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INTERLEUKIN-6 PRODUCTION AND EPITHELIAL CELL SIGNALING BY TELOMERASE IMMORTALIZED GINGIVAL KERATINOCYTES IN *PORPHYROMONAS GINGIVALIS* INFECTION

By

Himabindu Vuddaraju

BDS

A Thesis Submitted to the Faculty of the

School of Dentistry of the University of Louisville in

Partial Fulfillment of the Requirements for the Degree of

Master of Science

Department of Oral Health and Systemic Disease

University of Louisville,
School of Dentistry
Louisville, Kentucky

August 2014

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A Thesis approved on

August 5, 2014

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ABSTRACT

INTERLEUKIN-6 PRODUCTION AND EPITHELIAL CELL SIGNALING BY TELOMERASE IMMORTALIZED GINGIVAL KERATINOCYTES IN *PORPHYROMONAS GINGIVALIS* INFECTION

Himabindu Vuddaraju, BDS

August 5, 2014

P. gingivalis is a major periodontal disease causing pathogen. Gingival epithelial cells being the first line of defense, interact and internalize the bacteria evoking a cell signaling response that finally leads to the release of cytokines. IL-6 is a potent pro- inflammatory cytokine, which is one of the responsible factors for bone resorption, a clinical manifestation of periodontitis. The experimental model study hypothesizes that *P. gingivalis* infection increases the production of IL-6 in telomerase immortalized gingival keratinocytes (TIGKs) through up-regulation of c-Jun (AP-1 reporter). We observed the IL-6 production post-infection with *P. gingivalis* WT ATCC33277 and mutants Δ0482, ΔNdk and ΔluxS. The *ndk* gene in *P. gingivalis* controls ATP scavenging and persistence of the bacterium intracellularly. The mutant was therefore expected to be less efficient in the intra

cellular environment. The *luxS* gene in the bacterium plays a role in maintaining several physiological functions like hemin uptake, quorum sensing etc. The mutant was hence expected to show reduced activation of cell signaling pathways and IL-6 production. The cells were infected with bacteria at 4h and 24h time points and concluded that IL-6 production is increased in infected samples, compared to controls. Also, western blots indicated that c-Jun phosphorylation was up regulated in infected samples, providing a possible evidence that *P. gingivalis* stimulates IL-6 production through phosphorylation of c-Jun in gingival epithelial cells, finally leading to IL-6 secretion.

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CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

The Periodontal pathogen *P. gingivalis*

Periodontitis is a chronic multifactorial oral disease which affects a large group of adult population throughout the world. It is an inflammatory infectious disease, where the host immune reponses also play a huge role in its progression. Periodontitis progresses by marked destruction of host periodontal tissue and eventually leads to exfoliation of tooth. The periodontal pathogen, *Porphyromonas gingivalis* is an anaerobic, Gram negative bacterium that is considered to be a major etiological factor in chronic periodontitis. It is a non-spore forming bacterium, belonging to the phylum Bacteroidetes. This opportunistic pathogen is an inhabitant of the sub gingival crevice and is known to cause disease by disturbing the host immune homeostasis (dysbiosis) [1]. *P. gingivalis* grows in vitro by forming smooth, raised colonies on blood agar plates. The colonies appear creamy to white in color initially and turn to black in three to four days with a specific unpleasant odor. The odor has been suggested due to the conversion of L-methionine to methyl mercaptan along with other volatile sulfur compounds that play a role in the virulence of the bacterium [2]. Bacterial colonies turn black due to the uptake of hemin from blood in the plates. *P. gingivalis* is non-motile, lacking any special locomotory organelles. Morphologically, the bacterium appears cocco-bacillar (small rods) and is

asacchrolytic and thus metabolizes the host proteins for its growth and survival. Most of the strains are encapsulated.

P. gingivalis is a stealth pathogen that down regulates specific aspects of host immune responses and thereby survives proficiently by masking the immune system. The pathogen has a wide spectrum of virulence factors including capsule, outer membrane vesicles, lectin like adhesins, lipopolysaccharide, fimbriae and cysteine proteases- gingipains which make it a very potent periodontal pathogen. It is considered a "keystone" pathogen in periodontal disease as it has a disproportionately large effect on its biofilm community when compared to its abundance. The total percentage of abundance of *P. gingivalis* in few cases was found to be less than 0.01% of oral commensal bacteria in humans [3]. The bacterium is also responsible for perturbing the normal host-bacterial inter play in the periodontium. It suppresses the complement system and there by provides an excellent niche for other commensal bacteria to grow and invade further, leading to periodontitis. It is thus associated with the shift in the periodontal biofilm from aerobic organisms to anaerobic perio-pathogens [3]. The capsule of *P. gingivalis* has a significant contribution to virulence of the bacterium. Non encapsulated strains of *P. gingivalis* have shown less virulence in a mouse abscess model, causing localized infection and lacking tissue invasiveness in comparison to their counterparts [4]. The non-encapsulated strains would probably be less efficient in progression of periodontitis due to their reduced ability of both biofilm participation and tissue invasiveness. The capsule increases resistance to

phagocytosis [5]. The capsule polysaccharides (CPS) down regulate the expression of several pro-inflammatory cytokines including IL-6, IL-1β and IL-8 expression levels, thereby compromising the host immune system and enhancing bacterial tissue invasion [6]. *P. gingivalis* has two kinds of fimbriae major and minor. The fimbriae play a critical part in the bacterial virulence. Six variants of major fimbriae are known to exist depending on the genetic diversity of the gene coding them (*fim A*). Type II fimbriae are known to be associated with higher adhesion and invasive potential in comparison to others [7]. The minor fimbriae *(mfa1)* aid in cohesion with other bacteria (*Streptococcus gordonii*) and thereby biofilm development and growth. The lipopolysaccharide (LPS) of *P. gingivalis* is considered to be a potent virulence factor too. The LPS stimulates the host inflammatory pathway by interacting with the Toll like Receptor 4 (TLR 4). Interestingly, the LPS of the bacterium exists in varying chemical structure, each with different properties. The tetra-acylated form of LPS is a TLR 4 antagonist whereas the penta-acylated form is a TLR 4 agonist. The shift from penta-to tetra-acylated forms is regulated by the presence of hemin (increased hemin levels up regulate tetra-acylation) [8].

Gingipains are cysteine proteases that provide maximal advantage to the bacterium in the exacerbation of periodontal disease. More than 85% of the proteolytic activity of the bacterium is accountable to the existence of gingipains [9]. These are encoded by 3 genes *rgpA, rgpB* and *kgp*. RgpA and RgpB specifically cleave at arginine peptides and Kgp at lysine residue. These play a multi-functional role

in the bacterium and are thereby considered vital for the survival of the pathogen. The RgpA is required for the maturation of long/major fimbriae [10]. Thus, gingipains indirectly play a role in the attachment of *P. gingivalis* in the biofilm. These proteases also enhance the non-fimbrial adhesion mechanism by binding closely to epithelial cells, fibroblasts and extra cellular matrix proteins including fibrinogen, type V collagen, laminin and fibronectin [10]. Several studies have shown the involvement of gingipains (mainly RgpA and Kgp) in the co-aggregation and interaction with other bacteria in vivo [9], [11]. The destruction of extra cellular matrix leading to progression of periodontitis is attributed to gingipains [9], [11]. Studies have shown the fragmentation of human extra cellular matrix proteins fibronectin and tenascin-C by gingipains [9], [11]. Additionally, these proteolytic enzymes help the bacterium in nutrient acquisition. Iron uptake by *P. gingivalis* is attributed to the presence of hemagglutinins, which in turn are regulated by gingipains. Furthermore, the assacchrolytic bacterium also obtains peptides as a source of carbon and nitrogen through gingipains. The gingipains, being endonucleases cleave the host proteins which are then converted to di-tripeptides by the respective peptidases. Finally, the di-tri peptides are transported into the cell and then metabolized for energy needs [9]. Gingipains also neutralize and subvert the host immune responses by cleaving several products in the pathway of complement activation in the host.

Pathogenic strains of *P. gingivalis* are active participants of the complex oral biofilms. Bacterial interactions through quorum sensing, cross feeding, co-

aggregation, contact dependent signaling aid in the metabolic exchange, alteration of gene expression and efficient survival of the organism in the biofilms. The interplay not only effectively improves the pathogenicity of *P. gingivalis* but also of the entire community. The initial colonization of *P. gingivalis* in the oral cavity occurs by the adhesion to primary colonizers *Streptococcus gordonii*. The minor fimbrial antigen (*mfa1*) of *P. gingivalis* adheres to the streptococcal antigens I and II [12] , [13]. Biofilm formation experiments on polystyrene micro titer dishes have suggested that minor fimbriae might play a role in micro colony formation by preventing the shearing forces on the bacterium [14]. The interactions also aid in the survival of the bacteria in adverse conditions. *P. gingivalis* being an obligate anaerobe, obtains support from *Fusobacterium nucleatum* (anaerobic bacterium with high oxygen resistance) in aerated and carbon dioxide depleted environment which is unsuitable for its growth [15]. *P. gingivalis* when co-cultured with *Filifactor alocis* (another important bacterium associated with periodontal disease), increases the biofilm formation as well as invasion of epithelial cells by the latter [16]. The ability of the bacterium to co-aggregate and associate with several other bacteria in the biofilm, in turn up regulates the virulence of the entire community.

P. gingivalis, being a facultative intracellular bacterium, invades several host cell types including gingival epithelial cells, endothelial cells and smooth muscle cells [17]. The inter-cellular spreading of *P. gingivalis* is through a unique mechanism. The bacteria spread between the cells through actin based membrane protrusions.

This mode of inter cellular translocation prevents the bacterium from being exposed to the host's humoral immune responses. Studies on the invasion process have suggested the presence of a unique mechanism of invasion into the host. The serine phosphatase enzyme of *P. gingivalis* is involved in this process. The enzyme activity leads to de phosphorylation and activation of cofilin, a protein responsible for actin remodeling through de polymerization [18]. Although the bacterium can spread without cell-to-cell contact, the tight cell contact hastens the invasion process [17]. *P. gingivalis* establishes a long term cohabitation with gingival epithelial cells, which in turn can survive up to eight days after infection with the bacteria[19]. Inside the cell, the bacteria migrate to the peri-nuclear area and accumulate. The bacterium resides in the host cells, overcoming the major challenges like oxidative stress. There is an enormous change in the regulation of genes that code for the production of bacterial virulence factors (including the gingipains), inside the host cell [20].

P. gingivalis is known to regulate several metabolic pathways, within the bacterial cell after infection to be able to maintain harmony with the host cell. There is a shift in the metabolic pathways within the bacterium to reduce the extensive damage inside the host cell. Studies have elicited that the pathways leading to the production of propionate take over the regular butyrate, the latter being more apoptotic [20]. Additionally, the nucleoside diphosphate kinase enzyme secreted by *P. gingivalis* inside the host cell, scavenges the ATP and thereby masks the signal produced for an immune response. Nucleotide diphosphate kinase (NDK) is

secreted extracellularly by the bacterium that consumes the extra cellular ATP produced in response to the pathogen, inside the host cell. Consumption of ATP by the enzyme prevents apoptosis of GECs. Thus facilitating the survival of the bacterium inside the host cell [21]. Several studies have demonstrated that *P. gingivalis* internalizes into GECs by interaction between its fimbriae with integrins present on the cell surface followed by remodeling of actin and tubulin cytoskeleton [22]. *P. gingivalis* selectively targets the components of MAP kinase pathways [23]. The stealth bacterium thus grabs every opportunity to associate with the host initially, for effective disease progression finally.

Telomerase Immortalized Gingival Keratinocytes (TIGKs)

Introduction:

Keratinocytes form a predominant cellular component of the epithelial surface of oral mucosa. The culture of keratinocytes is necessary for wide applications like the treatment of burns, surgical correction of a mucosal defect, in developing model systems for *in vitro* research experiments, gene therapy etc. [24]. The gingival epithelium can broadly be classified as sulcular epithelium, junctional epithelium (which are non-keratinized) and oral gingival epithelium (keratinized). The junctional epithelium lies between the sulcus and the soft and hard tissues beneath it and is structurally unique. The junctional epithelium has wider intercellular spaces that create a pathway for leukocytes to transmigrate into the gingival sulcus. The oral gingival epithelium is stratified keratinized and lines the

gingival sulcus on the outside [25]. The Gingival epithelial cells (GECs) constitute a very important tool in simulating the interaction between periodontal pathogens and host cells *in vitro.* They are the first to encounter the colonizing bacteria in the oral cavity and are thereby, well-known to play an important role in the host-bacterial environment. Several studies have shown the significant role of gingival epithelial cells in the immunological interplay, alongside being the first line of defense *i.e.* barriers against pathogens. The immunological modulation of epithelial cells is essential for the initiation and progression of periodontitis [24].

Epithelial cell responses to *P. gingivalis* infection:

P. gingivalis is known to alter the innate immunity levels at the mucosal surface by altering the epithelial cell signaling in gingival epithelial cells [26]. The serine phosphatase (SerB) of *P. gingivalis* is responsible for suppression of IL-8 (a chemookine) production by altering the NF-kB pathway, which in turn is a very important pathway for inflammation. This is accomplished by the bacterium through de-phosphorylation of Serine 536 residue of P-65 subunit of NF-kB [26]. Furthermore, studies have also shown that the suppression of T-cell chemokines IP-10, Mig (CXCL9) and ITAC (CXCL11) in the gingival epithelial cells infected by *P. gingivalis* in mixed infections by stimulatory bacteria like *Fusobacterium nucleatum* [27]. Also, the bacterium by itself, did not induce the production of T-cell chemokines by transcriptional regulation of the Stat1-IRF1 genes. The decreased production of T cell chemokines is marked by reduced generation and trafficking of effector T cells, thereby leading to immune dysregulation. Studies

have also demonstrated the delay in the normal apoptosis in primary gingival epithelial cells *in vitro* after infection with *P. gingivalis* [28]. The bacterium down regulates pro-apoptosis gene Bax while up regulating the anti-apoptosis genes Bcl-2 and Bfl-1. This is achieved by the activation of JAK/STAT and PI3k/Akt signaling pathways (two important anti-apoptotic pathways) in gingival epithelial cells and culminates by inhibition of Caspase-3, thus prolonging apoptosis and enhancing bacterial survival inside the host cell. *P. gingivalis* infection leads to alteration in the expression of microRNAs in gingival epithelial cells [29]. MicroRNA 203 (miR-203) is one such significant microRNA that is regulated by gingival epithelial cells and affected during *P. gingivalis* infection. MiR203 is responsible for regulation of cytokine induced signaling in gingival epithelial cells. Studies have found that infection with *P. gingivalis* leads to increased expression of miR-203 which in turn suppresses the SOCS3 and SOCS6, responsible for suppressing the cytokine production. Thus, miR203 up regulation leads to overproduction of cytokine IL-6 one of the responsible cytokines that promotes the bone loss in periodontitis.

Primary gingival epithelial cells and their limitations:

Primary gingival epithelial cells are obtained from the gingival tissue of healthy human subjects and then isolated and cultured in the laboratory .Primary gingival epithelial cells have been used as model systems for several studies. However, the cells have a finite lifespan and cease to grow after 9 passages approximately and are then challenged to senescence [30] and stop replicating any further. The

limited life span poses a huge disadvantage in their usage. Also, there can be dissimilarities in their phenotype from donor to donor, questioning their use in *in vitro* experiments [30]. To overcome the issue of finite life span, viral oncoproteins E6 and E7 have been used to immortalize the cell line [31]. The HIGKs showed similar cytokeratin expression profile when compared to the parent gingival epithelial cells. Also, the HIGKs were shown to demonstrate a similar trans-epithelial electrical resistance (a marker to compare cell to cell junctions), like the GECs [31]. Evidences from a study, suggest that *P. gingivalis* when co-cultured with other oral commensals like *S. gordonii*, reduces the pathogenicity of the pathogen (*P. gingivalis*) in HIGKs *in vitro* [32]. Also, the increased rate of proliferation of HIGKs after *P. gingivalis* infection is inhibited by *S. gordonii*. But HIGKs have shown to induce several chromosomal abnormalities at high passages and thus have some limitations as model systems [30], [33].

Techniques of producing TIGKs:

The normal growth of a regular human cell is ceased by senescence in culture, thereby limiting the replicative lifespan of the cell. Senescence, thus leads to a permanent arrest in the cell cycle eventually. Several studies have demonstrated that senescence is triggered by telomere shortening. Telomerase is responsible for maintenance of the telomere length during replication and is present in germ cells and other immortal cancerous cell lines. Studies have suggested that the Human Telomerase Reverse Transcriptase (hTERT) catalytic domain of telomerase, was the active component of the enzyme. Experiments which induced ectopic expression of hTERT in keratinocytes concluded that only hTERT was not sufficient to attain immortality in these cell lines. A close association with an increase in $p16^{INKa}$ protein was found to be responsible for senescence, working independently without being affected by telomere shortening [34]. Evidence has suggested that $p16^{INKa}$ could be repressed by Bmi-1 which indeed prevents senescence in keratinocytes [35]. Thus, a TIGK cell line was produced by transduction with Bmi-1 and hTERT and sub cultured in keratinocyte serum free media.

Comparison of in vitro behavior of TIGK and GECs:

According to a recent study, the GECs and TIGKs when seeded at similar conditions, showed similar patterns of growth and morphological changes [30].

The GECs were slightly ahead of TIGKs in attachment and cytoplasmic spread. However, TIGKs showed similarities in morphology throughout culture. After 72 h of incubation, a monolayer of 80% confluence was achieved by both the cell lines with no chromosomal aberrations at lower passages [30]. The rate of growth was found to be similar. The TIGKs were found to show similar cytokeratin profile when compared with GECs. Both the cell lines expressed cytokeratins 13, 14 and 19, with cytokeratin 13 being expressed in slightly higher levels by GECs and cytokeratin 19 was expressed in higher levels by TIGKs. Neither of the cell lines expressed Cytokeratin 1/10, the cytokeratins that mark differentiation of epithelial cells. In mono layers, TIGKs show all the typical characteristics of the basal cell layer of the keratinized tissue. During prolonged culture, to more than 35 passages (approximately), the TIGKs stratified and still exhibited the behavior of primary gingival epithelial cells induced to stratify. The TIGKs simulate the junctional epithelium phenotype of the gingival epithelium.

From the above comparisons, it can be concluded that TIGKs are an effective and promising cell line to simulate the junctional epithelium *in vitro.* TIGKs retain all the necessary characteristics of the parent cell line including, morphology, karyotype, growth pattern and marker proteins. Thus, they overcome the disadvantage of non-standardization of primary GECs, thus making it easy to reproduce assays.

P. gingivalis Mutants

Δ*Ndk* Mutant (PGN_1018):

P. gingivalis is a stealth pathogen that can replicate very well inside the host cells hidden from the host's immune responses and also by prolonging life. The pathogen does it by down regulating the host's immune responses and activating the anti-apoptotic pathways. Studies have suggested that the pathway involved is the down regulation of P13K/Akt pathway and up regulation of JAK/STAT pathways that govern apoptosis in the host cell [36]. At cellular level, gingival epithelial cells have P2X7 (purinergic) receptors, that are involved in apoptosis and inflammatory cytokine production by binding to the extracellular ATP released at infection sites. Intracellular bacteria like *Mycobacterium tuberculosis* survive successfully due to the presence of ATP scavenging nucleotide diphosphate kinase (NDK) enzyme, to inhibit ATP induced cell death. Recent studies have revealed the PGN 1018 protein of *P. gingivalis* performed a very similar function homologous to the NDK of other intracellular bacteria and is specifically responsible for ATP scavenging [21]. ATP serves as a threat signal to the host cell post-infection and when released, evokes immune response and thereby stimulates the pro-apoptotic pathways. The NDK enzyme, thus consumes ATP and prolongs survival of the host cell from apoptosis and also controls the innate immune responses through suppressing the IL-1β release [36]. The *ndk* deficient mutant was found to be less efficient in suppressing the anti-apoptotic pathway, when compared to wild type *P. gingivalis*. Also, studies have shown that

epithelial cells treated with purified recombinant NDK showed reduction in the ATP- mediated plasma membrane permeability of the host cell in a dose dependent manner. The enzyme could probably be essential for the intracellular survival of the pathogen. Although the NDK enzyme plays a critical role in the intra cellular survival of the bacterium in GECs, not much has been studied regarding its role in cytokine production. We, therefore assumed that ΔNdk mutant will probably produce less IL-6 when compared to wild type bacteria.

Δ*luxS* mutant (PGN_1474):

P. gingivalis depends on hemin and inorganic iron uptake through LuxS/AI-2 based signaling which is regulated by the *luxS* gene [37]. The gene is also known to play a role in quorum sensing (a bacterial inter communication system in which bacterial density is monitored and auto inducers are secreted accordingly) [37]. Studies have suggested the role of auto inducer-2 (AI-2) in differentially regulating the hemin/iron uptake mechanisms in *P. gingivalis*. Expression of *hmuR, feoB1, fetB* and *ftn* are up regulated by AI-2 whereas, *tlr* and *kgp* are down regulated. The differential regulation aids the bacterium to survive in variable hemin availability conditions. HmuR plays a vital role in the uptake between hemin availability in surplus. On the contrary, *tlr* is expressed only while hemin is scarce. Kgp is a protease that degrades heme for easy uptake during low availability of hemin. *Tlr* mutants have shown to show adequate uptake of hemin

when supplies are surplus whereas *HmuR*-null mutants did not show good growth even in the presence of surplus hemin. FetB is a lipoprotein that binds hemin and plays a role in its transportation. FeoB1 is an iron transport protein, which aids in the iron uptake across inner membrane. Iron acquisition has been found to be positively related to virulence of the bacterium, in a mouse model study. Studies have suggested that LuxS is responsible for heterotypic biofilms, between *P. gingivalis* and *S. gordonii* [38].The *luxS* gene in the bacterium plays a role in maintaining several physiological functions like hemin uptake, quorum sensing etc. The mutant was hence expected to effect cell signaling pathways and IL-6 production.

Δ0482 mutant (PGN_0482):

The 0482 protein of *P. gingivalis* is a 23kDa probable immunoreactive antigen consisting of 209aa. Studies have shown the 0482 gene to be down regulated in the LuxS mutant, indicating regulation by LuxS [39]. The down regulation of the 0482 hypothetical protein is independent of the auto inducer-2 (AI-2) signaling activity, which was confirmed when ΔluxS mutant grown in the presence of exogenous 4,5-dihydroxy-2,3-pentanedione (DPD, a precursor of AI-2), did not increase the expression of PGN_0482. As studies have suggested that 0482 gene of *P. gingivalis* is regulated by *luxS* and not much has been studied regarding the

0482 protein, we assumed that Δ0482 mutant might show a similar pattern in IL-6 production as ΔluxS.

Cell signaling Pathways and Targets in IL-6 production

Eukaryotic cells have an organized way to respond to the external stimulus by transmitting the signal from the extra cellular receptors, to the transcription factors located intracellularly, through a cascade of events named the cell signaling pathway. The cell signaling pathways are responsible for secretion of several cytokines. *P. gingivalis*, being an intracellular pathogen, modulates the pathways significantly to aid in its survival. The activation of anti-apoptotic pathway inside the host cell and consumption of e-ATP through nucleotide diphosphate kinase (NDK) enzyme secreted by the bacterium are a few to name [36]. The Mitogen Activated Protein Kinase (MAPK) cell signaling is a very important pathway that plays a role in stress, immune responses, cell survival and differentiation[40] [Figure 1 MAP Kinase pathway]. The pathway inherits its name from mitogenic response to growth factors and also plays a major role in cytokine expression, stress responses, cytoskeletal reorganization (ex. during bacterial internalization) etc. The pathway can broadly be sub-divided into 3 groups- Extra cellular signal regulated Kinases (ERK), P38 MAP kinase [Figure 2 P 38 MAP Kinase pathway] and c-jun N Terminal kinase (JNK) pathways. MAP Kinase pathways have been known to be involved in signaling during invasion by bacteria in monocytes [41].

Studies have shown the differential activation of MAP kinase pathways in *P. gingivalis* infection in human gingival epithelial cells (GECs) by *P. gingivalis*.c-Jun N-terminal kinase (JNK) is activated during invasion. ERK1/2 however, was found to be down regulated by internalized bacteria. P38 was not shown to be activated by *P. gingivalis* [23].

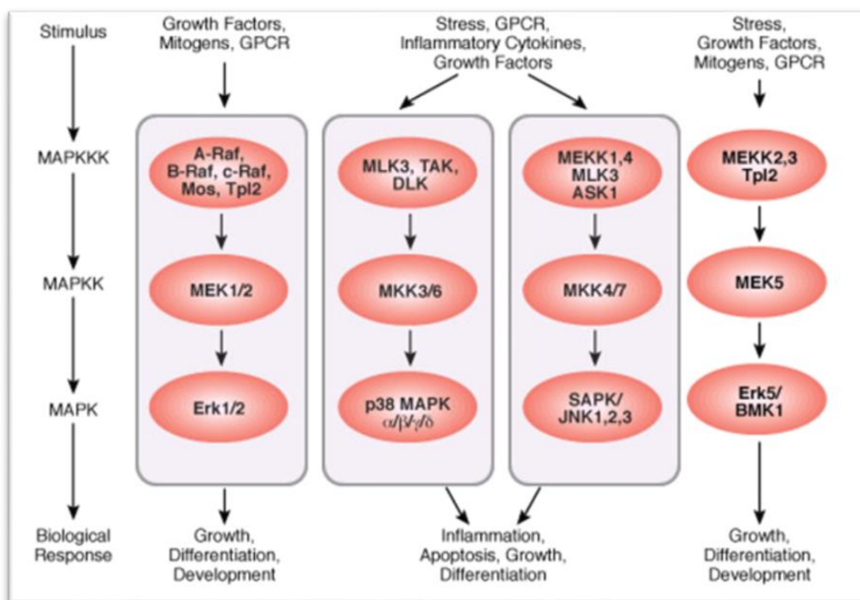

Figure 1 MAP Kinase pathway

The figure represents the MAP Kinase pathway of cell signaling. The pathway can be broadly divided into ERK pathway, p38 MAPK Pathway and JNK Pathway. Picture taken from Cellsignaling.com

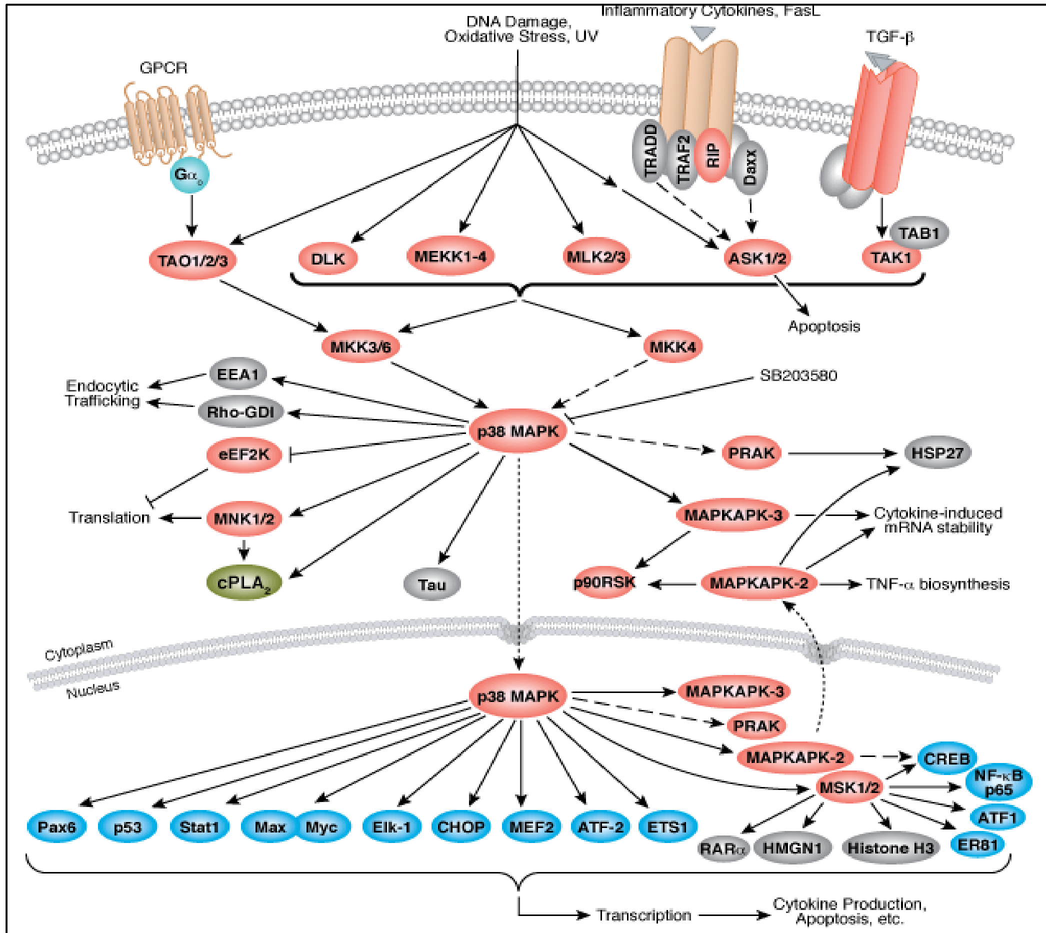

Figure 2 P 38 MAP Kinase pathway

The picture indicates the P 38 MAP kinase pathway (Cell Signaling) with targets P38 and CREB that have been chosen in the study.

IL-6 is a potent pro-inflammatory cytokine. IL-6 secretion in gingival epithelial cells is at least controlled by 3 transcription factors NF-κB, CREB and AP-1. NF-κB and AP-1 when activated, stimulate the secretion of IL-6 through the promoter region of IL-6 but CREB is antagonistic to the cytokine secretion. Studies have suggested that *P. gingivalis* interacts with TLR-2 and C5aR of macrophages (a fragment of the complement protein C5) and the cross talk leads to production of cyclic AMP, which in turn up regulates CREB (transcription factor) that further competes for the targets of NF-κB pathway and thus inhibits the secretion of IL-6 [42].

CREB:

CREB (Cyclic AMP Response Element Binding protein) is a cellular transcription factor of molecular weight 43kDa that is activated by phosphorylation at Serine133 through several pathways like ERK1/2 activation, stress and Ca^{2+} [Figure 3 CREB]. CREB when activated (phosphorylated) is known to compete with p65 sub unit of NF-κB pathway for common transcriptional factors [42]. The NF-κB being an important inflammatory pathway, CREB thus has a huge role in anti-inflammatory pathways in the immune system [43].

Figure 3 CREB (Mol wt- 43kDa)

CREB (Cell Signaling) is a 43kDa transcription factor. It has an anti-inflammatory role and it competes for p65 subunit of NFκB pathway reducing the inflammatory process (Cell signaling).

P 38:

P38 is a 43kDa kinase that participates in the signaling cascade of p38 MAP kinase pathway. Phosphorylation of p38 at Thr180/ Tyr182 leads to several

changes downstream finally activating the transcription factors. Studies have suggested that p38 is not significantly stimulated in gingival epithelial cells during *P. gingivalis* infection [44]. However, studies have shown a strong co relation between p38 MAP kinase pathway and IL-6 mRNA stability in osteoblasts [45]. The half-life of IL-6 mRNA (which is usually short lasting) is elevated in a p38 dependent manner. Inhibitors of p38, have thereby been unsuccessful in stabilizing the IL-6 mRNA in osteoblasts.

ERK1/2:

ERK family is a subgroup under the MAP Kinase pathway that control several cell responses to various stimuli. ERK1/2 is a 42-44kDa dimer consisting of 44kDa ERK1 and 42Kda ERK 1/2 [Figure 4 ERK 1/2]. When phosphorylated the dimer can signal downstream genes and activate several transcription factors like NF-κB and ATF-2. Studies have shown that ERK1/2 is down regulated in *P. gingivalis* infection in gingival epithelial cells[44]. The down regulation was found to increase with increasing time. However, at 5min, the inhibition of ERK1/2 activity was found only at MOI 10

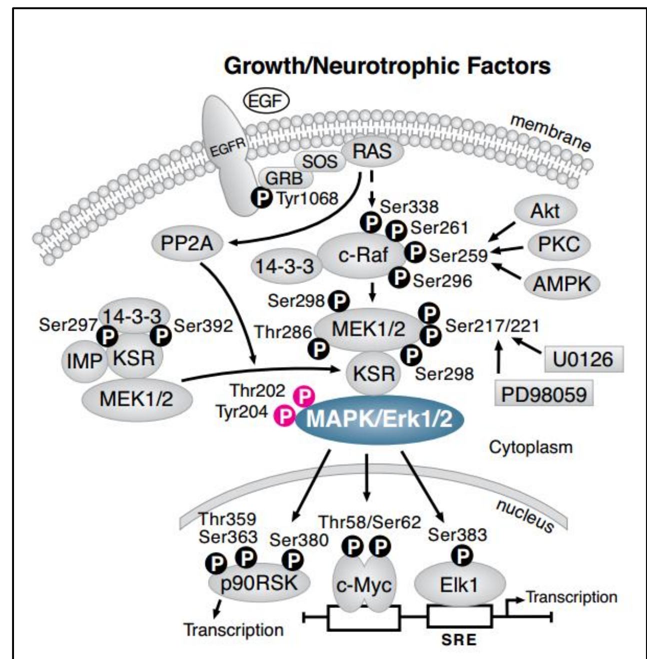

Figure 4 ERK 1/2

The picture represents ERK 1/2 target of MAPK pathway. It is a 42-44kDa protein (Cell signaling)

and higher MOI on the contrary activated ERK1/2. This could probably be due to presence of multiple pathways for ERK1/2 activity depending on the number of bacteria. ERK was not found to play a role in the invasion, as the bacteria could internalize despite ERK inhibition.

C-JUN:

C-Jun is a 48kDa member of the Jun family and is a component of the transcription factor Activator Protein-1 (AP-1). AP-1 had 2 dimers, c-Fos and c-Jun[46] [Figure 5 C-JUN]. c-Jun is activated by the JNK group of MAP kinase pathway. Phosphorylation of SAPK/JNK at Thr183/ Tyr185 leads to activation of c-Jun, which is phosphorylated at Ser63 and then activates the AP-1 reporter. Studies have shown that phosphorylation of c-Jun through JNK and then AP-1 reporter activation leads to the activation of RANKL mRNA expression in osteoblasts during infection by *P. gingivalis* that further leads to bone loss in periodontal disease [46]. The maximal activation of AP-1 reporter explain was found after 15min of infection with the bacteria. Phosphorylation of c-Jun and activation of AP-1 reporter was consistent with the activation of upstream target JNK. Activation of AP-1 leads to secretion of RANKL in osteoblasts, which is associated with bone loss in periodontitis.

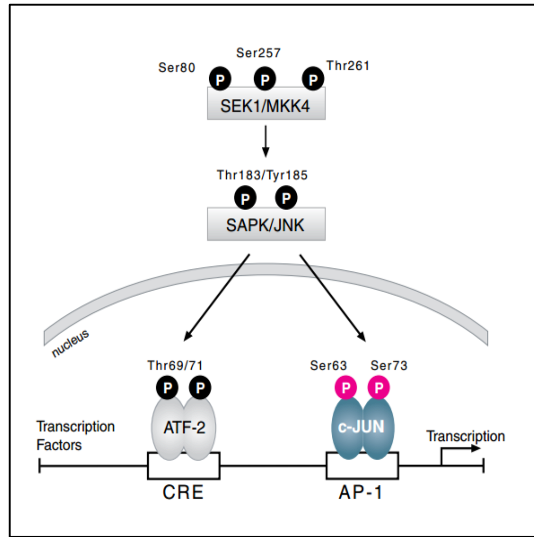

Figure 5 C-JUN

C- Jun is a 48kDa protein that when phosphorylated is activated. Phosphorylation of c-Jun at Ser63 activates the AP-1 transcription factor (Cell Signaling).

CHAPTER TWO: MATERIALS AND METHODS

Bacterial culture

P. gingivalis strains WT ATCC33277 (which is the type strain of *P. gingivalis* and widely studied for understanding the pathophysiological features of the species)[47] and mutants Δ0482, ΔNdk and ΔluxS were cultured anaerobically at 37^0C in Trypticase soy broth (BBL-BD) supplemented with 1 mg/ml yeast extract, 5 ug/ml hemin and 1 ug/ml menadione[30]. 5 ug/ml erythromycin was added to the broth in which the mutants were grown. An overnight subculture of OD~1, without antibiotics was used for infection.

Cell culture

Telomerase immortalized gingival keratinocytes (TIGKs) were grown in Dermalife-K serum free keratinocyte culture medium (Lifeline cell tech.) containing the supplements, Transferrin, rh TGF-α, rh Insulin, Epinephrine, L- Glutamine, Hydrocortisone and Extract-P[29]. Cells were cultured at 37^0C, 5% CO_2 between passages 22- 40 and supplemented with fresh culture media after every 48 h. The cells were seeded into 6-well plates at 20% confluence and infected when 80% confluence was attained (approx. $1x10^6$ cells/well).

Infection Conditions for Western Blots

The TIGKs at 80% confluence were infected with *P. gingivalis* strains WT ATCC33277, Δ0482, ΔNdk and ΔluxS at multiplicity of infection (MOI)-10, 50

and 100 ($1x10^7$, $5x10^7$ & $1x10^8$ bacteria/well in 6- well plates containing $1x\ 10^6$ cells approx. per well. The bacteria were infected for the following time-points, 5 min, 15 min, 30 min and 1 h. Infections were carried out in 6-well plates (2 or 3 wells per sample). Non-infected TIGKs served as a control for all conditions and sonicated lysates (approx. O.D~1 in PBS) of *Fusobacterium nucleatum* were used as a positive control for the experiment (Studies have suggested that *Fusobacterium nucleatum* activates P38 and JNK MAP Kinase pathways in gingival epithelial cells) [48] and others. Protease inhibitors 0.1 mM of TLCK (N-α-tosyl-l-lysine chloromethyl ketone hydrochloride) and 0.1 mM of Leupeptin (Sigma aldrich) were added to all the wells including controls. The cell culture medium was washed post-infection, the cells were treated with 10% Trichloro Acetic Acid (TCA) and lysates were incubated at 4^0C for 20 min, scrapped (using TPP plastic cell scrappers from MidSci) and treated with Lysis buffer (75-150ul) containing 2M thiourea, 7M urea, 1% Triton. BCA protein analysis was done following the protocol of BCA protein assay kit (Thermoscientific). Samples were equalized in concentration, treated with sample buffer, boiled and added to 12% poly acrylamide gels. 15 μg of protein from each sample was loaded into each well. The gels were run for SDS-PAGE at 120 V for 2 h and transferred at 70 V for 1 h on ice. The membranes were blocked with 5% skim milk for 2 h and probed with the respective primary antibodies, phospho-P38, P38, phospho ERK, ERK, phospho CREB, CREB, phospho c-Jun and c-Jun (Cell Signaling) at optimized concentrations at 4^0C overnight [Table 1 Primary antibody optimization

Table] . The secondary antibody anti-rabbit was added at a concentration of 1:5000 for 1 ½ h. Phosphate buffer saline with 0.05% Tween (PBST) was used to wash the membrane in between primary and secondary for 5 min each and 3 washes. Blots were viewed using Enhanced Chemiluminescence substrate (ECL-Thermoscientific). Blots were probed with β-Actin antibodies as the loading control. The densitometry comparison was done as Phospho/Total of each target using Image lab, Biorad XRS+ imaging system.

Table 1 Primary antibody optimization Table

S.No	Primary Antibody	Molecular wt	Company name	Concentration used
1	P-CREB	43kDa	Cell Signaling	1:2000
2	CREB	43kDa	Cell Signaling	1:2000
3	P-ERK	42-44kDa	Cell Signaling	1:5000
4	ERK	42-44kDa	Cell Signaling	1:5000
5	P-P38	43kDa	Cell Signaling	1:2000
6	P38	43kDa	Cell Signaling	1:2000
7	P-C Jun	48kDa	Cell Signaling	1:1000
8	C-Jun	48kDa	Cell Signaling	1:1000
9	B-Actin	45kDa	Cell Signaling	1:5000

The table indicates the concentration of primary antibodies used for western blots diluted in PBST (with 0.05% Tween). The antibodies were stored at 4^0C for repeated use (maximum of 2 times re-used). 10ml of solution was used for each blot.

Infection Conditions for ELISA

The TIGKs were infected with bacteria when 80% in confluence (approx. $1x10^6$ cells per well). Bacterial subcultures of overnight growth (without antibiotics) give final nuber1 were centrifuged and suspended in PBS and incubated for 30min in cell culture media with protease inhibitors, TLCK and Leupeptin at a concentration of 0.1mM each (including controls)[49]. The cells were challenged

with bacteria for 24 h, at multiplicity of infection (MOI) of 10. The supernatant was collected and ELISA was performed for IL-6 detection following the protocol of Rand D systems IL-6 ELISA Kit [Figure 6 Flow chart for ELISA MOI 10, 4 h and 24 h.]. The results were quantified by Perkin Elmer ELISA plate reader at absorbance 450nm (as per instructions). Each experiment was done in triplicate of each sample and IL-6 concentrations and statistical analysis was doing using unpaired t- tests was used to compare the samples and graphs were plotted Graph Pad Prism software [Figure 6 Flow chart for ELISA MOI 10, 4 h and 24 h.].

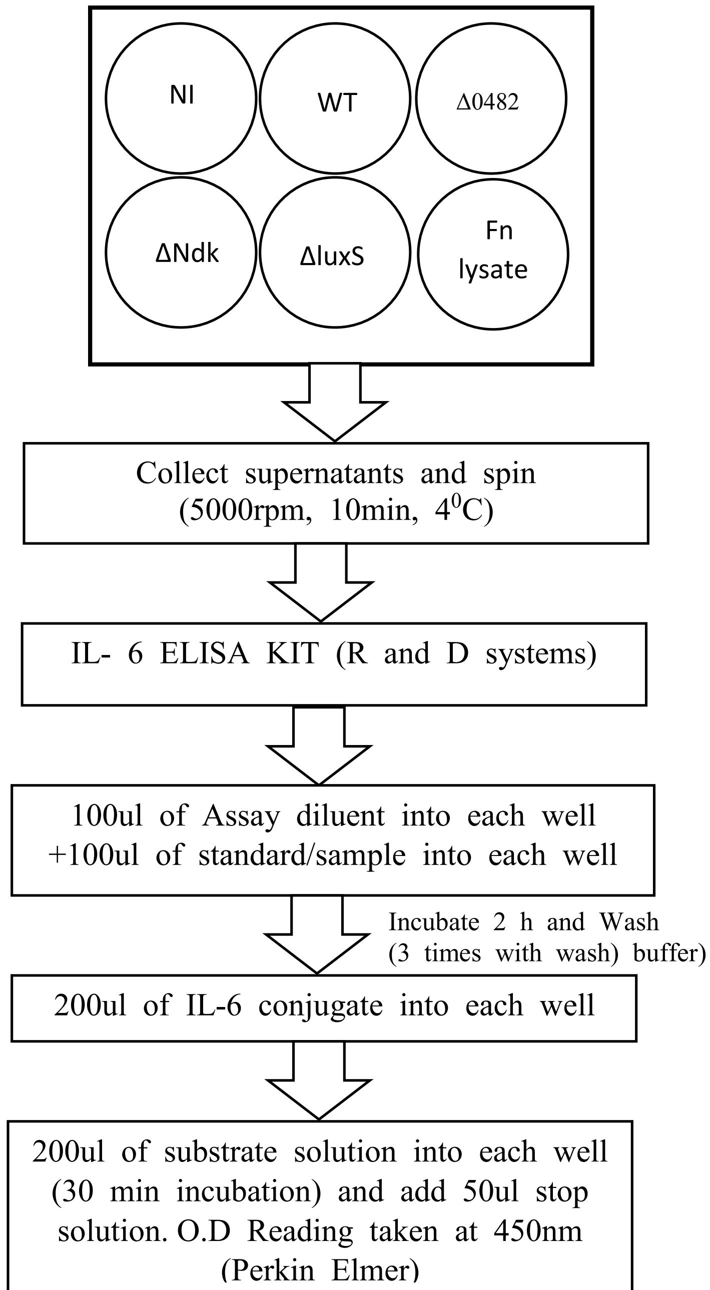

Figure 6 Flow chart for ELISA MOI 10, 4 h and 24 h.

The flow chart indicates the protocol followed for ELISA in the experiment for IL-6 detection as per the instruction provided in the kit (Rand D systems)

CHAPTER THREE: IL-6 PRODUCTION IN P. GINGIVALIS INFECTION BY TIGKS

RESULTS

ELISA

IL-6 ELISA of TIGK supernatants infected with *P. gingivalis* at MOI 10, for 4 h (with protease inhibitors) showed increased expression of IL-6 in wild type ATCC33277 *P. gingivalis* and Δ0482 mutant when compared to the control non-infected TIGKs [Figure 7A IL-6 ELISA 4 h Figure 7B IL-6 ELISA 24 h]. However, the IL-6 expression in ΔNdk and ΔLux S mutant samples was found to be less than the control. The secretion of IL-6 increased after 24 h in ΔNdk and ΔLux S mutant samples compared to WT and control (24 h incubation time, non-infection TIGKS). There was almost no difference in IL-6 WT sample at 4h and 24 h time point.

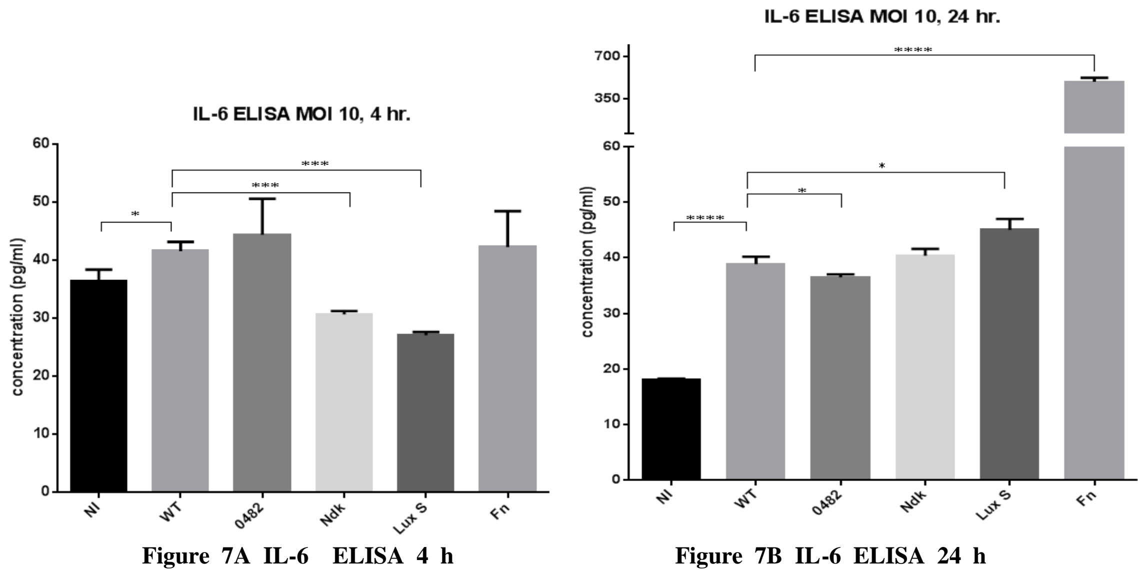

Figure 7A IL-6 ELISA 4 h **Figure 7B IL-6 ELISA 24 h**

TIGKs were infected with *P. gingivalis* at MOI for 4 h (Fig 7A) and 24 h (Fig 7B) in the presence of protease inhibitors (TLCK and Leupeptin, 0.1mM each). The supernatants were collected and ELISA was performed for Il-6 detection. Results obtained are from a single experiment done in triplicates. *, *** and ****, P< 0.05, P< 0.01 and P< 0.005 respectively (Unpaired *t* test), compares with *P. gingivalis* WT infected cells.

NI in the figures represent Non-infected TIGKs, 0482- Δ0482 sample, Ndk- ΔNdk sample, Lux S- ΔluxS sample and Fn represents *Fusobacterium nucleatum* lysates.

WESTERN BLOTS

P CREB/ TOTAL CREB

The blots probed with P- CREB and Total CREB antibodies separately were analyzed using Biorad XRS+ imaging software and graphs are plotted using graph pad prism. The blots were re probed for Actin that served as a loading control. The data is a blot from a single experiment. Densitometry analysis of blots of *P. gingivalis* infected TIGKs protein samples, at various infection time

points revealed that phosphorylation of CREB and activation by *P. gingivalis* infection was found to be maximum at 15min time point in both MOI 10 and 50. The MOI 10, 5 min samples show lesser P-CREB/Total CREB ratio when compared to the control. The MOI 50, 5min samples showed slight up regulation of WT and ΔluxS while Δ0482 and ΔNdk were slightly lesser than the control. The samples at MOI 10, 15 min show up regulation of CREB in all infected samples, although ΔluxS and ΔNdk were ahead of WT and Δ0482 samples in CREB activation. The pattern was very similar with MOI 50, 15min time point. In the MOI 10, 30min samples, the signal was still up regulated when compared with the control. The MOI 50, 30min samples were almost comparable with the control. But at MOI 10, 50 1h time point, all the samples reached the baseline in comparison with the control. The blots can be summed up saying CREB is activated by phosphorylation at MOI 10, 50 at 15min time point and then it comes back to baseline by down regulation represented by de- phosphorylation or reduced P-CREB/ Total CREB levels [Figure 8 P CREB/ Total CREB, MOI 10 (5min, 15min, 30min, 1h), Figure 9 P CREB/ Total CREB, MOI 50 (5min, 15min, 30min, 1h)].

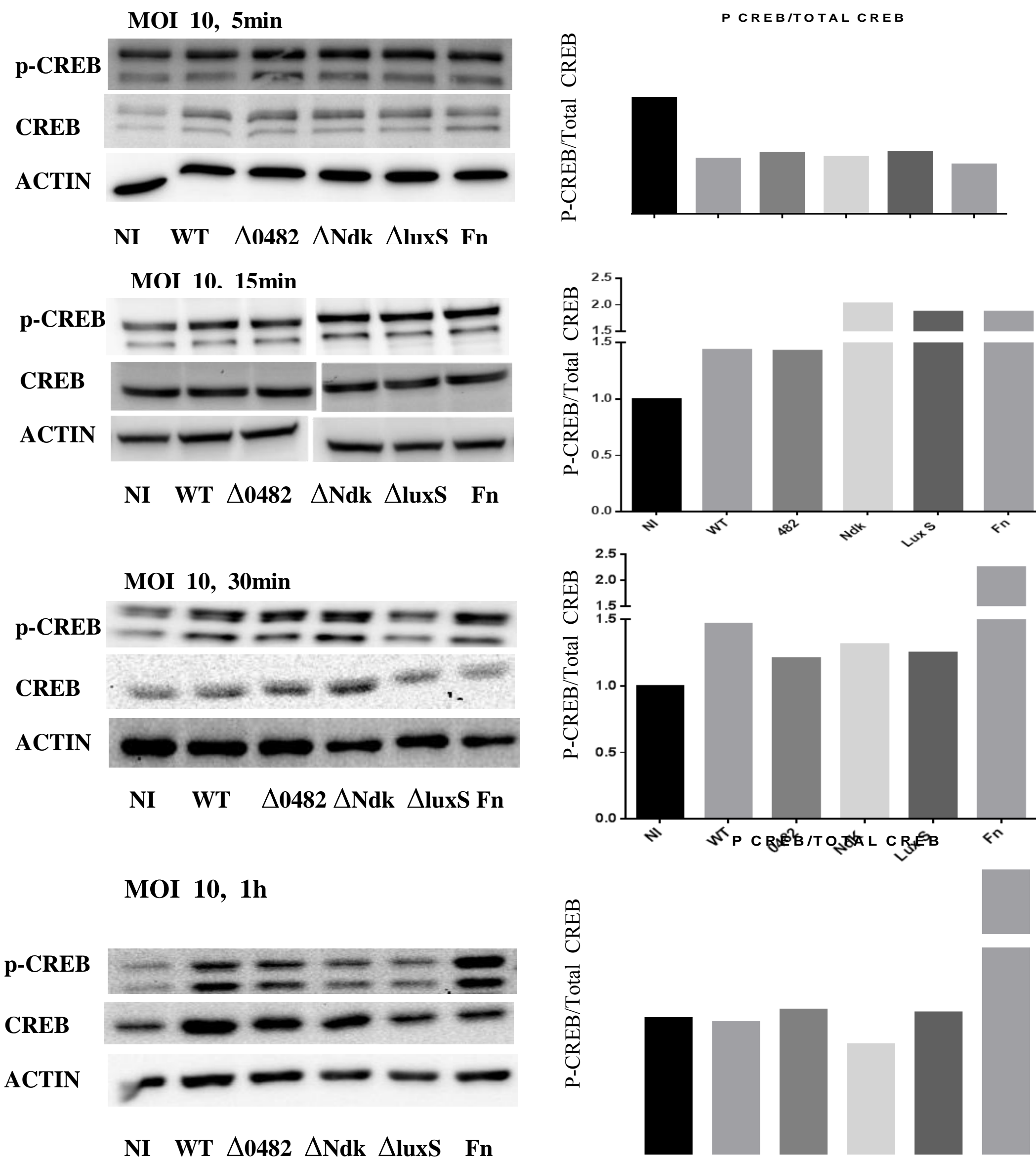

Figure 8 P CREB/ Total CREB, MOI 10 (5min, 15min, 30min, 1h)

Western blots on TIGK cell lysates infected with *P. gingivalis* (with protease inhibitors Leupeptin and TLCK, 0.1mM each) at MOI 10 for 5min, 15min, 30min and 1 h. *Fusobacterium nucleatum* lysates act as positive control. The blots were probed for the targets P-CREB and Total CREB (Mol wt=43kDa) separately and stripped for Actin (loading control). Densitometry was done using Biorad XRS+ software (BIORAD) and graphs plotted on Graph Pad Prism. All the infected samples are compares with Non-infected TIGKS (control). Result is representative of a single experiment. NI refers to Non-infected TIGKs and Fn refers to *Fusobacterium nucleatum* lysate.

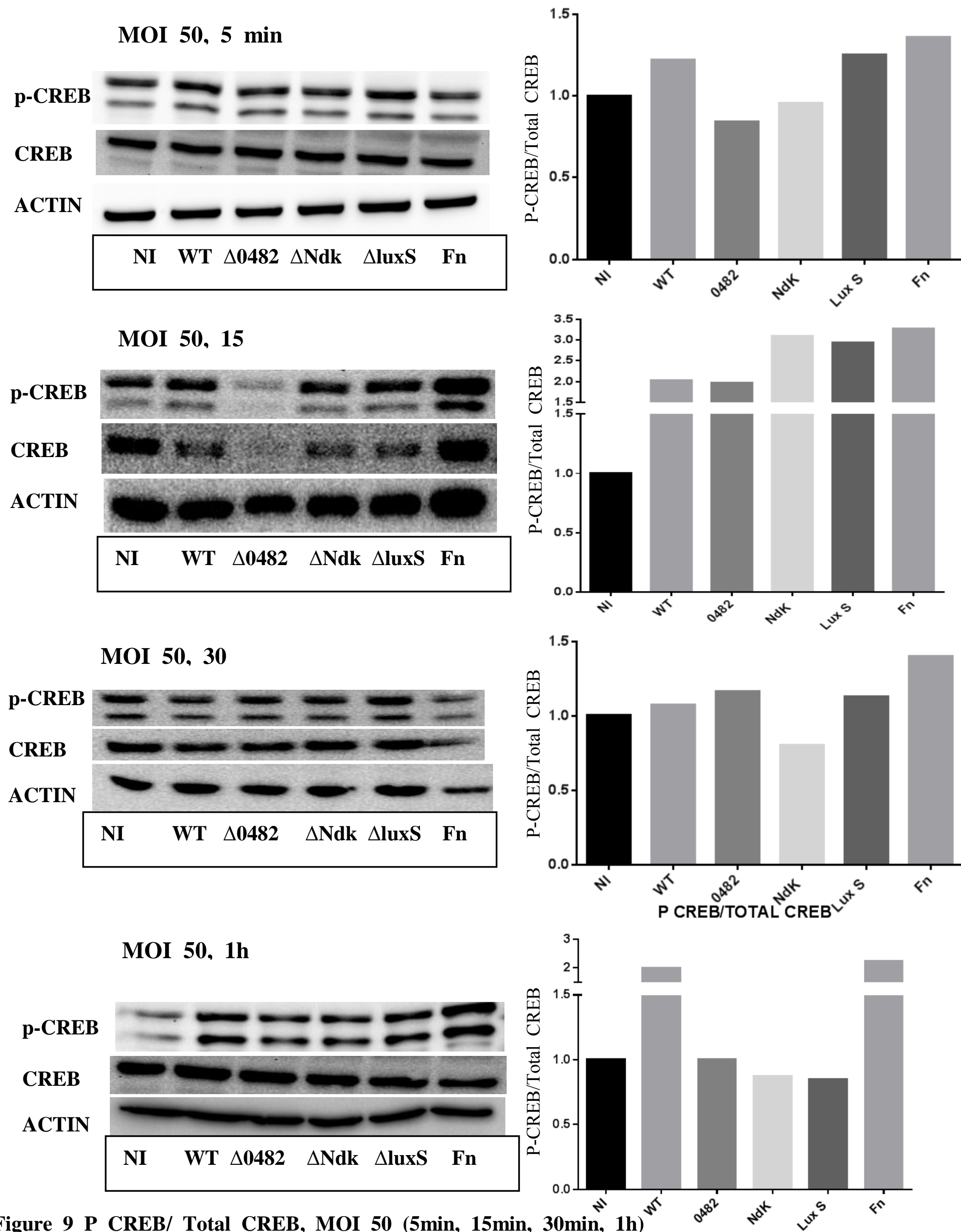

Figure 9 P CREB/ Total CREB, MOI 50 (5min, 15min, 30min, 1h)

Western blots on TIGK cell lysates infected with *P. gingivalis* (with protease inhibitors Leupeptin and TLCK, 0.1mM each) at MOI 50 for 5min, 15min, 30min and 1 h. *Fusobacterium nucleatum* lysates act as positive control. The blots were probed for the targets P-CREB and Total CREB (Mol wt=43kDa) separately and stripped for Actin (loading control). Densitometry was done using Biorad XRS+ software (BIORAD) and graphs plotted on Graph Pad Prism. All the infected samples are compares with Non-infected TIGKS (control). Result is representative of a single experiment.

P ERK/ TOTAL ERK

The blots probed for P-ERK and Total ERK were analyzed separately and stripped to probe for Actin that served as a loading control. At MOI 10, 5 min, 15min, 30min and 1 h, the samples showed no change in comparison with the control.

At MOI 50, 5min, the samples showed slight up regulation of ERK (increased P-ERK/Total ratio). At 15min there was increased activation of ERK in the infected samples when compared to the control. At 30min, the WT was down regulated that could be due to de-phosphorylation of activated P-CREB indicated by decreased P-ERK/ Total ERK. After 1 h, the WT and Δ0482 samples were almost equal to the control where as ΔNdk and ΔluxS showed continued up regulation in comparison with the control. Hence ERK did not seem to get phosphorylated at lower MOI. However, increased MOI seem to up regulate it.

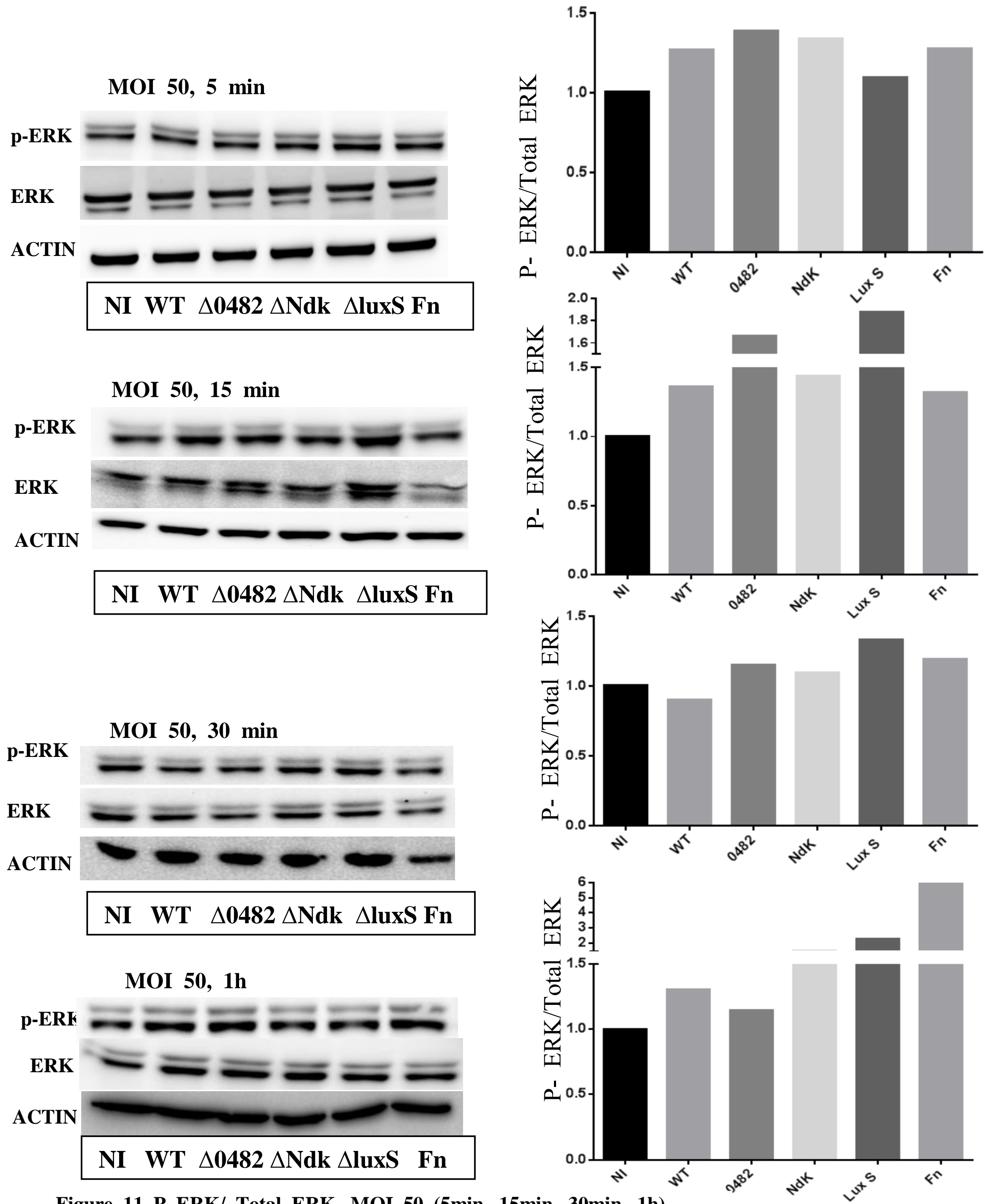

Figure 11 P ERK/ Total ERK, MOI 50 (5min, 15min, 30min, 1h)

Western blots on TIGK cell lysates infected with *P. gingivalis* (with protease inhibitors Lepeptin and TLCK, 0.1mM each) at MOI 50 for 5min, 15min, 30min and 1 h. *Fusobacterium nucleatum* lysates act as positive control. The blots were probed for the targets P-ERK and Total ERK (Mol wt=42-44 kDa) separately and then stripped for Actin (loading control). Densitometry was done using Biorad XRS+ software (BIORAD) and graphs plotted on Graph Pad Prism. All the infected samples are compares with Non-infected TIGKS (control). Result is representative of a single experiment.

P P38/ TOTAL P38

Densitometry of P P38/ Total P38 show that P38 was not activated at MOI 10 5min, 15min as well as MOI 50 5min and 15 min. The MOI 10 30min samples, however showed up regulation of both WT and all mutants with ΔluxS being lower in up regulation compared to others. At 1 h, the MOI 10 samples were not much different compared to the control again. Similar to the MOI 10 5 min. and 15min samples, there was no up regulation when compared to the control. At MOI 50, 30min infection, Δ0482 and ΔNdk were up regulated while WT and ΔluxS did not show much change. Also, at MOI 50, 1h time point not much change was seen and ΔluxS was down regulated compared to the control. The Lux S mutant does not seem to have up regulated P 38 phosphorylation at any time point and MOI in the experiment. The other infected samples seemed to activate P 38 at 30 min and reach the baseline by 1 hour [Figure 12P P38/ Total P38, MOI 10 (5min, 15min, 30min, 1h) Figure 13P P38/ Total P38, MOI 50 (5min, 15min, 30min, 1h)]

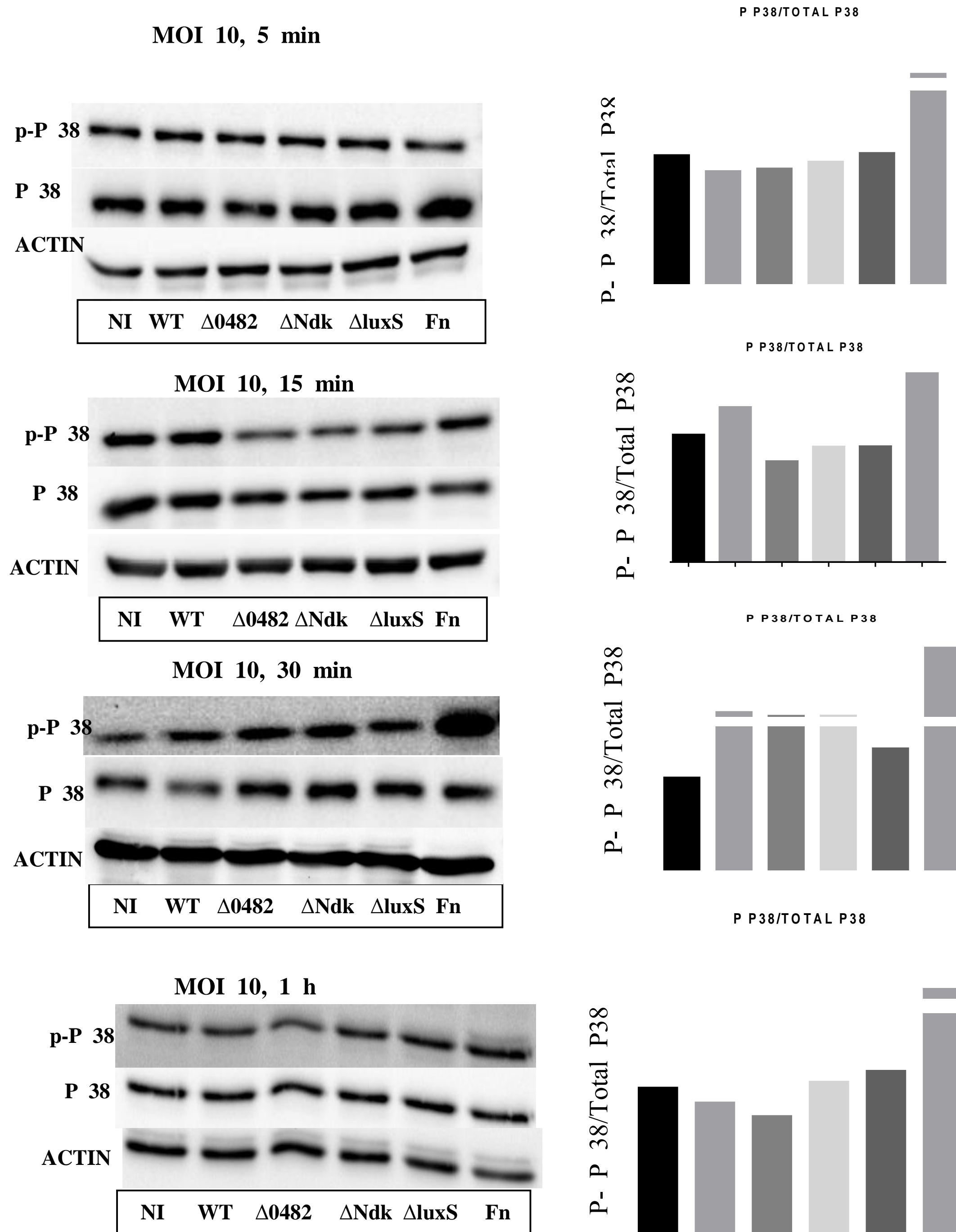

Figure 12P P38/ Total P38, MOI 10 (5min, 15min, 30min, 1h)

Western blots on TIGK cell lysates infected with *P. gingivalis* (with protease inhibitors Leupeptin and TLCK, 0.1mM each) at MOI 10 for 5min, 15min, 30min and 1 h. *Fusobacterium nucleatum* lysates act as positive control. The blots were probed for the targets P-P38 and Total P38 (Mol wt= 43 kDa) separately and then stripped for Actin (loading control). Densitometry was done using Biorad XRS+ software (BIORAD) and graphs plotted on Graph Pad Prism. All the infected samples are compares with Non-infected TIGKS (control). Result is representative of a single experiment.

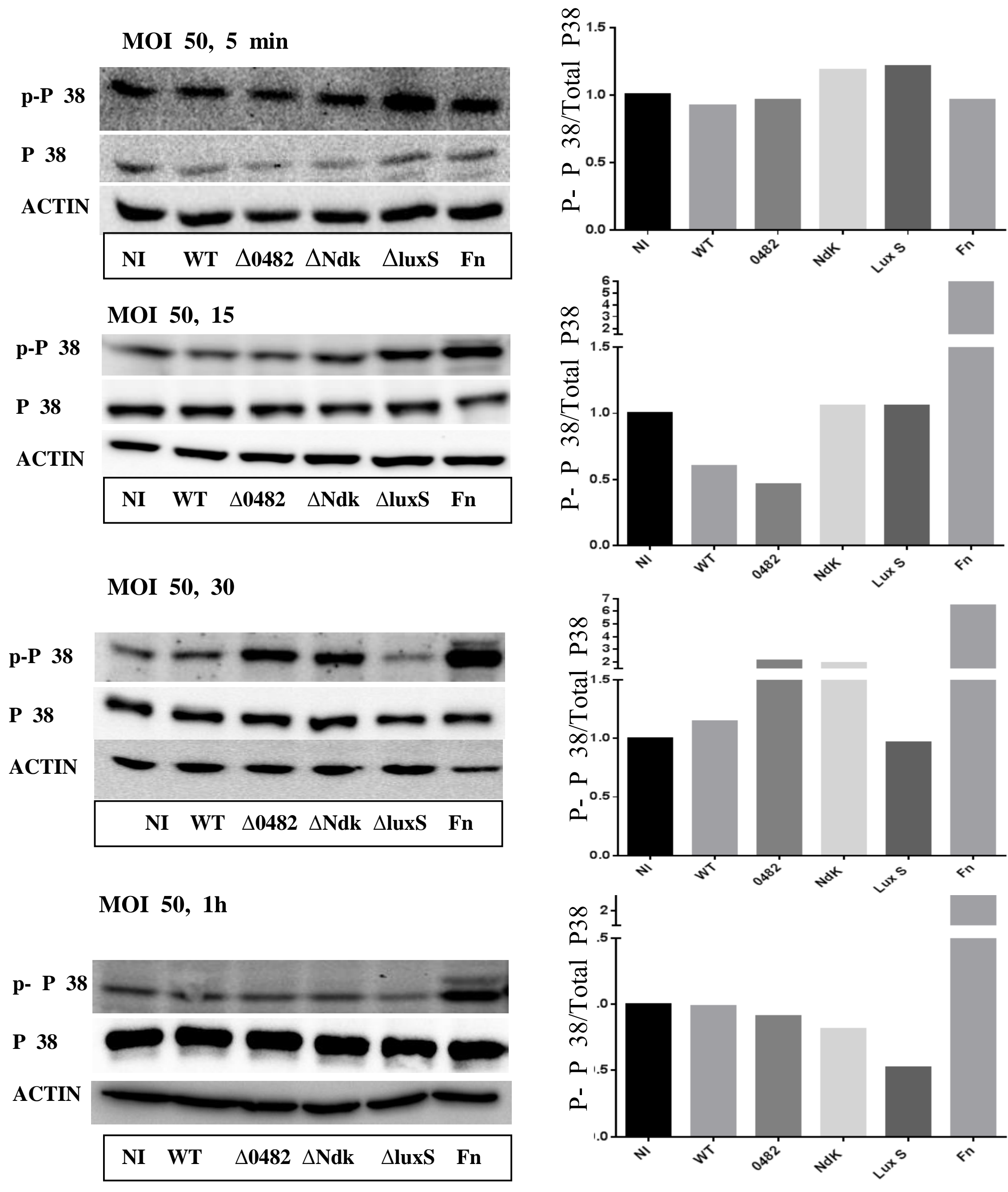

Figure 13P P38/ Total P38, MOI 50 (5min, 15min, 30min,

Western blots on TIGK cell lysates infected with *P. gingivalis* (with protease inhibitors Leupeptin and TLCK, 0.1mM each) at MOI 50 for 5min, 15min, 30min and 1 h. *Fusobacterium nucleatum* lysates act as positive control. The blots were probed for the targets P-P38 and Total P38 (Mol wt= 43 kDa) separately and then stripped for Actin (loading control). Densitometry was done using Biorad XRS+ software (BIORAD) and graphs plotted on Graph Pad Prism. All the infected samples are compares with Non-infected TIGKS (control). Result is representative of a single experiment.

P c-JUN/ TOTAL c-JUN

The densitometry of P c-Jun and Total C-Jun blots reveal that at MOI 10 5min, Δ0482 showed slight up regulation also ΔNdk but the WT was not much different from the control. ΔluxS was slightly down regulated. The signal was not detected for p-c Jun at MOI 10 15min and 30min. However, at MOI 10 1h, the mutants showed up regulation with ΔluxS showing the highest but no difference was seen in the WT [Figure 14 P C-Jun/ Total C-Jun, MOI 10 (5min, 15min, 30min, 1h)]. At, MOI 50 5min, Δ0482 showed highest up regulation among the mutants but the WT did not show any difference. At MOI 50 15 min, the phosphorylation of c-Jun could not be detected. At MOI 50, 30 min, all the infected samples were up regulated when compared with the control with Δ0482 being the showing the highest up regulation. At MOI 50, 1h the Δ0482 was still up regulated but all the other infected samples were not much different when compared to the control. Overall, c-Jun activity was observed to be maximal during late time points 30min and 1 h. The highest up regulation was at MOI 50, 30 min [Figure 15 Pcjun/ Total c Jun MOI 50 (5min, 15min, 30min, 1h)].

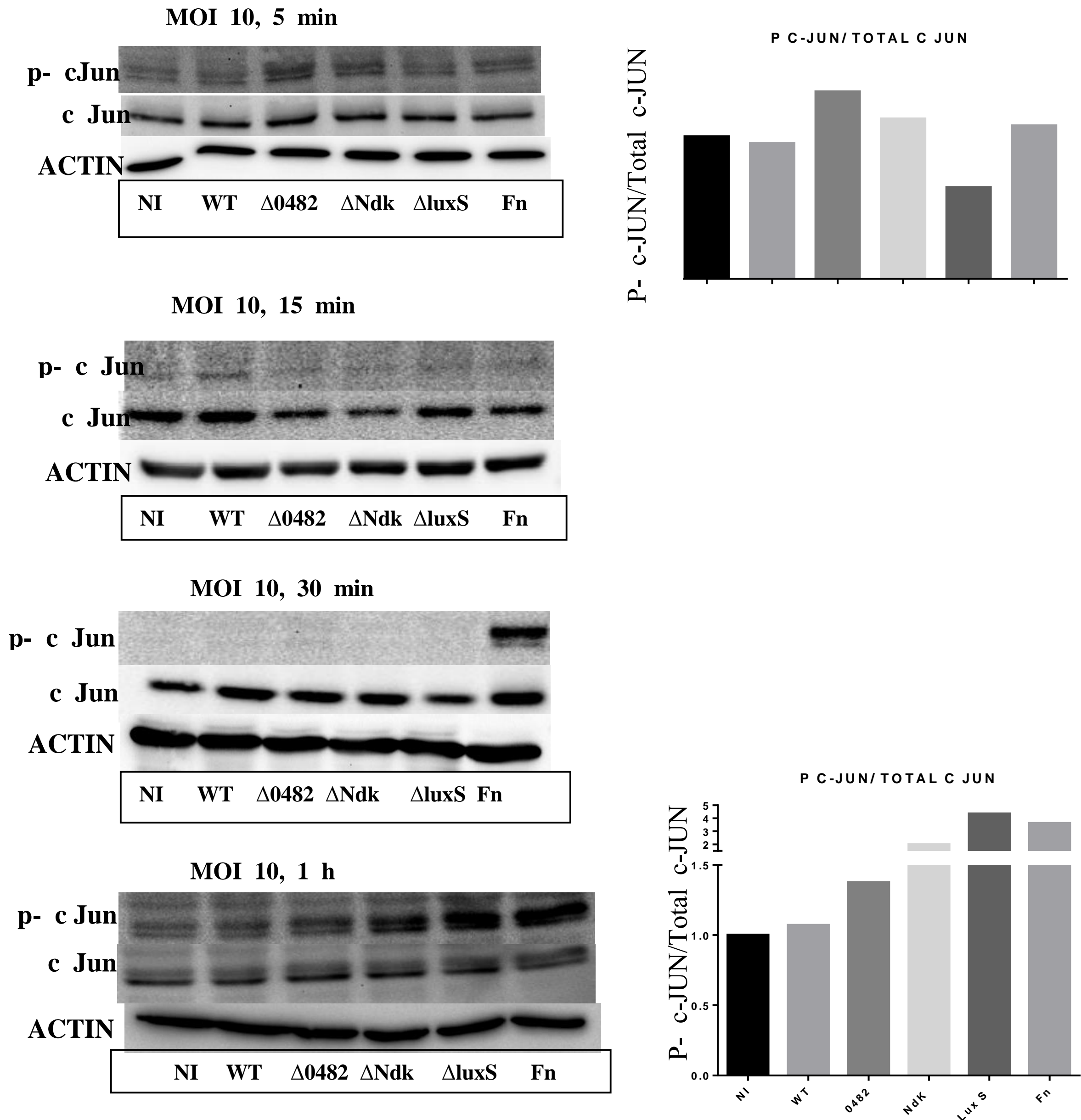

Figure 14 P C-Jun/ Total C-Jun, MOI 10 (5min, 15min, 30min, 1h)

Western blots on TIGK cell lysates infected with *P. gingivalis* (with protease inhibitors Leupeptin and TLCK, 0.1mM each) at MOI 10 for 5min, 15min, 30min and 1 h. *Fusobacterium nucleatum* lysates act as positive control. The blots were probed for the targets P- c Jun and Total c Jun (Mol wt= 48 kDa) separately and then stripped for Actin (loading control). Densitometry was done using Biorad XRS+ software (BIORAD) and graphs plotted on Graph Pad Prism. All the infected samples are compares with Non-infected TIGKS (control). Result is representative of a single experiment.

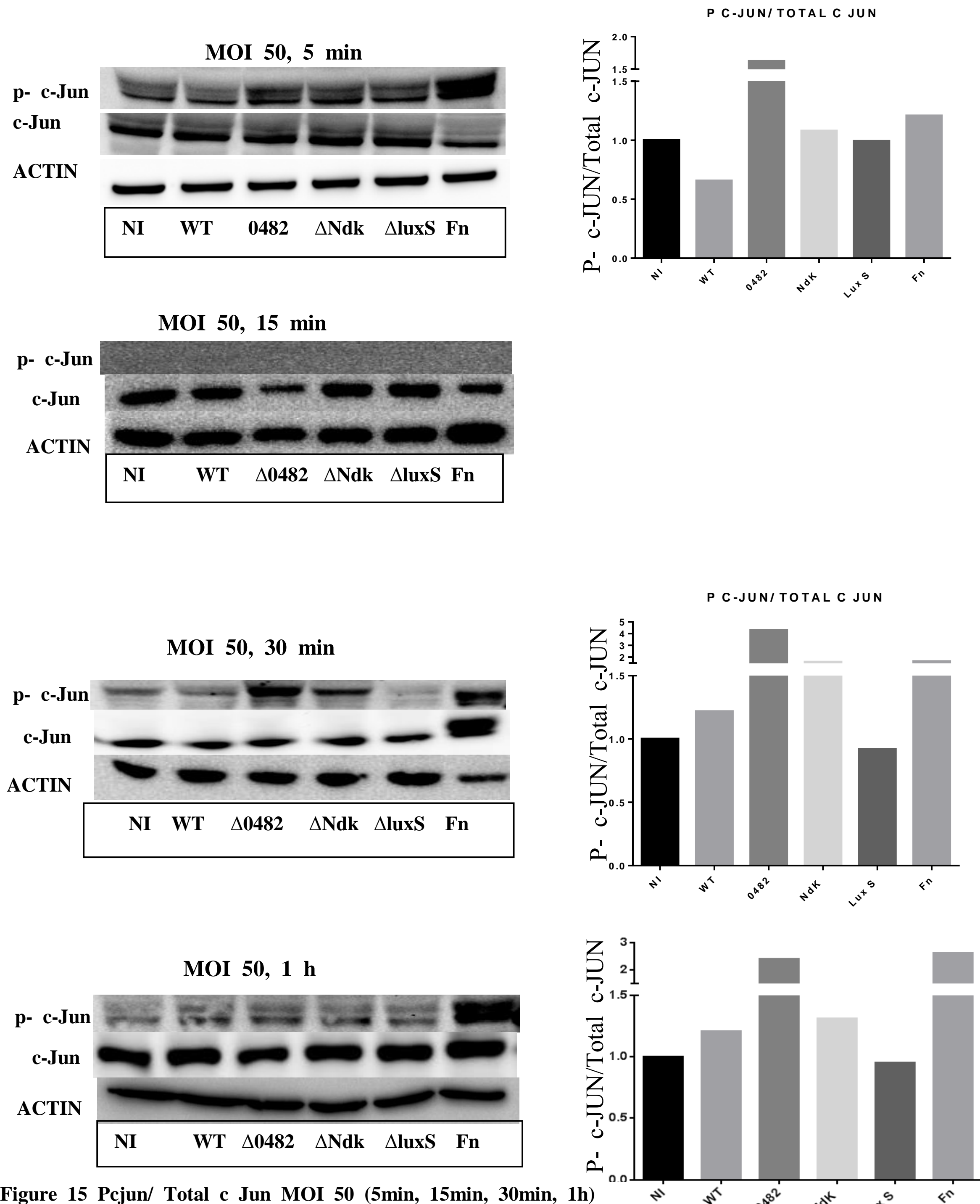

Figure 15 Pcjun/ Total c Jun MOI 50 (5min, 15min, 30min, 1h)

Western blots on TIGK cell lysates infected with *P. gingivalis* (with protease inhibitors Leupeptin and TLCK, 0.1mM each) at MOI 10 for 5min, 15min, 30min and 1 h. *Fusobacterium nucleatum* lysates act as positive control. The blots were probed for the targets P- c Jun and Total c Jun (Mol wt= 48 kDa) separately and then stripped for Actin (loading control). Densitometry was done using Biorad XRS+ software (BIORAD) and graphs plotted on Graph Pad Prism. All the infected samples are compares with Non-infected TIGKS (control). Result is representative of a single experiment.

MOI and Time point	Target	Mutants compared to control			
		WT	Δ0482	ΔNdk	ΔluxS
MOI 10, 5min	CREB	↓	↓	↓	↓
	P38	———	———	———	———
	ERK	———	———	———	———
	c-JUN	———	↑	———	↓
MOI 10, 15min	CREB	↑	↑	↑	↑
	P38	↑	———	———	———
	ERK	———	———	———	———
	c-JUN				
MOI 10, 30min	CREB	↑	———	———	———
	P38	↑	↑	↑	↑
	ERK	———	———	———	———
	c-JUN				
MOI 10, 1 h	CREB	———	———	———	———
	P38	———	———	———	———
	ERK	———	———	———	———
	c-JUN	———	↑	↑	↑

Table 2 Summary of Western blots activation MOI 10

The table represents the activation of pathway targets at various tie points in the mutants when compared to the No infection sample (control). ↑ indicates Up regulation when compared to control and ↓ indicates down regulation when compared with the control and --- indicates no change compared to the control. The results compared are from a single experiment.

MOI and Time point	Target	Mutants compared to control			
		WT	**Δ0482**	**ΔNdk**	**Δlux S**
MOI 50, 5min	CREB	———	———	———	———
	P38	———	———	———	———
	ERK	↑	↑	↑	———
	c-JUN	↑	↑	———	———
MOI 50, 15min	CREB	↑	↑	↑	↑
	P38	↓	↓	———	———
	ERK	↑	↑	↑	↑
	c-JUN				
MOI 50, 30min	CREB	———	———	↓	———
	P38	———	↑	↑	———
	ERK	———	———	———	↑
	c-JUN	↑	↑	↑	↑
MOI 50, 1 h	CREB	———	———	———	———
	P38	———	———	———	↓
	ERK	↑	———	↑	↑
	c-JUN	↑	↑	↑	———

Table 3 Summary of Western Blots activation MOI 50

The table represents the activation of pathway targets at various tie points in the mutants when compared to the No infection sample (control). ↑ indicates Up regulation when compared to control and ↓ indicates down regulation when compared with the control and --- indicates no change compared to the control. The results compared are from a single experiment.

DISCUSSION

Periodontitis, being an infectious inflammatory disease of bacterial origin that widely affects a large population throughout the globe. Host immune responses plays a huge role in the disease progression. The progression is marked by inflammation of gingiva, loss of attachment and bone resorption until the tooth is finally lost. The innate and adaptive immune responses of the host are the contributing factors in determining the disease progression. Several studies have suggested the presence of certain inflammatory biomarkers associated with periodontitis [50]. Cytokines are among such biomarkers. Although, cytokines are multi-functional, one of their important functions is inflammation [51]. Interleukin (IL-6) is seen in increased amounts in the gingival crevicular fluid and bloodstream of patients with periodontitis [52]. Pro inflammatory cytokines like Interleukin-1β and Interleukin-6 have been reported to be seen in elevated levels in patients with Periodontitis. IL-1β increases the production of IL-6 by increasing the antigen mediated stimulation of T cells which in turn up regulates osteoclastogenesis further leading to bone loss, which is the basic pathology leading to progression of periodontitis [53].

The IL-6 ELISA results indicate that *P. gingivalis* WT ATCC33277 induces the production of IL-6 in gingival epithelial cells at 4h of infection but the mutants ΔluxS and ΔNdk showed lesser IL-6 production when compared

to WT. However, after 24h of infection, the difference was much lesser. This indicates that ΔluxS and ΔNdk genes might be essential in the early expression of IL-6. The involvement of Lux S gene in several physiological functions like hemin uptake, autoinducer-2 production for multi-speices quorum sensing could be a possible explanation [37]. Also, the decreased IL-6 production at early time points for Ndk mutant was in accordance with a previous study that proposed the decreased IL-1β secretion in Ndk mutant as the gene is responsible for its production [36]. There could also be a possibility of alternate mechanisms playing role for Ndk and LuxS deficient mutants that lead to increased production of IL-6 after 24h.

As the ELISA results were positive for IL-6 production by *P. gingivalis*, we performed western blots targeting few important transcription factors and targets in IL-6 production. IL-6 expression is controlled by at least 3 factors AP-1 reporter, NF-κB and CREB. Previous studies have shown that *P. gingivalis* infection down regulates NF-κB pathway in gingival epithelial cells [26]. The blots indicate the up regulation of CREB which was highest at 15min time point in both MOI 10 and MOI 50. This explains that CREB competes for common transcriptional factors with NF-κB, thus down regulating it [42]. The blots however show that despite down regulation of NF-κB by CREB, phosphorylation of c-Jun was sufficient to produce IL-6. Also, the c-Jun activity was maximum after 30min, in

contrast to the CREB activity which was maximum at 15min time point. This could indicate that CREB and c-Jun work antagonistically. The up regulation of the former could probably prevent the up regulation of the other. From the ELISA results, we anticipated the ΔNdk and ΔluxS samples to be up regulated at later time points in the blots. However, no such co relation as found in the phosphorylation of c-Jun or CREB from the blots. This could indicate the presence of more than one pathway for the phosphorylation of the targets.

Also, ERK1/2 did not show much difference, although there was slight down regulation initially, corresponding to the results shown previously in gingival epithelial cells during *P. gingivalis* infection [44].

As indicated by the up regulation of c-Jun it can be assumed that the AP-1 transcription factor is also up regulated in *P. gingivalis* infection [Figure 16 Pathway for IL-6 production in *P. gingivalis* infection in gingival epithelial cells.].

The difference in the mutants was not sufficient enough to conclude on the effect of the gene on the pathway. However, as assumed that Δ0482 and ΔluxS could be closely associated, the results do not indicate any similarity in the IL-6 expression or the up regulation of targets related to IL-6 [39]. The 0482 sample showed IL-6 production early at 4h time point, whereas the ΔluxS showed maximum IL-6 production at 24 h when compared with

the WT sample. This could indicate presence of multiple pathways of IL-6 production in the luxS mutant.

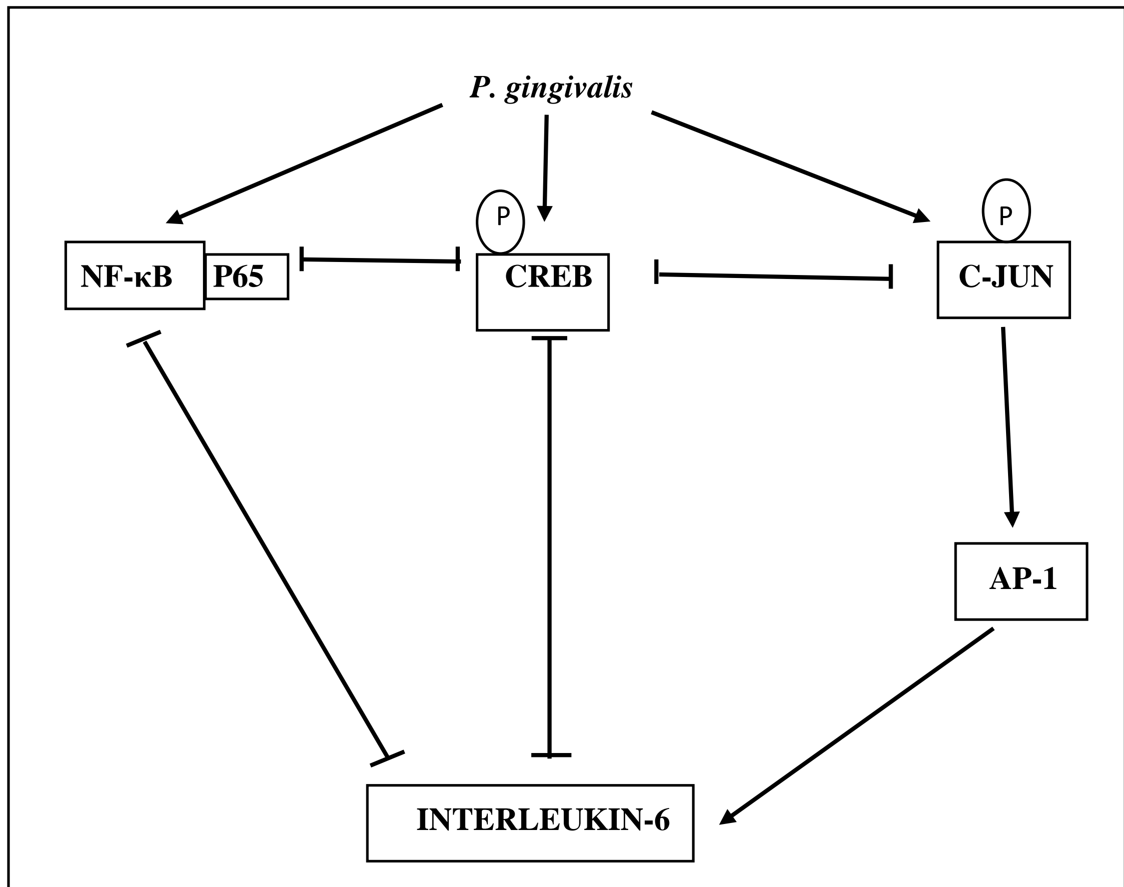

Figure 16 Pathway for IL-6 production in *P. gingivalis* infection in gingival epithelial cells.

The figure indicates the probable pathway from the study, for production of IL-6 in *P. gingivalis* infection in TIGKs. *P. gingivalis* infection activates the NF-κB pathway leading to the secretion of IL-6. However, it also phosphorylates CREB which competes with the P -65 subunit of NF-κB pathway that prevents the activation of NF-κB, reducing the IL-6 production. The third target involved is the phosphorylation of c-Jun that leads to activation of AP-1 transcription factor and produces IL-6. Due to antagonistic action of CREB on c-Jun, we proposed that

FUTURE DIRECTIONS

The above study has demonstrated that *P. gingivalis* induces the production of the pro-inflammatory cytokine IL-6. Although, the data is from a single experiment, the trends show that the pathway could be through the up-regulation of CREB and AP-1 transcription factor (through c –Jun Activation). We, therefore, would like to test the pathway by using inhibitors to CREB and observe if c-jun activity is enhanced. Also, if usage of CREB inhibitors could up regulate c-jun at early timepoints. Also, we would like to run RT-PCRs to confirm the m-RNA levels for IL-6 in the WT and the tested mutants to look for significant changes in m-RNA levels. As protease inhibitors were used in the experiments, we would like to test for Protease deficient mutants to know the way proteases affect IL-6 production and also to confirm if the results are similar between WT with protease inhibitors and protease deficient mutants. Internalization of bacteria inside the host cells is an important process in the IL-6 production. We, therefore would want to repeat experiments with non-invasive *P. gingivalis* strains to confirm it. The inability of the non-invasive to produce IL-6 would confirm that internalization of bacteria results in the activation of the pathway for IL-6 production. *P. gingivalis* is a potential periodontal pathogen with a diverse variety of adaptations and characteristics to efficiently switch-off and on the host's immune response at the pathogen's convenience. This study about tracing the IL-6 pathway can provide significant insights into understanding the important targets and bacterial genes involved in the pathway. Thorough understanding of the

pathway would be a huge breakthrough in the understanding the pathogenesis of periodontal disease better and also in establishing therapeutic targets in near future.

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