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THE ROLE OF KINASES AND PHOSPHATASES IN THE PATHOBIOLOGY OF

PORPHYROMONAS GINGIVALIS

By

Sarah Whitmore

A Dissertation

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THE ROLE OF KINASES AND PHOSPHATASES IN THE PATHOBIOLOGY OF

PORPHYROMONAS GINGIVALIS

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ABSTRACT

THE ROLE OF KINASES AND PHOSPHATASES IN THE PATHOBIOLOGY OF

PORPHYROMONAS GINGIVALIS

Sarah Whitmore

November 14, 2017

Periodontal diseases result from the interplay between the dysregulation of the host inflammatory response and the actions of a dysbiotic bacterial community. These chronic inflammatory conditions affect persons on a worldwide scale. *P. gingivalis* is strongly implicated as a key periodontal pathogen in the more severe manifestations of periodontal diseases such as periodontitis. A core element of *P. gingivalis* pathogenicity is its dysregulation of innate immunity, including suppression of IL-8 production by gingival epithelial cells. The NF-κB family of related transcription factors plays a central role in regulating many important aspects of innate immune responses. NF-κB RelA/p65 homodimers regulate transcription of IL-8. Here we show that SerB, a serine phosphatase secreted by *P. gingivalis*, is produced intracellularly and can specifically dephosphorylate S536 of p65 in gingival epithelial cells. A *P. gingivalis* mutant lacking SerB showed diminished ability to dephosphorylate p65 S536. Ectopically expressed SerB bound to p65 and co-localized with p65 in the cytoplasm. Ectopic expression of SerB also resulted in dephosphorylation of p65 with reduced nuclear translocation in TNF-stimulated epithelial cells. Conversely, the p105/50 subunit of NF-κB was unaffected by the presence of SerB. Co-expression of a constitutively active p65 mutant (S536D) relieved inhibition of nuclear translocation. IL-8 promoter activity and the

production of IL-8 were both found to be inhibited by SerB.   This study also characterized a bacterial tyrosine kinase in *P. gingivalis*, designated Ptk1, and demonstrated its involvement in interspecies signaling in the context of dual species *P. gingivalis*-*S. gordonii* communities.   Our findings show that Ptk1 is capable of autophosphorylation, and is one of the substrates of *P. gingivalis* tyrosine phosphatase Ltp1, as it is dephosphorylated by Ltp1.   In the Δ*ptk1* mutant (no active Ptk1) there was significantly reduced accumulation of *P. gingivalis* on the *S. gordonii* substrate indicating that tyrosine kinase activity is required for optimal polymicrobial synergy. Collectively these findings illustrate (1) the molecular mechanisms underlying a bacterial strategy to manipulate the innate host response and host inflammatory pathways, and (2) that Ptk1 is an important component in the signaling networks that regulate synergistic interactions between *P. gingivalis* and *S. gordonii*.

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INTRODUCTION

1.1 Periodontal Diseases

Periodontal diseases encompass a large spectrum of chronic, oral inflammatory conditions resulting from the interplay between the dysregulation of the host inflammatory response and the actions of a dysbiotic bacterial community. Epidemiological studies of periodontitis have identified numerous risk factors, both environmental and genetic; some of which include smoking, poor oral hygiene, stress, genetics, and defects in the host immune response. Periodontal diseases are purportedly the 6th most common condition affecting persons on a worldwide scale [1]. Periodontitis is the more severe clinical manifestation of periodontal diseases and can be classified into 2 distinct forms, chronic and aggressive. Chronic periodontitis is the most common form seen within all populations, usually presenting in individuals over 35 years of age. Periodontal diseases are now more accurately described as bacterial community based opportunistic infections [2]. The bacterial component is mediated by multispecies plaque biofilms which exist in the subgingival crevice of the oral cavity. The oral biofilm that develops on tooth surfaces, colloquially known as dental plaque, is an exemplar of a mixed microbial community. Oral biofilms can contain over 700 bacterial phyla [3]. A populational shift toward inflammophilic and tissue-destructive anaerobes increases the pathogenic potential of the community [4]. In the context of a pathogenic community, the oral organism most strongly implicated in chronic periodontitis is *Porphyromonas gingivalis*. However, the pathogenicity of *P. gingivalis* is only expressed in the context of a multispecies community (discussed further below). Periodontitis directly affects all tissues of the periodontium, more specifically, the alveolar bone, gingiva, and the periodontal ligament (PDL). Chronic damage to these

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supporting structures leads to tissue destruction, loss of tooth support, loss of alveolar bone and eventual tooth exfoliation.

1.2. Periodontitis and the Systemic Health Connection

The prevalence of periodontitis and its close association with systemic diseases certainly underscores the clinical significance of these chronic conditions. A recent review of the literature estimates there are 57 different systemic disease conditions actively being studied in relation to their association with periodontal diseases [5]. Some of the more frequently referenced conditions include diabetes mellitus, atherosclerosis, metabolic syndrome, preterm low birthweight babies, Alzheimers, coronary heart disease, stroke, pulmonary complications, and cancer [6] [7] [8] [9]. A mechanistic underpinning to this systemic connection is provided by findings that periodontal pathogens can cause an imbalance in the amounts and types of inflammatory mediators present within the host, thereby dismantling the normal inflammatory response of the host to infection [10] [11] [12]. A recent study has proposed that the shift towards the presence of more pro-inflammatory mediators is a potential reason linking periodontal disease and Alzheimers disease [13].

There is growing epidemiological evidence for an association between *P. gingivalis* and oral squamous cell carcinomas (OSCC) [14] [15]. Recent studies have shown a strong positive association between periodontal disease and the risk of developing oral, lung and pancreatic cancers [16] [17]. OSCC surfaces have been reported to harbor significantly higher levels of *Porphyromonas* and *Fusobacterium* compared with contiguous healthy mucosa [18]. Immunohistochemistry with *P. gingivalis* antibodies revealed higher levels of detection and intensity of staining in gingival carcinomas compared with healthy gingival tissue [19]. In addition to disruption

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of the inflammatory microenvironment, *P. gingivalis* can have direct effects on epithelial cells that affect cell cycle and cell survival. Suppression of apoptosis is discussed further below, and *P. gingivalis* can also accelerate progression through the S-phase of the cell cycle by manipulation of cyclin/CDK (cyclin-dependent kinase) activity and reducing the level of the p53 tumor suppressor [20]. One study reported that infection of gingival epithelial cells by *P. gingivalis* led to downstream activation of the beta-catenin signaling pathway [21]. Interestingly, this beta-catenin pathway is one of the key pathways involved in controlling cell proliferation and tumorigenesis.   Infection of gingival epithelial cells with *P. gingivalis* induces expression and nuclear localization of the ZEB1 transcription factor, which controls epithelial-mesenchymal transition [22]. ZEB1 levels were directly associated with an increase in the expression of mesenchymal markers, in particular, vimentin and MMP-9, and also with enhanced migration of epithelial cells into matrigel.

Recent research has also unveiled a role for periodontitis in autoimmune diseases. One study investigating the connections between Rheumatoid Arthritis and *P. gingivalis* found that *P. gingivalis* possesses a unique ability to citrullinate host proteins in periodontal lesions, thereby making these proteins into targets for the host immune system [23]. This potential role of periodontitis in the generation of host antigens targeted by the host immune system is supported by research which has identified the presence of citrullinated histone H3 proteins found in inflamed periodontal tissues [24].

Additionally, possible associations between periodontal disease and Parkinson's disease are also being investigated based on the premise that systemic inflammation is believed to be a central component responsible for the pathogenesis and progression of Parkinson's disease [25].

The periodontal-systemic health connection has clinically significant implications for the population as a whole, and is rapidly becoming an important public health issue

for the geriatric population as well. The prevalence of most chronic conditions that afflict the elderly, including periodontal diseases, increase with age [26]. Similarly, disease severity also increases with age and thus in older populations, periodontal diseases can have even more detrimental consequences in terms of their potential to contribute to or exacerbate systemic diseases.

1.3. *Porphyromonas gingivalis*

P. gingivalis is a gram-negative, assacharolytic black pigmented anaerobe. A secondary colonizer of subgingival plaque biofilms, *P. gingivalis* has an obligate requirement for iron in the form of hemin for growth, and possesses several hemin acquisition and storage systems with different activities and affinities [27]. *P. gingivalis* is best known for its role in periodontal diseases, as it is strongly implicated as a key periodontal pathogen in chronic and more severe manifestations of periodontal diseases [28-31]. *P. gingivalis* is a host-adapted opportunistic pathogen whose primary niche is the subgingival crevice of the oral cavity. Normally the host maintains a comfortable homeostatic balance between the host cells and microbial species. *P. gingivalis*, however, can disrupt this equilibrium, resulting in a dysbiotic host–microbiota interaction [32-34].

Contributions from both the host as well as the resident microorganisms are essential for transitioning of *P. gingivalis*-containing communities from commensal to pathogenic. *P. gingivalis* has the potential to express a wide array of virulence factors all of which contribute to its ability to effectively disrupt the host innate immune response and thus in turn, facilitate survival and persistence. One of the first host cells encountered by *P. gingivalis* are the gingival epithelial cells (GECs) of the oral cavity found deep within the subgingival crevice. *P. gingivalis* effectively and efficiently invades these epithelial cells and localizes in the perinuclear area [35]. Not only can this

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pathogen invade GECs but it can also spread intercellularly amongst them [36]. The *P. gingivalis*-epithelial cell interface is comprised of a dynamic interplay of events. During initial contact between *P. gingivalis* and GECs, the major fimbriae of *P. gingivalis*, FimA, mediates adhesion through β1 integrin receptors [37, 38]. During the process of invasion, key changes are evident within the epithelial cells including, induction of actin rearrangements, as well as activation of JNK kinases and conversely, the inactivation of ERK kinases [39]. Following invasion, inhibition of interleukin-8 (IL-8) production by GECs is also evident and this strategy has been coined "localized chemokine paralysis" [40, 41]). Complete internalization of *P. gingivalis* occurs within 20 minutes. Once internalized within epithelial cells, *P. gingivalis* utilizes all resources at its disposal in order to subvert the host immune surveillance and begin reprogramming the circuitry of the host cell signaling pathways, as well as modulating gene expression to further its own persistence and survival [42, 43]. *P. gingivalis* manipulation of epithelial cell signaling pathways is carried out by only a limited number of proteins, primarily the FimA-component fimbriae and the serine phosphatase SerB (discussed further below). In contrast, many other intracellular pathogens often rely on a much larger number of effector proteins to accomplish this same task. *Legionella pneumophila,* an intracellular pathogen, utilizes an array of effector molecules to infect and survive within amoebae along with human phagocytic and non-phagocytic cells. *Legionella pneumophila* primarily uses the Dot/Icm type IV secretion system to translocate these effectors into host cells, which then function to establish membrane-bound replication vacuoles through subversion of host process including vesicle trafficking, ubiquitylation, and lipid metabolism [44]. *Legionella pneumophila* injects approximately 300 effector proteins into host cells to facilitate the subversion and evasion of host cell signaling pathways [45]. Not only do these effector molecules represent more than 10% of the entire protein-encoding genome of *L. pneumophila,* but they also establish *Legionella* as the gram-

negative pathogen equipped with the largest supply of effector molecules [46]. While other intracellular pathogens may not have as large a number of effector proteins at their disposal as is seen with *Legionella*, many species still have a far greater number than those used by *P. gingivalis*. Consider for a moment *Salmonella enterica*, subspecies serovar Typhimurium. *S. typhimurium* can adhere to and invade the microfold cells of the epithelium in the distal part of the ileum. Coordination of this multi-step process is carried out by a minimum of 30 different effector proteins which are secreted into host cells via two distinct type III secretion systems that are encoded on Pathogenicity Islands 1 and 2 [47].

Another important feature is the capacity for *P. gingivalis* to suppress apoptosis in GECs [48-50]. In primary cultures of gingival epithelial cells, *P. gingivalis* is strongly anti-apoptotic and, indeed, can suppress chemically induced apoptosis [49]. *P. gingivalis* activates Jak1/Akt/Stat3 signaling that controls intrinsic mitochondrial apoptosis pathways [51]. At the mitochondrial membrane, the activity of pro-apoptotic Bad is inhibited, and the Bcl2 (anti-apoptotic):Bax (pro-apoptotic) ratio is increased, consequently curtailing the release of the apoptosis effector cytochrome c [52]. Remarkably, *P. gingivalis* possesses multiple mechanisms for inhibition of apoptosis in epithelial cells. Expression of microRNAs (miRs) is modulated, and up-regulation of miR-203 leads to inhibition of the negative regulator SOCS3 and subsequent suppression of apoptosis [53]. *P. gingivalis* secretes a nucleoside diphosphate kinase (NDK), which can function as an ATPase and prevent ATP-dependent apoptosis mediated through the purinergic receptor P2X7 [50]. The Ndk enzyme also confers protection to *P. gingivalis* against reactive oxygen species by raising levels of glutathione.

1.4 *P. gingivalis* Specific Virulence Factors

P. gingivalis is well equipped with a multitude of virulence factors, all of which have the potential to contribute to colonization and to periodontal tissue destruction. Collectively these virulence factors help *P. gingivalis* in a variety of ways that include attachment to host surfaces, survival in the oral cavity, tissue destruction and disruption of immune surveillance, to name a few. Some of the more well studied virulence factors include lipopolysaccharide (LPS), outer membrane vesicles, capsule, fimbriae, hemagglutinins and cysteine proteases.

Fimbriae are protein filaments found protruding from the cell surface of *P. gingivalis*. These filamentous structures are critical elements involved in mediating attachment of *P. gingivalis* to host tissues and other bacteria. They are also a necessary requirement for intracellular invasion. For successful adhesion and invasion into host cells, *P. gingivalis* possesses two types of fimbrial structures, commonly referred to as its major and minor fimbriae, FimA and Mfa1. These fimbriae are genetically distinct but exhibit similar structural properties.

The major fimbriae, comprised of the FimA (41 kDa) structural subunit, are long, peritrichous structures. FimA fimbriae mediate attachment to a number of host surfaces and stimulate expression of various inflammatory cytokines such as TNF, IL-6 and IL-1, in epithelial cells, monocytes, macrophages and endothelial cells [54]. The Mfa fimbriae mediate attachment to other oral biofilm bacteria [55], and contribute to auto-aggregation and monotypic biofilm formation [56, 57]. Mfa1 can also selectively engage the dendritic cell (DC) C-type lectin DC-SIGN, leading to evasion of antibacterial autophagy and lysosome fusion, and intracellular persistence in myeloid DCs [58], properties that may contribute to survival in vivo.

A well-studied example of the important role fimbriae play is exemplified by the coadhesion events that take place between *P. gingivalis* and *S. gordonii.* Binding is effectuated by two sets of interacting adhesins involving streptococcal surface proteins and the major and minor fimbriae of *P. gingivalis*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), while primarily a cytoplasmic enzyme, is also expressed on the streptococcal surface where it binds the FimA-subunit fimbriae of *P. gingivalis* [59]. The minor Mfa1-subunit fimbriae engage the SspA/B streptococcal surface proteins [57]. Interaction of the Ssp and Mfa1 proteins is required for the subsequent accumulation of *S. gordonii* and *P. gingivalis* into heterotypic communities [60].

Lipopolysaccharides (LPS) are lipoglycans found in the outer membrane of gram-negative bacteria. These consist of a polysaccharide, composed of an O-antigen, and an outer core and inner core, which is covalently attached to a lipid. *P. gingivalis* LPS plays a significant role in the pathogenesis of periodontal disease. The LPS of *P. gingivalis* is structurally unique even among other gram-negative bacterial species. Studies have shown that *P. gingivalis* can actively switch between various structural forms of its Lipid A component based on local environmental cues related to inflammation that are received in the form of hemin concentrations. Moreover, this heterogeneous pool of Lipid A structures from which *P. gingivalis* can choose from, each affect the Toll-Like Receptor (TLR) proteins differently [61]. Select isoforms of *P. gingivalis* LPS directly activate osteoclasts, stimulate the expression of a number of pro-inflammatory cytokines including TNF, IL-6 and IL-1β, and strongly activates complement. These pro-inflammatory cytokines can contribute to periodontal tissue destruction. Animal studies have shown *P. gingivalis* LPS to be a potent stimulator of pro-inflammatory responses as well as the alveolar bone resorption processes [62]. Moreover, the LPS of *P. gingivalis* has been shown to neutralize the stimulatory effects of LPS from other bacterial species [63]. *P. gingivalis* can produce various isoforms of its

LPS which differ structurally in their Lipid A cores and O-antigen regions [64]. These variations can modulate the host inflammatory response to *P. gingivalis* infection [65]. *P. gingivalis* expresses two major forms of its Lipid A component through differences in acylation patterns: tetra-acylated and penta-acylated. The penta-acylated form activates TLR4 and when hemin concentrations are high, this form will change into the tetra-acylated form [66]. Conversely, this tetra-acylated Lipid A acts as a TLR4 antagonist. The opposing actions that are evident between these two Lipid A structures and their effects on TLRs seems fitting for *P. gingivalis* which prides itself in its selective dysregulation of the host immune response to ensure its survival. *P. gingivalis* produces a second type of LPS known as anionic or A-LPS. A-LPS is a phosphorylated branched mannan [67], that is attached to many proteins translocated through the type IX secretion system (T9SS), thus anchoring them to the bacterial surface [68]. A mutant unable to incorporate A-LPS into T9SS substrates is less virulent in a mouse subcutaneous infection model [69]

Gingipains are yet another important aspect of *P. gingivalis* virulence. These enzymes play important roles in bacterial housekeeping and infection. Gingipains are trypsin-like cysteine proteases secreted by *P. gingivalis*. These proteases have been shown to play roles in *P. gingivalis* invasion, biofilm formation, evasion of host immune response, amino acid uptake from host proteins and fimbriae maturation [70]. Recent studies have proposed these gingipains may be involved in various systemic pathologies [71]. *P. gingivalis* secretes 3 separate but related cysteine proteases. Two of these enzymes are specific for cleaving after arginine residues and the third enzyme cleaves after lysine residues. These versatile proteases are capable of digesting a large variety of host proteins which in turn provide *P. gingivalis* with necessary nutrients for growth. These gingipains are responsible for as much as 85% of the proteolytic activity in *P. gingivalis* [72].

The capsular polysaccharide (CPS; capsule) or K-antigen of *P. gingivalis* is another major virulence factor which contributes to its pathogenicity. While not all *P. gingivalis* strains are encapsulated, those that do contain a capsule have been shown to be much more systemically invasive and demonstrate higher levels of resistance to phagocytosis [73]. Additionally, encapsulated strains have demonstrated the ability to incite a strong systemic IgG antibody response [74].

1.5 Polymicrobial Communities

In vivo, *P. gingivalis* is unlikely to cause disease individually, but rather in the context of a polymicrobial community in which the microbial constituents engage in synergistic interactions. Many organisms have evolved to live in polymicrobial communities where they flourish among other neighboring constituents. This is primarily because these organisms often derive greater benefits from this community lifestyle compared to living separately. This illustrates the idea of polymicrobial synergy. Each individual biofilm resident working in concert to elevate the overall pathogenic potential of the community as a whole. Communities of *P. gingivalis* and *S. gordonii* have demonstrated pathogenic synergism in a murine model of alveolar bone loss [75]. This polymicrobial synergy can also apply to the metabolic needs of organisms as well. For example, *P. gingivalis* and *T. denticola* display synergistic pathogenicity in murine models of periodontal disease [76]. The amino acid glycine is utilized as a major carbon source by *T. denticola* and is rapidly metabolized into acetate and lactate. *P. gingivalis* is an important source of free glycine [77]. Moreover, *T. denticola* has been shown to produce succinic acid that is used by *P. gingivalis* and incorporated into its lipids and phospholipids and important for growth stimulation [78]. In contrast, other polymicrobial communities exhibit antagonistic relationships such as those seen between *P. gingivalis* and *Streptococcus cristatus*. *S.*

cristatus produces arginine deiminase which inhibits the synthesis of the *P. gingivalis* FimA adhesin protein [79]. The dynamics at play in these polymicrobial microenvironments, both synergistic and antagonistic interactions, undoubtedly influence the overall pathogenicity of the resident biofilm. And as such, this directly impacts the "nososymbiocity" or disease causing potential of the biofilm as a whole [30]. This is a significant factor in treatment and prevention of human disease considering that the majority of skin and mucosal surfaces in humans are predominantly covered in bacterial biofilms.

1.6 Gingival Epithelial Cells (GECs)

The epithelial tissue covering the external surface of the gingiva plus the epithelium lining the junctional epithelium and gingival sulcus, are collectively known as the gingival epithelium of the oral cavity. The gingival epithelium can be identified as 3 separate histological regions: the oral epithelium, sulcular epithelium, and junctional epithelium. The oral epithelium establishes the primary barrier separating the oral environment from the deeper tissues. This epithelium is comprised of tightly attached epithelial cells organized into multiple layers. The oral epithelium is stratified squamous in shape, featuring keratinized as well as non-keratinized areas. Keratinized areas are primarily found on the hard palate and gingiva. Non-keratinized areas include buccal mucosa, soft palate and the floor of the mouth. Gingival epithelial cells have been used successfully for many years as an important study tool for investigating the interaction between oral bacteria, namely periodontal pathogens, and human host cells *in vitro*. The epithelial cells lining the gingival compartment are one of the first cells to make contact with the oral bacteria, both commensal and pathogenic species. These cells provide important mechanical barriers to microbial invasion, in addition to functioning as key

sensors of host infection and respond accordingly via expression of various chemical signals. For these reasons, GECs have been used extensively for research purposes as they have proven to be a reliable and accurate study tool for unveiling the intricacies and dynamics of the microbial-host interaction [80-85]. *P. gingivalis* rapidly internalizes within GECs, remaining viable for extended periods of time. The initial step involved in internalization begins with the *P. gingivalis* FimA fimbriae mediated attachment to the ß1-integrin receptors found on the GEC cell surface. This results in subsequent recruitment and activation of the host cell integrin focal adhesion complex. Concomitantly, *P. gingivalis* secretes the serine phosphatase SerB which is able to enter host cells and directly act on the host cell actin depolymerizing molecule cofilin. SerB dephosphorylates cofilin which leads to its activation. This transient and localized disruption in the host cell actin structure facilitates the intracellular entry of *P. gingivalis*. GEC integrin-dependent signaling intersects with cytoskeletal remodeling and thus can restore the overall actin structure. Once internalized, *P. gingivalis* rapidly localizes in the host cell cytoplasm and can also spread intercellularly via autophagosome trafficking [86, 87]. Intracellular *P. gingivalis* are notorious for their fast acting approach once having gained entry into host cells. GEC infection by *P. gingivalis* results in marked progression through the stages of the cell cycle as well as suppression of apoptosis. As mentioned above, *P. gingivalis* also activates various anti-apoptotic pathways within GECs. The long term co-existence of *P. gingivalis* within GECs gives rise to many subtle yet complex interactions that can directly impact innate immune status.

Our model cell line used for all study experiments were telomerase immortalized gingival epithelial cells (TIGKs). These were generated by immortalization of primary gingival epithelial cells with bmi1/hTERT (Bodnar *et al*., 1998).

1.7 Protein Phosphorylation

Regulation of protein activity, as orchestrated by the tightly co-ordinated and balanced dynamics between kinases and phosphatases, is one of the critical determinants of normal cellular growth and development. While the addition or removal of phosphoryl groups to/from serine, threonine or tyrosine residues has long been established as one of the predominant mechanisms of post-translational protein modification in eukaryotes, it was not until the 1970s that landmark studies demonstrated its importance in prokaryotes [88].   The first characterized phosphorylation systems in prokaryotic organisms were the two component systems (TCS) and the phosphotransferase system (PTS) [89]. In the basic configuration of TCS, a surface-exposed sensor kinase is first autophosphorylated in response to an external signal. The phosphoryl group is then transferred to the aspartyl residue of a response regulator, which in turn can modulate gene expression. In this manner, signal transduction is effectuated by phosphate flow. TCS are widespread in bacteria, and they control the response to a wide range of environmental stimuli. Remarkably, any one organism can possess up to 50 functionally isolated systems. In another phosphorylation system, the PTS system, a phosphoryl group from Phosphoenol Pyruvate is transferred along a chain of proteins by reversible phosphorylation of histidine residues. The final receptor for the phosphoryl group is a sugar; hence, the PTS is involved in carbohydrate uptake rather than signal transduction.

1.7.a. Serine/Threonine Phosphorylation

Bacterial serine/threonine specific phosphorylation as a post-translational modification is now known to be ubiquitous and is involved in a diverse array of physiological processes including secondary metabolism, catabolite repression, oxidative stress responses and

sporulation [90]. Serine/threonine kinases and phosphatases are also involved in bacterial virulence, in particular through their action on host cell substrates. For example, the YpkA/YopO kinase of *Yersinia* species is delivered into epithelial cells by type III secretion machinery, whereupon it disrupts actin microfilament structure [91]. Two autophosphorylated Ser/Thr protein kinases, NleH1 and NleH2, in enterohemorrhagic *Escherichia coli*, and the OspG protein in *Shigella flexneri,* inhibit activation of the pro-inflammatory transcription factor NF-κB [92, 93]. The secretion of serine kinases and/or phosphatases has thus afforded bacterial pathogens the means to interfere with host signal transduction pathways.

1.7.b. Tyrosine Phosphorylation

The first definitive evidence of protein tyrosine kinase activity in bacteria was discovered in *E. coli* with the identification of phosphotyrosine in partial acid hydrolysates of proteins [94]. Protein tyrosine phosphorylation subsequently was shown to direct many essential cellular processes, such as capsule production, growth, proliferation, migration, flagellin export, adaptation to stress and production of secondary metabolites. Moreover, the addition of a bulky, negatively charged phosphoryl group to a protein can influence both cellular location and the overall protein interactome [95]. A number of global phosphoproteome studies have now been conducted in bacteria, including *Escherichia coli*, *Helicobacter pylori*, *Bacillus subtilis*, *Streptomyces coelicolor*, *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Lactococcus lactis*, *Campylobacter jejuni* and *Pseudomonas species* [96-106]. These databases have shown an increasing number of bacterial proteins that are phosphorylated on Ser/Thr/Tyr residues; and, moreover, these proteins are involved in a variety of important cellular functions, including virulence and cell survival.

1.8. Bacterial Tyrosine Kinases

1.8.a. Structure

The bacterial tyrosine (BY) kinase family comprises the major group of bacterial enzymes endowed with tyrosine kinase activity. In most cases, BY kinases possess a transmembrane domain that can function both as an anchor and a sensor, as well as an intracellular catalytic domain [107]. The catalytic domain lacks the distinctive eukaryotic kinase motifs, and is defined by the presence of Walker A (P-loop) and B motifs. In addition, some BY kinases also contain a Walker A′ motif. BY kinases autophosphorylate at a tyrosine rich cluster in the C-terminal region using adenosine triphosphate as a phosphoryl donor, and the degree of phosphorylation in this region determines the interaction strength with other proteins. Some BY kinases also autophosphorylate on a tyrosine residue in close proximity to the Walker A′ box. Recent studies have identified other bacterial tyrosine kinases including those that closely resemble eukaryotic-like kinases, and those that utilize guanidino-phosphotransferase domains [107]. Additionally, in some cases, tyrosine can substitute for histidine in TCS.

1.8.b. Function

The majority of genes encoding BY kinases reside in operons responsible for regulating the synthesis and secretion of polysaccharides. The autophosphorylation state of the BY kinases exerts control over this process through phosphorylation, and activation, of UDP-sugar dehydrogenases and glucosyltransferases [95]. The extensive phosphotransfer reactions can also modulate a myriad of physiological processes that include resistance to cationic peptides and polymixin, along with heat shock responses. A greater appreciation for the role of tyrosine kinases in prokaryotes has emerged from

the application of global phosphoproteome technologies. For example, the PtkA BY kinase of *Bacillus subtilis* can phosphorylate at least nine different protein substrates [108]. Several of these substrates, most notably single-stranded DNA exonuclease YorK and aspartate semialdehyde dehydrogenase Asd, are activated via phosphorylation. Yet, the activity of many others, such as enolase, YjoA, YnfE, TvyG, Ugd and SsbA, remain unaffected by phosphorylation, and rather the cellular localization of these proteins is governed by phosphorylation status. Hence, BY action can not only regulate the activity of substrates, but also ensure the correct cellular localization of specific protein targets.

1.8.c. *P. gingivalis* Tyrosine Kinase-1 (Ptk1)

Previous studies discovered that the *P. gingivalis* protein PGN_1524 exhibited homology to a tyrosine kinase [55, 109]. This predicted tyrosine kinase was later confirmed and as the protein was named *P. gingivalis* tyrosine kinase-1 (Ptk1). Ptk1 was found to contain a cytoplasmic active domain spanning amino acid residues 541-821. Early investigations of this bacterial tyrosine kinase revealed it was involved in community development with the oral commensal organism, *Streptococcus gordonii*; however the function of the Ptk1 enzyme is unknown.

1.9. Bacterial Tyrosine Phosphatases

1.9.a. Structure

Bacterial tyrosine phosphatases catalyze the dephosphorylation of tyrosyl phosphorylated proteins, which in turn can result in either the propagation or inhibition of phospho-dependent signaling. Bacterial tyrosine phosphatases can be categorized into

three distinct families: (i) the eukaryotic-like phosphatases (PTPs) and dual-specific phosphatases that also display activity against phosphoserine and phosphothreonine; (ii) the low molecular weight protein-tyrosine phosphatases (LMW-PTPs), a family of small acidic enzymes also found in eukaryotes; and (iii) the polymerase–histidinol phosphatases (PHP), a family of phosphoesterases commonly found in Gram-positive bacteria. The PTP, dual-specific phosphatase and LMW-PTP enzymes utilize a common catalytic mechanism that involves the conserved signature $C(X)5R$ motif in the phosphate binding loop where cysteine, functioning as a nucleophile, attacks the phosphorus atom of the phosphotyrosine residue of the substrate. The arginine residue interacts with the phosphate moiety of the phosphotyrosine [110]. This motif is flanked, more remotely, by an essential aspartic acid residue, the location of which varies among the families. Protein tyrosine phosphatases are also capable of possessing dual functions, whereby, they can stimulate actions of cognate protein tyrosine kinases, yet in other cases, they may antagonize those actions [111, 112]. In Gram-negative bacteria, the gene encoding the LMW-PTP generally is upstream of the tyrosine kinase in the same operon. Conversely, in gram-positives, a PHP type phosphatase often is located in the same operon as the BY kinase alongside an adaptor protein, with the gene for the LMW-PTP at a remote site.

1.9.b. Function

While bacterial tyrosine phosphatases can be intimately involved in a number of cellular processes, two major themes have become apparent: involvement in polysaccharide production; and as secreted effector proteins with the potential for manipulation of host cell signal transduction pathways. Polysaccharide production, encompassing both exopolysaccharides and capsular polysaccharides, is also a key virulence determinant in

many organisms and thus tyrosine phosphatase activity is emerging as a central player in the information flow that controls pathogenic activity. Any type of tyrosine phosphorylation activity could prove to be a critical factor in the way an organism regulates its pathogenic mechanisms. A number of BY kinases have been studied previously and found to be involved in production and transport of extracellular polysaccharide [112]. For example, in *E. coli,* the Wzc BY kinase, regulated by its cognate phosphatase, sits in the membrane and is involved in Group 1 capsule biosynthesis [113]. The majority of BY kinases studied to date regulate the production and export of both extracellular and capsular polysaccharide. The interplay between bacterial tyrosine kinases and their cognate phosphatases confers major advantages in terms of allowing organisms to gain exquisite regulatory control over polysaccharide production based on constantly changing environmental conditions.

The YopH protein tyrosine phosphatase of *Yersinia,* a member of the PTP family, is an essential virulence factor that is injected into epithelial cells by type III secretion machinery. YopH can uncouple multiple signal transduction pathways [114] and in human epithelial cells, YopH dephosphorylates several focal adhesion proteins, including p130Cas (Cas), focal adhesion kinase and paxillin [115-117]. Similarly, *Salmonella* 'typhimurium' translocates the PTP tyrosine phosphatase SptP into epithelial cells where it is involved in reversing mitogen-activated protein kinase activation [118]. SptP is required for full virulence in murine models of disease [119]. *Shigella flexneri* produces a dually specific phosphatase, OspF, that dephosphorylates mitogen-activated protein kinase, which consequently prevents histone H3 phosphorylation [120]. A reduction in the level of histone 3 phosphorylation impedes access of the transcription factor NF-κB to the chromosome and hence transcription of NF-κB responsive genes such as IL-8 is reduced. Thus, OspF activity allows *S. flexneri* to modulate host cell

epigenetic information as a strategy for repressing innate immunity. In addition to physical protection, exopolysaccharide (EPS) such as capsule is often poorly immunogenic and can mask protein antigens and receptors for complement and phagocytic cells. In many cases, dephosphorylation of tyrosine kinases increases the level of polysaccharide synthesis [90], as evidenced by the activity of the *E. coli* K-12 BY kinase Wzc-ca, which is regulated by its cognate LMW-PTP, Wzb [121]. In this system, production of the capsular exopolysaccharide colonic acid is maximal when Wzc-ca is dephosphorylated by Wzb. Similarly, in *Streptococcus pneumoniae* autophosphorylation of the CpsD kinase, when in the presence of its cognate partner, CpsC, results in the attenuation of CpsD kinase activity, as well as a reduction in the level of encapsulation via a negative feedback regulatory loop [122]. Undoubtedly, the interplay among tyrosine kinases, phosphatases and exopolysaccharide is of a nuanced and subtle nature that may be reconfigured according to environmental conditions. Indeed, metabolic activity is one factor that has been shown to influence kinase to phosphatase ratios.

1.9.c. Role in Biofilms

A recent study in *Pseudomonas aeruginosa* demonstrated that tyrosine phosphatase activity is a unifying element that amalgamates polysaccharide production and biofilm formation with quorum sensing [123]. The PTP family tyrosine phosphatase, TpbA, is a negative regulator of 3,5-cyclic diguanylic acid (c-di-GMP), an important second messenger which suppresses transcription across the *pel* operon that encodes for extracellular matrix polysaccharide. Lower levels of exopolysaccharide in turn lead to reduced biofilm formation. In addition, TpbA responds to acyl homoserine lactone, and *tpbA* is regulated positively by the LasR transcriptional regulator. TpbA also regulates cell lysis as a means to control extracellular DNA that is used for complex biofilm

maturation [124]. These findings also reveal a previously unrecognized ability for phospho-dependent signaling to intersect with other important cellular second messenger systems.

Tyrosine phosphatases can also control heterotypic biofilm formation among oral organisms. *P. gingivalis* accumulates into heterotypic communities with the antecedent oral biofilm colonizer *S. gordonii*. In 2008, Maeda *et al.* identified a LMW-PTP, Ltp1, in *P. gingivalis* which functions as a negative regulator of EPS production, as well as community formation with *S. gordonii*. Transcription of *ltp1* is increased following contact with *S. gordonii [125]*, and Ltp1 is a component of a signaling pathway that converges on the LuxR family transcriptional regulator CdhR [126]. The expression of the *P. gingivalis* Mfa fimbriae and of LuxS, both of which contribute to community development with *S. gordonii*, are negatively regulated by CdhR. Thus, in both *P. aeruginosa* and *P. gingivalis*, tyrosine phosphatase activity results in arrested community development which may maintain optimal biofilm architecture.

1.9.d. *P. gingivalis* Serine Phosphatase, SerB

P. gingivalis SerB is the first serine phosphatase with a documented role in bacterial internalization [127]. SerB is a member of the Haloacid Dehalogenase (HAD) superfamily which is widespread in both prokaryotes and eukaryotes. The serine phosphatase SerB is vitally important for intracellular invasion and survival of *P. gingivalis* in gingival epithelial cells. *P. gingivalis* mutants deficient in SerB secretion were unable to internalize within epithelial cells nor were they able to survive intracellularly [127]. Studies have found that SerB is capable of inducing actin rearrangements in gingival epithelial cells [128]. Secretion of SerB by *P. gingivalis* facilitates invasion into epithelial cells through its activation of the host protein cofilin. It is established that infection of gingival epithelial cells with *P. gingivalis* results in the

suppression of Interleukin-8 (IL-8) production by the host cells. This inhibitory process, being of central importance to the ability of *P. gingivalis* to disrupt the host innate immunity, is associated with the serine phosphatase SerB. Further study into the mechanistic basis for SerB dependent inhibition of Interleukin-8 is one of the primary subjects of this study.

1.9.e. NF-κB Signaling

The Nuclear Factor kappa B (NF-κB) family of related transcription factors plays a central role in regulating the host response to microbial infection. Additionally, NF-κB regulates hundreds of genes which are involved in a myriad of cellular processes, including modulation of inflammatory and host innate immune responses, apoptosis, cell differentiation and proliferation. Activation of the NF-κB pathway is a central initiating event for host responses to any type of microbial invasion [129-131]. A key feature of this pathway is that the active NF-κB complex can be comprised of homodimers or heterodimers of p65 and p50 subunits. It is the different combinations of these subunits that lead to the regulation of distinct sets of genes [132]. In this way, NF-κB affords cells the versatility necessary to actively adjust their biological response to the outside world through the activation or repression of large subset of genes [133]. Various stimuli trigger activation of the NF-κB pathway which starts with the IKK activation leading to phosphorylation, subsequent ubiquitination and ultimately the degradation of these IκB inhibitory proteins. The NF-κB complex normally remains sequestered in the cytoplasm by inhibitory IκB proteins. Once these IκB proteins become targeted for degradation, they detach from and thereby activate the NF-κB complex. Active NF-κB can then freely move to the nucleus to induce transcription of important genes. Previous research has

found that the predominant control of IL-8 production is through the NF-κB p65 homodimers [134].

CHAPTER 2

STUDY RATIONALE, HYPOTHESIS AND SPECIFIC AIMS

Accumulating evidence reaffirms the notion that bacterial kinases and phosphatases display exquisite functional specificity; nevertheless, they are still capable of utilizing multiple protein substrates, both endogenous and exogenous, thereby providing versatility in phosphorelay signaling networks. To further advance our knowledge of the role of the specific tyrosine kinase, Ptk1 and the serine phosphatase SerB, of *P. gingivalis* in the selective manipulation of both host cell and bacterial phosphorelay signaling networks, we proposed the following hypothesis and aims:

1. *P. gingivalis* uses its protein serine phosphatase, SerB to selectively target specific host cell proteins and thus directly interfere with normal epithelial cell signaling to disrupt the host innate immune response.

2. The *P. gingivalis* tyrosine kinase Ptk1 is actively involved in the interspecies signaling between *P. gingivalis* and *S. gordonii* which underlies their dual species development.

Specific Aim 1: To determine if *P. gingivalis* SerB is affecting the secretion of Interleukin-8 (IL-8) via modulation of the NF-kB pathway.

Specific Aim 2: To investigate the presence and role of *P. gingivalis* Ptk1.

CHAPTER 3

MATERIALS AND METHODS

3.1. Bacterial Strains, Eukaryotic cells and Growth Conditions

P. gingivalis strain ATCC 33277 wild-type (WT) (obtained from the American Type Culture Collection), its isogenic mutant ΔserB, serB::FLAG, Ltp1 C10S, all Ptk1 mutant strains (Δ*ptk1* WT, Δ*ptk1* W83, Δ*ptk1*+p*ptk1*) and W83 were maintained as frozen stock cultures and cultured anaerobically in trypticase soy broth (TSB) supplemented with hemin (5 µg/ml), menadione (1 µg/ml) and yeast extract (1 mg/ml) at 37 C. Mutant strains were available in the laboratory. For solid media, TSB was supplemented with 5% sheep blood and 1.5% agar. The serB::FLAG strain expresses a three tandem FLAG-tag sequence inserted at the C-terminal end of the SerB protein. Overnight cultures were grown to mid exponential phase (OD600 nm; approx. 0.7). When necessary, erythromycin (10 µg/ml) and tetracycline (1 µg/ml) were added to the media. *S. gordonii* strain DL-1 was cultured in brain heart infusion (BHI) broth which contained 0.5% yeast extract. *E. coli* strains were grown aerobically with shaking at 37 C in Luria-Bertani Broth containing either kanamycin (50 µg/ml) or ampicillin (100 µg/ml) as needed. Our model cell line, telomerase immortalized gingival epithelial cells (TIGKs), were maintained in keratinocyte-SFM (K-SFM, serum free media) (Invitrogen) supplemented with 5 ng/ml of human recombinant epidermal growth factor and 50 µg/ml of bovine pituitary extract. For passage cells were washed twice with phosphate buffered saline (PBS) and then incubated with 0.05% Trypsin/0.53mM EDTA at 37 C for 5-10 min, at which point cells separated from the surface of the flask. K-SFM was added to cell suspensions to inhibit activity of Trypsin. Cells were centrifuged at 1000 rpm for 5 min. Supernatant was then removed and the cell pellet was re-suspended in K-SFM and

then reseeded at 1.0 x 10^6 cells/flask. Once 80% confluence was obtained, cells were used for experimentation.

3.2 *P. gingivalis-S. gordonii* Communities

S. gordonii (2x10^8 cells) were stained with the nucleic acid staining compound, hexidium iodide (15 µg/ml) and then incubated on glass coverslips for 16 h in anaerobic conditions at 37 C. *P. gingivalis* cells were stained with (5,6)-carboxyfluorescein succinimidyl ester (FITC; Invitrogen). Approximately 2x10^7 *P. gingivalis* cells were reacted with *S. gordonii* labeled cells for 18 h in pre-reduced PBS in an anaerobic environment at 37 C with rocking.   The resulting heterotypic biofilm community was washed in PBS and then analyzed via confocal scanning laser microscopy using an Olympus FV500 confocal microscope. Digital reconstruction of the X, Y, and Z stacks was accomplished using the Volocity analysis program (Perkin Elmer). In order to quantify the volume of *P. gingivalis* present in the mixed biofilm communities, the Find Objects Algorithm in the Volocity program was used. Similarly, the Find Objects algorithm also allowed analysis of the microcolony formation from the 3D digital confocal images. A threshold for 3D objects greater than 30 µm^3 was set.

3.3 Expression/Purification of Recombinant Proteins

The C-terminal region of Ptk1, spanning amino acids 541-821, was amplified by using the primers, 1524pet200F and 1524pet200R (Table 2), to generate a product 850 bp in length. This 850 bp product was cloned into the expression vector pET200 (Invitrogen) to create pET200-Fptk1, which was then transformed into *E. coli* TOP10 cells (Invitrogen). Successful transformants were analyzed by colony PCR, and the correct

insert was confirmed by sequencing. For protein purification, pET200-Fptk1 was transformed into the BL21 Star (Invitrogen) expression strain. N-dodecylphosphocholine detergent was used for solubilization and paramagnetic precharged nickel particles were used to isolate the histidine-tagged protein. The final purity of FPtk1 was determined by SDS-PAGE and Coomassie staining. Generation of recombinant Ltp1:C10S was accomplished by PCR amplification of the coding region of the catalytically inactive mutant Ltp1:C10S from the vector pTCOW-Ltp1:C10S [135] using the primers Ltp1C10SF and Ltp1C10SR (listed in Table 2). This recombinant protein was then cloned into the expression vector pET200. After sequencing, the expression vector containing Ltp1:C10S was transformed into BL21 Star and protein was purified with MagneHIS particles as described above. The purity of the resulting protein was determined by SDS-PAGE and Coomassie staining. The recombinant proteins Ltp1, PGN_0224, PGN_0613, and PGN_0261 were generated by PCR amplification of the total coding region from a *P. gingivalis* 33277 genomic template. Recombinant proteins were then cloned into the pET200 expression vector. Soluble protein was obtained as described above.

3.4 Kinase Assay

The Beacon Tyrosine kinase assay (Molecular Probes) was used to measure kinase activity. The catalytically active cytoplasmic fragment Ptk1:541-821 [55] was incubated with 0.5 mmol/L ATP and polyGlu:Tyr (4:1 ratio; 200 µg/mL) for 15 min. Following incubation, the level of fluorescence intensity was measured using a Victor X1 plate reader.

3.5 Immunoblotting

TIGK cells were lysed, centrifuged, separated by SDS-PAGE, and transferred onto nitrocellulose membranes by electroblotting. Membranes were blocked with TBS containing Tween 20 (0.1%), 1% skim milk and 10% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were incubated for 1 h at room temperature with primary antibodies. All primary antibodies were first diluted in PBST (PBS + Tween 20). These membranes were then washed three times with PBST followed by a second incubation with a 1:5000 dilution of HRP-conjugated secondary antibodies in PBST for 1 h at room temperature. After this second round of incubation, the membranes were again washed three times in PBST. Pierce Enhanced Chemiluminescence (ECL) Western Blotting Substrate (Thermo Scientific) and the ChemiDoc XRS Plus (Bio-Rad) were used to detect the presence of immunoreactive bands. All images were acquired using Image Lab Software version 3.0 from Bio-Rad.

3.6 Dephosphorylation Assay

Both Ltp1 and the catalytically inactive mutant, Ltp1:C10S (5 μg) [135]) were each pre-incubated with 5 μg of the catalytically active fragment, Ptk1:541-821for 1 h; after which standard immunoblotting procedures were carried out as described above. Membranes were blocked for 16 h with TBS containing 0.1% Tween-20 (TBST) and 10% BSA. Membranes were then probed with phosphotyrosine antibodies (Clone PY20; Sigma-Aldrich; 1:1000 dilution) for 2 h. The ChemiDoc XRS Plus imaging system (BioRad) was used to detect immunoreactive bands on the membranes.

3.7 Autophosphorylation Assay

Five micrograms of the catalytically active fragment, Ptk1:541-821, were treated with 20 units of calf intestinal alkaline phosphatase in buffer for 1 h at 37 C. 0.1 mmol/L ATP plus a phosphatase inhibitor cocktail (Sigma-Aldrich) were both added to the reaction for an additional 15 min prior to reactions being stopped. Reactions were quenched using equal volumes of 2x SDS-PAGE buffer and boiling for 10 min. Immunoblotting of protein samples was carried out as described previously. Phosphotyrosine antibodies were reacted with membranes. The ChemiDoc XRS imaging system (BioRad) was used to detect levels of phosphorylation.

3.8 Substrate Phosphorylation Assay

Five micrograms of the catalytically active fragment, Ptk1:541-821, were incubated with 10 μg of recombinant substrate. Similar to the autophosphorylation assay, ATP (5 mmols) and 0.5 μL of phosphatase inhibitor cocktail were both added to the reaction mixture and incubated for 30 min at 37 C. Addition of 2x SDS-PAGE buffer and boiling for 10 min halted the reaction.   Protein samples were analyzed by Western immunoblotting as described above.

3.9 Antibodies & Reagents for epithelial cell analysis

Mouse monoclonal anti-Myc and mouse monoclonal anti-β actin were obtained from Sigma-Aldrich. The following antibodies were obtained from Cell Signaling Technology: rabbit monoclonal anti-phospho-NFkB p65 S536, rabbit monoclonal anti-phospho-NFkB p105 S933, rabbit monoclonal anti-NFkB p105/p50, rabbit monoclonal anti-NF-kB p65

S468, mouse monoclonal anti-NF-kB p65, and rabbit monoclonal anti-calnexin. Rabbit monoclonal anti-β tubulin was obtained from Imgenex. The following antibodies were obtained from Invitrogen: mouse monoclonal anti-FLAG, Rhodamine Red-X-conjugated secondary antibody (goat anti-mouse IgG), Alexa Fluor 488-conjugated secondary antibody (goat anti-rabbit IgG). Rabbit polyclonal anti-NF-kB p65 S276 was obtained from Abcam. Horseradish Peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology. TNF was obtained from Pepro Tech and used for cell stimulation experiments. FITC-conjugated phalloidin obtained from Sigma-Aldrich was used for actin staining in fluorescent microscopy. *P. gingivalis* whole cell antibody and SerB antibodies were previously generated in the laboratory as described in Moffatt *et al.* (2012) and Hasegawa *et al.* (2008).

3.10. Transient Transfection

Plasmid constructs were available in the Lamont laboratory. In brief, PCR amplified *serB* from *P. gingivalis* was cloned into the vector pCMV-Myc (Clontech) using exogenously added EcoRI sites to generate a chimeric construct of Myc-SerB. GFP-tagged NF-kB p65 was created from TIGK cDNA and inserted into the pAcGFP1-C1 vector (Clontech) using exogenously added KpnI sites. A point mutation of GFP-tagged NF-kB p65 to S536D was introduced by overlapping fusion PCR. Again using TIGK cDNA, GFP-tagged NF-kB p105 was generated and inserted into the pAcGFP1-C1 vector using exogenously added EcoRI sites. GFP-tagged NF-kB p50 was constructed from amino acids 1 to 438 of NF-kB p105 and then subsequently cloned into the pAcGFP1-C1 vector. The pIL-8 kB-Luc reporter plasmid was constructed according to the published sequence [134]. The enhancer sequence of IL-8 was cloned into the pGL4.23 vector (luc2/minP) (Promega) using exogenously added KpnI and NheI sites.

All PCR products and mutations were confirmed by sequencing. For all transient transfections of TIGK cells, the FuGENE 6 Transfection Reagent was employed (Promega).

Primer sequences used in this study are listed in Table 1 below.

Table 1. Selected Primers used for SerB experiments

Name	Sequence
SerB-F	5'-CCGGAATTCTTATGAGTACAACACGA-3'
SerB-R	5'-CCGGAATTCCTATAATTTTTCCGTAAGCA-
NF-κB p65-F	5'-CGGGGTACCATGGACGAACTGTTC-3'
NF-κB p65-R	5'-CGGGGTACCTTAGGAGCTGATCTGACTC-3'
NF-κB p65 S536D-F	5'-AGACTTCTCCGACATTGCGGACAT-3'
NF-κB p65 S536D-R	5'-ATGTCCGCAATGTCGGAGAAGTCT-3'
NF-κB p105-F	5'-CCGGAATTCTTATGGCAGAAGATGATCC-3'
NF-κB p105-R	5'-CCGGAATTCCTAAATTTTGCCTTCTAGAGG-3'
NF-κB p50-R	5'-CCGGAATTCAGTGTCCATGGTTCC-3'
IL-8 κB-F	5'-CGGGGTACC(TGTGGAATTTCCGC)$_4$GCTAGCTAG-3'
IL-8 κB-R	5'-CTAGCTAGC(GCGGAAATTCCACA)$_4$GGTACCCCG-3'

3.11 Confocal Microscopy of TIGK cells

TIGK cells were fixed with 3% paraformaldehyde in PBS for 30 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature and blocked with 0.1% gelatin in PBS for 20 min at room temperature. All primary antibodies and the Rhodamine Red-X-conjugated secondary antibodies were diluted 1:400 in PBS. For labeling of DNA, cells were mounted onto glass slides using Vectashield Mounting Medium with DAPI (obtained from Vector laboratories). Cells

were examined using laser scanning confocal microscopy (Olympus FV500) and analyzed with FluoView Software.

3.12 NF-κB Nuclear Translocation

TIGK cells were transfected with either GFP-tagged NF-kB p65 or GFP-tagged phosphomimic-p65 S536D. This phosphomimic is a constitutively active construct mimicking the phosphorylated state and cannot be dephosphorylated. TIGKs were co-transfected with Myc-SerB. TIGKs underwent transient transfection for 36 h and were stimulated with TNF (5 ng/ml) for 30 min (unless otherwise indicated). All cells were collected and stained with DAPI and anti-Myc labels. Confocal microscopy was used to detect GFP and Myc tags.   >/= 90 GFP/Myc positive cells were counted in each condition.

3.13 Crosslinking

TIGK cells were cross linked with dithiobis (0.25 mM; Thermo Scientific) in PBS for 5 min at room temperature. Cells were then rinsed with a quenching buffer composed of 1 M glycine in PBS at a pH 7.4. Cells were washed in the quenching buffer 4 times after which the cell proteins were extracted using lysis buffer.   An anti-c-Myc immunoprecipitation kit (Sigma-Aldrich) was used in pull down reactions using cell lysates, following the manufacturer's protocol. All proteins that bound to the anti-c-Myc agarose were analyzed by immunoblotting.

3.14 Luciferase Reporter Assay

TIGK cells were transfected with pIL-8 kB-Luc, pRL-CMV Vector (Promega), along with other combinations of expression plasmids. The total plasmid amounts for each transfection were normalized. TIGK cells were first lysed and then reporter activity was measured using the Dual-Glo Luciferase Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity in the same extracts.

3.15 Enzyme-linked Immunosorbent Assay (ELISA)

A sandwich ELISA was used to measure Interleukin-8 (IL-8) concentrations in TIGK cell culture supernatants. The Quantikine Human CXCL/IL-8 kit by R&D Systems was used according to the manufacturer's protocol.

3.16 Quantitative Gene Expression

P. gingivalis cells were reacted with *S. gordonii* or with PBS under anaerobic conditions for 1 h. Total RNA was extracted using Trizol (Invitrogen). Extracted RNA from each of the samples was treated with DNase I. RNA template (20 ng) was converted to cDNA using the high capacity reverse transcription Kit from Applied Biosystems. Quantitative RT-PCR was performed on the StepOne Plus Real Time PCR system from Applied Biosystems. Power SYBR green PCR master mix along with 250 nmol/L of the following primers were used (Table 2 below).

Table 2. Primers used for Ptk1 experiments

Name	Primer sequence (5' – 3')	Function
A1524usF	GTATTGTCAGTAATTTTTTG	Generating fragment upstream of PGN_1524 for use with allelic exchange mutation
B1524usR	GGAAGCTATCGGGGGTACCTTCAATTAAGTTCATCTTTC	
C1524dsF	TGTCCCTGAAAAATTTCATCCATAGGATGTTTTCTATTTTCAAG	Generating fragment upstream of PGN_1524 for use with allelic exchange mutation
D1524dsF	CTGTATTATAAAAACGATCGA	
A436usF	TTGAGAAGGACGATGTGCTG	Generating fragment upstream of PG436 for use with allelic exchange mutation
B436usR	GGAAGCTATCGGGGGTACCGGCCAATACACTCGCTACAA	
C436dsF	TGTCCCTGAAAAATTTCATCCGCCCCATGAAGAAGAGCTAA	Generating fragment upstream of PG436 for use with allelic exchange mutation
D436dsR	ATTCGGAACCAGCCCTTATC	
ERMf	GGTACCCCCGATAGCTTCC	Erythromycin gene for fusion with upstream and downstream products above
ERMr	GGATGAAATTTTTCAGGGACA	
ptcow1524F	ATGAACTTAATTGAAGATTCAAAAAACAC	For cloning full length PGN_1524 into pTCOW
ptcow1524R	TTAGCTCTTCTTCATGGGGCC	
ltp1C10SF	ATGAAGCCACATAAAATC	Generation of recombinant Ltp1 C10S
ltp1C10SR	TCAGTCGCATGCGGAT	

Using the SYBR green channel from 60-95 C, melting curves were obtained. Relative quantities of mRNA were normalized to 16s rRNA using the $\Delta\Delta Ct$ method. A negative control of no RT was included in all experiments.

3.17 Statistics

All experiments were performed with at least 3 biological replicates and 2 technical replicates. Statistical analyses were performed using a two-tailed t-test for single

comparisons and ANOVA with the Tukey correction for multiple analyses. P-values

<0.05 were considered significant.

CHAPTER 4

P. GINGIVALIS SERINE PHOSPHATASE SERB: EXPERIMENTAL RESULTS

4.1 *P. gingivalis* SerB suppresses activation of the IL-8 Promoter

It has been well documented that Interleukin-8 (IL-8) production is predominantly regulated by p65 homodimers of NF-kB [134]. Previous research has demonstrated that *P. gingivalis* can not only inhibit the accumulation of IL-8 from gingival epithelial cells but also inhibits the accumulation of IL-8 in response to other bacteria, a phenomenon coined local chemokine paralysis [40]. Later research revealed that secretion of *P. gingivalis* serine phosphatase, SerB, was a necessary step required for maximal inhibition of IL-8 production by gingival epithelial cells [136]. We first sought to determine if SerB-dependent inhibition of IL-8 occurred at the transcriptional level. TIGK cells were transfected with a luciferase reporter plasmid linked to the IL-8 promoter. Transfected TIGK cells were infected with *P. gingivalis* 33277 wild type (WT), or *P. gingivalis* ΔserB mutant strains, and stimulated with TNF to induce transcription of the IL8 gene. Plasmid pRL-CMV, in which Renilla luciferase is driven by the cytomegalovirus promoter, was a transfection control. Luciferase activity was then measured. The fold changes presented are relative to the activity level of the unstimulated control group. The IL-8 promoter activity increased by a measure of 5-fold when stimulated with TNF, compared to the unstimulated control. When we infected with the *P. gingivalis* 33277 WT strain, the IL-8 promoter activity was significantly reduced (5-fold to 3.6 fold induction) compared to TNF stimulated, uninfected basal levels. Infection of TIGKs with *P. gingivalis* ΔserB mutant strain showed a marked inability to reduce TNF stimulated IL-8 promoter activity compared to the *P. gingivalis* WT strain (Figure 1). These results suggest that the ability of *P. gingivalis* SerB to inhibit IL-8 secretion by epithelial cells does occur at the transcriptional level.
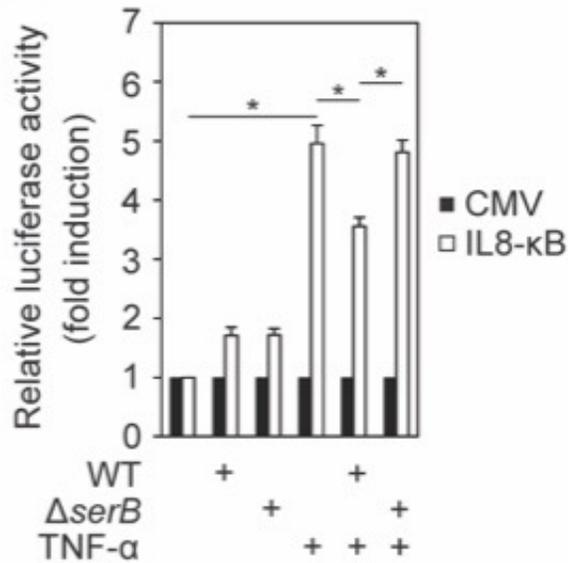
Figure 1. Infection of TIGK cells with *P. gingivalis* expressing SerB reduces IL-8 promoter activity. TIGKs were transiently transfected with pIL-8 kB-Luc luciferase reporter plasmid for IL-8 promoter activity, or pRL-CMV (CMV) where Renilla Luciferase driven by cytomegalovirus promoter, and infected with *P. gingivalis*. WT or ΔserB mutant (MOI=10). After 16 h infection, TIGKs stimulated with TNF (10 ng/mL) for 3 h or left unstimulated. Luciferase activity was measured and normalized to Renilla Luciferase. Results presented as fold relative to activity of unstimulated control. Values are mean +/- SD; n=3; *p<0.05.

4.2 *P. gingivalis* SerB dephosphorylates the NF-κB p65 subunit

We next wanted to ascertain whether the ability of SerB to suppress IL-8 promoter activity was related to the phosphorylation status of the NF-kB p65 subunit. As mentioned earlier, it has been reported that IL-8 production is predominantly controlled by the p65 homodimers of NF-kB. Previous studies performed in Jurkat cells have demonstrated that phosphorylation of serine residue S536 of p65 induces IL-8 transcription [137]. This led us to hypothesize that this S536 residue in p65 is also the target for dephosphorylation by *P. gingivalis* SerB phosphatase. We infected TIGKs with either *P. gingivalis* 33277 WT or *P. gingivalis* ΔserB mutant strains for 16 h. TIGKs were then stimulated with TNF or left unstimulated for 3 min or 10 min prior to cell lysis, and the phosphorylation status of S536 determined by immunoblotting. (Figure 2A). After 10 min of stimulation with TNF, the levels of phosphorylated (P)-S536 were significantly reduced when in the presence of *P. gingivalis* WT.   In contrast we saw that the ΔserB mutant strain showed diminished ability to reduce phosphorylation levels of the S536 residue compared to the *P.gingivalis* WT strain (Figure 2A). For quantification of these results, we performed a densitometric analysis of the immunoblots (Figure 2B), which showed a > 4-fold reduction of phosphorylation of S536 in *P. gingivalis* WT infected cells. We can conclude from these findings that NF-kB p65 S536 is in fact a target for dephosphorylation by *P. gingivalis* SerB. The importance of this finding is underscored by the fact that the phosphorylation status of this S536 residue is one factor that controls the dimerization and nuclear import of p65 into the nucleus.
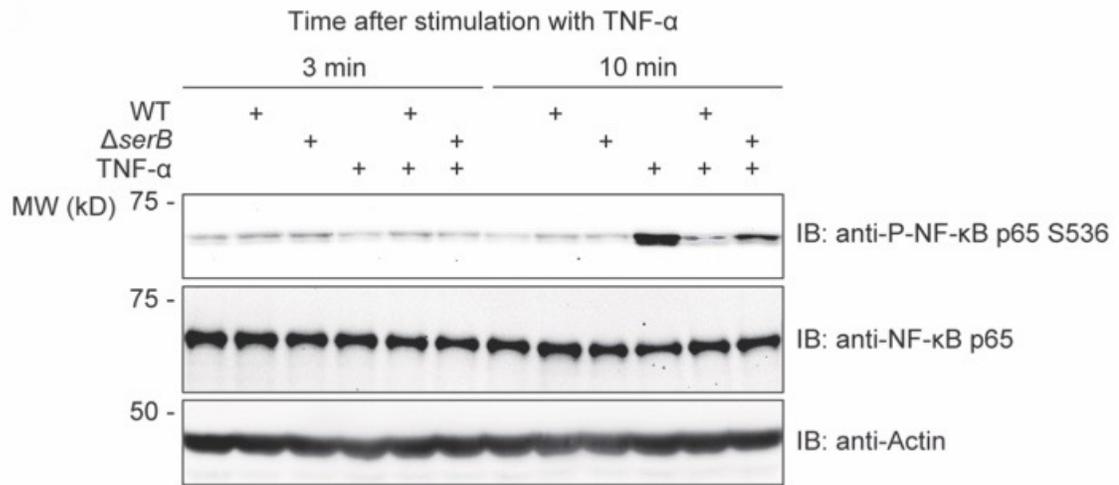
Figure 2A. Infection of TIGK cells with *P. gingivalis* expressing SerB diminishes phosphorylation of NF-kB p65.   Immunoblots of cell lysates of TIGKs infected with *P. gingivalis* WT or ΔserB (MOI=10).   16 h after infection TIGKs were stimulated with TNF (10 ng/mL) or left unstimulated for indicated timepoints prior to cell lysis. Immunoblots probed with indicated antibodies. Anti-total p65 shows no difference in the level of the protein. Actin was used as a loading control.
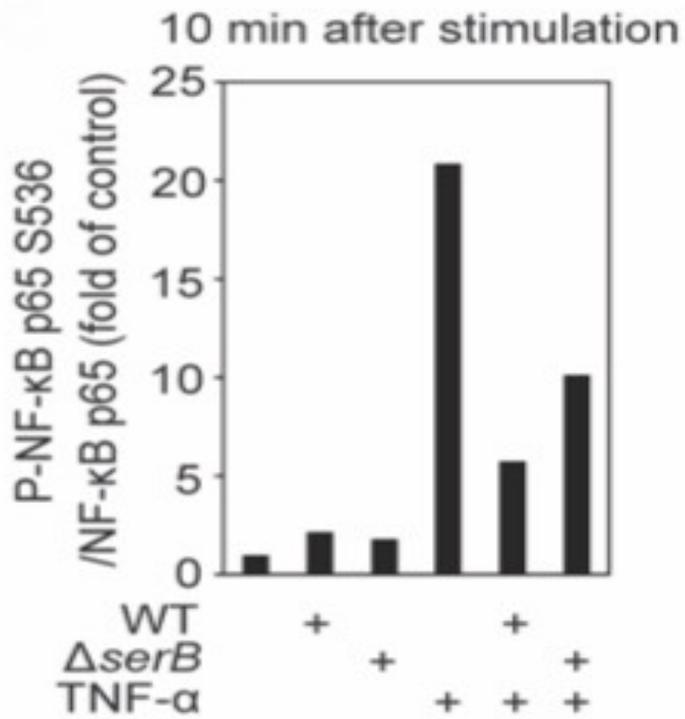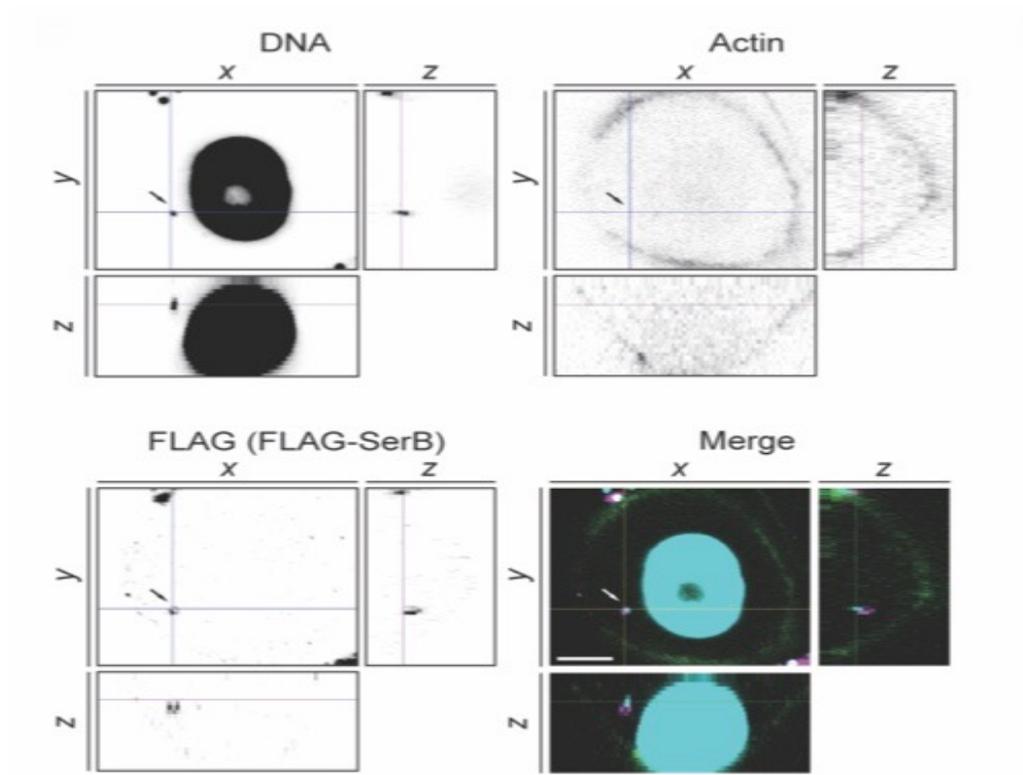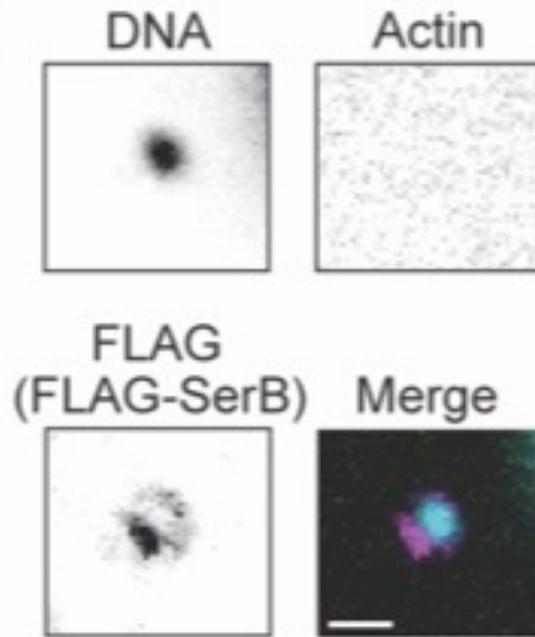
Figure 2B. Infection of TIGK cells with *P. gingivalis* expressing SerB diminishes phosphorylation of NF-kB p65. Scanning densitometry of immunoblots performed in A showing ratio of phosphorylated NF-kB p65 (S536) relative to total immunodetectable NF-kB p65.

4.3 SerB is produced and secreted by intracellular *P. gingivalis*

Previous research has established that intracellular invasion of gingival epithelial cells by *P. gingivalis* is a pre-requisite for NF-kB suppression [40]. In an attempt to establish that SerB itself is secreted within epithelial cells by *P. gingivalis* and thus potentially working directly on host cell components to effectuate these results, we made a construct in *P. gingivalis* where SerB was tagged with a FLAG-epitope. We then infected our model cell line of TIGKs with this *P. gingivalis* construct expressing FLAG-SerB. Cells were stained with DAPI (cyan) to detect the nuclear and bacterial DNA, FITC-phalloidin (green) to detect the actin, and anti-FLAG (magenta) to detect SerB (Figures 3A & 3B). Preliminary experiments confirmed that the detection of infected bacteria using DAPI proved to be just as specific as was seen when using *P. gingivalis* antibodies. Utilizing confocal microscopy, SerB was detected within the cytoplasm of TIGK cells. Here we can conclude that SerB is secreted and accessible for direct host cell interactions following *P. gingivalis* infection.

Figure 3A. TIGK cells infected with *P. gingivalis* expressing SerB show presence of SerB in cytoplasm of TIGKs. Confocal microscopy showing expression of SerB by intracellular *P. gingivalis*. TIGK cells were infected with *P. gingivalis* expressing FLAG-SerB (MOI=10) for 2 h, stained with DAPI (cyan), FITC-phalloidin (green) or anti-FLAG (magenta). Bar=5 um.

Figure 3B. TIGK cells infected with *P. gingivalis* expressing SerB show presence of SerB in cytoplasm of TIGKs. Higher magnification of *P. gingivalis* indicated by arrow shown in 3A. Bar=1 um.

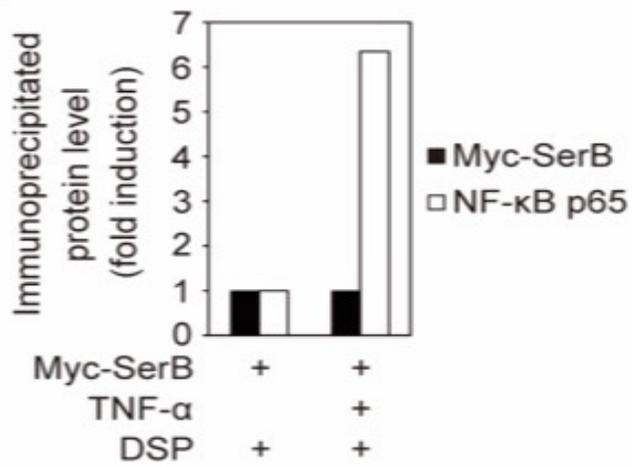4.4 SerB directly binds to and dephosphorylates NF-κB p65.

We wanted to investigate more thoroughly the precise function of SerB, independent of any other *P. gingivalis* effectors. First, we generated a mammalian expression vector, which expressed the fusion protein, Myc-SerB. TIGKs were transfected with an empty vector (Myc) or Myc-SerB, and then stimulated with TNF. Cells were cross-linked with DSP to stabilize all enzyme-substrate interactions. Cell lysates were immunoprecipitated with anti-Myc antibody. We found that Myc-SerB co-immunoprecipitated with endogenous NF-kB p65, whereas this was not seen with the empty Myc vector alone (Figure 4A). For quantification purposes, we performed scanning densitometry analysis of the immunoblots from Figure 4A showing the ratio of immunoprecipitated NF-kB p65 relative to immunoprecipitated Myc-SerB (Figure 4B). These findings suggest that *P. gingivalis* SerB can directly interact with p65. Yet it is also important to note that these results do not preclude the possibility that other *P. gingivalis* molecules might also possess the ability to interact with NF-kB components.

To further investigate the type of interaction between SerB and NF-kB p65 we used confocal microscopy to visualize the co-localization between SerB and p65 in TIGK cells. We constructed a GFP tagged NF-kB p65 (green) as well as a Myc expression vector, Myc-SerB (red), and transfected them into our model cell line of TIGKs. Transfected cells were then stimulated with TNF. Cells were fixed and stained with anti-Myc (red) and viewed with confocal microscopy (Figure 4C). Olympus FluoView software was used to measure the intensity of the fluorescent signals emitted from the GFP-NF-kB p65 (green), the Myc (Red, vector), and the Myc-SerB (red) (Figure 4D). Both Figures 4C and 4D clearly demonstrate that when Myc-SerB and GFP-NF-kB p65 are ectopically expressed in TIGK cells, Myc-SerB is found to co-localize with GFP-NF-kB p65 in the cytoplasmic region.

In an attempt to confirm that the phosphorylation status of NF-kB p65 is a critical factor for SerB activity, we constructed a constitutively active phosphomimic p65 S536D mutant. This phosphomimic is a mutant version of p65 whereby serine was replaced by glutamate, which . mimics the phosphorylated state but cannot be dephosphorylated. TIGK cells were transfected with GFP-NF-kB p65 or GFP-NF-kB p65 S536D, as well as with Myc-SerB or empty vector (Myc). Cells were cross-linked with DSP to stabilize substrate-enzyme interactions. Cells were not stimulated with TNF so as to ensure the phosphorylation of p65 was maintained at basal levels. Cell lysates were then immunoprecipitated with anti-Myc antibody (Figure 4E). We then performed quantification analysis by scanning densitometry of the immunoblots shown in Figure 4E showing the ratio of immunoprecipitated GFP-NF-kB p65 or the p65 S536D mutant relative to the total immunoprecipitated Myc-SerB (Figure 4F). These immunoprecipitation experiments revealed that Myc-SerB co-immunoprecipitated with GFP-NF-kB p65 S536D phosphomimic mutant. In contrast, we were unable to detect co-immunoprecipitation of Myc-SerB with the unphosphorylated NF-kB p65. These results suggest that the phosphorylation status of NF-kB p65 S536 is, in fact, a crucial element involved in the binding of SerB to NF-kB p65.
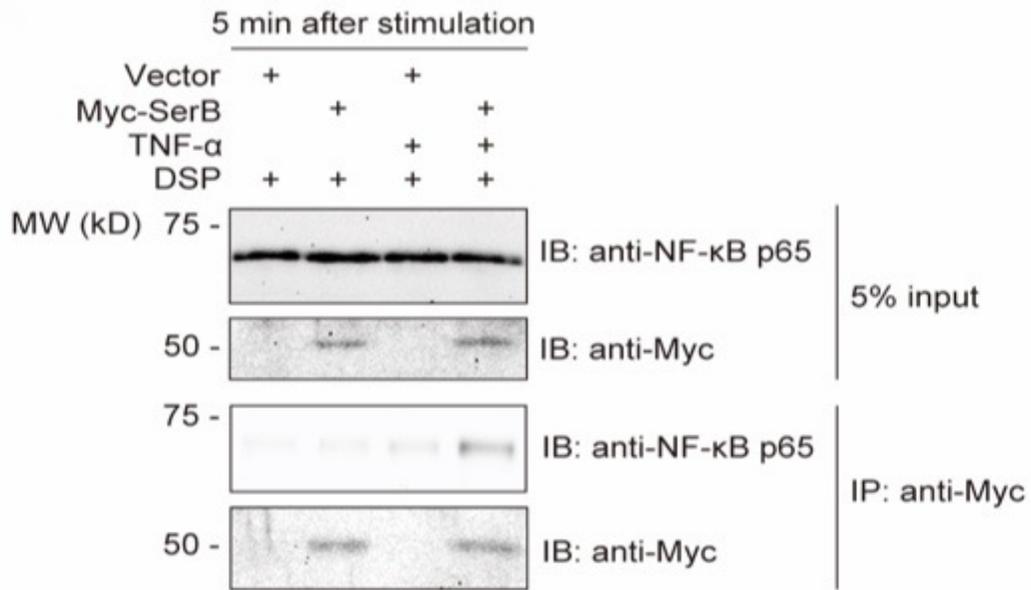
Figure 4A. Ectopically expressed SerB binds to and dephosphorylates NF-kB p65.
TIGK cells were transfected with Myc-SerB or empty vector, stimulated with TNF
(10 ng/mL) for 5 min or left unstimulated. TIGKs were cross-linked with DSP and
cell lysates immunoprecipitated (IP) with anti-Myc antibody. Immunoblots show
cell lysates prior to IP (5% input) and immunoprecipitate with Myc antibodies.
Results are representative of 3 biological replicates.

45

Figure 4B. Ectopically expressed SerB binds to and dephosphorylates NF-kB p65.

Scanning Densitometry of immunoblot in (A) showing ratio of immunoprecipitated

NF-kB p65 relative to immunoprecipitated Myc-SerB.

5 min after stiumlation with TNF-α

| GFP-NF-κB p65 | Myc | Merge |
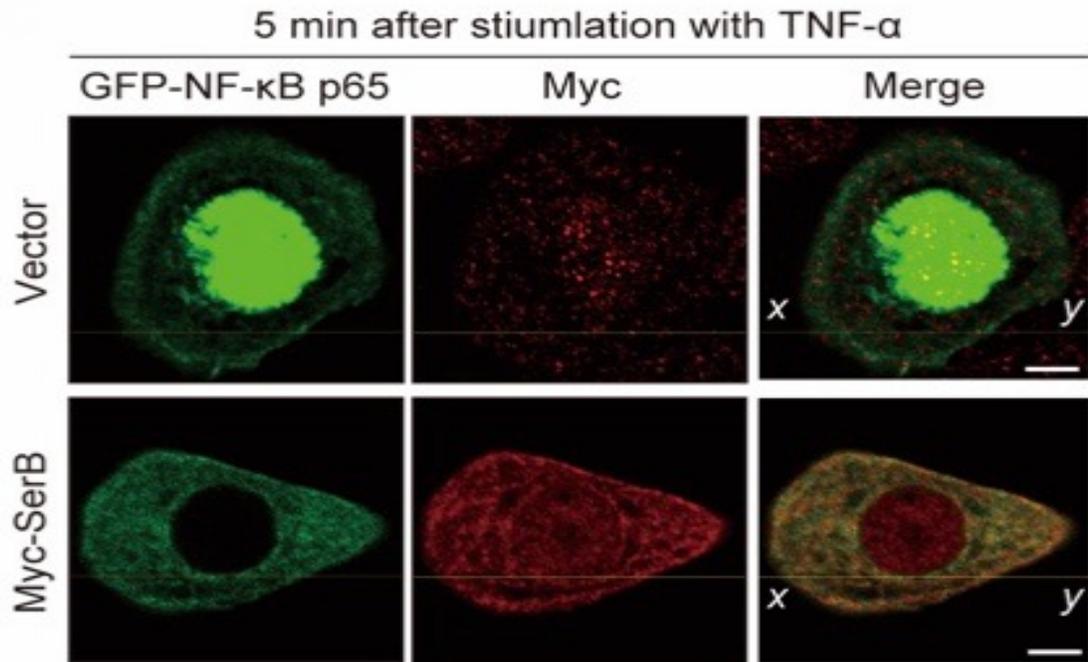
Vector

Myc-SerB

Figure 4C. Ectopically expressed SerB binds to and dephosphorylates NF-kB p65. Confocal microscopy of TIGKs expressing GFP-NF-kB p65 (green) and either Myc (vector) or Myc-SerB. 36 h after transfection, TIGKs were stimulated with TNF (10 ng/mL) for 5 min or left unstimulated. Cells were fixed and stained with anti-Myc (red). Bars=5 μm. Results are representative of 3 biological replicates.
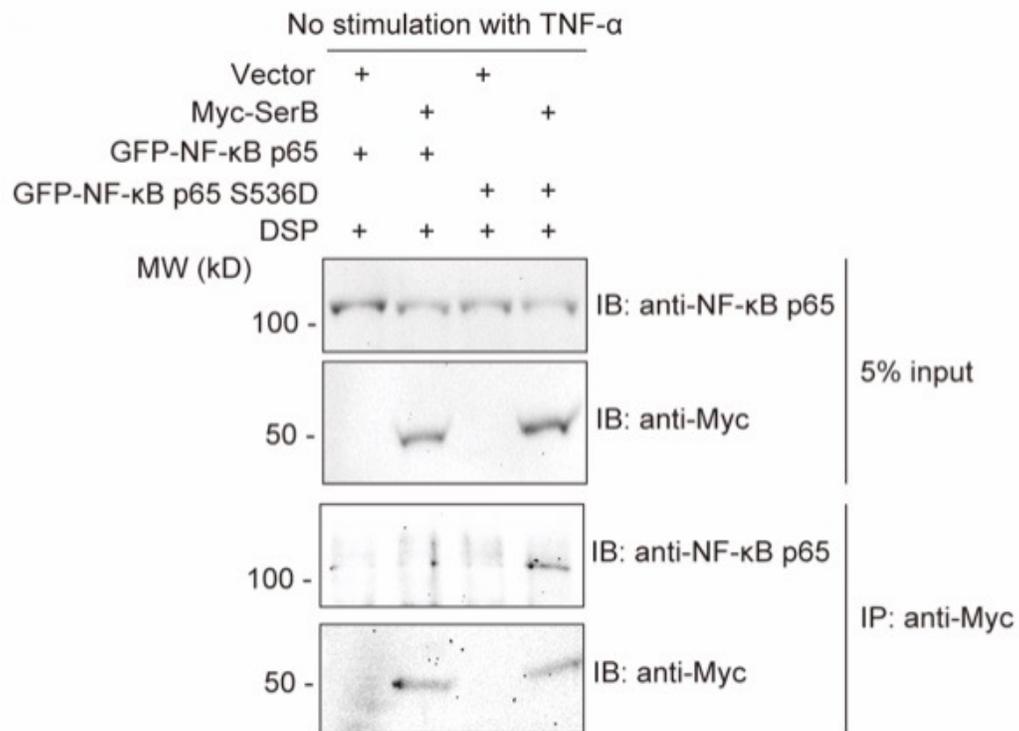
Figure 4D. Ectopically expressed SerB binds to and dephosphorylates NF-kB p65.
TIGKs transfected with empty vector or Myc-SerB, and GFP-NF-kB p65 or GFP-NF-
kB p65 S536D. Cells were cross-linked with DSP and cell lysates
immunoprecipitated with anti-Myc antibody. Immunoblots show cell lysates prior
to IP (5% input) and immunoprecipitate with Myc antibodies. Results are
representative of 3 biological replicates.
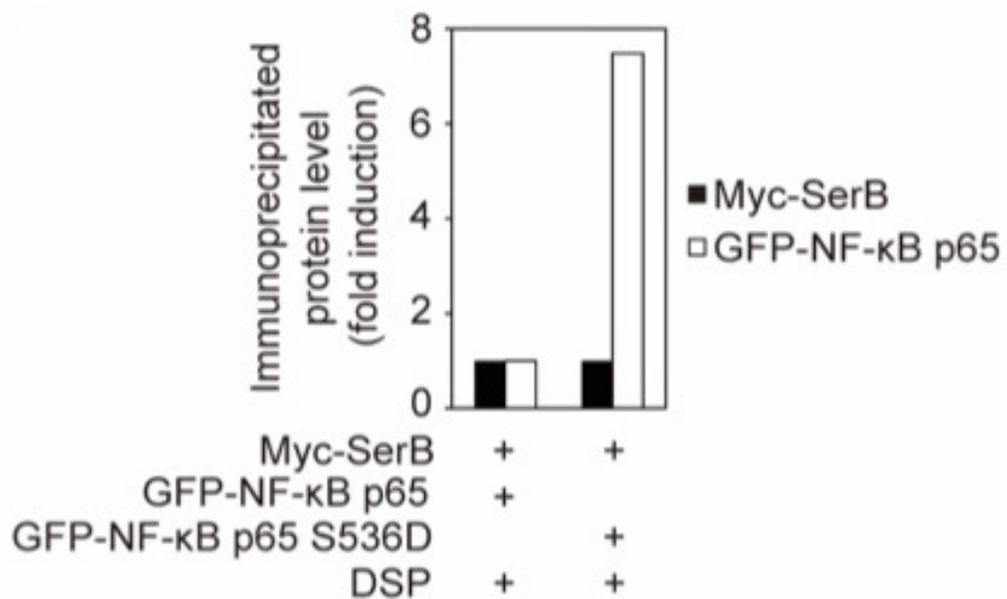
Figure 4E. Ectopically expressed SerB binds to and dephosphorylates NF-kB p65.
Densitometry of immunoblots from (D) showing ratio of immunoprecipitated GFP-
NF-kB p65 or p65 S536D relative to immunoprecipitated Myc-SerB.

Our next area of focus was to determine whether the binding of SerB itself to p65 is the primary event that triggers the subsequent dephosphorylation of the S536 residue. TIGK cells were transfected with an empty vector (Myc) or Myc-SerB. TIGKs ectopically expressing Myc-SerB were then stimulated with TNF for 0, 5, 30, 60 and 120 min. For each time point, cell extracts were prepared and immunoblotted with specific antibodies to (P)hosphorylated-NF-kB p65 S536, unphosphorylated NF-kB p65, Myc vector alone or Myc-SerB (Figure 5A). We quantified these immunoblots from Figure 5A by scanning densitometry (Figure 5B) showing the proportion of (P)-NF-kB p65 (S536) relative to total detectable NF-kB p65. Our findings showed that TIGK cells expressing Myc-SerB exhibited a lower level of (P) -NF-kB p65 between the time periods of 5 to 30 min following TNF stimulation when compared to TIGKs transfected with the empty Myc vector. Interestingly, levels of phosphorylated NF-kB p65 dropped in cells expressing Myc vector alone as well as those expressing Myc-SerB after 60 min of TNF stimulation. This can be attributed to the transitory effect of TNF stimulation. These findings indicate that the S536 residue of NF-kB p65 subunit can be directly dephosphorylated by *P. gingivalis* SerB.

To further explore the specificity of SerB for the p65 subunit, we examined whether SerB could target other NF-kB subunits such as the p105 subunit, specifically the S933 residue of p105. TIGK cells were transfected with empty vector (Myc) or Myc-SerB. After transfection, TIGKs were stimulated with TNF for various time periods. Cell extracts were then immunoblotted with antibodies specific for phosphorylated p105 S933, p105, and p50 (Figure 5C). Quantitative analysis of all immunoblots was performed using scanning densitometry (Figures 5D and 5E). These results showed that SerB did not dephosphorylate the NF-kB p105 S933 residue (Figures 5C and 5D). Previous research has established that processing of the precursor p105, resulting in

formation of the active subunit, p50, is also an important step in the activation of the NF-kB transcription factor [138]. To further substantiate our findings from p105 data, we verified that the total amount of the active p50 subunit remained unchanged in our TIGK cells expressing Myc-SerB and stimulated with TNF. When compared to the empty Myc vector control group, we confirmed that the amount of p50 was unchanged (Figures 5C and 5E).
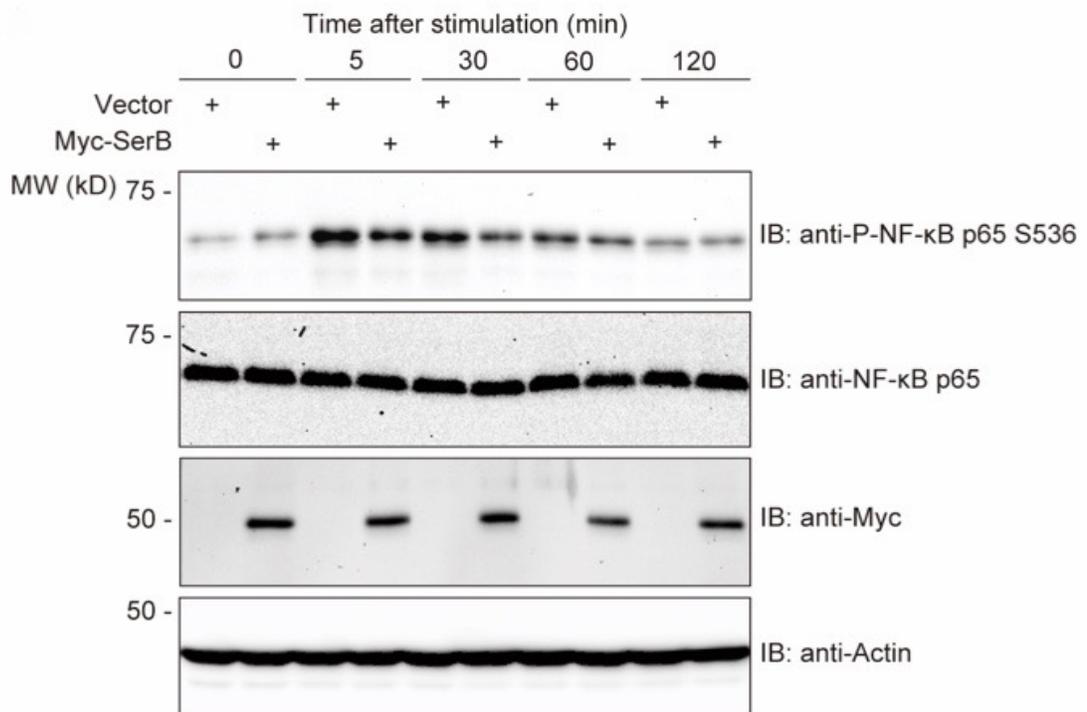
Figure 5A. *P. gingivalis* SerB dephosphorylates NF-kB p65 but not p105. TIGKs were transfected with empty Myc vector or Myc-SerB. 36 h after transfection, TIGKs were stimulated with TNF (5 ng/mL) and at time points shown cell extracts were prepared and immunoblotted with the antibodies indicated. Actin was used as the loading control. Results are representative of 3 biological replicates.
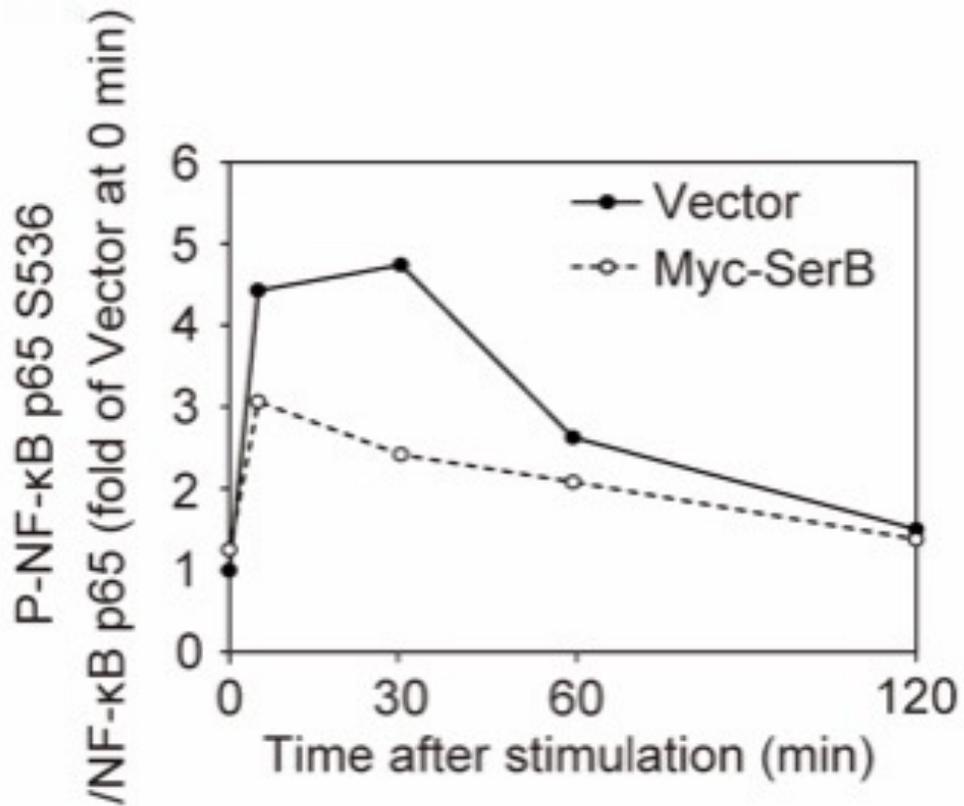
Figure 5B. *P. gingivalis* **SerB dephosphorylates NF-kB p65 but not p105. Densitometry of the immunoblots shown in (A) showing ratio of phosphorylated NF-kB p65 (S536) relative to total immunodetectable NF-kB p65.**
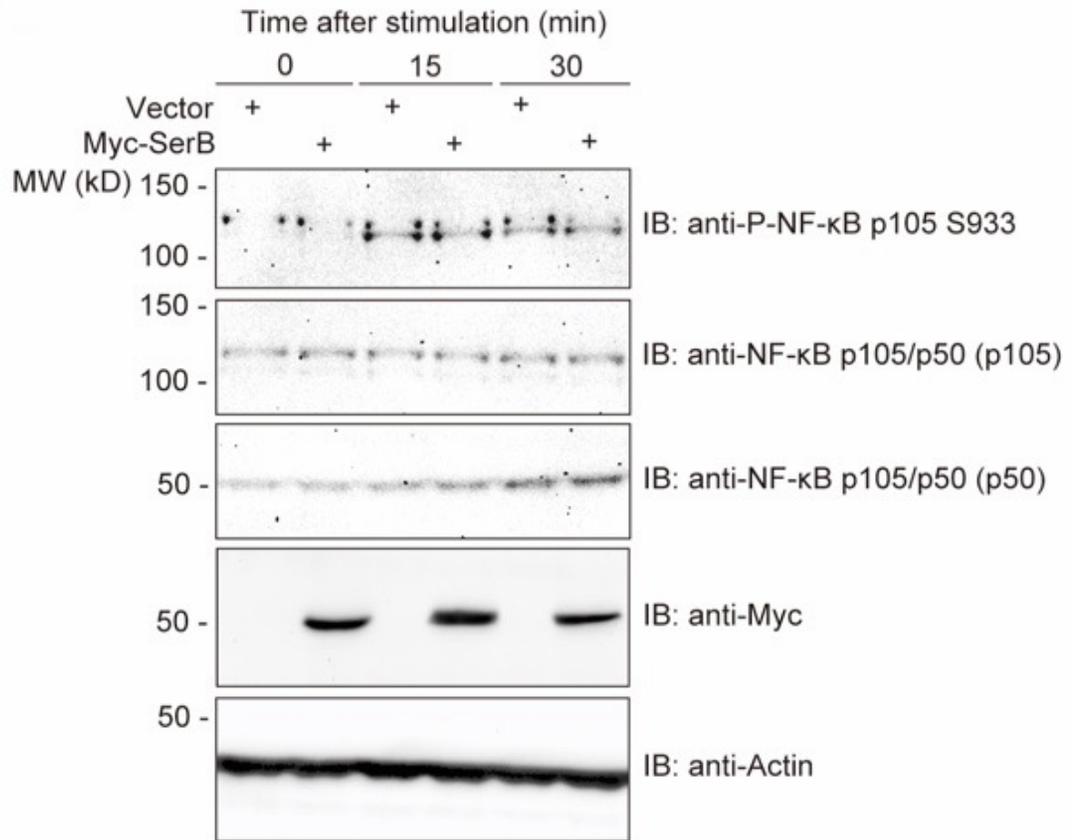
Figure 5C. *P. gingivalis* **SerB dephosphorylates NF-kB p65 but not p105. TIGKs were transiently transfected with empty Myc vector or Myc-SerB. After 36 h transfection, TIGKs were stimulated with TNF (5 ng/mL) and at specified time points cell extracts were prepared and immunoblotted with the antibodies indicated. Results are representative of 3 biological replicates.**
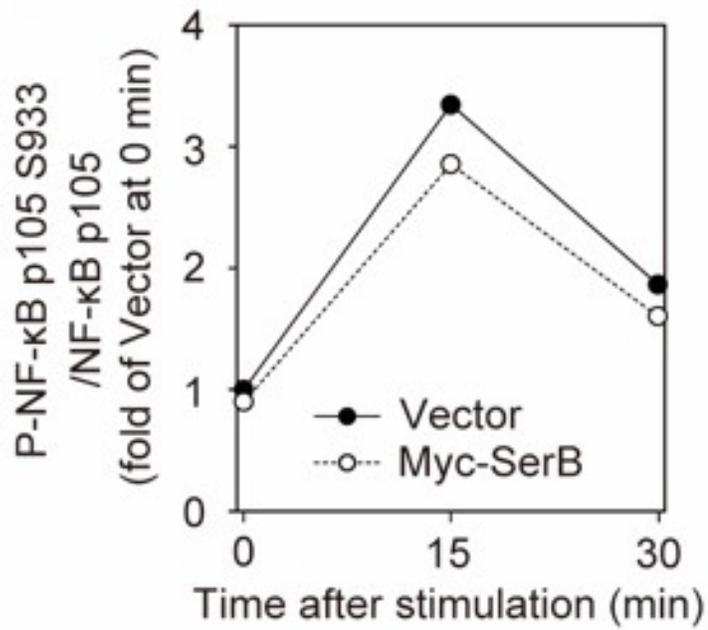
Figure 5D. *P. gingivalis* SerB dephosphorylates NF-kB p65 but not p105. Scanning densitometry of immunoblot from (C) showing ratio of phospho-NF-kB p105(S933) relative to total immunodetectable NF-kB p105.
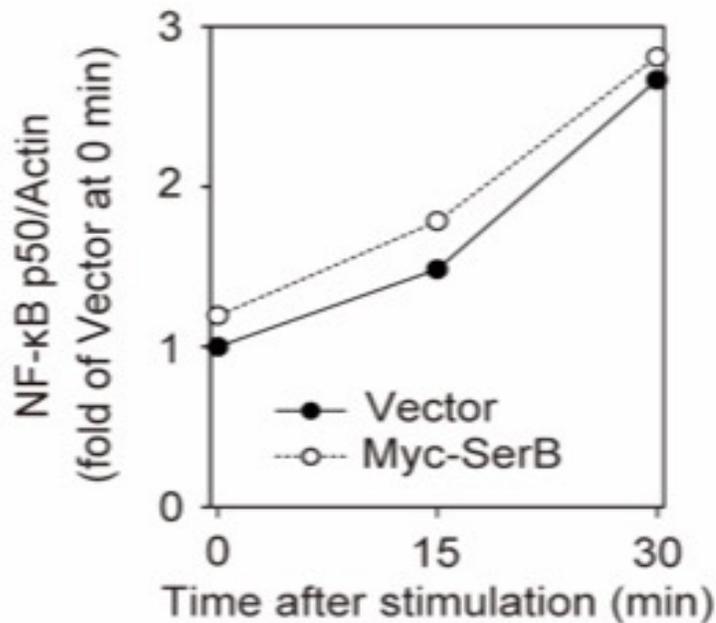
Figure 5E. *P. gingivalis* **SerB dephosphorylates NF-kB p65 but not p105. Densitometry of immunoblot in (C) showing ratio of phosphor-NF-kB p50 relative to actin.**

In order to fully address the specificity for dephosphorylation of p65 S536 residue by SerB, we examined whether this *P. gingivalis* enzyme could also dephosphorylate other serine residues, in particular, S276 and S468 (Figures 6 and 7). We transfected TIGK cells with empty Myc vector alone or Myc-SerB. TIGKs were then stimulated with TNF and cells lysates immunoblotted with phospho-specific antibodies (Figures 6A and 7A). For each experiment quantitative analysis of the immunoblots was performed using scanning densitometry to assess the ratios of (P)-NF-kB p65 S276 or S468 relative to

total immunodetectable NF-kB p65 (Figures 6B and 7B). Our results showed that SerB failed to significantly decrease the levels of phosphorylated S276 or S468 naturally induced by TNF stimulation.
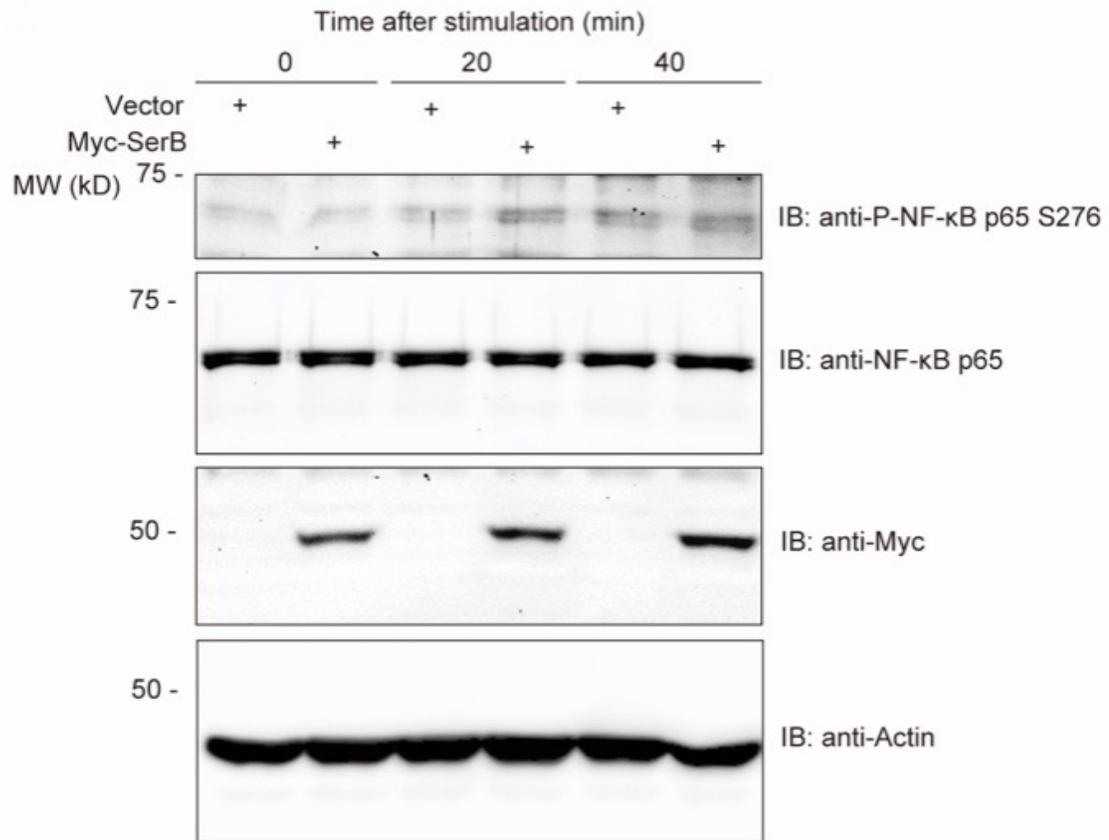
Figure 6A. Effects of ectopic expression of SerB on the phosphorylation of NF-kB p65 S276. TIGKs were transfected with empty vector or Myc-SerB. After 36 h transfection, cells were stimulated with TNF (5 ng/mL) for indicated time periods, then cell extracts were prepared and immunoblotted with the antibodies shown. Actin was used as the loading control. Results are representative of 2 biological replicates
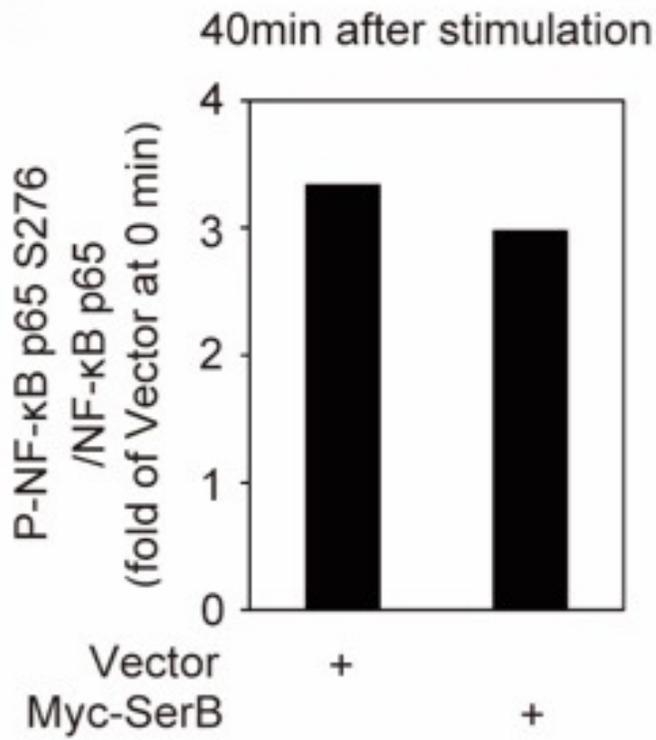
Figure 6B. Effects of ectopic expression of SerB on the phosphorylation of NF-kB p65 S276. Densitometry of immunoblots from (A) showing the ratio of phospho-NF-kB p65 (S276) relative to total immunodetectable NF-kB p65.

Time after stimulation (min)

	0	20	40
Vector	+	+	+
Myc-SerB	+	+	+

MW (kD)

75 - IB: anti-P-NF-κB p65 S468

75 - IB: anti-NF-κB p65

50 - IB: anti-Myc

50 - IB: anti-Actin
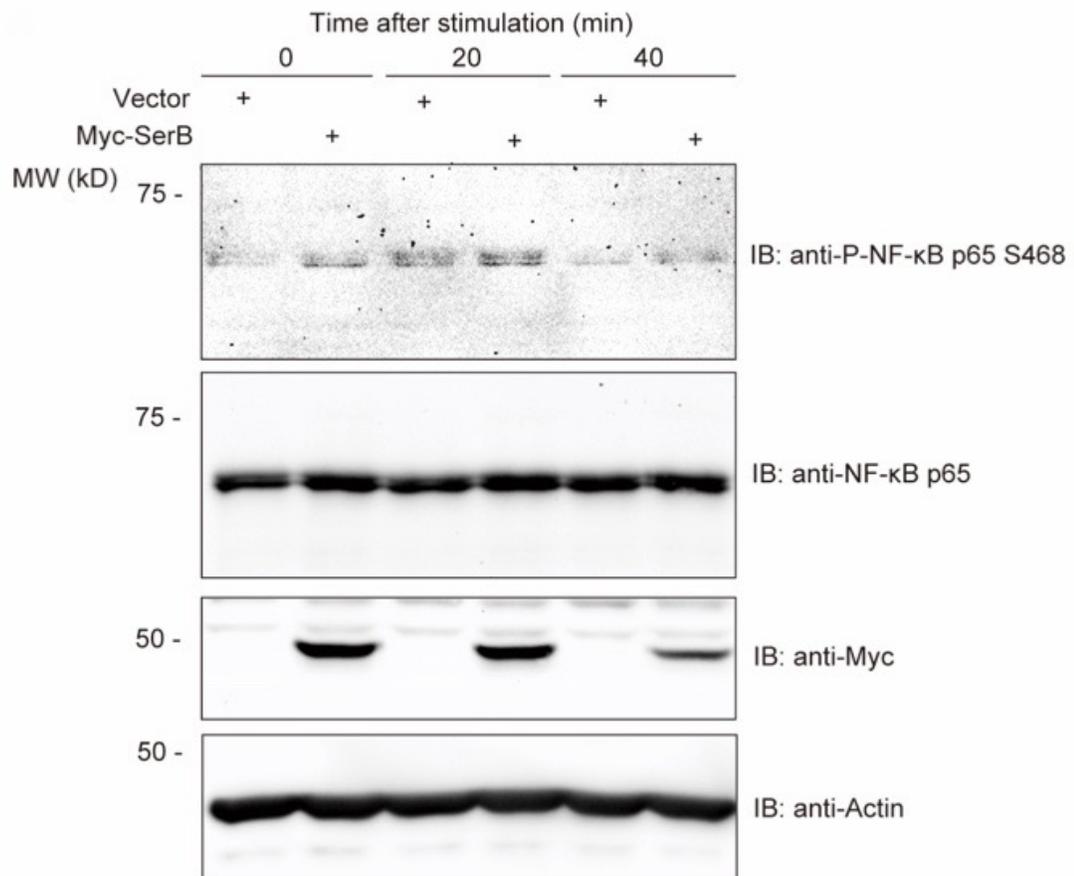
Figure 7A. Effects of ectopic expression of SerB on the phosphorylation of NF-kB p65 S468. TIGKs were transfected with empty vector or Myc-SerB. After 36 h transfection, cells were stimulated with TNF (5 ng/mL) for the indicated time periods. Cell extracts were then prepared and immunoblotted with the antibodies shown. Actin was used as a loading control. Results are representative of 2 biological replicates.
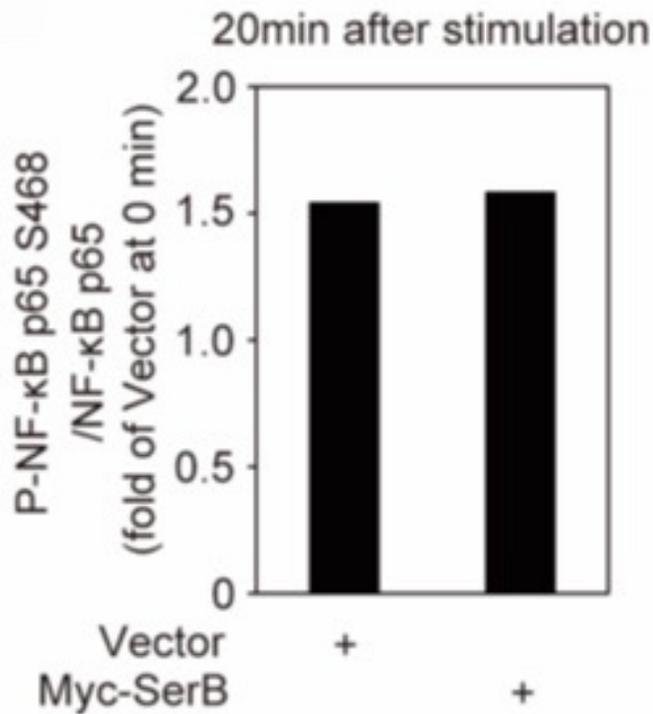
Figure 7B. Effects of ectopic expression of SerB on the phosphorylation of NF-kB p65 S468. Densitometry of immunoblots from (A) showing the ratio of phospho-NF-kB p65 (S468) relative to total immunodetectable NF-kB p65

Collectively, these findings illustrate that SerB does not directly target the phosphorylation status of either NF-kB p65 S468 or S276, nor does it impact NF-kB p105 S933. Furthermore, we can conclude that SerB does not affect the processing of phosphorylated p105 into the active form of NF-kB p50. Hence, the action of *P. gingivalis* SerB appears to be specific for the NF-kB p65 subunit, in particular the S536 residue.

4.5 *P. gingivalis* SerB prevents the nuclear translocation of NF-κB p65

Previous studies performed in Jurkat cells have shown that the kinetics of NF-kB p65 nuclear import can be controlled by the phosphorylation status of p65 S536 residue [139]. This led us to postulate that the phosphorylation status of p65 S536 would exhibit this same control over the nuclear import of p65 in gingival epithelial cells, and hence SerB-mediated dephosphorylation of p65 S536 would have biological relevance. We hypothesized that SerB could block the nuclear translocation of p65 in TNF-stimulated TIGK cells. To test our hypothesis, we first transfected TIGK cells with GFP-tagged NF-kB p65 or with GFP-tagged phosphomimic NF-kB p65 S536D mutant. This mutant p65 strain is constitutively active and therefore cannot be dephosphorylated by SerB. TIGK cells were then co-transfected with Myc vector alone or Myc-SerB. All cells were either stimulated with TNF or left unstimulated. Lastly, cells were fixed and stained with DAPI and anti-Myc and the location of the GFP-tagged NF-kB p65 was determined by confocal microscopy (Figure 8A). In Figure 8B we present quantification analysis of nuclear translocation of the cells shown in Figure 8A. In TIGK cells expressing Myc-SerB and GFP-NF-kB p65 and stimulated with TNF, we found 26% of counted cells to be positive for GFP-NF-kB p65 in the nucleus. Conversely, in stimulated TIGKs expressing Myc-SerB and GFP-tagged phosphomimic NF-kB p65 S536D, we found that the number of counted cells positive for nuclear p65 was in fact very similar to the levels seen in TIGKs not expressing SerB. Thus, we can infer that SerB-mediated dephosphorylation of S536 does inhibit nuclear translocation of NF-kB p65.

To further validate our findings that the action of SerB is specific for the NF-kB p65 subunit, thereby ruling out the possibility that SerB might also be targeting other NF-kB complexes we examined the effect of SerB on the nuclear translocation of the p50 and p105 subunits. TIGK cells were transfected with GFP-tagged NF-kB p50 or GFP-NF-kB

p105 fusions and then co-transfected with Myc vector or Myc-SerB. TIGKs were then left unstimulated or stimulated with TNF. Confocal microscopy was used to visualize the nuclear translocation of both the p50 and p105 subunits. As expected, the results from these experiments showed that the presence of SerB did not impact the nuclear translocation of either the p50 or p105 subunits (Figures 8C and 8D). Once again, corroborating our previous findings indicating that the action of this *P. gingivalis* serine phosphatase is specific for the NF-kB p65 subunit.
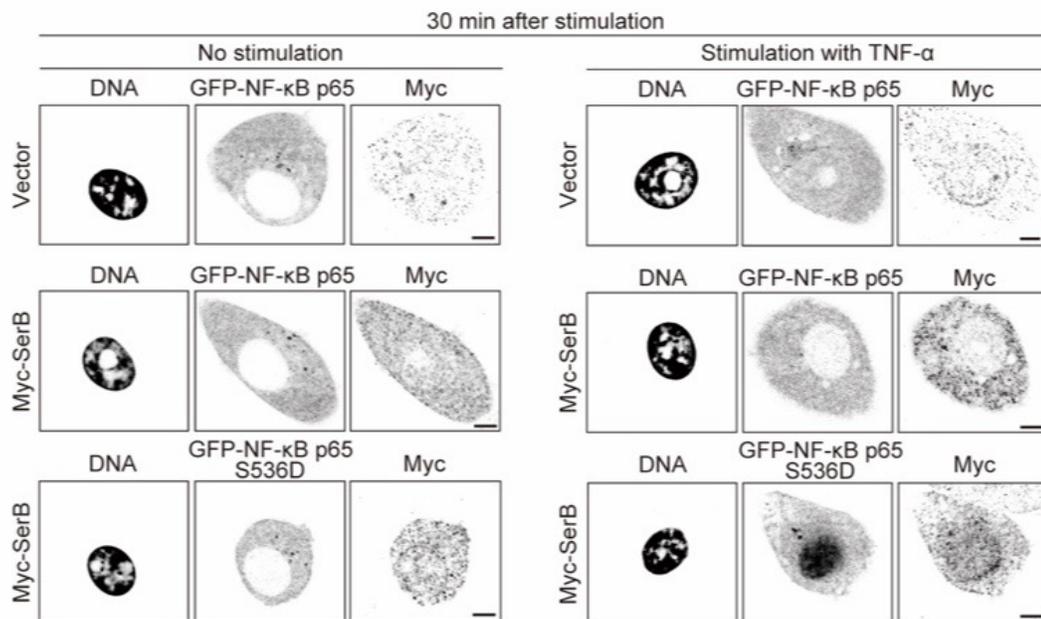
Figure 8A. *P. gingivalis* SerB inhibits nuclear translocation of NF-kB p65 but not p105/p50. Confocal microscopy of TIGKs co-transfected with empty Myc (vector) or Myc-SerB along with GFP-NF-kB p65 or GFP-NF-kB p65 S536D and left unstimulated or stimulated with TNF (5 ng/mL) for 30 min. Cells were fixed and stained with DAPI and anti-Myc. Bars=5 μm. Results are representative of 3 biological replicates.
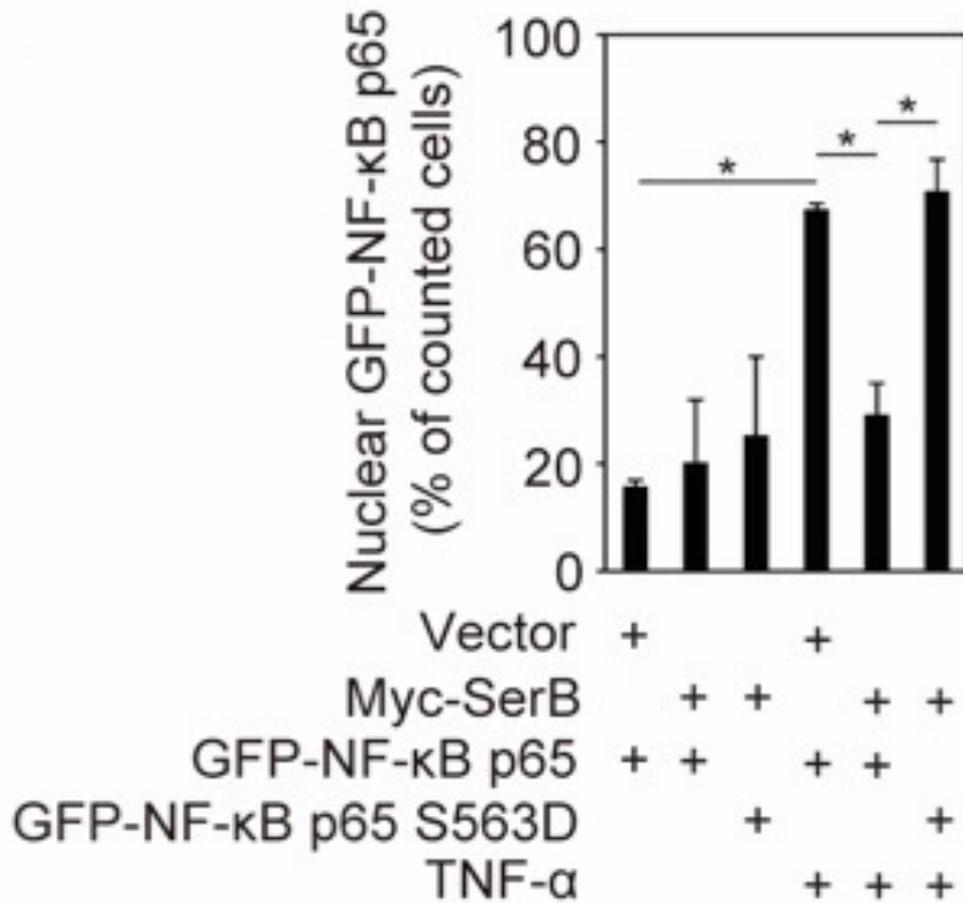
Figure 8B. *P. gingivalis* **SerB inhibits nuclear translocation of NF-kB p65 but not p105/p50. Quantification of nuclear translocation seen in cells from (A). Results are expressed as the percentage of Myc positive cells with nuclear GFP-NF-kB p65 and are the mean with SEM of 3 independent experiments. >/=90 Myc/GFP positive cells were counted per test. *p<0.05.**
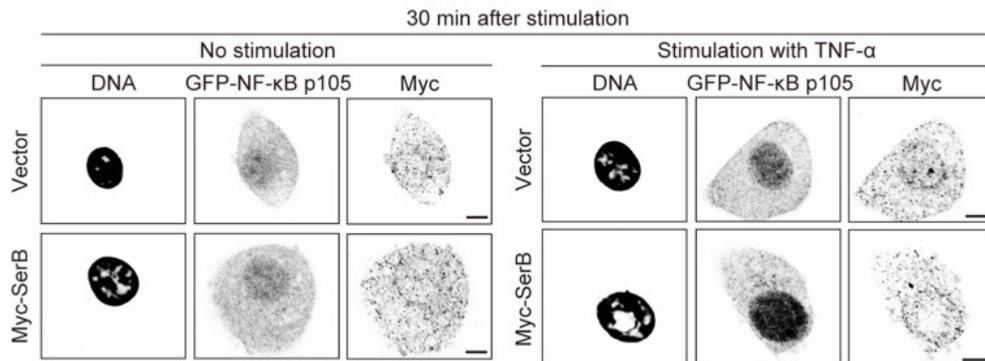
Figure 8C. *P. gingivalis* **SerB inhibits nuclear translocation of NF-kB p65 but not p105/p50. Confocal microscopy of TIGKs co-transfected with Myc (vector) or Myc-SerB along with GFP-NF-kB p105 and left unstimulated or stimulated with TNF (5 ng/mL) for 30 min. Cells were fixed and stained with DAPI and anti-Myc. Bars=5 μm. Results are representative of 3 biological replicates.**
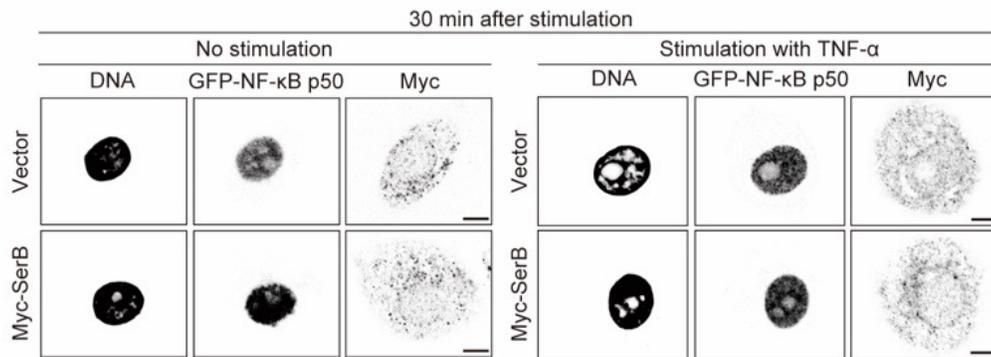
Figure 8D. *P. gingivalis* **SerB inhibits nuclear translocation of NF-kB p65 but not p105/p50. Confocal microscopy of TIGKs co-transfected with Myc (vector) or Myc-SerB along with GFP-NF-kB p50 and left unstimulated or stimulated with TNF (5 ng/mL) for 30 min. Cells were fixed and stained with DAPI and anti-Myc. Bars=5 μm.**

4.6 *P. gingivalis* SerB inhibits the IL-8 promoter response regulated by NF-κB p65

Previous studies have reported the possibility of distinct groupings of p65, which can be distinguished based on their phosphorylation status at S536 residue and on their association with p50 [137]. Additionally, it has also been reported that p65 independent of the p50 subunit is not regulated by IkB proteins and appears to follow a non-canonical pathway. To briefly summarize, there are 2 signaling pathways that lead to the activation of NF-κB, the canonical (classical) and non-canonical (alternative) pathways. The canonical pathway is activated by most physiological stimuli and is responsible for activating the NF-κB dimers comprising RelA, cRel, p50 and RelB. On the other hand, the non-canonical pathway is activated by only a very limited number of stimuli including lymphotoxin B and B cell activating factor. This non-canonical pathway is responsible for the activation of the p100/RelB complexes. These two pathways do, however, share a common regulatory step, and that is the activation of the IκB kinase (IKK) complex. One population of phosphorylated p65 subunits has been shown to translocate to the nucleus independently of p50 and can be recruited to the IL-8 promoter region following stimulation. We speculated that the action of SerB on the NF-kB p65 complex independent of any association with p50 was sufficient to block the recruitment of p65 as well as preventing its transcription of the IL-8 gene. To investigate this, we co-transfected TIGK cells with Myc vector or Myc-SerB, and also with either (1) GFP, (2) GFP-NF-kB p65, or (3) GFP-NF-kB p50. All cells were transfected with a Luciferase reporter plasmid, pIL-8 kB-Luc, to measure IL-8 promoter activity. TIGKs were subsequently stimulated with TNF for 3 h at which point, cells were then measured for IL-8 promoter driven luciferase activity and normalized to Renilla Luciferase. Results shown in Figure 9A are presented as fold induction relative to luciferase activity of our unstimulated control group. We found that the luciferase activity of TIGKs expressing

Myc-SerB was significantly reduced when stimulated with TNF when compared to TIGKs expressing empty Myc vector (Figure 9A). We also studied the effect of excess exogenous p65 or p50 on the IL-8 promoter activity. Accordingly, we found that the presence of exogenous p50 did not impact the SerB-mediated suppression of the IL-8 promoter activity. However, exogenous p65 introduced via GFP-NF-kB subunits into the TIGKs demonstrated the ability to relieve the suppression of the IL-8 reporter as evidenced by an increase in promoter activity from 0.76 to 55-fold induction of the control level compared with TNF-stimulated TIGKs expressing Myc-SerB and GFP (Figure 9A). To validate the relevance of this interaction between SerB and NF-kB p65 in terms of IL-8 production we next examined the amount of IL-8 secreted into TIGK culture media. TIGKs were transfected with Myc vector or Myc-SerB and stimulated with TNF for various timepoints. At each time period the levels of IL-8 present in the culture supernatants were measured by ELISA (Figure 9B). We found that after 2 h of TNF stimulation, IL-8 levels decreased by 74% in TIGKs expressing Myc-SerB. Likewise, after 4 hrs of stimulation, a similar trend was seen as IL-8 levels had decreased by 65% in TIGKs expressing Myc-SerB (Figure 9B). To further corroborate these results we co-transfected TIGKs with either Myc vector alone or Myc-SerB, and with either (1) GFP, (2) GFP-NF-kB p65, or (3) GFP-NF-kB p50. TIGKs were stimulated with TNF and then the IL-8 levels in the supernatants were measured by ELISA (Figure 9C). Once again, in support of our previous findings, exogenous p65 did function to relieve the inhibition of TNF stimulated IL-8 secretion. This trend however was not observed in TIGKs that had been transfected with GFP-NF-kB p50 subunit. Altogether, these findings indicate that exogenous intracellular SerB is capable of preventing IL-8 production. And, most importantly, inhibition of p65 directly corresponds to lower levels of IL-8 production.
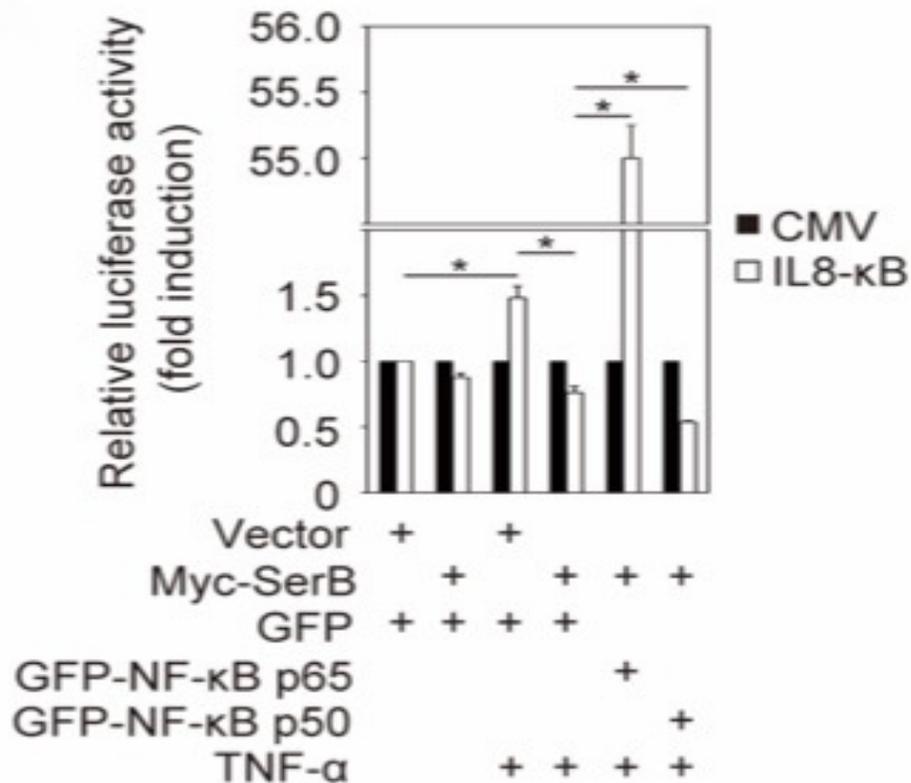
Figure 9A. SerB dephosphorylation of NF-kB p65 inhibits IL-8 promoter activity and IL-8 production. TIGK cells were co-transfected with Myc (vector) or Myc-SerB, and with either of GFP, GFP-NF-kB p65, GFP-NF-kB p50, along with pIL-8 kB-Luc luciferase reporter for IL-8 promoter activity or pRL-CMV Renilla Luciferase control. Cells were stimulated with TNF (40 ng/mL) as indicated and after 3 h stimulation, IL-8-kB luciferase activity was measured and normalized to Renilla luciferase. Results are presented as fold increase relative to the activity of the unstimulated control and are means +/-SD of 6 biological replicates. *p<0.05.
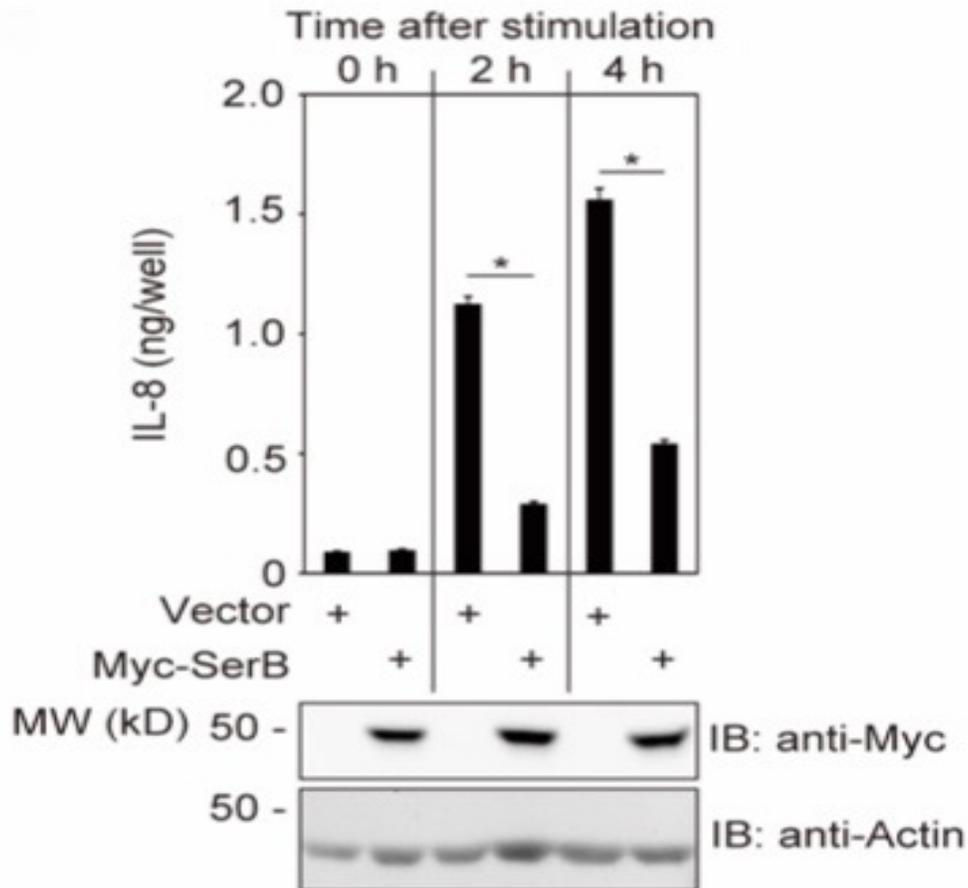
Figure 9B. SerB dephosphorylation of NF-kB p65 inhibits IL-8 promoter activity and IL-8 production. TIGKs were transfected with Myc (vector) or Myc-SerB and at 36 h after transfection cells were stimulated with TNF (5 ng/mL) for the indicated time periods. At each time point, the level of IL-8 in culture supernatants was measured by ELISA. Values are the mean +/-SD of 6 biological replicates. *p<0.05.
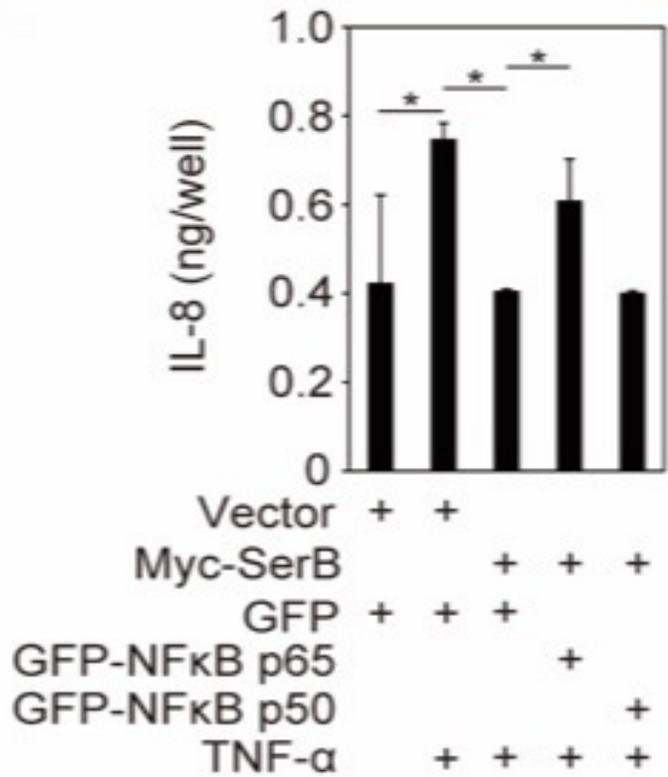
Figure 9C. SerB dephosphorylation of NF-kB p65 inhibits IL-8 promoter activity and IL-8 production. TIGKs co-transfected with Myc (vector) or Myc-SerB, and either of GFP, GFP-NF-kB p65, GFP-NF-kB p50. Cells were stimulated with TNF (5 ng/mL) as indicated and after 4 h the level of IL-8 in culture supernatants was measured by ELISA. Values are the mean +/-SD of 6 biological replicates. *p<0.05.

Consistent with all previous findings we have presented definitive evidence demonstrating that dephosphorylation of NF-kB p65 by *P. gingivalis* SerB serves to prevent its nuclear translocation which in turn inhibits activity of NF-kB p65 on the IL-8 promoter thereby resulting in reduced IL-8 secretion in TIGK cells.

P. GINGIVALIS TYROSINE KINASE-1: EXPERIMENTAL RESULTS

5.1 PGN_1524 is a bacterial tyrosine (BY) kinase present in *P. gingivalis*

Previous studies have shown that PGN_1524, originally identified as a hypothetical protein in *P. gingivalis*, was found to possess proteotypical features of a bacterial tyrosine (BY) kinase. This determination was made from in silico analysis of the genome of *P. gingivalis* 33277 [140]).   These aforementioned proteotypical features are well conserved among prokaryotic BY kinases and include Walker A and B motifs, a transmembrane domain, as well as a C-terminal tyrosine-rich cluster [109, 141]). Comparisons of the amino acid sequences between this hypothetical PGN_1524 protein and a previously identified BY kinase in *E. coli*, Wzc, showed a high degree of similarity. Given this information, our first course of action was to establish whether PGN_1524 possessed tyrosine kinase activity. Initially, we had difficulty attempting to express the full length recombinant PGN_1524 protein in *E. coli* due to toxicity issues. Previous studies have shown that the cytoplasmic domains of BY kinases often possess functional activity so we then decided to work with only recombinant cytoplasmic domain (aa residues 541-821) of PGN_1524, thus circumventing any toxicity issues. We tested this fragment for tyrosine kinase activity by performing a quantitative kinase assay. Our peptide cytoplasmic fragment (Ptk1: 541-821) was incubated with ATP and a poly:GluTyr substrate for 15 min. Measurement of fluorescence intensity indicated the amount of tyrosine phosphorylation activity. The results showed that residues 541-821 (labeled FPtk1 in Figure 10) possessed significant ($p<0.001$) kinase activity when compared to the negative kinase control (Figure 10A). To further test whether this kinase activity was dose-dependent we carried out a subsequent concentration-dependent kinase assay for FPtk1 where we tested fluorescence intensity with varying

amounts of FPtk1 (Figure 10B). Our negative control was kinase buffer. Here we found

that tyrosine kinase activity was strongly dose-dependent (Figure 10B). Based on these

initial findings we assigned PGN_1524 the name of Ptk1, *P. gingivalis* tyrosine kinase 1.

Autophosphorylation as well as substrate phosphorylation are key abilities exhibited by

bacterial tyrosine kinases [109, 142].   Thus, we explored the ability of FPtk1 to

autophosphorylate.   The recombinant purified protein fragment FPtk1: 541-821 was

heavily phosphorylated so we first treated this fragment with alkaline phosphatase. We

then added exogenous ATP along with a phosphatase inhibitor cocktail. Samples were

collected and prepared for Western blot analysis.   Immunoblots were probed with

phosphotyrosine antibodies. Our findings showed that with the addition of ATP, FPtk1

was restored to its heavily phosphorylated state observed in the untreated control

(Figure 10C). This provides us with two important pieces of information: (1) Ptk1 is

capable of utilizing ATP to autophosphorylate and (2) this autophosphorylation activity of

Ptk1 dwells within the cytoplasmic domain.   This finding was corroborated later by

research from our lab which showed that mutations in any of the Walker A, B, RK and

YC domains present in Ptk1 all prevented autophosphorylation of Ptk1. The Walker A

and B motifs are responsible for nucleotide binding and hydrolysis [143]. The C-terminal

YC domain is comprised of multiple closely spaced tyrosine residues which receive

phosphate groups from the catalytic domain and, in turn, increase kinase activity [107].

The RK cluster, rich in arginine and lysine residues, contributes to catalytic activity [144].
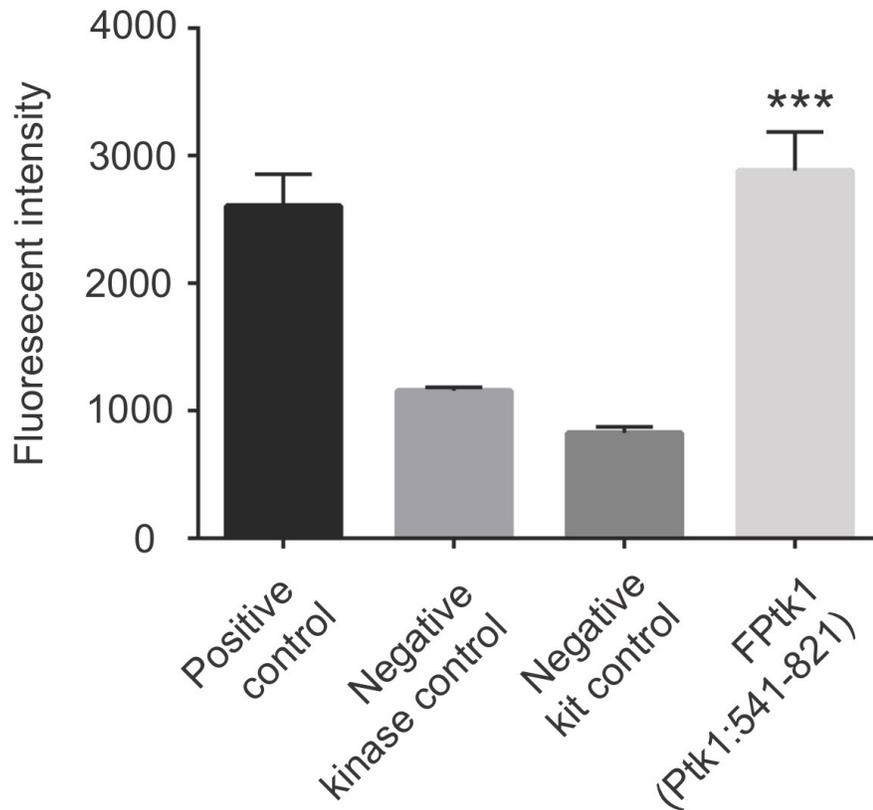
Figure 10A. Ptk1 demonstrates substrate- and auto- phosphorylation. Tyrosine kinase assay with the 541-821 C-terminal aa fragment of Ptk1 (FPtk1, 5 μg). Positive control and negative kit control supplied with the assay kit. Negative kinase control is buffer only. N=3. ***p<0.001 compared to kinase control.
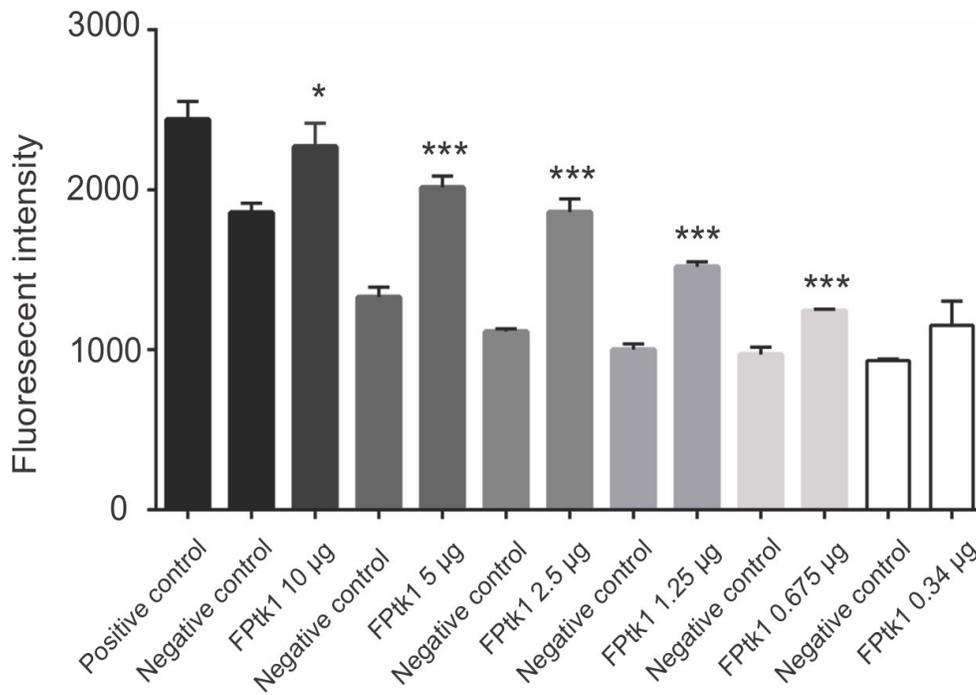
Figure 10B. Ptk1 demonstrates substrate- and auto- phosphorylation. Concentration-dependent kinase activity of FPtk1. Negative control is kinase buffer only. *p<0.05; ***p<0.001 compared to negative control.
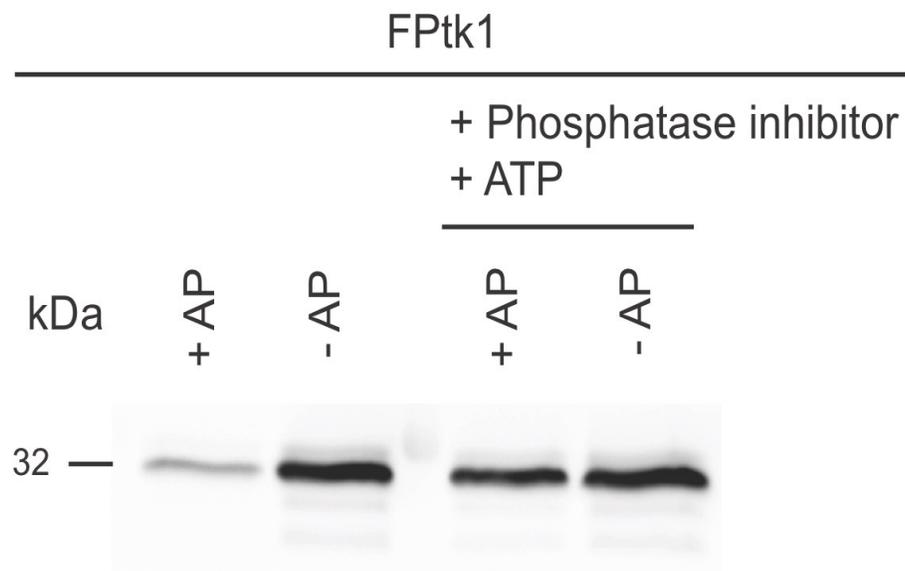
Figure 10C. Ptk1 demonstrates substrate- and auto- phosphorylation. **Autophosphorylation of FPtk1.** **FPtk1 was dephosphorylated with alkaline** **phosphatase (AP) then treated with phosphatase inhibitor and ATP or left** **untreated as a control. Phosphorylated FPtk1 was visualized with Western Blots** **using phosphotyrosine antibodies.**

5.2 *P. gingivalis* tyrosine phosphatase, Ltp1, dephosphorylates Ptk1

Currently, the only annotated tyrosine phosphatase and tyrosine kinase enzymes identified within the *P. gingivalis* 33277 genome are Ltp1 and Ptk1, respectively [135, 140, 143]. Previous research from our lab elucidated the role of tyrosine phosphatase, Ltp1, in *P. gingivalis-S. gordonii* dual species community development [126, 135]. We know that initial contact between *P. gingivalis* and *S. gordonii* cells initiates a streptococcal derived signal propagated through the minor Mfa1-component fimbriae, which activates Ltp1 and in turn, this transduces a signaling event that converges on the downstream transcriptional regulator, named Community Development and Hemin Regulator (CdhR). The main functions of Ltp1 and CdhR are to constrain *P. gingivalis* accumulation within the context of *P. gingivalis-S. gordonii* communities. Furthermore, we know from studies of other systems that these types of phosphatases usually have a kinase cognate pair as has been reported in *E. coli* as well as *S. pneumoniae*. Given that Ptk1 is currently the only identified tyrosine kinase in the *P. gingivalis* 33277 genome this raised our suspicions that perhaps Ptk1 is the cognate kinase of Ltp1 and may also be involved in this signaling cascade regulating the community formation process between *P. gingivalis* and *S. gordonii*. Our first task was to perform an initial mRNA screening of Ptk1 to assess whether it is regulated early on in the interaction between *P. gingivalis* and *S. gordonii*, thereby indicating its importance and involvement. Figure 11 shows the reverse transcriptase PCR of Ptk1 mRNA performed over a period of time in *P. gingivalis* when it is in association with *S. gordonii*. This experiment showed an early transcriptional increase (red bars=upregulation) in Ptk1 mRNA and thus supports our hypothesis that this kinase is important early on during *P. gingivalis-S. gordonii* community development.
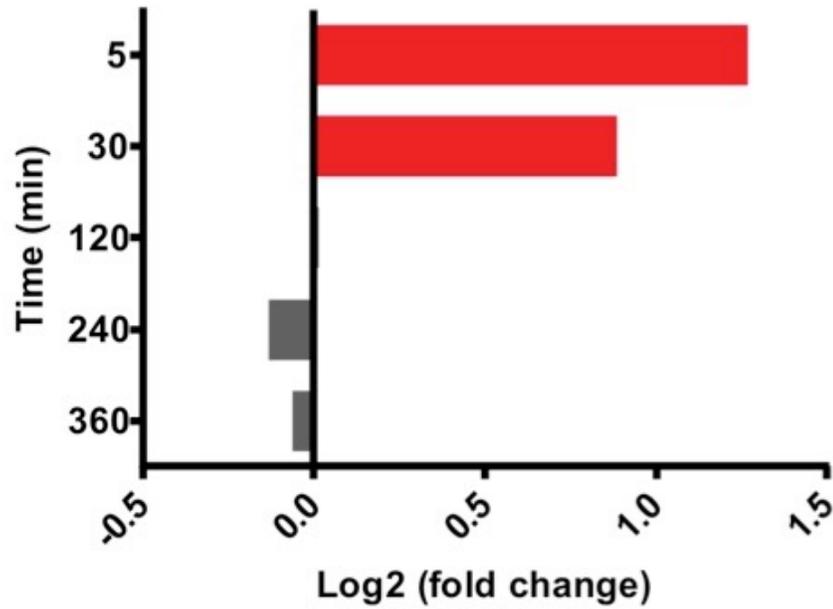
Figure 11. qRT-PCR of Ptk1 mRNA expression in *P. gingivalis + S. gordonii* compared to *P. gingivalis* alone over time. Results are expressed as log$_2$ fold change in *P. gingivalis* -*S. gordonii* compared to *P. gingivalis* alone at the times indicated. Statistically significant higher mRNA levels are represented by red bars.

We then wanted to examine the ability of Ltp1 to dephosphorylate the Ptk1 kinase. We carried this out by performing another tyrosine kinase assay where we found that incubation of the catalytically active domain of Ptk1 (FPtk1: 541-821) with Ltp1 resulted in significantly diminished kinase activity when compared to the FPtk1 group by itself (Figure 12A). When we substituted a Ser for Cys residue in the active domain of Ltp1, rendering it inactive, we found that when the FPtk1 was incubated with this inactive Ltp1 C10S mutant the kinase activity was restored back to normal levels seen previously when only FPtk1 activity was measured (Figure 12A). Consistent with these findings, we also observed reduced tyrosine phosphorylation of FPtk1following reaction with active Ltp1 by western blot analysis, probing with specific phosphotyrosine antibodies (Figures 12B and 12C). Moreover, addition of a phosphatase inhibitor served to block the action of Ltp1 thereby restoring the normal phosphorylated state of FPtk1. These results demonstrate that the catalytically inactive Ltp1 C10S mutant is unable to dephosphorylate FPtk1. In contrast, Ltp1 can successfully dephosphorylate and thus reduce kinase activity of Ptk1. We can conclude that Ptk1 is one of the substrates for Ltp1 and it is the phosphatase activity of Ltp1 which directly affects the kinase activity of Ptk1. Thanks to additional research carried out by our lab, it was determined that Ptk1 could also directly phosphorylate Ltp1, thus suggesting that Ptk1 and Ltp1 can phosphorylate and dephosphorylate each other, providing further evidence to support the notion that they are a cognate kinase-phosphatase pair potentially involved in a feedback mechanism important in regulating various signaling networks [143].
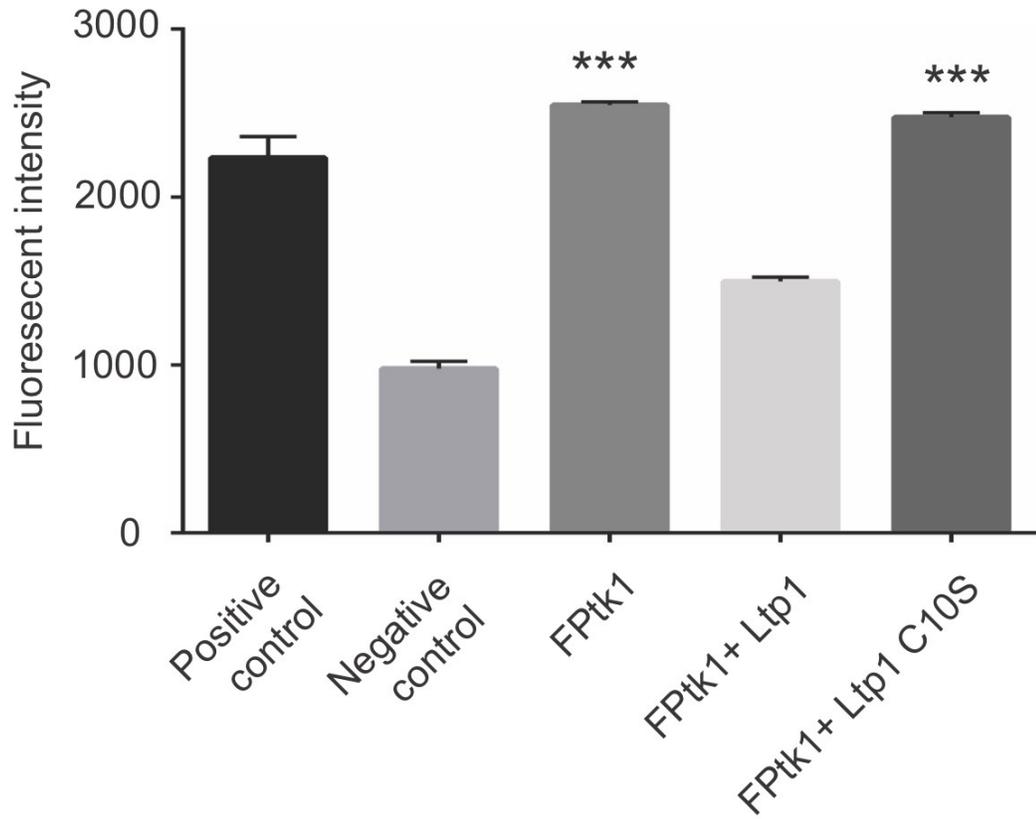
Figure 12A. Dephosphorylation of FPtk1 by Ltp1. Incubation of FPtk1 with Ltp1 results in dephosphorylation. ***p<0.001 compared to negative control.
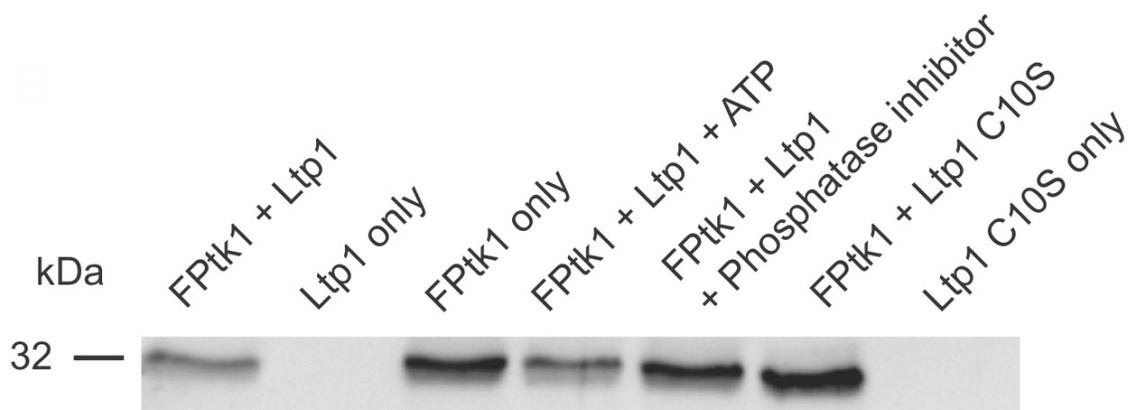
Figure 12B. Dephosphorylation of FPtk1 by Ltp1. Western immunoblot of FPtk1 incubated with Ltp1 or Ltp1 C10S, and with ATP or phosphatase inhibitor as indicated. Phosphorylation levels of FPtk1 determined by Western blots probed with phosphotyrosine antibodies as indicated.
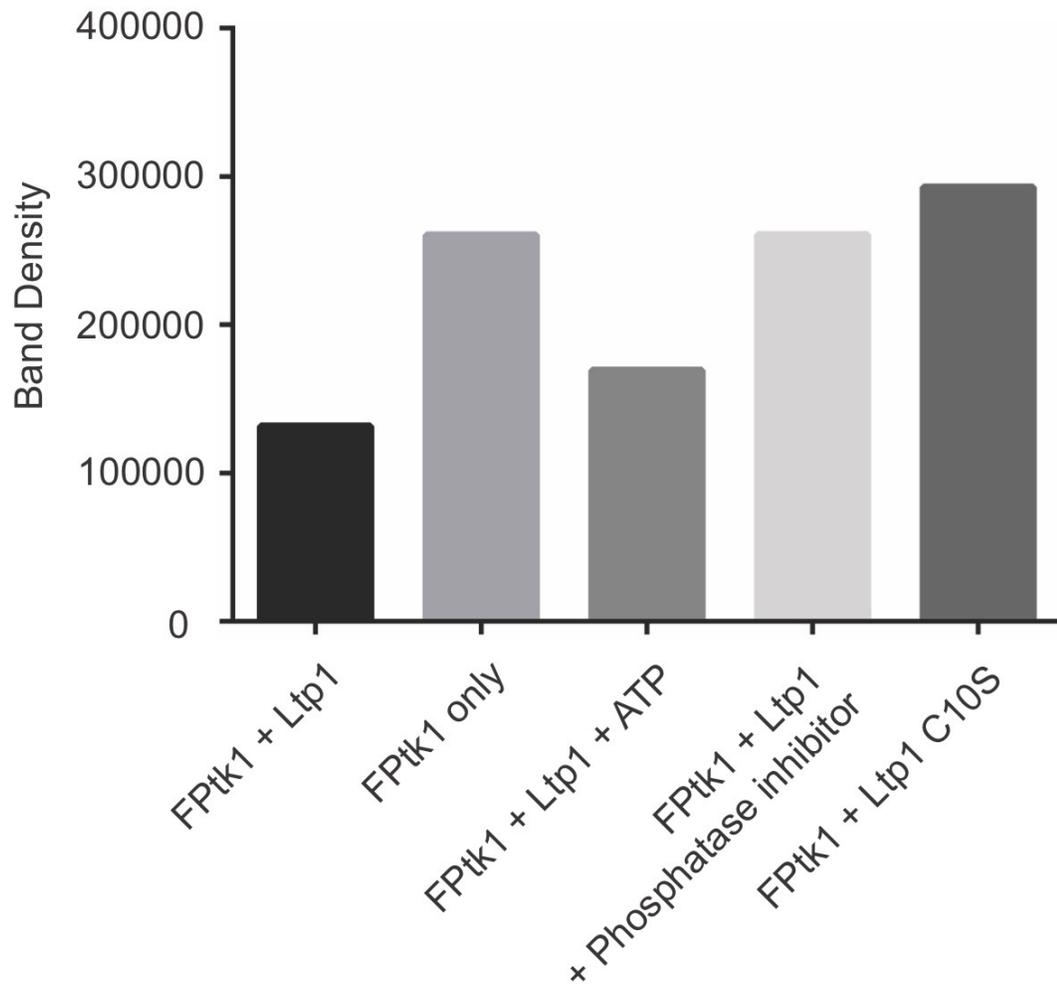
Figure 12C. Dephosphorylation of FPtk1 by Ltp1. Densitometry of immunoblots shown in (B) using ImageJ software. *p<0.05; ***p<0.001 compared to FPtk1+Ltp1.

5.3 Ptk1 is required for optimal *P. gingivalis-S. gordonii* community development

To further investigate the role of Ptk1 in *P. gingivalis-S. gordonii* community development we generated a mutant in the *ptk1* gene and then complemented the mutant with the functional allele in trans. These strains were tested for their abilities to form communities with *S. gordonii*. Since we already know from previous research generated by our lab that Ltp1 phosphatase activity leads to reduced accumulation of *P. gingivalis* with *S. gordonii* [126]), we predicted that this Ptk1 kinase would yield an opposite effect and thereby promote *P. gingivalis-S. gordonii* community formation. The *P. gingivalis* strains, 33277 (WT), Δ*ptk1*, and Δ*ptk1*+p*ptk1* complemented mutant, were all labeled with FITC (green). The *S. gordonii* substrate was labeled with hexidium iodide (red). The *P. gingivalis* strains were then reacted with the *S. gordonii* substrate for 18 hrs and these communities were visualized using confocal microscopy (Figure 13A). We found that with the Δ*ptk1* mutant (no active Ptk1) there was significantly reduced accumulation of *P. gingivalis* on the *S. gordonii* substrate. However, when we complemented the *ptk1* mutant, the levels of *P. gingivalis* accumulation were restored back to those seen when in the presence of *P. gingivalis* WT 33277 strain. We performed a quantitative image analysis of the results from Figure 13A in terms of *P. gingivalis* biovolume and microcolony counts (Figures 13B and 13C). Consistent with our previous findings, when comparing everything to our mutant *ptk1* strain we again see that the presence of Ptk1 promotes *P. gingivalis* accumulation on a *S. gordonii* substrate.
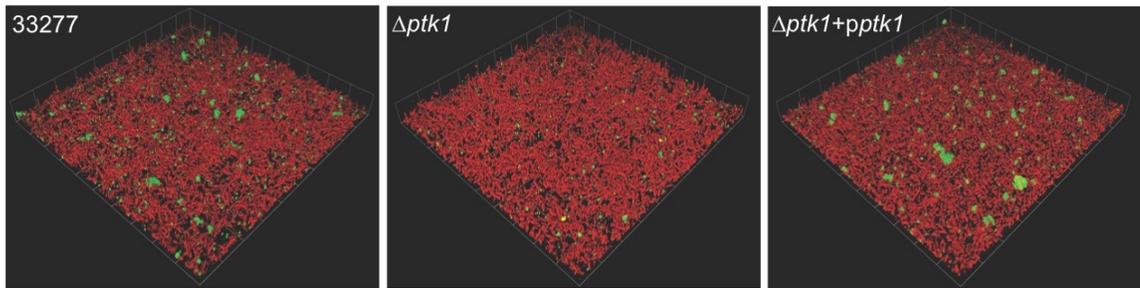
Figure 13A. *ptk1* **is required for maximal** *P. gingivalis-S. gordonii* **community development.** **Confocal microscopy of** *P. gingivalis* **33277, Δ***ptk1***, and Δ***ptk1* **+ p***ptk1* **(green) reacted with** *S. gordonii* **substrate (red) for 18 h.**
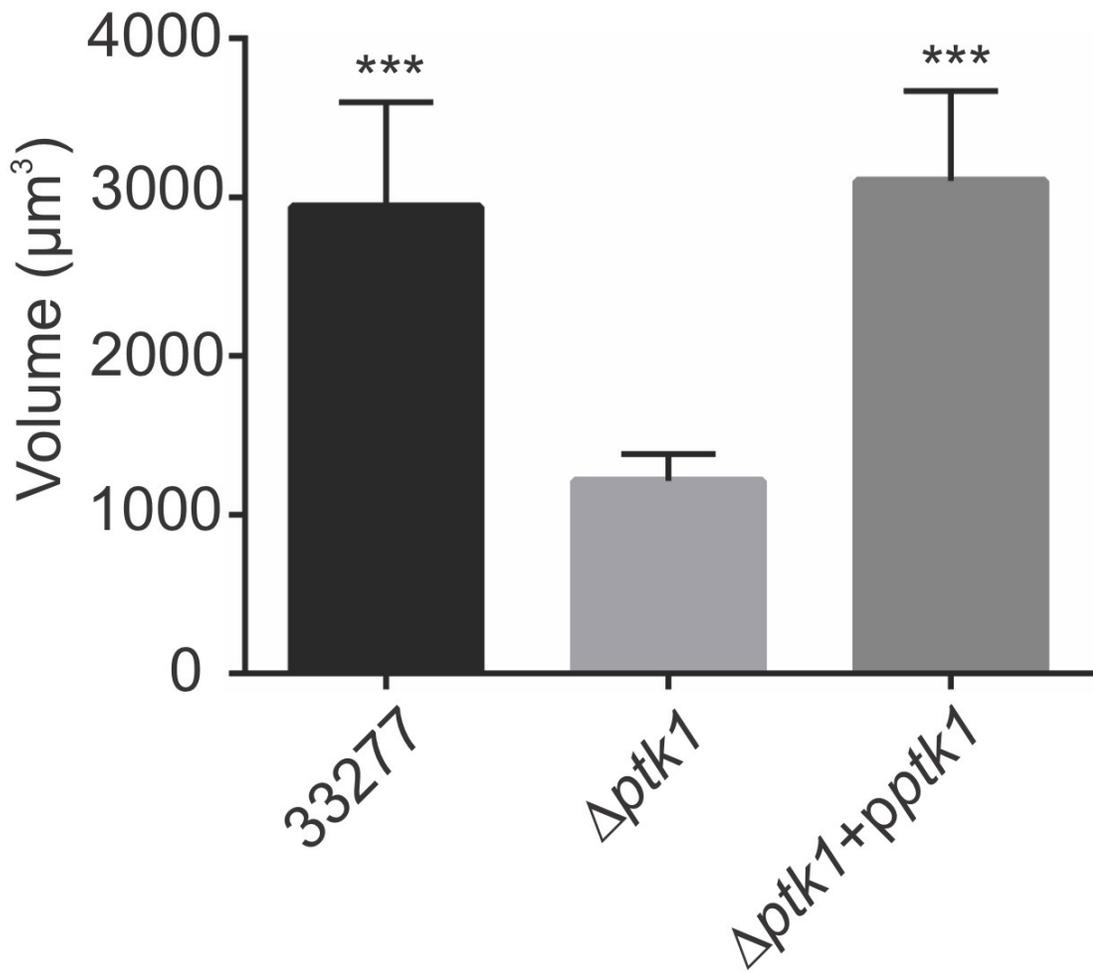
Figure 13B. *ptk1* is required for maximal *P. gingivalis-S. gordonii* community development. Total *P. gingivalis* biovolume was obtained with the 3D Find Objects function of Volocity software.
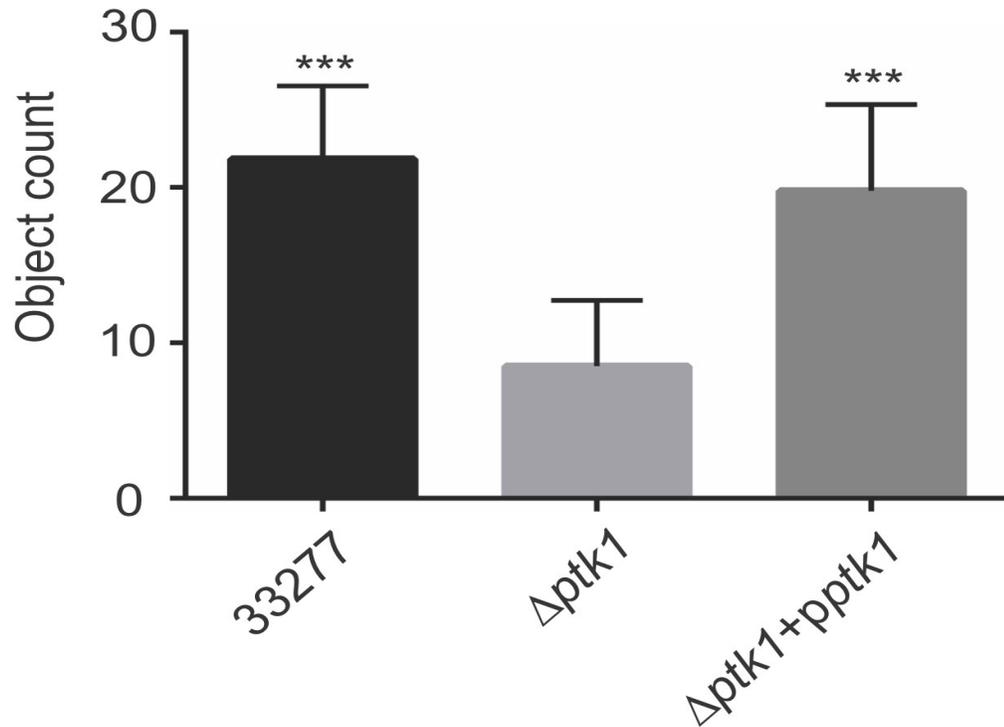
Figure 13C. *ptk1* **is required for maximal** *P. gingivalis-S. gordonii* **community development. Numbers of microcolonies of** *P. gingivalis* **using an object size cutoff algorithm of >30 μm for the total 3D volume for** *P. gingivalis* **fluorescence. Error bars represent SD. N=3; ***p<0.001 compared to Δ*ptk1*.**

CHAPTER 6

DISCUSSION

The ability of microorganisms to communicate and co-ordinate their behavior provides the basis for community living, and confers advantages to the bacterial population as a whole. The mechanisms of biofilm initiation and development have been extensively reviewed [145, 146]. These bacterial communities are comprised of multiple, physiologically diverse bacterial taxa [147]. Mixed microbial communities contain an endless number of inter- and trans-species signaling networks imparting on constituents the ability to collectively regulate activities including gene expression, nutrient acquisition, antibiotic resistance and DNA exchange. The physiology and potential pathogenicity of a mixed species community is contingent on the flow of information throughout the polymicrobial consortia. In a healthy state there is an equilibrium or homeostasis established between the community and the host. However, any significant populational shift towards higher numbers organisms with pathogenic potential, which can be a consequence of host or environmental influences, can lead to disease. Thus, in general, disease ensues when there is an ecological disruption in the normal host-microbe equilibrium. A vast amount of human diseases are caused by polymicrobial infections including periodontal disease, diabetic wound infections, osteomyelitis, urinary tract infections, and angular cheilitis [148]). Chronic periodontitis, the more severe form of periodontal disease, is a chronic inflammatory condition characterized by a continuous cycling between the induction and resolution phases of inflammation, thereby causing significant destruction of the hard and soft tissues of the periodontium. Periodontitis is a polymicrobial infection and one of the most strongly associated periodontopathogens implicated in the progression and severity of this disease is *Porphyromonas gingivalis.*

Porphyromonas gingivalis, a Gram-negative anaerobe harbors many unique virulence factors and pathogenic mechanisms. *P. gingivalis* plays a central role in the initiation and progression of chronic periodontitis [63]. Some of the more well studied pathophysiological properties of *P. gingivalis* include its invasion of epithelial cells, intercellular spread, inhibition of apoptosis in gingival epithelial cells, and suppression of cytokine production such as Interleukin-8 [40, 49, 81, 149]. In addition, *P. gingivalis* has an obligate requirement for iron in the form of hemin for growth, and possesses several hemin acquisition and storage systems with different activities and affinities [27]. The presence of early Gram-positive colonizers such as *Streptococcus gordonii* as well as other Gram-negative opportunistic bacterial species such as *Fusobacterium nucleatum*, favor further colonization by the more pathogenic species like *P. gingivalis. P. gingivalis* relies on these antecedent organisms for its attachment as well as for physiological support [150, 151]. In fact, recent research has identified a potential treatment therapy for periodontal disease through the use of small molecule inhibitors of this attachment. In particular, 1,2,3-triazole based inhibitors were found to effectively block *P. gingivalis* adherence to oral streptococci and thus prevent biofilm formation [152].

P. gingivalis is best described as a host-adapted opportunistic pathogen which possesses the ability to raise the pathogenicity of the entire microbial community. Hence, *P. gingivalis* is often regarded as a "keystone" pathogen [153]. Despite its pathogenic potential, *P. gingivalis* is commonly found in healthy patients [154]. The ability of *P. gingivalis* to persist and thrive in overt disease as well as in the absence of disease indicates the existence of various mechanisms capable of enhancing or restraining *P. gingivalis*' pathogenic potential in the context of a community. One study analyzing the pathogenic potential of *P. gingivalis* when colonized in germ free mice found that while *P. gingivalis* was able to successfully colonize this host, it failed to elicit

periodontal disease in the absence of an endogenous microbiota [155]. Thus, in addition to environmental parameters, *P. gingivalis* virulence is strongly influenced by its other accessory pathogens residing in the community. These other neighboring bacteria can establish the pathogenic potential of the entire community [156]. Furthermore, analysis of virulence gene expression in healthy versus diseased states shows the expression profile changes based on environmental cues and also between individuals [157].

The oral streptococci are a major component of both supra- and sub –gingival microbial communities. While some streptococci are antagonistic toward *P. gingivalis*, *S. gordonii* promotes *P. gingivalis* attachment and colonization. This is primarily due to the presence of a BAR motif found in streptococcal Ag I/II family adhesins that supports *P. gingivalis* attachment through its Mfa fimbriae [158, 159]. *P. gingivalis-S. gordonii* heterotypic community formation is initiated by the binding of the streptococcal SspA/B surface protein to the *P. gingivalis* Mfa1 fimbrial adhesin [159-161], and *P. gingivalis* exhibits mutualistic growth with *S. gordonii* in biofilm communities [162]. Interestingly, when *P. gingivalis* is exposed to tobacco smoke extract, it shows increased expression of FimA which enhances initial binding interactions and ultimately strengthens the Mfa1-SspB binding which promotes community formation [163]. Thus, both theoretical and experimental frameworks exist for enhanced pathogenicity of *P. gingivalis* when in a heterotypic community with *S. gordonii*. In vivo support comes from experiments where mice infected with *S. gordonii* followed by *P. gingivalis* displayed significantly greater bone loss compared to mice infected with either organism alone [75].

Inflammatory cytokines play a pivotal role in the control of infection and the host innate immune response to pathogens. Cytokines are important chemical messengers that function as immunomodulating agents and are primarily produced by immune cells.

Chemokines, a type of cytokine, are particularly important in that they can direct the chemotaxis of immune cells to respond to different environmental triggers or stimuli. Cytokines, both pro-inflammatory and anti-inflammatory, are largely responsible for regulating the host immune response to infection. The pro-inflammatory cytokines are necessary for controlling the initial infection. The acute inflammatory response is the initial protective response to bacterial pathogens, foreign stimuli or injury. This early stage is characterized by increased blood flow, vascular dilation, enhanced capillary permeability and increased recruitment of leukocytes into tissues. Some of the more commonly studied pro-inflammatory cytokines include TNF, IL-1β, IL-1α, IL-6, IL-8, and IL-17. These stimulate systemic inflammation. The secretion of these chemokines directly recruits and activates polymorphonuclear leukocytes (e.g. neutrophils) and macrophages. Neutrophils are paramount here as they are the most common leukocyte recruited to periodontal pockets [30]. The second grouping of cytokines, the anti-inflammatory cytokines, help to prevent any undesirable or harmful side effects of an exaggerated inflammatory reaction [164]. In general, the pro-inflammatory mediators are often associated with tissue damage and destruction; while the anti-inflammatory mediators have the ability to counteract this pro-inflammatory effect and ultimately arrest disease progression. Anti-inflammatory cytokines work to resolve the inflammation by suppression of inflammation. The net effect of these cytokines is to direct and coordinate the host immune response in a balanced way. Anti-inflammatory cytokines are often identified as "pro-resolving mediators" in the literature, as they can effectively halt disease progression through restricting leukocyte trafficking to inflamed sites, coordinating the clearance of expended leukocytes, and reversal of the signs of inflammation [12]. This balancing act between anti- and pro-inflammatory cytokines is a crucial element in regulating the overall inflammatory response because an overabundance of pro-inflammatory secretion can lead to severe tissue damage. The

disease progression and tissue destruction evident in periodontal disease is due to the periodontitis associated bacteria that exploit the normal secretion of inflammatory cytokines. Periodontal inflammation is not only a means by which host tissues are destroyed, rather it also functions as a means by which the periodontal bacteria can derive a wealth of nutrients important for their survival and persistence. It is this type of damage that culminates in the periodontitis phenotype characterized by a heightened and chronic inflammatory response that remains unresolved [165].

We know that the presence of bacteria alone is not sufficient for the progression and severity of periodontitis; rather it takes an immunologically susceptible host as well. The prolonged and persistent dysregulated host immune response to the polymicrobial biofilm communities is likely the primary cause of the tissue damage. This overwhelming and chronic inflammatory response by the host helps the persistence of periodontal bacteria as it inadvertently generates a continuous flow of nutrients supplied to the offending bacteria and this allows them to flourish and thrive. *P. gingivalis* has evolved to exploit this heightened inflammatory response to serve its own nutritional needs. It is the persistent host inflammatory response targeting pathogens that results in the irreversible destruction. Yet, by prolonging inflammatory reactions in the host, pathogens such as *P. gingivalis* ensure a continuous supply of host derived nutrients. In periodontally healthy subjects, both the innate and adaptive branches of immunity are functioning optimally, operating simultaneously in harmony. This establishes a neutral relationship between the human host and the microbiota. While the histopathogenesis of periodontal disease involves responses by the innate and adaptive branches of the immune system, it is the innate immunity that provides the first line of defense against any type of microbial challenge or insult. Even in the absence of disease, the host maintains control over commensal microbial inhabitants through its continuous and

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coordinated gradient of various cytokines/chemokines and cell adhesion molecules throughout the epithelial cell layers.

The primary resident cells involved in the host innate immune response include epithelial cells, periodontal ligament fibroblasts, osteoblasts, and dendritic cells [166]. In gingival epithelial cells (GECs), *P. gingivalis* inhibits the production of Interleukin (IL)-8, a member of the CXC (Cysteine X Cysteine) family of chemokines and neutrophil chemokine, along with the T-cell chemokines IL-10, IL-9, and IL-11 [40, 167], a phenomenon known as Localized Chemokine Paralysis. Conversely, *P. gingivalis* induces increased levels of secretion of IL-1β and IL-6 in GECs [168], and thus has both 'stealth-like' and pro-inflammatory properties depending on context. Recent research indicates that *P. gingivalis* gingipains play a role in modulating the innate immune response. Tada *et al*. found that *P. gingivalis* increased the expression of IL-33 mRNA and protein in GECs in a gingipain-dependent manner [169]. IL-33 is an important cytokine because it is also involved in regulating the inflammatory responses. In particular, IL-33 enhances Th2 cytokine mediated inflammation. Yet another recent study concluded that *P. gingivalis* (W83 strain) was a strong inducer of IL-6 secretion in monocytes only when all gingipain activity was entirely absent [170]. In fact, this study found that active gingipains degraded cytokines, as the accumulation of IL-6 was inversely dependent on the presence of active gingipains. The remarkable ability of *P. gingivalis* to differentially regulate the expression of various cytokines, helps to facilitate the dysregulation of the innate immune response. Despite its broad impact on a wide range of cytokines, *P. gingivalis* can also employ a much more targeted and selective approach when regulating individual cytokines, such as IL-8, produced by macrophages, epithelial cells, and endothelial cells among others. When epithelial cells secrete IL-8, this results in the recruitment of neutrophils and an increase in monocyte adhesion in

blood vessels. Neutrophils are continually being recruited into the subgingival area, and thus are very important for constraining the microbial challenge. This continuous gradient of neutrophils effectively helps to keep the bacterial community under control as they are responsible for inducing the secretion of many pro-inflammatory cytokines.

Considering the central role NF-κB plays in innate immune responses and regulation, it is not surprising that many different human pathogens have evolved a plethora of mechanisms to interfere with NF-κB activity and use this to their advantage [171]. For example, one common target of the NF-κB pathway is the IκB kinase complex (IKK), which is involved in the initial activation of NF-κB. *Listeria monocytogenes*, an intracellular pathogen capable of infecting both human and animal hosts, specifically targets this IKK complex to modulate innate immune responses. *Listeria monocytogenes* secretes the InlC protein intracellularly which directly interacts with a subunit of the IKK complex, IKKα. The interaction of InlC with IKKα blocks phosphorylation of IKKα, thereby restricting nuclear translocation of NF-κB and thus preventing transcription of genes [172]. The pathogen *Yersinia pseudotuberculosis* secretes an effector protein, YopJ which binds and acetylates the IKKβ inhibitory proteins. This prevents phosphorylation and degradation of the IKK complex thereby resulting in inhibition of NF-kB activation [173]. *Enteropathogenic E. coli* (EPEC) secrete effector proteins, NleH1 and NleH2, which attenuate the degradation of the IkBα subunit by preventing its ubiquitination. Ultimately this leads to inhibition of NF-κB activity and, in turn, a down regulation of pro-inflammatory cytokine secretion [93].

Bacterial effectors can also target components of the NF-κB signaling pathway both upstream and downstream of the IKK complex. While many of the mammalian NF-κB subunits are Rel proteins, other studies have identified a non-Rel subunit of NF-κB

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complexes, ribosomal protein S3 (RPS3), which is responsible for regulating NF-κB specificity for certain target genes [174]. The EPEC effector protein NleH1 can bind to this RPS3 subunit and prevent its phosphorylation [175]. This strategy allows EPEC to selectively inhibit the transcription of downstream RPS3-dependent target genes, amongst which include IL-8 and TNF. The novelty of this strategy is that the regulation or inhibition of those select target genes can be accomplished while at the same time no other NF-κB dependent gene expression is influenced. *Shigella flexneri* uses a Type 3 secretion system to secrete the effector protein OspG which is a kinase that binds ubiquitin substrates, thereby preventing degradation of phosphorylated inhibitory proteins such as IkBα. In doing this, activation of NF-κB is inhibited [176].

The serine phosphatase SerB protein of *P. gingivalis* plays a vital role in the entry and survival of *P. gingivalis* within GECs [127]. *P. gingivalis* SerB belongs to the haloacid dehalogenase (HAD) superfamily of hydrolytic dehalogenases [127]. SerB is required for optimal invasion of gingival epithelial cells, as well as IL-8 inhibition [42, 127, 136]. The results presented here have provided a much closer look into the role of *P. gingivalis* SerB in IL-8 suppression. Our data generated several layers of evidence supporting the concept that SerB antagonizes IL-8 production by dephosphorylating the serine-536 residue of the p65 subunit of NF-κB. Firstly, infection of epithelial cells with a Δ*serB* mutant resulted in lower levels of dephosphorylation of NF-κB p65 S536 as compared to the levels of dephosphorylation seen with infection by the *P. gingivalis* WT (33277) strain. Further, infection with the Δ*serB* mutant resulted in increased levels of IL-8 promoter activity when compared to the parental *P. gingivalis* strain. When expressed ectopically, SerB successfully bound to and dephosphorylated the NF-κB p65 subunit in epithelial cells that were stimulated with TNF. Exogenous SerB did, in fact, thwart the nuclear translocation of p65. And lastly, SerB was found to suppress IL-8

promoter activity and blocked IL-8 secretion from epithelial cells. These results describe for the first time a novel NF-κB subversion tactic employed by a periodontopathogen. *P. gingivalis* SerB can specifically dephosphorylate the p65 subunit at serine residue 536, thereby preventing its nuclear translocation. Previous studies have shown that phosphorylation of the p65 subunit at the serine 536 residue led to increased nuclear translocation and activity of NF-κB in monocytes and macrophages stimulated with lipopolysaccharide (LPS) [177]. Of course there is still ongoing research to better understand the full extent to which this phosphorylated serine residue in p65 broadly influences NF-κB regulation. It is believed that the full effect of serine phosphorylation of p65 on NF-κB transcription may vary depending on cell types; in addition, the exact mechanism of phosphorylation may also change (i.e. site-specific phosphorylation) [178]. Phosphorylation of serine residues is just one of many post-translational modifications that can happen to NF-κB and can result in distinct functional and biological consequences. Inhibition of IL-8 accumulation by *P. gingivalis* at sites of bacterial accumulation could have a devastating effect on innate host defense in the periodontium, where bacterial exposure is constant. The host may no longer be able to detect the presence of bacteria and direct leukocytes for their removal. The ability of *P. gingivalis* to selectively target p65 may also generate precision in terms of inhibiting IL-8 without impacting other functions controlled by NF-κB, as the IL-8 promoter is predominantly controlled by the p65 homodimers [134]. Our findings on *P. gingivalis* SerB represent the only current example of a bacterial HAD family phosphatase capable of reshaping innate immune responses by selective dephosphorylation of specific host signaling molecules. Furthermore, these results have provided the molecular mechanisms responsible for the localized chemokine paralysis induced by *P. gingivalis*

at mucosal surfaces; this being the selective targeting of IL-8 transcription carried out via dephosphorylation of p65 by SerB.

In order to fully investigate all aspects of *P. gingivalis* pathogenicity, the second major focus of my research was to evaluate *P. gingivalis* pathogenicity in the context of multispecies biofilms. In particular, the aspects of *P. gingivalis* pathogenicity when in a heterotypic community with the oral commensal *Streptococcus gordonii.* An overwhelming amount of literature pertains to monospecies biofilms and the molecular mechanisms underlying the development of these structures. However, there is substantially less research available on the complexities of multispecies biofilms as they relate to human disease and pathogenesis. However, a burgeoning interest in the complex mechanisms underlying multispecies bacterial biofilm communities has generated a surge in research on their implications for human disease. Many microorganisms have evolved to coexist within these diverse communities and often exhibit a harmonious, mutualistic relationship [179, 180]. Evidence for synergistic pathogenicity is emerging. For example, co-culture with *S. gordonii* elevates the pathogenic potential of *A. actinomycetemcomitans*) [181]. This increased pathogenic potential of *A. actinomycetemcomitans*-*S. gordonii* communities has been observed in vivo [179].

Early initiating organisms in the development of multispecies biofilm communities on tooth surfaces are the mitis group streptococci (MGS), that includes *S. gordonii, S. oralis*, *S. sanguinis* and related species [182]. *P. gingivalis* and *S. gordonii*, in particular, have a unique interaction and relationship when taking residence alongside each other. Interspecies binding is effectuated by two sets of interacting adhesins involving streptococcal surface proteins and the major and minor fimbriae of *P. gingivalis.* Interaction of the Ssp and Mfa proteins is required for the subsequent accumulation of *S.*

gordonii and *P. gingivalis* into heterotypic communities [60]. *S. gordonii* can also provide metabolic support to *P. gingivalis*, and *P. gingivalis* exhibits mutualistic growth with *S. gordonii* in biofilm communities [162]. While the full nature of the interspecies cross talk remains to be established, mutation of a large number of *S. gordonii* genes abolishes the ability of the organism to support a heterotypic community with *P. gingivalis* [183].

In community-associated *P. gingivalis*, there is upregulation of *ltp1*, a gene encoding a cytoplasmic eukaryotic-type Low Molecular Weight Tyrosine Phosphatase [125]. Ltp1 is responsible for restricting community development of *P. gingivalis* with *S. gordonii* by upregulating and activating the transcription factor CdhR (Community Development and Hemin Regulator), an orphan LuxR family transcriptional regulator [126, 135]. Once activated, CdhR negatively regulates expression of the *mfa1* gene encoding the Mfa1 co-adhesin [126]. Deletion of the *ltp1* gene, or loss of tyrosine phosphatase activity through expression of a catalytically inactive mutant, increases the level of *P. gingivalis* accumulation with *S. gordonii* [135]. Constraint of community development by Ltp1 may serve to maintain an optimal area:volume ratio [184], or to prevent the community from expanding into microenvironments that are more oxygenated and damaging to the obligately anaerobic *P. gingivalis*.

Preliminary work in the lab using RNA sequencing had shown that a putative tyrosine kinase (PGN_1524, Ptk1) was upregulated early on in the interaction of *P. gingivalis* with *S. gordonii,* immediately before the Ltp1 tyrosine phosphatase was upregulated. The enzymatic activity of this protein was confirmed through its ability to autophosphorylate and to phosphorylate both a small peptide and a *P. gingivalis* protein substrate. This is the first bacterial tyrosine kinase to be identified in *P. gingivalis*. Our findings are also the first to demonstrate the importance of a bacterial tyrosine (BY)

kinase in the signaling pathways that regulate the cooperative interactions in the context of a heterotypic biofilm community. The results showed that deletion of the *ptk1* gene caused a decrease in the levels of dual species *P. gingivalis*-*S. gordonii* community formation. As expected, restoration of active *ptk1* in the Δ*ptk1* mutant, promoted *P. gingivalis* accumulation on a *S. gordonii* substrate. This finding corroborated our initial prediction that Ptk1 would yield an opposing effect and serve to promote community formation, whereas the Ltp1 phosphatase inhibited *P. gingivalis* accumulation. Given the dynamic and opposing interaction between Ltp1 and Ptk1 it appears that these two proteins are involved in a signaling rheostat in the context of community development. We know from previous studies that phosphatases often have a cognate kinase that displays opposing effects. For example, in *E. coli* the BY kinases, Wzc and Etk are modulated by the cognate phosphatases, Web and Etp. These phosphatases are responsible for the cycling of the kinases between heavily phosphorylated and reduced phosphorylated states [185, 186]. Having a phosphatase-kinase cognate pair can confer many advantages to an organism in terms of its ability to selectively fine-tune its activities based on the fluctuating and dynamic changes/needs of its particular microenvironment.

It has been well established that the addition and/or removal of phosphoryl groups from Ser/Thr/Tyr residues is a common mechanism to carry out post-translational protein modifications. These changes serve to control a variety of important cellular functions in both eukaryotes and prokaryotes [112]. In terms of control of heterotypic *P. gingivalis*-*S. gordonii* community development, the studies reported here, along with subsequent work in the lab have led to the following model (Figure 14). Following initial contact of *P. gingivalis* with the *S. gordonii* surface, a streptococcal signal is propagated through the minor fimbria structural protein of *P. gingivalis*, Mfa1. This leads to the

activation of Ltp1 which results in subsequent dephosphorylation and inactivation of Ptk1. One substrate of Ptk1 is the transcriptional regulator CdhR (Community Development and Hemin Regulator), a negative regulator of *mfa1* transcription [126]. Phosphorylation of CdhR by Ptk1 diminishes activity and relieves repression of *mfa1* expression. Increased levels of Mfa1 then will be predicted to enhance interspecies binding and community development. In this manner the activation states of Ltp1 and Ptk1 constitute a signaling hub that controls community development. Recent research by the Lamont lab has also identified a specific metabolite produced by *S. gordonii*, para-amino benzoic acid (PABA) which is produced by the *cbe* gene. When *P. gingivalis* is in close proximity to *S. gordonii*, it can sense the release of this PABA metabolite and in fact takes up this PABA and feeds it into its folate metabolism. The presence of PABA serves to inactivate Ltp1 which means that Ptk1 is no longer inhibited. This increase in Ptk1 activity results in increased expression of Mfa1 since active Ptk1 inhibits CdhR. FimA expression is also increased as active Ptk1 normally induces FimA expression. The end result of *P. gingivalis'* sensing and uptake of PABA results in more attachment and thereby more community development [187]. The cumulative effect of PABA seems to function as a way of priming the *P. gingivalis* cell for physical attachment to the *S. gordonii* cells. Then over time *P. gingivalis* physically engages the *S. gordonii* cell through complementary fit between the Mfa1 and SspA/B proteins. Once *P. gingivalis* is physically attached to *S. gordonii*, this action suppresses the *cbe* gene that makes PABA and this priming effect is turned off and Ltp1 returns to its normal state of activation (refer to Figure 14 below).
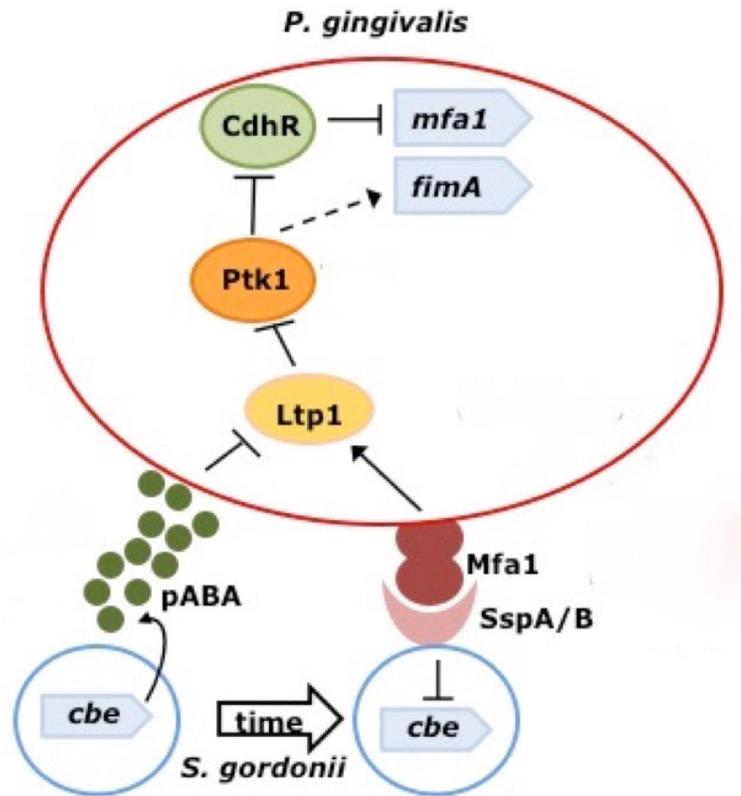
Figure 14. Partial rudimentary model of *P. gingivalis-S. gordonii* signaling related to community development and community pathogenicity

This work has implications for future strategies designed to prevent or treat periodontal infections. Targeting pathways responsible for the development of heterotypic communities may prevent an increase in the number of *P. gingivalis* and maintain the microbial community in a homeostatic state. Another potentially effective measure could involve targeted modulation of signaling pathways within epithelial cells that are subverted by *P. gingivalis* and fuel dysbiosis, such as chemokine paralysis. For example, blocking the action of SerB intracellularly could prevent antagonism of IL-8 production and maintain immune surveillance along with periodontal tissue homeostasis.

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