HHS Public Access

Author manuscript

Mol Oral Microbiol. Author manuscript; available in PMC 2017 February 01.

Published in final edited form as:

Mol Oral Microbiol. 2016 February ; 31(1): 18–32. doi:10.1111/omi.12121.

Effects of aging in the expression of NOD-like receptors and Inflammasome-related genes in oral mucosa

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SUMMARY

The molecular changes underlying the higher risk of chronic inflammatory disorders during aging remain incompletely understood. Molecular variations in the innate immune response related to recognition and interaction with microbes at mucosal surfaces could be involved in aging-related inflammation. We developed an ontology analysis of 20 NOD-like receptors (NLRs) and 7 inflammasome-related genes (IRGs) in healthy and inflamed/periodontitis oral mucosal tissues from young, adolescent, adult and aged nonhuman primates (*Macaca mulatta*) using the GeneChip® Rhesus Macaque Genome array. Validation of some of the significant changes was done by qRT-PCR. The expression of NLRB/NAIP, NLRP12, and AIM2 increased with aging in healthy mucosa whereas NLRC2/NOD2 expression decreased. Although higher expression levels of some NLRs were generally observed with periodontitis in adult mucosal tissues (*e.g.*, NLRB/NAIP, NLRP5, and NLRX1), various receptors (*e.g.*, NLRC2/NOD2, and NLRP2) and the inflammasome adaptor protein ASC, exhibited a significant reduction in expression in aged periodontitis tissues. Accordingly, the expression of NLR-activated innate immune genes, such as HBD3 and IFNB1, was impaired in aged but not adult periodontitis tissues. Both adult and aged tissues showed significant increase in IL-1β expression. These findings suggest that the expression of a subset of NLRs appears to change with aging in healthy oral mucosa, and that aging-related oral mucosal inflammation could involve an impaired regulation of the inflammatory and antimicrobial response associated with down-regulation of specific NLRs and IRGs.

Keywords

Aging; inflammasome; NOD-like receptors; oral mucosa; innate immunity

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1. Introduction

Reduced immunity in aging (also named immuno-senescence) is accompanied by chronic inflammation. This is reflected in a higher prevalence of inflammatory disorders or age-related diseases, including periodontal disease, whose prevalence is about 2–3 times higher among those aged >65 years compared to younger individuals (35 to 65 years) (Albandar 2011; Dye *et al.*, 2007). Periodontal disease is a common oral disease that involves inflammation of gingival tissues in response to bacterial species colonizing the oral mucosa, This process, leads to the destruction of the supporting tissues of the teeth (*i.e.*, gingival tissue, periodontal ligament, and alveolar bone) if it is not treated (Van Dyke and Serhan 2003), and is a risk factor for systemic conditions (*e.g.*, cardiovascular disease and diabetes) (Friedewald *et al.*, 2009; Lalla and Papapanou 2011). Humans and microbes have co-evolved during millions of years developing a mutually beneficial relationship with total estimates of about 10^{14} bacteria including more than 500 different species colonizing a healthy adult human. Similar to other mucosal surfaces, the oral cavity represents an example of this symbiotic relationship between host and bacteria whereby, despite the large and diverse microbial loads constantly challenging the oral mucosa across the life span (Dewhirst *et al.*, 2010), the host generally remains healthy. This is likely due to a balanced host immune response, whereby innate immune components appear to play a critical role (Darveau 2010).

It remains unclear if the higher prevalence of periodontitis seen in the elderly is a natural consequence of aging or the result of unique cellular and molecular changes that occur in the oral mucosa during aging, thus increasing the risk for developing the disease with age. Emerging evidence suggests that perturbations of innate immune mechanisms associated with aging could play a critical role in age-related chronic inflammatory disorders (Qian *et al.*, 2012; Shaw *et al.*, 2011). Accordingly, *ex vivo* evidence indicates that impaired expression and function of innate immune components (*i.e.*, Toll-like receptors-TLRs, cytokine/chemokine production, neutrophils and monocyte/macrophages) appears to be associated with aging and periodontitis (Hajishengallis 2010). Most recently, it was shown in mice that aging-associated periodontitis is accompanied by lower expression of an endogenous inhibitor of neutrophil adhesion dependent on the integrin LFA-1 (Del-1), which is a negative regulator for the recruitment of inflammatory cells into the tissues (Eskan *et al.*, 2012). Thus, an increased migration of neutrophils into the oral mucosa constantly challenged by bacteria would be associated with periodontitis in aged mice.

The nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) family of proteins is a growing group of cytosolic pattern recognition receptors (PRRs) involved in the regulation of the innate immune responses against pathogen- and danger-associated molecular patterns (PAMPs and DAMPs) (Geddes *et al.*, 2009). About 22 intracellular NLRs have been reported, which are classified into subfamilies based on their N-terminal domain into: (i) caspase-recruitment domain (CARD)-containing NLRCs or NODs (1 to 5), (ii) pyrin-domain (PYD)-containing NLRPs (1 to 14), and (iii) baculovirus inhibitor of apoptosis repeat (BIR)-containing domain NAIPs (neuronal apoptosis inhibitor proteins) (Franchi *et al.*, 2008). Among these NLRs, NLRC1/NOD1 and NLRC2/NOD2 are perhaps the most studied to date and are activated by the bacterial peptidoglycan motifs

diaminopimelic acid (DAP) from Gram-negative bacteria and muramyl dipeptide (MDP) from both Gram-positive and Gram-negative bacteria, which normally leads to NFκB activation and production of cytokines/chemokines (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003). Interestingly, the activation of several members of the NLR gene family (*i.e.*, NLRB/IPAF, NLRC4, NLRPs 1, 3, 5, 6 and 7) by a range of PAMPs such as MDP, poly(I-C), dsRNA from viruses, bacterial RNA and pore-forming toxins, as well as a range of DAMPs such as extracellular ATP, uric acid, asbestos, silica, aluminum hydroxide, and amyloid-β peptide, contributes to the assembly of a macromolecular protein complex termed the inflammasome. This process leads to activation of the cysteine protease, caspase-1, which in turn induces inflammation (*i.e.*, IL-1β, IL-18 and IL-33 secretion), pyroptosis (specialized type of pro-inflammatory cell death to control intracellular infectious niches), and repair and healing (FGF2 secretion and lipid membrane biogenesis) (Mariathasan and Monack 2007). In addition, reactive oxygen species (ROS) and lysosomal damage as well as activation of NLRC4/IPAF4 by flagellin have also shown the ability to enhance inflammasome activation (Martinon 2010; Zhao *et al.*, 2011). Of note, it has been recently suggested that inflammasome activation influences many metabolic disorders, such as atherosclerosis, type 2 diabetes, gout and obesity (Wen *et al.*, 2012).

In addition to some NLRs members, it was recently shown that absent in melanoma 2 (AIM2), a member of the family of hematopoietic IFN-inducible nuclear proteins with a 200-amino acid motif (HIN-200), and the cytoplasmic RIG-I-like helicase (RIG-I) also have the ability to activate the inflammasome in response to cytosolic double stranded DNA from viruses, bacteria or the host itself (Hornung *et al.*, 2009; Poeck *et al.*, 2010). In particular, NLRPs and AIM2 interact with the cytosolic adaptor protein called apoptosis-associated speck-like protein containing a CARD domain (ASC) to further activate caspase-1. In contrast, other NLR members (*e.g.*, NLRP2, NLRP10, NLRP12, and NLRX1) instead of being inflammasome activators, appear to be negative regulators of inflammation, decreasing NFkB activation and reducing type I interferon responses (Allen *et al.*, 2011; Williams *et al.*, 2005), as well as modulators of the adaptive immune response, controlling dendritic cell migration from tissues to lymphoid nodes (Arthur *et al.*, 2010). Thus, this emerging family of cytosolic sensors with the ability to regulate the host responses to bacteria, inflammation, and adaptive immunity appear to play a crucial role at mucosal surfaces highly exposed to PAMPs and DAMPs, to maintain tissue homeostasis. Most recently, it has been suggested that NLRs could be critical innate sensors for discriminating and controlling pathogenic species (*i.e.*, bacteria expressing specialized secretions systems, pore-forming toxins, and increased invasiveness ability) within the context of complex bacterial communities constantly colonizing the mucosal surfaces during health, mainly through a regulated activation of the inflammasome (Blander and Sander 2012). In fact, mutations in some NLRs (*e.g.*, NLRP3, NLRC4, NOD1 and NOD2) have been associated with disorders such as inflammatory bowel disease (e.g., Crohn's disease), atopic dermatitis and asthma [reviewed in (Geddes *et al.*, 2009)].

There is solid evidence indicating that cytokines belonging to the IL-1 family, such as IL-1β and IL-18, play a critical role in periodontitis (Graves and Cochran 2003; Orozco *et al.*, 2007) and recent *in vivo* and *in vitro* studies have shown variation in the expression of some

inflammasome genes, in response to oral biofilms and planktonic bacteria, as well as the ability of some oral periodontopathogenic species (e.g., *P. gingivalis, A. actinomycetemcomitans*) to activate the inflammasome (Belibasakis and Johansson 2012; Bostanci *et al.*, 2009; Yilmaz *et al.*, 2010). Since the aged population is at higher risk for infections and inflammatory disorders, we hypothesized that mucosal changes in the expression of NLRs and inflammasome-related genes occur with aging. In this study, we used the oral cavity as a model of a mucosal surface that naturally becomes constantly exposed to PAMPs and DAMPs across the lifespan to determine the changes in the expression of NLRs and inflammasome-related genes associated with aging during health and inflammation (*i.e.*, periodontitis). Importantly, the nonhuman primates develop periodontal disease naturally with age as it is observed in humans. Thus, in contrast to adult/aged animals, young and adolescent animals develop gingivitis but that does not progress to periodontitis (Schou *et al.*, 1993).

Methods

1.1. Animals and diet

Rhesus monkeys (*Macaca mulatta*) (n=34; 14 females and 20 males) housed at the Caribbean Primate Research Center (CPRC) at Sabana Seca, Puerto Rico, were used in these studies. Periodontally healthy animals (n=23) were selected by age based on the following criteria: ≤3 years (young; n=5), 3–7 years (adolescent; n=5), 12–16 years (adult; n=7) and 18–23 years (aged; n=6). Only adult (n=5; 3 males and 2 females) and aged (n=6; 5 males and 1 female) animals with periodontitis were used, since periodontitis does not occur naturally in younger animals. The nonhuman primates were typically fed a 20% protein, 5% fat, and 10% fiber commercial monkey diet (diet 8773, Teklad NIB primate diet modified: Harlan Teklad). The diet was supplemented with fruits and vegetables, and water was provided ad libitum in an enclosed corral setting.

1.2. Oral clinical parameters and gingival tissue sample collection

Following a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico, anesthetized animals were examined by a single investigator using a Maryland probe on the facial aspect of the teeth, 2 proximal sites per tooth (mesio- and disto-buccal), excluding the canines and 3rd molars. The clinical examination included probing pocket depth (PD), and bleeding on probing (BOP; 0–3 scale) (Ebersole *et al.*, 2008). Using a standard gingivectomy technique (a crevicular incision followed by an interdental incision at the base of the papillae using a #15 surgical blade), a buccal gingival papillae from either healthy or periodontitis-affected tissue from the premolar/molar maxillary region of each animal was taken and maintained frozen in RNAlater solution until RNA preparation for microarray analysis.

1.3. RNA Extraction, Reverse Transcription, and Gene Chip Hybridizations

Total RNA was isolated from each gingival tissue using Triazol reagent (Invitrogen, CA), and further cleaned up with the Qiagen RNeasy mini kit (Qiagen, Valencia, CA). All microarray RNA expression analyses were done at the University of Kentucky Microarray facility. Tissue RNA samples were submitted to the microarray core and RNA quality was

assessed with an Agilent 2100 Bioanalyzer. Reverse transcription of equal amounts of RNA from each sample was performed, followed by hybridization to the GeneChip® Rhesus Macaque Genome Array (Affymetrix) similar to methods we have described previously (Gonzalez *et al.*, 2013; Meka *et al.*, 2010). Briefly, 300ng of total RNA was labeled using Affymetrix GeneChip 3' IVT Express kit, from which 15μg of labeled cDNA was hybridized to the GeneChip Rhesus Macaque Genome Array (Affymetrix) following the Affymetrix protocol. Post-hybridization, washing and staining of arrays were performed in an Affymetrix GeneChip Fluidics FS450 station followed by scanning using an Affymetrix GeneChip 3000 7G Scanner and GeneChip Operating Software MAS 5.0. Individual samples were used for gene expression analyses of 20 NLRs, 7 inflammasome-related genes, and 3 antimicrobial genes (Table 1) in healthy and inflamed gingival tissues.

1.4. Quantitative RT-PCR

For qRT-PCR analysis, three mRNA samples for each group (*i.e.*, healthy or periodontitis) were individually analyzed in two independent experiments (n=6) from adult and aged animals. The cDNA synthesis was carried out starting with 1μg of mRNA and expression of specific transcripts for NLRs and IRGs analyzed using the LightCycler 480 (Roche, IN). Primers for each gene were designed using the software Primer Quest from Integrated DNA Technologies (IDT), and synthesized by the same company (www.idtdna.com, Coralville, IA). The primers sequences (5'-3') used were: NLRP14: Forward: CAAGATCTCTCCTCTGCTCTTATC and Reverse: CACTTAGGAGACTTCAGGACTTT; NLRP5: Forward: CACTCTCCTTGGCCCTTTC and Reverse: GCTGAACACAGCTTCATCATTC; NOD2: GAGGCAGTTCCATTTCATTTGT and Reverse: TGCTTAGAAGGAAGGGCTTAAT; ASC: Forward: AGGCCTGCACTTTGTAGAC and Reverse: TCCTGGTACTGCTGATCCT; IL-1B: Forward: GACAGGATCTGGAGCAACAA and Reverse: CCCAAGGCCACAGGTATTT; GADPH: Forward: GGTGTGAACCATGAGAAGTATGA and Reverse: GAGTCCTTCCACGATACCAAAG. Concentration ratios for the target genes were calculated by normalizing to the housekeeping gene GADPH.

1.5. Histopathologic Evaluation

Gingival tissues were formalin fixed (4% neutral buffered formalin) for 24 hours at room temperature and further placed in 70% ethanol. Tissues were processed by graded dehydration in alcohols using TissueTek VIP1000 and paraffin embedded. Serial sections of 5μm were obtained using Leica RM2255 Rotary Microtome and tissues were deparaffinized following standard protocol with xylene, and rehydrated with decreasing concentrations of ethanol. Sections of each biopsy were stained with Hematoxylin-Eosin (H&E). The areas of connective tissue under the epithelial basement membrane were analyzed under light microscopy at 200×. Each gingival tissue sample was divided into 10 fields and inflammatory cell infiltrate was determined in each field based on morphological characteristics by a blinded investigator.

1.6. Data analysis

The expression intensities for all genes across the samples were estimated using the Affymetrix PLIER algorithm. The GeneChip® Rhesus Macaque Genome Array contained matched and mismatched pairs allowing the MAS 5 algorithm to be used. For aging-related gene expression changes in healthy gingival tissues, a simple linear regression model was fit to the scatter plot of gene expression by age as a continuous variable. The 95% confidence bands for the fit line were included in the plots. For genes that had significant differences in expression with periodontitis, two sample t-tests were used. Statistical significance was considered by a p value ≤ 0.05. All statistical analysis was performed using the software JMP 10 (SAS, Inc., Cary, NC). Microarray data was uploaded into the ArrayExpress data base (www.ebi.ac.uk) under accession number: E-MTAB-1977.

2. Results

Clinical characterization for healthy and inflamed/periodontitis sites in each group included, young animals (mean PD 1.4±0.4 mm, mean BOP 0.4±0.5); adolescent animals (mean PD 1.9±0.2 mm, mean BOP 0.9±0.7), adult healthy group (mean PD 2.4±0.2 mm, mean BOP 0.9±0.4); aged healthy group (mean PD 2.5±0.4 mm, mean BOP 1.1±0.7); adult periodontitis group (mean PD 3.9±0.2 mm, mean BOP 2.6±0.6); and aged periodontitis group (mean PD 4.6±0.7 mm, mean BOP 2.7±0.4).

Among the NLRs and inflammasome-related genes evaluated by microarray, only the expression of 4 cytosolic receptors showed significant correlation with age, where the expression of NLRB/NAIP, NLRP12, and AIM2 increased, and NLRC2/NOD2 expression decreased with aging in healthy gingival tissues (Figure 1). Interestingly, the expression of inflammasome related genes including the adaptor protein ASC, as well as the Caspase 1 and its substrates pro-IL1β, and pro-IL18 did not change with age in healthy gingival tissues.

Although there was a similar trend with higher expression of the majority of genes during inflammation/periodontitis in adult and aged gingival tissues compared to healthy tissues, only NLRB, NLRP5, NLRX1, and Caspase-1 reached statistical significance in diseased adult but not aged tissues (Figs. 2A and 2B). Interestingly, inflamed tissues from aged animals, in contrast to the adult counterparts, exhibited a significant reduction in the expression of various NLRs (i.e., NLRC2/NOD2, NLRP2, and NLRP14) compared to healthy tissues of a similar age (Fig. 2A). Only NLRC1/NOD1 decreased expression was observed in periodontitis adult tissues. The presence of periodontitis was also associated with a significant reduction in the expression of the inflammasome adaptor protein ASC in aged, but not adult gingival tissues (Fig. 2B). Finally, the expression of the downstream substrates (*i.e.*, pro-IL-1β, pro-IL-33) of the inflammasome showed similar elevated expression with periodontitis in both adult and aged tissues; however, pro-IL18 mRNA levels were significantly diminished with disease in adults, but not aged gingival tissues (Fig. 2C).

Quantitative analyses of selected genes that showed significant differences with periodontitis in adult and aged gingival tissues by microarray were validated using qRT-PCR (Fig. 2D). GADPH expression determined by qRT-PCR was consistent between

healthy and periodontitis groups from both adult and aged gingival samples with the following crossing point (Cp) mean values ± standard deviations: adult healthy: 18.69±0.65, adult periodontitis: 18.35±0.35, aged healthy: 19.05±0.72, and aged periodontitis: 18.48±0.63. There were no statistically significant differences ($p \geq 0.05$) between healthy and periodontitis tissues. In general, these results are consistent with the overall trend of gene expression detected by microarray analysis, whereby higher levels in the expression of NLRs such as NLRP5 (5-fold) was seen with periodontitis in adult compared with aged tissues. Diminutions, albeit not reaching significance, in ASC and NOD2 were seen during periodontitis in particular related to aged diseased tissues, and increased IL-1β mRNA levels were observed in both adult and aged tissues with periodontitis. There were not significant changes in NLRP14 expression associated with periodontitis in aged tissues using qPCR; however, adult diseased tissues showed a significant increase in the expression of this NLR.

Histological characteristics determined by H&E staining of health and periodontitis gingival tissues from both adult and aged tissues, showed similar cellularity, whereby an increased inflammatory infiltrate was observed in both adult and aged periodontitis tissues compared with the healthy tissues (Figures 3A & 3B). In contrast to adult healthy tissues, inflammatory cells infiltrating clinically healthy aged tissues were more frequently observed, although it was not statistically significant. The histologic results were consistent with the bleeding on probing (BOP) levels as a clinical measure of inflammation with both adult and aged animals exhibiting similar increases in BOP scores with periodontitis (Figure 3C).

The expression levels of hBD2 were lower in aged but not periodontitis tissues compared to the healthy counterpart, albeit, this difference was not significant (Figure 4A). Although, both adult and aged periodontitis tissues showed lower levels of hBD3 expression with respect to healthy tissues, only aged diseased tissues reached significance with about 4-fold decrease in expression (Figure 4B). A significant increase in the expression of interferon beta (IFNB1) was observed with periodontitis in adult but not in aged periodontitis tissues, which exhibited an approximate 2-fold lower level (Figure 4C).

3. Discussion

Although there is solid evidence linking environmental factors, such as diet with aging and an increased risk for chronic inflammatory diseases (Gonzalez *et al.*, 2012; Omodei and Fontana 2011), less is known about how molecular variations in the innate immune response related to recognition and interaction with microbes at mucosal surfaces could increase this risk in aged populations. Microbes are a critical component in amplifying and perpetuating inflammation (*i.e.*, enhancing a continuous influx of neutrophils and macrophages), which can be harmful to the tissues as has been shown in several chronic inflammatory disorders, such as atherosclerosis, cardiovascular disease, diabetic chronic wounds, and periodontal disease (Grice and Segre 2012; Rosenfeld and Campbell 2011). Here, we presented a transcriptomic analysis of the age-related changes for an emerging group of innate genes that are crucial regulators of inflammation and infection at epithelial surfaces that are continually exposed to complex microbial communities in healthy and inflamed (*i.e.*, periodontitis) mucosal tissues (*i.e.*, gingiva).

The first observation from these analyses is that although the expression of most NLRs in healthy gingival tissues was not affected by aging, a significant positive correlation of the transcripts levels for 3 intracellular receptors (*i.e.*, NLRB/NAIP, NLRP12, and AIM2) with age was observed. Based on the emerging functions of these intracellular microbial receptors, one could hypothesize that the increased ability of gingival tissue to restrict replication of invasive microorganisms through NLRB-, NLRP12 or AIM2-induced inflammasome activation could be crucial for maintaining a healthy state in the periodontium. In particular, a unique feature that distinguishes NLRB/NAIP from other NLRs is the presence of BIR domains, which seems to be related to the ability to suppress apoptosis through activation of MAP kinase (JNK) (Sanna *et al.*, 2002). Thus, over-expression of NLRB/NAIP in healthy gingival tissue could be related to a reduction in apoptotic events normally occurring in gingival tissues from aged animals, as we have previously reported (Gonzalez *et al.*, 2013; Gonzalez *et al.*, 2011). Although the role of NLRP12 in periodontitis remains unknown, the anti-inflammatory effects of NLRP12 suggest that increased levels of this NLR in aged oral mucosa could also play an important role in balancing the host-microbes interactions across the life span with a central role in maintaining health at advanced age. Of note, increased expression of AIM2 also has been reported in senescent cells which could be related with a pro-inflammatory phenotype (*i.e.*, IL-1β secretion) associated with aging-associated inflammatory diseases (Duan *et al.*, 2011). Accordingly, aging-related up-regulation of AIM-2 in healthy gingival tissue could increase the risk for periodontitis in the elderly.

On the other hand, NLRC2/NOD2 expression significantly decreased with aging in healthy oral mucosa. Down-regulation of NLRC2/NOD2 with aging is of special interest, since growing evidence indicates that NOD2 negatively regulates TLR2 signaling in certain settings, it is essential for promoting intestinal epithelial barrier integrity, and NOD2 mutations have been related to reduced IL-10 production by cells from patients with Crohn's disease when stimulated with MDP and TLR agonists (Lala *et al.*, 2003; Noguchi *et al.*, 2009). Accordingly, aging-related periodontitis could involve an impaired regulation of inflammation and infection associated with decreased expression of some NLRs including NLRC2/NOD2 receptor. This hypothesis is supported by the aging-related decrease in the expression of antimicrobial genes such as hBD2 and hBD3, and the lack of difference in IFNB1 response observed in aged tissues during periodontitis.

It remains unknown, why the oral mucosa at an early age reacts to the accumulation of noxious biofilms with inflammation (*i.e.*, gingivitis), albeit this inflammatory response does not develop into periodontitis as is frequently observed in older individuals. It is tempting to hypothesize that an altered expression of NLRs such as NLRB (lower) and NOD2 (higher) in young/adolescent oral mucosa could be contributing to help maintain homeostatic host-microbes interactions through important regulatory mechanisms that control destructive inflammation and support epithelial barrier integrity, which may be weakened in an age-dependent manner.

In general, a higher expression of several NLRs and inflammasome-related genes was observed in inflamed/periodontitis gingival tissues from adult and aged animals compared to healthy tissues. These observations are consistent with recent evidence in humans showing

that some inflammasome-related genes are expressed at higher levels in gingival biopsies from periodontitis sites compared with healthy controls (Bostanci *et al.*, 2009), and the ability of subgingival biofilms to increase the expression of some inflammasome-activating receptors in gingival fibroblasts (Bostanci *et al.*, 2011). Nevertheless, specific aging-related differences were observed with periodontitis, where increased expression of NLRB, NLRP5, and NLRX1 was associated with inflamed tissues from adult but not aged animals when compared with healthy controls, and in contrast, a significant reduction in the expression of NLRC2, NLRP2 and NLRP14 was specifically observed in aged periodontitis tissues. Although the pathogenesis of periodontal disease has been historically considered similar at all ages, these differences in gene expression supports the idea that there could be important molecular differences in the pathogenesis of periodontitis related to age (Gonzalez *et al.*, 2011). Diminution in NLRP14 expression observed by microarray analysis in the aged periodontitis tissues compared to healthy tissues was not observed using the qPCR procedure. Although this discrepancy in gene expression results for this gene could involve fundamental methodological differences in these independent experimental approaches (Etienne *et al.*, 2004), further studies will be necessary to confirm variations in this NLR with disease and aging. Preliminary analysis of gingival transcriptomes of an ongoing longitudinal study conducted by our group using a ligature-induced periodontitis model in the same non-human primate model, indicates that NOD2 expression is significantly reduced, and NLRP14 expression is increased during initiation, and progression of periodontitis (unpublished) irrespective of aging, which re-enforces a potential change in the expression of these intracellular receptors with disease.

Altered expression of some NLRPs has been shown in mice and rhesus macaques (*M. mulatta*) (*i.e.*, NLRPs 2, 5 and 14) to be mainly in gametes and early embryos, suggesting that these receptors have specific functions in oocyte maturation and early embryonic development (Tian *et al.*, 2009). Our results indicate that some of these receptors are also expressed in healthy gingival tissues and their expression is specifically down-regulated in aged periodontitis tissues. In particular, NLRP2 appears to be a negative regulator of inflammation through the inhibition of TLR-driven NFκB activation in cells of myeloid origin (Fontalba *et al.*, 2007), and play a role as a mediator for the expression of antimicrobial peptides induced by oral bacteria (*i.e.*, *F. nucleatum*) in epithelial cells (Ji *et al.*, 2009). Therefore, down-regulation of NLRP2 with periodontitis in aged gingival tissue could also be increasing the likelihood for persistent infection and inflammation. Consistently, we found that hBD3 expression was significantly decreased during periodontitis in aged but not adult gingival tissues. The potential immunoregulatory role of NLRPs 5 and 14 at mucosal surfaces still needs to be determined.

As expected and broadly described, pro-IL-1β was up-regulated with disease in both adult and aged gingival tissues; however, pro-IL-18 expression was significantly decreased in adult but not aged periodontitis tissues. This difference in pro-IL-18 expression rather than being a unique characteristic of periodontitis in the adult tissues appears to be likely related to an age-related decrease of IL-18 expression, which although not statistically significant, is reflected in a negative correlation between the expression of this cytokine and age, as well as lower levels of IL-18 in aged healthy tissues compared to the adult tissues. Since both

IL-1β and IL-18 play prominent roles in polarizing T-cell helper responses, these age-related transcriptional changes could also be reflecting differences in the T helper responses during periodontal disease.

Finally, ASC expression was reduced with periodontitis in aged gingival tissues. As mentioned above, ASC is a crucial adaptor protein for successful inflammasome activation by NLRPs and AIM2, and most recently it has been shown that ASC-deficient mice exhibited defective antigen presentation by dendritic cells and lymphocyte migration due to impaired actin polymerization mediated by the small GTPase Rac (Ippagunta *et al.*, 2011). Decreased expression of this central adaptor protein for inflammasome assembly and activation observed in periodontitis gingival tissues when compared with healthy controls is consistent with previous evidence demonstrating the ability of the periodontopathogenic *P. gingivalis* to reduce ASC expression *in vitro* (Bostanci *et al.*, 2009). Thus, an impaired inflammasome response related to decreased ASC expression driven by oral pathogenic strains could be an interesting pathway to further explore in the pathogenesis of periodontal disease, where selected oral bacterial species could take advantage of this local altered host response leading to overgrowth and contributing to disease. An additional observation was that although both adult and aged periodontitis tissues exhibited a significant increase in mRNA IL-1β levels, aged tissues showed about 3 times higher message levels when compared with the adult periodontitis tissues. It could be hypothesized that a greater accumulation of IL-1β mRNA levels in aged tissues during periodontitis may occur, which would be consistent with a diminution of gingival cell/tissue ability to properly activate the inflammasome for the consequent maturation, translation and release of IL-1β.

It remains to be determined what cell types are involved in the age-related gene expression changes of NLRs and inflammasome-related genes that reflect the tissue milieu in health and periodontitis as reported in this study. Variations in the amount of inflammatory infiltrate with aging and periodontitis could not fully explain these gene expression differences, because despite of the higher presence of inflammatory cells normally observed with periodontitis, we found a significant reduction in the expression of a subset of NLRs and inflammasome-related genes in aging-periodontitis tissues. Moreover, it has been reported that some NLRs are expressed primarily by oral epithelial cells compared with the expression levels of fibroblasts and inflammatory cellular infiltrates in the tissues during health and periodontitis (Sugawara *et al.*, 2006). Thus, variations in gene expression, particularly at the oral epithelium, irrespective of variations in the immunoinflammatory infiltrate may be likely involved.

Age–related changes in the transcriptional profiles of several “sterile” tissues from mice and rhesus monkeys (*e.g.*, skeletal muscle, brain and heart) have shown up-regulation of transcripts involved in inflammation and oxidative stress related to aging (Lee *et al.*, 2002). We have demonstrated changes in gene expression of a group of critical innate regulators of inflammation and infection related with aging in a mucosal tissue constantly exposed to complex microbial communities during health and inflammation (Fig. 5). Whether variation in the expression of these innate genes is a cause or consequence of the disease, and the mechanisms by which these variations could be related to a higher prevalence and/or severity of periodontitis with aging need to be elucidated. The oral microbiome of *rhesus*

monkeys was recently characterized, exhibiting high similarity with the human oral microbiome (Ocon *et al.*, 2013). Therefore, detrimental changes of the immune response associated with aging (i.e., immunosenescence), including those described in this study, could be contributing to impair the symbiotic relationship between oral bacteria and the host leading to dysbiosis, persistent infection and chronic inflammation.

Although up-regulation of some of the genes studied here (e.g., IL-1β) is consistent with previous evidence showing increases at the protein level in the same animal model (Smith *et al.*, 1993), future immunohistochemistry studies to confirm these gingival transcriptional variations associated with aging are clearly necessary, given the fact that protein expression of some of these molecules has been shown to be post-transcriptionally regulated. The approach in this study focused our efforts on determining the inflammasome characteristics within the context of the multiple cellular interactions that would be occurring in the oral mucosa (*i.e.*, gingival tissues). It should be recognized that the literature is replete with studies of oral tissue responses employing reductionist approaches trying to understand disease. As such, these studies isolate single cell types removed from the tissue context and generally challenged with individual bacteria or their components, in attempting to describe the biology of the *in situ* situation in health and disease. However, as recently described by Dupré (Dupre), biological outcomes are not only dependent upon the activities of their individual constituents, but are critically affected by the complex systems of which they are a part. The evolving field of systems biology emphasizes the importance of moving science towards addressing the micro-environmental milieu that occurs *in situ* and attempt to reflect the “whole” of the system that defines health or disease.

ACKNOWLEDGMENTS

This work was supported by National Institute of Health (NIH) grants P20GM103538 and UL1TR000117. We express our gratitude to the Caribbean Primate Research Center (CPRC) supported by grant P40RR03640 and the Microarray Core of University Kentucky for their invaluable technical assistance. Dr. Stromberg was also funded by NIH grant 5P20GM103436-13.

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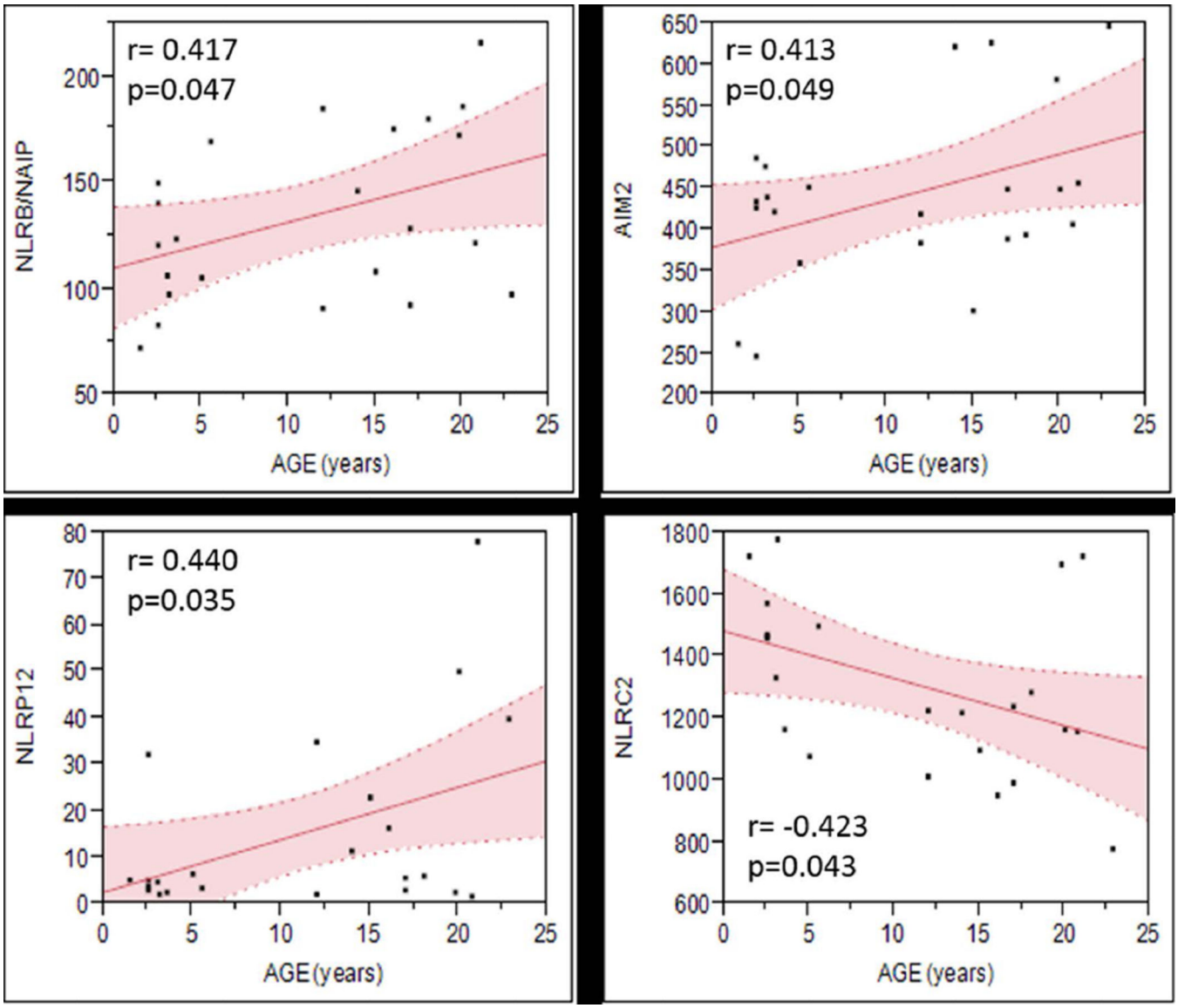
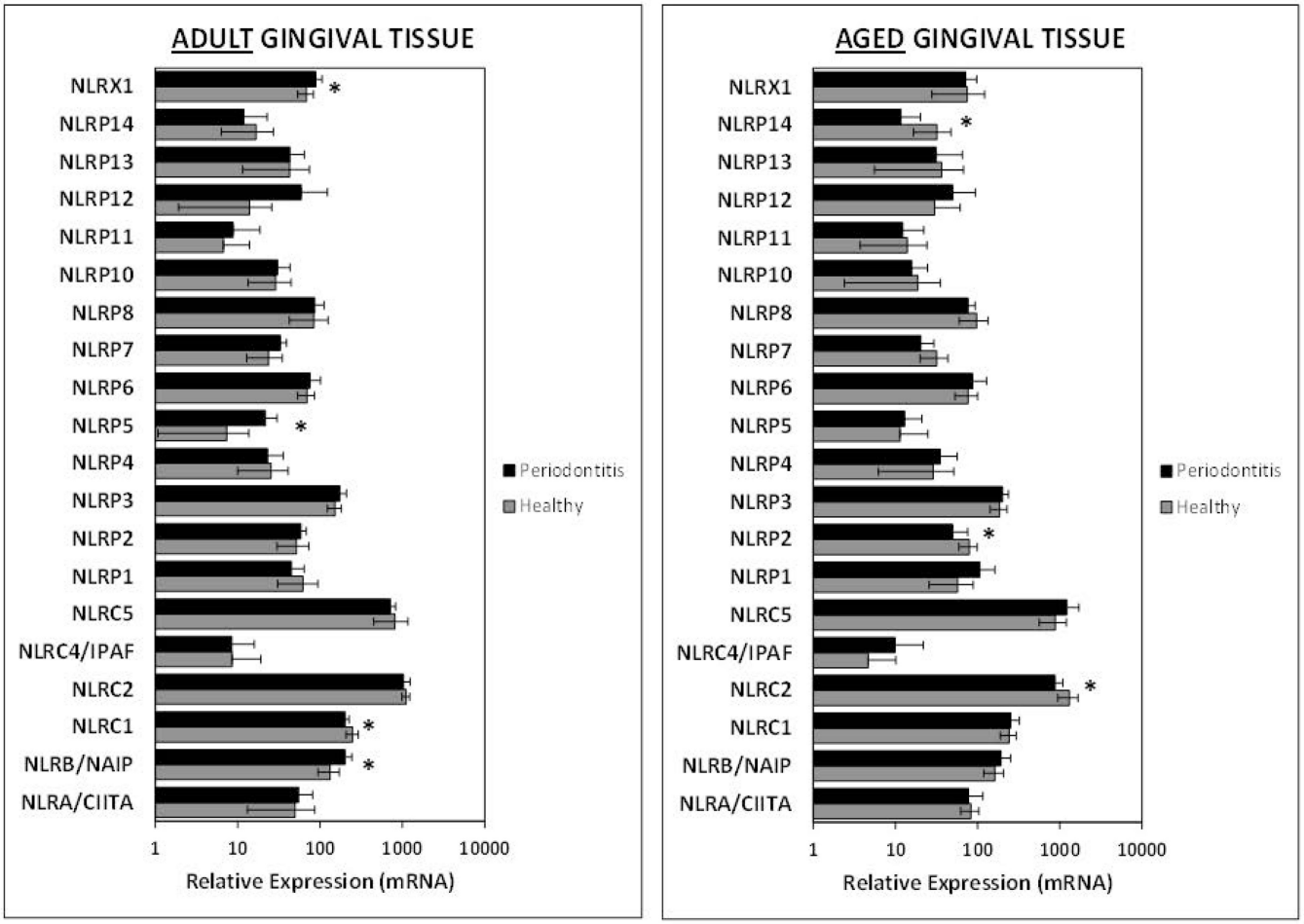


Figure 1. Scatterplot graphs showing the 95% confidence intervals for the regression fitting of NOD-like receptors and inflammasome related genes that significantly correlated with age

(A)



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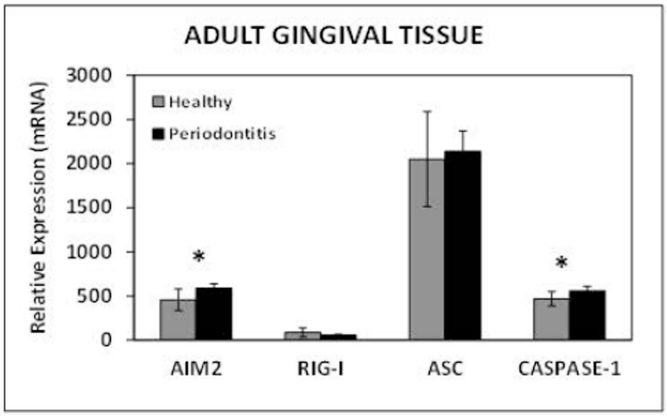

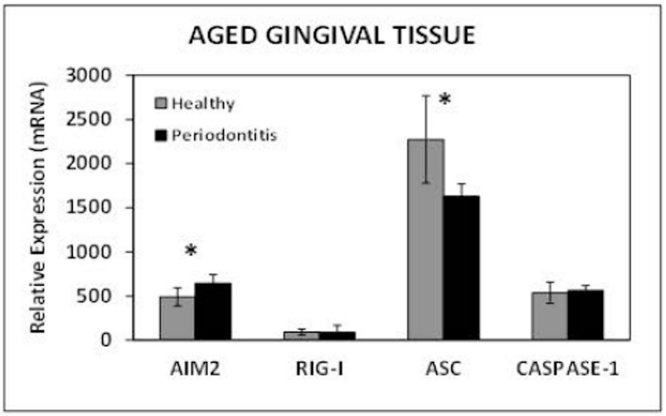

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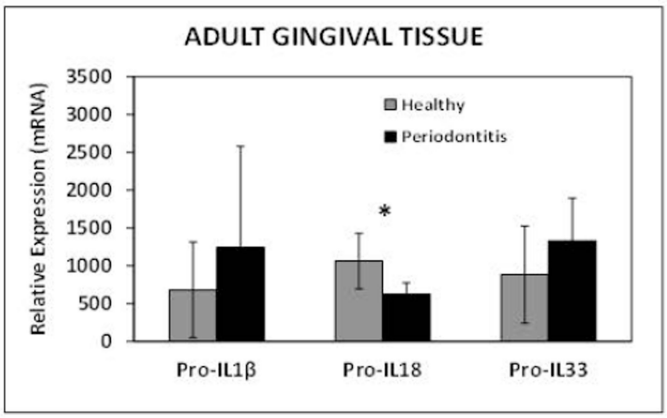
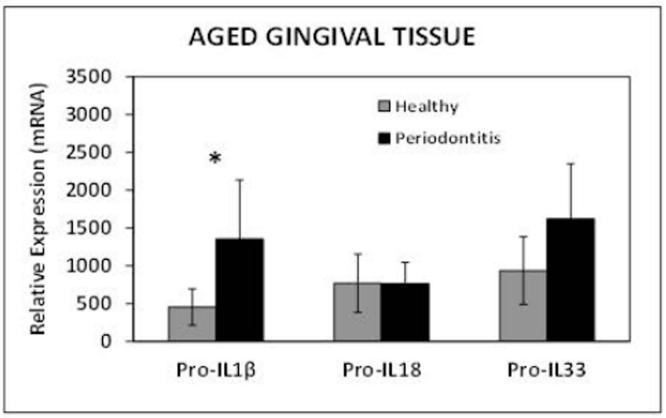




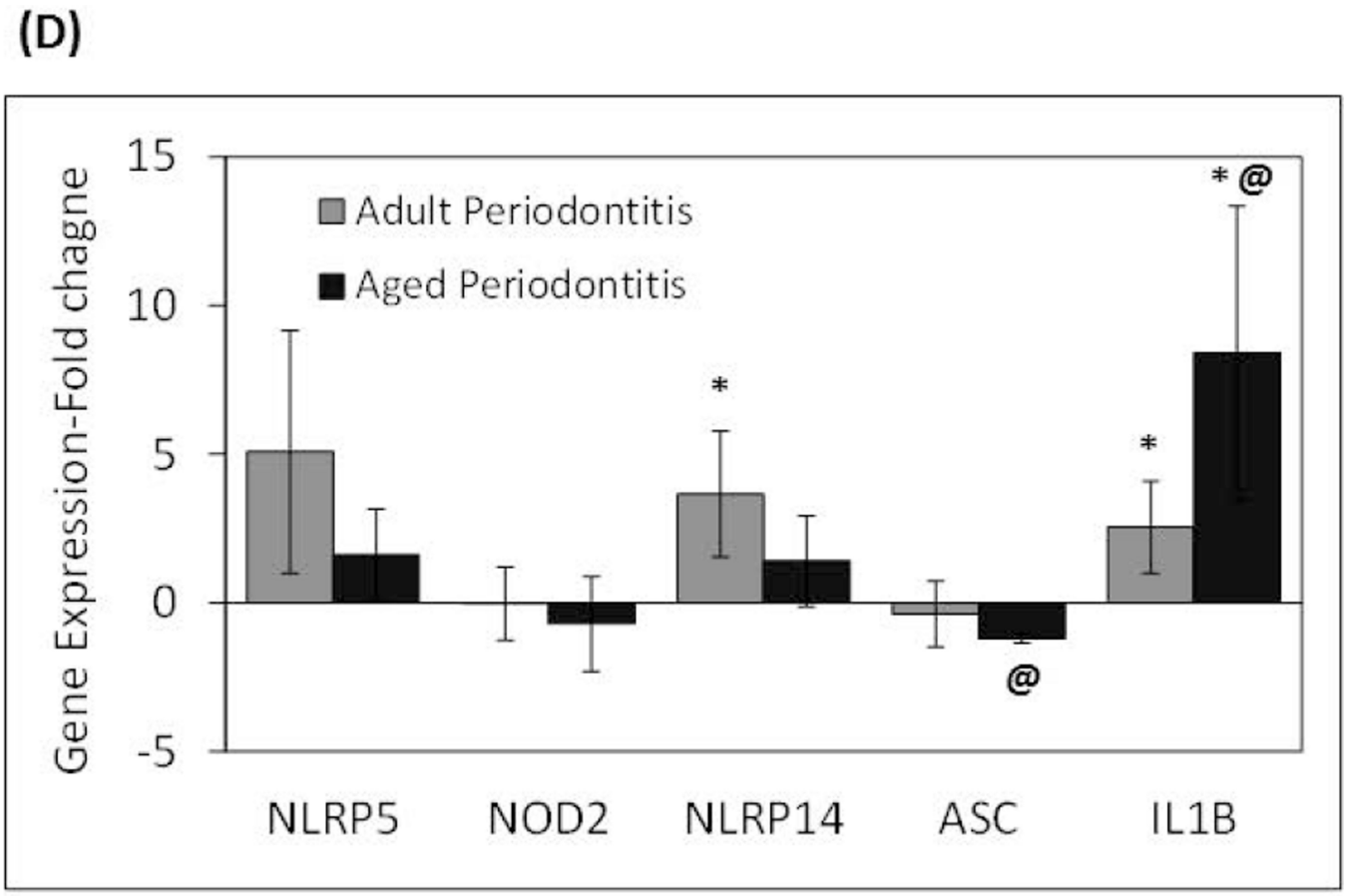
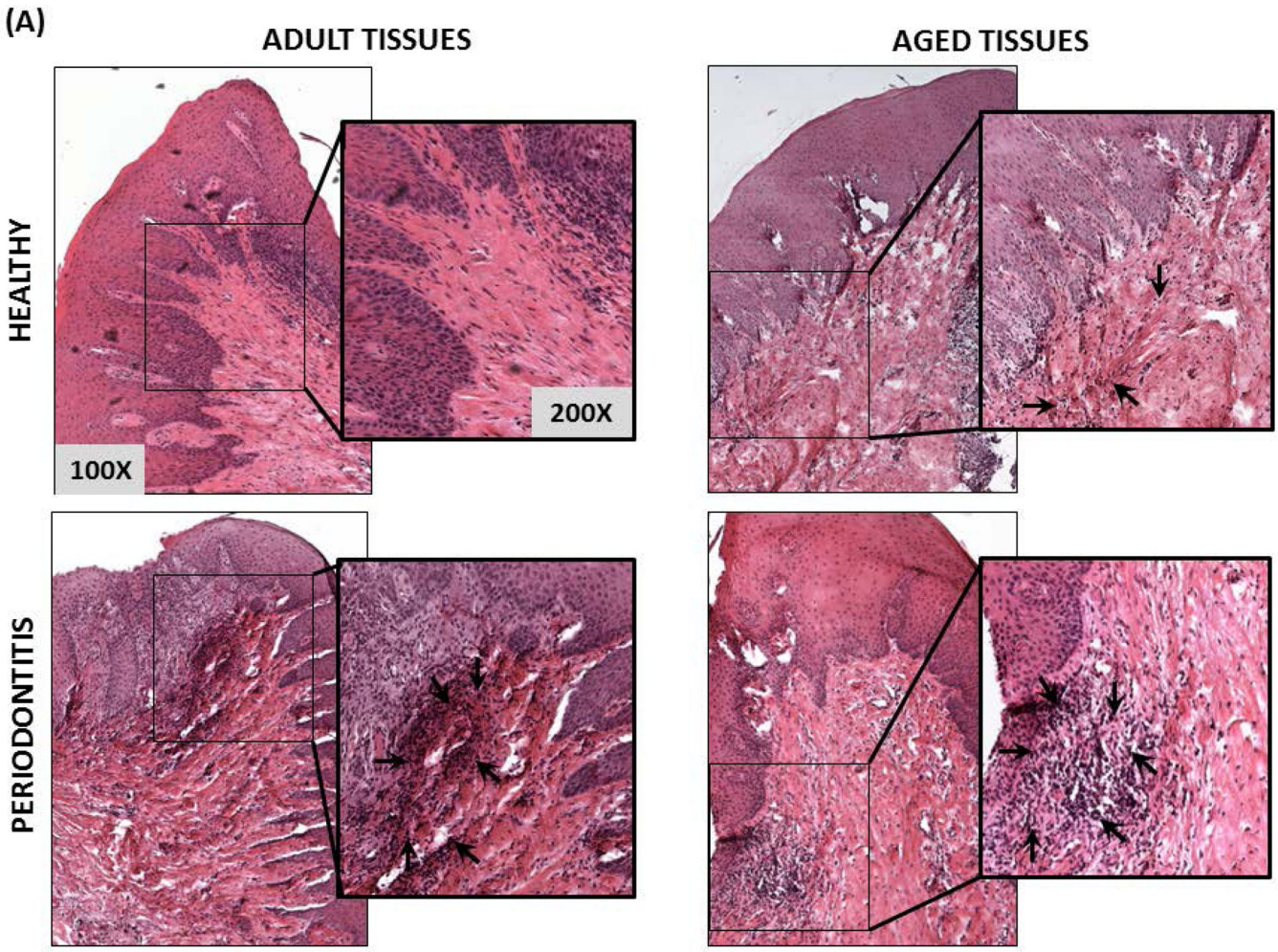


Figure 2. Age-related changes in the expression of NOD-like receptors and inflammasome-related genes in healthy and inflamed/periodontitis gingival tissues

The results shown are the Means ± Standard Deviation of gene expression for (**A**) NOD-like receptors, and (**B and C**) inflammasome-related genes of healthy and inflamed/periodontitis gingival tissues obtained from 5–6 animals per each age (*i.e.*, adult or aged non-human primates). Each sample was individually analyzed by microarray (**D**) *Gene expression levels determined by qRT-PCR*. Significant changes in gene expression with periodontitis in adult and aged tissues detected by microarray were validated by qRT-PCR in selected genes. Total mRNA from healthy (n=3) and periodontitis (n=3) gingival tissues obtained from adult and aged animals were individually analyzed in two independent experiments. Data are expressed as means ± standard deviations of fold changes in periodontitis vs. healthy tissues. *, $p \leq 0.05$ when periodontitis gene expression values where compared to healthy gene expression values, and @, $p \leq 0.01$ depicts significance between adult vs. aged periodontitis tissues, as determined by Student's *t* test.



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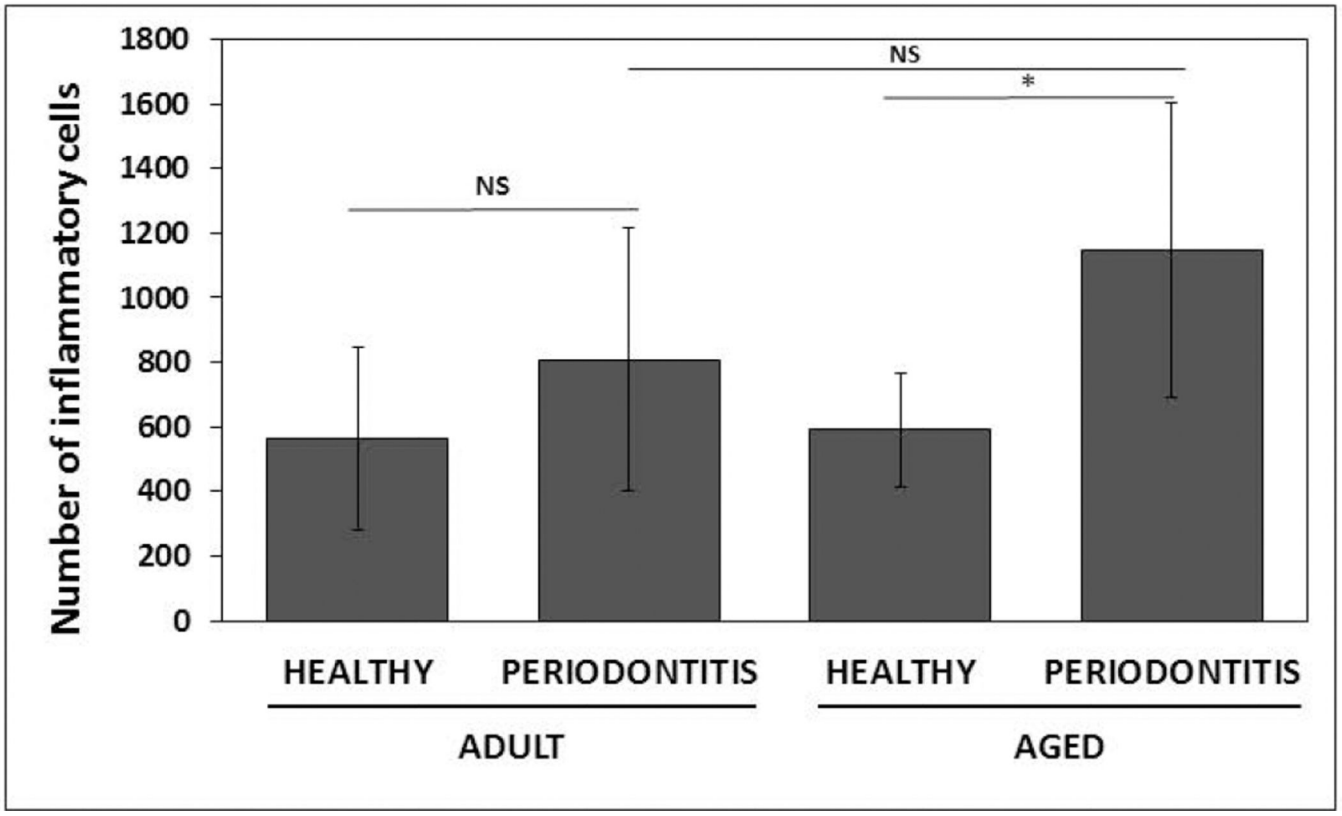

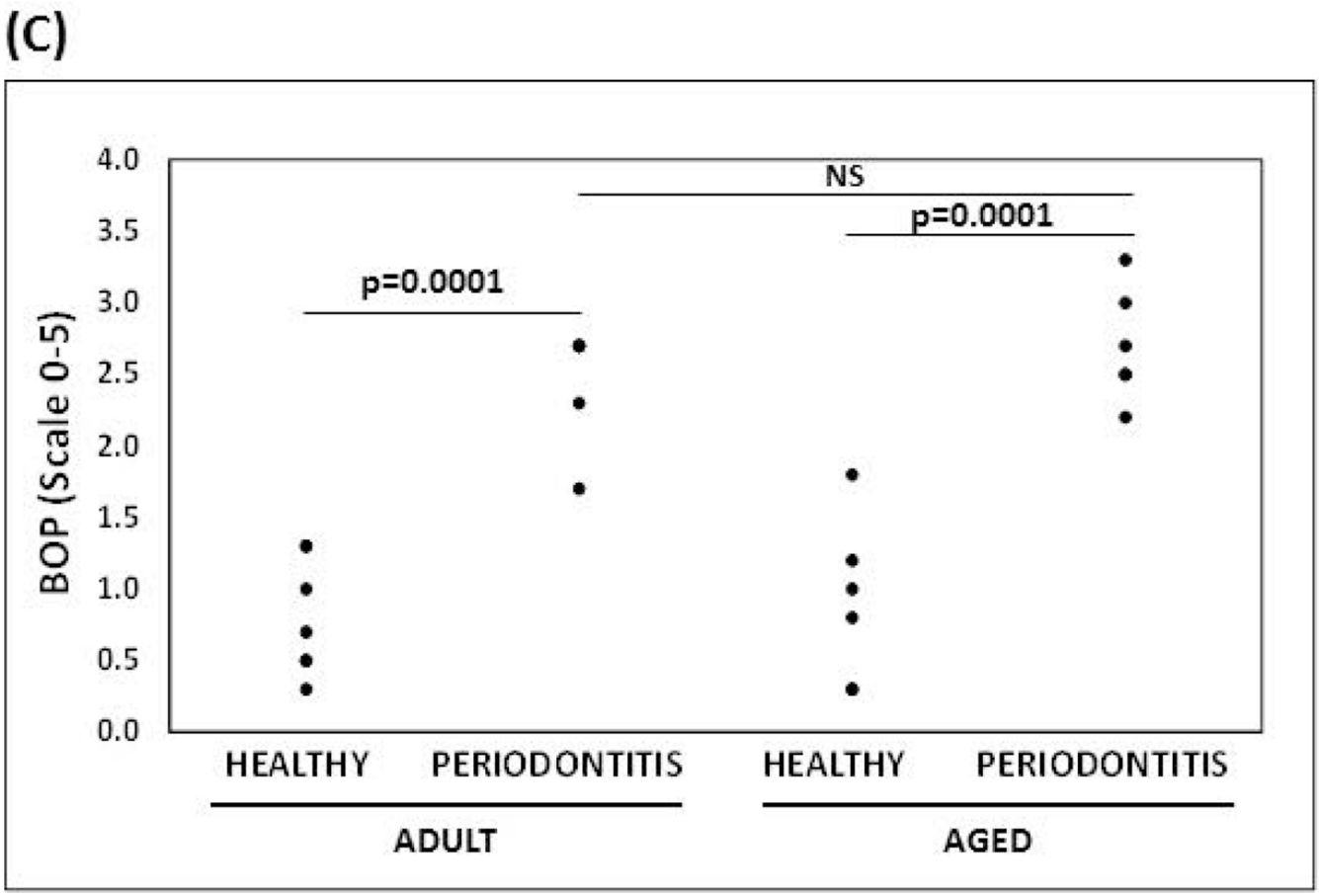

Figure 3. Histological and clinical measures of periodontal inflammation

(A) Micrographs of gingival biopsies from adult and aged animals with and without periodontitis stained with Hematoxylin and Eosin (H&E) illustrating cellularity at 100× and 200× magnification. Inflammatory cells are showing by black arrows **(B)** Scores of inflammatory infiltrate from healthy and periodontitis gingival biopsies (n=4/group) from adult and aged animals. *p≤0.05 healthy compared to periodontitis gingival tissues as determined by Student's *t* test. **(C)** Bleeding on probing scores (scale 0–5) from healthy and periodontitis sites from adult and aged animals (n=5–6) from which gingival biopsies were taken.

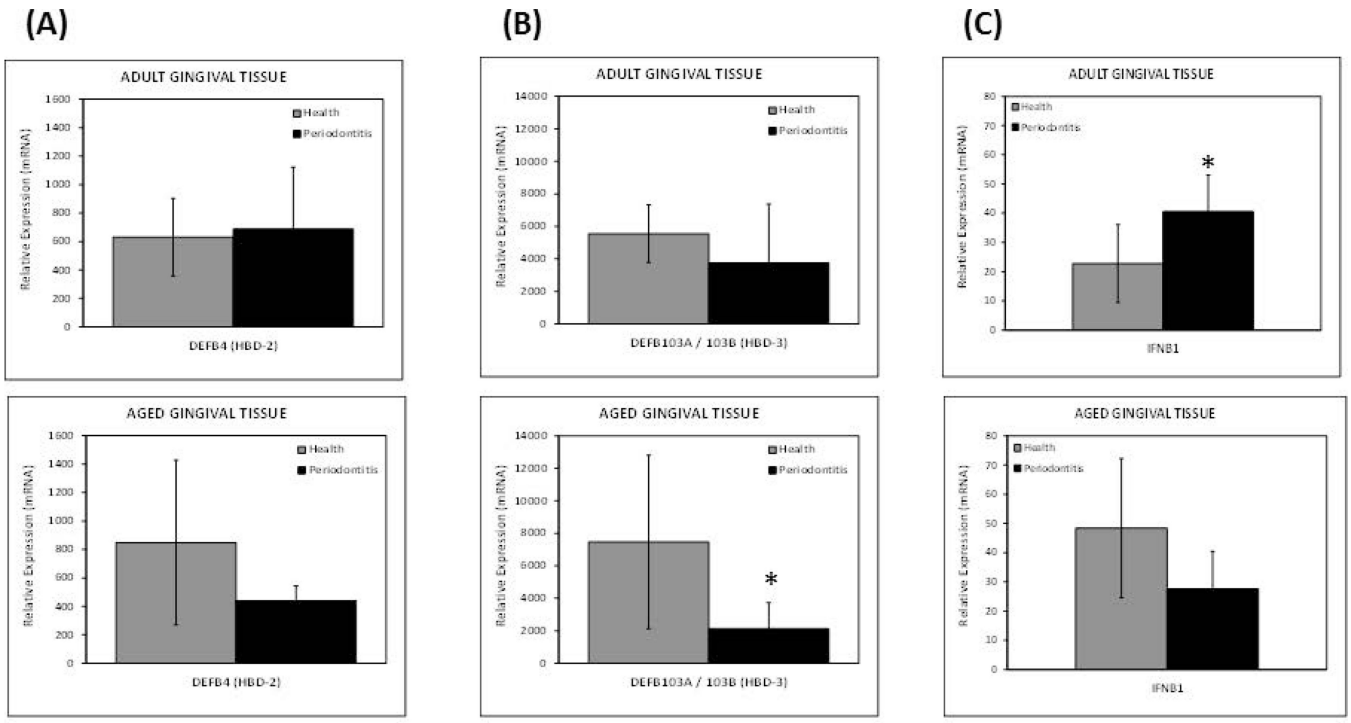

Figure 4. Expression levels of antimicrobial genes in healthy and inflamed/periodontitis gingival tissues

The results shown are the Means ± Standard Deviation of gene expression for **(A)** hBD2, **(B)** hBD3, and **(C)** IFNB1, of healthy and inflamed/periodontitis gingival tissues obtained from 5–6 animals per each age (*i.e.*, adult or aged non-human primates). Each sample was individually analyzed by microarray. *p≤0.05 healthy compared to periodontitis gingival tissues as determined by Student's *t* test.

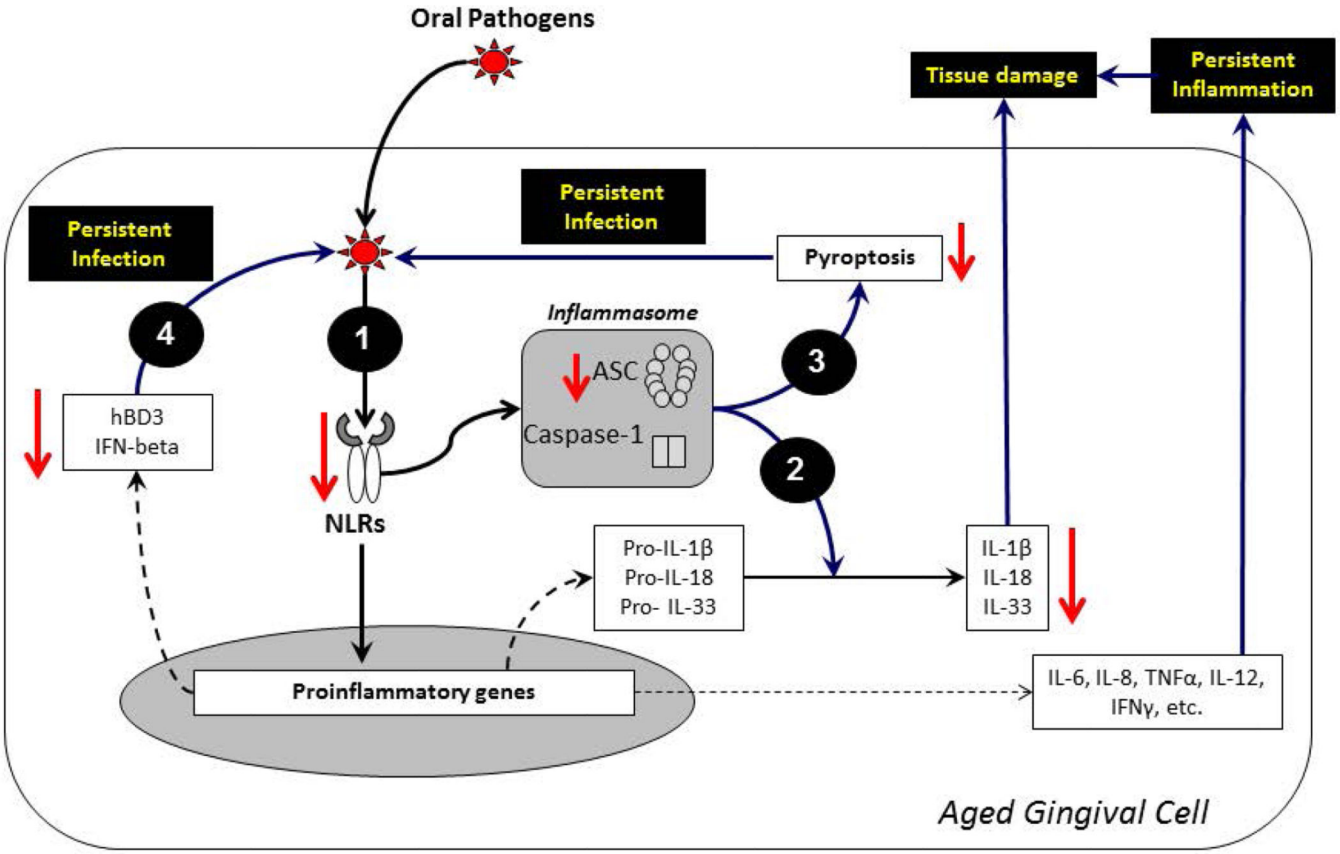

Figure 5. Schematic showing the potential impact of reduced expression of NOD-like receptors in persistent infection and inflammation of the oral mucosa during aging-related periodontitis

(**1**) An impaired expression of a subset of NLRs (*e.g.*, NLRPs 2, 5 and NOD2) and ASC during periodontitis in aged gingival tissues could lead to a weakened inflammasome activation which may drive: (**2**) mucosal tissue damage due to a decrease in the maturation and production of cytokines such as IL1β, IL-18, and IL-33 that are critical to maintain the mucosal integrity, and (**3**) persistent infection of invasive oral pathogens which could not be removed through pyroptosis (a central type of cell death to control intracellular pathogens). (**4**) Similarly, the aging-related decrease in NLRs expression during periodontitis could lead to persistent infection through reduced production of central innate antimicrobial factors (*e.g.*, hBDs and Type I IFNs) to protect oral mucosal tissues against invasive pathogens (*e.g.*, *P. gingivalis*). These molecular changes of the immunoinflammatory response with aging could increase the risk for oral infection/inflammation in aged individuals.

TABLE 1

NOD-like receptors (NLR), inflammasome-related genes, and antimicrobial genes evaluated by microarray in gingival tissues and their corresponding Probe Identification numbers.

NOD-LIKE RECEPTORS	Gene ID	Probe No.
NLR family, MHC class II transactivator	NLRA/CIITA	MmugDNA.20.1.S1_at
NLR family, apoptosis inhibitory protein	NLRB/NAIP	MmugDNA.16377.1.S1_at
NLR family, CARD domain containing 1	NLRC1/NOD1	MmugDNA.33152.1.S1_at
NLR family, CARD domain containing 2	NLRC2/NOD2	MmuSTS.3541.1.S1_at
NLR family, CARD domain containing 4	NLRC4/IPAF	MmugDNA.42771.1.S1_at
NLR family, CARD domain containing 5	NLRC5	MmugDNA.30929.1.S1_at
NLR family, pyrin domain containing 1	NLRP1	MmugDNA.34857.1.S1_at
similar to NACHT-, LRR- and PYD-containing protein 2 (PYRIN-containing APAF1-like protein 2)	NLRP2	MmugDNA.16291.1.S1_s_at
NLR family, pyrin domain containing 3	NLRP3	MmugDNA.4850.1.S1_at
NLR family, pyrin domain containing 4	NLRP4	MmugDNA.39919.1.S1_at
NLR family, pyrin domain containing 5	NLRP5	MmuSTS.1762.1.S1_at
NLR family, pyrin domain containing 6	NLRP6	MmugDNA.28440.1.S1_at
NLR family, pyrin domain containing 7	NLRP7	MmugDNA.41819.1.S1_at
NLR family, pyrin domain containing 8	NLRP8	MmugDNA.11176.1.S1_at
Similar to NLR family, pyrin domain containing 10	NLRP10	MmugDNA.41661.1.S1_at
NLR family, pyrin domain containing 11	NLRP11	MmugDNA.34108.1.S1_at
NLR family, pyrin domain containing 12	NLRP12	MmugDNA.25405.1.S1_at
NLR family, pyrin domain containing 13	NLRP13	MmugDNA.30103.1.S1_at
NLR family, pyrin domain containing 14	NLRP14	MmuSTS.1761.1.S1_at
NLR family member X1	NLRX1	MmugDNA.20879.1.S1_at
INFLAMMASOME-RELATED GENES	**Gene ID**	**Probe No.**
absent in melanoma 2	AIM2	Mmu.10556.1.S1_at
RIG-I-like receptor/ DEAD box polypeptide 58	RIG-I	MmugDNA.19189.1.S1_at
similar to PYD and CARD domain containing isoform b	ASC	MmuSTS.3453.1.S1_at
caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	CASPASE-1	MmugDNA.31375.1.S1_s_at
Interleukin 1, beta	IL-1B	MmuSTS.652.1.S1_at
Interleukin 18 (interferon-gamma-inducing factor)	IL-18	MmuSTS.2541.1.S1_at
Interleukin 33	IL-33	MmugDNA.20693.1.S1_at
ANTIMICROBIAL GENES	**Gene ID**	**Probe No.**
Defensin, Beta 4A/Human beta defensin-2	DEFB4	MmugDNA.33367.1.S1_at
Defensin, Beta 103B/Human beta defensin-3	DEF103A/103B	MmugDNA.18488.1.S1_at
Interferon, Beta 1	IFNB1	MmuSTS.625.1.S1_at