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Liver x receptors contribute to periodontal pathogen-elicited inflammation and oral bone loss

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SUMMARY

Periodontal diseases are chronic oral inflammatory diseases that are polymicrobial in nature. The presence of specific bacteria in subgingival plaque such as *Porphyromonas gingivalis* is associated with microbial dysbiosis and modulation of host immune response. Bacteria-elicited innate immune activation and inflammation are key elements implicated in the destruction of soft and hard tissues supporting the teeth. Liver X receptors (LXRs) are nuclear hormone receptors with important function in lipid homeostasis, inflammation, and host response to infection; however, their contribution to chronic inflammatory diseases such as periodontal disease is not understood. The aim of this study was to define the contribution of LXRs in the development of immune response to *P. gingivalis* and to assess the roles LXRs play in infection-elicited oral bone loss. Employing macrophages, we observed that *P. gingivalis* challenge led to reduced LXR α and LXR β gene expression compared to that observed with unchallenged wild type cells. Myeloid differentiation primary response gene 88 (MyD88)-independent, TIR-domain-containing adapter-inducing interferon- β (TRIF)-dependent signaling affected *P. gingivalis*-mediated reduction in LXR α expression, while neither pathway influenced the *P. gingivalis* effect on LXR β expression. Employing LXR agonist and mice deficient in LXRs, we observed functional effects of LXRs in the development of *P. gingivalis*-elicited cytokine response at the level of the macrophage, and participation of LXRs in *P. gingivalis*-elicited oral bone loss. These findings identify novel importance for LXRs in the pathogenesis of *P. gingivalis* infection-elicited inflammation and oral bone loss.

Keywords

Porphyromonas gingivalis; liver x receptor; cytokine; innate immunity; macrophage; oral bone loss

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INTRODUCTION

Inflammation is an essential component of the host response to infection; however, in the context of chronic infectious diseases, the elicited inflammation and host response do not resolve infection. Periodontal diseases are chronic inflammatory diseases of the oral cavity that lead to progressive destruction of the soft and hard tissues that support the teeth. It has been estimated that 40% of the adult population in the US may possess some measureable degree of periodontitis (Eke *et al.* 2012). Immune activation is a central element to the progression of periodontal diseases. The subgingival plaque is a highly complex microbial community (Paster *et al.* 2001). Bacteria present in the subgingival plaque including *Tannerella forsythia*, *Treponema denticola*, and *Porphyromonas gingivalis* are closely associated with periodontal diseases (Socransky *et al.* 1998). *P. gingivalis* is considered a keystone pathogen of chronic forms of periodontal disease due to its ability to modulate host immune factors associated with pathogen clearance, and stimulates microbial dysbiosis (Darveau *et al.* 2012; Hajishengallis *et al.* 2011).

The cellular infiltrate that accompanies periodontal disease is complex with neutrophils, lymphocytes and monocyte/macrophages predominating at different disease stages (Page and Schroeder 1976; Payne *et al.* 1975). Human data and experimental modeling are in general agreement that changes in the levels of cytokines and chemokines, interferon-associated molecules, cell adhesion molecules, complement receptors, and toll-like receptors (TLRs) are observed during periodontal disease, and in response to specific oral pathogens such as *P. gingivalis* (Garlet *et al.* 2003; Gemmell *et al.* 2001; Gemmell and Seymour 1998; Hajishengallis and Lambris 2012; Kajita *et al.* 2007; Kirby *et al.* 1999; Liang *et al.* 2011; Ramseier *et al.* 2009; Salvi *et al.* 1998). TLRs and their signaling pathways including myeloid differentiation primary response gene 88 (MyD88)-dependent and MyD88-independent signaling participate in different aspects of the elicited host response to *P. gingivalis* challenge (Burns *et al.* 2006; Burns *et al.* 2010; Costalonga *et al.* 2009; Gibson and Genco 2007; Papadopoulos *et al.* 2013).

In addition to its role in periodontal disease, *P. gingivalis* infection may play a role in non-oral diseases including obesity, diabetes, and cardiovascular diseases (Amar *et al.* 2007; Gibson *et al.* 2004; Graves *et al.* 2005; Lalla *et al.* 2003). The nuclear hormone receptors, liver X receptors (LXRs), are conditional transcriptional regulators involved in the expression of genes associated with the removal of excess lipid from cells such as hepatocytes and macrophages – a process termed cholesterol efflux (Chawla *et al.* 2001; Repa and Mangelsdorf 2002). LXRs play a critical role in normal physiological function, as well as in the pathogenesis of lipid-associated diseases such as obesity and cardiovascular disease (Beaven *et al.* 2013; Joseph *et al.* 2002). LXRs exist in two isoforms; LXR α (encoded by the gene *Nr1h3*) is primarily expressed in the liver, adipose tissue, and macrophages, while LXR β (encoded by the gene *Nr1h2*) is expressed in many tissues (Repa and Mangelsdorf 2002). In the nucleus LXRs are bound as heterodimers with retinoid X receptor, itself a nuclear hormone receptor, to DNA regulatory elements in a repressed state (Zelcer and Tontonoz 2006). Derivatives of fatty acid and cholesterol metabolism are endogenous activators of LXRs (Castrillo and Tontonoz 2004; Repa and Mangelsdorf 2002).

In addition to their role in lipid cycling, LXRs influence development of host inflammation (Castrillo and Tontonoz 2004). Macrophages treated with the LXR agonist develop a reduced inflammatory response to microbial antigens such as bacterial lipopolysaccharide (LPS) (Joseph *et al.* 2003). Interferon regulatory factor (IRF) 3 functions as a transrepressor of LXR function (Castrillo *et al.* 2003). IRF3 is activated via TLR signaling in a TIR-domain-containing adapter-inducing interferon- β (TRIF) dependent manner via TANK-binding kinase 1 (TBK-1) and I-kappa-B kinase epsilon (IKK ϵ) (Fitzgerald *et al.* 2003). Our group and others have reported that *P. gingivalis*, and *P. gingivalis* antigens stimulate IRF3 activation (Gaddis *et al.* 2009; Shaik-Dasthagirisahab *et al.* 2014). As such IRF3 may play a role in development of the host immune response to periodontal pathogens.

An unexpected finding that has shed important light on additional roles for LXR function reveals their involvement in host response to infection. A recent clinical study identified LXR polymorphisms associated with susceptibility to tuberculosis (Han *et al.* 2014). Mice deficient in LXRs are more susceptible to *Listeria monocytogenes* and *Mycobacterium tuberculosis* infection, and at the cellular level these pathogens stimulate LXR α gene expression (Joseph *et al.* 2004; Korf *et al.* 2009). LXR agonist treatment limits neutrophil recruitment to the lung and influenced host defense to *Klebsiella pneumoniae* challenge (Smoak *et al.* 2008). However, LXR deficient mice were more resistant to infection with the protozoan *Leishmania chagasi/infantum* (Bruhn *et al.* 2010). *In vitro Chlamydia pneumoniae* infection had no effect on LXR gene expression; yet, when investigated *in vivo* *C. pneumoniae* stimulated a 2.2-fold reduction in LXR α gene expression in aortic cells of hyperlipidemic mice (Bobryshev *et al.* 2011). LXRs have also been implicated in host response to viruses including HIV, and Hepatitis B (Hanley *et al.* 2010; Kim *et al.* 2008; Renga *et al.* 2012), as well as to bacterial LPS (Myhre *et al.* 2008; Wang *et al.* 2006).

Recently, LXR involvement in bone homeostasis has been identified. Employing LXR agonist treatment, RAW264.7 cells were impaired in their ability to form osteoclasts when cultured with LPS, yet agonist treatment had no effect on LPS-elicited pro-inflammatory cytokine expression (Robertson Remen *et al.* 2013a). LXR β is implicated in reduced effectiveness of receptor activator of nuclear factor kappa-B ligand (RANKL) in the pre-osteoclast to osteoclasts conversion (Remen *et al.* 2011). LXR agonist treatment also influences osteoblast function; however, in mice long-term *in vivo* agonist treatment had little effect on trabecular or cortical bone levels (Prawitt *et al.* 2011). Recent *in vivo* studies reported that LXR agonist treatment protected mice from LPS-elicited bone loss (Kim *et al.* 2013).

Although two studies have reported an association of LXRs with *P. gingivalis* infection and host lipid handling (Maekawa *et al.* 2011; Miyazawa *et al.* 2012); a functional role for LXRs in *P. gingivalis*-elicited inflammation and oral bone loss has not been determined. The aim of this study was to define the contribution of LXRs in the development of immune response to *P. gingivalis* and to assess the roles LXRs play in infection-elicited oral bone loss. Here we report that *P. gingivalis* challenge of wild type mouse macrophages led to reduced LXR α and LXR β gene expression. Pathogen-elicited change in LXR α gene expression was dependent on TRIF and IRF3, but not MyD88. Furthermore, LXRs participated in the development of the host inflammatory response to the *P. gingivalis*. Lastly, employing an

oral challenge model, we observed that in comparison to wild type mice, LXR deficient mice did not develop oral bone loss in response to *P. gingivalis* challenge. Collectively, our studies point to important roles for LXRs in inflammation, and periodontal pathogen-elicited oral bone loss.

MATERIALS AND METHODS

Mice

C57BL-6 wild type (C57BL-6 WT), MyD88^{-/-}, TRIF^{Lps2}, IRF3^{-/-}, as well as Sv129/BL6 wild type (Sv129/BL6 WT), and LXRαβ-double knockout (DKO) mice were used in these studies. Experiments employing animals were performed in accordance with Boston University Institutional Animal Care and Use Committee approvals. Animals were routinely monitored, and all efforts were made to minimize suffering. Sacrifice was performed in a humane manner by CO₂ asphyxiation. C57BL-6 mice were obtained from Jackson Laboratory (Bar Harbor, ME), MyD88^{-/-}, and TRIF^{Lps2} mice were obtained from Dr. S. Akira (Osaka University, Osaka, Japan) and Dr. B. Beutler (University of Texas Southwestern, Dallas, TX), respectively. IRF3^{-/-} mice were obtained from Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). Mixed background wild type Sv129/BL6, and LXRαβ-DKO mice were provided by Dr. D. Mangelsdorf (University of Texas Southwestern, Dallas, TX). All mice were maintained in the same facility, and received water and chow diet *ad libitum*.

Cultivation and *P. gingivalis*

Porphyromonas gingivalis strain 381 was used in these studies and was cultured as previously described (Shaik-Dasthagirisahab *et al.* 2014). In brief, bacteria were grown on anaerobic blood agar plates for 4–6 days, and placed into brain-heart infusion broth for 24h growth. Bacteria were harvested by centrifugation, washed, suspended in antibiotic-free RPMI-1640 medium + 10% heat-inactivated fetal bovine serum to OD₆₆₀ of 1 (1×10⁹ colony forming units/ml), and were added to cells at a multiplicity of infection (MOI) of 100. Bacterial culture purity was confirmed by Gram stain.

Growth and challenge of macrophages

Bone marrow was harvested from femurs of mice using aseptic technique as we have done previously (Shaik-Dasthagirisahab *et al.* 2010). In brief, bone marrow cells flushed from femurs were gently pipetted to generate single cell suspensions, and red blood cells were removed using RBC lysis buffer (eBioscience, San Diego, CA). The cells were suspended to 1×10⁶ cells/ml in fresh RPMI-1640 medium + 20% conditioned medium from L929 cells (source of macrophage-colony stimulating factor (M-CSF)), were placed in cell culture dishes and incubated in 5% CO₂ incubator for 7 days for macrophage differentiation. Adherent cells were harvested by scraping, washed, counted, and were seeded into wells of 24-well plates at 5×10⁵ cells/ml, and were used in experiments.

Agonist treatments

The LXR agonist GW3965 (Cayman Chemical, Ann Arbor, MI) was dissolved in dimethyl sulfoxide (DMSO), and working strength solutions of 2.0μM were made in antibiotic-free

complete RPMI-1640 medium, as previously reported (Joseph *et al.* 2003). Agonist treatments were applied to cells 18h prior to *P. gingivalis* challenge, and were present in culture medium during challenge.

Quantitative PCR (qPCR)

Following challenge, macrophages were washed, and lysed in RLT lysis buffer (Qiagen, Valencia, CA). RNA was harvested using RNEasy spin columns (Qiagen). Quality and quantity of RNA determined by Nanodrop. Complementary DNA (cDNA) was prepared from RNA with a RNA-to-cDNA kit (Life Technologies, Grand Island, NY) and qPCR assays were performed with StepOne Plus real-time PCR system (Life Technologies). Gene expression was measured using mouse Taqman assays (Life Technologies) for LXR α (Mm00443451), LXR β (Mm00437262), with β -actin (Mm00607939) serving as the housekeeping control. Following normalization to β -actin, fold-change in gene expression was determined using $\Delta\Delta$ CT method (Livak and Schmittgen 2001).

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatant fluid levels of murine tumor necrosis factor (TNF)- α and interleukin (IL)-6 were measured using commercially available DuoSet ELISA kits (R&D Systems, Minneapolis, MN).

Mouse oral challenge and bone loss

We employed the mouse oral bone loss model (Baker *et al.* 1994), with slight modification, as previously reported (Gibson *et al.* 2004). In brief, 8-week old Sv129/BL-6 WT and LXR $\alpha\beta$ -DKO mice were placed into groups (n=8 mice/group) and received chow diet and water *ad libitum*. Following 10-day sulphamethoxazole/trimethoprim treatment in drinking water, antibiotics were removed for three-days, at which time a blood sample was collected from each animal for serum. Mice were then orally challenged with *P. gingivalis* suspended in 2% carboxymethylcellulose in phosphate buffered saline (PBS) (vehicle) three-times in one week using a feeding needle attached to syringe, or received vehicle alone (unchallenged control). After six-weeks rest, a final blood sample was collected from each animal for serum, mice were then humanely sacrificed, and the head of each mouse harvested. Following extensive cleaning and methylene blue staining, digital micrographs were obtained for each hemi-maxilla, and measurements were made from the cementum-enamel junction (CEJ) to the alveolar bone crest (ABC) at 14 landmark sites for each animal, as reported previously (Baker *et al.* 1994). Onscreen measurements were obtained using ImageJ (Schneider *et al.* 2012).

P. gingivalis specific immunoglobulin (Ig)G measurement

ELISA was performed on all mouse sera to determine *P. gingivalis*-specific IgG levels as we have done previously (Gibson *et al.* 2005). Briefly, Immulon 4Hbx ELISA plates (Thermo Scientific, Waltham, MA) were coated with formaldehyde-fixed *P. gingivalis* 381 in carbonate-bicarbonate buffer (pH 9.6). Following blocking with 2% bovine serum albumin, serial two-fold dilutions of mouse sera were added to the wells and incubated overnight at room temperature. Goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma, St. Louis,

MO) was then added followed by substrate, and after 1h the resultant color intensity was recorded at 405 nm. A mouse IgG standard curve was generated to quantify the serum concentration of *P. gingivalis*-specific IgG present. For this, ELISA plates were coated with goat anti-mouse Fab IgG (Sigma), blocked, and then serial two-fold dilutions of mouse IgG (200 ng-10 pg/well) were added to the coated wells. These ELISA plates were processed in parallel with the mouse serum samples.

Statistical Analyses

Data were imported into and analyzed with Prism statistical analysis software (GraphPad, La Jolla, CA) and SAS version 9.3 (SAS Institute, Cary, North Carolina). Data sets analyzed by one-way analysis of variance (ANOVA) were first evaluated with Bartlett's test to ensure equal variance, and the Bonferroni method for multiple testing was used to control overall significance levels in post-hoc testing. For analysis of relative oral bone levels a generalized estimating equations (GEE) correction was used to account for the 14 correlated repeated measures within each mouse in a two-factor ANOVA model (wild type and LXR α β -DKO mice by *P. gingivalis* exposure). The natural log transformation was used to provide a more Gaussian distribution for these bone levels in analysis, and the resulting mean and confidence interval boundary estimates transformed back to the original scale. An overall significance level of 0.05 was used in all analyses.

RESULTS

P. gingivalis challenge limits LXR α and LXR β gene expression

To begin to understand if LXRs participate in host response to periodontal pathogen challenge, we employed qPCR to determine the effect *P. gingivalis* challenge had on LXR α and LXR β gene expression. Bone marrow macrophages from C57BL-6 WT mice cultured in medium alone served as reference controls for unchallenged cell expression of LXRs. Expression of LXR α and LXR β genes for both unchallenged and *P. gingivalis* challenged cells was accomplished by first normalizing all samples to β -actin, and we then calculated fold change in gene expression of the *P. gingivalis* challenged cells relative to the level measured for unchallenged genotype-matched cells. Employing this approach, we observed reduction in LXR α and LXR β gene expression as a consequence of *P. gingivalis* challenge (Fig. 1). These data support that *P. gingivalis* challenge of WT mouse macrophages led to reduced LXR α and LXR β gene expression.

Effect of TLR-signaling pathways on *P. gingivalis*-elicited LXR gene expression

As *P. gingivalis* challenge led to changes in LXR gene expression in macrophages relative to unchallenged cells, and TLR contribution to host response to *P. gingivalis* is established (Burns *et al.* 2006; Burns *et al.* 2010; Costalonga *et al.* 2009; Gibson and Genco 2007) we obtained macrophages from MyD88 $^{-/-}$, TRIF Lps2 , and IRF3 $^{-/-}$ and cultured these in medium, or with *P. gingivalis* to attempt to identify signaling pathways linked to *P. gingivalis*-influenced LXR gene expression. In comparison to WT, macrophages from MyD88 $^{-/-}$ mice presented with only a modest fold-change reduction in *P. gingivalis*-elicited LXR α gene expression ($P>0.05$ vs. WT; Fig. 1), and this mutation did not effect LXR β gene expression. Substantial inhibition of LXR α gene expression was observed by

macrophages from both TRIF^{Lps2}, and IRF3^{-/-} mice to *P. gingivalis* challenge ($P < 0.001$ and $P < 0.01$ vs. WT, respectively; Fig. 1). LXR β gene expression was not affected by either of these mutations (Fig. 1). From these studies we conclude that TLR signaling, principally via the TRIF arm, contributes to *P. gingivalis*-elicited reduction in LXR α gene expression, but does not alter the observed *P. gingivalis*-elicited reduction in LXR β gene expression.

LXR agonist treatment impacts macrophage cytokine response to *P. gingivalis*

To understand the potential impact of LXRs to host inflammatory response to *P. gingivalis*, we obtained bone marrow macrophages from C57BL-6 WT mice and treated them with the LXR agonist GW3965. Optimization of GW3965 dose (0.5, 1, and 2 μ M agonist) effect on *P. gingivalis*-elicited TNF- α was performed, and maximal reduction in TNF- α was achieved using a dose of 2 μ M GW3965 for 18h (data not shown, similar to prior reports (Joseph *et al.* 2003)). Next we incubated bone marrow macrophages with 2 μ M GW3965 to define the effect of agonist treatment on live *P. gingivalis* elicited pro-inflammatory cytokines production by macrophages. We observed that macrophages treated with GW3965 presented with reduced TNF- α production to *P. gingivalis* challenge ($P < 0.001$ vs. untreated; Fig. 2), but IL-6 production was not significantly affected (Fig. 2). These data support that LXR agonist treatment influences TNF- α cytokine response to *P. gingivalis* whole organism challenge.

Effect of LXR ablation on macrophage cytokine expression

To further understand the effect of LXRs on evolution of *P. gingivalis* elicited inflammatory response, we performed macrophage challenge studies employing a gene ablation approach. We obtained macrophages from Sv129/BL-6 WT and LXR $\alpha\beta$ -DKO mice and cultured these with *P. gingivalis* for 24h and measured inflammatory cytokine expression by ELISA. We observed no significant differences in TNF- α levels expressed by WT or LXR $\alpha\beta$ -DKO macrophages cultured with *P. gingivalis* ($P > 0.05$; Fig. 3). In contrast, supernatant levels of IL-6 elicited by *P. gingivalis* bacteria were reduced significantly in macrophages from LXR $\alpha\beta$ -DKO mice as compared with WT ($P < 0.01$ vs. WT; Fig. 3B).

LXR ablation effects *P. gingivalis*-elicited oral bone loss

To address the potential involvement of LXRs during *P. gingivalis* oral infection, we performed a mouse oral challenge study. Groups of Sv129/BL-6 WT and LXR $\alpha\beta$ -DKO mice were mock challenged, or challenged orally with *P. gingivalis*, and after 6-weeks rest, and extensive cleaning, we obtained measurements from ABC-CEJ at landmark sites from each group of animals (Fig. 4A). We observed significant differences in bone levels between unchallenged WT and LXR $\alpha\beta$ -DKO mice ($P < 0.0001$). As anticipated, *P. gingivalis* challenge stimulated oral bone loss in WT mice ($P < 0.0001$; Fig. 4B); however, *P. gingivalis* challenge failed to stimulate oral bone loss in LXR $\alpha\beta$ -DKO mice ($P > 0.05$ vs. genotype-matched unchallenged control; Fig. 4B). These findings identify a role for LXRs in development of oral bone loss in response to *P. gingivalis* challenge.

LXR deficiency and *P. gingivalis*-elicited IgG

To determine if LXRs play a role on elicitation of *P. gingivalis*-specific IgG, serum was separated from the blood of each animal at sacrifice, and the levels of *P. gingivalis*-specific IgG were determined by ELISA. As anticipated, IgG levels were elevated in the groups of mice orally challenged with live *P. gingivalis* ($P < 0.001$ vs. genotyped-matched unchallenged control; Fig. 5). Although a trend of reduced IgG levels were observed in the group of *P. gingivalis* challenged LXR α β -DKO mice as compared with WT *P. gingivalis* challenged mice, the levels did not reach significance ($P > 0.05$; Fig. 5).

DISCUSSION

LXRs were initially defined as transcriptional regulators of genes important in lipid homeostasis and cholesterol efflux (Zelcer and Tontonoz 2006). Recent studies have defined roles for LXRs in the development of inflammation, host response to infection, and bone remodeling. Based on this constellation of activities, we employed both *in vitro* and *in vivo* modeling approaches to determine whether LXRs are functionally involved in host response to the periodontal pathogen *P. gingivalis*. Our findings support a previously unknown contribution of LXRs to the host inflammatory response and oral bone loss elicited by *P. gingivalis*.

We observed reduced LXR α and LXR β gene expression by WT macrophages cultured with *P. gingivalis*. These data are in agreement with previous studies by Maekawa *et al.* (Maekawa *et al.* 2011) who reported that aortic tissue of wild type and hyperlipidemic mice orally challenged with *P. gingivalis* expressed lower levels of LXR α and LXR β as compared with sham challenge groups. Yet, our findings were not in agreement with findings from another study, where intraperitoneal injection of mice with *P. gingivalis* did not significantly influence LXR α or LXR β gene expression in the liver (Miyazawa *et al.* 2012). Our data, employing a macrophage challenge model with physiologically normal levels of lipids support that *P. gingivalis* infection downshifts LXR gene expression.

We observed divergence in the profile of LXR gene expression to *P. gingivalis* that was attributable to TLR adaptor molecule status. The reduction in LXR α gene expression elicited by *P. gingivalis* was lost when macrophages from TRIF^{Lps2} mice were challenged with this organism; yet was only partially influenced by MyD88 deficiency. When investigating LXR β gene expression, we found its levels were also reduced in macrophages of wild type mice cultured with *P. gingivalis*, but neither TRIF^{Lps2}, nor MyD88 disruption had any effect curtailing *P. gingivalis* infection-elicited LXR β gene expression. Crosstalk between LXR and TLR signaling was initially described as involving TLR3/TLR4 engagement, precedes in a MyD88-independent manner, and is under trans-repressive control by IRF3 (Castrillo *et al.* 2003). Interestingly, treatment of macrophages with LXR agonist had an impact on LPS (TLR4 ligand), as well as *C. pneumoniae* (TLR2 ligand) elicited macrophage foam cell formation (Cao *et al.* 2007). Both MyD88-dependent and independent processes are implicated in host response to *P. gingivalis* (Burns *et al.* 2010; Shaik-Dasthagirisahab *et al.* 2013). Our findings that the *P. gingivalis* elicited changes in LXR α , but not LXR β gene expression is influenced by TLR signaling via a MyD88-independent pathway are novel, and point to an important contribution of TRIF in the ability

of *P. gingivalis* to activate host LXR α gene expression. As such, our findings broadly agree with a recent study where MyD88-independent pathway involvement was shown to be important to different stages of the host response to *P. gingivalis* infection including development of cytokine response, and clearance of the organism (Burns *et al.* 2010; Shaik-Dasthagirisahab *et al.* 2013).

We also observed that IRF3 status impacted macrophage LXR α gene expression to *P. gingivalis* challenge. Others have implicated IRF3, and other IRFs including IRF7 in development of the host response to *P. gingivalis* (Gaddis *et al.* 2009; Zhou and Amar 2007); however, the precise role of IRF3 in periodontal disease is unclear. Recently, employing mouse macrophages we reported a partial role for IRF3 in the development of the host inflammatory response to *P. gingivalis* (Shaik-Dasthagirisahab *et al.* 2014). Thus, in the context of LXR gene expression, our data are in general agreement with findings that IRF3 impacts in the activation and control of LXR function (Castrillo *et al.* 2003).

We observed LXR-dependent effects on host pro-inflammatory cytokine response to *P. gingivalis*. LXR agonist treatment of macrophages reduced *P. gingivalis*-elicited TNF- α production, but did not significantly influence IL-6 production. Agonist treated cells cultured in medium alone possessed levels of TNF- α that were below the level of detection. LXR $\alpha\beta$ -deficient macrophages expressed TNF- α levels similar to wild type macrophages; however, a significant reduction in elicited IL-6 to *P. gingivalis* challenge was observed. In these experiments the levels of TNF- α and IL-6 produced by both WT and LXR $\alpha\beta$ -DKO macrophages cultured in medium alone were below the level of detection. In other studies, use of LXR agonist treatments has shown the ability to influence host cytokine and chemokine response to bacterial challenge. Use of synthetic LXR agonist treatment reduced LPS-elicited monocyte production of IL-1 β , IL-6, and TNF- α (Myhre *et al.* 2008). In a lung challenge model it was shown that LXR agonist treatment reduced early stage release of TNF- α in response to LPS or *K. pneumoniae* challenge (Smoak *et al.* 2008). Interestingly, Fontaine *et al.* (Fontaine *et al.* 2007) reported that while short term LXR pre-treatment of monocyte-derived human macrophages led to a reduced LPS elicited TNF- α and monocyte chemotactic protein 1 (MCP-1), long-term pre-treatment leads to a shift to enhanced cytokine and chemokine response to LPS challenge. In a rat endotoxemia model LXR agonist treatment was shown to reduce plasma levels of TNF- α , and prostaglandin E₂, but did not affect IL-6 or IL-10 production (Wang *et al.* 2006). Thus, when taken together, there is significant variation in the effects LXRs have on development of host inflammatory response, and likely reflect the nature of the challenge, and the anatomical niche investigated. Our findings support that LXRs play a role in development of an inflammatory response in the context of challenge with a defined periodontal pathogen.

The critical element in the pathogenesis of periodontal disease, and the target of periodontal therapy is preservation of the oral bone supporting the teeth. As multiple measures were made for each animal in our analysis of oral bone loss, we applied GEE analysis to account for this level of complexity (Hannigan and Lynch 2013). Interestingly, we observed that unchallenged mice deficient in LXRs had less oral bone, reflected by longer CEJ to ABC measurements, than was observed in the group of unchallenged wild type animals. This finding implicates LXR function in the establishment of bone levels in the oral cavity. In the

context of bone resorption and osteoclast activity Kim *et al.* (Kim *et al.* 2013) reported that LXR activation by agonist inhibited osteoclastogenesis. The inhibition elicited by LXR agonist treatment was associated with suppression of RANKL-elicited expression of the transcription factors FBJ osteosarcoma oncogene (c-Fos) and nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), and reduction in activator protein 1 (AP-1) activity. Interestingly, triggering LXR activity by agonist treatment elicited apoptosis of formed osteoclasts, and *in vivo* it was shown that this provided protection from LPS-elicited bone loss (Kim *et al.* 2013). This effect of LXR agonist treatment on osteoclast activity to LPS exposure was also observed by Robertson Remen *et al.* (Robertson Remen *et al.* 2013a) employing osteoclast phenotype-driven RAW264.7 cells; furthermore, LXR agonist treatment stimulated RAW cell expression of markers associated with high phagocytic capacity. Recently it was reported that the LXR agonist reduced osteoclastic activity of RANKL-primed cells and it occurred in an LXR β -dependent manner (Robertson Remen *et al.* 2013b). At the level of the osteoblast, LXR function is less understood. Short-term LXR agonist treatment improved bone formation, which correlated with reduction in osteocalcin; however, long-term treatments employing *in vivo* modeling did not appear to influence bone levels (Prawitt *et al.* 2011). At the molecular level LXR agonist treatment depresses osteopontin expression (Ogawa *et al.* 2005), a molecule associated with periodontal disease (Sharma and Pradeep 2007). At this stage we do not know what mechanisms are underlying the enhanced bone loss phenotype observed in the oral cavity of unchallenged LXR-deficient mice; however, the phenotype is consistent with observed effects of LXR ablation possibly leading to enhanced osteoclastic activity.

In the context of *P. gingivalis* oral infection, our data reveal that in relation to WT mice, LXR ablation led to inability of mice to develop oral bone loss to *P. gingivalis*. To the best of our knowledge our findings are the first to suggest a role for LXRs in the pathogenesis of oral bone loss associated with infection by a defined periodontal pathogen. As we also observed that the reduction of LXR α gene expression elicited by *P. gingivalis* challenge is related to the TLR adaptor molecule TRIF and to a lesser extend MyD88, there may be important linkage between TLR signaling and LXR α function. TLR-driven cytokine production has been shown to play a part in *P. gingivalis*-elicited inflammation and oral bone loss. TLR2 is implicated in *P. gingivalis*-elicited oral bone loss, and macrophage TNF- α production to *P. gingivalis* (Burns *et al.* 2006; Gibson and Genco 2007; Papadopoulos *et al.* 2013); however, it has also been reported that mouse genetic background may play a role in TLR4-mediated oral bone loss as Balb/C mice deficient in TLR4 failed to develop oral bone loss when infected with *P. gingivalis* (Costalonga *et al.* 2009). Interestingly, partitioning aspects of host response to *P. gingivalis* challenge including cytokine production, and pathogen clearance are attributed to MyD88-independent and MyD88-dependent pathways (Burns *et al.* 2010). TLR2 may also play a role in LXR function as the ability of the TLR2 agonist Pam3-Cys-Ala-Gly-OH and *C. pneumonia* to stimulate macrophage foam cell formation was affected by LXR agonist treatment (Cao *et al.* 2007). Additional studies are needed to determine if specific TLR receptors participates in LXR-mediated processes as part of a broader strategy aimed to define the precise signaling pathways involved, to characterize the inflammatory lesion, as well as the subgingival microbiota that develops during infection-elicited oral bone loss. However, our observations

extend an emerging literature linking LXR function to infections outcomes (Bruhn *et al.* 2010; Joseph *et al.* 2004; Korf *et al.* 2009).

Future studies are needed to precisely define the mechanisms underlying the role played by LXRs in *P. gingivalis*-elicited inflammation and oral bone loss, particularly in the context of defining the cellular, immunological, and bone modulating signatures that underlie our initial observations that LXRs play important roles in the host immune response to *P. gingivalis*.

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Fig. 1. *P. gingivalis* suppresses LXR gene expression in wild type macrophages, and TLR-signaling and IRF3 are involved in suppression of LXR α , but not LXR β

Bone marrow-derived macrophages were obtained from C57BL-6 (WT), as well as MyD88^{-/-}, TRIF^{Lps2}, and IRF3^{-/-} mice, and were cultured for 6h in medium or challenged with *P. gingivalis* at MOI 100. RNA was obtained for qPCR, and gene expression values were normalized to the internal control β -actin. Fold change in gene expression was calculated using the $\Delta\Delta$ CT method and dividing *P. gingivalis* challenge gene expression levels by the levels of untreated genotype-matched macrophage controls run in parallel targeting LXR α (A.) and LXR β (B.). Experiments were performed on macrophages obtained from individual animals and data were pooled from multiple experiments (N = 3); *** = P < 0.001, ** = P < 0.01, NS = not significant vs. WT by one way ANOVA with Bonferroni multiple comparison. Data presented as mean $\pm$ SEM.

Fig. 2. *P. gingivalis*-elicited inflammatory cytokine production is influenced by LXR agonist treatment

Bone marrow derived macrophages from C57BL-6 mice were cultured in medium alone, or medium containing 2 μ M LXR agonist GW3965 (GW3965) for 18h. Medium was removed and cells were cultured with medium (Cont) containing agonist alone or agonist + *P. gingivalis* strain 381 MOI 100 (Pg) for 24h. Culture supernatant fluids were harvested, and ELISA was used to measure levels of TNF- α (A.), and IL-6 (B.). Experiments were performed using macrophages obtained from individual animals and data were pooled from multiple experiments (N = 6); *** = P<0.001 between groups, # = P<0.05 vs. matched control, NS = not significant by one-way ANOVA with Bonferroni multiple comparison. ND = not detected. Data presented as mean $\pm$ SEM.

Fig. 3. LXR ablation influences *P. gingivalis*-elicited macrophage cytokine expression

Bone marrow derived macrophages were generated from Sv129/BL-6 mixed background wild type (WT), or LXR $\alpha\beta$ -DKO mice, and were cultured in medium alone (Cont.), or medium containing *P. gingivalis* at MOI 100 (Pg) for 24h. Culture supernatant fluid levels of the cytokines TNF- α (A.), and IL-6 (B.) were measured by ELISA. Experiments were performed using macrophages obtained from individual animals and data were pooled from multiple experiments (N = 5); ** = P<0.01 between groups, # = P<0.05 vs. matched control, NS = not significant by one-way ANOVA with Bonferroni multiple comparison. ND = not detected. Data presented as mean $\pm$ SEM.

Fig. 4. LXRs contribute to oral bone loss elicited by *P. gingivalis* challenge

Sv129/BL-6 mixed background wild type (WT) and LXR $\alpha\beta$ -DKO mice were either orally challenged with vehicle alone (Cont.; white bar), or vehicle + *P. gingivalis* (Pg; filled bar) three-times in one week. **(A.)** After six-weeks, oral bone levels (distance from ABC-CEJ) were measured onscreen at landmark sites on each hemimaxilla for each mouse (14 sites/mouse; N = 7–8 mice / group) using ImageJ. **(B.)** CEJ to ABC measurements (mm) were compiled and analyzed. *** = $P < 0.0001$, and NS = not significant by GEE ANOVA with Bonferroni multiple comparison. Data presented as mean with 95% confidence interval limits.

Fig. 5. *P. gingivalis*-elicited IgG is not significantly affected by LXR status

Quantities of total IgG (ng/ml) specific to *P. gingivalis* in sera of mock challenged (Cont.), or *P. gingivalis* (Pg) orally challenged Sv129/BL-6 mixed background wild type (WT) or LXR $\alpha\beta$ -DKO mice were determined by ELISA (N=8 / group). Absorbance values were applied to an IgG standard curve run in parallel with test samples to quantify total pathogen-specific IgG. # = $P < 0.05$ vs. unchallenged genotype-matched control, NS = not significant by one-way ANOVA with Bonferroni multiple comparison. Data presented as mean $\pm$ SEM.