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Porphyromonas gingivalis gingipains induce a pro-inflammatory extracellular microenvironment : the role of PAR-2 and fibronectin.

Jeffrey S. Marschall

PORPHYROMONAS GINGIVALIS GINGIPAINS INDUCE A PRO-INFLAMMATORY EXTRACELLULAR MICROENVRIONMENT: THE ROLE OF PAR-2 AND FIBRONECTIN

By Jeffrey S. Marschall
B.S., University of Louisville, 2011

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A Thesis Approved on

March 16th 2016

By the following Thesis Committee:

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Dr. Jan Potempa, Thesis Director

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Dr. Jesse Roman, Thesis Co-Director

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Dr. David Scott

DEDICATION

This thesis is dedicated to the memories of my maternal grandfather, Thomas Walker Senior; paternal grandmother, Alice Walker; paternal grandfather Kurt Marschall; and paternal grandmother, Virginia Marschall. In addition, I would like to dedicate this to my parents, David Marschall and Kimberly Marschall who have supported me through my entire education and have shown unconditional love and guidance.

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ABSTRACT

PORPHYROMONAS GINGIVALIS GINGIPAINS INDUCE A PRO-INFLAMMATORY EXTRACELLULAR MICROENVRIONMENT: THE ROLE OF PAR-2 AND FIBRONECTIN

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Periodontitis is a chronic inflammatory disease that is characterized by severe tissue destruction of the gingiva and other tooth supporting structures; if left untreated, tooth loss and disintegration of the alveolar bone occurs. This chronic inflammatory state has been linked to other systemic diseases such as cardiovascular disease, diabetes, rheumatoid arthritis, and Alzheimer's disease. *Porphyromonas gingivalis* is the major pathogenic microbe in periodontitis. The main virulence factors of *P. gingivalis* are the Arg-aa and Lys-aa gingipains, which are proteolytic enzymes implicated in a plethora of activities that allow *P. gingivalis* to subvert the human immune system in the oral cavity and cause host tissue destruction. Here, we report that Arg-aa gingipains, RgpB and HRgpA stimulate fibroblasts to express fibronectin, a glycoprotein associated with tissue injury and repair, through the transcription of its gene promoter in a dose and time dependent fashion. Interestingly, when using a protease activated receptor 2 antagonists, gingipain induced fibronectin promoter activity was attenuated. Furthermore, gingipains stimulate fibroblasts to produce a pro-inflammatory matrix triggering production of IL-6 and IL-8 by monocytes that correlated with

fibronectin-EDA expression. Taken together, our data suggests Arg-gingipains act on host fibroblasts to produce fibronectin which, stimulates immune cell activation. Taken together, these data suggest that gingipains promote and environment that disrupts host homeostasis.

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INTRODUCTION AND SPECIFIC AIMS

Periodontitis has been known for nearly 10,000 years as the diet shifted in Neolithic (Commonly referred as the "Stone Age") societies from what was one hunter gathers to plant and animal based diets [1, 2]. Periodontitis is a bacterially induced chronic inflammatory disease of the gums and other tooth supporting structures (i.e. periodontium). The traditional model of periodontitis implies that the disease develops when the microbial flora of the oral cavity transitions from healthy to what is considered the "red-complex" of pathogenic bacteria made up of *Tannerella forsythia*, *Treponema denticola*, and *Porphyromonas gingivalis*. However, recent meta-genomic data suggest a more diverse microbial community is involved in pathogenesis [3-6]. The transition from physiology to dysbiosis is an emerging area of research. Periodontitis affects millions of people and remains a major public health problem; in fact, it has been estimated 30% of individuals in developed countries [7] and up to 70% in undeveloped countries [8] have reported periodontitis symptoms. Considering this, many epidemiological studies have investigated the interplay of periodontitis as a risk factor for respiratory disease, cardiovascular disease, diabetes, osteoporosis [8], preterm birth and low birth rate [9, 10]. In addition, recent research has proposed a link between kidney disease [11-13], rheumatoid arthritis [14-16], Alzheimer's disease [17, 18], and metabolic syndrome [19-21]. However, several advances over the past 10 years have changed the wisdom about the microbial etiology of periodontitis [22]. More important for administrators, the economic cost of periodontitis is quite significant, with estimates ranging from $3.5 to $6

billion per annum or $4150 per patient per day [23]. Economic costs for periodontal disease are hard to determine, the World Health Organization (WHO) estimates that it could be as high as $260 billion in the US alone in 2010 (Data obtained from WHO and US Public Health Expenditures) considering 5-10% of public health expenditure relates to oral health. It is now well recognized the host response contributes significantly to tissue destruction and alveolar bone disintegration [22] which is hallmark of periodontitis. The host innate immune system is highly active in healthy tissues, and imbalance, corruption, and disruption in the expression of inflammatory mediators contributes greatly to the destruction of the tissue and bone supporting the root structures [24-26]. Despite periodontal disease's importance on overall health, public health, and economic impact the disease imposes, the exact mechanisms exacerbating periodontitis remain unclear. An essential component of the pathogenesis of periodontal disease is the destruction of the extracellular matrix (ECM). We hypothesize periodontal pathogens, such as *P. gingivalis* modulate the expression of ECM proteins to promote colonization, disease progression, and as a consequence jeopardize host wound healing and immunity. In addition we hypothesize, that these effects could be, in-part, mediated by *P. gingivalis* gingipains, and that these ECM interactions may contribute significantly to periodontitis progression. This hypothesis will be tested in specific aims designed to:

Aim 1. Examine the mechanisms by which *P. gingivalis* gingipains corrupt homeostatic ECM expression.

Aim 2. Investigate mechanisms underlying *P. gingivalis* ECM interactions with innate immunity

BACKGROUND & LITERATURE REVIEW

PART 1: PERIODONTITIS: ANATOMY, MICROBIOLOGY AND IMMUNOLOGY

Essential to mammalian survival is the ability to efficiently consume foodstuffs and convert it to ATP energy. The dentition plays a critical role in this process; thus, making the tissues that supports the dentition equally important. The periodontium (from the Greek, *peri* = around and *ondontos* = tooth) comprises the tissues that support the teeth and is made up of the gingiva, periodontal ligament, root cementum, and the alveolar bone (Figure 1). The periodontium is designed to attach the tooth to the bony tissue. Together, these tissues form a specific fibrous joint called a gomphosis.

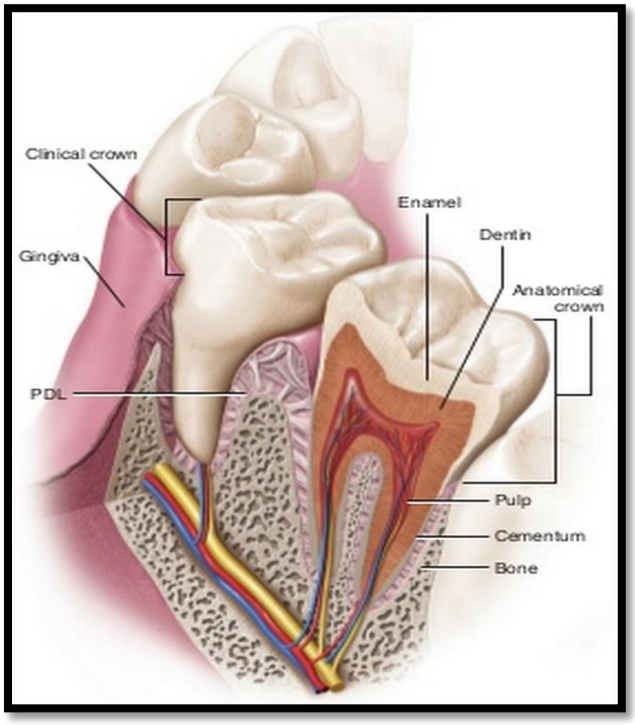

Figure 1. *The tooth and the periodontium (Image from Ten Cate's Oral Histology)*

Collectively, these individual components function together as a single unit. Furthermore, the extracellular matrix (ECM) composition can influence the cellular activities of adjacent structures. Hence, the pathologic changes that occur in one periodontal component can have significant ramifications for the maintenance, repair, and regeneration of the periodontium collectively [27].

Chronic periodontitis is defined as "inflammation of the gingiva and the adjacent attachment apparatus. The disease is characterized by loss of clinical attachment due to destruction of the periodontal ligament and loss of the adjacent supporting bone [28, 29]". The clinical features of chronic periodontitis consist of gingival bleeding, edema and more than 1/3 of the supporting periodontal tissue.

Chronic periodontitis is the most common form of periodontitis [30]. Although chronic periodontitis is most prevalent in adults, it can be observed in children. The hallmark association of chronic periodontitis is the accumulation of plaque and calculus clinically.

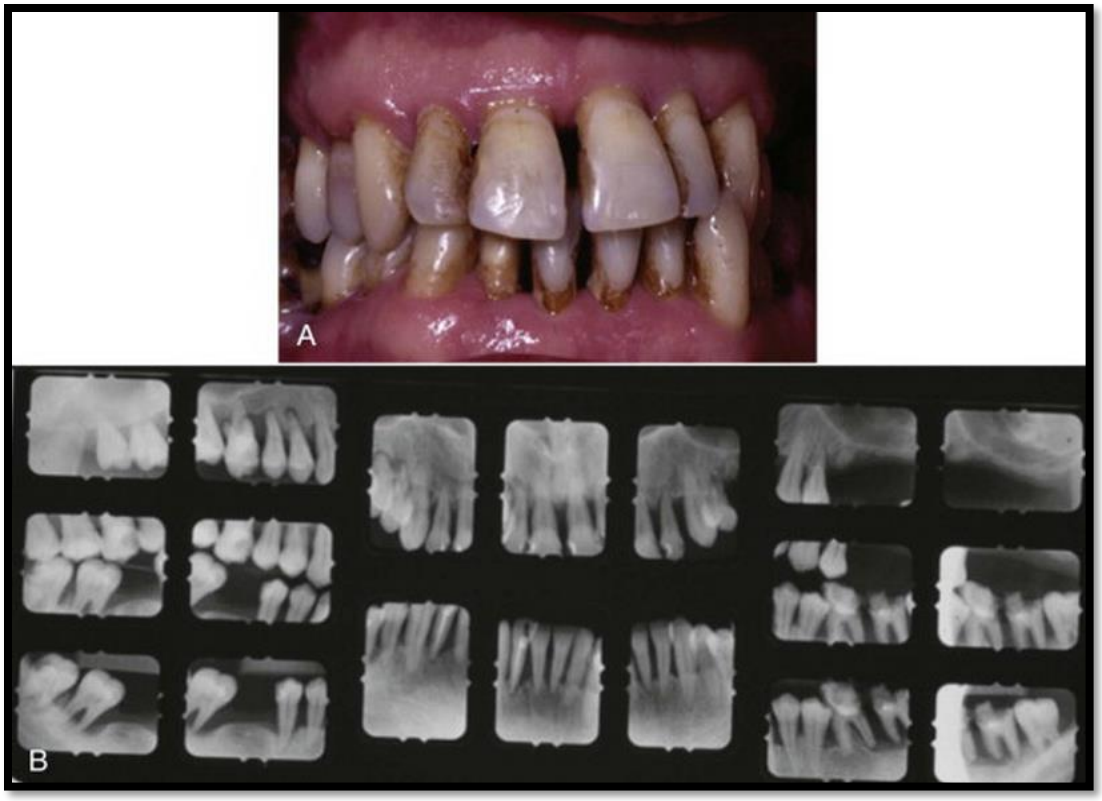

Figure 2. *Clinical Image and Radiographs of plaque-related chronic severe periodontitis with recession (Adapted from Carranza's Clinical Periodontics)*

As the plaque accumulates the microbial community shifts from one of periodontal health to disease. The symbiotic microbial community within the healthy, that is, homeostatic, periodontal sulcus is generally facultative bacteria such as *Actinomyces* and *Streptococci*. However, in dysbiosis the microbial community consists mostly of anaerobic bacteria from the phyla Firmicutes, proteobacteria, Spirochetes, Bacterioides, and Synergistetes [2]. These bacteria that facilitate a dysbiotic environment have evolved a plethora of virulence factors that allow life in a severely inflamed niche [31]. However, this environment can be broken down even further into the sub-gingival crevice, the gingival crevicular fluid, and the epithelium lining the crevice. The sub-gingival crevice is defined as the narrow space between the tooth surface and the free

gingiva [2]. Next, gingival crevicular fluid is defined as the serum exudate that originates in the gingival capillaries and flows into the gingival crevice carrying local produced immune and inflammatory mediators (cytokines and antimicrobial peptides) [2].

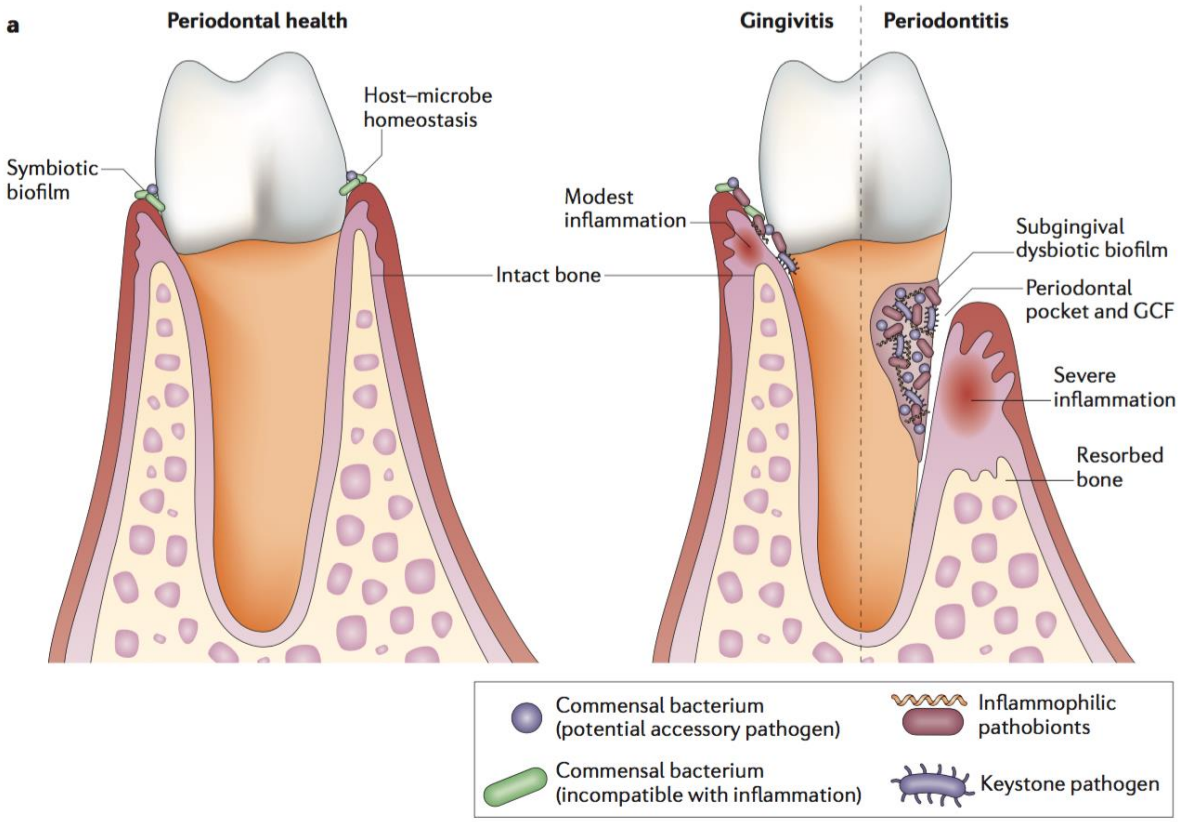

Figure 3. *Periodontal health with gingival pockets ≤2 mm vs. Periodontal disease with gingival pockets ≥4mm. (Adapted from Hajishengallis [2])*

The key to periodontal health is to maintain balance of pro-inflammatory modulators that in turn, keep a beneficial host-microorganism community the periodontium [32]. In periodontitis, the lack of oral hygiene, local factors, or immune-regulatory defects allow the growth of bacteria that can produce products that allow subversion of the immune system and propagation of disease [33]. The destruction of the local tissues can further enhance immune-imbalance and create self-perpetuating positive

feedback loop. It is important to note that these products from local tissue destruction are themselves immune-active.

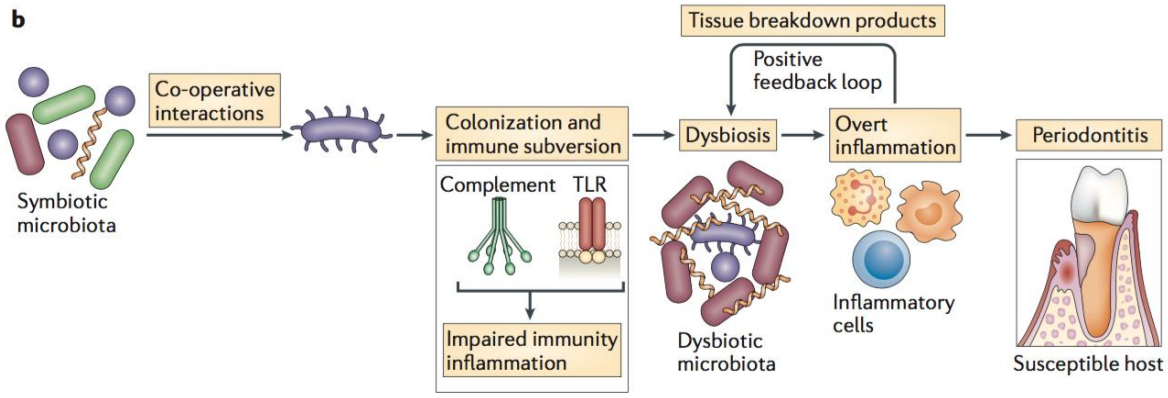

Figure 4. *Schematic of mechanisms associated with periodontitis. (Adapted from Hajishengallis [2]).*

The bulk of scientific studies investigating the main microorganisms in periodontitis have focused on *Porphyromonas gingivalis*. It is well known that *P. gingivalis* has the ability to produce many virulence factors that allow subversion of the human immune system. Traditionally, it was thought that these virulence factors (i.e. gingipains, which will be further discussed) were the main culprits in host tissue destruction and thus *P. gingivalis* directly causing periodontitis in animal models [2, 34]. Recent studies have shifted our understanding what role *P. gingivalis* plays within the periodontal microenvironment. In fact, it is essential that commensal microbiota to be present, that is, *P. gingivalis* cannot induce periodontitis in germ-free mice alone [6]. This study revealed that while *P. gingivalis* was present in comparatively low amounts compared to other species its pathogenicity was due to the ability to induce dysbiotic periodontal microbial communities [6]. Hajishengallis and his team referred to *P. gingivalis* as a keystone pathogen [6, 35], that is, certain species are non-pathogenic themselves but when together can initiate inflammatory periodontal bone loss.

The innate immune system is multifaceted in its mechanisms of protection from infections exogenous microorganisms. The first line of defense is the physical barrier provided by the integumentary system, or when considering the oral cavity, the gingival epithelium. With this system, defense occurs through the action of keratinized, waterproof, epithelium that impedes microorganism penetration to deeper tissues. This degree of impermeability is in part due to the evolution of tight junctions, secretion of heavily glycosylated mucous, and the secretion of bactericidal peptides (e.g., β-defensins, dermicidin, and psoriasin). Furthermore, most of the mucosal and gingival epithelium is associated with lymphoid tissues, such as Peyer's patches that house copious amounts of innate and adaptive immune cells. Considering the gingival epithelium are exposed to thousands of commensal and pathogenic microorganisms, these tissues have evolved as a very responsive system that aids in the protection of the host, one such mechanism are the Toll-like receptors (TLRs), a class of pattern recognition receptors.

TLRs are expressed by immune and non-immune cells in the oral cavity, and are responsible for the recognition of bacterial, viral, fungal, and protozoan molecules. These molecules include, but are not limited to, lipopolysaccharide (LPS), bacterial DNA, diacyl lipoproteins, and peptidoglycan, among others [36]. Thus far, 13 TLR receptors have been characterized in both humans and mice. Some TLRs are located on the cell surface, such as TLR-2 and TLR-4, which to date have been the most highly characterized; however, TLR-3, TLR-7, and TLR-9, which are nucleic acid detecting TLRs [37], can be intracellular and are expressed constitutively [38]. In fact, TLR-2 and 4 have been implicated in direct recognition of periodontal pathogens such as

Porphyromonas gingivalis, *Tennerella forsythensis*, and *Actinobacillus actinomycetemcomitans* [39, 40].

BACKGROUND AND LITERATURE REVIEW

PART 2: *PORPHYROMONAS GINGIVALIS* GINGIPAINS

Porphyromonas gingivalis has been implicated as one of the main pathogens in periodontitis as discussed above. *P. gingivalis* has several weapons in the form of virulence factors. These include bioactive metabolic products, fimbriae and proteolytic enzymes [41, 42]. Gingipains, which are cysteine proteases are responsible for up to 85% of the proteolytic activity of *P. gingivalis* [43]. Many studies indicate that gingipains are essential for *P. gingivalis* survival *in vivo* and experimental infection induction (Reviewed by Guo and Potempa [42]). In fact, it has been demonstrated that the concentration of RgpB in the periodontal pocket of patients with periodontitis can exceed 1μM [44].

Gingipains are classified into two categories: The arginine-specific gingipains, RgpA and RgpB, and the lysine-specific gingipain, Kgp. A conserved gene referred to as *rgpA*, *rgpB*, and *kgp* encodes each gingipain [45]. RgpA and RgpB share similar caspase-like protease domain and immunoglobulin-like domain. Both have their catalytic specificity towards Arg-aa peptide bonds. RpgA has a third domain, which is a hemagglutinin-adhesin domain. Kgp catalytic domain is specific for Lys-aa peptide bonds. The hemagglutinin-adhesin domain is absent from RgpB. The gingipains are either secreted in their monomeric form (only RgpB) or as a stable complex of protease and hemagglutinin-adhesin domain in the form of RgpA and Kgp [42]. Gingipains play

an essential role for *P. gingivalis* from colonization, nutrient acquisition, immune subversion and signaling. Each of these gingipain properties will be discussed.

Gingipains are not the main adhesins of *P. gingivalis* as fimbriae play the primary role; however, gingipains are potent non-fimbrial adhesins nonetheless. Gingipains bind extracellular matrix proteins, such as fibrinogen, fibronectin, laminin and collagen type V with high affinity [46]. Pathirana and others have also demonstrated that gingipains may mediate tight binding to gingival fibroblasts and epithelium [47, 48]. The hemagglutinin-adhesin domain exerts most of this binding capacity, of which, the RgpA/Kgp complex shows preference to immobilized matrix proteins [49]. These ECM-protease interactions may play a critical role in *P. gingivalis* colonization in the periodontal microenvironment. Interestingly, the proteolytic activity of Rgps yields cryptic ligand sites within cleaved ECM products that *P. gingivalis* fimbriae show preferential binding capacity to [50, 51]. Gingipains are essential in initial *P. gingivalis* infection and also play a crucial role in nutrient acquisition.

If the host is going to amount a proper attack against *P. gingivalis* initially, that is to say to initiate an inflammatory response, communication between the specific components of the innate immune system is required. This is completed via cytokines and their respective receptors on host tissues and immune cells. A popular mechanism that has evolved is for invading microorganisms to try and disrupt this communication between different immune cells and tissues of the host. As for *P. gingivalis*, gingipains play an integral part in cutting off communication in the host immune response. Gingipains have been shown to selectively cleave interleukin-1 beta, interleukin-6,

interleukin-8, interleukin-12, interleukin-4, interferon-gamma, tumor necrosis factor-alpha, CD4/CD8 complex, CD14, and intercellular adhesion molecule-1 [52-63]. Interestingly, gingipain proteolytic activity may not serve the organism well in all cases. For example, interleukin-8 is known to increase neutrophil chemotaxis [64] and respiratory burst [65]. However, the 72 amino acid form of interleukin-8 produced by local inflammatory cells is easily degraded by gingipains [55] but when epithelial cells are exposed to gingipains the less active form of 77 amino acid interleukin-8 they produce [66] is converted to the more active form of interleukin-8 [67]. Overtime eventually both forms of interleukin-8 were inactivated. Taken together, this suggests a relative concentration gradient of gingipains within the periodontium, that is, nearest to the periodontal pocket (where gingipains would be in highest concentration) the effect is inhibited neutrophil response caused by the degradation of interleukin-8 plus other cytokines. Conversely, further away form the periodontal pocket, where presumably lower concentration of gingipains would exist, tissue derived (from epithelial cells etc) 77 amino acid interleukin-8 would be activated to enhance vascular leakage and thus increase flow of nutrients to *P. gingivalis* and the microbial community [68]. Although this is just a sampling of the literature describing host immune signaling corruption, it is clear that gingipains play an integral role to manipulate hose defense and tailor the local microenvironment to suit *P. gingivalis* ecological needs.

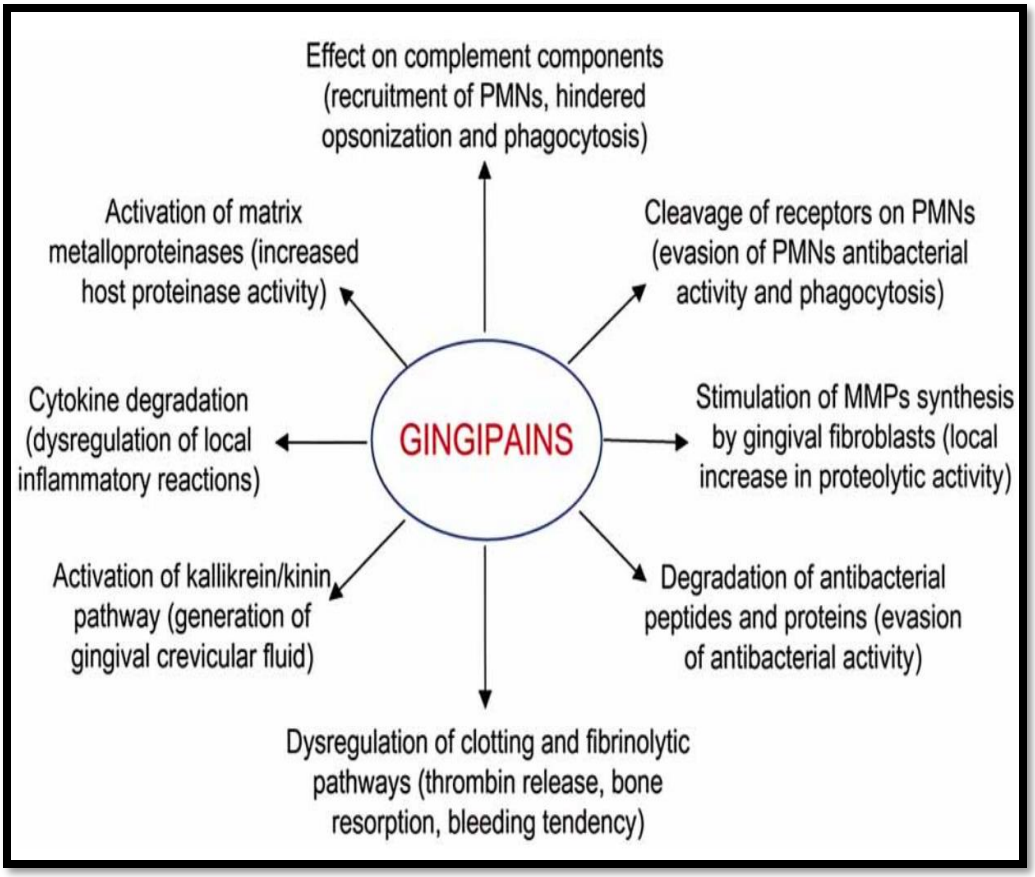

Figure 5. *Roles of gingipains in pathological tissue degradation and evasion of host defense mechanisms (adapted from Potempa J. et al[69])*

BACKGROUND AND LITERATURE REVIEW

PART 3: PROTEINASE-ACTIVATED RECEPTORS: PHYSIOLOGY AND GINGIPAIN TARGETS

Considering that proteases make up at least 2% of the human genome [70], it makes sense that humans have evolved receptors that capitalize on this enzymatic action. Proteinase-activated receptors (PARs) are a family of four seven-transmembrane domain G protein-coupled receptors. Unique to PARs is the activation mechanism consisting of a tethered ligand that stimulates the receptors when cleaved [71]. New data is emerging implicating PARs in several disorders including periodontal disease.

PARs are activated by proteolytic cleavage at the N-terminus, which uncovers a tethered ligand that interacts with one of the seven transmembrane domain external loops. This causes a conformation change that allows for signal transduction [72]. As mentioned above, there are currently four known PARs: PAR-1, PAR-2, PAR-3 and PAR-4. Thrombin is the endogenous physiological activator for PARs-1,-3 and -4. Whereas, PAR-2 is slightly more promiscuous being activated by trypsin, tryptase, coagulation factors VIIa, Xa, tissue kalikreins and neutrophil serine protease 3 [42]. PARs play an important role in many pathophysiological processes, which include but are not limited to, hemostasis, regulation of vascular permeability, vascular smooth muscle contraction and bone cell differentiation and proliferation [42, 73].

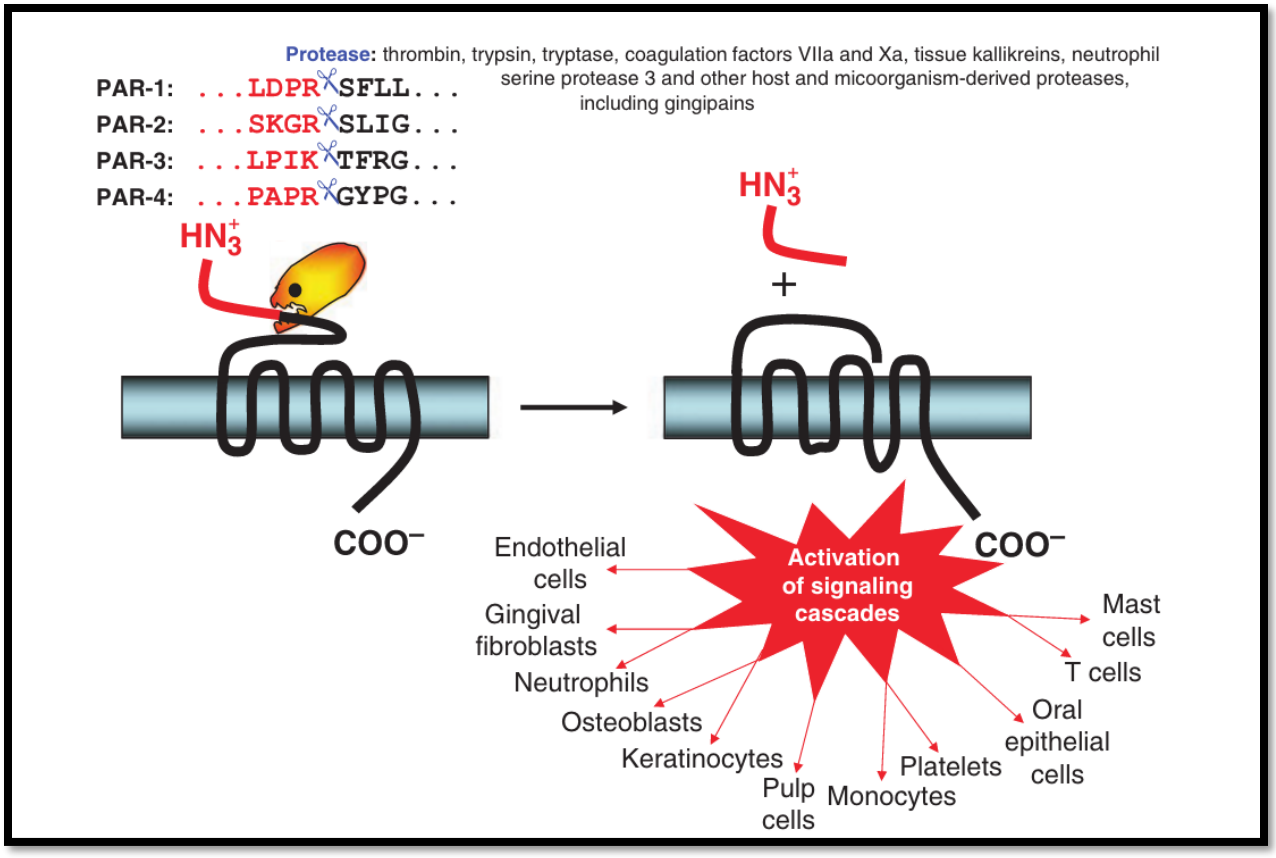

Figure 6. *Scheme of the signaling via PARs and cells in the periodontium affected by the gingipain-PAR activation. The inactive PAR has an un-cleaved and hidden N-terminal-tethered ligand sequence. The tethered ligand cannot interact with the body of the receptor until it is cleaved at the correct peptide bond (Adapted from Guo and Potempa [42]).*

Periodontal tissues express PARs. These cells include oral epithelial cells, gingival fibroblasts, mast cells, osteoblasts, vascular endothelial cells, macrophages, monocytes, neutrophils, and T-cells [42]. PARs seem to play a critical role in periodontal disease considering that PARs play integral roles in innate immunity and bone metabolism. For periodontal homeostasis to be maintained, extremely tight regulation of host protease-PAR signaling networks is controlled through multiple step cascade systems. This is aided by compartmentalization and through endogenous protease inhibitors [42]. However, once pathogenic plaque biofilm starts to accumulate, gingipains

concentration increases and host proteolytic cascades are undermined. The balance is shifted towards one that is increasingly inflammatory as host neutrophils begin to accumulate begin releasing their own proteolytic enzymes. Furthermore, gingipains can themselves cause cellular action via PAR cleavage further complicating the picture (Table 1).

Targeted Cell	Targeted PAR/gingipains	Response to PAR signaling	Pathophysiological consequence	References
Neutrophils	PAR-2 Rgp	Increase in intracellular Ca^{2+} concentration	Neutrophil activation	[74]
Osteoblasts	PAR-2 Rgp	Increase in intracellular Ca^{2+} concentration	Activation of osteoblastic bone resorption	[75]
Platelets	PAR-1, PAR-4, RgpA, RgpB	Platelet aggregation	A putative link between periodontitis and cardiovascular disease	[76]
Oral Epithelial Cells	PAR-1, PAR-2	Stimulation of IL-6 expression	Inflammatory events associated with periodontal disease	[77]
Pulp cells	PAR-2 RgpB	Induced expression of neuropeptides calcitonin gene related peptide and substance P	Link between periodontal disease and pulpal inflammation	[78]
Gingival fibroblasts	PAR-1, PAR-2 RgpB	Induced expression of hepatocyte growth factor	Effect on both inflammatory and tissue-repair processes	[79]
Rat kidney epithelial cells	PAR-2 *P. gingivalis* culture supernatants	Ca^{2+} mobilization		[80]
Keratinocytes	PAR-1, PAR-2 Rgp	Up-regulation of the HIV-1 co-receptor CCR5	Promotion of HIV infection of oral keratinocytes	[81]

Oral epithelial cells	PAR-2 Rgp	Induced expression of human beta defensing 2 and CC chemokine ligand 20	Enhancement of mucosal antibacterial defense	[82]
T-cells	PAR-1, PAR-2, PAR-4 RgpA	Induced expression of CD25 and CD65	T-cell activation	[60]
Vascular endothelial cells	PAR Rgp	Weibel-palade body exocytosis, enhanced production of IL-8 in response to LPS.	Enhanced pro-inflammatory responses to *P. gingivalis*	[83]
Oral epithelial cells	PAR-1, PAR-2 Rgp Kgp	RgpB up-regulate, while Kgp and RgpA down-regulate IL-8 expression	Attenuation of neutrophil migration and sustained chronic inflammation	[84]
Monocytic THP-1 cells	PARs Rgp Kgp	Up-regulation of IL-6, IL-8 and MCP-1	Synergistic effect of PAR-dependent and PAMP receptor signaling	[85]
ST2 mouse stromal and calvarial osteoblasts	PAR *P. gingivalis* cells	Pro-inflammatory response independent of TLR/MyD88 pathway	Pathogen recognition enhancement.	[86]
platelets	PARs Rgps	Platelet sensitization to aggregation by epinephrine	Mechanistic explanation of a direct connection between periodontitis and stress	[87]
Human oral keratinocytes	PAR-1, PAR-2 *P. gingivalis* cells	PAR-1 cleavage by Rgps medicated up-regulation of IL-1 alpha, IL-1 beta, IL-8, TNF-alpha	Osteoclast differentiation and alveolar bone resorption	[88]

Table 1. *PARs targeted by gingipains and the consequence of their action (adapted from Guo and Potempa [42]*

It is obvious that host proteases-gingipains-PAR signaling have many cellular effects. However, we must consider how this signaling axis plays into overall disease progression. PAR-1 and PAR-2 activation lead oral epithelial cells to produce IL-1alpha, IL-1beta, IL-6 and TNF-alpha and IL-8; in addition, monocytes release a similar pro-inflammatory cytokine profile when treated with Rgps [84]. Neutrophil activation via PAR-2 by gingipains also increase the expression of IL-1beta, IL-6, and IL-8 [74]. Not only do gingipain-PAR signaling enhance the expression of pro-inflammatory cytokines, they also induce the expression of mediators that are responsible for bone turnover. Oral fibroblasts when stimulated via PAR-1 and PAR-2 expressed hepatocyte growth factor, which is known to increase osteoclast activity. Furthermore, cytokines themselves can drive bone resorptive activities and this may be synergized by gingipains-PAR signaling via receptor activator of nuclear factor Kappa beta ligand (RANKL)/osteoprotegrin expression ration in osteoblasts [89]. For normal bone turnover to occur, osteoblasts most control osteoclastogenesis via tight control via RANKL (Stimulator of osteoclastogenesis) /Osteoprotegrin (inhibitor of osteoclastogenesis) expression. This balance is further undermined by *P. gingivalis* Kgp, which degrades osteoprotegrin [90].

Considering the signals that are released when PARs are activated by invading bacterial proteases, it is clear that they play an essential role in the surveillance of periodontal tissues [42]. However, periodontal pathogens are resistant to PAR mobilized innate immune cells [42]. Interestingly, one PAR has stood out to have an essential role in the pathogenesis of periodontitis. Several studies have demonstrated that PAR-2 plays a pivotal role in experimental periodontitis. One example, oral challenge with a PAR-2 agonist not only increased existing periodontitis in rats, but also caused the development

of the disease with the clinical hall marks of alveolar bone loss, gingival granulocyte infiltration accompanied by the overexpression of matrix metalloproteinase (MMP)-2, MMP-9, cyclooxygenase 1 and cyclooxygenase 2 [91]. Furthermore, Wong and team demonstrated PAR-2 knockout mice orally infected with *P. gingivalis* showed significantly reduced or no alveolar bone loss when compared to wild-type or PAR-1 knockout *P. gingivalis* infected mice [92]. The lack of alveolar bone loss in the PAR-2 knockout mice was followed by a large decrease in the infiltration of mast cells and type 1 helper T-cell dependent cytokines, such as interleukin-2 and interleukin-17 and TNF-alpha [92]. Considering these studies, there is no doubt about the important role PAR-2 plays in periodontal disease. The picture becomes more complicated when we consider the layers to PAR-2 activation. The first layer of PAR-2 activation is completed by the arginine specific gingipains. As discussed above, this leads to the expression of inflammatory cytokines, such as interleukin-6, that directly enhances bone resporption via osteoclasts [77, 92]. Another layer we need to consider is mast cells, which also recognize *P. gingivalis.* The mast cells could release tryptase, which can additionally activate PAR-2. Furthermore, mast cells can release TNF-alpha further adding to the inflammatory milieu [92]. Lastly, neutrophil infiltration would lead to the increase of proteinase-3, which also is known to activate PAR-2 [92, 93]. Considering that all three of these avenues of activation of PAR-2 could lead to T-cell involvement, it is no surprise that PAR-2 knockout mice lack the hallmarks of periodontitis. PAR-2 could be a potential therapeutic target in periodontitis.

Due to PARs unusual mechanism of action, that is the tethered ligand, a multitude of strategies has been used to develop antagonist. Ramachandran and colleagues

emphasized that the term antagonist is used loosely and doesn't necessarily fit the classical pharmacological definition [71]. The approaches used to abrogate PAR receptor signaling include: 1) blocking the docking of the tethered ligand sequence with a small-molecule receptor antagonist, 2) occluding the cleavage site of the tethered ligand with a PAR-selective antibody that prevents proteinase activation, or 3) interfering with the interactions between a PAR and its intracellular signal transduction proteins [71]. The therapeutics developed with these ideas are progressing; PAR-1 antagonists (Vorapaxar and Atopaxar) are late state clinical trials where as PAR-2 antagonists (i.e. ENMD-1068) are still being validated in animal trials [71].

Developing of a small-molecule non-peptide PAR-2 antagonist has not been as straightforward as it was for PAR-1. Two peptides were found to block trypsin induced PAR-2 signaling but not by PAR-2 activated peptides [94]. The PAR-2 antagonist peptide ENMD-1068 (N1-3-methylbutyryl-N4-6-aminohexanoyl-piperazine; Figure 8), which is used in this study, is also based on the tethered ligand motif of PAR-2. Ferrell and colleagues demonstrated that ENMD-1068 blocked trypsin-mediated receptor activation and attenuated PAR-2 mediated murine joint inflammation *in vivo* [95]. Unfortunately, in a clinical setting ENMD-1068 was not potent enough to match the preclinical results [96]. Taken together, PARs represent a relatively new avenue of therapeutic intervention in a host of diseases where inflammation plays a critical role. One avenue that has yet to be discussed is the role of the extracellular matrix plays in inflammation and periodontal disease.

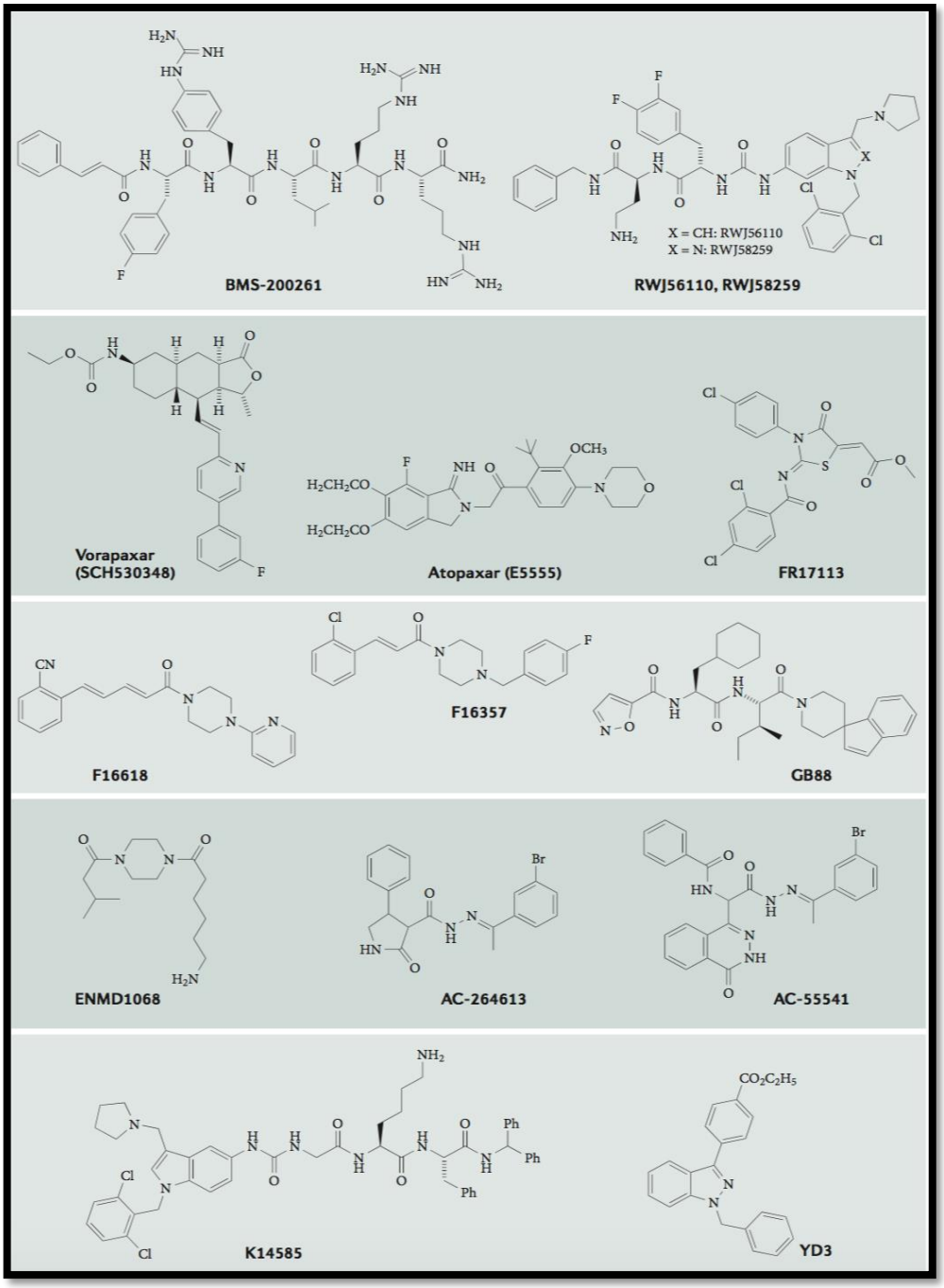

Figure 7. *Currently available Proteinase activated receptor antagonist. PAR-1 Antagonist: BMS-200261 (Used in this study), RWJ56110 and RWJ58259, FR17113, F16618, and F16357. PAR-2 Antagonist: GB88, ENMD-1068 (Used in this study), AC-264613, AC-55541, K14585. PAR-4 Antagonist: YD3. (Adapted from Ramachandran et al [71]).*

BACKGROUND AND LITERATURE REVIEW

PART 4: THE EXTRACELLULAR MATRIX

The extracellular matrix (ECM) consists of the secreted products of many cell types that form an organized scaffold for cellular support [97]. The ECM exists in many different biochemical and structural forms and its architecture is the product of extracellular and intracellular assembly from several cell types. The contents of the ECM are highly insoluble proteins that are made of specific conserved domains. Often, these domains contain relatively large amounts of glycosaminoglycans and sulphate leading to an overall negative charge [98, 99]. The negative charge and large surface area yields a plethora of interactions with other charged molecules such as growth factors and chemokines, effectively controlling the local concentration and accessibility to cells in close proximity [99].

The ECM can take two basic forms. These are the basement membrane or the interstitial matrix form. The basement membrane is a complex of thin networks of highly cross-linked glycoproteins. The interstitial form is loose and fibril like. Furthermore, there is specialized ECM structures that have a combination of both of the aforementioned forms, that is, the reticular fibers, which make up lymphoid organs [97]. Lastly, Eckes and others described a provisional matrix form that can be well-populated by immune cells during wound healing [100]. One of the main barriers immune cells have on their way to the site of infection is the basement membrane.

Tissue dynamics, that is its formation, function and regeneration after damage as well as its function in pathology is the result of an intricate interplay of temporal and spatial coordination of numerous individual cell fate processes, each of which is induced

by a myriad of signals originating from the ECM [101]. The extracellular milieu consists of a plethora of complex array of biophysical and biochemical signals that when bound to their respective receptors ultimately transduce an intracellular cascade that converges to regulate gene expression and cell phenotype [101]. The ECM, which surrounds cells and comprises the molecular signals, is a highly hydrated network hosting three main effectors: 1) insoluble hydrated macromolecules (Proteins such as collagen and fibronectin) 2) Soluble macromolecules (Growth factors) and 3) Proteins on the surface of neighboring cells (Integrins) [101]. Final cell fate, which is cellular differentiation, proliferation, migration or apoptosis, is the result of the individual cell and the molecular interactions with the ECM effector molecules [101].

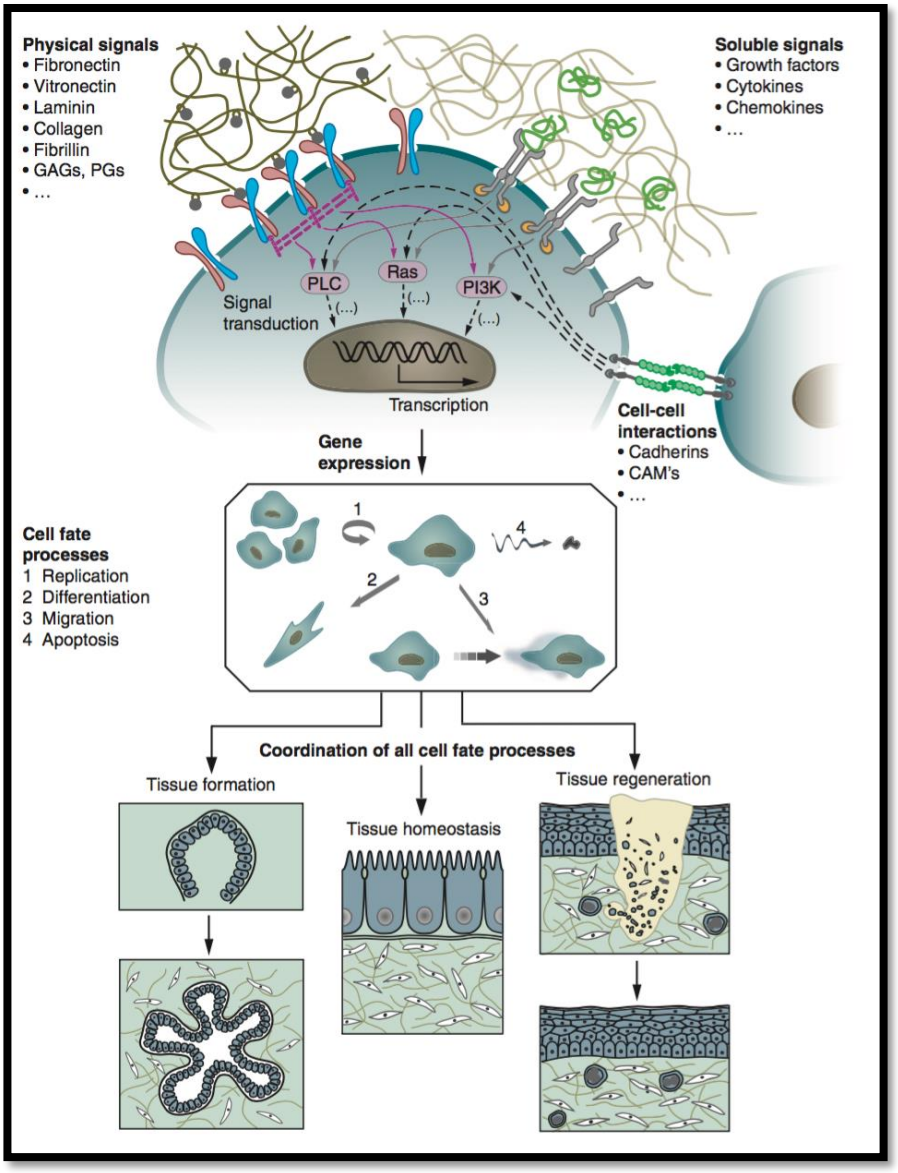

Figure 8. *The behavior of individual cells and the dynamic state of the multicellular tissues is regulated by reciprocal molecular interactions between cells and their surroundings (From Lutolf and Hubbell [101].*

The ECMs most elemental function provide nearby cells a structural scaffold that can resist tensile and compressive stress [101]. Structural ECM proteins include different forms of collagen. Some collagens are long and stiff while and function as structural proteins, whereas others serve connecting and recognition functions [101]. The fibrillar

structure of ECMs has been demonstrated to effect integrin clustering thus altering cellular behavior [101, 102]. The ECM provides bound adhesion ligands, which include fibronectin and vitronectin, that guide the development and maintenance of cell function [101]. The integrins, a family of transmembrane, heterodimeric, cell-surface molecules, function as the principle receptors of cells for many of these ECM adhesion molecules [101]. Integrins primarily link the macromolecules of the ECM with the cell's cytoskeleton, but are involved in cell-cell adhesion and binding to proteases, such as matrix metalloproteinase [101]. When bound to ECM ligands, integrins cluster and form associations with various signal-transducing molecules (PI3K etc.) to activate specific protein kinases such as mitogen activated protein kinase. Thus, integrins play an important role in relaying information from the cellular surface to genome, which ultimately brings about a phenotypic change [101, 103, 104].

Fibronectin is a large dimeric (2 x 230kDa) ubiquitous extracellular matrix protein [105]. Fibronectin is also present in the gingiva and periodontal ligament where it has multiple functions like those described in the previous paragraph [106]. *P. gingivalis* use its gingipains to proteolytically cleave integrin-gingipains interactions on fibroblast cell surface [107]. Previous studies demonstrate that gingival fibroblasts when co-cultured with a high concentration of purified gingipains lead to a decrease in fibronectin and α5β1 integrin, causing cell detachment and cell death [108]. Not surprisingly, samples taken from patients with periodontitis demonstrated increased amounts of fibronectin fragments, the result of host and pathogen proteolysis [109, 110]. Specially, the 120, 68 and 40 kDa, fibronectin fragments that include the main cell and heparin binding regions were greatly increased in periodontitis [109]. Feghali and Grenier

demonstrated that the smaller fibronectin fragments of 30 and 45 kDa were the most potent inflammatory inducers as they dose-dependently increased secretion of TNF-α, IL-1β, and IL-8 in human macrophages [110]. The 120-kDa fibronectin fragment did not induce the secretion of those same cytokines; whereas the whole 420-kDA fibronectin only increased IL-8 [110]. In addition, this study also demonstrated that the Arg and Lys specific gingipains purified from *P. gingivalis* modulate fibronectin fragmentation [110]. Furthermore, cellular fibronectins contain alternatively spliced exons encoding type III repeat extra domain A (fibronectin-EDA) and EDB are produced in response to tissue injury [111-113]. The response of cells exposed to recombinant EDA or EDA-containing fibronectin are similar to those observed when cells are treated with LPS, that is, they produce pro-inflammatory cytokines and other proteins such as matrix metalloproteinase [111-113].

Matrix metalloproteinases (MMPs) are a class of zinc dependent endopeptidases that consist of four conserved protein domains, which include the pro-domain, catalytic domain, haemopexin domain and the C-terminal domain. MMPs expression is regulated by pro-inflammatory cytokines, growth factors, and hormones [114-116]. MMPs are secreted as zymogens that require activation by removal of the amino terminal pro domain [117]. Interestingly, MMPs can reciprocally activate other MMPs and by furins [97]. However, the most important player is MMP regulation is the tissue inhibitor of metalloproteinase, or TIMPs, that bind and inactivate most MMPs. Importantly, MMP mRNA expression or even protein expression does not provide information on the *in vivo* localization of MMP activity, which is assessed by *in situ* gel zymography [118]. MMPs

are classified by their substrate that they can cleave that are identified by *in vitro* experimentation [97].

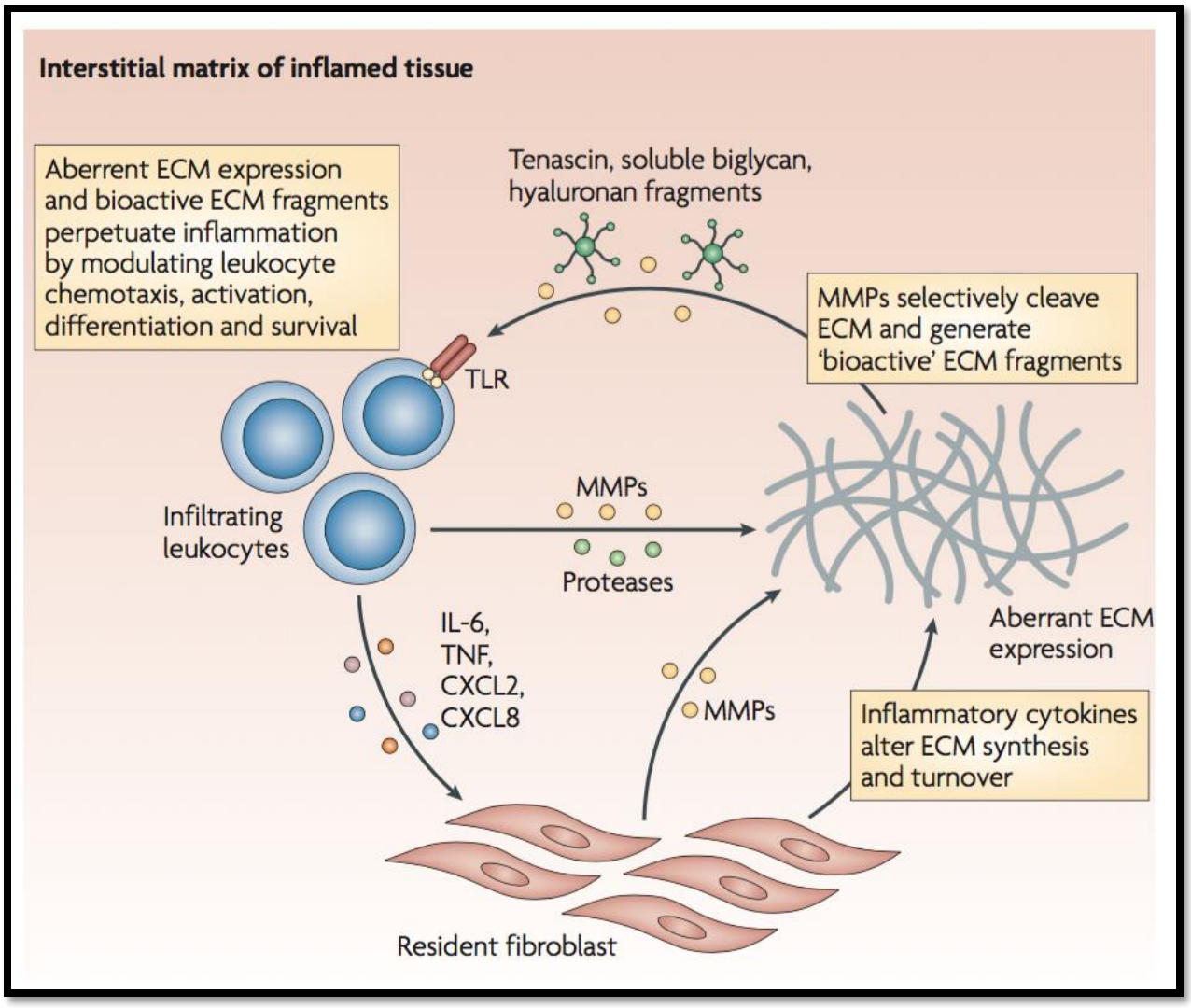

Figure 9. *Potential modes of ECM-mediated activation of immune cells (Adapted from Sorokin [97].*

The amount of evidence is increasing that demonstrates ECM turnover, by host or bacterial proteases, can act as chemo-attractants themselves that can alter immune cell

activity [97]. ECM fragments from type 1 collagen generated by proteolytic cleavage by MMP-8 or MMP-9 has been shown to have the same pro-inflammatory effects as a well-known pro-inflammatory stimuli, CXCL8, on neutrophils in lung inflammation [119]. In addition, MMP-12 and elastase generated from elastin induce chemotaxis in a model focused on monocytes in chronic inflamed lungs [120]. One of the main culprits that play a role in ECM induced activity of immune cells are the Toll-like receptors, which recognize defined molecular patters that are usually associated with pathogens and tissue damage. Activation of TLRs causes the activation of innate immune pathways which will ultimately have an effect on adaptive immune responses [97]. Data currently implicates fragments of the ECM as activators of TLR-4. Most of the data currently available has shown that the molecules involved are either up regulated or proteolytically processed in inflamed tissues and are mostly interstitial matrix molecules such as tenascin C, fibronectin-EDA, and heparin sulphate [97, 113] [121-123].

PORPHYROMONAS GINGIVALIS GINGIPAINS INDUCE A PRO-INFLAMMATORY EXTRACELLULAR MICROENVRIONMENT: THE ROLE OF PAR-2 AND FIBRONECTIN

INTRODUCTION

Periodontitis is a widespread chronic inflammatory disease of the periodontium. It has been estimated that 50% of adults greater than 30 years of age have manifestations of the disease in the United States [7]. If left untreated, gingival destruction, loss of alveolar bone, and eventually tooth loss will occur. In addition, periodontitis has been correlated as a risk factor with respiratory disease, cardiovascular disease, diabetes, osteoporosis [8], preterm birth and low birth rate [9, 10] Periodontal pockets, which are hallmark of periodontitis, allow for persistent anaerobic conditions that facilitate the colonization anaerobic pathogens such as *Porphyromonas gingivalis*. *P. gingivalis* is the causative pathogen for both adult periodontitis [124]. *P. gingivalis* posses an array of putative virulence factors that include lipopolysaccharide (LPS), fimbriae, toxic metabolites, and proteolytic enzymes; all of which stimulate the host immune system, inflammatory mediators, and promote periodontitis. Essential to *P. gingivalis* virulence are the proteolytic enzymes termed gingipains, which allow *P. gingivalis* to subvert the human immune system [125].

Here, we studied the proteolytic activity of two cysteine proteinases produced by *P. gingivalis* [126]. The arginine specific gingipains, RgpB and HRgpA, of 50 and 95 kDa which only cleave peptide bonds at Arg residues [127], and the lysine specific

gingipain, Kgp, of 105 kDa which cleaves specifically at Lys residues [128]. HRgpA and Kgp consist of a catalytic domain and a hemaglutinin/adhesion domain, which RgpB lacks. Gingipains take part in a plethora of physiological activities which have been shown to enhance vascular permeability through activation of the kinin pathway [129], activate complement [130], disrupt blood plasma clotting [131, 132] and cleave CD14 on human monocytic cells [133].

During inflammation and periodontal pocket formation, the extracellular matrix (ECM) becomes assessable to *P. gingivalis* gingipains. The periodontal ECM is made up of a plethora of proteins, such as collagen and fibronectin. The ECM and its corresponding network of proteins not only provides structure support, but also play a vital role in cell adhesion, migration, signaling, and ultimately cell survival. ECM and gingipain interaction has been shown to be important for colonization, that is, *P.gingivalis* preferentially binds to immobilized fibronectin (FN) in vitro [49]. FN is an essential adhesive glycoprotein that is important in physiologic and pathologic conditions associated with tissue injury and repair and a major component of the ECM. FN exists as either as an insoluble glycoprotein which polymerizes adding structure support, or alternatively, exists as a soluble disulphide-linked plasma protein which plays a more direct role in wound healing [134]. Both of which are found during tissue repair, cell migration, embryogenesis, and blood clotting. In addition, different splice variants, such as extra domain A of fibronectin (Fibronectin-EDA) can have vastly different biological activities. EDA of fibronectin stimulates toll-like receptor (TLR) 4 and induces NF-κB activation [113] In addition, during infection and inflammation, proteolytic cleavage of the ECM occurs; this has been well documented during rheumatoid arthritis [113, 135,

136]. In addition, it has also been demonstrated in other systems that degraded ECM molecules stimulate the host cells in the site of infection to produce a multitude of inflammatory cytokines [135, 137-142]. Considering FN fragments have been detected *in vivo* from lung lavages, individuals with periodontal disease [143, 144] a significant effort has been made towards the elucidation of the biologic activities of specific FN fragments. For example, 30kDa N-terminal FN fragments had greater chemotactic activity for monocytes when compared to un-fragmented FN and even decreased monocyte attachment to fibrin [145].

It has been established that bacterial adhesion/degradation of fibronectin is important in the virulence of different bacteria. For example, adhesion of fibronectin promotes the colonization of *Mycobacterium tuberculosis* to murine alveolar macrophages [146] and *Streptococcus pneumioniae* in meningeal inflammation [147]. In contrast, FN proteolysis provides a source of fermentable fuel [148, 149], or conversely, may jeopardize host wound healing [150]. Furthermore, interactions with FN allow pathogens to penetrate host tissues, thus avoiding some of the host immune system [151]. In addition, fibronectin binding has been shown in oral pathogens such as *Treponema denticola* [152]. In addition, the causative microbe of syphilis, *Treponema pallidum*, also interacts with FN [153]. Less is known about the specific molecular interactions *P. gingivalis* has with secreted ECM proteins such as fibronectin and the effect on the regulation of their corresponding genes. In addition, the downstream affect of pathologic proteolysis of ECM proteins, in particular fibronectin and the overall pathogenesis of periodontitis and the consequences for overall systemic health have yet to be elucidated.

MATERIALS & METHODS

Reagents and Cell Culture

NIH 3T3 fibroblasts and human gingival fibroblasts (ATTC, CRL-2014) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceburg, GA) 50 IU/ml penicillin/streptomycin and 1mg amphotericin (Corning Cellgro) at 37°C in a humidified 5% CO_2 incubator. U937 monocytes were incubated in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceburg, GA) 50 IU/ml penicillin/streptomycin and 1mg amphotericin (Corning Cellgro) at 37°C in a humidified 5% CO_2 incubator.

Purification and activation of gingipains

RgpB, HRgpA, and Kgp were purified from *P. gingivalis* HG66 culture supernatant, as described previously [128, 154]. Purity of each enzyme purification was checked by SDS-PAGE. In a 10% Tricine gel (Von Jaggow), RgpB showed a single band with an apparent molecular weight of 48kDa and the purity was > 95% as determined by laser densitometry scanning of the gel. HRgpA was composed of four major and one minor band on SDS-PAGE and each protein band was identified as a HRgpA component by N-terminal sequence analysis [128]. The amount of active enzyme from each gingipain was determined by active site titration using FPR-cmk and Z-FK-cmk for Rgps and Kgp, respectfully [43]. The concentration of fully activated gingipains with cysteine was calculated from the amount of inhibitor needed for complete inactivation of the

proteinases. The gingipains were activated in 0.2M HEPES, 5mM $CaCl_2$, and 10mM cysteine, pH 8.0, at 37°C for 10 minutes, and then diluted in media or buffer. To block enzymatic activity, activated gingipains were incubated with a 10mM concentration of KYT-1, (3S)- N- [(1S)- 5- Amino- 1- (N,N- dimethylcarbamoyl)pentyl]- 3- [(2S)- 6- amino- 2- (benzyloxycarbonyl) aminohexanoylamino]- 6- guanidino- 2- oxohexanamide, (PeptaNova, Germany).

Assay for fibronectin gene promoter activity

To evaluate fibronectin gene expression, NIH 3T3 cells expressing pFN(1.2kb)LUC containing ~1,200bp of the 5'-flanking region of the human fibronectin gene isolated from the human sarcoma cell line HT1080 were used. This construct includes 69bp of exon 1, a CAAT site located at -150bp, and the sequence ATATAA at -25bp from the transcription start site. It also contains several previously identified regulatory elements such as three cAMP response elements located at -415, -270, -170, and an SP-1 site at -102bp from the transcriptional start site. The promoter was sub-cloned in the *Sma* I site of pGL3 Basic Luciferase reporter vector (Promega, Fitchburg, WI). The pFN(1.2kb)LUC promoter construct was introduced into murine NIH 3T3 fibroblast via electroporation to create a stable transfectant. Fibroblasts were maintained in DMEM with 4.5g/l glucose supplemented with 10% heat-inactivated FBS and 1% antibiotic-antimycotic solution (100U/ml penicillin G sodium, 100U/ml streptomycin, and 0.25µg/ml amphotericin B) and incubated in a humidified 5%CO_2 incubator at 37°C. The cells were then treated with a 1:400 dilution of RgpB or a 1:100 dilution of HRgpA isolated for *P. gingivalis* for 2, 4 or 6 or 24 hours at varying concentrations. Afterward, the cells were tested for luciferase activity. For this, the cells were harvested by scraping, washed with PBS, resuspended, in

100µl of cell lysis buffer (Promega, Fitchburg, WI), sonicated, and a 20µl aliquot was tested by adding 50µl Luciferase assay reagent (Promega, Fitchburg, WI). Light intensity was measured using Labsystems Luminoskan Ascent Plate Luminometer. Results were recorded as normalized luciferase units adjusted to total protein concentration (Bradford method).

Western Blot Analysis

For western blotting of fibronectin, NIH 3T3 cells were treated with a 1:400 dilution of RgpB at 50, 100 or 200nm concentration or control and allowed to incubate at 37°C for 24 hours. The cells were lysed in homogenization buffer (50mM β-glycerophosphate, pH 7.4, 1mM EGTA, 1mM DTT, 2mM PMSF, 0.1mM sodium vanadate). Protein concentration was estimated by the Bradford method. 75µg of protein was loaded from each sample into a 5% SDS-polyacrylamide gel with a 3.9% stacking gel and ran at 150 volts for 1.5 hours. The separated proteins were transferred onto a nitrocellulose membrane using a BioRad Trans Blot Semi-dry transfer apparatus for 2 hours at 25 volts. The gels were blocked with 5% non-fat dry milk (0.5% Tween-20 and 10% 10xTBS) for 1 hour at room temperature and washed three times for 5 minutes with wash buffer (1x TBS, 0.5% Tween-20). The blots were incubated with primary antibody against fibronectin (Sigma, St. Louis, MO; 1:1000 dilution) overnight at 4°C. The blots were then washed 3 times for 5 minutes with wash buffer and incubated with secondary antibody to rabbit anti-goat IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; horseradish peroxidase-conjugated 1:20000) for 2 hours at room temperature. GAPDH (1:5000) was used as a loading control. Blots were developed using ECL solution (GE

Healthcare, formerly Amersham Pharmaceuticals). Protein bands were quantified by densitometry using laser densitometer (BioRad, GS-800). Means are reported ± SD.

Gelatin zymography

CHAPS buffer (0.15M NaCl, 10mM CHAPS, 20mM HEPES pH 7.5, 1mM EDTA, 2mM PMSF) was added to cell samples. Total protein concentration was determined using the Bradford method. 9% zymography gels were prepared with a final concentration of gelatin (Sigma Chemical Inc., St.Louis, MO) approximately 1mg/ml. The zymography gels were in 1x SDS-Page gel running buffer on ice at 50 volts for 30 minutes followed by 100 volts for an additional two hours. The gels were then incubated for 60 minutes in 2.5% Triton x-100 in distilled water at ambient temperatures. The gels were then incubated overnight at 37°C in substrate buffer (50mM Tris pH 8.8, 10mM $CaCl_2$), which activates the gelatinase MMPs. Next, the gels were stained with 0.5% Coomasie blue R-250 in water/methanol/acetic acid (45:45:10) for 30 minutes followed by destaining with 50% methanol, revealing the MMP bands while the rest of the gel remained stained. The MMP bands were quantified using a Bio-Rad laser scanner GS-800 densitometer. Data obtained was analyzed using one-way ANOVAs, and post hoc multiple comparisons, if warranted (Bonferrroni's Multiple comparison Test).

Testing the effect of fibroblast-derived ECM

Fibroblasts were cultured for 5 days, when confluence was reached in 6-well Costar cell culture plates (Corning). Afterwards, the fibroblasts were eliminated by osmotic lysis. Cells were washed once with PBS containing 1mM EDTA (3A solution), then treated for 30 minutes at 4°C with the 3B solution (0.25M NH_4OH, 1mM EDTA, 1mM PMSF). The cells were washed two additional times with solution 3A and then treated for 15 minutes

at 4°C with solution 3E (1M NaCl in 50 mM Tris (pH 7.4), 1mM EDTA, 1mM PMSF). Lastly, the culture plates were washed once with solution 3A. Isolated matrices were stored at 4°C with PBS. Matrix isolation was confirmed via direct immunoflouresnce of fibronectin.

Human monocytic U937 cells permanently transfected with the human IL-1β gene promoter fused to a luciferase reporter gene [155] were incubated in RPMI (Cellgro) on matrix-coated plates for 24 hours. Afterwards, the cells were harvested by scraping, washed with PBS, resuspended, in 100µl of cell lysis buffer (Promega, Fitchburg, WI), sonicated, and a 20µl aliquot was tested by adding 50µl Luciferase assay reagent (Promega, Fitchburg, WI). Light intensity was measured using Labsystems Luminoskan Ascent Plate Luminometer. Results were recorded as normalized luciferase units adjusted to total protein concentration (Bradford method).

Enzyme Linked Immunosorbant Assay

Enzyme linked immunosorbant assays (ELISAs) 96-well plates were pre-coated by the manufacturer with either human IL-6 or IL-8 antibodies from the manufacturer (Thermo Scientific, Rockford, IL). The manufactures instructions were followed. Before all experimental samples were tested, positive control was tested to make sure the value did not exceed the highest value of the standard curve (400pg/ml). All reagents were prepared fresh for each experiment. 30mL of 30x "Wash Buffer" was added to 870mL of sterile water for each plate used and was mixed thoroughly. Wash buffer was at room temperature before use in all assays. Standards for IL-6 or IL-8 were reconstituted with lyophilized standard. Standards were prepared using serial dilutions (contained 0.1% sodium azide). For IL-6 the standard dilutions were 400pg/ml, 160pg/ml, 64pg/ml

25.6pg/ml, 10.24pg/ml and 0pg/ml. For IL-8 standard dilutions were 1000pg/ml, 400pg/ml, 160pg/ml, 64pg/ml 25.6pg/ml, 10.24pg/ml and 0pg/ml. 50µL of biotinylated antibody reagent were added to each unknown sample well. 50µL of standards were added to the wells in duplicate. Plate was covered and allowed to incubate at room temperature for two hours. Plate was washed three times using wash buffer. 100µL of prepared streptavidin-HRP solution was added to each well. Plate was covered and allowed to incubate at room temperature for 30 minutes. Plate was then washed three times with wash buffer. 100µL of TMB substrate was added to each well and allowed to develop in the dark at room temperature for 30 minutes. The reaction was stopped by adding 100µL of stop solution (contains 0.16M sulfuric acid) to each well. Absorbance was measured with a plate reader set at 450nm. Results were calculated using Excel (Microsoft) and data was fit to the standard generated curve. Means are reported ± SD.

Immunofluorescence staining of fibronectin

Primary mouse lung fibroblasts were grown in 8-well chamber slides for 72 hours. After treatment with gingipains for 24 hours, media was aspirated and cells were covered with warm 4% paraformaldehyde in PBS for 15 minutes. Once cells were fixed, cells were incubated with blocking buffer (1x PBS, 5% normal goat serum, 0.3% Triton X-100) for 60 minutes. Blocking buffer was aspirated and cells were incubated with primary antibody against fibronectin-EDA (1:200; Sigma, St. Louis, MO) in antibody dilution buffer (1x PBS, 1%BSA, 0.3 Triton X-100) overnight at 4°C. Afterward cells were then washed 3 times with PBS. Cells were incubated with flourochrone-conjugated secondary

antibody (FITC) for 1 hour at room temperature in dark. DAPI was added at 1:5,000 in methanol for 5 minutes. Slides were mounted, cover slipped and examined.

Screening for lipopolysaccharide (LPS)

In order to avoid any confounding effects from LPS contamination, all treatment materials and culture media were screened with a limulus based endotoxin assay with a sensitivity of 0.06ng/ml (Lonza, Basel, Switzerland).

Statistical Analysis

Means plus standard deviations of the mean were calculated for all experimental values. To establish statistical significance, one-way ANOVAs were performed on data taken from experiments. Post-hoc multiple comparisons performed, if warranted (Bonferroni's Multiple Comparison Test). GraphPad Prism was used to analyze data (Software Licensed to Dr. Jan Potempa). Bars in graphs indicate post-hoc comparison to control.

RESULTS

Gingipains stimulate the expression of fibronectin through PAR-2

We first evaluated the effect of purified gingipains on the expression of fibronectin in fibroblasts using NIH 3T3 cells transfected with the human fibronectin gene promoter fused to a luciferase reporter gene. As depicted in Figure 1, we found that the Arg-specific gingipains, RgpB and HRgpA, greatly stimulated fibronectin gene expression; both RgpB and HRgpA stimulated fibronectin gene expression in a time- and dose-dependent manner when compared to control (Fig. 1A and B, respectively). Interestingly, both RgpB and HRgpA stimulated the fibronectin promoter approximately equally. When in the presence of KYT-1, a selective inhibitor of gingipain enzymatic activity, the observed effects were completely abrogated.

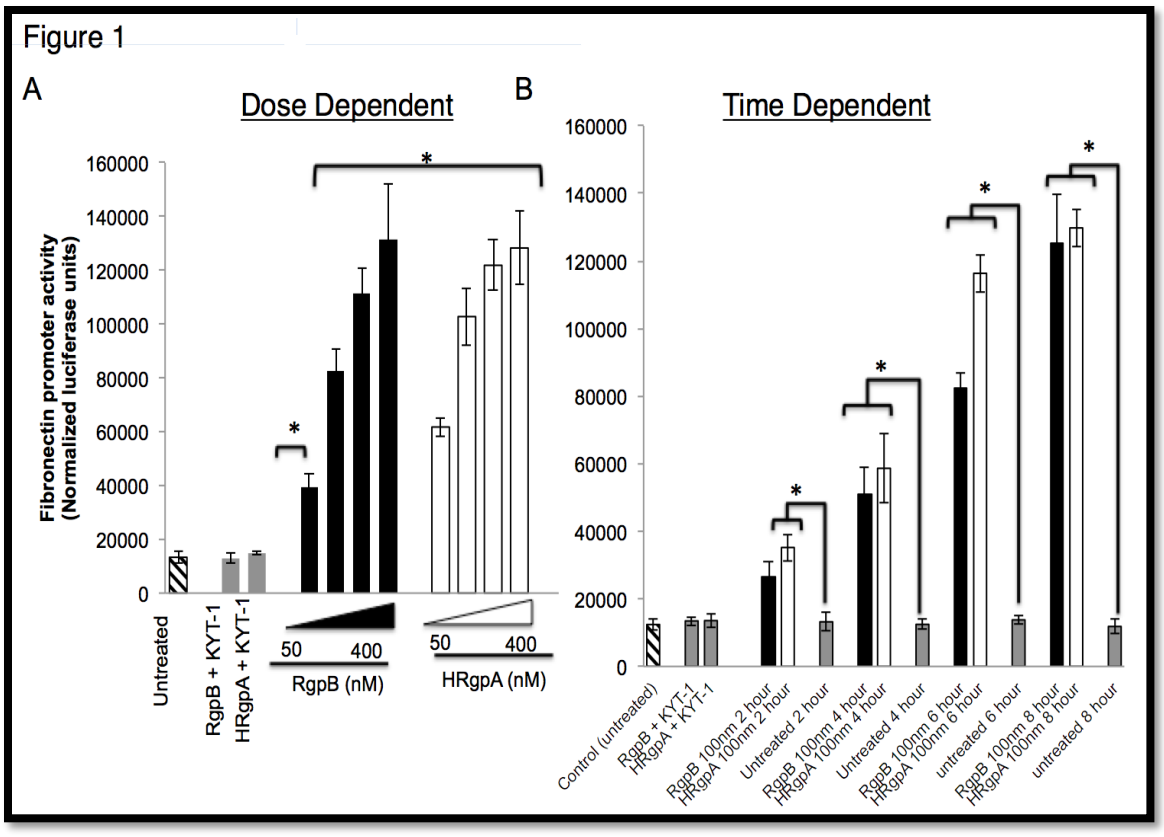

Figure 1. Gingipains induce fibronectin gene expression. (A) Dose dependent response of NIH 3T3 cells ($1.5x10^5$ cells) permanently transfected with the human fibronectin promoter were incubated with 50, 100, 200, or 400nm of RgpB, HRgpA, or RgpB or HRgpA plus KYT-1 (10μM) inhibitor for six hours at 37°C. Purified gingipains were pre-incubated with or without KYT-1 for 15 minutes at 37°C. Luciferase readings were taken after 6 hours. (B) Time dependent response of the human fibronectin promoter in NIH 3T3 cells ($1.5x10^5$ cells) stimulated with 50, 100, 200, or 400nm RgpB, HRgpA, or RgpB or HRgpA plus KYT-1 (10μM) inhibitor for 2, 4, 6, and 8 hours. Data depicted as means ± SD. Overall $P<0.0001$ (One-way ANOVA). Bars and asterisks indicated post-hoc comparison significance ($P <0.05$).

Considering that gingipains have been shown to signal through protease activated receptors (PARs; Lourbakos *et al.*, 2001), and PAR-2 activating peptides have been shown to promote fibronectin expression in human lung fibroblasts, we investigated the role PARs played in this system. Using a series of PAR antagonists, we systematically tested the role of PAR-1, 2, or 4 (Fig. 2). ENMN-1068 (Enzo Life Sciences), a selective

PAR-2 antagonist, completely abrogated the induction of the fibronectin gene via RgpB or HRgpA, suggesting that Arg specific gingipains cleave PAR-2 to stimulate fibronectin expression, whereas the PAR-1 and PAR-4 antagonists had no effect. Consistent with a role for PAR-2, we observed induction of fibronectin gene expression in cells treated with a PAR-2 agonist (Fig. 2). No observed effects were noted when gingipains were treated with a specific inhibitor or with the PAR antagonists.

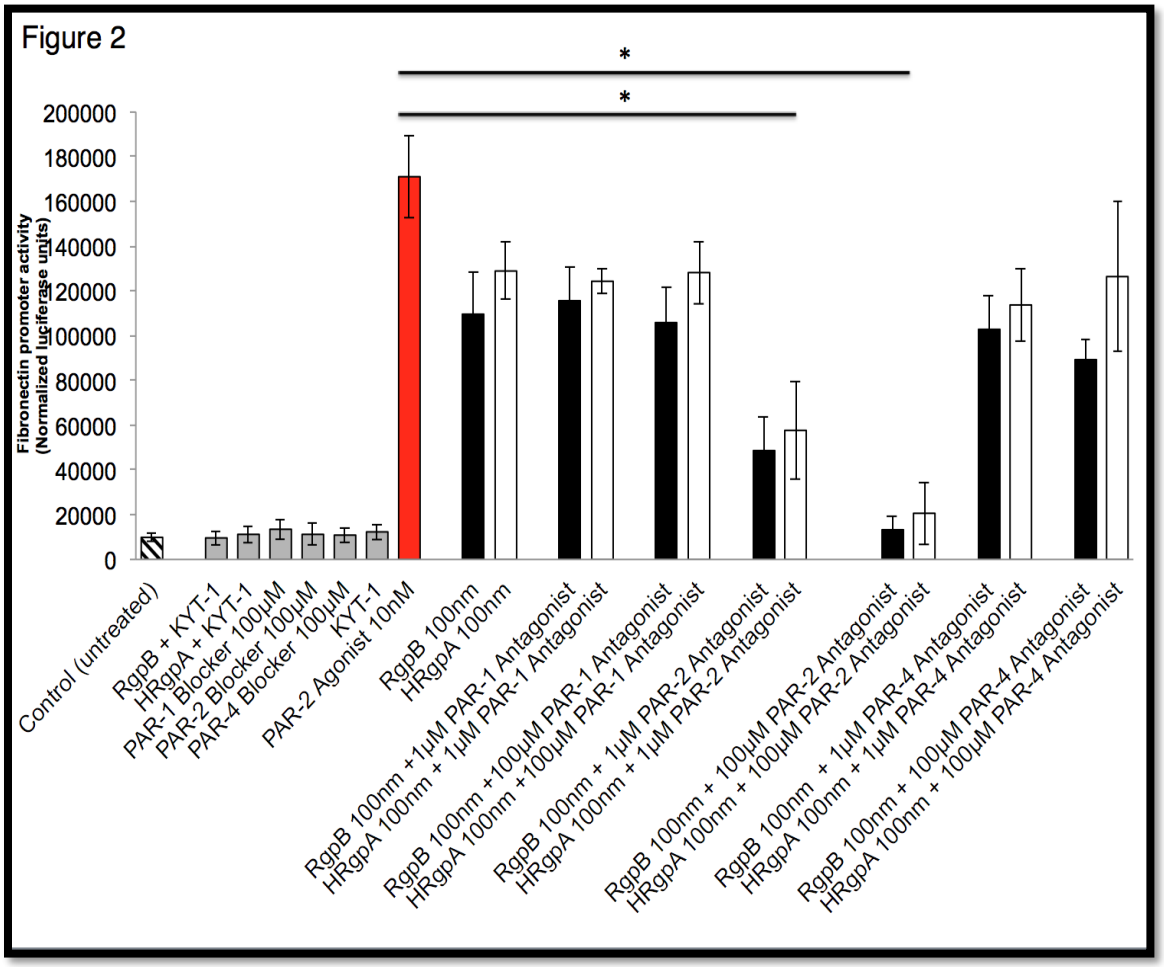

Figure 2. Gingipains induce fibronectin gene expression via PAR-2. NIH 3T3 fibroblasts ($1.5x10^5$ cells were pretreated with either AC55541 (PAR-2 agonist), BMS-200261 (PAR-1 antagonist), ENMN 1068 (PAR-2 antagonist), and trans-cinnamoyl-Tyr-Pro-Gly-Lys-Phe-NH2 (PAR-4 antagonist). Cells were subsequently treated with RgpB, HRgpA, or RgpB and HRgpA with KYT-1 inhibitor. Readings were taken after 6 hours of incubated at 37°C. Data depicted as means ±SD. Overall P<0.0001 (One-way

ANOVA). Bars and asterisks indicated post-hoc comparison significance when compared to control ($P<0.05$).

Next, we confirmed if increased promoter activity corresponded with an increase in secreted fibronectin protein. Exposure of NIH 3T3 cells to RgpB for 24 hours resulted in approximately a 2-fold increase in fibronectin protein accumulation as determined by densitometry readings of the blot normalized against GAPDH at a concentration of 50 nm; concentrations of 100 or 200 nm of RgpB caused no additional increase of fibronectin (Fig. 2A and B). In contrast, HRgpA did not increase fibronectin protein expression. HRgpA caused fibronectin fragmentation as determined by western blot (Fig. 2C). This might be related to the up-regulation of matrix metalloproteinase since RgpB increased MMP-9 related gelatinolytic activity as determined by gelatin zymography from NIH 3T3 fibroblasts cell culture supernatants (Fig. 3D).

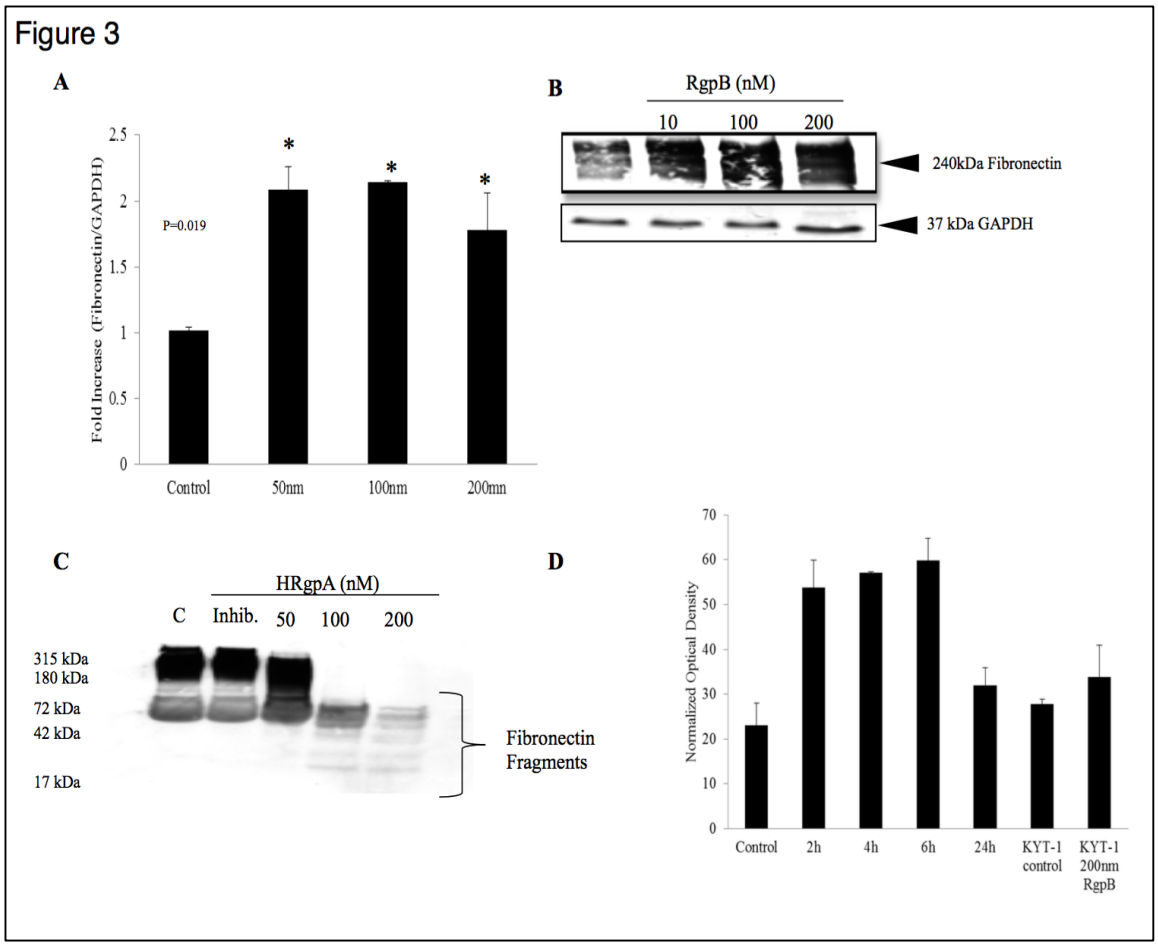

Figure 3. Gingipains induce fibronectin protein expression and MMP-9 activity. Western blot and gelatin zymography analysis. (A) Optical densities obtained from western blot shown in (B) depicted as fold increase over control (GAPDH) form NIH 3T3 fibroblasts treated with 50, 100 or 200 nm of RgpB, means ± SD depicted. P=0.019 (one-way ANOVA). (B) Western blot of fibronectin and GAPDH from NIH 3T3 Cells treated with RgpB for 24 hours (75µg total protein). (C) Western blot ran of a gradient gel for analysis of fibronectin fragmentation by 50, 100 and 200nm of HRgpA. (D) Gelatin zymography of NIH 3T3 cell culture supernatants depicting relative activity of MMP-9. NIH 3T3 cells were treated with 100nm of RgpB for 2, 4, 6 or 24 hours and with KYT-1 inhibitor. Gels were stained with 0.5% coomasie blue followed by destaining with 50% methanol. The gels were dried onto cellophane and scanned under a densitometer for determination of gelatinolytic activity. Data depicted as means ± SD. Overall P=0.0223 (One-way ANOVA). Data was normalized against total protein concentration (Bradford method). Asterisk indicated post-hoc comparison significance when compared to control ($P<0.05$).

Transforming Growth Factor β-1, a pro-fibrotic growth factor known for its ability to stimulate the expression of fibronectin and other tissue remodeling genes,

further enhanced the stimulatory effect of the gingipains (Fig. 4). As expected, purified TGF-β had a statically significant dose-dependent response on the fibronectin promoter in NIH 3T3 cells. Interestingly, when fibroblasts were treated with both TGF-β and with RgpB or HRgpA, and additive effect was observed in fibronectin promoter activity. The relative effect of the gingipains appeared to be attenuated in the presence of KYT-1. Important to note, however, we cannot rule of if gingipains induce TGF-β themselves, additional experimentation testing this is warranted.

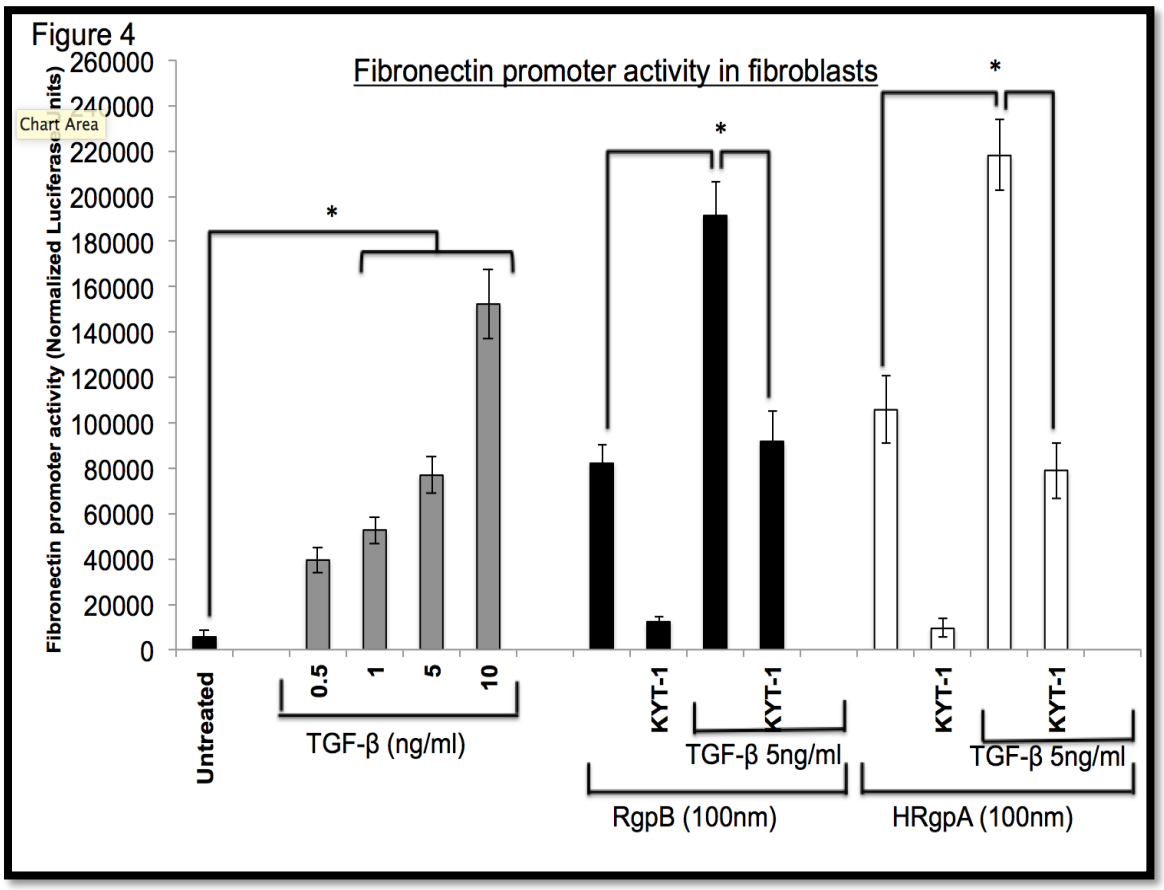

Figure 4. Transforming Growth Factor β and *Porphyromonas gingivalis* gingipains have an additive effect on activity of the human fibronectin promoter. NIH 3T3 cells ($1.5x10^5$ cells) were treated with varying amounts of TGF-β or gingipains with and without KYT-1 inhibitor. Means are reported ± SD. (Overall $P<0.001$, One-way ANOVA). Bars and asterisks indicated post-hoc comparison significance ($P<0.05$).

Gingipains stimulate fibroblasts to produce a pro-inflammatory matrix

Having established that gingipains stimulate fibronectin production in fibroblasts, we set out to investigate the implications of this with regards to inflammation. To this end, we first tested the effect of gingipains on IL-1β expression in U937 human monocytic cells transfected with the human IL-1β gene promoter fused to a luciferase reporter gene. As depicted in Figure 5, both RgpB and HRgpA stimulated IL-1β expression. Interestingly, this effect was further enhanced when cells were co-stimulated with gingipains and fibronectin EDA, a splicing variant of fibronectin implicated in tissue disrepair. Others have demonstrated that fibronectin EDA stimulates immune cells by actions on TLR4 [97] Consistent with this, we found that a selective TLR-4 blocker, CLI-095, inhibited the additive effect of fibronectin EDA (Figure 5).

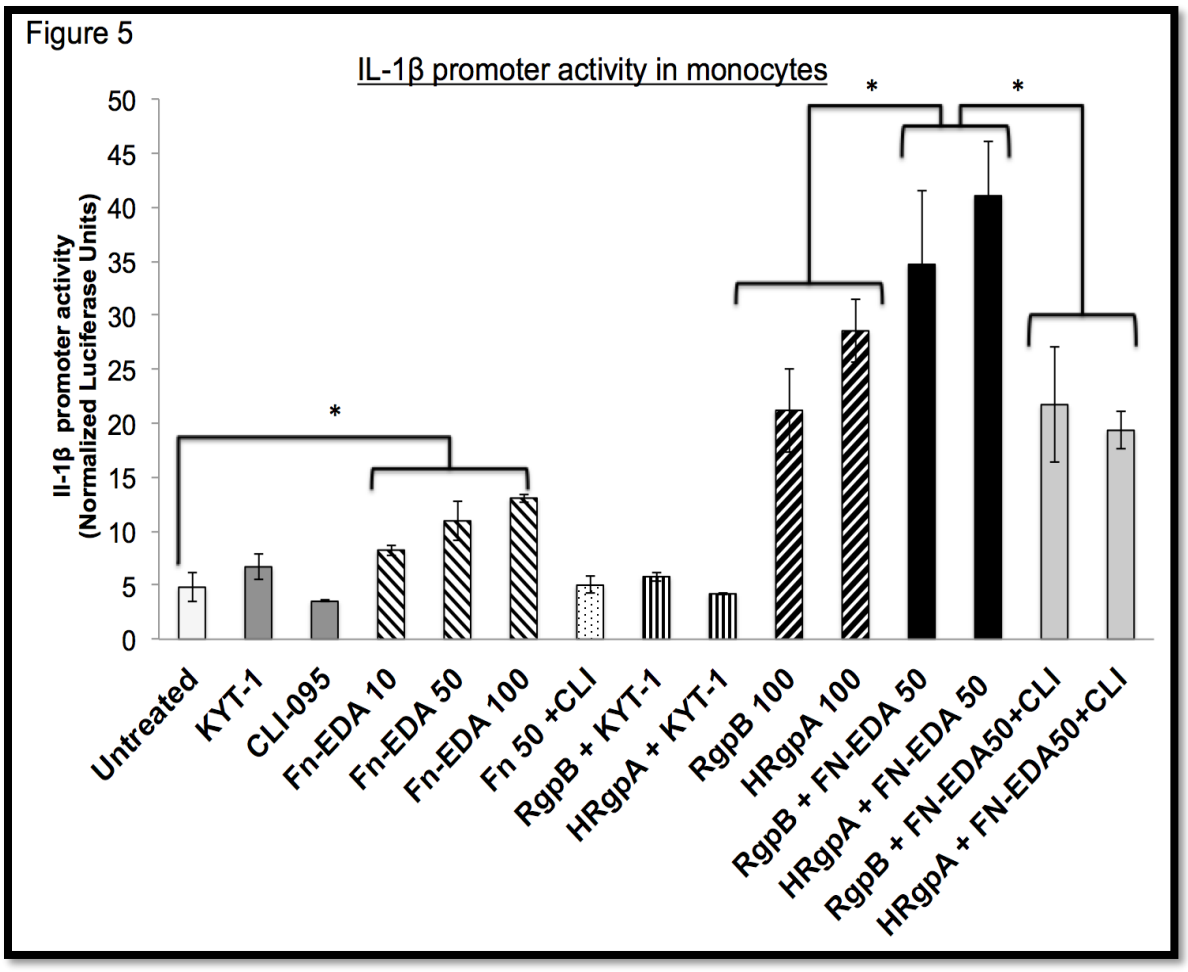

Figure 5. Fibronectin-EDA and gingipains have an additive effect on the IL-1β promoter which is in part mediated by toll-like receptor 4. Cells were treated with purified FN-EDA (10, 50 or 100 ng/ml), gingipains (100nm), gingipains plus KYT-1 (10μm), CLI-095 (InvivoGen, San Diego, CA; TLR-4 Receptor signaling inhibitor), or a combination. Means are reported ± SD. P< 0.0001 (One-way ANOVA); bars and asterisks indicated post-hoc comparison significance compared to control (P<0.05).

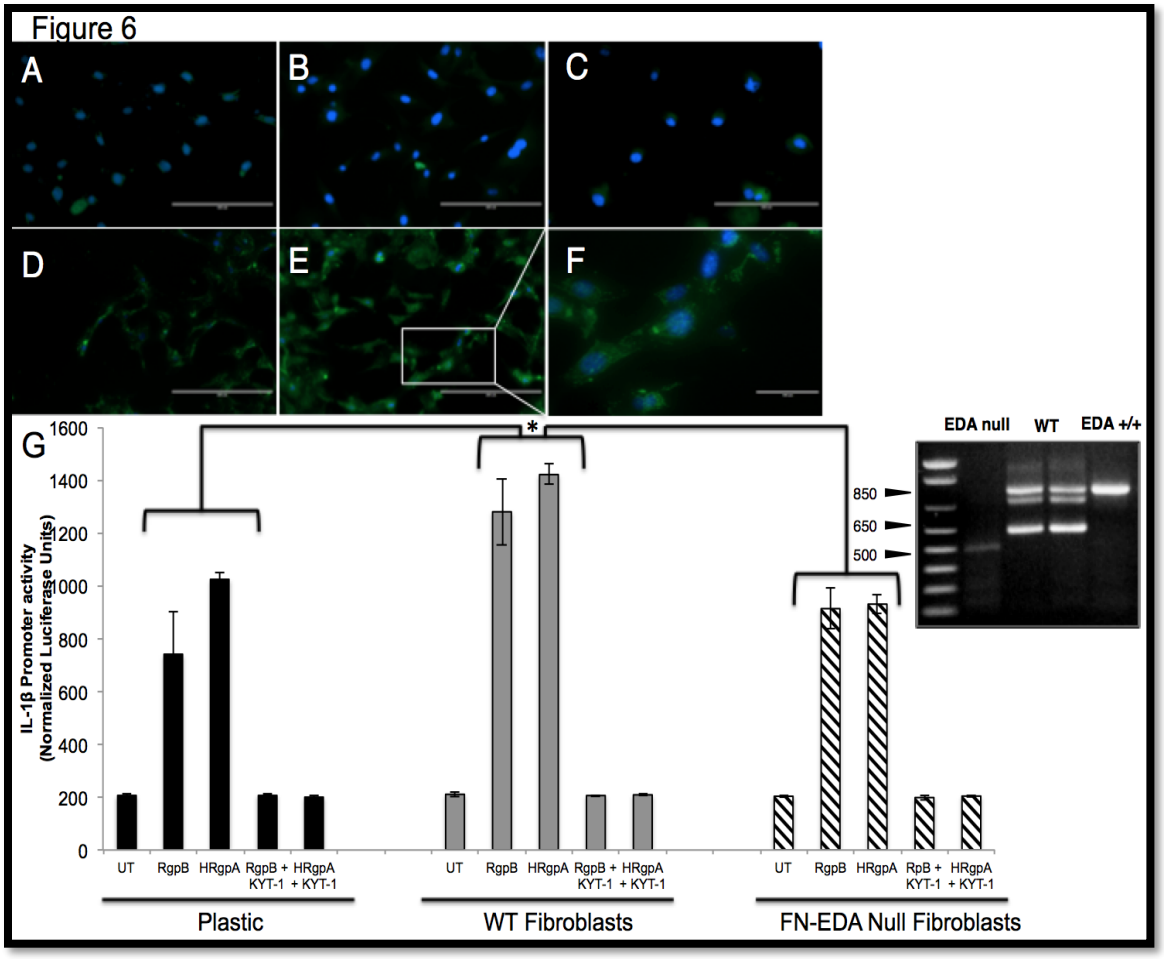

Figure 6. Gingipains induce FN-EDA expression. Immuflourescence staining of fibronectin-EDA from primary gingival fibroblasts. Primary gingival fibroblasts (1000 cell/well) were treated with either 100nm of RgpB, HRgpA with and without with out KYT-1 inhibitor (10μM) and probed with FN-EDA primary antibody (1:200) 24 hours post treatment. Alexa flour 488 was used as a secondary detection antibody (1:500). (A) Untreated primary gingival fibroblasts. (B) Secondary IgG control (C) Gingipains + KYT-1. (D) RgpB treated cells. (E) HRgpA treated cells. (F) 40x micrograph from box in E. (G) Gingipains induce a pro-inflammatory extracellular microenvironment. Extracellular matrices were isolated from primary fibroblasts after 5 days of growth. Isolated matrices were then pretreated with 100nm of RgpB, HRgpA with or without KYT-1 for 24 hours. Wells were copiously washed with PBS. U937 cells permanently transfected with the human IL-1β promoter fused to a luciferase reporter gene were then cultured onto the pre treated extracellular matrices from primary fibroblasts and FN-EDA null fibroblasts. Data depicted as means ± SD. Bars indicate post-hoc multiple comparisons significance. (H) RT-PCR of FN-EDA gene from primary fibroblasts.

We then tested the impact of fibroblast-derived matrices on the expression of cytokines IL-6 and IL-8. Cultured primary fibroblasts were allowed to deposit a matrix. Afterwards, the fibroblasts were eliminated via a series of washes; the newly derived matrices were cleared of possible contaminants with PBS and then treated with gingipains. After another thorough washing, U937 were cultured atop followed by analysis of IL-6 and IL-8 protein content in the conditioned media using enzyme-linked immunoassay. As a control, plastic did not show up-regulation of IL-6 or IL-8 (Figure 7A and D). However, U937s showed increased IL-6 and IL-8 protein secretion when cultured atop of matrices derived from fibroblasts treated with either gingipain (Figure 7A and B). This effect appeared mediated by fibronectin EDA present in the matrix since matrices derived from fibroblasts unable to produce fibronectin EDA (FN-EDA null) did not stimulate cytokine expression in U937 cells nearly to the same amount as their wild type counterparts. Immunoflourescent microscopy confirmed that primary fibroblasts treated gingipains increased expression of secreted fibronectin-EDA (figure 6).

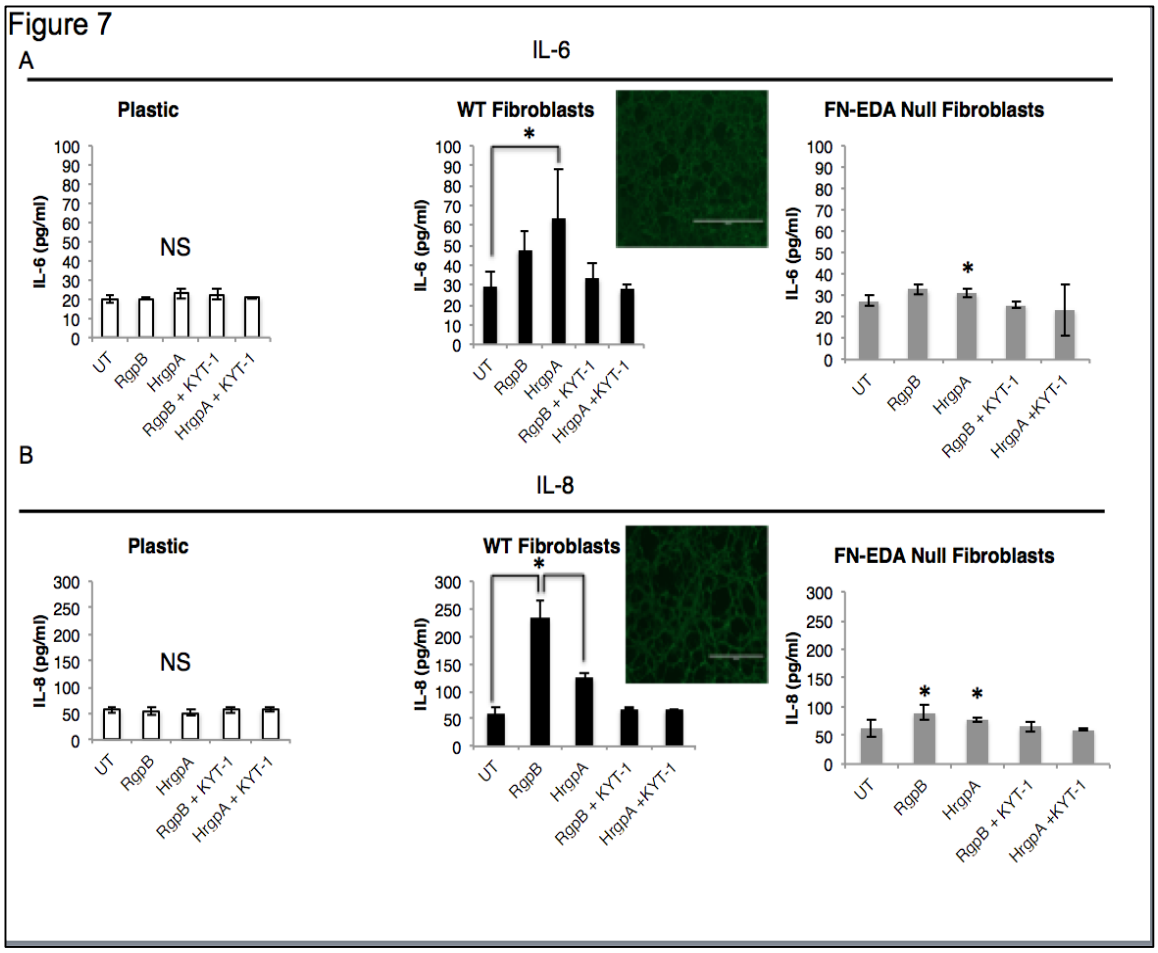

Figure 7. ECMs pretreated with gingipains induce pro-inflammatory cytokine production. ELISA of IL-6 and IL-8 secreted from U937 cells cultured on isolated primary fibroblasts extracellular matrices treated with gingipains. (A) Extracellular matrices were isolated from primary fibroblasts after 5 days of growth. Isolated matrices were then treated with 100nm of RgpB, HRgpA with or without KYT-1 for 24 hours. Wells were copiously washed with PBS. U933 cells were cultured on isolated matrices for 24 hours. Cell culture supernatants were analyzed for human IL-6. Data is depicted as means ±SD. P<0.0001. Bars indicate post-hoc multiple comparisons significance. (B) Extracellular matrices were isolated from primary fibroblasts after 5 days of growth. Isolated matrices were then pretreated with 100nm of RgpB, HRgpA with or without KYT-1 for 24 hours. Wells were copiously washed with PBS. U937 cells were cultured on pre treated isolated matrices for 24 hours. Cell culture supernatants were analyzed for human IL-8. Data is depicted as means ±SD. P<0.0001. Bars indicate post-hoc multiple comparisons significance.

DISCUSSION

An essential component of the pathogenesis of periodontal disease is the destruction of the extracellular matrix (ECM). We hypothesize periodontal pathogens, such as *P. gingivalis* modulate the expression of ECM proteins to promote colonization, disease progression, and as a consequence jeopardize host wound healing and immunity. Tissue damage caused by infection leading to periodontal pocket formation allows for ECM exposure and thus fibronectin and pathogen interaction. Alterations in fibronectin expression and variations in the gene sequence have been associated with many diseases [156, 157]. Furthermore, *P. gingivalis* anchoring to fibronectin may be essential for the pathogenesis of periodontal disease and other disease where *P. gingivalis* is a culprit; especially considering *P. gingivalis* fimbriae preferentially bind to fibronectin (and collagen) [158]. In addition, gingipains have been reported to bind to immobilized fibronectin in vitro [49]. Interestingly, *P. gingivalis* Arg-aa gingipains affect fibronectin even at the genomic level, that is, RgpB and HRgpA cause an increase in the transcription of the human fibronectin promoter. Importantly, this effect was dependent on the proteolytic activity of HRgpA and RgpB, suggesting that the specificity towards Arg residues may elicit increasing fibronectin transcription. Previous studies have demonstrated that gingival fibroblasts co-cultured with *P. gingivalis* culture supernatants lead to a rapid loss of fibronectin and α5β1 integrin from cell surfaces [109], our observation of increased fibronectin promoter activity could thus be a compensation

mechanism. Considering gingipains have already been shown to signal through PARs [76, 159], and PAR-2 activating peptides have been shown to stimulate fibronectin expression in human lung fibroblasts, investigation PARs was completed using a series of PAR antagonists. Interestingly, when using a potent selective PAR-2 antagonist, RgpB and HRgpA induced fibronectin promoter activity was completely abrogated.

Destruction of secreted and over-expression fibronectin can jeopardize host tissue integrity and cause chemotaxis and inflammation. However, not all *P. gingivalis* gingipains have the same effect on secreted fibronectin. Stimulation of the fibroblasts with RgpB caused a corresponding two-fold increase in secreted matrix fibronectin, as determined by western blot analysis. Conversely, HRgpA, which has a similar catalytic domain to RgpB, caused fragmentation of fibronectin at the same experimental concentrations, yet caused a similar increase in the transcription of the fibronectin promoter. This was confirmed by western blot. This differential affinity towards fibronectin is most likely due to the presence of the hemaglutinin domains in the HRgpA complex, which bind fibronectin facilitating proteolytic fragmentation. Our results of HRgpA dependent fibronectin fragmentation are consistent with the results of Ruggiero and others [108]. The presence of the hemaglutinin adhesion domain may serve as an important anchoring device to the extracellular milieu within the periodontal pocket.

Inflammatory mediators produced by immune cells and mucosal cells, alike, tightly regulate periodontal tissue homeostasis [160]. However, if chronic stimulation of the immune system either by periodontal pathogens or byproducts of periodontal infection, such as fibronectin fragments, can cause an unbalanced response in which host proteases become over stimulated, including MMPs. In fact, MMP-1, MMP-2, MMP-3,

MMP-8, MMP-9 and MMP-12 have been shown to contribute to tissue remodeling during periodontal infection [161]. Furthermore, both Arg- and Lys- gingipains have been shown to contribute to extracellular matrix metalloproteinase inducer (EMMPRIN), or CD147 shedding which can cause induction of inflammatory cytokines and MMPs.

Many studies have investigated the direct effect gingipains and proteases have on the extracellular matrix-fibroblasts dichotomy. First, gingipains can cleave fibronectin in the major cell-binding domain. Secondly, gingipains can also increase the anti-adhesive activity of tenascin C [108]. However, there is currently no data available on fibroblasts produced extracellular matrices treated with gingipains and immune cell interactions. Our data demonstrated that ECMs isolated and treated with gingipains can directly increase the IL-1β promoter activity and IL-6 and IL-8 protein secreted from U937 cells. Furthermore, fibroblasts that did not secrete FN-EDA had a less profound pro-inflammatory effect from the U937 cells. Considering this, it is easy to see how the production of a pro-inflammatory extracellular matrix can further potentiate the pro-inflammatory state in periodontitis. Not only are the ECM-fibroblasts interactions via integrins undermined from gingipains, which was not tested in this study [108], but also the actions of gingipains on the matrix itself lead to a proinflammatory state that can essentially act as a positive feedback loop of disrepair, tissue destruction and disease progression. Indeed, investigation of ECM, cell, and integrin cross talk could be a compelling avenue of research.

This is the first report to demonstrate that gingipains have a genomic effect on the fibronectin gene. The increased expression of fibronectin and fragmentation would allow

for increased colonization, tissue breakdown and increase in fermentable fuel for *P. gingivalis*. Furthermore, gingipains act on host fibroblasts to produce fibronectin which, when fragmented, stimulates immune cell chemotaxis and activation. In addition, ECMs that have been treated with gingipains stimulate base-line proinflammatory cytokines that could also potentially increase host-protease activity and disease progression. Further investigation is warranted into the exact mechanisms surrounding FN-EDA, PAR-2 and TLR-4 *in vivo*.

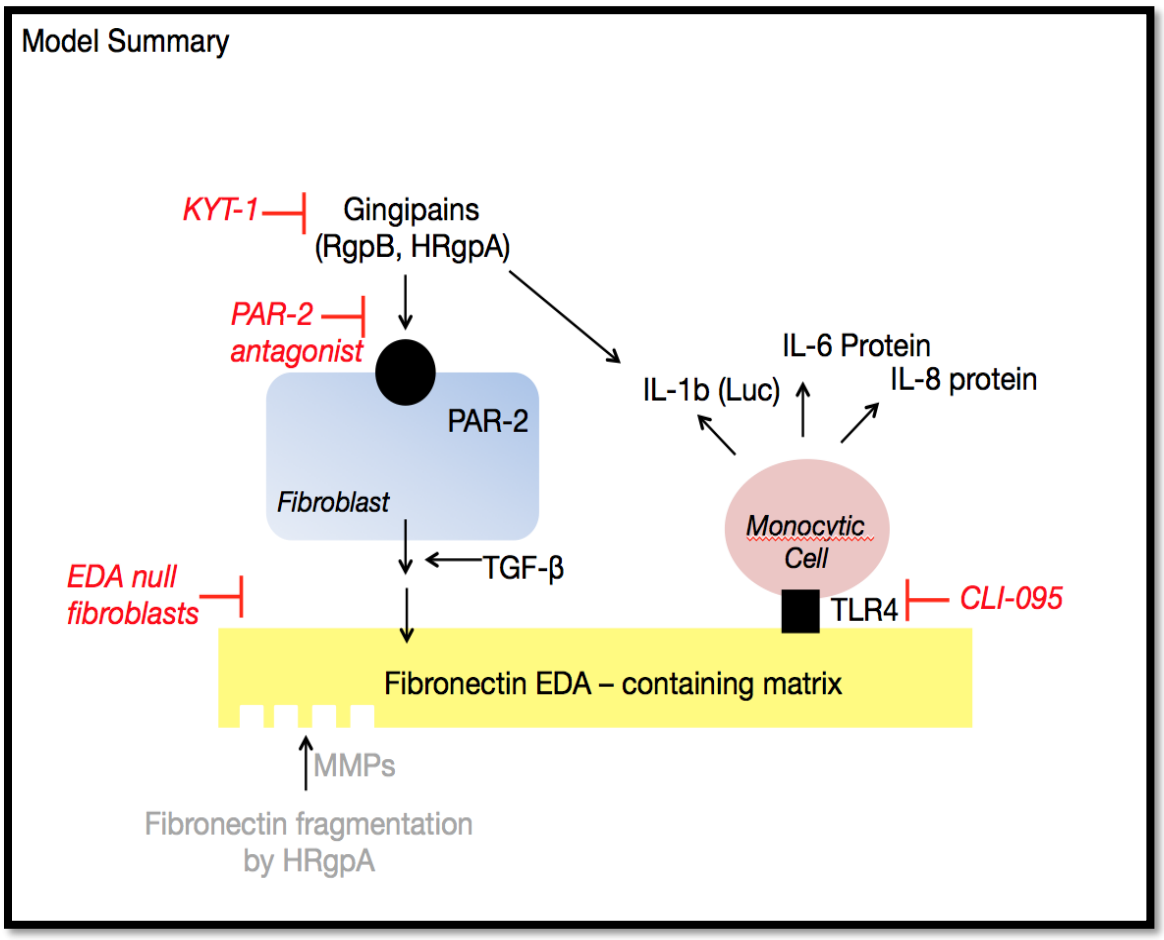

Figure 8. Thesis Model Summary Schematic.

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CURRICULUM VITA

NAME: Jeffrey S. Marschall

ADDRES: 2226 Highland Ave
Louisville, KY 40204

DOB: Cincinnati, Ohio-March 28th, 1989

SUMMARY:

I am a dual degree (DMD/MS) student pursuing a career in academic oral and maxillofacial surgery. I have research interests in tissue engineering/stem cell biology and extracellular matrix biology.

EDUCATION & TRAINING

2016-2022	Oral & Maxillofacial Surgery University of Louisville School of Dentistry	
2018-2019	University of Louisville School of Medicine Louisville, Kentucky	MD*
2012-2016	University of Louisville School of Dentistry Louisville, Kentucky	DMD
2012-2016	University of Louisville School of Dentistry Louisville, Kentucky	MS Oral Biology
2011-2012	UniversitätsSpital Zürich Zürich, Switzerland	Research Fellow Tissue Engineering
2007-2011	University of Louisville Louisville, Kentucky	BS Biology
2007	Boone County High School Florence, Kentucky	Commonwealth Diploma

*Advanced standing student, MD integrated into residency program.

EXAMS

08/2014	NBME CBSE: 65
05/2014	NBDE Part 1: Pass (No numerical score given)
09/2015	NBDE Part 2: Pass (No numerical score given)
05/2010	DAT 20 AA (90th Percentile) / 21 Total Science (93rd Percentile)

ORAL & MAXILLOFACIAL SURGERY EXTERNSHIPS

Dec 2014-1 week	University of Tennessee, Knoxville
Mar 2015-1 week	University of Cincinnati
May 2015-4 weeks	University of Louisville
May 2015-1 week	University of Texas, Houston

PEER-REVIEWED PUBLICATIONS

Largo, R.A, V.M. Ramakrishnan, **J.S. Marschall**, A. Ziogas, A. Banfi, D. Eberli and M. Ehrbar (2014). "Long-term biostability and bioactivity of "fibrin-linked" $VEGF_{121}$ *in vitro* and *in vivo" Biomaterial Science (*2), 581-590.

Sacchi, V., R. Mittermayr, J. Hartinger, M. M. Martino, K.M. Lorentz, S. Wolbank, A. Hofmann, R.A. Largo, **J.S. Marschall**, E. Groppa, R. Gianni-Barrera, M. Ehrbar, J.A. Hubbell, H. Redl and A. Banfi (2014). "Long-lasting fibrin matrices ensure stable and functional angiogenesis by highly tunable, sustained delivery of recombinant VEGF164." *Proc Natl Acad Sci* USA 111(19): 6952-6957

RESEARCH PROJECTS

06/2011-current Masters Degree- Immunology/Extracellular Matrix Biology

- Mentors: Jan Potempa, Ph.D. (Professor and University Scholar, Oral Health and Rehabilitation) and Jesse Roman, M.D. (Chairman, Dept. of Medicine University of Louisville).
- Project: Pathogen induced micro-environmental ECM changes

09/2011-05/2012: Research Fellowship – Tissue Engineering/

- Mentors: Daniel Eberli, M.D., Ph.D., and Martin Ehrbar, Ph.D., University Hospital

Zurich, Division of Urology & Division of Obstetrics
- Project: Pre-established Vascular Network for the Engineering of Functional Muscle Tissue

05/2008-05/2011: Undergraduate Research Project – Molecular Physiology
- Mentors: Cynthia Corbitt, Ph.D. (Dept. of Biology University of Louisville) and Jesse Roman, M.D. (Chairman, Dept. of Medicine University of Louisville).
- Project: Hormonal Effects on Lung Extracellular Matrix Proteins

ABSTRACT & POSTER PRESENTATIONS

1) "Engineering Biomimetic Hydrogels for induced recruitment of mesenchymal stem cells in vitro and in vivo" PS Lienemann, M Karlsson, A Sala, S Hoehnel, O Naveiras, **JS Marschall**, W Weber, MP Lutolf, and M Ehrbar. Tissue Engineering and Regenerative Medicine World Congress Vienna, Austria, (09/2012)

2) "Dichotomy of Porphyromonas gingivalis gingipains proteolytic activity: Differential degradation and stimulation of fibronectin in fibroblasts" **J.S. Marschall**, J.D. Ritzenthaler, B.A. Potempa, J Roman, J.S. Potempa, Research! Louisville September 2011 Annual Meeting, University of Louisville, Louisville, KY (10/2011)

3) "Porphyromonas gingivalis gingipains induce a pro-inflammatory extracellular microenvironment: The role of PAR-2 and fibronectin". **J.S. Marschall**, J.D. Ritzenthaler, B.A. Potempa, J Roman, J.S. Potempa; Research! Louisville September 2012 Annual Meeting, University of Louisville, Louisville, KY (09/2012)

4) "Porphyromonas gingivalis gingipains promote self propagating inflammation". **J.S. Marschall**, J.D. Ritzenthaler, B.A. Potempa, J. Roman, J.S. Potempa; Research!Louisville September 2014, University of Louisville, Louisville, KY (09/2014)

5) "Long-term Cell Demanded Release of TG-VEGF$_{121}$ for the Development of Mature Vascular Networks In Vivo," R.A. Largo*, **J.S. Marschall**, A. Banfi, V.M. Ramakrishnan, A. Ziogas, T. Sulser, K. Lorentz, J. Hubbell, M. Ehrbar, and D. Eberli, Tissue Engineering and Regenerative Medicine World Congress (TERMIS) 2012 Annual Meeting, Vienna, Austria (09/2012).

6) "Pre-establishment of a Vascular Network for Urinary Sphincter Engineering," **J.S. Marschall,** R.A. Largo, V.M. Ramakrishnan*, A. Ziogas, J. Plock, M. Ehrbar, and D. Eberli, TERMIS 2012 World Congress Meeting, Vienna, Austria (09/2012).

ACADEMIC AWARDS

2016	Omicron Kappa Upsilon Dental Honor Society
2015	Delphi Society University of Louisville Dental School award for excellence in the biomedical sciences and top 15% of the class
2013	Head and Neck Anatomy Honors
2012	1st place, Dental Student Division, Research Louisville
2011	*Magna cum Laude* University Honors Scholar Certificate of Honors, Department of Biology Honors Thesis Presentation • Title: "Hormonal Effects on Lung Extracellular Matrix Proteins"
2009	1st place, Kentucky Academy of Science, Undergrad Poster Competition-Biochemistry/Physiology division
2007-2011	Dean's List and Dean's Scholar

SCHOLARSHIPS AND RESEARH FELLOWSHIPS

2016	Hinman Scholar-$5,000
2013	Edward and Mary Smith Dental Scholarship- $5,000
2012	Humana Scholars Dental Scholarship-$1,800
2012	General Dentistry Scholarship-$10,000
2011-2014	Dental Student Research Grant-$12,000 (3,000/year)
2009	Summer Research Opportunity Program-$3500
2009	Undergraduate Research Grant (URIG)-$3,000
2008	US Airways Education Foundation Scholarship-$10,000

PROFESSIONAL ACTIVITIES

2015	Kentucky State Fair Head & Neck Screening Program
2015	Oral Pathology Teaching Assistant under Dr. Mark Bernstein
2015	Member, American Association for the Advancement of Science
2015	ADEA Louisville Chapter President
2015	ADEA Ohio Valley District Student Chair
2014	University of Louisville School of Dentistry Student Research Group-Web Master
2014	Skaggs Conference attendee hosted by University of Louisville OMFS
2013	Selection Committee for Clinical Outreach Scholars
2013-current	Member, International Association for Dental Research
2013-current	Academic Tutor-Histology, Physiology, Systemic Pathology, Head and Neck Anatomy, and Oral Pathology
2013-2014	American Dental Education Association Vice President
2011	featured in University Video (https://www.youtube.com/watch?v=fFCAxYWdlEc)
2009-2011	Alpha Epsilon Delta Vice President