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Human antimicrobial peptide LL-37 modulates proinflammatory responses induced by cytokine milieus and double-stranded RNA in human keratinocytes

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ABSTRACT

Epidermal keratinocytes produce proinflammatory cytokines/chemokines upon stimulation with cytokine milieus and Toll-like receptor ligands, which are considered to reflect epidermal environments in inflamed skin. The human antimicrobial peptide LL-37, besides having microbicidal functions, plays multiple roles as a "host defense peptide" in the immune system. Here, we examined the effect of LL-37 on proinflammatory responses induced by double-stranded RNA (dsRNA) and cytokines in primary human keratinocytes. LL-37 inhibited dsRNA-induced production of thymic stromal lymphopoietin (TSLP), CCL5/RANTES, CXCL10/IP-10, and CXCL8/IL-8, which was attributable to interaction between LL-37 and dsRNA, although LL-37 upregulated CXCL8 expression at an earlier time point (8 h). LL-37 inhibited the increase of CXCL10 and CCL5 induced by TNF-α- and/or IFN-γ but enhanced that of CXCL8. LL-37 and Th17 cytokines (IL-17 and IL-22) synergistically upregulated the expression of CXCL8 and IL-6. LL-37 showed the effects above at a high concentration (25 μg/ml, 5.6 μM). We also examined effects of a peptide with a scrambled LL-37 sequence, which has been frequently used as a negative control, and those of another peptide with the reversed LL-37 sequence, activities of which have not been well investigated. Interestingly, the reversed LL-37 had effects similar to LL-37 but the scrambled LL-37 did not. The modulation by LL-37 of the keratinocyte proinflammatory responses induced by cytokine milieus and dsRNA suggests novel roles for LL-37 in skin inflammation such as the promotion of IL17/IL-22/IL-6-associated psoriasis and suppression of TSLP-associated atopic dermatitis.

1. Introduction

The innate immune system in the skin and mucosal epithelia, the first line of defense against invading organisms, includes antimicrobial peptides such as the human cathelicidin hCAP18 (the precursor of LL-37) and human β-defensins [1–5]. hCAP18 is proteolysed to generate LL-37, a small mature C-terminal peptide of 37 amino acid residues starting with two leucine residues [6–8]. High cathelicidin concentrations are typically found at sites of inflammation and improper levels of LL-37 are associated with chronic respiratory diseases and autoimmune diseases. The expression of LL-37 and human β-defensins is augmented in various skin disorders including psoriasis but downregulated in atopic dermatitis, explaining why atopic dermatitis patients often demonstrate increased susceptibility to bacterial and viral infections in the skin [9]. Besides their antimicrobial function, antimicrobial peptides play multiple roles as "host defense peptides" in the immune system including chemotactic and immunomodulatory effects, wound healing, angiogenesis, and modulation of apoptosis [1–7]. LL-37 affects the production of proinflammatory cytokines/chemokines in various types of cells through the activation of multiple receptors [6] and can modulate responses to Toll-like receptor (TLR) ligands by forming complexes with LPS [10,11] and nucleic acids [12–15] or stimulating receptor expression [16].

Recent reports indicate critical roles for epidermal keratinocytes (KCs) and mucosal epithelial cells in disorders and homeostasis at barrier tissues [1,17–20]. KCs produce cytokines and chemokines including a Th2-inducing cytokine, thymic stromal lymphopoietin (TSLP) [19,21] upon stimulation with TLR ligands such as double-stranded RNA (dsRNA) [19,22,23]. KCs also respond to cytokines such as TNF-α, IFN-γ, IL-17, and IL-22, which are considered to reflect epidermal cytokine milieus associated with skin inflammation; for example, psoriasis is a Th17-associated inflammatory condition [24]. Although LL-37 is an important

Abbreviations: dsRNA, double stranded RNA; KC, keratinocyte; PolyI:C, polyinosinic–polycytidylic acid; TLR, Toll-like receptor; TSLP, thymic stromal lymphopoietin.

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component in the epidermis and can act on KCs to induce production of proinflammatory cytokines and chemokines [25,26], its effect on KC responses to TLR ligands and cytokines has not been well investigated. In the present study, we examine the effects of LL-37 on proinflammatory responses induced by dsRNA (TLR3 ligand) and cytokines in human KCs. We analyzed production of three representative KC-derived chemokines (CXCL8, CXCL10, and CCL5), dsRNA-induced production of a Th2-inducing cytokine TSLP, and Th17 cytokine-induced production of IL-6, which contributes to psoriasis.

2. Materials and methods

2.1. Reagents

The following concentrations of polyI:C (GE Healthcare, Buckinghamshire, United Kingdom), recombinant human cytokines (R&D Systems, Minneapolis, MN, USA) and synthetic peptides (Abgent, San Diego, CA) with wild-type (#SP2321a: NH_2-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-COOH), reversed (#SP2407a: NH_2-SETRPVLNRLFDKIRQVIRKFEKGIKEKSKRFFDGLL-COOH), and scrambled (#SP2246a: NH_2-GLKLRFEFSKIKGEFLKTPEVRFRDIKLKDNRISVQR-COOH) amino acid sequences of LL-37 were used to stimulate KCs: 0.1–10 μg/ml polyI:C, 20 ng/ml TNF-α, 100 ng/ml IFN-γ, 100 ng/ml IL-17A, 100 ng/ml IL-22, and 1–25 μg/ml (0.22–5.6 μM) LL-37 derivatives.

2.2. Cell culture and stimulation of KCs

Primary human KCs (Cascade Biologics, Portland, OR) were cultured in EpiLife KG2 (Invitrogen, Carlsbad, CA) with supplements including 300 nM hydrocortisone (Kurabo, Osaka, Japan). Cells were seeded at 25,000 and 10,000 cells/well in flat-bottomed 24-well and 96-well microculture plates, respectively. When the cell growth reached 90% confluence, the medium was changed to fresh medium without the hydrocortisone [27,28]. After further cultivation for 24 h, cells were incubated with LL-37 in fresh medium without hydrocortisone for 10–15 min and stimulated by adding medium containing polyI:C and cytokines. Culture supernatants for ELISA and mRNA were recovered at the time points indicated in the figures.

2.3. ELISA and real-time quantitative PCR

Concentrations of cytokines and chemokines were measured with ELISA kits (DuoSet; R&D Systems). Total RNA was extracted from the cells and cDNA was synthesized as described previously [22,23]. Real-time quantitative PCR was performed using a Taqman method with an ABI7500 (Applied Biosystems, Piscataway, NJ) [29]. The mRNA levels of target genes were normalized to that of β-actin and are shown relative to the control group.

2.4. Statistical analysis

A one-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used. Values of $P < 0.05$ were regarded as statistically significant.

3. Results

3.1. Modulation of dsRNA-induced KC responses by LL-37

KCs were stimulated with a synthetic dsRNA, polyI:C, mimicking viral and self dsRNA in the presence or absence of LL-37 (Supplementary Fig. S1). LL-37 at 25 μg/ml inhibited the polyI:C (1 and/or

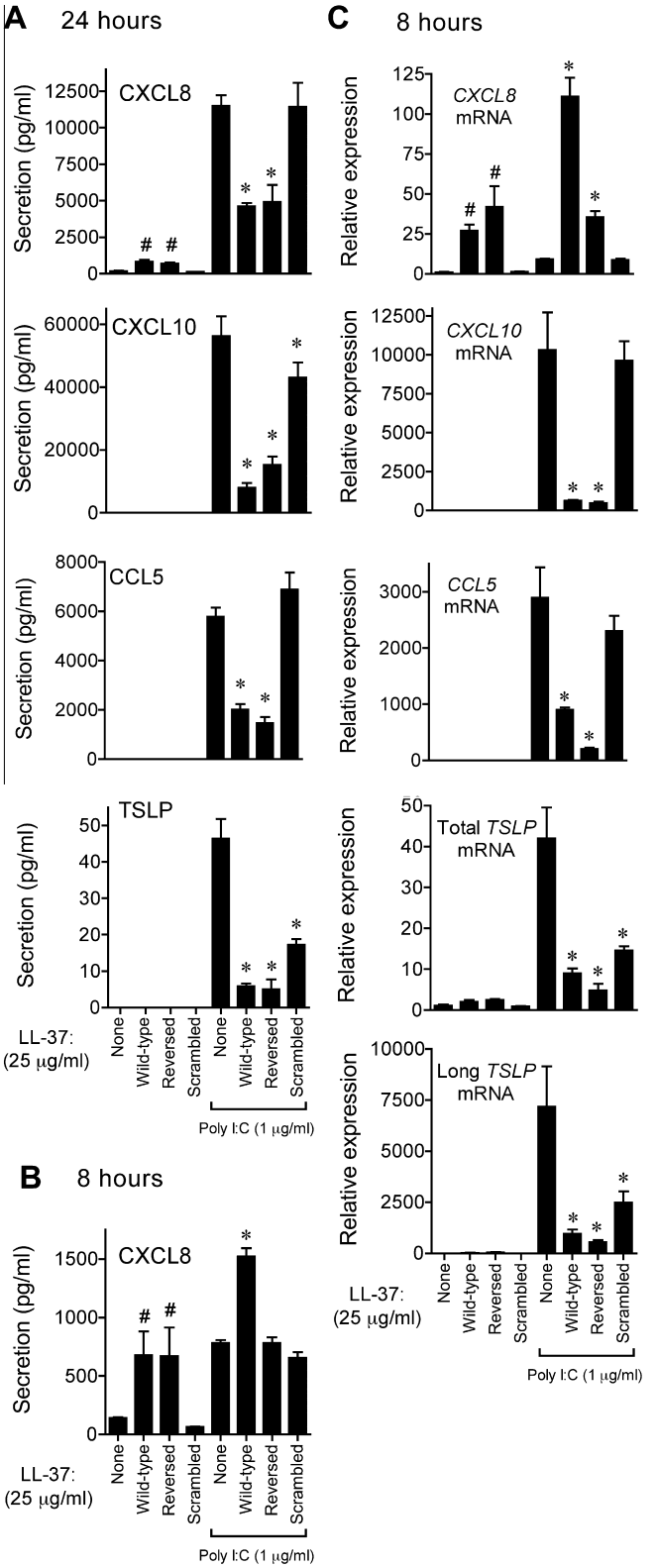

Fig. 1. Modulation of dsRNA-induced keratinocyte responses by LL-37. (A) Release of chemokines (CXCL8/IL-8, CXCL10/IP-10, and CCL5/RANTES) and a Th2-inducing cytokine TSLP at 24 h after the stimulation with synthetic dsRNA, polyI:C. (B) Release of CXCL8 at 8 h. (C) Gene expression of *CXCL8*, *CXCL10*, *CCL5*, total *TSLP* transcripts, and Long *TSLP* at 8 h. $^{*}P < 0.05$ compared with polyI:C stimulation without LL-37 (among the groups stimulated with polyI:C) and $^{\#}P < 0.05$ compared with the control without LL-37 and polyI:C (among the groups without polyI:C) by ANOVA with Tukey's multiple comparison test. Data shown are the means ± SDs for three wells and are representative of three independent experiments. *Wild-type*: LL-37. *Reversed*: the peptide with the reversed LL-37 sequence. *Scrambled*: the peptide with the scrambled LL-37 sequence.

10 μg/ml)-induced release of three chemokines (CXCL8/IL-8, CXCL10/IP-10, and CCL5/RANTES) and TSLP at 24 h after the stimulation (Supplementary Fig. S1A) and CXCL10 and CCL5 at 8 h (Supplementary Fig. S1B, CXCL10 and CCL5). At 8 h, LL-37 at 5 and 25 μg/ml enhanced the polyI:C (0.1, 1, and 10 μg/ml)-induced release of CXCL8 (Supplementary Fig. S1B, CXCL8). In the absence of dsRNA, LL-37 at 25 μg/ml induced the release of CXCL8 (Supplementary Fig. S1, CXCL8), supporting a result obtained previously using LL-37 at 13.5 μg/ml (3 μM) [25]. At 8 h, the release of TSLP did not reach the minimum detection limit of ELISA.

We compared the effects of LL-37 (wild-type) and its derivatives, each of which has the reversed (reversed LL-37) or scrambled (scrambled LL-37) sequence as described in Section 2, at the concentration of 25 μg/ml (Fig. 1). At 24 h, LL-37 and reversed LL-37 inhibited the polyI:C-induced release of CXCL8, CXCL10, CCL5, and TSLP and induced the release of CXCL8 in the absence of dsRNA (Fig. 1A, wild-type and reversed). Scrambled LL-37 showed no (CXCL8 and CCL5) or small/moderate (CXCL10 and TSLP) inhibition of the dsRNA-induced responses (Fig. 1A, scrambled). At 8 h, LL-37 and reversed LL-37 induced the release of CXCL8 but reversed

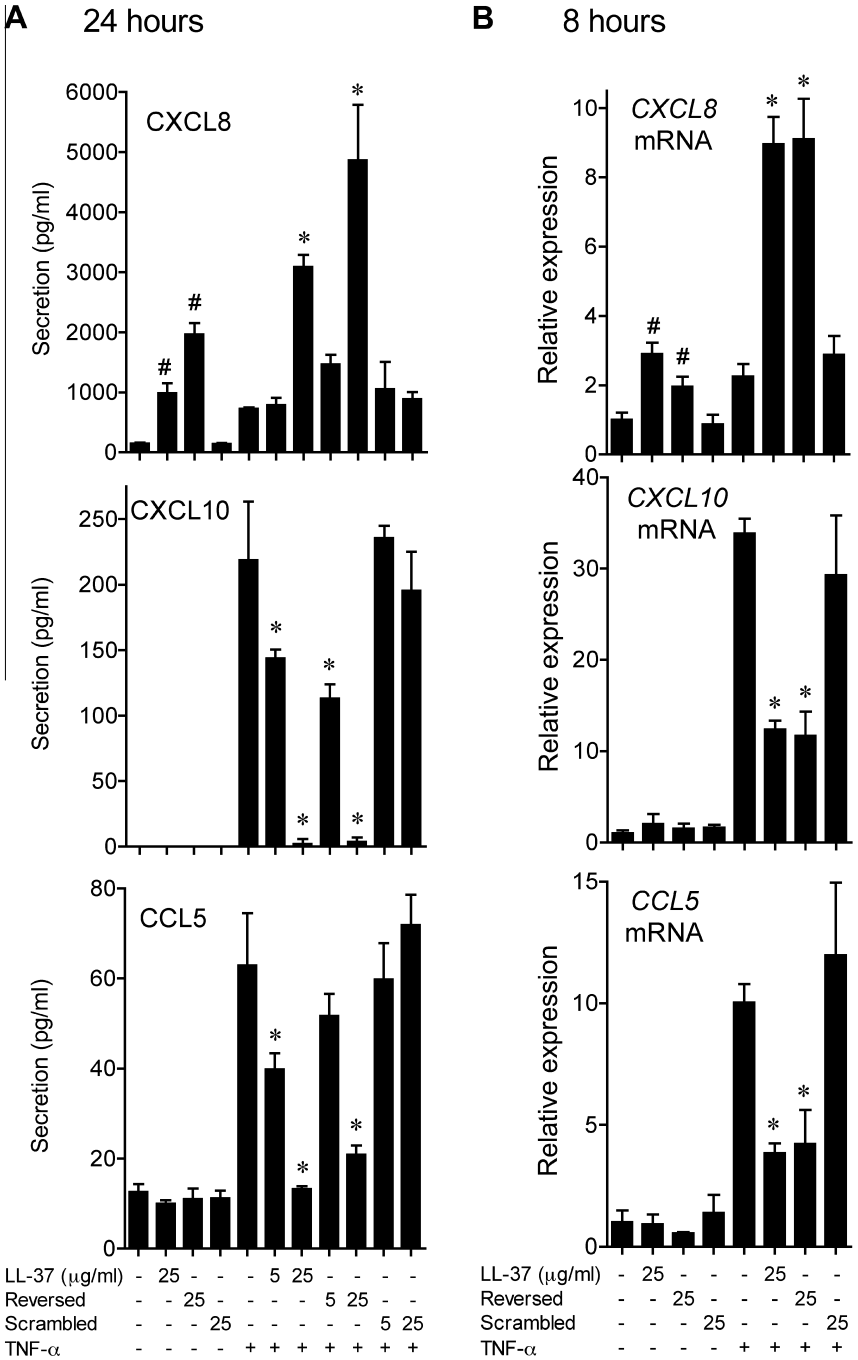

Fig. 2. Modulation of TNF-α-induced keratinocyte responses by LL-37. (A) Release of chemokines (CXCL8/IL-8, CXCL10/IP-10, CCL5/RANTES) at 24 h after the stimulation with TNF-α. (B) Gene expression at 8 h. $^{*}P < 0.05$ compared with TNF-α stimulation without LL-37 (among the groups stimulated with TNF-α) and $^{\#}P < 0.05$ compared with the control without LL-37 and TNF-α (among the groups without TNF-α) by ANOVA with Tukey's multiple comparison test. Data shown are the means ± SDs for three wells and are representative of three independent experiments. *Reversed*: the peptide with the reversed LL-37 sequence. *Scrambled*: the peptide with the scrambled LL-37 sequence.

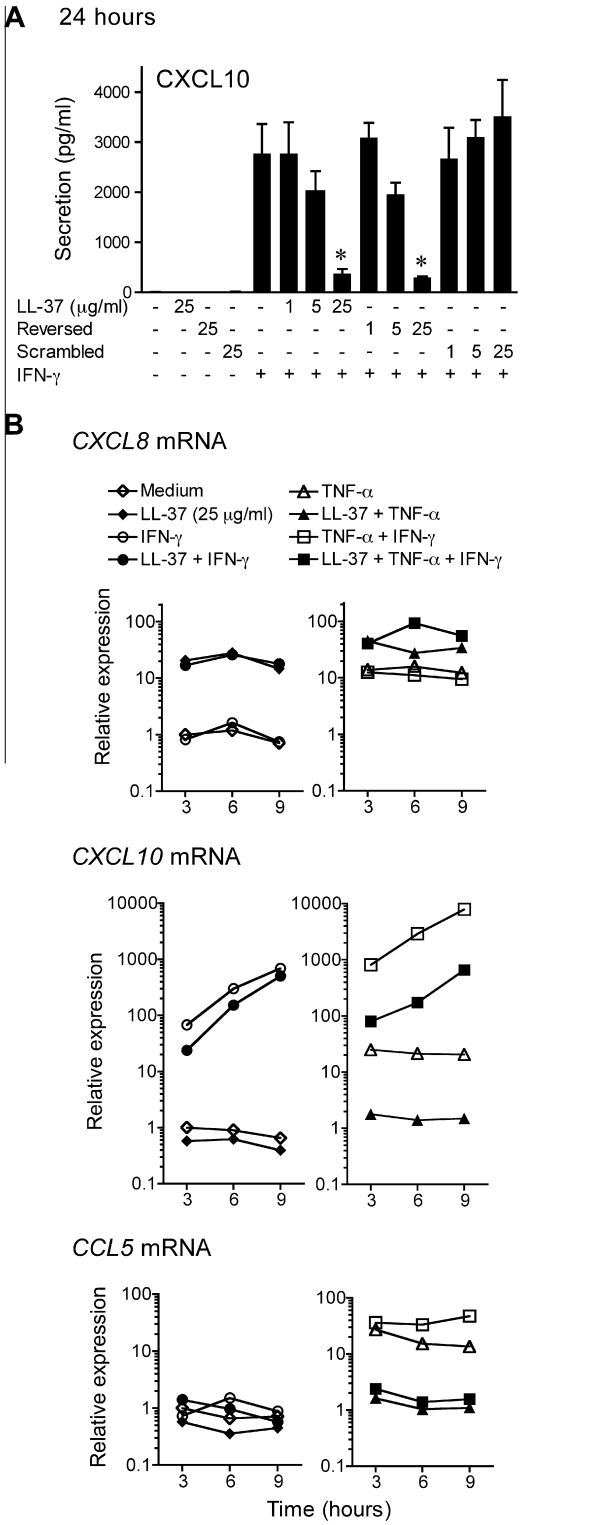

Fig. 3. Modulation of IFN-γ- and IFN-γ plus TNF-α-induced keratinocyte responses by LL-37. (A) Release of CXCL10/IP-10 at 24 h after the stimulation with IFN-γ. $^{*}P < 0.05$ compared with IFN-γ-stimulation without LL-37 by ANOVA with Tukey's multiple comparison test. Data shown are the means ± SDs for three wells and are representative of three independent experiments. *Reversed*: the peptide with the reversed LL-37 sequence. *Scrambled*: the peptide with the scrambled LL-37 sequence. (B) Gene expression at 3–9 h is presented as fold exchange relative to that in the control (*Medium*) at 3 h. Data shown are representative of three independent experiments.

LL-37 did not enhance the polyI:C-induced release of CXCL8. Scrambled LL-37 showed no induction or enhancement of the release of CXCL8 (Fig. 1B). The gene expression results at 8 h (Fig. 1C) supported the results regarding the release of the CXCL8 protein at 8 h (Fig. 1B) and the others at 24 h (Fig. 1A). Two forms of *TSLP* transcripts (long and short) have been reported and long-form *TSLP* expression is responsible for the release of the TSLP protein [29,30]. Long-form *TSLP* expression (Fig. 1C, Long *TSLP* mRNA) was measured using the long-form-specific probe set along with the total level of the *TSLP* transcripts (Fig. 1C, Total *TSLP* mRNA) analyzed by using the probe set, which does not distinguish between the long and short forms [29].

3.2. Modulation of TNF-α- and/or IFN-γ-induced KC responses by LL-37

TNF-α stimulated KCs to release CXCL8, CXCL10, and CCL5. LL-37 and reversed LL-37 at 25 μg/ml upregulated the TNF-α-induced release of CXCL8 (Fig. 2A, CXCL8) and at 5 and/or 25 μg/ml inhibited the TNF-α-induced release of CXCL10 and CCL5 (Fig. 2A, CXCL10 and CCL5). Scrambled LL-37 had no effect. The gene expression results (Fig. 2B) supported the results regarding the release of the proteins (Fig. 2A).

IFN-γ stimulated KCs to release CXCL10. LL-37 and reversed LL-37 at 25 μg/ml, but not scrambled LL-37, inhibited the IFN-γ-induced release of CXCL10 (Fig. 3A). The gene expression results (Fig. 3B, *CXCL10* mRNA, left panel) supported the results regarding the release of the CXCL10 protein (Fig. 3A). Stimulation with IFN-γ did not upregulate the release (our unpublished observations) or gene expression of CXCL8 and CCL5 (Fig. 3B, *CXCL8* and *CCL5*, left) but did enhance the TNF-α-induced gene expression of *CXCL8*, *CXCL10*, and *CCL5* (Fig. 3B, right). LL-37 at 25 μg/ml inhibited the gene expression of *CXCL10* and *CCL5* induced by TNF-α plus IFN-γ and enhanced that of *CXCL8* (Fig. 3B, right).

3.3. Modulation of Th17 cytokine-induced KC responses by LL-37

LL-37 at 25 μg/ml induced the release of CXCL8 and IL-6 (Fig. 4A). IL-17 at 11, 33, and/or 100 ng/ml also induced the release of CXCL8 and IL-6 but the amounts released were less than that induced by LL-37. LL-37 (25 μg/ml) and IL-17 showed remarkable synergy in the release of CXCL8 and IL-6 (1.2–100 and 3.7–100 ng/ml of IL-17 for the release of CXCL8 and IL-6, respectively) (Fig. 4A). The gene expression results (Fig. 4C, left panels) supported the results regarding the release of the proteins (Fig. 4A). Reversed LL-37 showed synergy with IL-17 in the release of IL-6 and CXCL8 but scrambled LL-37 did not (Fig. 4B). IL-22 is another Th17 cytokine, which is related to the pathogenesis of psoriasis along with IL-17 [24]. Th17 cytokines (IL-17 and IL-22) synergistically the enhanced gene expression of CXCL8 and IL-6 and addition of LL-37 further enhanced it (Fig. 4C).

4. Discussion

Braff et al. [25] reported that stimulation of human KCs with LL-37 at 13.5 or 45 μg/ml (3 or 10 μM, respectively) increased production of CXCL8 and IL-6 and secretion of IL-1α, and Niyonsaba et al. [26] reported that LL-37 induced production of IL-18. Although the concentrations of LL-37 in human body are not precisely known, it has been proven that the epithelial tissues contain high concentrations of LL-37, particularly at sites of infection or inflammation. For example, Ong et al. [9] reported an abundance of LL-37 in psoriatic skin lesions (median, 304 μM; range, 0–1605 μM). However, the effect of LL-37 on TLR ligand- and cytokine-induced KC responses had not been well investigated until recently when studies demonstrated that LL-37 enabled KC reactivity against TLR9 ligands [16] and LL-37 and flagellin (TLR5 ligand) or IL-1β synergistically

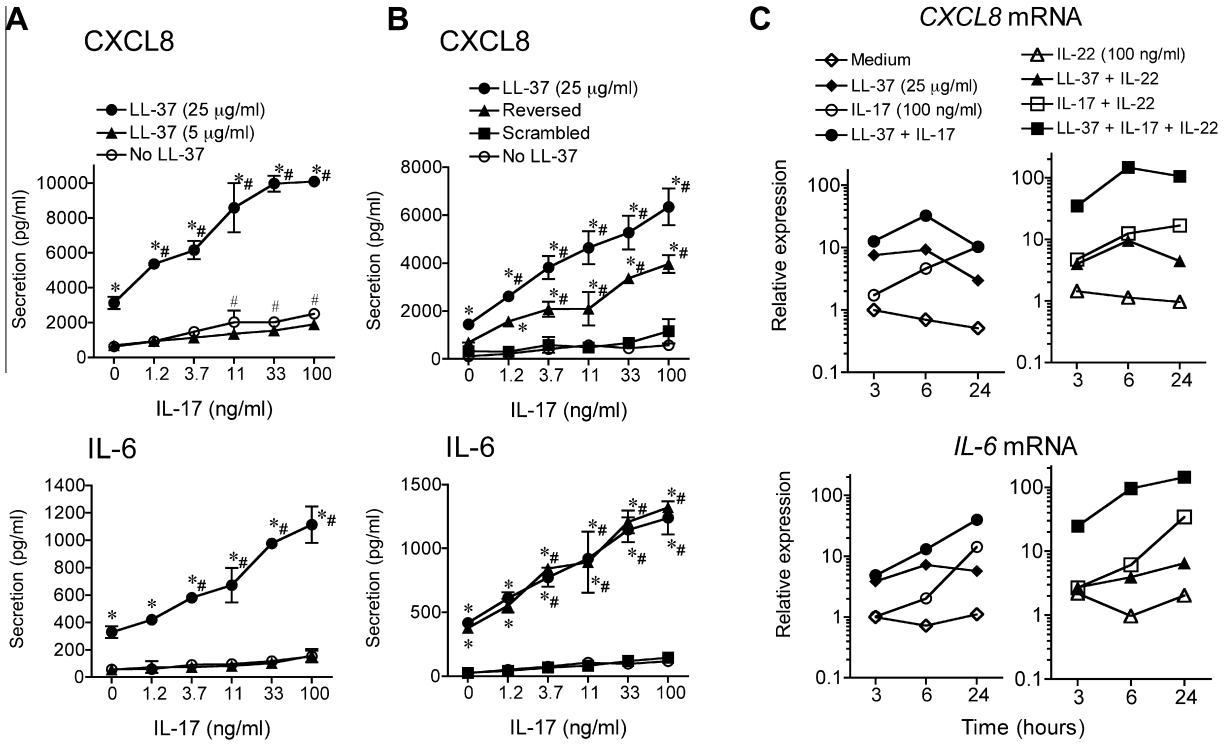

Fig. 4. Modulation of Th17 cytokine-induced keratinocyte responses by LL-37. (A and B) Release of CXCL8/IL-8 and IL-6 at 24 h after the stimulation with IL-17A. $^{*}P < 0.05$ compared with stimulation without LL-37 and $^{\#}P < 0.05$ compared with stimulation without IL-17A by ANOVA with Tukey's multiple comparison test. Data shown are the means ± SDs for three wells and are representative of three independent experiments. *Reversed*: the peptide with the reversed LL-37 sequence. *Scrambled*: the peptide with the scrambled LL-37 sequence. C, Gene expression at 3–24 h after the stimulation with IL-17A and/or IL-22 is presented as fold exchange relative to that in the control (*Medium*) at 3 h. Data shown are representative of three independent experiments.

stimulated CXCL8 production [31]. In the present study, we demonstrated that LL-37 at 25 μg/ml (5.6 μM) caused (i) inhibition of dsRNA (TLR3 ligand)-induced expression of CXCL8, CXCL10, CCL5, and TSLP (Supplementary Fig. S1 and Fig. 1), (ii) synergistic enhancement of TNF-α/IFN-γ-induced CXCL8 expression and unexpected inhibition of TNF-α/IFN-γ-induced expression of CXCL10 and/or CCL5 (Figs. 2 and 3), and (iii) remarkable, synergistic enhancement of IL-17/IL-22-induced expression of CXCL8 and IL-6 (Fig. 4), and that (iv) reversed LL-37 had the similar effects to LL-37 (Figs. 1, 2, 3A, and 4B, reversed) in human KCs.

Recent studies have shown that LL-37 inhibits dsRNA-mediated response in macrophages, microglial cells, and dendritic cells [14] but enhances that in bronchial epithelial cells and peripheral blood mononuclear cells [15], indicating that effects of LL-37 on dsRNA-mediated response differ among cell types and/or experimental conditions. In primary human KCs, LL-37 inhibited dsRNA-induced expression of CXCL8, CXCL10, CCL5, and TSLP (Supplementary Fig. S1 and Fig. 1). Interaction between LL-37 and dsRNA [14,15] might prevent the stimulation of KCs with dsRNA and/or unknown mechanisms might be involved. The upregulation of dsRNA-induced CXCL8 expression at an earlier time point (8 h) in the presence of LL-37 (Supplementary Fig. S1B, CXCL8; and Fig. 1B, CXCL8) might have resulted from synergy between the early signaling by dsRNA, which could not be quickly neutralized with LL-37, and the stimulation with free LL-37, which itself can stimulate KCs (Supplementary Fig. S1B, Figs. 1B and C and 2–4, CXCL8 and *CXCL8* mRNA). Interestingly, expression of LL-37 is downregulated [9] and TSLP is overexpressed [19,21] in the skin of atopic dermatitis patients, suggesting the possible involvement of LL-37 in the suppression of Th2 skin inflammation induced by viral or self dsRNA, the latter of which can be released from damaged cells [32,33].

LL-37 induced CXCL8 and IL-6 expression ([25] and Fig. 4A) and LL-37 and cytokines synergistically induced the expression of CXCL8 and IL-6 (Figs. 2–4), while LL-37 downregulated the TNF-α/IFN-γ-induced expression of CXCL10 and CCL5 via unknown mechanisms (Figs. 2 and 3). Interestingly, Th17 cells, IL-17, IL-22, IL-6, and LL-37 are highly accumulated/expressed in skin lesions in patients with psoriasis [1,24]. Moreover, IL-17 enhances vitamin D_3-induced expression of hCAP18, the precursor of LL-37, in human KCs [34]. We demonstrated remarkable synergy between LL-37 and Th17 cytokines in the induction of IL-6 and CXCL8 expression (Fig. 4). Taken together, the results suggest that IL-17 contributes to the induction of LL-37 expression and, in turn, LL-37 and Th17 cytokines (IL-17 and IL-22) show synergy in inducing the production of IL-6 and CXCL8, which contribute to skin inflammation in psoriasis, and this amplifies the capacity for host defense against invading organisms.

The scrambled LL-37 (see Section 2) used as a control, which did not influence KC proinflammatory responses (Figs. 1–4), had the same charge and net amino acid composition as LL-37 but lacked a significant α-helical structure [35]. The same scrambled sequence has been shown to lack activities that LL-37 can exhibit (for example, internalization into human dendritic cells [36] and inhibition of biofilm production by *Staphylococcus aureus* [35]). We also examined effects of another peptide with the reversed LL-37 sequence, activities of which have not been well investigated. Interestingly, the reversed LL-37 showed modulatory effects similar to LL-37 (Figs. 1–4). Many of the effects of LL-37 can be attributed to the cationic and hydrophobic nature of its linear α-helical structure [6]. Given that the reversed LL-37 formed the linear α-helical structure, it could retain the characteristic, cationic amphipathic α-helical structure essential to the function of LL-37

[6–8]. The reversed LL-37 sequence along with the wild-type sequence could be used as a template for developing new host defensive peptides or peptide-based antibiotics [37]. The results that the scrambled LL-37 showed moderate/small inhibition of the dsRNA-induced production of TSLP and CXCL10 (Fig. 1AC, Scrambled) suggest that the scrambled sequence might have a weak activity to interact with dsRNA and/or act on KCs.

In summary, we demonstrated that LL-37 modulates proinflammatory responses induced by cytokines (TNF-α, IFN-γ, IL-17, and/or IL-22) and dsRNA in human KCs. The results suggest greater involvement of LL-37 than expected in regulation of skin inflammation, for example, the vicious cycle of psoriasis, amplification of the capacity to defend against invading organisms, and suppression of dsRNA-induced Th2 inflammatory conditions such as atopic dermatitis. Additionally, we found that the reversed LL-37 sequence exhibited effects similar to LL-37.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.024.

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