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Biological

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The Antimicrobial Peptide, LL-37, Inhibits *in vitro* Osteoclastogenesis

ABSTRACT

Uncoupled bone resorption leads to net alveolar bone loss in periodontitis. The deficiency of LL-37, the only human antimicrobial peptide in the cathelicidin family, in patients with aggressive periodontitis suggests that LL-37 may play a pivotal role in the inhibition of alveolar bone destruction in periodontitis. We aimed to investigate a novel function of LL-37 in osteoimmunity by blocking osteoclastogenesis *in vitro*. Human osteoclast progenitor cells were isolated from a buffy coat of blood samples. The cells were cultured in the presence of various concentrations of LL-37 during an *in vitro* induction of osteoclastogenesis. Non-toxic doses of LL-37 could block multinuclear formation of the progenitor cells and significantly diminish the number of tartrate-resistant acid-phosphatase-positive cells and the formation of resorption pits ($p < 0.05$), whereas these concentrations induced cellular proliferation, as demonstrated by increased expression of proliferating cell nuclear antigen. Expression of several osteoclast genes was down-regulated by LL-37 treatment. It was demonstrated that nuclear translocation of nuclear-factor-activated T-cells 2 (NFAT2) was blocked by LL-37 treatment, consistent with a significant reduction in the calcineurin activity ($p < 0.005$). Collectively, our findings demonstrate that LL-37 inhibits the *in vitro* osteoclastogenesis by inhibiting the calcineurin activity, thus preventing nuclear translocation of NFAT2.

Abbreviations: CALCR, calcitonin receptor; ClC-7, chloride-proton exchanger; CTSK, cathepsin K; DAPI, 4′,6-diamidino-2-phenylindole; EGTA, ethylene glycol tetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; M-CSF/CSF1, macrophage-colony-stimulating factor; MMP-9, matrix metalloproteinase-9; MTT, [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; NFAT2, nuclear factor of activated T-cells 2; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; RANK, receptor activator of nuclear factor kappa-B; RANKL, receptor activator of nuclear factor kappa-B ligand; RT-PCR, reverse-transcription polymerase chain-reaction; TBS, Tris-buffered saline; TCIRG1, T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 subunit A3; TRAcP, tartrate-resistant acid phosphatase.

KEY WORDS: bone resorption, cathelicidin, innate immunity, nuclear factor of activated T-cells, osteoimmunity, periodontal disease.

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INTRODUCTION

LL-37, an antimicrobial peptide of the cathelicidin family, is synthesized by leukocytes and gingival epithelial cells (Dale *et al*., 2001; Zanetti, 2004). Previous studies have demonstrated antimicrobial actions of LL-37 against various periodontal micro-organisms (Ouhara *et al*., 2005; Ji *et al*., 2007). Furthermore, LL-37 displays immunomodulatory properties (Bowdish *et al*., 2006). Other biological actions of LL-37 include angiogenesis (Koczulla *et al*., 2003) and wound-healing promotion by inducing cell proliferation (Heilborn *et al*., 2003; Shaykhiev *et al*., 2005; Carretero *et al*., 2008). Some human disorders are associated with an aberrant expression of LL-37. For example, a morbus Kostman syndrome is caused by the lack of neutrophils (Pütsep *et al*., 2002), resulting in LL-37 deficiency that leads to repeated periodontal infections and severe alveolar bone resorption (Carlsson *et al*., 2006). Similarly, LL-37 cannot be detected in gingival crevicular fluid of patients with severe alveolar bone loss in aggressive periodontitis (Puklo *et al*., 2008). It was, therefore, hypothesized that LL-37 might negatively affect osteoclast formation and function.

Osteoclasts are derived from hematopoietic cells of monocyte/macrophage lineage that are induced by M-CSF (Tanaka *et al*., 1993) and RANKL (Jimi *et al*., 1999). The activation of dendritic cells derived from the same lineage as osteoclasts is inhibited by LL-37 (Kandler *et al*., 2006). However, it remains unknown whether LL-37 may also affect osteoclast formation and function. Since LL-37 deficiency is related to aggressive periodontitis, we hypothesized that LL-37 might inhibit osteoclastogenesis until LL-37 deficiency would cause the severe bone resorption observed in aggressive periodontitis.

MATERIALS & METHODS

Reagents

The synthesis and authenticity of LL-37 peptide were previously described (Montreekachon *et al*., 2011). Recombinant human M-CSF and RANKL were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Primary antibodies against PCNA and NFAT2 were from Abcam (Cambridge, UK), and those against ClC-7 and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Isolation

Osteoclast progenitor cells were isolated from human peripheral blood mononuclear cells (PBMCs). The whole-blood samples were obtained from healthy volunteers who provided informed consent. The human subject protocol was approved by the Human Experimentation Committee, Chiang Mai University. PBMCs were directly isolated from the buffy coats by density gradient centrifugation with Ficoll-Paque™ (GE Healthcare Bio-Sciences, Uppsala, Sweden), when a large number of cells was required. To obtain enriched monocytes, we incubated whole blood with antibody cocktail (RosetteSep™, STEMCELL Technologies, Vancouver, BC, Canada), which crosslinks the unwanted cells before density gradient centrifugation. PBMCs were cultured in αMEM (Lonza Walkersville, Inc., Walkersville, MD, USA), supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine (Lonza Walkersville, Inc.), and 1% penicillin/streptomycin (Invitrogen). Cells (5×10^5 cells/cm^2) were seeded on glass coverslips or well plates.

Cytotoxicity and Cell Proliferation Assays

The cytotoxicity of LL-37 was measured by an MTT assay. Enriched monocytes were seeded in 96-well plates (Nunc A/S, Roskilde, Denmark) and treated with 25 ng/mL of M-CSF and various concentrations of LL-37 for 3 days. Subsequently, a 20-μL quantity of MTT dye solution (5 mg/mL in PBS) (Sigma-Aldrich, St. Louis, MO, USA) was added, followed by 200 μL of dimethyl sulfoxide to solubilize formazan crystals. The absorbance was read at 540 nm with the Titertek Multiskan M340 multiplate reader (ICN Flow, Costa Mesa, CA, USA). The cell proliferation assay was determined by immunoblotting of PCNA expression.

In vitro Osteoclastogenesis and Quantification

Osteoclasts were generated from PBMCs by 25 ng/mL of M-CSF and 30 ng/mL of RANKL. Non-toxic concentrations of LL-37 (2-8 μM) were chosen to determine the effect of LL-37 on osteoclastogenesis by TRAcP and F-actin staining. On day 7, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS and stained with 1.5 mM Fast Red Violet LB salt (Sigma-Aldrich). Subsequently, cells were stained with 20 nM Alexa Fluor® 488-conjugated phalloidin (Invitrogen) and 1 μM DAPI (Biotium, Inc., Hayward, CA, USA). Stained cells were mounted and visualized by a fluorescence microscope (Olympus DP71, Tokyo, Japan). TRAcP-positive multinuclear and mononuclear cells were manually counted and calculated by ImageJ 1.45g software.

In vitro Dentin Resorption

To determine the osteoclast function, we performed the resorption pit assay by culturing PBMCs on dentin slices in 96-well chambers with or without LL-37 for 14 days. To detect the resorption area, we stained pits with India ink and observed them under a microscope. The resorption image was recorded and calculated by ImageJ 1.45g software.

RNA Expression

Total RNA was extracted from PBMCs on day 7 by means of an Aurum Total RNA Mini kit (Bio-Rad Laboratories, Hercules, CA, USA). cDNA was constructed from 250 ng of total RNA with the SuperScript First-Strand cDNA System (Fermentas, Hanover, MD, USA) (Krisanaprakornkit *et al*., 2008). A PCR was performed in a reaction mixture, containing specific primer pairs for the detection of transcripts of osteoclast genes and GAPDH (Appendix A). PCR products were resolved on 1.2% agarose gel and stained with ethidium bromide. Photographs were taken by a camera attached to a ChemiDoc XRS instrument (Bio-Rad Laboratories). A real-time PCR was conducted with the Light Cycler-FastStart DNA Master SYBR® Green I system (Roche Molecular Biochemicals, Mannheim, Germany) in the LightCycler® 480 System (Roche Molecular Biochemicals). To compare gene expression levels among samples, we calculated the relative gene expression from C_t of tested genes, normalized by that of GAPDH. The relative C_t (ΔC_t) of LL-37-treated samples was compared with that of the untreated sample to obtain $\Delta\Delta C_t$.

Immunoblotting

PBMCs underwent lysis in 1% Triton-X100 with protease inhibitor cocktail (Roche Molecular Biochemicals). A 10-μg quantity of total proteins was separated by 12% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in 0.1% Tween-20 (Bio-Rad Laboratories) in TBS. The blots were incubated with anti-ClC-7 (1:1,000), anti-PCNA (1:1,000), and anti-GAPDH (1:500) antibody. Immunoreactivity was detected by incubation of the membrane with horseradish-peroxidase-conjugated secondary antibody (KPL, Gaithersburg, MD, USA) and LumiGLO Reserve Chemiluminescence (KPL). The signal was captured by the ChemiDoc XRS instrument.

Nuclear and Cytoplasmic Extraction

PBMCs cultured with or without 4 or 8 μM of LL-37 for 4 days were extracted for nuclear and cytosolic proteins by an NE-PER® nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL, USA). Ten-μg quantities of nuclear and cytosolic proteins were subjected to immunoblotting as described above, with anti-NFAT2 antibody (1:1,000).

M-CSF ELISA

PBMCs were cultured without exogenously added M-CSF in the presence or absence of LL-37 for 3 days. The cell-free culture supernatants were collected for measurement of M-CSF levels by a Quantikine Human M-CSF Immunoassay kit (R&D Systems, Inc.) following the manufacturer's instructions. The M-CSF concentrations in supernatants were calculated from the standard curve of recombinant human M-CSF.

Calcineurin Activity Assay

The calcineurin phosphatase activity in cell lysates was measured by the colorimetric calcineurin cellular activity assay kit

(Enzo Life Sciences International, Inc., Plymouth Meeting, PA, USA). Briefly, PBMCs were cultured in osteoclast medium with or without LL-37 for 4 days, and then subjected to lysis. Free phosphate and nucleotides in the cell extracts were removed by de-salting gel filtration. The phosphatase activity of calcineurin was determined from free phosphate released from RII phosphopeptide based on the classic Malachite green assay. Human recombinant calcineurin was used as a positive control.

Immunofluorescence

PBMCs were fixed with 4% paraformaldehyde in PBS and incubated overnight at 4°C with anti-NFAT2 antibody. The localization of NFAT2 was detected by NorthernLights™ 557 anti-rabbit IgG (R&D Systems, Inc.) and co-stained with Alexa Fluor® 488-conjugated phalloidin and DAPI.

Statistical Analysis

Data were compared by mean ± standard deviation. To determine the differences among samples, we analyzed the variances by independent *t* test, with statistical significance levels at $p < 0.05$, $p < 0.01$, and $p < 0.005$.

RESULTS

LL-37 Promotes Cell Proliferation

We began our study by culturing enriched monocytes in osteoclast medium with LL-37. It was found that the monocytes did not differentiate into multinucleated osteoclasts (arrows) in the presence of LL-37 (Fig. 1A). The monocytes did not survive without the addition of M-CSF, whereas, surprisingly, they maintained their viability upon treatment with only LL-37 (Fig. 1A). To investigate the cytotoxicity of LL-37, we treated enriched monocytes with LL-37 from 2 to 30 μM. The cells mostly died if they were exposed to 20 μM (Fig. 1B), consistent with the significant decrease in viable cells, treated with 20 and 30 μM (Fig. 1C). However, low doses of LL-37 (2-6 μM) appeared to significantly increase the number of viable cells (Fig. 1C), confirmed by up-regulated PCNA expression in PBMCs, treated with 2 to 10 μM of LL-37 (Fig. 1D). M-CSF levels in cell-free culture supernatants of PBMCs were raised by LL-37 treatment, and the significance level was reached by treatment with 8 μM of LL-37 (Fig. 1E), indicating that LL-37 induces M-CSF production and secretion in PBMCs and maintains the cell viability even without exogenously added M-CSF.

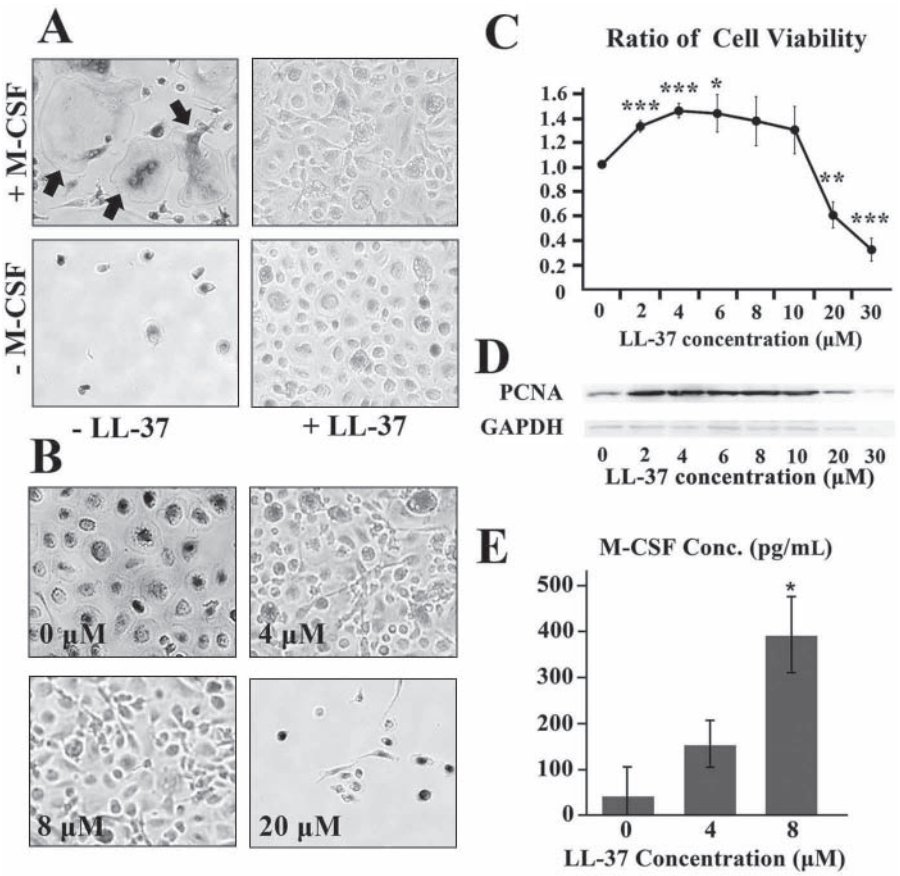

Figure 1. LL-37 inhibits osteoclastogenesis, but promotes proliferation of PBMCs by M-CSF induction. **(A)** TRAcP staining on *in vitro*-generated osteoclasts under RANKL stimulation with (+) or without (–) M-CSF and 4 μM of LL-37 for 7 days. The mature osteoclasts (arrows) are large cells that exhibit TRAcP. **(B)** TRAcP staining on viable PBMCs after being incubated with M-CSF and indicated doses of LL-37 for 3 days. Magnification power = 20x **(C)** MTT assay. PBMCs were incubated with M-CSF and indicated doses of LL-37 for 3 days. The linear graph demonstrates the ratios of cell viability in LL-37-treated samples relative to the untreated control, whose ratio was set to 1 (error bar = SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.005$, $n = 9$). **(D)** Immunoblot detection of PCNA and GAPDH expression in PBMCs treated with M-CSF and various LL-37 concentrations for 4 days. The data in A, B, and D are representative of 3 independent experiments. **(E)** M-CSF ELISA. PBMCs, cultured in the absence of exogenously added M-CSF, were treated with 0, 4, or 8 μM of LL-37 for 3 days. The cell-free culture supernatants were analyzed for M-CSF concentrations in pg/mL (error bar = SD, $*p < 0.05$, $n = 4$).

LL-37 Inhibits *in vitro* Osteoclastogenesis and Down-regulates Expression of Osteoclast Genes

To determine the effect of LL-37 on osteoclastogenesis, we examined TRAcP and F-actin staining and the expression of osteoclast genes. Mature osteoclasts were generated *in vitro* from enriched monocytes on day 7 in the presence or absence of LL-37. LL-37 inhibited osteoclast formation by diminishing TRAcP and F-actin staining in a dose-dependent manner (Figs. 2A and 2B, respectively). The TRAcP-positive multinuclear and mononuclear cells

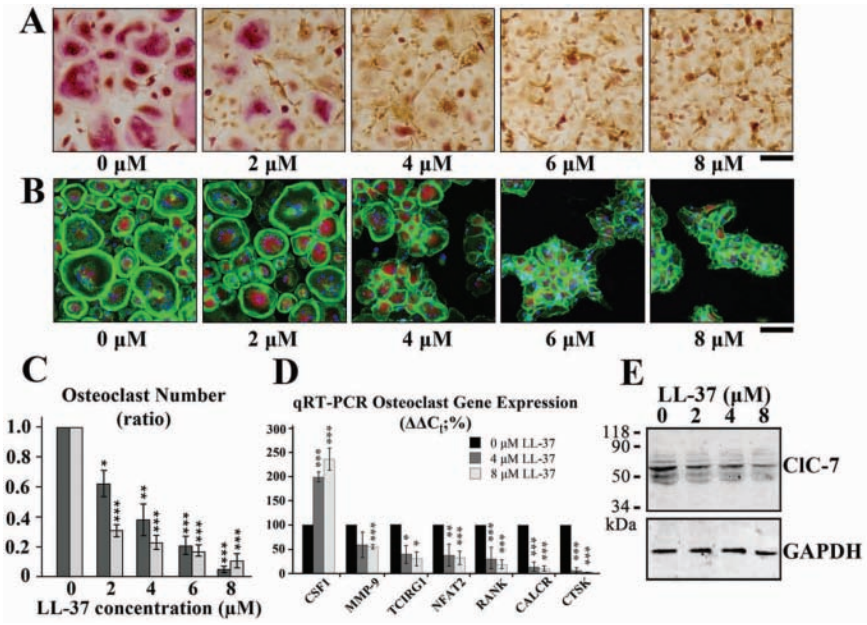

Figure 2. LL-37 inhibits *in vitro* osteoclastogenesis. Representative images from 5 separate experiments of differentiated cells induced by M-CSF and RANKL with or without indicated concentrations of LL-37 for 7 days and observed by **(A)** TRAcP staining and immunocytochemistry of PCNA under a light microscope and **(B)** TRAcP (red), F-actin (green), and DAPI staining under a fluorescence microscope. Bar = 100 μm. **(C)** The bar graph demonstrates the ratios of multinuclear (dark gray bars) and of mononuclear (light gray bars) TRAcP-positive cells in LL-37-treated samples relative to the untreated sample, whose ratio was set to 1 (error bar = SD, *p < 0.05, **p < 0.01, ***p < 0.005, *n* = 5). **(D)** The bar graph shows the percentages of CSF1, MMP-9, TCIRG1, NFAT2, RANK, CALCR, and CTSK mRNA expression in LL-37-treated samples (dark gray and light gray bars for 4 and 8 μM of LL-37, respectively) relative to the percentage of gene expression in the untreated sample (black bars), set to 100% (error bar = SD, *p < 0.05, **p < 0.01, ***p < 0.005, *n* = 4). **(E)** Immunoblot detections of ClC-7 and GAPDH in PBMC lysates during osteoclast induction in the presence or absence of indicated doses of LL-37 for 7 days. Note an intense band around 60 kDa from immunoreaction with anti-ClC-7 polyclonal antibody. The image is representative of 3 separate experiments.

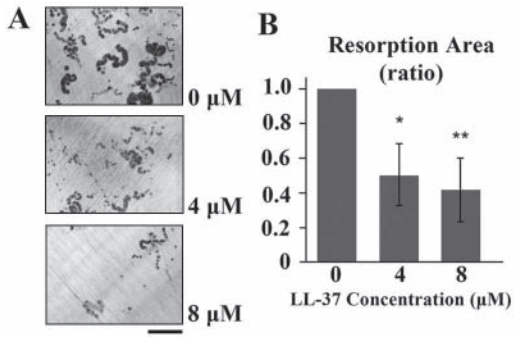

Figure 3. LL-37 inhibits *in vitro* dentin resorption. **(A)** Representative images from 7 independent experiments of dentin slices show resorption pits, formed by *in vitro*-generated osteoclasts for 14 days with 0, 4, or 8 μM of LL-37. Bar = 200 μm. **(B)** The bar graph shows the relative ratios of the quantity of resorptive areas in LL-37-treated samples to the untreated control, whose ratio was set to 1 (error bar = SD, *p < 0.05, **p < 0.01, *n* = 7).

were counted, and their ratios were significantly decreased by LL-37 treatment in a dose-dependent fashion compared with the untreated control, suggesting that LL-37 blocks both TRAcP expression and osteoclast fusion (Fig. 2C). From the time-course study, the significant inhibition of LL-37 on the formation of TRAcP-positive multinuclear cells was observed when LL-37 was added from days 0 to 4 (Appendix B). However, when LL-37 was added on day 6, no significant inhibition on TRAcP-positive cells was observed (Appendix B), suggesting that LL-37 negatively affects the early stage of osteoclastogenesis (days 0-4). To determine the inhibitory effect of LL-37 on osteoclast gene expression, we performed real-time PCR analyses. LL-37 significantly inhibited expression of MMP-9, TCIRG1, NFAT2, RANK, CALCR, and CTSK mRNAs in a dose-dependent manner, whereas it induced M-CSF/CSF1 expression (Fig. 2D). Since ClC-7 is required for osteoclasts to resorb mineralized tissue, this protein is suggested to be one of osteoclast markers (Kornak *et al.*, 2001). The expression of ClC-7 protein in PBMC lysates was determined, and the finding showed down-regulation of ClC-7 expression by LL-37 in a dose-dependent manner (Fig. 2E).

LL-37 Inhibits Dentin Resorption

To examine the effect of LL-37 on osteoclast function *in vitro*, we seeded enriched monocytes and induced cell differentiation in the presence or absence of LL-37 on dentin slices. The quantity of pits on the dentin slices was decreased by LL-37 treatment (Fig. 3A). The ratio of pit formation significantly declined in the LL-37-treated samples compared with that in the untreated sample (Fig. 3B).

LL-37 Prevents Nuclear Translocation of NFAT2 by Inhibiting Calcineurin Activity

To investigate the inhibitory mechanism of LL-37, we determined whether LL-37 could affect the function of NFAT2, a master transcription factor for many osteoclast genes, including itself (Chuvpilo *et al.*, 2002). The NFAT2 was localized in the cytoplasm of LL-37-treated PBMCs compared with the localization of NFAT2 in the nuclei of untreated PBMCs (Fig. 4A), indicating that LL-37 blocks nuclear translocation of NFAT2. The immunoblot analyses confirmed the absence of NFAT2 in the nuclear extract of PBMCs treated with LL-37, whereas several immunoreactive bands of NFAT2 (around 80-100 kDa) were detected in the cytosolic extracts, consistent with a previous finding (Garcia-Gomez *et al.*, 2012) (Fig. 4B). Accordingly, LL-37 treatment significantly inhibited NFAT2 mRNA expression in a dose-dependent manner, as determined by RT-PCR and

real-time PCR (Figs. 4C and 4D, respectively). To verify the blockade of NFAT2 nuclear translocation by LL-37, we measured the activity of calcineurin, controlling dephosphorylation of NFAT2, in LL-37-treated and untreated PBMCs. It was found that LL-37 treatment could significantly decrease the calcineurin activity around 50% compared with the untreated control (Fig. 4E).

DISCUSSION

LL-37 exerts an inhibitory effect on *in vitro* osteoclastogenesis, as evidenced by the inhibition of multinuclear formation, TRAcP expression, and resorption pit formation, down-regulating osteoclast-specific gene expression, suppressing the calcineurin activity, and then blocking NFAT2 nuclear translocation. To the best of our knowledge, this is the first detailed analysis of the inhibitory action on *in vitro* osteoclastogenesis of LL-37. Furthermore, low doses of LL-37 can increase proliferation of undifferentiated monocytes, as evidenced by induced PCNA expression. M-CSF is an essential molecule that promotes the proliferation of osteoclast progenitors (Tanaka *et al.*, 1993), and M-CSF-deficient mice demonstrate an osteopetrotic phenotype due to the lack of osteoclasts (Umeda *et al.*, 1996). Our study has demonstrated that M-CSF expression is enhanced by LL-37 in PBMCs, consistent with the inducible effect of LL-37 on M-CSF expression in the monocytic cell line RAW264.7 (Scott *et al.*, 2002). Therefore, LL-37 possibly maintains the viability of PBMCs by enhancement of M-CSF production. Our findings also correspond well with the previously reported function of M-CSF and LL-37 as anti-apoptotic molecules (Woo *et al.*, 2002; Chamorro *et al.*, 2009).

The concentrations of LL-37 less than 10 μM were not toxic to PBMCs, but the significant cell death was found at 20 μM or greater. This is in line with the finding that shows general toxicity of LL-37 to eukaryotic cells at concentrations above 13 μM (Johansson *et al.*, 1998). Low LL-37 concentrations have been recently described to convert monocytes to a special cell type capable of mineralization, called monoosteophils (Zhang and Shively, 2010). Instead of resorption pit formation, von

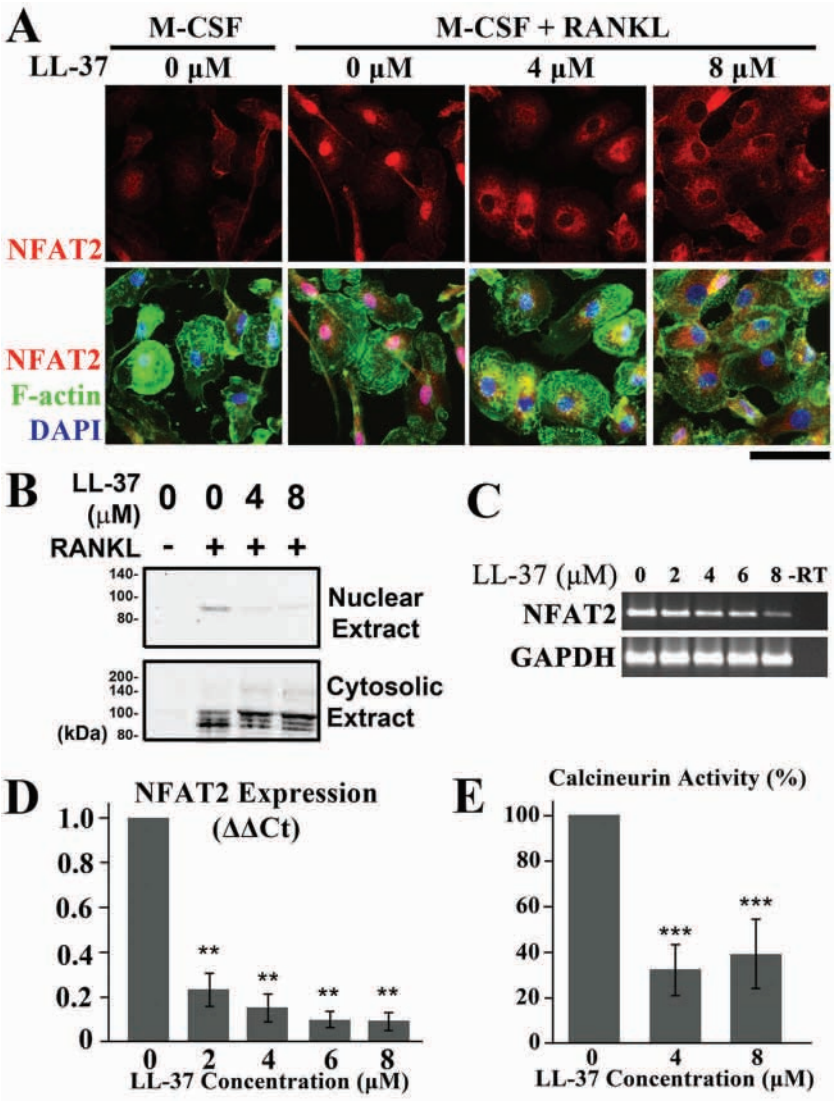

Figure 4. LL-37 blocks nuclear translocation of NFAT2 by decreasing the calcineurin activity. PBMCs were cultured in osteoclast medium, containing M-CSF with (+) or without (–) RANKL, as a negative control, in the presence or absence of indicated doses of LL-37 for 4 days. **(A)** Cells were stained with NFAT2 (red), F-actin (green), and DAPI (blue), and observed under a fluorescence microscope. Note no nuclear and weak cytoplasmic staining of NFAT2 in the PMBC culture without RANKL activation (the left-most column). Note the punctate staining of F-actin in PBMCs. Bar = 50 μm. **(B)** The nuclear and cytosolic protein fractions were extracted, subjected to immunoblotting, and probed with anti-NFAT2 antibody. Note the absence of immunoreactive bands in the nuclear and cytosolic extracts of PBMCs without RANKL activation (the left-most lane), consistent with the finding in (A). Further, the presence of several immunoreactive bands in the cytosolic extract may suggest post-translational modification of NFAT2. The findings in (A) and (B) are representative of 3 separate experiments. **(C)** An RT-PCR analysis. Total RNA was extracted from PBMCs, cultured in osteoclast medium with or without indicated doses of LL-37 for 4 days. –RT is a sample where the reverse-transcriptase enzyme was omitted in the reaction. **(D)** A real-time PCR analysis of NFAT2 expression was conducted with cDNA from (C). The bar graph illustrates the relative ratios (ΔΔCt) of LL-37-treated samples to that of the untreated sample, set to 1 (error bar = SD, $^{**}p < 0.01$, $n = 4$). **(E)** The calcineurin activity assay. PBMCs were cultured in osteoclast medium, containing 0, 4, or 8 μM of LL-37 for 4 days, and then underwent lysis. The bar graph shows the percentages of calcineurin activity in LL-37-treated samples relative to that of the untreated sample, set to 100% (error bar = SD, $^{***}p < 0.005$, $n = 4$).

Kossa-positive material was detected in monocytes incubated with LL-37 besides M-CSF and RANKL. The effect of LL-37 on osteoclastogenesis, however, was not characterized. The interpretation of these findings is currently very difficult. In our hands, there was no evidence for enhanced mineral deposition by monocytes under these conditions, which might be due to different culturing conditions.

The number of multinuclear cells is decreased, if the osteoclast formation is induced in the presence of LL-37, suggesting that non-toxic doses of LL-37 can block cell fusion of monocytes. It has been demonstrated that extracellular adenosine facilitates cell fusion by activating purinergic $P2X_7$ receptor (Pellegatti *et al*., 2011). The $P2X_7$ directs a release of adenosine triphosphate, which is degraded to adenosine and enhances cell fusion. Therefore, the $P2X_7$ receptor may be a candidate receptor for the inhibitory effect of LL-37 on osteoclastogenesis. However, from our study, the $P2X_7$ does not appear to act as a target receptor for the inhibition of cell fusion, since treatment with the neutralizing antibody against $P2X_7$ did not reverse the inhibition of osteoclastogenesis by LL-37 (Appendix C). Consequently, the candidate receptor for the inhibitory effect of LL-37 on osteoclastogenesis remains to be further investigated.

NFAT2 is the master regulator of osteoclastogenesis by transcribing many osteoclast-specific genes. Osteoclastogenesis requires NFAT2 nuclear translocation activated by RANKL that induces the calcineurin activity (Takayanagi *et al*., 2002). We have shown that LL-37 significantly decreases the calcineurin activity, resulting in the blockade of NFAT2 nuclear translocation and the down-regulation of mRNA expression for several osteoclast genes, including NFAT2. Consequently, we propose that the calcineurin-NFAT2 axis is a critical signaling pathway for the inhibition of *in vitro* osteoclastogenesis by LL-37.

In the oral cavity, the levels of LL-37 expression in tissues from aggressive periodontitis are lower than those from chronic periodontitis (Türkoğlu *et al*., 2011). This finding is consistent with ours, showing a novel inhibitory effect of LL-37 on osteoclastogenesis. We postulate that LL-37 is involved in bone metabolism during rapid bone destruction in aggressive periodontitis. Nevertheless, our *in vitro* findings should be interpreted with caution, since it is imperative to study the inhibition of *in vivo* osteoclastogenesis by LL-37 before any possible application of LL-37 in the therapeutic management of aggressive periodontitis.

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