Cathelicidin antimicrobial peptide LL-37 augments IFN-β expression and anti-viral activity induced by double-stranded RNA in keratinocytes

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What is already known about this topic?

- LL-37 enhances DNA uptake into intracellular compartments, which leads to TLR9- and TLR7-dependent overproduction of IFNs in dendritic cells
- In keratinocytes, LL-37 enhances TLR9 responsiveness.
- LL-37 modulates poly (I:C)-induced proinflammatory responses in keratinocytes.

What does this study add?

- LL-37 enhances IFN-β expression and anti-viral activity induced by poly (I:C) in keratinocytes.

Abstract

Background: Cathelicidin antimicrobial peptide LL-37 not only has the capacity to kill a wide variety of microbes, but also can modify host immunity. Recently our group has observed that the activation of keratinocytes by LL-37 and DNA greatly increases IFN-β through Toll-like receptor 9 (TLR9). On the other hand, the effect of LL-37 on IFN-β induction through TLR3, a sensor of double-stranded RNA, in keratinocytes is not well-known.

Objectives: To investigate whether LL-37 could affect TLR3 signaling and anti-viral activity in normal human epidermal keratinocytes (NHEKs).

Methods: We investigated IFN-β production in NHEKs stimulated with a TLR3 ligand, poly (I:C), in the presence of LL-37. To examine the effect of LL-37 and poly (I:C) on anti-viral activity, a virus plaque assay using herpes simplex (HS) virus type-1 was carried out. The uptake of poly (I:C) conjugated with FITC into the keratinocytes was observed in the presence of LL-37. Immunostaining for TLR3 and LL-37 was performed using skin samples from HS.

Results: LL-37 and poly (I:C) synergistically induced IFN-β expression in NHEKs. Furthermore, the co-stimulation with LL-37 and poly (I:C) significantly decreased the viral plaque numbers compared to poly (I:C) or LL-37 alone. LL-37 enhanced the

uptake of FITC-conjugated poly (I:C) into the cells. The immunohistochemical analysis demonstrated that TLR3 and LL-37 expression is up-regulated in lesions of HS.

Conclusion: Our findings suggested that LL-37 augments the anti-viral activity induced by double-stranded RNA in keratinocytes, which may contribute to the innate immune response to cutaneous viral infections such as HS.

Introduction

Skin is our first physical barrier against pathogens from the external environment. The innate immune systems immediately respond to prevent further invasion.[1] Antimicrobial peptides play an important role in innate immunity, and human epidermal keratinocytes produce a variety of antimicrobial peptides, including human β-defensin (HBD) and cathelicidin.[1] Cathelicidin antimicrobial peptide LL-37, a C-terminal 37-residue peptide derived from hCAP18, not only has the capacity to kill a wide variety of microbes, but also can modify host immune and growth responses.[1] On the other hand, Toll-like receptors (TLRs) play an essential role in the recognition of microbial components,[2] and 10 members of TLRs have been reported in human.[3-7] Among them TLR3, TLR7 and TLR8 are thought to be an important receptors in activating natural immune responses against viral infection.[2,8,9] TLR3 recognizes double-stranded RNA which is synthesized during viral replication,[10] which induces the activation of NF-κB and the production of type I interferons (IFNs).[8] Recently our group observed that the activation of keratinocytes by LL-37 and DNA greatly increase type I IFN through TLR9.[11] However, the effect of LL-37 on IFN-β induction through TLR3 signaling in keratinocytes has not been studied, although several groups have reported LL-37 modulates poly (I:C) (a synthetic analog of viral double-stranded RNA, TLR3 ligands)-induced immune

responses in keratinocytes.[12,13] Therefore we investigated whether LL-37 could affect IFN-β induction through TLR3 signaling in normal human epidermal keratinocytes (NHEKs).

Materials and methods

Cell culture and stimuli

NHEKs were obtained from Invitrogen/Cascade Biologics (Portland, OR, U.S.A.) and maintained in serum-free Epilife Medium containing 0.06 mM Ca^{2+}, 1× Epilife Defined Growth Supplement (EDGS; Cascade Biologics/Invitrogen), 100 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C, and the medium was replaced every 2 days. Subconfluent NHEK monolayers were cultivated in 24-well plates and then stimulated with LL-37 (Peptide Institute. Inc., Osaka, Japan), poly (I:C) (InvivoGen, San Diego, CA, U.S.A.) or their combination for 3 to 48 hours.

Immunohistochemistry

This study was approved by the Ethics Committee of Okayama University (No.1087). Human skin samples were collected from patients with herpes simplex and from healthy

volunteers at Okayama University Hospital. Informed consent was obtained for all procedures. Formalin-fixed, paraffin-embedded skin samples were cut into 4 μm sections. Deparaffinized sections were processed for rehydration, incubated with a peroxidase-blocking reagent for 5 min and incubated with mouse monoclonal anti-TLR3 antibody (Abcam, Cambridge, MA, U.S.A.) or rabbit polyclonal anti-LL37 antibody (Novus Biologicals, Littleton, CO, U.S.A.) at 4 °C overnight. After washing with PBS, sections were incubated with secondary antibody conjugated with streptavidin-HRP for 30 min at room temperature. Histochemical visualization was carried out with an LSAB 2 kit (Dako, Carpinteria, CA, U.S.A.) according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA was extracted from NHEKs using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.), and converted to cDNA using an iScript cDNA Synthesis Kit (BioRad, Hercules, CA, U.S.A.) as described by the manufacturer's protocol. TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, U.S.A.) were used to analyze the expression of human IFNB1 (assay ID: Hs01077958_s1), IL6 (assay ID: Hs00985639_m1), IL8 (assay ID: Hs00174103_m1),

CCL5 (assay ID: Hs00982282_m1), TLR3 (assay ID: Hs00152933_m1), DDX58 (assay ID: Hs00204833_m1), and IFIH1 (assay ID: Hs01070332_m1) as described by the manufacturer's instructions (the user bulletin provided by Applied Biosystems). GAPDH mRNA was detected using the probe VIC-CATCCATGACAACTTTGGTA-MGB and the primers 5'-CTTAGCACCCCTGGCCAAG-3' and 5'-TGGTCATGAGTCCTTCCACG-3, and was used as an internal control to validate RNA for each sample.[14] The expression level of each mRNA was calculated relative to that of GAPDH mRNA, and all data are presented as the fold change against the respective control (mean of non-stimulated cells).

ELISA

IFN-β protein in the NHEK culture media was measured by a commercial sandwich ELISA (PBL Interferon-Source, Piscataway, NJ, U.S.A.) following the manufacturer's instructions.

Virus plaque assays

Wild-type herpes simplex virus type 1 (HSV-1, KOS strain) was obtained from the

Research Institute for Microbial Diseases, Osaka University. Subconfluent NHEK monolayers were cultivated in six-well plates and treated with LL-37 (0.5 μM), poly (I:C) (0.1 μg/ml) or their combination for 24 hours. NHEKs were then infected with HSV-1 (MOI=2) and incubated for 1 hour at 37 °C to allow virus adsorption. The culture medium was then replaced with newly prepared medium containing γ-globulin (5 mg/ml) (Gammagard, Baxter Healthcare Corp, West Village, CA, U.S.A.). Forty-eight hours later, the viral plaque formation was visualized by crystal violet staining.

Confocal laser scanning microscopy analysis

NHEKs were treated with poly (I:C)-conjugated with FITC (2 μg/ml) in the presence or absence of LL-37 (10 μM) for 6 hours. Then the uptake of FITC-conjugated poly (I:C) was observed by confocal laser scanning microscopy (LSM780, Zeiss, Jena, Germany) in Central Research Laboratory, Okayama University Medical School. Nuclei were visualized with 4'-6-diamidino-2-phenylidole (DAPI).

Statistical analysis

All statistical analyses were conducted by using Graph Pad prism, version 4.03.

Student's *t*-test was used for statistical analysis and a value of $p<0.05$ was considered significant.

Results

LL-37 and poly (I:C) synergistically induce IFN-β expression in NHEKs.

In order to investigate the effect of LL-37 on TLR3 signaling in keratinocytes, we stimulated NHEKs with a TLR3 ligand, poly (I:C), in the presence or absence of LL-37. LL-37 and poly (I:C) in combination synergistically induced a higher IFNB1 mRNA compared with LL-37 or poly (I:C) alone (Fig. 1a).[11] Furthermore, we also analyzed other cytokines and chemokines. IL6 and IL8 mRNA expression was also synergistically induced but a chemokine CCL5 expression was significantly suppressed as recently reported by Chen et al. (Fig.1b-d)[13] A corresponding increase of IFN-β protein was also confirmed by ELISA (Fig.2a). A peak increase of IFNB1 mRNA or protein was observed 6 or 24 hours after the stimulation, respectively (Fig. 2b-c). In addition, the expression of IFNB1 mRNA was up-regulated by poly (I:C) and LL-37 in a dose-dependent manner (Fig. 2d-e).

LL-37 enhances anti-viral activity against HSV-1 induced by poly (I:C).

Poly (I:C)-induced TLR3 signaling is reported to enhance anti-viral activity in keratinocytes.[15] To investigate whether LL-37 could further up-regulate the anti-viral activity induced by TLR3 signaling, a virus plaque assay using herpes simplex virus type 1 (HSV-1) was performed. Although poly (I:C) or LL-37 alone significantly reduced the viral plaque numbers, the co-stimulation with LL-37 and poly (I:C) further decreased them compared with poly (I:C) or LL-37 alone (Fig. 3).

LL-37 does not increase the expression of poly (I:C) receptors.

Previously we reported that TLR9 expression is up-regulated by LL-37 in keratinocytes, and this up-regulation enhances TLR9 responsiveness.[11] Therefore we next examined if TLR3 expression could be induced by LL-37 at various concentrations in NHEKs. Unlike TLR9 expression, TLR3 expression was not increased by LL-37 although IFN-α and IL-29 significantly induced it in NHEKs as previously reported. (Fig. 4a)[16,17] We also analyzed whether LL-37 affects the poly (I:C)-induced up-regulation of expression of TLR3. Poly (I:C) induced TLR3 expression as previously described, but LL-37 did not change the up-regulation (Fig. 4b).[18] We further investigated the effect of LL-37 on other ds-RNA sensors RIG-I (DDX58) and MDA5 (IFIH1) expression in NHEKs. Similar to TLR3, the expression of these receptors was also significantly induced by

IFN-α and IL-29, but not by LL-37 (Fig. 4c, d).

The uptake of poly (I:C) into cells is increased by LL-37.

We next hypothesized that the uptake of poly (I:C) into the cells could be enhanced by LL-37, because nucleic acids are negatively charged and can easily bind to LL-37, and also because LL-37 can change the structure of the cell membrane.[19,20] To examine this hypothesis, we cultured NHEKs treated with poly (I:C) conjugated with FITC in the presence or absence of LL-37.

As shown in Fig 3, the addition of LL-37 increased dot-like FITC-conjugated poly (I:C) in the cytoplasm or on the cell membrane (Fig. 5), which suggests that LL-37 could increase the uptake of poly (I:C).

TLR3 and LL-37 expression is increased in keratinocytes surrounding herpetic vesicles.

The in vitro experiment suggested that TLR3 and LL-37 could affect keratinocytes in cutaneous viral infection. As expected, immunohistochemical analysis revealed that keratinocytes around herpetic vesicles abundantly expressed TLR3 and LL-37 compared with keratinocytes from normal skin. TLR3- or LL-37-positive cells were

also seen in the vesicles and the dermis of lesional skin (Fig. 6a-d).

Discussion

Antimicrobial peptides (AMPs) play an essential role in innate immunity against invasion of infectious agents, including bacteria, viruses, fungi and parasites. AMPs not only display antimicrobial activity but also play multiple roles in immune defense, including neutralization of endotoxins, chemokine-like activities, immunomodulating activities, and induction of both angiogenesis and wound repair.[21-23] Today, more than 2200 AMPs have been identified (The Antimicrobial Peptides Database: http://aps.unmc.edu/AP/main.php). LL-37 also affects the production of proinflammatory cytokines/chemokines in various types of cells through the activation of multiple receptors.[24] TLR3, TLR7/TLR8 and TLR9 are activated by viral double-stranded DNA, viral single-stranded RNA, and bacterial CpG, respectively, and these receptors are located within the endosome or other intracellular compartments.[5,8,25] LL-37 has been reported to form a complex with self-DNA and RNA, enhances DNA uptake into intracellular compartments, and stimulates TLR9- and TLR7-dependent overproduction of IFNs, which are the key mediator of myeloid dendritic cells activation in psoriasis.[26,27] On the other hand, other groups have reported

that TLR3 signaling is modulated by LL-37 in several types of cells.[12,13,28] In this study, we showed that LL-37 enhances IFN-β expression and anti-viral activity induced by poly (I:C) in keratinocytes.[20] Interestingly, IL6 and IL8 mRNA expression was similarly induced but that of CCL5 was significantly suppressed as previously described[13]. So far these modulation mechanisms have been not clarified well, thus further investigations are need to explain the difference of modulation among cytokines and chemokines.

Other dsRNA sensors, MDA5 and RIG-I, also might be involved in anti-HSV activity in keratinocytes. However, in our experiments, we simply added poly (I:C) into cultured media. Since MDA5 and RIG-I are located in the cytosol, transfection techniques should be needed to bind poly (I:C) to MDA5 and RIG-I. Therefore, we focused on TLR3 as dsRNA sensor in this study, and considered two possibilities of the enhancement of IFN-β induction by poly (I:C); (1) the expression of poly (I:C) receptor TLR3 was up-regulated by LL-37 and (2) the uptake of poly (I:C) into the cells was enhanced by LL-37. To investigate the first scenario, we examined whether LL-37 could affect TLR3 expression in NHEKs. However, we found that the expression was not changed by LL-37. Previously, our group reported that LL-37 increased TLR9 expression in normal human keratinocytes.[11] The reason for the differential effects of

LL-37 on TLR3 and TLR9 expression was not clear, because the mechanism of the induction is still not studied well. On the other hand, CLSM analysis showed that LL-37 enhanced the uptake of poly (I:C) into the cells. This result is consistent with previous studies showing that the antimicrobial peptide LL-37 is one protein that stabilizes and enhances uptake of self-nucleic acids into dendritic cells.[26,27] One of the reasons why LL-37 enhances the uptake of poly (I:C) might be associated with the previous report that the presence of LL-37 effectively protects plasmid DNA against degradation by serum nuclease and targets DNA plasmid to the nuclei of mammalian cells through caveolae-independent membrane raft endocytosis and cell surface proteoglycans.[29] On the other hand, the complex of DNA and LL-37 did not show a synergistic effect in keratinocytes.[11] Another possibility could be that LL-37 changes the structure of the cell membrane and thereby alters the efficacy of endocytosis. The mechanism of keratinocyte uptake into the endosome might be different from that of plasmacytoid dendritic cells or macrophages. Further investigations are needed to understand this mechanism.

Viral plaque assay revealed that LL-37 or poly (I:C) alone significantly suppressed HSV plaque formation. Poly (I:C)-induced TLR3 signaling is reported to enhance anti-viral activity in keratinocytes, and an antimicrobial peptide LL-37 itself

can kill the virus as already reported.[15,30] But the combination of LL-37 and poly (I:C) further decreased the viral plaque numbers compared with poly (I:C) or LL-37 alone. Since the combination of LL-37 and poly (I:C) synergistically induce IFN-β expression, the induction might enhance anti-viral activity in keratinocytes.

Immunohistochemical analysis showed increases in TLR3 and LL-37 expression in keratinocytes around herpetic vesicles. TLR3, a sensor of viral double-stranded RNA,[8] is an essential component of innate immunity in cutaneous viral infection.[31,32] Herpes simplex virus type 1 (HSV-1) is a double-stranded DNA virus but produces double-stranded RNA in infected cells.[10,33] On the other hand, TLR3 has been reported to recognize the double-stranded domains of self-RNA from damaged keratinocytes after injury or ultraviolet radiation.[34,35] Therefore, TLR3 might recognize not only viral RNA but also self-RNA released from degenerated keratinocytes in viral vesicles. In addition, it has recently been reported that TLR3 is vital for innate immunity to HSV-1 in the central nervous system.[36-38] Taken together with these evidences, our findings suggest that TLR3 and LL-37 could play an important role in skin viral infections such as herpes simplex. Herpes simplex often reoccurs, although acyclovir has been used effectively to suppress virus proliferation at the clinical level. Targeting TLR3 signaling and LL-37 might provide a new approach to the treatment of herpes

simplex.

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Figure legends

Fig. 1. (**a-d**) NHEKs were stimulated with LL-37 (5 μM), poly (I:C) (1.0 μg/ml) or their combination for 24 hours. IFNB1, IL6, IL8, and CCL5 mRNA expression was analyzed by quantitative real-time PCR. *P<0.05, **P<0.01. Data are the means ± SEM of triplicate samples and representative of three independent experiments.

Fig.2. (**a**) NHEKs were stimulated with LL-37 (5 μM), poly (I:C) (1.0 μg/ml) or their combination for 24 hours. IFN-β protein in the media was analyzed by ELISA. (**b,c**) NHEKs were stimulated with the combination of LL-37 (5 μM) and poly (I:C) (1.0 μg/ml) for 3 to 48 hours, and then the expressions of IFNB1 mRNA and IFN-β protein were analyzed by quantitative real-time PCR and ELISA. **(d, e)** NHEKs were treated with LL-37 (2-8 μM), poly (I:C) (0.1 to 10 μg/ml) or their combination for 24 hours and then the expression of IFNB1 mRNA was analyzed by quantitative real-time PCR. *P<0.05. Data are the means ± SEM of triplicate samples and representative of two independent experiments.

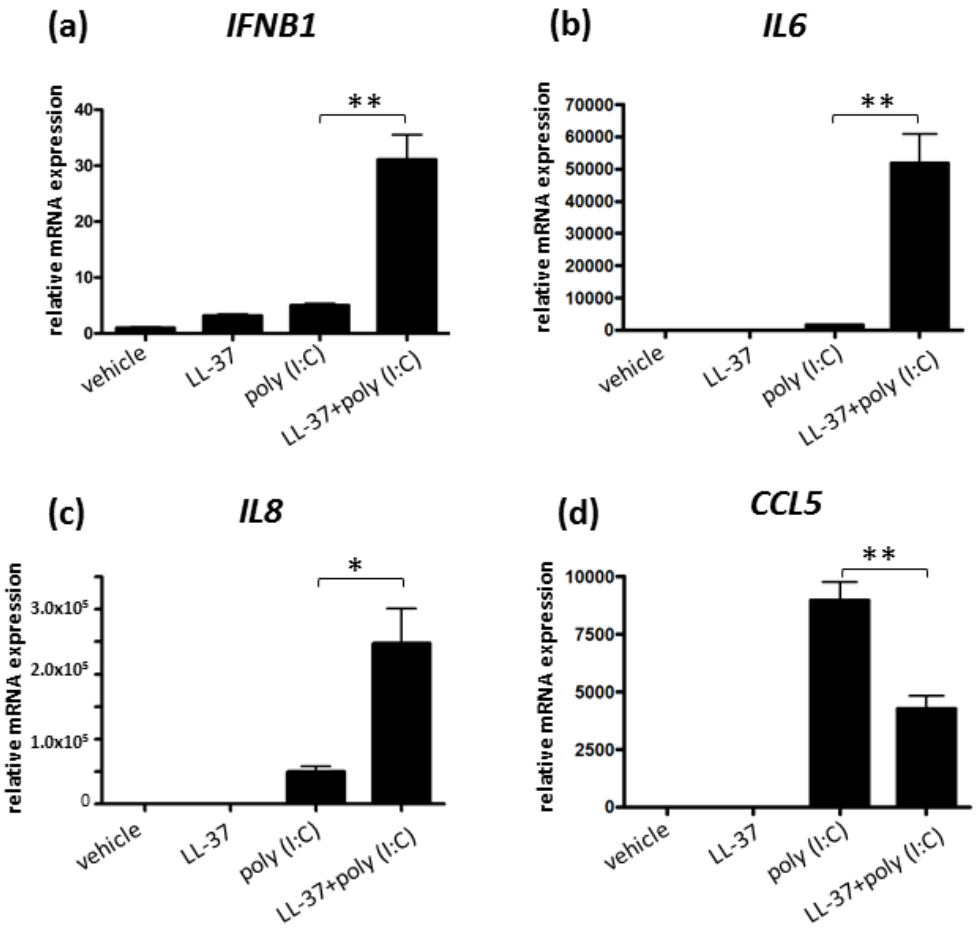
Fig. 3. NHEKs were treated with LL-37 (0.5 μM), poly (I:C) (0.1 μg/ml) or their combination for 24 hours and then infected by HSV-1 for 48 hours. Viral plaque

formation was visualized by crystal violet staining. ***, $p<0.001$. Data are the means ± SEM of triplicate samples and representative of three independent experiments.

Fig. 4. (**a,c,d**) NHEKs were treated with LL-37 (0.1-10 μM) for 24 hours. (**b**) NHEKs were stimulated with LL-37 (5 μM), poly (I:C) (1.0 μg/ml) or their combination for 24 hours. TLR3, DDX58 and IFIH1 mRNA expression was examined by quantitative real-time PCR. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Data are the means ± SEM of triplicate samples and representative of three independent experiments.

Fig. 5. NHEKs were treated with poly (I:C) conjugated with FITC (2 μM) in the presence or absence of LL-37 (10 μM) for 6 hours, and the uptake of poly (I:C) was examined by immunofluorescence. Nuclei were visualized with 4'-6-diamidino-2-phenylidole (DAPI) (scale bar =20 μm).

Fig. 6. (a-d) TLR3 and LL37 expression was examined by immunohistochemistry in lesional skin from herpes simplex (**a, c**) or normal skin (**b, d**). Data shown are from a single sample representative of three (scale bar =200 μm).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IFNB1
relative mRNA expression
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30
20
10
0
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vehicle
LL-37
poly (I:C)
LL-37+poly (I:C)
(b)
IL6
relative mRNA expression
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60000
50000
40000
30000
20000
10000
0
**
vehicle
LL-37
poly (I:C)
LL-37+poly (I:C)
(c)
IL8
relative mRNA expression
3.0x10⁵
2.0x10⁵
1.0x10⁵
0
*
vehicle
LL-37
poly (I:C)
LL-37+poly (I:C)
(d)
CCL5
relative mRNA expression
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2500
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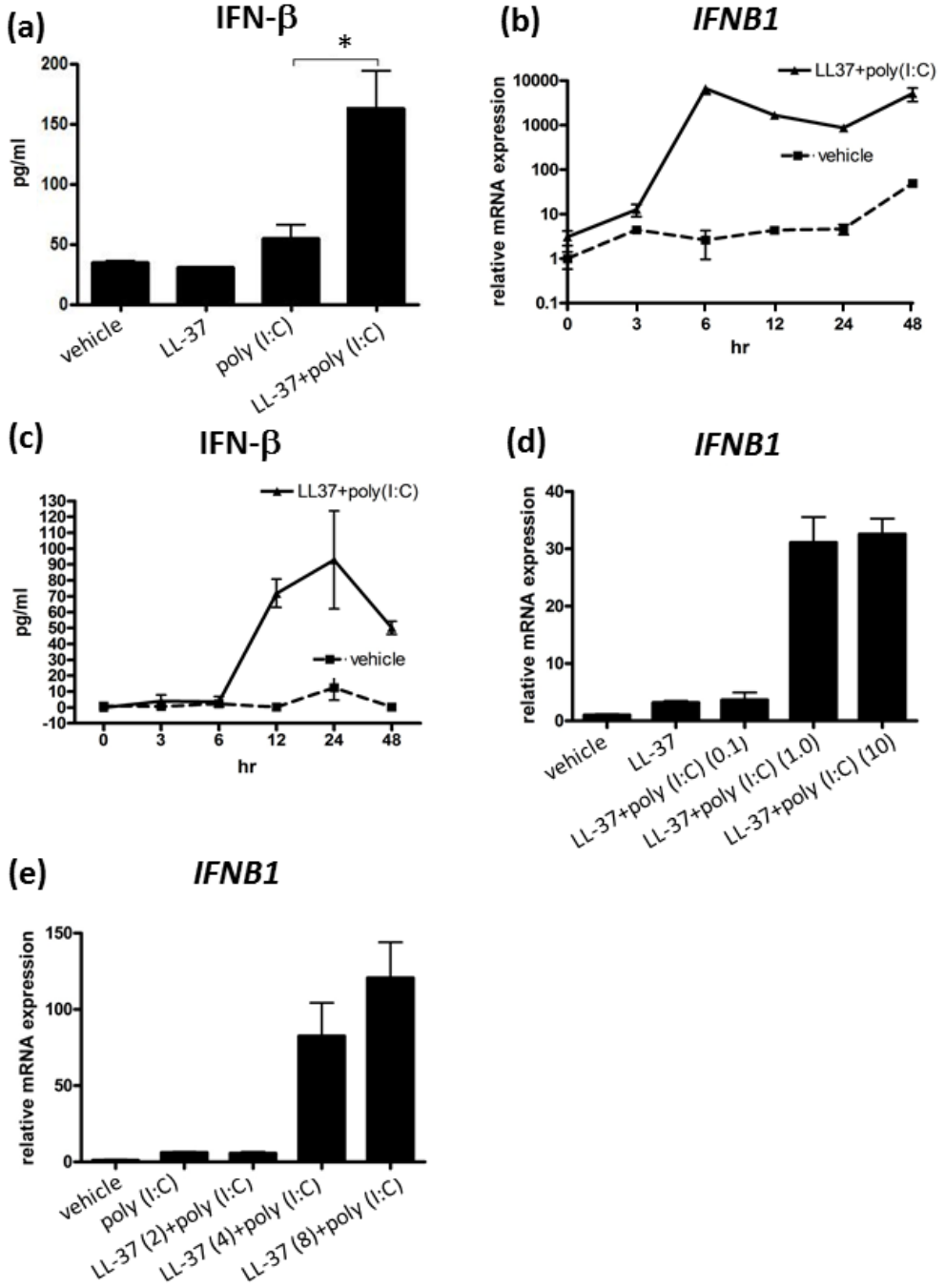
vehicle
LL-37
poly (I:C)
LL-37+poly (I:C)

Figure 1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IFN-β
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poly (I:C)
LL-37+poly (I:C)
(b)
IFNB1
LL37+poly(I:C)
vehicle
relative mRNA expression
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100
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12
24
48
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(c)
IFN-β
LL37+poly(I:C)
vehicle
pg/ml
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-10
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IFNB1
relative mRNA expression
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vehicle
LL-37
LL-37+poly (I:C) (0.1)
LL-37+poly (I:C) (1.0)
LL-37+poly (I:C) (10)
(e)
IFNB1
relative mRNA expression
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50
0
vehicle
poly (I:C)
LL-37 (2)+poly (I:C)
LL-37 (4)+poly (I:C)
LL-37 (8)+poly (I:C)

Figure 2

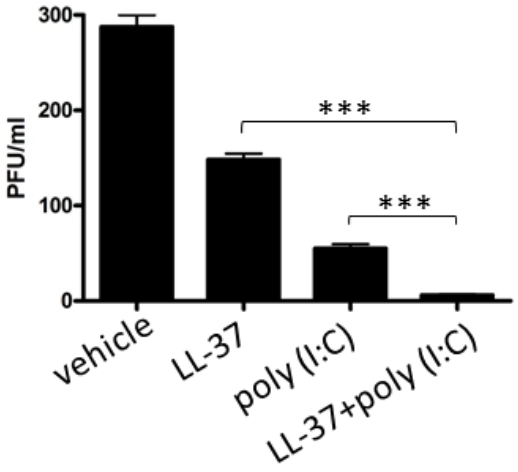

Figure 3

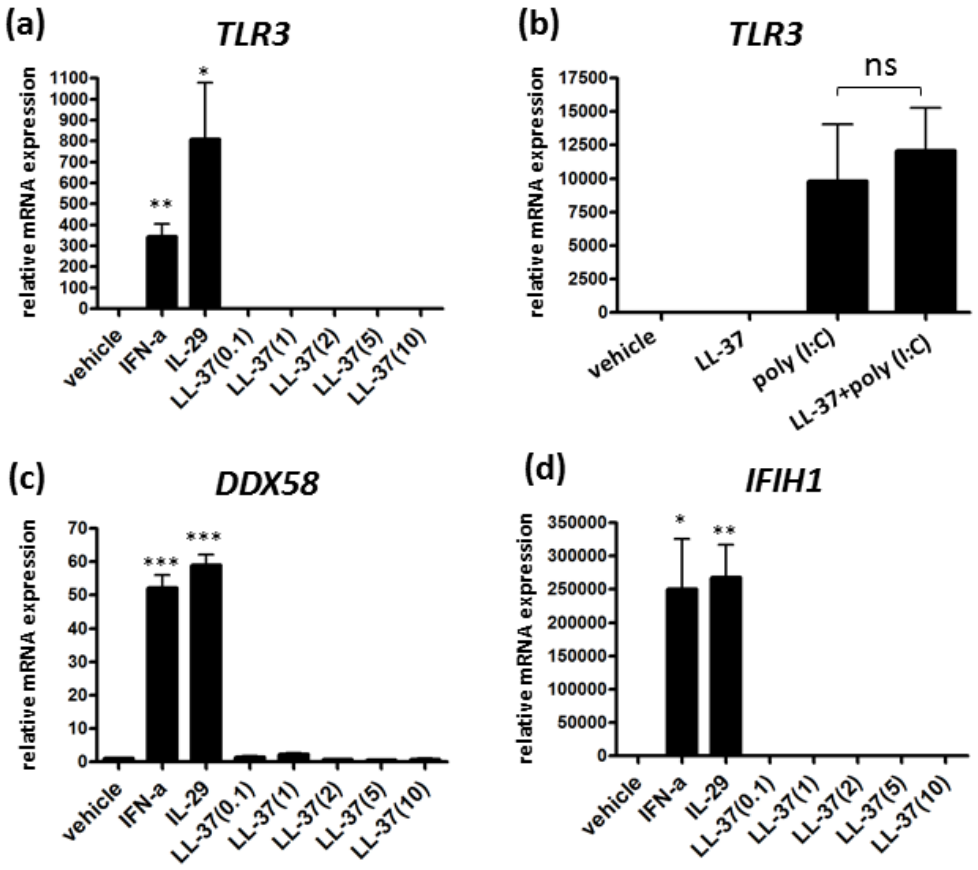
(a)
TLR3
relative mRNA expression
1100
1000
900
800
700
600
500
400
300
200
100
0
**
*
vehicle
IFN-a
IL-29
LL-37(0.1)
LL-37(1)
LL-37(2)
LL-37(5)
LL-37(10)
(b)
TLR3
ns
relative mRNA expression
17500
15000
12500
10000
7500
5000
2500
0
vehicle
LL-37
poly (I:C)
LL-37+poly (I:C)
(c)
DDX58
relative mRNA expression
70
60
50
40
30
20
10
0
***
***
vehicle
IFN-a
IL-29
LL-37(0.1)
LL-37(1)
LL-37(2)
LL-37(5)
LL-37(10)
(d)
IFIH1
relative mRNA expression
350000
300000
250000
200000
150000
100000
50000
0
*
**
vehicle
IFN-a
IL-29
LL-37(0.1)
LL-37(1)
LL-37(2)
LL-37(5)
LL-37(10)

Figure 4

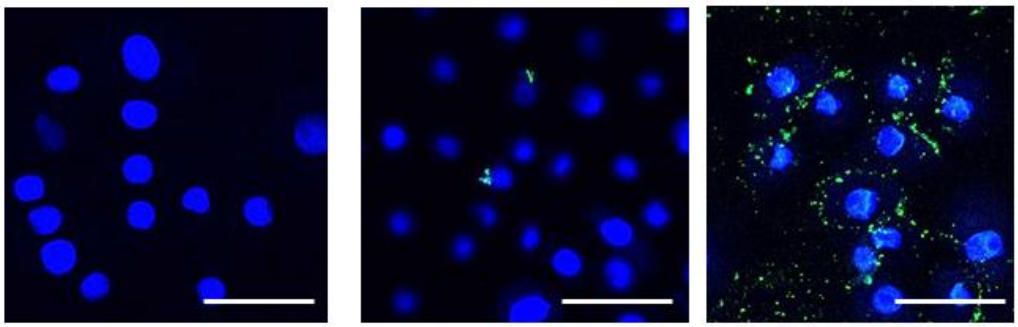

Figure 5

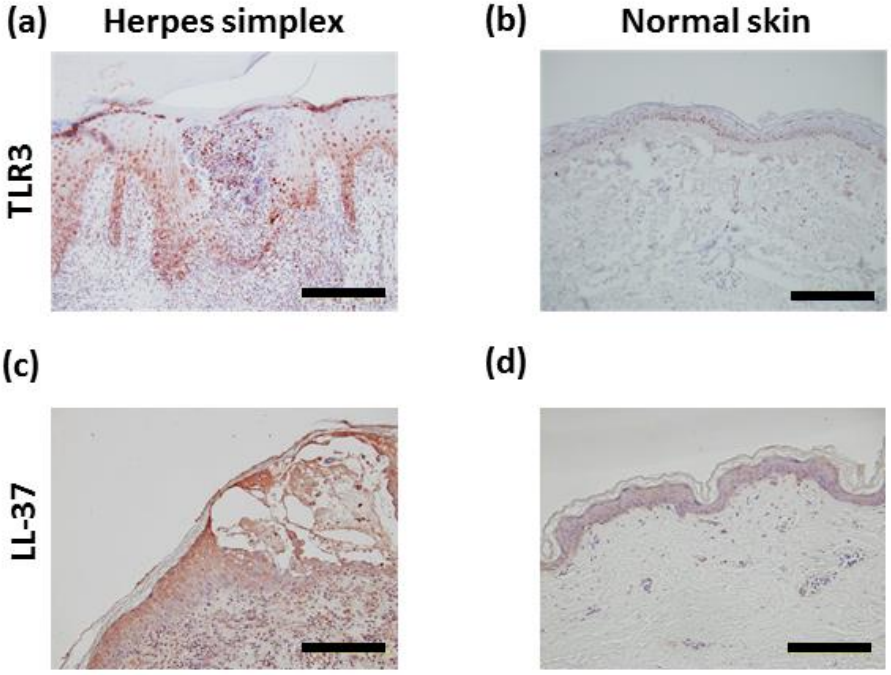

Figure 6