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## Sustained MAPK Activation with *Aggregatibacter actinomycetemcomitans* Causes Inflammatory Bone Loss

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### Summary

*Aggregatibacter actinomycetemcomitans* is a gram-negative facultative capnophile involved in pathogenesis of aggressive forms of periodontal disease. In the present study, we interrogated the ability of *A. actinomycetemcomitans* to stimulate innate immune signaling and cytokine production then established that *A. actinomycetemcomitans* causes bone loss in a novel rat calvarial model. In vitro studies indicated that *A. actinomycetemcomitans* stimulated considerable production of soluble cytokines, TNF- $\alpha$ , IL-6, and IL-10 in both primary bone marrow derived macrophages and NR8383 macrophages. Immunoblot analysis indicated that *A. actinomycetemcomitans* exhibits sustained activation of all major mitogen activated protein kinase (MAPK) pathways, as well as the negative regulator of MAPK signaling, MAPK phosphatase-1 (MKP-1), for at least 8 hours. In a rat calvarial model of inflammatory bone loss, high and low doses of formalin fixed *A. actinomycetemcomitans* were microinjected into the suprapariosteal calvarial space for 1 to 2 weeks. Histological staining and micro-computed tomography ( $\mu$ CT) of rat calvariae revealed a significant increase of inflammatory and fibroblast infiltrate and increased bone resorption as measured by total lacunar pit formation. From these data, we provide new evidence that fixed whole cell *A. actinomycetemcomitans* stimulation elicits a pro-inflammatory host response through sustained MAPK signaling leading to enhanced bone resorption within the rat calvarial bone.

### Keywords

periodontal disease; host immune response; cytokines; bone loss

### Introduction

Periodontal disease is a chronic inflammatory disease marked by inflammation in gingival tissue and alveolar bone resorption that commonly lead to tooth loss. In humans, a subgingival biofilm containing *Aggregatibacter actinomycetemcomitans*, a gram-negative capnophile, is associated with aggressive forms of periodontal disease in juveniles and adults (Kamma et al., 2004; Lopez et al., 1996; Slots and Ting, 1999; Takeuchi et al., 2003; Tinoco et al., 1997). In a susceptible host, *A. actinomycetemcomitans* endotoxin stimulates the

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release of pro-inflammatory mediators such as cytokines and prostaglandins that lead to destruction of periodontal tissues.

Bacterial constituents activate the innate immune response through toll-like receptors (TLRs) and promote subsequent release of cytokines. *A. actinomycetemcomitans* derived lipopolysaccharide (LPS), a gram-negative cell wall component, induces the mitogen-activated protein kinase (MAPK) pathway. MAPKs, a family of serine/threonine proteases, include p38, extracellular signal-related kinases (ERK1/2), and c-Jun N-terminal kinases (JNK). Specifically, MAPK-activated protein kinase 2 (MK2) and MAPK phosphatase-1 (MKP-1) regulate inflammatory bone loss in periodontal disease (Li et al., 2011; Yu et al., 2011a). Moreover, MK2 is targeted and phosphorylated by p38 MAPK. *In vivo*, silencing MK2 attenuates LPS-induced inflammatory bone loss in a rat periodontal disease model (Li et al., 2011). Contrary to MK2, MKP-1 regulates MAPKs by dephosphorylating proteins activated upon TLR signaling. MKP-1 is anti-inflammatory in *A. actinomycetemcomitans* LPS-induced bone loss in a periodontal disease rat model (Yu et al., 2011a).

Stress-induced activation of the MAPK pathway causes downstream regulation of host-derived inflammatory cytokines and prostaglandins such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, IL-1 $\beta$ , and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) that directly or indirectly cause subsequent bone loss (Chen et al., 1998; Ishimi et al., 1990; Rogers et al., 2007; Tsai et al., 1995; Zou and Bar-Shavit, 2002). These pro-inflammatory mediators lead to activation of matrix metalloproteinases (MMPs) that destroy the matrix in tissue surrounding the tooth (Rogers et al., 2007).

Inflammation in periodontal disease markedly enhances osteoclastogenesis in the alveolar bone (Hall and Chambers, 1996). Alveolar bone turnover that is normally coupled, is in disequilibrium in the periodontal disease state leading to net bone loss (Rogers et al., 2007). Interestingly, IL-1 and TNF- $\alpha$  can induce osteoclastogenesis directly by nuclear factor-kappa B ligand (RANKL), a TNF family cytokine, or indirectly through IL-6 (Rogers et al., 2007). Osteoblasts respond to IL-1 $\beta$ , PGE<sub>2</sub> and TNF- $\alpha$  to upregulate RANKL and stimulate osteoclast differentiation (Roux and Orcel, 2000).

In order to experimentally model the osteoimmunological host response in periodontal disease, small *animal* models are commonly used. For example, *A. actinomycetemcomitans* inoculation by oral gavage, *A. actinomycetemcomitans* LPS palatal injections, oral *A. actinomycetemcomitans* inoculation, or live *A. actinomycetemcomitans* feeding, mimic a periodontal disease host inflammatory response in rats or mice (Fine et al., 2009; Garlet et al., 2006; Yu et al., 2011a). Although *A. actinomycetemcomitans* LPS elicits a host response when injected into the palatal maxillae of rats, *A. actinomycetemcomitans* contains other virulence factors associated with aggressive periodontitis, such as cytolethal distending toxin that can contribute to pathogenesis by inhibiting both cellular and humoral immunity via apoptosis of immune response cells (Rogers et al., 2007; Tan et al., 2002). Therefore, whole *A. actinomycetemcomitans* stimulation is necessary to obtain a more comprehensive appreciation for the bacterial-induced host response.

In this study, we address the MAPK host intracellular signaling pathway, through stimulation with formalin-fixed *A. actinomycetemcomitans* in rat macrophages and *in vivo* using a rat calvarial model. *A. actinomycetemcomitans* expounds the contribution of the host immune response to periopathogenic bacterial stimulation and osteoimmunological impact on bone destruction.

## Methods

### Bacterial Preparation

*A. actinomycetemcomitans* Strain Y4 was grown on 5% Sheep Blood Agar plates (Becton Dickinson, Franklin Lakes, NJ USA) for 36-48 hours at 37°C with 10% CO<sub>2</sub> with large liquid culture prepared in NIH thioglycollate (Becton Dickinson, Franklin Lakes, NJ USA) under the same conditions. Harvested bacteria were centrifuged and fixed in 4% paraformaldehyde for 1 hour and then washed extensively with 5% glycerol in PBS. Final bacterial suspension in PBS was at a concentration of  $4 \times 10^9$  cells/ml. *A.*

*actinomycetemcomitans* LPS was extracted from strain Y4 by the hot phenol-water method as previously described (Rogers et al., 2007). The LPS used in this study contained <0.001% nucleic acid by spectrophotometry and approximately 0.7% protein by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL USA) as described previously (Rogers et al., 2007).

### Macrophage Culture

NR8383 cells (rat macrophage) were purchased from the American Type Culture Collection (ATCC, Manassas, VA USA). This rat macrophage cell line was established from a normal rat alveolar macrophage cells that were obtained by lung lavage. Cells were maintained in Ham's F-12 medium with 15% FBS. Primary bone marrow-derived macrophages (BMM $\phi$ ) were harvested from adult male Sprague-Dawley rats (Harlan Laboratories, Inc., Indianapolis, IN USA). The femurs and tibia were flushed with  $\alpha$ MEM (Invitrogen Life Technologies, Grand Island, NY USA). Total bone marrow cultures were incubated at in  $\alpha$ MEM containing 10% fetal bovine serum, 2mM glutamine, 100 $\mu$ l/ml penicillin and 100  $\mu$ g/ml of streptomycin overnight. Non-adherent cells post 24 hour culture were plated for macrophage differentiation using DMEM containing 10% fetal bovine serum, 2mM glutamine, 50 ng/ml murine macrophage-colony stimulating factor (R&D Systems, Minneapolis, MN USA), 100 $\mu$ l/ml penicillin and 100  $\mu$ g/ml of streptomycin. BMM $\phi$  were established after 7 days under these conditions as described previously (Yu et al., 2011b).

### Immunoblot and ELISA Analysis

Activation of the select MAPK/MKP/NF- $\kappa$ B signaling pathways were evaluated using total protein extracted from samples harvested at indicated time points following stimulation with *A. actinomycetemcomitans* LPS (100 ng/ml). Protein samples were obtained using a commercially available extraction buffer, supplemented with PMSF, protease inhibitors in SDS-PAGE buffer (BioRad, Hercules, CA USA). Total cellular lysate (25 $\mu$ g) was electrophoresed on 10% denatured SDS-PAGE gels and transferred to nitrocellulose membranes (BioRad). Antibodies against phosphorylated and non-phosphorylated forms of p38, JNK, ERK MAPK, MK2, NF- $\kappa$ B and NF- $\kappa$ B p65 (Cell Signaling Technologies, Danvers, MA USA), along with MKP-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used as primary antibodies in these studies. Primary antibodies were detected using Horseradish peroxidase (HRP)-conjugated secondary antibodies and exposure development against radiographic film using the chemiluminescence system (LumiGlo, Cell Signaling). Radiographic images were obtained on a Gel-Doc XR system and densitometric analysis was performed using Quantity One Software (Bio-Rad, Hercules, CA). Cytokine expression in cell culture supernatants was measured using the ELISA protocol DuoSet ELISA Development Systems to determine IL-6, TNF- $\alpha$ , and IL-10 (R&D Systems) according to manufacturer's instructions.

## Calvarial Model

The Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina approved all animal protocols. Adult male Sprague-Dawley rats (Harlan Laboratories) weighing ~250–274 g were used for *in vivo* studies. Rats were maintained under specific pathogen-free conditions in pairs with food and tap water *ad libitum*. Once weekly, animals were routinely weighed to ensure proper growth and nutrition. Following anesthesia with 5% isoflurane, Sprague-Dawley rats were injected once with 100  $\mu$ l of formalin-fixed *A. actinomycetemcomitans* at  $1 \times 10^9$  and  $1 \times 10^8$  cells/ml, *A. actinomycetemcomitans* LPS (250  $\mu$ g in 25  $\mu$ l), or PBS control in the mid-sagittal suture area near the suprapariosteal region of the rat calvarium via a 26-gauge needle through a Hamilton syringe. PBS + vehicle controls consisted of an empty 50ml tube treated under the same formalin fixation conditions without bacteria.

## Micro-computed tomography imaging

Harvested calvarial samples were fixed for 24 hours in 10% formalin and then changed to 70% ethanol. Samples were scanned by micro-computed tomography ( $\mu$ CT) (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Each scan was reconstructed at a mesh size of 18  $\mu$ m<sup>3</sup>, and three-dimensional digitized images were generated for each sample using GE Healthcare MicroView software (version viz. + 2.0 build 0029). By using  $\mu$ CT analysis it is possible to view the scans from virtually any angle or cross-section in order to retrieve an accurate bone volumetric fraction (BVF) analysis. Therefore, a standardized orientation of the images was necessary prior to measurement. Region of interest (ROI) landmarks for resorption pit enumeration were set using the coronal sutures adjacent to the occipital bone; the coronal sutures adjacent to the nasofrontal area; and the lambdoid sutures creating a 4-point connected square from the superior view. All  $\mu$ CT scans were measured and assessed by an independent examiner in a blinded manner.

## Histological Examination

Formalin-fixed, decalcified maxillae were paraffin embedded and serial sagittal sections (7  $\mu$ m) prepared. Some slides were stained routinely with H&E for descriptive histology. Histological images were acquired using an Olympus BX61 Research (Center Valley, PA USA) microscope outfitted with a DP71 digital camera. The inflammatory cell infiltrate and fibroblast scores were quantified using a pathological scoring system ranging from 0 to 4. A score of 0 indicated no significant inflammatory infiltrate, 1 was mild (<500), 2 was moderate (500-1000), 3 was severe (1001-1500), and 4 was extremely severe (>1501). A fibroblast score of 0 indicated no proliferation with no fibrotic band, 1 was mild with a thin band, 2 was moderate with a moderately thick band, 3 was severe with a thick band, and 4 was extremely significant fibroblast number with an extremely thick band.

## Osteoclast Measurement

To quantitate osteoclasts, tartrate-resistant acid phosphatase (TRAP) staining was performed in tissue sections using a leukocyte acid phosphatase kit (Sigma Aldrich). Mature osteoclasts were counted as TRAP-positive multinucleated (> 3 nuclei) in contact with the bone surface. Slides from serial sagittal sections were used to enumerate TRAP-positive cells. The tissue sections were counterstained with hematoxylin post TRAP staining.

## Statistical Analysis

Pairwise comparisons between experimental groups were performed using the student t-test with Welch's correction for unequal variances or identified by one-way analysis of variance (ANOVA). Post-hoc power analysis confirmed *in vivo* experimental trials. P-values less

than 0.05 were considered significant. All calculations were performed using Prism 4 software (GraphPad, Inc.).

## Results

### Induced Cytokine Expression post *A. actinomycetemcomitans* stimulation

For these studies we chose to elucidate activated signaling pathways by stimulating (NR8383) rat macrophage cell lines ( $1 \times 10^6$  cells/well) with formalin-fixed, whole *A. actinomycetemcomitans* bacteria at a high and low-dosage. Cells were harvested after 24 hours following microbial stimulation. Expression of candidate targets were tested independently enzyme-linked immunosorbent assay analysis (ELISA) using cell culture supernatant. Exposure to the periopathogenic stimulus resulted in significantly increased levels of TNF- $\alpha$  ( $p < 0.0001$ ), IL-6 ( $p < 0.0001$ ), and IL-10 ( $p < 0.0001$ ) in NR8383 cell lines, in comparison to controls (Figure 1A-C). To verify that these stimulatory effects were not limited to cell lines, primary rat BMM $\phi$  were treated in the same manner. As shown in Figure 1D-F, TNF- $\alpha$ , IL-6, and IL-10 ( $p < 0.0001$ ) cytokine levels were all significantly elevated post *A. actinomycetemcomitans* exposure.

### Activation of MAPK Signaling Pathway

Production of inflammatory cytokines, including TNF- $\alpha$  and IL-6 are closely related to the activation of the MAPK pathway and NF- $\kappa$ B (Rogers et al., 2007; Soloviev et al., 2002). NR8383 rat macrophage and primary BMM $\phi$  cells were exposed to 400 CFU of formalin-fixed *A. actinomycetemcomitans* or controls and harvested at indicated time points post-bacterial exposure. Previously, our group has shown that endotoxin stimulation activates protein kinases including, ERK, JNK and p38 MAPK in a manner consistent with formalin-fixed *A. actinomycetemcomitans* stimulation (Li et al., 2011; Yu et al., 2011a). Remarkably, immunoblotting analysis also revealed activation of targeted protein kinases, ERK, JNK, and p38 MAPK as indicated by an increase in phosphorylated expression starting at 30 minutes post-stimulation in tissue culture in both a rat macrophage cell line as well as primary cultured macrophages (Figure 2). In addition, these studies indicate that MKP-1, a negative regulator of MAPK signaling, and MK2, a downstream substrate of p38 MAPK has sustained activation up to 8 hours of bacterial exposure (Figure 2, Figure S1). This unexpected prolonged response was in contrast to the previous findings where cells displayed a significant increase in protein levels between 60 and 300 minutes following LPS stimulation, peaking from 30 to 120 minutes with subsequent downregulation (Li et al., 2011; Yu et al., 2011a). Results using primary macrophages indicate results are not cell line specific (Figure 2B).

### Induction of Bone Resorption

Inflammatory cytokine release can result in bone resorption as a consequence of the elicited host immune response to oral pathogens. In most cases this signaling activity is for protection, but if uncontrolled this may ultimately lead to alveolar bone loss. In the present study, *A. actinomycetemcomitans* with control samples was delivered to the mid-sagittal suture area near the suprapariosteal region of rat calvaria. Animals were sacrificed 1 or 2 weeks post injection and harvested calvaria and surrounding tissues were initially scanned by  $\mu$ CT to examine changes in calvarial bone resorption within the defined landmarks. Areas of resorption were identified as engraved pits (Figure 3A). We observed that bacterial stimulated rat calvarial tissues displayed greater lacunar formation in comparison to LPS and vehicle control samples (Figure 3B). Quantitatively, we found that areas of lacunar resorption dominated areas of the *A. actinomycetemcomitans* (high dosage) micro-delivery within the rat calvaria within specified landmarks of interest, in comparison to controls or LPS at the 2-week harvest (Figure 3B). The decrease in lacunae from 1 to 2 weeks with the

low dosage of *A. actinomycetemcomitans* compared to the high dosage at week 2 suggests that inflammation resolution and bone coupling is occurring. After 2 weeks post-injection the 400 CFU dosage *A. actinomycetemcomitans* is still sufficient to sustain an osteoclastic predominated event to maintain resorption craters compared with 100 CFU dosage *A. actinomycetemcomitans*.

### Inflammatory Cell Infiltration, Fibroblast Proliferation, and Osteoclastogenesis

Rat calvarial tissues were fixed and embedded for histological staining by hematoxylin and eosin (H&E). An influx of inflammatory cell infiltrate following *A. actinomycetemcomitans* microinjection was observed in representative sections from the harvested rat calvariae (Figure 4A). Following blinded pathological enumeration, cell types and tissue environment included robust inflammatory infiltrate numbers within the connective tissues, and numerous capillaries. In contrast, H&E staining indicated low levels of inflammatory cell infiltrate in control samples (Figure 4A). The inflammatory score was significantly increased in LPS ( $p < 0.05$ ) and 400 CFU dosage *A. actinomycetemcomitans* ( $p < 0.05$ ) treated rats 1-week post-injection when compared to the PBS (Figure 4B). Two weeks post-injection, the 100 CFU ( $p < 0.05$ ) and 400 CFU dosages of *A. actinomycetemcomitans* ( $p < 0.001$ ) produced a significant increase in inflammatory infiltrate compared to the control. The inflammatory infiltrate in the 400 CFU dosage group was also significantly different than LPS ( $p < 0.05$ ) and 100 CFU dosage groups ( $p < 0.05$ ) (Figure 4C). Upon microinjection, the fibroblast score increased in the 400 CFU dosage group compared to LPS during week 1 and 2 ( $p < 0.05$ ) and control groups at week 1 ( $p < 0.05$ ) and week 2 ( $p < 0.001$ ) post-stimulation (Figure 4B and C). By the second week, the 100 CFU amount of *A. actinomycetemcomitans* also produced an increase in the fibroblast proliferation score compared to the PBS and LPS treated groups ( $p < 0.05$ ) (Figure 2C). Inflammatory and fibroblast scores were dependent on potency of stimulus. Therefore, increased host response and healing was necessary during increased dosages. We also show that LPS and *A. actinomycetemcomitans* have a trend towards increased osteoclast formation (TRAP positive cells with 3 nuclei) (Figure 5A).

### Discussion

The hallmark of destructive periodontal disease is the overproduction of cytokines and other inflammatory mediators (reviewed in (Kirkwood and Rossa, 2009)). Production of cytokines and inflammatory mediators is usually a tightly controlled process that is initiated by external stimuli that are rapidly transduced through the cytoplasm and into the nucleus. Gene expression initiated within the nucleus is accompanied by a significant component of regulation occurring within the cytosol where signaling mechanisms help to regulate mRNA stability and translation thus amplifying and sustaining expression of immune cytokines. In the present study *A. actinomycetemcomitans* was able to robustly stimulate pro-inflammatory (TNF- $\alpha$  and IL-6) as well as the anti-inflammatory cytokine IL-10 consistent with previous studies using *A. actinomycetemcomitans*-derived LPS (Patil et al., 2006; Patil et al., 2004; Yu et al., 2011a; Yu et al., 2011b).

Following TLR engagement, pathogen-associated molecular patterns (PAMPs) stimulate NF- $\kappa$ B and MAPK signaling. Proximal signaling through the p38 MAPK pathway is a well-established cascade essential for inflammatory cytokine expression through regulation of mRNA stability (Patil et al., 2004; Zhao et al., 2011). Attenuation of MAPK signaling occurs through dephosphorylation of activated MAPKs by MKPs thus suppressing signaling and MAPK-dependent cytokine expression. Recently, loss- and gain-of-function studies have indicated that MKP-1 is a major signaling protein in *A. actinomycetemcomitans* LPS-induced alveolar loss (Sartori et al., 2009; Yu et al., 2011a). As part of these studies, a kinetic analysis indicated that LPS activated p38 occurs as early as 10 minutes but was quickly attenuated at the same time when MKP-1 expression was increased

(Sartori et al., 2009). Results from the present study using whole *A. actinomycetemcomitans* indicate a very different scenario. In contrast to the reciprocal regulation previously observed, data herein suggests that whole *A. actinomycetemcomitans* stimulates NF- $\kappa$ B and multiple MAPKs including p38, JNK, and ERK along with MK2, an immediate downstream kinase substrate of p38, with sustained MAPK activation for up to 8 hours in both NR8383 cell lines and primary BMM $\phi$  cell populations. We speculate that this sustained MAPK activation may be due to continued TLR activation with multiple PAMPs over time in culture, but additional experiments addressing TLR proximal activation and internalization are needed to substantiate these observations.

In addition to the sustained MAPK activation, we also observed that NF- $\kappa$ B is activated over the same time frame post whole *A. actinomycetemcomitans* exposure. TLR signaling requires a different set of adaptors, including myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor protein (TIRAP), which will recruit interleukin 1 receptor-associated kinase 1 (IRAK) or TRAF6. It is currently believed that the kinase TGF  $\beta$ -activated kinase 1 (TAK1) would link TRAF6 to the IKK complex, which is a critical regulator for the activation of NF- $\kappa$ B (Garcia de Aquino et al., 2009; Hayden and Ghosh, 2004; Tergaonkar, 2006). Thus, we suspect that activation of NF- $\kappa$ B canonical pathway occurs post *A. actinomycetemcomitans* exposure in macrophage populations.

A limitation of LPS-induced models of experimental periodontitis is that significant bone loss occurs in mice and rats only after multiple LPS injections (Li et al., 2011; Patil et al., 2006; Rogers et al., 2007; Rossa et al., 2006). In addition, some reports indicate difficulty in rodent colonization with the human pathogen including *A. actinomycetemcomitans* (Schreiner et al., 2011). Although some investigators have been successful using the oral gavage approach (Garlet et al., 2005; Garlet et al., 2008; Garlet et al., 2006; Gelani et al., 2009; Lima et al., 2010) success may vary depending on specific strains in rat models (Graves et al., 2008; Schreiner et al., 2011). Previous studies inducing periodontal disease require a combination of repeated oral inoculation and palatal injection of *A. actinomycetemcomitans* to elicit a host immune response and bone loss in mice (Garlet et al., 2005).

Thus, in the present study, formalin-fixed *A. actinomycetemcomitans* was used short-term following a single exposure of the periodontal pathogen to address host-bacteria interactions versus *A. actinomycetemcomitans* virulence. An innate immune response was observed in this model because the rats were naïve to the *A. actinomycetemcomitans* prior to inoculation making it less likely for an adaptive immune response to occur within the duration of the experiment (Graves et al., 2001). We noticed a sustained inflammatory infiltrate up to two weeks, which is inconsistent with prior findings using *P. gingivalis* calvarial injections that allowed for inflammation to decrease starting at day 5 post injection (Liu et al., 2004). In addition our model uses Sprague-Dawley rats with *A. actinomycetemcomitans*. Sprague-Dawley rats are highly susceptible to repeated inoculation of *A. actinomycetemcomitans* LPS that caused inflammatory bone loss in a periodontal disease model (Rogers et al., 2007). However, it is also possible that we missed peak inflammation at an earlier time-point and that the infiltrate seen at one and two weeks in our study is resolving. Therefore, it is possible that in this model rats have a diminished ability to resolve inflammation and continue to have sustained infiltrate 2 weeks post injection.

Following 1 to 2 weeks, we observed an increased number of resorption lacunae in rat calvariae after microinjection of *A. actinomycetemcomitans* in comparison to controls via  $\mu$ CT and TRAP staining for osteoclast formation. It is worth noting that TRAP staining only indicated a trend towards increased osteoclast number that was decreasing in week 2. This

may be the result of the osteoclasts undergoing apoptosis at the 2-week time frame and thus not detectable at this time point (Tanaka et al., 2006).

This *in vivo* study has identified the consequences of whole bacterial stimulation in rat calvaria in the context of innate immune response. Previously, *A. actinomycetemcomitans* LPS was found to stimulate RANKL, IL-6, and MMP-13 expression in several periodontal relevant cell types, including periodontal ligament fibroblasts, osteoblasts, and macrophages through transcriptional and post-transcriptional mechanisms (Patil et al., 2006; Rossa et al., 2006; Rossa et al., 2005). Since the calvarial bone is formed via intramembranous ossification, similar to alveolar bone, this is a suitable model to study short-term consequences of *A. actinomycetemcomitans* in the host immune interactions as a surrogate for a periodontal disease model similar to other studies (Assuma et al., 1998). In summary, we observed sustained MAPK activation and bone resorption in response to activated signaling pathways relevant to cytokine driven-inflammation and bone loss in response to *A. actinomycetemcomitans* stimulation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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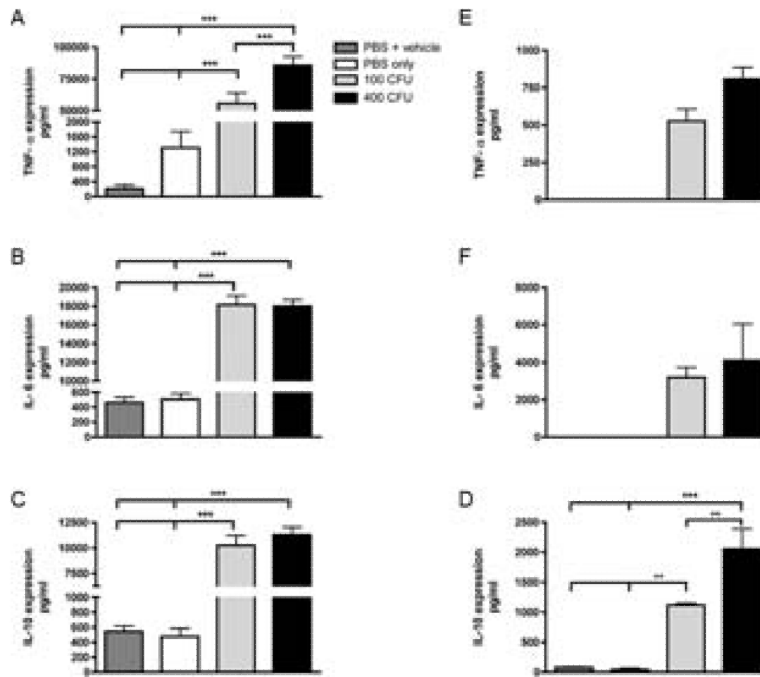
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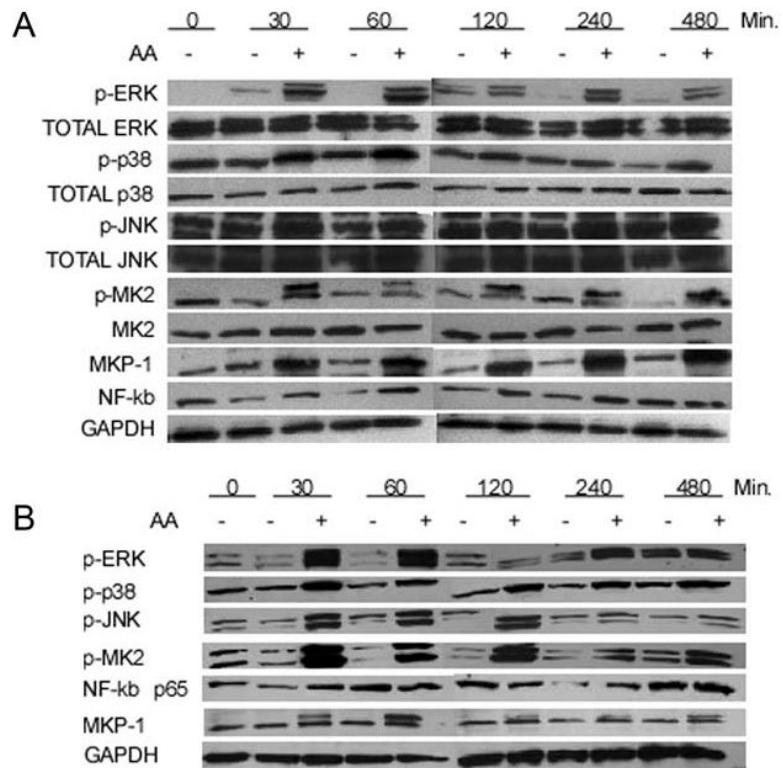


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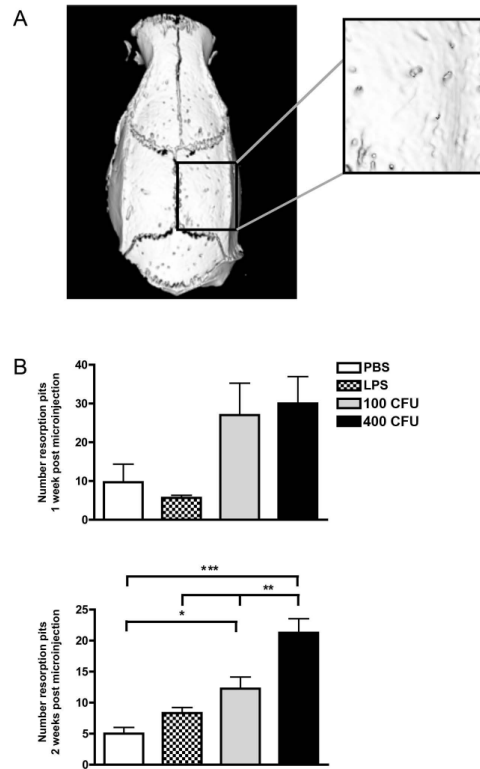


**Figure 1. Pronounced expression of inflammatory cytokines was observed in *A. actinomycetemcomitans* stimulated rat macrophage cells and primary BMMφ cells**  
 NR8383 cells were stimulated with *A. actinomycetemcomitans* for 24 hrs and cytokine expression measured via ELISA for TNF-α (A), IL-6 (B), and IL-10 (C). Following isolation and culturing for 7 days, BMMφ cells were stimulated with *A. actinomycetemcomitans* for 24 hrs and ELISA analysis used to measure TNF-α (D), IL-6 (E), and IL-10 (F). Representative data of 5 separate experiments is presented. (\*\*p<0.001, \*\*\*p<0.0001)

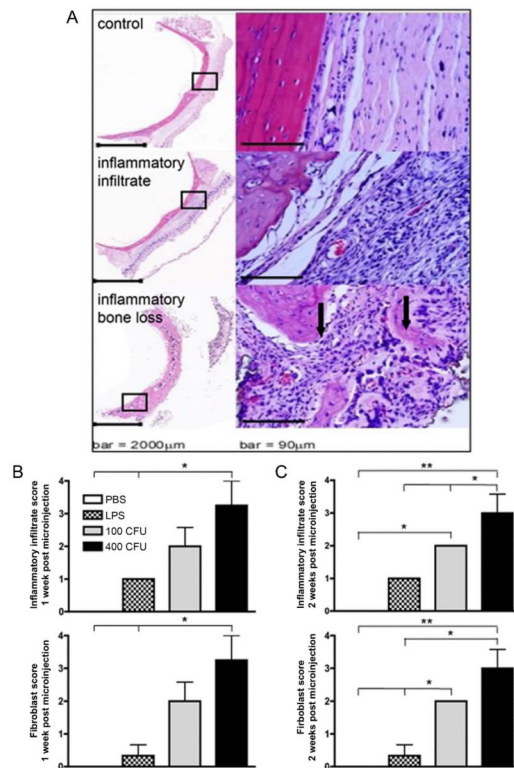


**Figure 2. Sustained activation of MAPK signaling pathway in rat macrophages and BMM $\phi$  cells following *A. actinomycetemcomitans* stimulation**

Sustained activation of intracellular signaling NF $\kappa$ B and MAPK/MKP pathways is observed post stimulation with *A. actinomycetemcomitans* in NR8383 rat macrophage cells (A) and BMM $\phi$ s (B). Western blot data representative of 3 separate experiments for panel A and 2 separate experiments in panel B.

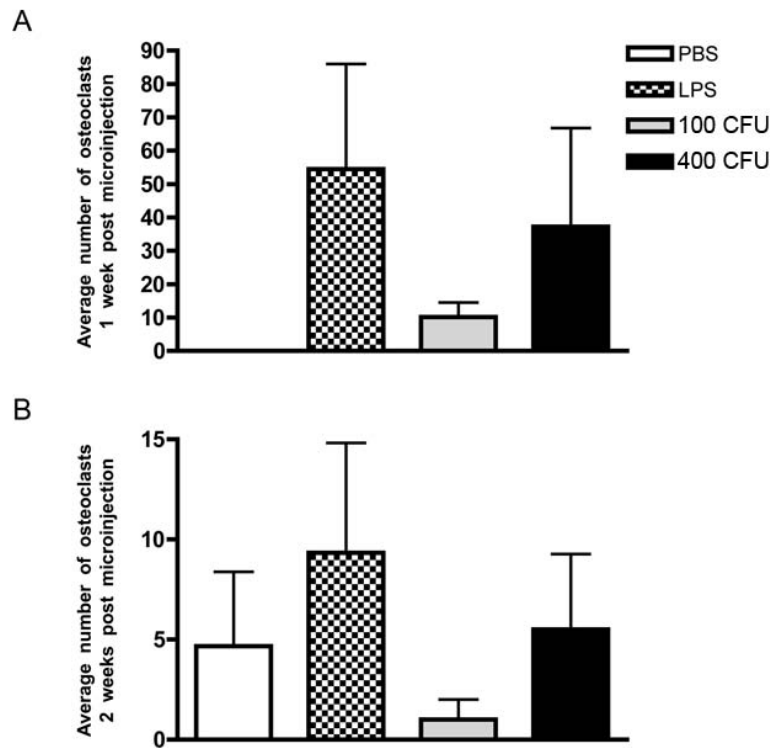


**Figure 3. Induction of bone resorption following *A. actinomycetemcomitans* microinjection**  
 Computer rendered micro-CT ( $\mu$ CT) images of representative calvaria sample post 2 weeks exposure to *A. actinomycetemcomitans*. Magnification indicates bone resorption lacunae (A). Enumeration of bone resorption areas at 1 (upper panel) and 2 weeks (lower panel) post bacterial stimulation (B). Data is expressed as mean number of resorption lacunae ( $n=6$  for controls and  $n=8$  for *A. actinomycetemcomitans* injected samples; \*\*\* $p<0.0001$ , \*\* $p<0.001$ , \* $p<0.05$ ).



**Figure 4. Pronounced inflammatory cell and fibroblast infiltration following *A. actinomycetemcomitans* stimulation**

Histological examination of rat calvarial tissues was used to evaluate the extent of inflammatory infiltrate and fibroblast infiltration. Representative H&E samples are presented indicating control (PBS) injected (upper panel), and *A. actinomycetemcomitans* injected samples (middle and lower panels) showing inflammation and bone resorption respectively. Inflammatory cell infiltration and fibroblast proliferation was enumerated by a pathological scoring system (B and C). Results are expressed as mean with S.E. (n=6 for controls and n=8 for *A. actinomycetemcomitans* injected samples; \*\*p<0.001, \*p<0.05).



**Figure 5. Increased osteoclast formation following LPS and *A. actinomycetemcomitans* stimulation**

Graphic representation of enumerated TRAP<sup>+</sup> multinucleated formation in calvarial tissue section is presented from indicated groups in after 1 week (A) or 2 weeks post stimulation (B). Horizontal bar indicates mean TRAP<sup>+</sup> cell counts.