

Decreased serum LL-37 and vitamin D3 levels in atopic dermatitis: relationship between IL-31 and oncostatin M

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Keywords

atopic dermatitis; IL-31; LL-37; oncostatin M; vitamin D3.

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Abstract

Background: Skin lesions with atopic dermatitis (AD) are associated with dysregulated expression of LL-37 and enhanced expression of IL-22, thymic stromal lymphopoietin (TSLP), IL-25, IL-31, and oncostatin M. Vitamin D3 enhances LL-37 production in keratinocytes. This study aimed to examine the serum levels of LL-37 and vitamin D3 and their regulation of cytokine production in patients with AD.

Methods: Serum levels of LL-37 and 25-hydroxyvitamin D3 were analyzed by ELISA. The effects of 1,25-dihydroxyvitamin D3 or LL-37 on cytokine production in T cells or keratinocytes were analyzed by ELISA and real-time PCR.

Results: Serum levels of LL-37 and 25-hydroxyvitamin D3 were decreased in patients with AD compared to normal donors and were correlated in both groups. Serum levels of LL-37 correlated with those of oncostatin M and IL-31 in normal donors and patients with AD, while 25-hydroxyvitamin D3 levels did so only in normal donors. 1,25-dihydroxyvitamin D3 increased LL-37 production in human keratinocytes and neutrophils. 1,25-dihydroxyvitamin D3 and LL-37 enhanced the oncostatin M and IL-31 production in CD3/28-stimulated T cells, but did not alter IL-25 and TSLP production in TNF- α -stimulated keratinocytes. In CD3/28-stimulated T cells, 1,25-dihydroxyvitamin D3 reduced the IL-22 production, while LL-37 enhanced it. These effects of 1,25-dihydroxyvitamin D3 and LL-37 were suppressed by vitamin D receptor antagonist and pertussis toxin, respectively.

Conclusions: Systemic vitamin D3 levels are reduced in patients with AD, which may contribute to decreased systemic LL-37 levels. LL-37 may systemically potentiate the oncostatin M and IL-31 production in normal donors and patients with AD, while vitamin D3 may do so only in normal donors.

Atopic dermatitis (AD) is a chronic inflammatory dermatosis associated with frequent cutaneous infection with *Staphylococcus aureus* or herpes simplex virus (1–4). Reduced

expression of antimicrobial peptides may cause recurrent infections (2, 3). LL-37, having antimicrobial activity against bacteria, viruses, and fungi, is produced by epidermal keratinocytes (2, 4, 5). Either decreased (3, 6) or increased (7) LL-37 expression is reported in AD lesions.

Vitamin D3 enhances LL-37 production in keratinocytes, because vitamin D response element (VDRE) occurs on the gene of LL-37 precursor cathelicidin (2). Oral vitamin D3 increases cathelicidin expression in AD lesions (8). However, the roles of vitamin D3 in the pathogenesis of AD are controversial (9); vitamin D3 supplementation improves AD symptoms (10), while vitamin D3 intake during infancy accelerates the development of AD (11). Vitamin D3 downregulates

Abbreviations

AD atopic dermatitis; BMI body mass index; DMSO dimethyl sulfoxide; eAD extrinsic AD; GAPDH glyceraldehyde 3-phosphate dehydrogenase; iAD intrinsic AD; KBM keratinocyte basal medium; 1,25(OH)2D3 1,25-dihydroxyvitamin D3; 25(OH)D3 25-hydroxyvitamin D3; OSMR oncostatin M receptor; SCORAD scoring of atopic dermatitis; STAT signal transducer and activator of transcription; TSLP thymic stromal lymphopoietin; VDR vitamin D receptor; VDRE vitamin D response element.

Th1/17 activities, induces regulatory T cells, and promotes or does not alter the Th2 activity (12, 13). Thus, *in vivo* effects of vitamin D3 in patients with AD may differ with timing or balance of T-cell subsets.

IL-22, oncostatin M, IL-31, IL-25, and thymic stromal lymphopoietin (TSLP) are recently implicated in the development of AD (14). IL-22, produced by T-cell subpopulation in AD lesions (15), induces the proliferation of keratinocytes (1, 15). IL-31, expressed by skin-homing T cells in AD lesions (16), is a pruritogen and mainly produced by Th2 cells (16, 17). IL-25 potentiates IL-4/5/13 production in T cells (18) and suppresses filaggrin synthesis in keratinocytes (19). T cells in AD lesions highly express oncostatin M (20), which induces eosinophilia in murine lung (21). TSLP, highly expressed in keratinocytes of AD lesions, stimulates dendritic cells to polarize naïve T cells to differentiate into Th2 cells (22). We recently analyzed the serum levels of these cytokines in patients with AD compared with normal donors (23). However, the relationships of their levels to vitamin D3 or LL-37 are unknown.

We examined serum LL-37 and vitamin D3 levels and their correlation with serum levels of IL-22, oncostatin M, IL-31, IL-25, and TSLP. We also investigated the effects of LL-37 and vitamin D3 on the production of these cytokines in T cells or keratinocytes.

Materials and methods

Patients and controls

This study was conducted according to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board (TUSM-RB 09-005). The profiles of 26 patients with AD and 27 normal volunteers are listed in Table S1. Patients with AD were categorized into two types: 22 extrinsic AD (eAD) patients with high total serum IgE levels (>200 U/ml) and positive serum IgE antibodies toward common inhalant and food allergens and four intrinsic AD (iAD) patients with low total serum IgE levels (<200 U/ml), without serum IgE antibodies for above-mentioned allergens, asthma, or rhinitis (24). There were no significant differences in age, body mass index (BMI), and scoring of AD (SCORAD) (25) between eAD and iAD, or between total AD and normal donors (by Mann–Whitney *U*-test).

Reagents

RPMI 1640 supplemented with 10% fetal calf serum (complete RPMI) was purchased from PromoCell (Heidelberg, Germany); synthetic LL-37, from Peptide Institute (Osaka, Japan); and pertussis toxin, from Merck (Darmstadt, Germany). Vitamin D receptor (VDR) antagonist TEI-9647 was donated by Teijin Pharma (Tokyo, Japan) and dissolved in dimethyl sulfoxide (DMSO) at 10 mM. Rabbit anti-LL-37 antibody was from AnaSpec (Fremont, CA, USA), and 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] from Sigma (St. Louis, MO, USA).

ELISA

Following ELISA kits were used: LL-37 (Hycult Biotech, Plymouth Meeting, PA, USA), 25-hydroxyvitamin D3 [25(OH)D3] (Uscn Life Science, Wuhan, China), IL-22, IL-31, TSLP, IL-4, and IFN- γ (R&D Systems, Minneapolis, MN, USA), oncostatin M (RayBiotech, Norcross, GA, USA), IL-25 (KOMA BIOTECH, Seoul, Korea), and IL-17A (GenProbe Life Sciences, Livingston, West Lothian, UK).

Keratinocytes

Human neonatal foreskin keratinocytes (Clonetics, Walkersville, MD, USA) were cultured in keratinocyte growth medium (Clonetics) containing keratinocyte basal medium (KBM) supplemented with 0.5 μ g/ml hydrocortisone, 5 ng/ml epidermal growth factor, 5 μ g/ml insulin, and 0.5% bovine pituitary extract. Keratinocytes were seeded into 24-well plates (5.0×10^4 cells/well), incubated in supplement-free KBM for 24 h, pretreated with 1 μ M TEI-9647 or 0.01% DMSO for 2 h, and treated with 1,25(OH)2D3 in 0.4 ml KBM. After 48 h, supernatants were assayed for LL-37. Keratinocytes, seeded and supplement-depleted, were treated with 10 ng/ml TNF- α (R&D) with LL-37 or 1,25(OH)2D3 for 48 h; supernatants were assayed for TSLP and IL-25.

Neutrophils and T cells

Neutrophils and T cells were isolated from heparinized blood of patients with AD and normal donors (4). Neutrophils (2.0×10^6 cells/well) were seeded in 2 ml complete RPMI in 24-well plates, pretreated as above, and treated with 1,25(OH)2D3; supernatants were assayed for LL-37.

T cells (2.0×10^6 cells/well) in 24-well plates were stimulated with plate-bound anti-CD3 (0.1 μ g/ml) and soluble anti-CD28 (2 μ g/ml) (BD Pharmingen, San Diego, CA, USA) with LL-37 or 1,25(OH)2D3 in 2 ml complete RPMI. After 48 h, supernatants were assayed for cytokines.

Real-time PCR

After 8-h incubation, total cellular RNA was extracted and reverse-transcribed. Real-time PCR was performed as described (26). mRNA levels of cathelicidin or cytokines were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Transfection

Vitamin D response element reporter (SABiosciences, Frederick, MD, USA), containing VDRE-responsive firefly luciferase and constitutive *Renilla* luciferase constructs, was mixed with Eugene HD (Roche, Indianapolis, IN, USA) and added to keratinocytes (3.0×10^4 cells/well) in 24-well plates. After 24 h, keratinocytes were supplement-depleted, pretreated as above, and incubated with 1,25(OH)2D3. After 18 h, firefly and *Renilla* luciferase activities of cell

extracts were quantified using Dual-Luciferase Assay System (Promega, Madison, WI, USA). Transcriptional activities of VDR are expressed as ratio of firefly to *Renilla* luciferase activities.

Flow cytometry

THP-1 cells (American Type Culture Collection, Rockville, MD, USA) (1.0×10^6 cells/well) in 24-well plates were

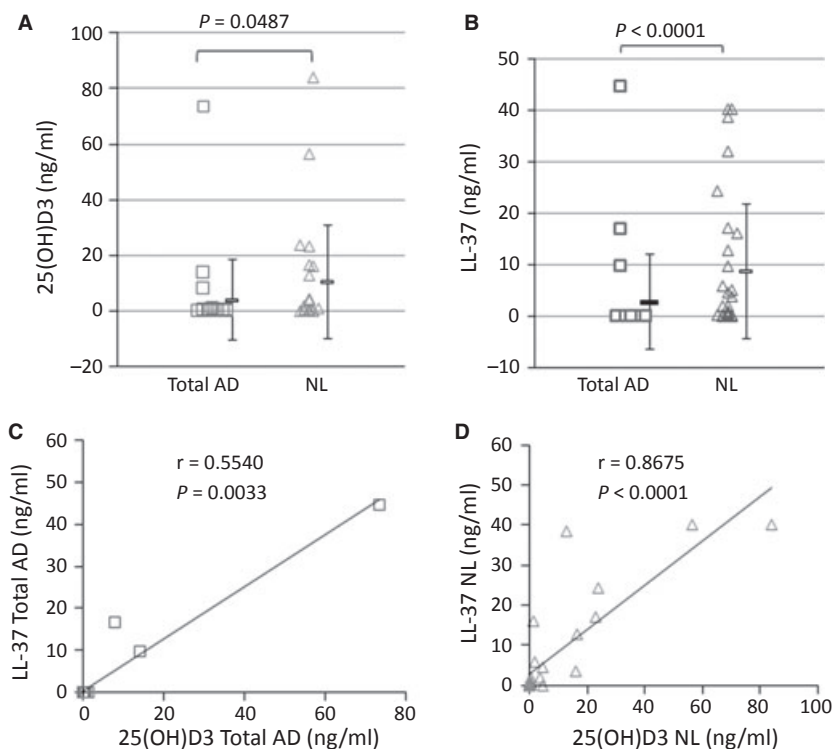


Figure 1 Serum 25(OH)D3 levels (A) and LL-37 levels (B) are lower in total patients with atopic dermatitis than in normal donors (NL) and are correlated in both groups (C, D). (A, B) Mann-Whitney U-test was used to determine the differences between two

groups, and the mean $\pm$ SD values are shown. (C, D) Correlations were analyzed by Spearman's rank test and linear regression analysis, and regression lines are shown.

Table 1 Correlations of serum LL-37 or 25-hydroxyvitamin D3 with BMI, SCORAD, or cytokine levels in normal donors and patients with AD

	LL-37				25-hydroxyvitamin D3			
	NL (n = 27)	Total AD (n = 26)	eAD (n = 22)	iAD (n = 4)	NL (n = 27)	Total AD (n = 26)	eAD (n = 22)	iAD (n = 4)
BMI	0.0736	-0.0164	0.0256	UC	-0.0287	0.0062	-0.0786	UC
SCORAD	-	0.2844	0.3185	UC	-	-0.0227	-0.1643	0.6000
IL-31	0.8755**	0.5554**	0.5964**	UC	0.7566**	0.2842	0.2808	0.4000
Oncostatin M	0.5503*	0.4611*	0.4724*	UC	0.6988**	0.2732	0.2358	UC
IL-25	-0.0128	0.2521	0.2849	UC	-0.0896	0.2832	0.4070	-0.4000
IL-22	0.2659	-0.1497	-0.1940	UC	0.2410	-0.1512	-0.0271	-0.4000
TSLP	-0.0551	0.1496	0.1312	UC	-0.1792	-0.0736	-0.0403	-0.3162
IL-17A	0.0554	-0.2546	-0.2887	UC	0.1873	-0.2589	-0.3003	0.8000
IL-4	0.2038	-0.0947	-0.1539	UC	0.0052	-0.1252	-0.0214	UC
IFN- γ	0.1322	-0.3368	-0.3931	UC	0.3202	-0.3703	-0.3402	0.0000

AD, atopic dermatitis; BMI, body mass index; eAD, extrinsic AD; iAD, intrinsic AD; NL, normal; SCORAD, scoring of AD; TSLP, thymic stromal lymphopoietin; UC, uncalculable.

Spearman's correlation coefficients are shown.

* $P < 0.05$.

** $P < 0.01$.

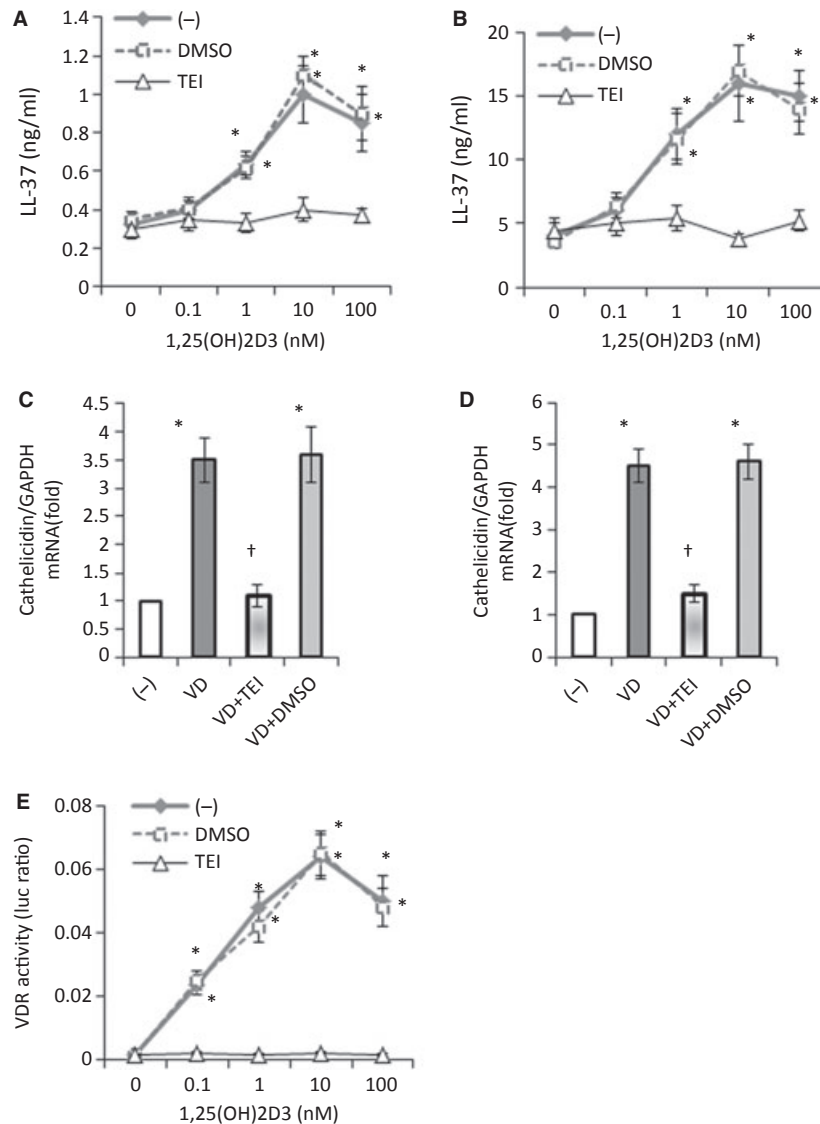


Figure 2 1,25(OH)2D3 increases LL-37 secretion and cathelicidin mRNA levels in keratinocytes (A, C) and neutrophils (B, D) and increases the transcriptional activity of vitamin D response in keratinocytes (E). (A, B) Keratinocytes or neutrophils were pretreated with 1 μ M TEI-9647 (TEI) or 0.01% DMSO for 2 h and treated with indicated concentrations of 1,25(OH)2D3 for 48 h, and the supernatants were analyzed for LL-37. (C, D) Keratinocytes or neutrophils were pretreated as above and treated with 10 nM 1,25 (OH)2D3 (VD) for

8 h. mRNA levels of cathelicidin are shown as fold inductions relative to control cells with the medium alone. (E) Keratinocytes transfected with firefly/*Renilla* luciferase vectors were pretreated as above and treated with indicated concentrations of 1,25(OH)2D3 for 18 h. (A, B, E) $*P < 0.05$ vs controls, by one-way ANOVA with Dunnett's test. (C, D) $*P < 0.05$ vs controls; $^{\dagger}P < 0.05$ vs VD alone, by one-way ANOVA with Scheffe's test. Data are mean $\pm$ SD values of triplicate cultures and represent four separate experiments.

incubated with LL-37 or 1,25(OH)2D3 or 1 μ g/ml LPS (Sigma) plus 10 ng/ml IFN- γ (R&D) in 1 ml complete RPMI for 24 h. Expression of IL-31RA or oncostatin M receptor (OSMR) was assessed by FITC-conjugated goat anti-IL-31RA or mouse PE-conjugated anti-OSMR antibody (Lifespan BioSciences, Seattle, WA, USA), respectively, and isotype controls (R&D). A minimum of 10^4 cells were analyzed on FACScan flow cytometer (BD Biosciences, Mountain View, CA, USA). mRNA levels of these receptors were analyzed at 8 h by real-time PCR.

Statistical analysis

Spearman's rank test was used to calculate the linear relationship. Mann-Whitney *U*-test or Kruskal-Wallis test was used to determine the differences between two or three groups, respectively. One-way ANOVA with Dunnett's test was used for Figs 2A,B,E and 3 and S1, and one-way ANOVA with Scheffe's test for Figs 2C,D and 4 and 5C,D and S2. $P < 0.05$ was considered significant.

Results

Decreased serum vitamin D3 and LL-37 levels in patients with AD

Serum 25(OH)D3 levels, representing systemic vitamin D3 status (26), were significantly reduced in total patients with AD compared to normal donors (Fig. 1A) without differences between eAD and normal donors, between iAD and normal donors, and between eAD and iAD (Table S2). Serum LL-37 levels were significantly reduced in total AD, eAD, and iAD compared to normal donors (Fig. 1B, Table S2). Serum LL-37 levels in all patients with iAD were below the detectable limit of ELISA kit (sensitivity 0.14 ng/ml), approximately corresponding to 0.00 ng/ml, although without differences between eAD and iAD. Serum LL-37 levels correlated with 25(OH)D3 levels in total AD (Fig. 1C), normal donors (Fig. 1D), and eAD ($r = 0.5952$, $P = 0.0035$). 25(OH)D3 or LL-37 levels did not correlate with SCORAD or BMI in any groups (Table 1). Because serum LL-37 levels were below the detectable limit in iAD, correlations of LL-37 levels with other factors could not be calculated in iAD.

We analyzed the serum levels of AD-related cytokines and their correlations with LL-37 or 25(OH)D3 levels. In total AD, serum IL-22 and oncostatin M levels were higher; IL-31 and IL-25 levels, lower; TSLP, IL-17A, IL-4, and IFN- γ levels, the same as in normal donors (23; Table S2). In eAD, IL-22 and oncostatin M levels were higher, while IL-31 levels were lower than in normal donors without differences in IL-25 levels (Table S2). Although levels of cytokines except for IL-22 and TSLP in iAD appeared lower than in eAD, differences were not significant between iAD and eAD and between iAD and normal donors. Serum IL-31 and oncostatin M levels correlated with LL-37 levels in normal donors, total AD, and eAD (Table 1). Serum IL-31 and oncostatin M levels correlated with 25(OH)D3 levels in normal donors, but not in total AD, eAD, and iAD (Table 1).

1,25(OH)2D3 increases LL-37 production in keratinocytes and neutrophils

We analyzed the effects of 1,25(OH)2D3, an active vitamin D3, on LL-37 secretion. 1,25(OH)2D3 increased LL-37 secretion from keratinocytes (Fig. 2A) and neutrophils (Fig. 2B), and the effects were counteracted by VDR antagonist TEI-9647, indicating VDR-mediated effects. 1,25(OH)2D3 enhanced mRNA levels of cathelicidin in keratinocytes (Fig. 2C) and neutrophils (Fig. 2D), and the effects were suppressed by TEI-9647, indicating transcriptional induction. In parallel with LL-37 production, 1,25(OH)2D3 enhanced the transcriptional activities of VDR in keratinocytes, and TEI-9647 suppressed the effects (Fig. 2E).

Effects of 1,25(OH)2D3 or LL-37 on cytokine production

We analyzed the effect of 1,25(OH)2D3 or LL-37 on cytokine secretion in T cells and keratinocytes. 1,25(OH)2D3 and LL-37 enhanced oncostatin M (Fig. 3A,F) and IL-31 secretion

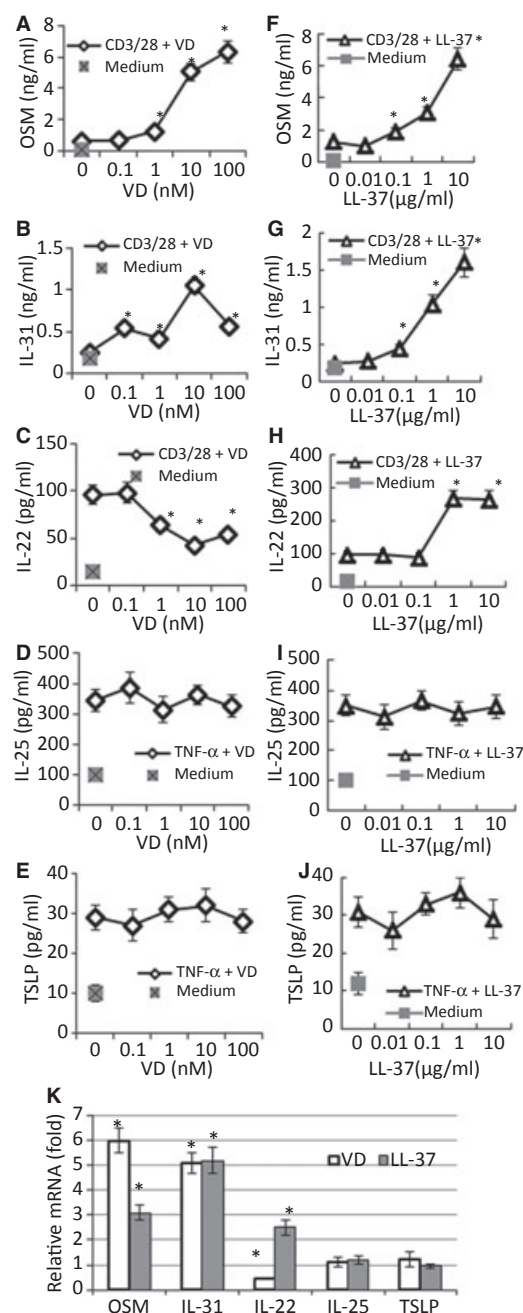


Figure 3 Effects of 1,25(OH)2D3 (VD) or LL-37 on the secretion and mRNA levels of oncostatin M (OSM), IL-31, and IL-22 in T cells and of IL-25 and TSLP in keratinocytes. (A–J) Normal donor T cells (A, B, C, F, G, H) or keratinocytes (D, E, I, J) were stimulated with CD3/28 or 10 ng/ml TNF- α , respectively, in the presence of the indicated concentrations of VD (A–E) or LL-37 (F–J). (K) mRNA levels of cytokines in T cells or keratinocytes after 8-h incubation with CD3/28 or TNF- α , respectively, in the presence of 10 nM VD or 1 μ g/ml LL-37 are shown as fold inductions relative to T cells with CD3/28 alone or keratinocytes with TNF- α alone, respectively. * $P < 0.05$ vs CD3/28 alone, by one-way ANOVA with Dunnett's test. Data are mean $\pm$ SD values of triplicate cultures and represent four separate experiments.

(Fig. 3B,G) in CD3/28-stimulated T cells. 1,25(OH)2D3 reduced the IL-22 secretion, while LL-37 increased it (Fig. 3C, H). Because IL-25 and TSLP are produced by epithelial cells in allergic diseases (18, 19), we examined IL-25 and TSLP secretion from TNF- α -stimulated keratinocytes, but observed no effects of 1,25(OH)2D3 or LL-37 (Fig. 3D,E,I,J). Upregulation or downregulation of individual cytokine mRNAs by 1,25(OH)2D3 or LL-37 (Fig. 3K) was similar to that of protein secretion, indicating transcriptional regulation.

1,25(OH)2D3 reduced the IL-17A secretion, while LL-37 did not alter IL-17A secretion in CD3/28-stimulated T cells (Fig. S1A,D). 1,25(OH)2D3 and LL-37 enhanced IL-4 secretion in these cells (Fig. S1B,E). 1,25(OH)2D3 reduced the IFN- γ secretion, while LL-37 increased it in these cells (Fig. S1C,F). Upregulation or downregulation of individual cytokine mRNAs by 1,25(OH)2D3 or LL-37 (Fig. S1G) was similar to that of protein secretion. T cells from patients with AD and normal donors showed similar responses to 1,25(OH)2D3 and LL-37 (data not shown).

To analyze the mutual dependency of the effects of 1,25(OH)2D3 and LL-37 on cytokine production, we examined the inhibition by VDR antagonist or anti-LL-37 antibody. TEI-9647, but not anti-LL-37 antibody, counteracted the effects of 1,25(OH)2D3 on oncostatin M, IL-31, IL-22 (Fig. 4A–C), IL-17A, IL-4, and IFN- γ secretion (Fig. S2A–C), suggesting the effects through VDR rather than LL-37. Anti-LL-37 antibody and $G_{i/o}$ protein inhibitor pertussis toxin, but not TEI-9647, counteracted the effects of LL-37 on oncostatin M, IL-31, IL-22 (Fig. 4–C), IL-4, and IFN- γ secretion (Fig. S2B,C), indicating the effects via $G_{i/o}$ proteins, but not VDR. Similar results were obtained on cytokine mRNAs (data not shown). These results suggest that 1,25(OH)2D3 and LL-37 may independently regulate the cytokine production.

Effects of 1,25(OH)2D3 or LL-37 on the expression of IL-31 receptors

IL-31 signaling receptor is a heterodimer of IL-31RA and OSMR (27). We examined whether 1,25(OH)2D3 or LL-37 alters IL-31RA and/or OSMR expression in monocytic THP-1 cells. 1,25(OH)2D3 or LL-37 did not alter the cell surface expression of IL-31RA or OSMR on THP-1 cells (Fig. 5A,B), while LPS plus IFN- γ enhanced their expression. 1,25(OH)2D3 or LL-37 did not alter mRNA levels of IL-31RA or OSMR in THP-1 cells, while those were increased by LPS plus IFN- γ (Fig. 5C,D).

Discussion

Serum LL-37 and vitamin D3 levels are decreased in total patients with AD, and their levels are correlated. Thus, the deficiency of vitamin D3 may contribute to that of LL-37 in total patients with AD and may be related to their recurrent cutaneous infection. As eAD occupied 84.6% of total AD in this study, above-mentioned features of total AD mostly reflected those of eAD. Serum levels of 25(OH)D3, LL-37, and cytokines in iAD appeared lower than in

eAD; however, differences were not significant possibly due to the small number of patients with iAD. Future studies on larger patients' population may elucidate the differences.

Further research should determine the cause of decreased vitamin D3 levels in patients with AD. One possibility is that patients with AD may have defects in vitamin D 25-hydroxylase, synthesizing 25(OH)D3, in the skin or liver, or in the absorption of ingested vitamin D3 precursors in the gut (26).

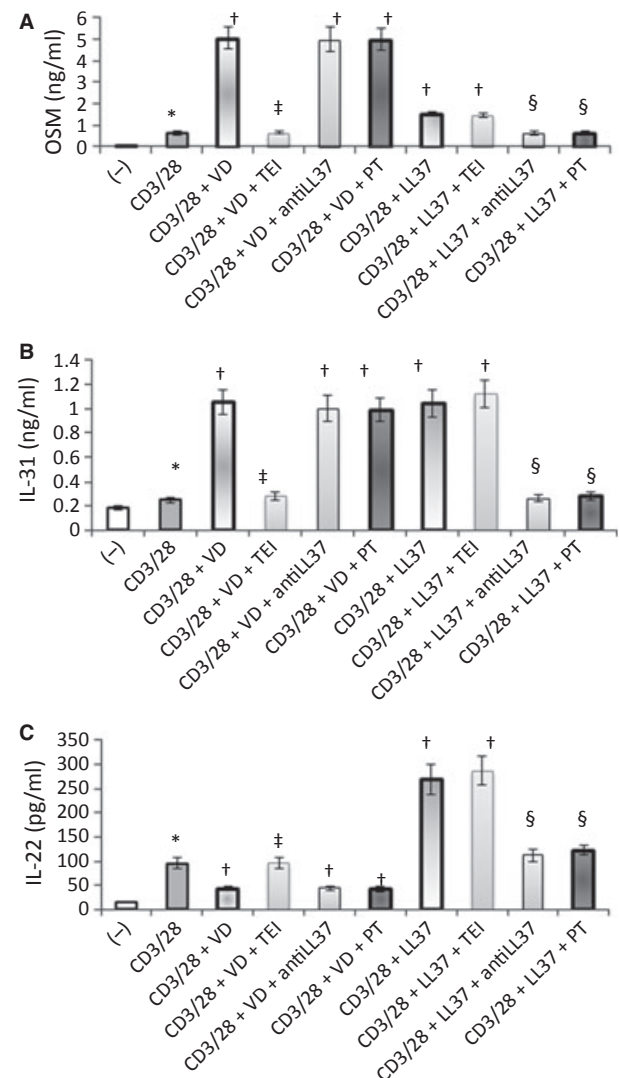


Figure 4 1,25(OH)2D3(VD) or LL-37 independently regulates oncostatin M (OSM) (A), IL-31(B), and IL-22 secretion (C) in T cells. T cells were pretreated with 1 μ M TEI-9647 (TEI), 10 μ g/ml anti-LL-37 antibody, or 100 ng/ml pertussis toxin (PT) for 2 h and treated with CD3/28 together with 10 nM VD or 1 μ g/ml LL-37 for 48 h, and supernatants were analyzed for cytokines. * P < 0.05 vs controls; $^{\dagger}P$ < 0.05 vs CD3/28 alone; $^{\ddagger}P$ < 0.05 vs CD3/28 plus VD; $^{\S}P$ < 0.05 vs CD3/28 plus LL-37, by one-way ANOVA with Scheffe's test. Data are mean $\pm$ SD values of triplicate cultures and represent four separate experiments.

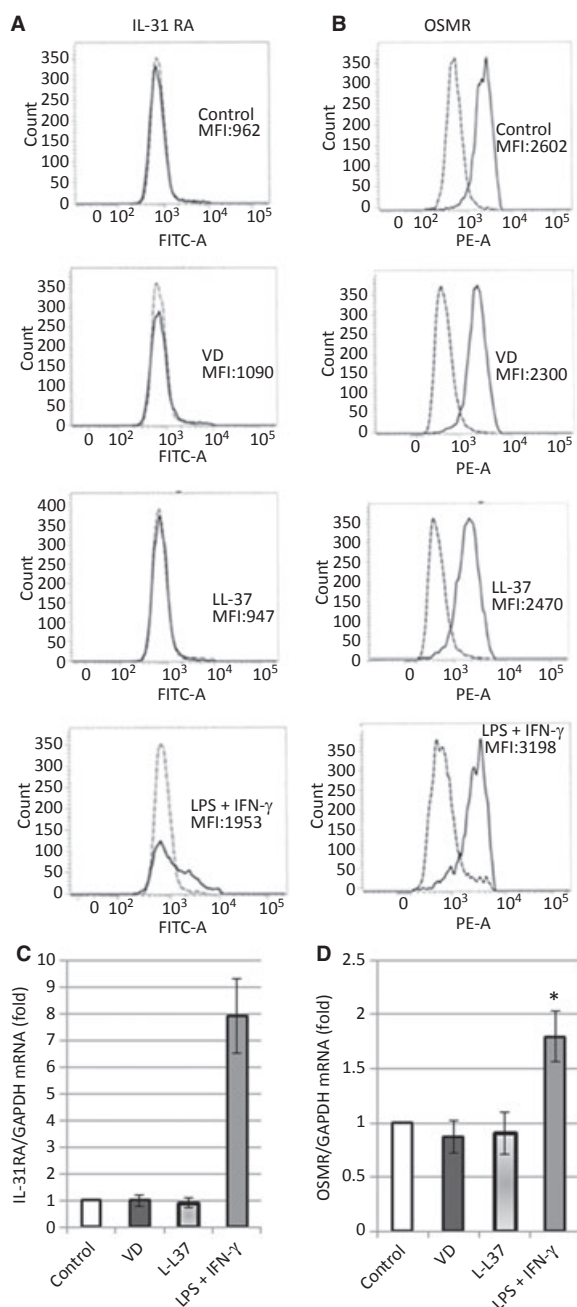


Figure 5 1,25(OH)2D3(VD) or LL-37 did not alter the cell surface expression or mRNA levels of IL-31RA (A, C) or oncostatin M receptor (OSMR) (B, D) in THP-1 cells. (A, B) THP-1 cells were treated with 10 nM VD, 1 μ g/ml LL-37, or 1 μ g/ml LPS plus 10 ng/ml IFN- γ for 24 h and were analyzed for IL-31RA or OSMR expression by flow cytometry. Solid lines are data of IL-31RA (A) or OSMR (B), while dotted lines are those of isotype controls. Data represent four separate experiments. MFI, mean fluorescence intensity. (C, D) mRNA levels of IL-31RA or OSMR after 8-h incubation are shown as fold inductions relative to controls with medium alone. * P < 0.05 vs controls, by one-way ANOVA with Scheffe's test. Data are mean $\pm$ SD values of triplicate cultures and represent four separate experiments.

Alternatively, the catabolism of vitamin D3, that is, activity of catabolic enzyme 25-hydroxyvitamin D-24-hydroxylase, may be accelerated in patients with AD (26).

In our study, neither serum vitamin D3 nor LL-37 levels in patients with AD correlated with SCORAD, indicating their independency from disease severity. However, recent study showed that while serum LL-37 levels in children with eczema did not differ from those in noneczema children, the levels increased with increasing severity of eczema (28). The discrepancy with our results may be due to differences in the patient population (race, age, etc.).

1,25(OH)2D3 promoted oncostatin M, IL-31, and IL-4 production, while it reduced IL-17A, IL-22, and IFN- γ production. These effects of 1,25(OH)2D3 on IL-17A, IL-22, IFN- γ , and IL-4 were consistent with those of previous studies (11, 12), indicating that 1,25(OH)2D3 may enhance Th2 activity, while it may suppress Th17, Th1, and Th22 activities. The stimulatory effects on IL-31 and oncostatin M production support Th2 polarization by 1,25(OH)2D3. Oncostatin M, although firstly identified as Th1 type, manifests Th2-favoring effects like signal transducer and activator of transcription (STAT)6 tyrosine phosphorylation (21). 1,25(OH)2D3 promotes the expression of STAT6 and GATA-binding factor 3 in human T cells (29), which may mediate IL-31 and oncostatin M production.

LL-37 enhanced IL-4, IL-31, oncostatin M, IFN- γ , and IL-22 production without altering that of IL-17A, indicating that LL-37 may favor Th2, Th1, and Th22 activities rather than Th17 because IL-22 is produced by Th22 cells and a subpopulation of Th1 as well as Th17 cells (15). The effects of LL-37 were suppressed by pertussis toxin, indicating the involvement of G_{i/o} protein-coupled receptors, which should further be identified. LL-37 enhances IL-31 production in human mast cells via the activation of PI3K, ERK, JNK, and p38 MAPK (30). Similar signals may mediate LL-37-induced promotion of IL-31 and oncostatin M in T cells. 1,25(OH)2D3 and LL-37 did not alter IL-31RA or OSMR expression on THP-1 cells. Thus, these agents may not affect responsiveness to IL-31 on THP-1 cells. LL-37-induced promotion or 1,25(OH)2D3-induced suppression of IL-22 production may be caused by the stimulation (5) or suppression (31) of STAT3 required for IL-22 production (32), respectively.

LL-37 promoted oncostatin M and IL-31 production in T cells, and serum levels of both cytokines correlated with LL-37 levels in normal donors and patients with AD. LL-37 may thus systemically potentiate oncostatin M and IL-31 production in both groups. On the other hand, serum IL-31 and oncostatin M levels correlated with vitamin D3 levels in normal donors, but not in patients with AD, indicating the contribution of vitamin D3 only in the former. We should further analyze whether vitamin D3 and LL-37 upregulate IL-31 and oncostatin M production in other cell types like dendritic cells.

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Authors contribution

Naoko Kanda performed all the experiments and described the manuscript. Carren S Hau and Yayoi Tada performed the experiments for the expression of IL-31RA and OSMR, and Shinichi Sato advised them about the experiments. Shinichi Watanabe revised the manuscript.

Conflict of interest

The authors, Naoko Kanda, Carren S Hau, Yayoi Tada, Shinichi Sato, and Shinichi Watanabe, have no conflicts of interest to be disclosed.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effects of 1,25(OH)2D3 (VD) or LL-37 on the secretion and mRNA levels of IL-17A, IL-4, and IFN- γ in T cells.

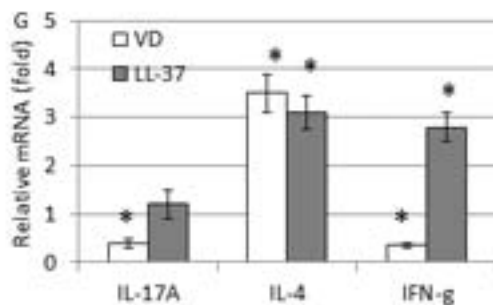
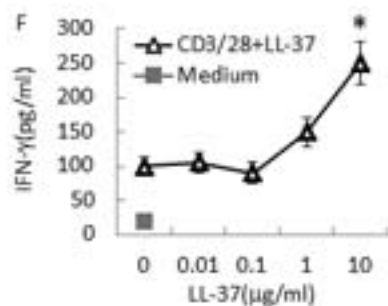
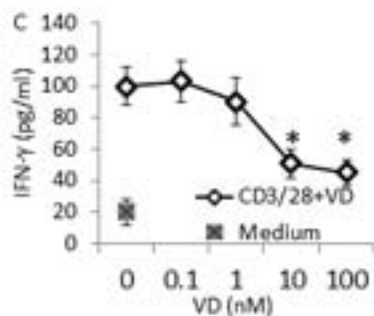
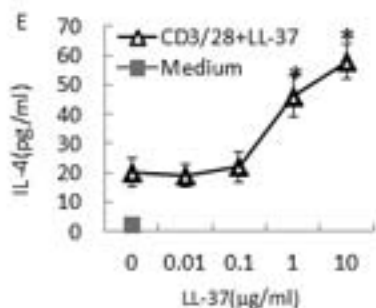
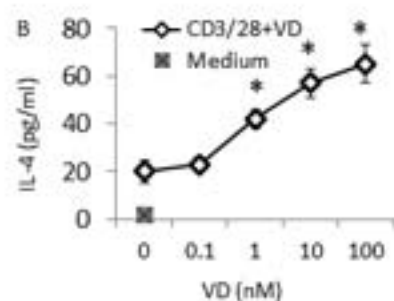
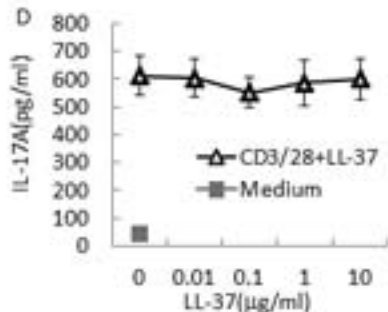
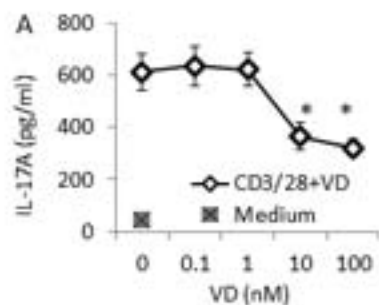
Figure S2. 1,25(OH)2D3(VD) or LL-37 independently regulates IL-17A (A), IL-4(B), and IFN- γ secretion (C) in T cells.

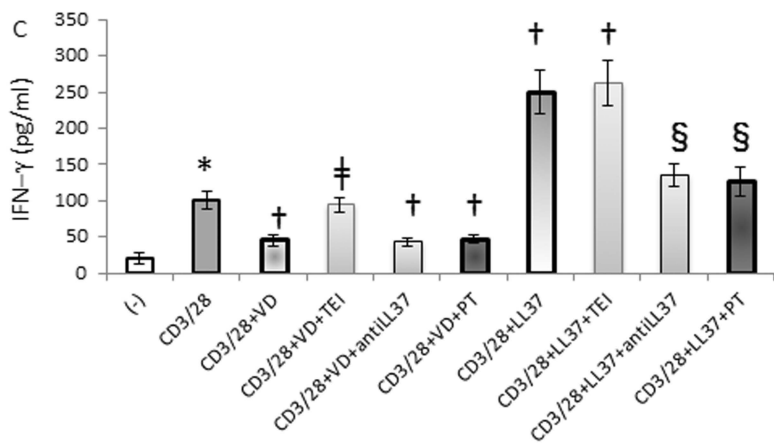
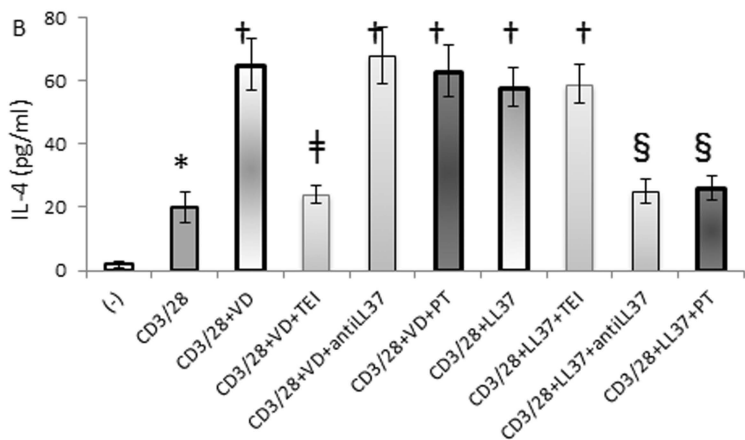
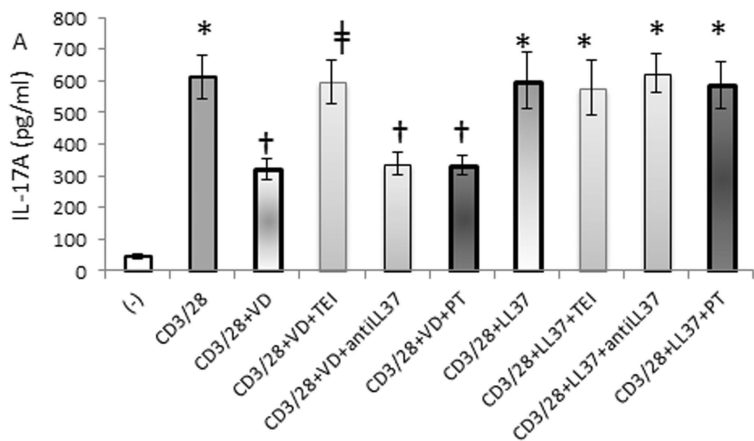
Table S1. Profiles of atopic dermatitis patients and normal donors.

Table S2. Comparisons of serum 25-hydroxyvitamin D3, LL-37 or cytokine levels in normal donors and atopic dermatitis patients.

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Supplemental Table 1 Profiles of atopic dermatitis patients and normal donors

	NL	Total AD	eAD	iAD
Number	27	26	22	4
Male:Female	19:8	16:10	13:9	3:1
Age	37.3 ± 6.3	38.3 ± 13.0	38.0 ± 12.4	40.3 ± 17.7
BMI	22.68 ± 3.75	22.69 ± 3.84	22.70 ± 4.02	22.63 ± 2.19
SCORAD	—	27.43 ± 17.88	27.61 ± 19.00	26.50 ± 12.23
Serum IgE (U/ml)	—	10,586 ± 15,906	12,209 ± 16,546	37.5 ± 41.8*

* $p < 0.05$ vs eAD by Mann-Whitney U test.

AD, atopic dermatitis; BMI, body mass index; eAD, extrinsic AD; iAD, intrinsic AD; NL, normal;

SCORAD, scoring of AD

Supplemental Table 2 Comparisons of serum 25-hydroxyvitamin D3, LL-37 or cytokine levels
in normal donors and atopic dermatitis patients

	Normal (<i>n</i> =27)	Total AD (<i>n</i> = 26)	eAD (<i>n</i> = 22)	iAD (<i>n</i> = 4)
25(OH)D3(ng/ml)	10.41 ± 20.31	3.88 ± 14.52*	4.51 ± 15.75	0.36 ± 0.23
LL-37 (ng/ml)	8.56 ± 13.16	2.73 ± 9.32**	3.23 ± 10.09**	0.0 ± 0.0*
IL-22 (pg/ml)	17.80 ± 13.40	37.97 ± 31.65**	38.34 ± 31.76*	35.96 ± 35.80
Oncostatin M (pg/ml)	3.6 ± 6.0	10.1 ± 7.0**	10.9 ± 7.2**	0.42 ± 0.11
IL-31(ng/ml)	10.8 ± 19.4	3.8 ± 16.3**	4.5 ± 17.7*	0.21 ± 0.18
IL-25 (pg/ml)	66.8 ± 118.0	38.8 ± 57.0*	41.8 ± 61.6	22.5 ± 7.9
TSLP (pg/ml)	22.2 ± 27.4	16.8 ± 3.7	16.8 ± 3.8	16.8 ± 3.3
IL-17A (pg/ml)	8.95 ± 32.4	3.94 ± 4.78	4.33 ± 5.08	1.77 ± 1.47
IL-4 (pg/ml)	0.612 ± 0.938	0.587 ± 0.632	0.635 ± 0.673	0.322 ± 0.214
IFN-γ (pg/ml)	15.01 ± 6.59	13.37 ± 15.84	14.29 ± 16.72	8.39 ± 9.69

*, $p < 0.05$, **, $p < 0.01$ vs normal donors, by Mann-Whitney U test for total AD, and by Kruskal Wallis test for eAD and iAD, respectively.
AD, atopic dermatitis; eAD, extrinsic AD; iAD, intrinsic AD; 25(OH)D3, 25-hydroxyvitamin D3; TSLP, thymic stromal lymphopoietin