Favorable interleukin-8 induction in human gingival epithelial cells by the antimicrobial peptide LL-37

Pattanin Montreekachon,[1] Sirikul Nongparn,[1] Thanapat Sastraruji,[2] Sakornrat Khongkhunthian,[1] Nuttapol Chruewkamlow,[3,4] Watchara Kasinrerk,[3,4] and Suttichai Krisanaprakornkit[2]

Summary

Background: LL-37, the only member of the antimicrobial peptide cathelicidin family in humans, exerts a variety of biological activities, especially immunomodulation through either direct chemotactic activity or up-regulation of several cytokines and chemokines in various cell types. In this study, we aimed to determine the immunoregulatory effect of LL-37 on Th1/Th2 cytokine expression and production in human gingival epithelial cells (HGECs).

Methods: Cultured HGECs were treated with different concentrations of LL-37 for different numbers of times. The cytotoxicity of LL-37 was determined by an MTT assay. Total RNA was isolated for RT-PCR and real-time PCR analyses of cytokine expression. Cell-free culture supernatants were assayed for Th1/Th2 cytokine levels by a cytokine bead array.

Results: Out of eleven Th1/Th2 cytokines tested, treatment of HGECs with non-toxic doses of LL-37 (2-6 μM) significantly raised only IL-8 levels in the cell-free culture supernatants, when compared to control untreated cells ($P < 0.05$). Consistent with the elevated IL-8 levels, IL-8 mRNA expression was remarkably and significantly induced by LL-37 treatment ($P < 0.05$), when compared to the modest mRNA induction of other three cytokines, including IL-1β, IL-6, and TNF-α. The time-course study demonstrated a cumulative IL-8 mRNA induction by LL-37 treatment within a 24-hour interval.

Conclusions: These findings indicate that LL-37 favorably induces IL-8 expression and secretion in HGECs, suggesting both direct and indirect involvement of LL-37 in neutrophil recruitment into an inflammatory site within diseased periodontal tissues. *(Asian Pac J Allergy Immunol 2014;32:251-60)*

Introduction

Periodontal disease is characterized as a chronic infection of the tooth-supporting tissues, leading to their destruction and eventual tooth loss. It is well known that dental plaque microorganisms existing in the biofilms are a primary etiological factor for periodontal disease,[1] but the severity of disease is driven by complex interactions between plaque bacteria and host immune responses, which result in overwhelming inflammatory responses involving both the innate and the adaptive immune responses.

Human gingival epithelium, a component of the tooth-supporting structures, functions as a critical part of the innate immune response.[2] In recent years, it has been increasingly recognized that gingival epithelium not only serves as a physical barrier to microbial challenges but also plays an active role in sensing and initiating host immune responses. Gingival epithelial cells can sense the presence of microorganisms by various pattern recognition receptors that recognize a variety of conserved microbial motifs, collectively termed as pathogen-associated molecular patterns. Thereafter, they can initiate host immune responses by production of cytokines, chemokines and other innate host defense molecules, such as antimicrobial peptides,[3] which

From 1. Department of Restorative Dentistry and Periodontology

2. Department of Oral Biology and Diagnostic Sciences, Faculty of Dentistry

3. Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences

4. Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Sciences and Technology Development Agency at the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand

Corresponding author: Suttichai Krisanaprakornkit

E-mail: suttichai.k@cmu.ac.th, suttichaikris@yahoo.com

are crucial for triggering both innate and adaptive immunity.

LL-37 is the only antimicrobial peptide that belongs to the cathelicidin family which occurs in humans.[4] LL-37, containing 37 amino acid residues and beginning with two leucine residues, is derived from the proteolytic cleavage at the C-terminal end of human cationic antimicrobial peptide 18. It is predominantly found in various tissues and fluids, such as airway surface fluid, gastric fluid, plasma and saliva, and is actively secreted from several types of cell, including neutrophils, keratinocytes and epithelial cells.[4] It has a broad range of antimicrobial activities typical of cathelicidin peptides.[5] Furthermore, LL-37 can exert the immunomodulatory effects on several immune and non-immune cells. For example, LL-37 can directly induce the chemotactic movement of neutrophils.[6] In addition, LL-37 can induce interleukin-8 (IL-8) expression via the purinergic $P2X_7$ receptor in gingival fibroblasts.[7] These suggest both direct and indirect involvement of LL-37 in neutrophil recruitment. LL-37 can also induce cyclooxygenase-2 expression and prostaglandin E_2 production in gingival fibroblasts,[8] which may be essential for initiating oral mucosal inflammation in periodontal disease.

Although the pro-inflammatory effect of LL-37 in up-regulation of IL-8 and of cyclooxygenase-2 is clearly demonstrated in gingival fibroblasts as mentioned above, the immunoregulatory effect of LL-37 in human gingival epithelial cells (HGECs) has not yet been investigated. Moreover, it is known that a complex network of cytokines has been considered to be important molecules in the inflammatory and immune responses during the progression of periodontal disease. However, it is still unclear whether or not LL-37 plays any role in controlling the expression and production of these cytokines within periodontal tissue, especially from human gingival epithelium. In this study we therefore aimed to investigate the immunoregulatory effect of LL-37 on Th1/Th2 cytokine expression and production in HGECs.

Methods

Reagents

LL-37 peptide (LLGDFFRKSKEKIGKEFKRIV QRIKDFLRNLVPRTES), a generous gift from Dr. Jan GM Bolscher, Academic Centre for Dentistry Amsterdam (ACTA), the Netherlands, was synthesized by Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry using a MilliGen 9050 peptide synthesizer (MilliGen/Biosearch, Bedford, Massachusetts, USA), as described previously.[9] Peptides were purified by preparative RP-HPLC. The purity of the peptides was at least 95% and the authenticity of the peptides was confirmed by a Microflex LRF matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer, equipped with an additional gridless reflectron (Bruker Daltonik, Bremen, Germany). Phorbol 12-myristate 13-acetate (PMA), a potent epithelial activator, was obtained from Sigma–Aldrich (St. Louis, Missouri, USA); interleukin-1β (IL-1β) was bought from R&D Systems, Inc. (Minneapolis, Minnesota, USA).

Culture of human gingival epithelial cells

HGECs were isolated from normal gingival tissue overlying impacted third molars from five different donors ($n = 5$), which were randomly recruited from the Department of Oral and Maxillofacial Surgery, Chiang Mai University, Chiang Mai, Thailand.[10] The research protocol was approved by the Human Experimentation Committee (#31/2008), Faculty of Dentistry, Chiang Mai University, and informed consent was obtained. Briefly, the gingival epithelium was separated from gingival connective tissue by 0.5 mg/ml Thermolysin® (Sigma-Aldrich, St. Louis, Missouri, USA) in HEPES-buffered saline for 90 min at 37ºC. After enzymatic separation, the epithelium was lifted off, and the epithelial sheets were further incubated in 5 ml of trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA; Invitrogen™, Grand Island, New York, USA) at 37ºC for 10 min, and the trypsinization was stopped by addition of an equal amount of DMEM (Invitrogen™), supplemented with 10% fetal bovine serum (Invitrogen™). The cell pellets were collected and resuspended in selective serum-free keratinocyte growth medium (BioWhittaker Inc., Walkersville, Maryland, USA), supplemented with human recombinant epidermal growth factor, hydrocortisone, bovine insulin, bovine pituitary extract, gentamycin sulfate, amphotericin B, and a low calcium concentration (0.03 mM). Resuspended epithelial cells, plated in 6-well plates (Corning, New York, USA) and grown in a humidified chamber at 37ºC and 5% CO_2, were treated with various doses of LL-37 for different numbers of times. Treatment with IL-1β or PMA served as positive controls.

MTT Assay

To examine 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, *i.e.*, succinate dehydrogenase, which can only occur in metabolically active cells. Cultured HGECs were seeded in a 96-well plate (Nunc™, Roskilde, Denmark) and incubated with various doses of LL-37 (0-30 μM) for 24 h. Subsequently, the medium was removed and 20 μl of MTT stock dye solution (5 mg/ml in PBS) (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 formazan crystals. The plate was shaken for 10 min and the optical density was read at 540 nm with a reference wavelength at 630 nm using the Titertek Multiskan M340 multiplate reader (ICN Flow, Costa Mesa, California, USA). Each sample was assayed in triplicate.

Cytokine bead array

Cell-free conditioned media from HGECs were collected and further analyzed for the levels of Th1 and Th2 cytokines, including IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF-α and TNF-β, by the Human Th1/Th2 11plex FlowCytomix Multiplex kit (FlowCytomix™, eBioscience® Vienna, Austria) together with flow cytometry. Briefly, a 25-μl quantity of each sample was added to the 96-well plate and 25 μl of bead mixture and 50 μl of biotin-conjugated mixture were then added. The plate was protected from the light and samples were incubated at room temperature for 2 h on a rotator [Gesellschaft für Labortechnik (GFL®), Burgwedel,

Germany] at 120 rpm. Subsequently, the plate was centrifuged by the microplate shaker at 2,500 rpm for 3 min. The supernatant was discarded and the plate was washed twice with 200 μl of the assay buffer and centrifuged by the microplate shaker at 2,500 rpm for 3 min. A100-μl quantity of assay buffer and 50 μl of streptavidin-phycoerythrin (PE) were added to all wells under light protection at room temperature for 1 h on a rotator at 120 rpm. The plate was washed again, as described above, and 200 μl of assay buffer was added to each well and transferred to FACS tubes. Thereafter, the assay buffer was added to each tube up to 400 μl and the cytokine concentrations were assessed by the FACSort flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA). The standard curves were generated by using reference concentrations, supplied by the manufacturer. The cytokine concentrations were calculated by the FlowCytomix™ Pro Software.

Total RNA Isolation and RT-PCR

HGECs were treated with non-toxic doses of LL-37 for various numbers of times and the total RNA was extracted using an Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, California, USA). The RT-PCR protocol has been previously described.[7] Briefly, two micrograms of total RNA were used for cDNA synthesis using a RevertAid™ Minus First Strand cDNA Synthesis kit (Thermo Scientific Life Science Research, Lafayette, Colorado, USA). The sequences of PCR primers and their conditions for IL-1β, IL-6, IL-8, TNF-α and GAPDH are summarized in Table 1. The PCR

Table 1. The sequences and annealing temperatures of oligonucleotide primers used in both RT-PCR and real time PCR analyses and the expected length [in base pairs (bp)] of each PCR product

	Primer sequence	Annealing Temperature (°C)	Product length (bp)
IL-1β	5'-CACGCTCCGGGACTCACAGC-3' (forward)	65.5	400
	5'-CTGGCCGCCTTTGGTCCCTC-3' (reverse)		
IL-6	5'-CGCCCCACACAGACAGCCAC-3' (forward)	65.5	417
	5'-AGCTTCGTCAGCAGGCTGGC-3' (reverse)		
IL-8	5'-TTTCTGATGGAGAGAGCTCTGTCTGG-3' (forward)	66.5	598
	5'-AGTGGAACAAGACTTGTGGATCCTGG-3' (reverse)		
TNF-α	5'-TTCTGCCTGCTGCACTTTGGA-3' (forward)	59.8	380
	5'-TTGATGGCAGAGAGGAGGTTG-3' (reverse)		
GAPDH	5'-ACCACAGTCCATGCCATCACTGC-3' (forward)	60.0	452
	5'-TCCACCACCCTGTTGCTGTAGC-3' (reverse)		

products were resolved on 1.2% agarose gel and photographs were taken by a CCD camera attached to a ChemiDoc XRS instrument (Bio-Rad Laboratories).

Real-time PCR

Real-time PCR was performed using 5% (vol/vol) of cDNA and the Maxima1 SYBR Green/ROX qPCR Master Mix (Thermo Scientific Life Science Research) and the Light Cycler 480 II (Roche Diagnostics, Ltd., Rotkreuz, Switzerland). The sequences of primers used in real-time PCR are shown in Table 1. The median ratio of IL-1β, IL-6, IL-8 and TNF-α expression relative to GAPDH expression in each sample was calculated from five separate experiments. The relative induction of each mRNA expression was determined by comparing the median ratio of an LL-37-treated sample with that of an untreated sample.

Statistical Analysis

The percentage of cell survival, cytokine expression from the real-time PCR, and cytokine concentrations from the fluorescent bead immunoassay were reported as mean ± standard deviation. The differences between LL-37-treated samples and a control sample in terms of the percentage of cell survival were determined using Student's t test. The differences between LL-37-treated samples and a control sample, in terms of cytokine expression and concentrations, were determined by One-way ANOVA with a Tukey test. Statistical analysis was performed with Statistics Package for the Social Sciences (SPSS) version 16.0. For all analysis, the P-values less than 0.05 were regarded as significant difference.

Results

LL-37 induced IL-8 production and secretion in HGECs

To determine the inducible effect of LL-37 on Th1/Th2 cytokine expression, we first investigated the non-toxic doses of LL-37 using the MTT assay. It was found that doses of LL-37 up to 6 μM did not significantly decrease the percentage of HGEC survival, whereas concentrations of LL-37 at 8 μM or greater significantly decreased the percentage of cell survival in comparison with untreated control HGECs ($P < 0.01$) (Figure 1). Therefore, the concentrations from 2 to 6 μM were chosen to treat HGECs in subsequent experiments.

We next treated HGECs with LL-37, IL-1β a pro-inflammatory cytokine, or PMA and the cell-free conditioned media were collected. It was demonstrated that there were only three cytokines whose levels were detectable in the conditioned media of HGECs, including IL-6, IL-8 and TNF-α, whereas the levels of remaining Th1/Th2 cytokines, including IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p70,
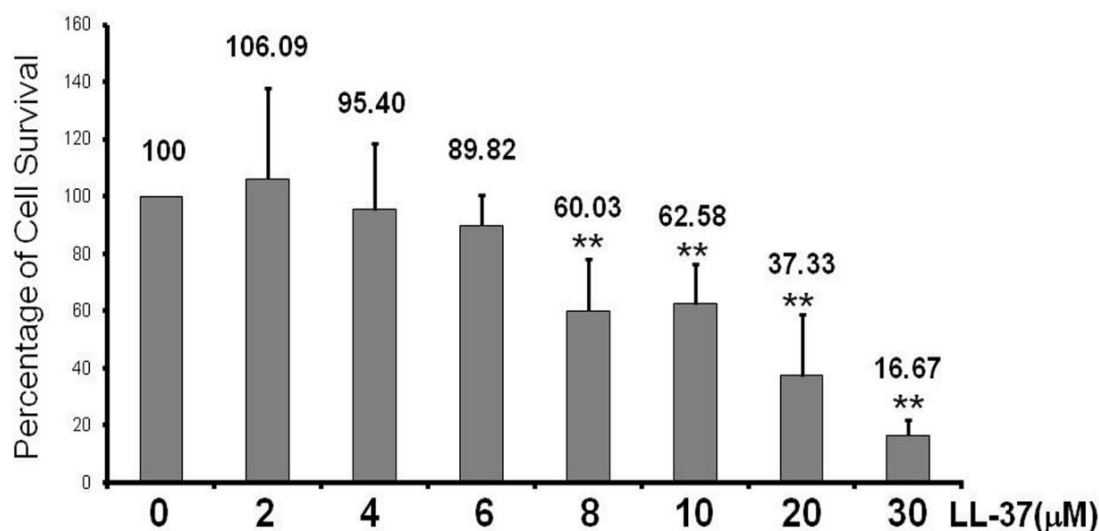

Figure 1. Survival analyses of human gingival epithelial cells (HGECs). HGECs were treated with LL-37 at the indicated doses for 24 h. After treatment, an MTT assay was conducted to determine the percentage of cell survival in treated HGECs, in comparison to that in untreated HGECs (control). Each of three separate experiments was performed in triplicate and the results were expressed as mean ± standard deviation. ** = statistically significant difference from untreated HGECs at P <0.01.

TNF-β, and IFN-γ, were not detectable in the conditioned media of both treated and untreated cells (data not shown), suggesting that HGECs are not the main cell type in the production of these cytokines. Moreover, LL-37 treatment preferentially induced significant production of IL-8 in HGECs with the maximal induction being observed at 4 μM ($P < 0.05$) (Figure 2A). Similarly, treatment with either IL-1β or PMA also significantly elevated IL-8

levels when compared to control untreated cells ($P < 0.05$), but in much greater levels than those induced by LL-37 (Figure 2A). In contrast to IL-8 induction, the levels of IL-6 and TNF-α released from LL-37-treated HGECs remained low and were not significantly enhanced when compared to those from the control untreated cells (Figure 2A). Note that PMA treatment could significantly enhance the levels of TNF-α ($P < 0.05$) in addition to the IL-8
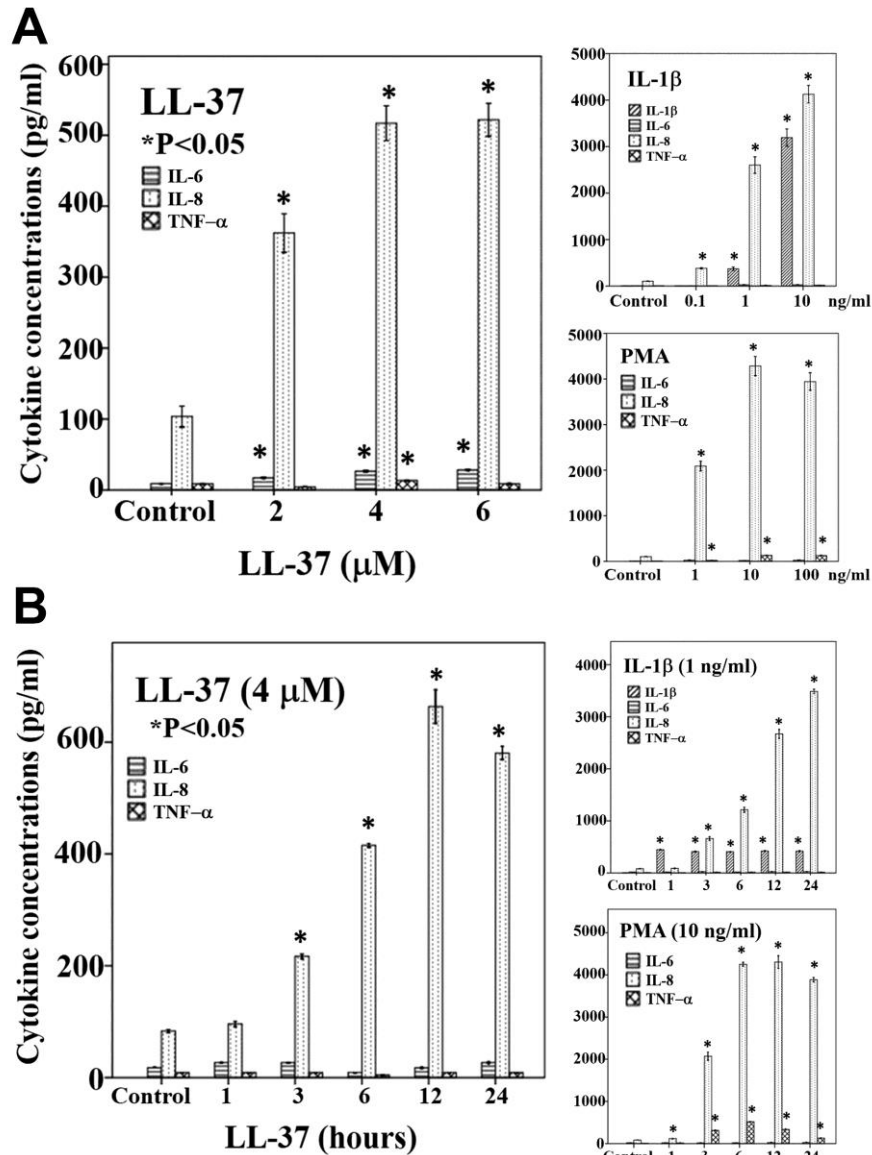

Figure 2. Th1/Th2 cytokine production by human gingival epithelial cells (HGECs). (A). HGECs were treated with indicated doses of LL-37, IL-1β or PMA overnight (16 h). (B). HGECs were treated with 4 μM of LL-37, 1 ng/ml of IL-1β or 10 ng/ml of PMA for various numbers of times. Cell-free conditioned media of HGECs were collected and further analyzed by the Human Th1/Th2 11plex FlowCytomix Multiplex kit. Data in bar graphs are presented as mean ± standard deviation, and N for each cell datum = 5. * = statistically significant difference between treated and control HGECs at $P < 0.05$. Note that IL-1β was only detected in IL-1β-stimulated HGECs, but not in LL-37- or PMA-treated HGECs, due to the exogenously-added IL-1β.

levels (Figure 2A). The time-course study showed different profiles of IL-8 induction between LL-37 treatment and IL-1β or PMA treatment. Specifically, LL-37 treatment resulted in significant IL-8 induction seen at 6 h, whereas IL-1β or PMA treatment did the same at 3 h (Figure 2B). Note that a transient and significant increase in TNF-α levels was shown by PMA treatment ($P < 0.05$), with the maximal increase being observed at 6 hours (Figure 2B).

Up-regulation of IL-8 mRNA by LL-37 treatment in HGECs

To determine mRNA expression of four cytokines whose levels were detectable in the conditioned media, including IL-1β, IL-6, IL-8 and TNF-α, HGECs were treated with various concentrations of LL-37, IL-1β or PMA overnight (16 hours). The RT-PCR analysis demonstrated that LL-37 induced mRNA expression of IL-1β, IL-6, IL-8 and TNF-α in a dose-dependent manner (Figure 3A). Further, mRNA expression of these four cytokines was up-regulated by treatment with 0.1–10 ng/ml of IL-1β or with 1-100 ng/ml of PMA (Figure 3A). The kinetic profiles of IL-8 mRNA induction by the three stimulants were different. Whereas a transient induction of IL-8 mRNA expression was observed in HGECs treated with PMA, cumulative induction of IL-8 mRNA expression was seen in both LL-37 and IL-1β treatment (Figure 3B), suggesting distinct intracellular signaling pathways mediating the inducible effect of IL-8 by different stimulants.

We further analyzed the levels of each cytokine induced by the quantitative real-time PCR analysis. Although LL-37 treatment could significantly up-regulate mRNA expression of all four cytokines, including IL-1β, IL-6, IL-8 and TNF-α mRNA in both dose- (Figure 4A) and time- (Figure 4B) dependent manners ($P < 0.05$), the induction of IL-8 mRNA was remarkable with an approximately 50 to100 fold increase, as compared to modest mRNA induction (up to 5 fold) of the other three cytokines, including IL-1β, IL-6, and TNF-α (Figures. 4A and 4B). This was consistent with the considerably elevated levels of IL-8 in the conditioned media of HGECs by LL-37 treatment, as compared to the low or unchanged levels of IL-6 and TNF-α in the conditioned media.

Discussion

In this study, we demonstrated that LL-37 exerted an inducible effect on mRNA expression of several cytokines in HGECs, including IL-1β, IL-6,
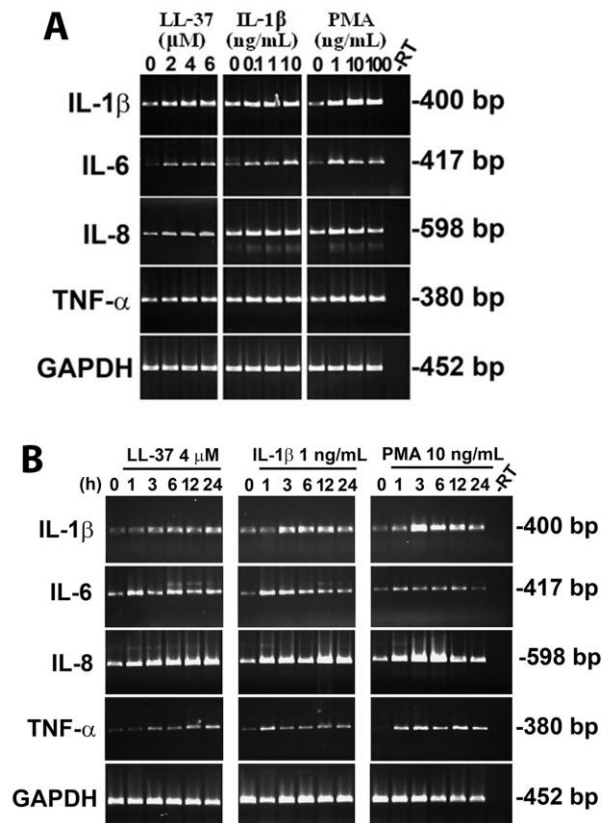

Figure 3. Th1/Th2 cytokine mRNA expression by human gingival epithelial cells (HGECs). (A). HGECs were treated with indicated doses of LL-37, IL-1β or PMA overnight (16 h). (B). HGECs were treated with 4 µM of LL-37, 1 ng/ml of IL-1β or 10 ng/ml of PMA for various numbers of times. RT-PCR was performed as described in Methods. GAPDH mRNA, serving as an internal control, was equally expressed. An -RT sample was a negative control in which the reverse transcriptase was omitted. The data shown in (A) and (B) are representative of 5 independent experiments. Note that some RT-PCR reactions in (A) reached a plateau level of PCR amplification, so the difference in terms of gene induction between the untreated control and LL-37-treated HGECs was not obvious.

IL-8 and TNF-α, but the greatest mRNA induction among these cytokines was IL-8. This result corresponds well with the induction of IL-8 by LL-37 in human airway smooth muscle cells,[11] and with the findings from two studies in human skin keratinocytes and in gingival fibroblasts that show the ability of LL-37 to up-regulate IL-8 mRNA and protein expression.[7,12] Moreover, another recent study has shown that low concentrations of LL-37
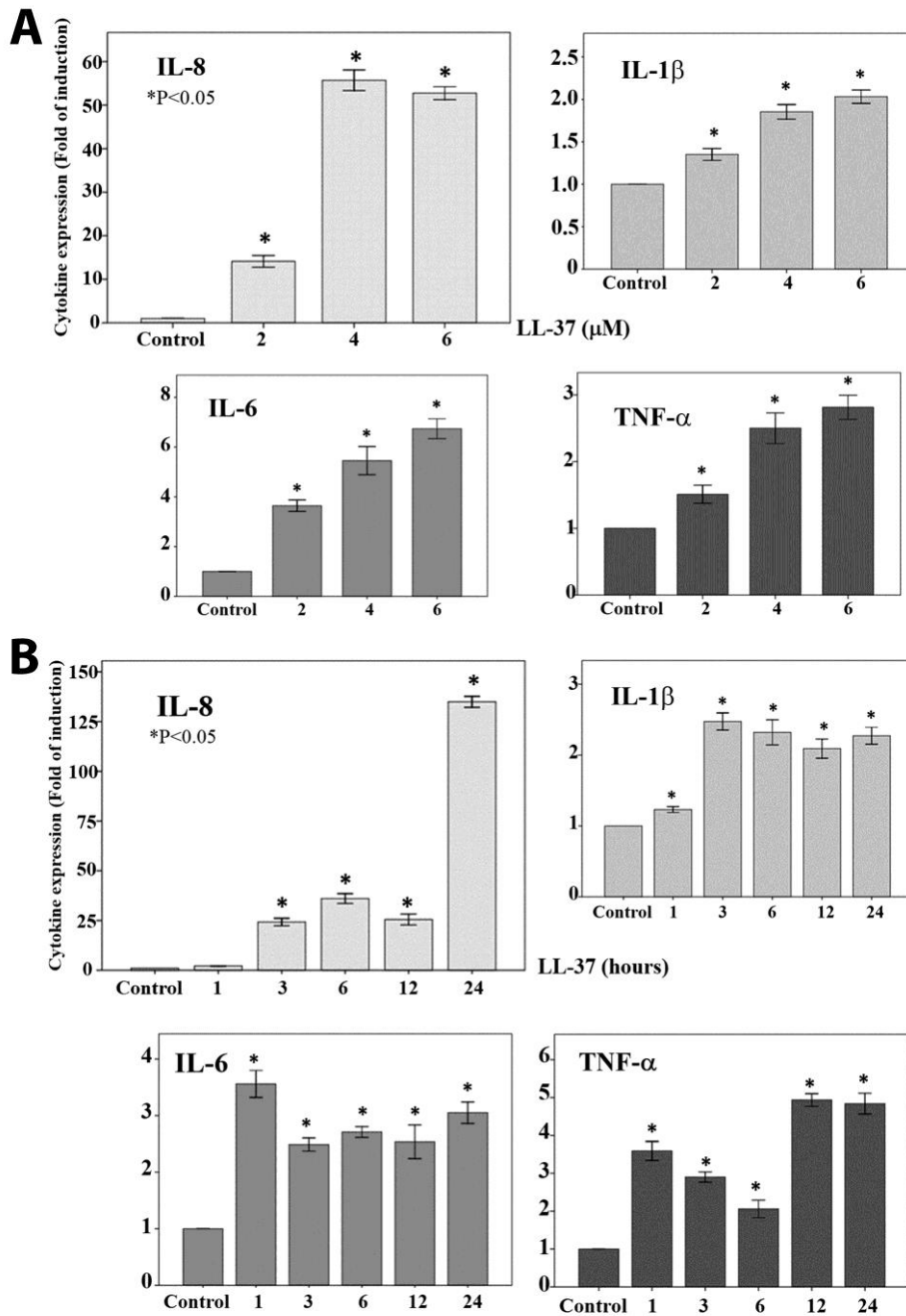
Figure 4. A quantitative analysis of cytokine mRNA induction by LL-37 treatment. (A). HGECs were treated with 0-6 µM of LL-37 overnight (16 h). (B). HGECs were treated with 4 µM of LL-37 for various numbers of times. Total RNA was isolated and a real-time PCR assay was conducted to quantify the degrees of cytokine mRNA induction. Data in bar graphs are presented as mean ± standard deviation, and N for each cell datum = 5 (*; P <0.05).

synergistically enhance the inducible effect of IL-1β on IL-8 production, both in proliferating and differentiating keratinocytes and in bronchial epithelial cells.[13] On the other hand, addition of LL-37 to LPS-primed monocytes has no influence on the release of IL-8, compared with the untreated LPS-primed monocytes,[14] reflecting the distinct responses to LL-37 treatment among different cell types.

It has been well recognized that the immunoregulatory control of Th1/Th2 cytokine profiles is fundamental to the pathogenesis of periodontal disease.[15] In the present study, we, therefore, attempted to determine the immunomodulatory

effect of LL-37 on the levels of Th1/Th2 cytokines by a cytokine array together with flow cytometric analysis. Our results demonstrated that, among eleven cytokines classified in the Th1 and Th2 profiles tested in our study, only the IL-8 levels in cell-free conditioned media of HGECs were detectable and dramatically elevated in response to LL-37 treatment, whereas the levels of IL-6 and TNF-α remained low and unchanged upon LL-37 treatment. These findings are consistent with the favorable induction of IL-8 mRNA by LL-37 treatment. In addition, they support the active and major function of HGECs in neutrophil migration and recruitment into the inflamed periodontal tissue by enhancement of IL-8 production in response to exposure to periodontal microorganisms[16] and the previously-reported function of LL-37 as an alarm signal, which has been shown to directly recruit neutrophils into the inflamed tissues[6] and to indirectly enhance expression of IL-8 in several different cell types.[7,11,13,17]

In periodontal tissues, LL-37 can be expressed in HGECs, but it is present in much higher amounts in specific 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*.[18] Upon activation by the inflammatory state, neutrophils can release hCAP18 into the periodontal connective tissue, which can be cleaved and activated by proteinase 3 into a mature LL-37 peptide and then accumulates in the tissue. Consequently, LL-37 levels are shown to be raised in gingival crevicular fluid (GCF) of patients with periodontitis and these levels correlate positively with the depth of the gingival crevice.[19]

The association between LL-37 deficiency and periodontitis is seen in syndromic periodontitis, including morbus Kostmann and Papillon-Lefèvre Syndrome,[20,21] and non-syndromic periodontitis, particularly aggressive periodontitis. In the syndrome-associated periodontitis, it is demonstrated that neutrophils from patients with morbus Kostmann are deficient in LL-37 and no LL-37 can be detected in plasma and saliva from these patients. Similarly, the impairment of cathepsin C function from a mutation of the *CTSC* gene that encodes cathepsin C results in the deficiency of the active form, LL-37. The deficiency of LL-37 is thus proven to cause repeated periodontal infections and severe periodontitis in

these two syndromes. In the non-syndromic periodontitis, it has been demonstrated that neutrophils from patients with aggressive periodontitis, but not chronic periodontitis, are deficient in LL-37 expression.[22] All of these studies point towards an important role of LL-37 as a major factor in alerting host innate immune defenses against periodontal pathogens,[23] possibly by exerting both a direct effect on promotion of neutrophil functions and an indirect inducible effect on IL-8 production in resident periodontal cells, especially in HGECs, as observed in our study. The lack of LL-37 may therefore cause severe breakdown of periodontal tissues seen in both syndromic and non-syndromic periodontitis.

The concentrations of LL-37 used in this study, ranging from 2 to 6 μM, which are not toxic to HGECs, are appropriate for evaluating the role of LL-37 in the pathogenesis of periodontal disease. This is because high concentrations of hCAP18/LL-37 (ranging from 8.4 to 28.6 μM) were measured in seminal plasma from ten healthy donors.[24] Moreover, it is reported that the concentrations of LL-37 in saliva are estimated to be 0.32 μM,[25] equivalent to the LL-37 levels in the airway fluid. However, the precise levels of LL-37 have not yet been quantified in more concentrated fluids like GCF. Nevertheless, it has been shown that the levels of LL-37 in GCF are elevated several fold and are significantly higher in patients with chronic periodontitis compared with those in healthy controls.[26,27] Consequently, it is likely that the concentrations of LL-37 are in the range of low μM within periodontal tissues during the pathophysiological processes of periodontal disease. Taken together, the selected concentrations in this study (up to 6 μM) are consistent with the detectable levels of LL-37 in GCF from patients with chronic periodontitis.[27]

Interestingly, we found mRNA expression of IL-1β by RT-PCR and real time PCR, but we were not able to detect IL-1β in cell-free culture supernatants. The possible explanation for the absence of IL-1β in cell-free culture supernatants may be owing to the limitation of fluorescent bead immunoassay, whose minimum detectable level is 4.2 pg/ml, but the IL-1β levels in cell-free culture supernatants might be less than this level. The other possible explanation is that it may be because of the fact that two required separate signals are required to produce and secrete mature IL-1β. The first signal initiated by bacterial PAMPs promotes intracellular production of immature IL-1β. The second signal,

derived from a danger signal, such as extracellular ATP, results in assembly of an inflammasome, activation of caspase-1, and secretion of mature IL-1β. It is probable that LL-37 treatment only provides the first signal but not the second, and cannot induce the formation of an inflammasome. In line with this explanation, Yilmaz and colleagues (2010)[28] report that *Porphyromonas gingivalis* can induce expression of the IL-1β gene and intracellular accumulation of IL-1β protein, but IL-1β is not secreted unless infected cells are subsequently stimulated with ATP. Therefore, it would be interesting to further determine whether or not HGECs can secrete IL-1β upon treatment with both LL-37 and extracellular ATP.

In conclusion, our results demonstrate that LL-37 treatment activates HGECs to induce mRNA expression of several cytokines, including IL-1β, IL-6, IL-8 and TNF-α. However, HGECs express and secrete IL-8 in much higher amounts than the other cytokines upon LL-37 treatment, suggesting a role for LL-37 as one of alarm signals for the host immune response by recruiting neutrophils to the site of infection through enhanced IL-8 production in HGECs. However, our *in vitro* study in a condition without the presence of bacteria may not mimic the pathogenesis of periodontal disease that mainly involves periodontal pathogens, since LL-37 has been previously shown to suppress production of pro-inflammatory cytokines, such as IL-6, IL-8, etc., in bacterially-stimulated gingival fibroblasts.[29] An improved insight into the mechanisms underlying the innate immune response will give us a better understanding of the immunopathogenesis of periodontal disease.

Acknowledgements

Conflicts of interest

All authors report no conflict of interest related to this study.

References

1. Dentino A, Lee S, Mailhot J, Hefti AF. Principles of periodontology. Periodontology 2000. 2013;61:16-53.

2. Jin L. An update on innate defense molecules of human gingiva. Periodontology 2000. 2011;56:125-42.

3. Dale BA. Periodontal epithelium: a newly recognized role in health and disease. Periodontology 2000. 2002;30:70-8.

4. Dürr UH, Sudheendra US, Ramamoorthy A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. Biochimica et biophysica acta. 2006;1758:1408-25.

5. De Smet K, Contreras R. Human antimicrobial peptides: defensins, cathelicidins and histatins. Biotechnology letters. 2005;27:1337-47.

6. Tjabringa GS, Ninaber DK, Drijfhout JW, Rabe KF, Hiemstra PS. Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. International archives of allergy and immunology. 2006;140:103-12.

7. Montreekachon P, Chotjumlong P, Bolscher JG, 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. Journal of periodontal research. 2011;46:327-37.

8. Chotjumlong P, Bolscher JG, Nazmi K, Reutrakul V, Supanchart C, Buranaphatthana W, et al. Involvement of the P2X7 purinergic receptor and c-Jun N-terminal and extracellular signal-regulated kinases in cyclooxygenase-2 and prostaglandin E2 induction by LL-37. Journal of innate immunity. 2013;5:72-83.

9. den Hertog AL, van Marle J, van Veen HA, Van't Hof W, Bolscher JG, Veerman EC, et al. Candidacidal effects of two antimicrobial peptides: histatin 5 causes small membrane defects, but LL-37 causes massive disruption of the cell membrane. The Biochemical journal. 2005;388(Pt 2):689-95.

10. Krisanaprakornkit S, Weinberg A, Perez CN, Dale BA. Expression of the peptide antibiotic human beta-defensin 1 in cultured gingival epithelial cells and gingival tissue. Infection and immunity. 1998;66:4222-8.

11. Zuyderduyn S, Ninaber DK, Hiemstra PS, Rabe KF. The antimicrobial peptide LL-37 enhances IL-8 release by human airway smooth muscle cells. The Journal of allergy and clinical immunology. 2006;117:1328-35.

12. Braff MH, Hawkins MA, Di Nardo A, Lopez-Garcia B, Howell MD, Wong C, et al. Structure-function relationships among human cathelicidin peptides: dissociation of antimicrobial properties from host immunostimulatory activities. Journal of immunology. 2005;174:4271-8.

13. Filewod NC, Pistolic J, Hancock RE. Low concentrations of LL-37 alter IL-8 production by keratinocytes and bronchial epithelial cells in response to proinflammatory stimuli. FEMS immunology and medical microbiology. 2009;56:233-40.

14. Elssner A, Duncan M, Gavrilin M, Wewers MD. A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37,

induces IL-1 beta processing and release. Journal of immunology. 2004;172:4987-94.

15. Gemmell E, Seymour GJ. Immunoregulatory control of Th1/Th2 cytokine profiles in periodontal disease. Periodontology 2000. 2004;35:21-41.

16. Tonetti MS. Molecular factors associated with compartmentalization of gingival immune responses and transepithelial neutrophil migration. Journal of periodontal research. 1997;32(1 Pt 2):104-9.

17. Tjabringa GS, Aarbiou J, Ninaber DK, Drijfhout JW, Sørensen OE, Borregaard N, et al. The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. Journal of immunology. 2003;171:6690-6.

18. Dale BA, Kimball JR, Krisanaprakornkit S, Roberts F, Robinovitch M, O'Neal R, et al. Localized antimicrobial peptide expression in human gingiva. Journal of periodontal research. 2001;36:285-94.

19. Hosokawa I, Hosokawa Y, Komatsuzawa H, Goncalves RB, Karimbux N, Napimoga MH, et al. Innate immune peptide LL-37 displays distinct expression pattern from beta-defensins in inflamed gingival tissue. Clinical and experimental immunology. 2006;146:218-25.

20. Pütsep K, Carlsson G, Boman HG, Andersson M. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. Lancet. 2002;360:1144-9.

21. de Haar SF, Hiemstra PS, van Steenbergen MT, Everts V, Beertsen W. Role of polymorphonuclear leukocyte-derived serine proteinases in defense against *Actinobacillus actinomycetemcomitans*. Infection and immunity. 2006;74:5284-91.

22. Türkoğlu O, Kandiloğlu G, Berdeli A, Emingil G, Atilla G. Antimicrobial peptide hCAP-18/LL-37 protein and mRNA expressions in different periodontal diseases. Oral diseases. 2011;17:60-7.

23. Lee SH, Jun HK, Lee HR, Chung CP, Choi BK. Antibacterial and lipopolysaccharide (LPS)-neutralising activity of human cationic antimicrobial peptides against periodontopathogens. International journal of antimicrobial agents. 2010;35:138-45.

24. Malm J, Sørensen O, Persson T, Frohm-Nilsson M, Johansson B, Bjartell A, 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. Infection and immunity. 2000;68:4297-302.

25. Tao R, Jurevic RJ, Coulton KK, Tsutsui MT, Roberts MC, Kimball JR, et al. Salivary antimicrobial peptide expression and dental caries experience in children. Antimicrobial agents and chemotherapy. 2005;49:3883-8.

26. 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 microbiology and immunology. 2008;23:328-35.

27. Türkoğlu O, Emingil G, Kütükçüler N, Atilla G. Gingival crevicular fluid levels of cathelicidin LL-37 and interleukin-18 in patients with chronic periodontitis. Journal of periodontology. 2009;80:969-76.

28. Yilmaz Ö, Sater AA, Yao L, Koutouzis T, Pettengill M, Ojcius DM. ATP-dependent activation of an inflammasome in primary gingival epithelial cells infected by *Porphyromonas gingivalis*. Cellular microbiology. 2010;12:188-98.

29. Inomata M, Into T, Murakami Y. Suppressive effect of the antimicrobial peptide LL-37 on expression of IL-6, IL-8 and CXCL10 induced by *Porphyromonas gingivalis* cells and extracts in human gingival fibroblasts. European journal of oral sciences. 2010;118:574-81.