

Role of Polymorphonuclear Neutrophils in the Clearance of *Enterococcus faecalis* Derived from Saliva and Infected Root Canals

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Abstract

Objectives: The goal of this study was to measure (1) the ability of polymorphonuclear neutrophil leukocytes (PMNs) to kill oral *Enterococcus faecalis* strains, (2) up-regulation of inflammatory mediators by PMNs in interaction with *E. faecalis*, and (3) the ability of *E. faecalis* to cause inflammation in mouse muscle tissue. **Methods:** Fifteen endodontic and nine saliva strains of *E. faecalis* were isolated and identified by specific 16S ribosomal RNA (16S rRNA) primers. The bacteria were grown in BHI broth and incubated with mouse PMN in appropriate media to determine the ability of the PMNs to kill the bacteria. In other experiments up-regulation of interleukin (IL)-1 α , tumor necrosis factor α (TNF- α), matrix metalloproteinase-8 (MMP-8), and cyclooxygenase (COX)-2 messenger RNA in the PMNs was measured after exposure of the leukocytes to the bacteria using real-time polymerase chain reaction. Finally, the inflammatory potential of and PMN response to *E. faecalis* suspension in mouse muscle tissue was examined from histological sections using hematoxylin-eosin staining and immunostaining. **Results:** Murine PMNs killed about 80% of the *E. faecalis* cells in 1 hour, irrespective of the source of isolation of the strains. Quantitative PCR results showed that IL-1 α , TNF- α , MMP-8, and COX-2 messenger RNA were markedly up-regulated in *E. faecalis*-stimulated PMNs or in *E. faecalis*-invaded muscular tissues. MMP-8 messenger RNA level was positively related to COX-2 messenger RNA level. Histological evaluation and immunostaining disclosed that all *E. faecalis* strains could recruit PMNs to the local infectious sites and cause abscess formation. **Conclusion:** *E. faecalis* strains from saliva and infected root canals have the potential to recruit PMNs in the infectious sites leading to inflammation via up-regulation of PMN IL-1 α ,

TNF- α , MMP-8, and COX-2. PMNs can play an important role in killing of *E. faecalis*. (*J Endod* 2011;37:346–352)

Key Words

Enterococcus faecalis, immunostaining, inflammatory cytokines, polymorphonuclear neutrophils

Infections in root canals have a polymicrobial nature. Many microorganisms including enterococci can be detected in the infectious root canals (1). Normally, enterococci inhabit the human and animal gastrointestinal tracts, the oral cavity, and vagina as normal commensals. They are also recognized as a major cause of nosocomial infections (2). Enterococci have been frequently implicated in endodontic infections because they are regularly found in obturated root canals with signs of continuing infection (3). They can also effectively invade dentinal tubules, which may allow reinfection of previously treated root canals (4). There are over 15 species of the *Enterococcus* genus; 80% of 90% of clinical isolates are *Enterococcus faecalis* (5). *E. faecalis* possesses a number of virulence factors that may relate to colonization, competition with other bacteria, resistance against defense mechanisms, and production of toxins directly or indirectly through induction of inflammation (2, 6). *E. faecalis* from infected root canals face a variety of stressful conditions in the root canal, such as nutrient limitations, toxins from other bacteria, and endodontic medicaments. Such environmental factors could have an influence on the expression of *E. faecalis* virulence factors (7). A better understanding of the role of *E. faecalis* in endodontic infection may help in the development of new strategies to prevent or eliminate the infection by this species.

Innate immunity plays a crucial role in determining the outcome of infection with many bacterial pathogens, including *E. faecalis*. Polymorphonuclear neutrophils (PMNs) are in general regarded as the most efficient first line of defense cells against invading microorganisms. PMNs play an important role in the early control of acute bacterial infections by killing bacterial pathogens through powerful oxidative and non-oxidative mechanisms and through the production of inflammatory and immunoregulatory cytokines and chemokines, such as tumor necrosis factor α (TNF- α), interleukin-1 (IL-1), and proteolytic enzyme, such as matrix metalloproteinases-8 (MMP-8, neutrophil collagenase) (8–10). These molecules lead to the influx and activation of PMNs and macrophages at the site of infection, which are important first steps in the development of inflammation (11).

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TABLE 1. Quantitative PCR Primers

Gene	Forward primers	Reverse primers
IL-1 α	CGAAGACTACAGTTCTGCCATT	GACGTTTCAGAGGTTCTCAGAG
TNF- α	GACGTGGAAGTGGCAGAAGAG	TTGGTGGTTTGTGAGTGTGAG
MMP-8	TCTTCCTCCACACAGCTTG	CTGCAACCATCGTGGCATT
COX-2	CTCCCTGAAGCCGTACACAT	ATGGTGTCCAAGCTCTACC
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

E. faecalis is frequently present in the infected root canal, whereas a mixed infiltrate of immune cells, in which a large proportion is PMNs, are present in the periapical lesions. (12). Previous studies have shown that human PMNs can kill most *E. faecalis* strains *in vitro* (13, 14), but some *E. faecalis* strains require opsonization to be killed by the PMNs (14). Moreover, there is still no direct evidence showing that PMNs can be recruited by invading *E. faecalis*, and what, if any, the role of PMNs in the host's resistance to local endodontic *E. faecalis* infection. Several studies have indicated that cyclooxygenase-2 (COX-2) is involved in the bacteria-induced inflammatory process (15). However, whether COX-2 is activated in PMNs during the *E. faecalis*-induced inflammatory process remains unknown.

The goal of this study was to measure (1) the ability of PMNs leukocytes to kill oral *E. faecalis* strains, (2) the up-regulation of some key inflammatory mediators by PMN cells in interaction with *E. faecalis*, and 3) the ability of *E. faecalis* to cause inflammation in mouse muscle tissue.

Materials and Methods

Special Reagents

Brain-heart infusion (BHI) broth was from Difco Laboratories (Lawrence, KS). RPMI 1640 medium and bovine serum were from Invitrogen (Carlsbad, CA). Casein, Histopaque-1077, and Histopaque-1119 were purchased from Sigma (Sigma, St Louis, MO).

Bacterial Strains

Approval for conducting the study was granted by the Peking University Institutional Review Board. Written consent to participate in the study was obtained from all participants. Patients aged 18 years or older who sought root canal retreatment for periapical lesions in the Hospital of Stomatology, Peking University, after root canal treatment that had been performed at least 2 years previously were recruited for this study. The exclusion criteria were the following: (1) patients who were pregnant; (2) patients who had active caries or periodontal disease (probing depth greater than 5 mm) or presence of a fistula; and (3) patients who had used local or systemic microbial agents during the 6 months before this study, smoked, or had diabetes or other systemic conditions. A paper point method was used in the root canal sampling process (16, 17), and the oral rinse method was used for saliva samples (18). Briefly, aseptic techniques were used throughout endodontic therapy and sample acquisition. Teeth were isolated from the oral cavity with a rubber dam and disinfected with 30% hydrogen peroxide and then 2.5% sodium hypochlorite, which was inactivated by 5% sodium thiosulfate. The root filling was removed with drills and endodontic files without chemical solvents. Irrigation was performed with sterile saline solution to moisten the canal before microbial sample collection. A sterile absorbent paper point was introduced into the full length of the canal (as determined with a preoperative radiograph), kept in place for 60 seconds, and transferred to transport medium. For saliva samples, patients rinsed their mouths for 60 seconds with 10 mL sterile distilled water and transferred the oral rinse sample to a 50-mL polypropylene tube. The identification of the strains was performed as previ-

ously described (17). Briefly, clinical samples were plated onto bile esculin azide agar (Becton Dickinson and Company, Sparks, MD) to selectively isolate enterococci. *E. faecalis* isolates were then identified by API 20 STREP (API Systems, La Balme les Grottes, France) and 16S ribosomal RNA (16S rRNA) polymerase chain reaction (PCR) method by using standard *E. faecalis* ATCC 29212 as the positive control (7). The sense *E. faecalis*-specific primer sequence was 5'-CCGAGTGCTTGCACTCAATTGG-3', and the antisense primer sequence was 5'-CTCTTATGCCATGCGGCATAAAC-3'. The PCR product size was 138 bp (19). *E. faecalis* was cultured in BHI broth at 37°C in air for 12 hours and then harvested, washed by phosphate-buffered saline (PBS), and serially diluted in PBS; the optical density was measured at 600 nm in order to establish the relationship between the turbidity and the viable count of the culture by the standard pour-plate method using BHI agar. Fresh *E. faecalis* cells were adjusted to a density of corresponding colony-forming units/mL (CFU/mL) in PBS and used in the following experiments.

Isolation of PMNs

Murine PMNs were isolated from peritoneal exudate cells by Histopaque density gradient centrifugation as previously described (20). Briefly, 1.0 mL 0.9% sterile casein solution was injected into the peritoneal cavity of each mouse 1 night before the isolation of PMNs. The inflammatory response was allowed to develop overnight, and 1.0 mL of casein solution was injected again in the next morning. Three hours later, the peritoneal fluid was harvested and centrifuged for 10 minutes at 200g at room temperature to collect the cells. The peritoneal exudate

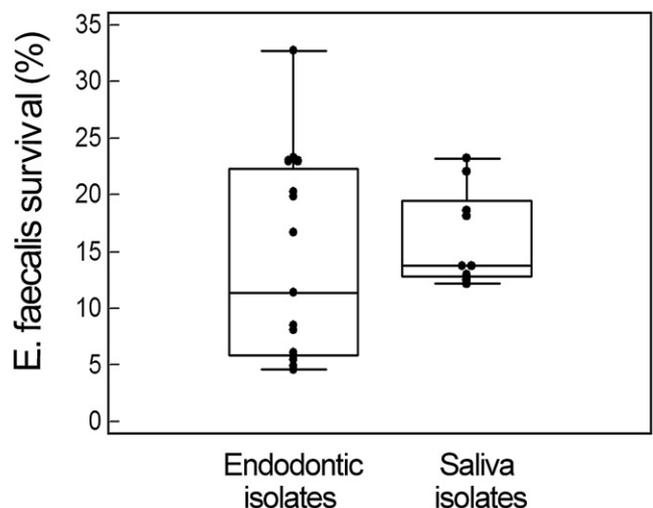


Figure 1. The comparison of PMN-mediated killing of endodontic and saliva isolates. PMNs and *E. faecalis* were cocultured at the ratio of 1:10 for 1 hour, then the nonphagocytosed *E. faecalis* and phagocytosed but still viable intracellular *E. faecalis* CFUs were determined by the pour-plate method using BHI agar. Each dot represents the viability of one *E. faecalis* strain in each group. There was no significant difference between endodontic and saliva strains ($p > .05$).

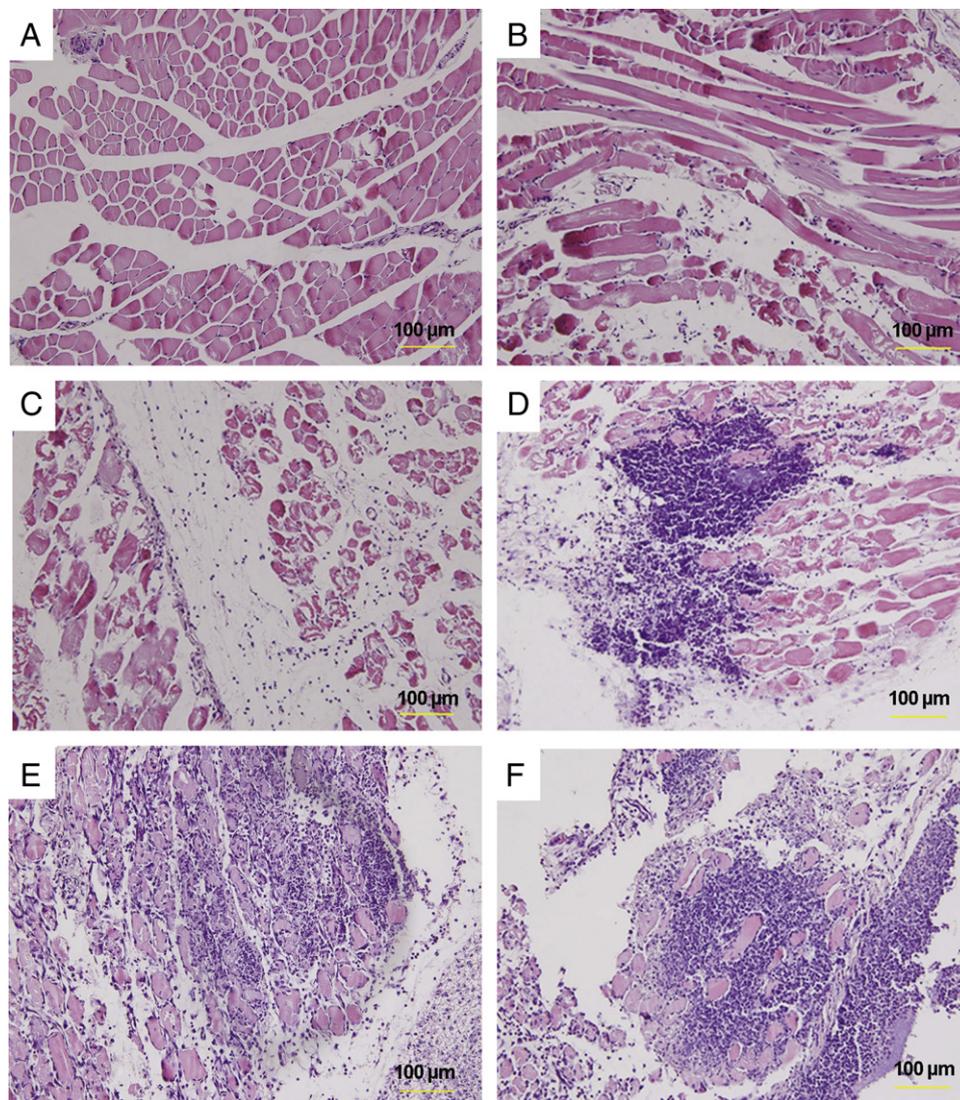


Figure 2. Representative microphotographs of pathological changes at indicated times post-intramuscular infections with endodontic *E. faecalis* eEf5. (A) Control, normal muscle injected with PBS; (B) 1 hour after eEf5 infection; (C) 2 hours after eEf5 infection; (D) 6 hours after eEf5 infection; (E) 24 hours after eEf5 infection; and (F) 48 hours after eEf5 infection. Original magnification 200 \times , bar =100 μ m.

cells were then washed three times with PBS, suspended in 1 mL of PBS, carefully layered over a density gradient comprised of Histopaque 1077 layered on top of Histopaque 1119, and centrifuged for 30 minutes at 700g at room temperature without using a brake. The PMN-rich fraction was collected and washed once with PBS. The density of PMN was adjusted to 2×10^7 cells/mL in RPMI 1640 medium containing 10% lipopolysaccharide low heat-inactivated fetal calf serum (Gibco, Gaithersburg, MD), 100 U/mL penicillin, and 100 μ g/mL streptomycin.

The PMN-Mediated Killing of *E. faecalis*

The interaction of PMNs and *E. faecalis* was performed as follows (13). Briefly, *E. faecalis* grown into logarithmic phase was harvested and washed twice in PBS and then diluted to a final concentration of 2×10^8 CFU/mL in PBS; 0.1 mL *E. faecalis* (2×10^7 CFU/mL) and 0.1 mL PMNs (2×10^6 cells/mL) were mixed well and placed at 37 $^\circ$ in an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany) with 300 rpm. After 1 hour of incubation, 10- μ L aliquots from all the reaction tubes were diluted with 190 μ L distilled water for 10 minutes to lyse the PMNs and release intracellular bacteria. Subsequently, the samples were serially diluted in sterile water, and colony counts were

determined by the pour-plate method using BHI agar. PMNs alone and bacteria alone served as the negative and positive controls, respectively. *E. faecalis* colonies were counted after 24 hours of incubation at 37 $^\circ$ C, and *E. faecalis* survival (%) was calculated from the number of viable *E. faecalis* after PMN incubation/number of initial viable *E. faecalis* \times 100%.

Quantitative PCR Analysis

The concentration of PMNs was adjusted at 1×10^6 cells /mL in RPMI 1640 complete medium, and 0.5 mL of PMN cell suspension was transferred into a 1.5-mL sterile tube; 1×10^7 endodontic *E. faecalis* strain eEf5 or saliva *E. faecalis* strain sEf64 were added to the corresponding tubes and incubated at 37 $^\circ$ C on the shaker. At 2 hours after interaction of *E. faecalis* and PMNs, the cells were separated by centrifugation (350g) for 10 minutes and stored for RNA extraction.

To detect messenger RNA expression of inflammatory mediators, the total RNA was extracted from the *E. faecalis*-stimulated PMNs and *E. faecalis*-invaded muscles using the Trizol reagent according to the manufacturer’s instruction (Invitrogen, Carlsbad, CA). Complementary DNA was reverse transcribed by the use of 2 μ g of RNA as template

and Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega, Madison, WI) in a total volume of 25 μ L. Real-time PCR was performed to detect the mRNA expression of IL-1 α , TNF- α , MMP-8, and COX-2; 0.5 μ L of complementary DNA was used as a template for amplification with 200 nmol/L of the following primers (Table 1). Reactions were performed by using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) and FastStart Universal SYBR Green PCR master mix (Roche) from Roche Applied Science (Indianapolis, IN).

Experimental Animals and Local Infection by *E. faecalis*

To explore the pathological role of *E. faecalis* in the local infection, we established the local infection model in mice. Briefly, adult male BALB/c mice (8 weeks old) were divided into 26 groups with five mice in each group; 10^8 *E. faecalis*/100 μ L PBS (15 endodontic isolates, 9 saliva isolates, and standard strain ATCC 29212) were inoculated into the right hind leg muscle of adult BALB/c mice, whereas 100 μ L PBS without bacteria was injected into the hind leg muscle of adult BALB/c mice used as the negative control. Twenty-four hours after infection, the infected tissues were removed, fixed by cool acetone, dehydrated through an ethanol series, treated with xylene, embedded in paraffin wax, and sectioned. The sections were stained with hematoxylin-eosin and evaluated by microscopic examination. In addition, one endodontic *E. faecalis* strain eEf5 and one salivary *E. faecalis* strain sEf64 were inoculated into the right hind leg muscle of adult BALB/c mice; 1, 2, 6, 24, and 48 hours after infection, the infected tissues, except for the 6-hour samples were removed, fixed by cool acetone, sectioned, and stained with HE for evaluation of the pathological changes in the tissue. The 6-hour fresh muscle samples were split in half. One half was fixed by cool acetone and subjected to immunostaining of Ly6-G, a PMN marker; the other half was subjected to RNA extraction to detect the messenger RNA expression of IL-1 α , TNF- α , MMP-8, and COX-2 by quantitative PCR as described earlier.

Immunostaining

The expression of Ly6-G antigen is specific to granulocytes; therefore, immunostaining of Ly6-G was performed to visualize the extent of PMN infiltration into the site of infection. Briefly, the sections were rinsed twice with PBS, and endogenous peroxidase was blocked by use of 3% hydrogen peroxide in PBS for 10 minutes. The samples were blocked with normal goat serum for 1 hour, incubated with rat anti-Ly6-G mAb (BD Pharmingen, San Jose, CA) for 1 hour at 37°C, followed by horseradish peroxidase-conjugated antirat immunoglobulin for 30 minutes, developed for color with peroxidase substrate 3-amino-9-ethylcarbazole (AEC; Zhongshan Golden Bridge Biotech Co, Ltd, Beijing, China), counterstained with hematoxylin, and recorded by using an Olympus DP controller (Olympus, Tokyo, Japan). PMNs were represented by red color staining.

Statistical Analysis

Data were expressed as mean \pm standard deviation and calculated for correlation coefficient r using Medcalc v.9.2.1.0 software (Frank Schoonjans, Mariakerke, Belgium); $p < .05$ was considered statistically significant.

Results

Prevalence of *E. faecalis*

A total of 24 isolates of *E. faecalis* were obtained among the 38 cases. Fifteen endodontic and 9 saliva strains of *E. faecalis* were from infected root canal and saliva samples, respectively. However, *E. faecalis* was isolated from both saliva and root canals only in 3

patients, most of the 24 isolates were isolated from different patients. API 20 STREP kits and 16S rRNA sequencing got the same results identifying *E. faecalis* in this study.

PMNs' Ability to Kill *E. faecalis*

In this study, we investigated the interaction of PMNs and saliva or endodontic *E. faecalis*. Murine PMNs were obtained from casein-stimulated mouse peritoneal cavity. When PMNs and *E. faecalis* cocultured at the ratio of 1:10, the unphaged *E. faecalis* and recovery of viable *E. faecalis* from the intracellular of PMNs were monitored. The survival of *E. faecalis* was reduced by 85.0% \pm 9.0% for endodontic isolates and by 83.7% \pm 4.3% for saliva isolates after 1-hour incubation with PMNs. There was no difference between strains from the two sources ($p > .05$, Fig. 1).

Pathological Observations

All *E. faecalis* strains caused a marked inflammatory reaction after injection and inoculation in mouse muscle tissue for 24 hours. When the mice were intramuscularly injected with 10^8 *E. faecalis*, injection site edema was detected at 1 hour after injection and developed into abscess during 24 to 48 hours. Histological examination revealed significant changes during *E. faecalis* infection. Infiltration of inflammatory cells was found at 1 hour after injection; necrosis started at 6 hours after infection and was found in most samples at 24 and 48 hours after injection. In contrast, PBS mock-infected mice did not show PMNs infiltration and necrosis at the indicated times (Fig. 2). Immunostaining of rat anti-Ly6-G monoclonal antibody revealed that large amount of infiltrated inflammatory cells were PMNs (Fig. 3). For the local inflammatory potential, there was no difference between the isolates of endodontic infection and saliva samples.

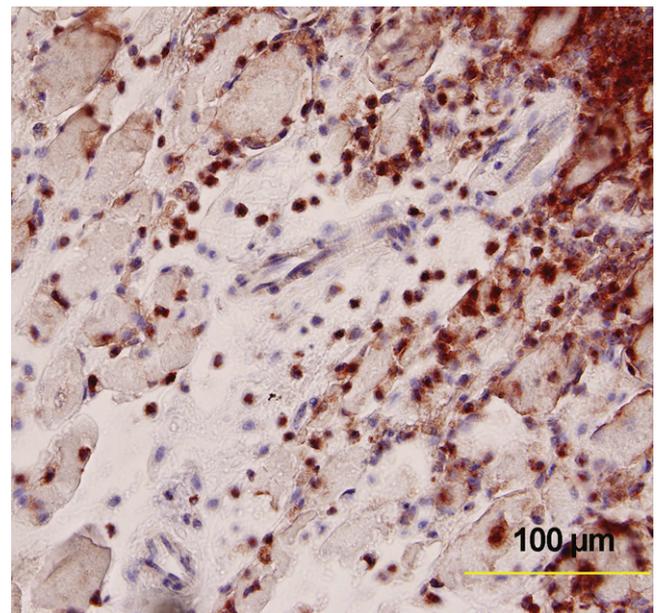


Figure 3. Immunostaining of Ly6-G. *E. faecalis* was intramuscularly inoculated into the hind leg of adult mice for 24 hours. The infectious tissues were sectioned, and immunostaining was performed by using anti-Ly6-G antibody (a PMN marker) and horseradish peroxidase-conjugated antirat immunoglobulin. Color development was with 3-amino-9-ethylcarbazole and counterstained with hematoxylin. Red color-labeled cells represent PMNs. Original magnification 400 \times , bar = 100 μ m.

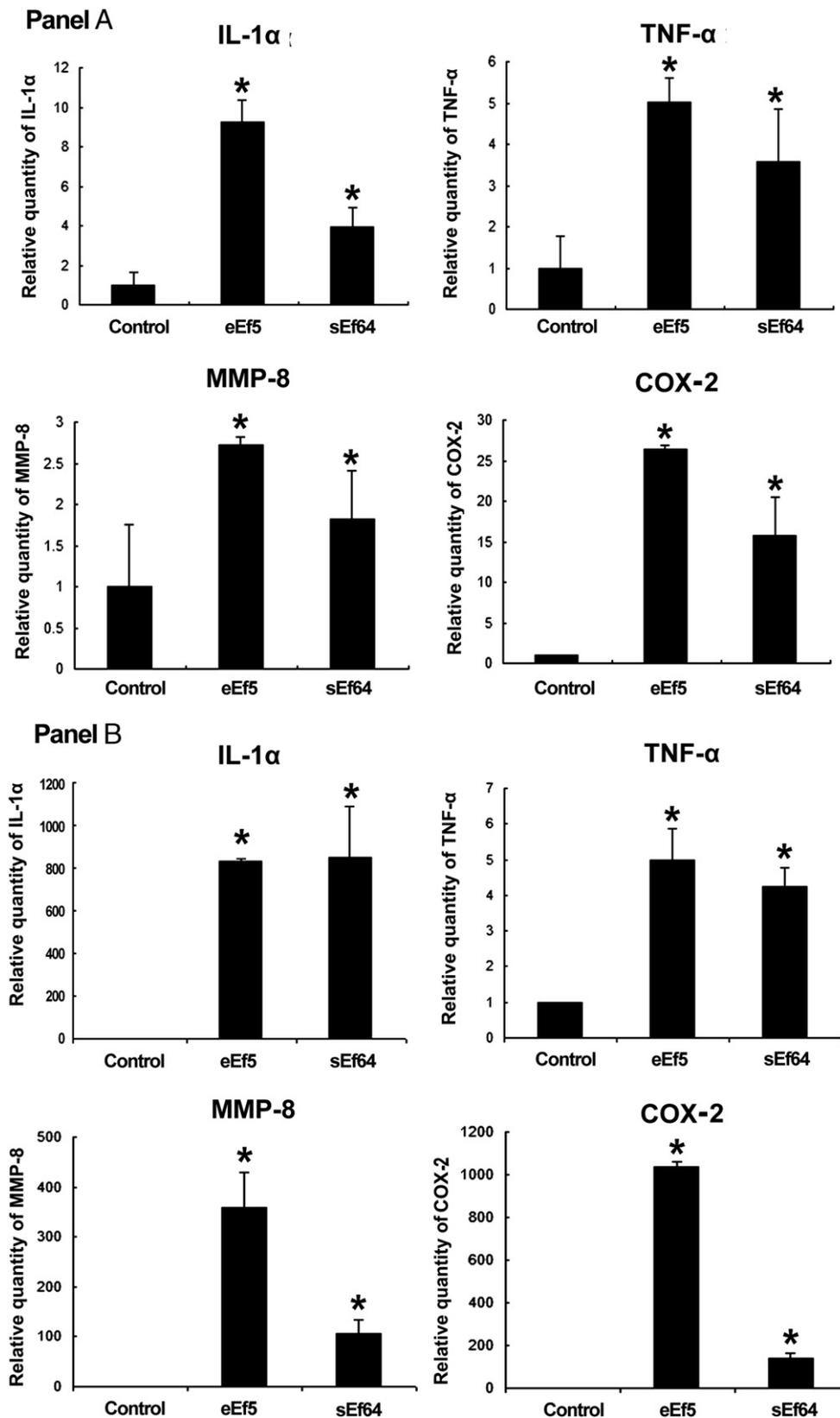


Figure 4. The induction of IL-1 α , TNF- α , MMP-8, and COX-2 messenger RNA expressions by endodontic *E. faecalis* strain eEf5 and saliva *E. faecalis* strain sEf64 in PMNs (A) *in vitro* and (B) in mouse muscle. The relative expressions of IL-1 α , TNF- α , MMP-8, and COX-2 messenger RNA were detected by quantitative PCR (A) after 2 hours of *E. faecalis*-stimulated PMNs at the ratio of 1:10 or (B) 6 hours of *E. faecalis*-invaded mouse muscle tissues. Glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNA served as an internal control. The histograms show increases over basal levels (control, unstimulated PMNs alone for A and normal mouse muscular tissue injected with sterile PBS for B). * $p < .05$ compared with corresponding controls.

Expression of TNF- α , IL-1 α , MMP-8, and COX-2 Messenger RNA in the *E. faecalis*-stimulated PMNs and in the *E. faecalis*-invaded Muscular Tissues

The inflammatory-related genes described previously were detected by quantitative PCR using total RNA derived from PMNs stimulated by an endodontic isolate eEF5 and a saliva isolate sEF64 for 2 hours and eEF5- or sEF64-invaded muscular tissues for 6 hours. Quantitative PCR results showed that eEF5 and sEF64 have the ability to release TNF- α , IL-1 α , MMP-8, and COX-2 from the PMNs *in vitro* (Fig. 4A) and in muscle tissue *in vivo* (Fig. 4B). The trends of the inflammatory mediators caused by *E. faecalis* *in vitro* and *in vivo* are similar, with the exception of IL-1 α . In the *in vitro* experiment, the IL-1 α expression level in eEF5-treated PMNs was a little bit higher than that in sEF64-treated PMNs, whereas its expression level in eEF5-invaded muscular tissues was as similar as that in sEF64-invaded muscular tissues. The MMP-8 messenger RNA level *in vitro* and in muscle tissue *in vivo* ($r = 0.91$, $p = 0.01$). eEF5 had more ability than sEF64 in the induction of IL-1 α , MMP-8, and COX-2 *in vitro* and MMP-8 and COX-2 *in vivo*. The differences were significant ($p < .05$).

Discussion

PMNs and monocyte/macrophages serve as phagocytic cells that are able to phagocytose and destroy infectious agents (8, 14, 15). Invading bacteria such as *E. faecalis* may be opsonized by complement proteins or antibodies and subsequently phagocytosed and killed by PMNs. However, some *E. faecalis* strains possess an aggregation substance that makes them resistant to killing by human PMNs (21). The stressful conditions in the root canal might influence endodontic *E. faecalis* virulence factors (7), and whether endodontic *E. faecalis* is more resistant to killing by PMNs needs to be investigated. A PMN-mediated *E. faecalis* killing experiment showed that 85.0% \pm 9.0% of endodontic isolates and 83.7% \pm 4.3% of saliva isolates were killed within 1 hour of incubation. The results indicated that whether endodontic *E. faecalis* carried more potential virulence factors or not, murine PMNs were equally effective in killing them than saliva strains. It is possible that PMNs from casein-stimulated murine peritoneal exudates cells were already activated and did not need assistance with complement activation. This opinion is supported by Itou et al' study, which showed that peritoneal exudate PMNs induced by thioglycolate or uric acid are already fully primed upon isolation from bone marrow (22).

Lipoteichoic acids isolated from strains of *E. faecalis* or from other gram-positive bacteria can stimulate leukocytes to release TNF- α , IL-1, and IL-8, and these factors have all been detected in periapical samples (2). Increased levels of several proteolytic enzymes such as elastase, cathepsin G, and MMP-8 released by PMNs in pulpitis and chronic apical periodontitis have been reported (9, 10). In this study, TNF- α , IL-1 α , and MMP-8 were clearly up-regulated when PMNs were incubated with *E. faecalis* *in vitro* (Fig. 2). Because only one endodontic and one saliva strain were tested, it is not possible to know if the difference between the two strains is normal variation between strains or whether it reflects different potential between strains from the two different sources. The experiments *in vivo* with 15 endodontic and 9 saliva strains and one control strain (ATCC 29212) showed similar ability by all strains to initiate inflammatory responses after intramuscularly injection in mice at the given dose. The development of the inflammatory responses caused by the *E. faecalis* strains was consistent, from edema at 1 hour after injection and abscess formation at 24 to 48 hours after infection. The trend of the IL-1 α expression caused by either eEF5 or sEF64 isolation *in vitro* is different from that *in vitro*. Our explana-

tion is that the different environment between PMNs and *E. faecalis* may contribute to the difference. In *in vitro* assay, there are only two kinds of cells, PMNs and *E. faecalis*, in the coculture system. The *in vitro* data of IL-1 α only represented PMNs' response to *E. faecalis*. But in the *in vivo* assay, *E. faecalis* not only recruits PMNs in the infectious tissues but also may recruit other inflammatory cells including macrophages, which may contribute to extra IL-1 α . In addition, tissue cells in the infectious site were also stimulated by *E. faecalis*, which may contribute to this response also.

Many studies have shown that COX-2 contributes to the inflammatory process (15, 23). In this study, COX-2 was markedly up-regulated by *E. faecalis* both in *E. faecalis*-stimulated PMNs and in muscle tissue. Moreover, MMP-8 messenger RNA level was positively correlated to COX-2 messenger RNA level. This indicates that COX-2 plays an important role in *E. faecalis*-induced infection. In previous studies, it has been shown that COX-2 is important for prostaglandins synthesis. Prostaglandin E2, in turn, can regulate the expression of MMP-1, MMP-3, and MMP-9 (24, 25). In refractory periodontitis, prostaglandin E2 levels were significantly higher in sites with high levels of MMP-8, and it was suggested these sites might be at risk for progression of periodontal disease (26). Selective COX-2 inhibitors and omega-3 fatty acids are potent prostaglandin inhibitors, and they also have an indirect inhibitory effect on MMP-8 (27). Histological examination in the present study showed that PMNs were mainly involved in *E. faecalis* infected muscular tissues, and elevated MMP-8 and COX-2 levels were also found. Higher MMP-8 levels during inflammation and wound healing evidently reflects the degree of connective tissue destruction, where inflammatory cells, particularly PMNs, play an important role in MMP-mediated tissue destruction (28). The results of present study showed that endodontic and saliva *E. faecalis* strains had similar ability to recruit PMNs to the local infectious sites.

In the present study, *E. faecalis* was relatively susceptible to PMN-mediated killing. The fact remains, however, that *E. faecalis* is the most common endodontic pathogen in root-filled teeth with persisting infection (3). The possible explanations include that PMNs and *E. faecalis* live in different environments in failed endodontic cases; they have less opportunity to interact with each other; even in apical area, the inflamed periapical lesion was encapsulated in collagenous connective tissue, which may affect enough PMNs to migrate the periapical lesion and thus may prevent direct PMN phagocytosis; or soluble products of *E. faecalis* may impair the bactericidal activity of PMNs (10, 29, 30). When PMNs enter the tissues, they will die within 1 to 2 days, whereas *E. faecalis* can survive long periods under harsh environmental stress and resuscitate upon returning to more favorable conditions (2). *E. faecalis* has the ability to invade and colonize dentinal tubules without the support of other bacteria (2, 4, 29, 31).

In conclusion, this study showed that both endodontic *E. faecalis* and saliva isolates have the potential to recruit PMNs in the infectious sites leading to inflammation via up-regulation of PMN IL-1 α , TNF- α , MMP-8, and COX-2. PMNs cells can play an important role in killing of *E. faecalis*.

Acknowledgments

The authors deny any conflicts of interest related to this study.

References

- Chavez de Paz LE. Redefining the persistent infection in root canals: possible role of biofilm communities. *J Endod* 2007;33:652–62.
- Kayaoglu G, Ørstavik D. Virulence factors of *enterococcus faecalis*: relationship to endodontic disease. *Crit Rev Oral Biol Med* 2004;15:308–20.

3. Love RM. *Enterococcus faecalis*—a mechanism for its role in endodontic failure. *Int Endod J* 2001;34:399–405.
4. Stuart CH, Schwartz SA, Beeson TJ, et al. *Enterococcus faecalis*: its role in root canal treatment failure and current concepts in retreatment. *J Endod* 2006;32:93–8.
5. Luis M, De la Maza. Color atlas of medical bacteriology. ASM Press, Herndon, VA; 2004:22.
6. Deng DM, Hoogenkamp MA, Exterkate RA, et al. Influence of Streptococcus mutants on *Enterococcus faecalis* biofilm formation. *J Endod* 2009;35:1249–52.
7. Pimentel LL, Semedo T, Tenreiro R, et al. Assessment of safety of enterococci isolated throughout traditional Terrincho cheesemaking: virulence factors and antibiotic susceptibility. *J Food Prot* 2007;70:2161–7.
8. van Faassen H, KuoLee R, Harris G, et al. Neutrophils play an important role in host resistance to respiratory infection with acinetobacter baumannii in mice. *Infect Immun* 2007;75:597–608.
9. Cootauco CJ, Rauschenberger CR, Nauman RK. Immunocytochemical distribution of human PMN elastase and cathepsin-G in dental pulp. *J Dent Res* 1993;72:1485–90.
10. Reynaud af Geijersstam A, Sorsa T, Stackelberg S, et al. Effect of *E. faecalis* on the release of serine proteases elastase and cathepsin G, and collagenase-2 (MMP-8) by human polymorphonuclear leukocytes (PMNs). *Int Endod J* 2005;38:667–77.
11. Dube PH, Revell PA, Chaplin DD, et al. A role for IL-1 alpha in inducing pathologic inflammation during bacterial infection. *Proc Natl Acad Sci U S A* 2001;98:10880–5.
12. Konttinen S, Ranta H, Lautenschlager I. Cells infiltrating human periapical inflammatory lesions. *J Oral Pathol* 1986;15:544–6.
13. Arduino RC, Murray BE, Rakita RM. Roles of antibodies and complement in phagocytic killing of enterococci. *Infect Immun* 1994;62:987–93.
14. Harvey BS, Baker CJ, Edwards MS. Contributions of complement and immunoglobulin to neutrophil-mediated killing of enterococci. *Infect Immun* 1992;60:3635–40.
15. Shen W, Li Y, Zhu J, et al. Interaction between macrophages, TGF-beta1, and the COX-2 pathway during the inflammatory phase of skeletal muscle healing after injury. *J Cell Physiol* 2008;214:405–12.
16. Pinheiro ET, Gomes BP, Ferraz CC, et al. Evaluation of root canal microorganisms isolated from teeth with endodontic failure and their antimicrobial susceptibility. *Oral Microbiol Immunol* 2003;18:100–3.
17. Pinheiro ET, Gomes BP, Ferraz CC, et al. Microorganisms from canals of root-filled teeth with periapical lesions. *Int Endod J* 2003;36:1–11.
18. Sedgley CM, Lennan SL, Clewell DB. Prevalence, phenotype and genotype of oral enterococci. *Oral Microbiol Immunol* 2004;19:95–101.
19. Sedgley CM, Molander A, Flannagan SE, et al. Virulence, phenotype and genotype characteristics of endodontic *Enterococcus spp.* *Oral Microbiol Immunol* 2005;20:10–9.
20. Luo Y, Dorf ME. Isolation of mouse neutrophils. *Curr Protoc Immunol* 2001;20:1–6.
21. Rakita RM, Vanek NN, Jacques-Palaz K, et al. *Enterococcus faecalis* bearing aggregation substance is resistant to killing by human neutrophils despite phagocytosis and neutrophil activation. *Infect Immun* 1999;67:6067–75.
22. Ito T, Collins LV, Thorén FB, et al. Changes in activation states of murine polymorphonuclear leukocytes (PMN) during inflammation: a comparison of bone marrow and peritoneal exudate PMN. *Clin Vaccine Immunol* 2006;13:575–83.
23. Wang XM, Wu TX, Lee YS, et al. Rofecoxib regulates the expression of genes related to the matrix metalloproteinase pathway in humans: implication for the adverse effects of cyclooxygenase-2 inhibitors. *Clin Pharmacol Ther* 2006;79:303–15.
24. Lindsey JD, Kashiwagi K, Boyle P. Prostaglandins increase proMMP-1 and proMMP-3 secretion by human ciliary smooth muscle cells. *Curr Eye Res* 1996;15:869–75.
25. McLaren J, Taylor DJ, Bell SC. Prostaglandin E(2)-dependent production of latent matrix metalloproteinase-9 in cultures of human fetal membranes. *Mol Hum Reprod* 2000;6:1033–40.
26. Söder B. Neutrophil elastase activity, levels of prostaglandin E2, and matrix metalloproteinase-8 in refractory periodontitis sites in smokers and non-smokers. *Acta Odontol Scand* 1999;57:77–82.
27. Vardar-Sengul S, Buduneli E, Turkoglu O, et al. The effects of selective COX-2 inhibitor/celecoxib and omega-3 fatty acid on matrix metalloproteinases, TIMP-1, and laminin-5gamma2-chain immunolocalization in experimental periodontitis. *J Periodontol* 2008;79:1934–41.
28. Buduneli N, Vardar S, Atilla G, et al. Gingival crevicular fluid matrix metalloproteinase-8 levels following adjunctive use of meloxicam and initial phase of periodontal therapy. *J Periodontol* 2002;73:103–9.
29. Lleó MM, Bonato B, Tafi MC, et al. Resuscitation rate in different enterococcal species in the viable but non-culturable state. *J Appl Microbiol* 2001;91:1095–102.
30. Nair PN. Pathogenesis of apical periodontitis and the causes of endodontic failures. *Crit Rev Oral Biol Med* 2004;15:348–81.
31. Sobrinho AP, Barros MH, Nicoli JR, et al. Experimental root canal infections in conventional and germ-free mice. *J Endod* 1998;24:405–8.