Harnessing dendritic cells to promote immune tolerance: Opportunities for allergen-specific immunotherapy

Bakdash, G.

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INTRADERMAL APPLICATION OF VITAMIN D3 INCREASES MIGRATION OF CD14⁺ DERMAL DENDRITIC CELLS AND PROMOTES THE DEVELOPMENT OF FOXP3⁺ REGULATORY T CELLS

Ghaith Bakdash¹⁺, Laura P. Schneider¹²⁺, Toni M. M. van Capel¹, Martien L. Kapsenberg¹, Marcel B. M. Teunissen² & Esther C. de Jong¹

¹These authors contributed equally
¹ Department of Cell Biology & Histology and
² Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

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ABSTRACT

The active form of vitamin D3 (VitD) is a potent immunosuppressive drug. Its effects are mediated in part through dendritic cells (DCs) that promote the development of regulatory T cells (Tregs). However, it remains elusive how VitD would influence the different human skin DC subsets, e.g. CD1a⁺/langerin⁺ Langerhans cells, CD14⁺ DDCs and CD1a⁺ DDCs upon administration through the skin route in their natural environment. We addressed this issue by intradermal (ID) administration of VitD in a human skin explant system that closely resembles physiological conditions. ID injection of VitD selectively enhanced the migration of CD14⁺ DDCs, a subset known for the induction of tolerance. Moreover, ID injection of VitD repressed the LPS-induced T cell stimulatory capacity of migrating DCs. These migrating DCs collectively induced T cells with suppressive activity and abolished IFN-γ productivity. Those induced T cells were characterized by the expression of Foxp3. Thus, we report the novel finding that ID injection of VitD not only modifies skin DC migration, but also programs these DCs in their natural milieu to promote the development of Foxp3⁺ Tregs.

KEYWORDS

Vitamin D, Skin dendritic cells, Tolerance, Regulatory T cells, Intradermal injection

ABBREVIATIONS

DC    dendritic cell
DDC   dermal dendritic cell
ID    intradermal
LC    Langerhans cell
LPS   lypopolysaccharide
KC    keratinocyte
SC    subcutaneous
SIT   allergen-specific immunotherapy
Treg  regulatory T cell
VitD  1,25-dihydroxyvitamin D3.
INTRODUCTION

Regulatory T cells (Tregs) are crucial for sustaining tissue homeostasis and preventing immunopathology in peripheral tissues. Defects in Treg numbers or functions contribute to the development and progression of autoimmunity (1,2). Restoring the immune balance in those diseases can be achieved through immunosuppressive agents such as 1,25-dihydroxyvitamin D3 (VitD) (3). Many autoimmune diseases are correlated with VitD deficiency, including multiple sclerosis, type I diabetes and Crohn's disease (4). VitD is a potent immunosuppressive drug in mouse models of autoimmunity, inflammatory and allergic diseases (5-9). Currently, VitD and its analogues are successfully used as a topical treatment of psoriasis, an inflammatory skin disease characterized by hyperproliferation of keratinocytes (KCs) and large lymphocyte infiltrations (10). VitD is thought to normalize the proliferation and differentiation of KCs (11), and to reduce the inflammation by modulating the functions of T cells and dendritic cells (DCs). Direct exposure to VitD inhibits T cell proliferation, IL-2 expression and inflammatory cytokines production and promotes the development of T cells into Tregs (12-16). VitD represses the expression of MHC-II and co-stimulatory molecules by DCs, leading to reduced immunostimulatory capacity (17,18). Furthermore, VitD renders DCs tolerogenic by downregulating the synthesis of IL-12, while boosting IL-10 secretion and inducing the expression of ILT3 and PD-L1, altogether leading to Treg priming and inhibiting Th1 cell development (19-21). Furthermore, VitD induces the expression of skin epidermal homing receptor CCR10 on T cells, which guides these cells to the epidermis (22).

The skin is an interesting site for vaccination as it is equipped with an extensive network of DCs. Mouse models have shown that topical or subcutaneous (SC) application of VitD induces significant migration of skin DCs to skin draining lymph nodes (23,24), abolishes T cell responses in draining lymph nodes (23,25) and expands antigen-specific Treg cells (26). SC application of VitD was shown to potentiate the regulatory effects of allergen-specific immunotherapy (SIT) in a mouse model of allergic asthma (9). However, skin contains different DC types — epidermal Langerhans cells (LCs) and CD1a+ and CD14+ dermal dendritic cells (DDCs) (27-30) — and it is unclear to what extent these individual DC types are influenced by VitD. We have recently shown that human skin-derived LCs and CD1a+ DDCs are differentially programmed by VitD to support the development of either TGF-β-dependent Foxp3+ Tregs (LCs) or IL-10-dependent IL-10 producing Tregs (DDCs) (21). In this study we analyzed the effect of VitD on the migratory and functional properties of skin DC subsets in a human skin explant model (31,32). We show that intradermal (ID) injection of VitD results in selective migration of CD14+ DDCs. The unseparated total DC population from VitD-injected skin promoted development of Foxp3+ Tregs that actively suppressed bystander T cells. These promising findings bare possibilities for the development of new therapies for autoimmune diseases, and improvement of current immunotherapies, like SIT.

MATERIALS AND METHODS

Human skin explants. Skin specimens were obtained from healthy subjects undergoing breast or abdominal reduction. ID injections with 50 µl of PBS, LPS (20 µg/ml; E. Coli, Sigma Aldrich, St.
Louis, MO), VitD (1,25(OH)2 Vitamin D3, 25 µM; Sigma Aldrich, St. Louis, MO) or LPS and VitD together were performed. Both LPS and VitD were diluted in PBS ID injections were applied by insulin needles (0.5x16 mm Microlance; BD Biosciences, Mountain View, CA). A biopsy, 6 mm in diameter and 3-4 mm in thickness, was taken from the injected site and placed temporarily for 1 h in 0.5 ml IMDM (Life Technologies, Paisley, UK) containing 1%FCS (HyClone, Logan, UT) and then cultured in 1 ml IMDM supplemented with 10%FCS and GM-CSF (100 ng/ml; Schering-Plough, Uden, The Netherlands). After 3 days, the migrating cells were harvested, while the medium was used to determine the levels of IL-6, IL-10, IL-12p70 and TNF-α as described previously (51).

Phenotypical analysis. We used anti-HLA-DR-PerCP, anti-CD1a-APC, anti-CD14-FITC (all from BD Biosciences, San Jose, CA), anti-CD11c-PE-Cy7 (eBioscience, San Diego, CA), and anti-langerin-PE (Beckman Coulter, Brea, CA) to identify DC subsets. As maturation markers we used anti-CD86-PE and anti-CD83-PE (BD Biosciences). Flow cytometry analysis was performed on a FACS Canto II (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR) was used for data analysis.

Isolation and stimulation of naive CD4+ T cells. Naïve CD4+ T cells were isolated as described previously (21). Prior to T cell stimulation by crawl-out cells, the latter were γ-irradiated (30 Gy) to prevent any possible proliferation of contaminating skin T cells. This procedure has no effect on T cell priming functions of DCs as reported previously (32). To determine the stimulatory capacity of DCs, 4 x 10^4 naive CD4+ T cells were cocultured with indicated numbers of γ-irradiated crawl-out cells in 200 µl IMDM 10%FCS in 96-well flat-bottom plates (Costar). After 5 days, 11 KBq/well [3H]-TdR (Radiochemical Center, Amersham, Little Chalfont, U.K.) was added and incorporation of [3H]-TdR was measured 16h later. Furthermore, 2 x 10^4 naive CD4+ T cells were stimulated by 2 x 10^4 γ-irradiated crawl-out cells in the presence of superantigen Staphylococcus aureus enterotoxin B (SEB, 10 µg/ml; Sigma Aldrich) and medium was refreshed at day 5 with culture medium supplemented with 40 U/ml of IL-2 (Chiron, Emeryville, CA). The intracellular expression of Foxp3 (BioLegend, San Diego, CA) and surface expression of CD127 (BD Biosciences) was determined around day 11. Furthermore, T cells were re-stimulated with phorbol 12-myristate 13-acetate (100 ng/ml) and ionomycin (1 µg/ml) (all purchased from Sigma-Aldrich) and analyzed for expression of IFN-γ and IL-4 by using anti-IFN-γ-FITC and anti-IL-4-APC (both from BD Biosciences). In parallel, 100x10^3 T cells were restimulated with plate bound αCD3 (16A9, 1 µg/ml) and αCD28 (15E8, 1ug/ml) (both from Sanquin, Amsterdam, Netherlands). 24 hours supernatants were taken for analysis of IL-10 and IFN-γ (U-Cytech, Utrecht, Netherlands) by ELISA.

T cell suppressor assay. The suppressive capacity was assessed as described previously (21). In brief, T cells induced by crawl-out DCs (test cells) were harvested after 5 days, extensively washed, counted, irradiated (30 Gy) to prevent expansion, and stained with the cell-cycle tracking dye PKH-26 (11.8 µM, Sigma-Aldrich). Memory T cells, from the same donor as test cells, were labeled with 5,6-carboxy fluorescein diacetate succinimidyl ester (CFSE) (0.5 µM; Molecular Probes, Eugene, OR) and subsequently used as bystander target cells. Test cells (5x10^3) were co-cultured with 25x10^3 target cells and 1000 LPS-matured monocyte-derived DCs. After 5-7 days, the proliferation of the target T cells was determined by flow cytometry.
Statistics. Data are presented as Mean±SEM. Student t tests were performed for paired measurements with GraphPad Prism software (GraphPad, La Jolla, CA). Values of \( P < 0.05 \) were considered significant.

RESULTS

ID-injected VitD selectively induces the migration of CD14^+ DDCs

We tested if ID administration of VitD, alone or together with LPS, would influence the migratory patterns of skin DCs. The numbers of DCs crawling out of injected skin biopsies were determined and compared to those obtained from PBS-injected skin. As shown previously (32), DCs were distinguished by their side scatter and forward scatter properties, in addition to high expression levels of both HLA-DR and CD11c (Supplementary Figure 1). Substantial DC migration from PBS-injected skin explants was observed, with an average of around 10000 DCs from each biopsy. LPS-injected biopsies showed similar numbers of crawl-out DCs, and injection of VitD, alone or together with LPS did not cause a significant change as compared to PBS or LPS (Fig. 1A).

Next, we evaluated whether the migration of the three skin DC subsets is differentially affected by ID-injected VitD, using the differential expression of CD1a, CD14 and langerin: CD1a^– CD14^+ DDCs (referred to as CD14^+ DDC), CD1a^{low} CD14^– DDCs (referred to as CD1a^+ DDC), and CD1a^{high} CD14^– langerin^+ LCs, as shown before (32), and as demonstrated in Supplementary Figure 2. In all conditions, the majority of migratory skin DCs belonged to the CD1a^+ DDC subset. Strikingly, VitD selectively boosted both the percentages and absolute numbers of migratory CD14^+ DDCs, when injected alone or with LPS in comparison to the control conditions with PBS or LPS alone (Fig. 1B). This increase was accompanied by significantly reduced percentages and counts of CD14^– DDCs. In line with our previous findings (32), injection of LPS selectively enhanced the percentages of migratory LCs and this enhancement persisted when VitD was concomitantly injected, while VitD by itself failed to induce any significant LC migration (Fig. 1B). A similar trend was observed with LC counts, though the LPS-induced increase was not significant (Fig. 1B, lower panel).

ID-injected VitD does not influence DC maturation, but modulates local cytokine production

VitD is able to block the maturation of monocyte-derived DDCs and LCs (21). In order to determine whether VitD maintained this capacity when injected directly in the skin, we determined the CD83 and CD86 expression on the different DC subsets. Regardless of the injected compounds, the CD14^+ DDC subset always appeared less mature compared to LCs and CD1a^+ DDCs, as reflected by the lower expression of both CD86 and CD83 (Fig. 2A). Mean fluorescence intensity (MFI) values of both markers were significantly lower in the CD14^+ DDC subset, when compared to LCs and CD1a^+ DDCs (Fig. 2B). Despite relatively high expression levels of CD86 by LCs and CD1a^+ DDCs upon PBS injection, administered LPS could further enhance this expression. Enhanced CD83 expression following LPS injection was only observed for the CD1a^+ DDC, albeit the increase in MFI values was not significant (Fig. 2B). LPS-induced maturation was also modestly observed for
Figure 1. ID injected VitD induces the migration of CD14+ DDCs from human skin explants. 6 mm biopsies were obtained from human skin immediately after injection of PBS, LPS, VitD or LPS/VitD followed by a 3 days culture. Skin crawl-out DCs were identified by their typical high side (SSC) and forward (FSC) scatter characteristics and the expression of HLA-DR and CD11c. (A) DC counts from one skin biopsy. (B) Representative dot plots from each treatment (upper panel), and the percentages (middle panel) and absolute numbers (lower panel) of the three skin DC subsets were determined based on their expression of CD1a, D14 and langerin. Results are the mean±SEM of 7 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
the CD14⁺ DDC subset, which maintained lower maturation level compared to the other subsets. However, this increase was not observed in the MFI values of several experiments (Fig. 2B). In contrast to our earlier findings with isolated skin DCs and LCs in vitro (21), ID-injected VitD was not able to inhibit LPS-induced maturation. On the contrary, VitD supported LPS-induced DC maturation by slightly upregulating the expression of CD86 in the CD14⁺ DDC subset and CD83 expression in the CD1a⁺ DDC subset (Fig. 2A). However, these changes were marginal and could not be observed in the MFI values of several experiments (Fig. 2B).

VitD is also known for modulating cytokine production by various tissue cells, including DCs. The levels of IL-6, IL-10, IL-12 and TNF-α, the last three known to be modulated by VitD (21), were determined in the medium surrounding the biopsies after 72 hours of injection. Injected VitD slightly reduced LPS-induced IL-6 production, yet this reduction was insignificant. The combined injection of LPS and VitD induced a significant 2-fold increase in IL-10 production (Fig. 2C), corroborating in vitro observations. We were not able to measure any detectable levels of IL-12 and TNF-α.

So, unlike in vitro studies, ID-injected VitD has a weak impact on skin DC maturation, but significantly induced IL-10 production in the injected tissue while causing slight and significant reduction in IL-6 levels.

**DCs from VitD-injected skin have enhanced capacity to induce regulatory T cells**

We determined whether VitD would influence the T cell stimulatory capacity of skin DCs in our ex vivo model. The co-administration of VitD and LPS significantly hampered the ability of crawl-out cells to promote the proliferation of allogenic naïve CD4⁺ T cells when compared to cellular crawl-outs obtained after injection of LPS alone (Fig. 3). This limited proliferation could be due to the presence of Tregs. Therefore, we assessed to what extent the VitD-exposed crawl-out cells supported the development of Tregs. Compared to T cells induced by LPS-driven crawl-out cells, those induced by LPS/VitD-driven crawl-out cells were significantly more efficient in suppressing bystander T cells as reflected by 30% reduced proliferation (Fig. 4A). These data show that presence of VitD during LPS injection promoted the development of Tregs. We have previously reported that under the influence of VitD, purified and cultured skin LCs and DDCs do induce Foxp3⁺ Tregs and IL-10 producing Tregs, respectively. Unfortunately, we were unable to perform such experiments due to the low number of crawl-out DCs from the skin explants. We have to take into account that the crawl-out population contained all three skin DC subsets, but that the ratio between CD14⁺ DDCs and CD1a⁺ DDCs is strongly increased by VitD. Next, we investigated the phenotype of the induced regulatory T cells by this particular blend of DCs. LPS/VitD-primed crawl-outs did not induce any significant IL-10 productivity in T cells (Fig. 4B). On the contrary, IL-10 levels in the supernatants of these cells tended to be lower, yet not significantly, than their LPS counterparts. Interestingly, T cells induced by LPS/VitD-primed crawl-out cells showed a significantly higher frequency of Foxp3⁺ T cells (Fig. 4C).

Finally, we investigated whether VitD would have any influence on the ability of the crawl-out cells to induce the expression of Th1 (IFN-γ) and Th2 (IL-4) cytokines in primed T cells. Crawl-out cells derived from LPS-injected skin mainly promoted Th1 cell development. However, adding VitD to LPS markedly reduced the capacity of crawl-out cells to induce IFN-
Figure 2. ID injection of VitD does not inhibit LPS-induced maturation and modifies cytokine production at the site of injection. The maturation status of migrating DC subsets was determined by the expression of CD86 and CD83. (A) Crawl-out cells were gated on HLA-DR+ and CD11c+ and the different DC populations were identified by their expression of CD1a and CD14. The expression of CD86 and CD83 by the three subsets following the injection of PBS (closed histograms), LPS or LPS/VitD (open histograms) were compared. (B) The mean fluorescence intensity (MFI) values of CD86 and CD83 by the three subsets: LCs (white bars), CD1a+ DDCs (black bars) and CD14+ DDCs (grey bars) were compared following the injection of PBS, LPS or LPS/VitD. (C) Fold induction of IL-10 or reduction of IL-6 (compared to LPS) in 72 h culture medium of skin biopsies. Mean values of IL-10 and IL-6 produced in LPS condition, which was set to 1, were 113.66 ± 57.41 pg/ml for IL-10 and 126.85 ± 58.06 ng/ml for IL-6. Results are a representative of 6 (A and C) or the mean±SEM of 6 independent experiments (B and C). *P < 0.05, **P < 0.01, ***P < 0.001.
γ-producing T (Fig. 5A). This was also accompanied by modest and insignificant reduction in IL-4-producing T cell percentages. Reduced IFN-γ levels were also measured in supernatants of T cells activated by LPS/VitD-primed crawl-out cells (Fig. 5B).

Collectively, ID-injected VitD modulated the function of resident skin DCs by enhancing the capacity to generate Foxp3⁺ Tregs, and concomitantly, reducing the potential of the DCs to induce IFN-γ production in T cells.

DISCUSSION

Exploiting skin as a route of vaccination is founded by Edward Jenner, who demonstrated that inoculating cowpox-causing vaccinia virus into human skin rendered protection against smallpox (33). Although ID application is efficient, the SC route is the most prominent administration method utilizing skin. Currently ID vaccines are limited to rabies and Bacille Calmette-Guérin (BCG) vaccines (30). Unlike the SC route, ID application allows utilizing the extensive skin network of DCs, which plays a central role in generating immune responses (34). By granting access to skin antigen presenting cells, ID administration may also be more superior to transcutaneous, i.e. topical, application of vaccines which is hindered by the barrier functions of stratum corneum, the most superficial layer of the epidermis. However, new approaches in transcutaneous vaccination are circumventing this obstacle by applying novel formulations like microemulsions and liposomes, and novel application techniques like microneedles (35). Although LCs are directly exposed to transcutaneous vaccines, neighboring KCs are also affected. Stimulated KCs may also influence LC migration and stimulation status (35). In contrast, ID application guarantees delivery to dermal DC subsets and further proof is required to demonstrate that ID vaccines actually reach the epidermis and that LCs are exposed directly to these vaccines. Based on these differences, we speculate that ID vaccination might be more efficient in inducing immune responses. In light of increased prevalence of autoimmune and allergic diseases, the concept of ID vaccination can be extended from promoting immunity to inducing tolerance. In this study we show for the first time that ID application of VitD modulates the functions of human skin DCs to induce the development of Tregs.

The human skin DC network comprises epidermal LCs and two types of DDCs having differential expression of CD1a and CD14 (27,29,30). We demonstrate a selective significantly increased number of migratory CD14⁺ DDCs accompanied by reduced numbers of migratory CD1a⁺ DDCs. A plausible explanation for this observation is that the CD1a⁺ DDC subset upregulated the expression of CD14 under the influence of VitD. This hypothesis is supported by previous in vitro studies showing that monocyte-derived DDCs and LCs, as well as CD1c⁺ blood DCs, exhibit enhanced CD14 expression under the influence of VitD (20,21,36). An alternative explanation could be that VitD caused retention of CD1a⁺ DDCs while promoting the migration of CD14⁺ DDCs. Regarding LC migration, we observed that VitD did not block the previously reported LPS-induced migration of LCs (32). The influence of injected VitD on skin DC migration may provide a mechanism by which VitD promotes tolerance, as it favors the migration of CD14⁺ DDCs that are known for their tolerogenic effects (36).
Figure 3. ID injection of VitD reduces the LPS-induced T cell stimulatory capacity of skin crawl-outs.
Proliferation of allogenic naïve CD4+ T cells stimulated with crawl-out cells derived from LPS or LPS/VitD injected skin was determined by [3H]-thymidine incorporation. Data are shown as mean±SEM of triplicates of 1 representative experiment (n=4). **P < 0.01.

In addition to phenotypical variations between the different skin DC populations, functional specialization of these populations has become more defined in recent years. Whereas human LCs seem to specialize in inducing cytotoxic CD8+ T cell responses (37,38), human CD1a+ DDCs, which constitute most of DDCs, mediate CD4+ T cell responses (39), while CD14+ DDCs are proposed to promote antibody production by B cells through the induction of follicular helper T cells (40). Compared to other crawl-out DC types, the CD14+ DDC subset expresses lower levels of the DC co-stimulatory molecules CD80 and CD86 and the DC maturation marker CD83 (41-43), which was also observed in this study. This maturation profile was accompanied by a lower T cell stimulatory capacity in comparison to other skin DC subsets (40,41), which may be a reflection of the tolerogenic nature of this CD14+ DDC subset. Indeed, the tolerogenic function of this subset was stressed in a recent study demonstrating that CD14+ DDCs induce IL-10-producing Tregs (36). Collectively, the tolerogenic effect resulting from ID-injected VitD may be caused by selective VitD-induced enhanced migration of the CD14+ DDC subset. However, we did not observe enhanced IL-10 production in T cells primed by DCs crawling out of LPS/VitD-injected skin. One explanation is that VitD not only promotes migration of CD14+ DDCs, but also primes these DDCs for the induction of Foxp3 Tregs that are suppressive without the contribution of IL-10. Another explanation is that the other two skin DC subsets are vital as well in inducing tolerance. We previously showed that under the effect of VitD, LCs would induce Foxp3+ Tregs in a TGF-β dependent mechanism; whereas CD1a+ DDCs would induce IL-10 producing Tregs in an IL-10 dependent manner (21). Although VitD-primed crawl-out DCs comprise high percentages of CD14+ DDCs, they also contain LCs and CD1a+ DDCs, also determining the type of induced Tregs. IL-10 is a major factor in driving the development of Tr1 type Tregs that are characterized by high IL-10 production. However, the mere presence of TGF-β during Tr1 priming by IL-10 would diminish IL-10 productivity by the induced T cells (44). Since LCs are a major source of TGF-β, they may hinder the induction of IL-10 producing T cells by the other two subsets and endorse the development of Foxp3+ Tregs. Purifying the three different subsets following ID
Figure 4. Skin DCs migrating out of VitD-injected skin induce the development of Foxp3+ Tregs. T cells induced by LPS- or LPS/VitD-primed skin crawl-out cells were analyzed for regulatory characteristics. (A) The suppressive capacity of induced T cells expressed as the proliferation of bystander T cells. This proliferation is depicted as the percentage of T cell proliferation of the LPS condition, which was set as a base line. (B) IL-10 fold induction in 24h-supernatants of T cells restimulated by αCD3/αCD28. (C) Foxp3 expression by resting T cells was determined by flow cytometry. Results are representative of 5 (A right panel), 6 (B right panel) or 7 (C right panel) or the mean±SEM of 5 (A left panel), 6 (B left panel) or 7 (C left panel) independent experiments. *P < 0.05, **P < 0.01.

% Proliferating cells

CFSE

IL-10 fold induction

IL-10 (ng/ml)

%Foxp3 Cells

CD127

84.3%

60%

18.6

23.5
injection of VitD would allow pinpointing the contribution of each subset to tolerance, but this was impossible in this study due to the limited number of in crawl-out cells.

The immunosuppressive effect exerted by VitD has been related to its influence on DCs. Several in vitro studies (17,18) demonstrated that VitD downregulates the expression of MHC-II and co-stimulatory molecules, to repress IL-12 production and boosts IL-10 production. In our skin explant model we were not able to observe this inhibitory effect on LPS-induced maturation. It is noteworthy that LC maturation was enhanced by ID-injection of LPS, though LCs do not express TLR4 (37). Both observations may be attributed to KCs, the main constituent of the epidermis, which express both TLR4 and vitamin D receptors (45,46). LPS-stimulated KCs were shown to release TNF-α, which may explain enhanced LC migration and maturation (45). Treatment of KCs with VitD also induces the release of TNF-α and thymic stromal lymphopoietin (TSLP) (47,48), the latter being known to instruct DCs to promote Th2 cell development (49). Yet, we were not able to detect enhanced Th2 priming by VitD-conditioned crawl-out cells, implying that the effect of TSLP may be overridden by other factors. In line with in vitro data, we were able to detect elevated IL-10 levels in the cultures of skin biopsies injected with VitD, though this elevation may also result from cells other than DCs, but not KCs (50). Nevertheless, KCs
may be a major contributor in shaping immune responses following ID injection of VitD through the release of mediators that can influence DC functions. Although intradermally injected VitD rendered skin DCs tolerogenic, VitD concentrations applied in this model were 10 times higher than usually used in vitro on monocyte-derived or purified DCs and LCs (21). Injecting lower concentrations did not have any effect (data not shown). However, it is noteworthy that LPS effects in this model are also observed at concentrations 10 times higher than those applied in vitro. This may have to do with the complexity of the skin model, as VitD being injected in a tissue and not simply applied on purified cells. This concentration factor should be addressed for any toxic effects, especially calcimic toxicity, before any clinical application of intradermal VitD.

We demonstrated that VitD is a potent inducer of Tregs when administered into the dermis. This may have great implications for innovating or potentiating old therapeutic approaches for the treatment of autoimmune and allergic diseases. SIT, currently the only cure for allergies, depends on the induction of Tregs to quench the Th2-led allergic responses. However, this therapy is characterized by poor efficiency and long treatment periods. Since SIT is usually applied through the skin, VitD would be a suitable regulatory adjuvant that maintains its tolerogenic effects when applied into the skin.
**SUPPLEMENTARY FIGURES**

Supplementary Figure 1. Skin crawl-out DCs were identified by their typical high sideward (SSC) and forward (FSC) scatter properties and their distinctive high expression of HLA-DR and CD11c.

Supplementary Figure 2. Within crawl-out DCs, three skin DC subsets could be distinguished based on their expression of CD1a, CD14 and langerin: LCs were langerin^+^CD1a^high^CD14^−^, whereas the two populations of DDCs both lacked expression of langerin and were either CD14^+^CD1a^−^ or CD14^−^CD1a^low^. 
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