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Comparative analysis of expression of microbial sensing molecules in mucosal tissues with periodontal disease

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ABSTRACT

Host-derived pattern recognition receptors (PRRs) are necessary for effective innate immune engagement of pathogens that express microbial-associated molecular patterns (MAMP) ligands for these PRRs. This study used a nonhuman primate model to evaluate the expression of these sensing molecules in gingival tissues. *Macaca mulatta* aged 12–24 with a healthy periodontium (n = 13) or periodontitis (n = 11) provided gingival tissues for assessment of naturally-occurring periodontitis. An additional group of animals (12–23 years; n = 18) was subjected to a 5 month longitudinal study examining the initiation and progression of periodontitis, RNA was isolated and microarray analysis conducted for gene expression of the sensing PRRs. The results demonstrated increased expression of various PRRs in naturally-occurring established periodontitis. Selected PRRs also correlated with both bleeding on probing (BOP) and pocket depth (PD) in the animals. The longitudinal model demonstrated multiple TLRs, as well as selected other PRRs that were significantly increased by 2 weeks during initiation of the lesion. While gene expression levels of various PRRs correlated with BOP and PD at baseline and resolution of disease, few correlated with these clinical parameters during initiation and progression of the lesion. These findings suggest that the levels of various PRRs are affected in established periodontitis lesions, and that PRR expression increased most dramatically during the initiation of the disease process, presumably in response to the juxtaposed microbial challenge to the tissues and goal of reestablishing homeostasis.

1. Introduction

The oral mucosa harbors a very complex microbial ecology that varies among the various niches in the oral cavity (Avila et al., 2009; Jenkinson, 2011; Alcaraz et al., 2012; Zarco et al., 2012). Furthermore in these niches there is an array of commensal bacteria that colonizes the sites during health, as well as a range of potential opportunistic pathogenic bacteria that can emerge within the microbiota and trigger disease processes (Wade, 2013; Darveau, 2009; Tlaskalova-Hogenova et al., 2004; Dixon et al., 2004; Cugini et al., 2013; Periasamy and Kolenbrander, 2009; Hajishengallis and Lamont, 2012).

The innate immune system has evolved to encompass an array of cellular receptors that are capable of recognizing a plethora of microbial components, enabling the cells to recognize, engage and respond to these microbial or pathogen-associated molecular patterns (MAMPs, PAMPs)(Hajishengallis, 2014; Ji and Choi, 2013; Hans and Hans, 2011). The pattern recognition receptors (PRRs) on host cells were originally described related to immune cells, such as macrophages (MΦ) and dendritic cells (DCs). However, it is now recognized that the PRRs are distributed more broadly across most cell types, including epithelial cells at mucosal surfaces (Walsh et al., 2013; Kumar et al., 2013; Jounai et al., 2012). The PRRs have now been delineated to exist as cell-surface associated [*e.g.* Toll-like receptors (TLRs)], intracellular [*e.g.* nucleotide-binding oligomerization domains (NODs)], and soluble PRRs (*e.g.* C-reactive protein, serum amyloid A) that comprise early warning system for innate immunity (Kumar et al., 2011). Thus, these microbial sensing molecules interact with a range of bacterial (*e.g.* lipopolysaccharide, lipoteichoic acid, flagella, DNA), viral (*e.g.* DNA,

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Table 1
Gene expression targets for microbial sensing molecules.

Gene ID	Gene Title
Surface Cell Associated PRRs	
CD14	Binds to monomeric lipopolysaccharide and delivers it to the MD-2/TLR4 complex; also soluble molecule
CD209 (DC-SIGN)	Surface of immature dendritic cells (DCs) and involved in initiation of primary immune response
CLEC7A	C-Type Lectin Domain Family 7, Member A, beta-1,3-linked and beta-1,6-linked glucans from fungi
CLEC4E	C-Type Lectin domain family 4, member E, mycobacteria is *via* trehalose 6,6'-dimycolate; SAP130, a nuclear protein that is released by dead or dying cells
CLEC4M (L-SIGN)	C-Type Lectin Domain Family 4, Member M, parasites to viruses
CLEC6A (Dectin-2)	C-Type Lectin Domain Family 6, Member A, alpha-mannans on *C.albicans* hypheas
COLEC12	Collectin Sub-Family Member 12, Scavenger receptor for phagocytosis of Gram-positive, Gram-negative bacteria and yeast
FCN2	Ficolin (Collagen/Fibrinogen Domain Containing Lectin) 2 (Hucolin), Complement-activating lectin, phagocytosis of *S.typhimurium* by neutrophils
MARCO	Macrophage Receptor With Collagenous Structure, class A scavenger receptor for both Gram-negative and Gram-positive bacteria
MRC1	Mannose Receptor, C Type 1, bind high-mannose structures on the surface of potentially pathogenic viruses, bacteria, and fungi for phagocytosis
TLR1	Toll-like receptor 1, diacylated and triacylated lipopeptides
TLR2	Toll-like receptor 2, bacterial lipoproteins and other microbial cell wall components
TLR4	Toll-like receptor 4, bacterial lipopolysaccharide (LPS)
TLR5	Toll-like receptor 5, bacterial flagellins
TLR6	Toll-like receptor 6, Gram-positive bacteria and fungi
Intracellular Associated PRRs	
AIM2	Cytosolic double-stranded DNA
ARHGEF2	Rho/Rac Guanine Nucleotide Exchange Factor (GEF) 2, intracellular sensing system along with NOD1 for the detection of microbial effectors during cell invasion
IFIH1 (MDA5)	Interferon induced with helicase C domain 1, RIG-1-like receptor family, viral sensor
LGP2 (DEXH58)	DEXH (Asp-Glu-X-His) Box Polypeptide 58, RIG-1-like receptor family, viral sensor
NAIP (BIRC1)	Baculoviral IAP repeat-containing protein 1, effects apoptosis
NLRC4 (IPAF)	NLR family CARD domain-containing protein 4, inflammasome
NOD1	Nucleotide-binding oligomerization domain 1, intracellular bacterial lipopolysaccharides (LPS), senses peptidoglycan (PGN)-derived muropeptides
NOD2	Nucleotide-binding oligomerization domain 2, intracellular bacterial lipopolysaccharides (LPS) by recognizing the muramyl dipeptide
RIG-1 (DDX58)	Retinoic acid-inducible gene 1, RIG-1-like receptor family, viral sensor
TLR3	Toll-like receptor 3, nucleotide-sensing for double-stranded RNA
TLR7	Toll-like receptor 7, nucleotide-sensing for single-stranded RNA
TLR8	Toll-like receptor 8, G-rich oligonucleotides
TLR9	Toll-like receptor 9, nucleotide-sensing for unmethylated cytidine-phosphate-guanosine (CpG) dinucleotides
ZBP1/DAI	Z-DNA Binding Protein 1, cytoplasmic sensor binds to foreign DNA and induces type-I interferon
Soluble PRRs	
CRP (PTX1)	C-reactive protein, promotes agglutination, bacterial capsular swelling, phagocytosis and complement fixation
FCN1	Ficolin (Collagen/Fibrinogen Domain Containing) 1, Complement-activating lectin, 9-O-acetylated 2-6-linked sialic acid derivatives and to various glycans
MBL2	Mannose-Binding Lectin (Protein C) 2, Soluble, soluble mannose-binding lectin or mannose-binding protein found in serum
PTX3	Pentraxin 3, Long, mediating agglutination, complement activation, and opsonization
PTX4	Pentraxin 4, Long, mediating agglutination, complement activation, and opsonization
SAA1	Serum Amyloid A1

RNA), and fungal (*e.g.* mannan) ligands.

It is well recognized that an array of PRRs are differentially expressed in periodontitis tissues (Ebersole et al., 2013; Benakanakere and Kinane, 2012). These response relationships have been developed conceptually in describing the disease as a host-mediated disruption of microbial homeostasis (Ji and Choi, 2013; Bartold and Van Dyke, 2013). The innate responses critical to this biologic interactions clearly (Fatemi et al., 2013)involve recognition of microbial components mediated by various PRRs, including the toll-like receptors in both resident and immune cells (Di Benedetto et al., 2013).

Multiple reports have identified alterations in TLR2 and TLR4 expression level in inflamed tissues of periodontitis (Myneni et al., 2013), associated with smoking (Fatemi, et al., 2013), and with diabetes as a co-morbidity (Promsudthi et al., 2014). These specific PRRs have also been demonstrated to be significant in a rodent model of *P. gingivalis* exacerbated ligature-induced RANKL-dependent periodontitis (Lin et al., 2014).

More recently, studies have broadened the perspective of PRRs contribution to the chronic inflammation of tissue destruction of periodontitis. MYD88 mRNA was increased in periodontitis tissues and was elevated to a greater degree in severe periodontitis. While this study did not find much effect on various TLRs, NLRs, or signaling molecules CD14 and TRIF, changes in MYD88 support a critical TLR transduction signaling change in periodontitis (Ghaderi et al., 2014). A recent report by Sahingur et al. (Sahingur et al., 2013) demonstrated upregulation of TLR9 and TLR8 in chronic periodontitis tissues, as well as significant correlations among PRRs, and detection of changes in intracellular PRRs, AIM2 and DAI, in diseased gingival tissues. These types of findings have been extended in attempts to better understand potential triggers and molecular mechanisms underlying these differences. Recent work has identified that gingival tissues in periodontitis displayed a differential TLR2 promoter methylation, thus suggesting a likelihood of altered modulation in the expression of this important PRR in inflamed tissues (Benakanakere et al., 2015). This PRR receptor changes connects with a report by Park et al. (Park et al., 2014) that demonstrated elevated inflammasome components were expressed in chronic periodontitis, including IL-1ß. These authors also showed that *P. gingivalis* induced IL-1ß and pyroptotic cell death in a macrophage cell line through engagement of NLRP3 and AIM2 inflammasome activation. Additionally, they described the detection of priming signals from TLR2 and TLR4 activation that preceded the *P. gingivalis* induced IL-1ß release representing a proinflammatory mediator activity in the periodontitis tissues.

Thus, while numerous PRRs have been identified in gingival tissues, the relationship of changes in the expression and levels of these innate immune molecules to health and disease processes remains unclear. This report uses a nonhuman primate model of naturally-occurring periodontitis and a prospective model of ligature-induced periodontitis to document alterations in expression of these microbial sensing molecules that are related to this mucosal disease.

2. Methods

2.1. Nonhuman primate model and oral clinical evaluation

Rhesus monkeys (*Macaca mulatta*) (n = 24; 10 females and 14

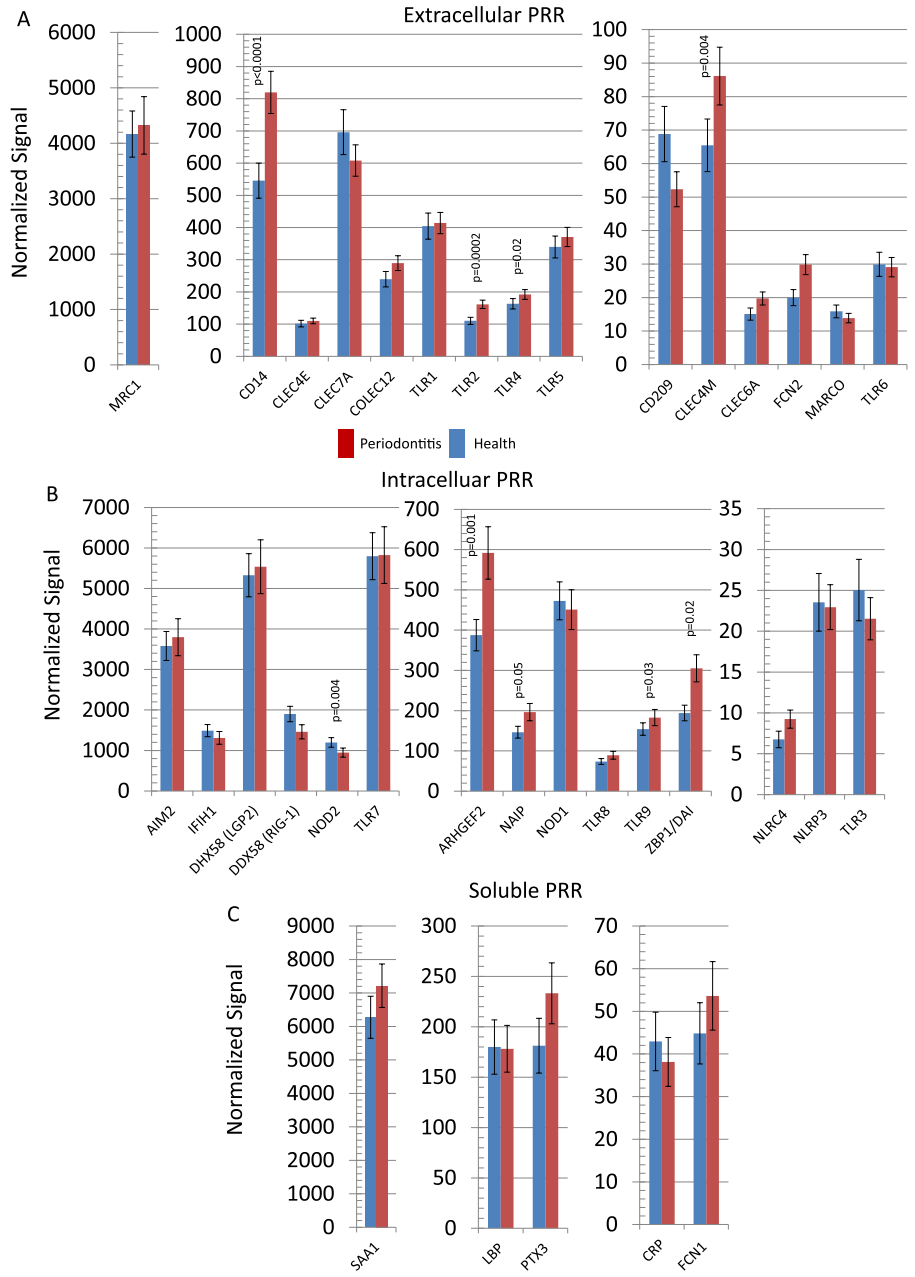

Fig. 1. Distribution of gene expression of PRRs in healthy and periodontitis gingival tissues. The bars denote the mean expression levels in healthy (n = 13), and periodontitis (n = 11) samples for Surface PRR (A), Intracellular PRR (B), and Soluble PRR (C). Significant differences are denoted by p-values.

males) housed at the Caribbean Primate Research Center (CPRC) at Sabana Seca, Puerto Rico, were used in these studies. Healthy animals (n = 13) and naturally-occurring periodontitis animals (n = 11) were 12–23 years of age. The 18 animals (10 females and 8 males) in the longitudinal study of ligature-induced disease were 12–23 years of age. The nonhuman primates are typically fed a 20% protein, 5% fat, and 10% fiber commercial monkey diet (diet 8773, Teklad NIB primate diet modified: Harlan Teklad). The diet is supplemented with fruits and vegetables, and water is provided *ad libitum* in an enclosed corral setting.

A protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico, enabled anesthetized animals to be examined for clinical measures of periodontal including probing pocket depth (PD), and bleeding on probing (BOP) as we have described previously (Ebersole et al., 2008).

2.2. Tissue sampling and gene expression microarray analysis

A buccal gingival sample from either healthy or periodontitis-affected tissue from the premolar/molar maxillary region of each animal was taken using a standard gingivectomy technique, and maintained frozen in RNAlater solution. Total RNA was isolated from each gingival tissue using a standard procedure as we have described and tissue RNA samples submitted to the microarray core to assess RNA quality analyze the transcriptome using the GeneChip® Rhesus Macaque Genome Array or GeneChip® Rhesus Gene 1.0 ST Array (Affymetrix) (Affymetrix) (Meka et al., 2010; Gonzalez et al., 2011). Individual samples were used for gene expression analyses. Table 1 lists the microbial sensing gene set examined in this report.

Based upon the microarray outcomes we selected 7 representative genes and performed a qPCR analysis using a standard technique in our laboratory employing a Roche 480 LightCycler (Gonzalez et al., 2014)

with Tm 62 °C. Primers were prepared for SAA1 (forward - CAGCGAT GCCAGAGAGAATATC; reverse – CAGCGATGCCAGAGAGAATATC; amplicon 121 bp), CD14 (forward – GCCCTAAACTCCCTCAATCTG; reverse – CAGTCTGTTGCAGCTGAGAT; amplicon 99 bp), NAIP (forward - GGCTCTTGGATGCAGATGATA; reverse – ATGATTGGAGAGAA CGGCAATA; amplicon 112 bp), TLR2 (forward - GATGCTGCCATTCT TGTTCTTC; reverse – CAGGTAGGTCTTGGTGTTCATT; amplicon 99 bp), ZBP1/DAI (forward – TCAGCCCATCACTCGAAAC; reverse – ATG AGGCTTCATCCACATAGTC; amplicon 113 bp), CLEC7A (forward – CAAGTGTCTTCCCAACCTGATA; reverse – GAATGTTGATCCATCCTC CCA; amplicon 93 bp), NOD2 (forward – GAGGCAGTTCCATTTCATT TGT; reverse – TGCTTAGAAGGAAGGGCTTAAT; amplicon 96 bp), and GAPDH (forward – GGTGTGAACCATGAGAAGTATGA; reverse – GAG TCCTTCCACGATACCAAAG; amplicon 123 bp) genes, designed using software PrimerQuest at Integrated DNA Technologies website (www.idtdna.com) and were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The level of message was determined and those levels compared across the RNA samples prepared from the healthy animals compared with the periodontitis group.

2.3. Data analysis

Normalization of values across the chips was accomplished through signal intensity standardization across each chip using Affymetrix PLIER algorithm. The arrays contained matched and mismatched pairs allowing the MAS 5 algorithm to be used. For each gene we first determined differences in expression across the groups using ANOVA (version 9.3, SAS Inc., Cary, NC). The healthy tissues were compared to the periodontitis tissues using a *t*-test and accepting a p-value ≤ 0.05 for significance. The choice of Least Significant Difference for multiple comparisons (ANOVA followed by t-tests) provided maximum power given our necessarily small sample sizes. We did determine a correlation between gene expression and clinical measures of periodontitis using a Spearman Rank correlation analysis that was fit to the gene expression by age. A p-value ≤ 0.05 was used to evaluate the significance of the correlation. A portion of the data has been uploaded into the ArrayExpress data base (www.ebi.ac.uk) under accession number: E-MTAB-1977.

3. Results

3.1. Profiles of PRRs

Both CD14 and CLEC4M expression levels were increased in periodontitis compared to healthy gingival tissues (Fig. 1A). TLR2, TLR4 and TLR9 levels were also increased significantly in the periodontitis samples, as was the intracellular sensor NAIP. NOD2 another intracellular detection receptor was significantly decreased, whereas ZBP1/DAI increased in the periodontitis samples (Figure 1A&B). An array of soluble PRRs was also examined in disease with no significant differences observed in established periodontitis lesions (Fig. 1C).

Fig. 2 A&B depict the correlations of the PRR gene expression in the periodontitis animals for both BOP and PD. The results demonstrate multiple sensing genes are correlated with increasing BOP during periodontitis, including CLEC isoforms and TLRs. Additionally, COLEC12, PTX3 and TLR1 were all significantly negatively correlated with this measure of inflammation. In contrast, very few of these genes were correlated with mean PD in the established periodontitis animals, although LBP expression levels were positively correlated with both BOP and PD.

3.2. PRRs in progressing periodontitis

We also evaluated the expression of the PRRs using a longitudinal model of progressing and resolving periodontitis. Following ligature placement, a substantial accumulation of microbial plaque occurs,

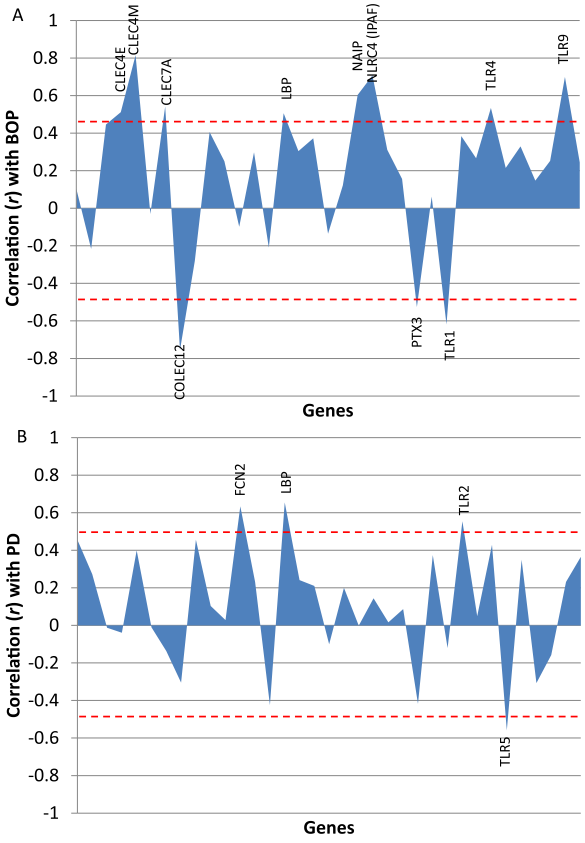

Fig. 2. Correlation of PRR gene expression in periodontitis gingival tissues with (A) Bleeding on Probing (BOP) and (B) Pocket Depth (PD). Red dashed lines denote the r-value required for reaching $p < 0.01$ for the 24 animals. Genes reaching statistical significance are identified. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which initiates an acute and chronic inflammatory response in the local tissues. This leads to progressing periodontitis through approximately 3 months (Smith et al., 1993; Moritz et al., 1998; Branch-Mays et al., 2008). Removal of the ligatures enables a clinical resolution within about 60 days. The clinical changes of inflammation (BOP) and tissue loss (PD) are demonstrated in Fig. 3.

Of the 36 PRRs evaluated, 15 demonstrated significant differences from baseline during the longitudinal study (Fig. 4). Interestingly, TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, and TLR9 all demonstrated a similar pattern with significant increases within 2 weeks of initiation of the disease. TLR1 levels dropped to baseline by 1 month, while TLR2, 4, 6, 7, 8, and 9 remained elevated throughout the disease progression (3 months). All TLR message levels decreased to baseline after resolution of the disease.

Other cellular PRRs, including NAIP (intracellular), CLEC4E and CD14 (surface) demonstrated a pattern similar to the TLRs with increases during the initiation of the disease that were, in some cases, maintained during disease progression and returned to baseline at 5 months (resolution). In contrast, both CLEC7A and NOD2 showed a pattern of changes with significant decreases during initiation and progression of disease, and returned to baseline at resolution. ZBP1/DAI exhibited a unique profile of expression with increases peaking at 1–3 months of disease; again returning to baseline by 5 months. AIM2 (intracellular) also showed a somewhat unique profile of expression with peak levels attained by 2 weeks that were maintained throughout the experiment, including remaining elevated after resolution of disease. Finally, the only soluble PRR level that was affected by the disease process was FCN1 (ficolin 1) that was significantly increased at 2 weeks

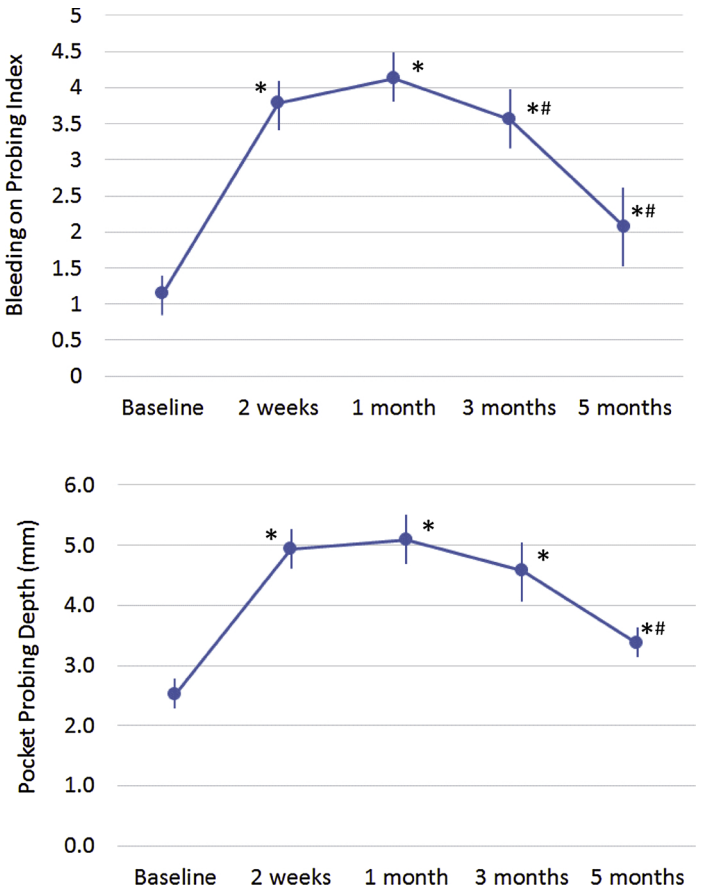

Fig. 3. Clinical features of (A) Bleeding on Probing and (B) Pocket Depth in longitudinal ligature-induced periodontitis (n = 18). Points denote mean level for the group at each sampling point. Vertical lines signify 1 SD. The asterisk (*) denotes significantly different from baseline at least at $p < 0.01$ and the hashtag (#) signifies a significant difference from peak levels at 1 mo. at least at $p < 0.01$.

during the acute inflammatory response but returned to baseline by 1 month during the progression of destructive disease.

The relationship of the gene expression profiles during the initiation, progression, and resolution of the disease was examined related to the baseline expression of these microbial sensing molecules. Table 2 shows that for a few of these sensing molecules, the changes in gene expression that occurred with disease were related to baseline gingival levels of the expression in the animals. Interestingly, the primary positive correlations with baseline levels of the expression of these genes occurred at 1 and 3 months of the disease process, suggesting some individual regulation of these responses based upon the existing interaction with the oral microbial ecology prior to disease initiation.

Table 3 summarizes the relationship of gene expression for the sensing molecules and clinical features of BOP and PD in the animals at baseline, during the disease process, and at resolution. Generally, a limited number of these host signaling genes were significantly correlated with BOP at baseline (3/36), although 9 were correlated with PD, not overlapping those related to BOP. These relationships were lost during disease initiation and progression, with no correlations detected. Of particular interest was the substantial frequency of gene expression levels that were significantly correlated with BOP (15/36) and PD (11/36) during the resolution phase of the disease.

Table 4 provides a summary of the qPCR analysis of selected genes as a validation of the microarray results. The findings demonstrate an agreement in level differences for 4/5 genes in the cross-sectional samples of established lesions and all 5 genes from the longitudinal study of periodontitis using these 2 independent techniques.

4. Discussion

Innate immune responses are controlled by the capacity of the host cells and biomolecules to recognize bacteria, viruses, and fungi through specific ligands (PAMPs, MAMPs) that are structural motifs of the microorganisms (Walsh et al., 2013; Kumar et al., 2013, 2011; Qian and Cao, 2013; Mogensen, 2009; Atkinson, 2008). This is accomplished through the expression and production of an array of pattern recognition receptors (PRR) that target specific components of these bacterial motifs. These interactions result in changes in cell functions and release of various molecules that more broadly engage host innate immune, inflammatory, and even adaptive immune cells and molecules (Kumar et al., 2013; Mogensen, 2009). Generally the PRRs appear to function in a nonspecific fashion, albeit individual PRRs have a predilection for certain structural motifs and are thus more individually effective within from a more limited range of microbes (Qian and Cao, 2013; Sukhithasri et al., 2013).

This report describes alterations in the expression of these PRRs in gingival tissues from nonhuman primates with established periodontal lesions, as well as evaluating the dynamics of the PRR changes in gingival tissues during the initiation, progression, and resolution of ligature-induced periodontitis (Gonzalez et al., 2012; Oz and Puleo, 2011; Struillou et al., 2010). We observed 9/35 sensing genes were significantly different in the established periodontitis tissues. These included cell surface (TLR2, TLR4, TLR9, CD14, and CLEC4M) and intracellular (NAIP, NOD2, ARHGEF2, and ZBP1/DAI) PRRs. TLR2 receptors are important PRRs in immune and non-immune host recognition of microbes. TLR2 primarily engages lipoteichoic acids from Gram-positive bacteria although it can forms heterodimers with other TLR and non-TLR receptors [*e.g.* CD36, CD14, CLEC7A (Dectin-1)] to bind additional specific molecular ligand structures on microbes (van Bergenhenegouwen et al., 2013). We also noted that TLR2 was positively correlated with mean pocket depth in established disease lesions. TLR4 and TLR9 expression was significantly positively correlated with the level of bleeding on probing. In contrast, TLR1 was negatively correlated with BOP and TLR5 was negatively correlated with pocket

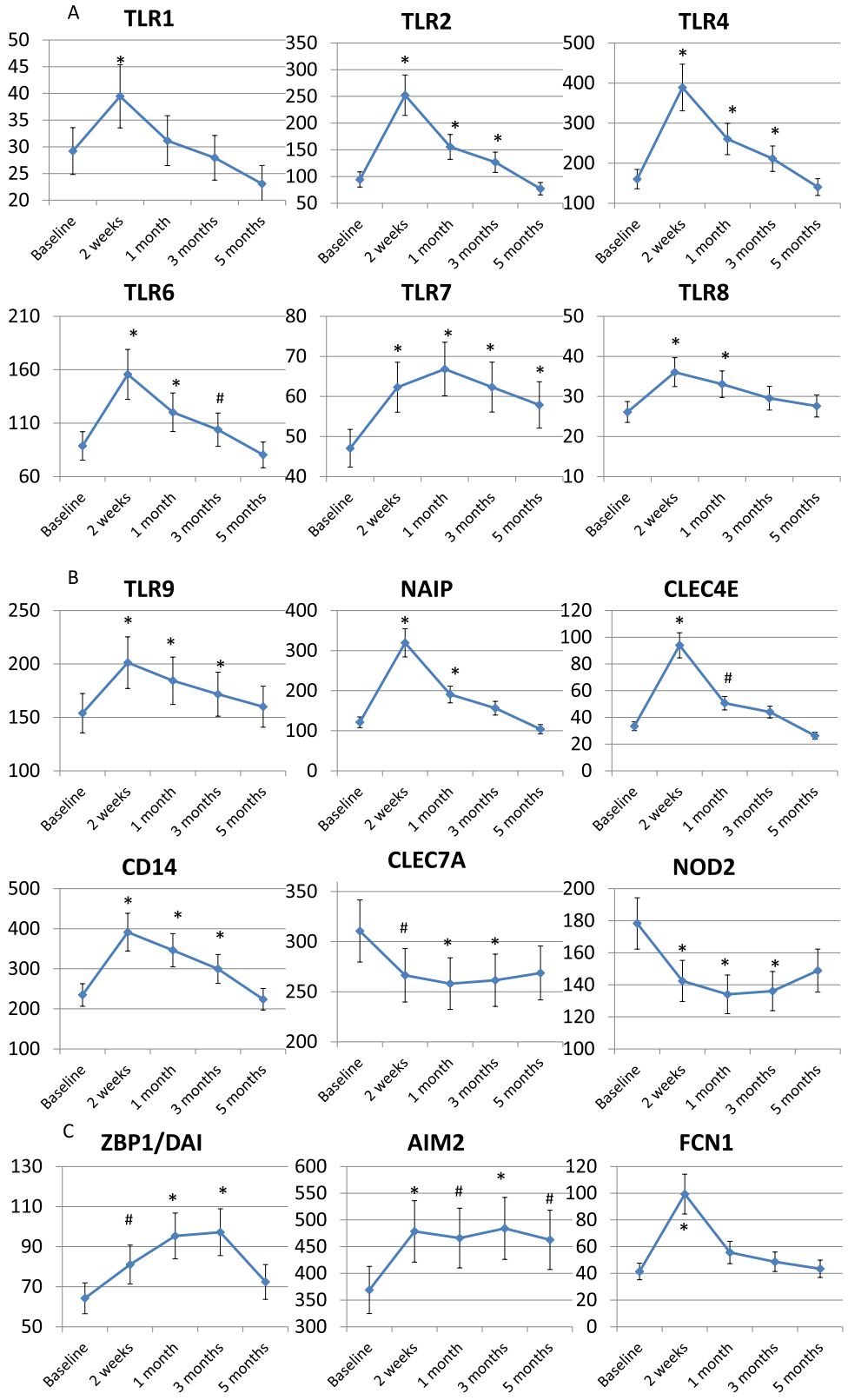

Fig. 4. PRRs whose gene expression changed significantly from baseline during initiation, progression and resolution of ligature-induced periodontitis. Points denote group means and vertical brackets enclose 1 SD. The asterisk (*; $p < 0.01$) and hashtag (#; $p < 0.05$) signify difference from baseline.

depth in established lesions. These findings supported potential roles for these PRRs in the diseased gingival tissues; however, there appeared to be some selectivity in their regulation reflecting the level of disease. This might be explained by the broader biologic processes occurring in the gingival tissues in trying to reestablish homeostasis, and/or it may reflect variations in the quality of the microbial biofilms that result in more selective regulatory influences on these cell signaling molecules. Extension of these findings to the longitudinal model of periodontitis provided additional evidence for the role of these PRRs in the process of inflammation and disease progression in the gingival tissues. TLR1,

Table 2
Correlation of gene expression levels with level of mRNA at baseline. R-values > 0.4200 or < -0.4200 were considered significant at $p < 0.05$.

	PERIODONTITIS			
Gene ID	2 wk	1 mo	3 mo	5 mo
Cell surface				
TLR2			0.5671	−0.5935
TLR3		0.6495	0.6574	
TLR4			0.4444	
CD209		0.4568	−0.4383	
CLEC4E			0.7392	
CLEC6A		0.5970		
FCN2				0.5267
MARCO	0.4879			
Intracellular				
AIM2		0.5277		
NLRC4			0.4468	−0.4258
TLR7		0.5482		
ZBP1/DAI		0.4391		
Soluble				
CD14		0.4313	0.6575	
LBP			0.4452	
TOTAL +	1	7	7	1
TOTAL -	0	0	1	2

TLR2, TLR4, TLR6, TLR8, and TLR9 all demonstrated significant increases by 2 weeks following initiation of disease. All except TLR1 remained elevated through the first month of disease, with TLR2, TLR4 and TLR6 remaining elevated through the 3 month progression of disease. All of these returned to baseline levels following disease resolution at 5 months. Only TLR7 demonstrated a different pattern with increases peaking at 1 month and remaining significantly elevated within gingival tissues even following resolution. TLR7, like TLR3 and TLR8 receptors, has been suggested to primarily engage nucleic acids as ligands, related to detection of viral infections (Wu et al., 2013; Szabo and Rajnavolgyi, 2013; Mansson Kvarnhammar et al., 2013; Kaiko et al., 2013). Its role as an intracellular sensing PRR may be related to increasing data supporting a role for bacterial invasion of gingival tissues related to the pathogenic potential and expression of disease by these bacteria (Darveau, 2009; Eick et al., 2006; Colombo et al., 2006; Vitkov et al., 2005; Atanasova and Yilmaz, 2014; Park et al., 2004). Additionally, correlation analyses showed that multiple TLRs at 1 and 3 months of disease were related to baseline gingival tissue levels. Additionally, TLR3, 5, and 9 were significantly positively correlated with BOP at resolution of the disease, while TLR4 and TLR8 were negatively correlated. TLR4 and TLR8 were significantly negatively correlated with pocket depth (PD) in the resolved lesions, supporting a potential role contributing to reestablishing tissue homeostasis with the

Table 4
Comparison of gene expression profiles using qPCR and microarray analyses. Values represent fold-difference compared to Healthy tissue message levels assigned a value of 1.0 from cross-sectional samples.

Gene ID	X-Sectional Periodontitis (Fold Δ vs. Health)	Longitudinal (Fold Δ vs. Baseline
SAA1	3.69 ± 0.25	
qPCR	2.57 ± 0.81	
GeneChip		
CD14	1.12 ± 0.48	2.16 ± 0.13[a]
qPCR	1.54 ± 0.30	1.76 ± 0.12
GeneChip		
NAIP	1.79 ± 0.06	
qPCR	1.37 ± 0.40	
GeneChip		
TLR2	0.97 ± 0.17	2.80 ± 0.17[a]
qPCR	1.56 ± 0.40	2.52 ± 0.30
GeneChip		
ZBP1/DAI	1.41 ± 0.54	2.68 ± 0.45[b]
qPCR	2.25 ± 0.84	1.58 ± 0.14
GeneChip		
CLEC7A		0.90 ± 0.11[a]
qPCR		0.84 ± 0.07
GeneChip		
NOD2		0.62 ± 0.04[a]
qPCR		0.78 ± 0.06
GeneChip		

[a] Denotes samples from 2 weeks compared with baseline.
[b] Denotes samples from 3 months compared with baseline.

Table 3
Correlation of gene expression levels with clinical parameters of bleeding on probing (BOP) and pocket depth (PD) at different states of disease. Values > 0.4200 or < -0.4200 for n = 18 were considered significant at $p < 0.05$ as highlighted in boldface type. Cells with no values showed no significant correlation related to BOP or PD.

Bleeding on Probing				**Pocket Depth**		
Gene ID	**B**	**Disease**	**Resolution**	**R-value**	**Disease**	**Resolution**
CD14				**0.4655**	−0.0258	−0.1814
CD209	−0.2233	−0.1697	**−0.5570**			
CLEC4E	0.2321	0.3004	**−0.5606**	0.1828	0.0956	**−0.6819**
CLEC6A				**0.5948**	0.1988	0.1613
COLEC12	0.2213	0.1251	**−0.6052**	−0.3121	−0.1615	**−0.7203**
CRP				−0.0399	−0.1067	**−0.6471**
FCN1	0.3966	0.2063	**−0.6687**			
FCN2	0.3174	0.0957	**0.4591**	**−0.5645**	0.2568	**0.7122**
IFIH1				**0.4573**	0.3536	**0.4213**
MBL2				**−0.5908**	−0.0192	−0.3087
NAIP	**0.5023**	0.3027	**−0.6901**	0.3658	0.1880	**−0.5163**
NOD1	−0.3031	0.1701	**0.5815**	−0.1197	0.0707	**0.6773**
NOD2	−0.2192	−0.1159	**−0.4445**	**0.5057**	−0.3414	0.2027
PTX3	−0.2574	0.0941	**−0.6679**	−0.3840	−0.0495	**−0.6538**
PTX4				**−0.4941**	−0.1414	**0.6646**
TLR3	−0.1057	0.1523	**0.4695**			
TLR4	**0.5312**	0.3030	**−0.6913**	0.2847	0.0608	**−0.6144**
TLR5	−0.1089	−0.0019	**0.5675**			
TLR6	**0.5960**	0.3712	−0.2864			
TLR7				**0.4538**	0.0949	−0.0439
TLR8	0.2647	0.3638	**−0.8853**	0.4059	0.1743	**−0.4872**
TLR9	0.2013	0.3023	**0.7415**			
ZBP1	0.0816	0.2028	**0.8422**	**0.5261**	−0.0679	0.2559

microbial ecology. Interestingly, while TLR message levels were related to baseline levels in individual animals, they were not quantitatively correlated with clinical disease parameters during disease initiation and progression of disease. These results suggest individual heterogeneity within this group of subject animals related to the characteristics of response to the microbial challenge. However, the complex microbial biofilms appear to uncouple TLR signaling potential related to clinical features of disease during initiation and progression of the lesion.

The CD14 molecule, a co-receptor for bacterial LPSs, exists both anchored to the membrane of immune cells and shed into a soluble form to interact with non-immune cells (Litvack and Palaniyar, 2010; Jin and Lee, 2008). The expression of CD14 was significantly increased in established periodontitis lesions. It demonstrated a response profile during disease initiation and progression similar to the TLRs. The level of CD14 mRNA was shown to be related to baseline levels of expression during disease initiation and progression, but was generally unrelated to the level of bleeding or pocket depth. As with the TLR transcription, regulation of CD14 appears in response to the increased microbial burden during disease initiation and progression, with levels seeming more animal specific rather than directly linked to the severity of the disease process, *e.g.* BOP, PD. Some information has been developed regarding levels of sCD14 and CD14 gene polymorphisms as related to periodontitis. The limited data available suggested that sCD14 levels in serum were elevated in periodontitis (Pussinen et al., 2007), while elevated levels of sCD14 in gingival crevicular fluid were correlated with fewer deep pockets in untreated chronic periodontitis patients (Jin and Darveau, 2001). Additionally, it appears that a particular polymorphism was related to higher serum levels of sCD14 and periodontitis (Gong et al., 2013). Our data implies that elevated levels of CD14 may reflect an initiating and progressing disease process.

Multiple C-type lectin family members are both endocytic and cell surface receptors that are linked to cell signaling directly through NF-κB activation (Yan et al., 2013). CLEC4E (Mincle) binds nuclear proteins from damaged cells, as well as carbohydrate residues of pathogens (Yamasaki et al., 2009; Miyake et al., 2013; Sharma et al., 2014). CLEC4M, which is similar to CD209 (DC-SIGN) recognizes various pathogens and interacts with ICAM-3 (Khoo et al., 2008; Rydz et al., 2013). CLEC7A (Dectin-1) is a PRR primarily expressed on immune cells and engages various glucan moieties on microbes, and contributes to development of Th17 cells (Sukhithasri et al., 2013; Rao et al., 2014; Plato et al., 2013; Gringhuis et al., 2012). Multiple of these receptors were significantly increased in established periodontitis lesions, and positively correlated with BOP as a measure of inflammation. Prospectively, CLEC4E showed a significant increase by 2 weeks, while CLEC7A was significantly decreased through the initiation and progression of periodontitis. Rather limited information exist examining C-type lectin PRRs in the gingival tissues related to health or disease. Dectin-1 expression has been shown to be increased in gingival tissues from aged mice unrelated to disease (Liang et al., 2010), while Tamai et al. (Tamai et al., 2011) demonstrated the importance of Dectin-1 on oral epithelial cells related to detection of *Candida albicans.* Carvalho et al. (Carvalho et al., 2012) have also identified that Dectin-1 appears to trigger a predominant Th17 response resulting from engagement with *C. albicans.* While this type of activity has not been identified in gingival tissues, and this type of fungal pathogen is not generally associated with periodontitis, alterations in the family of CLEC PRRs remains rather unexplored for a role in the disease process or resolution. Since Dectin-1 is primarily an immune cell PRR, the contrast between the developing lesion data and established natural lesion results provide a temporal contrast for how this type of receptor might function within the periodontium as part of the innate immune response to the microbial challenge.

The nucleotide-binding oligomerization domain-containing protein receptors (NLRs) are intracellular PRRs (NOD1, NOD2, NLRC4, NLRP3, and NAIP). The NOD PRRs bind intracellular bacterial peptidoglycan components leading to activation of NF-κB (Rasmussen et al., 2009; Wen et al., 2013). Various studies in host-pathogen responses in periodontitis have identified a role for NOD1/2 in sensing periodontal pathogens, regulating molecules that enhance interaction of neutrophils with endothelial cells, and triggering innate responses in periodontal ligament cells (Benakanakere and Kinane, 2012; Zelkha et al., 2010). NOD1 has been shown to be a critical molecule in bone loss in mice to a murine-specific oral microorganism (Jiao et al., 2013). Also, Yuan and colleagues (Yuan et al., 2013) have suggested a pivotal role for NOD2 in controlling inflammatory responses that may link periodontitis with chronic systemic inflammation, such as occurs in atherosclerosis. In our study, NOD2 was significantly decreased in established lesions and was decreased during initiation and progression of the experimental lesions, as well as negatively correlated with BOP at the resolution phase. In contrast, while NOD1 was not found to be significantly different or change with disease, it was highly positively correlated with both BOP and PD in the resolved lesions. Thus, the exact role for NOD involvement in periodontitis initiation and progression, and details on the components of the complex microbial ecology at disease sites remain to be evaluated, but would seem to hold some promise for understanding risk for expression of disease.

NAIP is a sensor component of the NLRC4 inflammasome that recognizes intracellular bacteria pathogens. This molecule is a part of a larger family of Inhibitors of Apoptosis (IAP) and links this biological function with the NLR aspects of the inflammasomes (Kofoed and Vance, 2012). Little information is available regarding NAIP alterations in periodontitis tissues; however, it appears to be increased during inflammatory responses (Beug et al., 2012) and has recently been reported to interact with bacterial type III secretion proteins to engage the NLRC4 inflammasomes in response to both respiratory and gastrointestinal pathogens (Gong and Shao, 2012). In our models of periodontitis, NAIP was significantly elevated in established periodontitis tissues and was positively correlated with BOP in disease. In addition, it increased significantly during the initiation of experimental periodontitis. This is consistent with our previous findings of decreased apoptotic gene profiles in periodontitis tissues (Gonzalez et al., 2011, 2013). NLRC4 (IPAF) indirectly senses specific proteins from pathogenic microbes and helps to assemble the inflammasome complex (Lupfer and Kanneganti, 2013). NLRP3, AIM2 (Absent in Melanoma 2), and NLRC4 inflammasomes have all been shown to contribute to caspase-1 activation and IL-1ß secretion by activated macrophages (Rathinam et al., 2012; Martinon et al., 2009). This process has been documented to contribute to mucosal immunity to both lung (Cai et al., 2012) and intestinal pathogens (Nordlander et al., 2014) in murine models of infection. Both the NLRP3 and AIM2 inflammasome complexes have been suggested to have a role in periodontitis with NLRP3 being activated in diseased tissues and macrophages (Bostanci et al., 2009), and AIM2 up-regulated in gingival fibroblasts (Belibasakis et al., 2013). However, *in vitro*, it has been shown that *P. gingivalis* can repress NLRP3 inflammasome activation that could be triggered by PAMPs and danger associated molecular pattern (DAMPs) signals that require endocytosis (Park, et al., 2014). Our results demonstrated no effects on NLRP3 in either established or progressing lesions in the nonhuman primates. However, NLRC4 was positively correlated with BOP in periodontitis. AIM2 is also a critical part of the inflammasome responses that contributes to host resistance through recognition of bacterial and viral DNA (Vilaysane and Muruve, 2009). IFNγ induces expression of AIM2 that has a role in processing of pro-inflammatory cytokines, like IL-1ß and IL-18 (Fang et al., 2014). Recent work by Sahingur and colleagues (Sahingur et al., 2013) reported an up-regulation of AIM2 in chronic periodontitis tissues, coupled with increases in TLR9 and DAI mRNA and products in the tissues. Our current study showed that AIM2 levels increased by 2 weeks following initiation of disease and maintained a significantly increased level throughout the entire longitudinal study. In contrast, the expression of this gene was unrelated to established lesions, or directly to the clinical features of progressing periodontitis. These findings support alterations in intracellular detection of

microbial PAMPs from both Gram-positive and Gram-negative bacteria with disease, and suggest that triggering of the inflammasome assembly relates to the gingival inflammation and bleeding that occurs in periodontitis. The results also suggest that increases in gene products related to inflammasome development and activity may be a process that takes place at earlier stages of the progression of disease, but is not necessarily sustained in established lesions. Alternatively, these findings could indicate that many clinical lesions in the nonhuman primates and humans could actually represent a biologically stable environment at the molecular level.

ARHGEF2 (Rho guanine nucleotide exchange factor 2) activates Rho-GTPases via extracellular stimuli. It has been suggested to be involved in epithelial barrier permeability, antigen presentation, and innate immune responses. It is a tight junction-associated molecule that forms an intracellular sensing system along with NOD1 for the detection of invasive bacterial pathogens that exploit tight junctions for entry/crossing epithelial barriers (Philpott et al., 2014). Involved not only in sensing peptidoglycan through NOD1, but also in the activation of NFκB and in innate immune signaling by promoting IL-6 and TNFα secretion by macrophages.

As with the NLRs, Z-DNA-binding protein 1 (ZBP1 or DAI/DLM-1) is an intracellular PRR that recognizes DNA in the cytoplasm, primarily considered an as antiviral mechanism. ZBP1 once activated, elevates the level of antiviral cytokines, such as type I interferons (Vilaysane and Muruve, 2009; Yanai et al., 2009). Expression of this PRR was significantly elevated in established periodontitis lesions. It was significantly increased during the initiation and progression of disease, albeit peak levels were seen at 1–3 months, which were somewhat different kinetics than other PRRs. It was also highly significantly positively correlated with BOP during disease resolution. Based on its DNA ligand, this PRR has been related to antiviral innate immunity, as well as its potential to signal biomolecules that are immunomodulatory. However, these functions could overlap in detecting intracellular periodontopathogens (Eick et al., 2006; Vitkov et al., 2005; Atanasova and Yilmaz, 2014; Park et al., 2004; Tamai et al., 2011), or is consistent with observations of the potential role of DNA viruses (*e.g.* CMV, EBV) in the human disease process (Slots, 2010).

Soluble PRRs are antimicrobial molecules that function by binding to the cell wall of various microorganisms, enhancing phagocytosis and complement activation, that lead to microbe destruction. Collectins, ficolins and the family of pentraxins [*e.g.* Serum Amyloid A (SAA), C-Reactive Protein (CRP)] are all secreted by cells as soluble PRRs. COLEC12 promotes binding and phagocytosis of Gram-positive, Gram-negative bacteria and yeast, and also interacts with oxidized LDLs (Canton et al., 2013; Taylor and Drickamer, 2007). COLEC12 was highly negatively correlated with BOP in the cross-sectional analysis comparing health to periodontitis tissues. Thus, this relationship could signify a contribution of elevated COLEC12 in the tissues towards modulating the intensity of the vascular inflammatory response in the gingival tissues. The ficolins (FCN1, FCN2) are secreted PRRs released from neutrophils following triggering of fMLP receptors (Endo et al., 2011; Thomsen et al., 2011) and can activate the lectin pathway of complement activation upon engagement of N-acetylglucosamine (GlcNAc) residues. We observed an increased levels of ficolins in periodontitis, with FCN2 positively correlated with pocket depth measures in naturally-occurring periodontitis and FCN1 demonstrating a significant increase in levels within 2 weeks of disease initiation in the longitudinal model, but then returning to baseline levels. These finding are consistent with the acute role of the neutrophils in responding to the infectious challenge of the accumulating microbial biofilms at sites of ligature placement. The lack of sustainability of the elevated gene expression may signify the transient role of these PRRs in the disease process and engagement of more robust biomolecules to help control the infection. Pentraxins are divided into short proteins, such as CRP and serum amyloid protein, and long proteins, such as PTX3 and PTX4. PTX3 binds to various microbes to activate the classical complement pathway and enhances the clearance of apoptotic cells (Cieslik and Hrycek, 2012; Deban et al., 2009). This PRR has been found to be increased in gingival crevicular fluid and plasma with disease progression from health to gingivitis to periodontitis (Pradeep et al., 2011). In addition, gingival tissues from patients with generalized aggressive periodontitis had a higher mean concentration of PTX3 than tissues from patients with generalized chronic periodontitis or control subjects (Lakshmanan et al., 2013). Finally, salivary levels of PTX3 correlated with plaque index and bleeding on probing in the chronic periodontitis patients, while serum and salivary PTX3 levels correlated with those of IL-1β in aggressive periodontitis individuals (Gumus et al., 2014). We observed a significant negative correlation in expression levels of PTX3 with both BOP and PD only during resolution of disease, which is consistent with a role in for the molecule in the reestablishment of homeostasis of the gingival tissues.

This study provides data that support a role for an array of PRRs during the initiation and progression of periodontal lesions. The results also indicated that the profiles of PRR gene expression during the initiation and early progression of a periodontal disease lesion are somewhat different than those detected in naturally occurring established lesions and suggest that the heterogeneity of local changes in human disease may be a reflection of fundamental differences in the "molecular stage" of the lesion that cannot be discerned from standard clinical measures of gingival index, bleeding on probing, and pocket depth.

Conflict of interest

The authors state no conflict of interest in the experimental design or data reported.

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