Vitamin D3–elicited CD14+ human skin dendritic cells promote thymic stromal lymphopoietin–independent type 2 T-helper responses

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**Vitamin D3-elicited CD14+ human skin dendritic cells
promote thymic stromal lymphopoietin-independent type 2
T-helper responses**

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Keywords:	atopic dermatitis, dendritic cells, T cells, basic immunology, dermatology

Point-by-point response

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Title of Article: Vitamin D3-elicited human skin dendritic cells promote thymic stromal lymphopoietin-independent type 2 T-helper responses

Name of the Corresponding Author: Dr. Vincent Flacher, Ph.D.

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Dear Dr. Traidl-Hoffmann,

Please find enclosed a new version for our manuscript "Vitamin D3-elicited human skin dendritic cells promote thymic stromal lymphopoietin-independent type 2 T-helper responses". We have applied the minor revision asked by Reviewer #2, and we have carefully followed the instructions of Allergy Graphics Editor Mrs. Globinska to edit the final version of figures.

Specific Responses:

Reviewer #1:

Comment:

*Dear Authors,
Thank you for your work and commitment.
Sincerely yours,*

Reply:

Dear Reviewer,
We thank you again for your helpful comments.
Best regards,
The authors

Reviewer #2:

1. General comments:

The authors of the manuscript "Vitamin D3-elicited CD14+ human skin dendritic cells promote thymic stromal lymphopoietin-independent type 2 T-helper responses" addressed all my major and minor concerns in an adequate manner and I think the manuscript greatly improved. Additional experiments provided strong evidence for their findings and the authors also worked on the manuscript and the graphical abstract to improve it even more. The new finding of

increased soluble CD14 is very interesting and I look forward to reading further studies from this group.

Reply: We are very grateful for your comments and suggestions.

2. Specific comments:

b) Minor:

- Please change in Methods part 100mg "calcipotriol" to "calcipotriol ointment" to avoid confusion, if this is what the authors meant.

Reply: We have modified the text in the Material and Methods section as well as in Figures, according to the Reviewer's suggestion.

We thank again the Editor and the Reviewers for evaluating our manuscript and providing helpful comments to improve its quality. We hope that the revised version will be suitable for publication in Allergy. We remain at your disposal for any other modification that would be important to achieve this goal.

Sincerely,
Vincent Flacher

900x600mm (96 x 96 DPI)

Vitamin D3-elicited CD14+ human skin dendritic cells promote thymic stromal lymphopoietin-independent type 2 T-helper responses

- Vitamin D3 consistently upregulates CD14 on human skin DCs, yet induces TSLP production in only 30% of skin explants
- Skin DCs from TSLP-producing donors shift naïve T cells into IL-13+/IL-4+ Th2 cells.
- Vitamin D3-elicited CD14+ DCs from donors that fail to release TSLP are sufficient to generate IL-4+ Th2 cells.

Abbreviations:

DC: Dendritic cell

IL: Interleukin

LC: Langerhans cell

Th2: T helper 2

TSLP: Thymic stromal lymphopoietin

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Vitamin D3-elicited CD14+ human skin dendritic cells promote thymic stromal lymphopoietin-independent type 2 T-helper responses

Short title: Vitamin D3 directs human skin DCs to prime Th2

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Author contributions

VF and AB conceived the study, designed experiments, and analyzed data. AB performed experiments, with the assistance of AH and QM for explant culture preparations. Quantifications of immunofluorescence microscopy images were performed by JDF. AB, CM and VF wrote the manuscript.

Word count: 3736 words

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Abstract (249 words)

Background

Immune modulation by vitamin D3 through dendritic cells (DCs) remains controversial. Human DCs exposed in vitro counteract type-1 T-helper (Th1) differentiation and induce regulatory T cells. However, cutaneous application on mice promotes Th2-driven inflammation resembling atopic dermatitis and relying on Thymic Stromal Lymphopoietin (TSLP) from keratinocytes and T-cell orientation by TSLP-stimulated skin DCs. We studied the effects of vitamin D3 in human skin, focusing on TSLP production and the role of skin DCs in T-cell differentiation.

Methods

Human healthy skin explants were exposed in vitro to vitamin D3 analogs. Migrating DCs were analyzed and TSLP quantified in the supernatant. Allogeneic naïve CD4+ T cells were cocultured with DCs to assess their proliferation and cytokine production.

Results

Vitamin D3 induced skin DCs to differentiate Th2 cells producing IL-4 and IL-13. Vitamin D3 triggered TSLP release in ~30% of skin explants, correlating with IL-13 detection in Th2 cells. In these donors, blocking TSLP receptor during skin explant cultures abrogated IL-13 production, yet IL-4+ Th2 cells were unaffected. Among skin DCs emerged CD14+ cells that had responded directly to vitamin D3 and differed from classical CD14+ dermal emigrants. Vitamin D3-elicited CD14+ DCs sufficed to promote IL-4+ Th2 cells in a TSLP-independent manner.

Conclusion

Vitamin D3, despite inducing TSLP in some donors, had a direct influence on skin DCs, affecting their phenotype and ability to drive Th2 responses independently of TSLP. Our findings pave the way

towards in vitro systems that accurately model human cutaneous Th2 responses, notably involved in atopic dermatitis.

Keywords (5)

Atopic dermatitis, Dendritic cell, T helper 2, Thymic stromal lymphopoietin, Vitamin D3.

For Peer Review

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Abbreviations

- vitD3: Vitamin D3
- cDCs: conventional dendritic cells
- DCs: Dendritic cells
- LCs: Langerhans cells
- MLR: Mixed leukocyte reaction
- MonoDCs: Monocyte-derived dendritic cells
- Th: T helper
- Treg: Regulatory T cell
- TSLP: Thymic stromal lymphopoietin
- TSLPR: Thymic stromal lymphopoietin receptor
- VDR: Vitamin D receptor
- CYP24A1: Cytochrome P450 family 24 subfamily A member 1
- UVB: type B ultraviolet light
- FCS: Fetal calf serum
- FVD: Fixable Viability Dye
- HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- RPMI: Roswell Park Memorial Institute medium
- LPS: Lipopolysaccharide
- PMA: Phorbol Myristoyl Acetate
- CFSE: Carboxyfluorescein succidiny ester
- HPRT1: Hypoxanthine phosphoribosyltransferase 1
- GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
- SEM: Standard error of the mean

INTRODUCTION

Bioactive 1,25-dihydroxy-vitamin D3 (calcitriol) triggers nuclear translocation and DNA binding of the vitamin D receptor (VDR), an ubiquitous transcription factor notably involved in the regulation of calcium and phosphate storage¹. In psoriasis, antiproliferative effects of calcitriol on keratinocytes have been harnessed to counteract acanthosis.

Immunoregulatory functions of vitamin D3 (vitD3) are a consequence of modulated gene expression in immune cells, in particular dendritic cells (DCs). Most studies have focused on the influence of vitD3 during in vitro differentiation of monocytes into monocyte-derived DCs (MonoDCs) with GM-CSF and IL-4²⁻⁶. The resulting CD1a^{low} CD14^{high} MonoDCs displayed low levels of MHC class II, CD80 and CD86 upon maturation and produced anti-inflammatory IL-10 rather than the type 1 T-helper (Th1)-driving cytokine IL-12²⁻⁶. Such features likely explain why they showed a primarily tolerogenic influence, yielding poor T-cell proliferation, limited Th1 polarization and an increase in regulatory T cell (Treg) differentiation^{4;7;8}.

Human skin harbors several subsets of DCs with distinct phenotypes and functions. Danger signals trigger their maturation, which is required for migration to lymph nodes and T-cell stimulation. The epidermis contains exclusively CD14⁻ CD207⁺ Langerhans cells (LCs), whereas CD14⁻ dermal DCs comprise CD1c⁺ type 2 conventional DCs (cDC2) and a smaller population of CD141⁺ cDC1^{9;10}. Part of dermal CD14⁺ cells exhibit some motility and thus can be harvested from skin explant cultures together with other DC subsets. However, they strongly resemble sessile CD14⁺ macrophages and lack the chemokine receptor CCR7 that allows cDCs and LCs to colocalize with naive T cells in lymph nodes^{11;12}. When exposed to maturation factors in the presence of vitD3, both dermal DCs and epidermal LCs promoted a moderate increase in Treg differentiation^{7;13}.

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Thymic stromal lymphopoietin (TSLP) is a proinflammatory soluble factor released mainly by suprabasal keratinocytes^{14;15}. The most commonly investigated form of TSLP is undetectable in healthy human skin¹⁶, although a shorter, alternatively-spliced TSLP variant might be present¹⁷. Within atopic dermatitis lesions, a basal expression of TSLP can be found¹⁶ and it can be induced in nonlesional skin by house dust mite extracts¹⁸.

In the mouse, cutaneous application of the vitD3 analog calcipotriol (MC903) promotes VDR-dependent TSLP expression as well as a Th2-driven inflammatory condition that mimicks many features of atopic dermatitis¹⁹. In both human and mouse models, TSLP stimulates DCs to support the development of Th2 responses^{16;20;21}. Despite this, there have been reports of in vivo Treg induction reminiscent of experiments with human DCs^{22;23}. Other discrepancies need to be addressed, such as poor TSLP production from human keratinocytes of healthy or nonlesional atopic skin upon stimulation with vitD3¹⁸, whereas calcitriol ointment has recently been shown to induce TSLP expression in lesional psoriasis skin²⁴.

Here, we applied vitD3 to human skin explants and investigated the consequences in terms of TSLP production, the phenotype of skin DCs and their functional ability to promote T-helper responses.

METHODS

Human skin explants

Skin specimens were obtained from anonymized healthy subjects undergoing abdominal reduction, with written informed consent and institutional review board approval, in agreement with the Helsinki Declaration and French legislation. 4cm², ~1mm-thick biopsies were placed at air-liquid interface onto a 40µm cell strainer (Biologix) in 6mL RPMI1640 medium supplemented with 10µg/mL gentamycin, 100U/mL penicillin, 100µg/mL streptomycin, 10mM HEPES (Lonza) and 10% FCS (Dutscher). After 3 days, migrating cells were harvested and analyzed by flow cytometry and immunofluorescence. If necessary, CD14⁺ and CD14⁻ migrating cells were separated using CD14 MicroBeads (Miltenyi-Biotec). Where indicated, 50ng/mL TSLP, 1µg/mL anti-human TSLPR neutralizing antibody, or polyclonal goat IgG (R&D Systems) were added during the culture.

Generation of MonoDCs

Human monocytes were purified from buffy coats of healthy blood donors (EFS, Strasbourg, France) by successive Ficoll and Percoll gradients (Sigma-Aldrich). MonoDCs were differentiated from monocytes in 5 days with 200ng/mL GM-CSF (Schering-Plough) and 10ng/mL IL-4 (ImmunoTools), and characterized as CD14^{low} CD1a^{high} DC-SIGN^{high}.

Vitamin D3 treatment

200nM calcitriol (R&D Systems) was added to the culture medium of skin explant (3 days), MonoDCs (24h), or skin migrating cells (24h). Alternatively, using a commercially available vitD3 ointment (Daivonex 50µg/g, Leo Pharma), 100mg of calcipotriol ointment was applied onto the skin surface during the whole culture period (3 days).

Mixed leukocyte reactions

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Naive CD4+ T cells (purity >95%) were isolated from peripheral blood mononuclear cells using human naive CD4+ T Cell Isolation Kit II (Miltenyi-Biotec). To determine the stimulatory capacity of vitD3-stimulated DCs, 2×10^5 naive CD4+ T cells were cocultured with 2×10^4 skin DCs in 200 μ L RPMI1640+10% pooled human sera (Corning) in 96-well U-bottom plates (Falcon). After 7 days, T cells were restimulated with 1 μ g/mL PMA and 1 μ M ionomycin (Sigma-Aldrich) in the presence of 10 μ g/mL brefeldin and 1 μ M monensin (BD-Biosciences) and analyzed by flow cytometry for intracellular expression of IFN- γ , IL-13 and IL-4. T-cell proliferation was determined by labelling naive CD4+ T cell with CFSE (Invitrogen) before starting the coculture.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants of skin explant cultures or MonoDCs were collected and analyzed for TSLP (eBioscience) and soluble CD14 (R&D Systems) production, respectively. After MLR, 10^5 T cells were restimulated with PMA/ionomycin for 1 day and supernatants were evaluated for IL-4 production (BD-Biosciences). ELISA were performed in duplicates according to the manufacturer's recommendations. The absorbance was detected at 450nm using a Thermo Fisher Scientific Multiskan FC Microplate Photometer.

Flow cytometry

Cells were incubated for 20 min at 4°C with the following antibodies: CD86-FITC (clone FUN-1), CD83-APC (HB15e), DC-SIGN/CD209-PerCP-Cy5.5 (DCN46), CD1a-PE (HI149), CD3-AF700 (UCHT1), CD206-FITC (19.2), CD163-PE (GHI/61), OX40L/CD252-PE (ik-1) from BD-Biosciences; CD4-APCviolet770 (REA623), CD141-APCviolet770 (AD5-14H12) and CD1c FITC (AD5-8E7) from Miltenyi-Biotec; CLEC10A-PE (H037G3) and TSLPR-APC (1B4) from Biolegend; HLA-DR-AF700 (L203, R&D Systems), CD207-PE (DCGM4, Beckman Coulter); CD14-PE or -APC (MEM-18, Immunotools). For intracellular staining, T cells were fixed and permeabilized with BD Fixation/Permeabilization Solution Kit then stained for 45

min at 4°C with IFN-γ-APC (B27), IL-4-PE (8D4-8) and IL-13-PE (JES10-5A2) (BD-Biosciences). Cells were collected using a Beckman-Coulter Gallios flow cytometer, and analyzed with FlowJo (Tree Star). Dead cells stained by Fixable Viability Dye-eFluor450 (eBioscience) were excluded from analysis.

RNA isolation, reverse transcription and qPCR

Total RNA of MonoDCs, skin CD14+ or CD14- DCs was isolated and purified with NucleoSpin RNA Plus kit (Macherey-Nagel). cDNA was synthesized by extension of oligo(dT) with ImPromII™ reverse-transcriptase (Promega) on a T100 thermal cycler (Bio-Rad). qPCR reactions were performed on a StepOnePlus™ thermocycler (Applied Biosystems) using the RT2SYBR Green ROX qPCR Master Mix (Qiagen). Relative expression levels were calculated with the comparative Ct method using the proprietary software. HPRT1 and GAPDH were chosen as housekeeping gene for normalization. Following primer sequences (forward/reverse) were designed with the Primer-BLAST software: HPRT1 (5'-CCTGGCGTCGTGATTAGTGAT-3'; 5'-AGACGTTTCAGTCCTGTCCATAA-3'), GAPDH (5'-GGAGCGAGATCCCTCCAAAAT-3'; 5'-GGCTGTTGTCATACTTCTCATGG-3'), CYP24A1 (5'-GATTTTCCGCATGAAGTTGGGT-3'; 5'-CCTTCCACGGTTTGATCTCCA-3').

Statistics

Data are presented as Mean ±SEM. After testing normality with d'Agostino-Pearson test, paired Student's t-tests (normally distributed population) or Wilcoxon's test (non-parametric) were performed with Prism software (GraphPad); p-values below 0.05 were considered significant.

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RESULTS

Vitamin D3-exposed skin DCs induce Th2 polarization.

To study the effect of vitD3 on cutaneous immune responses, we stimulated skin explants at the onset of ex vivo culture by adding calcitriol (200ng/mL) to the medium or by topical application of calcipotriol ointment (100mg). After 3 days, immune cells of untreated explants spontaneously migrate into the supernatant, including CD4+ and CD8+ resident memory T cells²⁶ and different subsets of HLA-DR+ DCs. With vitD3-treated skin, the viability of DCs was slightly increased (**Figure S1A**). To address the influence of vitD3 on T cell-priming capacities of DCs, we harvested total emigrating DCs without further stimulation and cultured them with CFSE-labelled allogeneic naïve CD4+ T cells (>95% viable, **Figure S1B**). After 7 days of mixed leukocyte reaction (MLR), T-cell proliferation and intracellular cytokine production were quantified by flow cytometry upon restimulation. No difference was observed in DC survival following MLR (**Figure S1C**). VitD3 exposure of DCs weakly altered their capacity to stimulate T-cell proliferation (**Figure 1A**), and this was not related to decreased T-cell survival (**Figure S1D**). However, calcitriol and calcipotriol consistently led DCs to promote differentiation of more IFN- γ - IL-13+/IL-4+ Th2 cells than DCs of untreated skin (**Figure 1B-E**). Th2 cells were detected only among proliferative cells (**Figure 1D-E**) and released IL-4 (**Figure 1F**). Conversely, the generation of IFN- γ + Th1 cells was slightly reduced in the presence of vitD3-exposed DCs (**Figure 1G**). DCs collected from vitD3-treated skin did not generate FoxP3+ Treg or support IL-10 production by T cells (**Figure 1H-I**).

TSLP from vitamin D3-treated human skin favors IL-13+ Th2 induction by DCs.

Epithelial TSLP is widely described as Th2 inducer through its effect on DCs^{16;27}. DCs migrating from skin explants stimulated with TSLP promoted differentiation of IFN- γ - IL-13+ Th2 cells, although this did not generate more IL-4+ Th2 cells (**Figure 2A**). Thus, we evaluated TSLP production by human skin

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3 samples cultured in the presence or absence of vitD3. Overall, TSLP was detected at significantly
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6 higher levels in the culture supernatant after either calcitriol or calcipotriol treatments (**Figure 2B**).
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8 Nevertheless, the TSLP concentrations of different donors were spread across a large range. To
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10 acknowledge this variability, we defined thresholds based on the Mean-minus-SEM for TSLP
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12 concentrations obtained in vitD3-treated skin. Accordingly, donors were split into two groups: those
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14 concentrations obtained in vitD3-treated skin. Accordingly, donors were split into two groups: those
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16 that showed strong TSLP production upon vitD3 exposure (responding: $[\text{TSLP}] > \text{Mean-SEM}$) and those
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18 that responded poorly or not at all (non-responding: $[\text{TSLP}] < \text{Mean-SEM}$). With thresholds set at
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20 56.63pg/mL for calcitriol and 62.73pg/mL for calcipotriol, the rates of responding donors were 29.4%
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22 and 32.1%, respectively.
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26 We took advantage of this dichotomy to evaluate the importance of TSLP for Th2 orientation. We
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28 reanalyzed the corresponding data from T-helper polarization assays (**Figure 1**). As compared with
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30 untreated skin, IL-13+ Th2 cells were significantly augmented when DCs from TSLP-producing skin
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32 were used, which was not the case for DCs from non-responding skin (**Figure 2C**). On the other hand,
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34 vitD3 did increase the capacity of skin DCs to promote IL-4+ Th2 cells, but this occurred even with
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36 donors that showed poor response in terms of TSLP production (**Figure 2D**). This suggested that only
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38 IL-13 production is positively influenced by TSLP-exposed skin DCs.
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43 To confirm the role of TSLP, we introduced an antagonistic anti-TSLPR antibody into the supernatant
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45 of skin explant cultures, in order to block potential effects of TSLP on skin DCs as early as possible. As
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47 expected, this did not alter the rate of IL-13+ Th2 cells obtained with vitD3-exposed DCs from non-
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49 responding skin (**Figure 2E, left**). Yet, blocking TSLPR abrogated the capacity of DCs from vitD3-
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51 treated, TSLP-producing skin explants to stimulate the generation of IL-13+ Th2 cells (**Figure 2E,**
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53 **right**). With respect to IL-4+ Th2 cells, no significant effect of TSLPR blocking could be observed with
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55 either non-responding or responding skin samples (**Figure 2F**). This demonstrates a link between the
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presence of TSLP in the supernatant of skin explants and IL-13 production by Th2 cells induced by vitD3-treated DCs.

Vitamin D3 alters the proportions of skin DC subsets but not their migration

To explore how vitD3 exposure affects cutaneous antigen-presenting cells, we examined emigrating HLA-DR+ skin DCs (**Figure 3A**). When obtained by explant culture of healthy, untreated skin, they comprise CD207+ CD14- epidermal LCs, CD14- dermal DCs (CD141+ cDC1 and CD1c+ cDC2)^{9;10}, and CD14+ dermal macrophage-like cells^{11;12}. The proportion and absolute numbers of CD207- CD14+ cells were markedly increased by vitD3 treatment (**Figure 3B**), in agreement with previous work¹³. Microscopic analysis showed that most of emigrating cells coexpressed HLA-DR and CD14 upon vitD3 exposure (**Figure S2**). Conversely, CD207+ CD14- LCs and CD207- CD14- dermal DCs were decreased as compared to untreated skin (**Figure 3B**). Within CD14- dermal DCs, CD141+ cDC1 were also missing from the supernatant of vitD3-treated skin (**Figure 3C**). The proportion of CD14+ cells was similar with responding and non-responding skin samples (**Figure 3D**) and was not affected by TSLPR blocking (**Figure 3E**). Therefore, the increase of CD14+ cells was independent of TSLP.

Then, we questioned whether vitD3 potentiates emigration of bona fide CD14+ dermal cells, as previously proposed¹³, or rather upregulates CD14 expression on CD14- DC subsets. We first noticed that identical numbers of total HLA-DR+ cells migrated from untreated and vitD3-treated skin explants (**Figure 3F**). Cryosections of skin samples after 3 days of culture were stained for HLA-DR and CD14 (**Figure S3A**) to quantify HLA-DR+ CD14+ cells that did not emigrate. Strikingly, CD14+ cell density was not diminished by vitD3 treatment, and was even increased with calcitriol (**Figure S3B**). This strongly suggests that there is no dramatic increase in the migratory capacity of CD14+ dermal cells.

Vitamin D3 affects the phenotype of skin DCs and MonoDCs

We thus suspected that vitD3 promoted phenotypical modifications of CD14⁻ DCs, including increased expression of CD14. To identify the cellular targets of vitD3 among skin DCs, we quantified mRNA of calcitriol 24-hydroxylase (CYP24A1), which degrades calcitriol into excreted calcitroic acid and is expressed under the control of VDR-mediated transcriptional activation¹. CYP24A1 was absent from all DCs migrating from untreated skin. However, following vitD3 treatment, a clear expression of CYP24A1 appeared, which was specific of CD14⁺ cells (**Figure 4A**). Therefore, the CD14⁺ population emigrating from vitD3-treated explants includes cells that had their gene expression directly modulated by vitD3 and that are different from bona fide CD14⁺ dermal cells of untreated skin.

To substantiate our hypothesis, we set out to analyze well-known markers of human skin DC subsets (**Figures 4 and S4**). CD14⁺ cells recovered from vitD3-treated skin explant cultures had a decreased expression of typical markers of macrophages and CD14⁺ macrophage-like cells (CD209, CD163, CD206), which are poorly expressed by CD14⁻ cDC2 of untreated skin. Nevertheless, vitD3-elicited CD14⁺ cells did not gain markers of cDC2 (CLEC10A, CD1c), and they increased CD141. As expected¹¹, CD14⁺ cells emigrating from untreated skin did not express CD86, as opposed to mature CD14⁻ DCs (**Figure 4B**). However, after vitD3 treatment, CD86 and CD83 were both present on CD14⁺ cells (**Figure 4B-C**). HLA-DR levels did not change in CD14⁺ cells but increased in CD14⁻ DCs (**Figure S4**, bottom panel). CD14⁺ cells from vitD3-treated skin had higher TSLPR surface levels (**Figure 4D**) and decreased OX40L expression (**Figure 4E**). Overall, the phenotype of CD14⁺ cells showed significant variations upon vitD3 treatment, whereas the remaining CD14⁻ cells were unaltered as compared to untreated skin (**Figure 4F**), in line with their lack of CYP24A1 expression. Therefore, CD14⁺ cells induced by vitD3 do not only comprise CD14⁺ macrophage-like cells found in untreated skin, but also transitional, vitD3-elicited CD14⁺ DCs. They may stem from CD14⁻ DCs, which are the main cells expressing CD86 and TSLPR in untreated skin¹¹.

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An elevated CD14 expression has been consistently reported for MonoDCs exposed to vitD3 throughout their differentiation from monocytes²⁻⁶. To verify whether vitD3 also affects fully differentiated cells such as skin DCs, MonoDCs generated with GM-CSF and IL-4 were stimulated for 24h with calcitriol. They showed a dose-dependent upregulation of CYP24A1 mRNA (**Figure S5A**), supporting that MonoDC gene expression is directly modulated by vitD3. Furthermore, CD14 upregulation by MonoDCs (**Figure S5B**) sustained the notion that this represents a primary effect of vitD3. The increased surface expression of CD14 did not reflect an impaired shedding of this receptor, since soluble CD14 was rather detected in higher amounts in the supernatant of vitD3-treated MonoDCs (**Figure S5C**).

Vitamin D3-elicited CD14+ skin DCs are TSLP-independent inducers of IL-4+ Th2 differentiation.

Based on the above-described phenotypic analyses of emigrating cells, we propose that vitD3 directly affects skin DCs, eliciting a new subset of transitional DCs displaying high CD14 levels. Thus, we asked whether these vitD3-elicited CD14+ DCs might contribute to TSLP-independent Th2 polarization. Choosing only non-responding donors to exclude any contribution of TSLP, CD14+ cells were isolated with anti-CD14 magnetic beads from total emigrating DCs. The enrichment procedure itself did not influence the maturation of positively selected CD14+ cells, as verified by their CD86 expression (data not shown). The resulting CD14+ and CD14- subsets were then separately cocultured with allogeneic naïve T cells. CD14+ DCs from vitD3-exposed skin induced less proliferation of total T cells than the CD14+ dermal cells of untreated skin (**Figure 5A**), without affecting T-cell viability (**Figure S1E**). As expected for a TSLP-independent process, untreated and vitD3-treated CD14+ cells had a similar capacity to promote IL-13+ Th2 cells (**Figure 5B**). However, both calcitriol and calcipotriol conferred to transitional CD14+ DCs a superior ability to induce the differentiation of IL-4+ Th2 cells (**Figure 5C-D**), and they did not support Th1 polarization (**Figure 5E**). On the other hand, CD14- DCs were

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3 unaffected by vitD3 for their influence on T-cell proliferation (**Figure 5F**) and polarization (**Figure 5G-**
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6 **J**), despite a slightly decreased T-cell viability in the calcitriol-treated condition (**Figure S1F**).
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8 Altogether, our findings indicate that CD14⁺ DCs arising in vitD3-treated skin acquire the capacity to
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10 induce IL-4⁺ Th2 responses independently of TSLP.
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DISCUSSION

In the here presented study, topically applied and soluble vitD3 potently affected the phenotype and function of cutaneous human DCs. In particular, in skin donors that presented the strongest capacity for TSLP production upon vitD3 treatment, TSLP-exposed DCs enhanced the production of IL-13 by Th2 cells. Nevertheless, vitD3 also promoted direct effects, eliciting CD14+ DCs that differed from the well-described CD14+ dermal cells of untreated skin¹¹ and supported TSLP-independent differentiation of IL-4+ Th2 cells.

VitD3 consistently drives DCs to inhibit Th1 polarization²⁻⁴, similarly to our findings. With skin DCs, it was proposed that differentiation of Treg could explain this outcome^{7;13}, which we could not confirm. A Th2-inducing bias that opposes Th1 differentiation appears to be the most likely explanation for the lack of Th1 cells in our experimental settings. In previous investigations involving human skin DCs, vitD3 was provided in addition to LPS¹³ or IL-1 β +TNF- α ⁷. When used alone, such potent maturation factors are known to favor Th1 priming²⁸. Furthermore, we noted that mature skin DCs did not sense vitD3 (data not shown). For these reasons, we focused on culturing untreated or vitD3-treated explants in the absence of additional adjuvants, which is sufficient to achieve spontaneous migration of DCs. With either method, DCs appeared similarly mature and capable of naïve T cell priming, yet functional differences are expected that very likely explain differences between our findings and published studies^{7;13}.

In mouse models, cutaneous exposure to calcipotriol has been convincingly demonstrated to induce TSLP *in vivo*²⁰, but a similar outcome had yet to be proven in human skin. In our assays, vitD3 induced potent TSLP production in only ~30% of skin samples, which could explain why this has been previously overlooked¹⁸. Differences in sensitivity to and/or consequences of vitD3 exposure may stem from the origin of human skin samples. Since they were collected as surgical waste, the clinical

history of donors could not be traced back, in accordance with our current ethical agreement. Therefore, the reasons for TSLP production variability remains unclear. Our samples might have included patients with atopic skin disorders. However, we obtained only healthy skin with no apparent atopic lesion, and atopic patients display TSLP staining within their active lesions but not in perilesional skin¹⁶. We may also suspect skin phenotype differences that modify the half-life of experimentally applied bioactive vitD3²⁹.

Segregating our data according to TSLP levels allowed us to reveal that skin DCs from TSLP-producing donors were more prone to shift Th2 differentiation towards IL-13+ cells. IL-13 expression by T cells can be provoked by TSLP directly in the mouse³⁰ or via human MonoDCs¹⁶ and LCs³¹. In line with this, we observed a strong bias for IL-13 production when DCs were collected from TSLP-exposed skin explants. A dominant IL-13 production has been proposed as a hallmark of pathogenic Th2 cells³², notably because IL-13R α is particularly present on epithelial cells and impairs expression of the epidermal barrier constituents³³.

Despite this clear TSLP-dependent promotion of IL-13+ cells, the poor correlation between TSLP amounts and the capacity of skin DCs to generate IL-4+ Th2 cells suggested a TSLP-independent effect. Among other consequences, an increase in the rate of CD14+ cells emigrating from skin explants was observed upon treatment with vitD3. This prominent effect has been presumed to result from increased migration of CD14+ dermal cells^{7;13}. Our interpretation differs because (1) total numbers of emigrating DCs were not modified by vitD3 treatment, (2) the massive increase in CD14+ cells in the supernatant did not correlate with a decrease of CD14+ cells remaining in the dermis and (3) immature MonoDCs exposed to vitD3 showed a consistent upregulation of CD14. The latter effect has been repeatedly pointed out upon vitD3 treatment of monocytes during in vitro differentiation into DCs^{2;3;5}. We confirmed that vitD3 led terminally differentiated MonoDCs to upregulate both

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surface and soluble CD14. Moreover, immature LCs and dermal DCs sorted from human skin and exposed to vitD3 also upregulate CD14 to some extent⁷. Therefore, we propose that vitD3 does not promote emigration of the well-described CD14+ dermal cells but rather leads skin DCs to phenotypical changes. Although we gathered strong evidence supporting this, CD14+ DCs that dominate migratory cell suspensions after vitD3 treatment remain of unclear origin. They do not express macrophage-like markers, but they also lack other typical features of dermal CD14- cDC2. These complex alterations, which were independent of TSLP but may not be only due to vitD3, result in a yet undescribed phenotype for skin DCs and, importantly, bring a strong bias in their T-cell priming function. Indeed, vitD3-elicited CD14+ DCs appeared sufficient to efficiently generate IL-4+ Th2 cells. Nevertheless, our phenotypic analyses did not reveal which alterations could be responsible for this function. The hallmark of vitD3 exposure, CD14, is unlikely to play a role in T-cell differentiation. OX40L, a known Th2 inducer^{16;20;21}, is rather decreased, and TSLPR, although increasing, should not be involved in skin explants that release little TSLP. Altogether, this intriguing differentiation pathway may be involved in the regulation of IL-4/IL-13 balance in the skin and should be further investigated for its potential to harness a relevant therapeutic approach limiting the atopic march and inflammation in skin allergies.

Overall, our findings provide several interesting insights for clinical research. Topical application of calcipotriol ointment is a well-established treatment for psoriasis¹. In addition to limiting keratinocyte proliferation and promoting their differentiation, this treatment induces TSLP in lesional psoriatic skin²⁴. A shift towards Th2 responses may be beneficial in this Th17/Th22-driven disease. Of note, dermatitis, erythema and xerosis, which are hallmarks of atopic dermatitis, may develop in 1-10% of patients as a side effect of the DaivonexTM calcipotriol ointment ([Medscape](#)). Moreover, a case report pointed out that erroneous prescription of this drug to an atopic patient results in a dermatitis flare³⁴. Finally, we demonstrate the potential of using vitD3 in the design of human skin

models reproducing early immune responses in atopic dermatitis³⁵. This strategy, aiming at reducing animal use and ameliorating the safety of clinical trials, currently represents an important goal in drug discovery.

For Peer Review

References

1. Tremezaygues L, Reichrath J. Vitamin D analogs in the treatment of psoriasis: Where are we standing and where will we be going? *Dermatoendocrinol* 2011;3:180-186.

2. Berer A, Stöckl J, Majdic O et al. 1,25-dihydroxyvitamin D₃ inhibits dendritic cell differentiation and maturation in vitro. *Experimental Hematology* 2000;28:575-583.

3. Piemonti L, Monti P, Sironi M et al. Vitamin D₃ affects differentiation, maturation, and function of human monocyte-derived dendritic cells. *J Immunol* 2000;164:4443-4451.

4. Penna G, Amuchastegui S, Giarratana N et al. 1,25-dihydroxyvitamin d3 selectively modulates tolerogenic properties in myeloid but not plasmacytoid dendritic cells. *J Immunol* 2007;178:145-153.

5. Sommer A, Fabri M. Vitamin D regulates cytokine patterns secreted by dendritic cells to promote differentiation of IL-22-producing T cells. *PLoS One* 2015;10:e0130395.

6. Pedersen AW, Holmstrom K, Jensen SS et al. Phenotypic and functional markers for 1alpha,25-dihydroxyvitamin D(3)-modified regulatory dendritic cells. *Clin Exp Immunol* 2009;157:48-59.

7. van der Aar AM, Sibiryak DS, Bakdash G et al. Vitamin D3 targets epidermal and dermal dendritic cells for induction of distinct regulatory T cells. *J Allergy Clin Immunol* 2011;127:1532-1540.

8. Nikolic T, Roep BO. Regulatory multitasking of tolerogenic dendritic cells - lessons taken from vitamin d3-treated tolerogenic dendritic cells. *Front Immunol* 2013;4:113.

9. Bigley V, McGovern N, Milne P et al. Langerin-expressing dendritic cells in human tissues are related to CD1c+ dendritic cells and distinct from Langerhans cells and CD141high XCR1+ dendritic cells. *J Leukoc Biol* 2015;97:627-634.

10. Haniffa M, Shin A, Bigley V et al. Human Tissues Contain CD141(hi) Cross-Presenting Dendritic Cells with Functional Homology to Mouse CD103(+) Nonlymphoid Dendritic Cells. *Immunity* 2012;37:60-73.

11. McGovern N, Schlitzer A, Gunawan M et al. Human dermal CD14(+) cells are a transient population of monocyte-derived macrophages. *Immunity* 2014;41:465-477.

12. Banchereau J, Thompson-Snipes L, Zurawski S et al. The differential production of cytokines by human Langerhans cells and dermal CD14(+) DCs controls CTL priming. *Blood* 2012;119:5742-5749.

13. Bakdash G, Schneider LP, Van Capel TM, Kapsenberg ML, Teunissen MB, De Jong EC. Intradermal application of vitamin D3 increases migration of CD14+ dermal dendritic cells and

- promotes the development of Foxp3⁺ regulatory T cells. *Hum Vaccin Immunother* 2013;9:250-258.
14. Liu Y-J, Soumelis V, Watanabe N et al. TSLP: An Epithelial Cell Cytokine that Regulates T Cell Differentiation by Conditioning Dendritic Cell Maturation. *Annu Rev Immunol* 2007;25:193-219.
 15. Varricchi G, Pecoraro A, Marone G et al. Thymic Stromal Lymphopoietin Isoforms, Inflammatory Disorders, and Cancer. *Front Immunol* 2018;9:1595.
 16. Soumelis V, Reche PA, Kanzler H et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002;3:673-680.
 17. Xie Y, Takai T, Chen X, Okumura K, Ogawa H. Long TSLP transcript expression and release of TSLP induced by TLR ligands and cytokines in human keratinocytes. *J Dermatol Sci* 2012;66:233-237.
 18. Landheer J, Giovannone B, Sadekova S et al. TSLP is differentially regulated by vitamin D3 and cytokines in human skin. *Immun Inflamm Dis* 2015;3:32-43.
 19. Li M, Hener P, Zhang Z, Kato S, Metzger D, Chambon P. Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis. *Proc Natl Acad Sci U S A* 2006;103:11736-11741.
 20. Li M, Hener P, Zhang Z, Ganti KP, Metzger D, Chambon P. Induction of thymic stromal lymphopoietin expression in keratinocytes is necessary for generating an atopic dermatitis upon application of the active vitamin D3 analogue MC903 on mouse skin. *J Invest Dermatol* 2009;129:498-502.
 21. Pattarini L, Trichot C, Bogiatzi S et al. TSLP-activated dendritic cells induce human T follicular helper cell differentiation through OX40-ligand. *J Exp Med* 2017;214:1529-1546.
 22. Ghoreishi M, Bach P, Obst J, Komba M, Fleet JC, Dutz JP. Expansion of antigen-specific regulatory T cells with the topical vitamin d analog calcipotriol. *J Immunol* 2009;182:6071-6078.
 23. Lechner TM, Satake A, Harrison VS et al. Skin-derived TSLP systemically expands regulatory T cells. *J Autoimmun* 2017;79:39-52.
 24. Sato-Deguchi E, Imafuku S, Chou B, Ishii K, Hiromatsu K, Nakayama J. Topical vitamin D(3) analogues induce thymic stromal lymphopoietin and cathelicidin in psoriatic skin lesions. *Br J Dermatol* 2012;167:77-84.
 25. Berg S, Kutra D, Kroeger T et al. ilastik: interactive machine learning for (bio)image analysis. *Nat Methods* 2019;16:1226-1232.
 26. Clark RA, Chong BF, Mirchandani N et al. A novel method for the isolation of skin resident T cells from normal and diseased human skin. *J Invest Dermatol* 2006;126:1059-1070.
 27. Leyva-Castillo JM, Hener P, Michea P et al. Skin thymic stromal lymphopoietin initiates Th2 responses through an orchestrated immune cascade. *Nat Commun* 2013;4:2847.

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28. Schuler-Thurner B, Schultz ES, Berger TG et al. Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. *J Exp Med* 2002;195:1279-1288.

29. Carlberg C, Haq A. The concept of the personal vitamin D response index. *J Steroid Biochem Mol Biol* 2018;175:12-17.

30. Rochman Y, Dienger-Stambaugh K, Richgels PK et al. TSLP signaling in CD4(+) T cells programs a pathogenic T helper 2 cell state. *Sci Signal* 2018;11.

31. Ebner S, Nguyen VA, Forstner M et al. Thymic stromal lymphopoietin converts human epidermal Langerhans cells into antigen presenting cells that induce pro-allergic T cells. *J Allergy Clin Immunol* 2007;119:982-990.

32. Ito T, Wang YH, Duramad O et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med* 2005;202:1213-1223.

33. Bieber T. Interleukin-13: Targeting an underestimated cytokine in atopic dermatitis. *Allergy* 2020;75:54-62.

34. Turner MJ, Dasilva-Arnold SC, Yi Q, Mehrotra P, Kaplan MH, Travers JB. Topical application of a vitamin D analogue exacerbates atopic dermatitis and induces the atopic dermatitis-like phenotype in Stat6VT mice. *Pediatr Dermatol* 2013;30:574-578.

35. Bernard M, Carrasco C, Laoubi L et al. IL-1beta induces thymic stromal lymphopoietin and an atopic dermatitis-like phenotype in reconstructed healthy human epidermis. *J Pathol* 2017;242:234-245.

FIGURE LEGENDS

Figure 1: Vitamin D3-exposed skin DCs induce Th2 responses.

Human skin explants were stimulated 3 days with 200nM soluble calcitriol (into the medium) or 100mg topical calcipotriol ointment. Emigrating skin DCs were collected and cultured with CFSE-labelled allogeneic naïve CD4⁺ T cells. Intracellular cytokines were detected by flow cytometry upon restimulation with PMA+Ionomycine. **(A)** Percentage of proliferative (CFSE^{low}) T cells after 7 days of MLR with total cells emigrating from untreated or vitamin D3-exposed skin explants (n=26). Representative dot plots and percentages of cells in thick-lined gates define Th2 subsets: **(B)** IFN- γ - IL-13⁺ (n=40) **(C)** IFN- γ - IL-4⁺ (n=30) **(D)** proliferating IL-13⁺ (n=23) or **(E)** proliferating IL-4⁺ cells (n=19). **(F)** IL-4 released in 24h-restimulated MLR supernatant (n=28). **(G)** Percentages of IFN- γ ⁺ Th1 cells (n=37) **(H)** FoxP3⁺ Treg (n=10) and **(I)** IFN- γ - IL-10⁺ T cells (n=10). Data are presented as Mean $\pm$ SEM. n= total number of skin explant donors. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (paired Student's t-tests except **(F)** Wilcoxon non-parametric tests).

Figure 2: TSLP production in vitamin D3-treated human skin regulates the capacity of Th2 cells to produce IL-13.

(A) Percentages of IFN- γ - IL-13⁺ (n=6) and IFN- γ - IL-4⁺ (n=5) CD4⁺ T cells after MLR with emigrating DCs from TSLP-stimulated skin explants **(B)** TSLP released after 3 days of skin explant culture (n=53). Dashed lines depict thresholds defined from Mean-SEM values, allowing to split donors into responding and non-responding populations. **(C)** IFN- γ - IL-13⁺ Th2 percentages for MLR with skin DCs from non-responding (n=24) or responding (n=8) donors. **(D)** IFN- γ - IL-4⁺ Th2 percentages for MLR with skin DCs from non-responding (n=20) or responding (n=6) donors. **(E, F)** Anti-TSLPR antibody or isotype control were added into the supernatant during skin explant cultures. **(E)** IFN- γ - IL-13⁺ and **(F)** IFN- γ - IL-4⁺ (n=19 for non-responding, n=7 for responding). Data are presented as Mean $\pm$ SEM. n= total number of skin explant donors. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (paired Student's t-tests except **(F)** Wilcoxon non-parametric tests).

Figure 3: Vitamin D3 alters DC populations emigrating from skin explants

(A) Gating strategy to determine the phenotype of emigrating HLA-DR⁺ cells by flow cytometry. Representative dot plots and quantification are shown for **(B)** CD14⁺ CD207⁻, CD14⁻ CD207⁻ (n=44 for percentages, n=16 for absolute numbers), CD14⁻ CD207⁺ LCs (n=8 for both) and **(C)** CD14⁻ CD207⁻ CD141⁺ cDC1s (n=15 for percentages, n=7 for absolute numbers). **(D)** Percentages of CD14⁺ cells emigrating from non-responding (n=36) and responding skin explants (n=15). **(E)** Percentages of CD14⁺ cells emigrating from calcitriol- or calcipotriol-responding skin with or without anti-TSLPR antibody or isotype control (n=4) **(F)** Total number of migrating cells for one skin explant (n=16). Data are presented as Mean $\pm$ SEM. n= total number of skin explant donors. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (paired Student's t-tests).

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Figure 4: Vitamin D3 induces phenotypic modifications of skin DCs into a CD14+ subset

(A) Relative expression levels for CYP24A1 mRNA in CD14+ and CD14- emigrating subsets (n=4). (B) Representative histogram overlays and mean fluorescence intensities (MFI) for CD86 (n=19). (C) Representative histogram overlays and MFI for CD83 (n=4). (D) Representative histogram overlays and MFI for TSLPR (n=4). (E) Representative histogram overlays and MFI for OX40L (n=5). (F) Radar plots depicting the average MFI of all tested donors for CD1c, CD141, CD86, CD163, CD209, TSLPR, CLEC10A, CD83, HLA-DR, OX40L and CD206 for CD14+ and CD14- populations (see detailed analysis in Figure S4). Data are presented as Mean ± SEM. n= total number of skin explant donors. ns: non-significant, * p<0.05, **p<0.01, ****p<0.0001 (paired Student's t-tests).

Figure 5: Vitamin D3-elicited CD14+ cells induce IL4+ Th2 cells independently of TSLP.

Magnetically separated CD14+ and CD14- cells from untreated or vitamin D3-exposed skin explants were cocultured with allogenic naïve T cells. Data presented here only shows results from skin explants that did not respond to vitamin D3 by TSLP production (non-responding donors). (A-J) Percentages of (A, F) proliferative (CFSE^{low}) T cells (n=12 for CD14+, n=9 for CD14-) (B, G) IFN-γ- IL-13+ (n=13 for CD14+, n=10 for CD14-) or (C, H) IL-4+ Th2 cells (n=12 for CD14+, n=7 for CD14-). (D, I) IL-4 in 24h restimulated supernatants (n=13 for CD14+, n=9 for CD14-). (E, J) Percentages of IFN-γ+ Th1 cells (n=13 for CD14+, n=10 for CD14-). Data are presented as Mean ± SEM. n= total number of skin explant donors. ns: non-significant, *p<0.05, **p<0.01 (paired Student's t-tests).

SUPPORTING INFORMATION

Supplementary methods

Immunofluorescence microscopy

Skin explant samples were frozen embedded in O.C.T Compound (CellPath) and 16µm sections were prepared, mounted on SuperFrost-Plus™ glass slides (Thermo Scientific), fixed in cold acetone for 20min and air-dried. Sections were incubated overnight at 4°C with antibodies against HLA-DR (L203) and CD14 (AB383) (R&D Systems), diluted in PBS-10% FCS. Thereafter, they were incubated 2h at room temperature with DAPI (100ng/mL) and their corresponding secondary antibody: Donkey anti-Mouse IgG AlexaFluor 555 (Molecular Probes, A31570) and Donkey anti-Goat IgG AlexaFluor 647 (Jackson ImmunoResearch, 705-605-147). Sections were then mounted with Fluoromount G medium (EBioscience, 00-4958-02). Images were acquired on a Zeiss Axio-Observer Z1 epifluorescent inverted microscope driven by the Metamorph software, using a 10x EC Plan Neofluar objective. Quantification was based on 85 images obtained from 11 different donors. Nuclei prediction maps were realized using the pixel classification module in Ilastik software²⁵ on the DAPI staining channel. Based on this prediction maps, the object classification module of Ilastik was used to train the software to determine to which of the 4 following classes belongs each nuclei: epidermal cells, HLA-DR+ cells, HLA-DR+ CD14+ cells, unstained cells. We then determined the number of nuclei for each class using the ImageJ software.

Skin-emigrating DCs were fixed with paraformaldehyde and stained as described above. Images were acquired with a spinning disk confocal head (Yokogawa, CSU-X1), using a 100x Plan Apochromat oil immersion NA1.4 objective and analyzed with ImageJ software.

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7 **Figure S1: Vitamin D3 weakly alters viability of skin DCs and T cells**
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9 (A-F) Survival of HLA-DR+ or CD3+ cells before (A,B) and after (C-F) MLR. Data are presented as Mean
10 ± SEM. n= total number of skin explant donors. ns: non-significant, **p<0.01, ***p<0.001 (paired
11 Student's t-tests).
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15 **Figure S2: Vitamin D3 alters emigrating skin DC populations**
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17 Representative immunofluorescence of cells emigrating from untreated, calcitriol- or calcipotriol-
18 treated skin explant (3 days). Scale bars: 25µm. Cells were stained with DAPI (blue), anti-CD14 (green)
19 and anti-HLA-DR (red) (n=2).
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24 **Figure S3: CD14+ cells are not depleted from the dermis after skin explant culture with vitamin D3.**
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26 (A) Representative immunofluorescence images of skin explants after 3 days of culture. Cryosections
27 were stained with DAPI (blue), anti-CD14 (green) and anti-HLA-DR (red) monoclonal antibodies.
28 Insets correspond to the regions highlighted in the larger images. Arrows point to HLA-DR+ CD14+
29 (yellow) cells. HLA-DR+ CD14- (red) and HLA-DR low CD14+ (green) are also visible. Scale bars: 50µm.
30 (B) Numbers of CD14+ cells in skin sections and percentages of CD14+ cells among total dermal cells
31 (n=11). Data are presented as Mean ± SEM. n= total number of skin explant donors. ns: non-
32 significant, **p<0.01 (paired Student's t-tests).
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38 **Figure S4: Vitamin D3 alters the phenotype of skin-emigrating HLA-DR+ cells**
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40 Representative dot plots and MFI quantification of CD209 (n=8), CD163 (n=6), CD206 (n=8), CLEC10A
41 (n=5), CD1c (n=7), CD141 (n=13), HLA-DR (n=20) expression on emigrating CD14+ and CD14- cells.
42 Data are presented as Mean ± SEM. n= total number of skin explant donors. ns: non-significant,
43 *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (paired Student's t-tests).
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48 **Figure S5: Vitamin D3-treated MonoDCs upregulate CD14 expression**
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50 Immature MonoDCs were stimulated for 24h with vitamin D3 or left untreated. (A) Relative
51 expression of CYP24A1 after 24h-stimulation with different doses of calcitriol: 2nM, 20nM (n=3) and
52 200nM (n=5). (B) Mean fluorescence intensity of CD14 after 200nM calcitriol treatment (n=12). (C)
53 soluble CD14 release (n=5). Data are presented as Mean ± SEM. n= total number of skin explant
54 donors. ns: not significant, *p<0.05, ****p<0.0001 (paired Student's t-tests).
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Figure 1: Vitamin D3-stimulated skin DCs induce Th2 responses.

600x840mm (96 x 96 DPI)

Figure 2: TSLP production in vitamin D3-treated human skin regulates the capacity of Th2 cells to produce IL-13.

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600x840mm (96 x 96 DPI)

Figure 5: Vitamin D3-elicited CD14+ cells induce IL4+ Th2 cells independently of TSLP.

600x840mm (96 x 96 DPI)

Figure S1: Vitamin D3 weakly alters viability of skin DCs and T cells

600x840mm (96 x 96 DPI)

Figure S2: Vitamin D3 alters emigrating skin DCs populations

600x840mm (96 x 96 DPI)

Figure S3: CD14+ HLA-DR+ cells are not depleted from the dermis after skin explant culture with vitamin D3.

600x840mm (96 x 96 DPI)

Figure S4: Vitamin D3 alters the phenotype of skin-emigrating HLA-DR+ cells

600x840mm (96 x 96 DPI)

Figure S5: Vitamin D3-treated MonoDCs upregulate CD14 expression

600x840mm (96 x 96 DPI)

Vitamin D3-elicited CD14⁺ human skin dendritic cells promote thymic stromal lymphopoietin-independent type 2 T-helper responses

Short title: Vitamin D3 directs human skin DCs to prime Th2

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26 BATMAN).

28 ***Author contributions***

29 VF and AB conceived the study, designed experiments, and analyzed data. AB performed
30 experiments, with the assistance of AH and QM for explant culture preparations. Quantifications of
31 immunofluorescence microscopy images were performed by JDF. AB, CM and VF wrote the
32 manuscript.

33 **Word count:** 3736 words

For Peer Review

Abstract (249 words)

Background

Immune modulation by vitamin D3 through dendritic cells (DCs) remains controversial. Human DCs exposed in vitro counteract type-1 T-helper (Th1) differentiation and induce regulatory T cells. However, cutaneous application on mice promotes Th2-driven inflammation resembling atopic dermatitis and relying on Thymic Stromal Lymphopoietin (TSLP) from keratinocytes and T-cell orientation by TSLP-stimulated skin DCs. We studied the effects of vitamin D3 in human skin, focusing on TSLP production and the role of skin DCs in T-cell differentiation.

Methods

Human healthy skin explants were exposed in vitro to vitamin D3 analogs. Migrating DCs were analyzed and TSLP quantified in the supernatant. Allogeneic naïve CD4+ T cells were cocultured with DCs to assess their proliferation and cytokine production.

Results

Vitamin D3 induced skin DCs to differentiate Th2 cells producing IL-4 and IL-13. Vitamin D3 triggered TSLP release in ~30% of skin explants, correlating with IL-13 detection in Th2 cells. In these donors, blocking TSLP receptor during skin explant cultures abrogated IL-13 production, yet IL-4+ Th2 cells were unaffected. Among skin DCs emerged CD14+ cells that had responded directly to vitamin D3 and differed from classical CD14+ dermal emigrants. Vitamin D3-elicited CD14+ DCs sufficed to promote IL-4+ Th2 cells in a TSLP-independent manner.

Conclusion

Vitamin D3, despite inducing TSLP in some donors, had a direct influence on skin DCs, affecting their phenotype and ability to drive Th2 responses independently of TSLP. Our findings pave the way

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56 towards in vitro systems that accurately model human cutaneous Th2 responses, notably involved in
57 atopic dermatitis.

58 **Keywords (5)**

59 Atopic dermatitis, Dendritic cell, T helper 2, Thymic stromal lymphopoietin, Vitamin D3.

For Peer Review

Abbreviations

vitD3:	Vitamin D3
cDCs:	conventional dendritic cells
DCs:	Dendritic cells
LCs:	Langerhans cells
MLR:	Mixed leukocyte reaction
MonoDCs:	Monocyte-derived dendritic cells
Th:	T helper
Treg:	Regulatory T cell
TSLP:	Thymic stromal lymphopoietin
TSLPR:	Thymic stromal lymphopoietin receptor
VDR:	Vitamin D receptor
CYP24A1:	Cytochrome P450 family 24 subfamily A member 1
UVB:	type B ultraviolet light
FCS:	Fetal calf serum
FVD:	Fixable Viability Dye
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
RPMI:	Roswell Park Memorial Institute medium
LPS:	Lipopolysaccharide
PMA:	Phorbol Myristoyl Acetate
CFSE:	Carboxyfluorescein succidiny ester
HPRT1:	Hypoxanthine phosphoribosyltransferase 1
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
SEM:	Standard error of the mean

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85 **INTRODUCTION**

86 Bioactive 1,25-dihydroxy-vitamin D3 (calcitriol) triggers nuclear translocation and DNA binding of the
87 vitamin D receptor (VDR), an ubiquitous transcription factor notably involved in the regulation of
88 calcium and phosphate storage¹. In psoriasis, antiproliferative effects of calcitriol on keratinocytes
89 have been harnessed to counteract acanthosis.

90 Immunoregulatory functions of vitamin D3 (vitD3) are a consequence of modulated gene expression
91 in immune cells, in particular dendritic cells (DCs). Most studies have focused on the influence of
92 vitD3 during in vitro differentiation of monocytes into monocyte-derived DCs (MonoDCs) with GM-
93 CSF and IL-4²⁻⁶. The resulting CD1a^{low} CD14^{high} MonoDCs displayed low levels of MHC class II, CD80
94 and CD86 upon maturation and produced anti-inflammatory IL-10 rather than the type 1 T-helper
95 (Th1)-driving cytokine IL-12²⁻⁶. Such features likely explain why they showed a primarily tolerogenic
96 influence, yielding poor T-cell proliferation, limited Th1 polarization and an increase in regulatory T
97 cell (Treg) differentiation^{4;7;8}.

98 Human skin harbors several subsets of DCs with distinct phenotypes and functions. Danger signals
99 trigger their maturation, which is required for migration to lymph nodes and T-cell stimulation. The
100 epidermis contains exclusively CD14⁻ CD207⁺ Langerhans cells (LCs), whereas CD14⁻ dermal DCs
101 comprise CD1c⁺ type 2 conventional DCs (cDC2) and a smaller population of CD141⁺ cDC1^{9;10}. Part of
102 dermal CD14⁺ cells exhibit some motility and thus can be harvested from skin explant cultures
103 together with other DC subsets. However, they strongly resemble sessile CD14⁺ macrophages and
104 lack the chemokine receptor CCR7 that allows cDCs and LCs to colocalize with naive T cells in lymph
105 nodes^{11;12}. When exposed to maturation factors in the presence of vitD3, both dermal DCs and
106 epidermal LCs promoted a moderate increase in Treg differentiation^{7;13}.

Thymic stromal lymphopoietin (TSLP) is a proinflammatory soluble factor released mainly by suprabasal keratinocytes^{14;15}. The most commonly investigated form of TSLP is undetectable in healthy human skin¹⁶, although a shorter, alternatively-spliced TSLP variant might be present¹⁷. Within atopic dermatitis lesions, a basal expression of TSLP can be found¹⁶ and it can be induced in nonlesional skin by house dust mite extracts¹⁸.

In the mouse, cutaneous application of the vitD3 analog calcipotriol (MC903) promotes VDR-dependent TSLP expression as well as a Th2-driven inflammatory condition that mimicks many features of atopic dermatitis¹⁹. In both human and mouse models, TSLP stimulates DCs to support the development of Th2 responses^{16;20;21}. Despite this, there have been reports of in vivo Treg induction reminiscent of experiments with human DCs^{22;23}. Other discrepancies need to be addressed, such as poor TSLP production from human keratinocytes of healthy or nonlesional atopic skin upon stimulation with vitD3¹⁸, whereas calcitriol ointment has recently been shown to induce TSLP expression in lesional psoriasis skin²⁴.

Here, we applied vitD3 to human skin explants and investigated the consequences in terms of TSLP production, the phenotype of skin DCs and their functional ability to promote T-helper responses.

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METHODS

Human skin explants

Skin specimens were obtained from anonymized healthy subjects undergoing abdominal reduction, with written informed consent and institutional review board approval, in agreement with the Helsinki Declaration and French legislation. 4cm², ~1mm-thick biopsies were placed at air-liquid interface onto a 40µm cell strainer (Biologix) in 6mL RPMI1640 medium supplemented with 10µg/mL gentamycin, 100U/mL penicillin, 100µg/mL streptomycin, 10mM HEPES (Lonza) and 10% FCS (Dutscher). After 3 days, migrating cells were harvested and analyzed by flow cytometry and immunofluorescence. If necessary, CD14⁺ and CD14⁻ migrating cells were separated using CD14 MicroBeads (Miltenyi-Biotec). Where indicated, 50ng/mL TSLP, 1µg/mL anti-human TSLPR neutralizing antibody, or polyclonal goat IgG (R&D Systems) were added during the culture.

Generation of MonoDCs

Human monocytes were purified from buffy coats of healthy blood donors (EFS, Strasbourg, France) by successive Ficoll and Percoll gradients (Sigma-Aldrich). MonoDCs were differentiated from monocytes in 5 days with 200ng/mL GM-CSF (Schering-Plough) and 10ng/mL IL-4 (ImmunoTools), and characterized as CD14^{low} CD1a^{high} DC-SIGN^{high}.

Vitamin D3 treatment

200nM calcitriol (R&D Systems) was added to the culture medium of skin explant (3 days), MonoDCs (24h), or skin migrating cells (24h). Alternatively, using a commercially available vitD3 ointment (Daivonex 50µg/g, Leo Pharma), 100mg of calcipotriol ointment was applied onto the skin surface during the whole culture period (3 days).

Mixed leukocyte reactions

Naive CD4⁺ T cells (purity >95%) were isolated from peripheral blood mononuclear cells using human naive CD4⁺ T Cell Isolation Kit II (Miltenyi-Biotec). To determine the stimulatory capacity of vitD3-stimulated DCs, 2x10⁵ naive CD4⁺ T cells were cocultured with 2x10⁴ skin DCs in 200μL RPMI1640+10% pooled human sera (Corning) in 96-well U-bottom plates (Falcon). After 7 days, T cells were restimulated with 1μg/mL PMA and 1μM ionomycin (Sigma-Aldrich) in the presence of 10μg/mL brefeldin and 1μM monensin (BD-Biosciences) and analyzed by flow cytometry for intracellular expression of IFN-γ, IL-13 and IL-4. T-cell proliferation was determined by labelling naive CD4⁺ T cell with CFSE (Invitrogen) before starting the coculture.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants of skin explant cultures or MonoDCs were collected and analyzed for TSLP (eBioscience) and soluble CD14 (R&D Systems) production, respectively. After MLR, 10⁵ T cells were restimulated with PMA/ionomycin for 1 day and supernatants were evaluated for IL-4 production (BD-Biosciences). ELISA were performed in duplicates according to the manufacturer's recommendations. The absorbance was detected at 450nm using a Thermo Fisher Scientific Multiskan FC Microplate Photometer.

Flow cytometry

Cells were incubated for 20 min at 4°C with the following antibodies: CD86-FITC (clone FUN-1), CD83-APC (HB15e), DC-SIGN/CD209-PerCP-Cy5.5 (DCN46), CD1a-PE (HI149), CD3-AF700 (UCHT1), CD206-FITC (19.2), CD163-PE (GHI/61), OX40L/CD252-PE (ik-1) from BD-Biosciences; CD4-APCviolet770 (REA623), CD141-APCviolet770 (AD5-14H12) and CD1c FITC (AD5-8E7) from Miltenyi-Biotec; CLEC10A-PE (H037G3) and TSLPR-APC (1B4) from Biolegend; HLA-DR-AF700 (L203, R&D Systems), CD207-PE (DCGM4, Beckman Coulter); CD14-PE or -APC (MEM-18, Immunotools). For intracellular staining, T cells were fixed and permeabilized with BD Fixation/Permeabilization Solution Kit then stained for 45

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3 168 min at 4°C with IFN-γ-APC (B27), IL-4-PE (8D4-8) and IL-13-PE (JES10-5A2) (BD-Biosciences). Cells
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6 169 were collected using a Beckman-Coulter Gallios flow cytometer, and analyzed with FlowJo (Tree Star).
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8 170 Dead cells stained by Fixable Viability Dye-eFluor450 (eBioscience) were excluded from analysis.
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11 171 **RNA isolation, reverse transcription and qPCR**
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14 172 Total RNA of MonoDCs, skin CD14+ or CD14- DCs was isolated and purified with NucleoSpin RNA Plus
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16 173 kit (Macherey-Nagel). cDNA was synthesized by extension of oligo(dT) with ImPromII™ reverse-
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19 174 transcriptase (Promega) on a T100 thermal cycler (Bio-Rad). qPCR reactions were performed on a
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21 175 StepOnePlus™ thermocycler (Applied Biosystems) using the RT2SYBR Green ROX qPCR Master Mix
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24 176 (Qiagen). Relative expression levels were calculated with the comparative Ct method using the
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26 177 proprietary software. HPRT1 and GAPDH were chosen as housekeeping gene for normalization.
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29 178 Following primer sequences (forward/reverse) were designed with the Primer-BLAST software:
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31 179 HPRT1 (5'-CCTGGCGTCGTGATTAGTGAT-3'; 5'-AGACGTTTCAGTCCTGTCCATAA-3'), GAPDH (5'-
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34 180 GGAGCGAGATCCCTCCAAAAT-3'; 5'-GGCTGTTGTCATACTTCTCATGG-3'), CYP24A1 (5'-
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36 181 GATTTTCCGCATGAAGTTGGGT-3'; 5'- CCTTCCACGGTTTGATCTCCA-3').
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39 182 **Statistics**
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44 184 Student's t-tests (normally distributed population) or Wilcoxon's test (non-parametric) were
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47 185 performed with Prism software (GraphPad); p-values below 0.05 were considered significant.
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RESULTS

Vitamin D3-exposed skin DCs induce Th2 polarization.

To study the effect of vitD3 on cutaneous immune responses, we stimulated skin explants at the onset of ex vivo culture by adding calcitriol (200ng/mL) to the medium or by topical application of calcipotriol ointment (100mg). After 3 days, immune cells of untreated explants spontaneously migrate into the supernatant, including CD4⁺ and CD8⁺ resident memory T cells²⁶ and different subsets of HLA-DR⁺ DCs. With vitD3-treated skin, the viability of DCs was slightly increased (**Figure S1A**). To address the influence of vitD3 on T cell-priming capacities of DCs, we harvested total emigrating DCs without further stimulation and cultured them with CFSE-labelled allogeneic naïve CD4⁺ T cells (>95% viable, **Figure S1B**). After 7 days of mixed leukocyte reaction (MLR), T-cell proliferation and intracellular cytokine production were quantified by flow cytometry upon restimulation. No difference was observed in DC survival following MLR (**Figure S1C**). VitD3 exposure of DCs weakly altered their capacity to stimulate T-cell proliferation (**Figure 1A**), and this was not related to decreased T-cell survival (**Figure S1D**). However, calcitriol and calcipotriol consistently led DCs to promote differentiation of more IFN- γ ⁻ IL-13⁺/IL-4⁺ Th2 cells than DCs of untreated skin (**Figure 1B-E**). Th2 cells were detected only among proliferative cells (**Figure 1D-E**) and released IL-4 (**Figure 1F**). Conversely, the generation of IFN- γ ⁺ Th1 cells was slightly reduced in the presence of vitD3-exposed DCs (**Figure 1G**). DCs collected from vitD3-treated skin did not generate FoxP3⁺ Treg or support IL-10 production by T cells (**Figure 1H-I**).

TSLP from vitamin D3-treated human skin favors IL-13⁺ Th2 induction by DCs.

Epithelial TSLP is widely described as Th2 inducer through its effect on DCs^{16;27}. DCs migrating from skin explants stimulated with TSLP promoted differentiation of IFN- γ ⁻ IL-13⁺ Th2 cells, although this did not generate more IL-4⁺ Th2 cells (**Figure 2A**). Thus, we evaluated TSLP production by human skin

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3 209 samples cultured in the presence or absence of vitD3. Overall, TSLP was detected at significantly
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6 210 higher levels in the culture supernatant after either calcitriol or calcipotriol treatments (**Figure 2B**).
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8 211 Nevertheless, the TSLP concentrations of different donors were spread across a large range. To
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11 212 acknowledge this variability, we defined thresholds based on the Mean-minus-SEM for TSLP
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13 213 concentrations obtained in vitD3-treated skin. Accordingly, donors were split into two groups: those
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16 214 that showed strong TSLP production upon vitD3 exposure (responding: [TSLP]>Mean-SEM) and those
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18 215 that responded poorly or not at all (non-responding: [TSLP]<Mean-SEM). With thresholds set at
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21 216 56.63pg/mL for calcitriol and 62.73pg/mL for calcipotriol, the rates of responding donors were 29.4%
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23 217 and 32.1%, respectively.
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26 218 We took advantage of this dichotomy to evaluate the importance of TSLP for Th2 orientation. We
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28 219 reanalyzed the corresponding data from T-helper polarization assays (**Figure 1**). As compared with
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31 220 untreated skin, IL-13+ Th2 cells were significantly augmented when DCs from TSLP-producing skin
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33 221 were used, which was not the case for DCs from non-responding skin (**Figure 2C**). On the other hand,
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36 222 vitD3 did increase the capacity of skin DCs to promote IL-4+ Th2 cells, but this occurred even with
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38 223 donors that showed poor response in terms of TSLP production (**Figure 2D**). This suggested that only
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41 224 IL-13 production is positively influenced by TSLP-exposed skin DCs.
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44 225 To confirm the role of TSLP, we introduced an antagonistic anti-TSLPR antibody into the supernatant
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46 226 of skin explant cultures, in order to block potential effects of TSLP on skin DCs as early as possible. As
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48 227 expected, this did not alter the rate of IL-13+ Th2 cells obtained with vitD3-exposed DCs from non-
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51 228 responding skin (**Figure 2E, left**). Yet, blocking TSLPR abrogated the capacity of DCs from vitD3-
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53 229 treated, TSLP-producing skin explants to stimulate the generation of IL-13+ Th2 cells (**Figure 2E,**
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56 230 **right**). With respect to IL-4+ Th2 cells, no significant effect of TSLPR blocking could be observed with
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58 231 either non-responding or responding skin samples (**Figure 2F**). This demonstrates a link between the
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presence of TSLP in the supernatant of skin explants and IL-13 production by Th2 cells induced by vitD3-treated DCs.

Vitamin D3 alters the proportions of skin DC subsets but not their migration

To explore how vitD3 exposure affects cutaneous antigen-presenting cells, we examined emigrating HLA-DR⁺ skin DCs (**Figure 3A**). When obtained by explant culture of healthy, untreated skin, they comprise CD207⁺ CD14⁻ epidermal LCs, CD14⁻ dermal DCs (CD141⁺ cDC1 and CD1c⁺ cDC2)^{9;10}, and CD14⁺ dermal macrophage-like cells^{11;12}. The proportion and absolute numbers of CD207⁻ CD14⁺ cells were markedly increased by vitD3 treatment (**Figure 3B**), in agreement with previous work¹³. Microscopic analysis showed that most of emigrating cells coexpressed HLA-DR and CD14 upon vitD3 exposure (**Figure S2**). Conversely, CD207⁺ CD14⁻ LCs and CD207⁻ CD14⁻ dermal DCs were decreased as compared to untreated skin (**Figure 3B**). Within CD14⁻ dermal DCs, CD141⁺ cDC1 were also missing from the supernatant of vitD3-treated skin (**Figure 3C**). The proportion of CD14⁺ cells was similar with responding and non-responding skin samples (**Figure 3D**) and was not affected by TSLPR blocking (**Figure 3E**). Therefore, the increase of CD14⁺ cells was independent of TSLP.

Then, we questioned whether vitD3 potentiates emigration of bona fide CD14⁺ dermal cells, as previously proposed¹³, or rather upregulates CD14 expression on CD14⁻ DC subsets. We first noticed that identical numbers of total HLA-DR⁺ cells migrated from untreated and vitD3-treated skin explants (**Figure 3F**). Cryosections of skin samples after 3 days of culture were stained for HLA-DR and CD14 (**Figure S3A**) to quantify HLA-DR⁺ CD14⁺ cells that did not emigrate. Strikingly, CD14⁺ cell density was not diminished by vitD3 treatment, and was even increased with calcitriol (**Figure S3B**). This strongly suggests that there is no dramatic increase in the migratory capacity of CD14⁺ dermal cells.

Vitamin D3 affects the phenotype of skin DCs and MonoDCs

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3 255 We thus suspected that vitD3 promoted phenotypical modifications of CD14- DCs, including
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6 256 increased expression of CD14. To identify the cellular targets of vitD3 among skin DCs, we quantified
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8 257 mRNA of calcitriol 24-hydroxylase (CYP24A1), which degrades calcitriol into excreted calcitroic acid
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11 258 and is expressed under the control of VDR-mediated transcriptional activation¹. CYP24A1 was absent
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13 259 from all DCs migrating from untreated skin. However, following vitD3 treatment, a clear expression
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16 260 of CYP24A1 appeared, which was specific of CD14+ cells (**Figure 4A**). Therefore, the CD14+ population
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18 261 emigrating from vitD3-treated explants includes cells that had their gene expression directly
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21 262 modulated by vitD3 and that are different from bona fide CD14+ dermal cells of untreated skin.
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24 263 To substantiate our hypothesis, we set out to analyze well-known markers of human skin DC subsets
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26 264 (**Figures 4 and S4**). CD14+ cells recovered from vitD3-treated skin explant cultures had a decreased
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28 265 expression of typical markers of macrophages and CD14+ macrophage-like cells (CD209, CD163,
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31 266 CD206), which are poorly expressed by CD14- cDC2 of untreated skin. Nevertheless, vitD3-elicited
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33 267 CD14+ cells did not gain markers of cDC2 (CLEC10A, CD1c), and they increased CD141. As expected¹¹,
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36 268 CD14+ cells emigrating from untreated skin did not express CD86, as opposed to mature CD14- DCs
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38 269 (**Figure 4B**). However, after vitD3 treatment, CD86 and CD83 were both present on CD14+ cells
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41 270 (**Figure 4B-C**). HLA-DR levels did not change in CD14+ cells but increased in CD14- DCs (**Figure S4**,
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43 271 bottom panel). CD14+ cells from vitD3-treated skin had higher TSLPR surface levels (**Figure 4D**) and
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46 272 decreased OX40L expression (**Figure 4E**). Overall, the phenotype of CD14+ cells showed significant
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48 273 variations upon vitD3 treatment, whereas the remaining CD14- cells were unaltered as compared to
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50 274 untreated skin (**Figure 4F**), in line with their lack of CYP24A1 expression. Therefore, CD14+ cells
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53 275 induced by vitD3 do not only comprise CD14+ macrophage-like cells found in untreated skin, but also
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55 276 transitional, vitD3-elicited CD14+ DCs. They may stem from CD14- DCs, which are the main cells
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58 277 expressing CD86 and TSLPR in untreated skin¹¹.
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An elevated CD14 expression has been consistently reported for MonoDCs exposed to vitD3 throughout their differentiation from monocytes²⁻⁶. To verify whether vitD3 also affects fully differentiated cells such as skin DCs, MonoDCs generated with GM-CSF and IL-4 were stimulated for 24h with calcitriol. They showed a dose-dependent upregulation of CYP24A1 mRNA (**Figure S5A**), supporting that MonoDC gene expression is directly modulated by vitD3. Furthermore, CD14 upregulation by MonoDCs (**Figure S5B**) sustained the notion that this represents a primary effect of vitD3. The increased surface expression of CD14 did not reflect an impaired shedding of this receptor, since soluble CD14 was rather detected in higher amounts in the supernatant of vitD3-treated MonoDCs (**Figure S5C**).

Vitamin D3-elicited CD14+ skin DCs are TSLP-independent inducers of IL-4+ Th2 differentiation.

Based on the above-described phenotypic analyses of emigrating cells, we propose that vitD3 directly affects skin DCs, eliciting a new subset of transitional DCs displaying high CD14 levels. Thus, we asked whether these vitD3-elicited CD14+ DCs might contribute to TSLP-independent Th2 polarization. Choosing only non-responding donors to exclude any contribution of TSLP, CD14+ cells were isolated with anti-CD14 magnetic beads from total emigrating DCs. The enrichment procedure itself did not influence the maturation of positively selected CD14+ cells, as verified by their CD86 expression (data not shown). The resulting CD14+ and CD14- subsets were then separately cocultured with allogeneic naïve T cells. CD14+ DCs from vitD3-exposed skin induced less proliferation of total T cells than the CD14+ dermal cells of untreated skin (**Figure 5A**), without affecting T-cell viability (**Figure S1E**). As expected for a TSLP-independent process, untreated and vitD3-treated CD14+ cells had a similar capacity to promote IL-13+ Th2 cells (**Figure 5B**). However, both calcitriol and calcipotriol conferred to transitional CD14+ DCs a superior ability to induce the differentiation of IL-4+ Th2 cells (**Figure 5C-D**), and they did not support Th1 polarization (**Figure 5E**). On the other hand, CD14- DCs were

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301 unaffected by vitD3 for their influence on T-cell proliferation (**Figure 5F**) and polarization (**Figure 5G-**
302 **J**), despite a slightly decreased T-cell viability in the calcitriol-treated condition (**Figure S1F**).
303 Altogether, our findings indicate that CD14+ DCs arising in vitD3-treated skin acquire the capacity to
304 induce IL-4+ Th2 responses independently of TSLP.

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DISCUSSION

In the here presented study, topically applied and soluble vitD3 potentially affected the phenotype and function of cutaneous human DCs. In particular, in skin donors that presented the strongest capacity for TSLP production upon vitD3 treatment, TSLP-exposed DCs enhanced the production of IL-13 by Th2 cells. Nevertheless, vitD3 also promoted direct effects, eliciting CD14⁺ DCs that differed from the well-described CD14⁺ dermal cells of untreated skin¹¹ and supported TSLP-independent differentiation of IL-4⁺ Th2 cells.

VitD3 consistently drives DCs to inhibit Th1 polarization²⁻⁴, similarly to our findings. With skin DCs, it was proposed that differentiation of Treg could explain this outcome^{7;13}, which we could not confirm. A Th2-inducing bias that opposes Th1 differentiation appears to be the most likely explanation for the lack of Th1 cells in our experimental settings. In previous investigations involving human skin DCs, vitD3 was provided in addition to LPS¹³ or IL-1 β +TNF- α ⁷. When used alone, such potent maturation factors are known to favor Th1 priming²⁸. Furthermore, we noted that mature skin DCs did not sense vitD3 (data not shown). For these reasons, we focused on culturing untreated or vitD3-treated explants in the absence of additional adjuvants, which is sufficient to achieve spontaneous migration of DCs. With either method, DCs appeared similarly mature and capable of naïve T cell priming, yet functional differences are expected that very likely explain differences between our findings and published studies^{7;13}.

In mouse models, cutaneous exposure to calcipotriol has been convincingly demonstrated to induce TSLP *in vivo*²⁰, but a similar outcome had yet to be proven in human skin. In our assays, vitD3 induced potent TSLP production in only ~30% of skin samples, which could explain why this has been previously overlooked¹⁸. Differences in sensitivity to and/or consequences of vitD3 exposure may stem from the origin of human skin samples. Since they were collected as surgical waste, the clinical

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3 329 history of donors could not be traced back, in accordance with our current ethical agreement.
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6 330 Therefore, the reasons for TSLP production variability remains unclear. Our samples might have
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8 331 included patients with atopic skin disorders. However, we obtained only healthy skin with no
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11 332 apparent atopic lesion, and atopic patients display TSLP staining within their active lesions but not in
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13 333 perilesional skin¹⁶. We may also suspect skin phenotype differences that modify the half-life of
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16 334 experimentally applied bioactive vitD3²⁹.
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19 335 Segregating our data according to TSLP levels allowed us to reveal that skin DCs from TSLP-producing
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21 336 donors were more prone to shift Th2 differentiation towards IL-13+ cells. IL-13 expression by T cells
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23 337 can be provoked by TSLP directly in the mouse³⁰ or via human MonoDCs¹⁶ and LCs³¹. In line with this,
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26 338 we observed a strong bias for IL-13 production when DCs were collected from TSLP-exposed skin
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28 339 explants. A dominant IL-13 production has been proposed as a hallmark of pathogenic Th2 cells³²,
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31 340 notably because IL-13R α is particularly present on epithelial cells and impairs expression of the
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33 341 epidermal barrier constituents³³.
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36 342 Despite this clear TSLP-dependent promotion of IL-13+ cells, the poor correlation between TSLP
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39 343 amounts and the capacity of skin DCs to generate IL-4+ Th2 cells suggested a TSLP-independent
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41 344 effect. Among other consequences, an increase in the rate of CD14+ cells emigrating from skin
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43 345 explants was observed upon treatment with vitD3. This prominent effect has been presumed to
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46 346 result from increased migration of CD14+ dermal cells^{7;13}. Our interpretation differs because (1) total
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48 347 numbers of emigrating DCs were not modified by vitD3 treatment, (2) the massive increase in CD14+
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51 348 cells in the supernatant did not correlate with a decrease of CD14+ cells remaining in the dermis and
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53 349 (3) immature MonoDCs exposed to vitD3 showed a consistent upregulation of CD14. The latter effect
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56 350 has been repeatedly pointed out upon vitD3 treatment of monocytes during in vitro differentiation
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58 351 into DCs^{2;3;5}. We confirmed that vitD3 led terminally differentiated MonoDCs to upregulate both
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surface and soluble CD14. Moreover, immature LCs and dermal DCs sorted from human skin and exposed to vitD3 also upregulate CD14 to some extent⁷. Therefore, we propose that vitD3 does not promote emigration of the well-described CD14⁺ dermal cells but rather leads skin DCs to phenotypical changes. Although we gathered strong evidence supporting this, CD14⁺ DCs that dominate migratory cell suspensions after vitD3 treatment remain of unclear origin. They do not express macrophage-like markers, but they also lack other typical features of dermal CD14⁺ cDC2. These complex alterations, which were independent of TSLP but may not be only due to vitD3, result in a yet undescribed phenotype for skin DCs and, importantly, bring a strong bias in their T-cell priming function. Indeed, vitD3-elicited CD14⁺ DCs appeared sufficient to efficiently generate IL-4⁺ Th2 cells. Nevertheless, our phenotypic analyses did not reveal which alterations could be responsible for this function. The hallmark of vitD3 exposure, CD14, is unlikely to play a role in T-cell differentiation. OX40L, a known Th2 inducer^{16;20;21}, is rather decreased, and TSLPR, although increasing, should not be involved in skin explants that release little TSLP. Altogether, this intriguing differentiation pathway may be involved in the regulation of IL-4/IL-13 balance in the skin and should be further investigated for its potential to harness a relevant therapeutic approach limiting the atopic march and inflammation in skin allergies.

Overall, our findings provide several interesting insights for clinical research. Topical application of calcipotriol ointment is a well-established treatment for psoriasis¹. In addition to limiting keratinocyte proliferation and promoting their differentiation, this treatment induces TSLP in lesional psoriatic skin²⁴. A shift towards Th2 responses may be beneficial in this Th17/Th22-driven disease. Of note, dermatitis, erythema and xerosis, which are hallmarks of atopic dermatitis, may develop in 1-10% of patients as a side effect of the DaivonexTM calcipotriol ointment ([Medscape](#)). Moreover, a case report pointed out that erroneous prescription of this drug to an atopic patient results in a dermatitis flare³⁴. Finally, we demonstrate the potential of using vitD3 in the design of human skin

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376 models reproducing early immune responses in atopic dermatitis³⁵. This strategy, aiming at reducing
377 animal use and ameliorating the safety of clinical trials, currently represents an important goal in
378 drug discovery.

For Peer Review

References

1. Tremezaygues L, Reichrath J. Vitamin D analogs in the treatment of psoriasis: Where are we standing and where will we be going? *Dermatoendocrinol* 2011;3:180-186.
2. Berer A, Stöckl J, Majdic O et al. 1,25-dihydroxyvitamin D₃ inhibits dendritic cell differentiation and maturation in vitro. *Experimental Hematology* 2000;28:575-583.
3. Piemonti L, Monti P, Sironi M et al. Vitamin D₃ affects differentiation, maturation, and function of human monocyte-derived dendritic cells. *J Immunol* 2000;164:4443-4451.
4. Penna G, Amuchastegui S, Giarratana N et al. 1,25-dihydroxyvitamin d3 selectively modulates tolerogenic properties in myeloid but not plasmacytoid dendritic cells. *J Immunol* 2007;178:145-153.
5. Sommer A, Fabri M. Vitamin D regulates cytokine patterns secreted by dendritic cells to promote differentiation of IL-22-producing T cells. *PLoS One* 2015;10:e0130395.
6. Pedersen AW, Holmstrom K, Jensen SS et al. Phenotypic and functional markers for 1 α ,25-dihydroxyvitamin D(3)-modified regulatory dendritic cells. *Clin Exp Immunol* 2009;157:48-59.
7. van der Aar AM, Sibiryak DS, Bakdash G et al. Vitamin D3 targets epidermal and dermal dendritic cells for induction of distinct regulatory T cells. *J Allergy Clin Immunol* 2011;127:1532-1540.
8. Nikolic T, Roep BO. Regulatory multitasking of tolerogenic dendritic cells - lessons taken from vitamin d3-treated tolerogenic dendritic cells. *Front Immunol* 2013;4:113.
9. Bigley V, McGovern N, Milne P et al. Langerin-expressing dendritic cells in human tissues are related to CD1c+ dendritic cells and distinct from Langerhans cells and CD141^{high} XCR1+ dendritic cells. *J Leukoc Biol* 2015;97:627-634.
10. Haniffa M, Shin A, Bigley V et al. Human Tissues Contain CD141(hi) Cross-Presenting Dendritic Cells with Functional Homology to Mouse CD103(+) Nonlymphoid Dendritic Cells. *Immunity* 2012;37:60-73.
11. McGovern N, Schlitzer A, Gunawan M et al. Human dermal CD14(+) cells are a transient population of monocyte-derived macrophages. *Immunity* 2014;41:465-477.
12. Banchereau J, Thompson-Snipes L, Zurawski S et al. The differential production of cytokines by human Langerhans cells and dermal CD14(+) DCs controls CTL priming. *Blood* 2012;119:5742-5749.
13. Bakdash G, Schneider LP, Van Capel TM, Kapsenberg ML, Teunissen MB, De Jong EC. Intradermal application of vitamin D3 increases migration of CD14+ dermal dendritic cells and

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2
3 413 promotes the development of Foxp3+ regulatory T cells. Hum Vaccin Immunother 2013;9:250-
4 414 258.
5
6
7 415 14. Liu Y-J, Soumelis V, Watanabe N et al. TSLP: An Epithelial Cell Cytokine that Regulates T Cell
8 416 Differentiation by Conditioning Dendritic Cell Maturation. Annu Rev Immunol 2007;25:193-219.
9
10 417 15. Varricchi G, Pecoraro A, Marone G et al. Thymic Stromal Lymphopoietin Isoforms, Inflammatory
11 418 Disorders, and Cancer. Front Immunol 2018;9:1595.
12
13
14 419 16. Soumelis V, Reche PA, Kanzler H et al. Human epithelial cells trigger dendritic cell mediated
15 420 allergic inflammation by producing TSLP. Nat Immunol 2002;3:673-680.
16
17 421 17. Xie Y, Takai T, Chen X, Okumura K, Ogawa H. Long TSLP transcript expression and release of
18 422 TSLP induced by TLR ligands and cytokines in human keratinocytes. J Dermatol Sci 2012;66:233-
19 423 237.
20
21
22 424 18. Landheer J, Giovannone B, Sadekova S et al. TSLP is differentially regulated by vitamin D3 and
23 425 cytokines in human skin. Immun Inflamm Dis 2015;3:32-43.
24
25 426 19. Li M, Hener P, Zhang Z, Kato S, Metzger D, Chambon P. Topical vitamin D3 and low-calcemic
26 427 analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic
27 428 dermatitis. Proc Natl Acad Sci U S A 2006;103:11736-11741.
29
30 429 20. Li M, Hener P, Zhang Z, Ganti KP, Metzger D, Chambon P. Induction of thymic stromal
31 430 lymphopoietin expression in keratinocytes is necessary for generating an atopic dermatitis
32 431 upon application of the active vitamin D3 analogue MC903 on mouse skin. J Invest Dermatol
33 432 2009;129:498-502.
34
35
36 433 21. Pattarini L, Trichot C, Bogiatzi S et al. TSLP-activated dendritic cells induce human T follicular
37 434 helper cell differentiation through OX40-ligand. J Exp Med 2017;214:1529-1546.
38
39 435 22. Ghoreishi M, Bach P, Obst J, Komba M, Fleet JC, Dutz JP. Expansion of antigen-specific
40 436 regulatory T cells with the topical vitamin d analog calcipotriol. J Immunol 2009;182:6071-6078.
41
42
43 437 23. Lechner TM, Satake A, Harrison VS et al. Skin-derived TSLP systemically expands regulatory T
44 438 cells. J Autoimmun 2017;79:39-52.
45
46 439 24. Sato-Deguchi E, Imafuku S, Chou B, Ishii K, Hiromatsu K, Nakayama J. Topical vitamin D(3)
47 440 analogues induce thymic stromal lymphopoietin and cathelicidin in psoriatic skin lesions. Br J
48 441 Dermatol 2012;167:77-84.
49
50
51 442 25. Berg S, Kutra D, Kroeger T et al. ilastik: interactive machine learning for (bio)image analysis. Nat
52 443 Methods 2019;16:1226-1232.
53
54 444 26. Clark RA, Chong BF, Mirchandani N et al. A novel method for the isolation of skin resident T cells
55 445 from normal and diseased human skin. J Invest Dermatol 2006;126:1059-1070.
56
57
58 446 27. Leyva-Castillo JM, Hener P, Michea P et al. Skin thymic stromal lymphopoietin initiates Th2
59 447 responses through an orchestrated immune cascade. Nat Commun 2013;4:2847.
60

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2
3 448 28. Schuler-Thurner B, Schultz ES, Berger TG et al. Rapid induction of tumor-specific type 1 T helper
4 449 cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-
5 450 loaded monocyte-derived dendritic cells. *J Exp Med* 2002;195:1279-1288.
6
7
8 451 29. Carlberg C, Haq A. The concept of the personal vitamin D response index. *J Steroid Biochem*
9 452 *Mol Biol* 2018;175:12-17.
10
11 453 30. Rochman Y, Dienger-Stambaugh K, Richgels PK et al. TSLP signaling in CD4(+) T cells programs a
12 454 pathogenic T helper 2 cell state. *Sci Signal* 2018;11.
13
14
15 455 31. Ebner S, Nguyen VA, Forstner M et al. Thymic stromal lymphopoietin converts human epidermal
16 456 Langerhans cells into antigen presenting cells that induce pro-allergic T cells. *J Allergy Clin*
17 457 *Immunol* 2007;119:982-990.
18
19 458 32. Ito T, Wang YH, Duramad O et al. TSLP-activated dendritic cells induce an inflammatory T helper
20 459 type 2 cell response through OX40 ligand. *J Exp Med* 2005;202:1213-1223.
21
22
23 460 33. Bieber T. Interleukin-13: Targeting an underestimated cytokine in atopic dermatitis. *Allergy*
24 461 2020;75:54-62.
25
26 462 34. Turner MJ, Dasilva-Arnold SC, Yi Q, Mehrotra P, Kaplan MH, Travers JB. Topical application of a
27 463 vitamin D analogue exacerbates atopic dermatitis and induces the atopic dermatitis-like
28 464 phenotype in Stat6^{VT} mice. *Pediatr Dermatol* 2013;30:574-578.
29
30
31 465 35. Bernard M, Carrasco C, Laoubi L et al. IL-1 β induces thymic stromal lymphopoietin and an
32 466 atopic dermatitis-like phenotype in reconstructed healthy human epidermis. *J Pathol*
33 467 2017;242:234-245.
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FIGURES

Figure 1: Vitamin D3-exposed skin DCs induce Th2 responses.

Human skin explants were stimulated 3 days with 200nM soluble calcitriol (into the medium) or 100mg topical calcipotriol ointment. Emigrating skin DCs were collected and cultured with CFSE-labelled allogeneic naïve CD4⁺ T cells. Intracellular cytokines were detected by flow cytometry upon restimulation with PMA+Ionomycine. (A) Percentage of proliferative (CFSE^{low}) T cells after 7 days of MLR with total cells emigrating from untreated or vitamin D3-exposed skin explants (n=26). Representative dot plots and percentages of cells in thick-lined gates define Th2 subsets: (B) IFN- γ ⁻ IL-13⁺ (n=40) (C) IFN- γ ⁻ IL-4⁺ (n=30) (D) proliferating IL-13⁺ (n=23) or (E) proliferating IL-4⁺ cells (n=19). (F) IL-4 released in 24h-restimulated MLR supernatant (n=28). (G) Percentages of IFN- γ ⁺ Th1 cells (n=37) (H) FoxP3⁺ Treg (n=10) and (I) IFN- γ ⁻ IL-10⁺ T cells (n=10). Data are presented as Mean $\pm$ SEM. n= total number of skin explant donors. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (paired Student's t-tests except (F) Wilcoxon non-parametric tests).

Figure 2: TSLP production in vitamin D3-treated human skin regulates the capacity of Th2 cells to produce IL-13.

(A) Percentages of IFN- γ - IL-13+ (n=6) and IFN- γ - IL-4+ (n=5) CD4+ T cells after MLR with emigrating DCs from TSLP-stimulated skin explants (B) TSLP released after 3 days of skin explant culture (n=53). Dashed lines depict thresholds defined from Mean-SEM values, allowing to split donors into responding and non-responding populations. (C) IFN- γ - IL-13+ Th2 percentages for MLR with skin DCs from non-responding (n=24) or responding (n=8) donors. (D) IFN- γ - IL-4+ Th2 percentages for MLR with skin DCs from non-responding (n=20) or responding (n=6) donors. (E, F) Anti-TSLPR antibody or isotype control were added into the supernatant during skin explant cultures. (E) IFN- γ - IL-13+ and (F) IFN- γ - IL-4+ (n=19 for non-responding, n=7 for responding). Data are presented as Mean $\pm$ SEM. n= total number of skin explant donors. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (paired Student's t-tests except (F) Wilcoxon non-parametric tests).

Figure 3: Vitamin D3 alters DC populations emigrating from skin explants

(A) Gating strategy to determine the phenotype of emigrating HLA-DR+ cells by flow cytometry. Representative dot plots and quantification are shown for **(B)** CD14+ CD207-, CD14- CD207- (n=44 for percentages, n=16 for absolute numbers), CD14- CD207+ LCs (n=8 for both) and **(C)** CD14- CD207- CD141+ cDC1s (n=15 for percentages, n=7 for absolute numbers). **(D)** Percentages of CD14+ cells emigrating from non-responding (n=36) and responding skin explants (n=15). **(E)** Percentages of CD14+ cells emigrating from calcitriol- or calcipotriol-responding skin with or without anti-TSLPR antibody or isotype control (n=4) **(F)** Total number of migrating cells for one skin explant (n=16). Data are presented as Mean ± SEM. n= total number of skin explant donors. ns: non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (paired Student's t-tests).

Figure 4: Vitamin D3 induces phenotypic modifications of skin DCs into a CD14+ subset

(A) Relative expression levels for CYP24A1 mRNA in CD14+ and CD14- emigrating subsets (n=4). **(B)** Representative histogram overlays and mean fluorescence intensities (MFI) for CD86 (n=19). **(C)** Representative histogram overlays and MFI for CD83 (n=4). **(D)** Representative histogram overlays and MFI for TSLPR (n=4). **(E)** Representative histogram overlays and MFI for OX40L (n=5). **(F)** Radar plots depicting the average MFI of all tested donors for CD1c, CD141, CD86, CD163, CD209, TSLPR, CLEC10A, CD83, HLA-DR, OX40L and CD206 for CD14+ and CD14- populations (see detailed analysis in Figure S4). Data are presented as Mean ± SEM. n= total number of skin explant donors. ns: non-significant, * p<0.05, **p<0.01, ****p<0.0001 (paired Student's t-tests).

Figure 5: Vitamin D3-elicited CD14+ cells induce IL4+ Th2 cells independently of TSLP.

Magnetically separated CD14+ and CD14- cells from untreated or vitamin D3-exposed skin explants were cocultured with allogenic naïve T cells. Data presented here only shows results from skin explants that did not respond to vitamin D3 by TSLP production (non-responding donors). (A-J) Percentages of (A, F) proliferative (CFSE^{low}) T cells (n=12 for CD14+, n=9 for CD14-) (B, G) IFN-γ- IL-13+ (n=13 for CD14+, n=10 for CD14-) or (C, H) IL-4+ Th2 cells (n=12 for CD14+, n=7 for CD14-). (D, I) IL-4 in 24h restimulated supernatants (n=13 for CD14+, n=9 for CD14-). (E, J) Percentages of IFN-γ+ Th1 cells (n=13 for CD14+, n=10 for CD14-). Data are presented as Mean ± SEM. n= total number of skin explant donors. ns: non-significant, *p<0.05, **p<0.01 (paired Student's t-tests).

SUPPORTING INFORMATION

Supplementary methods

Immunofluorescence microscopy

Skin explant samples were frozen embedded in O.C.T Compound (CellPath) and 16µm sections were prepared, mounted on SuperFrost-Plus™ glass slides (Thermo Scientific), fixed in cold acetone for 20min and air-dried. Sections were incubated overnight at 4°C with antibodies against HLA-DR (L203) and CD14 (AB383) (R&D Systems), diluted in PBS-10% FCS. Thereafter, they were incubated 2h at room temperature with DAPI (100ng/mL) and their corresponding secondary antibody: Donkey anti-Mouse IgG AlexaFluor 555 (Molecular Probes, A31570) and Donkey anti-Goat IgG AlexaFluor 647 (Jackson ImmunoResearch, 705-605-147). Sections were then mounted with Fluoromount G medium (EBioscience, 00-4958-02). Images were acquired on a Zeiss Axio-Observer Z1 epifluorescent inverted microscope driven by the Metamorph software, using a 10x EC Plan Neofluar objective. Quantification was based on 85 images obtained from 11 different donors. Nuclei prediction maps were realized using the pixel classification module in Ilastik software²⁵ on the DAPI staining channel. Based on this prediction maps, the object classification module of Ilastik was used to train the software to determine to which of the 4 following classes belongs each nuclei: epidermal cells, HLA-DR+ cells, HLA-DR+ CD14+ cells, unstained cells. We then determined the number of nuclei for each class using the ImageJ software.

Skin-emigrating DCs were fixed with paraformaldehyde and stained as described above. Images were acquired with a spinning disk confocal head (Yokogawa, CSU-X1), using a 100x Plan Achromat oil immersion NA1.4 objective and analyzed with ImageJ software.

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554 **Supplementary figures:**

Figure S1: Vitamin D3 weakly alters viability of skin DCs and T cells

(A-F) Survival of HLA-DR+ or CD3+ cells before (A,B) and after (C-F) MLR. Data are presented as Mean $\pm$ SEM. n= total number of skin explant donors. ns: non-significant, **p<0.01, ***p<0.001 (paired Student's t-tests).

Figure S2: Vitamin D3 alters emigrating skin DC populations

Representative immunofluorescence of cells emigrating from untreated, calcitriol- or calcipotriol-treated skin explant (3 days). Scale bars: 25 μm. Cells were stained with DAPI (blue), anti-CD14 (green) and anti-HLA-DR (red) (n=2).

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Figure S3: CD14+ cells are not depleted from the dermis after skin explant culture with vitamin D3.

(A) Representative immunofluorescence images of skin explants after 3 days of culture. Cryosections were stained with DAPI (blue), anti-CD14 (green) and anti-HLA-DR (red) monoclonal antibodies. Insets correspond to the regions highlighted in the larger images. Arrows point to HLA-DR+ CD14+ (yellow) cells. HLA-DR+ CD14- (red) and HLA-DR low CD14+ (green) are also visible. Scale bars: 50µm.

(B) Numbers of CD14+ cells in skin sections and percentages of CD14+ cells among total dermal cells (n=11). Data are presented as Mean ± SEM. n= total number of skin explant donors. ns: non-significant, **p<0.01 (paired Student's t-tests).

Figure S4: Vitamin D3 alters the phenotype of skin-emigrating HLA-DR+ cells

Representative dot plots and MFI quantification of CD209 (n=8), CD163 (n=6), CD206 (n=8), CLEC10A (n=5), CD1c (n=7), CD141 (n=13), HLA-DR (n=20) expression on emigrating CD14+ and CD14- cells. Data are presented as Mean $\pm$ SEM. n= total number of skin explant donors. ns: non-significant, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 (paired Student's t-tests).

Figure S5: Vitamin D3-treated MonoDCs upregulate CD14 expression

Immature MonoDCs were stimulated for 24h with vitamin D3 or left untreated. **(A)** Relative expression of CYP24A1 after 24h-stimulation with different doses of calcitriol: 2nM, 20nM (n=3) and 200nM (n=5). **(B)** Mean fluorescence intensity of CD14 after 200nM calcitriol treatment (n=12). **(C)** soluble CD14 release (n=5). Data are presented as Mean ± SEM. n= total number of skin explant donors. ns: not significant, *p<0.05, ****p<0.0001 (paired Student's t-tests).