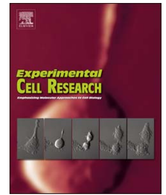
Experimental Cell Research 362 (2018) 152–158

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr



# *Enterococcus faecalis* immunoregulates osteoclastogenesis of macrophages

Zhezhen Xu[a,b,1], Zhongchun Tong[a,b,1], Prasanna Neelakantan[c], Yanling Cai[a,b], Xi Wei[a,b,*]

[a] *Department of Operative Dentistry and Endodontics, Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, 56 Lingyuan Xi Road, Guangzhou 510055, Guangdong, China*
[b] *Guangdong Province Key Laboratory of Stomatology, No. 74, 2nd Zhongshan Road, Guangzhou 510080, Guangdong, China*
[c] *Discipline of Endodontology, Faculty of Dentistry, The University of Hong Kong, Pok Fu Lam, Hong Kong SAR, China*

ARTICLE INFO

*Keywords:*
*Enterococcus faecalis*
Inflammation
Macrophage
Osteoclast
Osteoclastogenesis
Persistent apical periodontitis

ABSTRACT

Persistent apical periodontitis (PAP) is characterized by refractory inflammation and progressive bone destruction. *Enterococcus faecalis* infection is considered an important etiological factor for the development of PAP, although the exact mechanisms remain unknown. This study aimed at investigating the role of *E. faecalis* in cell proliferation, inflammatory reactions and osteoclast differentiation of macrophages using an *in vitro* infection model of osteoclast precursor RAW264.7 cells. A cell viability assay of cultured RAW264.7 cells exposed to live *E. faecalis* at a multiplicity of infection of 100 for 2 h, indicated that the infection exhibited no cytotoxic effect. Transmission electron microscopy images revealed no apoptotic changes but a rise of metabolic activity and phagocytic features in the infected RAW264.7 cells. Confocal laser scanning microscopic and flow cytometric analysis indicated that the phagocytosis of RAW264.7 cells was activated by *E. faecalis* infection. Furthermore, quantitative real-time PCR assays demonstrated that the expression of inflammatory cytokines was remarkably elevated in infected RAW264.7 cells. Differentiation of infected RAW264.7 cells into osteoclasts was remarkably attenuated, and expression of osteoclast marker genes as well as fusogenic genes significantly dropped. In summary, *E. faecalis* appears to attenuate osteoclastic differentiation of RAW264.7 precursor cells, rather stimulates them to function as macrophages.

## 1. Introduction

Bone destruction in the periradicular region due to persistent apical periodontitis (PAP) is the result of chronic inflammation [1] that creates an imbalance between bone formation and resorption [2]. Fusion of monocytes and macrophages results in formation of osteoclasts, which are highly specialized cells for bone absorption [3]. The role of macrophages in mediating immune response is well-known: once localized to a site of infection, macrophages phagocytize bacterial invaders, recognize bacterial virulence factors through pattern-recognition receptors, and secrete an abundance of immuno-regulatory products [4,5]. Macrophages have garnered significant attention due to their dual-ability to serve as osteoclast precursors as well as immune response modifiers. In part, this is because of the release of pro-inflammatory cytokines, particularly IL-1β, IL-6, and TNF-α, which promote osteoclast formation and activity, resulting in periradicular bone resorption [6,7].

Although root canal and periradicular infections are characterized by a complex microbiome, the most frequently isolated pathogen is *Enterococcus faecalis* [8,9], ranging in prevalence from 24–77% [10]. This bacterium resists harsh environmental conditions such as alkalinity, malnutrition and endodontic medicaments, thereby surviving contemporary treatment modalities resulting in persistent infection [11–13]. Furthermore, it possesses an array of virulence factors such as lipoteichoic acid (major cell wall constituent), ace (collagen binding protein), cylA (hemolysin activator), esp (protein surface), and gelE (gelatinase) which facilitate its adherence and invasion, thereby triggering or exacerbating inflammatory responses [14].

The effects of *E. faecalis* in mediating persistent apical periodontitis are unclear. While two studies showed that purified LTA of *E. faecalis* or

*Abbreviations:* PAP, persistent apical periodontitis; LEF, live *E. faecalis*; MOI, multiplicity of infection; TEM, transmission electron microscopy; qRT-PCR, quantitative real-time PCR assays; IL-1β, interleukin-1β; TNF-α, tumor-necrosis factor-α; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; iNOS, inducible nitric oxide synthase; LTA, lipoteichoic acid; HKEF, heat-killed *E. faecalis*; BHI, brain heart infusion; α-MEM, Alpha-minimal essential medium; CCK-8, Cell Counting Kit-8; CLSM, confocal laser scanning microscopy; SCID, immunodeficient mice

* Corresponding author at: Postal Address: Department of Operative Dentistry and Endodontics, Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, 56 Lingyuan Xi Road, Guangzhou 510055, Guangdong, China.

*E-mail addresses:* czxuzz@126.com (Z. Xu), t_z_c1977@163.com (Z. Tong), prasanna@hku.hk (P. Neelakantan), yanling0822@yahoo.com (Y. Cai), weixi@mail.sysu.edu.cn (X. Wei).

[1] Zhezhen Xu and Zhongchun Tong contributed equally to this work.

https://doi.org/10.1016/j.yexcr.2017.11.012
Received 25 September 2017; Received in revised form 7 November 2017; Accepted 8 November 2017
Available online 10 November 2017
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heat-killed *E. faecalis* (HKEF) attenuated the differentiation of macrophages into osteoclasts [15,16], another paper indicated that HKEF enhanced osteoclastogenesis [17]. This divergent data demonstrates an urgent need to elucidate the impact of *E. faecalis* on osteoclastogenesis. The aim of this present study was to investigate the effect of *E. faecalis* on cell proliferation, inflammatory reactions and osteoclastogenesis of RAW264.7 macrophages *via* an *in vitro* model using live *E. faecalis* (LEF).

## 2. Materials and methods

### 2.1. Bacterial culture and preparation of heat-killed E. faecalis

*E. faecalis* strain OG1RF was used in this study. Following routine streaking on brain heart infusion (BHI) agar (Difco, Sparks, MD, USA) and aerobic culture, a single bacterial colony was inoculated into BHI broth up to the exponential phase. To obtain HKEF, bacteria were collected by centrifugation and exposed to a temperature of 85 °C for 1 h, following a protocol described previously [17]. HKEF was then recultivated on BHI agar to confirm the heat-killing process.

### 2.2. RAW264.7 cells infected with E. faecalis

RAW264.7 murine macrophage cell line (ATCC, Manassas, VA) was cultured in Alpha-minimal essential medium (α-MEM; Gibco, New York, NY, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified 5% $CO_2$, 95% air atmosphere.

RAW264.7 cells were seeded overnight in six-well culture plates at a density of $6 \times 10^5$/well. *E. faecalis* was added to the culture at a multiplicity of infection (MOI) of 100 to allow adhesion and internalization. After 2 h of exposure, unbound bacteria were discarded. Adherent extracellular bacteria were subsequently killed by incubation with medium containing nisin (4000 μg/mL) and ciprofloxacin (8 μg/mL) for 1 h. The validity of this process was confirmed by the absence of bacterial growth when inoculated on BHI agar. RAW264.7 cells were then incubated in two media: α-MEM and in a medium supplemented with recombinant murine receptor activator of nuclear factor κB ligand (RANKL; R&D Systems, Minneapolis, MN, USA).

### 2.3. Cell viability assay

RAW264.7 cells infected with *E. faecalis* were seeded in 96-well plates at $2.5 \times 10^3$/well and infected with LEF or HKEF for 2 h. At 0, 24, 48, 72, and 96 h after infection, 10 μl CCK-8 (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan) was added into every well following manufacturer's instructions and absorbance of the supernatant was recorded at 450 nm with a microplate reader (Tecan, Reading, UK). RAW264.7 cells exposed to bactericides (nisin and ciprofloxacin) for 1 h served as negative controls, and cells without exposure to *E. faecalis* and bactericides served as blank controls. All determinations were performed in quintuplicate.

### 2.4. Transmission electron microscopy

RAW264.7 cells were infected with LEF or HKEF for 2 h. At 24 h post infection, the cells were harvested and fixed with a solution containing 2% paraformaldehyde and 0.5% glutaraldehyde for 4 h, stained with 1% osmic acid, dehydrated and embedded in resin and cut using a diamond knife on a microtome (Sorvall MT-5000; Du Pont, Palo Alto, CA, USA) to obtain 100 nm ultra-thin sections. The sections were collected on copper grids and observed under a TEM (JEM-1400 Plus, JEOL Ltd., Tokyo, Japan).

### 2.5. Confocal laser scanning microscopy (CLSM) and flow cytometric analysis of phagocytosis

HKEF ($1 \times 10^8$ colony-forming units, CFU) were stained with 20 μg/mL FITC (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37 °C. RAW264.7 cells were infected with LEF or HKEF for 2 h. At 6 and 24 h after infection, FITC-labeled HKEF was added to the infected RAW264.7 culture (MOI = 100). Following incubation for 1 h, a solution containing 0.2 mg/mL trypan blue (pH 4.4) was added to quench the extracellular FITC-fluorescence [18]. Infected cells were then fixed, stained with rhodamine phalloidin (Cytoskeleton, USA) and DAPI, and were observed under a CLSM (Carl Zeiss, Thornwood, NY, USA). Images were processed using commercial software (ZEN 2012 Light Edition, Carl Zeiss). The same labeled cells were quantitatively evaluated using FACscalibur (BD Biosciences, San Jose, CA, USA). Raw data were analyzed using Flowjo (Tree Star Inc., Ashland, OR, USA).

### 2.6. Quantitative real-time polymerase chain reaction (qRT-PCR) for analysis of inflammatory cytokines

RAW264.7 cells were cultured for 6 or 24 h after 2 h of infection with *E. faecalis*. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and purified. RNA concentrations were determined spectrophotometrically. The cDNA synthesis reaction was carried out using the Reverse Transcription Kit (Takara, Kyoto, Japan). The qRT-PCR was performed with LC480 SYBR Green I master mix (Roche, Indianapolis, IN, USA), using universal cycling conditions on LightCycler 480 (Roche). Each plate contained β-actin as the housekeeping gene, and the relative expressions for the test gene were normalized using the $2^{-\Delta\Delta Ct}$ method.

The primer sequences were as follows:

*IL-1β*, 5′(GAAATGCCACCTTTTGACAGTG)3′, and 5′(TGGATGCTCTCATCAGGACAG)3′; *TNF-α*, 5′(CTGAACTTCGGGGTGATCGG)3′, and 5′(GGCTTGTCACTCGAATTTTGAGA)3′; *IL-6*, 5′(TCTATACCACTTCACAAGTCGGA)3′, and 5′(GAATTGCCATTGCACAACTCTTT)3′; *MCP-1*, 5′(CACTCACCTGCTGCTACTCATT)3′, and 5′(CACTGTCACACTGGTCACTCCT)3′; *iNOS*, 5′(GTTCTCAGCCCAACAATACAAGA)3′, and 5′(GTGGACGGGTCGATGTCAC)3′; and

*β-actin*, 5′(CATCCGTAAAGACCTCTATGCCAAC)3′, and 5′(ATGGAGCCACCGATCCACA)3′.

### 2.7. Osteoclast differentiation

RAW264.7 cells were infected with LEF or HKEF for 2 h and incubated with 60 ng/mL of RANKL for 4 d to induce differentiation [19]. The culture medium was replaced every other day. Uninfected cells served as positive control. Osteoclast differentiation was detected by TRAP staining using the leukocyte acid phosphatase kit (Sigma-Aldrich). Only TRAP-positive, multinucleated (≥ 3 nuclei) cells were identified as mature osteoclasts. Mature osteoclasts in each well with 6 ≥ nuclei ≥ 3, 10 ≥ nuclei ≥ 7, and nuclei ≥ 10 were counted.

Expression of the genes related with osteoclastogenesis *i.e., Acp5* (TRAP), *Ctsk* (cathepsin K), *c-fos*, *Dcstamp*, and *Atp6v0d2*, were examined by qRT-PCR. Uninfected and non-RANKL induced RAW264.7 cells served as negative control. Total RNA was extracted from the infected cells and the negative control, and then reverse-transcribed to cDNA. Primer sequences were designed as follows:

*Acp5*, 5′(ACCTTGGCAACGTCTCTGCAC)3′, and 5′(GTCCAGCATAAAGATGGCCACA)3′; *Ctsk*, 5′(CTCGGCGTTTAATTTGGGAGA)3′, and 5′(TCGAGAGGGAGGTATTCTGAGT)3′; *c-fos*, 5′(CGGCATCATCTAGGCCCAG)3′, and 5′(TCTGCTGCATAGAAGGAACCG)3′; *Dcstamp*, 5′(TACGTGGAGAGAAGCAAGGAA)3′, and 5′(ACACTGAGACGTGGTTTAGGAAT)3′; *Atp6v0d2*, 5′(CAGAGCTGTACTTCAATGTGGAC)3′, and 5′(AGGTCTCACACTGCACTAGGT)3′.

### 2.8. Statistical analysis

Data were represented as mean ± standard deviation (SD) from at least three independent experiments. The distribution of all the data had been confirmed to be normal by a normality test using SPSS 20.0. Comparison of data between the control and treatment groups was performed using one-way ANOVA followed by Tukey HSD for multiple comparisons with the alpha error set at $P = 0.05$.

## 3. Results

### 3.1. Cell viability in RAW264.7 cells

The highest cell viability was observed in the LEF-infected group at 24 and 48 h after 2 h of infection, and this was significantly higher than the blank control ($P < 0.05$). However, at the 72 and 96 h post-infection, no significant differences were observed between the LEF, HFEF and control groups ($P > 0.05$). The HKEF-infected group demonstrated no significant difference compared to the blank and negative control at any time point of the experiment ($P > 0.05$) (Fig. 1).

### 3.2. Ultrastructural characterization

Representative images of RAW264.7 cells observed under the TEM have been presented in Fig. 2. Cells of the control groups showed normal morphology including a large nucleus with clear membrane boundary, uniform cytoplasm with rare cytoplasmic vacuoles, and well-organized organelles (Fig. 2**A, a**). Cells infected with *E. faecalis* showed internalization in the cytoplasm of the macrophages and envelopment with the unit membrane (Fig. 2**B, C**) which was either intact or damaged (Fig. 2**b, c**). The infected cells were characterized by abundant organelles associated with phagocytosis and metabolic activity, such as lysosomes and mitochondria (Fig. 2**b, c**). This intracellular machinery appeared more pronounced in the LEF-infected cells (Fig. 2**b**) compared with HKEF-infected ones (Fig. 2**c**). Furthermore, no mitochondrial swelling or nuclear condensation was observed in these cells indicating absence of apoptotic change.

### 3.3. Phagocytosis of RAW264.7 cells

CLSM revealed that the FITC-labeled HKEF could be effectively engulfed by the RAW264.7 cells (Fig. 3**A**). More fluorescent bacteria were observed in the LEF- and HKEF-treated cells compared to the control at 6 h and 24 h post-infection. LEF-infected cells demonstrated evidence of more phagocytic activity than HKFF-treated cells, especially at 24 h post-infection (Fig. 3**A**). Flow cytometry revealed similar results. The number of FITC-labeled cells was significantly higher in the

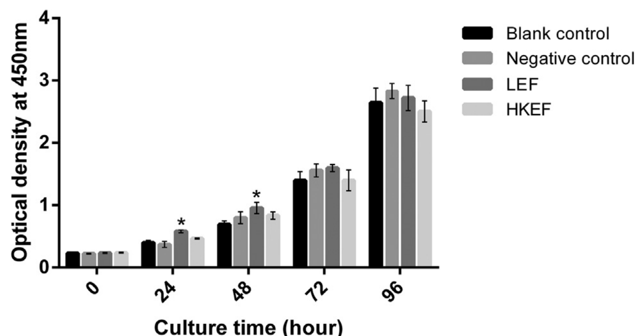


**Fig. 1.** Effect of *E. faecalis* on cell viability in RAW264.7 cells. The proliferation ability of LEF or HKEF infected RAW264.7 cells at MOI = 100 was measured using the CCK-8 assay at 0, 24, 48, 72 and 96 h post infection. RAW264.7 cells exposed to bactericides (nisin and ciprofloxacin) for 1 h served as negative controls, and cells without any treatment were used as blank controls. *$P < 0.05$, compared with the blank controls.

LEF group compared to the HKEF and control groups ($P < 0.01$). There was also evidence of increased rate of phagocytosis of infected cells at 24 h (Fig. 3**B, C**).

### 3.4. Expression of inflammatory cytokines

There was significant upregulation in the expression of all inflammatory cytokines in the infected cells ($P < 0.01$). Expression of the tested genes was higher at the growth phase of 6 than at 24 h after infection with the exception of *iNOS* (Fig. 4). At 6 and 24 h after infection, expression of *IL-1β*, *IL-6*, and *MCP-1* increased in the LEF group compared to the HKEF group ($P < 0.01$) (Fig. 4**A, C, D**).

### 3.5. Osteoclast differentiation

Differentiation of RAW264.7 to multinuclear osteoclasts was significantly prevented by both LEF and HKEF infection with the effect of LEF being more significant (Fig. 5**A**). Both the number and size of cells was significantly affected in the LEF group compared to the HKEF group and controls ($P < 0.05$).

The number of multinucleated osteoclasts was very low in the LEF group (Fig. 5**A**). Additionally, a significant reduction in the number of TRAP+ osteoclasts was noted in this group (Fig. 5**B**). The size of osteoclasts was negatively influenced by infection with *E. faecalis*. The number of large osteoclasts (> 10 nuclei per cell) was remarkably lower in both *E. faecalis*-infected groups compared to the controls (Fig. 5**C**). The qRT-PCR results reflected the same, wherein genes related to osteoclastogenesis being significantly downregulated in both LEF and HKEF groups compared to the positive control, with the downregulation in the LEF group being more significant ($P < 0.05$) (Fig. 5**D**).

## 4. Discussion

Several aspects of the role of *E. faecalis* in pathogenesis of PAP have been researched extensively [8,9], but, the literature is severely lacking in demonstrating the precise effects of this bacterium on osteoclastogenesis. Osteoclasts are multinucleated cells derived from monocyte/macrophage precursors [20]. Recent osteo-immunological studies have shown a close relationship between cells of the immune system (such as macrophages, T cells and NK cells) and osteoclasts [21–24]. Signaling pathways involved in the regulation of antigen-presenting cells also play an important role in osteoclastogenesis [22], suggesting that osteoclast precursors play an important role in the bone resorption process, as well as in regulating inflammation. To this effect, there is an increasing interest in exploring the “dual identity” of macrophages but most available research on this aspect centers around transitory inflammatory reaction of macrophages activated by *E. faecalis* [25,26]. Changes in osteoclastogenesis due to *E. faecalis* infection have not been extensively explored.

In the present study, we established an *in vitro* model to evaluate the effect of *E. faecalis* on proliferation, inflammatory reactions, and osteoclastogenesis of RAW264.7 macrophages, with an aim to observe the global impact of this pathogen, including components of the bacteria and its secretory molecules. Interactions between bacteria and host cells are much more complex than that of bacterial components and host cell membrane. This is justified by the results of previous studies that used purified bacterial by-products and inactivated *E. faecalis* as individual stimulants for osteoclast precursors with inconsistent results [15–17]. Indeed, while the LTA of *E. faecalis* has been consistently shown to contribute to the inflammatory responses and chemokine production, there is also a TIR domain containing protein (Tcp) in *E. faecalis* which appears to limit host cell immune activation [27,28]. Cells treated with HKEF were established synchronously as controls in our study. We observed that the HKEF contributed partially to the induced responses compared to LEF, implying that the heat process may denature and

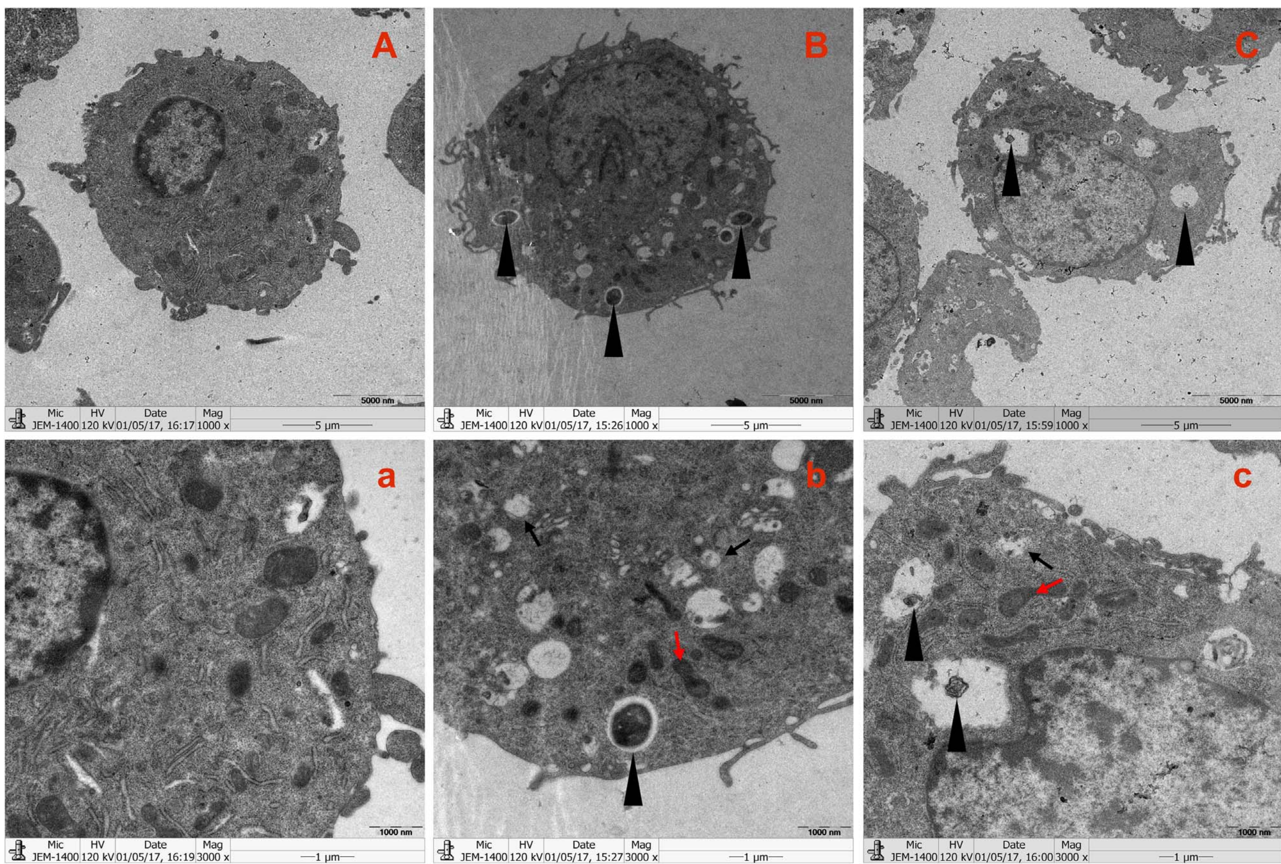


**Fig. 2.** Transmission electron microscopy of ultrastructural changes in RAW 264.7 cells infected with LEF or HKEF (24 hours post-infection). (A) Normal RAW 264.7 cells, Bar = 5 µm; (a) Ultrastructural observations in normal RAW cells, Bar = 1 µm. (B) RAW 264.7 cells infected with LEF, Bar = 5 µm; (b) Ultrastructural observations in cells infected with LEF, Bar = 1 µm. (C) RAW 264.7 cells infected with HKEF, Bar = 5 µm; (c) Ultrastructural observations in cells infected with HKEF, Bar = 1 µm. The arrowheads indicate the bacteria that were internalized *i.e.,* enclosed in phagosomes, either intact or damaged. The black and red arrows point to the lysosomes and mitochondria respectively, both of which showed an increase in infected cells.

consequently, inactivate some virulence factors.

To develop a robust *in vitro* infection model, it is essential to identify strategies that can rapidly and effectively kill *E. faecalis*; considering the ability of *E. faecalis* to resist (both intrinsic and acquired) a wide array of antibiotics. We utilized nisin in conjunction with ciprofloxacin. In our experiments (data not shown), we identified that ciprofloxacin (8 µg/mL) in combination with nisin (4000 µg/mL) could exterminate *E. faecalis* ($5\times 10^7$ CFU/mL) within 1 h. Cell viability measurements confirmed this, indicating that neither the brief duration of infection nor the bactericide exhibited cytotoxic effects on RAW264.7 cells.

In our study, 2 h infection by LEF was considered appropriate because the faster growth rate of *E. faecalis* after 2 h, with an increase in MOI, makes it challenging to harvest macrophages. Cell viability of macrophages did not significantly decrease after 2 h of infection. LEF stimulation significantly increased the cell viability at 24 and 48 h of infection. However, TRAP staining showed that both LEF and HKEF at 2 h of infection could inhibit osteoclastogenesis of macrophages indicating that the cytotoxic effect of infection was irrelevant to the inhibition of osteoclastogenesis.

The present study demonstrated that RAW264.7 cells infected with LEF and HKEF significantly upregulated expression of inflammatory cytokine iNOS and stimulated phagocytic activity of macrophage. Phagocytosis of disease-relevant particles inhibits RANKL-mediated osteoclastogenesis of human monocytes [29], hence we hypothesized that infection of osteoclast precursors by *E. faecalis* may demonstrate a pattern to maintain the infected cells as part of the inflammatory system by inhibiting their differentiation into mature osteoclasts while upregulating the production of inflammatory cytokines, thus promoting osteoclast differentiation. Our results showed that *E. faecalis* infection not only significantly reduced the RANKL-dependent induction of osteoclastogenic marker genes, including *Acp5, Ctsk*, and *c-fos*, but also down-regulated osteoclast fusogenic genes *Dcstamp* and *Atp6v0d2*. Inhibition of osteoclastogenesis by *E. faecalis* infection was also demonstrated by TRAP activity measurements. Rather than accelerating the differentiation of osteoclast precursors into osteoclasts, *E. faecalis* infection appeared to enhance the immune response by promoting cytokine production and bacterial phagocytosis of osteoclast precursors, thus contributing to bacterial clearance.

The findings of our study corroborate with earlier findings [15,16] showing that both HKEF and purified LTA of *E. faecalis* inhibited osteoclast differentiation of macrophages. Such results have also been demonstrated with pathogens such as *Staphylococcus aureus* [30], *Porphyromonas gingivalis* [31], or even inanimate particles such as latex beads [29]. In the case of bone destruction in periodontitis, immunocytes T lymphocytes and B cells grow abundantly in the presence of periodontopathogens, along with an increase in the production of proinflammatory mediators IL-1 and TNF-α [32]. These proinflammatory cytokines act as pro-osteoclastogenic factors to activate osteoclasts through RANKL production by osteoblasts, eventually leading to alveolar bone loss. This has also been elucidated by the lack of marked bone destruction in immunodeficient mice (SCID), confirming the role of immune regulation in bacteria-mediated osteoclastogenesis [33]. Based on this knowledge, we speculated that bone destruction caused by *E. faecalis* infection could be primarily mediated by the host cell immune response. Indeed, considering the upregulated gene expression of various inflammatory cytokines *IL-1β, TNF-α, IL-6,* and *MCP-1*, all of which have been reported to promote the osteoclast differentiation in the induction of RANKL [7,34,35], *E. faecalis* infection

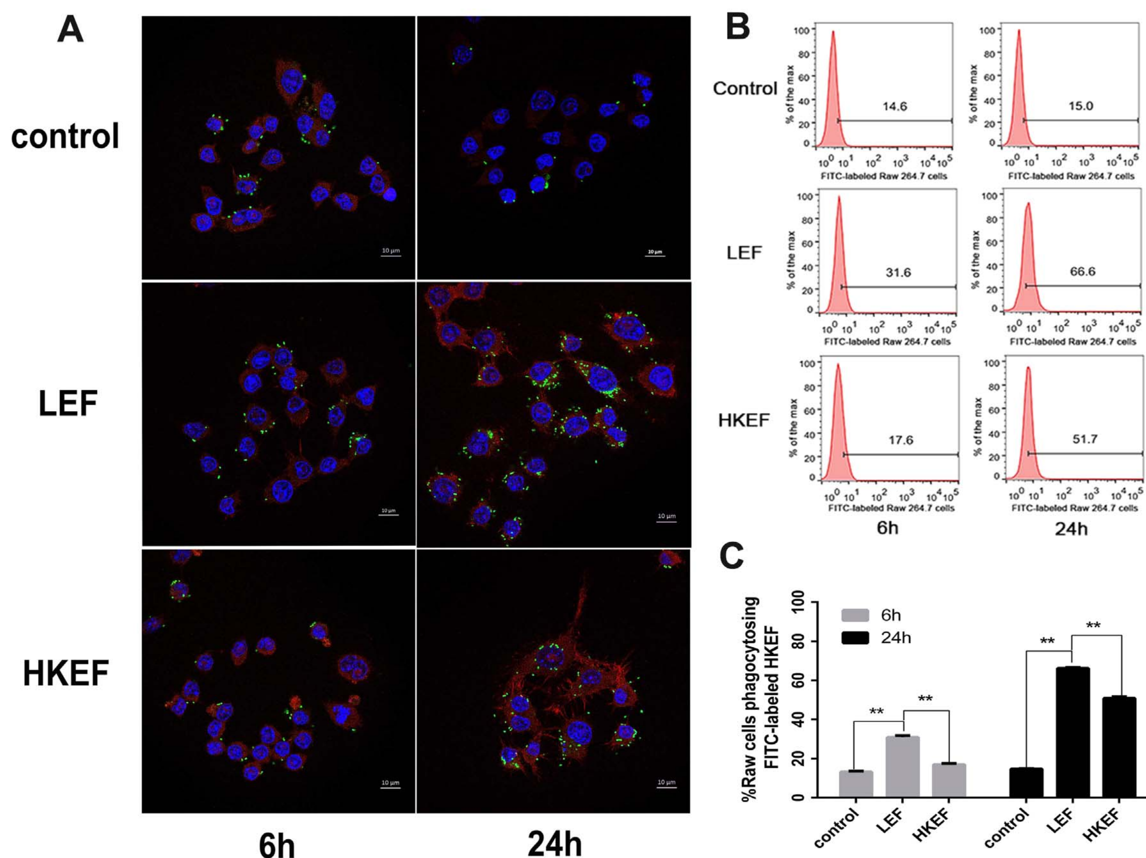


**Fig. 3.** Phagocytic capacity of infected RAW264.7 cells. RAW264.7 cells were infected with LEF or HKEF for 2 h at a MOI of 100. At 6 h and 24 h post-infection, cells were incubated with FITC-labeled HKEF at a MOI of 100 for 1 h, and studied using confocal microscopy and flow cytometry. (A) Representative confocal microscopic image of phagocytosis of FITC-labeled (green) HKEF by RAW 264.7 cells. The actin cytoskeleton of cells was counterstained with rhodamine phalloidin (red), and nuclei were stained with DAPI (blue). Bars represent 10 µm. (B) Flow cytometric detection of FITC-labeled HKEF by RAW 264.7 cells. Non-treated cells were blank controls. (C) Number of RAW 264.7 cells phagocytosing FITC-labeled HKEF was quantitatively analyzed. Statistically significant differences between groups were indicated with asterisks (**P < 0.01).

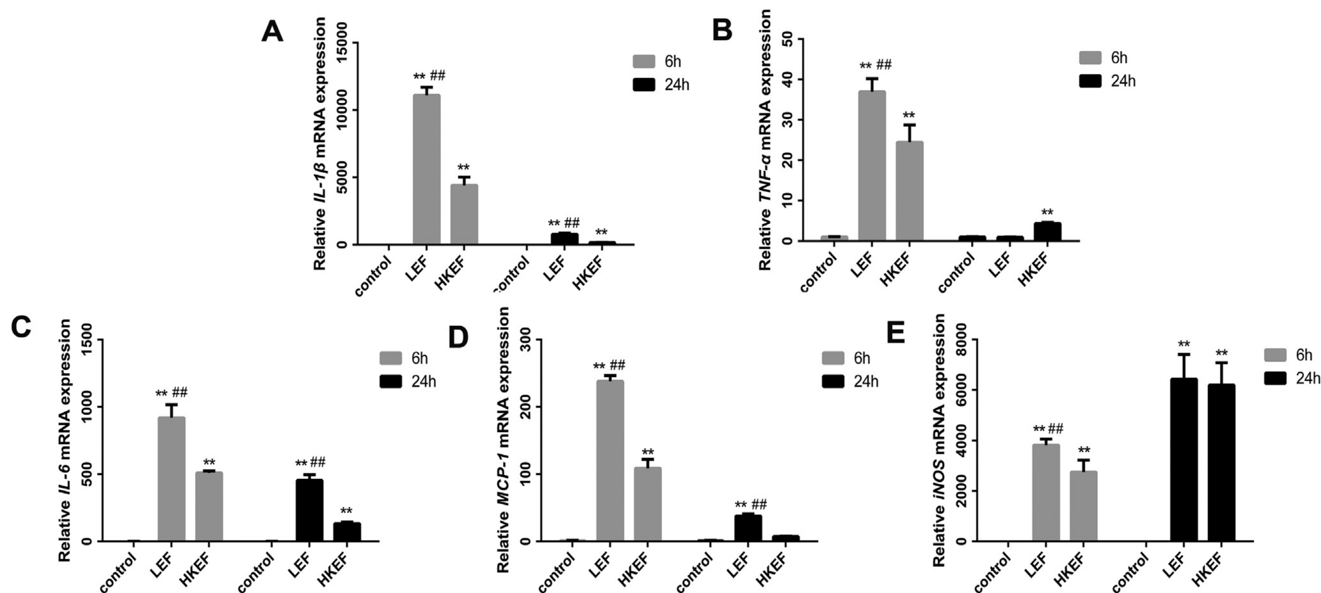


**Fig. 4.** *E. faecalis* induces expression of (A) *IL-1β*, (B) *TNF-α*, (C) *IL-6*, (D) *MCP-1*, and (E) *iNOS* in RAW264.7 cells. RAW264.7 cells were infected with LEF or HKEF (MOI = 100:1) for 2 h. Total RNA was extracted 6 h and 24 h post stimulation and expression level of the genes was quantified with qRT-PCR assay. The data represent one experiment that was performed in triplicate and are representative of the other two experiments. The expression of genes in LEF infected cells was significantly higher than that in the HKEF infected cells; gene expression in the infected cells (LEF or HKEF) was higher than that in non-infected cells (**P < 0.01, compared with the control group; ##P < 0.01, compared with the group infected by HKEF at the same time point). Abbreviations: *IL-1β*, interleukin-1β; *TNF-α*, tumor-necrosis factor-α; *IL-6*, interleukin-6; *MCP-1*, monocyte chemoattractant protein-1; and *iNOS*, inducible nitric oxide synthase.

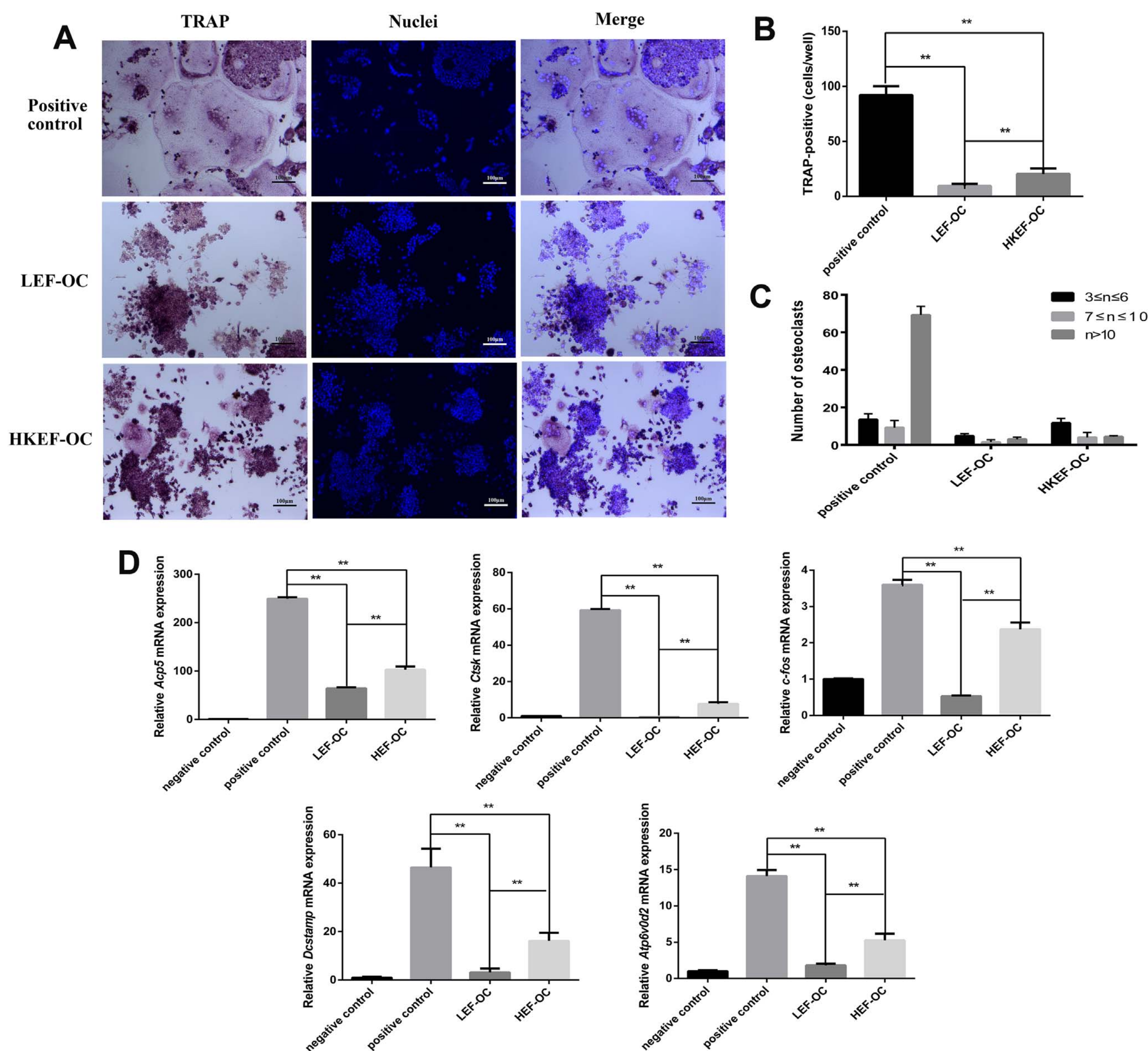


**Fig. 5.** Infection of RAW264.7 cells by live or heat-killed *E. faecalis* inhibits osteoclastogenesis. (A) RAW264.7 cells were infected with LEF or HKEF for 2 h at a MOI of 100 and then grown in the presence of recombinant murine RANKL. After 4 days, cells were fixed and stained for TRAP activity. Cells were counterstained with DAPI to visualize the nuclei. Scale bars represent 100 μm. (B) $TRAP^{+}$ multinucleated cells containing 3 and more nuclei were counted as osteoclasts. (C) Osteoclasts with 3–6 nuclei (small), 7–10 nuclei (medium), and > 10 nuclei (large) were counted from experiments in panel B. (D) Gene expression of osteoclast differentiation induced in infected cells (LEF or HKEF). Expression of genes *Acp5*, *Ctsk*, *c-fos*, *Dcstamp*, and *Atp6v0d2* were assayed using real-time PCR. The mean and SD are shown. Cells without any treatment served as negative controls, and cells without infection served as positive controls. Statistical significance is indicated with $^{**}P < 0.01$. Abbreviations: OC, osteoclasts.

of macrophages appeared to keep their abilities as activated macrophages, actively secreting pro-osteoclastogenic factors, thus exerting an indirect effect on bone destruction.

## 5. Conclusion

In summary, this *in vitro* model to study the interaction between live *E. faecalis* and osteoclast precursors revealed that infection by *E. faecalis* (i) upregulated the expression of various inflammatory factors, (ii) enhanced the phagocytosis of osteoclast precursors and, (iii) markedly attenuated their differentiation into mature osteoclasts. Collectively, *E. faecalis* infection of osteoclast precursors redirects their differentiation from mature osteoclasts to retain their roles in immunoregulation as activated macrophages. Such a mechanism may be significant in host defense in the pathogenesis of persistent apical periodontitis. Further studies are needed to precisely define the role of *E. faecalis* in the bone remodeling process.

## Acknowledgments

This work was supported by the National Natural Science Foundation of China (Number: 81670983), Natural Science Foundation of Guangdong Province (Number: 2017A030308011), and Guangdong Science and Technology Department (Number: 2015A020212004, 201607010271). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

## References

[1] N. Kawashima, T. Okiji, T. Kosaka, H. Suda, Kinetics of macrophages and lymphoid cells during the development of experimentally induced periapical lesions in rat molars: a quantitative immunohistochemical study, J. Endod. 22 (1996) 311–316,

http://dx.doi.org/10.1016/S0099-2399(96)80266-4.

[2] U.H. Lerner, Osteoblasts, osteoclasts, and osteocytes: unveiling their intimate-associated responses to applied orthodontic forces, Semin. Orthod. 18 (2012) 237–248, http://dx.doi.org/10.1053/j.sodo.2012.06.002.

[3] W.J. Boyle, W.S. Simonet, D.L. Lacey, Osteoclast differentiation and activation, Nature 423 (2003) 337–342, http://dx.doi.org/10.1038/nature01658.

[4] M. Locati, A. Mantovani, A. Sica, Macrophage activation and polarization as an adaptive component of innate immunity, Adv. Immunol. 120 (2013) 163–184, http://dx.doi.org/10.1016/B978-0-12-417028-5.00006-5.

[5] F.C. Martinho, W.M. Chiesa, F.R. Leite, J.A. Cirelli, B.P. Gomes, orrelation between clinical/radiographic features and inflammatory cytokine networks produced by macrophages stimulated with endodontic content, J. Endod. 38 (2012) 740–745, http://dx.doi.org/10.1016/j.joen.2012.02.021.

[6] M.N. Michalski, L.K. Mccauley, Macrophages and skeletal health, Pharmacol. Ther. 174 (2017) 43–54.

[7] P.P. Souza, U.H. Lerner, The role of cytokines in inflammatory bone loss, Immunol. Investig. 42 (2013) 555–622, http://dx.doi.org/10.3109/08820139.2013.822766.

[8] Y. Takahashi, A. Yoshida, M. Nagayoshi, C. Kitamura, T. Nishihara, S. Awano, et al., Enumeration of viable *Enterococcus faecalis*, a predominant apical periodontitis pathogen, using propidium monoazide and quantitative real-time polymerase chain reaction, Microbiol. Immunol. 55 (2011) 889–892, http://dx.doi.org/10.1111/j.1348-0421.2011.00390.x.

[9] G.O. Zoletti, J.F. Siqueira, K.R. Santos, Identification of *Enterococcus faecalis* in root-filled teeth with or without periradicular lesions by culture-dependent and-independent approaches, J. Endod. 32 (2006) 722–726, http://dx.doi.org/10.1016/j.joen.2006.02.001.

[10] C.H. Stuart, S.A. Schwartz, T.J. Beeson, C.B. Owatz, *Enterococcus faecalis*: its role in root canal treatment failure and current concepts in retreatment, J. Endod. 32 (2006) 93–98, http://dx.doi.org/10.1016/j.joen.2005.10.049.

[11] I. Portenier, T. Waltimo, D. Ørstavik, M. Haapasalo, The susceptibility of starved, stationary phase, and growing cells of *Enterococcus faecalis* to endodontic medicaments, J. Endod. 31 (2005) 380–386, http://dx.doi.org/10.1097/01.don.0000145421.84121.c8.

[12] D. Richards, J.K. Davies, D. Figdor, Starvation survival and recovery in serum of Candida albicans compared with *Enterococcus faecalis*, Oral. Surg. Oral. Med. Oral. Pathol. Oral. Radiol. Endod. 110 (2010) 125–130, http://dx.doi.org/10.1016/j.tripleo.2010.03.007.

[13] A. Rincé, B.Y. Le, N. Verneuil, J.C. Giard, A. Hartke, Y. Auffray, Physiological and molecular aspects of bile salt response in *Enterococcus faecalis*, Int. J. Food Microbiol. 88 (2003) 207–213, http://dx.doi.org/10.1016/S0168-1605(03)00182-X.

[14] G. Kayaoglu, D. Ørstavik, Virulence factors of *Enterococcus faecalis*: relationship to endodontic disease, Crit. Rev. Oral. Biol. Med. 15 (2004) 308–320, http://dx.doi.org/10.1177/154411130401500506.

[15] O.J. Park, J. Yang, J. Kim, C.H. Yun, S.H. Han, *Enterococcus faecalis* attenuates the differentiation of macrophages into osteoclasts, J. Endod. 41 (2015) 658–662, http://dx.doi.org/10.1016/j.joen.2014.12.015.

[16] J. Yang, O.J. Park, J. Kim, J.E. Baik, C.H. Yun, S.H. Han, Lipoteichoic acid of Enterococcus faecalis inhibits the differentiation of macrophages into osteoclasts, J. Endod. 42 (2016) 570–574, http://dx.doi.org/10.1016/j.joen.2016.01.012.

[17] S. Wang, Z. Deng, C.J. Seneviratne, G.S. Cheung, L. Jin, B. Zhao, et al., Enterococcus faecalis promotes osteoclastogenesis and semaphorin 4D expression, Innate Immun. 21 (2015) 726–735, http://dx.doi.org/10.1177/1753425915593162.

[18] S. Sahlin, J. Hed, I. Rundquist, Differentiation between attached and ingested immune complexes by a fluorescence quenching cytofluorometric assay, J. Immunol. Methods 60 (1983) 115–124, http://dx.doi.org/10.1016/0022-1759(83)90340-X.

[19] J. Tian, W. Qi, Y. Zhang, M. Glogauer, Y. Wang, Z. Lai, et al., Bioaggregate inhibits osteoclast differentiation, fusion, and bone resorption in vitro, J. Endod. 41 (2015) 1500–1506, http://dx.doi.org/10.1016/j.joen.2015.05.018.

[20] S. Takeshita, K. Kaji, A. Kudo, Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts, J. Bone Miner. Res. 15 (2000) 1477–1488, http://dx.doi.org/10.1359/jbmr.2000.15.8.1477.

[21] H. Liu, T. Luo, J. Tan, M. Li, J. Guo, Osteoimmunology' offers new perspectives for the treatment of pathological bone loss, Curr. Pharm. Des. (2017), http://dx.doi.org/10.2174/1381612823666170511124459.

[22] F.A. Del, A. Teti, The tight relationship between osteoclasts and the immune system, Inflamm. Allergy Drug Targets 11 (2012) 181–187, http://dx.doi.org/10.2174/187152812800392733.

[23] H. Takayanagi, New immune connections in osteoclast formation, Ann. N. Y. Acad. Sci. 1192 (2010) 117–123, http://dx.doi.org/10.1111/j.1749-6632.2009.05303.x.

[24] H. Takayanagi, The unexpected link between osteoclasts and the immune system, Adv. Exp. Med. Biol. 658 (2010) 61–68, http://dx.doi.org/10.1007/978-1-4419-1050-9_7.

[25] O.J. Park, J.Y. Han, J.E. Baik, J.H. Jeon, S.S. Kang, C.H. Yun, et al., Lipoteichoic acid of *Enterococcus faecalis* induces the expression of chemokines via TLR2 and PAFR signaling pathways, J. Leukoc. Biol. 94 (2013) 1275–1284, http://dx.doi.org/10.1189/jlb.1012522.

[26] J. Zou, N. Shankar, Roles of TLR/MyD88/MAPK/NF-κB signaling pathways in the regulation of phagocytosis and proinflammatory cytokine expression in response to E. faecalis infection, PLoS One 10 (2015) e0136947, http://dx.doi.org/10.1371/journal.pone.0136947.

[27] J. Zou, A.S. Baghdayan, S.J. Payne, N. Shankar, A TIR domain protein from E. faecalis attenuates MyD88-mediated signaling and NF-κB activation, PLoS One 9 (2014) e112010, http://dx.doi.org/10.1371/journal.pone.0112010.

[28] T.D. Kraemer, O.D. Quintanar Haro, E. Domann, T. Chakraborty, S. Tchatalbachev, The TIR domain containing locus of *Enterococcus faecalis* is predominant among urinary tract infection isolates and downregulates host inflammatory response, Int. J. Microbiol. 2014 (2014) 918143, http://dx.doi.org/10.1155/2014/918143.

[29] D.E. James, B.J. Nestor, T.P. Sculco, L.B. Ivashkiv, F.P. Ross, S.R. Goldring, et al., The relative timing of exposure to phagocytosable particulates and to osteoclastogenic cytokines is critically important in the determination of myeloid cell fate, J. Immunol. 185 (2010) 1265–1273, http://dx.doi.org/10.4049/jimmunol.0902808.

[30] S. Trouilletassant, M. Gallet, P. Nauroy, J.P. Rasigade, S. Flammier, P. Parroche, et al., Dual impact of live Staphylococcus aureus on the osteoclast lineage, leading to increased bone resorption, J. Infect. Dis. 211 (2015) 571–581, http://dx.doi.org/10.1093/infdis/jiu386.

[31] Y. Fujimura, H. Hotokezaka, N. Ohara, M. Naito, E. Sakai, M. Yoshimura, et al., The hemoglobin receptor protein of Porphyromonas gingivalis inhibits receptor activator NF-κB ligand-induced osteoclastogenesis from bone marrow macrophages, Infect. Immun. 74 (2006) 2544–2551, http://dx.doi.org/10.1128/IAI.74.5.2544-2551.2006.

[32] S.A. Hienz, S. Paliwal, S. Ivanovski, Mechanisms of bone resorption in periodontitis, J. Immunol. Res. 2015 (2015) 615486, http://dx.doi.org/10.1155/2015/615486.

[33] Y.T.A. Teng, H. Nguyen, X. Gao, Y.Y. Kong, R.M. Gorczynski, B. Singh, et al., Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection, J. Clin. Investig. 106 (2000) R59–R67, http://dx.doi.org/10.1172/JCI10763.

[34] K. Miyamoto, K. Ninomiya, K.H. Sonoda, Y. Miyauchi, H. Hoshi, R. Iwasaki, et al., MCP-1 expressed by osteoclasts stimulates osteoclastogenesis in an autocrine/paracrine manner, Biochem. Biophys. Res. Commun. 383 (2009) 373–377, http://dx.doi.org/10.1016/j.bbrc.2009.04.020.

[35] M.S. Kim, C.J. Day, N.A. Morrison, MCP-1 is induced by receptor activator of nuclear factor-κB ligand, promotes human osteoclast fusion, and rescues granulocyte macrophage colony-stimulating factor suppression of osteoclast formation, J. Biol. Chem. 280 (2005) 16163–16169, http://dx.doi.org/10.1074/jbc.M412713200.