Periodontal bacterial-DNA initiated immuno-inflammatory responses in human osteoblastic cells

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PERIODONTAL BACTERIAL-DNA INITIATED IMMUNO-INFLAMMATORY RESPONSES IN HUMAN OSTEOBLASTIC CELLS

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Physiology at Virginia Commonwealth University

by

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# Table of Contents

| Acknowledgements                                                                 | ii    |
| List of Tables                                                                   | vi    |
| List of Figures                                                                   | vii   |
| List of Abbreviations                                                            | ix    |
| Abstract                                                                        | xii   |

## INTRODUCTION

A. Pathogenesis of periodontal disease ................................................. 1

B. Periodontal PAMPs and their role in periodontal disease .......................... 5

C. Interaction of bacterial DNA (bDNA) with TLR9 and their role in periodontal disease ......................................................... 8

D. The role of osteoblast-osteoclast in periodontal disease .......................... 11

E. Periodontal microbiology and the microorganisms used in this study .............. 17

F. Study objective ..................................................................................... 19

## METHODS AND MATERIALS

A. Morphology and characteristics of MG-63 cells ........................................ 21

B. MG-63 cell culture .................................................................................. 23

C. MG-63 cell passenging ........................................................................... 24

D. MG-63 cell storage .................................................................................. 24

E. Bacterial cell culture conditions ........................................................... 26
F. Bacterial DNA (bDNA) isolation .................................................................28
G. Gel electrophoresis ....................................................................................30
H. Limulus Amoebocyte Lysate (LAL) assay .................................................30
I. Determination of optimum time for osteoblasts (MG-63 cells) to release
cytokines in response to bDNA .................................................................32
J. Determination of optimum concentration for osteoblasts (MG-63 cells) to
release cytokines in response to bDNA ....................................................33
K. Stimulation of MG-63 cells by bDNA and heat killed whole bacteria ..........33
L. Determination of the amount of IL-6 and IL-8 released from MG-63 cells by
enzyme-linked immunosorbent assay (ELISA) ........................................34
M. Statistical analysis ....................................................................................35
RESULTS .........................................................................................................36
A. Bacterial DNA purification and Limulus Amoebocyte Lysate (LAL) assay....36
B. Bacterial DNA concentration and purity ....................................................39
C. Determination of the quality of bacterial DNA preparations using agarose gel
electrophoresis ..........................................................................................45
D. Production of IL-6 from MG-63 cells in response to 100ng/µl bDNA at different
time points ..................................................................................................47
E. Production of IL-6 from MG-63 cells in response to 100ng/µl P.g DNA versus
100ng/µl LPS at different time points ..........................................................49
F. Production of IL-6 from MG-63 cells in response to different concentrations of bDNA ................................................................................................................51
G. IL-6 production from MG-63 cells in response to bDNA ........................................53
H. IL-6 production from MG-63 cells in response to heat killed whole bacteria .......55
I. Effect of chloroquine treatment on IL-6 production from MG-63 cells stimulated with bDNA ........................................................................................................57
J. Effect of chloroquine treatment on IL-6 production from MG-63 cells stimulated with heat killed whole bacteria ..............................................................59
K. IL-8 production from MG-63 cells in response to bDNA .......................................61
L. IL-8 production from MG-63 cells in response to heat killed whole bacteria .......63
M. Effect of chloroquine treatment on IL-8 production from MG-63 cells stimulated with bDNA ........................................................................................................65
N. Effect of chloroquine treatment on IL-8 production from MG-63 cells stimulated with heat killed whole bacteria ..............................................................67

DISCUSSION ................................................................................................................69
LITERATURE CITED ......................................................................................................74
VITAE ..........................................................86
### List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Characteristics of the MG-63 cell line</td>
<td>22</td>
</tr>
<tr>
<td>Table 2</td>
<td>List of bDNA and LPS used to stimulate osteoblasts</td>
<td>26</td>
</tr>
<tr>
<td>Table 3</td>
<td>Reagents used in the isolation of bDNA</td>
<td>28</td>
</tr>
<tr>
<td>Table 4</td>
<td>LAL assay, endotoxin standards preparation</td>
<td>31</td>
</tr>
<tr>
<td>Table 5</td>
<td>Endotoxin levels after the addition of Polymyxin-B</td>
<td>38</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Bacterial invasion of periodontal structures, and host immune response</td>
<td>3</td>
</tr>
<tr>
<td>Figure 2</td>
<td>The different TLRs and their location within a cell</td>
<td>6</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Structure of TLRs and their signaling pathway</td>
<td>10</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Preostoblastic/stromal cells regulation of osteoclastogenesis</td>
<td>13</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Effect of IL-6 on osteoclastogenesis</td>
<td>15</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Microscopic view of the MG-63 cells morphology</td>
<td>21</td>
</tr>
<tr>
<td>Figure 7</td>
<td>LPS standard curve</td>
<td>37</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Spectrophotometric analysis of bDNA isolated from <em>Aa</em> bacteria</td>
<td>41</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Spectrophotometric analysis of bDNA isolated from <em>Pg</em> bacteria</td>
<td>42</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Spectrophotometric analysis of bDNA isolated from <em>Ec</em> bacteria</td>
<td>43</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Spectrophotometric analysis of bDNA isolated from <em>S.s</em> bacteria</td>
<td>44</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Agarose gel electrophoresis showing the genomic bacterial DNA</td>
<td>46</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Time experiment: IL-6 production</td>
<td>48</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Time experiment, <em>Pg</em> LPS vs <em>Pg</em> DNA: IL-6 production</td>
<td>50</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Concentration experiment: IL-6 production</td>
<td>52</td>
</tr>
<tr>
<td>Figure 16</td>
<td>IL-6 release in response to bDNA</td>
<td>54</td>
</tr>
<tr>
<td>Figure 17</td>
<td>IL-6 release in response to heat killed whole bacteria</td>
<td>56</td>
</tr>
<tr>
<td>Figure 18</td>
<td>IL-6 release in response to bDNA from chloroquine treated MG-63 cells</td>
<td>58</td>
</tr>
</tbody>
</table>
Figure 19: IL-6 release in response to heat killed whole bacteria from chloroquine treated MG-63 cells ...........................................................................................................................................60

Figure 20: IL-8 release in response to bDNA ........................................................................62

Figure 21: IL-8 release in response to heat killed whole bacteria ........................................64

Figure 22: IL-8 release in response to bDNA from chloroquine treated MG-63 cells ......66

Figure 23: IL-8 release in response to heat killed whole bacteria from chloroquine treated MG-63 cells ...........................................................................................................................................68
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>Aa</td>
<td>Aggregatibacter actinomycetemcomitans</td>
</tr>
<tr>
<td>AIM-2</td>
<td>Absent In Melanoma-2</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>bDNA</td>
<td>bacterial DNA</td>
</tr>
<tr>
<td>BspA</td>
<td>putative virulence factor of the bacterium T.forsythia</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphate-Guanosine</td>
</tr>
<tr>
<td>CpG-ODN</td>
<td>Cytosine-phosphate-guanine Oligodeoxynucleotides</td>
</tr>
<tr>
<td>c-Fms</td>
<td>colony stimulating factor 1 receptor</td>
</tr>
<tr>
<td>DAI</td>
<td>DNA-dependent Activator of Interferon-regulatory factors</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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</tbody>
</table>
Hr   hours
IL-1β  interleukin-1β
IL-6  interleukin-6
IL-8  interleukin-8
K  Potassium
LAL  Limulus Amoebocyte Lysate
LB  Luria Bertani broth
LPS  Lipopolysaccharide
LRR  Leucine rich repeats
M  Molar
min  minutes
mL  millilitre
MG-63  human osteosarcoma cell line
M-CSF  Macrophage Colony Stimulating Factor
MOI  multiplicity of infection
MyD88  myeloid differentiation primary response gene 88
NF-κB  nuclear factor-kappa B
ng  nanogram
OD  optical density
OPG  osteoprotegerin
PAMP  pathogen-associated molecular patterns
PBS  Phosphate-buffered saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pg</td>
<td><em>Porphyromonas gingivalis</em></td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>p-value</td>
<td>Probability value</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor-kB</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor-kB ligand</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>Ss</td>
<td><em>Streptococcus sanguinis</em></td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA buffer</td>
</tr>
<tr>
<td>THP-1</td>
<td>human monocyctic leukemia cell line</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-interleukin-1 domain</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor-necrosis factor alpha</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
Periodontal bacterial-DNA initiated immunoinflammatory responses in human osteoblastic cells

By Najib J. Bou Chebel, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Physiology at Virginia Commonwealth University.

Virginia Commonwealth University, 2010

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Periodontitis is a chronic inflammatory disease initiated by gram negative anaerobic bacteria. These bacteria possess pathogen-associated molecular patterns (PAMPs) that interact with various receptors including Toll like receptors (TLRs). Bacterial DNA (bDNA) is one of the PAMPs mainly recognized by TLR9. Interaction of bDNA and its receptors leads to activation of inflammatory signaling pathways potentially resulting in periodontal bone destruction. The aim of this study was to determine the production of IL-6 and IL-8 in response to periodontal bDNA from human osteoblastic cells (MG-63). MG-63 cells were stimulated in duplicate for 20 hours with 100ng/µl of bDNA from various pathogens including Porphyromonas gingivalis, Esherichia coli, Streptococcus sanguinis, Aggregatibacter actinomycetemcomitans as well as heat killed whole bacteria (1:100).
E.coli LPS (10ng/µl) was used as a positive control in each experiment. To block TLR9 signaling, further experiments were carried out by treating MG-63 cells with chloroquine (10ng/µl) for 2 hours at 37ºC prior to stimulations. Cytokine levels were determined using enzyme linked-immunosorbent assay. Although IL-6 and IL-8 production was increased in response to periodontal bDNA in MG-63 cells, the results were not significant compared to unstimulated controls. As expected, E.coli DNA, E.coli LPS and heat killed whole bacteria stimulated significantly increased cytokine production (p<0.05). Blocking TLR9 with chloroquine did not affect the amount of cytokine production in bDNA stimulated cells suggesting that TLR9 may not be operant in triggering IL-6 and IL-8 production from MG-63 cells. In conclusion, periodontal bDNA did not trigger significantly increased IL-6 and IL-8 production from MG-63 cells. Considering the involvement of several inflammatory mediators in periodontal bone destruction, further studies are warranted to assess the production of other cytokines in response to periodontal bDNA in human osteoblastic cells.
Introduction

A. Pathogenesis of periodontal disease

Periodontitis is an inflammatory disease of the periodontium characterized by a progressive destruction of the tissues supporting the tooth. Its primary etiology is a poorly characterized series of microbial infections including some of the 300 or more species of bacteria identified in the oral cavity (Darveau, Tanner et al. 1997). Oral health has been improving and new treatments and technologies are being implemented, but in spite of these advancements, periodontitis still affects a large number of adults and a small number of children and teenagers worldwide (Y.-T.A.Teng 2005). Recent studies showed that periodontal disease not only leads to tooth loss, but also is a kind of early warning system associated with various systemic diseases such as diabetes (Southerland et al. 2005), kidney disease (Kshirsagar et al. 2005), preterm labor (Pretorius et al. 2007; Michalowicz and Durand 2007), osteoporosis, Alzheimer’s disease and certain types of cancer (Dietrich and Garcia 2005).

Periodontal diseases are initiated by a number of gram negative, anaerobic bacterial species which form dental plaque biofilms. These bacterial biofilms have been described as multispecies ecosystems where oral bacteria cooperatively interact with each other. Bacterial cells will either bind to the hard tissues including tooth and root surfaces and/or adhere to other bacteria that have already colonized on these surfaces, continuously
releasing cell surface components into the oral cavity and the gingival sulcus causing inflammation (Hojo, Nagaoka et al. 2009). In addition some of these bacterial species can invade soft tissues causing damage in deeper periodontal tissues. Up to now, no single bacterial species has been identified as the primary species responsible for periodontal disease. However it is widely accepted that gram negative, anaerobic bacteria such as *Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans* initiate periodontitis, and the unbalanced host immune response to these pathogenic stimuli is responsible for the periodontal tissue destruction.

Gram negative anaerobic bacterial cells have the ability to penetrate the gingival epithelium. As such, their virulence factors like lipopolysaccharide, lipids, proteins, endotoxin, immunologically active compounds and cytotoxic enzymes are presented directly to the host’s inflammatory cells (Darveau, Tanner et al. 1997). Once bacterial invasion takes place, the periodontal epithelium is breached, leading to inflammation and the destruction of the connective tissue and eventually causing bone loss and possible tooth loss (Dale 2002). Figure 1 is a schematic diagram briefly summarizing the initial immune responses that take place in the periodontium.
Figure 1. Bacterial invasion of periodontal structures, and host immune response to this antigenic challenge (adapted from Dale 2002).
There is a dynamic equilibrium between dental plaque and the host immune response. Dental plaque bacteria have adapted survival strategies that favor growth in this environment, while the host limits growth by a combination of innate and adaptive immune responses (Darveau, Tanner et al. 1997). The host immune system will respond to invading pathogens by detecting conserved pathogen associated molecular patterns (PAMPs) through a variety of receptors called pattern recognition receptors (PRRs) that include Toll-like receptors (TLRs). A microbial PAMP binds to its TLR and transmits a signal to the host cell’s nucleus inducing the expression of genes coding for the synthesis of several mediators called cytokines and chemokines (M. Azuma, 2006). Chemokines, such as IL-8, are a family of small polypeptides and are supposed to act as potent chemoattractants enhancing inflammatory cell infiltration and thus contributing in bone destruction (Suva and Bendre 2005). They play an important role in the pathogenesis of periodontitis. Other cytokines such as IL-6, IL-1β and tumor necrosis alpha (TNF-α) undergo biological activities that are known to be involved in the progress of this disease. These activities include the activation of osteoblasts and fibroblasts (Yumoto, Nakae et al. 1999). The disease progresses as periodic, i.e. relatively short episodes of rapid tissue destruction followed by some repair and then prolonged intervening periods of disease remission. But despite the apparent random distribution of episodes of disease activity, the tissue breakdown exhibits a symmetrical pattern of alveolar bone loss and pocket formation common to several forms of periodontitis. The distribution of the affected teeth and surfaces may vary among diseases.
B. Periodontal PAMPs and their role in periodontal disease

As mentioned before, bacteria and their cell wall components may initiate a host immune response, which includes innate and adaptive immunities. PAMPs trigger innate immunity in the early phase of infection through pattern recognition receptors (PRRs) such as the TLRs that are expressed on a number of cells including phagocytic cells, dendritic cells, endothelial cells, mucosal epithelial cells and lymphocytes (Akira, 2006; Akira et al. 2006). Structurally, TLRs are single membrane non-catalytic receptors which comprise a family of type I transmembrane proteins and are characterized by an extracellular leucine-rich repeat domain and an intracellular toll/interleukin-1 receptor (TIR) domain (M. Azuma, 2006; Kumagai, Takeuchi et al. 2007). There are at least 11 different types of TLRs; those recognizing structure components of pathogens such as proteins, lipoproteins, polysaccharides (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10) are extracellular and those that are specialized to recognize polynucleotides such as RNA and DNA (TLR3, TLR7, TLR8, TLR9) are intracellular and meet their ligand in the endosomal compartment (Amcheslavsky et al. 2005; Azuma 2006) as depicted in Figure 2.
**Figure 2.** The different TLRs and their location within a cell (adapted from Takeda and Akira 2005).
Some of the PAMPs associated with periodontal pathogens include LPS, gingipains (specific to the bacterium *P. gingivalis*), bacterial fimbriae, BspA protein of *T. forsythia* and bacterial DNA (bDNA) (Darveau, Tanner et al. 1997; Madianos, Bobetsis et al. 2005; Tatakis and Kumar 2005; Uehara et al. 2008; Sahingur et al. 2010). These PAMPs each play important role in periodontal disease pathogenesis mainly by promoting production of inflammatory mediators. Among these PAMPs, bacterial DNA (bDNA), the focus of our study, is one of the newly emerging PAMPs that might be involved in inflammation-induced tissue destruction associated with periodontal disease (Sahingur et al. 2010).

Bacterial DNA (bDNA) is mainly recognized through TLR9 and bDNA-TLR9 interactions have been involved in inflammation-induced tissue destruction in several diseases such as sepsis (Plitas et al., 2008; Sjolinder et al., 2008), systemic lupus erythematosus (Komatsuda et al., 2008; Wu et al., 2009; Zorro et al., 2009), and rheumatoid arthritis (Loos et al., 2006, Nakano et al., 2008; Rudnika et al., 2009), most of which share a common pathophysiology with periodontal disease. The studies conducted in our laboratory (Sahingur et al. 2010) as well as by other investigators point out a possible involvement of bDNA-initiated and TLR9 mediated inflammatory responses in periodontal pathology (Takeshita et al. 1999; Nonnenmacher et al. 2003). The next section will briefly summarize the interaction of bDNA with host immune cells and the evidence linking bDNA-initiated immune responses in periodontal disease.
C. Interaction of bacterial DNA (bDNA) with TLR9 and their role in periodontal disease

Microbial DNA sequences containing unmethylated CpG (Cytosine-phosphate-Guanosine) dinucleotides mainly activate Toll-like receptor 9 (TLR9) leading to the production of pro-inflammatory cytokines as shown in figure 3 below (Ewaschuk, Backer et al. 2007; Kumagai, Takeuchi et al. 2007). Recent studies also implicated other receptors, such as AIM-2 (Absent In Melanoma-2) and DAI (DNA–dependent Activator of Interferon-regulatory factors) in foreign DNA recognition (Takaoke et al. 2007; Muruve et al. 2008; Hornung et al. 2009). The stimulatory effect of bDNA is conferred by unmethylated CpG dinucleotides in particular base contexts (CpG motifs) that also determine the species-specific activity of the nucleic acids. Accumulating evidence suggests that bDNA and TLR9 interact in intracellular compartments. As bDNA is internalized through endocytosis, TLR9 relocates. The accumulation of bDNA and TLR9 in the endosomes leads to their co-localization within the same vesicles, and induces the recruitment of MyD88 to initiate signaling (Takehita F. et al. 2004; Kumagai, Takeuchi et al. 2007). TLR9 recognizes specifically bDNA that is unmethylated and single stranded (ss). Methylation of the cytosine within the CpG motif strongly reduces the affinity of TLR9 (Rutz M et al. 2004). Due to the abundance of unmethylated/hypomethylated cytosine-phosphate-guanosine (CpG) dinucleotides (CpG motifs) in the bacterial genome (Bauer et al., 2001), host cells have been shown to distinguish pathogenic DNA from host DNA. These motifs are found to be mostly methylated and rare in eukaryotic DNA (Ewaschuk, Backer et al. 2007; Kumagai, Takeuchi et al. 2007).
The majority of the studies thus far have used synthetic CpG oligonucleotides to investigate the receptor-ligand interactions in bDNA-mediated immune responses (Dalpke et al. 2002; Kreig, 2006; Heeg et al. 2008). Varying from synthetic CpG oligonucleotides, the structures of naturally-occurring TLR9 activators such as bacterial genomic DNA are more heterogeneous. Moreover, besides differences encountered between native DNA and oligonucleotides, the extent of the immune response to different bacterial nucleic acids may show differences depending on cell type (Dalpke et al. 2006; Mogensen et al. 2006). Thus, it remains important to ascertain the immunostimulatory effect of native bDNA from different pathogens in various cell types to assess the contribution of these molecular structures to the pathologic processes. Previous studies investigated periodontal bacterial DNA triggering cytokine (IL-6 & TNF-α) release from human gingival fibroblasts and murine macrophages (Takeshita et al. 1999; Nonnenmacher et al. 2003). Moreover, the expression of TLR9 is increased in periodontally diseased tissues compared with healthy sites supporting a possible role of TLR9 and its ligand in periodontitis (Kajita et al. 2007; Beklen et al. 2008). Most recently, studies in our laboratory demonstrated increased pro-inflammatory cytokine production from human monocytes (THP-1) in response to various periodontal pathogenic DNA via TLR9 and NF-kappa B (NF-κB) signaling pathway (Sahingur et al. 2010). Considering differential responses to bDNA in various cells, this study is designed to investigate the possible effect of periodontal bDNA on human osteoblastic cells.
Figure 3. Structure of Toll-like receptors and their signaling pathway (adapted from Azuma 2006)
D. The role of the osteoblast-osteoclast balance in periodontal disease

As mentioned before, periodontitis is characterized by periodontal pocket formation and alveolar bone resorption eventually leading to tooth loss. That occurs due to the impairment of the normal bone remodeling via unbalanced inflammatory responses. Bone remodeling is regulated by the balance between osteoclast and osteoblast formation and activity, known as alveolar bone homeostasis (Mori, Brunetti et al. 2007).

Osteoblast’s function is to synthesize the organic matrix components and to direct the events resulting in mineralization. The osteoblast becomes an osteocyte once it is surrounded with the mineralized matrix and these osteocytes which are known to maintain the mineralized matrix, work cooperatively with osteoblasts to produce proteases that are involved in degrading and remodeling the extracellular matrix resulting in its maturation and calcification. In addition, osteoblasts are involved in the production of paracrine and autocrine factors such as cytokines and growth factors which deeply influence both the formation and resorption of bone. In contrast, osteoclasts, cells of hematopoietic origin, function in the resorption of the mineral and organic phases of the bone ultimately working in concert with osteoblasts and osteocytes to remodel and maintain skeletal integrity throughout life (Schwartz et al. 1997; Spyrou et al. 2001; Stanley et al. 2006). In short, a balance between the activities of both osteoblasts and osteoclasts determine the level of bone mass.

Receptor activator of nuclear factor-κB ligand (RANKL) and its decoy receptor, osteoprotegerin (OPG) are essential molecules for the differentiation of osteoclasts regulated by osteoblasts. Other resident cells besides osteoblasts such as periodontal
ligament fibroblasts and gingival fibroblasts also regulate RANKL and osteoprotegerin behavior in periodontal tissues (Nagasawa, Kiji et al. 2007). Preosteoblastic cells and stromal cells express RANKL, which binds to its receptor RANK on the surface of osteoclasts and their precursors as shown in figure 4.
Figure 4. Preosteoblastic/stromal cells regulation of osteoclastogenesis (adapted from Khosla 2001).
Macrophage colony stimulating factor (M-CSF) which binds to its receptor c-Fms on preosteoclastic cells is essential for the development of osteoclasts because it is the primary determinant of the pool of these precursor cells. RANKL, however, is critical for the differentiation, fusion into multinucleated cells, activation and survival of osteoclastic cells. OPG is secreted by osteoblasts and osteogenic stromal stem cells and protects the skeleton from excessive bone resorption by binding to RANKL preventing it from interacting with RANK and blocking its effectiveness (Khosla 2001; Boyce and Xing, 2007). A number of proresorptive cytokines and chemokines such as IL-6 and IL-8 modulate this system primarily by stimulating M-CSF production thereby increasing the pool of preosteoclastic cells and by directly increasing RANKL expression. Furthermore a number of other cytokines (TNF-α and IL-1β) and hormones exert their effects on osteoclastogenesis by regulating the RANKL/OPG system (Schwartz et al. 1997). While our main focus is on both IL-6 and IL-8, other cytokines/chemokines will be further looked at in future studies.

IL-6 is known to modulate the differentiation of osteoblasts and osteoclasts (Marriott et al. 2008). It is believed to play a positive regulatory role in osteoclast differentiation by inducing the expression of RANKL on the surface of osteoblasts (Marriot et al. 2008). It has been demonstrated in recent studies that IL-6 can also directly act on osteoclast progenitors to suppress their differentiation via an inhibition of RANK signaling pathways as shown in figure 5 (Yoshitaki et al. 2003).
Figure 5. Effect of IL-6 on osteoclastogenesis (adapted from Yoshitaki et al. 2008)
IL-8 expression is elevated during a number of inflammatory processes including periodontal disease which is associated with osteoclast activation and bone destruction (Suva and Manali 2005). IL-8 is able to regulate the expression of the essential osteoclastogenic factor (RANKL), by osteoblastic stromal cells, and can also up-regulate parathyroid hormone (PTH) production by cells of the parathyroid gland. Regulation of parathyroid hormone, a hormone involved in the normal calcium metabolism coupled with the production of IL-8 by a variety of different cell types in bone suggests that IL-8 may be an important mediator in bone homeostasis (Suva and Manali 2005).

Recent studies have shown that not all regulation of the osteoclast is exclusively via the osteoblast because calcitonin acts directly on osteoclastic cells, and estrogen has been shown to induce apoptosis of osteoclasts as well as inhibit osteoclast differentiation by interfering with RANK signaling (Yoshitaki et al. 2003). Moreover, RANK expression can also be stimulated by TGF-β on preosteoclastic cells enhancing osteoclastic sensitivity to RANKL. The RANKL/OPG ratio in bone marrow is thus an important determinant of bone mass in normal and disease state (Khosla 2001).
E. Periodontal microbiology and the microorganisms used in this study

The supragingival biofilm of healthy gingivae is composed of a few (1-20) layers of oral streptococci, gram positive rods, and a few gram negative cocci. However, dental plaque associated with clinical gingivitis is composed of 100-300 layers, predominantly anaerobic gram-negative rods and filaments. Depending on environmental characteristics, the species found in bacterial biofilms can vary but patterns of colonization are fairly stable (Stingu, Eschrich et al. 2008). *Streptococcus sanguinis*, is a gram positive facultative coccus species of bacteria. It is a normal inhabitant of the healthy human mouth where it is found in dental plaque. It modifies the environment to make it less hospitable for other strains of streptococcus that cause cavities, such as *streptococcus mutans*. *S.sanguinis* may enter the blood stream when the opportunity presents itself. Once there, it can colonize the heart valves. It is the most common cause of subacute bacterial endocarditis (Li et al. 2003). *S.sanguinis* strain ATCC 10556 was selected as a non-pathogenic oral bacterial control in this study.

*P.gingivalis* w83 was chosen because it is known to be a virulent strain. It is a gram negative rod associated with severe and chronic periodontal diseases (Tachibana-ono, Yoshida et al. 2007). Progression of the disease is caused by the colonization of this organism in an anaerobic environment in host tissues and severe progression can eventually lead to tooth loss. The black pigmentation of this bacterium comes from iron acquisition that does not use the typical siderophore system of other bacteria that accumulate hemin (Tachibana-ono et al. 2008; Lin, Li et al. 2009). Peptides appear to be the predominant carbon and energy source of this organism, perhaps in keeping with its
ability to destroy host tissue. Oxygen tolerance systems play a part in establishing the organism in the oral cavity, including a superoxide dismutase (Najjar 2008). Pathogenic factors include extracellular adhesins that mediate interactions with other bacteria as well as the extracellular matrix, and a host of degradative enzymes that are responsible for tissue degradation and the spread of the organism including the gingipains, which are trypsin-like cysteine proteases. Proteinases attack extracellular matrix proteins, cell adhesion molecules, and immune system proteins such as cytokines and gamma interferon (Andrian, Grenier et al. 2006).

*Aggregatibacter actinomycetemcomitans*, previously *Actinobacillus actinomycetemcomitans*, is a gram negative facultative non-motile rod found in severe infections in the oral cavity, mainly the periodontium. It possesses certain virulence factors that enable it to invade tissues such as leukotoxin (Shimada, Nishihara et al. 2008; Kachlany, 2010). Leukotoxin has destructive effect on neutrophiles, monocytes, and T-lymphocytes, and in that way leads to the local immunosuppression in supragingival area which has a central role in development of periodontal lesions in juvenile periodontitis. This is why leukotoxin from *A. actinomycetemcomitans* is an essential virulent factor, and this microorganism has been used in our study since it is an important periodontal pathogen (Kachlany, 2010).

*Escherichia coli* is a gram-negative rod shaped bacterium that is commonly found in the lower intestine of warm blooded organisms (Todar 2004). The non-oral microorganism *E. coli* served as a source of ubiquitous, non-periodontal disease-associated antigens.
F. Study objective

The cellular and molecular events related to bone cells constitute the end stage of periodontal disease. Hence, a better understanding of the interaction of bDNA with osteoclasts and osteoblasts might potentially lead to new therapies to treat bone disease, including periodontal disease. Bacterial DNA is one of the newly emerging pathogen-associated molecular patterns (PAMPs) that might be involved in inflammation-induced tissue destruction associated with periodontal disease. Therefore, in this study, we seek to determine pro-inflammatory cytokine production, specifically cytokines/chemokines IL-6 and IL-8 in response to periodontal bacterial DNA in the osteoblastic cell line MG-63, and compare that with responses generated from whole bacterial cells.

Most of the previous studies have employed CpG oligodeoxynucleotides (CpG-ODNs) that are known to mimic bacterial DNA to elucidate the role of DNA recognition by osteoblastic and osteoclastic cells (Zou et al. 2003; Amcheslavsky et al. 2004; Amcheslavsky et al. 2005); however, in our study whole genomic bacterial DNA was isolated from different types of bacteria and used in the stimulation of the osteoblastic cell line MG-63. In this study, we also characterize the interactions of bDNA with osteoblasts in comparison with LPS as well as whole bacteria.

Varying from synthetic CpG oligonucleotides, the structures of naturally-occurring TLR9 activators are more heterogeneous. Hence, it is sometimes hard to predict the effects of bacterial nucleic acid initiated immune responses by extrapolating results obtained with synthetic oligonucleotides. Because of this, it remains important to ascertain the immunostimulatory effect of native bDNA from different pathogens in various cell types.
to assess the contribution of these molecular structures to the pathologic processes of periodontal diseases. This will be the first study that will determine the effect of periodontal bDNA in osteoblastic cells.
Materials and Methods

A. Morphology and Characteristics of MG-63 cells

Figure 6. Microscopic view of the MG-63 cell’s morphology
Table 1. Characteristics of the MG-63 cell line

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line designation</td>
<td>MG-63</td>
</tr>
<tr>
<td>ATCC catalog #</td>
<td>CRL-1427</td>
</tr>
<tr>
<td>Organism</td>
<td>Homo sapiens</td>
</tr>
<tr>
<td>Organ</td>
<td>Bone</td>
</tr>
<tr>
<td>Disease</td>
<td>Osteosarcoma</td>
</tr>
<tr>
<td>Morphology</td>
<td>Fibroblast</td>
</tr>
<tr>
<td>Growth properties</td>
<td>Adherent</td>
</tr>
<tr>
<td>cellular products</td>
<td>Interferon</td>
</tr>
</tbody>
</table>
B. MG-63 Cell culture

The base medium for this cell line is ATCC-formulated Eagle’s Minimum Essential Medium, catalog # 30-2003. To make the complete growth medium, heat-inactivated fetal bovine serum (FBS) (Gibco, Invitrogen) was supplemented to a final concentration of 10% and 1% Penicillin-Streptomycin (Gibco, Invitrogen) was added. In order to make FBS heat-inactivated, fetal bovine serum (not heat activated) was put in a water bath for 30 min at a temperature of 56°C.

The main cell line that was used in this research project was MG-63. These cells were originally isolated from a human osteosarcoma (Billiau et al., 1975) and purchased from ATCC catalog # CRL-1427 (Manassas, VA). The cell vial was removed from storage at -140°C and thawed by gentle agitation in a 37°C water bath. Both the O-ring and cap were kept out of the water to reduce contamination. The vial was then removed from the water bath as soon as the content was thawed and decontaminated by spraying 70% ethanol. The vial content was transferred to a centrifuge tube containing 9.0 ml complete culture medium and centrifuged approximately 125 x g for 7 minutes. The cell pellet was re-suspended with the recommended complete medium and dispensed into a 75 cm² flask (Corning CellBind Surface). The cells were maintained at 37°C in a fully humidified atmosphere at 5% CO₂ in air.
C. MG-63 Cell passaging

The culture medium was removed and discarded from the flask containing the cells. The cell layer was briefly rinsed with 0.25% (w/v) Trypsin-0.53mM EDTA (Gibco) solution to remove all traces of serum which contains trypsin inhibitor. 3.0 ml of Trypsin-EDTA solution was added to the flask which was then placed at 37°C for 5 to 10 minutes allowing the cells to detach. The cells were observed under an inverted microscope making sure the cell layer has dispersed. 6.0 to 8.0 ml of complete growth medium was added and the cells were aspirated by gently pipetting. Appropriate aliquots of the cell suspension were added to new culture vessels with a subcultivation ratio of 1:4 to 1:8. New cultures were incubated at 37°C. The media was changed every 48 hours and the cells were passaged with trypsin-EDTA every 3 days.

D. MG-63 Cell storage

The culture medium was removed from four flasks and discarded. The cell layer from each flask was briefly rinsed with 0.25% trypsin, 0.03% EDTA solution to remove all traces of serum which contained trypsin inhibitor. An additional 2 to 3 ml of trypsin-EDTA solution was added to each flask. The flasks were allowed to sit in the incubator at 37°C for 5 to 10 min until the cells detach. 10 ml of fresh culture medium was then added to each flask, transferred into a 50 ml centrifuge tube and centrifuged at 800 x rpm for 7 min. The cells were counted (freeze about 2x10^6 cells/ml. At least 1x10^6 cells and up to 5.4x10^6 cells). Media was added to the cells to make the concentration around 2x10^6 cells/ml and mixed well. DMSO (BP231-100, Fisher BioReagents) was added to the cell suspension.
until it reached 5%. 1.5 ml of cell suspension (with 5% DMSO) was added into a 2 ml vial and put on ice immediately. The vials were transferred to the -80°C freezer then transferred to the -140°C freezer the next day.
## E. Bacterial cell culture conditions

**Table 2.** List of bDNA and LPS used to stimulate osteoblasts

<table>
<thead>
<tr>
<th>bDNA &amp; LPS</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ec</em></td>
<td>Strain DH-10B</td>
<td>Dr. Kitten's lab, VCU, Richmond, VA</td>
</tr>
<tr>
<td><em>Pg</em></td>
<td>Strain W83</td>
<td>Dr. Schenkel's lab VCU, Richmond VA</td>
</tr>
<tr>
<td><em>Aa</em></td>
<td>Strain D045-D40</td>
<td>Dr. Schenkel's lab VCU, Richmond VA</td>
</tr>
<tr>
<td><em>Ss</em></td>
<td>Strain 10556</td>
<td>Dr. Schenkel's lab VCU, Richmond VA</td>
</tr>
<tr>
<td><em>Pg LPS</em></td>
<td>Strain 184</td>
<td>Dr. Shifferle’s lab UB, Buffalo, NY</td>
</tr>
<tr>
<td><em>Pg LPS</em></td>
<td>Strain 381</td>
<td>Dr. Shifferle’s lab UB, Buffalo, NY</td>
</tr>
<tr>
<td><em>Ec LPS</em></td>
<td>EH100</td>
<td>SIGMA St. Louis, MO</td>
</tr>
</tbody>
</table>
All of the bacteria (P. gingivalis, S. sanguinis, A. actinomycetemcomitans) except E. coli were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% yeast extract (Difco Laboratories), hemin (5µg/ml), vitamin K (0.5µg/ml), and cysteine (0.1%) (Sigma-Aldrich, St. Louis, Mo.). All cultures were incubated at 37°C in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Mich.) in 10% H₂, 10% CO₂, and 80% N₂.

The E. Coli medium was made of 10g Difco LB Broth ref # 240230 (Becton, Dickenson and Company) added into 500ml autoclaved water. One vial of E. coli was taken from the -80°C freezer, thawed, seeded into a 50ml LB broth medium, then incubated at 37°C in the shaking incubator overnight (16 hours) at a speed of 250 rpm.
F. Bacterial DNA (bDNA) isolation

**Table 3.** Reagents used in the isolation of bDNA

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1)</strong></td>
<td>RNase, lysozyme and proteinase were added to digest the proteins making it easier for the extraction of bDNA</td>
</tr>
<tr>
<td><strong>2)</strong></td>
<td>Phenol: chloroform: isoamyl alcohol (25:24:1)</td>
</tr>
<tr>
<td><strong>3)</strong></td>
<td>Chloroform</td>
</tr>
</tbody>
</table>
| **4)** | 3M Sodium Acetate (pH 5.0) prepared by adding glacial acetic acid to 3M Sodium acetate until this pH is obtained.  
12.3 grams of Sodium acetate was added into 50 ml of sterile water (pH 5.0), autoclaved and stored at 4°C |
| **5)** | Ice cold 100% ethanol |
| **6)** | 70% ethanol |
Bacterial cells were grown overnight in 500 ml of broth medium and the pellet cells were collected by centrifugation and re-suspended in 10 ml 50mM Tris at a pH of 8.0, and 50 mM EDTA. The cell suspension was placed in the freezer at -20°C. One day later, 0.2 ml of 50 mg/ml lysozyme was added to the frozen suspension and let thaw at room temperature. Once thawed, the cell suspension was placed on ice for 45 minutes. Also, 2ml of 2.0% SDS, 10 mM Tris (pH 7.5), 10 M EDTA and 0.25 ml of 10 mg/ml of proteinase K were added to the cell suspension, and placed in a 50°C water bath for 60 minutes; 400µl (30 tube X2) of the bacterial lysis mixture were extracted with equal volume of phenol:chloroform:isoamyl alcohol, mixed by vortex for 10 seconds and centrifuged at 10,000X g for 15 minutes. The top layer was transferred to a new tube (avoiding interface) and an equal volume of chloroform was added and mixed by vortex for 10 seconds and centrifuged at 10,000X g for 15 minutes. Again, the top layer was transferred to a new tube (avoiding interface) and 0.1 volume of 3M Na acetate was added and mixed gently, then 2 volume of 95% ethanol was added, which was mixed by inverting; the DNA was spooled out and transferred to 4 ml of autoclaved water, and was dissolved by vortex. After bacterial DNA was extracted and purified, polymyxin-B solution EC # 2157747 (Sigma-Aldrich) was added for further purification. 100µl of polymyxin-B was added for every 300µl DNA and set for 30 min to allow the complete binding of LPS to polymyxin-B. DNA was transferred to a new tube and re-precipitated. The purity and concentration of the DNA was determined by using spectrophotometric analysis (Nanodrop model # ND-1000 spectrophotometer) and by undergoing gel electrophoresis. The amount of LPS was measured by the Limulus Amoebocyte Lysate assay (LAL) described below.
G. Gel electrophoresis

Reagents used in gel electrophoresis included; 1xTBE (prepared from 10xTBE), 0.5 grams of agarose and ethidium bromide.

Gel electrophoresis technique:

50 ml of 1xTBE (catalog # B52, Fermentas life science) were put in a sterile beaker and 0.5g agarose (catalog # 162-0022, Bio-Rad laboratories) were added. The mixture was heated in the microwave and 2µl of Ethidium Bromide (CAS 1239-45-8, OmniPur) was added. The gel was then poured into the gel casting tray and was allowed to set for at least 45 min. Once the gel has set, the combs and casting gates were removed, the gel was transferred to the gel tank and 1xTBE solution was poured into the gel tank until the gel was completely covered. Different DNA samples were mixed with dye and loaded into the wells in the gel (3µl DNA + 5µl dye +5µl H2O). The ladder was also loaded and the gel was run at 100 volts for 45 min. The gel was photographed under ultraviolet light.

H. Limulus Amoebocyte Lysate (LAL) assay

Limulus Amoebocyte Lysate (LAL) QCL-1000 catalog number: 50-647U was purchased from Lonza (Walkerville, MD USA) and the amount of LPS in the bacterial DNA samples was determined in all the DNA samples according to the manufacturer’s instructions. The chromagenic substrate was reconstituted with 6.5ml LAL water and kept in 4°C. The following 4 standards of endotoxin were made as shown in table 4.
### Table 4. LAL assay; Endotoxin standards preparation

<table>
<thead>
<tr>
<th>Standards</th>
<th>Concentration</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard #1</td>
<td>1.0 EU/ml</td>
<td>1.4ml LAL water was added to 0.05ml original stock endotoxin solution and vortexed well</td>
</tr>
<tr>
<td>Standard #2</td>
<td>0.5 EU/ml</td>
<td>0.2ml of standard #1 was added to 0.2ml LAL water and vortexed well</td>
</tr>
<tr>
<td>Standard #3</td>
<td>0.25 EU/ml</td>
<td>0.2ml of standard #1 was added to 0.6ml LAL water and vortexed well</td>
</tr>
<tr>
<td>Standard #4</td>
<td>0.1EU/ml</td>
<td>0.1ml of standard #1 was added to 0.9ml LAL water and vortexed well</td>
</tr>
</tbody>
</table>
The microplate was pre-equilibrated at 37°C. 50µl of standards and samples were added to the appropriate wells. 50µl of water was added to the two blanks. 50µl of LAL was added to the wells at the same time. The plate was tapped briefly and incubated at 37°C for 10 minutes. 100µl of substrate was then added to the wells, the plate was briefly tapped again and incubated at 37°C for 6 minutes. 100µl of stop reagent (25% glacial acetic acid) was added to each well and the absorbance was read at 405-410 nm using the original multiscan MCC manufactured for Fisher Scientific (reference # 5111340).

1. Determination of optimum time for osteoblasts (MG-63 cells) to release cytokines in response to bDNA.

MG-63 cells were plated at a concentration of 4x10⁵ cells/well in a 48 well plate and incubated overnight under an atmosphere of 5% CO₂ at 37°C. Cells were infected with 100ng/µl P.gingivalis DNA in duplicates as well as E.coli DNA (100ng/µl), S.sangunis DNA (100ng/µl), A. actinomycetemcomitan DNA (100ng/µl), E.coli LPS(10ng/µl), P.gingivalis LPS 381 (10ng/µl ), P.gingivalis LPS 184(10ng/µl ), however un-stimulated cells were used as a control. Cells were incubated with bacterial DNA and LPS for 1 hour, 2 hour, 4 hour, 8 hour, 12hour and 24 hour time points at 37°C in 5% CO₂. Supernatants were collected and frozen to further determine IL-6 and IL-8 release by Enzyme-linked immunosorbent assay (ELISA).
J. Determination of optimum bDNA concentration for osteoblasts (MG-63 cells) to release cytokines in response to bDNA.

MG-63 cells were plated at a concentration of $4 \times 10^5$ cells/well in a 48 well plate and incubated overnight under an atmosphere of 5% CO$_2$ at 37°C. Cells were infected at different concentrations (100ng/µl, 200ng/µl and 400ng/µl) in duplicates with $P. gingivalis$ DNA, $E. coli$ DNA, Ss DNA, Aa DNA. They were also infected with $E. coli$ LPS at different concentrations (10ng/µl, 25 ng/µl, 50 ng/µl) and un-stimulated cells were used as a control. Cells were incubated for 24 hours at 37°C in 5% CO$_2$. Supernatants were collected and frozen to further determine IL-6 and IL-8 release by Enzyme-linked immunosorbent assay (ELISA).

K. Stimulation of MG-63 cells by bDNA and heat killed whole bacteria

Bacterial DNA was diluted with a proportional amount of autoclaved water, boiled for 5 minutes then put on ice for 1min. An additional amount of polymyxin-B (25%) was added to the DNA to eliminate any remaining LPS traces. All the stimulators were incubated for 30min at 37°C. Bacterial DNA (100ng/µl) and LPS (10 ng/µl) were added to the MG-63 and cells were incubated for 20 hours. For cell stimulations involving whole bacterial cells, bacteria were heat-killed and added to the MG-63 cells at a multiplicity of infection (MOI) of 100:1 (bacteria to cell ratio). This ratio indicates that this amount of bacteria is sufficient for osteoblasts activation without damaging the host cell. Prior to stimulating the osteoblastic cell line, bacteria were harvested at an OD$_{660}$ of 0.9. 1ml of bacteria culture suspension was taken and centrifuged at 12,000xg for 10 minutes. The
supernatant was discarded while the bacterial pellet was resuspended in OD_{660}/2 ml of cell culture medium then put in a water bath for 15 min at a temperature of 80°C in order to kill the bacteria. The appropriate number of bacteria (1x10^9) was added to the MG-63 cells and incubated at 37°C in an atmosphere of 5% CO_2. The supernatant was collected after 20 hours and frozen for further study.

L. Determination of the amount of IL-6 and IL-8 released from MG-63 cells by Enzyme-linked immunosorbent assay (ELISA).

Human IL-6 enzyme linked immunosorbent assay (ELISA) Kit catalog # 88-7966-29 was purchased from e.bioscience (San Diego, CA). This ELISA reagent set is specifically engineered for accurate and precise measurements of human IL-6 protein levels from cell cultures’ supernatants.

5x assay diluent was diluted to 1x assay diluent. Standards and reagents were made according to the manufacturer’s instruction manual.

Human IL-8 enzyme linked immunosorbent assay (ELISA) Kit catalog # 88-7087-22 was purchased from e.bioscience (San Diego, CA). This ELISA reagent set is specifically engineered for accurate and precise measurements of human IL-8 protein levels from cell cultures’ supernatants.

5x assay diluent was diluted to 1x assay diluent. Standards and reagents were made according to the manufacturer’s instruction manual.

The ELISA assay was conducted as follows: 100µl of standards and samples (2µl sample + 98µl diluent) were added to the wells according to the experiment’s layout. The
plate was covered with a plate sealer, mixed gently and incubated at room temperature for 2 hours. Each well was aspirated by 300µl of wash buffer; this step was repeated for another 4 times with 1 min interval. 100µl of detection antibody was added to each well; the plate was sealed and incubated for another hour. The wells were aspirated again and washed with another 300µl of wash buffer; this step was also repeated for another 4 times with 1 min interval. 100µl of Avidin-HRP diluent solution was added to each well, the plate was sealed and incubated for another 30 min. The wells were aspirated and washed with 300µl of wash buffer; this step was repeated for another 6 times with 2 min interval. 100µl of substrate solution was added to each well and the plate was sealed and incubated for 15 min. 50µl of stop solution was added to each well and the plate was read at 450 nm within 30 min using the original multiscan MCC manufactured for Fisher Scientific (reference # 5111340).

M. Statistical analysis

Cytokine levels were determined in supernatants using ELISA (R&D Systems). Data were analyzed by analysis of variance, using the Tukey and Dunn method with GraphPad Prism (version 4.0) software and the results were presented as the mean ± SEM. The results represent at least five independent experiments which were run in duplicate. A value of P ≤ 0.05 was considered significant.
Results

A. Bacterial DNA purification and Limulus Amoebocyte Lysate (LAL) assay

Whole bacterial genomic DNA was isolated by repeated phenol/chloroform extraction and ethanol precipitation as described previously to ensure pure DNA preparations. However, it is plausible to assume that bDNA isolated from gram negative bacteria would be contaminated with some LPS. In order to further purify bacterial DNA, Polymyxin-B, a natural peptide, was used during DNA isolation to inactivate and remove any residual LPS. Polymyxin-B is a potent antibiotic that has the ability to bind and neutralize LPS. It is a decapeptide cyclic cationic antibiotic containing lipophilic and hydrophilic groups that binds to lipid-A, the major component of the endotoxin (Cardoso et al. 2007). By ensuring that the LPS is further neutralized via the polymyxin-B treatment, we can ensure that bDNA is stimulating the osteoblast not the LPS. The lipopolysaccharide (LPS) concentration of all bacterial DNA preparations was determined using the Limulus Amoebocyte Lysate kit. Results were quantified using the graphical method protocol from the manufacturer’s handbook. The more polymyxin-B was added the less the amount of LPS found in bDNA as shown in table 5.
Figure 7. LPS standard curve
Table 5. Endotoxin levels after the addition of Polymyxin-B

<table>
<thead>
<tr>
<th>Samples &amp; Std</th>
<th>OD</th>
<th>ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLANK</td>
<td>0.364</td>
<td>0</td>
</tr>
<tr>
<td>S1</td>
<td>2.34</td>
<td>0.1</td>
</tr>
<tr>
<td>S2</td>
<td>1.458</td>
<td>0.05</td>
</tr>
<tr>
<td>S3</td>
<td>0.926</td>
<td>0.025</td>
</tr>
<tr>
<td>S4</td>
<td>0.577</td>
<td>0.001</td>
</tr>
<tr>
<td>Control</td>
<td>2.61</td>
<td>2.785</td>
</tr>
<tr>
<td>20µl polymyxinB+bDNA</td>
<td>0.853</td>
<td>0.0572</td>
</tr>
<tr>
<td>100 µl polymyxinB+bDNA</td>
<td>0.681</td>
<td>0.0356</td>
</tr>
</tbody>
</table>
All of the samples were below 1EU/ml. 1 EU= 0.0001µg. Thus for the infection of MG-63 cells with 100µg/ml bacterial DNA, my samples contained 0.0356 ng/ml of LPS. Although the LPS concentration remained below stimulatory levels (<0.05 ng/ml) throughout the study, we still treated the bacterial DNA with polymyxin-B to further reduce the effect of LPS before stimulation of the cells.

B. Bacterial DNA concentration and purity

The quantity and the quality of the bacterial DNA preparations were assessed using two different methods: absorbance (optical density) by a spectrophotometer equipped with a UV light and agarose gel electrophoresis.

The spectrophotometric method involves performing absorbance readings at 260nm (\(A_{260}\)) where DNA absorbs light most strongly. The number generated allows one to estimate the concentration of the solution and DNA purity can be estimated from the \(A_{260}/A_{280}\) ratio. \(A_{260}/A_{280}\): ratio of sample absorbance at 260 and 280 nm. A ratio between 1.7 and 2.0 generally represents a high quality DNA sample. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280nm. \(A_{260}/A_{230}\): ratio of sample absorbance at 260 and 230nm. This is a secondary measure of nucleic acid purity. The 260/230 values for “pure” nucleic acid are often higher than the respective 260/280 values. They are commonly in the range of 1.8-2.3. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants. In other words, DNA quality measurement is based on the fact that optical density (OD) at 260 nm is twice that at 280 nm if the solution contains pure DNA. In the
presence of a contaminant, there will be some additional OD which decreases the OD ratio between 260 and 280 nm.

The figures 8-11 below show the purity and concentration of each bacterial DNA used in this study after purification with Polymyxin-B.
Figure 8. Spectrophotometric analysis of the genomic DNA isolated from *A. actinomycetemcomitans* (*Aa*) bacteria
Figure 9. Spectrophotometric analysis of the genomic DNA isolated from *P.gingivalis* (*Pg*) bacteria
Figure 10. Spectrophotometric analysis of the genomic DNA isolated from *E.coli* (*Ec*) bacteria
Figure 11. Spectrophotometric analysis of the genomic DNA isolated from *S. sanguinis* (Ss) bacteria
C. Determination of the quality of bacterial DNA preparations using agarose gel electrophoresis

Bacterial DNA preparations were further visualized using agarose gel electrophoresis that allowed us to determine possible degradation products. Figure 12 represents an agarose gel showing bacterial DNA used in this study. As can be seen in figure 12, all the bacterial DNA preparations were intact and did not show any degradation that would interfere with our experiments.
Figure 12. Agarose gel electrophoresis showing the genomic bacterial DNA
D. Production of IL-6 from MG-63 cells in response to 100ng/µl bDNA at different time points

The time experiment was performed to determine the optimal time for the release of inflammatory cytokines, specifically IL-6 and IL-8. Bacterial DNA (E.Coli DNA, Pg DNA, Ss DNA and E.Coli LPS) treated MG-63 cells were collected at designated time points (1hr, 2hr, 4hr, 8hr, 12hr and 24hr) and cytokine production was determined using ELISA. The results showing the IL-6 production from MG-63 cells stimulated with bDNA and LPS are shown in Figure 13 below.
**Figure 13.** Increased IL-6 production was observed in MG-63 cells stimulated by bDNA and LPS as early as 8 hour.
E. Production of IL-6 from MG-63 cells in response to 100ng/µl *Pg* DNA versus 100ng/µl *Pg* LPS at different time points

To further confirm our previous data, MG-63 cells were stimulated again with *Pg* DNA and also *Pg* LPS 184 and *Pg* LPS 381. *Pg* LPS 184 is a less virulent strain than *Pg* LPS 381. The supernatant was collected at different time points (4hr, 8hr, 12hr, and 24hr). IL-6 concentration was determined by ELISA and the results were summarized in Figure 14.
Figure 14. Peak increase of IL-6 shown within 12-24 hours
The results in figure 14 show release of IL-6 by osteoblasts once challenged with bDNA. *Pg* LPS 184 shows less release than *Pg* LPS 381 as expected since *Pg* LPS 184 is less virulent than *Pg* LPS 381. Since the amount of cytokine release is very close between 12 hours and 24 hours, 20 hours was the optimal stimulating time selected for the release of inflammatory cytokines. This has been also reported by different studies done previously in our laboratory (Sahingur et al. 2010).

**F. Production of IL-6 from MG-63 cells in response to different concentrations of bacterial DNA**

The bacterial DNA concentration that was used to stimulate the cells in our experiments was determined by stimulating MG-63 cells with different concentration of DNA (100µg/ml, 200µg/µl, and 400µg/µl) as shown below in Figure 15.
Figure 15. Consistent with the previous reports (Takeshita et al., 1999; Nonnenmacher et al. 2003; Dalpke et al. 2006; Sahingur et al. 2010), we also determined 100µg/ml bDNA as the optimal stimulating concentration and therefore used this concentration in our assays.
G. IL-6 production from MG-63 cells in response to bDNA

Recent studies from our laboratory indicated that human monocytes (THP-1 cells) can produce the key inflammatory cytokine IL-6 following exposure to periodontal bacterial DNA (Sahingur et al. 2010). To determine whether osteoblasts produce IL-6 in response to bacterial DNA, we have used the osteoblast cell line MG-63 and stimulated them with 100μg/ml of the following bacterial DNA (Ss DNA, Aa DNA, Pg DNA, and E.coli DNA). E.Coli LPS (1ng/ml) and E.coli DNA served as positive controls while Ss DNA served as a non pathogenic oral bacterial control. The cell culture supernatants were collected after 20 hours and the IL-6 levels were determined using ELISA. Figure 16 summarizes IL-6 levels produced by MG-63 cells stimulated with various bacterial DNA.
Figure 16. The level of the inflammatory cytokine Interleukin-6 was increased significantly in MG-63 cells treated with *E.coli* DNA and *E.coli* LPS compared with unstimulated cells. On the other hand, even though *Ss* DNA, *Aa* DNA and *Pg* DNA induce IL-6 production from MG-63 cells compared to unstimulated cells, the results did not reach significance.

* (p<0.05)
H. IL-6 production from MG-63 cells in response to heat killed whole bacteria

In addition to bDNA, The MG-63 cells were also stimulated with heat killed whole bacteria. Figure 17 shows the release of IL-6 from MG-63 cells treated with different heat killed whole bacteria and bacterial DNA.
The results showed a significant release of IL-6 when the osteoblasts are stimulated with *E.coli* DNA and *E.coli* LPS compared to unstimulated osteoblasts. Stimulation with heat killed whole bacteria also induced significant IL-6 release in MG-63 cells. Again, although periodontal bacterial DNA (*Pg* DNA and *Aa* DNA induced IL-6 production from MG-63 cells, the results were not significant compared to unstimulated control cells. (* p<0.05)

**Figure 17.**
I. Effect of chloroquine treatment on IL-6 production from MG-63 cells stimulated with bDNA

To identify the involvement of TLR9 signaling in periodontal bacterial DNA initiated IL-6 production in human osteoblasts, MG-63 cells were treated with chloroquine prior to bDNA stimulations. Chloroquine, a non-specific TLR9 signaling inhibitor, is known to inhibit immune stimulation by bacterial DNA. The inhibition of TLR9 occurs at endosomes where chloroquine was shown to block CpG and TLR9 interaction by altering endosomal acidification. Figure 18 shows the effect of bacterial DNA on the osteoblasts in the presence of chloroquine.
Figure 18. Chloroquine treatment had no significant effect on IL-6 production in MG-63 cells stimulated with bacterial DNA. Surprisingly, the chloroquine treatment slightly increased IL-6 levels in cells stimulated with *E.coli* DNA, but the difference was not statistically significant.
J. Effect of chloroquine treatment on IL-6 production MG-63 cells stimulated with heat killed whole bacteria

MG-63 cells were stimulated with heat killed whole bacteria in the presence of chloroquine and the results are shown in Figure 19.
Figure 19. Chloroquine treatment of MG-63 cells slightly increased the release of IL-6 when compared with osteoblasts not treated with chloroquine, but the results were not statistically significant.
K. IL-8 production from MG-63 cells in response to bDNA

Interleukin IL-8 is a chemokine produced by a variety of tissue and blood cells, and is a potent inducer of neutrophil chemotaxis and activation (Waugh & Wilson, 2008). Aberrant and persistent production of IL-8 has been demonstrated in various inflammatory diseases, including periodontal disease (Yang et al. 2003). To date the interactions of bacteria and human osteoblasts are under extensive investigation. The objective of this experiment was to investigate the IL-8 production by human osteoblastic cells stimulated with bacterial DNA including *Ss* DNA, *Aa* DNA, *Pg* DNA, *E.coli* DNA and *E.coli* LPS. The IL-8 levels as measured in cell culture supernatants by ELISA are summarized in Figure 20.
Figure 20. The level of the inflammatory chemokine Interleukin-8 was increased significantly in MG-63 cells treated with \textit{E.coli} DNA and \textit{E.coli} LPS compared with unstimulated cells. \textit{Ss} DNA, \textit{Aa} DNA and \textit{Pg} DNA show some release compared with unstimulated cells but the results did not reach significance. (*p<0.05)
L. IL-8 production from MG-63 cells in response to heat killed whole bacteria

MG-63 cells were also challenged with heat killed whole bacteria in comparison with bacterial DNA. IL-8 production was assessed in cell culture supernatants using ELISA and the results are summarized in Figure 21.
Figure 21. IL-8 production was significantly increased in response to *E.coli* DNA and *E.coli* LPS, although heat killed whole bacteria also induced increased cytokine production from MG-63 cells the results were not significant.
M. Effect of chloroquine treatment on IL-8 production from MG-63 cells stimulated with bacterial DNA

MG-63 cells were treated with chloroquine (10µg/ml) for two hours before DNA stimulation. As mentioned previously, chloroquine is an antimalarial agent and widely used to block TLR9 signaling by affecting endosomal acidification (El Kebir et al., 2009). IL-8 levels were determined in culture supernatants using ELISA (eBiosciences) following the manufacturer’s instructions and the results are displayed in figure 22 shown below.
Figure 22. Chloroquine treatment of MG-63 cells slightly decreased cytokine production in osteoblast when challenged with all bacterial DNA but *E. coli* DNA. Increased release of IL-8 in MG-63 cells was seen in response to *E. coli* DNA, but the results were not significant.
N. Effect of chloroquine treatment on IL-8 production from MG-63 cells stimulated with heat killed whole bacteria

MG-63 cells were again stimulated with heat killed whole bacteria but in the presence of chloroquine. The results are show in figure 23 below.
Figure 23. Chloroquine treatment of MG-63 cells prior to bacterial stimulations resulted with increased IL-8 production compared to the cells not treated with chloroquine, but the results were not significant. However, compared to un-stimulated control cells, the chloroquine treatment resulted in significantly increased IL-8 production in response to *A. actinomyctemcomitans* and *S. sanguinis*. (* p<0.05*)
Discussion

Impaired bone homeostasis that results in periodontal bone destruction is commonly attributed to chronic inflammation that is initiated by multiple periodontal pathogens. The mechanisms responsible for such clinical outcomes are still under extensive investigation (Nair et al. 1996; Liu et al. 2009). The generation of proinflammatory cytokines and chemokines is thought to be pivotal in the recruitment and activation of infiltrating immune cells. It is also pivotal in the net balance between bone resorption and bone formation (Madianos et al. 2005). Periodontal pathogens possess several virulence factors that have the ability to trigger inflammatory responses and one of the newly emerging periodontal PAMPs, include bacterial DNA. Our studies as well as studies conducted by other researchers point out a possible role of bDNA-initiated immune responses in periodontal disease pathogenesis (Takeshita et al. 1999; Nonnenmacher et al. 2003; Sahingur et al. 2010). Considering the multibacterial etiology of periodontal disease and the presence of several bacteria in the oral environment, it is plausible to think that the oral tissues represent an environment where bacterial DNA can be abundant and contribute to the inflammatory responses.

Periodontal diseases are unique not only because of their multibacterial etiology but also involvement of different cell types and immune responses in different stages of the disease. Previous studies conducted in our laboratory (Sahingur et al, 2010) demonstrated increased production of key inflammatory cytokines, IL-1β, IL-6 and TNF-α, in response
to periodontal pathogenic DNA in human monocytes. While it is likely that infiltrating immune cells, such as monocytes/macrophages, are an important source of these inflammatory mediators, there are other cell types that are exposed to these pathogenic bacteria and their PAMPs. Osteoblasts are one of these cells that come into play at the bone-bacteria interface of periodontal inflammation. Therefore in this study, we seek to determine whether periodontal bacterial DNA triggers the production of two key inflammatory cytokines, IL-6 and IL-8, in human osteoblastic cells (MG-63). Here, we showed the production of both IL-6 and IL-8 in osteoblasts (MG-63 cells) infected with periodontal bDNA and whole periodontal bacteria using ELISA. To our knowledge, this is the first study that investigated the effect of periodontal bacterial DNA and whole periodontal bacteria in inflammatory cytokine production from these cell lines.

Osteoblasts stimulated by bacterial DNA (Aa DNA, Ss DNA and Pg DNA) released IL-6 and IL-8 cytokines but not in amounts significantly different from unstimulated osteoblasts (Figures. 16 & 20). However, E.coli DNA and E.coli LPS - which were used as positive controls triggered the release of IL-6 and IL-8 significantly more, due to their inherent virulence (Figures. 16 & 20) (p<0.05). Osteoblasts stimulated by heat killed whole bacteria also showed a significantly higher release of cytokines (Figures. 17 & 21) (p<0.05). This finding was expected since whole bacteria possess several virulence factors. Thus, we would expect these different PAMPs to take different routes by binding to their specific receptors, together releasing inflammatory cytokines. In our case, E.coli DNA and E.coli LPS triggered osteoblasts to produce more IL-6 and IL-8 than heat killed whole bacteria. It is possible that it might take longer for the whole bacterial cell to generate
immune responses and therefore further experiments can be planned stimulating MG-63 cells with whole bacteria for longer periods. Also it is possible that heat killed bacteria may not possess the same abilities as live bacteria which again may warrants further investigations using live bacteria to determine the differences.

TLR9 is considered to be the major receptor that is involved in foreign DNA initiated immune responses in several cell types including osteoblasts. However, previous studies that investigated TLR9-DNA interactions solely tested the effect of synthetic oligonucleotides that are known to mimic bDNA (Zou et al. 2003; Amcheslavsky et al. 2004; Amcheslavsky et al. 2005). Here we employed whole bacterial genomic DNA to assess cytokine production in MG-63 cells that better represents physiological responses. One of the drawbacks of using genomic DNA is the possibility of contaminating proteins during purification processes, specifically LPS contamination in gram negative bacterial cells. We employed several procedures which are described in methods section to obtain pure bDNA which is free of any contaminants and we are confident that our DNA preparations were pure (Table 5).

To further investigate the role of TLR9 in periodontal bDNA-induced cytokine production in human osteoblastic cells, cells were treated with chloroquine prior to stimulations. Our results showed that inhibition of TLR9 signaling by chloroquine resulted in slightly decreased IL-8 production (Fig. 22) and slightly increased IL-6 production (Figs. 18 & 19) from human osteoblasts. Although, none of the differences were statistically significant, it might be speculated that different receptors and signaling pathways are involved in triggering IL-6 and IL-8 production in response to periodontal
bacterial DNA from MG-63 cells. It is also important to note that chloroquine exerts its effect as a non-specific inhibitor of TLR9 signaling by affecting endosomal acidification. It is possible that TLR9 signaling inhibition was not completely inhibited by chlororoquine which again warrants further investigations creating cells in which TLR9 was knocked down using transfections.

As mentioned in the Introduction section, there are other cytosolic DNA sensors such as DNA –dependent activator of interferon-regulatory factors (DAI) and absent in melanoma-2 (AIM-2) for inflammasome (Takaoke et al. 2007; Muruve et al. 2008; Burck, Stummer et al. 2009; Fernandes, Alnemri et al. 2009; Hornung et al. 2009; Roberts et al. 2009) that may play a role in bDNA- induced immune responses in osteoblastic cells. So far there are no studies that investigated the expression of these receptors in osteoblastic cells, but it can be speculated that inhibition of TLR9 signaling by chloroquine would create an environment for other DNA receptors and signaling pathways to be more effective resulting in increased production of the inflammatory mediators. An increase in cytokines release was also seen when osteoblasts were stimulated with heat killed whole bacteria in the presence of chloroquine which may again be as a result of other signaling pathways being more operant when TLR9 signaling is inhibited.

Although we demonstrated increased IL-6 and IL-8 production in response to periodontal bacterial DNA from MG-63 cells, the differences were not significant compared to unstimulated controls. IL-6 and IL-8 are undoubtfully important in periodontal pathology, however, there are also other inflammatory mediators that can contribute to tissue destruction such as RANKL (Zou et al. 2003), IL-12 (Hiroyoki et al.
The production of these inflammatory mediators by bDNA stimulated-osteoblasts may contribute to the development of progressive inflammatory damage and aberrant bone remodeling associated with the pathology of periodontal disease. Treatment of MG-63 cells with chloroquine prior to bDNA stimulation resulted in decreased IL-8 and increased IL-6 production. These results indicate that different signaling pathways might be involved in bDNA-initiated cytokine production in osteoblasts for different inflammatory mediators. Hence, although our results were not significant for IL-6 and IL-8, it is safe to suggest that human osteoblasts can respond to periodontal bDNA. Thus, further studies are needed that will assess the production of other inflammatory mediators in response to periodontal bDNA to fully elucidate the role of bDNA-initiated immune responses in periodontal disease pathology.


VITAE

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