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Tobacco-induced changes to Porphyromonas gingivalis gene expression, phenotype and host-pathogen interactions.

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TOBACCO-INDUCED CHANGES TO *PORPHYROMONAS GINGIVALIS* GENE EXPRESSION, PHENOTYPE AND HOST-PATHOGEN INTERACTIONS.

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ABSTRACT

TOBACCO-INDUCED CHANGES TO *PORPHYROMONAS GINGIVALIS* GENE EXPRESSION, PHENOTYPE AND HOST-PATHOGEN INTERACTIONS.

By

Juhi Bagaitkar

15th December, 2010

Tobacco smoke is a strong and independent risk factor for several chronic systemic diseases and also increases susceptibility to a multitude of bacterial infections, including periodontal infections. Periodontitis is a chronic inflammatory disease of the supporting tissues of the periodontium caused by its chief etiological agent, *Porphyromonas gingivalis*. Smokers are more prone to persistant infections by *P. gingivalis*, and harbor higher numbers of *P. gingivalis* than non-smokers. However, smokers show reduced clinical signs of inflammation compared to non-smokers, making diagnosis of periodontal disease in smokers problematic. While several studies delineate the different mechanisms of how tobacco smoke alters host response to periodontitis,

very little is know about its effects on the virulence profile of *P. gingivalis*. We hypothesize, that tobacco smoke presents an environmental stress to which *P. gingivalis* adapts by altering its gene expression, which in turn will influence its interaction with the host.

Indeed, *P. gingivalis* microarray and qRT-PCR data show that about 7% of *P. gingivalis* genes are differentially regulated on exposure to cigarette smoke extract (CSE) including major and minor fimbrial antigens (FimA and Mfa1, respectively) and capsule. CSE- induced phenotypic alterations are consistent with increased biofim formation and reduced pro-inflammatory potential of intact *P. gingivalis*. Furthermore chronic exposure to *P. gingivalis* FimA leads to the abrogation of the pro-inflammatory response in a TLR2- and IRAK1 dependent manner.

These studies provide some of the first information to explain, mechanistically, how tobacco smoke changes the *P. gingivalis* phenotype in a manner likely to promote *P. gingivalis* colonization and infection while simultaneously reducing the host response to this major mucosal pathogen.

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CHAPTER ONE: INTRODUCTION

Tobacco smoking is the single and most common preventable cause of premature mortality in the United States (1, 2). Tobacco users account for one third of the global adult population and it is estimated that tobacco-related health complications will result in premature death in 50% of this group (3). About 5-6 million people are killed annually by tobacco use and most of these deaths (70%) occur in middle- to low- income countries (4).

Tobacco smokers are at an increased risk for various types of cancers, stroke, vascular dysfunction, oral diseases, allergies and multiple chronic inflammatory and degenerative disorders (5). Cigarette smoke toxicity is attributed to the presence of more than 4000 chemicals, including multiple allergens, irritants, toxins, mutagens and carcinogens in the particulate/tar and gaseous phases of tobacco smoke (6). Polycyclic aromatic hydrocarbons, benzopyrenes, hydrozxyquinone, acrolein, formaldehyde, nitrogen oxides, acetone, ammonia, nicotine, heavy metals like lead and cadmium, free radicals etc, all contribute to the disease burden through multiple adverse effects on the innate and adaptive immune systems (7) .

Active smoking is more hazardous than second hand smoke. Inhaled tobacco is associated with more diverse types of diseases than oral tobacco use. However, even second hand exposure is indicated in increased susceptibility to, and severity of, multiple diseases (8). Exposure to cigarette side stream smoke, a major environmental pollutant, for example, significantly increases risk for cardiovascular disease (9), adverse pregnancy outcomes (10, 11), infertility (12, 13), genetic aberrations (14) and persistent infections (15). Thus tobacco smoke contributes significantly to the global disease burden. However, there has been surprisingly little research focusing on how tobacco causes diseases, particularly chronic inflammatory and infectious diseases. Therefore, the specific underlying mechanisms are still largely unknown.

Chronic Inflammatory Diseases and Smoking

Tobacco smoking substantially increases the risk for chronic, inflammatory diseases (16-19). This is particularly true for atherosclerosis, acute myocardial infarction, stroke and peripheral vascular disease (20, 21), inflammatory bowel disease, chronic kidney disease (5, 19), and periodontal diseases (22). Smokers are also pre-disposed to autoimmune inflammatory disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS). Again, it is important to point out that despite convincing epidemiological associations between tobacco use and such diseases, mechanistic insight is poor. For example, in RA, polycyclic aromatic hydrocarbons from cigarette smoke are known to induce a proinflammatory cytokine response from fibroblast-like synoviocytes (23). Moreover, there is a strong association between tobacco use and joint destruction in RA patients who smoke (24, 25). In SLE

patients, the mutagenic nature of tobacco smoke is partly responsible for accumulation of DNA abnormalities, particularly double strand breaks and DNA-adduct formation, contributing to anti-DNA antibody production. In MS patients, nicotine interferes with axonal conduction, exacerbating disease (26). However, deeper mechanistic understanding is lacking.

Smoking induces alterations in several systemic inflammatory markers associated with chronic inflammation. Elevated levels of serum fibrinogen, C-reactive proteins (CRP), pro-inflammatory cytokines, aberrant expression of inflammatory markers and adhesion molecules are common in smokers with increased numbers and chemotaxis of neutrophils, monocytes and macrophages in the periphery (26). Such systemic changes could account for overt susceptibility to chronic inflammatory diseases in smokers.

The endothelial dysfunction associated with vascular diseases is induced by many tobacco smoke components, such as nicotine, carbon monoxide, nitric oxide and free radicals. Indeed, tobacco-induced increases in vascular permeability, adhesion molecule expression, leukocyte adherence, and platelet aggregation all contribute towards vascular disease progression and atherosclerotic lesion formation (21, 27, 28). Even passive smoking increases cardiovascular disease risk by about 30% (29).

Cigarette smoking is also directly responsible for the majority of chronic inflammatory diseases of the airways, such as bronchitis, chronic obstructive pulmonary disease (COPD), and emphysema. Smoking is the main etiological factor associated with COPD, a progressive disease characterized by airway limitation (30). Increased pro-inflammatory cytokine expression by airway epithelial cells, immune cell sequestration, reactive oxygen species (ROS) generation and matrix metalloprotease (MMP) release, all

contribute to perpetuation of inflammation that results in destruction of bronchial tissue, chronic lung injury and emphysema (as reviewed by MacNee et al (31)). Besides destructive lung diseases, both active and passive smoking has been associated with compromised renal function, nephropathies, and glomerular dysfunction (32) leading to chronic kidney disease (5, 19). Smoking also increases risk for inflammatory bowel disease, and several oral diseases particularly gingivitis and periodontitis (22).

Infectious Diseases and Smoking

Tobacco smoke adversely affects the immune response to pathogenic insults, making smokers more susceptible to a multitude of infectious diseases compared to non-smokers (15). These include respiratory tract infections by several bacterial pathogens, including *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae* and *Legionella pneumophila* (33-37). Tuberculosis is also common in smokers and is often exacerbated in a dose-dependent manner (38, 39) with quicker rates of disease progression, relapse and higher mortality in smokers compared to non-smokers (40). Smoking is also significantly associated with the development of bronchitis and bacterial pneumonia (41).

Besides respiratory illnesses, smokers show higher prevalence of meningitis (42), post-surgical or nosocomial bacterial infections (43) and sexually-transmitted bacterial infections, such as chlamydia and gonorrhoea (44). Smokers also have an increased predisposition to bacterial-induced periodontal diseases (45, 46). Shifts in periodontal plaque composition from one primarily constituted by Gram positive, aerobic commensals to a more anaerobic, Gram negative microflora that includes overt and

opportunistic pathogens is also common in smokers (47). A summary of bacterial infections associated with tobacco smoking is listed in Table 1.

Table 1: Bacterial infections associated with tobacco smoking.

Infection	Odds Ratio (95% CI)	Additional references
Nasopharyngeal and respiratory pathogens (such as *S. pneumonia*, *N. meningitidis*, *H. influenzae*, *L. pneumophila*)	2.5, (1.1-6.0) (36)	(33-35, 37, 48-51)
Group A streptococcus sore throat	-	(52)
Legionnaires disease	3.6 (2.1-5.8) (37)	(37, 53)
Cystic fibrosis	*Increased severity on smoke exposure* (54, 55)	(56, 57)
Pneumonia	2.6 (1.9-3.5)(58, 59)	(34, 41, 60)
Tuberculosis	1.8 *Light* (38) 3.2 *Moderate* (38) 3.7 *Heavy* (38)	(39, 40, 61, 62)
	4.1 (2.4 to 7.3) *Active* (34) 2.5 (1.2 to 5.1) *Secondhand* (34)	
Meningococcal carriage	2.2 (1.0-4.8) *Light* (63)	(36, 42, 64-

	7.2 (2.3-22.9 *Heavy* (63)	68)
Bacterial vaginosis	**2.7 (69)**	**(44, 70, 71)**
Helicobacter pylori	**1.9 (1.4-2.5) (72)**	**(73-76)**
Periodontitis	**3.3 (2.3-4.5) *Light* (77)** **7.3 (5.1-10.3) *Heavy* (77)**	**(78-89)**
Ulcerative colitis	**0.6 (0.4-0.8) (90)**	**(91-93)**
Crohn's disease	**3.6 (2.5 - 5.0) *Active (94)*** **2.0 (1.3 - 3.3) *Secondhand*** **(95)**	**(91, 96, 97)**
Otitis media	**4.12, 1.5-11.9 *Secondhand***	**(33, 98-100)**
Surgical infections	**1.2 (1.1, 1.3) (101)**	**(43, 102, 103)**

*** The specific OR (95% CI) presented is selected from a single reference. The "Additional references" column provides more complete information.**

Periodontal diseases

Tobacco smokers are more susceptible than non-smokers to plaque-induced gingivitis and periodontitis. Periodontitis is a chronic inflammatory, infectious disease with a majority of the cases found in developing nations (104, 105). It is also highly prevalent in the US affecting, more than 100 million people (106) . Inflammatory periodontal diseases represent a risk factor for several chronic and serious systemic conditions, such as vascular diseases including stroke (107-110), pulmonary disease (111-113), nephritis (114) and pre-term birth (115, 116).

Gingivitis is reversible and is characterized by redness, swelling, and bleeding (117). It can proceed into the more severe, chronic inflammatory periodontitis characterized by inflammatory destruction of the supporting soft tissues surrounding the teeth, loss of periodontal ligaments, and resorption of the alveolar bone that can lead to tooth loss and even edentulism (105, 118). Increased subgingival infection by several Gram negative species such as *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Campylobacter rectus*, and the red complex bacteria (*Tannerella forsynthia*, *Treponema denticola* and *Porphyromonas gingivalis*) inhabiting the largely anaerobic niche between the tooth surface and the junctional epithelium of the periodontium is a hallmark of inflammatory periodontal disease (119).

Subgingival colonization by pathogens is aided by several immune subverting virulence factors expressed by these periodontal pathogens. Bacterial persistence and the inflammatory response to such pathogens combine to promote inflammatory tissue destruction in periodontitis. Multiple pro-inflammatory cytokines (i.e. IL-1β, IL-6, TNFα, IL-8, and RANKL) lead to osteoclast activation and, subsequently, alveolar bone

loss (120). The degree and extent of the inflammatory response to these pathogens is determined by several host-specific factors like age, race, genetic polymorphisms, underlying immune abnormalities, etc (121). However tobacco smoke is the most important environmental risk factor associated with chronic, inflammatory periodontal disease (45, 46, 122).

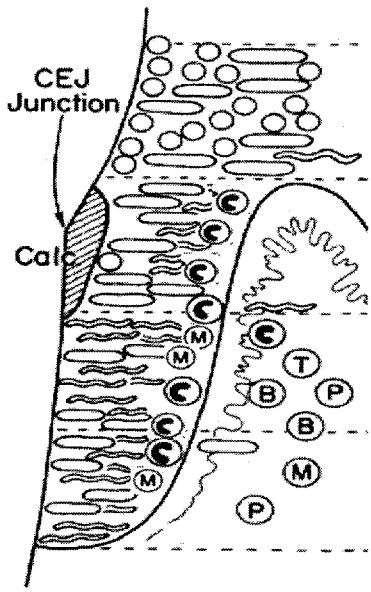

Figure 1: Immune response to oral biofilm bacteria: T –T cells, B- B cells, M-Monocytes/Macrophages, P- platelets and C -neutrophils. (*Source Adapted from a figure appearing on the website of Dr. Loesche, University of Michigan)*

Smoking and Periodontitis

Smoking is the single most important risk factor for periodontitis (22). There is a dose dependent relationship between smoking status and deteriorating periodontal health (123). Smokers are highly susceptible to periodontitis and the disease is often more severe compared to non-smokers with increased alveolar bone loss (124), attachment loss, percentage of sites with significant attachment loss (125), tooth mobility, and tooth loss (126). Patients who smoke are also much more likely to be refractory to treatment than non-smokers (88).

Although cigarette smoking causes both systemic and local changes, components of cigarette smoke are found at their highest concentrations intraorally and particularly in the gingival crevicular fluid – the serum-derived exudate that enters the mouth via the gingival crevice. Several studies report nicotine, and its primary metabolite cotinine, both adversely affect proliferation, attachment and chemotactic responsiveness in human periodontal ligament fibroblasts (127, 128). Nicotine induced stress response in these cells is characterized by elevated levels of c-fos expression (129). Arecoline, another toxic component in cigarette smoke, significantly inhibits cell proliferation in a dose-dependent manner and depletes intracellular thiol levels. Thiols are protective against oxidative damage and their depletion may increase susceptibility to oxidative damage by oxidants present in cigarette smoke (130). In human gingival fibroblasts, nicotine and cigarette smoke condensate both promote membrane-associated MMPs-dependent collagen degradation, which may promote attachment loss (131, 132). Chronic angiogenic suppression and reduced oxygen tensions (133) also contributes to tobacco-induced predisposition to periodontal disease. However, very little is known about how

tobacco smoke affects periodontal pathogens and, particularly, host-pathogen interactions.

Despite some conflicting data that smoking may not influence the sub-gingival micoflora (134-137), the balance of recent data strongly suggests that tobacco-induced susceptibility to periodontitis is associated with shifts in the microbial composition of complex periodontal plaque communities (72, 138-145). For example, Zambon et al showed a higher prevalence of *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia* and *Porphyromonas gingivalis* in smokers (138). Umeda et al (142) reported an increased risk for harbouring *Treponema denticola* in smokers. Haffajee and Socransky (146) showed an increased prevalence of eight species, including *P. gingivalis*, in current smokers, while Eggert et al (141) have shown a higher prevalence and proportion of *T. forsythia*, *Campylobacter rectus*, *P. gingivalis* and *Peptostreptococcus micros* in plaque samples from smokers. *P. gingivalis* is found in significantly higher numbers in smokers compared to non-smokers and the infection is more persistent (88, 147). The evidence for tobacco smoke increasing susceptibility to *P. gingivalis* infection and in increasing the *P. gingivalis* infectious load is particularly strong.

Thus tobacco smoke alters the oral biofilm bacterial composition while simultaneously dysregulating host immune response. A summary of cigarette smoke induced alterations in the context of periodontal disease are shown in Figure 2.

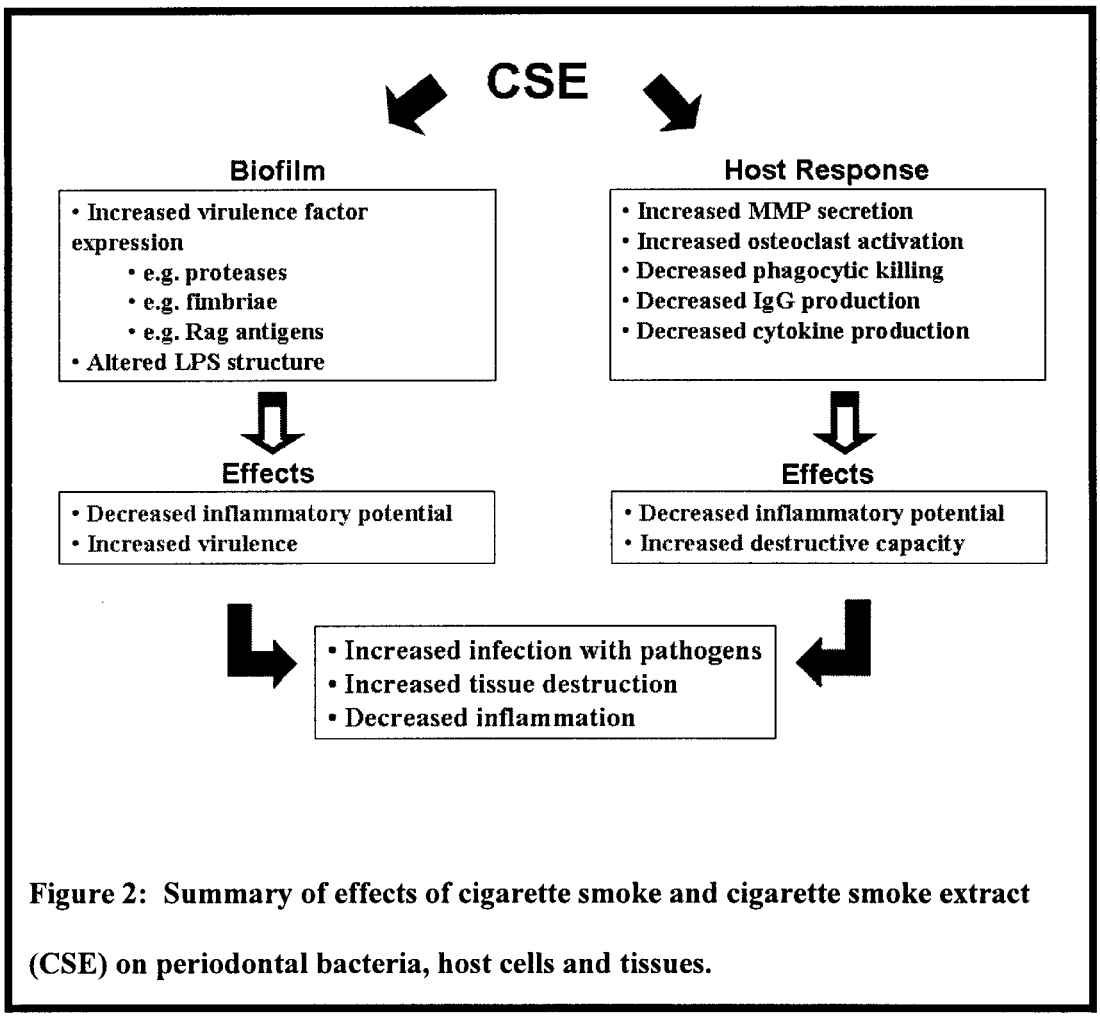

Figure 2: Summary of effects of cigarette smoke and cigarette smoke extract (CSE) on periodontal bacteria, host cells and tissues.

Porphyromonas gingivalis

Porphyromonas gingivalis is a Gram-negative, proteolytic, asaccharolytic anaerobe associated with chronic periodontitis in humans (148, 149). It is an opportunistic pathogen and colonizes the gingival cavity via several key virulence factors such as gingipains, fimbriae, lipopolysaccharide (LPS), capsule, hemagglutinins and other outer membrane proteins which aid in attachment and invasion, degradation of extracellular matrix proteins, and circumvention of a normally strong pro-inflammatory

response to infection (150, 151). The major *P. gingivalis* virulence factors are discussed in the next section.

Major fimbrial antigen (FimA)

The major fimbriae of *P. gingivalis* are long (0.5-1μm (152)), hair-like, peritrichous, adhesive, filamentous structures that project away from the cell surface and are primarily comprised of a 41 kD protein (FimA, fimbrillin) encoded by the *FimA* gene (153). Based on the genomic variations in FimA gene, fimbriae are classified into Types I, II, III, IV and V (150). *P. gingivalis* strains with Type II fimbriae are most commonly associated with periodontitis and have been found to be more virulent in mouse models of systemic infection compared to TypeI/IV carrying strains (154). Several accessory proteins also associate with the major fimbriae such as Fim C, D and E encoded by genes downstream of *FimA* gene in the FimA operon (155, 156). FimCDE deficient strains show reduced invasion and persistence in macrophages and induce diminished bone loss in mouse models of periodontitis (157).

P. gingivalis major fimbriae are essential virulence factors in host colonization. Fimbriae mediate adhesion to several host proteins such as fibronectin, laminin, collagen, and salivary proteins (158); aid attachment to, and invasion of, oral epithelial cells, endothelial cells and gingival fibroblasts (159, 160); co-aggregate with other plaque bacteria (161); are key in internalization and persistence in macrophages through exploitation of complement receptor 3 (CR3) (162); induce pro-inflammatory cytokines such as TNF-α, IL-6, but inhibit IL-12 in monocytes and macrophages, thus limiting immune clearance (163). They are thought to directly induce alveolar bone loss in murine

models (164, 165). Several studies have delineated their role in upregulating adhesion molecules such as ICAM-1, VCAM-1, P- and E-selectins in endothelial cells (166). However, their capacity to induce pro-inflammatory mediators from immune cells is controversial. The ability of FimA to generate an inflammatory response might be cell type dependent. Contrary to human monocytes, primary human gingival epithelial cells stimulated with purified FimA from *P. gingivalis* 33277 failed to generate a proinflammatory response as indicated by low levels of IL-6 and IL-8 (167). Fimbriae-deficient strains are also found to be as inflammatory as wildtype strains in mouse scalp challenge model indicating FimA is not entirely responsible for the inflammatory response associated with *P. gingivalis* (168).

Minor Fimbrial Antigen (Mfa1)

The minor fimbriae of *P. gingivalis* are shorter filaments (18-120nm – (169)) composed of a 67-kDa protein encoded by the *mfa1* gene (170). Mfa1 is co-transcribed with Mfa2, the product of the *mfa2* gene, which is postulated to be important in anchoring Mfa1 in the *P. gingivalis* membrane and in regulating fimbrial length (171). Native minor fimbriae have been shown to contain several putative glycosylation sites (172).

The role of Mfa1 in *P. gingivalis* virulence is beginning to be elucidated. Mfa1 is key in co-adhesion to the primary periodontal colonizer, *Streptococcus gordonii*, through specific interaction between Mfa1 and the SspB protein expressed on the surface of *S. gordonii* (173). Mfa1 is also important in single species biofilm maturation and microcolony development (174). Unlike FimA, Mfa1 is highly inflammatory and

induces IL-6, IL-1β, TNF-α in mouse peritoneal macrophages, endothelial cells (175, 176) and is associated with alveolar bone loss in various mouse models of periodontitis (164, 165). Zeituni et al have shown, using *mfa1*-deficient strains of *P. gingivalis*, that minor fimbriae are important in internalization by monocyte-derived dendritic cells through interaction with surface DC-SIGN. Such Mfa1-dependent targeting into DC-SIGN-rich intracellular compartments results in lowered inflammatory cytokine levels thus preventing DC maturation and inducing a Th2 biased immune response (177).

P. gingivalis LPS

P. gingivalis is an important virulence factor recognized by the host receptors TLR4 and TLR2 (178, 179). It is associated with inflammatory cytokine production in human gingival fibroblasts (HGF) (180), macrophages (181), and neutrophils (182, 183), as well as direct osteoclast activation and promotion of bone resorption in several *in vitro* and *in vivo* models (184). It has also been implicated in increasing the risk for other systemic conditions in periodontitis patients such as atherosclerosis by inducing foam cell formation in murine macrophages (185).

P. gingivalis LPS is atypical in structure, consisting primarily of tetra- and penta-acylated Lipid A with one or two phosphate groups. Unlike *E. coli* LPS (the canonical TLR4 agonist), *P. gingivalis* LPS displays significant amount of Lipid A structural heterogeneity, such as differing numbers of phosphate groups, amounts and positions of fatty acids and variations in acyl chain lengths (178). These structural alterations can be strain specific and / or altered according to environmental conditions but are directly responsible for variations in the immune response to *P. gingivalis* LPS (186). Both

penta- and tetracylated forms of lipidA present are recognized by TLR4, however it is the pentaacylated form that is associated with NF-κB activation and E-selectin upregulation in endothelial cells (187, 188). Tetraacylated forms can antagonize TLR4 activation and may be responsible for modulating and / or subverting the host inflammatory response (as reviewed by Hajishengallis (189)).

P. gingivalis LPS is significantly less inflammatory than *E. coli* LPS. Various mechanisms have been proposed to explain the lower biological activity of *P. gingivalis* LPS, such as decreased affinity for LPS-binding protein (LBP) and subsequent recognition by TLR4 (186); increased activation of transcriptionally inactive p50 subunit of NF-κB rather than p65 subunit which is associated with transcription of proinflammatory genes in Monocyte derived Dendritic cells (MoDCs) (190); reduced activation of IRAK1 and increased production of IRAK-M (negative regulator of TLR signaling) (191).

P. gingivalis Capsule

Some strains of *P. gingivalis* produce an extracellular capsule composed of negatively charged polysaccharide residues. This capsular layer provides resistance against phagocytic clearance by neutrophils (192) and complement-mediated lysis or opsonization (193). Except for a recent report by Brunner et al (194) stating that the presence of capsule reduced inflammatory response to *P. gingivalis* in human gingival fibroblasts, the literature ascribes highly pro-inflammatory potential to *P. gingivalis* capsule, which is associated with increased abscess formation, compared to non-encapsulated strains, in animal models of systemic infection (195, 196). Dentritic cells

challenged *in vitro* with encapsulated strains of *P. gingivalis* (K1-K6 serotypes) produces a proinflammatory response associated with elevated levels of IL-1β, IL-6, IL-12p35, IL-12p40, and IFN-γ. The K1 serotype of *P. gingivalis* W83, used in our microarray experiments, was found to be most inflammatory of all *P. gingivalis* capsule subtypes (197).

Long-term exposure to capsular polysaccharides is associated with a decreased ability of periodontal ligament fibroblasts to attach to the tooth root surface (198). *P. gingivalis* serotype K5 capsular polysaccharide mediates co-aggregation between *P. gingivalis* and another periodontal pathogen, *Fusobacterium nucleatum* (199). Furthermore, mice immunized with purified *P. gingivalis* capsular polysaccharides elicited a strong humoral response and were protected from oral bone loss on secondary challenge with the whole bacterium (196). The presence of capsular polysaccharides, thus, is important in *P. gingivalis* virulence and likely contributes to the progression of periodontitis.

P. gingivalis Proteases (Gingipains)

P. gingivalis produces trypsin-like cysteine proteases – the Arg- and Lys-gingipains encoded by *rgpA*, *rgpB*, and *kgp* genes. Gingipains play a role in multiple virulence mechanisms of *P. gingivalis*. In addition to providing energy through degradation, and subsequent metabolism, of extracellular matrix proteins and stimulating the expression of matrix metalloproteinases (MMPs), they subvert the host response by degrading cytokines, complement proteins and several key immune receptors, including macrophage CD14 and T cell CD4 and CD8 receptors. Moreover they perpetuate

inflammation and activate the blood coagulation system, enhancing vascular permeability, promoting gingival edema and inducing platelet aggregation (as reviewed by (200, 201)).

Smoking and inflammatory response in Periodontitis

Chronic exposure to tobacco smoke adversely affects host reponse in periodontitis. More than 50% of cases of periodontitis are found in current or ex-smokers. Tooth and bone loss in smokers is directly influenced by tobacco consumption in a dose dependent manner (202). Contrary to the increased severity of disease, smokers consistently show reduced signs of clinical inflammation such as bleeding on probing, gingival redness (203) accompanied by reduced levels of pro-inflammatory markers (47, 133, 204).

Inflammatory dysregulation in smokers can be attributed to the debilitating effects of tobacco smoke on both innate and adaptive immune responses. Smoking is associated with inappropritate activation of neutrophils (205), neutrophil elastase and matrix metalloproteinases release (206-208); inhibition of key endogenous protease inhibitors (a1-antitrypsin and a2-macroglobulin) (209) contributing to destruction of gingival tissues. Periodontal breakdown in smoking patients has recently been associated with increased T-cell proliferation (210). Furthermore, smoking contributes to alveolar bone loss (and osteoporotic bone resorption) by promoting the differentiation and activation of osteoclasts (211-213) and it may also adversely influence fibroblasts by inhibiting cell proliferation and attachment (127, 130). Thus, inflammatory dysregulation is an

important contributing factor to the increased severity of periodontitis and is likely to influence disease progression in periodontitis.

Tobacco smoke components are readily available in the gingival crevicular fluid and are likely to alter the host pathogen interactions of plaque bacteria. Smoking causes a shift in the normal microbial flora, increased prevalence, numbers and persistence of *P. gingivalis* in infected individuals (47). Thus it is likely that the tobacco smoke influences *P. gingivalis* virulence in smokers while maintaing inflammatory response suppression. While several studies delineate the different mechanisms of how tobacco smoke alters host response to periodontitis very little is known about its effects on the virulence profile of *P. gingivalis*.

Our main objective in this dissertation was to identify differentially expressed proteins and lipids of *P. gingivalis* regulated by cigarette smoke focusing primarily on secreted and cell surface components since these represent likely candidates that can influence virulence and interactions with host tissues. Membrane proteins play an essential role in colonization, biofilm formation and development of disease as well as the immune response to *P. gingivalis* while gingipain activity is central to many aspects of *P. gingivalis* physiology and contributes to nutrient acquisition, virulence and the processing of cell surface components. Thus cigarette smoke induced changes in the key pathogenic determinants of *P. gingivalis* are likely to alter host pathogen interactions in a manner that promotes reduced inflammatory response but increased infection.

Our main hypothesis is that tobacco smoke presents an environmental stress to which *P. gingivalis* adapts by altering its gene expression and altering phenotypic traits that are likely to influence host-pathogen interactions. Our studies would help determine

how *P. gingivalis* responds to cigarette smoke at the molecular level and delineate the mechanisms that contribute to the observed suppression of inflammatory response in smokers.

CHAPTER TWO: MATERIALS AND METHODS

Reagents and antibodies:

DMEM, RPMI 1640 and keratinocyte-serum-free (KSF) media, Dulbecco's PBS, bovine pituitary extract, Amphotericin B and Nu-Page 4-12% Bis-Tris gels and all other SDS-PAGE chemicals were obtained from Invitrogen (Carlsbad, CA). FBS came from HyClone (Logan, UT). THP-1, THP-1 Blue, TLR-transfected Human Embryonic Kidney (HEK) 293 and vector control cells as well as Pam3CSK4, FSL, LPS, normocin, blastocidin, zeocin, hygrogold and QUANTI-Blue NF-κB assays were purchased from Invivogen (San Diego, CA). Penicillin–streptomycin came from Mediatech (Manassas, VA). RIPA buffer, PhosSTOP and complete Mini EDTA-free protease inhibitor cocktail tablets were bought from Roche (Indianapolis, IN). BCA Protein Assay kits came from Pierce (Rockford, IL). SuperSignal West Pico Chemiluminescent Substrate kits and Bacterial Protein Extraction Reagent (B-PER) were purchased from Thermo Scientific (Rockford, IL). FimA-specific antibodies were custom generated by Cocalico Biologicals, Reamstown, PA, while FITC-labeled mouse anti- human CD16b and mouse IgM-FITC isotype control antibodies came from AbD Serotec (Oxford, UK). The E38

monoclonal anti-RagB antibody was a kind gift of Dr. Mike A. Curtis of The London, Queen Mary's School of Medicine and Dentistry, UK. Fibronectin pre-coated microplates were from BD Biosciences (San Jose, CA). Anti-*P. gingivalis* antibodies were generated in house, while HRP-linked anti-rabbit IgG and all other antibodies were purchased from Cell Signaling Technology (Beverly, MA). Gifu anaerobic medium (GAM) was bought from Nissui Pharmaceuticals (Tokyo, Japan). Lymphocyte separation medium (LSM) was purchased from MP Biologicals (Solon, OH). Dextran, PBS and tetramethylbenzidine were obtained from Fisher Scientific (Fair Lawn, NJ), EDTA from AA Hoefer Inc. (San Francisco, CA), while trypan blue, insulin, transferrin, 2-mercaptoethanol, 2-aminoethanol, sodium selenite, proteinase K acrylic beads and phosphotongstic acid were purchased from Sigma-Aldrich (St. Louis, MO). HiTrap Chelating HP affinity columns came from Amersham Biosciences Corp., (Piscataway, NJ). Isopropyl-*p*-D-thiogalactopyranoside (IPTG) was bought from RPI Corporation (Prospect, IL). Limulus amoebocyte lysates assay kit was purchased from Cape Cod Inc. (Falmouth, MA). IL-8 ELISA kits were from Cell Sciences (Canton, MA). TNF-α, IL-6 and IL-10 ELISA kits and FITC conjugated anti-human TLR2 and appropriate istoype control antibodies were purchased from eBioscience (San Diego, CA). Formavar coated copper grids were from Electon Microscopy Sciences (Hatfield, PA). Finally, standard reference cigarettes were obtained from the Kentucky Tobacco Research and Development Center (Lexington, KY).

Lysozyme was purchased from Sigma, St. Louis, MO; RNA Midi and PCR purification kits from Qiagen, Valencia, CA; random hexamers from Biosynthesis, Lewiston, TX; RT buffer, DTT, RT-PCR mastermix and Superscript II from Invitrogen,

Carlsbad, CA; 2:3 aminoallyl dUTP:dTTP ratio labeling mix from Amersham Biosciences, Piscataway, NJ. *P. gingivalis* W83 microarrays were obtained from The J. Craig Venter Institute through the NIDCR Oral Microbe Microarray Initiative (NOMMI).

Bacterial culture and *in vitro* modeling of tobacco exposure:

Porphyromonas gingivalis W83 and ATCC 33277 were purchased from the American Type Culture Collection (Manassas, VA) and maintained as frozen stocks. *P. gingivalis* was grown in GAM or GAM conditioned with CSE (GAM-CSE) under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) at 37°C in a Coy Laboratories anaerobic chamber. Bacteria were harvested at mid- to late-exponential phase (O.D. of 1 corresponds to 10^9 cells ml^{-1}). To prepare GAM-CSE, cigarette smoke was drawn through 50 ml GAM by using a three-way stopcock and a syringe, with 35ml 'drags' performed over a period of 2 sec, one drag every 2 sec. Cigarette smoke extract-conditioned medium was filtered (0.22 μ), and adjusted to pH 7.2. The nicotine content of GAM-CSE was determined by gas–liquid chromatography, as previously described (214) and GAM-CSE adjusted to physiologically relevant doses (214-217). Unless otherwise noted, GAM-CSE was employed at a concentration of 4000 ng nicotine equivalents /ml. For all experiments *P. gingivalis* was either cultured in GAM or CSE for two passages. CSE grown *P. gingivalis* was cultured back in GAM for two more passages for the reconditioned group.

Human PBMC, neutrophil and gingival epithelial cell isolation and maintenance:

Human peripheral blood mononuclear cells (PBMCs) were isolated from de-

identified whole, citrated (10%) venous blood obtained from healthy donors by separation and collection of buffy coat and elimination of erythrocyte contamination with Histopaque 1077 density gradients, as previously described (218). Written informed consent was obtained from all the donors, and studies performed in compliance with University of Louisville, Institutional Review Board, Human Subjects Protection Program, (study number 503.05). Viability of all cells was routinely >98%, as determined by trypan blue exclusion. PBMCs were maintained in RPMI-1640 supplemented with 10% heat inactivated FBS, 100u/ml penicillin G and 100μg/ml streptomycin, at 37°C, 5% CO_2. Cells were allowed to rest overnight before addition of agonists.

Neutrophils were isolated from the erythrocyte-neutrophil pellet by collection from 1% Dextran- DPBS suspensions and elimination of erythrocyte contamination by hypotonic lysis. On restoring isotonicity, cells were washed once with cold DPBS and once with plasma buffer (2% plasma in DPBS). Neutrophils were suspended in RPMI-1640 supplemented with 10% heat inactivated FBS, 100u/ml penicillin G and 100μg/ml streptomycin, at 37°C, 5% CO_2. Viability was routinely >98%, as determined by trypan blue exclusion. Purity of neutrophils was routinely >95%, as determined by flow cytometry using FITC-labeled anti-CD16b.

The human gingival epithelial cell line, OBA-9, was obtained from Dr. Denis Kinane (University of Pennsylvania, PA) and maintained at 37°C, 5% CO_2 in KSF medium supplemented with 10 μg/ml of insulin, 5 μg/ml of transferrin, 10 μM 2-mercaptoethanol, 10 μM of 2-aminoethanol, 10 nM of sodium selenite, 50 μg/ml of bovine pituitary extract, 100 units/ml of penicillin/streptomycin and 50 ng/ml

Amphotericin B.

TNF-α release from *P. gingivalis*-stimulated PBMCs:

PBMCs (0.5 x 10^6 cells per well) were stimulated with 10^6 – 10^9 *P. gingivalis* grown in GAM or GAM-CSE. Cell-free supernatants were harvested by centrifugation and TNF-α cytokine levels were determined by ELISA.

***P. gingivalis* W83 microarrays:**

P. gingivalis W83 microarrays were obtained from The J. Craig Venter Institute through the NIDCR Oral Microbe Microarray Initiative (NOMMI). To determine how exposure to CSE influences the activity of the *P. gingivalis* genome, we compared gene expression in *P. gingivalis* W83 grown in control (GAM) and conditioned (CSE-GAM) media. Microarrays were performed on triplicate GAM and GAM-CSE cultures. Total RNA from control and experimental cultures was isolated by extraction from washed cells with hot phenol followed by chloroform extraction and isopropanol precipitation. RNA was purified and genomic DNA was removed using a Qiagen Midi kit with on-column DNAse treatment followed by further digestion with RNAse-free DNAse to remove any trace contamination of genomic DNA. The synthesis of labeled cDNA was carried out by methods established by The J. Craig Venter Institute (www.jcvi.org). Briefly, 2-2.5μg of total RNA was mixed with 500ng of random hexamers, incubated at 70^0C for 10' and transferred to ice. A 50 x 2:3 aminoallyl dUTP:dTTP ratio labeling mix containing 25 mM remaining nucleotides, 5 x RT buffer, 3 μl of 0.1 M DTT and 2 μl Superscript II (200U/μl) were added. Samples were then incubated for 10' at room temperature and transferred to 42^0C, overnight. Reactions were stopped with 10 μl of 1N

NaOH and 10 µl of 0.5 M EDTA for 15' at 65^{0}C and neutralized with 25 µl 1M Tris, pH 7.4. Unincorporated aa-dUTP and free amines were removed using a Qiagen QIAquick PCR purification kit protocol with phosphate buffers. The cDNA was then dried down in a speed vacuum and resuspended in 4.5 µl of 0.1 M sodium carbonate buffer, pH 9.0. The appropriate NHS-ester Cy3 or Cy5 dye in DMSO was added (4.5 µl) and the reaction incubated at room temperature in the dark for 2 hours. The reaction was neutralized by adding 35µl of 100 mM sodium acetate, pH 5.2. The labeled cDNA was purified to remove any uncoupled dye using the Qiagen PCR purification kit with Minelute columns and an extra wash. The labeled cDNA was quantified and evaluated by spectrophotometry and equivalent pmol of Cy3 and Cy5 samples were combined, dried down, and stored at -80°C. Hybridization and subsequent analysis of the arrays was carried out following protocols established by The J. Craig Venter Institute, essentially as described by (219). Arrays were pre-hybridized at 42^{0}C for 1 hr in 5 x SSC/0.1%SDS/ 1% BSA and washed several times with MilliQ water. Washed arrays were then dipped into isopropanol and dried by centrifugation at 1000 rpm for 10 minutes. For hybridization, Cy3/Cy5 probe mixtures were suspended in 50µl hybridization buffer (50% formamide, 5 x SSC, 0.1%SDS, 0.1 mM DTT, 0.56 µg/ml sheared salmon sperm DNA), heated then applied to the array under a Lifter^{TM} cover slip. The array was incubated at 42^{0}C in a sealed chamber for 15-20 hr. After hybridization, the array was washed twice in 2 x SSC/0.1% SDS at 55°C, twice in 0.1 x SSC/0.1%SDS, once with 0.1 x SSC, water rinsed, and dried. Arrays were then scanned using an Axon slide reader, scanning in the Cy5 channel and the Cy3 channel. Identification and calculation of spots was carried out using the TIGR Spotfinder software

(http://www.jcvi.org/cms/research/software/). Subsequent normalization of fluorescent data was conducted using TIGR-MIDAS (also freely available from TIGR). Subtraction of local background and integration of Cy5 and Cy3 fluorescent intensities was calculated using Excel. Array experiments were carried out in triplicate using independently isolated RNA samples. Genes that are up- or down-regulated by greater than 2-fold in the post-normalized consensus were classified as differentially expressed, as presented and further detailed in Figure 3.

Validation of array data:

Differentially expressed genes of interest were confirmed by quantitative real-time reverse transcription-PCR using an Applied Biosystems 7500 Real-Time PCR system, essentially as described previously (220). Primers were designed using the real-time PCR primer design software provided by GenScript (www.genscript.com/ssl-bin/app/primer):

PG1055 (F: CCTACAGATTGGAGGTGGCT, R: ATAGGCATGGTATGCTGCAA);

PG2102 (F: ATCGTTTGGTCTGATACGCA, R: CTGCACGTTCAGCCTGTATT);

PG2100 (F: ATTACAAGATGGCTGTGGCA, R: TGCTGTCATGACTGTCCAAA);

PG2008 (F: ATTCTTAGGAACGAGCGCAT, R: GGGATTCCCTTGATCGAGTA);

PG1286 (F: AGCGATCAATGACCAAATCA, R: CACGGCATCGATAGCTTCT);

PG1552 (F: GATTTGAAGCCGGAGAAGAG, R: TAAGGCAGTACCACATTCGC);

PG0111 (F: AGGCAACGGAGAAGTATCGT, R: AAAGCACCATCAATGACGAA);

PG1432 (F: ACAGCTCGAACTGCATCAAC, R: CTGCATATAAGTGCGGGCTA);

PG0117 (F: TCTCGAACGACCAATAGTGC, R: CATTCTCTGCAATCGGCTTA);

PG2132 (F: GTAAATCTGGAGCCAACCGT, R: CGAGTAACGGCAAGAGGAGT); and

PG1548 (F: TGGAAGATGCCTCATGGATA, R: GTAATGCTGAAAGTCGGCAA).

***P. gingivalis* W83 outer membrane protein profiling:**

Outer membrane preparations were obtained from *P. gingivalis* cells grown in GAM or GAM-CSE. Briefly, the cell pellet was suspended in 10mM Tris, pH 7.5, 1mM EDTA (TE) containing 100 μg/ml lysozyme and protease inhibitor cocktail and was incubated for 15 minutes at 25^0C. The cell suspension was sonicated three times on ice and cellular debris was removed by centrifugation at 1000 x g for 10 minutes. The supernatant was subsequently centrifuged at 100,000 x g for 60 minutes. The membrane pellet was suspended in 5 mls TE containing 0.5M NaCl and centrifuged again at 100,000 x g for 60 minutes. After discarding the supernatant, the membrane pellet was suspended in 5 ml $MgCl_2$ containing 1% Triton X-100. Triton X-100 insoluble material was then collected by centrifugation at 100,000 x g. The resulting outer membrane fraction was suspended in TE containing 0.5% SDS and analyzed by PAGE. Outer membrane preparations (20 μg protein) were electrophoresed through 4-15% gradient SDS-PAGE gels and protein bands were visualized by staining with Coomassie brilliant blue R-250. Gel image analysis and densitometry were performed using the Kodak 4000MM Image Station system (Eastman Kodak, New Haven, CT). Protein identification was achieved by excision of the protein bands of interest (see Figure 8) from SDS-PAGE gels, followed by in-gel trypsin digestion, peptide preparation, MALDI-MS analysis, and

bioinformatic identification by peptide mass fingerprinting using the core facilities at the University of Louisville core proteomics laboratory.

Expression of RagB in response to CSE:

P. gingivalis cells were sequentially passaged in GAM, GAM-CSE and or reconditioned back in GAM, respectively. Bacterial cells were lysed (10mN Tris, 100mM NaCl and 1mM EDTA) and probed by western blot (20 μg protein per lane). Total protein estimated using a BCA protein assay kit. MAbE38, an anti-RagB antibody, was a kind gift from Mike A. Curtis. Anti-Rabbit HRP-linked IgG was used as the secondary antibody. Probing and visualization of immunoreactive bands was performed by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate kit as per the manufacturer's protocol. Gel image analysis and densitometry were performed using the Kodak 4000MM Image Station system.

Analysis of capsule production and FimA expression on CSE-exposure:

Mid- to late-log phase *P. gingivalis*, grown in GAM or GAM-CSE, were mounted on a formvar-coated copper grid, negatively stained with phosphotungstic acid at pH 7.0, and the bacteria visualized using a Philips CM-10 Transmission Electron Microscope. Total *P. gingivalis* lysate (1×10^5 mid-late log cells) for Western blots were obtained from cells passaged twice in GAM, twice in GAM-CSE, or twice in GAM-CSE then reconditioned in fresh GAM. Western blots were probed with rabbit anti-FimA sera and HRP-linked anti-rabbit IgG. Immunoreactive bands were visualized by chemiluminescence. Imaging and densitometry were performed using the Kodak

4000MM Image station.

Analysis of FimA binding activity and surface availability:

Adhesion of mid- to late log phase CSE-exposed and control *P. gingivalis* cells to the extracellular matrix protein and FimA ligand, fibronectin, was measured by ELISA using fibronectin coated microplates plates, rabbit anti-*P. gingivalis* antibody followed by HRP-linked anti-rabbit IgG antibody and tetramethylbenzidine as the chromogenic substrate, essentially as previously described by Pierce et al (221). Similarly, surface accessibility of FimA presented on mid- to late- log phase CSE-exposed and control *P. gingivalis* cells was estimated by ELISA using rabbit anti-FimA antibody.

Analysis of *P. gingivalis* biofilm formation:

Formation of mono-species *P. gingivalis* biofilms was examined, using a similar procedure to that described by Daep et al for bi-species biofilms (222). Briefly, *P. gingivalis* cultures grown to late log phase either in GAM or GAM-CSE were introduced into BST FC 71 flow cells (Biosurface Technologies Corp., Bozeman, MT) with saliva coated cover slips at a flow rate of 6 ml/hr for 2hr. Flow was maintained using a Manostat Carter 4/8 cassette peristaltic pump (Fisher Scientific, Suwanee, GA). Bacteria were then fed with either GAM or GAM-CSE for 48hr. Resulting *P. gingivalis* biofilms were visualized by FITC staining. The depth of FITC-labeled *P. gingivalis* biofilm was determined on an Olympus Fluoview confocal laser scanning microscope (Olympus, Pittsburgh, PA) from 5 frames randomly chosen by FluoView (Olympus, Pittsburgh, PA). Microcolony depth was determined by performing *Z*-plane scans from 0 μm to 50 μm

above the cover glass surface. Biofilm characteristics (depth, total surface area covered, substratum coverage, average and total thickness) were computed using Matlab v7.5.0 and Comstat (The Mathworks Inc., Natick, MA) (169).

Purification of rFimA and rMfa1:

Recombinant FimA and Mfa1 proteins were induced in *Escherichia coli* vectors containing FimA or Mfa1 cloned into pET-30 expression system, as previously described by Park et al . The C-terminal penta-histidine tagged recombinant proteins were induced in *Escherichia coli* by IPTG and purified using HiTrap chelating HP affinity columns. Purity of recombinant proteins was confirmed by SDS-PAGE. The lack of contamination by LPS, or other inflammatory mediators, was confirmed by several measures, that is, assessment of pro-inflammatory activity (TNF-α production by PBMCs) of recombinant proteins digested with proteinase K attached to acrylic beads; assessment of pro-inflammatory activity (TNF-α production by PBMCs) of recombinant proteins following boiling in 2% SDS; and the limulus amebocyte lysate assay.

Pro-inflammatory profiling of FimA and Mfa1:

0.5 x 10^6 PBMCs or neutrophils per well were seeded in 96-well plates and stimulated with 1μg/ml rFimA, rMfa1, or TLR-specific agonists (*E. coli* LPS, Pam3CSK4 and FSL). TNF-α, IL-6, and IL-10 (PBMC) and IL-8 (neutrophil) levels were determined in 24 hr supernatants by ELISA. OBA-9 cells were seeded at 0.1 x 10^6 cells per well in 12-well plates. On reaching confluence (48 hr) the epithelial cells were

stimulated with rFimA, rMfa1, LPS, Pam3CSK4 and FSL. 24 hr OBA-9 culture supernatants were assayed for IL-8 by ELISA.

TLR-specificity of FimA and Mfa1:

Human Embryonic Kidney (HEK) 293 cells expressing either TLR2/1, TLR2/6, TLR4, TLR4-CD14-MD2 and HEK TLR null cells were maintained at 37°C in 5% CO_2 in DMEM medium supplemented with 10% FBS, 100μg/ml normocin and 10μg/ml blastocidin. 50μg/ml hygrogold was added to the additionally to the above media for TLR4-CD14-MD2 cell maintenance. TLR activation in each cell line was measured as IL-8 induction 24 hr post-stimulation with rFimA, rMfa1 or specific TLR positive control agonists.

Induction of TLR2 tolerance by FimA:

To determine if rFimA induced TLR2-specific tolerance, PBMCs (1 x 10^6 per well) were stimulated, or not, with rFimA (1μg/ml) in 5 ml polypropylene tubes. 24 hr culture supernatants were collected, PBMCs washed once with DPBS and re-suspended in RPMI. The cells were then restimulated with FimA, Mfa1, Pam3CSK4 or *E. coli* LPS (all 1μg/ml) for an additional 24 hrs. TNF-α, IL-6 secretion was measured by ELISA.

NF-κB induction by FimA and Mfa1:

0.5 x 10^6 THP-1 Blue cells, maintained at 37°C, 5% CO_2 in RPMI supplemented with heat inactivated FBS, 100u/ml penicillin G and 100μg/ml streptomycin and 200μg/ml zeocin, were stimulated with FimA, Mfa1 or TLR agonists in 96 well plates for

24hrs. Nf-κB activation was quantified by using the QUANTI-Blue assay, according to the manufacturer's protocol.

TLR2 surface expression following FimA stimulation:

PBMCs (1 x10^6 cells) were treated with FimA (1μg / ml) or left unstimulated for 24hrs. Surface expression or redistribution of TLR2 was quantified by flow cytometry using FITC-conjugated anti-TLR2 and istoype control antibodies.

Involvement of IRAK-1, in FimA-induced TLR2 tolerization:

Whole cell lysates from 2.5 million PBMCs stimulated with 1 μg/ml FimA, and unstimulated controls, were prepared using RIPA lysis buffer with a phosphatase and protease inhibitor cocktail. Total protein was determined by BCA assay according to the manufacturer's protocol. Western blots (30 μg protein) were probed for IRAK-1, IRAK-M, IκBα and β-actin. Immunoreactive bands were visualized by chemiluminescence. Image analysis and densitometry were performed using the Kodak 4000MM Image Station. Band intensities were normalized to the loading control (β-actin) and expressed as relative intensities.

Statistical analysis:

All experiments were carried out a minimum of three times, unless otherwise noted. Statistical significance between groups was evaluated by one-way nonparametric ANOVA and the Tukey multiple-comparison test using the InStat program (Graph-Pad Software, San Diego, CA). Differences between groups were considered significant at the

level of $P < 0.05$.

CHAPTER THREE: TOBACCO-INDUCED ALTERATIONS TO *P. GINGIVALIS*-HOST INTERACTIONS.

Introduction

Cigarette smokers, and those exposed to secondhand smoke, are more susceptible to multiple infectious diseases than unexposed individuals (58, 223-227). Tobacco smoking is considered to be a major environmental risk factor for periodontitis, a common infectious oral disease that results in detachment of the gingival tissue cuff, destruction of the collagen attachment to the root surface of the tooth, and resorption of the supporting alveolar bone. Patients who smoke exhibit not only increased susceptibility to periodontitis but are also more likely than non-smokers to display severe disease and to be refractory to treatment (88). Although periodontitis is caused by a polymicrobial infection of the gingiva, the Gram negative, anaerobic bacterium, *Porphyromonas gingivalis*, has been strongly associated with diseased sites in the oral cavity and is considered to be a causative agent of chronic periodontitis (146, 228). In addition, *P. gingivalis* infection elicits systemic inflammation (229) and may increase susceptibility to systemic vascular diseases (230, 231). Multiple studies have shown that

smokers are more likely to be infected with *P. gingivalis,* to harbor higher numbers of *P. gingivalis*, and to exhibit more persistent infection (138-141, 147) relative to non-smokers.

Despite increased *P. gingivalis* infection rates and susceptibility to disease progression, smokers consistently display reduced clinical inflammation, measured as angiogenesis, edema, inflammatory index, and/or gingival bleeding compared to non-smokers (86, 88, 133, 203, 232). Smokers also exhibit lower levels of pro-inflammatory cytokines at diseased sites (233-237). This represents a clinical conundrum to oral health professionals who must diagnose periodontal diseases, and a scientific enigma to those attempting to understand the mechanisms underlying tobacco-induced and / or exacerbated periodontitis.

It is known that tobacco smoke and specific smoke components and/or metabolites can exert a profound negative influence on the host response (86, 88, 217, 238-241). For example, previous work in the lab in myelocytic cells demonstrated that nicotine and/or its primary metabolite, cotinine, suppresses the pro-inflammatory cytokine response to *P. gingivalis* while promoting the release of the anti-inflammatory cytokine IL-10 (241). Nicotine also suppresses the production of reactive oxygen species in response to Gram negative stimuli, concomitant with a reduced capacity to kill phagocytosed *P. gingivalis* cells (242).

In contrast to what is known about host responses, there are essentially no data available on how components in cigarette smoke may influence the organisms that comprise the oral microbial biofilm. Thus, it is possible that smoking not only influences the host response but also the organisms in the dental biofilm and that the

increased severity of periodontitis in smokers may be attributed to both of these effects. We hypothesized that cigarette smoke extract (CSE) represents an environmental stress to *P. gingivalis* and that the organism adapts to this stress by altering its pattern of gene expression. In this report, we show that the pro-inflammatory response of primary human peripheral blood mononuclear cells (PBMCs) against *P. gingivalis* is significantly reduced when the bacteria are pre-treated with CSE and that this effect was reversed when CSE treated bacteria were sub-cultured in fresh growth medium without CSE. The response of *P. gingivalis* to CSE exposure was examined using both biochemical and molecular biologic approaches. Numerous genes, including those encoding outer membrane proteins and virulence determinants were differentially expressed upon CSE exposure. These results may explain at least in part the altered virulence and host-pathogen interactions associated with cigarette smoking.

Results

P. gingivalis growth in CSE-conditioned medium:

To determine if a physiologically relevant dose of CSE (500 ng ml^{-1} nicotine equivalents [see Experimental Procedures]; (214-217) was overtly toxic to *P. gingivalis* W83 cells, we compared the growth rates of bacteria in CSE-conditioned and non-conditioned medium. As shown in Figure 3, similar growth characteristics were observed, suggesting that *P. gingivalis* W83 tolerates this level of CSE. Thus, for all subsequent experiments, *P. gingivalis* was cultured in medium containing 500 ng ml^{-1} nicotine equivalents.

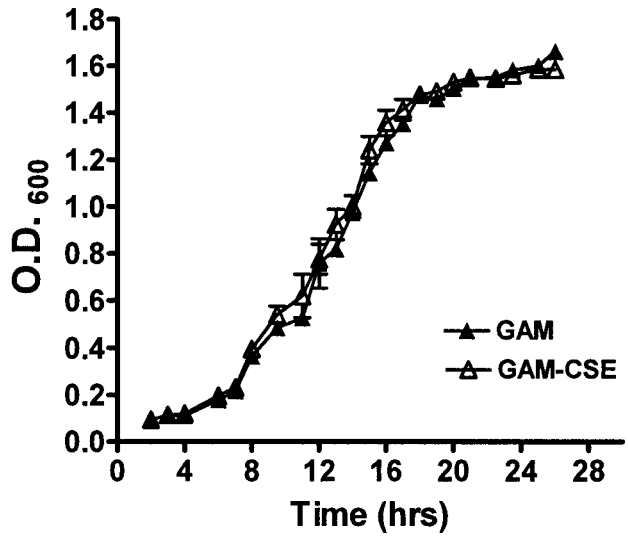

Figure 3. Effect of CSE on *P. gingivalis* growth: Typical growth curves of *P. gingivalis* W83 in CSE conditioned and unconditioned medium. Growth of *P. gingivalis* was compared in GAM medium and GAM conditioned with CSE (500 ng ml-1 nicotine equivalents). Closed triangles represent growth in GAM. Open triangles represent growth in GAM-CSE. Error bars represent the mean (SD) of three experiments. There were no significant differences in the growth characteristics of *P. gingivalis* cultured in GAM or GAM-CSE ($P>0.05$).

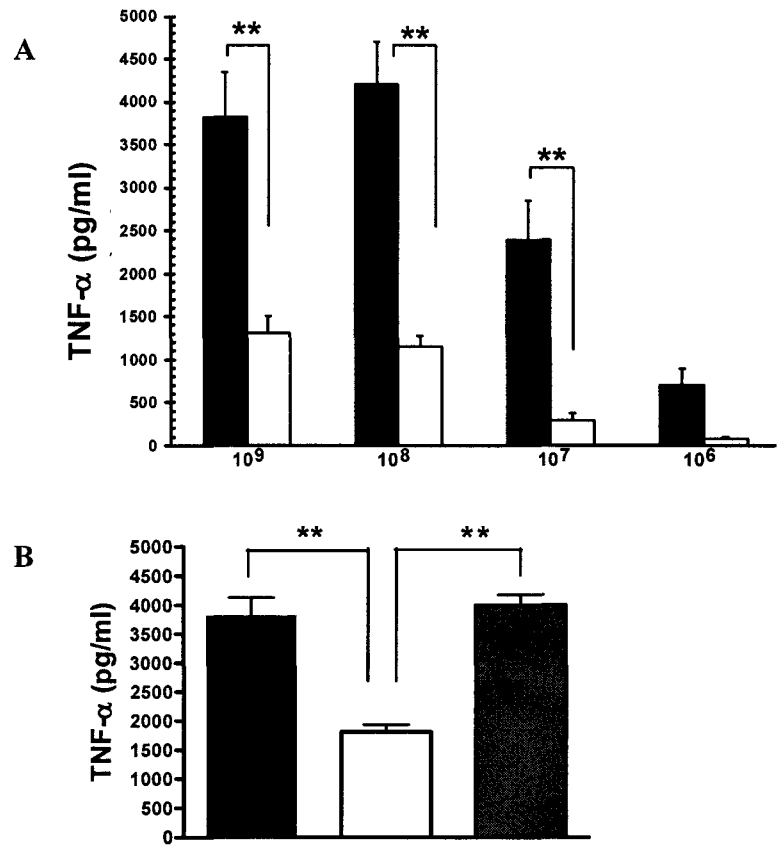

Figure 4. Reversible suppression of TNF-α release from human monocytes: **(A)** Primary human monocytes (0.5×10^6) were stimulated with 10^6-10^9 cells of control *P. gingivalis* (black bars) or GAM-CSE (white bars) for 20h and TNF-α levels determined in cell-free supernatants by ELISA (** represents $P < 0.01$). **(B)** Reconditioning of *P. gingivalis* in GAM rescues pro-inflammatory potential: Primary human monocytes (0.5×10^6) were stimulated for 20 h with GAM-cultured *P. gingivalis* (Black bars), CSE-treated *P. gingivalis*, or *P. gingivalis* cells that were first grown in GAM-CSE for two passages then reconditioned in untreated GAM for two passages (107 *P. gingivalis* cells). TNF-α levels were determined in cell-free supernatants by ELISA (** represents $P < 0.01$).

Induction of TNF-α release from PBMCs by CSE-exposed *P. gingivalis*:

Evidence from *in vivo* studies in humans have shown that smokers exhibit reduced clinical inflammation in response to pathogenic plaque bacteria (88, 203) and exhibit decreased gingival crevicular fluid (GCF) concentrations of major pro-inflammatory mediators, such as TNF-α and IL-1 (233-237) and increased GCF levels of anti-inflammatory cytokines including IL-10 and TGF-β1 (235, 243). To determine if exposure of *P. gingivalis* to CSE alters its inflammatory potential, primary human PBMCs were challenged with control and CSE-treated bacteria. As shown in Figure 4a, the release of TNF-α was significantly lower when PMBCs were incubated with CSE-treated bacteria. Interestingly, when CSE-treated organisms were sub-cultured in fresh growth medium without CSE, their inflammatory potential increased back to the level of untreated bacteria (Figure 4b), suggesting that the CSE-induced effect is reversible. Thus, *P. gingivalis* appears to reversibly respond to CSE as an environmental stress. The production of multiple proinflammatory cytokines (TNF-a, IL-6 and IL-12 p40) by CSE-exposed and control *P. gingivalis* was also screened in PBMCs. Production of TNF- α and IL-12 p40 was reduced in PBMCs stimulated with CSE-exposed bacteria compared with untreated *P. gingivalis* controls and, again, the inflammatory potential of *P. gingivalis* was restored on reconditioning in fresh GAM (Fig. 5).

***P. gingivalis* genes up-regulated on CSE-exposure:**

In order to further characterize the response of *P. gingivalis* to CSE-exposure, we performed microarray analyses to identify genes that are differentially expressed in CSE-

treated versus control bacteria. A total of 104 genes (approximately 4.7% of the *P. gingivalis* genome) were found to be differentially expressed by 2-fold or more ($p < 0.05$); 58 genes were induced and 46 were suppressed upon CSE treatment (see Figure 6). Of the 58 genes that were induced by CSE treatment, multiple genes within several predicted operons (based upon the gene annotation of the *P. gingivalis* genome sequence

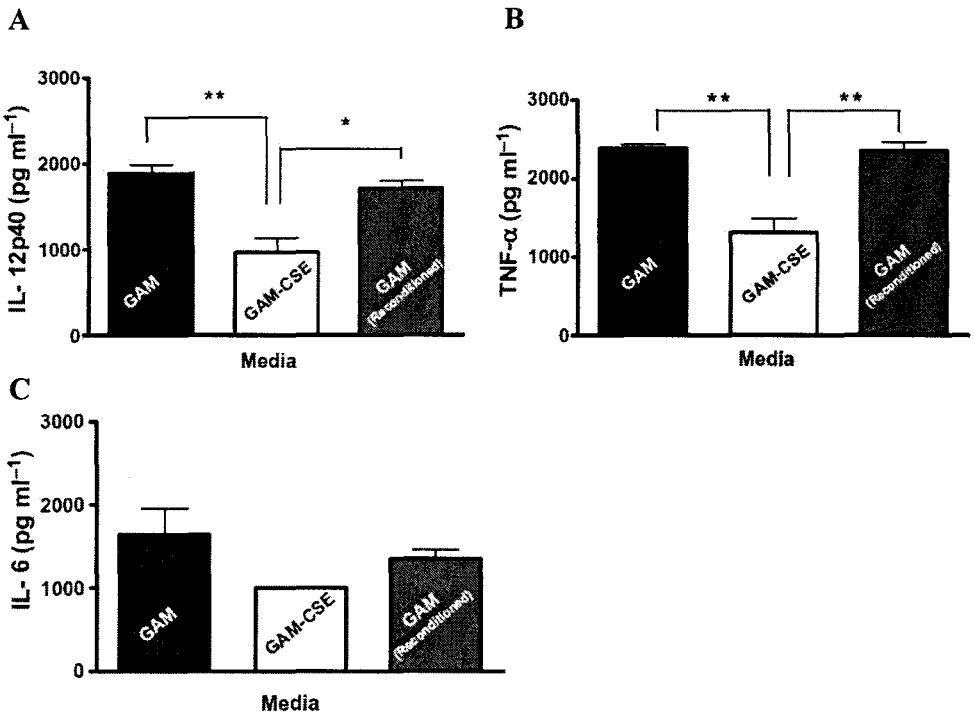

Figure 5: Reversible suppression of multiple proinflammatory cytokines in human PBMCs stimulated with CSE-treated *P. gingivalis*. Primary human PBMCs (0.5×10^6) were stimulated for 20 h with GAM-cultured *P. gingivalis*, CSE-treated *P. gingivalis*, or *P. gingivalis* cells that were first grown in GAM-CSE for two passages then reconditioned in untreated GAM for two passages (10^7 *P. gingivalis* cells). PBMCs stimulated with reconditioned *P. gingivalis* produced levels of **(A)** IL-12p40 **(B)** TNF-α **(C)** IL-6, that were similar to PBMCs that were stimulated with bacteria from control cultures (not exposed to CSE). Error bars represent the mean (SD) of three experiments. One asterisk (*) indicates statistical significance at $P < 0.05$; double asterisks (**) indicate statistical significance at $P < 0.01$.

by TIGR) were identified, including the major fimbrial operon (2 genes), an operon encoding outer membrane antigenic lipoproteins (4 genes); the transfer (*tra*) gene cluster (4 genes) and an operon encoding an ABC transporter of unknown function [PG0682 – PG0685; 3 genes]. Within the CSE-induced genes, several functional families predominate. One group of genes encode proteins that may be involved in DNA replication and repair (e.g. *ruvC* [PG1324, holliday junction endonuclease]; PG0817 encoding a putative integrase; and *radC* [PG0894, DNA repair protein]) or in the insertion and transposition of genetic material (e.g., *traE* [PG1483]; *traF* [PG1482]; and *traK* [PG1478], each encoding proteins associated with a conjugative transposon). Several other transfer genes (*traG* [PG1481] and *tra*Q [PG1473]) from this same region of the *P. gingivalis* genome were differentially expressed but fell just below the 2-fold limit used in the analysis. In addition, several of the insertion sequences that are present in the *P. gingivalis* genome were differentially expressed (ISPg3 [PG0299]; ISPg5 [PG0943]; ISPg6 [PG1061].

A second group of CSE-induced genes encode proteins involved in potential pathogen-host interactions and virulence. These include several proteases (encoded by *tpr*, *trpT* and PG0317), an efflux transporter (PG0283) related to the bacterial secretion system I protein HlyD, and a putative polysaccharide transport protein related to O-antigen flippase (PG0912). In addition, two genes encoding putative lipoproteins (PG2133 and PG2134) that are required for the assembly of the major fimbriae of *P. gingivalis* (244) were induced by CSE. These genes are co-expressed with *fimA* encoding the fimbrial subunit protein. Several other genes encoding cell surface or outer membrane polypeptides were also differentially expressed including the co-expressed

Figure 6. *Porphyromonas gingivalis* genes up-regulated following CSE exposure. The microarray data of Log2 ratios were uploaded onto MultipleExperiment viewer 4.1v (January 8, 2008 release) software and evaluated by *t*-test with *P*-value based on't' distribution with significance (alpha cut-off) set at $P < 0.01$. The subset of significant genes was divided into two groups based on (i) upregulated genes (> 1.5-fold increase) and (ii) downregulated genes (< 0.5-fold). The distance was calculated based on Euclidean distance with average linkage and the tree was generated based on hierarchical clustering. Cigarette smoke extract-induced genes (significantly induced genes > 1.5-fold compared with baseline; $P < 0.01$ are represented in yellow to blue colour with the lower limit = 1.5; midpoint value = 2.5; and upper limit = 5.

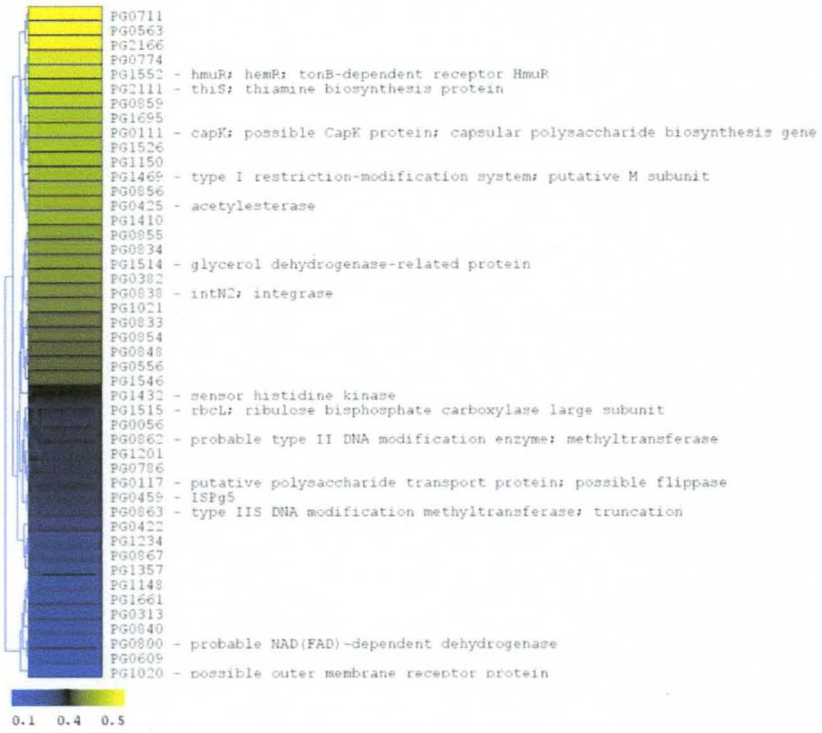

Figure 7. *Porphyromonas gingivalis* genes down-regulated following CSE exposure. The microarray data of Log2 ratios were uploaded onto MultipleExperiment viewer 4.1v (January 8, 2008 release) software and evaluated by *t*-test with *P*-value based on't' distribution with significance (alpha cut-off) set at $P < 0.01$. The subset of significant genes was divided into two groups based on (i) upregulated genes (> 1.5-fold increase) and (ii) downregulated genes (< 0.5-fold). The distance was calculated based on Euclidean distance with average linkage and the tree was generated based on hierarchical clustering. Cigarette smoke extract-suppressed genes (significantly downregulated genes < 0.5-fold compared with baseline; $P < 0.01$) are represented in yellow to blue colour with the lower limit = 0.1; midpoint value = 0.4; and upper limit = 0.5.

major immunoreactive 61 and 63 KDa surface antigens encoded by PG2102 and PG2100, respectively, putative lipoproteins (PG1233, PG1234 and PG0722), and an outer membrane TonB-dependent receptor (PG2008). Other genes were differentially expressed but fell just below the 2-fold limit used in the analysis, including the lpt-1 (ptp1, PG1641) gene that has recently been shown to be a multifunctional regulator of virulence (245).

To verify the results of the microarray experiments, the expression of select genes from the array dataset were analyzed by RT-PCR. As shown in Table 2, the RT-PCR results of selected genes that were tested were consistent with the increased expression of these genes observed in the microarray analyses of CSE-treated cells.

***P. gingivalis* genes down-regulated on CSE-exposure:**

Many of the 46 genes (2.1% of the *P. gingivalis* genome) that were suppressed by two-fold or more ($p < 0.05$) encode hypothetical proteins (see Figure 7) whose functions have yet to be determined. However, two genes in the capsular biosynthesis locus (246) were down-regulated - a putative capsular polysaccharide synthesis gene (*capK*, PG0111) and a polysaccharide transport protein related to a flippase (PG0117). The expression of two other genes in the capsular biosynthesis locus, encoding a glycosyltransferase (PG0118) and a UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase (wecC, PG0108) were down-regulated just below the 2-fold limit used in the analysis. Also down-regulated in response to CSE was a sensor histidine kinase (*fimS*) which together with the co-expressed response regulator FimR has been reported to regulate the expression of the major and minor fimbrial operons of *P. gingivalis* (247). Lastly, the expression of *hmuR* (PG1552) encoding a tonB-dependent hemoglobin

receptor HmuR was reduced by approximately 2-fold by CSE treatment. Selected down-regulated genes were analyzed by RT-PCR (see Table 2) with consistent results.

Table 2: Real time PCR analysis of selected CSE-regulated *P. gingivalis* genes

Locus name [a]	Putative identification in TIGR and/or Oralgen database	Microarray [fold (mean, SD)]	RT-PCR [fold (mean, SD)]
PG1055	Thiol protease (Tpr)	13.93, 4.73	108.69, 21.43
PG2102	Lipopolysaccharide-modified immunoreactive 61kDa antigen	10.58, 1.47	36.62, 1.44
PG2100	Immunoreactive 63 kDa antigen	6.00, 1.23	30.79, 1.47
PG2008	tonB-linked outer membrane receptor P90	2.50, 2.00	1.74, 0.09[b]
PG1286	Ferritin (ftn)	2.02, 0.85	2.23, 0.21
PG1552	tonB-dependent receptor (HmuR, HemR)	0.47, 0.29	0.72, 0.02[c]
PG0111	Putative capsular polysaccharide biosynthesis gene (CapK)	0.45, 0.04	0.78, 0.02[c]
PG1432	Sensor histidine kinase	0.39, 0.10	0.62, 0.05[b]
PG0117	Putative polysaccharide transport protein	0.36, 0.14	0.50, 0.08

a. The J. Craig Venter Institute annotations; **b.** > 1.5, < 2.0-fold change in gene activity; **c.** Reduced activity not reaching threshold (< 1.5-fold).

CSE alters expression of *P. gingivalis* outer membrane proteins RagA and RagB:

In addition to the transcriptome analysis described above, a membrane fraction that was enriched for *P. gingivalis* outer membrane proteins was isolated from CSE-treated and control cells and analyzed by SDS gel electrophoresis. As shown in Figure 8, the Triton X-100 insoluble membrane fraction contained approximately ten major polypeptide bands, of which three were clearly overrepresented in the extract from CSE-

treated bacteria. These bands were excised from the gel and analyzed by MALDI-MS after digestion with trypsin. Tryptic peptides were identified for the RagA (43% sequence coverage), RagB (66% coverage) and a putative lipoprotein (PG0179, 53% coverage) that is likely co-expressed with the minor fimbrial antigen of *P. gingivalis* (248). Interestingly, western blots of RagB proteins revealed that reconditioning of *P. gingivalis* in GAM, following culture in GAM-CSE, resulted in RagB expression levels returning to control levels (Figure 9).

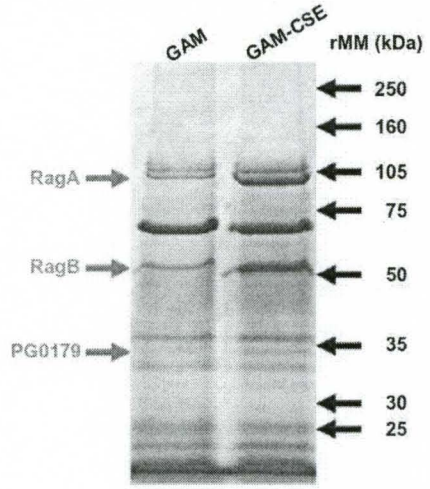

Figure 8: Upregulation of specific outer membrane protein in CSE-exposed *P. gingivalis*. Outer membrane proteins of *P. gingivalis* grown in GAM and GAM-CSE were isolated by differential extraction with Triton X-100. 20μg of total outer membrane protein extracts was run through a 4–15% gradient gel. MALDI-MS of excised bands followed by comparison with the open reading frame database of *P. gingivalis* W83 from The J. Craig Venter Institute identified the major CSE upregulated bands, as shown.

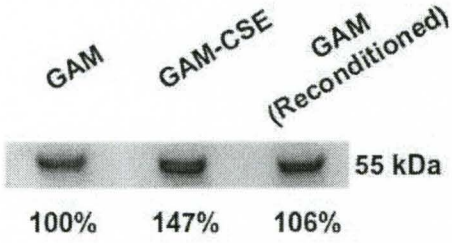

Figure 9: Cigarette smoke extract-induced RagB upregulation in *P. gingivalis* is reversible. A typical Western blot of RagB in lysates of cells sequentially passaged in GAM, GAM-CSE and GAM, respectively, is shown. Culture in GAM-CSE resulted in an increase in relative RagB expression levels (147%). Relative RagB expression returned to near control levels on reconditioning in GAM.

Discussion

Cigarette smoking increases vulnerability to *P. gingivalis* infection and increases susceptibility to periodontitis, but reduces clinical signs of overt inflammation. The mechanisms that cause the apparent dichotomy between increased infection by *P. gingivalis*, increased severity of disease, but reduced inflammatory response in smokers remain to be determined. Our results provide some of the first information to explain such phenomena and are consistent with many previous *in vivo* observations.

We show that *P. gingivalis* exposed to physiologic concentrations of CSE induce a significantly lower pro-inflammatory response from PMBCs than control cultures grown without CSE. Furthermore, this effect is reversed when CSE-treated bacteria are

sub-cultured in medium without CSE, suggesting that CSE exposure may represent an environmental stress that *P. gingivalis* is able to specifically respond to. Thus, the reduced inflammatory potential of CSE-exposed *P. gingivalis* cells represents a mechanism that may contribute, at least in part, to the reduced inflammation observed in periodontitis patients who smoke.

Such a reduced *P. gingivalis*-elicited inflammatory response is likely to aid these bacterial cells in immune evasion which, in turn, would help explain the increased levels of *P. gingivalis* infection and reduced clearance rates noted in smokers compared to non-smokers (138-141, 147).

Our results showing suppressed TNF-α induction from innate cells by smoke-exposed *P. gingivalis* are also in keeping with several *in vivo* reports of reduced levels of pro-inflammatory cytokines at diseased sites (233-237). Furthermore, the upregulation of several virulence factors in the periodontopathogen, *P. gingivalis*, are in keeping with the increased disease severity known to occur in smokers compared to non-smokers. Specific dysregulated virulence factors are discussed further below.

Our results clearly show that the response of *P. gingivalis* to CSE exposure is varied and genome wide. Approximately 7% of the genes in the *P. gingivalis* genome were found to be differentially expressed by transcriptome analyses. Furthermore, a number of the differentially expressed genes have been associated with aspects of *P. gingivalis* virulence. Several functionally-related genes were dysregulated, including multiple genes in the major fimbrial and capsular polysaccharide operons, as well as genes encoding transcriptional regulators; efflux pump and transport proteins; proteases and cell envelope proteins. The major fimbriae have been shown to be important for

numerous aspects of *P. gingivalis* virulence, including bacterial adhesion and invasion of epithelial and endothelial cells (Lamont & Jenkinson, 1998 and 2000). PG2133 and PG2134 encode putative lipoproteins that are co-expressed with FimA, the major fimbrial subunit protein. Wang et al. recently showed that inactivation of these genes results in the expression of significantly shorter fimbriae, suggesting that these putative lipoproteins play an essential role in fimbrial biogenesis. The mutant strains also exhibited reduced invasion and intracellular persistence in macrophages and were less virulent in a mouse model of periodontitis (244).

There are at least six different capsular serotypes (K1 to K6) expressed by *P. gingivalis* and altered virulence has been associated with K^+ and K^- strains (195, 249-251). While the contribution of *P. gingivalis* capsule to inflammation is poorly understood, recent evidence shows that capsular polysaccharide from several strains of *P. gingivalis*, including W83 elicits a substantial pro-inflammatory cytokine response from murine innate cells (252). The down regulation of several genes in the capsular biosynthesis operon (PG0111 and PG0117) suggests that capsule synthesis may be reduced upon exposure to CSE, consistent with the reduced inflammatory potential of CSE-treated bacteria.

In addition to the differential expression of genes described above, several other cell surface or outer membrane proteins, i.e., RagA, RagB and PG0179 were shown by biochemical approaches to be present at higher levels after CSE treatment. Interestingly, the genes encoding for these components were not identified as being differentially expressed in the micro array experiments. This suggests that post-transcriptional events may also be involved in the response to CSE, or alternatively may reflect inherent

limitations in the microarray approach. Each of the differentially expressed proteins has been associated with aspects of *P. gingivalis* virulence. The *ragAB* locus was likely acquired by horizontal gene transfer (253). RagA is a putative tonB-dependent outer membrane receptor whereas RagB is an immunodominant lipoprotein. Both genes have reported to be up-regulated under conditions of thermal stress (254) and both represent significant *P. gingivalis* virulence factors (255, 256), although their specific contribution to periodontal disease remains to be determined. However, it is interesting that the RagA homolog (designated OmpA) from the closely related bacterium *Bacteroides caccae* has been associated with inflammatory mucosal (bowel) disease (257). PG0179 is a gene that is co-expressed with the minor fimbrial antigen (*mfa1*). The minor fimbriae play a key role in *P. gingivalis* autoaggregation (174) and are critical mediators of the interspecies interactions between *Porphyromonas gingivalis* and oral streptococci that facilitate biofilm formation (173, 218, 258). Thus, stimulation of the minor fimbrial operon by CSE may facilitate increased *P. gingivalis* colonization of the periodontia.

Lastly, a number of genes that were differentially expressed encoded proteins involved in DNA replication, DNA repair and the transfer or mobilization of genetic material. Comparison of the recently completed genome sequence of *P. gingivalis* ATCC 33277 with that of strain W83 indicates that mobile genetic elements have contributed greatly to genomic diversity among *P. gingivalis* strains (Naito et al., DNA Res. 2008). The up regulation of transfer genes and several insertion sequence elements in CSE-exposed cells suggests that the environmental stress imposed by cigarette smoke may stimulate genetic rearrangements. In addition, considering the highly carcinogenic potential of cigarette smoke, the up-regulation of DNA-repair and -control genes is not

surprising. Cigarette smoke is also a major oxidative stressor (259, 260). A single cigarette is estimated to contain 10^{16} oxidant molecules (261). Thus, the up regulation of ferritin [PG1286], an iron sequestrator, may provide protection against free-iron-related oxidative stress (262). In addition, the response regulator, RprY (also up regulated after CSE treatment) has recently been shown to be responsive to reactive oxygen species and iron (263). It is also interesting to note that several other anti-oxidant genes were differentially expressed ($p<0.05$) when the threshold for induction in the array experiment was reduced from 2-fold to 1.5-fold. In addition, the up-regulation of efflux pump and other ABC transport systems may confer resistance to potentially harmful chemicals present in CSE. Taken together, these results suggest that CSE may represent a potent environmental stressor that induces a protective response from *P. gingivalis*.

In summary, smokers are more prone to infection with *P. gingivalis* and to develop periodontitis, yet exhibit reduced clinical inflammation. Consistent with this, we have found that CSE-exposed *P. gingivalis* exhibits a reduced capacity to elicit an inflammatory response from immune cells. *P. gingivalis* responds globally to CSE exposure and multiple virulence associated genes are differentially expressed. These results may explain in part the altered virulence and host-pathogen interactions that occur in smokers with periodontal disease and provide some of the first information illustrating how *P. gingivalis* responds at the molecular level to cigarette smoke.

CHAPTER FOUR: TOBACCO UPREGULATES *P. GINGIVALIS* FIMBRIAL PROTEINS THAT INDUCE TLR2 HYPOSENSITIVITY.

Introduction

Tobacco smokers are more susceptible than non-smokers to multiple infectious diseases, particularly mucosal infections such as tuberculosis, pneumonia, Chlamydiasis, gonorrhoea, otitis media and periodontitis (264). The mechanisms underlying such increased susceptibility are not well understood. Several groups have shown that tobacco smoke as well as individual smoke components induce physiological and structural changes, e.g. reduction of mucociliary clearance (265, 266), and dysregulate specific elements of immune function, e.g. inhibition of the respiratory burst and phagocytosis (242, 267, 268) and interference in antigen presentation (269, 270). However, the influence of tobacco on bacterial virulence is – essentially – unstudied.

Periodontitis is a bacteria-induced, irreversible chronic inflammatory mucosal disease characterized by the destruction of the soft and hard supporting structures of the teeth. Tobacco smokers are more susceptible than non-smokers to infections with periodontal pathogens (264) and are more likely to develop severe periodontitis and to

prove refractory to treatment (88). Paradoxically, smokers show reduced clinical signs of inflammation in response to dental plaque than non-smokers, particularly the key diagnostic indices of gingival bleeding on probing and edema (88, 203). Again, the mechanisms underlying this phenomenon are poorly characterized.

Porphyromonas gingivalis, a Gram negative, asaccharolytic anaerobe, is a key periodontal pathogen whose numbers are increased in tobacco smokers (88, 203). There is some evidence that components of tobacco smoke augment *P. gingivalis* pathogenesis. Nicotine and its primary metabolite - cotinine - have been shown to increase the lethality of cell-free extracellular toxins and cell lysates from *P. gingivalis* in the chick embryo model (271, 272). The combination of benzopyrene, a tobacco smoke aryl hydrocarbon, and *P. gingivalis* lipopolysaccharide (LPS) significantly increase the inhibition of osteogenesis in a rat bone marrow cell model, compared to either agonist alone (273).

We have recently shown that *P. gingivalis* adapts to the environmental stress presented by cigarette smoke extract (CSE) by altering the expression of several genes and outer membrane proteins (274). Concomitant with this adaptive response to CSE, *P. gingivalis* induces a lower inflammatory response (TNF-α, IL-6 and IL-12 p40) from human innate cells compared to unexposed, control bacteria (274). Furthermore, the inflammation-inducing potential of *P. gingivalis* is restored when cells are sub-cultured back into fresh medium without CSE. Interestingly, microarray analyses determined that specific genes (PG2133 and PG2134) in operons coding for the synthesis and assembly of major and minor fimbrial antigens (FimA and Mfa1) of *P. gingivalis* were induced on exposure to CSE, while several genes in the capsular biosynthesis locus (*capK*, PG0117, PG0118 and *wecC*) were suppressed (274).

It is important to note that capsule polysaccharides and major fimbrial protein will be the two *P. gingivalis* features that first engage the host response and, thus, are likely to play critical roles in directing initial host-pathogen interactions. Capsule production is associated with tissue invasiveness (195) and has been reported to be inversely related to biofilm growth (275), while capsular polysaccharides represent potent cytokine-inducing stimuli (252). The major fimbrial antigen or FimA is also an important virulence factor that facilitates the adhesion and initial attachment of *P. gingivalis* to junctional epithelial cells, thus aiding sub-gingival colonization (276). FimA appears to signal via TLR2 and induces the expression of several pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β in innate immune cells (150). However, the potency of FimA as a pro-inflammatory agonist is controversial (157, 168, 277, 278). Thus, alterations in *P. gingivalis* capsule and fimbriae production would be expected to exert marked effects on virulence and host-pathogen interaction.

We hypothesized that CSE-regulation of capsule and fimbrial genes is reflected at the ultrastructural and functional levels, alters the nature of host-pathogen interactions, and contributes to the reduced pro- inflammatory potential of smoke exposed *P. gingivalis*. We establish that CSE up-regulates *P. gingivalis* FimA at the protein level, suppresses the production of capsular polysaccharides at the ultrastructural level, and creates conditions that promote biofilm formation. We further show that while FimA is recognized by TLR2/6, it has only minimal inflammatory activity in several cell types (PBMCs, neutrophils, and epithelial cells) and, furthermore, FimA stimulation chronically abrogates the pro-inflammatory response to subsequent TLR2 stimulation by other TLR2-specific agonists (Pam3CSK4, Mfa1) in an IRAK-1-dependent but NF-κB-

independent manner. These studies provide some of the first information to explain, mechanistically, how tobacco smoke changes the *P. gingivalis* phenotype in a manner likely to promote *P. gingivalis* colonization and infection while simultaneously reducing the host response to this major mucosal pathogen.

Results

Tobacco smoke exposure alters expression of *P. gingivalis* fimbriae and capsule:

Our previous microarray data indicated that CSE induced the expression of genes key to the synthesis and assembly of FimA (PG2133 and PG2134), concomitant with a suppression expression of several genes in capsular biosynthesis locus (*capK*, PG0117, PG0118 and *wecC*) (274). We now show that CSE-exposure reversibly increases FimA protein, as shown in *Figures 10A* and *B*. Transmission electron micrographs clearly establish that these CSE-regulated transcriptional and translational activities are reflected at the ultrastructural level, as shown in *Figures 10C* and *10D*. Furthermore, CSE-induced FimA is both surface exposed, as assessed by availability to FimA-specific antibodies (*Figure 10F*) and functional, as assessed by binding to the established FimA ligand – fibronectin (155) (*Figure 10E*).

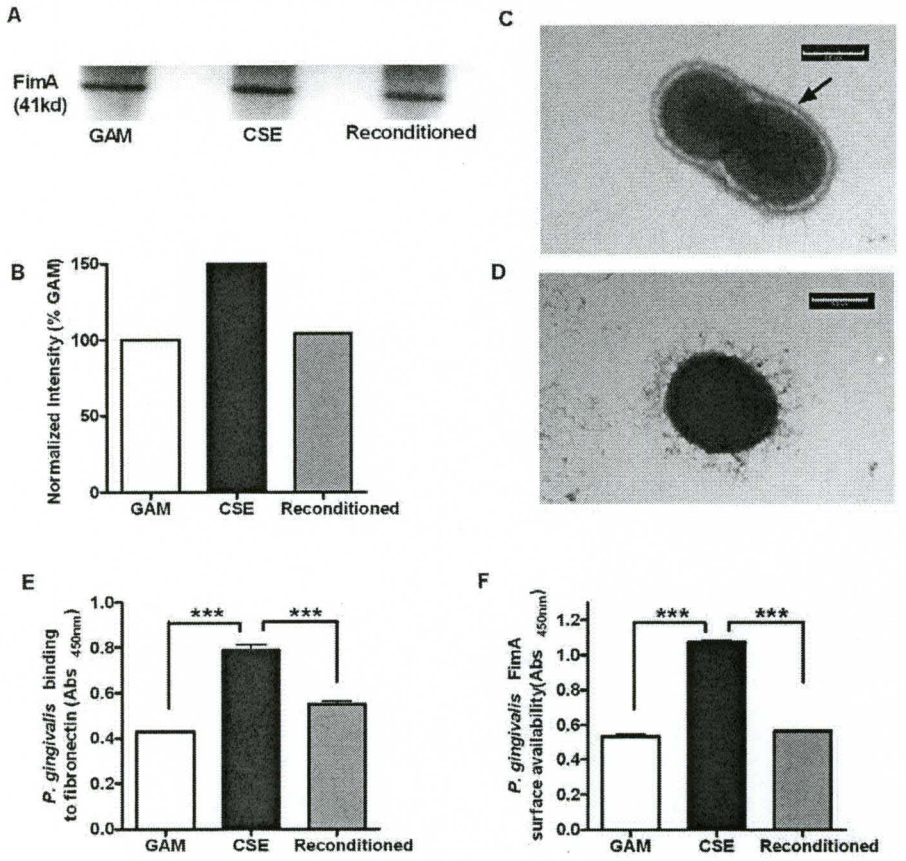

Figure 10: CSE induces phenotypic surface changes in *P. gingivalis*. **(A)** Typical Western blot of FimA in lysates of $1x10^6$ *P. gingivalis* cells sequentially passaged in GAM, GAM-CSE, and then fresh GAM, respectively. **(B)** Typical relative band intensities establish that FimA expression is increased on CSE-exposure, but that FimA expression reverts to control levels upon sub-culturing *P. gingivalis* back into fresh GAM. Representative transmission electron images of *P. gingivalis* grown in GAM **(C)** or GAM-CSE **(D)**. The black arrow indicates the *P. gingivalis* capsule, which is greatly reduced in presence of CSE. These CSE-induced phenotypic changes are concomitant with an increased binding of *P. gingivalis* to the FimA ligand, fibronectin **(E)** and surface availability of FimA **(F)**.

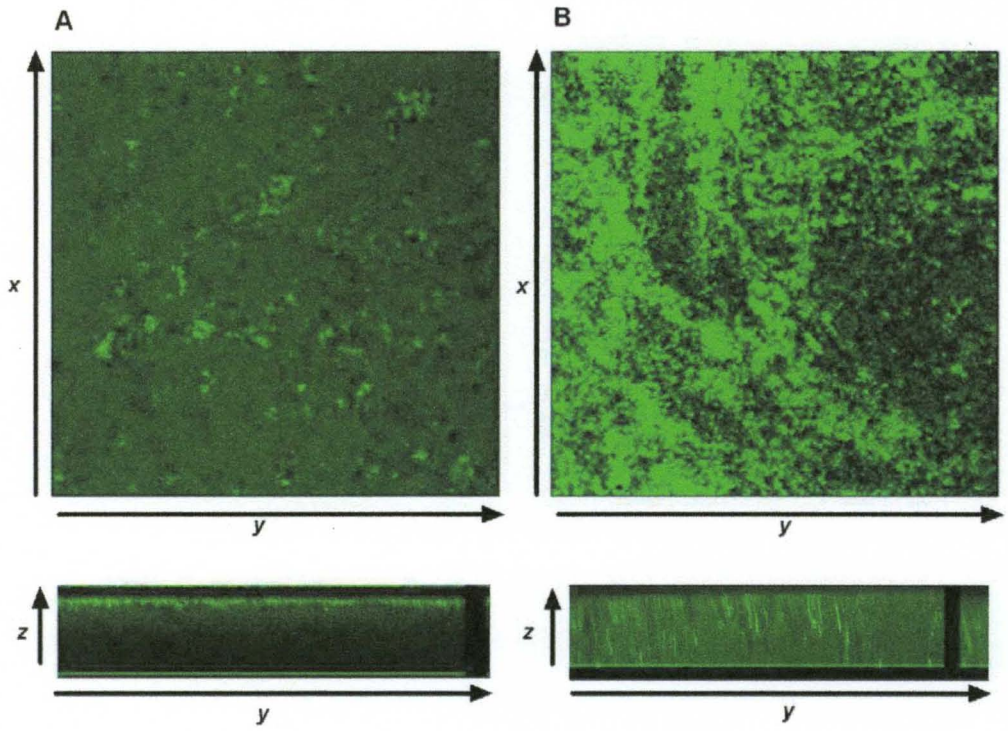

Figure 11: Visualization of homotypic biofilm formation by *P. gingivalis* grown in GAM or CSE. 48 hr *P. gingivalis* biofilms, formed in an open flow system, were stained with FITC. Optical sections were obtained along the x-y and z axis at 1 μm intervals. **(A)** and **(B)** are representative images of biofilms formed in GAM or GAM-CSE, respectively. Top panels represent x-y images while the bottom panel shows x-z stacks, reflecting biofilm thickness formed in GAM or GAM-CSE, respectively. Z-stack images were obtained from 5 randomly selected fields per biofilm and image data recorded along the x-y-z planes.

CSE augments *P. gingivalis* biofilm formation:

Biofilms play an important role in the pathology of periodontal disease by mechanisms that include protection of plaque bacteria against phagocytosis and against antibiotics (249). FimA plays a critical role in *P. gingivalis* biofilm formation. Indeed, *P. gingivalis* strains lacking FimA cannot form biofilms (279). Furthermore capsule synthesis has been shown to inversely correlate with biofilm growth (275). To establish if our initial discoveries that CSE upregulates FimA production but downregulates capsule production (274) influence biofilm formation, CSE-exposed and unexposed *P. gingivalis* was grown in an open flow biofilm system. Representative biofilm images are presented in *Figure 11A-B*. There was a significant increase in overall homotypic biofilm formation in the presence of CSE (*Table 3*), with increased biomass, substratum coverage, maximum and mean thickness apparent (all $p < 0.01$).

Table 3: Quantitative characteristics of *P. gingivalis* biofilms

48h Biofilm	Biomass ($\mu m^3/\mu m^2$)	Substratum coverage (μm^2)	Average thickness (μm)
P. gingivalis GAM	1.869 ± 1.15	0.315 ± 0.11	2.505 ± 0.443
P. gingivalis GAM-CSE	5.708 ± 0.38***	0.679 ± 0.06	12.66 ± 1.487**

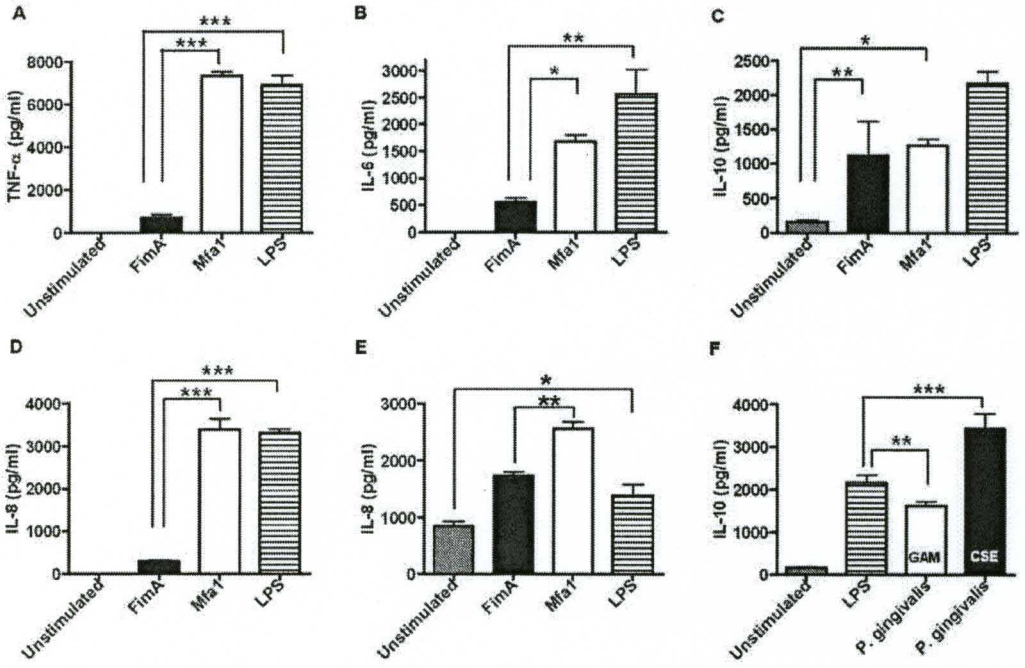

Figure 12: Inflammatory potential of rFimA and rMfa1. $0.5x10^6$ primary human PBMCs were stimulated with 1 μg/ml of rFimA, rMfa1 or the TLR2 and -4 specific agonists Pam3CSK4 and *E. coli* LPS, respectively. **(A)** TNF-α; **(B)** IL-6; and **(C)** IL-10 release was quantified by ELISA in 20 hr cell-free supernatants, harvested by centrifugation. **(D)** $0.5x10^6$ primary human neutrophils were stimulated with 1 μg/ml of rFimA, rMfa1, Pam3CSK4 or *E. coli* LPS. IL-8 release was quantified in 20 hr cell-free supernatants by ELISA.**(E)** Confluent OBA-9 epithelial cells (0.46106) were stimulated with 1 μg/ml of rFimA, rMfa1, Pam3CSK4 or E. coli LPS. IL-8 release was quantified in 20 hr cell-free supernatants by ELISA. **(F)** Similar to purified FimA, whole, CSE exposed *P. gingivalis* also induced increased IL-10 secretion from primary human PBMCs compared to control bacteria. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

FimA and Mfa1 exhibit differential pro-inflammatory potential:

While the innate response to the major fimbrial antigen of *P. gingivalis*, FimA, has been partially characterized (45) the inflammatory potential of the minor fimbrial antigens (Mfa1) are not well understood. Therefore, to better understand the relevance of CSE-upregulation of FimA and Mfa1, particularly in the context of the reduced inflammatory response to plaque that is consistently observed in human smokers (280), we quantified the cytokine production elicited by these predominant, CSE-regulated surface antigens in PBMCs (Figure 12A-C), neutrophils (Figure 12D) and gingival epithelial cell line OBA-9 (Figure 12E). While FimA did promote a cytokine response from each cell type tested, the concentration of pro-inflammatory cytokines induced (TNF-α; IL-6; IL-8) was minimal when compared to the classic bacterial-derived pro-inflammatory agonists, LPS and Pam3CSK4. In contrast, FimA did induce high levels of the anti-inflammatory cytokine, IL-10 (Figure 12C). Mfa1, on the other hand, induced a robust pro-inflammatory response that was comparable or greater in magnitude to that of LPS in PBMC's (Figures 12A and 12B), neutrophils (Figure 12D) and epithelial cells (Figure 12E). These results are similar to those seen with intact bacteria. We have previously shown that whole, smoke-exposed *P. gingivalis* exhibit reduced inflammatory potential than control (unexposed) cells, as measured by decreased induction of multiple pro-inflammatory cytokines (TNF-α, IL-6 and IL-12 p40) (281). We extend these observations to show that CSE-exposed *P. gingivalis* induce increased IL-10 in innate cells, compared to unexposed bacteria (Figure 12F).

FimA and Mfa1 are TLR2-specific agonists:

In order to understand the differential pro-inflammatory potential of FimA and Mfa1 at the mechanistic level, we first established the TLR-specificity of each of these two specific CSE-dysregulated *P. gingivalis* surface proteins. IL-8 production by rFimA or rMfa1 stimulated HEK293 cells stably expressing variant TLRs was quantified. As shown in Figure 13A, significantly higher levels of IL-8 were produced by HEK clones expressing TLR2/1 and, particularly, TLR2/6 compared to all other clones (both $p > 0.001$).

As FimA induced lower levels of pro-inflammatory cytokines in innate immune cells than Mfa1 we hypothesized that FimA may not be a strong activator of NF-κB. However, in the THP-1 blue cell model, FimA and Mfa1 each proved to be effective inducers of NF-κB transcription (see Figure 13B).

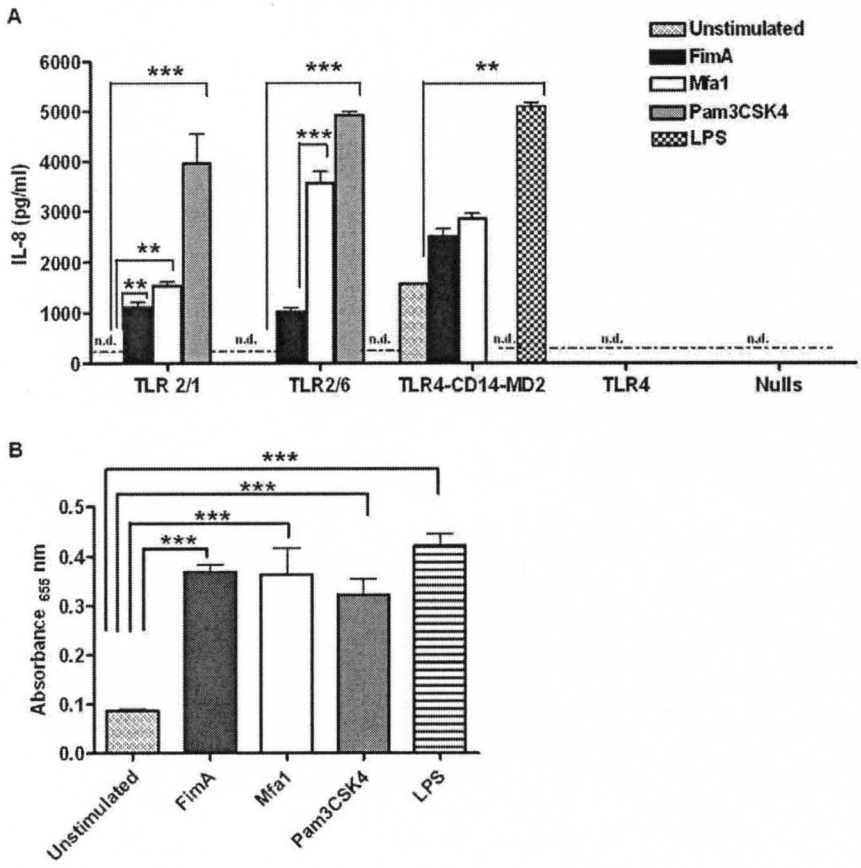

Figure 13: rFimA and rMfa1 signal preferentially through TLR2/6. (A) HEK 293 cells stably expressing TLR2, 4, 2/1, 2/6 or TLR4-CD14-MD2 were stimulated with 1 μg/ml of rFimA, rMfa1, the classic TLR2-specific agonist, Pam3CSK4 or the classic TLR4-specific agonist *E. coli* LPS. IL-8 release was quantified in 20 hr cell-free supernatants by ELISA. n.d. = not detected (below assay threshold); *p< 0.05; **p<0.01; ***p<0.001. **(B)** THP-1 Blue cells were stimulated with 1 μg/ml of rFimA, rMfa1, Pam3CSK4 or *E. coli* LPS. Relative expression levels of SEAP (reflecting NF-kB) in cell-free supernatants were determined by spectrophotometric analysis of SEAP activity at 655 nm. Unstimulated cells represent the 100% control. Here we show that all TLR-agonists employed are equally capable of inducing NF-kB. ***p< 0.001 compared to unstimulated cells.

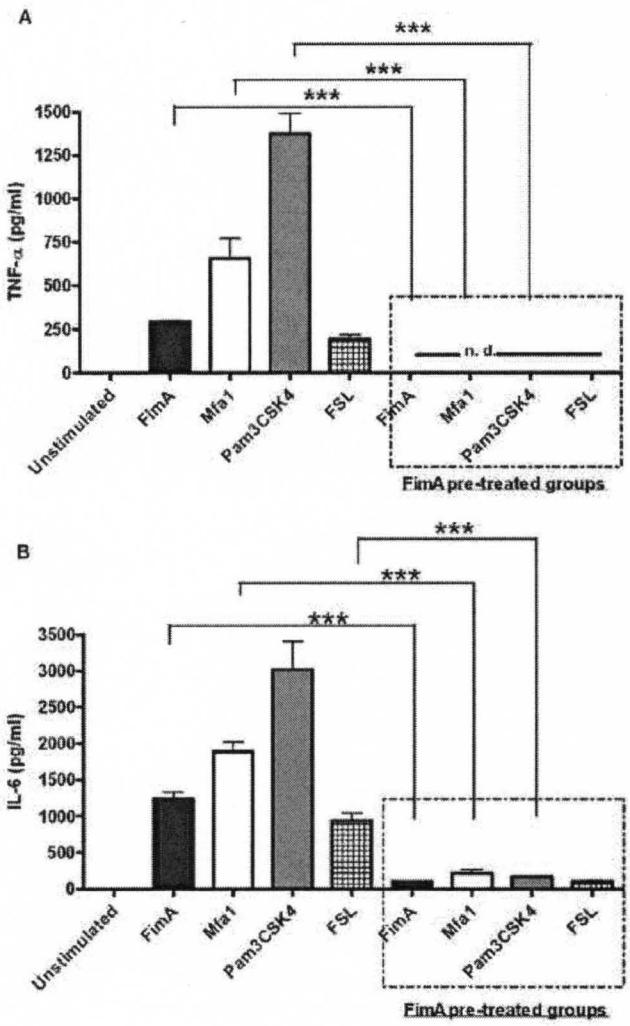

Figure 14: FimA induces TLR2-specific innate tolerance. $0.5x10^6$ primary human PBMCs were pre-incubated with rFimA for 24 hrs before stimulation with 1 μg/ml of rFimA, rMfa1 or the TLR2 and -4 specific agonists Pam3CSK4 and *E. coli* LPS. Responses were compared to cells treated with the same agonists without any pre-incubation. **(A)** TNF-α; **(B)** IL-6. n.d. = not detected (below assay threshold); ***p< 0.001.

FimA induces TLR2-specific innate tolerance:

We next examined if exposure to low-activity FimA influenced cytokine production (IL-6, TNF-α) in PBMCs on subsequent TLR stimulation by potent pro-inflammatory agonists (Mfa1 and the TLR2/1 and TLR2/6 specific agonists, Pam3CSk4 and FSL respectively). Pre-incubation with FimA inhibited cytokine production in response to all TLR2 specific agonists tested, as shown in Figure 14. Thus, FimA induces a state of inflammatory hypo-responsiveness in PBMCs. TLR2 surface expression by PBMCs in response to FimA stimulation was monitored by flow cytometry. There was no significant difference in TLR2 surface expression levels up to 24hrs post-FimA engagement (*data not shown*). Thus, FimA stimulation does not alter surface expression of TLR2 in PBMCs.

FimA stimulation does not induce IκBα degradation but inhibits IκBα degradation by Mfa1:

NF-κB transcription factors are complexed with IκB proteins in the cytosol. On receiving specific, but varied, extracellular signals IκBα is phosphorylated and targeted for proteasome-mediated degradation, resulting in the release and nuclear translocation of transcriptionally effective NF-κB (82, 88, 203, 232, 282, 283). We compared Iκ-Bα protein levels in PBMCs exposed to rMfa1, with or without pre-incubation with rFimA. As shown in Figure 15, while the potent pro-inflammatory agonist, Mfa1, induced rapid and extensive Iκ-Bα degradation, pre-incubation with rFimA efficiently abrogated this Mfa1-triggered Iκ-Bα degradation. Thus, FimA appears to promote TLR2 hyposensitivity

by inhibiting TLR2 agonist-induced degradation of Iκ-Bα. Essentially identical results were found for a second TLR2 agonist, FSL (*data not shown*).

FimA induced innate tolerance is IRAK-1-mediated:

To further understand mechanisms underlying FimA-induced TLR hypo-responsiveness, we monitored cytosolic IRAK-M and IRAK-1 in PBMCs. In unstimulated PBMCs, exposure to TLR2 agonists resulted in the rapid (within minutes) degradation of the upstream NF-κB regulator, IRAK-1, as shown for Mfa1 in (Figure 16A). However, FimA stimulation not only degrades IRAK-1, without leading to induction of pro-inflammatory cytokine production (Figure 12) but IRAK-1 levels remain minimal 24 hours after FimA stimulation and, therefore, unavailable for signaling on secondary TLR2 stimulation, as is shown for Mfa1 in (Figure 16b). Essentially identical results were found for IRAK-M (*data not shown*).

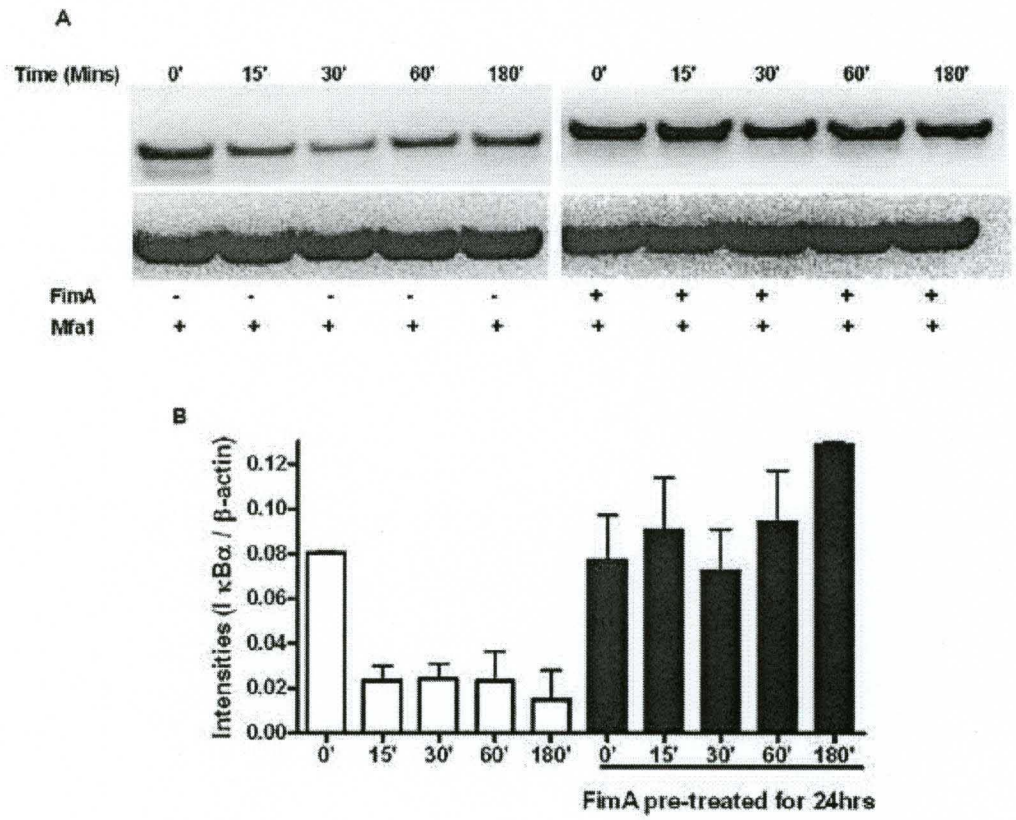

Figure 15: FimA induced tolerance reduces Iκ-Bα degradation. (A) Human PBMCs were pre-treated with 1 μg/ml rFimA for 24 hrs before stimulation with 1 μg/ml of rMfa1 for various timepoints. Immunoblots (25 μg protein per well) were probed for IkBα and re-probed β -actin to ensure equal loading. **(B)** Mean (S.E.) normalized band intensities.

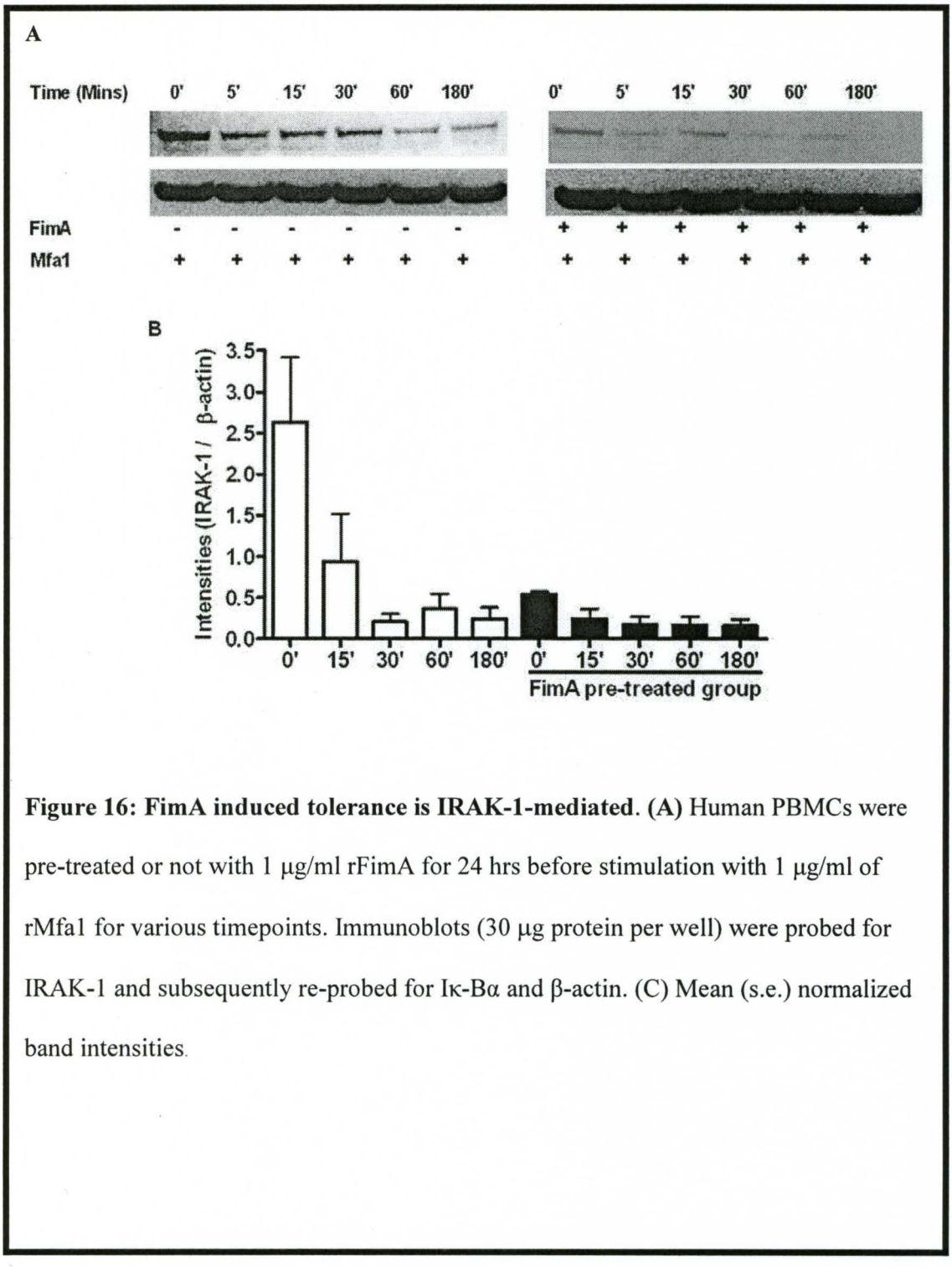

Figure 16: FimA induced tolerance is IRAK-1-mediated. (A) Human PBMCs were pre-treated or not with 1 μg/ml rFimA for 24 hrs before stimulation with 1 μg/ml of rMfa1 for various timepoints. Immunoblots (30 μg protein per well) were probed for IRAK-1 and subsequently re-probed for Iκ-Bα and β-actin. (C) Mean (s.e.) normalized band intensities.

Discussion

Smokers are more susceptible to periodontitis and exhibit more severe disease, yet the normally overt inflammatory response to plaque bacteria is suppressed (274). We have previously shown that human monocytes challenged with tobacco smoke-exposed *P. gingivalis* respond with reduced levels of pro-inflammatory cytokines (274) and now show that tobacco smoke-exposed *P. gingivalis* promote increased IL-10 production. However, the specific mechanisms of tobacco-induced innate immune suppression remain unknown. Initial microarray data suggested that cigarette smoke extract suppressed the production of *P. gingivalis* capsular polysaccharides while promoting the expression of fimbrial proteins (155, 158). As both capsular polysaccharides and fimbrial proteins of this key periodontal pathogen have been reported to influence cytokine production and because these specific bacterial structures will be the first to engage the host response, we hypothesized that such smoke-induced changes to *P. gingivalis* surface may contribute to the lower pro-inflammatory potential of smoke-exposed bacteria.

We have herein established that tobacco smoke increases expression of FimA, the major fimbrial protein, reduces *P. gingivalis* capsular layer, and promotes the growth of *P. gingivalis* biofilms of increased biomass. FimA has been shown to play a critical role in *P. gingivalis* colonization of the periodontium through strong interactions with several host proteins, including collagen, laminin and fibronectin (284), and by promoting adherence to other plaque bacteria, such as *Streptococcus spp.* (285, 286), and the oral epithelia (279). Furthermore, it is long established that biofilms represent immunoprotective structures. FimA is an important player in *P. gingivalis* biofilm formation (287). Indeed, FimA deficient strains show reduced biofilm formation (275),

while non-encapsulated mutants of *P. gingivalis* W83 exhibit enhanced biofilm formation (138, 139, 264). These results are clearly in keeping with, and provide mechanisms to support, established clinical observations that show that, compared to non-smokers, smokers are more likely to be infected with *P. gingivalis* (140, 141), to harbor higher numbers of *P. gingivalis* (141), and for such *P. gingivalis* infections to be more persistent (288).

It has been previously shown that *P. gingivalis* FimA interacts with TLR2 (175, 277, 278, 289). However, the capacity of FimA to subsequently elicit an inflammatory response is controversial. Some research groups have shown that FimA induces the production of pro-inflammatory cytokines from mononuclear innate cells (158, 175) and have hypothesized that this contributes to the development of pathological inflammation (277, 289, 290). However, other studies suggest that the pro-inflammatory response to FimA in mononuclear innate cells may be significantly lower than that expected from comparable doses of classic TLR agonists (168) and is unlikely to play a major role in *P. gingivalis*-induced inflammation (244). Moreover, FimA has even been suggested to be anti-inflammatory (280). We now show that the CSE upregulated *P. gingivalis* fimbrial proteins, rFimA and rMfa1, each signal though TLR2/1, and particularly, TLR 2/6; both strongly activate NF-κB transcription; but that rFimA induces a minimal pro-inflammatory cytokine (TNF-α, IL-6) response in multiple innate cell types (human PBMCs, neutrophils and gingival epithelial cells) compared to rMfa1 and to classic TLR2 agonists; and also induces significant quantities of the potent anti-inflammatory cytokine, IL-10. These results mimic the cytokine profiles that we have previously shown in innate cells stimulated with intact, CSE-exposed *P. gingivalis* (88, 203, 291).

Furthermore, these observations are in keeping with multiple *in vivo* studies in smokers that have shown that, despite increased susceptibility to plaque-induced periodontitis, the inflammatory response is suppressed in smokers compared to non-smokers (233, 234, 236, 237). This inflammatory suppression is reflected in lower concentrations of pro-inflammatory mediators (IL-1 β, IL-6 and, perhaps, TNF-α) in the gingival crevicular fluid and periodontal tissues of smokers, compared to non-smokers (292, 293). Inefficient TLR stimulation and the promotion of a less vigorous inflammatory response is an established means of immune evasion for several pathogens, including *Bordetella,* Shigella and pathogenic *Escherichia* (294-297) .

While CSE-induces functional, surface-exposed FimA expression, we further demonstrate that FimA induces a state of hyporesponsiveness, or tolerance, to secondary stimulation with Mfa1, and other TLR2 specific agonists, in PBMC's - as demonstrated by an ablation of TNF-α and IL-6. This FimA-induced hyporesponsiveness was not associated with an alteration to TLR2 expression on the surface of innate cells. Rather, we show that FimA-induced, TLR2-specific tolerance is mediated by a suppression of IκBα degradation, which is expected to be concomitant with reduced NF-κB translocation.

TLR2 activation leads to the rapid recruitment of MyD88 to TLR2 and the subsequent recruitment of IRAK-1 and IRAK-4 (298, 299). IRAK-4 phosphorylates IRAK-1 that is in association with TLR2, allowing IRAK-1 to interact with TRAF6 (295), leading to IκBα degradation, NF-κB nuclear translocation and the induction of multiple pro-inflammatory cytokine genes (300). We show, however, that stimulation with FimA, leads to rapid IRAK-1 degradation and the long-term unavailability of this

positive regulator of the TLR-2/MyD88 pathway, helping to explain FimA-induced tolerance. IRAK-M is a negative regulator of NF-κB translocation that acts by preventing the dissociation of IRAK-1 and IRAK4 from MyD88 (301). While FimA stimulation also leads to the rapid degradation and chronic suppression of IRAK-M, this would appear inconsequential with respect to NF-κB activation because of the unavailability of IRAK-1.

This FimA-induced, IRAK-1- and IκBα-involved suppression of TLR2 pro-inflammatory cytokine signaling provides one mechanism by which tobacco smoke dysregulates the inflammatory response and suppresses cytokine production in smokers, as discussed above. This mechanism of suppression may be similar to what has been previously reported for Pam3CSK4 (301). Monocytes stimulated with Pam3CSK4 have been shown to be refractory to secondary stimulation with the same agonist (280). This occurs due to reduced IRAK-1 protein in these cells, just as we show in FimA-tolerized PBMCs.

It is important to note that there are limitations inherent in using recombinant proteins, particularly as they may behave entirely different outside the context of the whole bacterium and because of potential vector-specific alterations in the processing of the recombinant protein. The advantages, however, are that recombinant technology is long established and allows simple purification of a large amount of protein that can be readily characterized, that is free of accessory molecules (such as associated fimbrial proteins FimB through E) and that allows for effective and reproducible elucidation of signaling pathways. Moreover FimA and Mfa1 are simultaneously expressed (150, 287), raising the possibility that Mfa1 may nullify the anti-inflammatory effectiveness of

FimA. However, FimA-comprised fimbriae are much longer appendages than the minor Mfa1 fimbriae (150, 287) and are, thus, likely to interact with host cells prior to Mfa1. Furthermore, it is been suggested that FimA appendages may be shed from the *P. gingivalis* surface (302) further increasing the likelihood of early FimA host interaction. Importantly, intact CSE-exposed *P. gingivalis* that, presumably, express both Mfa1 and FimA, exhibit similar anti-inflammatory properties to recombinant FimA. While tobacco smoke produces multiple phenotypic and genotypic changes in *P. gingivalis* (280) and, thus, other yet to be identified mechanisms way well contribute to the overall reduced inflammatory response, the anti-inflammatory effects of recombinant FimA are potent and are likely to be a major factor in CSE-induced inflammatory suppression.

In summary, CSE-exposure results in the upregulation of FimA. FimA suppresses the inflammatory response by at least two different mechanisms. One is passive, i.e. FimA itself engages TLR2 but has minimal inflammatory potential. The second is active, i.e., FimA induces TLR2 hyporesponsiveness by a mechanism involving IRAK-1 degradation and the stabilization of IκBα.

CSE mediated stress induces alterations in key virulence factors of *P. gingivalis*. While down-regulated capsular polysaccharides increased biofilm formation, upregulated fimbriae promoted TLR2 hyposensitivity in an IκBα-, IRAK-1- dependent manner. Both mechanisms, contribute towards altering host-pathogen interactions and reducing host-induced inflammatory response consistent with both our prior observations that CSE-exposed whole P. *gingivalis* cells have reduced inflammatory potential compared to control bacteria (274). These observations are also consistent with the clinical profiles of

smokers of severe periodontal disease who lack overt signs of clinical inflammation (88, 203).

CHAPTER FIVE: SUMMARY & FUTURE DIRECTIONS

Tobacco smoking is a major risk factor for multiple and varied types of diseases, including persistent infections, cancers, autoimmune disorders, and chronic inflammatory diseases – such as chronic periodontitis. The toxicity and / or carcinogenic nature of various chemicals found in cigarette smoke is compounded by their composite debilitating effects on host innate and adaptive immune responses.

Tobacco smoke is the single most important environmental risk factor associated with periodontal diseases with more than 50% of cases of periodontitis found in current or former smokers. Compared to non-smokers, smokers show increased severity and incidence of periodontal disease and are highly susceptible to persistent periodontal infections by the Gram negative bacterial pathogen, *P. gingivalis*. Seemingly contrary to increased severity, smokers exhibit reduced signs of clinical inflammation (bleeding on probing, redness and edema). This thesis explains the underlying mechanisms that might play a role in this apparent contradiction.

While several studies delineate the role of tobacco smoke in the skewing of immune response to infection very little is known about how it affects the virulence of

potential periodontal pathogens such as *P. gingivalis* and its subsequent interaction with host. Components of tobacco smoke are available systemically and compromise host immune response. However they are readily available in the oral cavity for interaction with both host tissue and resident pathogenic bacteria. In this dissertation, we show that tobacco smoke presents an environmental stress to *P. gingivalis* and it adapts to this stress by suppressing its inflammatory profile and altering the expression of several genes and outer membrane proteins. Tobacco induced changes to *P. gingivalis* gene expression, phenotype and host pathogen interactions were determined to help better understand why exacerbated disease is accompanied by a reduced inflammatory response in smokers.

Gross, tobacco-induced alterations caused to the *P. gingivalis* genome, transcriptional profile and phenotype were identified. We have found that *P. gingivalis* cells exposed to cigarette smoke extract (CSE) induce a reduced inflammatory profile (reduced levels of proinflammatory cytokines like TNF-α, IL-6, IL-12p40) in monocytes and peripheral blood mononuclear cells than unexposed bacteria. This inflammatory suppression was consistent with identified cigarette smoke-induced transcriptional and post-transcriptional alterations. Microarray analysis of CSE exposed *P. gingivalis* revealed altered expression of several genes including detoxification and oxidative stress-related genes; DNA repair genes; and multiple genes related to *P. gingivalis* virulence, including genes in the major fimbrial and capsular operons. Exposure to CSE also altered the expression of outer membrane proteins, most notably by inducing the virulence factors RagA and RagB, and a putative lipoprotein cotranscribed with the minor fimbrial antigen.

CSE exposure also resulted in phenotypic alterations such as increased fimbrial expression and downregulated capsular layer. Upregulated fimbriae play an important role in increasing persistence by forming significantly more robust biofilms. We further show that, while FimA is recognized by TLR2/6, the major fimbrial antigen has minimal inflammatory activity in several cell types. Furthermore, FimA stimulation chronically abrogates the pro-inflammatory response to subsequent TLR2 stimulation by other TLR-2-specific agonists (Pam3CSK4, FSL, and Mfa1) in an IκBα- and IRAK-1-dependent manner. These changes may explain, in part, the altered virulence and host-pathogen interactions that have been documented *in vivo* in smokers with periodontal disease.

Our studies demonstrate that the recombinant Mfa1 (rMfa1) is highly proinflammatory. However recent work by Chris Cutler's group (172, 177) demonstrates that native Mfa1, isolated from *P. gingivalis* 381 is a glycosylated protein and is important in binding to DC-SIGN receptors on Monocyte derived Dendritic cells (MoDC). When these cells are stimulated with either wild-type Pg381 or isogenic major (DPG-3)-, minor (MFI)-, or double fimbriae (MFB)-deficient mutant *P. gingivalis* strains, MFI was the most potent inducer of proinflammatory cytokines (IL-1β, IL-8, IL-6, and TNF-α) while DPG-3 induced significantly lower levels of TNF-α and IL-1β indicating a role of Mfa1 in lowering proinflammatory cytokine induction from these cells. It would be important to determine whether glycosylation is important in reducing the inflammatory potential Mfa1 and to determine whether the glycosylation patterns found in rMfa1 which was expressed in *E. coli* are different than those expressed by *P. gingivalis* strains. Alternatively, CSE exposure might result in addition of different

types/amounts of sugars on Mfa1 resulting in shifts in glycosylation patterns *P. gingivalis* proteins in a manner that promotes reduced inflammatory potential of the pathogen.

Glycosylation of several proteins in *P. gingivalis* is carried out by the *vim* family of genes and inactivation of genes in the *vim* operon such as *vimA or vimE* results in inactivation of gingipain activity due to ineffective glycosylation of these key virulence-associated proteases (303). Particularly, inactivation of the *vimA* gene, which codes for a 39kD putative acyl-CoA *N*-acyltransferase, resulted in a loss of Arg-gingipain activity, downregulation of capsule and detection of FimA protein in the *vimA* defective mutant strain (FLL92). This is an interesting finding as the wildtype W83 strain has been previously shown to lack functional fimbriae and is often cited as a non-fimbriated strain (304). The authors also suggested that the presence of capsule might actually interfere with the production of fimbriae in *P. gingivalis*. Thus the lack of a capsular layer might allow for fimbrial presentation on the surface and allow for subsequent fimbriae dependent interactions such as biofilm formation, adherence, invasion etc. In the context of our studies we find that CSE exposure downregulates capsular polysaccharides while simultaneously promoting major fimbrial expression which is consistent with the *vimA* defective mutant strain (FLL92) phenotype. Thus it is likely that CSE alters the expression or activity of *vimA* gene product.

Gingipains have been shown to play an important role in the processing of fimbrillin, the monomeric unit of the major fimbriae that is essential for the formation of functional fimbriae on *P. gingivalis* surface (305). Gingipain activity is central to *P. gingivalis* virulence and they are essentially involved in several degradative processes (host tissue associated proteins as well as immune response proteins such as complement

components and specific cytokines). Although gingipain expression was not altered in microarray data it is possible that the components found is CSE might alter their enzymatic activity. Toxic chemicals found in cigarette smoke have an inhibitory effect on the enzyme activity of several host enzymes like alkaline phosphatase, gluoce-6-phosphatase, 5'-nucleotidase, and cholinesterase in mouse liver and small intestine (306). While alpha, beta-unsaturated aldehydes (acrolein, crotonaldehyde) and saturated aldehydes (acetaldehyde) significantly inhibited salivary amylase activity due to the interaction between aldehydes and -SH groups of these enzymes (307). Thus it is likely that noxious chemicals found in cigarette smoke might alter the enzymatic activity of gingipains which are extracellular secreted proteases and are likely to interface with cigarette smoke associated toxins.

P. gingivalis major fimbriae have been shown to be essential for inducing such Nf-κB downregulation and reducing the inflammatory response from macrophages that is associated with pathogen clearance by inducing TLR2-CXCR4 crosstalk (157). Induction of TLR2-CXCR4-C5aR crosstalk promotes cAMP production and subsequent cAMP dependent activation of protein kinase A (PKA) that is associated with downregulation of NF-κB activity (308, 309). In this thesis we show that rFimA has modest inflammatory activity but induces significantly higher amounts of IL-10 compared to Mfa1. The downstream signaling mechanisms behind such induction remain to be determined. One possible mechanism causing FimA dependent IL-10 activation could be cAMP/cAMP-dependent PKA activation that has been previously shown to induce IL-10 production in monocytes via phosphorylation of cAMP response element binding protein (CREB) (310, 311). Thus competition between NF-κB and CREB for binding to the coactivator CREB-

binding protein (CBP) is important in regulating transcription of proinflammatory cytokines (TNFα, IL-6, IL-8, IL-1β) or the anti-inflammatory cytokine IL-10. Whole *P. gingivalis* cells passaged in CSE also induced significantly lower levels of proinflammatory cytokines (IL-6, TNF-α) and higher levels of IL-10 than control non-exposed cells. Thus it is possible that FimA upregulation contributes towards IL-10 production by CSE-exposed *P. gingivalis* in a cAMP-PKA-CREB dependent manner and downregulation of Nf-κB associated transcription of proinflammatory genes.

There are several other negative-feedback mechanisms and signaling molecules that regulate TLR associated signal transduction to avert chronic TLR activation which might cause harmful immunological responses. In our studies we looked at one such regulator IRAK-M that prevents TRAF6 induced activation of IRAK1 by preventing its dissociation from IRAK1-IRAK4 complex (300) . FimA stimulation also leads to the rapid degradation and chronic suppression of IRAK-M. However this would appear inconsequential with respect to NF-κB activation because of the unavailability of IRAK-1. It is possible that FimA in addition to IRAK1 and IRAK-M degradation might induce TLR2 hyporesponsivensess through other negative regulators like Suppressor of cytokine signalling-1 (SOCS-1) that has been shown to regulate TLR2 and 4 by targeting Mal, the adaptor protein important for Myd88 signaling, for polyubiquitination and subsequent proteosomal degradation (312).

Besides inducing inflammatory suppression, *P. gingivalis* fimbriae are important in persistence. Persistence, in the context of the oral cavity, would not only involve host inflammatory suppression but also successful interaction with other colonizers of oral plaque by effective recruitment into oral biofilms. We have shown that the phenotypic

alterations caused by CSE promote *P. gingivalis* existence in a biofilm. Specifically CSE induced loss of capsular layer and upregulated fimbriae. While presence of a capsular layer is inversely proportional to biofilm formation (275) fimbriae are important in biofilm formation and FimA deficient strains cannot form effective biofilms (287). In mature dental plaque *P. gingivalis* clearly interacts with multiple middle and late colonizers, such as *Actinomyces viscosus* (313), *Treponema denticola* (314, 315) and *Tannerella forsynthus* (316). However, adhesive interactions with primary colonizers, such as *Streptococcus gordonii*, are critical for *P. gingivalis* colonization of the supragingival niche (284, 317). The early colonizers, like *S. gordonii*, initiate biofilm formation by binding to the tooth pellicle and providing an attachment surface for bacterial secession through multimodal receptor-ligand mediated coadhesive interactions. For example, the long fimbriae (FimA) of *P. gingivalis* bind to *S. gordonii* Glyceraldehyde-3 phosphate deydrogenase (GAPDH) (284, 318) while the shorter fimbriae (Mfa1) bind to streptococcal SspB protein (169). An 80 amino acid sequence on the *S. gordonii* surface protein, SspB, is critical for Mfa1 adhesion, with a synthetic peptide of this SspB region, named BAR, a potent inhibitor of Mfa1-dependent *P. gingivalis-S. gordonii* biofilms formation (319). As CSE upregulates *fimA* gene activity, it would be of interest to determine CSE exposure augments *P. gingivalis* - *S. gordonii* biofilms formation via a FimA-dependent mechanism.

Presence of a persistent *P. gingivalis* infection leads to gingival ulceration and local vascular changes that promote transient, low level bacteremias (106) that help spread *P. gingivalis* and other periodontal bacteria from the oral cavity making periodontitis an important risk factor for heart disease. Viable *P. gingivalis* cells, as well

as *P. gingivalis* DNA, have been found in atheromatous plaques (320, 321) while *P. gingivalis* can invade endothelial cells as well as smooth muscle cells and spread from infected cells to uninfected cells (322). Tobacco smoke-mediated changes to *P. gingivalis* are important contributing factors to the increased persistence of this pathogen in smokers and, subsequently, an increased risk for vascular inflammation and atherosclerotic progression.

These studies provide some of the first information to explain, mechanistically, how tobacco smoke changes the *P. gingivalis* phenotype in a manner likely to promote *P. gingivalis* colonization and infection while simultaneously reducing the host response to this major mucosal pathogen. The mechanisms responsible for periodontal disease induction and progression may actually be different in smokers compared to non-smokers, demonstrating a need for differential treatment regimens.

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Journal publications:

1) **Bagaitkar J**, Patel C, Renaud DE, Daep-Amorin C, Demuth DR, Scott DA. Tobacco exposure augments *Porphyromonas gingivalis* aggregation and Mfa1-dependent biofilm formation. *Environmental Microbiology* (*under review*, 2010).
2) Scott DA, Palmer RM, **Bagaitkar J.** Mechanisms underlying tobacco-induced suppression of inflammation in the gingiva. *Journal of Clinical Periodontology* (*under review*, 2010).
3) Buduneli N, Larsson L, Biyikoglu B, **Bagaitkar J,** Renaud DE, Scott DA*. Lipid

A-derived C12 and C14 3-OH fatty acids are decreased in relation to C13 3-OH fatty acid in smokers with chronic periodontitis. *Journal of Dental Research* (*in press*, 2010).

4) **Bagaitkar J**, Demuth DR, Daep-Amorin C, Renaud DE, Pierce DL, Scott DA. Tobacco upregulated *P. gingivalis* fimbrial proteins which induce TLR2 hyposensitivity. *PLoS One* 2010;5:e9323.
5) **Bagaitkar J**, Williams LR, Renaud DE, Benakanakere MR, Martin M, Scott DA, Demuth DR. Tobacco induced alterations to *Porphyromonas gingivalis*– host interactions. *Environmental Microbiology* 2009;11:1242-53.
6) **Bagaitkar J**, Demuth DR, Scott DA. Tobacco use and susceptibility to bacterial infection. *Tobacco Induced Diseases* 2008;4:e12.
7) Liu W, **Bagaitkar J**, Watabe K. Roles of AKT signal in breast cancer. *Frontiers in Biosciences* 2007; 12:4011-9.
8) Whitehurst B, Flister MJ, **Bagaitkar J**, Volk L, Bivens CM, Pickett B, Castro-Rivera E, Brekken RA, Gerard RD, Ran S. Anti-VEGF-A therapy reduces lymphatic vessel density and expression of VEGFR-3 in an orthotopic breast tumor model. *International Journal of Cancer* 2007; 121:2181-91.

Book Chapter:

Scott DA, **Bagaitkar J.**

Tobacco smoke and susceptibility to infectious bacterial diseases. In: "**Cigarette Smoke Toxicity**" David Bernhard, Editor. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim,

Germany (*in press*, 2010).

Abstracts:

1. "Tobacco exposure augments *Porphyromonas gingivalis* Mfa1-dependent biofilm formation" **Bagaitkar J**, Patel C, Renaud DE, Daep-Amorin C, Demuth DR, Scott DA.
2. *Tobacco alkaloids inhibit pro-inflammatory signaling initiated through multiple cell surface TLRs.*" **Bagatikar J**, Renaud DE, Scott DA. 2010, ASM, International Society for the Prevention of Tobacco-Induced Diseases, Boston, MA.
3. *Tobacco-regulated changes to P. gingivalis fimbrial proteins and capsule polysaccharides induce TLR2 hyposensitivity.*" **Bagaitkar J**, Demuth DR, Daep C, Pierce DL Renaud DE, Scott DA. AADR, Washington D.C., 2010 ***and*** Gordon Conference on Periodontal Diseases, 2009 ***and*** James Graham Brown Annual Research Retreat. Louisville, KY, 2009.
4. *Cigarette smoke extract alters the structure and immune function of Porphyromonas gingivalis LPS.*" Aronica Y. Boyle, **Juhi Bagaitkar**, Diane E. Renaud, Don R. Demuth, David Scott. James Graham Brown Annual Research Retreat. Louisville, KY, 2009 ***and*** AADR, Washington D.C., 2010.
5. *Tobacco smoking, innate immunity and susceptibility to bacterial infection."* **Bagaitkar J,** Demuth DR, Scott DA. 2008, ASM, International Society for the Prevention of Tobacco- Induced Diseases, Kyoto, Japan.
6. *Tobacco smoke-exposure alters Porphyromonas gingivalis W83-innate cell interactions.*" **Bagaitkar J,** Williams LR, Daep C Demuth DR, Scott DA. 2008, ASM, Society for Leukocyte Biology, Denver, Colorado, USA.

INVITED TALKS/PRESENTATIONS:

- *Invited Speaker* at the AADR, Washington D.C., 2010.
- *Invited Speaker at the* Society for Leukocyte Biology ASM, Vancouver, 2010. Presidential Research Award finalist.
- *Invited Presenter* at the Gordon Conference on Periodontal Diseases, 2009.
- *Invited Presenter* to the Senate and Congress of the Commonwealth of Kentucky. 'Annual Graduate Student Research Day', Frankfurt, Kentucky., 2008.

AWARDS/HONORS:

- ***Travel Award*** for the 43rd Annual meeting of *Society for Leukocyte Biology*, Vancouver, Canada, 2010.
- ***First Place:*** *Society for Leukocyte Biology* Presidential Research Award, Vancouver, Canada, 2010.
- ***First Place: Condict Moore Graduate Student Research Prize*** at the *James Graham Brown Cancer Center Annual Research Retreat.* Louisville, KY, 2009.
- ***Travel Award*** for the 41st Annual Meeting of *Society for Leukocyte Bio*logy, Denver, Colorado, 2008.
- Multiple ***travel awards*** from the *Graduate Student Council* (GSC) at the University of Louisville.
- ***President*** (2008-2009), *Microbiology and Immunology Student Association.*
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