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The current state of play of rodent models to study the role of vitamin D in UV-induced immunomodulation.

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Title: The current state of play of rodent models to study the role of vitamin D in UV-induced immunomodulation.

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Abbreviations:

1,25(OH)₂D₃ = 1,25-dihydroxyvitamin D

25(OH)D₃ = 25-hydroxyvitamin D

DC = dendritic cell

PGE₂ = prostaglandin E₂

UVR = ultraviolet radiation

VDD = vitamin D deficiency

VDR = vitamin D receptor

Abstract

Ultraviolet radiation (UVR) from sunlight is immunomodulatory and the main source of vitamin D for humans. Vitamin D can also regulate adaptive immunity, through mechanisms that involve the induction or activation of regulatory T cells. Similar mechanisms have also been proposed for the induction of regulatory T cells after skin exposure to UVR. Here we discuss the converging and diverging immunoregulatory pathways of UVR and vitamin D, including the molecular pathways for regulatory T cell induction, non-genomic pathways regulated by vitamin D, antimicrobial peptides, skin integrity and potential interactions between vitamin D and other UVR-induced mediators. We then discuss potential *in vivo* approaches that could be used to demonstrate a direct (or otherwise) role for vitamin D in mediating the immunosuppressive effects of UVR such as the use of dietary vitamin D restriction to induce vitamin D deficiency, gene knockout mice or drugs to block enzymes of vitamin D metabolism. We end with discussion of the epigenetic effects of vitamin D and UVR for immunosuppression.

1 Introduction

Ultraviolet radiation (UVR), a component of sunlight, has many potent effects on human health.¹ UVR is immunomodulatory, such that skin exposure may perturb the development of immune-driven disorders like multiple sclerosis² and allergic asthma.³ Exposure to UVR is also the primary means by which humans obtain vitamin D.⁴ Vitamin D is essential for the correct regulation of calcium throughout our bodies, and is also integral for the control of immunity.⁵ Sufficient vitamin D status is linked with reduced severity of many disorders in

which over-zealous immune responses and inflammation contribute towards disease pathogenesis.^{1,5} There is much interest in the potential for vitamin D supplementation to reduce the burden of allergic, autoimmune, cardiovascular and carcinogenic disorders. Many trials are currently underway to assess the efficacy of vitamin D supplementation in reducing disease burden (>400, clinicaltrials.gov, using vitamin D and supplementation as key words). However, the results of many of the trials performed to date have been disappointing, and have not shown a clear positive effect for vitamin D supplementation.^{6,7}

2 UV-induced immunomodulation and vitamin D

UVR induces a suite of immune mediators (aside from vitamin D) that can suppress immune responses.⁵ There is new evidence that sun exposure and vitamin D may be independent risk factors for the development of diseases like multiple sclerosis.² The lack of success of vitamin D supplementation in clinical trials may indicate that vitamin D is a biomarker for recent sunlight exposure. The ability of UVR to regulate immunity independently of vitamin D production seems to be a factor largely ignored by many working on the effects of vitamin D on immunity. In addition, it is not known whether vitamin D is directly responsible for UVR-induced immunoregulation. Here, we discuss the potential use of rodent models to assess the importance of vitamin D in UV-induced immunomodulation.

UV-induced immunomodulation was first recognised by Margaret Kripke in 1974.⁹ She reported that UVR suppressed anti-tumour immune responses to enable the development of skin tumours. The cellular and molecular pathways through which UVR regulates immunity are still incompletely characterised, but involve the release of soluble products by skin cells like keratinocytes and mast cells, altered antigen presenting cell precursors at distant sites like the bone marrow and the induction of regulatory T and B cells (for review⁵). Soluble

mediators include interleukin-6 (IL-6), IL-10, nerve growth factor, platelet activating factor, prostaglandin E₂ (PGE₂), tumour necrosis factor and *cis*-urocanic acid.⁵ Another potential immune regulator induced by UVR is vitamin D. For humans, much of our vitamin D is obtained from processes subsequent to the absorption of UVB photons by 7-dehydrocholesterol in skin.⁴ The active form of vitamin D, 1,25(OH)₂vitamin D (1,25(OH)₂D), is then produced both locally by keratinocytes¹⁰ and systemically by sequential hydroxylations in the liver and kidney. Post-UV irradiation (or dietary intake), serum levels of the metabolite 25-hydroxyvitamin D (25(OH)D) are used as a measure of vitamin D status.

3 Converging and diverging pathways by which vitamin D and UVR regulate immunity

Here we discuss the current evidence for the role of vitamin D in mediating (or otherwise) the effects of UVR on immunity.

3.1 RANKL expression in keratinocytes and regulatory T cells

There is significant indirect evidence to support a role for vitamin D in UVR-induced immunomodulation. Indeed, UVR and vitamin D activate similar cellular and molecular pathways to regulate immunity. Central to the effects of UVR and vitamin D on immunity are dendritic cells (DCs), which are important sentinel cells that present newly-acquired antigen to T cells to establish acquired immunity. UVR and vitamin D both induce a tolerogenic phenotype in DCs, which then control the induction of regulatory T cells. Exposure of skin-resident DCs to UVR damages their DNA,^{11,12} and causes their migration into the skin-draining lymph nodes.¹² These DCs then increase the suppressive activity and/or the numbers of lymph node-resident CD4+CD25+Foxp3+ regulatory T cells.¹²⁻¹⁴ Similarly, topically-applied 1,25(OH)₂D induces skin DCs to migrate to the draining lymph nodes to control regulatory T cells.^{15,16} RANKL (receptor activator of NFκB ligand) is enhanced in

keratinocytes by UVR¹⁷ or after topical application of a vitamin D analogue (calcipotriol),¹⁵ which then causes an expansion of antigen-specific regulatory T cells. These observations support a model of UVR-induced immunosuppression that is mediated through 1,25(OH)₂D regulation of RANKL in keratinocytes with downstream effects on DCs and regulatory T cells. Increased numbers of DEC-205+ DCs have been detected in the skin-draining lymph nodes, 1-2 days post UVR¹⁸ or topical 1,25(OH)₂D treatment¹⁶, and DEC-205 expression by DCs may be important for augmenting the suppressive capacity or numbers of lymph node-resident regulatory T cells.¹⁹ Induction of regulatory T cells and epidermal RANKL expression by UVR is also dependent upon expression of the vitamin D receptor (VDR).¹⁵ Other immune modulators induced by UVR may also regulate the RANKL pathway. Indeed, after erythral UVR, PGE₂ signals through prostaglandin E receptor subtype 4 (EP4) to increase regulatory T cell numbers by enhancing epidermal RANKL expression.¹⁸ In these studies, an EP4 antagonist prevented UVR-induced immunosuppression by inhibiting UV-induced increases in CD4+Foxp3+ regulatory T cell and DEC-205+ DC numbers and was associated with reduced expression of RANKL in the epidermis.¹⁸ These observations indicate that UVR may modulate immunity by inducing the expression of multiple molecules (i.e.; PGE₂ and 1,25(OH)₂D) which could redundantly modify regulatory T cell activities through the same cellular pathway (see Figure 1).

3.2 Non-genomic pathways regulated by vitamin D

1,25(OH)₂D interacts with the VDR in the nucleus to exert many of its biological effects.⁴ The VDR is then phosphorylated and heterodimerizes with the retinoid X receptor, and binds to vitamin D response elements to regulate the transcription of vitamin D-responsive genes. A non-genomic 'rapid response' pathway also exists, mediated by a variety of receptor types located near or associated with the plasma membrane.²⁰ This pathway is important for the

photoprotective effects of vitamin D compounds in reducing UVR-induced immunosuppression, and skin carcinogenesis in hairless mice (*Skh:hr1*).^{21,22} A *cis*-locked analogue of 1,25(OH)₂D (1 α ,25-dihydroxy-luminsterol, JN), which is only capable of non-genomic responses was as effective as 1,25(OH)₂D in protecting against UV-induced immunosuppression in these mice.²¹ In contrast, in humans and hirsute (hairy) mice, topical 1,25(OH)₂D is immunosuppressive (reviewed in²³). We propose that there may be a fundamental difference in receptor configurations in mouse strains and the signalling induced upon 1,25(OH)₂D binding to the alternate receptors may explain the different observations of photoprotection²¹ and immunosuppression for topical 1,25(OH)₂D.²³ The alopecia observed in both *Skh:hr1* and VDR^{-/-} mice (see below) may indicate that VDR expression is dysregulated in *Skh:hr1* mice. Indeed, the hairless protein (hr1) directly interacts with the VDR to repress VDR-mediated transcription.²⁴ The opposing effects of 1,25(OH)₂D may be explained if the non-genomic pathway is responsible for photoprotection, and the genomic pathway is responsible for immunosuppression.²⁵ In our studies in which topical 1,25(OH)₂D was applied directly after UVR of the shaved dorsal skin of BALB/c mouse, 1,25(OH)₂D and UVR had additive effects to suppress contact hypersensitivity responses.²³ This observation suggests that UVR and topical 1,25(OH)₂D may act through different pathways to suppress immunity.

3.3 Antimicrobial peptides

Antimicrobial peptide (AMP) expression is regulated in skin exposed to UVR or treated topically with vitamin D. AMPs are a family of small molecules, which classically exhibit antimicrobial activity, but have other functions, such as inducing chemotaxis of immune cells.

²⁶ Two principal families of AMPs exist, the cathelicidins and the defensins. AMPs, including the cathelicidin peptide LL-37 and β -defensin-2 (BD-2), enhance microbe killing through disruption of bacterial and viral membranes and can also activate other anti-microbial

pathways within infected cells.²⁷ Infecting microbes initiate AMP induction through a complex pathway, whereby toll-like receptor (TLR) signaling induces 1,25(OH)₂D production. Active vitamin D then signals through the VDR to further amplify AMP synthesis.²⁸ The mouse homologue of the LL37 is the cathelin-related antimicrobial peptide (CRAMP). The murine VDR lacks a vitamin D response element required for CRAMP induction.²⁹ Interestingly, AMP expression has been examined in the skin of hairless mice that received 3 daily doses of sub-erythral UVR. Increased mBD-2, mBD-3 and CRAMP were detected in the skin 24 h after the final irradiation.³⁰ Blocking cutaneous 1,25(OH)₂D synthesis by using a chemical inhibitor, ketoconazole immediately post-UVR prevented the UVR-induction of AMP expression, including CRAMP, in skin.³⁰ Topical 1,25(OH)₂D also enhances dermal AMP expression downregulated by corticosteroid treatment.³¹ These results suggest that 1,25(OH)₂D may indirectly increase CRAMP expression. An indirect mediator of this pathway may include dermal mast cells, that can be activated by 1,25(OH)₂D to release immunomodulators like IL-10.³² Mast cells can also be stimulated to produce CRAMP.^{33,34} A VDR-independent pathway that regulates CRAMP also exists, in which endoplasmic reticulum stress signaling activates NF-KB pathways.³⁵ These results indicate that in mice, UVR-induced 1,25(OH)₂D indirectly affects AMP production to regulate innate immune responses to potentially control skin infections.

3.4 Skin integrity

Maintenance of skin integrity is vital for the prevention of infection. Low-dose UVR is used to treat atopic dermatitis to improve defective permeability barriers in affected skin. UVR improved skin integrity (in naïve mice) through a vitamin D-dependent pathway, where ketoconazole treatment immediately post-UVR reversed the benefits of low-dose UVR on epidermal permeability barrier function.³⁰ Similar studies with topical 1,25(OH)₂D on

reduced epidermal permeability induced by corticosteroids also show a protective effect of vitamin D on skin integrity.³¹ Thus at low doses, UVR induces 1,25(OH)₂D to maintain skin integrity.

3.5 Potential interactions between vitamin D and other UVR-induced mediators

One mediator induced by UVR implicated in immunosuppression is platelet activating factor (PAF).^{2,36} While both vitamin D and PAF may contribute towards UVR-induced immunosuppression, they have opposing effects on the formation of DNA damage in UV-irradiated skin.³⁶ PAF and other UVR-induced molecules like *cis*-urocanic acid contribute towards DNA damage (eg. cyclobutane pyrimidine dimers) and ROS formation.³⁷ In contrast, 1,25(OH)₂D can repair UVR-induced CPD formation if administered topically before and/or after UV treatment.^{14,21,38} Other studies suggest that vitamin D may induce the expression of PAF to regulate immunity. In human decidual macrophages, 1,25(OH)₂D (≥1 nM) or 25(OH)D (≥100 nM) reduced the enzymatic activity of the platelet activating factor (PAF) deactivation enzyme, PAF-acetylhydroxylase.³⁹ In addition, expression of the PAF-receptor was increased in NOD (non-obese diabetic) mice treated with 1,25(OH)₂D (5 µg/kg ip, every 2 days), in which severe shock was induced by foot-pad injection of hen egg white lysozyme.⁴⁰ Whether UVR-induced 1,25(OH)₂D contributes towards PAF formation has yet to be shown.

4 Rodent models to examine whether vitamin D is responsible for UV-induced immunosuppression

While there is indirect evidence that UVR may suppress immunity at least partially through vitamin D, direct evidence is lacking. Here we discuss possible strategies using rodent models

for assessing whether vitamin D may be a mediator of UVR-induced immunosuppression.

4.1 Dietary vitamin D restriction to induce vitamin D deficiency

A physiologically-relevant approach could be to exclude vitamin D from the diets of rodents to effectively reduce serum 25(OH)D levels to those equivalent to clinical deficiency in humans (<25 nmol/L). In our studies, serum 25(OH)D levels fall to <20 nmol/L four weeks after vitamin D is first removed from the diet of mice.^{41,42} One important factor to consider is the potential effects of vitamin D deficiency (VDD) on calcium. Indeed, controlling for VDD-induced hypocalcemia is important as the severity of inflammatory bowel disease and multiple sclerosis observed in murine models has been attributed to the hypocalcemia induced by vitamin D deficiency.⁴³⁻⁴⁵ In models where serum calcium did not change with VDD (Table 1), a vitamin D-null diet increased the disease severity^{42,46,47} with the exception of mice with autoimmune encephalomyelitis (EAE, a murine model of multiple sclerosis)⁴⁸ (Table 1). For assays of contact hypersensitivity, VDD enhanced responses in male but not female mice with no difference in serum calcium levels observed in the male mice.⁴⁹ We are currently using this approach of dietary-induced VDD to determine whether vitamin D contributes towards the capacity of UVR to suppress immune responses in mice.

Experimental mouse models could also be used to determine whether dietary vitamin D supplementation has the same effects as UVR on systemic immunity. UVR suppressed EAE when there were minimal changes in serum 25(OH)D, suggesting that vitamin D may not be a central mediator of UVR-induced systemic immunosuppression.⁵⁰ However, it is likely that serum 25(OH)D levels were already optimal as these mice had initial levels of ≥ 50 ng/mL 25(OH)D (≥ 125 nmol/L) prior to UVR exposure, which in humans would be indicative of the upper range of vitamin D sufficiency (>75 nmol/L).⁵ We anticipate that experimental

approaches will be more informative if mice are vitamin D-deficient before experimentation such that more robust correlations could be measured between increases in 25(OH)D by UVB exposure and alterations to immune outcomes.⁵ In similar experiments, up to 1000 µg/kg 25(OH)D significantly delayed the onset and severity of EAE in hypercalcemic mice, where the degree of suppression was comparable with that observed for chronic UVR, but significantly less than observed in mice administered 2.5 µg/kg 1,25(OH)₂D.⁵⁰

4.2 Gene knockout mice

An alternative methodology could be to use knockout mice that cannot respond to, or cannot make or breakdown 1,25(OH)₂D through ablating expression of the VDR, or hydroxylating enzymes that synthesise or breakdown 1,25(OH)₂D, the 1α-hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1), respectively.

4.2.1 VDR^{-/-} mice

The VDR is expressed by many cells in the skin, the immune system and in various tissues throughout the body. As a result, a loss of VDR expression has catastrophic effects, and there are many problems associated with using mice with the VDR knocked out globally, or even in a tissue-specific manner. VDR^{-/-} mice have serious developmental problems that lead to dermatological, skeletal, reproductive and immune dysfunction.⁴ For adequate bone development and survival, VDR^{-/-} mice need to be fed a rescue diet consisting of 2% calcium and 20% lactose,⁵¹ which may induce hypercalcaemia. VDR^{-/-} mice also have alopecia and exhibit keratinocyte stem cell defects.⁴ This alopecia is independent of 1,25(OH)₂D (ligand) expression and we anticipate that these ligand-independent effects could still occur in the skin of mice with keratinocyte-specific knockdown of expression of the VDR. Other studies indicate that the VDR acts as a tumour suppressor independently of 1,25(OH)₂D. VDR^{-/-} mice

exhibit reduced rates of thymine-dimer repair and apoptosis in UV-exposed skin and these dysfunctional epidermal repair pathways coincide with an enhanced susceptibility to the development of skin tumours induced by chronic UV irradiation or by a chemical carcinogen.⁵² The effects of dietary-induced VDD are not always reproduced in $VDR^{-/-}$ mice (see Table 1). For example, a reduced asthma phenotype occurs in $VDR^{-/-}$ mice⁵³ while allergic sensitisation is enhanced in VDD mice.⁴² Similarly, in non-obese diabetic mice, increased diabetes was observed in VDD⁴⁷ but not $VDR^{-/-}$ mice.⁵⁴ These observations highlight how mice with a global knockout in VDR gene expression are not appropriate to use to investigate whether vitamin D contributes towards UVR-induced immunomodulation. In other studies vitamin D or VDR deficiency can have the same effects; increased disease severity was observed in VDD or $VDR^{-/-}$ mice with experimental arthritis^{55,56} and colitis^{46,57-59}, while reduced EAE disease severity observed in VDD⁴⁸ or $VDR^{-/-}$ mice⁶¹ (Table 1).

4.2.2 *CYP27B1^{-/-} mice*

Another option could be to use mice with a global deficiency in the expression of the enzyme responsible for hydroxylating 25(OH)D to active 1,25(OH)₂D (CYP27B1). However, like the $VDR^{-/-}$ mice, $CYP27B1^{-/-}$ mice also have serious developmental problems⁴ and need to be fed a rescue diet for adequate bone development and fertility.⁶² $CYP27B1^{-/-}$ mice do not have alopecia, but their keratinocytes exhibit reduced expression of differentiation markers and have an inhibited ability to recover normal skin barrier function after an acute perturbation.⁴ Unlike, $VDR^{-/-}$ mice, $CYP27B1^{-/-}$ mice do not develop skin tumours in response to a chemical carcinogen.⁵² Normal bone phenotype in $CYP27B1^{-/-}$ mice is partially restored by treatment with vitamin D. In particular, body weight and bone-related parameters were restored by chronic intramuscular treatment with 10,000 IU vitamin D₃/week for 4 weeks.⁶³ These effects were mediated in the absence of conversion to 1,25(OH)₂D, where 25(OH)D may be another

(albeit lower-affinity) ligand of the VDR. These studies suggest that CYP27B1^{-/-} mice can still respond to vitamin D, and are thus an incomplete model to study the effects of ‘vitamin D deficiency’ on disease, and sub-optimal for investigating the effects of UVR on skin immunity. Like mice with VDD and VDR^{-/-} mice, CYP27B1^{-/-} mice have more severe disease in models of experimental colitis (Table 1).⁶⁴

4.2.3 CYP24A1^{-/-} mice

The CYP24A1 knockout mouse, in which expression of the enzyme that inactivates both 25(OH)D and 1,25(OH)₂D is negated, could be used for examining a role for vitamin D in UVR-induced immunomodulation. Like the VDR^{-/-} and CYP27B1^{-/-} mice, CYP24A1^{-/-} mice have poor viability but these mice have a tendency towards hypercalcemia (reviewed in⁶⁵). The CYP24A1 gene is regulated by the VDR, and in VDR^{+/-} mice, 1,25(OH)₂D expression in serum is enhanced due to impaired ligand-dependent activation of CYP24A1. VDR^{-/-} may represent the equivalent of a partial CYP24A1 deficiency.⁶⁵ CYP24A1^{-/-} mice exhibit increased plasma and tissue levels of 1,25(OH)₂D after exogenous 1,25(OH)₂D administration.⁶⁵

4.3 Specific deletion or upregulation of genes involved in vitamin D biology

A more targeted and potentially informative approach could be to use transgenic mice with inducible deletion or over-expression of the VDR, CYP27B1, CYP24A1 or other vitamin D-related gene of interest within specific cell populations of the skin (eg. keratinocytes, Langerhans cells, mast cells) via the *Cre-LoxP* system. In the epidermis, gene expression changes as keratinocytes differentiate from basal cells into squamous cells of the stratum spinosum, granular cells of the stratum granulosum and cornified cells of the stratum corneum.⁶⁶ Basal, granular and cornified cells can be identified by differential expression of

keratin 5 and 14, involucrin and loricrin, respectively.⁶⁶ The development of mice with inducible knockdown (or upregulation) of VDR, CYP27B1 or other vitamin D-related genes within these specific keratinocyte populations, could reveal whether vitamin D is important for their differentiation. This strategy could also determine which keratinocyte population is responsible for 1,25(OH)₂D synthesis in skin after UVR exposure as keratinocytes contain all the enzymatic machinery required for vitamin D synthesis, from 7-dehydrocholesterol to 1,25(OH)₂D.⁶⁷

Vitamin D₃ is detected in UV-irradiated rat skin, 30 minutes post-UVR.⁶⁸ However, it may take 4 days for equilibrium to be reached, with 80% of pre-vitamin D converted to vitamin D₃.⁶⁹ The timing of vitamin D₃ synthesis may be critical for the ability of 1,25(OH)₂D to modulate immunity post-UVR. This might be especially relevant after irradiation with erythema quantities of UVR, where the production of significant concentrations of mediators like PGE₂ may overwhelm the activities of 1,25(OH)₂D. Delaying increased expression of the CYP27B1 gene after UVR by using an inducible keratinocyte promoter may reveal how the timing of 1,25(OH)₂D production contributes towards UVR-induced immunosuppression.

4.4 Chimeric mice and adoptively transferring cells

Gamma-irradiation can be used to ablate the immune system of mice, removing bone marrow, splenic and lymph node immune cell populations, with the exception of radio-resistant cells like Langerhans cells. Mice can then be reconstituted with bone marrow from other animals, which gradually repopulate hematopoietic cell populations with time. These chimeric mice can be used to examine mechanisms of UVR-induced immunomodulation. Indeed, Fukunaga *et al.* (2010) used the notion that Langerhans cell precursors are radioresistant, and dermal DC precursors are radiosensitive to examine skin DC migration and their role in UVR-induced

immunosuppression.⁷⁰ Chimeric mice reconstituted with bone marrow from VDR^{-/-} mice were used to show the importance of the VDR expression on lung epithelium for the development of asthma.⁷¹ Similarly, the effects of UVR-induced immunosuppression could be examined in mice reconstituted with whole bone marrow cells or more defined progenitor populations from vitamin D-deficient or VDR^{-/-} or other knockout mice.

Adoptive transfer of cells from vitamin D-deficient or gene knockout mice could also be a useful strategy for examining the role of vitamin D in mediating the effects of UVR on immunity. Using this approach, a role for the VDR in controlling the anti-inflammatory capacity of mast cells in UV-irradiated skin was identified.³² Inflammation induced by chronic UVR (2 kJ/m², 15 doses over 30 days) was enhanced in the skin of mast cell-deficient mice (WBB6F₁-Kit^{W/W^v}) that were engrafted with bone marrow-derived mast cells from VDR^{-/-} mice relative to wild type controls.³² These studies suggested that the VDR may be important for the ability of mast cells to suppress skin inflammation caused by sub-erythral UVR. Skin mast cells also control susceptibility of different mouse strains to UVR-induced immunosuppression,⁷² with fewer numbers of mast cells observed in the skin of more resistant strains like BALB/c mice, and greater numbers in more susceptible strains, like C57Bl/6 mice.⁷³ Indeed, UVR was unable to suppress contact hypersensitivity responses in mast cell-deficient mice (*Wf/Wf*), but was able to suppress these responses following the reconstitution of the skin of *Wf/Wf* mice with mast cells derived from the bone marrow of wild type mice.⁷³ In addition, the ability of mast cells to limit skin inflammation, and to enhance cutaneous levels of IL-10, both induced by chronic UVR depends, at least in part, on the expression of functional VDRs by mast cells.³² It is not known whether the expression of the VDR by mast cells or skin synthesis of 1,25(OH)₂D₃ controls susceptibility to UVR-induced immunosuppression. As dermal mast cells correlate with the extent of UVR-induced

immunosuppression, it would be interesting to determine their numbers in mice with developmental VDD (*in utero*) or in CYP27B1^{-/-} mice.

4.5 Drugs to block vitamin D pathway enzymes

A final method to examine the role of vitamin D in UVR-induced immunomodulation could employ the use of chemicals that block the production or activity of 1,25(OH)₂D. One such molecule is ketoconazole, which has been employed to examine UVR-induced production of lipids, antimicrobial peptides and modulation of permeability barrier homeostasis.³⁰ However, in the same study, ketoconazole inhibited the expression of not only the CYP27B1 gene but also the VDR gene, indicating that its specificity may be questionable.³⁰ Other chemicals of interest include inhibitors of the 7-dehydrocholesterol-Δ-reductase, the enzyme responsible for the conversion of 7-dehydrocholesterol to cholesterol. Using an inhibitor of 7-dehydrocholesterol-Δ-reductase, serum levels of 25(OH)D were induced in UV-irradiated cats, who naturally have low skin levels of 7-dehydrocholesterol and are normally unable to alter their serum levels of 25(OH)D in response to UVR.⁷⁴

5. Epigenetic effects of vitamin D deficiency

The lack of positive results in vitamin D supplementation trials for reducing disease burden may also be explained by epigenetic effects of VDD on immunity that may not be rescuable.⁸ In particular, VDD *in utero* may establish epigenetic changes that are difficult to reverse with supplementation. In a recent study, the generation of invariant natural killer T cells was inhibited by *in utero* VDD, which was not recovered by treatment with 1,25(OH)₂D from just before birth.⁸ Genome-wide scans of human monocyte⁷⁵ and lymphoblastoid cell lines⁷⁶ have revealed that the VDR binds to multiple sites across the genome, mostly proximal to promoter regions and exons.⁷⁵ With 1,25(OH)₂D treatment, the VDR binds to regions distal to their

previous locations,⁷⁵ with increased binding within introns,⁷⁶ suggesting that genome-wide epigenetic changes occur with 1,25(OH)₂D to enable gene expression. These observations indicate that vitamin D can modify the expression of multiple genes through genome-wide epigenetic changes.

UVR may modulate the development of non-melanoma skin cancers (NMSC) through epigenetic mechanisms. UV-specific p53 gene mutations in NMSC occur preferentially within 5'-CG-3' dinucleotide (CpG) sequences, where CpGs are the consensus target motif for epigenetic DNA methylation⁷⁷ This preferential epigenetic change may affect the formation of UV-induced DNA damage or mutagenesis especially in genes with a coding sequence long enough to include many CpG sites.⁷⁷ Epigenetic mechanisms of UVR-induced immunosuppression are yet to be fully appreciated. However, we have shown that skin exposure to UVR reduces the ability of DC progenitor cells from the bone marrow to develop into effective antigen-presenting cells.⁷⁸ One potential mechanism for this effect could be through epigenetic modification of committed progenitor cells in the bone marrow to differentiate into less immunogenic DCs, where vitamin D or other UVR-induced immune mediators like prostaglandins⁷⁸ could play a role.

6. Conclusion

The immunomodulatory effects of UVR are not just dependent upon vitamin D, and involve a number of other mediators, like PGE₂, that could redundantly control the same immunoregulatory cellular pathways as vitamin D. We have yet to directly determine whether vitamin D contributes towards the immunomodulatory effects of UVR. This is an important issue, as there are many expensive vitamin D supplementation trials underway, and clinical trials performed to date have had limited success at reducing the burden of diseases

characterised by over-zealous immune responses. If vitamin D is not required for or contributes minimally to the effects of UVR on immunity, then more emphasis should be placed on the capacity of UVR or other UVR-induced mediators to treat immune-driven diseases.

Conflict of Interest

The authors have no conflict of interest.

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References

1. M. Norval, R. M. Lucas, A. P. Cullen, F. R. de Gruijl, J. Longstreth, Y. Takizawa and J. C. van der Leun, The human health effects of ozone depletion and interactions with climate change, *Photochem. Photobiol. Sci.* 2011, **10**, 199-225.
2. R. M. Lucas, A. L Ponsonby, K. Dear, P. C. Valery, M. P. Pender, B. V. Taylor, T. J. Kilpatrick, T. Dwyer, A. Coulthard, C. Chapman, I. van der Mei, D. Williams, and A. J. McMichael, Sun exposure and vitamin D are independent risk factors for CNS demyelination. *Neurology*, 2011, **76**, 540-548.

3. J. P. McGlade, S. Gorman, G. R. Zosky, A. N. Larcombe, P. D. Sly PD, J. J. Finlay-Jones, D. J. Turner, and P. H. Hart, Suppression of the asthmatic phenotype by UVB-induced, antigen-specific regulatory cells. *Clin. Exp. Allergy* 2007, **37**, 1267-1276.
4. R. Bouillon, G. Carmeliet, L. Verlinden, E. van Etten, A. Verstuyf, H. F. Luderer, L. Lieben, C. Mathieu, and M. Demay, Vitamin D and human health: Lessons from vitamin D receptor null mice, *Endocr. Rev.* 2008, **29**, 726-776.
5. P. H. Hart, S. Gorman, and J. J. Finlay-Jones, Modulation of the immune system by UV radiation: more than just the effects of vitamin D? *Nat. Rev. Immunol.* 2011, **11**, 584-596.
6. C. S. Maxwell, and R. J. Wood, Update on vitamin D and type 2-diabetes, *Nutrition Rev.* 2011, **69**, 291-295.
7. C. J. Lavie, J. H. Lee, and R. V. Milani, Vitamin D and cardiovascular disease. Will it live up to its hype? *J. Am. Coll. Cardiol.* 2011, **58**, 1547-1556.
8. S. Yu, and M. T. Cantorna, Epigenetic reduction in invariant NKT cells following in utero vitamin D deficiency in mice. *J. Immunol.* 2011, **186**, 1384-1390.
9. M. L. Kripke, Antigenicity of murine skin tumors induced by ultraviolet light, *J. Natl. Cancer Inst.* 1974, **53**, 1333-1336.

10. B. Lehmann, T. Genehr, P. Knuschke, J. Pietzsch, and M. Meurer, UVB-induced conversion of 7-dehydrocholesterol to 1 α ,25-dihydroxyvitamin D₃ in an in vitro human skin equivalent model, *J. Invest. Dermatol.* 2001, **117**, 1179-1185.
11. A. A. Vink, A. M. Moodycliffe, V. Shreedhar, S. E. Ullrich, L. Roza, D. B. Yarosh, and M. L. Kripke, The inhibition of antigen-presenting activity of dendritic cells resulting from UV irradiation of murine skin is restored by in vitro photorepair of cyclobutane pyrimidine dimmers, *Proc. Natl. Acad. Sci. U. S. A.* 1997, **94**, 5255-5260.
12. A. Schwarz, A. Maeda, K. Kernebeck, H. van Steeg, S. Beissert, and T. Schwarz, Prevention of UV radiation-induced immunosuppression by IL-12 is dependent on DNA repair, *J. Exp. Med.* 2005, **201**, 173-179.
13. M. Ghoreishi, and J. P. Dutz, Tolerance induction by transcutaneous immunization through ultraviolet-irradiated skin is transferable through CD4⁺CD25⁺ T regulatory cells and is dependent on host-derived IL-10, *J. Immunol.* 2006, **176**, 2635-2644.
14. 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.
15. M. Ghoreishi, P. Bach, J. Obst, J. C. Fleet, and J. P. Dutz, Expansion of antigen-specific regulatory T cells with the topical vitamin D analog calcipotriol, *J. Immunol.* 2009, **182**, 6071-6078.

16. S. Gorman, M. A. Judge, and P. H. Hart, Topical 1,25-dihydroxyvitamin D₃ subverts the priming ability of draining lymph node dendritic cells, *Immunology* 2010, **131**, 415-425.
17. K. Loser, A. Mehling, S. Loeser, J. Apelt, A. Kuhn, S. Grabbe, T. Schwarz, J. M. Penninger, and S. Beissert, Epidermal RANKL controls regulatory T-cell numbers via activation of dendritic cells, *Nat. Med.* 2006, **12**, 1372-1379.
18. K. Soontrapa, T. Honda, D. Sakata, C. Yao, T. Hirata, S. Hori, T. Matsuoka, Y. Kita, T. Shimizu, K. Kabashima, and S. Narumiya, Prostaglandin E₂-prostaglandin E receptor subtype 4 (EP4) signaling mediates UV irradiation-induced systemic immunosuppression, *Proc. Nat. Acad. Sci. U. S. A.* 2011, **108**, 6668-6673.
19. K. Loser, and S. Beissert, Regulation of cutaneous immunity by the environment: An important role for UV irradiation and vitamin D, *Inter. Immunopharmacol.* 2009, **9**, 587-589.
20. A. W. Norman, M. T. Mizwicki, D. P. Norman, Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model, *Nat. Rev. Drug Discov.* 2004, **3**, 27-41.
21. K. M. Dixon, S. S. Deo, A. W. Norman, J. E. Bishop, G. M. Halliday, V. E. Reeve, R. S. Mason, *In vivo* relevance for photoprotection by the vitamin D rapid response pathway, *J. Steroid Biochem. Mol. Biol.* 2007, **103**, 451-456.
22. K. M. Dixon, A. W. Norman, V. B. Sequeira, R. Mohan, M. S. Rybchyn, V. E. Reeve, G. M. Halliday, R. S. Mason, 1 α ,25(OH)₂-vitamin D and a nongenomic vitamin D analogue

inhibit ultraviolet radiation-induced skin carcinogenesis. *Cancer Prev. Res. (Phila.)* 2011, **4**, 1485-1494.

23. S. Gorman S, M. A. Judge, P. H. Hart, Immune-modifying properties of topical vitamin D: Focus on dendritic cells and T cells, *J. Steroid Biochem. Mol. Biol.* 2010, **121**, 247-249.

24. J. C. Hsieh, S. A. Slater, G. K. Whitfield, J. L. Dawson, G. Hsieh, C. Sheedy, C. A. Haussler, M. R. Haussler, Analysis of hairless corepressor mutants to characterize molecular cooperation with the vitamin D receptor in promoting the mammalian hair cycle, *J. Cell Biochem.* 2010, **110**, 671-686.

25. L. A. Kuritzky, J. J. Finlay-Jones, P. H. Hart, The controversial role of vitamin D in the skin: immunosuppression vs. photoprotection, *Clin. Exp. Dermatol.* 2008, **33**, 167-170.

26. T. Tecle, S. Tripathi, K. L. Hartshorn, Review: Defensins and cathelicidins in lung immunity, *Innate Immun.* 2010, **16**, 151-159.

27. J. S. Adams, S. Ren, P. T. Liu, R. F. Chun, V. Lagishetty, A. F. Gombart, N. Borregaard, R. L. Modlin, M. Hewison, Vitamin D-directed rheostatic regulation of monocyte antibacterial responses, *J. Immunol.* 2009, **182**, 4289-4295.

28. P. T. Liu, S. Stenger, H. Li, L. Wenzel, B. H. Tan, S. R. Krutzik, M. T. Ochoa, J. Schaubert, K. Wu, C. Meinken, D. L. Kamen, M. Wagner, R. Bals, A. Steinmeyer, U. Zugel, R. L. Gallo, D. Eisenberg, M. Hewison, B. W. Hollis, J. S. Adams, B. R. Bloom, R. L.

Modlin, Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response, *Science* 2006, **311**, 1770 -1773.

29. M. Hewison, Vitamin D and the intracrinology of innate immunity, *Mol. Cell Endocrinol.* 2010, **321**, 103-111.

30. S. P. Hong, M. J. Kim, M.-Y. Jung, H. Jeon, J. Goo, S. K. Ahn, S. H. Lee, P. M. Elias, and E. H. Choi, Biopositive effects of low-dose UVB on epidermis: coordinate upregulation of antimicrobial peptides and permeability barrier reinforcement, *J. Invest. Dermatol.* 2008, **128**, 2880-2887.

31. S. P. Hong, Y. Oh, M. Jung, S. Lee, H. Jeon, M. Y. Cho, S. H. Lee, E. H. Choi EH. Topical calcitriol restores the impairment of epidermal permeability and antimicrobial barriers induced by corticosteroids, *Br. J. Dermatol.* 2010 **162**, 1251-1260.

32. L. Biggs, C. Yu, B. Fedoric, A. F. Lopez, S. J. Galli, and M. A. Grimaldeston, Evidence that vitamin D(3) promotes mast cell-dependent reduction of chronic UVB-induced skin pathology in mice, *J. Exp. Med.* 2010, **207**, 455-463.

33. A. Di Nardo, K. Yamasaki, R. A. Dorshner, Y. Lai, R. L. Gallo, Mast cell cathelicidin antimicrobial peptide prevents invasive group A Streptococcus infection of the skin, *J. Immunol.* 2008, **180**, 7565-7573.

34. Z. Wang, Y. Lai, J. J. Bernard, D. T. Macleod, A. L. Cogen, B. Moss, A. Di Nardo, Skin mast cells protect mice against vaccinia virus by triggering mast cell receptor S1PR2 and releasing antimicrobial peptides, *J. Immunol.* 2012, **188**, 345-357.
35. K. Park, P. M. Elias, Y. Oda, D. Mackenzie, T. Mauro, W. M. Holleran, Y. Uchida, Regulation of cathelicidin antimicrobial peptide expression by an endoplasmic reticulum (ER) stress signaling, vitamin D receptor-independent pathway, *J. Biol. Chem.* 2011, **286**, 34121-34130.
36. G. Halliday, Common links among the pathways leading to UV-induced immunosuppression, *J. Invest. Dermatol.* 2010, **130**, 1209-1211.
37. C. S. Sreevidya, A. Fukunaga, N. M. Khaskhely, T. Masaki, R. Ono, C. Nishigori, S. E. Ullrich, Agents that reverse UV-induced immune suppression and photocarcinogenesis affect DNA repair, *J. Invest. Dermatol.* 2010, **130**, 1428-1437.
38. D. Damian, Y. J. Kim, K. M. Dixon, G. M. Halliday, A. Javeri, R. S. Mason, Topical calcitriol protects from UV-induced genetic damage but suppresses cutaneous immunity in humans, *Exp. Dermatol.* 2010, **19**, e23-30.
39. H. Narahara, I. Miyakawa, and J. M. Johnston, The inhibitory effect of 1,25-dihydroxyvitamin D3 on the secretion of platelet-activating factor acetylhydroxylase by human decidual macrophages, *J. Clin. Endocrinol. Metab.* 1995, **80**, 3121-3126.

40. L. Overbergh, B. Decallonne, D. D. Branisteanu, D. Valckx, A. Kasran, R. Bouillon, and C. Mathieu, Acute shock induced by antigen vaccination in NOD mice, *Diabetes* 2003, **52**, 335-341.
41. G. R. Zosky, L. J. Berry, J. G. Elliot, A. L. James, S. Gorman, and P. H. Hart, Vitamin D deficiency causes deficits in lung function and alters lung structure, *Am. J. Respir. Crit. Care Med.* 2011, **183**, 1336-1343.
42. S. Gorman, D. H. W. Tan, M. J. M. Lambert, N. M. Scott, M. A. Judge, and P. H. Hart, Vitamin D(3) deficiency enhances allergen-induced lymphocyte responses in a mouse model of allergic airway disease, *Pediatric Allergy Immunol.* 2012, **23**, 83-87.
43. Y. Zhu, B. D. Mahon, M. Froicu, and M. T. Cantorna, Calcium and 1 alpha,25-dihydroxyvitamin D3 target the TNF-alpha pathway to suppress experimental inflammatory bowel disease, *Eur. J. Immunol.* 2005, **35**, 217-235.
44. K. M. Spach, and C. E. Hayes, Vitamin D3 confers protection from autoimmune encephalomyelitis only in female mice, *J. Immunol.* 2005, **175**, 4119-4126.
45. M. T. Cantorna, J. Humpal-Winter, and H. F. DeLuca, Dietary calcium is a major factor in 1,25-dihydroxycholecalciferol suppression of experimental autoimmune encephalomyelitis in mice, *J. Nutr.* 1999, **129**, 1966-1971.

46. V. Lagishetty, A. V. Misharin, N. Q. Liu, T. S. Lisse, R. F. Chun, Y. Ouyang, S. M. McLachlan, J. S. Adams, and M. Hewison, Vitamin D deficiency in mice impairs colonic antibacterial activity and predisposes to colitis, *Endocrinol.* 2010, **151**, 2423-2432.
47. A. Giulietti, C. Gysemans, K. Stoffels, E. van Etten, B. Decallonne, L. Overbergh, R. Bouillon, and C. Mathieu, Vitamin D deficiency in early life accelerates Type 1 diabetes in non-obese diabetic mice, *Diabetologica* 2004, **47**, 451-462.
48. D. A. Fernandes de Abreu, E. C. Ibrahim, J. Boucraut, M. Khrestchatisky, F. Feron, Severity of autoimmune encephalomyelitis is unexpectedly reduced in mice born to vitamin D-deficient mothers, *J. Ster. Biochem. Mol. Biol.* 2010, **121**, 250-253.
49. R. C. Malley, H. K. Muller, M. Norval, G. M. Woods, Vitamin D3 deficiency enhances contact hypersensitivity in male but not female mice, *Cell Immunol.* 2009, **255**, 33-40.
50. B. R. Becklund, K. S. Severson, S. V. Vang, and H. F. DeLuca, UV radiation suppresses experimental autoimmune encephalomyelitis independent of vitamin D production, *Proc. Natl. Acad. Sci. U. S. A.* 2010, **107**, 6418-6423.
51. Y. Song, S. Kato, and J. C. Fleet, Vitamin D receptor (VDR) knockout mice reveal VDR-independent regulation of intestinal calcium absorption and ECaC2 and calbindin D9k mRNA, *J. Nutr.* 2002, **133**, 370-380.

52. T. I. Ellison, M.K. Smith, A. C. Gilliam, and P. N. MacDonald, Inactivation of the vitamin D receptor enhances susceptibility of murine skin to UV-induced tumorigenesis, *J. Invest. Dermatol.* 2008, **128**, 2508-2517.
53. A. Wittke, V. Weaver, B. D. Mahon, A. August, and M. T. Cantorna, Vitamin D receptor-deficient mice fail to develop experimental allergic asthma, *J. Immunol.* 2004, **173**, 3432-3436.
54. C. Gysemans, E. van Etten, L. Overbergh, A. Guilietti, G. Eelen, M. Waer, A. Verstuyf, R. Bouillon, C. Mattieu, Unaltered diabetes presentation in NOD mice lacking the vitamin D receptor, *Diabetes*, 2008, **57**, 269-275.
55. M. Moghaddami, G. Mayrhofer, P. H. Anderson, H. A. Morris, M. Van Der Hoek, L. G. Cleland, Efficacy and mechanisms of action of vitamin D in experimental polyarthritis, *Immunol. Cell Biol.* 2012, **90**, 168-177.
56. K. Zwerina, W. Baum, R. Axmann, G. R. Heiland, J. H. Distler, J. Smolen, S. Hayer, J. Zwerina, G. Schett, Vitamin D receptor regulates TNF-mediated arthritis, *Ann. Rheum. Dis.*, 2011, **70**, 1122-1129.
57. M. T. Cantorna, C. Munsick, C. Bemiss, B. D. Mahon, 1,25-Dihydroxycholecalciferol prevents and ameliorates symptoms of experimental murine inflammatory bowel disease, *J. Nutr.* 2000, **130**, 2648-2652.
58. C. J. Bemiss, B. D. Mahon, A. Henry, V. Weaver, M. T. Cantorna, Interleukin-2 is one of

the targets of 1,25-dihydroxyvitamin D₃ in the immune system., *Arch. Biochem. Biophys.* 2002, **402**, 249-254.

59. M. Froicu, M. T. Cantorna, Vitamin D and the vitamin D receptor are critical for control of the innate immune response to colonic injury. *BMC Immunol.* 2007, **8**, 5.

60. M. Froicu, V. Weaver, T. A. Wynn, M. A. McDowell, J. E. Welsh, M. T. Cantorna, A crucial role for the vitamin D receptor in experimental inflammatory bowel diseases, *Mol. Endocrinol.* 2003, **17**, 2386-2392.

61. T. F. Meehan, and H. F. DeLuca, The vitamin D receptor is necessary for 1 α ,25-dihydroxyvitamin D₃ to suppress experimental autoimmune encephalomyelitis in mice, *Arch. Biochem. Biophys.*, 2002, **42**, 200-204.

62. W. Sun, H. Xie, J. Ji, X. Zhou, D. Goltzman, and D. Miao, Defective female reproductive function in 1,25(OH)₂D-deficient mice results from indirect effect mediated by extracellular calcium and/or phosphorus, *Am. J. Physiol. Endocrinol. Metab.* 2010, **299**, E928-E9235.

63. Z. L. Zhang, X. F. Ding, J. Tong, B. Y. Li, Partial rescue of the phenotype in 1 α -hydroxylase gene knockout mice by vitamin D₃ injection, *Endocr. Res.* 2011, **36**, 101-108.

64. N. Liu, L. Nguyen, R. F. Chun, V. Lagishetty, S. Ren, S. Wu, B. Hollis, H. F. DeLuca, J. S. Adams, and M. Hewison, Altered endocrine and autocrine metabolism of vitamin D in a mouse model of gastrointestinal inflammation, *Endocrinol.*, 2008, **149**, 4799-4808.

65. S. Masuda, V. Byford, A. Arabian, Y. Sakai, M. B. Demay, R. St-Arnaud, G. Jones, Altered pharmacokinetics of 1 α ,25-dihydroxyvitamin D₃ and 25-hydroxyvitamin D₃ in the blood and tissues of the 25-hydroxyvitamin D-24-hydroxylase (Cyp24a1) null mouse, *Endocrinol.*, 2005, **146**, 825-834.
66. C. Adolphe, B. Wainwright, Pathways to improving skin regeneration, *Expert Rev. Mol. Med.* 2005, **7**, 1-14.
67. B. Lehmann, The vitamin D₃ pathway in human skin and its role for regulation of biological processes, *Photochem. Photobiol.* 2005, **81**, 1246-1251.
68. T. Okano, M. Yasumura, K. Mizuno, T. Kobayashi T, Photochemical conversion of 7-dehydrocholesterol into vitamin D₃ in rat skins, *J. Nutr. Sci. Vitaminol. (Tokyo)*. 1977, **23**, 165-168.
69. A. R. Webb, M. F. Holick, The role of sunlight in the cutaneous production of vitamin D₃, *Annu. Rev. Nutr.* 1988, **8**, 375-399.
70. A. Fukunaga, N. M. Khaskhely, Y. Ma, C. S. Sreevidya, K. Taguchi, C. Nishigori, S. E. Ullrich, Langerhans cells serve as immunoregulatory cells by activating NKT cells, *J. Immunol.* 2010, **185**, 4633-4640.
71. A. Wittke, A. Chang, M. Froicu, O. F. Harandi, V. Weaver, A. August, R. F. Paulson, M. T. Cantorna, Vitamin D receptor expression by the lung micro-environment is required for maximal induction of lung inflammation, *Arch. Biochem. Biophys.* 2007, **460**, 306-313.

72. F. P. Noonan, H. A. Hoffman, Susceptibility to immunosuppression by ultraviolet B radiation in the mouse, *Immunogenetics*, 1994, **39**, 29-39.
73. P. H. Hart, M. A. Grimbaldston, G. J. Swift, A. Jaksic, F. P. Noonan, 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.
74. J. G. Morris, Ineffective vitamin D synthesis in cats is reversed by an inhibitor of 7-dehydrocholesterol-delta7-reductase, *J. Nutr.* 1999, **129**, 903-908.
75. S. Heikkinen, S. Vaisanen, P. Pehkonen, S. Seuter, V. Benes, C. Carlberg, Nuclear hormone 1 α , 25-dihydroxyvitamin D₃ elicits a genome-wide shift in the locations of VDR chromatin occupancy, *Nucleic Acids Res.* 2011, **39**, 9181-9193.
76. S. V. Ramagopalan, A. Heger, A. J. Berlanga, N. J. Maugeri, M. R. Lincoln, A. Burrell, L. Handunnetthi, A. E. Handel, G. Disanto, S. M. Orton, C. T. Watson, J. M. Morahan, G. Giovannoni, C. P. Ponting, G. C. Ebers, J. C. Knight, A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution, *Genome Res.* 2010, **20**, 1352-1360.
77. H. Ikehata, T. Ono, Significance of CpG methylation for solar UV-induced mutagenesis and carcinogenesis in skin, *Photochem. Photobiol.* 2007, **83**, 196-204.
78. R. L. Ng, J. L. Bisley, S. Gorman, M. Norval, P. H. Hart, Ultraviolet irradiation of mice

reduces the competency of bone marrow-derived CD11c⁺ cells via an indomethacin-inhibitable pathway, *J. Immunol.* 2010, **185**, 7207-7215.

Table: Comparison of the effects of dietary-induced vitamin D deficiency (VDD) or VDR/CYP27B1 gene deficiency on immune-related disease outcomes in rodents

Disease model	Dietary-induced VDD	VDR ^{-/-}	CYP27B1 ^{-/-}
Allergic asthma (allergic airway disease)	Increased allergic sensitisation induced by ovalbumin and aluminium hydroxide ^{42(b)}	Reduced airway inflammation, airway hyper-reactivity but increased IgE induced by ovalbumin and aluminium hydroxide ^{53(b)}	Not reported
Arthritis	Increased arthritis severity induced by transfer of CD45RB+ cells from synovium-rich tissues ^{55(a)}	Increased arthritis severity in human tumor necrosis factor transgenic mice VDR ^{-/-} mice ^{56(a)}	Not reported
Inflammatory bowel disease (experimental colitis)	Increased colitis severity induced by dextran sodium sulfate ^{46(b)}	Increased colitis severity induced by dextran sodium sulfate ^{59(b)}	Increased colitis severity induced by dextran sodium sulfate ^{64(a)}

	Increased colitis severity in IL-10 ^{-/-} mice ^{57(c)}	Increased colitis severity in IL-10 ^{-/-} VDR ^{-/-} mice ^{60(b)}	
	No affect on colitis severity in IL-2 ^{-/-} mice ^{58(c)}		
Multiple sclerosis (Experimental autoimmune encephalomyelitis)	Disease onset delayed and severity reduced following injection of myelin oligodendrocyte glycoprotein peptide in Freund's complete adjuvant ^{48(b)}	Reduced incidence in disease induced by injection of myelin basic protein in Freund's complete adjuvant ^{61(b)}	Not reported
Type-1 diabetes	Increased diabetes incidence and glycaemia in non-obese diabetic mice ^{47(b)}	No affect on diabetes severity or insulitis in non-obese diabetic mice ^{54(c)}	Not reported

^aCalcium levels not reported, ^bNormal calcium levels, ^cHypocalcemia

Figure legend

Figure: UV-induced immunomodulation via increased RANKL expression in skin can be mediated by 1,25(OH)₂D or PGE₂. UVR from sunlight induces the synthesis of 1,25(OH)₂D from 7-dehydrocholesterol in keratinocytes. 1,25(OH)₂D increases the expression of RANKL on epidermal Langerhans cells. UVR also induces the expression of PGE₂, which may signal through the EP4 receptor to increase RANKL expression by Langerhans cells. 1,25(OH)₂D- or PGE₂-modified Langerhans cells then migrate to the draining lymph nodes to induce or increase the suppressive activity of CD4+CD25+ regulatory T cells. These cells may regulate immunity in a systemic fashion. Abbreviations: 1,25(OH)₂D = 1,25-dihydroxyvitamin D, 25(OH)D = 25-hydroxyvitamin D, 7-DHC = 7-dehydrocholesterol, LC = Langerhans cells, LV = lymphatic vessel, PGE₂ = prostaglandin E₂, RANK(L) = receptor activator of NFκB (ligand), UV = ultraviolet.

Figure

Here we discuss the converging and diverging immunoregulatory pathways of ultraviolet radiation and vitamin D, and potential *in vivo* approaches to determine if vitamin D mediates the immunosuppressive effects of UVR.

160x199mm (300 x 300 DPI)

352x528mm (240 x 240 DPI)

Point-by-point response to referees' comments

PP-PER-04-2012-025108

We thank the referees' for their critique of our manuscript. Please note that all changes to the original text are shown by underlining the next text.

Referee: 1

1. Page 8, para 2, the first sentence needs restructuring: 'A physiologically-relevant approach excludes vitamin D from....'

Response: Modified to: A physiologically-relevant approach could be to exclude vitamin D from the diets of rodents to effectively reduce serum 25(OH)D levels to those equivalent to clinical deficiency in humans (<25 nmol/L).

2. Page 9, para 2: '...or the enzymes that hydroxylate 25(OH)D and 1,25(OH)₂D, the 1 α -hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1) respectively.'

Response: Modified to: An alternative methodology could be to use knockout mice that cannot respond to, or cannot make or breakdown 1,25(OH)₂D through ablating expression of the VDR, or hydroxylating enzymes that synthesise or breakdown 1,25(OH)₂D, the 1 α -hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1), respectively.

3. Is it possible that there is an association between platelet activating factor's role in UV immunosuppression and vitamin D?

Response: Yes, there may be an association. Treatment of human decidual macrophages with 1,25(OH)₂D (≥ 1 nM) or 25(OH)D (≥ 100 nM) reduced the expression of the platelet activating factor (PAF) deactivation enzyme, PAF-acetylhydroxylase (Nahara et al 1995, J Clin Endocrinol Metabolism 80, 3121). In addition, expression of the PAF-receptor was increased in NOD (non-obese diabetic) mice treated with 1,25(OH)₂D (5 μ g/kg ip, every 2 days), in which severe shock was induced by foot-pad injection of hen egg white lysozyme (Overbergh et al 2003, Diabetes 52, 2003). These studies suggest that vitamin D can induce the expression of PAF to regulate immunity. However, vitamin D and PAF have opposing contributions towards the formation of DNA damage after UV irradiation of skin (reviewed in Halliday, 2010). PAF and other UVR-induced molecules like cis-urocanic acid contribute towards DNA damage (eg. cyclobutane pyrimidine dimers) and ROS formation (Sreevidya et al 2010, Journal of Investigative Dermatology 130, 1428-37). In contrast, 1,25(OH)₂D can repair UVR-induced CPD formation if administered before and/or after UV treatment (Damian et al 2010, Exp Dermatol 19, e23, Gorman et al, 2007, ref14). Whether 1,25(OH)₂D contributes towards PAF formation after UV irradiation has yet to be shown.

We have discussed these observations in a new section:

3.5 Potential interactions between vitamin D and other UVR-induced mediators

One mediator induced by UVR implicated in immunosuppression is platelet activating factor (PAF).^{2,36} While both vitamin D and PAF may contribute towards UVR-induced immunosuppression, they have opposing effects on the formation of DNA damage in UV-irradiated skin.³⁶ PAF and other UVR-induced molecules like cis-urocanic acid contribute towards DNA damage (eg. cyclobutane pyrimidine dimers) and ROS formation.³⁷ In contrast, 1,25(OH)₂D can repair UVR-induced CPD formation if administered topically before and/or after UV treatment.^{14,21,38} Other studies suggest that vitamin D may induce the expression of PAF to regulate immunity. In human decidual macrophages, 1,25(OH)₂D (≥ 1 nM) or 25(OH)D (≥ 100 nM) reduced the enzymatic activity of the platelet activating factor (PAF) deactivation enzyme, PAF-acetylhydroxylase.³⁹ In addition, expression of the PAF-receptor was increased in NOD (non-obese diabetic) mice treated with 1,25(OH)₂D (5 μ g/kg ip, every 2 days), in

which severe shock was induced by foot-pad injection of hen egg white lysozyme.⁴⁰ Whether UVR-induced 1,25(OH)₂D contributes towards PAF formation has yet to be shown.

New references:

36. G. Halliday, Common links among the pathways leading to UV-induced immunosuppression, *J. Invest. Dermatol.* 2010, 130, 1209-1211.

37. C. S. Sreevidya, A. Fukunaga, N. M. Khaskhely, T. Masaki, R. Ono, C. Nishigori, S. E. Ullrich, Agents that reverse UV-induced immune suppression and photocarcinogenesis affect DNA repair, *J. Invest. Dermatol.* 2010, 130, 1428-1437.

38. D. Damian, Y. J. Kim, K. M. Dixon, G. M. Halliday, A. Javeri, R. S. Mason, Topical calcitriol protects from UV-induced genetic damage but suppresses cutaneous immunity in humans, *Exp. Dermatol.* 2010, 19, e23-30.

39. H. Narahara, I. Miyakawa, and J. M. Johnston, The inhibitory effect of 1,25-dihydroxyvitamin D₃ on the secretion of platelet-activating factor acetylhydroxylase by human decidual macrophages, *J. Clin. Endocrinol. Metab.* 1995, 80, 3121-3126.

40. L. Overbergh, B. Decallonne, D. D. Branisteanu, D. Valckx, A. Kasran, R. Bouillon, and C. Mathieu, Acute shock induced by antigen vaccination in NOD mice, *Diabetes* 2003, 52, 335-341.

Referee: 2

Just a few typos to correct:

P5 last line immunosuppression is spelt immunosuppresison

P7 second last line integrity is spelt intergrity

P10 Line 11 VDR-/- the -/- is not superscripted

P11 Line 15 VDR-/- the -/- is not superscripted

p14 4th line from the bottom finvitamin should be 'in vitamin'

1. Reference climate is missing an e

Response: All typos have been corrected.

Referee: 3

1. In the abstract, there is the statement that "Vitamin D can also regulate immunity.... activation of regulatory T cells."

It would be sensible to add the word "adaptive" as in regulate adaptive immunity, since vitamin D can also regulate innate immunity via a different mechanism, not regulatory T cells. This is actually discussed in 3.3.

Response: The word adaptive added as suggested. We have also added a few phrases to the abstract to help further define the contents of our paper.

2. In 4.5, 'Drugs to block the effect of vitamin D' Inhibitors of 7-dehydroreductase would increase the 7-dehydrocholesterol present in skin and so could potentially increase 25OHD levels(as stated in relation to cats), rather than "block" the effects of vitamin D. Perhaps alter the title to 'Drugs to block vitamin D pathway enzymes' or similar.

Response: Title of section 4.5 altered as suggested.