Regulation of inflammasome signaling pathway by antimicrobial peptide LL-37

Sung-Hyun Yoon

Department of Medical Science

The Graduate School, Yonsei University

Regulation of inflammasome signaling pathway by antimicrobial peptide LL-37

Sung-Hyun Yoon

Department of Medical Science

The Graduate School, Yonsei University

Regulation of inflammasome signaling pathway by antimicrobial peptide LL-37

Directed by Professor Je-Wook Yu

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Sung-Hyun Yoon

December 2019

This certifies that the Master's Thesis
of Sung-Hyun Yoon is approved.

Thesis supervisor: Je-Wook Yu

Thesis Committee Member#1: Ho-Keun Kwon

Thesis Committee Member#2: Tae-Gyun Kim

The Graduate School
Yonsei University

December 2019

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Regulation of inflammasome signaling pathway by antimicrobial peptide LL-37

Sung-Hyun Yoon

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Je-Wook Yu)

Antimicrobial peptides (AMPs) are compounds serving as natural antibiotics. LL-37, the member of cathelicidin antimicrobial peptides, is known to be mainly produced by epithelial cells and crucial role in innate immune response from the first line of host defense after bacterial infection. LL-37 increases or decreases pro-inflammatory cytokines including IFN-β, IL-6 or IL-1β. However, the role of LL-37 in the immune system is not yet clearly understood. Recent studies have proposed that LL-37 is excessively produced in chronic skin diseases such as rosacea and psoriasis. But, the pathogenesis of rosacea has not been studied in detail yet. In this study, I examined the role of LL-37 in the innate immune responses. Here, I demonstrated that LL-37 induces NLRP3 inflammasome activation. I also had found that the mechanism of LL-37-mediated NLRP3

inflammasome activation in bone marrow-derived macrophages (BMDMs). In addition, inhibition of potassium efflux and reduction of intracellular calcium levels failed to trigger the activation of caspase-1 and IL-1β secretion from BMDMs. I had also shown that internalized LL-37 caused lysosomal leakage and cathepsin B-mediated NLRP3 inflammasome activation in BMDMs. Subsequently, I examined the potential effect of the NLRP3 inflammasome complex in rosacea mice model, which induced by LL-37 injection. Interestingly, I observed that the lesion and the release of IL-1β were markedly reduced in *Nlrp3*-deficient mice, but not in wild type mice. Taken together, I suggest that LL-37-induced NLRP3 inflammasome activation potentially contributes to the pathogenesis of rosacea.

Key words : LL-37, Rosacea, Inflammasome, Caspase-1, IL-1β

Regulation of inflammasome signaling pathway by antimicrobial peptide LL-37

Sung-Hyun Yoon

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Je-Wook Yu)

Ⅰ. INTRODUCTION

Innate immune system constitutes the first line of host defense to avoid pathogens. It is mainly happened by macrophages and dendritic cells (DCs) as well as non-professional cells such as epithelial cells and endothelial cells.[1] These cells have Pattern recognition receptors (PRRs), which recognize the pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMP). There are four types of PRR families, including the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs).[2,3] In response to infection and tissue injuries, PRRs up-regulate the transcription of genes associated with inflammatory response. PRRs-mediated inflammation mediators include cytokines such as type I interferons (IFNs), pro-interleukin 1-beta (pro-IL-1β), tumor necrosis factor (TNF) and interleukin-6 (IL-6).[4,5] Whereas TNF and IL-6 are released as it produced, the expulsion of IL-1β requires another PRRs activation steps, called as “inflammasome”.[6,7]

Inflammasome is a cytosolic multi-protein complex. In response to various stimuli, it is assembled, promotes the cleavage of caspase-1, subsequently processes IL-1β and IL-18, responsible for the initiation of inflammation.[8,9] Five sensor proteins have been identified as inflammasome components, including NLR family pyrin domain-containing 1, 3 (NLRP1, NLRP3), NLR family CARD domain-containing protein 4 (NLRC4), the proteins absent in melanoma 2 (AIM2) and pyrin.[10] Among them, NLRP3 inflammasome responds to diverse stimuli, including crystalline, particulate matter (such as MSU, silica, asbestos and alum), extracellular ATP and nigericin.[11-13] Although the underlying mechanism of NLRP3 activation is still poorly understood, two-step activation mechanism by priming signal (signal 1) and activation signal (signal 2) is generally accepted. Priming signal provided by inflammatory stimuli such as TLR4 agonists induces the IL-1β and NLRP3 expression and then Second signal triggered by DAMPs and PAMPs, promotes NLRP3 inflammasome assembly and caspase-1 mediated-IL-1β and IL-18 and pyroptosis.[14,15]

Antimicrobial peptides (AMPs) are the part of innate immune response to protect the host against invading microbes.[16] The cathelicidin family of AMPs play an essential role in the immune defense especially in mammals. It is abundantly produced in damaged tissues. They participate in the immune system by directly killing microbes or increasing host defense mechanisms.[17-19] Although several cathelicidin peptides exist, LL-37 is the only member of the human cathelicidin family. LL-37 is produced by cleavage of hCAP18 that is a cationic antimicrobial 18-kDa polypeptide.[20,21]

Previous reports showed that LL-37 mediates Type I interferon production by forming self-RNA or DNA complexes.[22-25] Production of type I IFNs is generally promoted by the recognition of TLR3, TLR7 and TLR9 in endosomes as well as DAI and RIG-I in cytosol.[26] This mechanism is caused by DNA and RNA of the invading microbe, but not by self-DNA and RNA.[24] However, the self-DNA and RNA from the damaged cells are complexed with LL-37 to be recognized by TLR3, TLR7, TLR9 or RIG-I for the production of type I IFNs. In additions, LL-37 can induce process and release of IL-1β in

monocytes and bone marrow-derived macrophages (BMDMs). However, the specific mechanisms how LL-37 induces IL-1β release remain unclear.

On the other hand, LL-37 has also an anti-inflammatory effects.[27] LL-37 can form a complex with lipopolysaccharide (LPS) to prevent LPS from interacting with TLR4.[20,28,29] The LL-37/LPS complex reduces IL-6 production as a result of TLR4 signaling. Moreover, LL-37 can interact with DNA to form LL-37/DNA complex which do not trigger the activation of caspase-1.[30] Therefore, Effects of LL-37 on the inflammation still remains controversial.

Rosacea, atopic dermatitis and psoriasis are characterized by chronic inflammatory skin diseases.[31-33] Although the exact pathogenesis of these diseases is not completely understood, innate immune response plays an important role in these disease. Since some studies shown abnormal expression or function of LL-37 inflamed lesion of skin disease, LL-37 have been proposed as an important molecule in the progression of inflammatory skin diseases including rosacea. Rosacea is characterized by erythema, pustules and telangiectasia and affects an estimated 14 million Americans (1 in 20 people).[34] Although the cause of rosacea is unknown, It is mainly characterized by an excessive increase in active LL-37 as well as abnormalities in vascularity.[21,32,35] However, the undoubting mechanisms of LL-37 on the pathogenesis of rosacea still remains unclear.

LL-37 is one of the molecule to protect host against external microbes. The original role is beneficial to the host, but LL-37 can cause inflammation in certain environments. A recent study has revealed that LL-37 is associated with PRRs signaling. As mentioned above, LL-37 mediates both pro-inflammatory and anti-inflammatory effects.[27] In this report, I tried to identify the relation between LL-37 and PRRs signaling. Of particular interest, LL-37/LPS complex decreases IL-6 production. However, surprisingly, LL-37 and LPS increase IL-1β production. Here, I examined that LL-37 is related to inflammasome and also provided that LL-37 plays a potential role in rosacea disease.

Ⅱ. MATERIAL & METHODS

1. Mice

C57BL/6, Nlrp3$^{-/-}$ were from the Jackson Laboratory. All mice strains were administered in Yonsei University College of medicine. Entire mice were maintained under specific pathogen-free conditions. All experiments were in compliance with the Guide for Care and Use of Laboratory Animals and approved by the Institutional Ethical Committee, Yonsei University College of Medicine.

2. Reagents and antibodies

LL-37 was obtained from Invivogen (San Diego, CA, USA). Lipopolysaccharide (LPS), ATP, nigericin, glibenclamide, BAPTA-AM and poly (dA;dT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CA-074 was purchased from ENZO Life Sciences (Farmingdale, NY, USA). Acridine orange was obtained from Thermo Fisher scientific (Waltham, MA, USA).

Mouse monoclonal anti-caspase-1 (p20) antibody and mouse monoclonal anti-NLRP3/NALP3 antibody were purchased from Adipogen (San Diego, CA, USA). Goat polyclonal anti-IL-1β/IL-1F2 antibody was purchased from R&D systems (Minneapolis, MN, USA). Rabbit monoclonal anti-ASC antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal anti-β-actin antibody and rabbit monoclonal anti-caspase-1 (p10) antibody was purchased from Santa Cruz (Santa Cruz, CA, USA).

3. Cell cultures

Primary BMDMs were isolated from femurs and tibias of C57BL/6 mice or Nlrp3 $^{-/-}$ mice. Harvested bone marrow progenitor cells were maintained in 8% L929 conditional Dulbecco's modified Eagle's medium (DMEM) (Corning, NY, USA) media, supplemented with 10% fetal bovine serum (FBS) (Corning), 100 U/mL penicillin and streptomycin (Gibco, Gran Island, NY, USA). After 4 days, culture medium was replaced with 5% L929-conditional fresh complete DMEM and then adherent cells were plated in appropriated plates. All cells were incubated in a 37℃ incubator filled with 5% CO_2.

4. Mice treatment and measurement of skin thickness

The back of adult mice (6-10 weeks) were shaved using a razor 24 hrs before treatment and removed completely with hair removal cream. The thickness of the back tissue was measured before LL-37 injection. LL-37 were intradermally injected on the back with 40 μl of peptide (50, 300 μM) twice a day. 48 hrs after the initial injection, skin was measured thickness and then biopsied for western blotting and hematoxylin-eosin (H&E) staining.

5. Immunoblot analysis

Cells were lysed by cross-linking buffer containing 20 mM HEPES (pH 7.5), 0.5 % NP-40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, phosphatase inhibitors and protease inhibitors. Cell culture supernatants were precipitated by methanol-chloroform extraction method. Soluble cell lysates and culture supernatants were fractionated by SDS-PAGE gels and then transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA).

6. Quantification of mRNA

To measure mRNA production, total RNA was extracted using TRIzol reagent (Invitrogen) and reverse transcribed using PrimeScript^{TM}RT Master Mix (Takara clontech, Mountain view, CA, USA) according to the manufacturer's method. Quantitative real-time PCR was performed using SYBR Premix Ex Taq (Takara) and detected by a StepOnePlus real-time PCR system (Applied Biosystems, Foster city, CA, USA).

7. Measurement of cytokine production

Secreted cytokines were measured in cell-free supernatants using ELISA. Mouse IL-1β and IL-6 enzyme-linked immunoassay (ELISA) kits were obtained from R&D (Minneapolis, MN, USA).

8. Immunofluorescence assay

Cells grown on coverslip in a 12-well plate or 24-well plate were fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. After blocking with 4% BSA, cells were incubated with primary antibodies targeting LL-37 (Santa Cruz), phalloidin (Thermo Fisher), LAMP-1 (Santa Cruz) followed by the Cy3- or Alexa Fluor 488-conjugated anti-mouse or anti-rabbit IgG (Jackson Immuno Research or Invitrogen). Cells were then observed by confocal microscopy (Zeiss, LSM700).

9. Measurement of ions by ICP-OES

To measure ions inside cells, treated cells were washed by triple distilled water (T.D.W) and dissolved with 5% Nitric acid. Dissolved cells were analyzed by using Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) (OPTIMA 8300,

perkin elmer, MA, USA).

10. Measurement of lysosomal rupture by flow cytometry

For flow cytometry-based monitoring of lysosomal rupture, treated cells were washed in fluorescence-activated cell sorting (FACS) buffer (PBS with 10 % FBS) and stained with 0.3 μg/ml acridine orange reagent (Thermo Fisher scientific, Waltham, MA, USA) for 10 mins, 37℃. Next, stained cells were collected by centrifugation (500 g, 3 mins) and resuspended with FACS buffer for analysis using flow cytometry (FACS Verse, Beckman coulter, CA, USA).

11. Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM) of individual samples. Groups were compared using t-test or one-way ANOVA with a bonferroni post-test. P-value of < 0.05 demonstrates statistically significant. Analyses were performed using GraphPad Prism program.

Table 1. Primer sequence for PCR

Primer sequence	
Target	Sequence
Mouse IL-6	Forward: 5'- AGTTGCCTTCTTGGGACTGA -3'
	Reverse: 5'- TCCACGATTTCCCAGGAGAC -3'
Mouse IL-1β	Forward: 5'- GCCCATCCTCTGTGACTCAT -3'
	Reverse: 5'- AGGCCACAGGTATTTTGTCG -3'
Mouse IFN-β	Forward: 5'-TTCCTGCTGTGCTTCTTCAC -3'
	Reverse: 5'- CTTTCCATTCAGCTGCTCCA -3'
Mouse Rn18s	Forward: 5'- CGCGGTTCTATTTTGTTGGT -3'
	Reverse: 5'- AGTCGGCATCGTTTATGGTC -3'
Mouse VEGF-A	Forward: 5'- CA GGCTGCTGTAACGATGAA -3'
	Reverse: 5'- TTTCTTGCGCTTTCGTTTT -3'

Ⅲ. RESULT

1. LL-37 enhances dsRNA-triggered IFN-β production in macrophages

TLR3, one of the pattern recognition receptors (PRRs) is located in the endolysosome, recognizes dsRNA to produce type Ⅰ IFNs through TRIF, TBK1 signaling.[36] Previous studies suggested that LL-37 promotes IFN-β production by dsRNA in keratinocytes or plasmacytoid dendritic cell (pDC).[23,25] However, the production of interleukin-6 (IL-6) and interleukin-8 (IL-8) upon TLR3 is reduced by LL-37.[25] To investigate the production of IFN-β and other pro-inflammatory cytokines in BMDMs, cells other than keratinocytes or pDC, BMDMs were pre-treated with LL-37 for 1 hr, and then poly(I:C), similar to dsRNA treatment. The IFN-β mRNA was induced by poly(I:C) treatment, but higher amounts were produced when pre-treatment with LL-37 (Fig. 1A). In contrast, the IL-1β and IL-6 mRNA were decreased in LL-37 pre-treated BMDMs (Fig. 1B, C). Similar with previous reports, this results demonstrate that the complex of LL-37 and dsRNA helps to produce IFN-β.

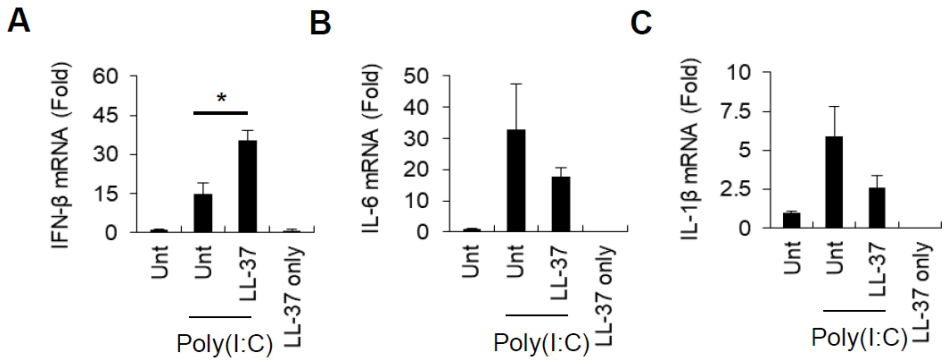

Figure 1. Pre-treatment with LL-37 increases IFN-β production by dsRNA in macrophages. BMDMs were pre-treated with LL-37 (20 μg/ml) for 1 hr, then followed by poly(I:C) (20 μg/ml) treatment for 6 hrs. The cells were used for the quantification of IFN-β (A), IL-6 (B) and IL-1β (C) mRNA levels by quantitative real-time PCR. The changes in the levels of each mRNA were normalized to the Rn18s and then the fold-changes were calculated in comparison to corresponding untreated cells (n = 2). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (*P<0.05).

2. LL-37 regulates LPS-triggered pro-inflammatory cytokine production in macrophages

In order to examine the association of the other TLR ligand with LL-37, BMDMs were treated with LPS, TLR4 agonists.[37,38] The activation of TLR4 by LPS produces the pro-inflammatory cytokines such as IL-6, TNFα and pro-IL-1β. Recent studies have proposed pro-inflammatory cytokines are decreased in LL-37 pre-treated dendritic cells.[28,29] To confirm the previous studies, the amount of secreted cytokines was confirmed by ELISA. BMDMs were treated with LL-37, followed by LPS treatment for 6 hrs. Contrary to the finding that LL-37 induces more IFN-β production, it is seen that LL-37 inhibits IL-6 (Fig. 2A). However, Release of IL-1β is increased by LL-37 (Fig. 2B). These results predict that LL-37 is associated with inflammasome.

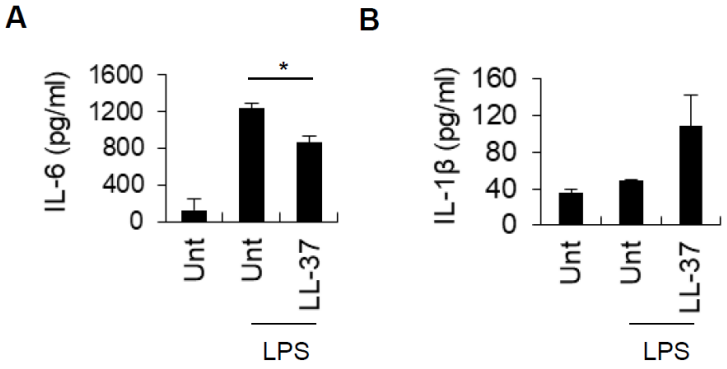

Figure 2. LL-37 regulates the production of pro-inflammatory cytokine produced by LPS. BMDMs were treated with LL-37 (20 μg/ml) and then LPS (0.25 μg/ml) treatment for 6 hrs. Cell culture supernatants were measured for the quantification of IL-6 (A) and IL-1β (B) by ELISA (n = 2). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (*P<0.05).

3. LL-37 induces the activation of NLRP3 inflammasome in LPS-primed macrophages

Since previous data showed an increase in IL-1β by LL-37 (Fig. 2B), I tested the association with inflammasome that mainly releases IL-1β. The cells were stimulated with LPS, LL-37 and ATP in various ways, inflammasome is activated when LL-37 is treated after LPS priming (Fig. 3). In the culture supernatants of LPS and LL-37 treated BMDMs, the secretion of IL-1β was confirmed by western blot.

The previous data confirmed the activation of inflammasome by LL-37 (Fig. 3). The following experiment was conducted to determine which inflammasome was dependent. BMDMs obtained from NLRP3 knock-out mice were used in the previous method (Fig. 4A). NLRP3 deficient BMDMs did not show inflammasome activity by LL-37. I also confirmed that ASC deficient BMDMs did not secrete the cleavage caspase-1 (p20) and IL-1β in culture supernatant (Fig. 4B). These data was shown that LL-37 induces NLRP3 dependent inflammasome activation in LPS-primed BMDMs.

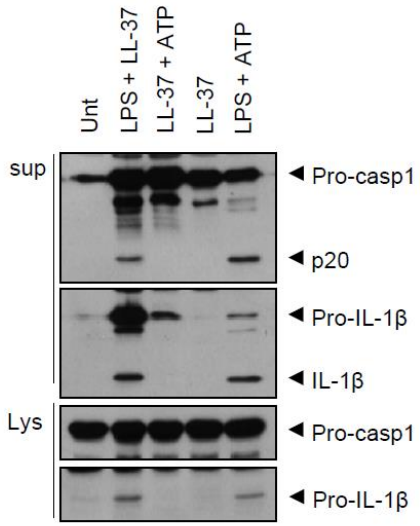

Figure 3. LL-37 mediates the secretion of IL-1β and the cleavage of caspase-1 (p20) in LPS-primed macrophages. Immunoblots of BMDMs with LL-37 (15 μg/ml) treatment under various conditions. BMDMs were treated with LPS (0.25 μg/ml) for 3 hrs and then LL-37 for 3.5 hrs (line 2). After pre-treatment of LL-37 for 3.5 hrs in BMDMs, ATP (2.5 mM) was treated for 35 mins (line 3). BMDMs were treated with LL-37 for 3.5 hrs (line 4). LPS-primed BMDMs were treated with ATP for 35 mins. Cell culture supernatants (sup) or cellular lysates (Lys) were immunoblotted with the indicated antibodies. Unt: untreated, Pro-casp1: pro-caspase-1, p20: active caspase-1.

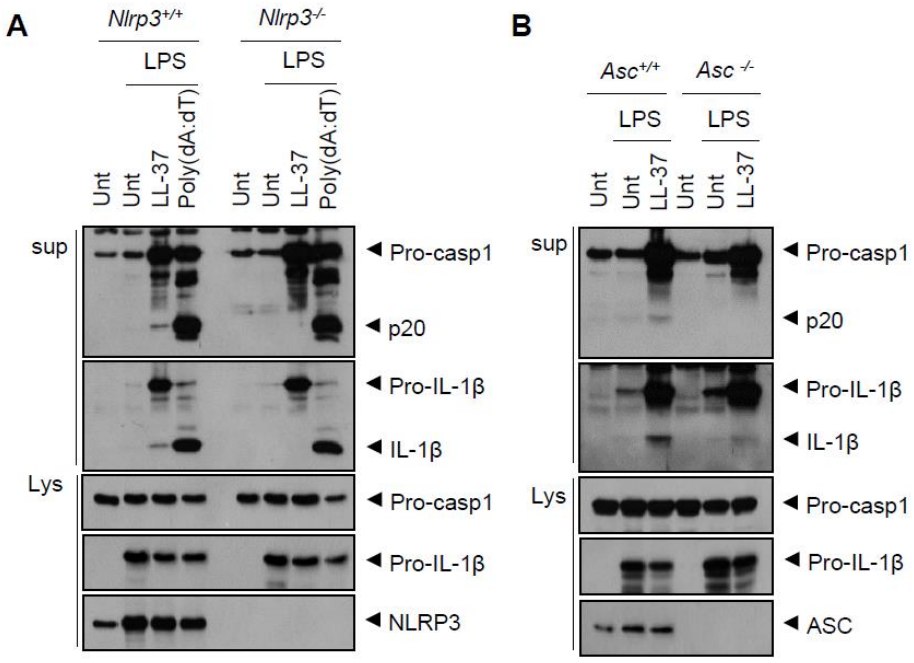

Figure 4. LL-37 treatment promotes NLRP3-dependent caspase-1 activation in the presence of LPS priming. BMDMs were treated with LPS (0.25 μg/ml) for 3 hrs, followed by LL-37 (15 μg/ml) for 3.5 hrs. (A) Cell culture supernatants (sup) or cellular lysates (Lys) from wild type BMDMs and NLRP3 knock-out BMDMs were immunoblotted with the indicated antibodies cultures. (B) Immunoblots from wild type BMDMs and ASC knock-out BMDMs cultures. The cells were treated in the same way as (A). Unt: untreated, Pro-casp1: pro-caspase-1, p20: active caspase-1.

4. LL-37 treatment triggers potassium efflux and calcium influx for NLRP3 inflammasome activation

Signal 2–induced efflux of intracellular potassium and influx of extracellular calcium is generally considered a pivotal common phenomenon for the activation of NLRP3 inflammasome.[39,40] To examine whether LL-37 treatment induced ions flux, the experiment was conducted using inhibitors such as KCl, glibenclamide and BAPTA-AM. KCl and glibenclamide both block potassium efflux and inhibit NLRP3 inflammasome activation. BAPTA-AM is a calcium chelator that lowers intracellular calcium levels. When three inhibitors were used in each experiment, all NLRP3 inflammasome acivation induced by LL-37 was inhibited in LPS-primed BMDMs (Fig. 5A-C). To determine whether the treatment of LL-37 is associated with ions transport, ion concentrations in cells were measured. LL-37 treatment caused a decrease in intracellular potassium and increase in intracellular calcium levels (Fig. 6). When LL-37 was treated, immunofluorescence (IF) was used to determine where it is located in the cell. The filamentous actin (F-actin) was stained with phalloidin and the location of LL-37 was confirmed. LL-37 began to adhere to the plasma membrane of the cells within 10 mins and confirmed entering into the cells within 2 hrs (Fig. 7). These results indicate that LL-37 treatment is mediated by ions movement for the activation of NLRP3 inflammasome.

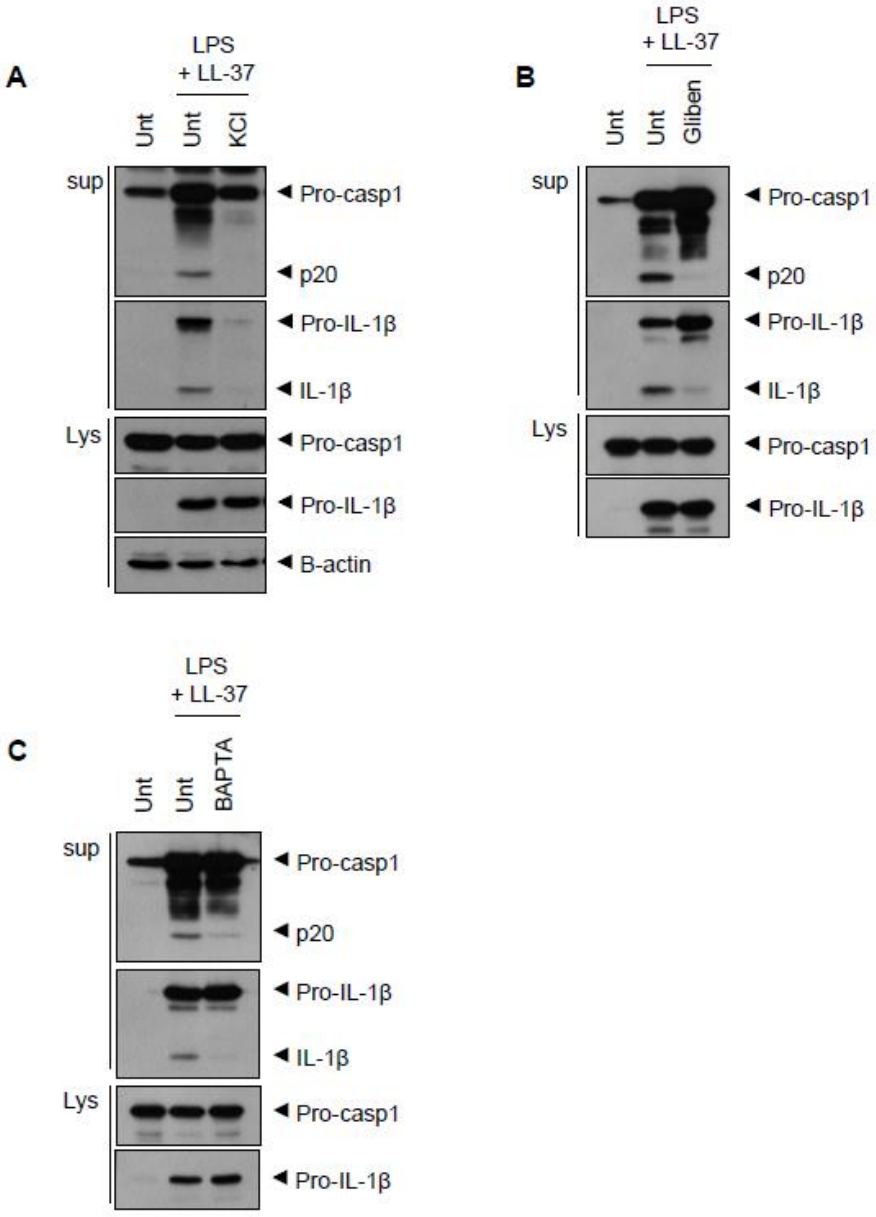

Figure 5. Inhibition of ions flux prevents inflammasome activation by LL-37. BMDMs were treated with LPS (0.25 μg/ml) for 3 hrs. Then the cells were treated with KCl (40 mM, 1 hr) (A), Glibenclamide (30 μM, 1 hr) (B) or BAPTA-AM (20 μM, 1 hr) (C), followed by treatment with LL-37 (15 μg/ml) for 3.5 hrs. Cell culture supernatants (sup) or cellular lysates (Lys) were immunoblotted with the indicated antibodies. Unt: untreated, Pro-casp1: pro-caspase-1, p20: active caspase-1.

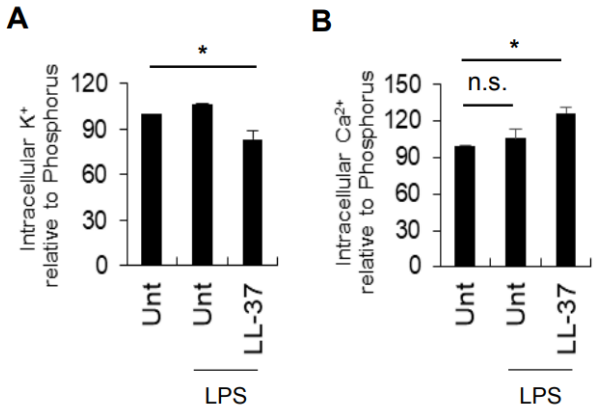

Figure 6. LL-37 induces the intracellular potassium efflux and extracellular calcium influx in LPS-primed macrophages. BMDMs were stimulated with LPS (0.25 μg/ml) for 3 hrs, followed by LL-37 (15 μg/ml) treatment for 1 hr. Intracellular potassium (A) and calcium (B) levels were measured by ICP-OES (n = 2). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (n.s.. not significant; *P<0.05).

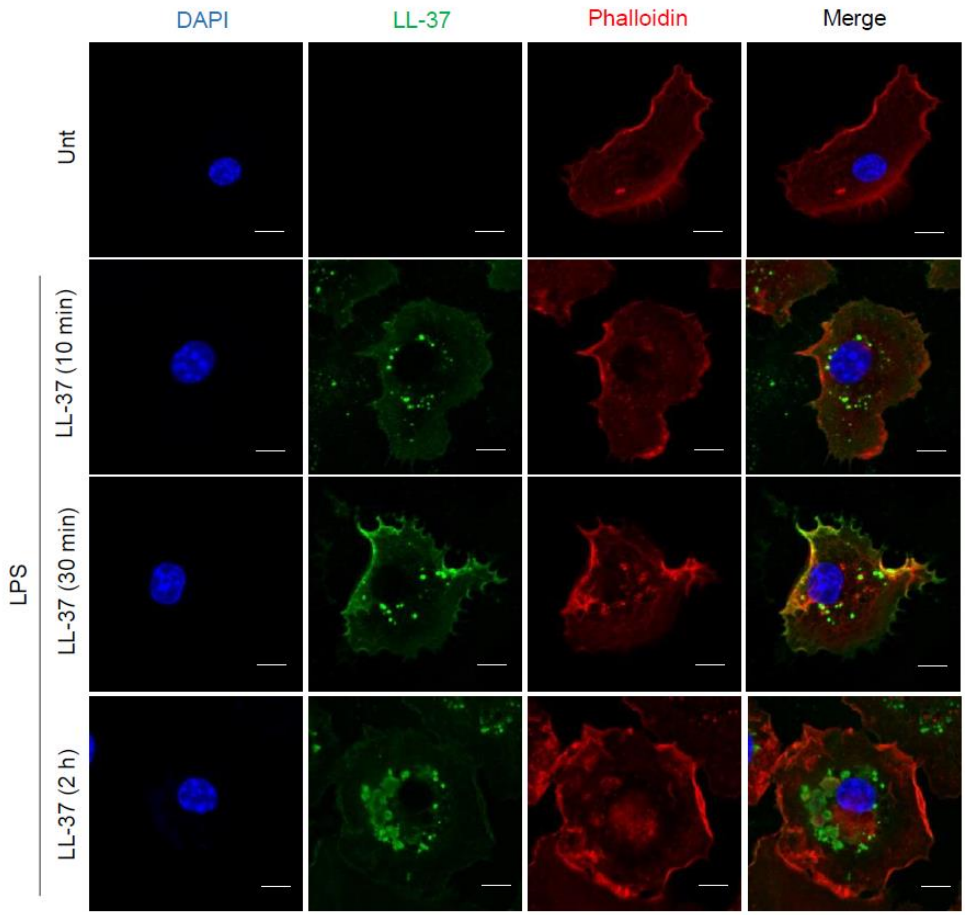

Figure 7. LL-37 enters into the intracellular cytosol in LPS-primed macrophages. BMDMs were stimulated with LPS (0.25 μg/ml) for 3 hrs, followed by LL-37 (15 μg/ml) treatment for 10, 30 mins, 2hrs after staining with phalloidin (red) and anti-LL-37 (green). DAPI represents the nuclear signal (blue). Representative immunofluorescence images of BMDMs were observed by confocal microscopy.

5. LL-37 promotes the activation of NLRP3 inflammasome via inducing lysosomal rupture

In the previous data (Fig. 7), I confirmed that LL-37 enters into the cells, so I examine the role of LL-37 in the cells in terms of inflammasome activation. Crystallin, such as MSU and Alum, is also known to activate inflammasome.[41] These types of stimuli are engulfed by phagocytosis and induce lysosome rupture in cells, resulting in cytosolic release of lysosomal contents.[42] One of these contents, cathepsin B, activates NLRP3 inflammasome signaling. To examine whether LL-37 is associated with lysosomal rupture, the cathepsin B inhibitor, CA-074, is used. CA-074 significantly reduced activation of inflammasome in LPS-primed BMDMs (Fig. 8). I used acridine orange to check if LL-37 releases cathepsin B through lysosomal rupture. Acridine orange detects pH changes in cells. Lysosomal rupture changes intracellular pH level. Flow cytometry data showed an increase in acridine orange staining after LL-37 treatment in LPS-primed BMDMs (Fig. 9). Next, I confirmed that LL-37 was co-located with the lysosome in the cells. The cells were stained with lysosomal-associated membrane protein 1 (LAMP1) antibody and confocal microscopy confirmed that LL-37 co-localized with lysosome (Fig. 10). Summarized, LL-37 enters the cells and encounters lysosomes, inducing lysosomal rupture, resulting in the release of cathepsin B, one of the lysosomal contents. As a result, the released cathepsin B activates LL-37-mediated NLRP3 inflammasome signaling.

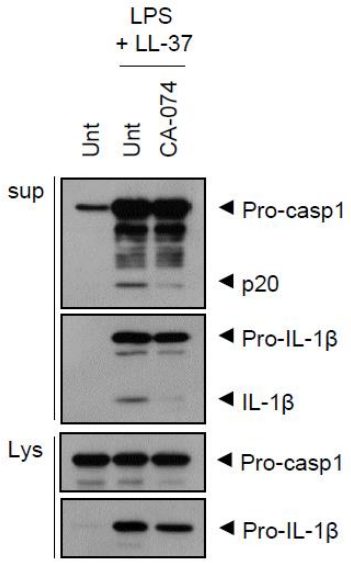

Figure 8. LL-37 treatment activates inflammasome via cathepsin B in macrophages. BMDMs were stimulated with LPS (0.25 μg/ml) for 3 hrs. After then the cells were treated with CA-074 (40 μM) for 1 hr, followed by LL-37 (15 μg/ml) treatment for 3.5 hrs. Cell culture supernatants (sup) or cellular lysates (Lys) were immunoblotted with the indicated antibodies. Unt: untreated, Pro-casp1: pro-caspase-1, p20: active caspase-1.

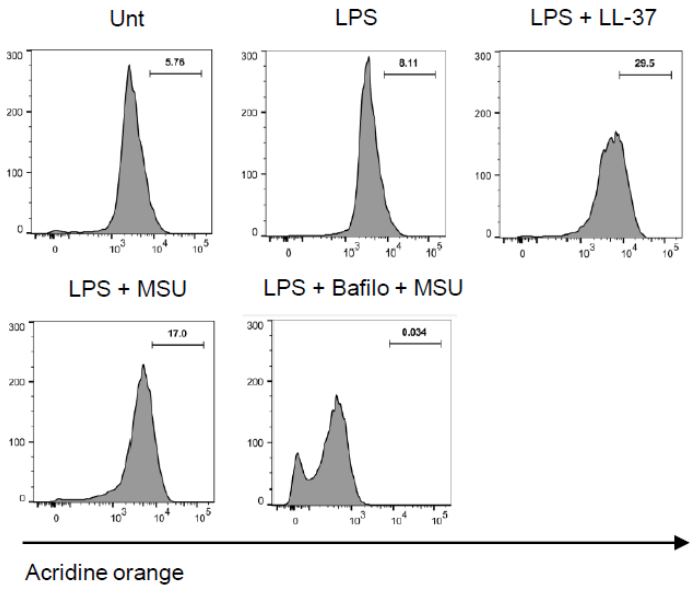

Figure 9. LL-37 induces lysosomal rupture in LPS-primed macrophages. The cells were treated with LPS (0.25 μg/ml) for 3 hrs, followed by LL-37 (15 μg/ml, 3.5 hrs) or MSU (150 μg/ml, 3.5 hrs) treatment in absence or presence of bafilomycin (100 nM, 1 hr). Flow cytometric analysis to measure pH changes by LL-37 treatment in BMDMs.

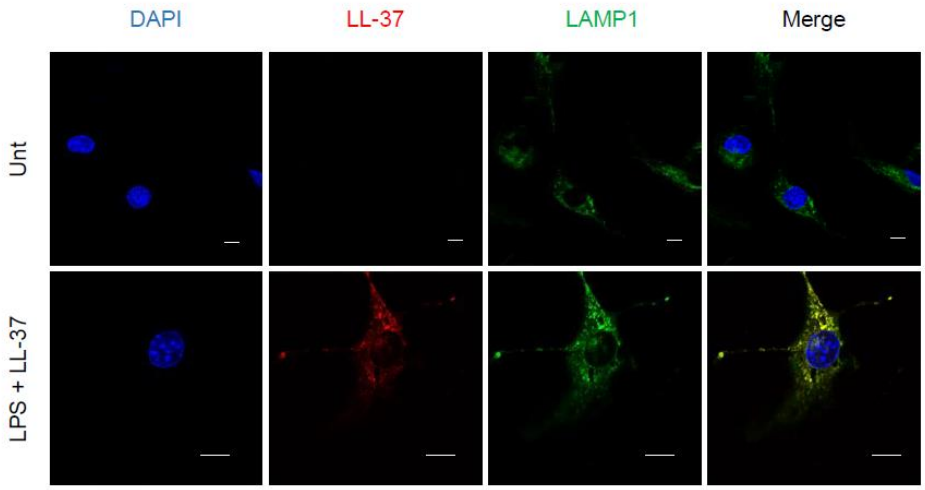

Figure 10. LL-37 co-localizes with the lysosome. BMDMs were treated with LPS (0.25 μg/ml, 3 hrs), followed by LL-37 (15 μg/ml, 4 hrs) after staining with anti-LAMP1 (green) and anti-LL-37 (red). DAPI represents the nuclear signal (blue). Representative immunofluorescence images of BMDMs were observed by confocal microscopy.

6. NLRP3 deficiency alleviates skin redness and inflamed blood vessels in rosacea mice model

LL-37 is a peptide known to be mainly produced by epithelial cells and has been reported to be associated with the pathogenesis of skin diseases.[43,44] Since the previous data confirmed that LL-37 is related to inflammasome activation (Fig. 4), a type of innate immune response, it is predicted that the inflammasome is related to skin disease. Among the many skin disorders, rosacea is not well-known for the cause of the disease and the treatment is not clear. In order to check the relationship between rosacea and inflammasome, I examined the rosacea mice model. To induce rosacea symptoms, I administered intradermal injection of LL-37 to wild type (*Nlrp3*$^{+/+}$) and NLRP3 knock-out (*Nlrp3*$^{-/-}$) mice and then examined the severity of disease via observation and skin thickness (Fig. 11A, B). As expected, administration of LL-37 induced skin redness and thickness like rosacea in wild type mice. In contrast, NLRP3 knock-out mice showed relatively fewer redness and thickness compared to wild type mice. Next, I observed changes in the amount of protein and RNA in skin tissues. Protein obtained from skin tissues was compared wild type and NLRP3 knock-out using western blot (Fig. 12A) and ELISA (Fig. 12B) and checked the messenger RNA (mRNA) expression levels of inflammatory cytokine, IL-1β (Fig. 12C). Notably, LL-37 induced skin lesion was significantly protected by the NLRP3 knock-out mice. Furthermore, a significant reduction of IL-1β mRNA was observed in the LL-37 treated NLRP3 knock-out mice, but not in wild type mice. Then, I checked the histopathological changes using hematoxylin-eosin (H&E) staining (Fig. 12D). H&E Staining showed that the skin lesion of NLRP3 knock-out mice is much thinner than wild type mice.

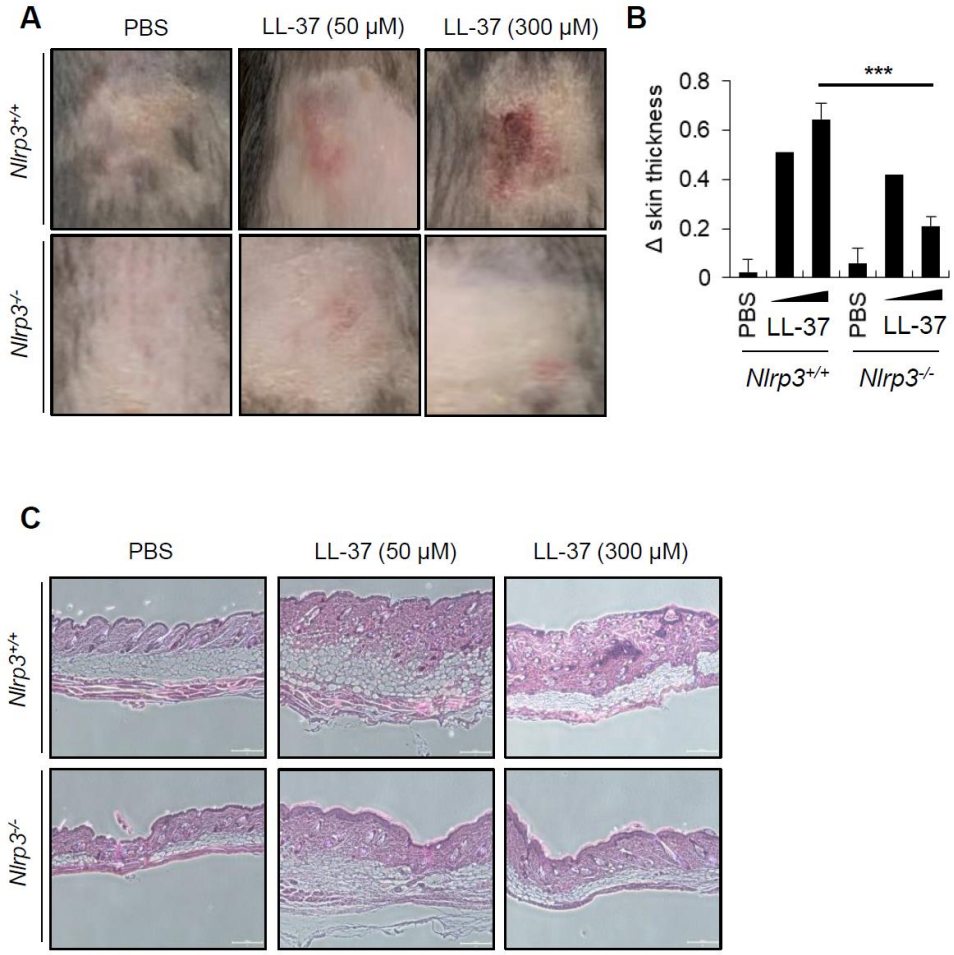

Figure 11. NLRP3 deficiency attenuates skin redness and inflammation in LL-37 injected mice. Wild type and NLRP3 knock-out mice were intradermally administered with PBS and LL-37 (50 or 300 μM, 40 μl) for 2 days and then observed skin surface images (A) and measured the skin thickness (B) (n = 5). (C) Skin tissue was isolated for hematoxylin-eosin (H&E) staining. Statistical significance was determined by one-way ANOVA with a bonferroni post-test (***P<0.001).

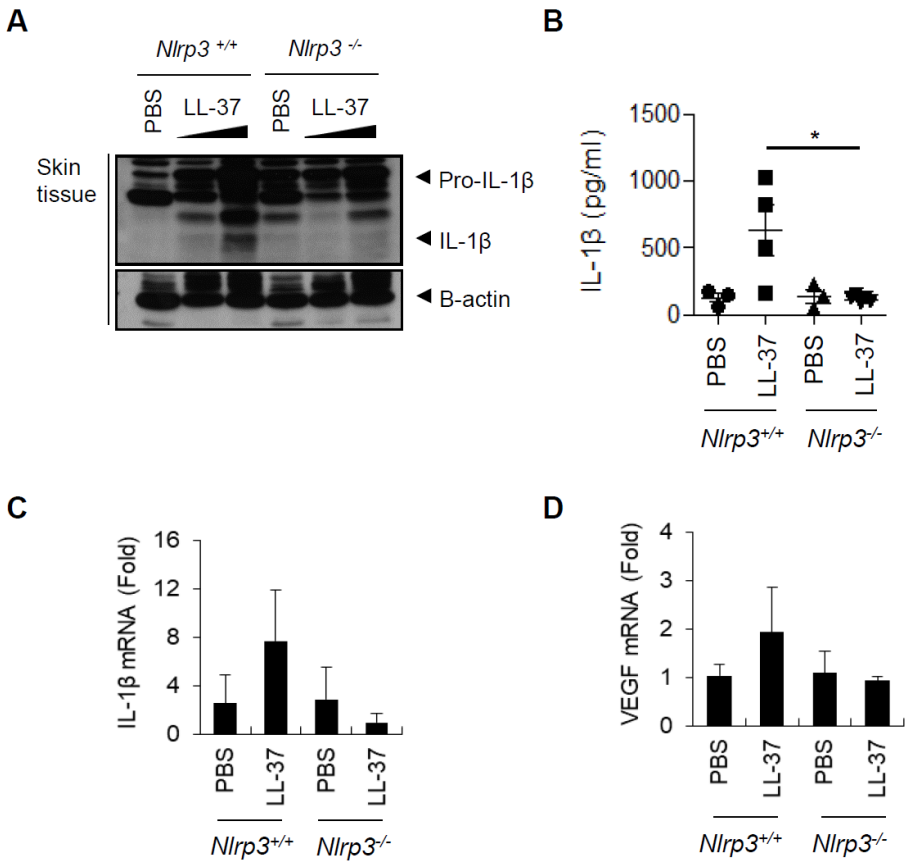

Figure 12. NLRP3 deficiency prevents the production of IL-1β and the abnormality of vascular in rosacea mice model. Mice were intradermally administered with PBS and LL-37 (50 or 300 μM, 40 μl) for 2 days and then skin tissue was isolated for western blot (A), ELISA (B), qPCR (C). (A) Immunoblot from skin tissue in wild type or NLRP3 knock-out mice. (B) ELISA of IL-1β protein levels in skin tissue treated with LL-37 (300 μM, 40 μl) (n = 4). (C, D) Quantification of IL-1β and VEGF mRNA levels in mice skin tissue treated with LL-37 (300 μM, 40 μl) as determined by qPCR (n = 3). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (*P<0.05).

Ⅳ. DISCUSSION

Recent studies suggested that LL-37 affects both pro-inflammatory response and anti-inflammatory response. Among them, LL-37 has been reported to be associated with the release of IL-1β, a major cytokine processed by inflammasome activation. However, the mechanisms underlying the contribution of LL-37 to inflammasome pathways remains unclear. In this study, I tried to demonstrate the mechanism of NLRP3 inflammasome activation by LL-37 and confirm that the activation of NLRP3 inflammasome affect the disease pathogenesis of rosacea.

It has been verified that LL-37 increases the production of IFN-β as previously reported. The anti-inflammatory effect of LL-37 was also confirmed by LL-37/LPS complex, which reduced the cytokine produced by binding LPS to TLR4. In this study, it was confirmed that IL-1β was produced in BMDMs upon LPS and LL-37 treatment. The activation of inflammasome requires priming by extracellular stimuli such as LPS or Pam3CSK4, which results in the transcriptional induction of pro-IL-1β and NLRP3. After the priming, inflammasome is activated given the variety of DAMP stimuli, such as ATP or MSU. The following step was confirmed to determine whether the secretion of IL-1β is caused by inflammasome activation. The main potential is that LL-37 is associated with the inflammasome known to be primarily IL-1β production. Although LL-37 has been reported to produce IL-1β by activating the NLRP3 inflammasome, the molecular mechanism remains unclear and controversial.

At first, the NLRP3 knock-out BMDMs were used to determine whether LL-37 is associated with the NLRP3 inflammasome. NLRP3 deficient BMDMs significantly reduced IL-1β production by LL-37 compared to wild type BMDMs. In additions, the same result was observed in ASC deficient BMDMs. Then, to check the molecular mechanism for LL-37-mediated IL-1β production, I identified

ions movement, known as a common trigger for NLRP3 inflammasome activation. Several inhibitors such as KCl, glibenclamide or BAPTA-AM were used to confirm the possibility and LL-37-mediated Inflammasome activation was inhibited by these inhibitors. This result suggests that the activation of inflammasome by LL-37 was associated with ion transport. Furthermore, confocal microscopy data confirmed that LL-37 was attached to the plasma membrane. These results indicate that LL-37 is involved in ions movement of cells. Based on the confocal microscopy data, I found LL-37 enters into the cells. A recent study reported that LL-37 was internalized into cell through $P2X_7$ receptor-mediated endocytosis. However, because details of the internalization process underlying how LL-37 moves inside the cells are not found in this study, further studies are required to determine whether endocytosis-mediated internalization of LL-37 triggers NLPR3 dependent inflammasome activation.

It has been reported that several skin diseases are associated with the overexpression of LL-37. Previous data have shown that LL-37 induces the production of IL-1β by activating inflammasome, suggesting the association between inflammasome and skin disease. In this study, I used rosacea mice model, which is a skin disease with excessive increase of LL-37. Recent studies showed that rosacea was induced by intradermal injection of LL-37 on the mice back and confirmed by H&E staining or vascular abnormalities factor such as VEGF or CD31. To determine whether NLRP3 inflammasome affect rosacea disease induction, rosacea was induced by intradermal injection of LL-37 in both wild type and NLRP3 knock-out mice. The severity of the rosacea phenotype was assessed, based on the skin surface images and thickness change. It was confirmed that rosacea was induced normally in wild type mice, but not in NLRP3 knock-out mice, indicating that NLRP3 has a critical role in the pathogenesis of rosacea. Currently, There is no the proper treatment available for rosacea, except for laser treatment, powerful oral

acne drugs such as isotretinoin or oral antibiotic such as doxycycline. This study indicates that the activation of NLRP3 inflammasome is a novel factor for rosacea pathogenesis. Therefore, it suggests that regulation of NLRP3 inflammasome is a potent therapeutic target for rosacea disease. Whether MCC950 or IL-1 receptor antagonist (IL-1Ra), anakinra, which can be used as a therapeutic agent for inflammasome, is effective for rosacea, this study is not found. Therefore, further investigation are required to determine possibility of MCC950 or anakinra as treatment for rosacea.

Collectively, this study reveals the mechanism for how LL-37 activates NLRP3 inflammasome and also indicates that NLRP3 inflammasome plays a crucial role in rosacea disease. Therefore, I suggest that NLRP3 inflammasome signaling might be proposed as a therapeutic target for rosacea.

Ⅴ. CONCLUSION

Here, I demonstrated that LL-37 activates NLRP3 dependent inflammasome. Moreover, NLRP3 inflammasome activation by LL-37 is mediated by potassium efflux, calcium influx and lysosomal rupture. I also showed that NLRP3 deficiency in mice had protective effects on LL-37 injected rosacea mouse model. I observed LL-37 injected NLRP3 deficiency mice attenuated skin redness and abnormality of vascular compared to LL-37 injected wild type mice. Collectively, all these data produce evidence that the activation of NLRP3 inflammasome has a crucial role in rosacea skin disease. Because the alleviation of rosacea disease was observed in NLRP3 knock-out mice, further studies is needed to determine whether MCC950 or IL-1 receptor antagonist (IL-1Ra), anakinra associated with NLRP3 inflammasome inhibitor treatment can attenuate rosacea. If further studies are conducted to confirm that MCC950 or anakinra treatment can be attenuated in rosacea, then it could be the therapeutic target.

REFERENCE

1. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev 2009;22:240-73, Table of Contents.
2. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 2010;140:805-20.
3. Thompson MR, Kaminski JJ, Kurt-Jones EA, Fitzgerald KA. Pattern recognition receptors and the innate immune response to viral infection. Viruses 2011;3:920-40.
4. Grandvaux N, tenOever BR, Servant MJ, Hiscott J. The interferon antiviral response: from viral invasion to evasion. Curr Opin Infect Dis 2002;15:259-67.
5. Swiecki M, Colonna M. Type I interferons: diversity of sources, production pathways and effects on immune responses. Curr Opin Virol 2011;1:463-75.
6. Guo H, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. Nat Med 2015;21:677-87.
7. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. Nat Rev Immunol 2013;13:397-411.
8. Kanneganti TD. Central roles of NLRs and inflammasomes in viral infection. Nat Rev Immunol 2010;10:688-98.
9. Sharma D, Kanneganti TD. The cell biology of inflammasomes: Mechanisms of inflammasome activation and regulation. J Cell Biol 2016;213:617-29.
10. Broz P, Dixit VM. Inflammasomes: mechanism of assembly, regulation and signalling. Nat Rev Immunol 2016;16:407-20.
11. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature 2006;440:237-41.
12. Gasse P, Riteau N, Charron S, Girre S, Fick L, Petrilli V, et al. Uric acid is a danger signal activating NALP3 inflammasome in lung injury inflammation and fibrosis. Am J Respir Crit Care Med 2009;179:903-13.

13. Griffith JW, Sun T, McIntosh MT, Bucala R. Pure Hemozoin is inflammatory in vivo and activates the NALP3 inflammasome via release of uric acid. J Immunol 2009;183:5208-20.
14. Hiscott J, Marois J, Garoufalis J, D'Addario M, Roulston A, Kwan I, et al. Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. Mol Cell Biol 1993;13:6231-40.
15. Schroder K, Tschopp J. The inflammasomes. Cell 2010;140:821-32.
16. Reinholz M, Ruzicka T, Schauber J. Cathelicidin LL-37: an antimicrobial peptide with a role in inflammatory skin disease. Ann Dermatol 2012;24:126-35.
17. Schauber J, Gallo RL. Expanding the roles of antimicrobial peptides in skin: alarming and arming keratinocytes. J Invest Dermatol 2007;127:510-2.
18. Murakami M, Ohtake T, Dorschner RA, Schittek B, Garbe C, Gallo RL. Cathelicidin anti-microbial peptide expression in sweat, an innate defense system for the skin. J Invest Dermatol 2002;119:1090-5.
19. Dorschner RA, Pestonjamasp VK, Tamakuwala S, Ohtake T, Rudisill J, Nizet V, et al. Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A Streptococcus. J Invest Dermatol 2001;117:91-7.
20. Larrick JW, Hirata M, Balint RF, Lee J, Zhong J, Wright SC. Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. Infect Immun 1995;63:1291-7.
21. Yamasaki K, Schauber J, Coda A, Lin H, Dorschner RA, Schechter NM, et al. Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. Faseb j 2006;20:2068-80.
22. Zhang X, Oglecka K, Sandgren S, Belting M, Esbjorner EK, Norden B, et al. Dual functions of the human antimicrobial peptide LL-37-target membrane

perturbation and host cell cargo delivery. Biochim Biophys Acta 2010;1798:2201-8.

23. Chamilos G, Gregorio J, Meller S, Lande R, Kontoyiannis DP, Modlin RL, et al. Cytosolic sensing of extracellular self-DNA transported into monocytes by the antimicrobial peptide LL37. Blood 2012;120:3699-707.
24. Ganguly D, Chamilos G, Lande R, Gregorio J, Meller S, Facchinetti V, et al. Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. J Exp Med 2009;206:1983-94.
25. Zhang LJ, Sen GL, Ward NL, Johnston A, Chun K, Chen Y, et al. Antimicrobial Peptide LL37 and MAVS Signaling Drive Interferon-beta Production by Epidermal Keratinocytes during Skin Injury. Immunity 2016;45:119-30.
26. Stetson DB, Medzhitov R. Type I interferons in host defense. Immunity 2006;25:373-81.
27. Kahlenberg JM, Kaplan MJ. Little peptide, big effects: the role of LL-37 in inflammation and autoimmune disease. J Immunol 2013;191:4895-901.
28. Rosenfeld Y, Papo N, Shai Y. Endotoxin (lipopolysaccharide) neutralization by innate immunity host-defense peptides. Peptide properties and plausible modes of action. J Biol Chem 2006;281:1636-43.
29. Di Nardo A, Braff MH, Taylor KR, Na C, Granstein RD, McInturff JE, et al. Cathelicidin antimicrobial peptides block dendritic cell TLR4 activation and allergic contact sensitization. J Immunol 2007;178:1829-34.
30. Dombrowski Y, Peric M, Koglin S, Kammerbauer C, Goss C, Anz D, et al. Cytosolic DNA triggers inflammasome activation in keratinocytes in psoriatic lesions. Sci Transl Med 2011;3:82ra38.
31. Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T, et al. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. N Engl J Med 2002;347:1151-60.

32. Yamasaki K, Di Nardo A, Bardan A, Murakami M, Ohtake T, Coda A, et al. Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea. Nat Med 2007;13:975-80.
33. Hollox EJ, Huffmeier U, Zeeuwen PL, Palla R, Lascorz J, Rodijk-Olthuis D, et al. Psoriasis is associated with increased beta-defensin genomic copy number. Nat Genet 2008;40:23-5.
34. Chosidow O, Cribier B. Epidemiology of rosacea: updated data. Ann Dermatol Venereol 2011;138 Suppl 3:S179-83.
35. Murakami M, Lopez-Garcia B, Braff M, Dorschner RA, Gallo RL. Postsecretory processing generates multiple cathelicidins for enhanced topical antimicrobial defense. J Immunol 2004;172:3070-7.
36. Hemmi H, Takeuchi O, Sato S, Yamamoto M, Kaisho T, Sanjo H, et al. The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. J Exp Med 2004;199:1641-50.
37. Chamorro S, Garcia-Vallejo JJ, Unger WW, Fernandes RJ, Bruijns SC, Laban S, et al. TLR triggering on tolerogenic dendritic cells results in TLR2 up-regulation and a reduced proinflammatory immune program. J Immunol 2009;183:2984-94.
38. Parker LC, Whyte MK, Vogel SN, Dower SK, Sabroe I. Toll-like receptor (TLR)2 and TLR4 agonists regulate CCR expression in human monocytic cells. J Immunol 2004;172:4977-86.
39. Gong T, Yang Y, Jin T, Jiang W, Zhou R. Orchestration of NLRP3 Inflammasome Activation by Ion Fluxes. Trends Immunol 2018;39:393-406.
40. Yang Y, Wang H, Kouadir M, Song H, Shi F. Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors. Cell Death Dis 2019;10:128.
41. Swanson KV, Deng M, Ting JP. The NLRP3 inflammasome: molecular activation and regulation to therapeutics. Nat Rev Immunol 2019;19:477-89.

42. Tschopp J, Schroder K. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? Nat Rev Immunol 2010;10:210-5.
43. Lee HM, Shin DM, Yuk JM, Shi G, Choi DK, Lee SH, et al. Autophagy negatively regulates keratinocyte inflammatory responses via scaffolding protein p62/SQSTM1. J Immunol 2011;186:1248-58.
44. Li N, Yamasaki K, Saito R, Fukushi-Takahashi S, Shimada-Omori R, Asano M, et al. Alarmin function of cathelicidin antimicrobial peptide LL37 through IL-36gamma induction in human epidermal keratinocytes. J Immunol 2014;193:5140-8.

ABSTRACT (in Korean)

항균펩티드 LL-37 의 인플라마좀 신호경로 조절

<지도교수 유제욱>

연세대학교 대학원 의과학과

윤성현

항균펩티드는 천연 항생제 역할을 할 수 있는 물질이다. LL-37 은 항균펩티드 중에서 카델리시딘의 종류 중 하나로써, 주로 표피세포에서 많이 생산되고, 박테리아 감염이 일어났을 때, 빠르게 일어나는 방어 기작으로 선천면역반응에 있어 중요한 역할을 한다. LL-37 은 IFN-β 의 생산은 증가시키고, IL-6 의 생산은 감소시킨다는 보고가 있다. 그러나 현재까지 면역반응에서의 LL-37 의 역할은 명확하게 밝혀진 것이 없다. 최근 연구에서 LL-37 이 주사나 건선과 같은 피부 질환에서 과도하게

증가되어 있다라는 것이 밝혀졌다. 그 중에서도 주사 피부질환의 병인기전은 아직까지 밝혀진 것이 없다. 본 연구에서는 선천 면역반응에서 LL-37 의 역할을 확인해보고자 하였다. 마우스 골수 대식세포에서 LL-37 에 의해 NLRP3 인플라마좀의 활성이 유도된다는 것을 확인하였고, 또한 NLRP3 인플라마좀의 활성이 유도되는 과정을 밝혔다. LL-37 에 의해 나타나는 NLRP3 인플라마좀 활성 측면에서 칼륨의 유출과 칼슘의 유입의 억제가 인플라마좀 활성을 저해하는 것을 확인하였고, LL-37 이 세포 내부로 들어가 리소좀을 파괴시켜 cathepsin B 가 유출되어 NLRP3 인플라마좀이 활성화 된다는 것을 확인하였다. 그 이후에 LL-37 을 주입하여 주사 질병을 유도하였을 때, NLRP3 인플라마좀의 활성이 잠재적인 역할을 할 것이라고 확인하였다. 흥미롭게 NLRP3 결핍 마우스에서 병변 완화와 IL-1β 의 유출의 감소를 확인하였다. 위의 결과들을 토대로 본 연구에서는 LL-37 에 의한 NLRP3 인플라마좀의 활성이 주사 질병에 중요한 역할을 하고 있다는 것을 제시한다.

핵심되는 말: LL-37, 주사 질병, 인플라마좀, 대식세포, NLRP3

PUBLICATION LIST

1. Hong S, Hwang I, Gim E, Yang J, Park S, **Yoon SH**, Lee WW, Yu JW. Brefeldin A-sensitive ER-Golgi vesicle trafficking contributes to NLRP3-dependent caspase-1 activation. FASEB J, doi: 10.1096/fj.201801585R