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Cathelicidin antimicrobial peptide LL-37 in psoriasis enables keratinocyte reactivity against TLR9 ligands

Shin Morizane[1,2], **Kenshi Yamasaki**[1,3], **Beda Mühleisen**[1], **Paul F. Kotol**[1], **Masamoto Murakami**[4], **Yumi Aoyama**[2], **Keiji Iwatsuki**[2], **Tissa Hata**[1], and **Richard L. Gallo**[1]

[1]Division of Dermatology, Department of Medicine, University of California, San Diego and VA San Diego Healthcare System, San Diego, CA, USA

[2]Department of Dermatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan

[3]Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan

[4]Department of Dermatology, Asahikawa Medical College, Asahikawa, Japan

Abstract

Here we show that keratinocytes in psoriatic lesional skin express increased Toll-like receptor (TLR) 9 that similarly localizes with elevated expression of the cathelicidin antimicrobial peptide LL-37. In culture, normal human keratinocytes exposed to LL-37 increased TLR9 expression. Furthermore, when keratinocytes were exposed to LL-37 and subsequently treated with TLR9 ligands such as CpG or genomic DNA, keratinocytes greatly increased production of type I interferons. This response mimicked observations in the epidermis of psoriatic lesional skin as keratinocytes in psoriatic lesions produce greater amounts of interferon-β than normal skin lacking LL-37. The mechanism for induction of type I interferons in keratinocytes was dependent on TLR9 expression but not on a DNA-LL-37 complex. These findings suggest that keratinocytes recognize and respond to DNA and can actively participate in contributing to the immunological environment that characterizes psoriasis.

Introduction

Keratinocytes are a well-described source of inflammatory mediators that influence T-cell and other leukocyte functions. However, it remains unclear how the production of a variety of cytokines and chemokines by keratinocytes influences the development of inflammatory skin diseases. For example, although the pathogenesis of psoriasis is incompletely understood, altered T-cell mediated immune responses are thought to be critical to

Address correspondence to: Shin Morizane, M.D., Ph.D., Department of Dermatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences. 2-5-1, Shikata-cho, Kita-ku, Okayama, 700-8558, Japan, Phone: +81-86-235-7282 Fax: +81-86-235-7283, zanemori@cc.okayama-u.ac.jp.

Conflict of Interest

The authors state no conflict of interests.

manifestations of the disease. While it is clear that T-cell function is a major determinant of the local inflammatory events that lead to the characteristic manifestations of psoriasis, recent studies of skin immune defense systems have highlighted the importance of innate immunity, a primary responsibility of epithelial cells and granulocytes. This study sought to better define the potential role of the innate immune system of keratinocytes in triggering inflammation.

Keratinocytes following injury, or in psoriatic lesional skin, are characterized by the excessive production of antimicrobial peptides (AMPs) such as β-defensins and cathelicidin (Dorschner *et al.*, 2001; Gallo *et al.*, 1994; Harder *et al.*, 1997; Liu *et al.*, 1998; Ong *et al.*, 2002). These antimicrobial molecules are known for integral roles of killing pathogenic microorganisms, but AMPs also modify host inflammatory responses by a variety of mechanisms (Lai and Gallo, 2009). The cathelicidin peptide LL-37 has been described as enabling plasmacytoid dendritic cells (pDCs) to recognize self-DNA through Toll-like receptor (TLR) 9 (Lande *et al.*, 2007). The importance of this stimulation of DC gains relevance to psoriasis in the context of the capacity of these DCs to influence T-cell polarization critical to manifestation of the disease, and include inflammatory cytokines such as type I interferons (IFNs), IL-17A, IL-22 and IL-23 (Nestle *et al.*, 2009). The significance of Th17 cytokines to psoriasis has been confirmed by observations of the efficacy of therapeutic approaches directed to neutralization of the Th17 axis (Di Cesare *et al.*, 2009) Thus, keratinocytes and neutrophils, through the release of LL-37, may provide a critical co-factor for recognition of self-DNA by DCs, and this in turn can be a critical element in the pathophysiology of the disease.

Since keratinocytes produce and are directly exposed to AMPs in psoriatic skin, are the most numerous cell type present in psoriatic lesions, and also have the capacity to influence T cell polarization through production of type I interferons and other cytokines (Lebre *et al.*, 2007; Ong *et al.*, 2002), we investigated here if epidermal keratinocytes might also be responsive to self-DNA and contribute to production of cytokines critical to the development of psoriasis and inflammation. We show here for the first time that keratinocytes recognize and respond to DNA and can actively participate in contributing to the immunological environment that characterizes psoriasis.

Results

Increased LL-37 is associated with elevated TLR9 in psoriatic epidermis

We hypothesized that the expression and function of TLRs by epidermal keratinocytes might be a critical element in amplifying the immune response. Measurement of TLR mRNA abundance in skin biopsies of psoriatic lesion revealed that TLR9, but not the other TLRs, was significantly elevated in psoriatic lesional skin compared to psoriatic non-lesional skin or lesional skin of atopic dermatitis (Figure 1a–1d), which are consistent with a previous report (Miller *et al.*, 2005). TLR3 expression was significantly decreased in lesional skin of atopic dermatitis compared to psoriatic lesional skin. The increase of TLR9 expression in psoriatic lesional skin corresponded with increased TLR9 production in keratinocytes as immunohistochemical staining revealed that TLR9 protein expression was greatly enhanced in keratinocytes (Figure 1e–1g). TLR9-positive cells are also seen in the

dermis of psoriatic lesional skin. Co-immunostaining revealed that cathelicidin was similarly located with TLR9 in the psoriatic epidermis (Figure 1h–1j).

Since immunohistochemical identification of cathelicidin in this study and prior studies (Frohm *et al.*, 1997) has not defined the peptide form of cathelicidin present in psoriasis, and these various proteolytically processed forms are critical to the function of the peptide (Braff *et al.*, 2005; Yamasaki *et al.*, 2007), we performed surface-enhanced laser desorption-ionization time-of-flight mass spectrometry (SELDI-TOF-MS) on whole extracts of psoriatic lesional skin to define the peptide mass and thus its sequence. Unlike normal skin or skin from other inflammatory disorders such as rosacea (Yamasaki *et al.*, 2007), all cathelicidin detectable in psoriasis was in the form of LL-37 (Figure 1k).

LL-37 induces TLR9 expression in keratinocytes

We next evaluated if the presence of LL-37 could influence the expression of TLR9 in keratinocytes. Cultures of normal human epidermal keratinocytes (NHEKs) were exposed to LL-37 at concentrations within the physiologic range of cathelicidin previously measured in psoriatic skin (Ong *et al.*, 2002). Keratinocytes cultured with LL-37 increased expression of mRNA for TLR9, but not TLR1, TLR2 or TLR3 (Figure 2a), a pattern similar to the expression of TLRs found in psoriatic epidermis. TLR5 and TLR6 were not significantly increased, and TLR4, TLR7 and TLR8 were not consistently detectable in cultured keratinocytes (data not shown). We also examined the effect on TLR9 expression by inflammatory cytokines including IL-22, but under these culture conditions interferon-γ, IL-4, IL-17A, IL-22 or TNFα did not induce TLR9 mRNA in keratinocytes (Figure 2b). LL-37 induced TLR9 expression in a dose-dependent manner (Figure 2c), and a peak increase of TLR9 mRNA was observed 12 hours after the stimulation (Figure 2d). TLR9 protein increase paralleled TLR9 mRNA induction by LL-37 (Figure 2e–2g).

LL-37 enhances type I IFN expression from keratinocytes exposed to DNA

To evaluate the functional significance of keratinocyte TLR9, IFNA2 and IFNB1 mRNA abundance was measured in the presence of the TLR9 ligand CpG DNA. As previously reported (Miller *et al.*, 2005), CpG by itself had minimal effect on keratinocytes but greatly increased type I IFNs (IFNA2 and IFNB1) mRNA in the presence of LL-37 (Figure 3a and 3b). Whole genomic DNA had a similar effect to simulate IFN expression in the presence of LL-37 (Figure 3c). The induction of IFNB1 expression was dose-dependent of LL-37 (Figure 3d). A corresponding increase of IFN-β protein was also confirmed by ELISA (Figure 3e, 3f) and the induction was observed 24 hours after the stimulation (Figure 3g). This effect was significantly inhibited by the TLR9 antagonist G-ODN (Figure 3h).

Psoriatic keratinocytes express IFN-β

The *in vitro* experiments predicted that epidermal keratinocytes affected by LL-37 would produce type I IFNs. As expected, keratinocytes in lesional skin from psoriatic patients showed abundant expression of IFN-β compared to keratinocytes from normal skin that had small amounts of LL-37 (Figure 4a–4c). IFN-β-positive cells were also observed in the upper dermis (Figure 4d–4f). A corresponding increase of IFNB1 mRNA was confirmed by real-time PCR of skin biopsies (Figure 4g).

Keratinocytes respond to sequential exposure to LL-37 and CpG, but not to LL-37 complexed to CpG

LL-37 and DNA have been previously proposed to form a complex between the peptide and nucleic acids to activate TLR9 in pDCs, thus LL-37 protects DNA from degradation and facilitates DNA entry to the early endosome (Lande *et al.*, 2007). The methods used in Figure 3 relied on the addition of LL-37 to keratinocytes followed by sequential addition of CpG. This approach may have enabled formation of a complex between the cationic peptide and anionic DNA. Alternatively, LL-37 may have directly interacted with the keratinocyte membrane to enhance entry of CpG. To distinguish these events, LL-37 and CpG were first mixed together to allow complex formation. Assembly of these two molecules into a complex was confirmed by HPLC. CpG alone was seen as the expected single peak (Figure 5a). Once CpG was pre-incubated with LL-37 the assembly into a multimeric complex could be directly visualized by the disappearance of the single peak of CpG and the appearance of several broad peaks (Figure 5b). However, the CpG/LL-37 complex was not able to enhance type I IFNs production from keratinocytes. In contrast, sequential addition of LL-37 followed immediately by CpG at the same concentrations as used in the complex was required to stimulate keratinocyte IFN expression (Figure 5c).

We also examined if other cathelicidin antimicrobial peptides have similar capacity to increase IFNs production from keratinocytes. KS-30, a derivative of LL-37 that has previously shown the capacity to activate IL-8 expression in keratinocytes (Braff *et al.*, 2005), showed a similar ability to increase type I IFN when added before CpG (Figure 5d). Several other cationic peptides including the murine cathelicidin peptide mCRAMP (GL-33), other human cathelicidin peptides such as FA-29 and DI-27, or the cationic but unstructured proline-rich antimicrobial porcine cathelicidin peptide PR-39 did not enhance type I IFNs induction. Although these cationic AMPs could not enhance type I IFNs in keratinocytes they were still able to bind and form complexes with CpG as observed by HPLC analysis (data not shown).

Overexpression of TLR9 enhances type I IFN induction by the ligands in keratinocytes

We further investigated the role of TLR9 in keratinocyte recognition of DNA by transient overexpression of TLR9 in keratinocytes. Plasmid DNA encoding human TLR9 (pUNO-hTLR9-HA) was transfected into keratinocytes. These cells (or controls) were then exposed to CpG or LL-37 then CpG. Transfection of pUNO-hTLR9 increased TLR9 mRNA expression several fold (Figure 6a). Addition of CpG alone increased IFNB1 approximately 3-fold in keratinocytes transfected with TLR9 (pUNO-TLR9) but not the control (pUNO-mcs) (Figure 6b). As seen previously, LL-37 treatment of control keratinocytes greatly enhanced IFNB1 expression by the sequential stimulation with CpG. (Figure 6c). This increase occurred to a significantly larger extent when cells were transfected with pUNO-TLR9-HA. Genomic DNA had a similar effect in the presence of LL-37 (Figure 6d).

Discussion

In the current study we have shown for the first time that keratinocytes will respond to DNA with a large increase in release of type I IFNs, and that this occurs in a LL-37 and TLR9-

dependent manner. This previously unknown phenomenon, combined with the importance of type I IFNs in the pathogenesis of psoriasis, suggests that the abundant keratinocytes expressing LL-37 in psoriatic lesional skin could make a critical contribution to disease pathogenesis.

LL-37 is the carboxy-terminal peptide fragment derived from the human cathelicidin precursor protein hCAP18. This peptide not only has the capacity to kill a wide variety of microbes, but also can modify host immune and growth responses (Lai and Gallo, 2009). LL-37 has been reported to form a complex with self-DNA and self-RNA to induce IFN-α in pDCs through TLR9 and TLR7, and hypothesized to drive autoimmunity in psoriasis by this peptide-nucleic acid binding phenomenon (Ganguly *et al.*, 2009; Lande *et al.*, 2007). In the present study, we have observed that LL-37, or the alternatively processed cathelicidin peptide KS-30, can both greatly increase type I IFNs production from keratinocytes while other AMPs of similar cationic charge and antimicrobial potency can not.

The expression and processing of the cathelicidin precursor protein is specific for the cell, tissue and disease state. Cathelicidin is found at low levels in normal skin and is not in the form of LL-37 (Yamasaki *et al.*, 2007). In contrast, excess cathelicidin is seen in the inflammatory skin disease rosacea, but in contrast to normal skin these cathelicidin peptides are found in several unique forms (Yamasaki *et al.*, 2007). Our findings here of elevated cathelicidin in psoriasis is consistent with previous observations in this disease (Frohm *et al.*, 1997; Ong *et al.*, 2002) Furthermore, we now show by SELDI-TOF-MS of psoriatic lesional skin that the predominant peptide form of cathelicidin is indeed in the form of LL-37. It was not previously known that LL-37 was the exclusive detectable form of cathelicidin in psoriasis. In this way psoriasis differs significantly with rosacea where many cathelicidin peptide forms other than LL-37 are present. The significance of observing different processed forms of the cathelicidin is that different peptide forms have different antimicrobial activities and proinflammatory functions (Braff *et al.*, 2005). Thus, although psoriasis and rosacea both have elevated cathelicidin expression, the balance of peptides with different and potentially competitive actions may partially explain differences in phenotype.

Keratinocytes in psoriatic lesional skin have previously been shown to express TLR9, and cultured keratinocytes express it in a weakly functional form (Lebre *et al.*, 2007; Miller *et al.*, 2005). The present study shows *in vitro* that in addition to enhancing of the capacity of TLR9 to respond to DNA, LL-37 also induces an increase in TLR9 expression in keratinocytes. The increase in TLR9 seen after LL-37 is not likely to be the only reason for increased TLR9 responsiveness since increased function was seen immediately after LL-37 addition, thus not allowing time for increased expression of TLR9. The ability of LL-37 to increase TLR9 expression is consistent with the elevated abundance of and similar location of cathelicidin and TLR9 seen by immunostaining of psoriatic epidermis.

The mechanism through which TLR9 is induced by LL-37 is unclear at present. LL-37 is known to induce IL-8 in keratinocytes through G_i protein-coupled receptor or EGFR-dependent signaling events (Braff *et al.*, 2005). The G_i protein inhibitor pertussis toxin, or EGFR tyrosine kinase inhibitor AG1478, however, did not inhibit the increase in TLR9 by

LL-37 (Supplemental Figure S1), suggesting that induction of TLR9 by LL-37 is independent of G_i protein and EGFR signaling. Thus, further investigation is required to understand how LL-37 enables keratinocytes to increase TLR9.

The consequence of increased type I IFN production by keratinocytes may be that these cells become an important and active participant in exacerbating psoriasis. Much evidence has previously been assembled to suggest that type I IFNs contribute to this disease (Nestle *et al.*, 2005). pDCs are currently thought to be major type I IFNs-producing cells, and produce large amount of IFN-α in psoriasis (Nestle *et al.*, 2005). A complex between LL-37 and DNA has been previously proposed to be necessary for activation of TLR9 in pDCs (Lande *et al.*, 2007), and we examined if this mechanism is also involved in the keratinocyte TLR9 response. We confirmed that CpG and LL-37 can form a complex. This is not surprising as all cationic peptides tested have this capacity to bind anionic nucleic acids. However, these complexes did not induce type I IFNs in keratinocytes. Only LL-37 and a unique derivative of this peptide were active, and this occurred only when the keratinocytes were first treated with the peptide. Therefore, in keratinocytes it appears that LL-37 must first interact with the cell membrane, an interaction that is known to alter membrane fluidity (Di Nardo *et al.*, 2007). Presumably, this interaction enables subsequent recognition by TLR9. It is unclear if this membrane permeability phenomenon also takes place in pDCs in addition to or in lieu of the DNA binding and protection hypothesis that has been previously put forward. Thus, unlike that previously reported for pDCs, the present findings suggest that the binding of LL-37 to DNA is not the mechanism of action to stimulate keratinocyte TLR9 activation.

Overexpression of TLR9 in keratinocytes enhances the synergistic induction of type I IFNs, thus supporting that the response to CpG was TLR9-dependent. LL-37 then further enhanced the response of keratinocytes transfected with a TLR9-expressing vector. These results indicate that TLR9 expression levels alter the sensitivity to its ligands. Combined with the results that LL-37 increased TLR9 expression in keratinocytes, and that LL-37 and TLR9 are similarly located and highly expressed in the epidermis of psoriasis, these suggest that keratinocytes are poised to respond TLR9 ligands *in vivo* and together may be critical to pathogenesis of the disease. In support of this hypothesis are recent observations that psoriatic patients who improve on etanercept therapy also show a decrease in TLR9 expression but maintain elevated levels of LL-37 in their lesional skin (Miller *et al.*, 2011).

In summary, we have shown that the activation of keratinocytes by LL-37 enhances TLR9 responsiveness to DNA and greatly increases the production of type I IFNs. The epidermis is a rich source of genomic DNA that can be released by trauma, infection and normal differentiation. Thus, the stimulus for these events and the responding cells all exist in the epidermis. Recently Dombrowski et al. have reported that LL-37 also serves as an anti-inflammatory agent by blocking activation of the DNA-sensing inflammasomes, which might be a mechanistic explanation why vitamin D, a strong cathelicidin inducer in keratinocytes, is effective for psoriasis (Dombrowski *et al.*, 2011). They showed that intracellular LL-37 blocks the DNA-triggered formation of AIM2 inflammasomes in keratinocytes, inhibiting IL-1β release. Because our current study demonstrated that extracellular or endosomal LL-37 enhances TLR9 expression and function in keratinocytes, leading to increased IFN-α/β production, the location of LL-37 and DNA might be crucial

for their pro- or anti- inflammatory effect. Thus, LL-37/DNA complex formation may be important because it is required for the anti-IL-1β effect but sequential exposure leads to a pro-IFN-α/β effect in keratinocytes. These findings therefore uncover a previously underappreciated role for keratinocytes in modulating the immune response in psoriasis and suggest that targeting these superficial events for therapy could provide a mechanism to improve disease without the risks of systemic immunosuppressive approaches.

Materials & Methods

Skin Biopsies

This study was approved by the Human Research Protection Program at the University of California, San Diego and performed in accordance with the Declaration and Helsinki Principles. Skin was collected from patients with psoriasis or atopic dermatitis and from healthy volunteers. For all procedures, informed consent was obtained. After the injection of local anesthesia, 2 or 4 mm punch biopsies were taken from the untreated lesional skin of individuals with psoriasis or atopic dermatitis and from healthy volunteers. All samples were stored at −80 °C until use.

Peptide synthesis

Cathelicidin peptides, LL-37, GL-33, PR-39, KS-30, FA-29 and DI-27 were commercially prepared by Synpep (Dorschner *et al.*, 2001; Murakami *et al.*, 2004; Yamasaki *et al.*, 2007).

Cell culture and stimuli

NHEKs were cultured as previously described (Morizane *et al.*, 2010). Cells were stimulated with synthetic cathelicidin peptides (LL-37, GL-33, PR-39, KS-30, FA-29, DI-27, 0.1–10 μM), IFN-γ (2 ng/ml, R&D Systems, Minneapolis, MN), IL-4, IL-17A, IL-22 (50 ng/ml, R&D Systems), TNF-α (50 ng/ml, Chemicon, Temecula, CA), type C CpG ODN M362 (2 μM, Invivogen), genomic DNA (10 μg/ml), or G-ODN (4 μM, Invivogen) in 24-well flat bottom plates (Corning Incorporated Life Sciences, Lowell, MA) for 3–72 hours. Genomic DNA was purified from HaCaT cells by Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Pertussis toxin (100 ng/ml, Sigma-Aldrich) and AG1478 (2 μM, Calbiochem) were used to pre-incubate for 1 hour.

Plasmid DNA transfection

Transfection was performed by Amaxa Human Keratinocytes Nucleofector Kit (Lonza Cologne AG). NHEKs at 60–70 % confluency were harvested by trypsinization and 1×10^6 cells were transfected with 3 μg plasmid DNA, pUNO-mcs (control plasmid) or pUNO-hTLR9-HA (Invivogen), following the protocol. Transfected cells were incubated in 6-well plates at 37 °C under standard tissue culture conditions for 24 hours. The cells were then stimulated with CpG or LL-37 then CpG or genomic DNA for 24 hours.

Quantitative real-time PCR (qRT-PCR)

Total RNA from skin samples and cultured keratinocytes was extracted, and cDNA was synthesized as previously written (Buchau *et al.*, 2009). TaqMan Gene Expression Assays

(Applied Biosystems ABI, Foster City, CA) were used to analyze mRNA expression as described by manufacturer instruction. GAPDH and 18s ribosomal RNA were examined as previously written (Morizane *et al.*, 2010; Taylor *et al.*, 2007). mRNA expression was calculated as relative expression to *GAPDH* mRNA (cultured cell samples) or *18S* ribosomal RNA (skin samples), and data are presented as fold change against each control (mean of non-stimulated cells or mean of psoriatic non-lesional skins).

ELISA

IFN-β in culture media was measured by a commercial sandwich ELISA following the manufacturer's instructions (PBL InterferonSource, Piscataway, NJ).

Immunostaining

For immunohistofluorescence, frozen sections (6 μm) were fixed with 4% paraformaldehyde, blocked with 3% BSA in PBS, and incubated with mouse monoclonal anti-TLR9 antibody (IMGENEX, San Diego, CA), rabbit anti-LL-37 antibody, or mouse monoclonal anti-IFN-β antibody at 4 °C overnight. Normal mouse and rabbit IgG were used as negative control. After washing with PBS, FITC-conjugated goat anti-mouse IgG antibody (Sigma) for TLR9 or IFN-β staining or FITC-conjugated goat anti-rabbit IgG antibody (Jackson Immuno Research LABORATORIES, INC) and AlexaFluor568-conjugated goat anti-mouse IgG antibody (Molecular Probes/Invitrogen) for double staining of TLR9 and LL-37 were used as second antibody. For immunocytofluorescence, keratinocytes were grown on chamber slides. After 4% paraformaldehyde fixation and blocking with 3% BSA in PBS, TLR9 staining was performed as described above. Sections were mounted in ProLong Gold Anti-Fade reagent with DAPI (Molecular Probes/Invitrogen). Images were obtained using an Olympus BX41 fluorescent microscope (Scientific Instrument Company, Temecula, CA).

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS)

Cathelicidin peptides were analyzed by SELDI-TOF-MS as previously written (Yamasaki *et al.*, 2007).

Size-exclusion HPLC

Superdex™75 Columns (Amersham Biosciences) and AKTA purifier HPLC system (Amersham Biosciences) were used for analysis. CpG (500 pmol) was pre-incubated with LL-37, GL-33 or PR-39 (2.5 nmol) in 100 μl PBS overnight. The samples were eluted with PBS at a flow rate of 1.0 mL/min. The detection wavelength was 214 nm.

Statistics

Results are expressed as the mean ± SEM. Student's t test was used to determine significance. Mann-Whitney U test was used to compare IFN-β protein expression and mRNA expression in skin samples between each group. $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AMP	antimicrobial peptides
IFN	interferon
NHEKs	normal human epidermal keratinocytes
pDCs	plasmacytoid dendritic cells
SELDI-TOF-MS	surface-enhanced laser desorption-ionization time-of-flight mass spectrometry
TLR	Toll-like receptor

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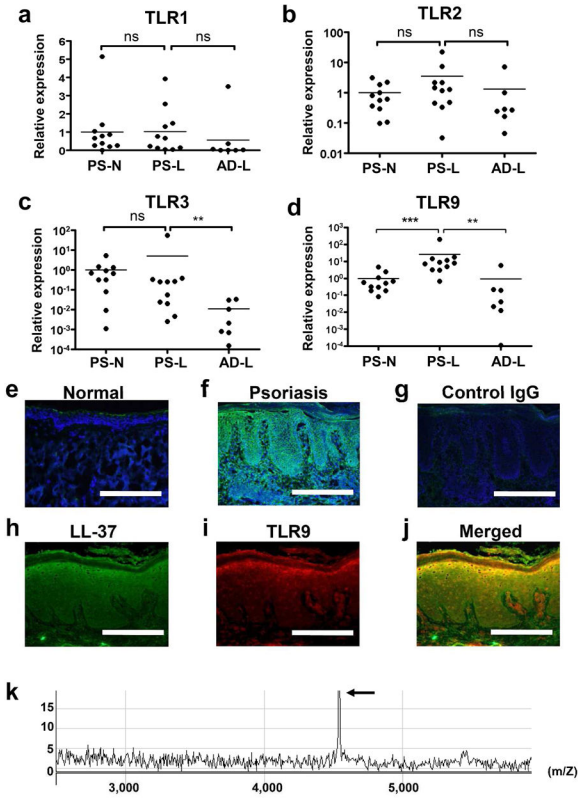

Figure 1. TLR9 is increased in psoriatic epidermis and similarly localizes with elevated cathelicidin processed as LL-37

(**a**–**d**) TLR mRNA expression was analyzed and normalized with the mean of non-lesional skin from psoriasis patients. PS-N; non-lesional skin from psoriasis patients (N = 11), PS-L; lesional skin from psoriasis patients (N = 5), AD-L; lesional skin from atopic dermatitis patients (N = 7). **: $P < 0.01$, ***: $P < 0.001$. (**e**–**g**) TLR9 protein expression was examined by immunofluorescence, and DAPI visualized nuclei. Scale bar = 250 μm. (**h**–**j**) The localization of cathelicidin (green) and TLR9 (red) in psoriatic lesional skin was visualized by immunofluorescence. Scale bar = 250 μm. Data are representatives of three samples (e–j). (**k**) Mass of cathelicidin peptides extracted from psoriatic lesional skin were examined by SELDI-TOF-MS. The arrow indicates the peak of LL-37. Data is a representative of five samples.

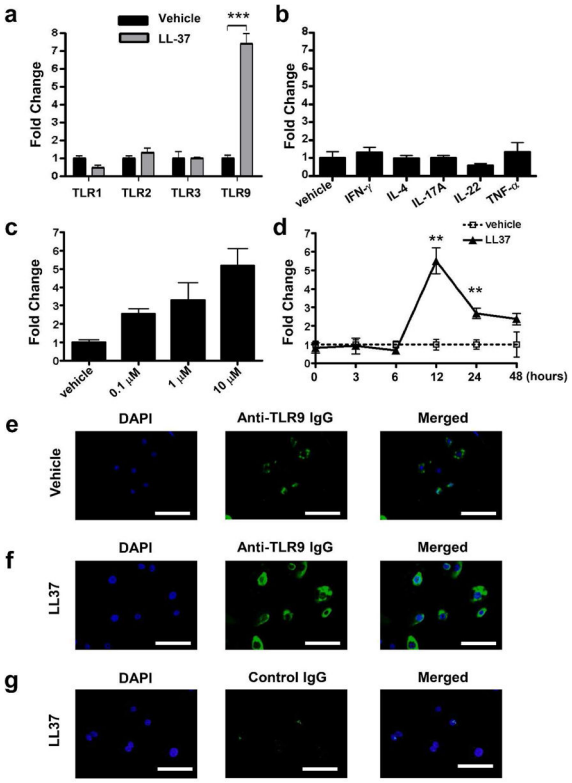

Figure 2. LL-37 induces TLR9 expression in keratinocytes

(**a**–**d**) NHEKs were treated with LL-37 (0.1 – 10 μM) or cytokines for 3 – 48 h, and then TLRs mRNA expression was analyzed by RT-qPCR. Data are mean ± SEM of triplicate samples and representative of three independent experiments. ***: $P < 0.001$. (**e**–**g**) TLR9 protein expression was examined by immunofluorescence, and nuclei were visualized with DAPI. Scale bar = 100 μm.

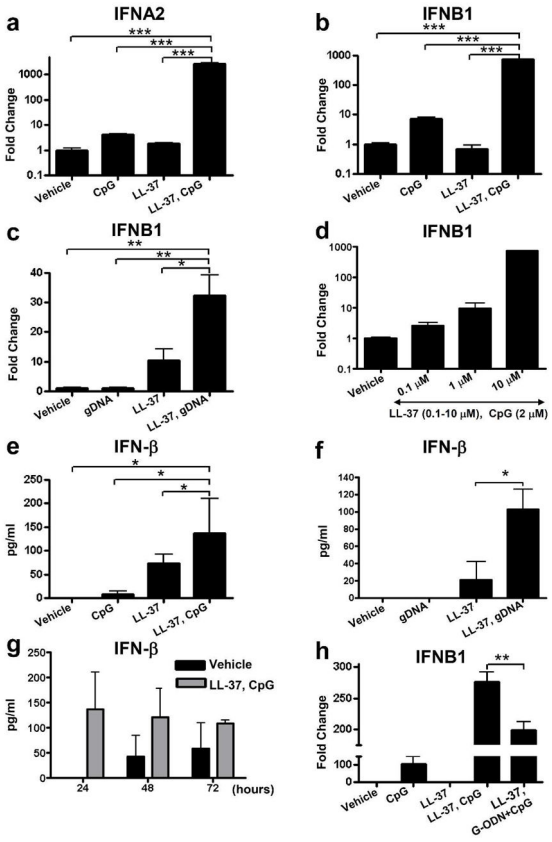

Figure 3. LL-37 enhances TLR9 responsiveness in keratinocytes

(**a**–**h**) NHEKs were stimulated with LL-37 (0.1 – 10 μM) then CpG (2 μM), genomic DNA (10 μg/ml), G-ODN (4 μM) or their combinations for 24 –72 h. (**a**–**d, h**) The expression of IFNA2 and IFNB1 mRNA were analyzed by RT-qPCR. (**e**–**g**) Protein expression of IFN-β in the media was measured by ELISA. gDNA; genomic DNA. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. Data are mean ± SEM of triplicate samples and representative of three independent experiments

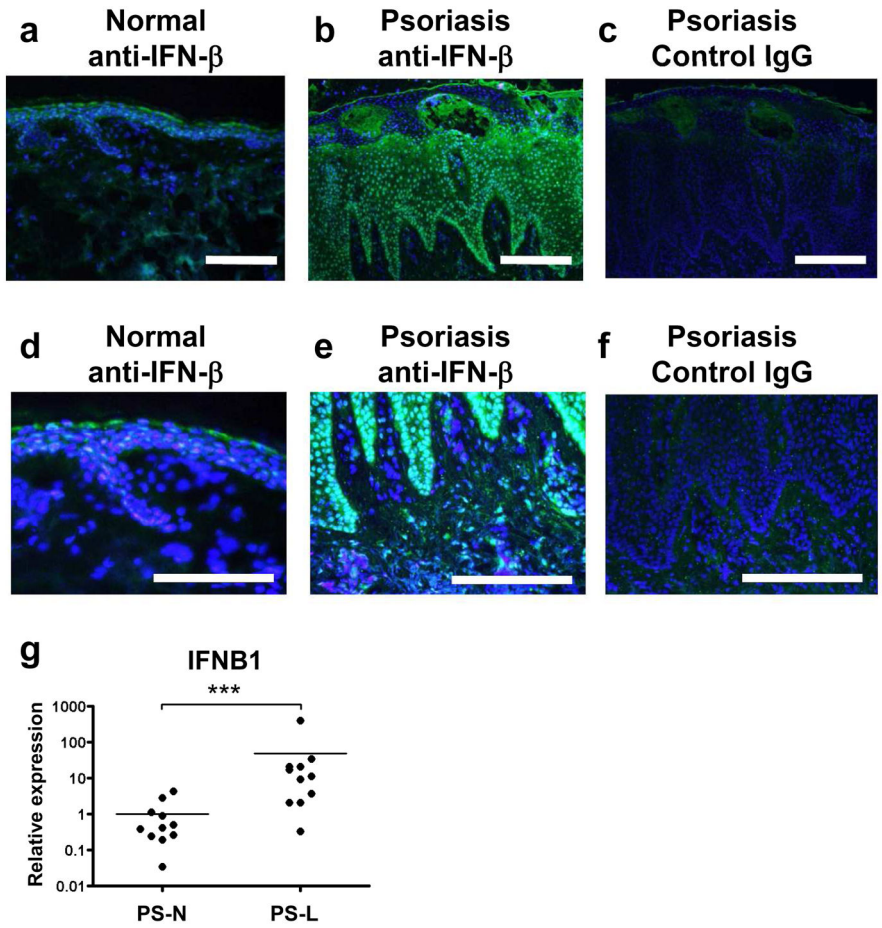

Figure 4. IFN-β is increased in keratinocytes from psoriatic lesional skin

(**a**–**f**) The expression of IFN-β was examined by immunofluorescence in normal skin or lesional skin from psoriatic patients, and nuclei were visualized with DAPI. Scale bar = 200 μm (a–c), 100 μm (d–f) Data shown are from a single sample representative of three. (**g**) IFNB1 mRNA expression was analyzed and normalized with the mean of non-lesional skin from psoriasis patients. PS-N; non-lesional skin from psoriasis patients (N = 11), PS-L; lesional skin from psoriasis patients (N = 11). ***: $P < 0.001$.

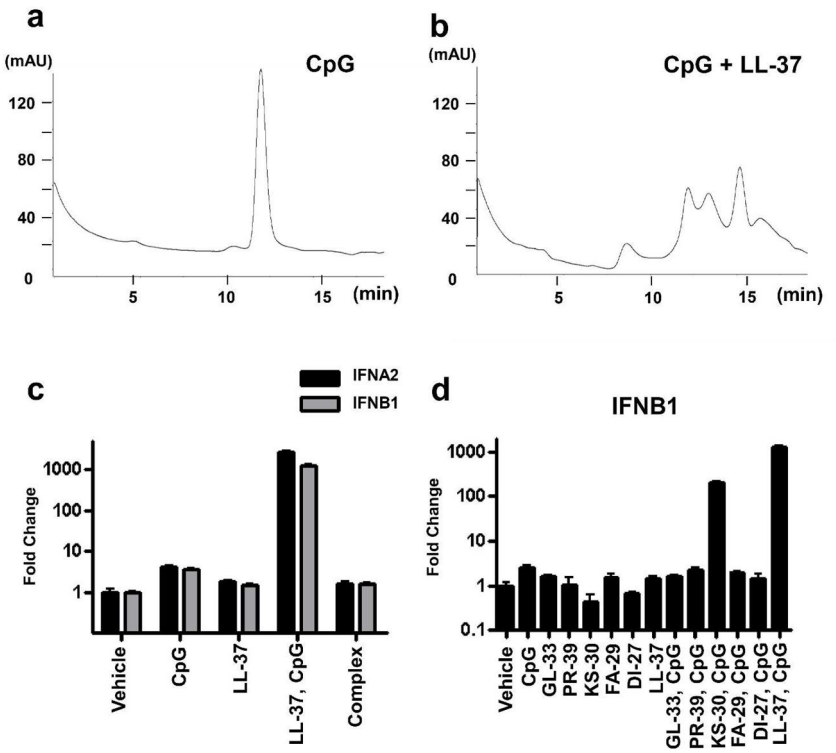

Figure 5. Keratinocytes respond to sequential exposure to LL-37 and CpG, but not to LL-37 complexed to CpG

(**a, b**) Size-exclusion HPLC was performed to analyze potential formation of a complex between CpG and LL-37. X axis indicates elution time (min) and Y axis indicates OD214 values (mAU). (**c, d**) NHEKs were stimulated for 24 h with 2 μM of CpG alone (ex. 'CpG'), 10 μM of cathelicidin peptides alone (ex. 'LL-37'), the sequential addition of both (ex. 'LL-37, CpG') or the complex formed as shown in the Figure 5b (ex. 'complex'). The IFNA2 or IFNB1 mRNA expression was analyzed by RT-qPCR. Data are mean ± SEM of triplicate samples and representative of two independent experiments.

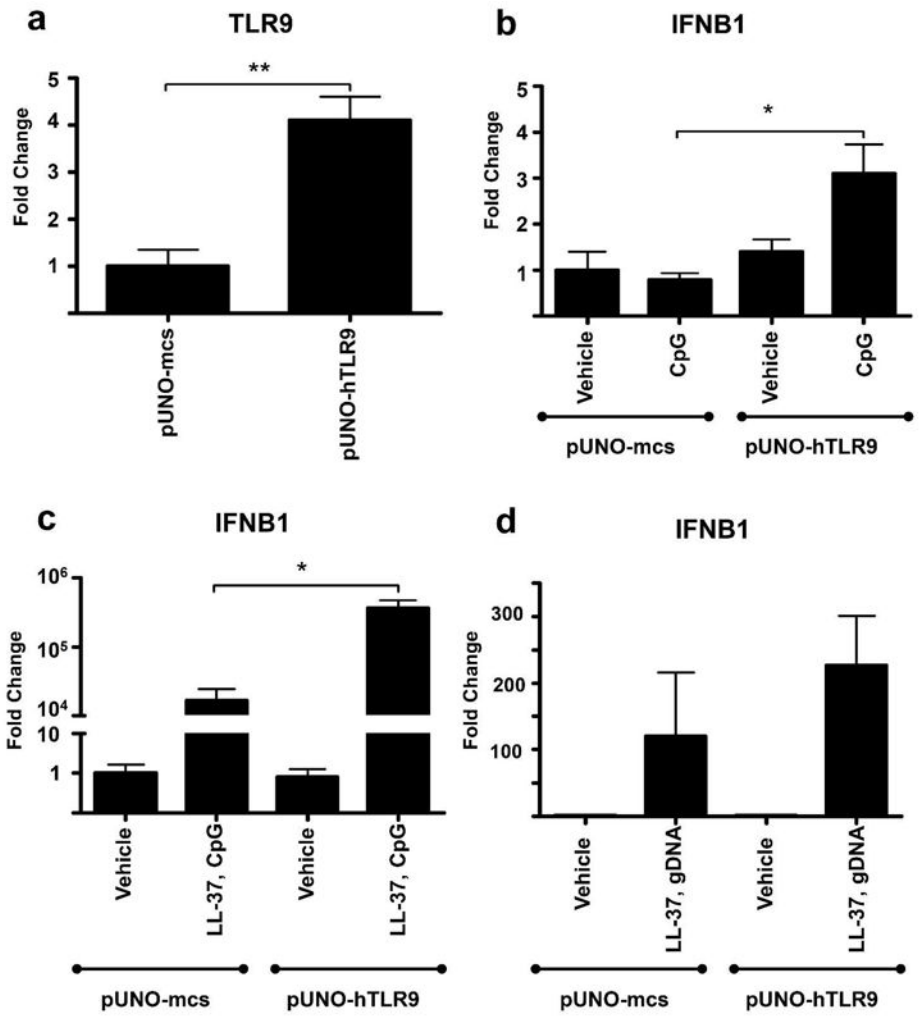

Figure 6. Overexpression of TLR9 enhances IFN-β production by the ligands in keratinocytes

(**a**) NHEKs were transfected with pUNO-mcs or pUNO-hTLR9-HA using Amaxa Human Keratinocyte Nucleofector Kit. 24 h after the transfection TLR9 mRNA expression were examined. (**b**–**d**) 24 h after the transfection with pUNO-mcs or pUNO-hTLR9-HA, NHEKs were stimulated with CpG alone (2 μM) (**b**) or LL-37 (10 μM) then CpG (2 μM) (c), genomic DNA (10 μg/ml) (**d**) for 24 h. The expression of IFNB1 mRNA was analyzed by RT-qPCR. *: $P < 0.05$, **: $P < 0.01$. Data are mean ± SEM of triplicate samples and representative of two independent experiments.