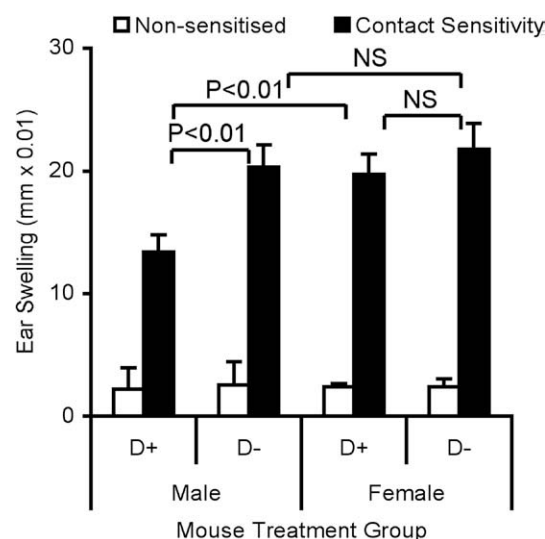


Fig. 1. Vitamin D3 levels as well as sex influence the contact hypersensitivity response. Vitamin D3-deficient (D–) and replete (D+) male and female mice were treated with oxazalone or vehicle and 1 week later the ears were challenged with 0.25% oxazalone. The CHS response (ear swelling) was measured 24 h later. The mean increase in ear thickness of sensitised groups averaged over eight separate experiments (+SEM) is shown; each group contained at least four mice. The mean (+SEM) of the inflammatory response (non-sensitised mice) averaged over 2–3 separate experiments is also shown; each group contained at least three mice. There was no significant difference in the inflammatory response between any non-sensitised group. NS=not significant.

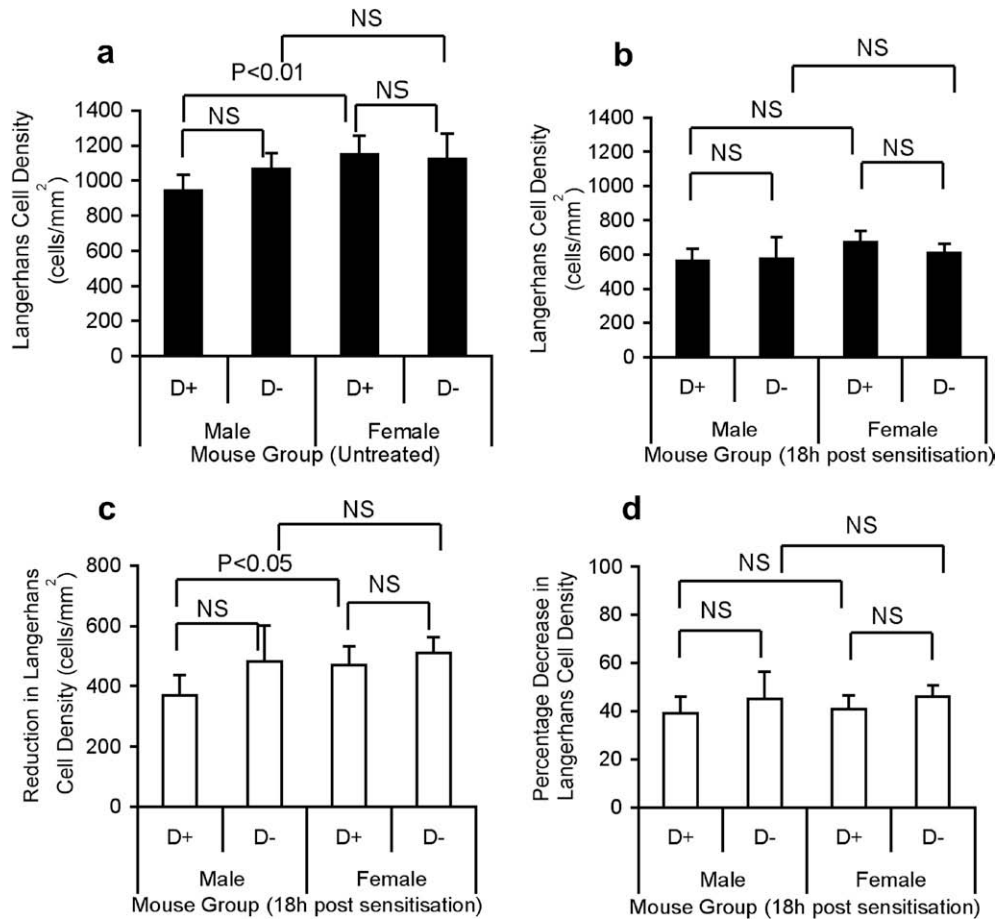


Fig. 2. Vitamin D3 does not influence oxazolone-induced Langerhans cell (LC) migration. (a) Mean LC density (+SD) in epidermal sheets from D+ and D- male and female mice, six mice per group; (b) mean LC density (+SD) in epidermal sheets 18h post oxazolone application; (c) reduction in LC density 18h post oxazolone exposure, and (d) mean percentage decrease in LC density (+SD) of oxazolone treated mice compared with vehicle (un)-treated mice 18h following treatment, six mice per group. NS=not significant.

application of the contact sensitizer, FITC (Fig. 3). Two populations of FITC⁺ cells (based on forward and side scatter) were identified which were distinguished by high (FITC^{Hi}) or low (FITC^{Lo}) fluorescent intensity. Gate R3 contained the DC population of FITC^{Hi} cells identified by CD11c⁺ in Fig. 4. Similar percentages and numbers of FITC^{Hi} and FITC^{Lo} cells, that included DCs, were present in the D+ and D- male and female mice. An analysis of the FITC^{Hi} and FITC^{Lo} cells identified by gate R3 revealed no difference in absolute numbers of whole lymph node populations when comparing D+ to D- male and female mice (data not shown). When the lymphoid population (R8) within the skin draining lymph node was analysed, less than 1% of cells were FITC^{Hi} and approximately 10% were FITC^{Lo}. There was no significant difference in the proportions of these cell populations, or their absolute number in D+ or D- male or female mice (data not shown). This indicates that vitamin D3 did not alter the numbers of cells carrying antigen or the amount of antigen reaching the skin draining lymph nodes during the sensitisation phase of the CHS response.

3.5. Vitamin D3 does not alter costimulatory molecule expression in FITC⁺CD11c⁺ DCs in the skin draining lymph nodes

A second requisite for induction of the CHS response is the appropriate costimulation of hapten-specific naïve T cells by DCs in the draining lymph node. Antigen carriage by, and costimulatory molecule expression on, enriched DCs was tested 18h after FITC

application. FITC⁺ DC populations based on forward and side scatter characteristics, as indicated in Fig. 3 (R3), were first analysed for the mouse DC marker CD11c. Two populations of FITC⁺CD11c⁺ DCs were identified, CD11c^{Hi} and CD11c^{Lo}. Fig. 4 demonstrates the antigen carriage and costimulatory molecule expression in these two subsets. FITC⁺CD11c^{Hi} DCs largely carried high amounts of antigen and no difference was found between D+ and D- male and female mice. FITC⁺CD11c^{Lo} DCs tended to have a slightly increased proportion of lower antigen-carrying DCs but there was no difference between D+ and D- male and female mice. This demonstrates, as already seen in Fig. 3, that vitamin D3 status did not alter antigen carriage by DCs in the sensitisation phase of the CHS response. Fig. 4 also shows that FITC⁺CD11c^{Hi} and CD11c^{Lo} DCs within the skin draining lymph nodes of D+ and D- male and female mice have similar levels of CD40, CD80 and CD86 expression. Thus vitamin D3 does not modify DC costimulatory molecule expression and this parameter does not account for the reduced CHS response in the D+ males.

3.6. Sex alters, but vitamin D3 does not alter, lymphocyte recruitment to the skin draining lymph node during the sensitisation phase of the CHS response

The entire lymphocyte population within lymph nodes that drain the skin influence the potential for interaction between DCs and CHS effector cells during the sensitisation phase. To determine

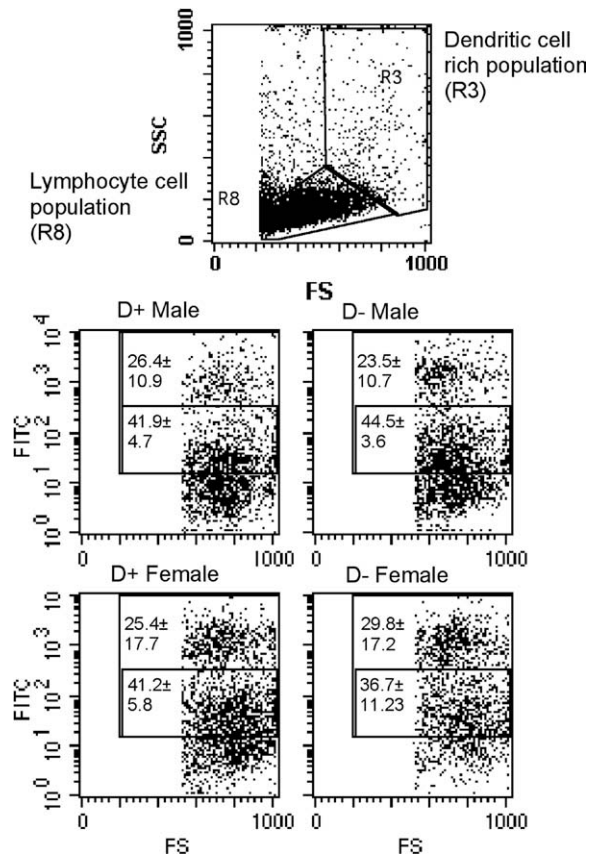


Fig. 3. Vitamin D3 does not alter antigen carriage to the skin draining lymph nodes. 18 h after treatment with FITC, the DC rich (R3) populations in the skin draining lymph nodes were analysed by flow cytometry. The dot-plot shows a representative experiment. The numbers show the average percentage of DC rich FITC⁺ cells within the category indicated by the boxes of three separate experiments ($\pm$ SD). There was no significant difference of antigen carriage identified in the DC rich population between any of the D+ and D– male and female groups.

if vitamin D3 affects the lymphocyte populations within the draining lymph nodes during sensitisation, cells were analysed 18 h after the application of oxazolone or vehicle alone (Fig. 5).

In the vehicle treated groups, there was no significant difference in total lymphocyte numbers or subsets between D+ male and female mice or between D– male and female mice. Similarly, no significant differences were noted between the lymph node populations from vehicle treated mice in the D+ when compared to the D– mice in either males or females. However, in the oxazolone treated groups, there was a significant increase in all the LN cell populations (CD4⁺, CD4⁺CD25⁺, CD8⁺ and B220⁺) in D+ female compared with D+ male mice, and a significant increase in CD4⁺ and CD8⁺ lymphocyte numbers in D– female mice compared with D– male mice. No significant differences were noted between the lymph node populations from oxazolone treated mice in the D+ when compared to the D– mice in either males or females.

3.7. Vitamin D3 does not alter cellular proliferation or IFN- γ production in the skin draining lymph nodes during the sensitisation phase of CHS

Following topical treatment with vehicle or oxazolone, the draining lymph nodes were removed and cultured for 72 or 96 h. As tested at 96 h, there was no significant difference in the proliferation following vehicle or oxazolone application between D+ and D– male and female mice (Fig. 6a). Cell supernatants harvested at 72 h were assessed for IFN- γ , IL-12, IL-10 and IL-4. Only IFN- γ

was identified within the culture supernatants. No difference was found in IFN- γ production between D+ and D– male or D+ and D– female mice, although D+ females showed a tendency for higher IFN- γ levels than D+ males but this was not statistically significant (Fig. 6b).

4. Discussion

In vitro findings that $1\alpha,25(\text{OH})_2\text{D}_3$ alters DC function have led to the hypothesis that vitamin D modulates the immune system by influencing the DCs. One such study isolated LCs from $\text{VDR}^{-/-}$ and wild type mice and compared their maturation on culture with $1\alpha,25(\text{OH})_2\text{D}_3$ [10]. The up-regulation of CD40, CD80 and CD 86 in LCs from wild type mice was inhibited whereas LCs isolated from $\text{VDR}^{-/-}$ mice matured normally, indicating signalling through the VDR could affect LC maturation. Consequently the VDR was proposed to play an important role as signalling via this receptor *in vitro* inhibited LC maturation, thus correlating with a reduced capacity to stimulate an allogeneic T cell response [10]. In animal models of autoimmune disease, supplementation with vitamin D3, $1\alpha,25(\text{OH})_2\text{D}_3$ or its analogues leads to a significant reduction in autoimmune disease incidence/severity (reviewed in [16]). Despite such observations, DCs have yet to be shown *in vivo* as mediators of the immunomodulatory effects of the vitamin D endocrine system in autoimmune disease models. To determine whether the vitamin D3 endocrine system influences DC function *in vivo* we utilised CHS, and compared skin DCs from vitamin D3-deficient mice with those isolated from vitamin D3-supplemented (normal) mice. The results of these investigations show that the vitamin D3 endocrine system acts to regulate the CHS response in male but not female mice, and the mechanism was not by modulation of DC function.

An initiating step in the sensitisation phase of CHS is the LC-mediated antigen carriage from the skin to the draining lymph nodes. The signals for LC migration include the production of cytokines by keratinocytes as well as the expression of lymphoid homing receptors, particularly CCR7, by skin DCs, which culminate in chemotaxis of LC to the draining lymph nodes. In an *in vitro* study the chemotaxis of LCs towards CCL21 (the ligand for CCR7, expressed in the high endothelial venules of the lymph node) is reduced by 50% in the presence of $1\alpha,25(\text{OH})_2\text{D}_3$ [11]. In the present study LC migration from antigen treated skin was similar in normal and vitamin D3-deficient mice. As $1\alpha,25(\text{OH})_2\text{D}_3$ *in vitro* can stimulate apoptosis of LCs [11] and other DC subsets, it was important to ascertain that antigen-carrying DCs were reaching the draining lymph nodes and not undergoing apoptosis in the skin. Employing the fluorescent sensitiser, FITC, to track the antigen carriage, similar numbers of FITC⁺ DCs and lymphoid cells carrying similar amounts of FITC were identified in the lymph nodes. A proportion of FITC^{Lo} cells are believed to be the B220⁺ cells identified by Matsumura et al. (2006) [17]. When further assessed, by enriching for FITC⁺CD11c⁺ DCs, similar levels of CD80, CD86 and CD40 expression were identified within the vitamin D3-deficient and normal mice.

The results presented in this study differ from *in vitro* work assessing the influence of $1\alpha,25(\text{OH})_2\text{D}_3$ on DC function. *In vitro* models have largely used monocyte-derived DC rather than the DCs present within non-lymphoid tissues. $1\alpha,25(\text{OH})_2\text{D}_3$ can act directly and indirectly on DCs via the modulation of epithelial and mesenchymal cells in the local tissue microenvironment. These indirect actions of $1\alpha,25(\text{OH})_2\text{D}_3$ on DC function are difficult to assess in the skin immune system. For example, $1\alpha,25(\text{OH})_2\text{D}_3$ reduces keratinocyte production of granulocyte macrophage colony stimulation factor *in vitro*, resulting in reduced LC maturation [18]. Vitamin D3-induced alterations in mediators produced by keratinocytes may also exist *in vivo*, and would not be identified *in vitro*.

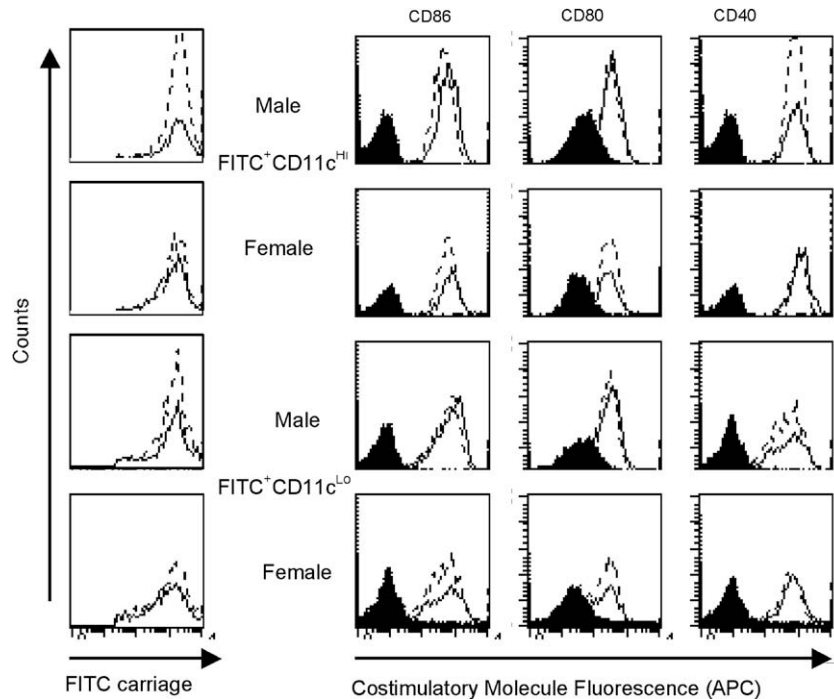


Fig. 4. Vitamin D3 does not influence the costimulatory molecule expression on or antigen carriage by FITC⁺ CD11c⁺ dendritic cells (DCs) in the skin draining lymph nodes. FITC⁺ cells were purified from the skin draining lymph nodes 18 h following the topical application of FITC. FITC⁺DC were gated based on FS characteristics and CD11c⁺ (Hi or Lo) expression and their costimulatory molecule expression was analysed using three-colour flow cytometry. Solid filled lines represents the isotype control. Solid line represents cells from D+ mice and hashed line cells from D- mice. No significant difference in the level of costimulatory molecule expression of FITC⁺CD11c^{Hi} or CD11c^{Lo}DC between D+ and D- male and female mice was found. Results are representative of three separate experiments.

A major finding of the present study was that the vitamin D3 endocrine system regulates cutaneous immune responses in male, but not in female, mice. This sexual dimorphism was not secondary to alterations in DC function *in vivo* between normal male and female mice. Nor was it due to a higher level of 25(OH)D3 in females

when compared to males. The metabolism of 1 α ,25(OH)₂D3 by immune cells may be altered by local cytokine and sex steroid levels, an interaction that may not be detected *in vitro*. This phenomenon has been demonstrated in an animal model of multiple sclerosis, experimental autoimmune encephalitis (EAE). Dietary

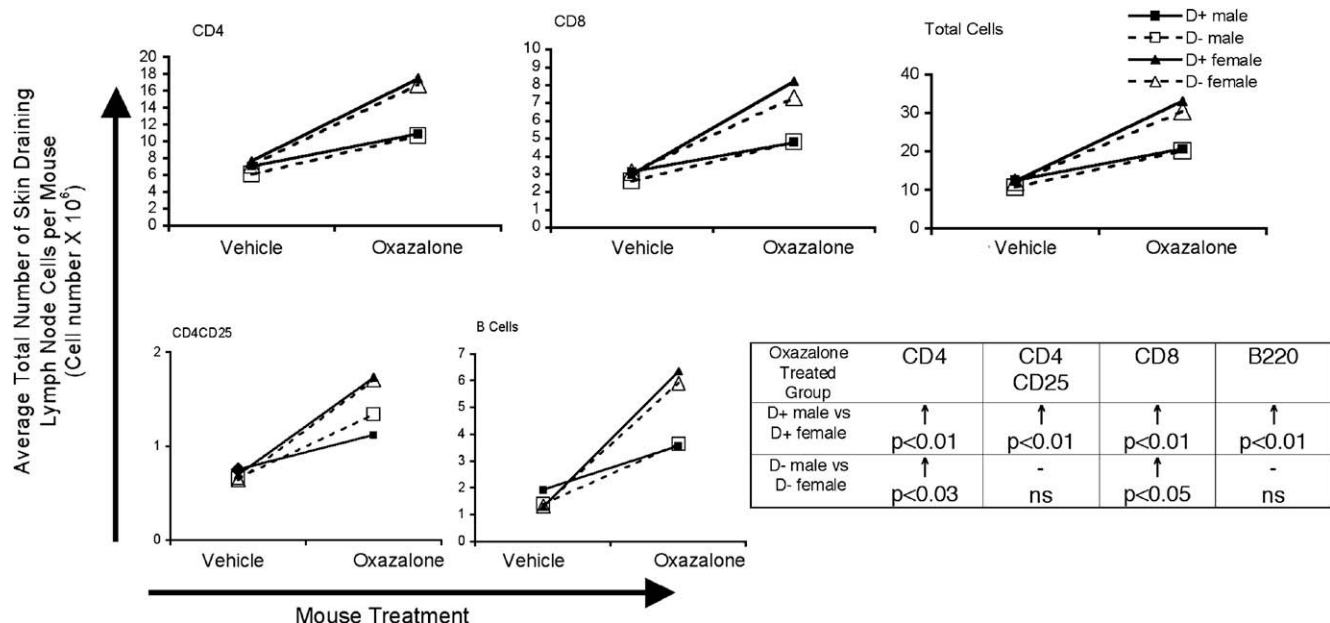


Fig. 5. Vitamin D3 does not alter lymph node populations during the sensitisation phase of the contact hypersensitivity response but sex does. 18 h after skin exposure to vehicle or oxazalone, lymph node cell populations were analysed by flow cytometry. There was no significant difference between D+ and D- female and male mice in the vehicle treated groups. There was a significant increase in all lymph node cell populations (indicated in the table) in D+ female compared with D+ male mice in the oxazalone treated groups, and a significant increase in CD4 and CD8 lymph node populations in D- female mice compared with D- male mice in the oxazalone treated groups. Each point represents the average number of cells/mouse in the skin draining lymph nodes. SD for all experiments was not greater than 30%. Results represent a minimum of 3 experiments.

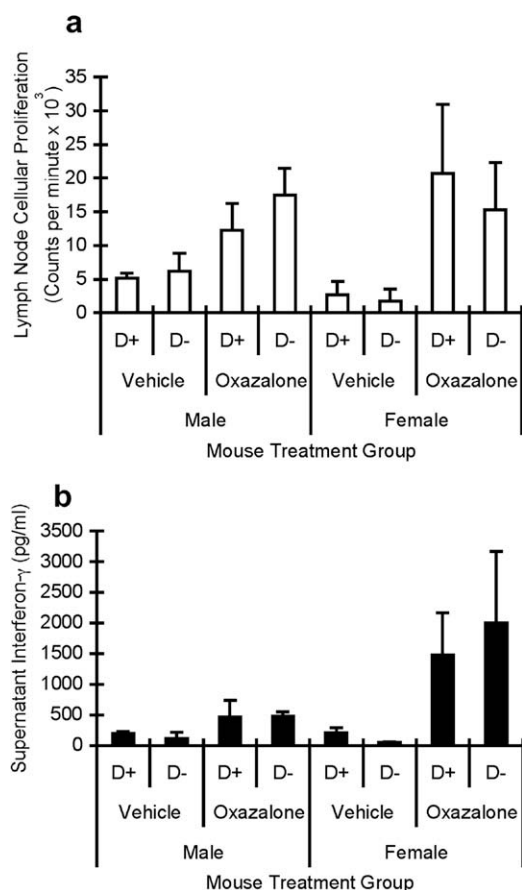


Fig. 6. Vitamin D3 does not alter lymph node cell proliferation or IFN- γ production following contact sensitisation. Mice were treated with vehicle or oxazalone and 18 h later lymph nodes were removed and cultured. No significant differences in (a) proliferation or (b) IFN- γ production between D+ and D- male and female mice were found. Results show the mean ($\pm$ SD) of 3–4 separate experiments.

vitamin D3 protected female, but not male, mice from developing EAE due to the higher macrophage production of $1\alpha,25(\text{OH})_2\text{D}_3$ in the central nervous system (CNS) of female mice. The increased $1\alpha,25(\text{OH})_2\text{D}_3$ production was a consequence of higher IFN- γ production within the CNS, which inhibited the induction of the 24-hydroxylase responsible for inactivating $1\alpha,25(\text{OH})_2\text{D}_3$ [19]. We demonstrated that females had increased (though not significantly) IFN- γ production in cultured lymph node suspensions from normal and vitamin D3-deficient mice compared with their male counterparts. Female mice, where there is no difference in the CHS response, had no differences in cellular proliferation or IFN- γ production. In contrast, despite a difference in the CHS between normal and vitamin D3-deficient male mice, there was no difference in IFN- γ or cellular proliferation. This suggests that differences in the contact hypersensitivity response between normal and vitamin D3-deficient male mice were not due to alterations in IFN- γ production or cellular proliferation. However, higher levels of IFN- γ may be achieved in the local lymphocyte environment, particularly in females, and this, in turn, may alter local $1\alpha,25(\text{OH})_2\text{D}_3$ production.

The CHS response is a measure of both the sensitisation and elicitation phases of an immune response. If the vitamin D3 endocrine system is not acting on the DC during the sensitisation phase, it may be acting on effector cells/regulatory cell development and their recruitment to the elicitation site. The reduction in the CHS response in normal males, compared with normal females, may reflect different contributions of $1\alpha,25(\text{OH})_2\text{D}_3$ to

humoral and T cell components of the CHS response. Our study demonstrates that normal females have significantly increased numbers of B cells when compared to normal male mice 18 h following sensitisation. Similar increases in B cell numbers (although not statistically significant) were also demonstrated in vitamin D3-deficient females when compared to vitamin D3-deficient males. Females tend to have stronger humoral immune responses than males [20,21]. It appears that oestrogen and the vitamin D3 endocrine system may contribute to greater humoral immune responses. These differences in B cell numbers are likely to reflect lymphocyte recruitment as lymph node cell proliferation *in vitro* was not increased in female compared with male mice. As $1\alpha,25(\text{OH})_2\text{D}_3$ is believed to suppress Th1 immune responses and promote regulatory T cell development, rather than suppress humoral immune responses, this could partially explain why the female CHS response remained high while the males was suppressed.

We found that $\text{CD4}^+\text{CD25}^+$ cell induction in the sensitisation phase was significantly higher in normal females when compared with that in males, whereas no differences were seen in the vitamin D3-deficient males when compared with vitamin D3-deficient females. A subset of these cells serve to regulate the CHS at the effector phase by limiting the recruitment of inflammatory T cells to the elicitation site [22]. More activated T cells, rather than regulatory T cells, may be present in the normal females than in males. As the numbers of $\text{CD4}^+\text{CD25}^+$ T cells in deficient female mice were not greater than their male counterparts, it implies that vitamin D and oestrogen contribute to the development of this regulatory population during cutaneous immune responses. Further investigations to elucidate the sex differences in proportion and function of $\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$ regulatory T cells will help to resolve this issue.

While an effect on DC function, particularly cytokine production, by the vitamin D3 endocrine system has not been fully excluded in this study, the *in vivo* involvement of DCs in modulating the CHS response has not identified any differences in migration, antigen carriage or costimulatory molecule expression, all demonstrated *in vitro*. Further investigations of how the vitamin D3 endocrine system modulates the immune system *in vivo* are in progress, in particular to explain why males and females respond differently to UVB-induced immunosuppression and autoimmune disease development.

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References

- [1] M.L. Kripke, Immunological unresponsiveness induced by ultraviolet radiation, *Immunol. Rev.* 80 (1984) 87.
- [2] M. Kripke, Immunological mechanisms in UV radiation carcinogenesis, *Adv. Cancer. Res.* 34 (1981) 69–106.
- [3] T. Schwarz, Mechanisms of UV-induced immunosuppression, *Keio J. Med.* 54 (2005) 165–171.
- [4] A.W. Norman, S. Ishizuka, W.H. Okamura, Ligands for the vitamin D endocrine system: different shapes function as agonists and antagonists for genomic and rapid response receptors or as a ligand for the plasma vitamin D binding protein, *J. Steroid Biochem. Mol. Biol.* 76 (2001) 49–59.
- [5] C.M. Veldman, M.T. Cantorna, H.F. DeLuca, Expression of 1, 25-dihydroxyvitamin D(3) receptor in the immune system, *Arch. Biochem. Biophys.* 374 (2000) 334–338.

- [6] J.W. Morgan, N. Kouttab, D. Ford, A.L. Maizel, Vitamin D-mediated gene regulation in phenotypically defined human B cell subpopulations, *Endocrinology* 141 (2000) 3225–3234.
- [7] E. May, K. Asadullah, U. Zugel, Immunoregulation Through 1, 25-dihydroxyvitamin D3 and its Analogs, *Curr. Drug Targets Inflamm. Allergy* 3 (2004) 377–393.
- [8] B. Lehmann, K. Querings, J. Reichrath, Vitamin D and skin: new aspects for dermatology, *Exp. Dermatol.* 13 (Suppl. 4) (2004) 11–15.
- [9] B. Lehmann, W. Sauter, P. Knuschke, S. Drebler, M. Meurer, Demonstration of UVB-induced synthesis of 1 α ,25-dihydroxyvitamin D3 (calcitriol) in human skin by microdialysis, *Arch. Dermatol. Res.* 295 (2003) 24–28.
- [10] S. Meindl, A. Rot, W. Hoetzenecker, S. Kato, H.S. Cross, A. Elbe-Burger, Vitamin D receptor ablation alters skin architecture and homeostasis of dendritic epidermal T cells, *Br. J. Dermatol.* 152 (2005) 231–241.
- [11] H. Fujita, A. Asahina, M. Komine, K. Tamaki, The direct action of 1 α , 25(OH) $_2$ -vitamin D3 on purified mouse Langerhans cells, *Cell. Immunol.* 245 (2007) 70–79.
- [12] K.K. Hanneman, H.M. Scull, K.D. Cooper, E.D. Baron, Effect of topical vitamin D analogue on in vivo contact sensitization, *Arch. Dermatol.* 142 (2006) 1332–1334.
- [13] Z. Guo, H. Okamoto, S. Imamura, The effect of 1, 25(OH) $_2$ -vitamin D3 on Langerhans cells and contact hypersensitivity in mice, *Arch. Dermatol. Res.* 284 (1992) 368–370.
- [14] J. Reichrath, G. Rappl, Ultraviolet light (UV)-induced immunosuppression: is vitamin D the missing link?, *J. Cell. Biochem.* 89 (2003) 6–8.
- [15] H. McGee, D.K. Scott, G.M. Woods, Neonatal exposure to UVB radiation leads to a large reduction in Langerhans cell density, but by maturity, there is an enhanced ability of dendritic cells to stimulate T cells, *Immunol. Cell. Biol.* 84 (2006) 259–266.
- [16] L. Adorini, Intervention in autoimmunity: the potential of vitamin D receptor agonists, *Cell. Immunol.* 233 (2005) 115–124.
- [17] Y. Matsumura, S.N. Byrne, D.X. Nghiem, Y. Miyahara, S.E. Ullrich, A role for inflammatory mediators in the induction of immunoregulatory B cells, *J. Immunol.* 177 (2006) 4810–4817.
- [18] A. Kowitz, M. Greiner, R. Thieroff-Ekerdt, Inhibitory effect of 1 α , 25-dihydroxyvitamin D3 on allogeneic lymphocyte stimulation and Langerhans cell maturation, *Arch. Dermatol. Res.* 290 (1998) 540–546.
- [19] K.M. Spach, C.E. Hayes, Vitamin D3 confers protection from autoimmune encephalomyelitis only in female mice, *J. Immunol.* 175 (2005) 4119–4126.
- [20] T.J. Lang, Estrogen as an immunomodulator, *Clin. Immunol.* 113 (2004) 224–230.
- [21] N. Gleicher, D.H. Barad, Gender as risk factor for autoimmune diseases, *J. Autoimmun.* 28 (2007) 1–6.
- [22] S. Ring, S.C. Schafer, K. Mahnke, H.A. Lehr, A.H. Enk, CD4 $^+$ CD25 $^+$ regulatory T cells suppress contact hypersensitivity reactions by blocking influx of effector T cells into inflamed tissue, *Eur. J. Immunol.* 36 (2006) 2981–2992.