In Vitro Evaluation of Virulence Characteristics of Lipopolysaccharide from Two Different Clinical Isolates of *Tannerella forsythia*

By

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ABBREVIATIONS

Akt/PKB: Protein Kinase B

AP: apical periodontitis

APCs: Antigen presenting cells

CLR: C-type lectin receptor

CVD: Cardiovascular disease

ELISA: Enzyme-linked immunosorbent assay

ERK: Extracellular-signal-regulated kinase

FDF: Forsythia detachment factor

LPS: Lipopolysaccharide

MMPs: matrix metalloproteases

MOI: Multiplicity of infection

PCR: Polymerase chain reaction

PD: Periodontitis

PGE2: prostaglandin-2

rLPS: rough-type LPS

RANKL: Receptor activator of nuclear factor kappa-B ligand

sLPS: smooth-type LPS
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Tf: *Tannerella forsythia*

TLR: Toll-like receptor

WT: Wild type; Laboratory type strain ATCC 43037 of *Tannerella forsythia*
ABSTRACT

Lipopolysaccharide (LPS) is one of the dominant virulence factors of Gram-negative bacteria that is implicated in the stimulation of inflammatory cytokines and inflammatory bone resorption. The inflammatory activity of an LPS molecule depends on its structure and chemical composition. In the context of periodontal diseases, LPS derived from Gram-negative periodontal bacteria can initiate inflammatory alveolar bone loss. In this regard, the knowledge about the virulence potential of different forms of LPS from the periodontal pathogen *Tannerella forsythia* is lacking.

The aim of this study was to investigate if LPS molecules from different clinical isolates of *T. forsythia* differ in their inflammatory activity and ability to promote osteoclastogenesis. To reach this aim, LPS was isolated from two different clinical isolates (namely, UB4 and UB20) and a laboratory strain (ATCC 43037) of *T. forsythia* and analyzed by SDS-polyacrylamide gel electrophoresis. THP-1 derived macrophages were then stimulated with different LPS molecules and the secreted cytokines were analyzed by ELISA. In addition, mouse RAW 264.7 macrophages were challenged with LPS molecules and osteoclast differentiation was observed by light microscopy following TRAP staining. The results showed that macrophages stimulated with LPS from the clinical isolate UB20 induced increasing levels of IL-1β, TNFα, and IL-6 and osteoclast differentiation in a dose dependent manner as compared to the LPS from the clinical isolate UB4 as well as the laboratory strain ATCC 43037.

It was deduced from this study that UB20 is a potential virulent strain of *T. forsythia*, and infection by this strain might possibly lead to intense periodontal and peri-
apical inflammation as well as periodontal alveolar bone loss due to elevated induction of proinflammatory cytokines and enhanced osteoclastogenesis process.
Chapter 1

INTRODUCTION

T. forsythia is strongly associated with apical periodontitis (AP) and chronic periodontitis (PD), both are inflammatory diseases that affect periodontal tissues. AP is an endodontic disease that is defined as an inflammation of the tissues surrounding the apical part of the root resulting in clinical and/or radio-graphical symptoms. The primary cause of apical periodontitis is micro-organisms infecting the root canal space (1-3). Endodontic infections are mostly endogenous infections that develop when the normal oral microorganisms invade the devitalized pulp. The seminal study by Kakehashi et al (1) proved that pulp necrosis and peri-apical inflammation resulted in conventional rats when the pulp tissue was exposed to oral microorganisms. Conversely, in experimental rats when the pulp tissues remain germ-free, no pulp necrosis and peri-apical inflammation developed. Moller and his colleagues (2) showed that in monkeys’ teeth, infected pulp tissue induced peri-apical inflammatory reactions, while no peri-apical inflammation was observed in non-infected necrotic pulp tissue. These results have been replicated by Lin and co-workers (3) when they indicated that uninected pulp tissues in dogs did not cause peri-apical inflammation. However, peri-apical inflammation was developed in teeth with infected necrotic pulp tissues. Taken together, these studies provided a considerable evidence that bacteria are the major etiologic factor for AP. Although AP is primarily an infectious disease, the peri-apical tissue is usually bacteria-free as bacteria are confined to the intra-canal spaces (4), except in
certain cases such as the presence of draining sinus tract (5) or extra-radicular endodontic infection (6, 7). Bacteria themselves may not directly interact with the peri-apical tissues, but rather bacterial by-products by diffusing into tissues can induce inflammation (8). Bacterial endotoxins (Lipopolysaccharides or LPS) are capable of spreading from the root canal system into the peri-apical tissues and rouse a peri-apical inflammatory reaction (9). Thus, apical periodontitis can be caused either by bacteria invading the root canal system or indirectly by the ingress of bacterial toxins, enzymes, and noxious metabolic by-products into the peri-apical tissues.

Several studies have been conducted to assess the nature and composition of microorganisms present in the endodontic infection using different methods of assessment. These studies indicated that the bacterial composition associated with the primary endodontic infection is a mixture of different bacterial species that is predominated by anaerobic Gram-negative bacteria (10-13). Although there is no association between a specific species of bacteria and any form of AP, some species are isolated from the root canal system more frequent than other species, such as Tannerella forsythia, Fusobacterium nucleatum, and Porphyromonas species (14). Moreover, although several studies have showed that there is no association between specific kind of bacteria and the presence of clinical signs and symptoms (15, 16), Gomez et al reported that T. forsythia is more frequently identified in cases where patients complain of pain on percussion (17).

PD is an inflammatory disease characterized by a progressive destruction of the tooth supporting structures, that if kept without dental treatment, it can eventually result
in tooth loss. The prevalence of PD in the United States was recorded to be over 40% in adults, affecting more than 64 million people (18). PD is induced by a subgingival polymicrobial community in which a bacterial triad known as the red-complex comprising Porphyromonas gingivalis (Pg), Treponema denticola (Td) and Tannerella forsythia (Tf) is strongly represented (19).

The etiology and pathogenesis of AP are similar to that of PD as both diseases are caused by bacterial infection and involve pathologic changes of alveolar bone, periodontal ligament and cementum. The main difference is that PD affects coronal periodontal tissues, whereas AP affects apical periodontal tissues. In both the situations, bone loss is one of the characteristic features.

Pathogenic bacteria acquire diverse virulence factors that challenge the host immune system and play an important role in their pathogenicity. Normally, as the first line of defense to eradicate the invading pathogen, the host recruits neutrophils (primary phagocytes) and produces anti-inflammatory cytokines which help to amplify the phagocytic cascades and prime the immune system to secrete pathogen specific antibodies (20). However, if the bacterial challenges remain for a long period of time as it happens in chronic infections such as different forms of periodontitis, the host defense mechanisms are undermined, ending in tissue damage due to the release of destructive enzymes such as proteases, prostanoids and reactive oxygen species (21).
BACKGROUND

Both AP and PD are inflammatory diseases caused by bacterial infections where *T. forsythia* has been strongly implicated. AP is caused by an endodontic infection that develops inside the root canals following pulp necrosis. When endodontic infections reach a progressive stages, bacterial biofilm is observed on the root canal walls (22). Subsequently, apical periodontitis has been considered as one of the biofilm-related oral diseases (22, 23). Similarly, PD is caused by a chronic bacterial infection that comprises the subgingival plaque in the periodontal pockets. While more than 700 bacterial species have been identified in the subgingival plaque, the red complex triad including *T. forsythia* is the most significant contributor to the development of PD (19). Although bacteria are the primary etiological factor of AP and PD, some studies have isolated viruses (24, 25) and fungi (26, 27) in both diseases as well, especially in immunocompromised patients.

The peri-apical and periodontal tissues consist of cementum, periodontal ligament and alveolar bone, and the response of these tissues to any insult is similar to that of other connective tissues elsewhere in the body. When these tissues are challenged by bacteria and/or their byproducts, the host immune system will be activated resulting in inflammatory reaction that consequently will result in the development of AP or PD. Although microbial infection of the root canals is the primary cause of AP (1-3), the pathologic changes of the peri-apical tissues in AP are usually not directly caused by microbes themselves, but rather by their toxins, and noxious metabolic byproducts.
The adversity of these diseases is not limited to the oral cavity, but it also can negatively affect the patient’s systemic health. In this regard, it is hypothesized that oral bacteria ‘piggy-back’ on inflammatory cells or their virulence factors can be systemically carried to distant organs and affect the systemic health of the patients (28). A number of studies have been conducted to investigate the association between AP and PD with different systemic disorders. In concern to AP, some studies have reported a strong association between systemic disorders and the development of endodontic pathologies (29-31). However, other studies reported uncertain or no association (32, 33). In a systematic review by Gomez et al, they stated that apical periodontitis is associated with an elevated level of systemic inflammatory molecules (34), indicating that endodontic disease may contribute to a systemic inflammation. Another recent systematic review by Khalighinejad and co-workers, reported a possible association between some systemic diseases and the pathogenesis of endodontic disease such as cardiovascular and blood disorders (35).

On the other hand, in regard to chronic PD, research in this topic is more exhaustive and detailed compared to AP. For instance, it was found that blood glucose level is affected by PD in diabetic patients as bacterial products may interfere with the insulin activity (36). Also, cardiovascular diseases have strong association with PD. The relationship between these two conditions could be attributed to the chance of bacterial translocation from the periodontal pocket through circulation to the cardiovascular system (37, 38). PD was also found to be related to pre-term low-birthweight infants. This was explained by the increased level of systematic prostaglandin (PGE2) and matrix metalloproteases (MMPs) in patients having periodontal disease. PGE can
activate uterus contractions, and MMPs may result in chorioamniotic membrane rupture and subsequently lead to pre-term low-birthweight (39). Furthermore, patients diagnosed with PD are noticed to have other systemic conditions such as rheumatoid arthritis, obesity and respiratory diseases than healthy patients.
**Tannerella forsythia**

*T. forsythia* is a major bacterium implicated in endodontic and periodontal infections that carries unique virulence factors to enable it of escaping the immune system. *T. forsythia* is a strictly anaerobic, Gram-negative, rod-shape bacterium, that used to be named *Bacteroides forsythus* (40). This microorganism is difficult to culture because it requires fastidious and anaerobic growth conditions, therefore, *T. forsythia* is not well studied and the knowledge about its pathogenicity in periodontal disease is not well appreciated. It requires exogenous N- acetylmuramic acid (MurNAc), which is essential for the synthesizes of its cell-wall (peptidoglycan layer) along with N-acetyl glucosamine (GlcNAc) (41). Also, in order to support the growth of *T. forsythia*, it requires hemin as an additional nutrient in the medium (40). *T. forsythia* besides the other 2 members of the red complex play a major role in exacerbating the severity of the periodontal disease, as they work with each other to provide the needed nutrition. For example, the proteolytic effect of *P. gingivalis* on the host tissue is able to stimulate the production of several peptides and amino acids that are used by *T. forsythia* to grow. In an opposite way, *P. gingivalis* utilizes a succinate that is produced from *T. forsythia* to synthesize a membrane phospholipids which is essential for its growth (42). One of the earliest studies on the virulence potential of *T. forsythia* has indicated that *T. forsythia* in a mixed culture with *P. gingivalis* and *T. denticola* are able to form an abscess in rabbits, while a pure infection with one of these bacteria was not abscessogenic (43).

*T. forsythia* is the only Gram-negative bacteria that possess glycosylated Surface (S)-layer which is a water-insoluble protein layer that covers the bacterial cell as a saw
tooth like serrated lattice (44, 45). It is composed of 2 glycoprotein molecules, TfsA and TfsB. TfsA and TfsB genes encode the protein parts (45), and wecC gene cluster expresses the glycan part of this glycoprotein (46, 47). Researches have indicated that the S-layer protects the *T. forsythia* against host’s immunity (48, 49). Studies were directed in our laboratory to understand the S-layer role in modifying host’s immunity. These studies indicated that the S-layer glycoproteins have trisaccharide branch that is responsible for modulating the activity of antigen presenting cells (APCs; macrophages and dendritic cells) by weakening Th17 skewing cytokines IL-6 and IL-23. This is related to the ability of surface polysaccharides in modulating the lectin-like pathogen recognition receptors on APCs and promoting the survival of pathogens within the APCs. In addition to this, Th2 cell differentiation is controlled by cytokines produced in APCs when stimulated by BspA protein that activates the pattern recognition receptor TLR2. Thus, when *T. forsythia* weakens Th17 cytokines, it at the same time induces APCs to secrete Th2 cytokine. *In vivo* model of mouse periodontitis model showed that infection with *T. forsythia* ATCC 43037 resulted in alveolar bone loss through TLR2-Th2 dependent inflammatory axis (50). Sharma et al (51) showed that, mice infected by BspA-deficient mutant *T. forsythia*, had significantly less alveolar bone loss than the wild-type *T. forsythia*. This has proved the role of BspA protein as virulence factor in the pathogenesis of periodontal bone loss (51).
**T. forsythia Virulence factors and their role in pathogenesis**

**Proteolytic enzymes**

*T. forsythia* possesses proteolytic enzymes such as trypsin-like protease and PrtH protease, that are capable of breaking down the host’s proteins to amino acids, which are essential for the bacterium to grow. These enzymes can also activate the host immune response. Moreover, PrtH protease is known as *forsythia detachment factor* (FDF). As the name indicates, FDF detach the epithelial cells from the substratum and induce the release of IL-8 that is a main contributor in the pathogenesis of periodontitis (42).

**Surface (S)-layer**

*T. forsythia* possesses a unique surface layer (S-layer) glycoprotein lattice. S-layers are water-insoluble proteins intrinsically capable of self-assembling into crystalline lattices on bacterial cell surfaces, and are believed to provide selective advantages to bacteria, such as resistance to predation or immune clearance. The S-layer in *T. forsythia* surface plays a role in inhibiting or deferring the initiating of the immune response (52). It also facilitates the adhesion and invasion of *T. forsythia* into oral epithelial tissues (42). Moreover, the S-layer acts by stimulating the inflammatory cells to release pro-inflammatory cytokines and induce cellular apoptosis (42).

**BspA protein**

*T. forsythia* expresses a cell surface-associated and secreted protein, designated
BspA, which belongs to the leucine-rich repeat (LRR) and bacterial immunoglobulin-like protein families. It can interact with a variety of host cells, including monocytes. Similar to the S-layer, BspA protein is also essential for the bacterial adhesion and invasion into the host epithelial cells. BspA protein activates the host immune cells to release pro-inflammatory cytokines and chemokines. This protein helps *T. forsythia* to co-aggregate with other bacteria. Further, BspA protein can bind to Toll-like receptor 2 (TLR-2) and consequently results in bone resorption (42). This was proven in a mice study which demonstrated that *T. forsythia* BspA protein is able to activate the TLR-2 on the antigen presenting cells and causes a Th-2 response that induces the alveolar bone resorption (50).

**Glycosidases**

*T. forsythia* releases glycosidase enzymes that are able to break down oligosaccharides and proteoglycans to produce nutrients for other bacteria, facilitate bacterial adhesion and colonization, and affect the integrity of the periodontal tissues (42).

**Lipopolysaccharides (LPS)**

The LPS, also called endotoxins is a component of Gram-negative bacteria. Depending on the type of LPS (rough vs smooth), it may consist of 3 components;

i) **lipid A**, which consists of a phosphorylated N-acetyl glucosamine (NAG) molecule with 6-7 hydrophobic fatty acids (FA) that anchor the LPS to the bacterial membrane.
ii) Core-oligosaccharides, which is attached to the lipid A domain and consist of oligosaccharides.

iii) O-antigen, which is attached to the core unit. It consists of few oligosaccharides and forms the outermost layer of the LPS. There is usually a great variation in the O antigen structure among different species which gives immunological specificity for different species. Also, the presence of the O- chain is responsible for the smoothness of the LPS, where it’s absence makes rough LPS as depicted in Figure 1.

A recent study conducted in our laboratory has revealed that the expression of CXCL-10 chemokine in macrophages is differentially regulated in response to challenge with different *T. forsythia* strains (unpublished). CXCL-10 is a chemokine that has the ability to modulate the immune response as it is highly potent in attracting, recruiting and directing leukocytes to the inflamed or infected tissues. The same study has suggested that differences in the nature and composition of LPS structure among different strains is responsible for differential macrophage activation and CXCL-10 secretion. Therefore, CXCL-10 secretion in macrophages infected with *T. forsythia* is likely controlled by LPS-macrophage interactions likely involving TLR4 receptor.

Based on the above studies we speculated that differences in lipopolysaccharide (LPS) among different strains may be involved in the induction of CXCL-10 and the severity of the disease. The role of LPS in yielding bone resorption is well recognized (53). LPS stimulates the differentiation and maturation of osteoclasts and bone resorption by initiating the host response to recruit inflammatory cells that induce the release of diverse cytokines and inflammatory mediators (54). Moreover, osteoclast
maturation and bone resorption process is regulated by the OPG, RANK and RANKL system. RANKL is secreted by osteoblast cells, and it can also be released from T- cells when they are activated (55). Maturation of pre-osteoclast to osteoclasts requires high expression of RANKL that interact with RANK receptors on the precursor cells and promote the differentiation to mature osteoclasts.

To date, knowledge about the virulence factors, including LPS, of *Tannerella* species has come exclusively from the laboratory type strain ATCC 43037. Thus, it is important to identify and characterize the virulence factors such as LPS, of these species from other strains/clinical isolates.

**SPECIFIC AIM**

The present study was performed to determine whether LPS of different clinical strains of *T. forsythia* differentially activate proinflammatory cytokines production and osteoclast differentiation.
Chapter 2

MATERIALS & METHODS

LPS isolation and analysis by SDS-PAGE

Briefly, the bacterial cell (T. forsythia laboratory strain ATCC 43037 and clinical isolates UB4 and UB20) pellets were suspended in distilled water (1 g wet weight per 1.5 ml water) and an equal volume of 90% phenol was added. The suspension was stirred at 65-70°C for 20 min, cooled, centrifuged and aqueous phase was dialyzed in a dialysis tubing (Snake Skin, 3.5 K MWCO, Pierce, Rockford, IL) at 4°C against distilled water with at least 3 changes. The solution was brought to 0.15 M NaCl/50 mM Tris-Cl/4 mM Mg\(^{2+}\)/1 mM Ca\(^{2+}\) and then treated with deoxyribonuclease and ribonuclease A (Sigma-Aldrich, St. Louis, MO; each at 0.01 mg/ml) for 2 h. Endonuclease digested samples were then treated with proteinase K (Sigma-Aldrich) at 0.04 mg/ml at 60°C for 1 h. Finally, the digested samples were dialyzed as above and lyophilized. Different concentrations of T. forsythia LPS from ATCC 43037, UB4 and UB20 strains were separated on SDS-polyacrylamide gels, which was followed by silver-staining using a protocol routinely used in the Sharma’s laboratory.

Monocyte derived macrophages

THP-1 cells are human monocytes that were derived from patients diagnosed with acute monocytic leukemia. This cell line is usually used as an in vitro model to
study the monocyte/macrophage biology and to explore immune responses of monocyte/macrophage cells to different challenges (56). The morphology of THP-1 cells appears to be similar to the morphology of the primary monocytes and macrophages. Importantly, THP-1 cells provide an important in vitro model for analyzing the role of variety of pattern-recognition receptors such as C-type lectin receptors (CLR’s) and Toll-like receptors (TLR’s) in pathogen-associated molecular patterns (PAMPs).

THP-1 cells were obtained from the American Type Culture Collection (ATCC) and their growth was maintained in ATCC modified RPMI-1640 medium containing 2 mM L-glutamine, 10 mM HEPES, 1.0 mM sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate. The medium was enriched with 0.05 mM of 90% 2-mercaptoethanol, 10% of fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated in 5% CO2 at 37° C. THP-1 cells were differentiated into macrophages upon treatment with 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 48 h. After differentiation, the culture media was aspirated and replaced by fresh RPMI-1640 with 10% FBS. Differentiated cells were then challenged with LPS preparations.

**Enzyme-linked immunosorbent assay**

Macrophages (PMA stimulated THP1 cells) were stimulated with LPS (1-5 μg/ml) from different strains of *T. forsythia*. (*T. forsythia* 43037 UB20 and UB4), or left unstimulated (Control). Supernatants were collected at 16h post-stimulation and the concentrations of IL-1β, TNF-α and IL-6 were determined using sandwich based ELISA.
kit (e Biosciences), as per the manufacturer’s instructions. Standard curve for the cytokines were obtained by using the recombinant protein provided in the kit.

**Osteoclasts formation and Tartrate Resistant Acid Phosphatase (TRAP) staining**

Mouse RAW264.7 cell line (purchased from ATCC) was used for analyzing the ability of LPS molecules to induce macrophage differentiation into osteoclasts. RAW 264.7 cells are a macrophage-like, Abelson leukemia virus transformed, cell line derived from BALB/c mice. This cell line is commonly used as a model cell line in order to study the cellular responses and intracellular signaling in macrophage to microbes and their products. In addition, RAW 264.7 cells are routinely used as osteoclasts precursors as they can be readily induced with factors that promote osteoclast formation. Briefly, in this study, RAW 264.7 macrophages were pre-incubated with sub-optimal concentrations of recombinant murine RANKL (50ng/ml) and M-CSF 50 ng/ml (Cat #) for 4 days and then treated with LPS from Tf strains at different concentrations (1µg/ml and 5µg/ml) for 3 days in the presence of M-CSF (50ng/ml) and RANKL (50 ng/ml). After 7 days, cells were washed with phosphate-buffered saline (PBS) and fixed in 4% (v/v) paraformaldehyde at room temperature for 15 min. The cells were stained with TRAP staining kit (Sigma-Aldrich, USA) as per manufacturer instructions. Stained cells were then applied for microscopy (Olympus IX71, Olympus Optical, Tokyo, Japan). Osteoclasts were identified as TRAP-positive cells with ≥3 nuclei in each cell.
Statistical analysis

Data are shown as mean ± standard deviation of mean (SD) and were analyzed by ANOVA followed by post-hoc t-test for comparison between specific groups. A p-value of less than 0.05 was considered significant.

RESULTS

LPS purification and characterization

Different concentrations of LPS from \textit{T. forsythia} (ATCC 43037, UB4 and UB20) were subjected to electrophoresis in polyacrylamide gels in the presence of SDS and visualized by silver staining. Results (Fig. 2) showed a ladder-like pattern characteristic of a smooth-type LPS due to the presence of O-polysaccharide repeats in the case of UB20 LPS. On the other hand, the LPS isolated from Tf 43037 and UB4 migrated as a single band close to the dye front, indicating the absence of O-polysaccharide chains (rLPS).

IL-1β, TNF-α and IL-6 secretion by macrophages stimulated with LPS

ELISA was used to assay the concentration of cytokine proteins in the supernatants from THP-derived macrophages following stimulation with LPS from different strains of \textit{T. forsythia}. The ELISA results showed that the UB20 LPS was the
most potent LPS compared to either Tf 43037 or UB4 LPS in stimulating IL-1β, TNF-α or IL-6 from macrophages; UB20 LPS induced several-fold higher levels of IL-1β, TNF-α and IL-6 in comparison to other LPS. (Fig. 3).

*T. forsythia* LPS promotes osteoclastic activity

The number of mature osteoclasts (multi-nucleated TRAP⁺ cells) increased significantly in wells with M-CSF and RANKL primed RAW 264.7 macrophages following LPS treatment than without LPS treatment (Fig. 4). Strikingly, LPS from UB20 induced significantly higher numbers of multi-nucleated TRAP⁺ cells in a dose dependent manner than LPS from either Tf 43037 or UB4 (Fig. 4).
DISCUSSION

The current study was designed to evaluate the potential of LPS from different strains of *T. forsythia* in promoting inflammatory cytokine secretion and osteoclastogenesis, and to determine whether LPS presentation by different clinical strains is responsible for their virulence potential during the pathogenesis of AP and PD. The data presented in this thesis indicated that the LPS of UB20 clinical strain stimulated macrophages to secrete higher levels of proinflammatory cytokines IL-1β, TNFα and IL-6 and enhanced the formation of osteoclasts from RANKL-primed macrophages as compared to the LPS from other strains. A recent study conducted in our laboratory also reported that UB20 strain is highly potent in inducing CXCL-10 chemokine secretion from macrophages (57); CXCL-10 is a chemokine protein that attracts leukocytes to the site of inflammation (58). Together, these findings indicate that infection with UB20 may result in an intense periodontal inflammation and alveolar bone loss by promoting osteoclastic activity and recruitment of inflammatory leukocytes into the periodontium.

The above findings suggest that the ability of UB20 to promote inflammatory alveolar bone loss could be attributed to its highly potent smooth-type LPS (sLPS). LPS is an important virulence factor involved in the pathogenesis of inflammatory diseases such as chronic periodontitis and apical periodontitis. In inflammatory bone loss diseases, osteoclastogenesis induced by LPS is mediated by different ways. For example, LPS stimulates osteoblast cells to produce osteoclastogenic factors that have
the ability to activate and promote the differentiation of osteoclasts. These factors include RANKL, IL-1β, prostaglandin E2 (PGE2) and TNF-α, each of which are involved in LPS-mediated bone resorption pathways (59). Chiang and colleagues showed that in calvarial bone resorption models, high concentrations of LPS can drive bone resorption by stimulating TNF-α and IL-1 secretion (60). Another way is that, LPS can induce osteoclastogenesis by directly stimulating osteoclast precursors to secrete TNF-α, which in an autocrine manner can promote differentiation of these cells into mature osteoclasts (61). In addition, LPS can directly activate TLR4 and enhance the survival of osteoclasts by blocking apoptosis (62). TLR4 stimulation by LPS results in the activation of intracellular signaling pathways that activate Akt (also known as Protein Kinase B), NF-κB, and ERK (extracellular signal-regulated kinase) that ultimately promotes the proliferation and survival of osteoclasts. LPS also induces the expression of NFATc1 (nuclear factor of activated T-cells, cytoplasmic 1) that is essential for regulating osteoclastogenesis in RANKL-pretreated cells (53). In addition, LPS stimulate osteoclast differentiation by enhancing the phosphorylation of c-Jun N-terminal kinases (JNK) and ERK1/2 in pre-osteoclasts which are implicated in cell proliferation (63). Furthermore, Islam et al demonstrated that not only osteoclast precursor cells but also macrophages have the ability to differentiate into osteoclasts when challenged by LPS independent of RANKL (64). These observations advise that LPS plays multiple roles in the process of osteoclastogenesis.

Several studies have been conducted in an attempt to understand the mechanism by which LPS induces osteoclastogenesis and these studies have revealed knowledge about this process. TLRs, a class of pattern-recognition receptors,
expressed by several human cells such as monocytes, tissue macrophages, dendritic cells, endothelial cells, epithelial cells, lymphocytes and other cells including gingival fibroblasts (65) are the major receptors that detect LPS and subsequently initiate an inflammatory response by releasing inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNFα (59, 66). These inflammatory cytokines promote the expression of RANKL on osteoblasts, T- and B-cells (67, 68). RANKL can then bind to RANK presented on osteoclast precursor surface and induce osteoclast differentiation.

While LPS is one of the known virulence factors of many bacteria, there is a lack of understanding how LPS structure and composition (smooth/rough LPS or lipidA variants) contribute to LPS activity and virulence. To this end, this study and a study by Posch et al, (47) has shown that T. forsythia ATCC 43037 has a rough-type LPS (rLPS). The study of Posch et al (47) indicated that the rLPS of T. forsythia 43037 required serum factors for its biological activity since it was found to be significantly weaker in inducing the release of inflammatory cytokines from macrophages in the absence of serum. Here we showed that LPS of UB20 was significantly more potent than LPS from other strains in inducing the secretion of inflammatory cytokines IL-1β, TNFα and IL-6 by macrophages and this activity was not dependent on serum. These findings together suggest that the presentation of sLPS may render the T. forsythia more virulent in regard to bone destruction. Our results indicated that the sLPS which is composed of three functional parts; lipid portion (lipid A), polysaccharide (core region), and O-antigen, is potentially more virulent than the rLPS which lacks the O-antigen part. These findings suggest that the structure of LPS may influence its virulence. However, it should be noted that LPS structural analysis was not performed in the current study,
therefore, our data is inconclusive in regard to how does the LPS composition properties control the virulence of *T. forsythia*. Future research will be conducted in our laboratory to focus on the compositional analysis of the LPS parts.

We speculate from the results of our study that sLPS may be responsible for the bone destruction process. However, other virulence factors that were not investigated in our study could possibly participate in this process. It was confirmed in a previous study by Myneni et al (50) that BspA protein as well has the ability to stimulate TLRs and initiate a pronounced inflammatory response which in turn leads to bone destruction. However, up to now, the mechanism of how UB20 acts on increasing the release of IL-1β, IL-6 and TNFα inflammatory cytokines and consequently resulting in bone destruction remains unclear.

*In conclusion,* we speculate that infection by UB20 or strains with a similar LPS serotype would lead to intense periodontal and peri-apical inflammation with periodontal alveolar bone loss due to elevated levels of induction of inflammatory cytokines and enhanced osteoclastogenesis process. Consequentially, we predicted that UB20 is a potential virulent strain of *T. forsythia* and it would be interesting to evaluate the prevalence of this strain in patients presenting severe forms of periodontitis.
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**Fig. 1:** Lipopolysaccharides consist of: 1- lipid A component that is made of hydrophobic fatty acids chain, 2- core component that is composed of oligosaccharides, 3- O-antigen that is the outermost layer of the LPS. The presence or absence of O chains determines the type of LPS; Smooth LPS carries O-antigen chain while rough LPS lacks the presence of the chain.
Fig. 2: SDS-PAGE analysis of LPS molecules. The electrophoretic profiles indicate that UB20 expresses a smooth type (a ladder-like pattern due to O-chain repeats) and ATCC 43037 and UB4 express rough type LPS molecules.
Fig. 3: Cytokine secretion by THP-1 derived macrophages in response to LPS from different *T. forsythia* strains. ***, P<0.001 versus Tf 43037 and UB4.
**Fig. 4:** *T. forsythia* LPS promotes differentiation of RANKL-primed macrophages to osteoclasts. (Left) TRAP staining of LPS treated cell to assess osteoclast formation. (Right) Bar graph showing the numbers of osteoclasts (multinucleated TRAP$^+$ cells) per sq. mm. LPS from UB20 as compared to that from Tf 43037 or UB4 induced higher osteoclast formation in a dose dependent manner. *P < 0.05 versus no LPS; # P < 0.05 versus UB4 or UB20