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Effect of the Antimicrobial Peptide LL-37 on Gene Expression of Chemokines and 29 Toll-like Receptor-Associated Proteins in Human Gingival Fibroblasts Under Stimulation with *Porphyromonas gingivalis* Lipopolysaccharide

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Abstract

The antimicrobial peptide LL-37 neutralizes the biological activity of lipopolysaccharide (LPS), while it upregulates the expression of several immune-related genes. We investigated the effect of LL-37 on gene regulation of human gingival fibroblasts (HGFs), stimulated with or without *Porphyromonas gingivalis*-derived LPS, a ligand for Toll-like receptor (TLR). LL-37 was non-toxic to HGFs up to a concentration of 10 μg/ml. *P. gingivalis* LPS upregulated the expression of *IL8*, *CXCL10*, and *CCL2*, whereas LL-37 reduced this upregulation. In absence of LPS, LL-37 itself upregulated the expression of *IL8* and *CCL2*. LL-37 increased the expression of P2X7, which was constitutively expressed in HGFs. The P2X7 antagonist A-438079 suppressed the cytotoxicity and upregulatory effect of LL-37 on chemokine response, but not its downregulatory effect on *P. gingivalis* LPS–induced chemokine response. Whether LL-37 alters the expression of 29 genes that encode TLR-associated proteins, including TLRs, co-receptors, signaling molecules, and negative regulators, in HGFs, under stimulation with LPS, was examined. Among TLRs, *P. gingivalis* LPS upregulated the level of *TLR4*, whereas LL-37 reduced it. In co-receptors, LL-37 downregulated the level of *CD14*. Among signaling molecules, LL-37 augmented the LPS-upregulated expression of *IRAK1*. Similar effects were observed in the specific negative regulators *TNFAIP3*, *RNF216*, *TOLLIP*, and *SIGIRR*. Our results suggest that LL-37 exerts cytotoxicity and upregulation of chemokine response via the P2X7 receptor, while it induces downregulation of *P. gingivalis* LPS–induced chemokine response through alteration in the expression of 7 specific TLR-associated genes: downregulation of *TLR4* and *CD14* and upregulation of *IRAK1*, *TNFAIP3*, *RNF216*, *TOLLIP*, and *SIGIRR*.

Key words LL-37 · Human gingival fibroblasts · Toll-like receptor · *Porphyromonas gingivalis* lipopolysaccharide

Introduction

Antimicrobial peptides form a part of the innate defense system in the oral cavity [1]. The antimicrobial peptide LL-37 is a member of the conserved antimicrobial peptide family cathelicidin, and the only member of human cathelicidin [2]. LL-37 is generated by cleavage of the C-terminus of CAMP (also called CAP18) by serine proteases and consists of 37 amino acids starting with two leucine residues [3]. This peptide is actively secreted from a variety of cells, such as neutrophils and gingival epithelial cells [1, 2, 4] and can be found in saliva, gingival crevicular fluids, and periodontal tissues [5, 6]. Given that LL-37 possesses antimicrobial activity typical of cathelicidin [2, 7], it is effective against oral bacterial species, including the periodontopathic Gram-negative bacteria *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitance*, and *Prevotella intermedia* [1, 8]. Patients with periodontitis exhibit increased levels of LL-37 in gingival crevicular fluids [9]. In addition, deficiency of LL-37 or lowered production of LL-37 in humans is thought to be correlated with development of periodontal diseases [6, 10]. Thus, LL-37 production in the periodontal tissue acts as an important innate defense factor especially against periodontopathic bacteria.

In addition to its antimicrobial activity, LL-37 possesses the ability to regulate innate immune responses. LL-37 has been found to neutralize the biological activities of lipopolysaccharide (LPS) derived from Gram-negative bacteria, including

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P. gingivalis, *A. actinomycetemcomitance*, and *P. intermedia* [11–14]. We have previously reported that LL-37 potently attenuates *P. gingivalis* LPS–induced chemokine production in human gingival fibroblasts (HGFs) [15]. The LPS-neutralizing effect of LL-37 is mediated through direct binding of LL-37 to LPS leading to the inhibition of CD14 recognition of LPS in macrophages [12, 16] and increase in LPS uptake and lysosomal degradation in liver sinusoidal endothelial cells [17]. This effect is also probably exerted through the regulation of expression of several genes in macrophages [13]. Contrary to its LPS-neutralizing effect of LL-37, LL-37 itself upregulates the expression of several immune-related genes in various cell types [13]. For instance, LL-37 activates the expression of IL-8 in HGFs, airway epithelial cells, smooth muscle cells, and skin keratinocytes in addition to inducing cyclooxygenase-2 and prostaglandin E2 in HGFs [18–22]. The mechanism by which LL-37 modulates responses of HGFs stimulated with or without *P. gingivalis* LPS is, however, not fully understood.

Initiation of TLR-mediated responses involves different categories of proteins, including Toll-like receptors (TLRs), co-receptors, signaling molecules, and negative regulators. For example, in LPS-induced TLR4-mediated responses, LPS first binds to the TLR co-receptor CD14 [23]. The dimerized TLR4-MD-2 complex then recognizes the structure of LPS and subsequently triggers signal transduction through the interaction of the TIR domain-containing adaptor proteins with the intracellular TIR domain of TLR4 [24]. The adaptor protein MyD88-dependent pathway activates downstream signaling using IRAK proteins and TRAF6 for activation of the MAP kinase cascade and the transcription factor NF-κB, whereas the adaptor protein TICAM1-dependent pathway activates downstream signaling using TRAF3 for activation of the transcription factor IRF3 [25, 26]. Simultaneously, various types of negative regulators suppress TLR signal transduction to avoid induction of excessive inflammatory responses [26–28]. It has not fully been elucidated whether LL-37 regulates the expression of genes that encode TLR-associated proteins. In this study, we investigated the effect of LL-37 on the gene regulatory responses of HGFs stimulated with or without *P. gingivalis* LPS. Also, we examined whether LL-37 alters the expression levels of 29 TLR-associated genes, including genes coding for TLRs, co-receptors, signaling molecules, and negative regulators, in HGFs stimulated with *P. gingivalis* LPS or co-stimulated with *P. gingivalis* LPS and LL-37.

Materials and Methods

Reagents

The synthetic LL-37 peptide (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was purchased from AnaSpec (Fremont, CA, USA). *P. gingivalis* LPS (tlrl-pglps) was obtained from InvivoGen (San Diego, CA, USA). A-438079 hydrochloride was purchased from Wako (Osaka, Japan).

Cell Culture

HGFs isolated under the approval by the Ethics Committee of Asahi University [15, 29] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. These cells were originally isolated from clinically healthy gingival tissues of donors, who had no history or current signs of systemic diseases and had received no medication within the previous 6 months. Cells from passages 3 to 6 were used for experiments. For the experiment, HGFs were grown in antibiotic-free DMEM supplemented with 10% inactivated FBS overnight and then treated with LL-37 in the presence or absence of *P. gingivalis* LPS for 24 h.

Lactate Dehydrogenase Release Assay

Cytotoxicity of LL-37 towards HGFs was assessed by the LDH released in the culture supernatants. Before LL-37 treatment, the culture medium was changed to DMEM without phenol red (Wako). Released LDH was colorimetrically measured by means of CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA). Cytotoxicity (%) was calculated as 100× [(experimental LDH release) - (control LDH release) / (maximum LDH release) - (control LDH release)], where values for LDH released from the control and maximum LDH release were obtained from non-stimulated cells and completely lysed cells using 0.9% Triton X-100, respectively.

Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from HGFs using PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) and 1 μg of total RNA was reverse transcribed using ReverTraAce reverse transcriptase (Toyobo, Otsu, Japan) with a mixture of an oligo$(dT)_{21}$ primer and random hexamer primers. Real-time qRT-PCR was conducted using SYBR Premix Ex Taq (TaKaRa, Otsu, Japan) on the thermal cycler Dice Real Time System TP800 (TaKaRa). The predesigned primer sets for all the genes listed in Table 1 were acquired from TaKaRa. Since primer sequences were provided by the manufacturer, they are not displayed. Reaction conditions comprised pre-denaturation at 95 °C for 30 sec, denaturation at 95 °C for 5 sec, and annealing at 60 °C for 30 sec, for a total of 40 cycles. The assessment of gene expression levels was performed using the $\Delta\Delta C_t$ method. The results are shown as the relative expression levels of the genes of interest were normalized to the levels of *ACTIN*.

Table 1 List of the genes investigated in this study

Category	Gene Symbol	Gene Name	Protein Name
TLRs	*TLR1*	Toll-like receptor 1	TLR1
	TLR2	Toll-like receptor 2	TLR2
	TLR3	Toll-like receptor 3	TLR3
	TLR4	Toll-like receptor 4	TLR4
	LY96	lymphocyte antigen 96	MD2, MD-2
	TLR5	Toll-like receptor 5	TLR5
	TLR6	Toll-like receptor 6	TLR6
	TLR7	Toll-like receptor 7	TLR7
	TLR8	Toll-like receptor 8	TLR8
	TLR9	Toll-like receptor 9	TLR9
	TLR10	Toll-like receptor 10	TLR10
TLR co-receptors	*CD14*	CD14 molecule	CD14
	CD36	CD36 molecule	CD36
TLR signaling molecules	*MYD88*	myeloid differentiation primary response 88	MyD88
	TICAM1	Toll-like receptor adaptor molecule 1	TRIF, TICAM1
	TICAM2	Toll-like receptor adaptor molecule 2	TRAM, TICAM2
	TIRAP	TIR domain containing adaptor protein	TIRAP, Mal
	IRAK1	interleukin 1 receptor associated kinase 1	IRAK1, IRAK-1
	IRAK2	interleukin 1 receptor associated kinase 2	IRAK2, IRAK-2
	IRAK4	interleukin 1 receptor associated kinase 4	IRAK4, IRAK-4
	TRAF6	TNF receptor associated factor 6	TRAF6
	TRAF3	TNF receptor associated factor 3	TRAF3
TLR negative regulators	*TNFAIP3*	TNF alpha induced protein 3	A20, TNFAIP3
	RNF216	ring finger protein 216	Triad3A, RNF216
	TOLLIP	Toll-interacting protein	TOLLIP, IL-1RAcPIP
	CYLD	CYLD lysine 63 deubiquitinase	CYLD
	ITCH	itch E3 ubiquitin protein ligase	ITCH
	IRAK3	interleukin 1 receptor associated kinase 3	IRAKM, IRAK-M
	SIGIRR	single Ig and TIR domain containing	SIGIRR, TIR8, IL-1R8

Statistical Analysis

Results, expressed as mean ± standard deviation (SD) of triple wells, were representative of three independent experiments. Data were subjected to two-way analysis of variance (ANOVA) followed by Bonferroni test. Values of $p < 0.05$ were considered statistically significant.

Results

Cytotoxicity of LL-37 in HGFs

We have previously demonstrated that LL-37 does not exhibit cytotoxicity in HGFs, at concentrations of 0.1 and 1 μM (approximately 0.5 and 4.5 μg/ml, respectively) as assessed by ATP production [15]. In accordance with this, when evaluated through LDH release, 10 μg/ml of LL-37 was found to be non-toxic to HGFs while concentrations of more than 20 μg/ml of LL-37 were slightly toxic (Fig. 1).

Effect of LL-37 on the Expression of Chemokines in HGFs Under Stimulation With or Without *P. gingivalis* LPS

We investigated the effect of LL-37 on the responses of HGFs stimulated with or without *P. gingivalis* LPS. Stimulation of HGFs with *P. gingivalis* LPS alone strongly induced the expression of the chemokine genes *IL8*, *CXCL10*, and *CCL2* (Fig. 2a), while a non-toxic concentration of LL-37 dramatically reduced this upregulation (Fig. 2a). The LPS neutralization effect of LL-37 is consistent with previous results [14, 15,

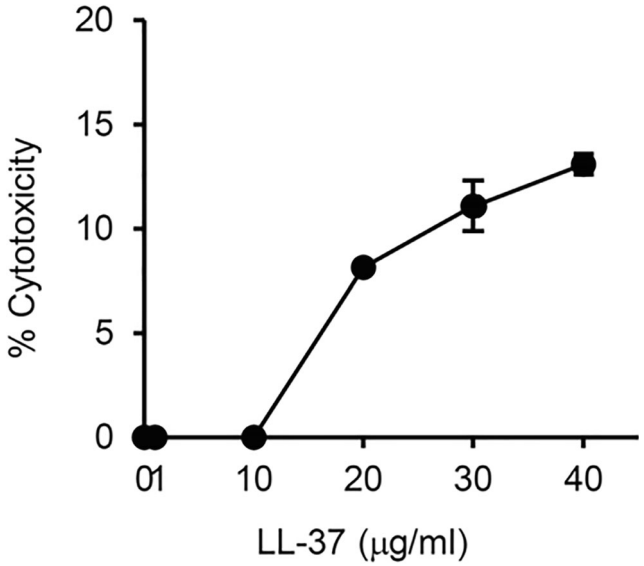

Fig. 1 Cytotoxicity of different concentrations of LL-37 towards HGFs. HGFs were treated with various concentrations of LL-37 for 24 h, and then assayed for LDH released in the culture supernatant

30]. In the absence of *P. gingivalis* LPS, LL-37 itself induced the expression of *IL8* and *CCL2* but did not induce expression of *CXCL10* (Fig. 2b and data not shown). These results suggest that LL-37 exerts a downregulatory effect on *P. gingivalis* LPS–induced chemokine response, namely LPS-neutralization effect, whereas it induces an upregulatory effect on chemokine response in the absence of *P. gingivalis* LPS.

Involvement of the $P2X_7$ Receptor on LL-37-Induced Cytotoxicity and the Upregulatory and Downregulatory effect on Chemokine Response in HGFs

In HGFs, LL-37 itself has been reported to be recognized by the $P2X_7$ purinergic receptor, which induces the expression of IL-8, cyclooxygenase-2 and prostaglandin E2 [31, 32]. $P2X_7$ was found to be constitutively expressed in HGFs, and its expression was upregulated by LL-37 treatment (Fig. 3a). We, therefore, examined whether the $P2X_7$ receptor is involved in the effects of LL-37 on HGFs, including cytotoxicity and its upregulatory and downregulatory effects on chemokine response. The cytotoxicity of LL-37 was inhibited by the $P2X_7$ antagonist A-438079 in a dose-dependent manner (Fig. 3b). In addition, the upregulatory effect of LL-37 on *IL8* and *CCL2* expression was suppressed by A-438079 (Fig. 3c); however, the downregulatory effect of LL-37 on *P. gingivalis* LPS–induced *IL8*, *CXCL10*, and *CCL2* expression was not affected by A-438079 (Fig. 3d and data not shown). These results suggest that the cytotoxicity and upregulatory effect, but not downregulatory effect, of LL-37 on chemokine response is mediated through the $P2X_7$ receptor.

Effect of Co-stimulation with LL-37 and *P. gingivalis* LPS on the Expression of TLRs, Co-receptors, Signaling Molecules, and Negative Regulators in HGFs

To investigate the mechanism by which LL-37 downregulates the *P. gingivalis* LPS–induced response of HGFs, we examined whether LL-37 alters the expression levels of selected 29 TLR-associated genes in HGFs in the presence with *P. gingivalis* LPS. These genes (listed in Table 1) were divided into four categories: (1) TLRs, (2) TLR co-receptors, (3) TLR signaling molecules, and (4) negative regulators.

In the category of TLRs, the expression levels of *TLR4* and *LY96* were relatively higher than those of the others (Fig. 4a). The expression level of *TLR9* was considerably low, and *TLR8* was not detectable (Fig. 4a). The expression level of *TLR4* was slightly upregulated by *P. gingivalis* LPS stimulation, but this upregulation was diminished by *P. gingivalis* LPS–LL-37 co-stimulation (Fig. 4a). No remarkable changes in the expression levels of other TLRs were observed after stimulation with *P. gingivalis* LPS and *P. gingivalis* LPS–LL-37 co-stimulation (Fig. 4a). In case of TLR co-receptors, the expression level of *CD14* was higher than that of *CD36*, and it was decreased by *P. gingivalis* LPS–LL-37 co-stimulation (Fig. 4b). In the expression levels of TLR-signaling molecules, the expression levels of *MyD88*, *IRAK1*, *IRAK4*, and *TRAF3* were relatively higher than those of others (Fig. 4c). The expression levels of *TICAM1*, *TIRAP*, *IRAK2*, and *TRAF6* were considerably low, and *TICAM2* was not detectable (Fig. 4c). *IRAK1* was upregulated by *P. gingivalis* LPS stimulation, and the *P. gingivalis* LPS–LL-37 co-stimulation further enhanced this level (Fig. 4c). Among the TLR negative regulators, the expression level of *TOLLIP* was relatively high, while the expression level of *SIGIRR* was considerably low (Fig. 4d). The expression levels of *TNFAIP3*, *RNF216*, *TOLLIP*, and *SIGIRR* were upregulated by *P. gingivalis* LPS stimulation, and *P. gingivalis* LPS–LL-37 co-stimulation further increased these levels (Fig. 4d). The expression levels of *CYLD*, *ITCH*, and *IRAK3* were not significantly altered by *P. gingivalis* LPS or *P. gingivalis* LPS–LL-37 co-stimulation (Fig. 4d). These results suggest that LL-37 modulates particular 7 TLR-associated gene expression under stimulation with *P. gingivalis* LPS.

Discussion

In this study, we demonstrated that LL-37 itself has an upregulatory effect on the expression of *IL8* and *CCL2* in HGFs via the $P2X_7$ receptor, while it exerts a downregulatory effect on the expression of *IL8* and *CXCL10* induced by *P. gingivalis* LPS, i.e., LPS-neutralizing effect. *CAMP* is expressed in oral epithelial cells, but not in HGFs, despite stimulation with *P. gingivalis* LPS [15]. This suggests that

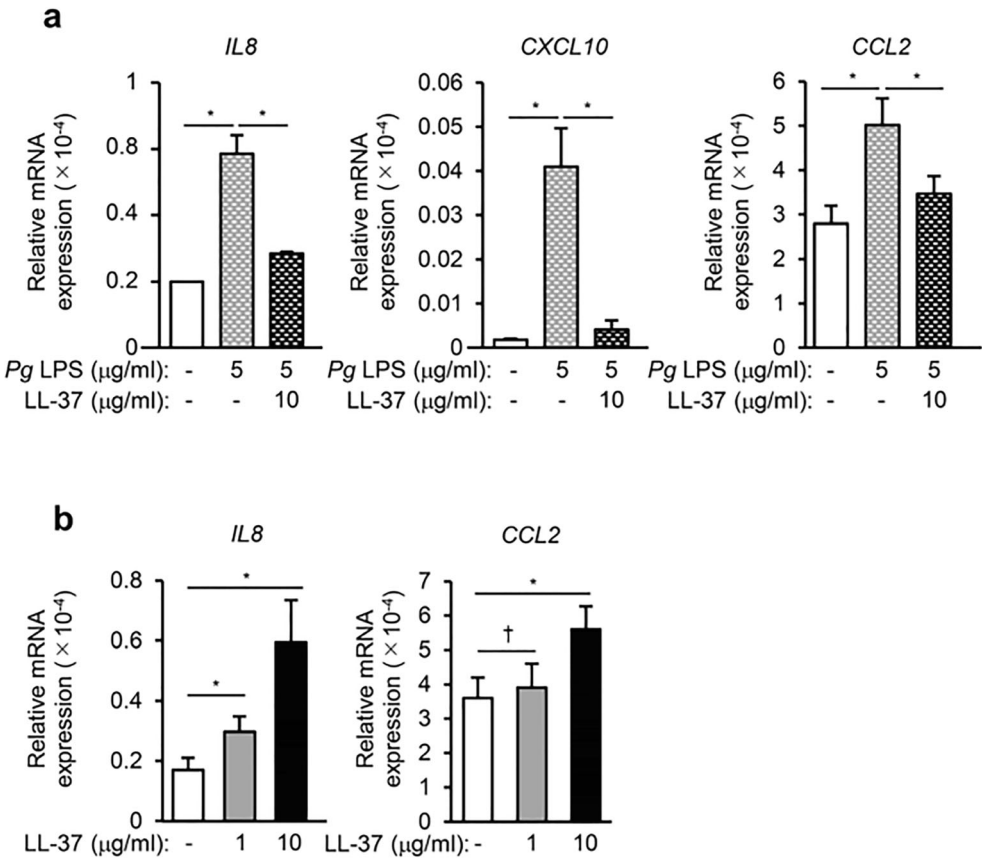

Fig. 2 Effect of LL-37 on chemokine responses in HGFs under stimulation with or without *P. gingivalis* LPS. **a** HGFs were incubated with 10 μg/ml of LL-37 in the presence or absence of 5 μg/ml of *P. gingivalis* LPS for 24 h, followed by RNA extraction. Relative expression levels of *IL8*, *CXCL10*, and *CCL2* were determined by qRT-PCR. *$p < 0.01$. **b** HGFs were incubated with LL-37 for 24 h, after which the RNA was extracted. The expression levels of *IL8* and *CCL2* were determined by qRT-PCR. *$p < 0.01$, $^{\dagger}p < 0.05$

LL-37 exerts an upregulatory effect on the chemokine response of HGFs in a paracrine manner, by which immune cells are recruited to the site of infection, resulting in the clearance of the infection. On the other hand, LL-37 can act to suppress *P. gingivalis* LPS-induced chemokines to inhibit excessive inflammatory response in HGFs under stimulation with *P. gingivalis* LPS. Since LL-37 is also the antimicrobial peptide, our results suggest that LL-37 works as a multifunctional modulator of innate immune responses in HGFs.

To investigate the mechanism by which LL-37 induces the downregulatory effect on *P. gingivalis* LPS–induced chemokines, we examined whether LL-37 alters the expression levels of 29 TLR-associated genes, which we divided into four categories, in HGFs stimulated with *P. gingivalis* LPS that serves as a ligand for TLR. Among TLRs, *P. gingivalis* LPS stimulation upregulated the level of *TLR4*, but LL-37 treatment diminished this upregulation. In co-receptors, LL-37 treatment downregulated the level of *CD14*. During infection by periodontopathic Gram-negative bacteria such as *P. gingivalis*, TLR4 and CD14 stimulate periodontal inflammation by recognizing LPS, the most common component of Gram-negative bacteria [33], suggesting that regulation of TLR4 and *CD14* genes by LL-37 exerts an initial suppressive effect on inflammatory responses of HGFs against periodontopathic bacteria. After the TLR4 recognition of LPS, TLR4 on the cytoplasmic membrane activates downstream signaling using the signaling molecules TIRAP and MyD88, whereas TLR4 internalized in endosomes activates downstream signaling using the signaling molecules TICAM1 and TICAM2 [24, 26]. In the MyD88-dependent signaling pathway, activated MyD88 binds with the signaling molecules IRAK1 and IRAK4 to form the signaling complex "myddosome" which subsequently binds with TRAF6 to activate the downstream NF-κB-activating signaling [26]. IRAK1 is known to be degraded after signal transduction, thereby regulating signal transduction [34] and LPS-induced cellular tolerance [35]. In the TICAM1-dependent signaling pathway, TICAM1 interacts with TRAF3 to activate the downstream IRF3-dependent type I interferon-producing pathway [26]. Among TLR signaling molecules, we found that the expression level of *IRAK1* is increased not only by *P. gingivalis* LPS alone but also by *P. gingivalis* LPS–LL-37 co-stimulation, suggesting that LL-37 alters the cellular reactivity of HGFs by increasing IRAK-1 levels to preferentially activate the MyD88-dependent signal transduction. The ligand recognition and signal transduction mechanisms of TLR are "fine-tuned" by various types of negative regulators so as to avoid induction of excessive inflammatory responses [27, 36]. The *TNFAIP3*-encoded protein A20 is known to be a zinc finger protein with ubiquitin-editing activity [36]. A20 is rapidly produced in response to inflammatory signals, including TLR signaling, and potently suppresses activation of the

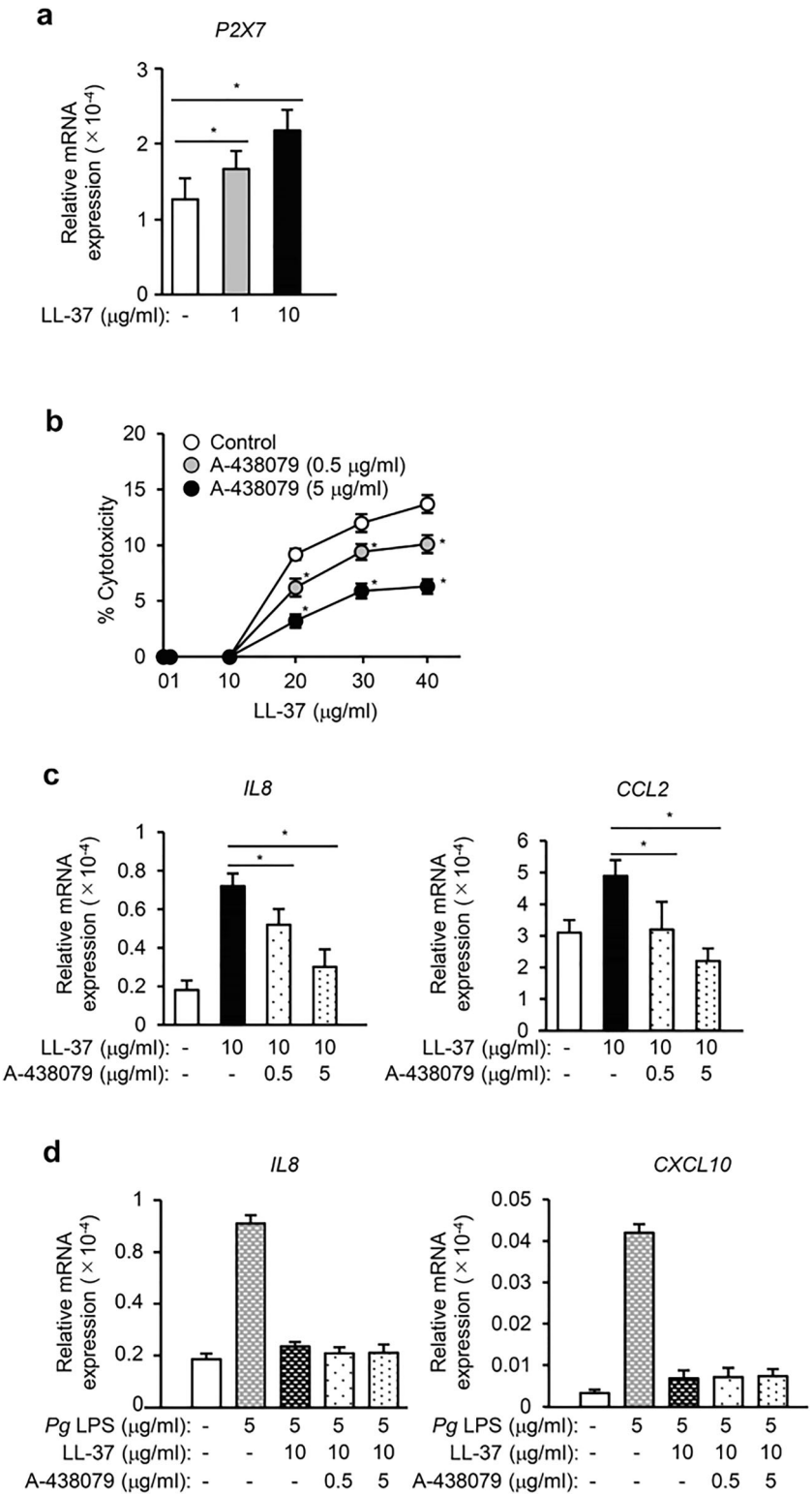

Fig. 3 Involvement of $P2X_7$ in LL-37-induced effects on HGFs. **a** HGFs were incubated with LL-37 for 24 h, after which the RNA was extracted. The expression level of *P2X7* was determined by qRT-PCR. *$p < 0.01$. **b** HGFs were pretreated with or without the indicated doses of A-438079 for 30 min. Then the cells were incubated with various concentrations of LL-37 for 24 h, and then assayed for LDH released in the culture supernatant. *$p < 0.01$ versus control. **c** HGFs pretreated with or without the indicated doses of A-438079 for 30 min were incubated with 10 μg/ml LL-37 for 24 h, after which the RNA was extracted. Relative expression levels of *IL8* and *CCL2* were determined by qRT-PCR. *$p < 0.01$. **d** HGFs pretreated with or without the indicated doses of A-438079 for 30 min were incubated with 10 μg/ml of LL-37 in the presence or absence of 5 μg/ml of *P. gingivalis* LPS for 24 h, followed by RNA extraction. Relative expression levels of *IL8* and *CXCL10* were determined by qRT-PCR

NF-κB pathway [26, 37]. The *RNF216*-encoded protein Triad3A is an E3 ubiquitin ligase that mediates ubiquitination and subsequent degradation of TLRs, thereby inhibiting TLR-mediated ligand recognition and signal transduction [38]. TOLLIP is an inhibitory adaptor protein that can interact with several TLRs, including TLR4 [24, 39]. SIGIRR is a Toll/IL-1 receptor family member that serves as an intracellular decoy receptor for negative regulation of IL-1 and TLR signaling [24, 40]. In this study, upregulation of expression of negative regulators *TNFAIP3*, *RNF216*, *TOLLIP*, and *SIGIRR* was

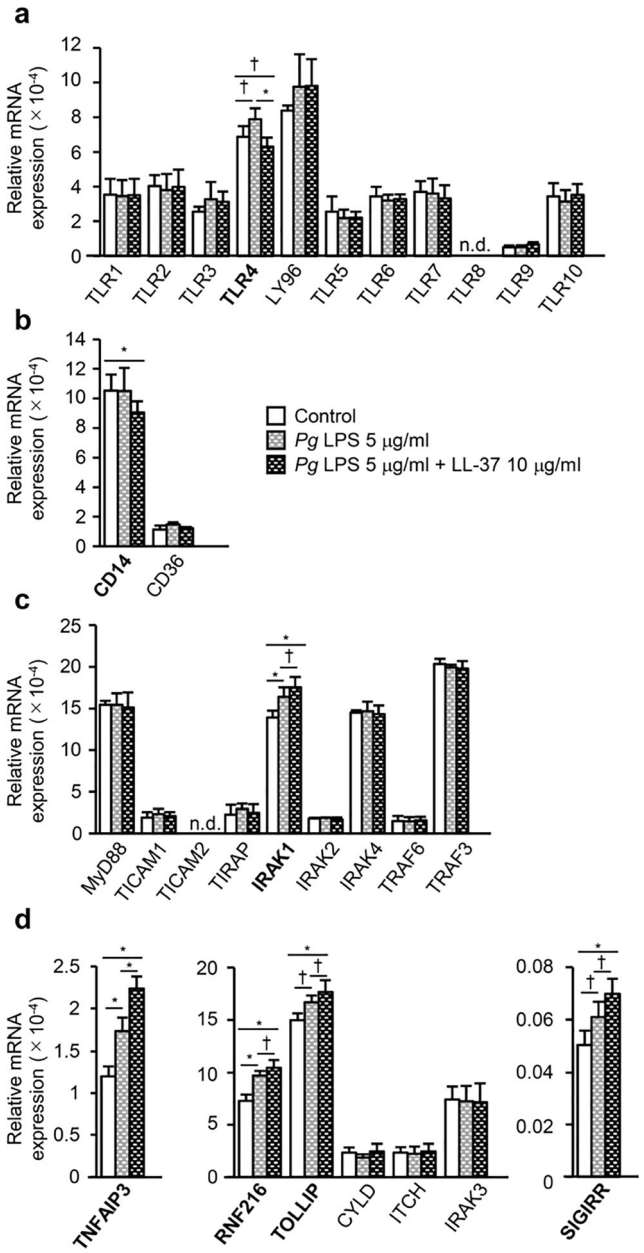

Fig. 4 Effect of *P. gingivalis* LPS–LL-37 co-stimulation on the expression of 29 TLR-associated genes in HGFs. HGFs were incubated with 10 μg/ml of LL-37 in the presence or absence of 5 μg/ml of *P. gingivalis* LPS for 24 h, after which the RNA was extracted. Relative expression levels of *TLRs* and *LY96* (**a**), co-receptors (**b**), signaling molecules (**c**), and negative regulators (**d**) as determined by qRT-PCR. $*p < 0.01$, $^{\dagger}p < 0.05$; n.d., not detected

observed upon *P. gingivalis* LPS stimulation and this increase was further enhanced by *P. gingivalis* LPS–LL-37 co-stimulation. On the other hand, the levels of the negative regulators *CYLD* [28], *ITCH* [41], and *IRAKM* [42] were not altered by LL-37 treatment or *P. gingivalis* LPS stimulation. These results indicate that LL-37 upregulates specific negative regulators, by which mediates suppression of *P. gingivalis* LPS–induced proinflammatory responses in HGFs. Collectively, our results suggest that LL-37 exerts the downregulatory effect on *P. gingivalis* LPS–induced cellular responses in HGFs through alteration in the expression of 7 particular TLR-associated genes: downregulation of *TLR4* and *CD14* expression; upregulation in the expression of *IRAK1*, *TNFAIP3*, *RNF216*, *TOLLIP*, and *SIGIRR*.

P. gingivalis LPS has been indicated to alter the gene expression levels of *TLR2* and/or *TLR4* in HGFs but results for these have been inconsistent [43–45]. In our experiment, the expression level of *TLR2* was not significantly affected by *P. gingivalis* LPS and/or LL-37. The results shown by Herath et al. [46] suggest that this inconsistency may be attributed to heterogeneity of *P. gingivalis* LPS and acute alteration of the expression level of *TLR2* in HGFs.

Previous reports have shown that LL-37 is recognized by the $P2X_7$ receptor and induces activation of the MAP kinase cascade leading to the induction of IL-8, cyclooxygenase-2, and prostaglandin E2 in HGFs [18, 22]. The expression of $P2X_7$ was found to be increased by LL-37 treatment in HGFs. Indeed, the $P2X_7$ antagonist abrogated the LL-37-induced chemokines. However, unexpectedly, the $P2X_7$ antagonist did not affect the downregulatory effect of LL-37 on *P. gingivalis* LPS–induced chemokines. It has been reported that LL-37 can directly bind to LPS and resulting conjugate LL-37–LPS is not recognized by $P2X_7$ in liver sinusoidal endothelial cells [17]. Collectively, these observations suggest that the $P2X_7$ receptor is involved in the recognition of LL-37 alone in HGFs. The mechanism underlying this recognition of LL-37 by HGFs in the presence of *P. gingivalis* LPS needs to be addressed in a future study.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflicts of interest.

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