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INVESTIGATIVE REPORT

Calcipotriol Increases hCAP18 mRNA Expression but Inhibits Extracellular LL37 Peptide Production in IL-17/IL-22-stimulated Normal Human Epidermal Keratinocytes

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Interleukins (IL)-17A and -22 are involved in the pathogenesis of psoriasis. Cathelicidin LL37 serves as not only antimicrobial peptide but also as autoinflammatory mediator. 1,25-Dihydroxyvitamin D3 analogues, such as calcipotriol, are used as topical treatment for psoriasis. However, the effect of calcipotriol on the mRNA expression/production of human cathelicidin antimicrobial protein (hCAP18) and LL37 peptide by IL-17A/IL-22-stimulated keratinocytes remains controversial. To evaluate the modulatory action of calcipotriol on the production of hCAP18 and LL37, we analysed hCAP18 mRNA expression and hCAP18/LL37 peptide production in IL-17A/IL-22-stimulated cultured human keratinocytes by real-time qPCR, ELISA, western blotting, and immunocytostaining. By western blotting, hCAP18 protein was detected in keratinocytes cultured for 72 h with IL-17/IL-22. Calcipotriol increased hCAP18 mRNA expression in IL-17/IL-22-stimulated keratinocytes. However, LL37 peptide in the culture supernatants was reduced by calcipotriol. Immunostaining revealed that the overproduced LL37 resides within the cells. LL37 promotes psoriasis via interaction with extracellular DNA, but may suppress psoriasis by interfering cytosolic DNA. ***Key words: psoriasis; cytokine; 1,25-dihydroxyvitamin D3; antimicrobial peptide; ELISA; interleukins.***

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Recent evidence has clearly shown that Th17 cells are involved in the pathogenesis of psoriasis. Th17 cells produce IL-17A and IL-22, which are crucial for the activation of STAT3 and resultant hyperproliferation of keratinocytes (1). Both cytokines cooperate with each other for Th17 to function as an immunomodulator in certain conditions (2). IL-17A and/or IL-22 induce the production of cytokines/chemokines such as interleukin (IL)-8/CXCL8 (3), and the production of antimicrobial peptides such as cathelicidin (4). Cathelicidin antimicrobial peptide (CAMP; NM 004345.4), also known as hCAP18 in humans, is an important molecule in the pathogenesis of psoriasis. hCAP18 is composed of a signal peptide domain, a cathelin domain and an LL37 domain (5), and this peptide is generated by neutrophils, monocytes, mast cells and epithelial cells. LL37 is produced when the hCAP18 protein is cleaved extracellularly by proteinase 3 or kallikrein 5 (6, 7). LL37 peptide exerts broad antimicrobial activity against bacteria and is a chemotactic agent for neutrophils, monocytes and T cells (8–10). hCAP18 protein and LL37 peptide are strongly expressed in lesional psoriatic skin and may play an important role as both an antimicrobial peptide and as an autoinflammatory mediator in psoriasis (4). Furthermore, the LL37 peptide induces multiple immunomodulatory effects on host cells (11).

1,25-Dihydroxyvitamin D3 (VitD3) regulates the proliferation and differentiation of keratinocytes, and its analogues are widely used as topical applicants for the treatment of psoriasis. While stimulating the keratinocyte differentiation, VitD3 can exert immunomodulatory actions. VitD3 (12, 13) and its analogues, 22-oxacalcitriol (13) and tacalcitol (14), inhibit the production of interleukin (IL)-6 and/or IL-8 by stimulated keratinocytes. VitD3 and calcipotriol modulate antigen-presenting dendritic cells, which consequently leads to the proliferation of regulatory T cells (15, 16). Furthermore, VitD3 regulates innate immunity by controlling the expression of antimicrobial peptides. However, the effect of VitD3 analogues on the expression and production of LL37 by stimulated keratinocytes remains controversial.

In this study, we investigated the regulatory action of calcipotriol, a synthetic VitD3 analogue with a high affinity for the vitamin D receptor, on the mRNA expression/protein production of hCAP18 and LL37 peptide in IL-17A and IL-22-stimulated human keratinocytes. We also monitored IL-8, because it targets neutrophils and participates in the epidermal collection of neutrophils and the formation of pustular psoriasis. Results suggest the unique action of the VitD3 analogue on the production and distribution of hCAP18 and LL37 peptide.

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MATERIALS AND METHODS

Cell culture

Normal human epidermal keratinocytes (NHEK) were purchased from Lifeline Cell Technology (Frederick, MD, USA). They were grown in serum-free keratinocyte growth medium Epilife (Invitrogen, Carlsbad, CA) and used at third passage in all experiments (17). Growth supplement was omitted 48 h before experiments. As a control, IL-17A and IL-22 (R&D Systems, Minneapolis, MN, USA) were either added or not added to the cells. Cultured NHEK cells were stimulated with IL-17A (200 ng/ml) and/or IL-22 (200 ng/ml) followed by co-incubation in the presence or absence of calcipotriol (Leo Pharma, Ballerup, Denmark) at 0.2–40 nM to test its modulatory effect. Cells were harvested 3 days later and subjected to real-time quantitative PCR (qPCR) as described previously (18). Culture supernatants were also collected and frozen at –80°C until use for ELISA.

Quantitative real-time qPCR for hCAP18 and IL-8 mRNA expression

Total mRNA was extracted from NHEK cells with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. cDNA was reverse transcribed from total RNA using the TaqMan RT reagents (Applied Biosystems, Foster City, CA, USA). The evaluation of mRNA expression was analysed with SYBR®GeenER™ qPCR Reagent system (Invitrogen) using the ABI PRISM 7000 sequence detection system (Applied Biosystems). For IL-8 mRNA expression the following primer pairs were used: IL-8F; 5'-TGCAGC-TCTGTGTGAAGGTG-3' and IL-8R; 5'-GGTCCACTCT-CAATCACTCTCAG-3' and for hCAP18 mRNA expression the primers CAMP-F; 5'-GAAGGACGGGCTGGTGAAG-3' and CAMP-R; 5'-ACCCAGCAGGGCAAATCT-3' were used. As an endogenous reference glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene expression was measured using primers GAPDH-F; 5'-ACCACAGTCCATGCCATCAC-3' and GAPDH-R; 5'- TCCACCACCCTGTTGCTGTA-3'. The expression of the target gene normalised to the endogenous reference and relative to calibrator was given by the formula $2^{-\Delta\Delta C_T}$.

Quantification of LL37 peptide and IL-8 in culture supernatants by enzyme-linked immunosorbent assay (ELISA)

In addition, 3-day culture supernatants were measured for LL37 using LL37 ELISA kit (Hycult Biotech, Plymouth Meeting, PA, USA) and IL-8 using Quantikine® human CXCL8/IL-8 immunoassay kit (R&D Systems) according to the manufacturer's protocol. The absorbance at 450 nm was monitored with iMark microplate reader (Bio-Rad, Hercules, CA, USA).

Western blotting

Proteins were extracted from NHEK cells with cell lysis buffer (80mM Tris-HCl (pH7.6), 2% SDS, 10% glycerol, 0.1 mM PMSF, complete protease inhibitor cocktail (Roche, Mannheim, Germany)). Protein concentration was measured by Lowry assay with DCTM Protein assay kit (Bio-Rad), 40 µg sample proteins were used for the assay. CAMP 293T Cell Transient Overexpression Lysate (Abnova, Taipei, Taiwan) was used as a positive control. Proteins were separated on a 4–12% NuPAGE® Bis-Tris gel (Invitrogen) by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were then stained with rabbit polyclonal antibodies to human CAP18/LL37 (1:500; Hycult Biotech) or a polyclonal antibody to human β-actin (1:2,000; Cell Signalling Technology, Inc., Boston, MA, USA). Proteins were detected with the ECL plus or ECL Western blot detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Immunocytostaining for hCAP18 and LL37 peptide in NHEK cells

Cultured NHEK cells were fixed with 4% paraformaldehyde for 15 min followed by cell permeabilisation for 15 min with 0.2% TritonX-100 solution. Fixed cells were incubated for 1 h in 1% bovine serum albumin (BSA) in PBS to block non-specific binding sites. Cells were incubated for 1 h with a mouse anti-human LL37/CAP-18 monoclonal primary antibody (3D11, 1:100; HM2070, Hycult Biotech, Plymouth Meeting, PA, USA). An Alexa Fluor 594 conjugated-goat anti-mouse antibody (Molecular probes, Eugene, OR, USA) was used as a secondary antibody. Nuclei were stained with DAPI.

Statistical analysis

Student's *t*-test (impaired) was employed to determine statistical differences between means. Correlations were studied by Pearson's product-moment correlation coefficient.

RESULTS

Increased production of hCAP18 by IL-17A and IL-22

We first examined the ability of IL-17A/IL-22 to increase the production of hCAP18 protein by NHEK cells in various culture periods. We have previously reported that cytokine production by NHEK cells peaks at 72 h after the addition of IL-17A/IL-22 (3). To confirm the time course of hCAP18 production, we cultured NHEK cells with IL-17A/IL-22 for 6, 24, or 72 h, and the lysates were then subjected to western blot analysis. As positive control, CAMP 293T Cell Transient Overexpression Lysate was used. We found that hCAP18 protein was produced in 72 h cultured NHEK cells treated with IL-17A/IL-22, but not in 6 or 24 h cultured cells (Fig. S1[1]). Thus, we used 72 h as the culture period in the following experiments.

Increase of hCAP18 mRNA expression and decrease of IL-8 mRNA expression by calcipotriol in IL-17A and IL-22-stimulated NHEK cells

Given that keratinocytes are exposed to Th17-derived cytokines in the psoriatic lesion, it is reasonable to investigate the *in vitro* effect of calcipotriol on the expression of hCAP18 mRNA in keratinocytes stimulated with IL-17A and IL-22. As control, IL-8 was also monitored in parallel, because VitD3 and its analogues are known to suppress IL-8 expression (12–14). In NHEK cells that were not stimulated with IL-17A or IL-22, calcipotriol at 0.2-20 nM did not substantially affect mRNA expression of hCAP18 (Fig. 1a). The addition of IL-17A/IL-22 remarkably increased hCAP18 expression (Fig. 1b). More dramatically, further addition of calcipotriol increased the hCAP18 mRNA expression in the presence of IL-17A/IL-22 in a dose-dependent manner. Calciptriol at 20 nM yielded the maximum response.

We have shown the synergistic effects of IL-17 and IL-22 on the production of IL-8 in NHEK cells (3). When NHEK cells were not stimulated with IL-17A or IL-22, calcipotriol slightly enhanced (0.2 nM) IL-8

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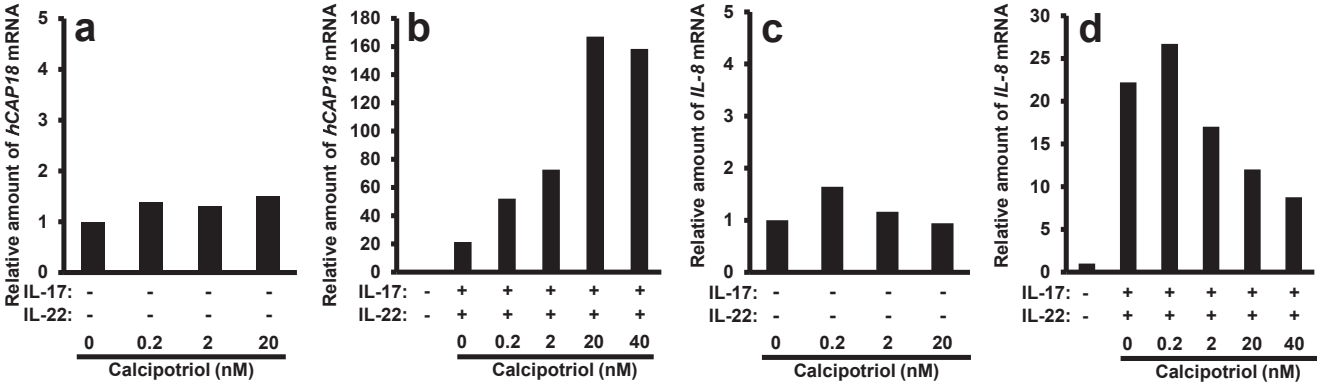

Fig. 1. Calcipotriol increases mRNA expression of hCAP18, but reduces the expression of IL-8 in IL-17A and IL-22-stimulated NHEK cells. NHEK cells were cultured with the indicated concentration of calcipotriol in the presence of IL-17A and IL-22. After a 3-day culture, they were subjected to real-time qPCR. (a, b) hCAP18 mRNA expression (n=3) and (c, d) IL-8 mRNA expression (n=3).

mRNA expression or had no effect (2–20 nM) (Fig. 1c). The addition of IL-17A and IL-22 markedly increased the mRNA expression of IL-8 (Fig. 1d), confirming our previous study (3). This enhanced IL-8 mRNA expression was suppressed by calcipotriol at 2, 20 and 40 nM in a dose dependent manner (Fig. 1d).

Thus, calcipotriol decreases IL-8 mRNA expression but increases hCAP18 mRNA expression in IL-17A/IL-22-stimulated NHEK cells.

Inhibition of extracellular production of LL37 cathelicidin peptide in IL-17A and IL-22-stimulated NHEK cells

We measured the LL37 peptide concentration by ELISA in the culture supernatants of NHEK cells after 72 h incubation with IL-17A, IL-22, and/or calcipotriol. Both cytokines enhanced the LL37 peptide production synergistically, as the LL37 concentration of supernatants from IL-17A/IL-22-stimulated NHEK cells was significantly higher than that from non-stimulated NHEK cells (Fig. 2a). This cytokine-enhanced, extracellular production of LL37 peptide was reduced by calcipotriol at 20 nM. On the other hand, the downmodulatory effect of calcipotriol on IL-8 mRNA expression was also observed at protein level (Fig. 2b). The results suggest that calcipotriol increases hCAP18 mRNA expression, but the extracellular production of LL37 peptide is inhibited in IL-17A and IL-22-stimulated NHEK cells.

hCAP18/LL37 peptide immunoreactivity in NHEK cells stimulated with IL-17A/IL-22 and/or calcipotriol

NHEK cells were cultured with IL-17A/IL-22, calcipotriol, or both, and immunoreactivity with anti-LL37 antibody was observed by immunofluorescence microscopy (Fig. 3). It is considered that both hCAP18 protein and LL37 peptides that are cleaved from hCAP18 can be detected with anti-LL37 antibody. Compared with the no treatment group (hCAP18$^+$/LL37 peptide$^+$ cells, 86/high power view), the calcipotriol-added group had a comparable number of positive cells (79/high power view). The IL-17A/IL-22-added group exhibited a high number of hCAP18$^+$/LL37 peptide$^+$ cells (182/high power view). Further addition of calcipotriol together with IL-17A/IL-22 enhanced the hCAP18$^+$/LL37 peptide$^+$ cell number (280/high power view). These findings suggest that, in calcipotriol-treated and IL-17A/IL-22-stimulated NHEK cells, the overproduced hCAP18 protein and LL37 peptide resides within the cells and its release to the culture is downmodulated, resulting in the elevation of intracellular hCAP18/LL37 peptide.

We performed western blot analysis of LL37 with anti-hCAP18/LL37 as shown in Fig. S1[1]. Although hCAP18 protein was found, LL37 peptide (4 kDa) could not be detected even at 72 h after incubation with IL-17A/IL-22. It is possible that a large part of the cleaved proteins from hCAP18 are dissimilar to LL37. Alternatively, it might be difficult to detect LL37 by western blotting because of its small molecular weight or difficulty in extraction.

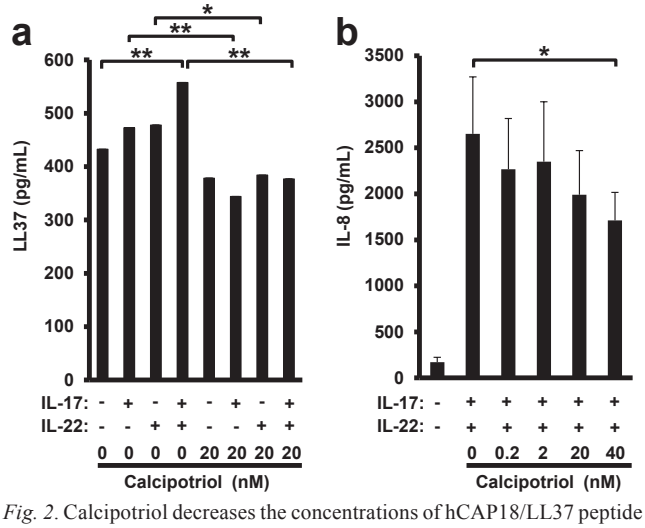

Fig. 2. Calcipotriol decreases the concentrations of hCAP18/LL37 peptide and IL-8 in the culture supernatants from IL-17A/IL-22-stimulated NHEK cells. NHEK cells were cultured with the indicated concentration of calcipotriol in the presence or absence of IL-17A and IL-22. After a 3-day culture, the supernatants were collected and subjected to ELISA. *p<0.05, **p<0.01. (a) LL37 protein concentration (n=3) and (b) IL-8 protein concentration (n=3).

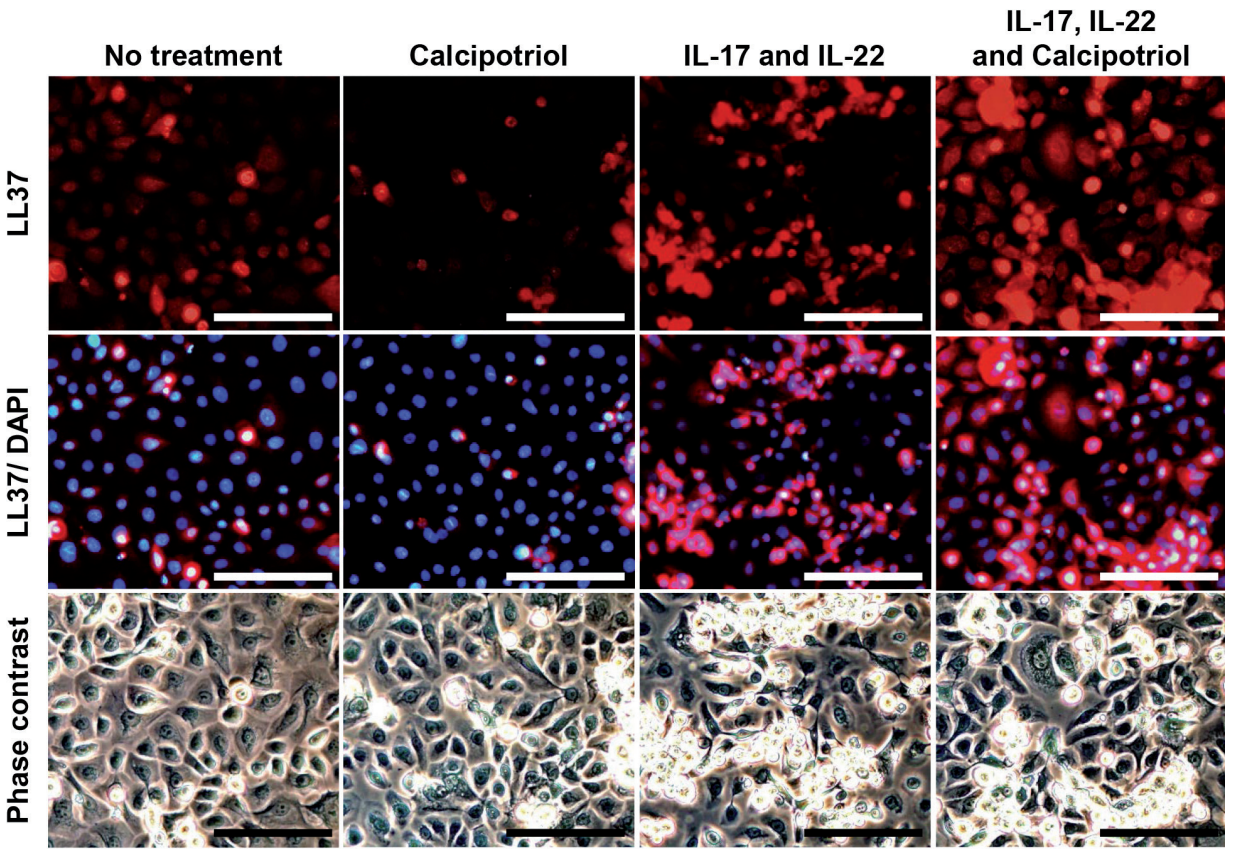

Fig. 3. Calcipotriol increases the number of intracellularly hCAP18^{+}/LL37 peptide^{+} cells in IL-17A and IL-22-stimulated NHEK cells. NHEK cells were cultured with the indicated concentration of calcipotriol in the presence or absence of IL-17A and IL-22. After a 3-day culture, cells were fixed by 4% paraformaldehyde. Cells were then lysed with TritonX-100 solution. A mouse anti-human LL37/CAP-18 monoclonal antibody and an Alexa Fluor 594 conjugated-goat anti-mouse antibody were used as the primary and secondary antibodies, respectively. Nuclei were stained with DAPI. Scale bar represents 100 μm. The photograph is a representative of 3 independent experiments.

DISCUSSION

Stimulation with the combination of IL-17A and IL-22 may yield cutaneous inflammation as seen in psoriasis. By using cultured keratinocytes stimulated with IL-17A/IL-22, we found that the expressions and/or productions of hCAP18/LL37 peptide and IL-8 are differentially modulated by calcipotriol. While IL-8 was suppressed by calcipotriol at both mRNA and protein levels, hCAP18 mRNA expression was enhanced, but its extracellular release as hCAP18 or LL37 peptide was downmodulated by calcipotriol.

It has been reported that VitD3 (6, 13) and its analogues, 22-oxacalcitriol (13) and tacalcitol (14), inhibit the production of IL-8 by keratinocytes. In these previous studies, keratinocytes were stimulated with TNF-α, or synergistically with TNF-α and IFN-γ (13, 14). In addition to these well-known keratinocyte-stimulatory cytokines, we found that IL-17A and IL-22 synergistically stimulated keratinocytes to produce IL-8 (3), and that calcipotriol suppresses this overproduction. In fact, topical application of calcipotriol reduces the IL-8 concentration in the skin (19). Thus, calcipotriol is therapeutically beneficial for psoriasis by suppressing IL-8 production and resultant inhibition of neutrophil accumulation.

Of particular interest is the observation that the IL-17A/IL-22-augmented expression of hCAP18 was further enhanced by calcipotriol. Another study using different stimulants, i.e. UVB, lipopolysaccharide and TNF-α, showed that calcipotriol oppositely suppressed the expressions of LL37 and human β defensin-2 (HBD-2) (20). In agreement with our study, however, there is a report demonstrating that the simultaneous addition of IL-17A and VitD3 markedly induced the expression of cathelicidin peptide in NHEK cells (21). In their study, VitD3 blocked the induction of IL-8 and HBD-2 (21), consistent with our finding. In *in vivo* studies, topical treatment with calcipotriol enhanced cathelicidin expression, but reduced HBD-2 and HBD-3 expression in human skin (22, 23). Therefore, our data, together with previous findings, collectively suggest that calcipotriol or VitD3 decreases IL-8 but increases hCAP18 mRNA expression, when keratinocytes are stimulated especially with Th17-derived cytokines.

However, our study showed that the overexpressed LL37 cathelicidin peptide was not released extracellularly but resided within the cells. Extracellular LL37 can interact with self-DNA in psoriatic skin, and the production of self-DNA/LL37 complexes activate plasmacytoid dendritic cells, thereby inducing psoria-

tic lesions (11). On the other hand, cytosolic DNA has recently been identified as a danger signal that activates inflammasomes containing DNA sensor AIM2 and converting pro-IL-1β to IL-1β (24, 25). LL37 cathelicidin peptide neutralises cytosolic DNA in keratinocytes and blocks inflammasome activation (26). Thus, within keratinocytes, LL37 peptide interferes with DNA-sensing inflammasomes, suggesting an anti-inflammatory function for this cathelicidin peptide.

Therefore it seems that LL37 peptide has contrasting effects in the pathogenesis of psoriasis. On one hand, released LL37 peptide promotes psoriasis *via* interaction with extracellular DNA, but on the other hand, it may suppress psoriasis by interfering cytosolic DNA within the cell. The effect of calcipotriol to increase the intracellular LL37 cathelicidin peptide, along with its potential to decrease the extracellular concentration of LL37, is likely beneficial for the treatment of psoriasis.

The authors declare no conflicts of interest.

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