Journal of
PERIODONTAL RESEARCH

J Periodont Res 2011; 46: 327–337

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2011.01346.x

# Involvement of $P2X_7$ purinergic receptor and MEK1/2 in interleukin-8 up-regulation by LL-37 in human gingival fibroblasts

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*Montreekachon P, Chotjumlong P, Bolscher JGM, Nazmi K, Reutrakul V, Krisanaprakornkit S. Involvement of $P2X_7$ purinergic receptor and MEK1/2 in interleukin-8 up-regulation by LL-37 in human gingival fibroblasts. J Periodont Res 2011; 46: 327–337. © 2011 John Wiley & Sons A/S*

*Background and Objective:* The antimicrobial peptide LL-37, derived from human neutrophils, can directly chemoattract leukocytes and up-regulate the expression of several immune-related genes in various cell types. In this study, we wanted to determine the immunoregulatory effect of LL-37 on interleukin-8 (IL-8) expression in human gingival fibroblasts (HGFs) and to characterize intracellular signaling pathway(s) and receptor(s) involved in IL-8 induction.

*Material and Methods:* Cultured fibroblasts were treated with different concentrations of LL-37 or interleukin-1β (IL-1β), as a positive control, for specific periods of time in the presence or absence of various inhibitors. RT-PCR and real-time PCR were conducted to analyze the expression of IL-8 mRNA, and the IL-8 levels in cell-free culture media were measured using ELISAs. The MTT assay was performed to determine the cytotoxicity of LL-37.

*Results:* Nontoxic concentrations of LL-37 (up to 10 μM) and IL-1β significantly up-regulated the expression of IL-8 mRNA in a dose-dependent manner ($p < 0.05$). The IL-8 protein levels were consistently significantly elevated in conditioned media of LL-37-treated HGFs ($p < 0.05$). IL-8 up-regulation by LL-37 was completely abrogated by 20 μM U0126, consistent with transient phosphorylation of p44/42 MAP kinases. Moreover, pretreatment with Brilliant Blue G (a selective antagonist of the $P2X_7$ receptor) and the neutralizing antibody against $P2X_7$ blocked IL-8 up-regulation in a dose-dependent manner, consistent with expression of the $P2X_7$ receptor in HGFs.

*Conclusion:* These findings indicate that LL-37 induces IL-8 expression via the $P2X_7$ receptor and the MEK1/2-dependent p44/42 MAP kinases in HGFs, suggesting both direct and indirect involvement of LL-37 in neutrophil recruitment into an inflammatory site within diseased periodontal tissues.

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Key words: LL-37; interleukin-8; p44/42 MAP kinases; MEK1/2; $P2X_7$ purinergic receptor

Accepted for publication December 15, 2010

Human cationic antimicrobial peptide 18 (hCAP18), the only member of the cathelicidin family expressed in humans, consists of a highly conserved N-terminal cathelin domain and a variable C-terminal cationic antimicrobial domain. Upon proteolytic cleavage by proteinase 3, an active LL-37 peptide is liberated from hCAP18 (1).

LL-37 was first identified in neutrophils, and later found in epithelial cells, keratinocytes, lymphocytes, monocytes and mast cells (2). In the oral cavity, LL-37 is mainly synthesized by neutrophils (3) and to a lesser extent by buccal and tongue mucosal epithelia (4). In addition, the concentrations of LL-37 are raised in the gingival crevicular fluid collected from patients with chronic periodontitis (5). In contrast, only low levels, of mature LL-37 peptide are found in gingival crevicular fluid collected from patients with aggressive periodontitis (5), which highlights the importance of LL-37 as a key effector molecule of innate immune responses in periodontitis.

The deficiency of LL-37 may cause an aberration in host immune responses that can enhance the severity and progression of periodontal disease, because it is now recognized that LL-37 also acts a potent immune modulator by functioning as a chemoattractant for various types of hematopoietic cells (6) and by up-regulating gene expression, particularly of several chemokines and their receptors, resulting in greater responsiveness to the inflammation (7). For example, it has been demonstrated that LL-37 activates expression of interleukin-8 (IL-8) in airway epithelial cells (8) and smooth muscle cells (9) in the respiratory tract through distinct receptors and in skin keratinocytes (10). Therefore, it is possible that LL-37 may also affect IL-8 expression in some resident periodontal cells.

Periodontitis is a multifactorial disease that causes destruction of tooth-supporting structures, eventually leading to tooth loss. One of its etiologies is infections with specific gram-negative microorganisms in plaque biofilm, which have utilized different strategies to survive and thrive in the biofilm. They can overwhelmingly elicit a host response, finally leading to periodontal tissue damage. Host immune responses in periodontal disease include a complex set of inflammatory mediators and effector molecules, such as antimicrobial peptides, that are synthesized and released from transmigrating leukocytes and resident epithelial cells (3). The antimicrobial peptides may then interact with other adjacent cells of the periodontium, especially human gingival fibroblasts (HGFs), in a paracrine manner. Consequently, it is probable that HGFs can synthesize and release various inflammatory cytokines and chemokines in response to the antimicrobial peptides.

In inflamed periodontal tissues, IL-8 is mainly synthesized by epithelial cells, macrophages and fibroblasts (11), and can be induced by stimulation with bacterial lipopolysaccharide and several cytokines in HGFs (12). IL-8 can attract neutrophils from blood vessels to the site of inflammation and activate neutrophils to undergo a metabolic burst and to degranulate upon arrival at the site of the challenge (13). As a result, the amounts of mature LL-37 peptide, stored in neutrophil granules as a pro-peptide, can be accumulated in the inflamed tissues. Therefore, it was logical to hypothesize that such higher amounts of LL-37 would induce IL-8 expression in HGFs, which would further enhance neutrophil recruitment and LL-37 release in a positive amplification loop. Moreover, in this study, the involvement of potential candidate receptor(s) and intracellular signaling pathway(s) in IL-8 up-regulation by LL-37 was further extrapolated by using different selective inhibitors.

## Material and methods

### Materials

LL-37 peptide (LLGDFFRKSKEKIG KEFKRIVQRIKDFLRNLVPRTES) was synthesized by Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry using a MilliGen 9050 peptide synthesizer (MilliGen/Biosearch, Bedford, MA, USA), as described by den Hertog *et al*. (14). Peptides were purified by preparative reverse-phase HPLC. The purity of the peptides was at least 95%, and the authenticity of the peptides was confirmed by ion-trap mass spectrometry with an LCQ Deca XP spectrometer (Thermo Finnigan, San Jose, CA, USA). Interleukin-1β (IL-1β) was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). A specific p38 MAP kinase inhibitor (SB203580) and its inactive analog (SB202474); a specific JNK inhibitor (SP600125) and its inactive analog (-JNK); a highly selective MEK1/2 inhibitor (U0126) and its inactive analog (U0124); a broad-spectrum antagonist of P2 purinergic receptors (suramin) and the suramin analog (NF279); and a selective blocker of tyrosine phosphorylation of epidermal growth factor receptor (EGFR) (i.e. AG1478), were purchased from Calbiochem (Darmstadt, Germany). All chemical reagents were dissolved in dimethylsulfoxide (DMSO). Brilliant Blue G (BBG), a selective $P2X_7$ receptor antagonist, was purchased from Sigma-Aldrich (St Louis, MO, USA), and dissolved in water at a stock concentration of 100 mM. The polyclonal antibodies against each MAP kinase and its phosphorylated form were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), and those against $P2X_4$, $P2X_7$ and actin were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The neutralizing antibody that binds to the extracellular portion of the $P2X_7$ receptor to inhibit activation was obtained from Alomone Laboratories (Jerusalem, Israel).

### Culture of HGFs

Gingival biopsies were collected from tissues overlying impacted third molars from three different donors. The research protocol was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University, and informed consent was obtained. Primary HGFs were cultured according to an established procedure (15). In brief, the gingival explants were immediately washed, cut into a smaller dimension (approximately $2 \times 2$ mm) and placed in a 60-mm tissue-culture dish (Nunc A/S, Roskilde, Denmark). HGFs, cultured in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen), at the fourth to eighth passages were used throughout this study. In each cell line, two separate experiments were

conducted. Confluent HGFs were starved of serum for 6 h before treatment with various doses of LL-37 or IL-1β (as a positive control for IL-8 induction), for the times indicated and in the presence or absence of inhibitors.

### Total RNA isolation and RT-PCR

Total RNA was extracted using an Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA, USA). The RT-PCR protocol has been previously described (16). Briefly, 2 μg of total RNA was used for cDNA synthesis using the Super-Script™ First-Strand cDNA System (Fermentas, Hanover, MD, USA). The sequences of PCR primers and the amplification conditions for IL-8, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and P2X purinergic receptors have been previously described (17–20) and are summarized in Table 1. The PCR products were resolved by electrophoresis on a 1.2% agarose gel, and photographs were taken by a camera attached with a charge-coupled device, attached to a ChemiDoc XRS instrument (Bio-Rad Laboratories).

### Real-time PCR

The real-time PCR was performed using 5% (vol/vol) of cDNA and the Light Cycler-FastStart DNA Master SYBR® Green I system (Roche Molecular Biochemicals, Mannheim, Germany) (16). The median ratio of IL-8 relative to GAPDH expression in each sample was calculated from six separate experiments. The relative induction (multiples) of IL-8 mRNA was determined by comparing the median ratio of an LL-37-treated sample with that of an untreated control (Fig. 1B and 1F).

### Western blot analysis

HGFs were treated with the indicated doses of LL-37 for various periods of time and whole-cell lysates were then extracted by RIPA buffer (21). Forty micrograms of cell lysate was resolved by electrophoresis on a 12% sodium dodecyl sulfate–polyacrylamide gel and then transferred to nitrocellulose membranes. The membranes were blocked, incubated with primary antibody against p38, JNK, or p44/42 MAP kinases (1 : 1000 dilution), against the phosphorylated form of p38, JNK, or p44/42 MAP kinases (1 : 1000 dilution), against actin, $P2X_4$ or $P2X_7$ (1 : 500 dilution), and then reacted with horseradish peroxidase-conjugated secondary antibody (1 : 2000 dilution) (KPL, Gaithersburg, MD, USA). LumiGLO Reserve Chemiluminescence (KPL) was used as a substrate, and the signal was captured by the ChemiDoc XRS instrument.

### Determination of IL-8 levels

The concentrations of IL-8 in cell-free conditioned media, collected from HGFs after treatment with different concentrations of LL-37 for various periods of time, in the presence or absence of specific inhibitors, were measured by ELISA using a commercially available kit (Thermo Science, Rockford, IL, USA) according to the manufacturer's instructions. In brief, samples or standards of known IL-8 concentrations were added to the wells of a 96-well plate and incubated for 90 min. Subsequently, the wells were washed and the second biotinylated antibody was added. After incubation for 90 min, the second antibody was removed and washed, and the streptavidin-conjugated peroxidase was added and further incubated for 30 min. Then, the wells were washed to remove the unbound enzyme, and the substrate solution was added for 30 min at room temperature in the dark. A stop solution was added to each well, and the absorbance was read at 405 nm using the Titertek Multiskan M340 multiplate reader (ICN Flow, Costa Mesa, CA, USA). Each sample was assayed in triplicate.

*Table 1.* Oligonucleotide primer sequences, annealing temperatures and the expected length [in base pairs (bp)] of each PCR product of interleukin-8 (IL-8), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and P2X receptors

| | Primer sequence | Annealing temperature (°C) | Product length (bp) |
|---|---|---|---|
| IL-8 | Forward: 5′-TTTCTGATGGAGAGAGCTCTGTCTGG-3′<br>Reverse: 5′-AGTGGAACAAGACTTGTGGATCCTGG-3′ | 60.0 | 598 |
| GAPDH | Forward: 5′-ACCACAGTCCATGCCATCACTGC-3′<br>Reverse: 5′-TCCACCACCCTGTTGCTGTAGC-3′ | 60.0 | 452 |
| $P2X_1$ | Forward: 5′-GTTTGGGATTCGCTTTGA-3′<br>Reverse: 5′-TGGCTGAGAGGGTAGGAGAC-3′ | 57.4 | 384 |
| $P2X_2$ | Forward: 5′-GCATCGGAGTGCAACCCCAA-3′<br>Reverse: 5′-TCACAGGCCAGCTACCTGAG-3′ | 55.0 | 780 |
| $P2X_3$ | Forward: 5′-GAGAGTGAGGAGAAATACCG-3′<br>Reverse: 5′-CACTGGTCCCAGGCCTTG-3′ | 59.0 | 437 |
| $P2X_4$ | Forward: 5′-CCTTCTGCCCCATATTCCGTCT-3′<br>Reverse: 5′-GTTGATCATAGTGGGGATGATGTCA-3′ | 55.0 | 341 |
| $P2X_5$ | Forward: 5′-GCTGGAAACGGAGTGAAG-3′<br>Reverse: 5′-GCCTCCTGGGAACTGTCT-3′ | 55.7 | 665 |
| $P2X_6$ | Forward: 5′-CGCCAGCCCAAGTTCAGG-3′<br>Reverse: 5′-GCCTACAGAGCCACCCAG-3′ | 57.9 | 440 |
| $P2X_7$ | Forward: 5′-AGATCGTGGAGAATGGAGTG-3′<br>Reverse: 5′-TTCTCGTGGTGTAGTTGTGG-3′ | 60.0 | 399 |

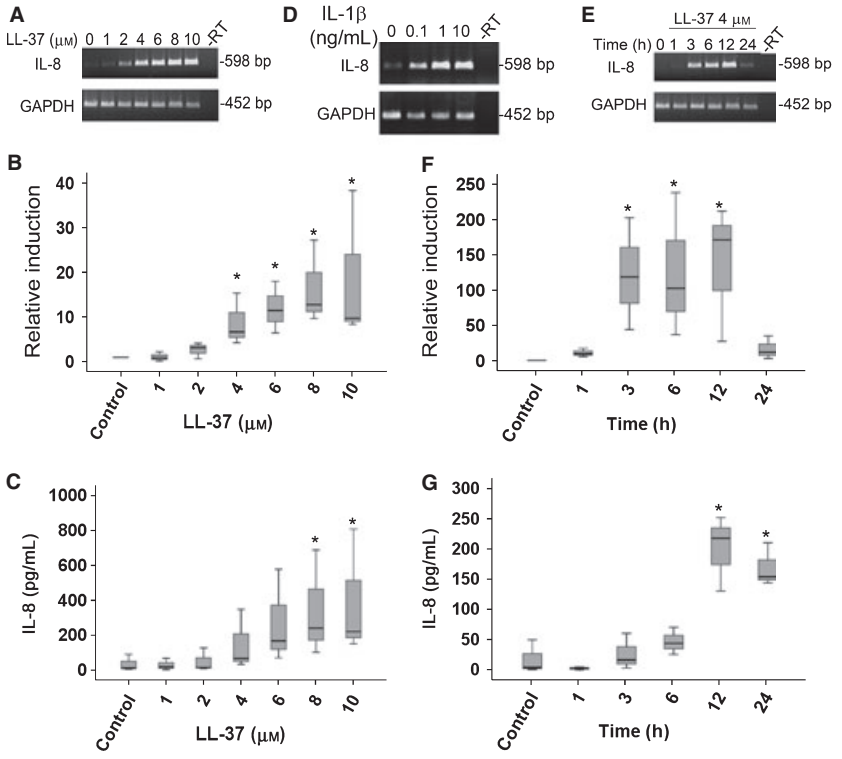


*Fig. 1.* Up-regulation of interleukin-8 (IL-8) mRNA and protein in a dose-dependent manner. (A) Human gingival fibroblasts (HGFs) were treated with 0–10 μM of LL-37 for 6 h. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression (serving as a housekeeping gene control) was similar at all concentrations of LL-37. –RT, negative control where the reverse transcriptase was omitted. (B) Real-time PCR assay. cDNA samples from (A) were used to quantify the median induction (multiples) of IL-8 relative to expression of GAPDH mRNA. (C) The IL-8 levels in cell-free culture supernatants from (A) were measured using an ELISA, as described in the Material and methods. (D) HGFs were treated with 0.1–10 ng/mL of interleukin-1β (IL-1β) for 12 h. Total RNA isolation and RT-PCR were performed as described in the Material and methods. (E) HGFs were treated with 4 μM LL-37 for 0–24 h (h). (F) Real-time PCR assay. cDNA samples from (E) were used to quantify the median induction (multiples) of IL-8 relative to GAPDH mRNA expression. (G) The IL-8 levels in cell-free culture supernatants from (E) were measured using an ELISA. The data in box plots are presented as medians with 25th and 75th percentiles, and $n = 6$ for each cell datum (*$p < 0.05$).

### MTT assay

To examine the cell toxicity, an indirect measurement of cell survival by an MTT assay was employed by determining the reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by a mitochondrial enzyme (e.g. succinate dehydrogenase), which can only occur in metabolically active cells. Cultured HGFs were seeded in a 96-well plate (Nunc A/S) and incubated with various concentrations of LL-37 (0–30 μM) or with 5 mM $H_2O_2$, as a positive control for cytotoxicity, for 24 h. Subsequently, the medium was removed and 20 μL of MTT stock dye solution (5 mg/mL in phosphate-buffered saline) (Sigma-Aldrich) was added and incubated at 37°C, in a humidified chamber with 5% $CO_2$ and 95% air, for 4 h. Then, the solution was removed and 200 μL of DMSO was added to each well to solubilize the formazan crystals. The plate was shaken for 10 min, and the absorbance was read at 540 nm with a reference wavelength at 630 nm using the Titertek Multiskan M340 multiplate reader (ICN Flow).

### Statistical analysis

The differences in expression of IL-8 mRNA and protein, between controls and samples treated with LL-37, as shown in Fig. 1B, 1C, 1F and 1G, the differences in the ratios of the intensities of each phosphorylated MAP kinase to those of respective MAP kinases between controls and samples treated with LL-37 in Fig. 2C or with IL-1β in Fig. 2D, and the inhibition of IL-8 protein synthesis between LL-37- and inhibitor-treated samples, as shown in Figs 3B, 4C and 6B, were tested by the Mann–Whitney *U*-test at a significance level of $p < 0.05$. The resulting values are illustrated as medians (horizontal lines), 25th and 75th percentiles, and the highest and lowest values in the box plots. In addition, the differences in the percentage of cell survival between a control sample and LL-37-treated samples or an $H_2O_2$-treated sample were tested using the Student's *t*-test at the significance levels of $p < 0.05$ and $p < 0.01$.

## Results

### Up-regulation of IL-8 mRNA and protein by LL-37 in HGFs

To determine the inducible effect of LL-37 on IL-8 expression, we first investigated the effect of nontoxic doses of LL-37 using the MTT assay. It was found that doses of LL-37 up to 10 μM were not toxic to HGFs, whereas at 20 and 30 μM, LL-37 significantly decreased the percentage of cell survival in comparison with untreated control HGFs (Table 2). As anticipated, treatment with 5 mM $H_2O_2$ significantly reduced the percentage of cells surviving ($p < 0.01$) (Table 2). Therefore, concentrations of LL-37 of 1–10 μM were chosen as the optimal doses to treat HGFs in this study.

Subsequently, confluent HGFs were treated with various concentrations of LL-37 for the periods of time indicated (0–24 h). RT-PCR analysis demonstrated that LL-37 induced IL-8 mRNA expression in a dose-dependent manner (Fig. 1A), with a significant induction of IL-8 mRNA expression observed by exposure to 4–10 μM of LL-37 ($p < 0.05$) (Fig. 1B). Consistent with induction of IL-8 mRNA, IL-8 protein levels in cell-free conditioned

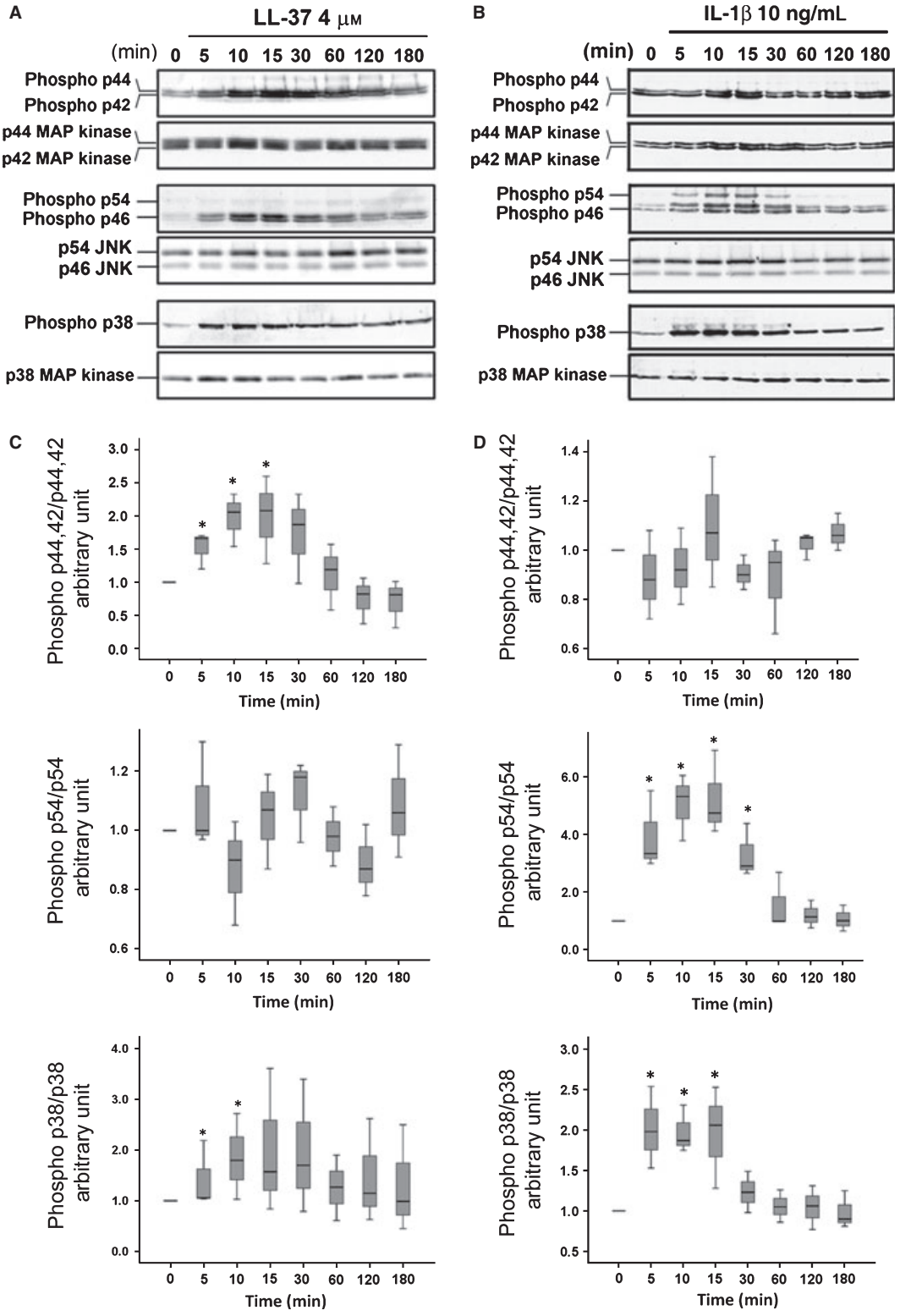


*Fig. 2.* Phosphorylation of MAP kinase pathways by LL-37 or interleukin-1β (IL-1β) treatment in human gingival fibroblasts (HGFs). HGFs were treated with (A) 4 μM LL-37 or with (B) 10 ng/mL of IL-1β for 0–180 min (min). Forty micrograms of whole-cell lysates were resolved by electrophoresis on a 12% sodium dodecyl sulfate gel and probed with antibodies against p44/42, JNK (p54/46), p38, or each phosphorylated form (phospho). (C and D) The densitometric analyses were conducted to measure the intensities of each phosphorylated MAP kinase relative to those of its total MAP kinase from samples in (A) and (B), respectively, and the ratios are presented as an arbitrary unit on the *y*-axis. The data in box plots are presented as medians with 25th and 75th percentiles, and $n = 6$ for each cell datum (*$p < 0.05$).

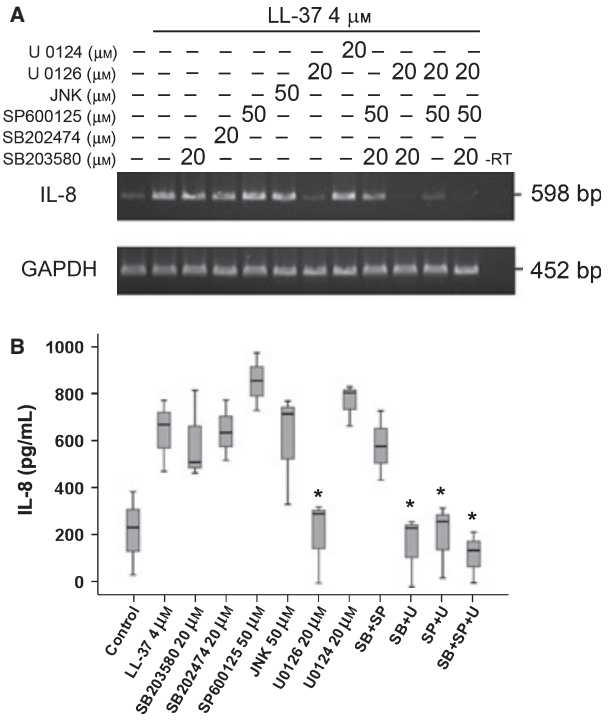


*Fig. 3*. Involvement of p44/42 MAP kinases in interleukin-8 (IL-8) up-regulation. (A) Human gingival fibroblasts (HGFs) were pretreated with indicated doses of the specific inhibitor of each MAP kinase pathway, its inactive analog, or various combinations of SB203580 (SB), SP600125 (SP) and U0126 (U), for 30 min before treatment with 4 μM LL-37 for 12 h. Total RNA isolation and RT-PCR were performed. –RT, negative control where the reverse transcriptase was omitted. (B) The IL-8 levels in cell-free culture supernatants from (A) were assayed by ELISA. Data in box plots are presented as medians with 25th and 75th percentiles, and $n = 6$ for each cell datum (*$p < 0.05$).

media of LL-37-treated samples were elevated in a dose-dependent manner, with a significant increase observed in the samples treated with 8–10 μM of LL-37 for 6 h (Fig. 1C). As a positive control, IL-8 mRNA was up-regulated by 0.1–10 ng/mL of IL-1β (Fig. 1D). The time-course study demonstrated an obvious and significant induction of IL-8 mRNA as early as 3 h after treatment of HGFs with LL-37 ($p < 0.05$) (Fig. 1E and 1F), and IL-8 mRNA induction became maximal at 12 h (Fig. 1F). However, this induction decreased to almost baseline control levels of IL-8 expression by 24 h of treatment with LL-37, indicating a transient up-regulation of IL-8 (Fig. 1E and 1F). Consistent with the transient induction of IL-8 mRNA, the IL-8 levels were significantly raised ($p < 0.05$) and reached their maxima after 12 h of cell treatment, and the levels then decreased at 24 h (Fig. 1G). Consequently, for subsequent experiments on cell activation, the conditions of 4 μM LL-37 for 12 h of stimulation were selected.

### IL-8 induction by LL-37 is dependent on MEK1/2-dependent p44/42 MAP kinases

As LL-37 was previously shown to activate MAP kinase pathways in airway epithelial cells (8) and monocytes (22), the involvement of MAP kinases in IL-8 induction by LL-37 in HGFs was examined. HGFs were treated with either 4 μM LL-37 or 10 ng/mL of IL-1β for the indicated periods of time (0–180 min), or untreated, and both phosphorylated and total forms of p38, p44/42 and JNK MAP kinases were determined by Western blot analysis, using specific antibodies. After treatment with LL-37, transient phosphorylation was observed of components of all three MAP kinase pathways, including p44/42 MAP kinases, p38 MAP kinase and p46 JNK, of approximately 5–60 min' duration (Fig. 2A). However, unlike the phosphorylation of p54 JNK by *Fusobacterium nucleatum* cell wall extract in human gingival epithelial cells (21) or by IL-1β in HGFs (Fig. 2B), p54 JNK was not phosphorylated by LL-37 in HGFs (Fig. 2A). This was confirmed by densitometric analyses, which showed a transient and significant increase in the phosphorylation of p44/42 and p38 MAP kinases by LL-37 treatment, while no significant increase in the phosphorylation of p54 MAP kinase was observed (Fig. 2C). In contrast, a transient and significant phosphorylation of p54 and p38 MAP kinases was observed in IL-1β-treated HGFs (Fig. 2D).

To further investigate the role of each MAP kinase in the LL-37-stimulated up-regulation of IL-8, HGFs were pretreated for 30 min with SB203580, SP600125 or U0126, with combinations of these inhibitors, or with each of their inactive analogs, before treatment with LL-37 for 12 h.

*Table 2*. Survival analyses of human gingival fibroblasts (HGFs) treated with different concentrations of LL-37

| Conditions | Percentage cell survival |
|---|---|
| Control | 100 |
| LL-37 1 μM | 102.76 ± 9.3 |
| LL-37 2 μM | 106.18 ± 14.6 |
| LL-37 4 μM | 94.49 ± 8.7 |
| LL-37 6 μM | 97.97 ± 16.4 |
| LL-37 8 μM | 98.31 ± 9.7 |
| LL-37 10 μM | 91.14 ± 6.3 |
| LL-37 20 μM | 47.52 ± 8.9* |
| LL-37 30 μM | 27.87 ± 6.1** |
| $H_2O_2$ 5 mM | 22.36 ± 1.5** |

The HGFs were treated with LL-37 at the doses indicated, or with $H_2O_2$ (a positive control for cell toxicity) for 24 h. After treatment, the MTT assay was conducted to determine the percentage of cell survival in treated HGFs in comparison to that in untreated HGFs (control). Each of three separate experiments was performed in triplicate, and the results were expressed as means ± standard deviations.
*Statistically significant difference from untreated HGFs at $p < 0.05$.
**Statistically significant difference from untreated HGFs at $p < 0.01$.

It was demonstrated that up-regulation of expression of IL-8 mRNA and protein by LL-37 was significantly inhibited by 20 μM U0126, a highly potent inhibitor of MEK1 and MEK2 (upstream molecules of p44/42 MAP kinases), and by all combinations that included U0126 ($p < 0.05$), whereas its inactive analog (U0124) did not block IL-8 up-regulation (Fig. 3A and 3B). It is interesting to note that induction of IL-8 by LL-37 was minimally blocked by 20 μM SB203580 but was not blocked at all by 50 μM SP600125, or by their inactive analogs, SB202474 and -JNK (Fig. 3A and 3B). Taken together, all of these results suggest the involvement of MEK1/2-dependent p44/42 MAP kinases in the LL-37-stimulated induction of IL-8 in HGFs.

### IL-8 induction by LL-37 is mediated by the $P2X_7$ purinergic receptor

To examine the receptor that mediates the induction of IL-8 by LL-37 in HGFs, two potential candidate receptors, including EGFR and purinergic receptors, which have been shown to be involved in IL-8 up-regulation by LL-37 in airway epithelial cells (8) and smooth muscle cells (9), were initially explored. HGFs were pretreated with various doses (0.3–10 μM) of AG1478, a selective tyrosine kinase inhibitor of EGFR, for 30 min before treatment with LL-37 for 12 h. It was demonstrated that none of the tested doses of AG1478 inhibited IL-8 mRNA induction in HGFs (Fig. 4A), suggesting that EGFR has no role in mediating IL-8 induction by LL-37 in HGFs, and that an investigation for EGFR expression in HGFs is not necessary. We then investigated the possible role of P2 purinergic receptors, which exist as two distinct families, including the P2X ligand-gated ionotropic channel family, which consists of seven members, and the P2Y metabotropic heptahelical G-protein-coupled-receptor family, which consists of at least four known members, in IL-8 up-regulation by LL-37. HGFs were pretreated with either suramin, a broad-spectrum antagonist of P2 purinergic receptors (9), or NF279, a selective P2X inhibitor (23), at the indicated doses for 30 min, before treatment with LL-37 for 12 h. It was found that both inhibitors, particularly 10 and 100 μM of suramin and 1 and 10 μM of NF279, could significantly abrogate the expression of IL-8 mRNA and protein ($p < 0.05$) in a dose-dependent manner (Fig. 4B and 4C), suggesting the involvement of P2X purinergic receptors in IL-8 up-regulation by LL-37 in HGFs, similarly to their involvement in human airway smooth-muscle cells (9).

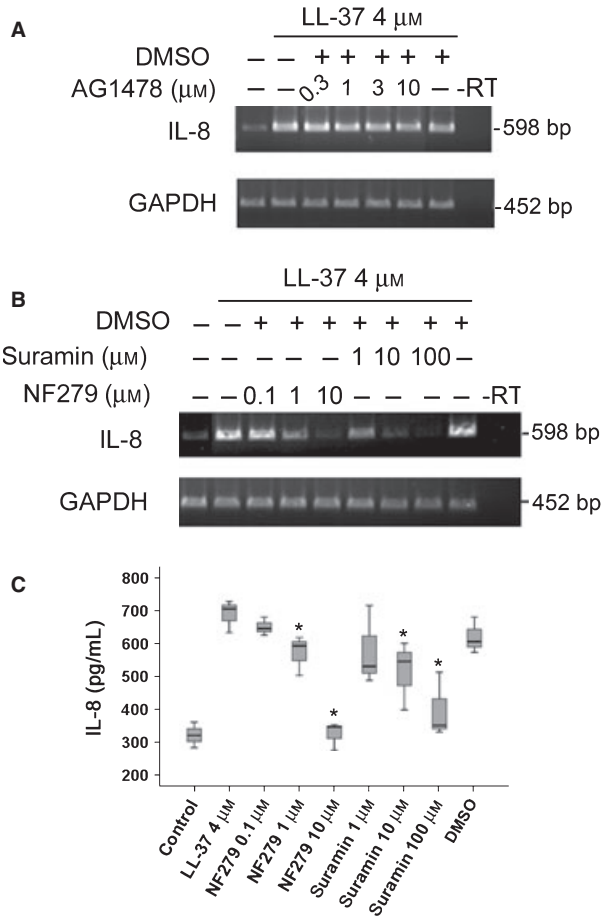


*Fig. 4.* Involvement of P2X receptors, but not an EGF receptor, in interleukin-8 (IL-8) induction. (A) Human gingival fibroblasts (HGFs) were pretreated with the indicated doses of AG1478 for 30 min before treatment with 4 μM LL-37 for 12 h. Dimethylsulfoxide (DMSO) was used as a solvent control, at a final concentration in the culture medium of < 0.1% (vol/vol). Total RNA isolation and RT-PCR were performed. (B) HGFs were pretreated with the indicated doses of either NF279 or suramin for 30 min before treatment with 4 μM LL-37 for 12 h. DMSO was used as a solvent control. (C) The IL-8 levels in cell-free culture supernatants from (B) were assayed using an ELISA. The data in box plots are presented as medians with 25th and 75th percentiles, and $n = 6$ for each cell datum (*$p < 0.05$).

Moreover, expression of all seven members of the P2X receptor family was examined at both mRNA and protein levels. It was shown that $P2X_4$ and $P2X_7$ mRNAs were constitutively expressed, while $P2X_1$ mRNA was minimally expressed and other P2X receptors were not expressed in HGFs (Fig. 5A). However, expression of the $P2X_4$ protein at the expected size (around 70 kDa) was weak, compared with much stronger expression of the $P2X_7$ protein at the predicted size (70 kDa) in the same cell (Fig. 5B), implying the significance of $P2X_7$ in the up-regulation of IL-8 by LL-37 in HGFs. It was interesting to note that intense immunoreactive bands with the primary antibody against the $P2X_4$ receptor were detected at approximately 100 and

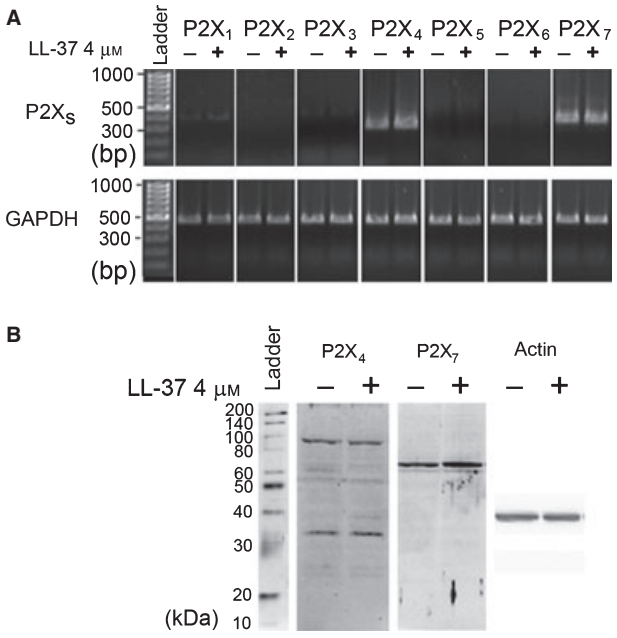


*Fig. 5.* Constitutive expression of the $P2X_7$ receptor in human gingival fibroblasts (HGFs). (A) HGFs were treated with 4 μM LL-37 (+), or were left untreated (–). Total RNA isolation and RT-PCR were performed. Primer sequences and conditions of $P2X_1$ to $P2X_7$ were as described in Table 1. The expected sizes of PCR products were as predicted. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was similar in all treatment conditions. Note that $P2X_4$ and $P2X_7$ mRNAs were expressed more strongly than mRNAs for other P2X receptors. (B) Forty micrograms of whole-cell lysates from the same experimental design as (A) were resolved by electrophoresis on a 12% sodium dodecyl sulfate–polyacrylamide gel and reacted with the antibody against $P2X_4$, $P2X_7$, or actin, a housekeeping gene control. Note the presence of immunoreactive bands of the $P2X_7$ receptor at the expected size of around 70 kDa. The data shown are representative of three independent experiments.

30 kDa (Fig. 5B). This was in agreement with the finding from a previous study that demonstrated the *in vivo* expression of the $P2X_4$ receptor in the gingiva, mainly in fibroblasts, by immunohistochemical staining (24). To further verify the role of the $P2X_7$ receptor in the induction of IL-8 by LL-37, HGFs were pretreated with either various doses of BBG or 10 μg/mL of the neutralizing antibody against $P2X_7$ before treatment with 4 μM LL-37 for 12 h. It was demonstrated that both BBG and the neutralizing antibody blocked IL-8 mRNA up-regulation in a dose-dependent manner (Fig. 6A) and significantly abrogated the induction of IL-8 protein by LL-37 ($p < 0.05$) (Fig. 6B).

## Discussion

In this study, we demonstrated that LL-37 can induce IL-8 mRNA and protein expression via the $P2X_7$ receptor and the MEK1/2-dependent p44/42 MAP kinase pathway in HGFs. This result corresponds well with the induction of IL-8 by LL-37 in several other types of nonimmune cells, including airway epithelial cells (8) and smooth muscle cells (9) in the respiratory tract and in skin keratinocytes (10). On the other hand, LL-37 does not alter the release of IL-8 in macrophages (25) and even inhibits IL-8 production by serum amyloid A in human neutrophils (26), reflecting the nature of distinct responses to LL-37 among different cell types. The involvement of the $P2X_7$ receptor, but not of EGFR, in the induction of IL-8 in gingival fibroblasts, differs from that of EGFR in airway epithelial cells (8) and in skin keratinocytes (10), but is similar to that of purinergic receptors in smooth muscle cells (9), indicating the same cell lineage between smooth muscle cells and fibroblasts and the dependence on each specific cell type for receptor mediation of the inducible effect on chemokine expression by LL-37.

In periodontal tissue, although gingival epithelial cells do express and may release LL-37 into saliva and gingival crevicular fluid, LL-37 is present in much higher amounts in granules of neutrophils that transmigrate through the junctional epithelium into the gingival sulcus during the inflammatory state. Consequently, LL-37, detected in the junctional epithelium by immunohistochemistry, appears to be the product of neutrophil migration through the tissue rather than of the epithelial cells *per se* (27). Upon activation by the inflammatory state, neutrophils can release hCAP18 into the periodontal connective tissue, which can be activated by proteinase 3 into a mature LL-37 peptide and then accumulate in the tissue in sufficient amounts to activate gingival fibroblasts in a paracrine manner. Therefore, consistent with our *in vitro* results, both IL-8 and LL-37 levels have been shown to be elevated in gingival crevicular fluid from patients with periodontitis (28,29) and to correlate positively with the depth of the gingival crevice (29).

The LL-37 concentrations used in the present and in other studies (8–10, 22), ranging from 1 to 10 μM, and which were not toxic to HGFs, may be appropriate for evaluating the physiological roles of LL-37 *in vivo*, because high concentrations of hCAP18 (ranging from 8.4 to 28.6 μM) have been detected in seminal plasma from 10 healthy donors (30). Furthermore, the concentrations of LL-37 can show a massive increase, of up to 300 μM, in psoriatic skin lesions (31). In the oral cavity, the concentrations of LL-37 in a diluted solution, such as saliva, are estimated to be 0.32 μM (32), equivalent to those of the airway fluid, yet the precise levels of LL-37 in a more concentrated solution (i.e. gingival crevicular fluid) have not been quantified. However, it was demonstrated that the levels of LL-37 in gingival crevicular fluid were elevated severalfold and were significantly higher in chronic

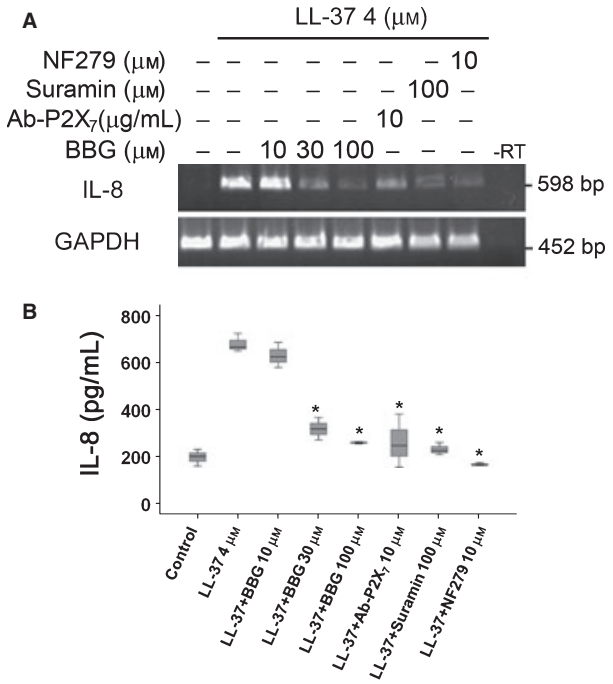


*Fig. 6.* Involvement of $P2X_7$ receptor in interleukin-8 (IL-8) up-regulation. (A) Human gingival fibroblasts (HGFs) were pretreated with the indicated doses of Brilliant Blue G (BBG), the $P2X_7$ neutralizing antibody (Ab-$P2X_7$), suramin, or NF279 for 30 min before treatment with 4 μM LL-37 for 12 h. Total RNA isolation and RT-PCR were performed. – RT, negative control where the reverse transcriptase was omitted. (B) The IL-8 levels in cell-free culture supernatants from (A) were assayed by ELISA. The data in box plots are presented as medians with 25th and 75th percentiles, and $n = 6$ for each cell datum (*$p < 0.05$).

periodontitis compared with those in healthy controls (5). Consequently, the concentrations of LL-37 are likely to be low (i.e. lowμM levels) within oral mucosal tissue during the pathophysiological processes of periodontal disease.

As prolonged activation of the $P2X_7$ receptor leads to apoptotic cell death in mesangial cells (33), it is likely that treatment with toxic doses of LL-37 (> 20 μM) results in a significant reduction in the percentage of cell survival by induced cell death via $P2X_7$ activation, as shown in another study (34). Therefore, the role of high doses of LL-37 in cell death, via possible $P2X_7$ activation in gingival fibroblasts as well as in other cell types, should be the subject of further investigations.

In this study, IL-8 induction by LL-37 was completely abrogated by pretreatment with U0126, a selective inhibitor of MEK1 and MEK2, which can phosphorylate the p44/42 MAP kinases. This is in agreement with the transient phosphorylation of p44/42 MAP kinases by treatment with LL-37. Although LL-37 could phosphorylate p38 MAP kinase and p46 JNK, pretreatment with SB203580 or SP600125 did not inhibit IL-8 up-regulation in HGFs, indicating the sole involvement of the MEK1/2-dependent p44/42 MAP kinase pathway in IL-8 induction by LL-37 in this cell type. These findings correspond with others that demonstrate IL-8 up-regulation by LL-37 via ERK1/2 in human airway epithelial cells (8) and smooth muscle cells (9), but differ from one study that revealed involvement of both p44/42 and p38 MAP kinases in IL-8 up-regulation by LL-37 in human monocytes (22). This discrepancy may be explained by the unique nature of each individual cell type in signaling gene expression, even in response to the same agent. Furthermore, it remains to be elucidated whether activator protein-1 is involved as a transcription factor in IL-8 up-regulation by LL-37 in gingival fibroblasts, similarly to its implication in skin keratinocytes (35). It would also be interesting to further investigate the role of nuclear factor-κB (NF-κB) in IL-8 expression, as it has been reported that LL-37 can induce NF-κB activation in human bronchial epithelial cells (36) and the NF-κB pathway is also essential for IL-8 production.

In this study, we showed the involvement of the $P2X_7$ receptor in the LL-37-stimulated induction of IL-8 by HGFs using NF279, previously reported as a selective $P2X_1$ receptor antagonist (37), which has very recently been demonstrated also to fully inhibit the human $P2X_7$ receptor (38). Furthermore, the role of the $P2X_7$ receptor in IL-8 induction is suggested by our findings that have shown not only much higher expression of both $P2X_7$ mRNA and protein at the expected size (around 70 kDa; 39) but also minimal and insignificant mRNA expression of the $P2X_1$ receptor in gingival fibroblasts. In line with these observations, pretreatment of HGFs with BBG (the selective antagonist of the $P2X_7$ receptor) and the $P2X_7$ neutralizing antibody (which binds to the extracellular portion of the receptor) (40) can considerably inhibit IL-8 induction by LL-37. Taken together, our study suggests that the $P2X_7$ receptor is involved in IL-8 up-regulation by LL-37 in gingival fibroblasts, similarly to its suggested involvement in IL-8 induction by LL-37 in airway smooth muscle cells (9).

The involvement of $P2X_7$ receptors in the LL-37-stimulated induction of IL-8 in HGFs is in agreement with an essential role of $P2X_7$ in the immune system, particularly its involvement in inflammation via the unique ability to induce the rapid activation of caspase-1 with subsequent release of pro-inflammatory cytokine IL-1β from activated macrophages and microglia (41,42). In addition, it has been demonstrated that LL-37 can directly activate the $P2X_7$ receptor by a mechanism that is independent of the release of endogenous ATP (25). Nevertheless, it has been previously demonstrated that activation of purinergic receptors with ATP can result in IL-8 up-regulation in

human keratinocytes (43). Therefore, it would be interesting to further determine whether treatment with LL-37 can cause an extracellular release of ATP that can bind to the $P2X_7$ receptor and induce IL-8 expression, or whether LL-37 can directly bind to the $P2X_7$ receptor in gingival fibroblasts. In addition, it has been reported that the $P2X_7$ receptor mediates the receptor–ligand interaction via the phosphorylation of several intracellular signaling molecules. This phosphorylation is blocked by pretreatment with AG126 (44), a tyrosine kinase inhibitor. This finding is consistent with phosphorylation of several MAP kinase pathways by treatment with LL-37, as observed in our study.

In summary, our findings suggest that LL-37 can induce IL-8 expression via the $P2X_7$ receptor and the MEK1/2-dependent p44/p42 MAP kinase pathway in HGFs, suggesting an indirect involvement of LL-37 in neutrophil recruitment and migration into an inflammatory site through the induction of IL-8, in addition to its direct effect on chemotactic movement of neutrophils via formyl peptide receptor-like 1 (45). Therefore, the antimicrobial peptide LL-37, similarly to its closely related molecule of the β-defensin antimicrobial peptide family (human β-defensin-3) (15), should be viewed as a multifunctional molecule, rather than as a simple antimicrobial peptide, that can play a pivotal role in alerting, mobilizing and amplifying the innate and adaptive immunity of the host to periodontal disease.

## Acknowledgements

We thank Dr M. Kevin O Carroll, Professor Emeritus of the University of Mississippi School of Dentistry, USA, and Faculty Consultant at Chiang Mai University Faculty of Dentistry, Thailand, for his critical reading of this manuscript. The support from the Thailand Research Fund to S.K. (grant no. RMU5380014) and V.R. (Senior Research Scholar Award), the Center of Excellence for Innovation in Chemistry (PERCH-CIC), and the National Science and Technology Development Agency (P-10-11290), Ministry of Science and Technology, Thailand, is gratefully acknowledged.

## References

1. SØrensen OE, Follin P, Johnsen AH *et al*. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood* 2001;**97:**3951–3959.
2. Niyonsaba F, Ogawa H. Protective roles of the skin against infection: implication of naturally occurring human antimicrobial agents β-defensins, cathelicidin LL-37 and lysozyme. *J Dermatol Sci* 2005;**40:** 157–168.
3. Dale BA, Fredericks LP. Antimicrobial peptides in the oral environment: expression and function in health and disease. *Curr Issues Mol Biol* 2005;**7:**119–133.
4. Frohm Nilsson M, Sandstedt B, SØrensen O, Weber G, Borregaard N, Ståhle-Bäckdahl M. The human cationic antimicrobial protein (hCAP-18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6. *Infect Immun* 1999;**67:**2561–2566.
5. Puklo M, Guentsch A, Hiemstra PS, Eick S, Potempa J. Analysis of neutrophil-derived antimicrobial peptides in gingival crevicular fluid suggests importance of cathelicidin LL-37 in the innate immune response against periodontogenic bacteria. *Oral Microbiol Immunol* 2008;**23:**328–335.
6. Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* 2004;**75:**39–48.
7. Scott MG, Davidson DJ, Gold MR, Bowdish D, Hancock RE. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J Immunol* 2002;**169:**3883–3891.
8. Tjabringa GS, Aarbiou J, Ninaber DK *et al*. The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. *J Immunol* 2003;**171:**6690–6696.
9. Zuyderduyn S, Ninaber DK, Hiemstra PS, Rabe KF. The antimicrobial peptide LL-37 enhances IL-8 release by human airway smooth muscle cells. *J Allergy Clin Immunol* 2006;**117:**1328–1335.
10. Braff MH, Hawkins MA, Di Nardo A *et al*. Structure-function relationships among human cathelicidin peptides: dissociation of antimicrobial properties from host immunostimulatory activities. *J Immunol* 2005;**174:**4271–4278.
11. Tonetti MS, Freiburghaus K, Lang NP, Bickel M. Detection of interleukin-8 and matrix metalloproteinases transcripts in healthy and diseased gingival biopsies by RNA/PCR. *J Periodontal Res* 1993;**28:** 511–513.
12. Sakuta T, Tokuda M, Tamura M *et al*. Dual regulatory effects of interferon-alpha, -beta, and -gamma on interleukin-8 gene expression by human gingival fibroblasts in culture upon stimulation with lipopolysaccharide from *Prevotella intermedia*, interleukin-1alpha, or tumor necrosis factor-alpha. *J Dent Res* 1998;**77:**1597–1605.
13. Baggiolini M, Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett* 1992;**307:**97–101.
14. den Hertog AL, van Marle J, van Veen HA *et al*. Candidacidal effects of two antimicrobial peptides: histatin 5 causes small membrane defects, but LL-37 causes massive disruption of the cell membrane. *Biochem J* 2005;**388:**689–695.
15. Chotjumlong P, Khongkhunthian S, Ongchai S, Reutrakul V, Krisanaprakornkit S. Human beta-defensin-3 up-regulates cyclooxygenase-2 expression and prostaglandin E2 synthesis in human gingival fibroblasts. *J Periodontal Res* 2010;**45:**464–470.
16. Krisanaprakornkit S, Chotjumlong P, Kongtawelert P, Reutrakul V. Involvement of phospholipase D in regulating expression of anti-microbial peptide human beta-defensin-2. *Int Immunol* 2008;**20:**21–29.
17. Krisanaprakornkit S, Kimball JR, Weinberg A, Darveau RP, Bainbridge BW, Dale BA. Inducible expression of human beta-defensin 2 by *Fusobacterium nucleatum* in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. *Infect Immun* 2000;**68:** 2907–2915.
18. Montreekachon P, Chotjumlong P, Reutrakul V, Krisanaprakornkit S. Involvement of cytosolic phospholipase A2alpha in MMP-9 up-regulation. *J Dent Res* 2009;**88:**1031–1036.
19. Banfi C, Ferrario S, De Vincenti O *et al*. P2 receptors in human heart: upregulation of $P2X_6$ in patients undergoing heart transplantation, interaction with TNFalpha and potential role in myocardial cell death. *J Mol Cell Cardiol* 2005;**39:**929–939.
20. Solini A, Santini E, Chimenti D *et al*. Multiple P2X receptors are involved in modulation of apoptosis in human mesangial cells: evidence for a role of $P2X_4$. *Am J Physiol Renal Physiol* 2007;**292:** 1537–1547.
21. Krisanaprakornkit S, Kimball JR, Dale BA. Regulation of human beta-defensin-2 in gingival epithelial cells: the involvement of mitogen-activated protein kinase pathways, but not the NF-kappaB transcription factor family. *J Immunol* 2002;**168:** 316–324.

22. Bowdish DM, Davidson DJ, Speert DP, Hancock RE. The human cationic peptide LL-37 induces activation of the extracellular signal-regulated kinase and p38 kinase pathways in primary human monocytes. *J Immunol* 2004;**172:**3758–3765.
23. Damer S, Niebel B, Czeche S *et al*. NF279: a novel potent and selective antagonist of P2X receptor-mediated responses. *Eur J Pharmacol* 1998;**350:** R5–R6.
24. Binderman I, Bahar H, Jacob-Hirsch J *et al*. $P2X_4$ is Up-regulated in gingival fibroblasts after periodontal surgery. *J Dent Res* 2007;**86:**181–185.
25. Elssner A, Duncan M, Gavrilin M, Wewers MD. A novel $P2X_7$ receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. *J Immunol* 2004;**172:**4987–4994.
26. Lee HY, Kim SD, Shim JW, Lee SY, Yun J, Bae YS. LL-37 inhibits serum amyloid A-induced IL-8 production in human neutrophils. *Exp Mol Med* 2009;**41:**325–333.
27. Dale BA, Kimball JR, Krisanaprakornkit S *et al*. Localized antimicrobial peptide expression in human gingiva. *J Periodontal Res* 2001;**36:**285–294.
28. Tsai CC, Ho YP, Chen CC. Levels of interleukin-1 beta and interleukin-8 in gingival crevicular fluids in adult periodontitis. *J Periodontol* 1995;**66:**852–859.
29. Hosokawa I, Hosokawa Y, Komatsuzawa H *et al*. Innate immune peptide LL-37 displays distinct expression pattern from beta-defensins in inflamed gingival tissue. *Clin Exp Immunol* 2006;**146:**218–225.
30. Malm J, SØrensen O, Persson T *et al*. The human cationic antimicrobial protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa. *Infect Immun* 2000;**68:**4297–4302.
31. Ong PY, Ohtake T, Brandt C *et al*. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med* 2002;**347:**1151–1160.
32. Tao R, Jurevic RJ, Coulton KK *et al*. Salivary antimicrobial peptide expression and dental caries experience in children. *Antimicrob Agents Chemother* 2005;**49:** 3883–3888.
33. Schulze-Lohoff E, Hugo C, Rost S *et al*. Extracellular ATP causes apoptosis and necrosis of cultured mesangial cells via $P2Z/P2X_7$ receptors. *Am J Physiol* 1998;**275:**F962–F971.
34. Aarbiou J, Tjabringa GS, Verhoosel RM *et al*. Mechanisms of cell death induced by the neutrophil antimicrobial peptides alpha-defensins and LL-37. *Inflamm Res* 2006;**55:**119–127.
35. Wu S, Gao J, Dinh QT, Chen C, Fimmel S. IL-8 production and AP-1 transactivation induced by UVA in human keratinocytes: roles of D-alpha-tocopherol. *Mol Immunol* 2008;**45:**2288–2296.
36. Pistolic J, Cosseau C, Li Y *et al*. Host defence peptide LL-37 induces IL-6 expression in human bronchial epithelial cells by activation of the NF-kappaB signaling pathway. *J Innate Immun* 2009;**1:**254–267.
37. Rettinger J, Schmalzing G, Damer S, Müller G, Nickel P, Lambrecht G. The suramin analogue NF279 is a novel and potent antagonist selective for the $P2X_1$ receptor. *Neuropharmacology* 2000;**39:** 2044–2053.
38. Donnelly-Roberts DL, Namovic MT, Han P, Jarvis MF. Mammalian $P2X_7$ receptor pharmacology: comparison of recombinant mouse, rat and human $P2X_7$ receptors. *Br J Pharmacol* 2009;**157:**1203–1214.
39. Aga M, Johnson CJ, Hart AP *et al*. Modulation of monocyte signaling and pore formation in response to agonists of the nucleotide receptor $P2X_7$. *J Leukoc Biol* 2002;**72:**222–232.
40. Li J, Liu D, Ke HZ, Duncan RL, Turner CH. The $P2X_7$ nucleotide receptor mediates skeletal mechanotransduction. *J Biol Chem* 2005;**280:**42952–42959.
41. Ferrari D, Pizzirani C, Adinolfi E *et al*. The $P2X_7$ receptor: a key player in IL-1 processing and release. *J Immunol* 2006;**176:**3877–3883.
42. Di Virglio F. Liaisons dangereuses: $P2X_7$ and the inflammasome. *Trends Pharmacol Sci* 2007;**28:**465–472.
43. Pastore S, Mascia F, Gulinelli S *et al*. Stimulation of purinergic receptors modulates chemokine expression in human keratinocytes. *J Invest Dermatol* 2007;**127:**660–667.
44. Shemon AN, Sluyter R, Stokes L, Manley PW, Wiley JS. Inhibition of the human $P2X_7$ receptor by a novel protein tyrosine kinase antagonist. *Biochem Biophys Res Commun* 2008;**365:**515–520.
45. Yang D, Chertov O, Oppenheim JJ. Participation of mammalian defensins and cathelicidins in anti-microbial immunity: receptors and activities of human defensins and cathelicidin (LL-37). *J Leukoc Biol* 2001;**69:**691–697.