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MyD88-mediated innate sensing by oral epithelial cells controls periodontal inflammation

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ABSTRACT

Periodontal diseases are a class of non-resolving inflammatory diseases, initiated by a pathogenic subgingival biofilm, in a susceptible host, which if left untreated can result in soft and hard tissue destruction. Oral epithelial cells are the first line of defense against microbial infection within the oral cavity, whereby they can sense the environment through innate immune receptors including toll-like receptors (TLRs). Therefore, oral epithelial cells directly and indirectly contribute to mucosal homeostasis and inflammation, and disruption of this homeostasis or over-activation of innate immunity can result in initiation and/or exacerbation of localized inflammation as observed in periodontal diseases. Dynamics of TLR signaling outcomes are attributable to several factors including the cell type on which it engaged. Indeed, our previously published data indicates that oral epithelial cells respond in a unique manner when compared to canonical immune cells stimulated in a similar fashion. Thus, the objective of this study was to evaluate the role of oral epithelial cell innate sensing on periodontal disease, using a murine poly-microbial model in an epithelial cell specific knockout of the key TLR-signaling molecule MyD88 ($B6^{K5Cre.MyD88plox}$). Following knockdown of MyD88 in the oral epithelium, mice were infected with *Porphorymonas gingivalis* and *Aggregatibacter actinomycetemcomitans* by oral lavage 4 times per week, every other week for 6 weeks. Loss of oral epithelial cell MyD88 expression resulted in exacerbated bone loss, soft tissue morphological changes, soft tissue infiltration, and soft tissue inflammation following polymicrobial oral infection. Most interestingly while less robust, loss of oral epithelial cell MyD88 also resulted in mild but statistically significant soft tissue inflammation and bone loss even in the absence of a polymicrobial infection. Together these data demonstrate that oral epithelial cell MyD88-dependent TLR signaling regulates the immunological balance within the oral cavity under conditions of health and disease.

1. Introduction

Periodontal diseases are a class of inflammatory diseases that if left untreated can result in soft and hard tissue destruction surrounding the tooth (Blinkhorn et al., 2009). While microbes are etiological agents of periodontal disease, they do not directly cause disease, but rather induce harmful inflammatory responses in a susceptible host (Page & Kornman, 1997). Specifically, in periodontal disease, subgingival bacteria initiate and sustain a non-resolving inflammation that is ineffective at controlling the infection (Gaffen & Hajishengallis, 2008).

Oral epithelial cells are the first line of defense against microbial infection within the oral cavity. These important cells serve multiple roles including: 1. a physical barrier to microbes; 2. a source of antimicrobial peptides which inhibit microbe growth; 3. innate immune cells which produce cytokines to trigger anti-microbial immunity; and 4. tolerogenic immune cells which can produce immunoregulatory cytokines or remain quiescent in response to non-pathogenic commensal microorganisms (McCormick & Weinberg, 2010). We and others have demonstrated that the oral epithelial cell express several innate immune receptors including toll-like receptors (TLRs), whereby their ligation induces immune and anti-microbial factors (Dale et al., 2001; Diamond, Kimball, Krisanaprakornkit, Ganz, & Dale, 2001; Ford, Gamonal, & Seymour, 2010; Kollisch et al., 2005; Mahanonda & Pichyangkul, 2007; Sugawara et al., 2006; Weinberg, Krisanaprakornkit, & Dale, 1998). To this end, our previously published data indicates that oral epithelial cells respond in a unique manner when compared to canonical immune cells stimulated in a similar fashion (Wallet et al., 2013), but the relative role of this innate immune

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function to the protection from or induction of periodontal disease was not evaluated.

TLRs have two distinct functions on epithelial cells – protection from infection (*immune activation*) and tissue homeostasis (*immune regulation*) (Shibolet & Podolsky, 2007). Therefore, over-activation of innate immunity or disruption of this homeostasis can result in initiation and/or exacerbation of localized inflammation as seen in periodontal diseases. TLRs utilize both MYD88-depedent and MYD88-independent signaling pathways which, at least in macrophages, are responsible for induction of pro-inflammatory cytokines or type 1 interferon and interferon-inducible genes, respectively (Lu, Yeh, & Ohashi, 2008). Here we tested the contribution of the MyD88-dependent innate sensing of oral epithelial cells to periodontal disease using a novel cell-specific knockdown. Inducible knockout of MyD88 in oral epithelial cells exacerbated bone loss and soft tissue inflammation following a polymicrobial oral infection. Additional data indicates that MyD88-dependent signaling in oral epithelial cells also plays a role in controlling inflammation even in the absence of infection. Together these data suggest that innate immune sensing by oral epithelial cells is key to controlling inflammation under conditions of health and disease.

2. Materials and methods

2.1. Murine models

All experimental procedures were conducted in accordance with the guidelines of the University of Florida Institutional Animal Care and Use Committee. All mice were maintained in a specific pathogen-free (SPF) environment at the breeding facilities of the University of Florida. C57Bl/6 (B6) Keratin CrePR mice ($B6^{K5Cre}$) were a kind gift from Dr. Xiao-Jing Wang from the University of Colorado Denver Health Sciences Center (Malkoski, Cleaver, Lu, Lighthall, & Wang, 2010). Keratin CrePR mice express a Cre recombinase progesterone receptor (PR) fusion protein whose expression is restricted by a Keratin 5 (K5) promoter. The CrePR is inducible by a progesterone antagonist but not by endogenous progesterone (Malkoski et al., 2010). Upon induction with RU486, CrePR translocates into the nucleus and excises DNA sequences flanked by Lox P sites. These mice were crossed with the B6.Cg-*Myd88*^{tm1Defr}/J ($B6^{MyD88plox}$) from Jackson Laboratories (Bar Harbor, ME), which bare *loxP* sites on either side of exon 3 of the *MyD88* gene. When these mutant mice are bred to $B6^{K5Cre}$ mice, offspring ($B6^{K5Cre.MyD88plox}$) will have *MyD88* deleted in cells with an active K5 promotor upon the administration of a progesterone antagonist.

2.2. Induction of periodontal disease

MyD88 in the oral epithelial cells was either knocked down or not through the oral administration of 500 ug of RU486 (Sigma Aldrich, St. Louis, MO) solubilized in 25 uL of sesame oil (Publix, Gainesville, FL) administered one time a day for three consecutive days. After which all mice were lavaged with 25 ul of 0.12% chlorhexidine gluconate (3M, St. Paul, MN) for three days. Infection consisted of an 25 ul oral lavage with 2.5×10^9 *Porphoromonas gingivalis* strain 381 and 2.5×10^9 *Aggregatibacter actinomycetemocomitans* strain 29522 (ATCC, Manassas, VA) resuspended in 2% low viscosity carboxy-methyl-cellulose (Sigma-Aldrich) on 4 consecutive days, every other week for 6 weeks. Each week of infection, on the first day of infection, prior to infection, microbial sampling of the oral environment was performed with calcium alginate swabs (Fisher Scientific). One week following the last infection, the maxillae and mandibles were harvested to evaluate bone morphometric analysis, soft tissue infiltration, and/or soluble mediator expression.

2.3. Bacterial growth

Both bacterial strains were grown under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37 °C in a Coy anaerobic chamber. All media were fully reduced for 24–48 h prior to inoculation. *P. gingivalis* strain 381 was grown for 3 days on anaerobic 5% sheep blood agar plates (Remel, Lenexa, KS), and the resultant growth scraped using a sterile inoculating loop and placed in trypticase soy broth supplemented with 0.5% yeast extract, 0.05% L-cysteine, hemin (5 mg ml^{-1}), and vitamin K_1 (1 μg ml^{-1}) and allowed to culture anaerobically at 37 °C until reaching an OD of 0.53. *A. actinomycetemocomitans* strain 29522 was grown in Trypticase Soy Broth supplemented with 0.5% yeast extract (TSB-YE) in a humidified, 10% CO_2 atmosphere, at 37 °C until reaching an OD of 0.70.

2.4. Alveolar bone loss

The maxilla of mice were de-fleshed and immersed in 3% hydrogen peroxide (Fisher Scientific). To quantify bone loss, color digital images were captured under a 10× stereo dissecting microscope (Leica, Buffalo Grove, IL). Using morphometry image analysis software, the area of lingual bone resorption in mm^2 was determined. The volumes of interests (VOI) were identified and an appropriate and uniform threshold was applied to all specimens after comparing grayscale and binarized images in all groups. After thresholding, the bone volume/bone volume of WT was quantified.

2.5. Periodontal tissue inflammatory scoring

The left maxilla of all mice were removed and fixed in 10% buffered formalin, decalcified, embedded and sectioned for histological analysis. 5-μm sections were deparafinzed in xylene (25 min), followed by stepwise rehydration in 100% (5 min) 95% (5 min), and 70% (5 min) ethanol. Rehydrated sections were then rinsed in distilled water (5 min) followed by equilibration in PBS (5 min). Sections were stained with hematoxylin and eosin (Sigma-Aldrich). Images were captured at 20× magnification and inflammation scored using PMN/mononuclear cell infiltration (0, no inflammatory cells; 1, minimal inflammation (scattered inflammatory cells close to the junctional epithelium); 2, moderate inflammation (numerous inflammatory cells in the gingival connective tissue); and 3, severe inflammation).

2.6. Soft tissue soluble mediator expression

The right maxilla with both soft tissue and bone were subjected to bead beating at two 2 min minute intervals with 2 min of cooling in between using 1.0 mm diameter zirconia silica beads (BioSpec, Bartlesville, OK) in cell extraction buffer (ThermoFisher, Waltham, MA) prepared with a protease inhibitor cocktail (mini cOmplete, Roche, Basel, Switzerland) and PMSF protease inhibitor (Abcam, Cambridge, United Kingdom) to allow for dissociation and lysis of all soft tissue while leaving the hard tissues intact. MILLIPLEX® Multiplex Assays (EMD Millipore, Billerica, MA) were used to probe resulting lysates for TNFα, IL6, IL8, TGFβ1, and IL10, according to the manufacturer protocols. Data was acquired on a Luminex 200® system running xPONENT® 3.1 software (Luminex, Austin, TX) and analyzed using a 5-paramater logistic spline-curve fitting method using Milliplex® Analyst V5.1 software (Vigene Tech, Carlisle, MA). All data are presented as pg/ml normalized to total protein as determined by BCA assay (Thermo Scientific Pierce, Waltham, MA).

2.7. Real-time PCR of bacterial specific 16S expression

On the first day of infection, prior to infection, and at the beginning of each week of infections, samples of the oral environment were taken with calcium alginate swabs (Fisher Scientific). After which the gDNA was isolated using a DNeasy Kit (Qiagen) according to the manufacturers' instructions. The gDNA was then probed for *P. gingivalis* 16S, *A. actinomycetemocomitans* 16S and total 16S using real time PCR. The