Alveolar Bone Resorption after Ecological Time-sequential Polybacterial Periodontal Infection (ETSPPI) in the TLR4$^{-/-}$ Mice.

Sandhya Rani Nadakuditi[1]*, Syam Jeepipalli[1], Luz de Aquino Martins AR[1], Tushar Desaraju[1], Eduardo Colella[1], Parvathi Gurusamy[1], Edward K. L. Chan[2] and Lakshmyya Kesavalu[1,2].

[1]Department of Periodontology, College of Dentistry, University of Florida, Gainesville, FL, USA.

[2]Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, FL, USA.

ABSTRACT

INTRODUCTION: Periodontitis (PD) is a polymicrobial dysbiotic chronic inflammatory disease caused by microbes interacting in the host subgingival sulcus/pockets. *Streptococcus gordonii, Fusobacterium nucleatum, Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia* are common co-colonizers of the supra- and subgingival sulcus/pockets in humans. The purpose of this investigation was to determine alveolar bone resorption (ABR) after ecological time-sequential polybacterial periodontal infection (ETSPPI) with partial human mouth microbes (PAHMM) *S. gordonii, F. nucleatum, and P. gingivalis+T. denticola+T. forsythia* in TLR4$^{-/-}$ mice. **METHODS:** Ten-week-old male and female TLR4$^{-/-}$ mice (n=20) and C57BL6 mice (n=20) were used for polymicrobial ETSPPI infection (Groups 1 and 3, respectively) and sham infection (Groups 2 and 4, respectively). Both maxilla (left and right) and mandible (left and right) were harvested after 17 weeks of (ETSPPI) infection. **RESULTS:** TLR4$^{-/-}$ mice exposed to 19-week-polymicrobial infection did not show significant ABR in the right maxillary and mandibles compare to TLR4$^{-/-}$ sham infection. In contrast, wild-type C57BL6/J infected mice developed significant ABR compared to sham infection.

CONCLUSION: Periodontal bacteria have colonized on the gingival surfaces of infected mice but did not induce a significant ABR in the TLR4$^{-/-}$ infected mice.

INTRODUCTION

- *S. gordonii* (early bacterial colonizer), *F. nucleatum* (intermediate colonizer and bridging bacterium), *P. gingivalis*, *T. denticola*, and *T. forsythia* (late bacterial colonizers) are common co-colonizers of the supra- and subgingival sulcus in humans and are considered leading opportunistic bacteria.
- The purpose of this study was to determine the impact of TLR4 signaling on ABR after ecological time-sequential polybacterial periodontal infection (ETSPPI) with PAHMM such as *S. gordonii, F. nucleatum, P. gingivalis+T. denticola+T. forsythia* in TLR4$^{-/-}$ mice.

MATERIALS AND METHODS

MICROORGANISMS: *S. gordonii* DL1, *F. nucleatum* ATCC 49256, *P. gingivalis* 381, *T. denticola* ATCC 35405, *and T. forsythia* ATCC 43037 were used in this study. *P. gingivalis, S. gordonii, and F. nucleatum* were grown in Brucella blood agar plates supplemented with hemin and vitamin K. The oral spirochete *T. denticola* was grown in GM-1 broth and *T. forsythia* was grown in TSB broth supplemented with N-acetyl muramic acid and hemin. All the bacteria were cultured and harvested in a Coy anaerobic chamber for 2 to 3 days. **ANIMALS:** Ten-week-old male (n=16) and female (n=16) TLR4$^{-/-}$ mice and C57BL6 mice were used and all procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida (IACUC protocol #202200000223).

ECOLOGICAL TIME-SEQUENTIAL POLYBACTERIAL PERIODONTAL INFECTIONS: To suppress the oral microbiota, Kanamycin (500 mg/mL) was administered for 3 days in the drinking water followed by rinsing with 0.12% chlorhexidine gluconate. Mice were randomly assigned to the infected (PD) and the sham-infected groups. Mice were infected in order first with *S. gordonii* and second with *F. nucleatum* and a sequential three microbes of *P. gingivalis, T. denticola, and T. forsythia* together by oral lavage for 10 infection cycles in the total 19 weeks infection time (inoculating 4 times per week every alternate week) to induce periodontitis. Detailed experimental procedure is given in the schematic diagram (Figure 1). Sham-infected mice were inoculated with vehicle carboxy methyl cellulose (6% CMC). Oral plaque swabs were collected post-infection.

PREPARATION OF JAWS: The right mandibles and maxilla were autoclaved, defleshed, and immersed in 3% hydrogen peroxide for 30 min and air-dried. **Image capture procedure:** Two-dimensional molar teeth images were captured using a stereo dissecting microscope (Stereo Discovery V8, Carl Zeiss Microimaging, Inc, Thornwood, NY, USA).

ALVEOLAR BONE MORPHOMETRY: The area between the cemento-enamel junction to the alveolar bone crest of the buccal and the palatal surfaces of the maxillary jaws was measured by using the line tool (AxioVision LE 29A software version 4.6.3, Thornwood, NY, USA). Examiners blinded to the study performed all morphometric measurements twice at separate times.

STATISTICS: Ordinary two-way ANOVA with Turkey's multiple comparison tests with a single pooled variance was performed for ABR measurements to identify the statistical significance using Prism 9.4.1 (GraphPad Software, San Diego, CA, USA). All the data in the graphs were presented as mean ± SD.

EXPERIMENTAL DESIGN

FIGURE 1: Schematic diagram: Ecological Time-Sequential polybacterial Periodontal infections in TLR4$^{-/-}$ and C57BL6 mice.

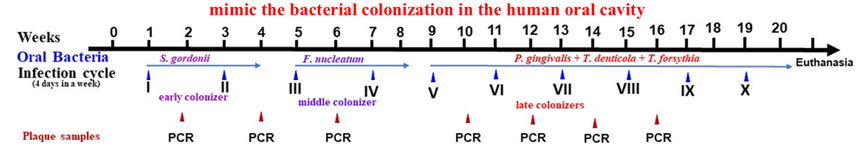

TABLE 1: Animal groups and gingival plaque samples positive for bacterial gDNA by PCR.

Group / Mice / Infection	Positive gingival plaque samples (N=16 mice)				
	2 weeks (Sg)	6 weeks (Fn)	8 weeks (Fn)	10 weeks *Pg/Td/Tf*	14 weeks *Pg/Td/Tf*
Group I / TLR4$^{-/-}$ mice / ETSPPI infection	16	9	16	10/9/9	14/13/15
Group II / TLR4$^{-/-}$ mice / Sham-infection	0	0	NC	0/0/0	NC
Group III / C57BL6/J mice / ETSPPI infection	16	12	16	12/11/9	15/13/14
Group IV / C57BL6/J mice / Sham-infection	0	0	NC	0/0/0	NC

RESULTS

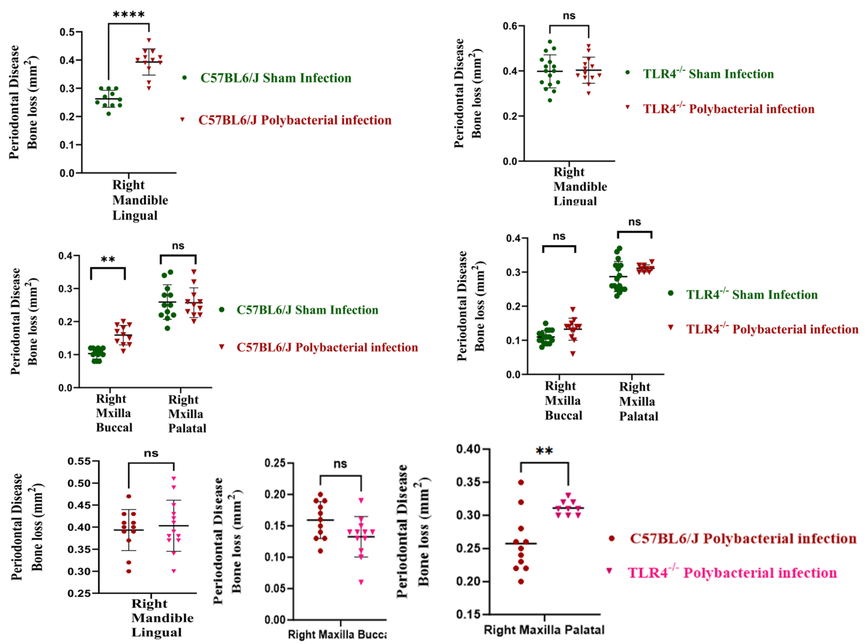

FIGURE 2: Morphometric analysis of the Mandibles and maxillary ABR in mice. No significant ABR was observed in the mandibles and maxilla of TLR4$^{-/-}$ infected mice.

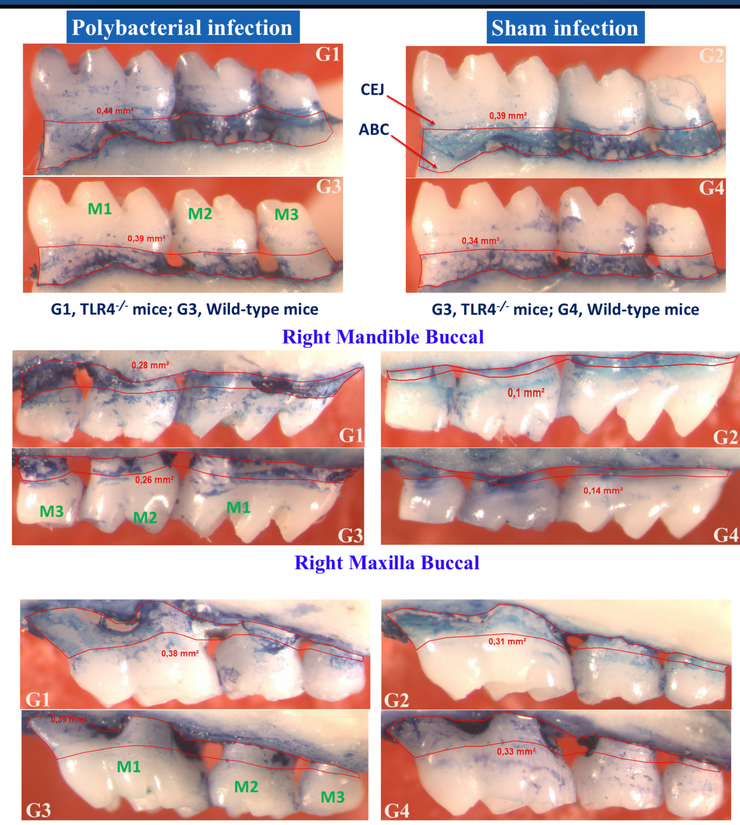

FIGURE 3: Representative mandible (lingual) and maxilla (buccal and palatal) ABR images. M: Molar teeth; CEJ: Cemento Enamel Junction; and ABC: Alveolar Bone Crest.

CONCLUSION

- Gingival plaque samples from TLR4$^{-/-}$ and C57BL6/J infected mice after polybacterial infection [colony PCR] showed the presence of bacterial 16S rRNA gene amplicons.
- Male and female C57BL6/J mice infected with PAHMM had ABR in the mandible & maxilla.
- ETSPPI infection with PAHMM did not lead to a significantly enhanced ABR in the TLR4$^{-/-}$ knockout mice mandibles.
- Mice lacking TLR4 receptor have dampened periodontal inflammatory responses and ABR.
- Genetic deficiency of TLR4 receptor significantly abrogates inflammation in the periodontium and reduces ABR (periodontitis).

FUNDING: National Institute of Dental and Craniofacial Research (R01 DE028536).