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Engagement of specific innate immune signaling pathways during *Porphyromonas gingivalis* induced chronic inflammation and atherosclerosis

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1. ABSTRACT

Toll-like receptors (TLRs) are a group of pathogen-associated molecular pattern receptors, which play an important role in innate immune signaling in response to microbial infection. It has been demonstrated that TLRs are differentially up regulated in response to microbial infection and chronic inflammatory diseases such as atherosclerosis. The expression of TLRs are markedly augmented in human atherosclerotic lesions and this occurs preferentially by endothelial cells and macrophages in areas infiltrated with inflammatory cells. Furthermore polymorphisms in the human gene encoding one TLR receptor (TLR4) which attenuates receptor signaling and diminishes the inflammatory response to Gram-negative pathogens, is associated with low levels of certain circulating mediators of inflammation and a decreased risk for atherosclerosis in humans. Recent advances have established a fundamental role for inflammation in mediating all stages of atherosclerosis. However, the triggers that initiate and sustain the inflammatory process have not been definitively identified. Although definitive proof of a role of infection contributing to atherogenesis is lacking, multiple investigations have demonstrated that infectious agents evoke cellular and molecular changes supportive of such a role. Evidence in humans suggesting that periodontal infection predisposes to atherosclerosis is derived from studies demonstrating that the periodontal pathogen *Porphyromonas gingivalis* resides in the wall of atherosclerotic vessels and seroepidemiological studies demonstrating an association between pathogen-specific IgG antibodies and atherosclerosis. Our recent work with P. gingivalis has demonstrated the effectiveness of specific intervention strategies (immunization) in the prevention of pathogen-accelerated atherosclerosis. We have also established that the inflammatory signaling pathways that P. gingivalis utilizes is dependent on the cell type and this specificity clearly influences innate immune signaling in the context of local chronic inflammation versus distant chronic inflammation. We postulate that bacterial infection mediates inflammatory responses that involve specific innate immune pathways in defined host cells. Furthermore, these inflammatory responses can be correlated with atherosclerosis and ultimately thrombotic complications.

2. INTRODUCTION

Periodontal diseases comprise a group of inflammatory diseases of the gingiva and supporting structures of the periodontium. It has been suggested that greater than 100 million people in the US possess measurable periodontal bone loss (1, 2), making this disease one of the most common chronic infectious diseases of humans. Despite identification of over 500 different bacterial species in the oral cavity, it is now recognized that bacterial load does not necessarily result in establishment of periodontal disease; but rather the presence of a select few bacterial species resident in the subgingival niche are apparently periodontopathic (3), and include *Fusobacterium nucleatum*, *Tannerella forsythensis*, *Prevotella intermedia*, *Treponema denticola*, and *Porphyromonas gingivalis*. *P. gingivalis* is a Gram-negative anaerobe and is considered the principal agent associated with chronic generalized forms of periodontal disease. Currently, the underlying theme of understanding is that this organism, along with the host immune response to this organism is critical to the destruction of the supporting structures of the teeth (3). During periodontal health, the tissues adjacent to and beneath the gingival epithelium possess a modest accumulation of neutrophils. These cells are thought to be important in clearing transient bacteria that gain access to this site; however, in patients in the acute or active stage of periodontal disease, the periodontium presents with a significant neutrophilic cellular infiltrate that switches to a predominating monocytic and lymphocytic cellular infiltrate in chronic lesions. Understanding the complex cellular interactions that occur during periodontal disease is critical to defining which bacterial factors and inflammatory events might be responsible for this chronic inflammatory disease. Gingival crevicular fluid obtained from diseased sites possess elevated levels of interleukin (IL)-1-alpha, IL-8, and IL-10, the chemokine regulated upon activation normal T cell expressed and secreted (RANTES) (4), IL-6, transforming growth factor, prostaglandin E2 (PGE2), IL-2, tumor necrosis factor (TNF)-alpha, and interferon (IFN)-gamma (5). The mechanisms by which *P. gingivalis* stimulate cytokine and chemokine production are not well known, but recent *in vitro* studies have been performed with *P. gingivalis* as well as with purified antigens from this organism. Results from these studies have collectively determined that the host cell type, the number of bacteria, or amount of antigen being tested is critical to the reported observations.

Mounting evidence has accumulated supporting a role for periodontal disease and infection with *P. gingivalis* as a risk factor for several systemic diseases including, diabetes, pre-term birth, stroke, and atherosclerotic cardiovascular disease (6-9). Infection with *P. gingivalis* induces local inflammation in the oral cavity and this inflammatory response can lead to gingival ulceration and local vascular changes, which have the potential to increase the incidence and severity of transient bacteremias (10). Patients with periodontal disease have higher systemic levels of C-reactive protein (CRP) and IL-6 (11). *P. gingivalis*, has been detected in atherosclerotic plaque (12-15). Recent studies have identified *P. gingivalis* together with *Chlamydiophila pneumoniae*, another organism associated with accelerated atherosclerosis in atheromatous plaques by PCR (16). A recent report has demonstrated viable *P. gingivalis* in human atherosclerotic plaque (13). Despite these observations the precise molecular mechanisms by which *P. gingivalis* infection contributes to the progression of atherosclerosis and the links among lipids and innate immune and inflammatory responses are not well understood. Here we will focus on potential mechanisms by which *P. gingivalis* infection and the subsequent inflammation can lead to acceleration of atherosclerosis. We will focus on the role of defined *P. gingivalis* antigens as well as the specific host cells and signaling inflammatory pathways in these cells.

3. EPIDEMIOLOGICAL ASSOCIATIONS OF INFECTION WITH SYSTEMIC DISEASES

3.1. Infectious Agents and Association with Atherosclerosis

The notion that chronic infections influence or cause complex diseases such as cardiovascular disease has been proposed for over a century (17); however, this perspective has met opposition due in part to conflicting data and medical perceptions. The challenge of medical dicta regarding peptic ulcers by Drs. Robin Warren and Barry Marshall changed the medical perception due their seminal studies in the early 1980's demonstrating the cause of gastritis in the 'sterile' environment of the stomach was due to *Helicobacter pylori* infection rather than the result of stress or diet and was treatable with antibiotics (18). Even despite their contributions, the medical community was slow to adopt this position and it was not until the mid 1990s that antibiotic therapy was widely implemented for treatment of peptic ulcers. Interestingly, it now appears that *H. pylori* infection may influence other chronic disease including gastric carcinoma (19) and cardiovascular disease (20). Thus, these and other investigations have now provoked clinicians and basic researchers alike to consider the hypotheses that either unidentified organisms or sequelae of known diseases could significantly influence or cause diseases without apparent infectious etiology.

Based on the current consensus of the epidemiological, clinical, and experimental literature it appears that several chronic infectious diseases and / or the organisms responsible for causing these infections influence cardiovascular disease. Evidence in humans suggesting that infection predisposes to atherosclerosis is derived from studies that infectious agents reside in the wall of atherosclerotic vessels, and that an association between the pathogen-specific antibodies and atherosclerosis exists (21-24). Furthermore, several studies have reported detection of pathogen-specific 16S rRNA gene in blood vessel walls and atherosclerotic plaques, supporting that several of these putative candidate organisms gain access to the vasculature and localize to the vessel wall and atherosclerotic plaques (25, 26). However, it must be pointed out that an association with infectious disease may not exist, or alternatively is only weakly correlated. In addition, indirect consequences such as the

stimulated immune response or the genotype of the host as well as other undefined complexities of infection may play an important role in the mechanism underlying infection-accelerated atherosclerosis. Despite these conflicting data it remains that infection may represent an important set of risk factors for CVD. Those organisms / diseases best associated with atherosclerotic cardiovascular disease include *C. pneumoiae*, Cytomeglovirus infection, and periodontal disease.

The bulk of data to date support that individual pathogens, groups of pathogens, or the total number of infections incurred by an individual are associated with acceleration of atherosclerotic cardiovascular disease. In the multiple pathogen hypothesis the prognosis of patients with atherosclerosis has been independently correlated with the number of infections an individual has been exposed and that this cumulative burden contributes to the progression of carotid atherosclerosis (22), and CVD independent of classical risk factors (27). In a prospective population based study on the pathogenesis of atherosclerosis Kiechl *et al.* (28) determined that chronic infections accentuated the risk for atherosclerosis. Among subjects with chronic infection, atherosclerosis risk was highest in patients with elevated levels of soluble adhesion molecules, circulating bacterial endotoxin, and elevated levels of soluble human HSP60. CRP levels and infectious burden have also been associated with atherosclerosis risk in young women (29). Neureiter *et al.* (30) evaluated the detection of *C. pneumoniae* and *H. pylori* in normal carotids versus atherosclerotic carotids and compared these findings with serology, plaque morphology, apoptosis, and inflammatory cell infiltrate. In this study *C. pneumoniae* was found more frequently in atherosclerotic than in normal carotids, and this correlated with elevated *C. pneumoniae* IgG in the sera of diseased individuals (30). Although *H. pylori* was not detected in carotids, elevated *H. pylori* antibody levels were associated with the degree of atherosclerosis. Based on these observations the authors concluded that *C. pneumoniae* contributes to increased inflammation in coronary heart disease and that this contribution is even more pronounced when present in combination with *H. pylori* IgA antibodies. In addition to evoking pro-inflammatory signaling that may influence systemic disease, one mechanism by which multiple pathogens stimulate atherosclerosis could be the result of inflicting damage to the endothelial cell resulting in the endothelial cell dysfunction (31).

3.2. Periodontal Disease and *P. gingivalis*

Generalized forms of periodontal disease are characterized by chronic inflammation in the periodontium at sites of alveolar bone resorption and in severe cases this can lead to early loss of dentition (32). Several case control studies have concluded that there is correlation between cardiovascular disease and periodontal disease after adjusting for confounding factors including cholesterol levels, smoking, hypertension, social class, and body mass index (15, 24, 33). However, in a self-report patient study this association was not observed (34). Recently, in renal transplant patients it was reported that gingivitis, in the absence of systemic inflammation, was associated with atherosclerosis (35). In addition, tooth number has been reported to be inversely related to aortic valve sclerosis (36). Results from the Oral Infections and Vascular Disease Epidemiology Study (INVEST) investigating the relationship between periodontal bacteria and early atherosclerosis focusing on intima-media thickness revealed that of 657 subjects there was association between periodontal pathogens and sub-clinical atherosclerosis (37). Employing radiographic analysis of periodontal disease Engebretson *et al.* (38) reported that severe periodontits was independently associated with carotid atherosclerosis. In addition to these clinical studies, further evidence of linkage comes from reports that *P. gingivalis*-specific DNA has been detected in atherosclerotic plaque (14, 39). In addition to direct result of infection, it has been suggested that the inflammation accompanying *P. gingivalis* infection may be responsible for the association of this organism with acceleration of atherosclerosis. Indeed, periodontal disease could promote chronic low level bacteremia, this in turn could elevate white cell counts, and the accompanying systemic endotoxemia could affect endothelial integrity, metabolism of plasma lipoproteins, blood coagulation, and platelet function. Patients with periodontal disease have higher systemic levels of CRP and IL-6 (33, 40). Thus, in the oral cavity this inflammatory response could facilitate gingival ulceration and local vascular changes, which in turn would have the potential to increase the incidence and severity of transient bacteremias. The systemic consequences of periodontal infection may increase the risk for vascular thromboembolic events, such as that seen with acute myocardial infarction and stroke (41). Recently, Amar *et al.* (42) reported that periodontal disease was associated with aberrant vasomotor function, supporting that periodontal disease affects endothelial cell function. Interestingly, treatment of periodontal disease has been reported to improve endothelial dysfunction (43).

In addition to clinical studies several groups using various animal model systems have reported that mono-microbial challenge of these animals with *P. gingivalis* stimulates acceleration of atherosclerosis (44-48). Furthermore a number of *in vitro* studies have documented the potential of *P. gingivalis* to induce robust inflammation responses in defined host cells. These are discussed in detail below.

4. INNATE RESPONSE OF RELEVANT HOST CELLS *IN VITRO* TO *P. GINGIVALIS*

4.1. Oral epithelial cells

Sulcular and junctional epithelium, represents one of the initial host barriers to *P. gingivalis* when this organism is present in the gingival sulcus. Several studies have begun to characterize the host response of oral epithelial cells to *P. gingivalis*. Challenge of oral epithelial cells with *P. gingivalis* elicits a TNF-alpha and IL-1beta response. Studies have shown that these cells also express cell adhesion molecules on their surface in response to *P. gingivalis* antigens and include Intracellular Adhesion Molecule (ICAM-1) and Vascular Cell Adhesion Molecule (VCAM-1) (49). The innate immune receptor, toll-like Receptor (TLR2), but not TLR4 or CD14, has been

implicated in *P. gingivalis* FimA protein stimulation of human gingival epithelial cells (50, 51). TLR4 polymorphisms Asp299Gly and Thr399Ile polymorphisms have been reported to render hyporesponsiveness of oral epithelial cells to *P. gingivalis* (52). Employing an oral mucosal model, expression of matrix metalloproteinase (MMP)2 and MMP9 have been shown to be upregulated in response to *P. gingivalis* providing for a mechanism of tissue destruction mediated by MMPs (53). Interestingly MMPs are also linked to atherosclerotic plaque rupture (54), and this may represent an important link between these two diseases. Work by Darveau *et al.* (55) has demonstrated that gingival epithelial cells challenged with *P. gingivalis* LPS fail to produce IL-8; furthermore, *P. gingivalis* LPS stimulation functions as a potent inhibitor of subsequent *E. coli* LPS stimulation of IL-8. In addition to these antagonistic properties, *P. gingivalis* infection of gingival epithelial cells can inhibit IL-8 production in response to other bacteria present in dental plaque including *F. nucleatum* (55). This mechanism has been coined "localized chemokine paralysis". The nature of this inhibition is not well understood, but appears to relate to the heterogeneity of *P. gingivalis* LPS and the varied ability of the gingival epithelial cells to recognize these variants of *P. gingivalis* LPS. *P. gingivalis* also appears to travel from cell to cell without passing through extracellular spaces (56), and this mechanism may play an important role in tissue invasion by this pathogen.

4.2. Polymorphonuclear leukocytes (neutrophils)

Anatomically, healthy individuals possess a layer of neutrophils that reside immediately below the epithelium of the gingival sulcus that likely perform immune surveillance and quickly clear bacteria that enter the sub-epithelial tissues. During the switch from periodontal health to disease, these infected sites typically present with an influx of PMNs. *In vitro*, human PMNs challenged with live *P. gingivalis* produce the pro-inflammatory cytokines TNF-alpha, IL-1beta, and IL-6. PMNs also express IL-10 (a cytokine commonly associated with regulation of the host immune response) in response to *P. gingivalis*. More recent studies employing purified *P. gingivalis* fimbriae, LPS and gingipains have demonstrated that these virulence factors alone influence host response. *P. gingivalis* LPS up-regulates complement receptor 3 expression on PMNs (57), as well as stimulating expression of TNF-alpha, IL-1beta, and IL-8 (58). Purified fimbrillin monomer (43-kDa protein), possesses potent cytokine and chemokine stimulating activities (59). Recent studies using defined gingipain mutants of *P. gingivalis* indicate that gingipains down regulate the transcription of genes responsible for chemokine production (60). Harokopakis and Hajishengallis (61) reported that neutrophils respond to *P. gingivalis* FimA in a TLR2-dependent manner and that this recognition augments CD11b-CD18 and that CD14 facilitates recognition of *P. gingivalis* FimA protein.

4.3. Dendritic cells

A relatively new area of investigation is the role that dendritic cells (DCs) may play in *P. gingivalis*-mediated periodontal disease. Studies by Cutler *et al.* (62) suggest that there is an aggregation of dendritic cells and T-cells at sites of periodontal damage. Aroonrerk *et al.* (63) demonstrated that autologous DCs generated from human mononuclear cells act as potent antigen presenting cells (APCs), presenting *P. gingivalis* antigens to T cell clones. Human monocyte-derived DCs respond to *P. gingivalis* antigens such as LPS by expressing a variety of inflammatory mediators including TNF-alpha and IL-10 (albeit to a lesser extent than *E. coli* LPS), yet these cells failed to express IL-12 or IP-10. *P. gingivalis* LPS-pulsed human monocyte-derived DCs elicited IFN-gamma, IL-2, and IL-5 (64). Notably, IL-13 was elaborated from naive CD4+ T cells in response to *P. gingivalis* LPS-pulsed MDDC, supporting that *P. gingivalis* LPS polarizes human monocyte-derived DCs to induce Th2 responses (64). Similar results were reported by Kanaya *et al.* (65) showing that *P. gingivalis* LPS evoked CD14, CD16, soluble CD14, elevated CD40, CD80, CD83 and CD86 albeit less than *E. coli* LPS, and IL-6, IL-8, IL-10, IL-12 and RANTES production from DC and T cell proliferation. The role of fimbriae in the ability of *P. gingivalis* to gain entry into human DC and subsequent stimulation has also been assessed. It was observed that fimbriae were necessary for efficient entry of *P. gingivalis* into DCs (66). DCs cultured with wild type *P. gingivalis* underwent maturation, express elevated levels of the co-stimulatory molecules HLA-DR, CD80, CD83, and CD86, as well as the pro-inflammatory mediators IL-1, IL-6, TNF-alpha, IL-10, and IL-12 (66). In an autologous mixed lymphocyte reaction (MLR) wild type *P. gingivalis* evoked a more robust MLR and with a Th1 response as evidenced by elevated IFN- gamma (66). Interestingly recombinant FimA protein stimulated DC maturation, and evoked autologous CD4+ IFN- gamma secretion in MLR (66). Collectively these data support that DCs respond specifically to *P. gingivalis* and due to their ability to act as APCs for *P. gingivalis* and *P. gingivalis* antigens it is apparent that these cells play an important role in the directing the host response to *P. gingivalis*. Moreover, the importance of TLRs, specifically TLR2 in DC response to *P. gingivalis* lipopeptide indicates that TLRs may be necessary for proper antigen presentation during the development of PD (67).

4.4. T-cells

Little is known about the T- cell-specific responses that occur during *P. gingivalis*-mediated periodontal disease. The number of T- cells present in periodontal lesions extracted from patients with adult periodontal disease are elevated as compared to normal subjects (68). The use of T cell clones established from periodontal disease patients has demonstrated that these cells produce MCP-1, MIP-1-alpha, and RANTES, and the cytokines IFN- gamma, IL-4 and IL-10 in response to *P. gingivalis* challenge, supporting the importance of IFN-GAMMA and a Th1-like response in the progression of periodontal disease (69). CD4+ T cell specific responses are critical to the progression of oral bone loss in response to *P. gingivalis* oral challenge, and mice deficient in IFN-gamma and IL-6 are also resistant to *P. gingivalis*-mediated oral bone loss (70, 71). On the basis of antibody data, it appears that a *P. gingivalis*-specific Th1 response predominates in humans during periodontal disease as compared with normal control subjects. What is not

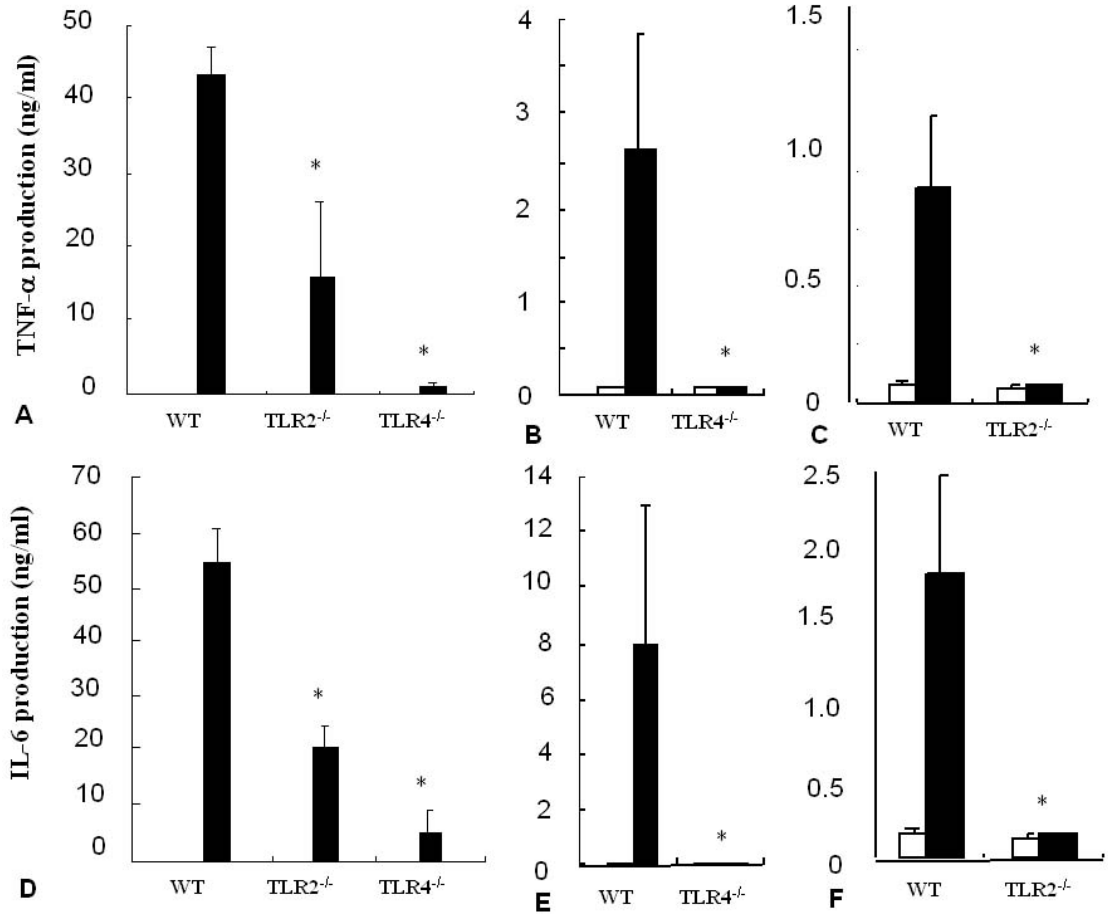

Figure 1. *P. gingivalis* fimbriae induces TNF-alpha and IL-6 production in macrophages via both TLR2 and TLR4 dependent pathways. Thioglycollate elicited peritoneal macrophages from C57BL-6 (WT), TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were cultured with *P. gingivalis* major fimbriae (10 - ml) (A,D)(black) prepared as described (76), *E. coli* LPS (100ng/ml) (B,E)(black), SLTA (2 µg/ml)(C,F) (black) or medium (A-F)(open) for 24hours. Culture medium were harvested for ELISA analysis of TNF-alpha and IL-6. Data are shown as the mean and SDs * = P<0.01 compared with the stimulated WT macrophages.

known; however, is why this mechanism is not protective. It is intriguing to speculate that modification of the host T cell-mediated immune response, could serve to limit human periodontal disease progression.

4.5. Monocytes

Monocytes represent a primary cell population present in chronic periodontal lesions. A number of studies have focused on the use of both human and murine monocytic cells, and despite some differences which may be attributable to the different strains used, the trend is that *P. gingivalis* stimulation of monocytes leads to the expression of TNF-alpha, IL-1beta, MCP-1, IL-6, IL-8, nitric oxide, as well as PGE2. After the initial interaction of *P. gingivalis* fimbriae with macrophages, there is the rapid induction of the neutrophil chemotactic factor and protein kinase C (72, 73). Associated with this interaction is the formation of a 68-kDa phosphorylated protein within the confines of the macrophage, the induction being mediated by prior induction of protein kinase C. We have also observed that human plastic adherent peripheral blood monocytes cultured with wild type *P. gingivalis* 381 elicited a more robust IL-8 response as compared with that elicited by a FimA- mutant (74). Employing antibody protein arrays Zhou *et al.* (75) reported that the host response of macrophages to *P. gingivalis* is complex and that many of the mediators observed in *P. gingivalis* challenged macrophages are detected in human periodontal lesions. Moreover, it was shown that *P. gingivalis* LPS and FimA elicit robust chemokine profiles that differ from than of whole organism (75). These data support that fimbriated *P. gingivalis* stimulate monocytes to produce chemokines that may signal the recruitment of inflammatory cells to the site of *P. gingivalis* infection.

4.6. *P. gingivalis* interactions with macrophages-*P. gingivalis* fimbriae activates macrophages in a TLR2 and TLR4-dependent manner

Much more is needed to determine the nature of the interaction of *P. gingivalis* with macrophages; however, our data support that *P. gingivalis* elicits an innate immune response from human monocytes that is consistent with that observed during the development of atherosclerosis. We previously determined that in murine macrophages the IL-

and TNF-alpha response to the *P. gingivalis fimA*- mutant (DPG3) was less than that observed with the wild-type *P. gingivalis* strain 381 (manuscript in preparation). We thus assessed the stimulatory effect of purified fimbriae on macrophage pro-inflammatory cytokine production, and examined the role of TLR2 and TLR4 in this response. For these studies we isolated the major fimbriae from, the minor fimbriae mutant *P. gingivalis* MF1 as described previously (76). Fractions were analyzed by 12% SDS-PAGE, and those containing fimbriae were pooled and dialyzed against 5 mM Tris buffer, pH 8.0. Fimbriae were then concentrated utilizing Centriprep centrifugal filter devices (Millipore) and quantitated by the Lowry protein estimation method. Fimbriae preparations were analyzed for LPS contamination through electrophoresis on polyacrylamide gels and visualized with Silver Stain Plus (Bio-Rad). In addition, each fimbriae preparation was verified to be LPS free by the *Limulus* amebocyte lysate assay (<0.1 endotoxin U ml^{-1}; Cambrex; data not shown). Peritoneal macrophages were harvested from C57BL-6 (WT), $TLR2^{-/-}$ and $TLR4^{-/-}$ mice and were cultured in medium alone, or medium containing purified fimbriae protein (10µg/ml). We observed that WT macrophages cultured with the fimbriae secreted a significant amount of TNF-alpha and IL-6 (Figure 1A and 1D). $TLR2^{-/-}$ mouse macrophages expressed significantly less TNF-alpha and IL-6 as compared with WT macrophages (Figure 1A and 1D). $TLR4^{-/-}$ macrophages cultured with fimbriae protein expressed even less TNF-alpha and IL-6 than the $TLR2^{-/-}$ macrophages Figure 1A and 1D. As expected $TLR4^{-/-}$ and $TLR2^{-/-}$ mouse macrophages failed to respond to *E. coli* LPS and *S. aureus* LTA, respectively Figure 1B, 1C, 1E and 1F). These results demonstrate that fimbriae stimulates pro-inflammatory cytokines repertoire and that the macrophage response to fimbria is dependent on both TLR2 and TLR4. Signaling through TLR2 appears to require additional accessory proteins as demonstrated in transfected cell lines (77). A separate report has also documented the ability of the FimA protein of *P. gingivalis* to activate monocytes and demonstrated that TLR2 is responsible for FimA-dependent monocyte activation (61).

4.7. *P. gingivalis* stimulates TLR expression on macrophages

Recent studies have demonstrated that TLRs are regulated in response to microbial products (78). To determine if *P. gingivalis* could alter TLR expression on macrophages, we stimulated macrophages with *P. gingivalis,* LPS or fimbriae and measured TLR expression. *P. gingivalis* LPS was purified as previously described (76). We found that cell surface TLR2 was increased in response to live *P. gingivalis* (data not shown). We also found the *P. gingivalis* LPS and fimbriae stimulated TLR2 expression (Figure 2-upper panels). Neither *P. gingivalis* fimbriae nor LPS appeared to up regulate TLR4 in mouse macrophages (Figure 2-lower panels); rather we observed a slight decrease in TLR4 expression. We also found the increased TLR2 expressed on the surface of mouse macrophages following stimulation with fimbriae was functionally able to induce a TNF-alpha response following subsequent challenge with live *P. gingivalis* or SLTA (Figure 3). We also observed a down regulation of the response to *E. coli* LPS indicating down regulation of TLR4. Similar results were obtained in human monocytes following stimulation with fimbriae (data not shown). We observed that there were no obvious changes in TLR2 and TLR4 transcript levels in mouse macrophages following stimulation with fimbriae (Figure 4). Based on these results we postulate that the increase in cell surface expression of TLR2 may result from the transport of preformed TLR2 to the cell surface. In future studies we will examine the requirement for the uptake of fimbriae into the cell for the observed increase in cell surface TLR2. *P. gingivalis* are found in vacuoles within the cell where they can potentially interact with intracellular TLRs (79). It is possible that as a part of trafficking within vacuoles that TLRs can be transported to the cell surface. Likewise the uptake of fimbriae into phagocytic and non-phagocytic cells would suggest that fimbriae may also interact with intracellular TLRs, although what happens to fimbriae once it is taken up by cells is not known. We have demonstrated the inhibitory effect of cytochalasin D on MCP-1 and IL-8 production by endothelial cells in response to fimbriae suggesting that internalization is necessary for the observed responses (95). Collectively these results indicate macrophages respond to fimbriae stimulation by increasing cell surface expression of TLR2 which can then respond to other TLR2 ligands. The slight decrease in TLR4 following fimbriae stimulation may result from the induction of tolerance in these cells.

The phenomenon of tolerance has been well described for endotoxin and TLR4 (80). Several groups have shown that pre exposure of cells to endotoxin induces suppression of a variety of pro-inflammatory responses when a second endotoxin challenge is administered. This includes inhibition of pro-inflammatory cytokine production, such as TNF, IL-1, and IL-6, as well as other inflammatory mediators such as cyclooxegenase-2 activation, mitogen-activated protein kinase (MAPK) activation, and impaired translocation of NF-kappabeta. Muthukuru *et al* (81) have also demonstrated that human macrophages respond to an initial stimulus of *P. gingivalis* LPS or *E. coli* LPS by upregulation of TLR2 and TLR4 mRNA and protein. However restimulation of macrophages with either *P. gingivalis* LPS or *E. coli* LPS was shown to down regulate TLR2 and TLR4 mRNA and protein. It has also been shown that cross-tolerance can be induced when primary and secondary stimuli are directed at parallel signaling pathways, as would be the case for Gram-positive and Gram-negative products that trigger different TLRs (78, 82). However little is known about tolerance of other TLRs (including TLR2) and in response to other TLR ligands.

4.8. *P. gingivalis* influences macrophage conversion to foam cell-like cell

The earliest defined pathological lesion observed in atherosclerosis is the fatty streak. Lipid-engorged cells, primarily macrophages, which make up this lesion, characterize the fatty streak. The accumulation of macrophage foam cells is the hallmark of the fatty streak, yet this lesion type does not cause clinical consequences; rather, the accumulation of macrophages within the arterial intima sets the stage for progression of atheroma to more

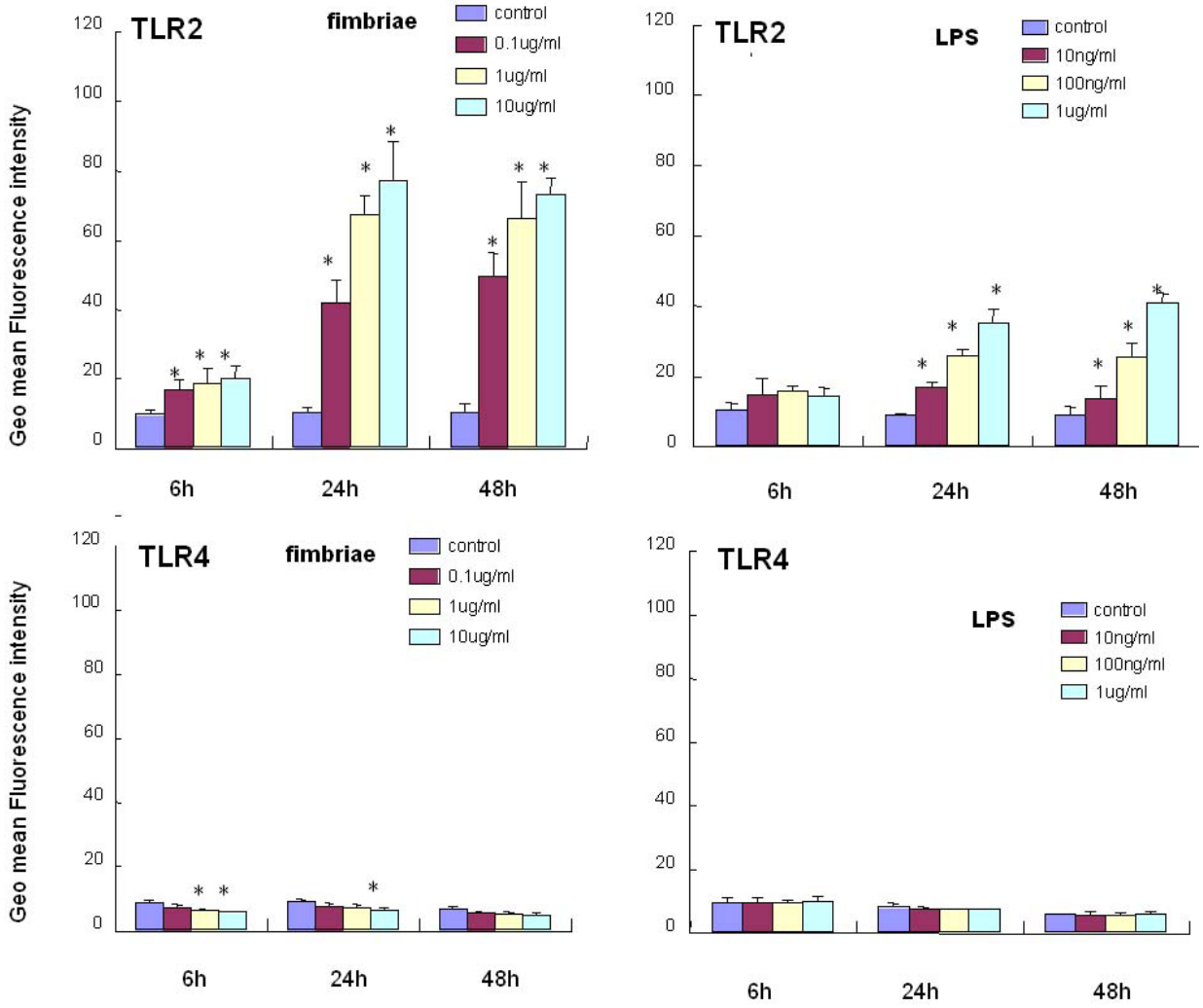

Figure 2. *P. gingivalis* fimbriae and LPS regulate TLR2 expression on mouse macrophages. Thioglycollate elicited peritoneal macrophages from C57BL-6 mice were cultured with medium alone (control), or *P. gingivalis* fimbriae (left panel) or *P. gingivalis* LPS (right panel) at the indicated concentrations and temporal expression of TLR2 (top panel) and TLR4 (bottom panel) were detected by FACS. *P. gingivalis* LPS was purified as previously described (76). Data is expressed as the geo-mean fluoresce intensity and SDs * = $p<0.01$ compared with medium control alone.

complicated plaques that may indeed cause disease (83). The stimuli that evoke foam cell formation are poorly understood; however, a balance between lipid loading and lipid transport out of the cell (cholesterol efflux) govern this process (84). Passive diffusion was thought to be the principal mechanism governing cholesterol accumulation in the vascular wall. Indeed, LDL levels in these tissues can reach 100 mg/dL (85). More recently, the non-specific hypothesis of LDL accumulation in cells has lost favor as a significant number of studies have identified that accumulation of lipids inside cells such as macrophages by scavenger receptor (SR)-mediated uptake of modified LDL species (85). SRs are a broad group of surface expressed proteins that function as pattern-recognition receptors (86). Brown and Goldstein (87) coined the term SR based on the scavenging role of these receptors for uptake of modified LDL by macrophages. Subsequent cloning has revealed that SRs are trans-membrane, multi-domain structures. SRs are regulated on the surface of macrophages in response to stimulation, and demonstrate broad ligand specificity for polyanionic molecules (86). In addition to host derived LDL, SRs are capable of binding Gram-negative and Gram-positive bacteria, bacterial structures such as LPS and LTA, as well as advanced glycation end-products (86). The most notable SRs (SR-A and CD36) participate in lipid accumulation. Furthermore, as demonstrated by knockout animal models, approximately 80% of ac-LDL and 50% ox-LDL uptake is mediated by SR-A (88). CD36 is a principal receptor for ox-LDL (89). Generation of mice lacking SR-AI / SR-AII and CD36 demonstrate that SR-A and CD36 account for approximately 90% of macrophage uptake of ac-LDL and ox-LDL (90). SRs have been detected in human atherosclerotic lesions supporting that these molecules play

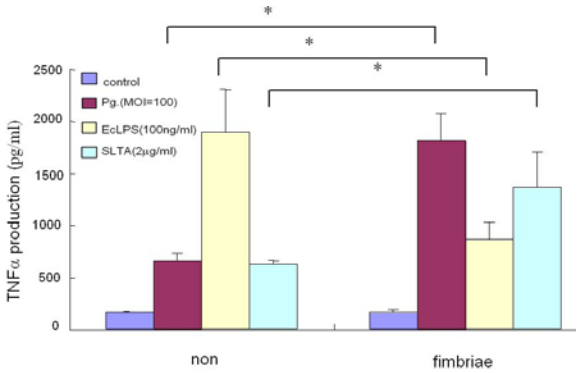

Figure 3. *P. gingivalis* fimbriae prime mouse macrophages making them responsive to TLR2 ligands. Thioglycollate elicited peritoneal macrophages from C57BL-6 mice were un stimulated (non) or stimulated with *P. gingivalis* fimbriae (1.0 μg/ml) for 6 h, washed, and incubated with medium, *P. gingivalis*, *E. coli* LPS, *S. aureus* lipoteichoic acid (SLTA) for 3 h, as indicated, and TNF-alpha production was assessed by ELISA. *p <0.05, significant difference.

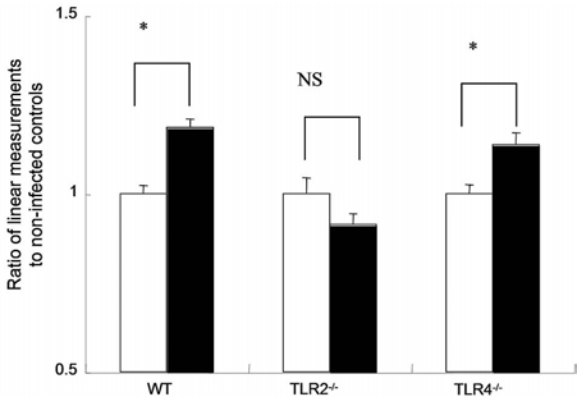

Figure 4. Temporal assessement of TLR2 and TLR4 mRNA levels in mouse macrophages stimulated with *P. gingivalis* major fimbriae. Thioglycollate-elicited peritoneal macrophages from C57BL-6 mice were cultured with medium alone (control) or 1 ug/ml of *P. gingivalis* 381 major fimbriae, and TLR-specific mRNA levels were analyzed by reverse transcriptase-PCR at 1, 3, 6, 24 and 48 hours. beta -actin served as a loading control. Primers for TLR2 and TLR4 and beta-actin were as described (44).

a role in atherosclerosis (91). Emerging data suggest that *C. pneumoniae* and *P. gingivalis* stimulate foam cell formation (92-94), and it is supported that surface antigens such and LPS, heat shock proteins, and fimbria participate in this process (92, 94, 95). However, in the context of challenge with bacteria such as *P. gingivalis*, the cellular events that lead to macrophage foam cell formation are poorly defined. One mechanism by which bacteria are now known to stimulate foam cells involves TLR2. *Cao et al.* (96) recently reported that murine macrophages deficient in TLR2 failed to develop into foam cells when challenged with *C. pneumoniae.* It will be interesting to determine if this TLR-dependent mechanism of macrophage foam cell formation holds true for *P. gingivalis*.

4.9. *P. gingivalis* interactions with endothelial cells

Because of the importance of the endothelial cell in the formation of an inflammatory atheroma, much work has focused on understanding the interactions of *P. gingivalis* or components of the organism with these cells. These studies have collectively established that *P. gingivalis* has the potential to accelerate atherosclerosis via specific stimulatory activities in these cells. *P. gingivalis* is invasive for HUVEC and has a requirement for fimbriae for the attachment to these cells and for invasion (97). In HUVEC active *P. gingivalis* invasion mediated via the major fimbriae stimulates surface expression of cell adhesion molecular expression as well as the expression of the chemokines IL-8 and MCP-1 (60, 97). Furthermore *P. gingivalis* can temporally modulate the chemokine response in endothelial cells through both fimbriae and gingipain mediated mechanisms (60). *P. gingivalis* has been reported to modulate the cytokine response in HUVEC and disrupts the adhesion activity through the cooperative action of the arginine and lysine specific cysteine proteases (Rgp and Kgp) (98). Choi *et al* (99) recently demonstrated that MCP-1 expression is up regulated in *P. gingivalis* infected HUVEC via reactive oxygen species, p38 kinase, JNK, NF-kappaB, and Ap-1.

We have been particularly interested in the specific interactions of *P. gingivalis* with human aortic endothelial cells (HAEC) because of the documented association of this organism with the acceleration of atherosclerosis. We previously demonstrated that *P. gingivalis* infection of HAEC with fimbriae expressing *P. gingivalis* results in increased cell surface expression of TLR2 and TLR4, and this enhances the response to TLR2 and TLR4 specific ligands (100). We have also established using high-density oligonucleotide micro arrays that the response to invasive *P. gingivalis* is highly specific to genes involved in inflammation. We observed the up regulation of 68 genes (101). Of note only 4 of these 68 genes were also up regulated in HAEC infected with the non-invasive *P. gingivalis fimA* mutant. Utilizing major and minor fimbriae mutants we demonstrated that major fimbriae are required for *P. gingivalis* invasion of HAEC and that only invasive *P. gingivalis* strains induced the production of pro-inflammatory molecules IL-1beta, IL-8, MCP-1, ICAM-1, VCAM-1 and E-selectin. We also demonstrated that following major fimbriae-mediated initial attachment, the minor fimbriae appear to play an important role in more intimate attachment facilitating endocytosis of the bacteria. The purified native forms of the major and minor fimbriae induced chemokine and adhesion molecule expression similar to invasive *P. gingivalis*, but failed to elicit IL-1beta production. We have also demonstrated that in HAEC, fimbriae can signal through TLR2 and TLR4 (manuscript in preparation). Finally we reported that *P. gingivalis* can antagonize the pro-inflammatory effects of fimbriae when cells are exposed simultaneously to both molecules (76).

The interactions of *P. gingivalis* with primary human coronary artery endothelial cells (HCAEC) has also been examined. Following invasion *P. gingivalis* can be found within autophagosomes and may use components of

the autophagocytic pathway as a means to survive within these cells (102, 103). Furthermore, *P. gingivalis* evades the endocytic pathway to lysosymes and instead traffics to the autophagosome (104). It has been postulated that the survival of *P. gingivalis* within these cells depends on the activation of autophagy, which provides a replicative niche for the organism (105). The hemagglutinin HagB appear to be required for the adherence of *P. gingivalis to* HCAEC (106). *P. gingivalis* also appears to differently regulate host genes during invasion of HCAEC (107). Rolling and the resulting adhesion of monocytes to endothelial cells are important in the development of the initiation of inflammation characteristic of atherosclerosis. It has been recently reported that live invasive *P. gingivalis* strain 381 increases the adhesion of mononuclear leukocytes to HAEC (108). These investigators also demonstrated that the non-invasive *fimA* strain DPG3 was unable to induce significant immune cell adhesion to HAEC. It has also been recently shown that *P. gingivalis* infection of HAEC induces enhanced tissue factor expression and decreased levels and activity of tissue plasminogen activator and enhanced plasminogen activator inhibtor-1 antigen and activity (109).

5. MODELS OF PATHOGEN-ACCELERATED ATHEROSCLEROSIS AND CHRONIC DISTANT INFLAMMATION

To date 4 working models have emerged to define the mechanisms governing pathogen-accelerated atherosclerosis and include: 1- Direct invasion of vascular endothelium; 2- Immunological sounding; 3- Pathogen trafficking; and 4- Autoimmunity. It is important to note that one or a combination of these different mechanisms may collectively contribute to pathogen-accelerated atherosclerosis. Direct invasion of and / or replication of a pathogen within endothelial cells *in vitro* have been demonstrated for *P. gingivalis* as discussed in detail above. In the immunological sounding model, local disease such as oral infection with *P. gingivalis* can signal systemic changes in the host inflammatory response via molecules secreted from the site of infection including: acute phase mediators, cytokines, and chemokines. Indeed, it is well established that atherosclerosis is an inflammatory disease, and it is feasible that persistent local infections, such as those in the oral cavity could promote atherosclerosis via chronic up-regulation of inflammatory cascades.

Conceptually similar to the second model, the third model to assess the role of infection as a risk factor for pathogen-accelerated atherosclerosis is via trafficking of pathogens from the local site of infection (such as the oral cavity) to the developing atheroma via inflammatory cells. In this model, as a result of the tissue damage caused by the infection and subsequent inflammatory response, the inflammatory cells present at the local infection ingest the pathogens, and upon re-emergence of these pathogen-laden inflammatory cells into the vasculature with subsequent localization at the site of developing atheroma may occur. The final hypothesis by which infectious agents may accelerate atherosclerosis is by stimulation of an autoimmune response via molecular mimicry. Molecular mimicry requires infection by a pathogen that possesses molecules with significant homology to a host structure. The host response is initiated against the pathogen; however, the response then presents as an autoimmune insult against those host tissues that possess these cross-reactive epitopes. Future studies will likely shed light in this important area of study regarding the mechanisms of infection-accelerated atherosclerosis.

6. ANIMAL MODELS OF *PORPHYROMONAS GINGIVALIS* INFECTION AND CHRONIC INFLAMMATION

6.1. Local Chronic Infection and Inflammation

No one animal model effectively mimics all aspects of host response / pathology observed in human periodontal disease. Thus numerous model systems including non-human primate, dog, rat, rabbit, mouse, hamster, and sheep have been successfully employed to model this disease (110). The seminal animal study defining *P. gingivalis* as a periodontal pathogen was performed in the non-human primate *Macaca fascicularis*. This study demonstrated that mono-microbial oral challenge of these non-human primates resulted in a rapid host adaptive response to *Bacteroides gingivalis* (*P. gingivalis*) with subsequent oral bone loss (111). More recently, murine models have become one of the principal animal systems employed to assess microbiological and immunological mechanisms governing host response to *P. gingivalis* challenge and virulence attributes of this organism. A variety of challenge models have been developed to take advantage of specific attributes of mice and to define the host response to challenge. These include subcutaneous injection abscess model (112), subcutaneous wire chamber challenge model (113), calvaria challenge model (114-116), intravenous challenge (112), and oral challenge model (117). Moreover, the explosion of available gene knockout and transgenic mice which have been generated have allowed for detailed mechanistic studies to be performed in these various models (Table 1).

The utility of the mouse for assessment of oral bone loss in response to *P. gingivalis* was first reported by Baker *et al.* (117). Since this time, this model has proven effective for assessing the contribution of host response to *P. gingivalis*-elicited oral bone loss as well as proven useful in studies assessing putative vaccine candidates for there ability to intercede in this process. An overwhelming consensus of the mouse knockout results support that the host innate immune response plays an important role in inflammation to *P. gingivalis* as knockout of genes generally results in modulation of endpoints such as expression of pro-inflammatory cytokines or immuno-regulatory cytokines and oral bone loss. Indeed, Baker *et al.* (70) reported that severe combined immune deficient (SCID) mice which do not possess functional B or T cells do not develop oral bone loss in response to *P. gingivalis* challenge. In this study, it was observed that CD4+ T cells played a significant role in regulating oral bone loss during *P. gingivalis* oral challenge and that IFN-GAMMA and IL-6 were important mediators of this process (70). Follow up

Table 1. Innate immune system knockout mouse models assessing host response to *P. gingivalis* challenge

Gene	Outcome relative to wild type mice	Study
iNOS	• Enhanced lesions and chamber rejection; • Greater numbers of *P. gingivalis* in chamber fluids; • Similar PGE_2, TLF-alpha, IL-1beta, IL-6 levels • Similar *P. gingivalis*-specific IgG levels • Increase in numbers of dead leukocytes in chamber fluid • Reduced soft tissue damage • Fail to develop oral bone loss	129 130 130, 131
IFN-gamma	• Attenuated inflammatory infiltrate in chambers • Blunted TNF-alpha and IL-1beta • Elevated IgG1 response Th2 dominant response • Less oral bone loss	132 70
TNFr-dKO	• Reduced fibroblast apoptosis • Reduced PMN recruitment • Reduced osteoclastogenesis	115
IL-6	• Less oral bone loss	70
IL-10	• Unchanged gingival levels of TNF-alpha or IL-1beta • Enhanced oral bone loss	133
IL-17	• Enhanced oral bone loss	120
CXCR2	• Reduction in oral bone loss	120
ICAM-1	• Reduction in oral bone loss	121
P-selectin	• Reduction in oral bone loss	121
CD18	• No reduction in oral bone loss	121
TLR2	• Reduced TNF-alpha, and IL-10 response in chamber fluids • Undetectable IL-1beta and IFN-gamma in chamber fluids • More rapid clearance of bacteria, no bacteremia • Resist oral bone loss	123
TLR4	• Elevated TNF-alpha response	123
SCID	• Reduction in oral bone loss • Effect localized to MHCII CD4+ T cell population	70
TCR-alpha chain	• No change in bacterial colonization • Reduced oral bone loss	118

studies have confirmed an important role for T cells in oral bone loss in mice orally challenged with *P. gingivalis* (71, 118). The anti-inflammatory cytokine IL-10 has been implicated in *P. gingivalis* elicited oral bone loss. Comparing oral bone levels from groups of wild type and IL-10-deficient mice challenged with bacteria associated with periodontal disease revealed that in the absence of IL-10 these organisms stimulated more robust oral bone loss (119). An interpretation of these results supports a role for IL-10 in down-regulating the inflammatory response which occurs during bacterially-elicited inflammatory oral loss. IL-17 (a mediator of granulopoesis and neutrophil trafficking) deficiency leads to greater oral bone loss than that observed in wild type mice orally challenged with *P. gingivalis* (120). CXCR2 mice displayed reduction in oral bone loss (120). In addition to soluble mediators, it has been determined that various components involved in cell-to-cell adhesion play a role in *P. gingivalis* oral bone loss. Mice deficient in ICAM-1 or P-selectin, but not CD18, all demonstrated reduction in oral bone loss (121).

6.2. TLR2 plays a role in *P. gingivalis*-elicited oral bone loss.

To examine the role of TLR2 and TLR4 in *P. gingivalis*-elicited oral bone loss we orally infected C57BL-6 (WT), $TLR2^{-/-}$ and $TLR4^{-/-}$ mice with *P. gingivalis* strain 381, as reported previously (117, 122) and examined oral bone loss in these mice. Following 3 oral challenges (10^9 CFU / challenge) with *P. gingivalis* over a one week period, and a 6 week rest period, all mice were sacrificed at 17 weeks of age and oral bone loss was determined by linear measurement of the maxillary molars from the alveolar bone crest (ABC) to the cementum-enamal junction (CEJ) at 7 landmark sites (117). To normalize for differences in ABC-CEJ measurements between the 3 mouse strains we present data as the ratio of the measurement compared with unchallenged mice which = 1. We observed that WT mice orally challenged with *P. gingivalis* presented with an increase in ABC-CEJ ratio (equivalent to an increase in measurement), which is indicative of oral bone loss (Figure 5). *P. gingivalis*-infected $TLR2^{-/-}$ mice failed to present with oral bone loss, while $TLR4^{-/-}$ mice presented with oral bone loss in response to *P. gingivalis* that was similar to WT mice. A recent report has also demonstrated that TLR2 deficient mice failed to develop oral bone loss in response to *P. gingivalis* oral challenge (123). Collectively, these results demonstrate that oral bone loss elicited by *P. gingivalis* oral infection in mice is dependent on TLR2.

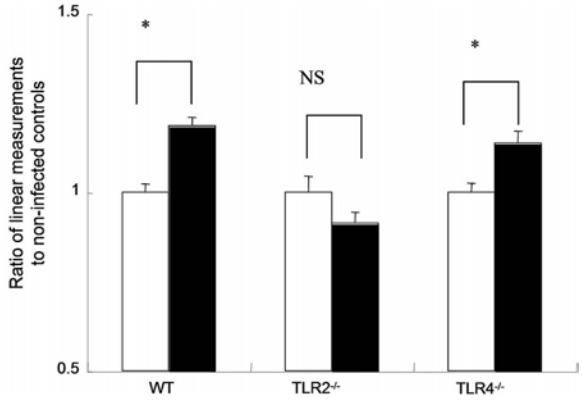

Figure 5. TLR2 is required for inflammatory oral bone loss in response to *P. gingivalis*. (A) WT, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice (all of C57BL-6 background) were orally infected with *P. gingivalis* (black bars). All mice, including age matched, non-infected controls (open bars), were sacrificed six weeks following the final oral challenge. Linear measurements of bone loss (n = 14 sites) were obtained from the maxillary molars of each mouse. Data are expressed as means and SEMs of the ratio between infected vs. control mice for each of the groups tested (8 mice per group). *, $P< 0.01$ by unpaired *t*-test. NS, no significant difference.

6.3. Animal Models of Atherosclerosis

Prior to the generation of genetically modified mice that are spontaneously hyperlipidemic such as the ApoE$^{-/-}$ mouse, the mouse dietary model of atherosclerosis had some utility for mechanistic studies into the processes governing deposition of atherosclerotic plaque. However, with the development of new models such as the ApoE-deficient mice and dietary models including LDLR-deficient mice, detailed mechanistic understandings of the processes underlying atherosclerosis have emerged. It is now known that atherosclerosis is primarily a chronic inflammatory disease and that inflammation impacts all aspects of atherosclerosis from endothelial cell activation to rupture of vulnerable plaques. Histopathology has revealed many inflammatory molecules present in atherosclerotic lesions in mice, and many of these have subsequently been detected in human plaques. Generation of mice deficient in these inflammatory mediators such as MCP-1, IFN-gamma, IL-1, TNF-alpha, IL-6, ICAM-1, VCAM-1, MMPs, and numerous others have demonstrated a role for each of these mediators in different aspects of atherosclerosis. More recently it has become apparent that innate immune signaling, particularly via pattern recognition receptors profoundly influences atherosclerosis. Indeed, TLR2, TLR4, and the MyD88 adaptor molecule-deficient mice provide evidence that these pattern recognition receptors play a role in atherosclerosis (124, 125).

6.4. *P. gingivalis* oral infection accelerates atherosclerosis

Several investigators have utilized various animal models to test the hypothesis that *P. gingivalis* impacts atherosclerosis. Li *et al.* (45) employing an ApoE$^{+/-}$ heterozygous mouse demonstrated that injection of *P. gingivalis* into the tail vein accelerated atherosclerotic plaque deposition. This was followed by the studies of Lalla *et al.* (46) who demonstrated that providing ApoE$^{-/-}$ mice with *P. gingivalis* via the oral route of exposure was sufficient to accelerate atherosclerosis, and that mice challenged by *P. gingivalis* presented with elevated serum levels of the chemokine MCP-1, a mediator known to positively influence atherosclerotic plaque deposition. These data suggest that mice orally infected with *P. gingivalis* respond to infection by elevating expression of systemic inflammatory molecules known to play a role in atherosclerosis.

To begin to determine the contribution of defined *P. gingivalis* virulence factors to the acceleration of atherosclerosis we challenged groups of ApoE$^{-/-}$ mice with wild type *P. gingivalis* strain 381 or the isogenic FimA-deficient mutant DPG3 by oral gavage (44). During the course of oral challenge, we observed by 16S rRNA PCR that both the wild type and FimA- mutant were capable of gaining access to the blood and localizing in the wall of the aortic arch, albeit that the FimA- mutant was attenuated (44). Despite both organisms being able to gain access to the vasculature and localizing in the aortic arch, only ApoE$^{-/-}$ mice challenged with wild type *P. gingivalis* presented with acceleration of atherosclerotic plaque deposition (Figure 6C-F). No differences were observed in cholesterol or triglycerides in all 3 groups of mice (Figure 6A). In addition we demonstrated that both the wild-type *P.gingivalis* the *fimA* mutant challenged mice expressed elevated levels of *P. gingivalis* specific IgG (Figure 6B). The mechanism involved in *P. gingivalis*-accelerated atherosclerotic plaque accumulation involved a specific immune response to invasive *P. gingivalis*, as aortic tissue from ApoE$^{-/-}$ mice orally challenged with wild type *P. gingivalis* presented with enhanced expression of the host innate immune system receptors TLR2 and TLR4 (Figure 7). This was detected by both RT-PCR (Figure 7A) and immunohistochemistry (Figure 7B) of the aortic lesions. Collectively these results demonstrate that *P. gingivalis* can stimulate fimbriae expressing TLR expression in aortic tissue.

6.5. Immunization against whole *P. gingivalis* inhibits *P. gingivalis* accelerated atherosclerotic plaque formation

As immunization with various preparations of *P. gingivalis* including whole killed cells preparations to purified antigens of this organism has been demonstrated to prevent oral bone loss (126), we hypothesized that specific immunization could also inhibit *P. gingivalis*-accelerated atherosclerosis. For these studies we immunized ApoE$^{-/-}$ mice prior to challenge with invasive *P. gingivalis* (44). ApoE$^{-/-}$ mice immunized with whole cell preparation of heat-killed *P. gingivalis* and then challenged with *P. gingivalis* were protected from both oral inflammation as well as the progression of atherosclerotic lesion formation. As shown in Figure 8A-D, we observed protection from accelerated atherosclerosis in immunized animals at 6-weeks following completion of oral challenge. We have also recently determined that immunization protects mice from *P. gingivalis* accelerated atherosclerosis early after

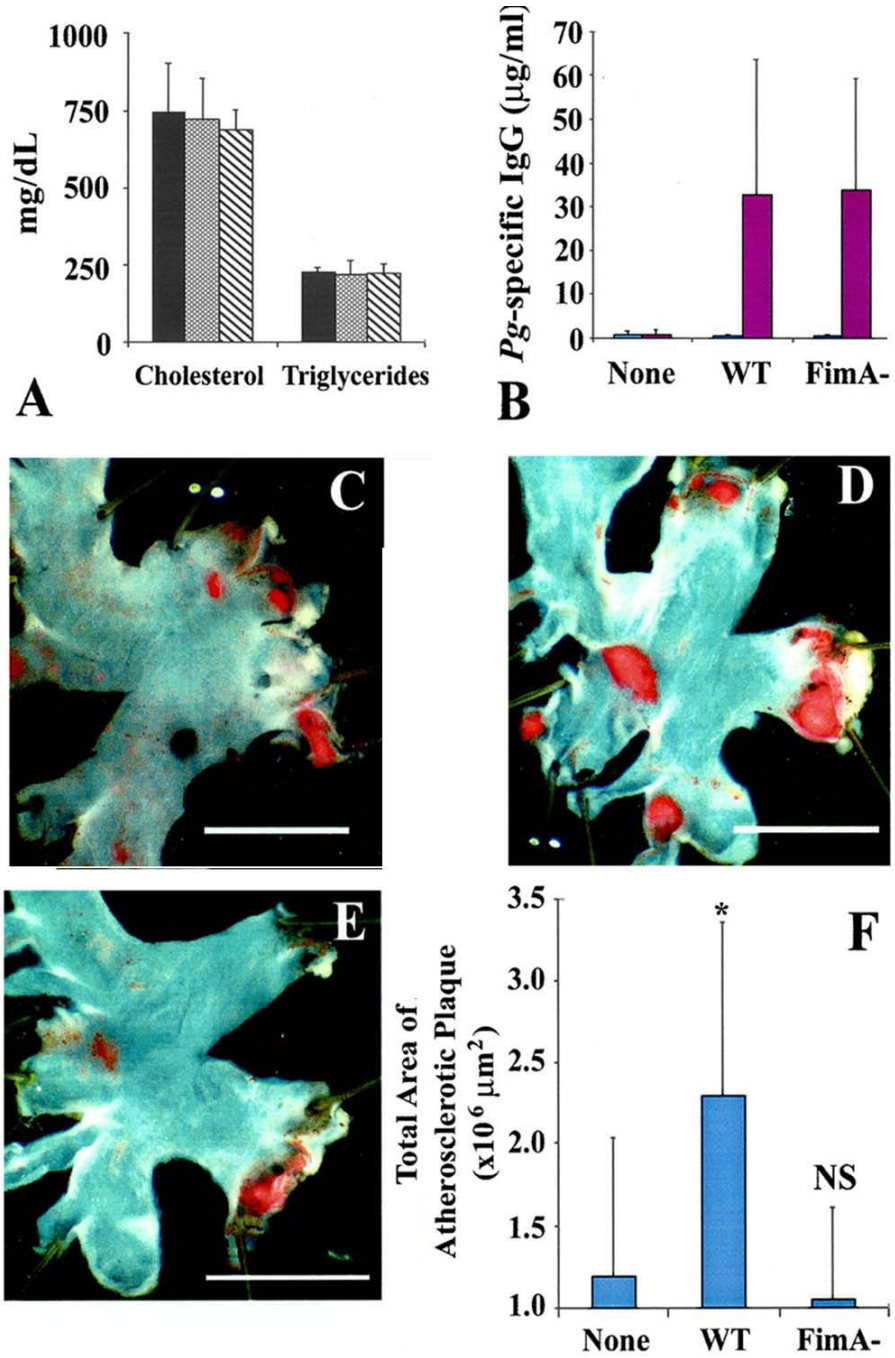

Figure 6. Oral infection with *P. gingivalis* accelerates atherosclerotic plaque formation. (A) Serum analysis (n=10 mice/group) of total cholesterol and triglyceride levels from unchallenged (black bars), WT *P. gingivalis* (gray bars), or *P. gingivalis* FimA– mutant (hatched bars) challenged mice. Mice were challenged as described (44). B, Serum levels of *P. gingivalis*-specific IgG before oral challenge (blue bars) or at 6 weeks after challenge (purple bars) with WT *P gingivalis* (WT) or FimA– mutant (FimA–). (C) Unchallenged, (D) WT *P. gingivalis*, and (E) FimA– mutant are representative of atherosclerotic plaque present on intimal surface of aortic arches of mice. F, Morphometric analysis of total area of atherosclerotic plaque of unchallenged (None), WT *P. gingivalis*, or FimA– mutant challenged mice (FimA–). **P*<0.05; NS indicates not significant vs unchallenged mice. Magnification x40; bar=1 mm.

bacterial challenge (127). In the context of specific bacterial challenge these data are the first to demonstrate that vaccination prevents infection-accelerated atherosclerosis.

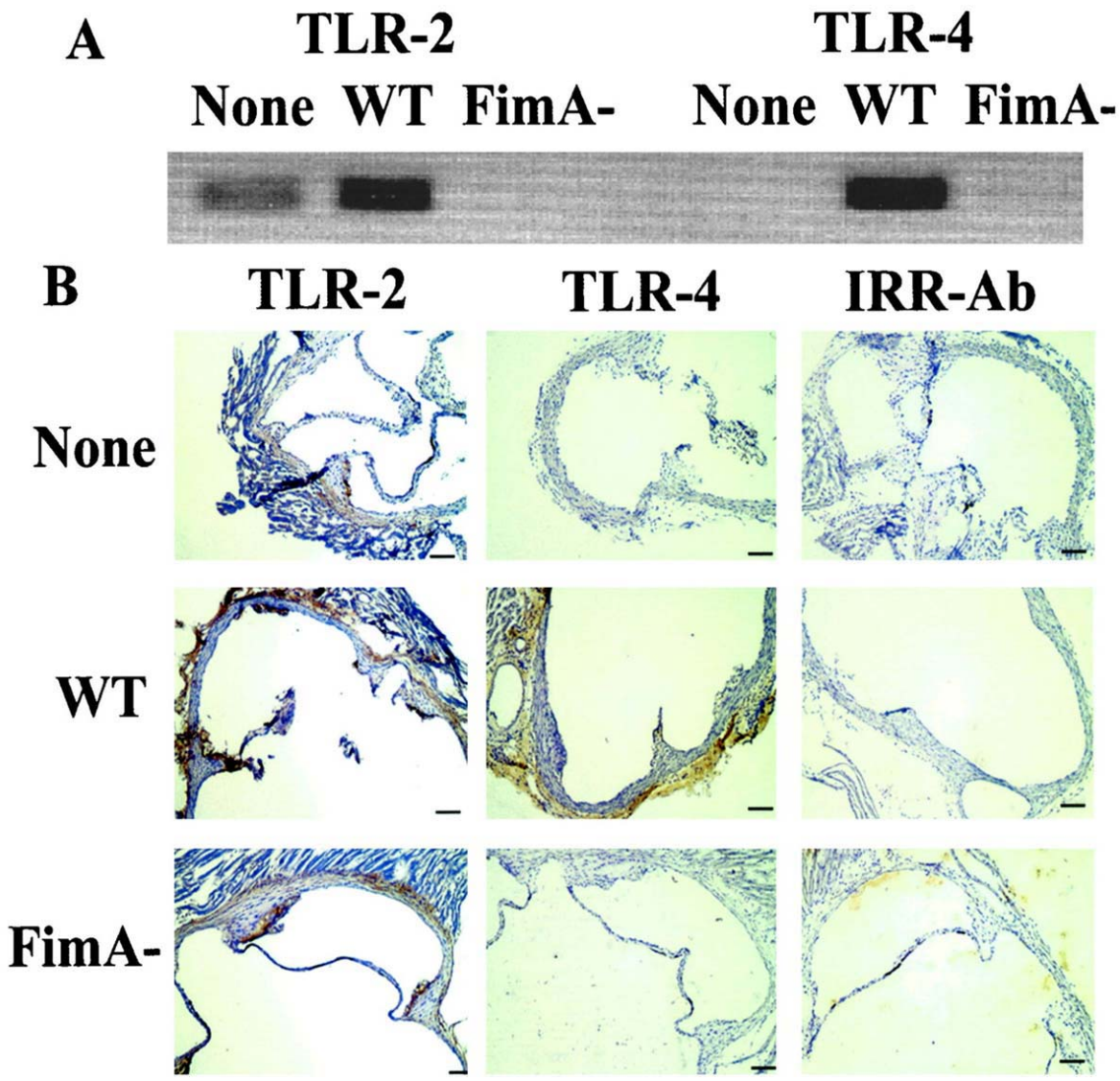

Figure 7. ApoE$^{-/-}$ mice orally challenged with *P. gingivalis* express increased TLR2 and TLR4 in aortic arch tissue. (A) RT-PCR amplification of TLR-2, and TLR-4 mRNA from aortic arch tissue of unchallenged (None), WT *P. gingivalis* (WT), or mutant *P. gingivalis* (FimA–) challenged mice. (B) Immunohistochemical confirmation of TLR-2 and TLR-4 expression in aortic tissue of mice. IRR-Ab indicates irrelevant isotype-matched antibody. Magnification x100; bar=10 μm. Primers and immunohistochemistry protocols were as described (44).

7. SUMMARY / FUTURE DIRECTIONS

Cardiovascular disease and the associated pathology of inflammatory atherosclerosis is a major cause of morbidity and mortality in industrialized societies (128). Available data underscore the current lack of a complete understanding of the molecular mechanisms that link infection to innate immunity and trigger the signals for enhanced inflammation and atherogenesis. Accumulating evidence supports a role for periodontal disease and infection with *P. gingivalis* as a risk factor for atherosclerotic cardiovascular disease. We have demonstrated that ApoE$^{-/-}$ mice infected with *P. gingivalis* present with increased atherosclerotic plaque and increased expression of TLR2 and TLR4 in aortic tissue early following infection. *In vitro* studies support a role for both TLR2 and TLR4 in the macrophage and endothelial cell signaling response to *P. gingivalis* and LPS and fimbriae from this organism. In mouse macrophages the addition of *P. gingivalis* or fimbriae stimulates TLR2 surface expression and makes these cells hyper responsive to TLR2 ligands. Likewise, *P. gingivalis* infection of human aortic endothelial cells stimulates TLR2 and TLR4 surface expression and these cells then become hyper responsive to TLR2 and TLR4 ligands. Collectively our studies together with published studies support a role for TLR2 and TLR4 in the signaling response to *P. gingivalis* and fimbriae. Based on our findings we propose that *P. gingivalis* infection can modulate TLR2 and TLR4 expression on

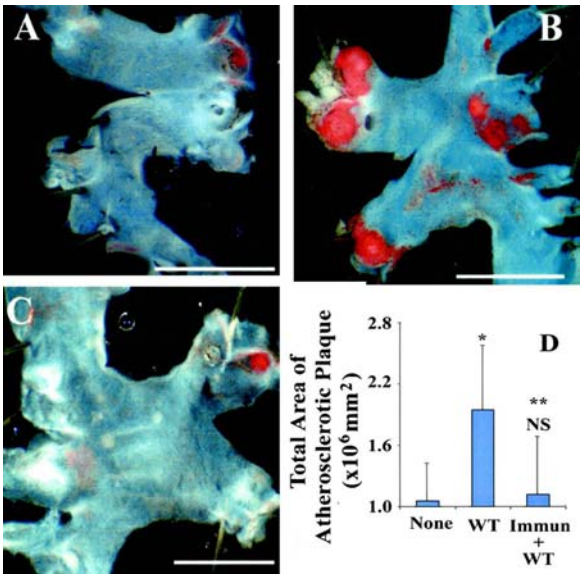

Figure 8. Immunization against whole P. gingivalis inhibits P. gingivalis accelerated atherosclerotic plaque formation. Immunization of mice with heat-killed P. gingivalis prevents P. gingivalis-accelerated atherosclerosis. A (unchallenged), B (WT P gingivalis), and C (heat-killed P. gingivalis immunized and WT P. gingivalis challenge) are representative of atherosclerotic plaque on intimal surface of aortic arch of apoE–/– mice 6 weeks after oral challenge. D, Morphometric analysis of total area of atherosclerotic plaque deposited on intimal surface of aortic arch of unchallenged (None), WT P. gingivalis (WT) challenged, or immunized and WT challenged apoE–/– mice. Mice were immunized and challenged as described (44). *P<0.05 vs unchallenged; **P<0.05 vs WT challenged animals. NS indicates not significant vs unchallenged mice. Magnification x40; bar=1 mm.

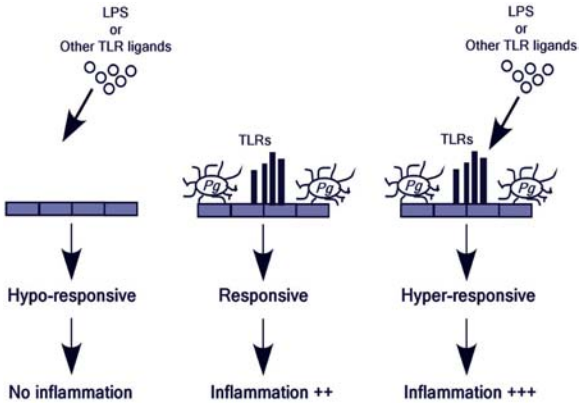

Figure 9. Model of the direct and indirect effects of *P. gingivalis* infection on the local inflammation of the endothelium. Our studies indicate that *P. gingivalis* stimulates TLR expression on the surface of endothelial cells and macrophages and these cells become hyper-responsive. We hypothesize that *P. gingivalis* infection can modify the levels of TLRS expressed on the surface of endothelial cells and macrophages and that infection can convert these cells from TLR ligands hypo-responsive to TLR ligand hyper-responsive cells. This in turn can accelerate atherosclerosis in a mouse model.

aortic endothelial cells and macrophages and that these cells can then respond to subsequent stimulation with other TLR2 and TLR4 ligands including *P. gingivalis* and other pathogens such as *C. pneumoniae*. This in turn can result in increased acceleration of atherosclerotic plaque accumulation (Figure 9).

Our studies are the first studies with any bacterial pathogen, which have demonstrated a link between innate immune markers, and infection-accelerated atherosclerosis in an animal model. Our findings raise the possibility that enhanced TLR expression and signaling following *P. gingivalis* infection may play a role in inflammation in atherosclerosis. Improved understanding of the molecular mechanisms driving TLR over-expression and signaling and the role of chronic inflammation during atherosclerosis may ultimately provide new targets for therapy.

8. ACKNOWLEDGEMENTS

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