Epithelial mechanisms in the microbial pathogenesis of periodontal disease

Iancu, Simona Ioana

Awarding institution: King's College London

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Epithelial mechanisms in the microbial pathogenesis of periodontal disease

Simona Ioana Iancu

Thesis submitted for the degree of Doctor of Philosophy

Mucosal and Salivary Biology Division
King’s College London
2016
Abstract

Periodontitis, a major cause of tooth loss, is a bacterially induced inflammatory disease that has been associated with certain bacterial species. The aim of this thesis was to identify the epithelial mechanisms activated by commensal and periodontal pathogens to determine which signalling pathways, transcription factors, and pro-inflammatory cytokine responses are associated with periodontitis.

The H400 gingival epithelial cell line was infected with the health-associated Actinomyces naeslundii and periodontopathogens Fusobacterium nucleatum and Porphyromonas gingivalis. Differential pathway activation was observed between the three species. A. naeslundii induced phosphorylation of JNK and NF-κB, similarly to F. nucleatum which activated all MAPK pathways (including p38 and ERK1/2) and NF-κB. P. gingivalis induced minimal levels of p-JNK. Differential transcription factor activation was observed in response to the three bacteria. A. naeslundii and F. nucleatum induced activation of c-Fos and c-Jun, while P. gingivalis transiently activated binding of ATF-2. Notably, F. nucleatum was the most potent activator. Both A. naeslundii and F. nucleatum induced a pro-inflammatory response, together stimulating release of IL-1α, IL-1β, IL-6, GM-CSF and G-CSF. P. gingivalis was the least stimulatory bacterium, an observation supported by a lack of cytokine production, IL-8 down-regulation and a reduction in lactate dehydrogenase release (measure of damage). To determine the functional role of these signalling pathways in inducing effector responses, the MAPK and NF-κB pathways were inhibited. Results indicate that the p38 MAPK pathway is the main regulator of inflammatory responses in A. naeslundii and F. nucleatum infections while in P. gingivalis infections, the JNK pathway appears to be the major regulator of oral epithelial responses. Furthermore, the possible involvement of P. gingivalis virulence factors in the bacterium’s ability to prevent epithelial cell activation was investigated. It was observed that the Lys-gingipain (Kgp) of P. gingivalis plays a role in supressing activation of the MAPK and NF-κB pathways and the c-Fos transcription factors in oral epithelial cells.

Overall, the data in this thesis suggests that epithelial cells recognise and respond differently to commensal bacteria compared to periodontal pathogens and that P. gingivalis Kgp may be a key virulence factor involved in immune subversion.
Acknowledgements

I would like to express my sincere gratitude to my supervisor, Prof. Julian Naglik, for the continuous support throughout my PhD, for his patience, motivation and immense knowledge. I would also like to thank Dr. Jonathan Richardson and Dr. David Moyes for encouraging my research and for allowing me to grow as a scientist. Your help and advice throughout the last three years have been priceless.

I want to thank my fellow groupmates Mariana Blagojevic, Dr. Shirley Tang and Dr. Nessim Kichik as well as everyone in the Mucosal and Salivary Biology Division for their precious support, friendship and encouragement. You have all filled this journey with fun and laughter.

My sincere thanks also go to Dr. Veronica Booth, for her advice and insightful comments.

I especially want to thank my amazing family, Mama, Tata si Coca, for their unconditional love, support and inspiration. Voi sunteti pilonii mei, iar fara voi nu as fi ajuns aici. Va iubesc!

Last but not least, Andrei – thank you for believing in me, for always being there and for bringing out the best in me. Cu tine alaturi, orice e posibil
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<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>°</td>
<td>Degree</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>AIM2</td>
<td>non-NLR absent in melanoma 2</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein – 1</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BA</td>
<td>Blood agar</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculovirus inhibitor repeat</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
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<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DUSP/MKP</td>
<td>Dual-specificity protein phosphatase</td>
</tr>
<tr>
<td>eATP</td>
<td>Extracellular ATP</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polymeric substances</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAA</td>
<td>Fastidious anaerobe agar</td>
</tr>
<tr>
<td>FAD-I</td>
<td>Fusobacterium-associated defensing inducer</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box protein</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>hBD</td>
<td>Human beta-defensin</td>
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<td>HbR</td>
<td>Haemoglobin receptor protein</td>
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<td>Human cytomegalovirus</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<td>HMGB</td>
<td>High-mobility group box</td>
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<td>HSP</td>
<td>Heat shock protein</td>
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<tr>
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<td>Herpes simplex virus</td>
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<td>HTLV</td>
<td>Human T-cell lymphotrophic virus</td>
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<tr>
<td>ICAM</td>
<td>Intercellular expression molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Type I interferon</td>
</tr>
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<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>Interferon gamma-induced protein</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Kgp</td>
<td>Lysine gingipain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LBP</td>
<td>Lipopolysaccharide binding protein</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>LTA</td>
<td>Lipoteichoic acid</td>
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<td>Molar</td>
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<td>MAL</td>
<td>MyD88-adaptor-like</td>
</tr>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
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<td>mL</td>
<td>Milliliter</td>
</tr>
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<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mROS</td>
<td>Mitochondrial ROS</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NDK</td>
<td>Nucleoside diphosphate kinase</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa light chain enhancer of activated B cells</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod-like receptor</td>
</tr>
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<td>NLRC</td>
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<td>Nuclear factor erythroid 2</td>
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<td>OMV</td>
<td>Outer membrane vesicle</td>
</tr>
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<td>OSCC</td>
<td>Oral squamous cell carcinoma</td>
</tr>
<tr>
<td>P2X</td>
<td>Ligand-gated ion channel</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease-activated receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDL</td>
<td>Periodontal ligament</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PPAD</td>
<td><em>P. gingivalis</em> peptidylarginine deiminase</td>
</tr>
<tr>
<td>PPRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PSD</td>
<td>Polymicrobial synergy and dysbiosis</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin domain</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
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<tr>
<td>Rgp</td>
<td>Arginine gingipain</td>
</tr>
<tr>
<td>RIG</td>
<td>Retinoic-acid-inducible gene 1 receptor</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio immuno-precipitation assay buffer</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SARM</td>
<td>Sterile-alpha and armadillo-motif-containing protein</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TIR</td>
<td>TLR cytoplasmic Toll/IL-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour-necrosis factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>vs</td>
<td>Versus</td>
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</table>
Chapter 1: Literature review

1.1 Host-pathogen interactions

Basic concepts regarding host-microbe interactions have seen substantial changes since microorganisms were first described to cause disease in humans. The popular and scientific view was that pathogens were primarily responsible for host-microbe interactions resulting in disease. The later finding that microbes can exist within hosts without causing disease, increased knowledge about commensal, mutualistic and opportunistic organisms represent a breakthrough in the understanding that host-pathogen interactions are in many aspects essential in health and immune homeostasis.

Microorganisms colonise various sites in a mammalian host, including the skin, mouth, intestine and respiratory tract (Dethlefsen, McFall-Ngai et al. 2007). The effect of this colonisation on the host is dependent on microbial adaptation and can be positive, displaying acts of commensalism and mutualism such as in the gut (Clemente, Ursell et al. 2012), or can be negative, as seen with pathogens. Adaptation of pathogens to particular host niches is driven by virulence factors, with an end goal of survival and transmission to another host. A highly successful example is the influenza virus which is renowned for its ability to continually evolve, leading to emergence of novel strains each year (Taubenberger and Kash 2010).

Different strategies of microbial adaptation and change result in varying degrees of damage to the host. This presents a constant challenge to the immune system in trying to prevent and control disease. Two main types of defence, innate and adaptive immunity, cooperate to protect the host (Hoebe, Janssen et al. 2004). The innate immune system provides a first line of defence against microorganisms with recognition being mediated by pattern recognition receptors (PRRs) (Medzhitov 2007). These receptors, however, are limited to the recognition of molecules shared by most pathogens. Adaptive immune recognition is mediated by antigen receptors on lymphocytes. Each lymphocyte carries antigen receptors of a single specificity allowing for the recognition of an almost infinite diversity of antigens (Schatz, Oettinger et al.
The cells of the innate immune system play a crucial role in directing the secondary, adaptive response, which is activated when the primary response fails to clear an infection (Hoebe, Janssen et al. 2004).

Apart from being effective at discriminating between self and non-self, the innate immune system has to constantly differentiate between commensal and pathogenic organisms. This is particularly important at mucosal surfaces, which are colonised by a vast number of microbes, and in responses to polymicrobial infections and diseases (Srinivasan 2010). The mechanisms governing the ability of the innate immune system to tolerate commensals while mounting a protective response against pathogens remain unclear and therefore represent an attractive target for research.

1.2 Polymicrobial diseases

Polymicrobial diseases are clinical and pathological manifestations caused by multiple microorganisms such as bacteria, fungi, viruses and other parasites (Brogden, Guthmiller et al. 2005). One microorganism can generate a niche that favours the growth of another, whether it be a commensal or a pathogen. At the same time, a microorganism generated niche can suppress the colonisation by other organisms; known as microbial interference. This interplay can occur across organisms from the same or different kingdoms, genera, species and strains (Brogden and Guthmiller 2003), making polymicrobial diseases more common than previously thought. Many diseases formerly believed to be driven by single aetiological agents are now recognised as polymicrobial infections. A few examples include otitis media (Bakaletz 1995), hepatitis (Mathurin, Thibault et al. 2000), gastroenteritis (Bettelheim, Bowden et al. 1999), vaginosis (Cherpes, Meyn et al. 2003), necrotising fasciitis (Fontes, Ogilvie et al. 2000) and periodontitis (Rickard, Gilbert et al. 2003).

The molecular mechanisms of most polymicrobial infections remain poorly defined and are unique to each disease. There are, however, common underlying predisposing factors leading to disease. Physiological and psychological stress, as well as metabolic diseases can favour the onset of polymicrobial infections. Stress can increase susceptibility to upper respiratory tract infections (Graham, Douglas et al. 1986, Cobb
and Steptoe 1996) while metabolic disorders such as diabetes can increase the risk for periodontitis (Moore, Orchard et al. 2000).

Alterations of mucosal surfaces caused by one organism can favour the colonisation of another. Cell surface changes in receptor expression owing to microbial activity can increase adherence and replication of other microbes (Miedzobrodzki and Macura 1993). For example, influenza virus infections can damage the respiratory epithelium, contributing to adherence of *Streptococcus pneumoniae* (Hament, Kimpen et al. 1999). Further to this, influenza virus-infected cells can become susceptible to lipopolysaccharide (LPS) induced tumour necrosis factor (TNF)-α and interleukin (IL)-1 cytokine release (Nain, Hinder et al. 1990, Bender, Sprenger et al. 1993). In patients with human immunodeficiency virus (HIV), LPS from systemic Gram-negative bacteria of gut or oral origin can up-regulate pro-inflammatory cytokine secretion from monocytes and re-activate latent HIV infected cells (Baqui, Jabra-Rizk et al. 2000, Moriuchi, Moriuchi et al. 2000).

Synergy between commensal and weakly pathogenic microorganisms can also lead to disease and this can be attributed to sharing of virulence factors. An example is *Bordetella pertussis* and its pertussis toxin which enhances the adherence of *H. influenzae, S. pneumoniae* and *Staphylococcus aureus* to respiratory cilia (Tuomanen 1986). Probably the most consequential underlying factor leading to polymicrobial infections is microbe-induced immunosuppression. Infections with human T-cell lymphotrophic virus (HTLV) and HIV, lead to extensive immunosuppression with T cell depletion, dysregulation of B cells and loss of macrophage function. This increases the host’s susceptibility to a multitude of secondary infections involving other viruses, bacteria, fungi and parasites (Bakaletz 2004).

Polymicrobial diseases are caused by polyviral, polybacterial and polymycotic infections, parasitic co-infections, viral and bacterial co-infections, as well as viral-bacterial-mycotic co-infections (Brogden and Guthmiller 2003). These diseases present with complex interactions among different microorganisms, triggering a plethora of host responses. An archetype of polymicrobial synergy is periodontitis, a disease that is the primary focus of this study.
1.3 Periodontitis

Periodontitis is a highly prevalent disease worldwide, affecting between 10 – 40% of older adults (Eke, Dye et al. 2015). Periodontal diseases are microbially induced inflammatory diseases which can affect the epithelial, connective and bone tissues surrounding and supporting the teeth (Darveau 2010). Plaque induced gingivitis is reversible gingival inflammation and the mildest of periodontal diseases, which is caused by the accumulation of dental plaque on teeth adjacent to the gingiva (Pihlstrom, Michalowicz et al. 2005). Gingivitis presents with red, swollen and bleeding gums but does not affect the underlying supporting structures of the teeth. Simple, effective oral hygiene will reverse gingivitis. However, if left untreated, the disease can progress to periodontitis. Periodontitis is characterised by the loss of connective tissue attachment to the tooth, resulting in the formation of deep periodontal pockets allowing for the accumulation of a more anaerobic dental-plaque biofilm into the periodontal pocket (Darveau 2010). The inflammatory process leads to an irreversible loss of supporting bone around the teeth and eventually to tooth loss. A schematic representation of periodontal disease progression is shown in Figure 1-1.

![Figure 1-1. Progression from periodontal health to gingivitis or periodontitis.](image)

In periodontal health, the oral biofilm is in a state of homeostasis with the host, without inflammation. In gingivitis, a build-up of bacteria causes mild inflammation but bone tissues remain intact. In periodontitis, bacteria form a dysbiotic biofilm, leading to the formation of deep periodontal pockets, further inflammation and bone resorption.
1.3.1 Periodontitis: a polymicrobial disease

Although previously perceived as a ‘single aetiologic agent disease’, periodontitis is now recognised as being caused by an extensive list of potential aetiological agents. Apart from bacteria, which are the primary aetiologic factor of periodontal disease, viral-bacterial and mycotic-bacterial co-infections have been described (but not necessarily found to be aetiological). Herpesviruses, including human cytomegalovirus (HCMV), Epstein-Barr virus (EBV) and herpes simplex virus (HSV) have been shown to occur with high frequency in active periodontal lesions (Kamma and Slots 2003, Rodrigues, Teixeira et al. 2015). Sites presenting with HCMV and HSV had a higher occurrence of Porphyromonas gingivalis (Slots, Kamma et al. 2003) and sites infected with active HCMV were reported to harbour an increased occurrence of Aggregatibacter actinomycetemcomitans (Teughels, Sliepen et al. 2007). Herpesviruses infect host defence cells and are strong inducers of pro-inflammatory responses. This herpesvirus-bacterial periodontal infection leaves the host susceptible to increased bacterial load while promoting tissue destruction. Oral colonisation by fungi has generally been assumed to be associated with pathology. However, a study by Ghannoum and co-workers identified 101 fungal species in healthy individuals belonging to seven genera; only four of which are known to be pathogenic to humans – Candida, Aspergillus, Fusarium and Cryptococcus (Ghannoum, Jurevic et al. 2010). Fungi are ubiquitously present in the oral cavity and by far the most prevalent fungal species are the Candida spp. (Naglik 2013). Candida spp. form biofilms with several bacterial species including Staphylococcus aureus (Peters, Jabra-Rizk et al. 2010), Streptococcus mutans (Pereira-Cenci, Deng et al. 2008) and Streptococcus gordonii (Holmes, Gopal et al. 1995, Silverman, Nobbs et al. 2010). These Candida-bacterial interactions may play both beneficial and detrimental roles in host homeostasis. Candida-bacterial adherence can favour commensal colonisation of oral surfaces and promote health (Krom, Kidwai et al. 2014) but can also unfavourably alter the local microbiome to promote inflammation (Xu, Jenkinson et al. 2014) and render bacteria less susceptible to antibiotic treatment (Harriott and Noverr 2009).

Studies of inter-kingdom interactions have suggested new concepts in the complexity of periodontal diseases; however, their implication in pathogenesis remains uncertain. With over 700 bacterial species in the oral cavity (Aas, Paster et al. 2005), the
significance of bacteria in the development of all types of chronic inflammatory periodontal disease is undisputable. The transition from periodontal health to gingival inflammation is associated with growth and maturation of dental plaque. Current thoughts about the transition from gingivitis to periodontitis suggest a dramatic transition from a mature symbiotic community to a dysbiotic one.

1.4 Dental plaque development
Surfaces of oral tissues are permanently coated with salivary pellicles, which are layers of proteins and glycoproteins of salivary origin. Bacterial adhesion to the pellicle is the first step of biofilm formation and, therefore, it is not surprising that many oral bacteria possess mechanisms of adherence to these surfaces. The predominant early colonisers are the *Streptococcus* spp., *Actinomyces* spp., *Veillonella* spp., *Haemophilis* spp., and *Lactobacillus* spp. (Li, Helmerhorst et al. 2004, Diaz, Chalmers et al. 2006, Dige, Raarup et al. 2009). These pioneer bacteria recognise molecules on the salivary pellicle such as statherin, proline-rich proteins, salivary α-amylase, sialylated mucins and salivary agglutinin; all of which originate from the host. Some of the best characterised adhesins of oral streptococci are the proteins of the antigen I/II family, designated Ssp proteins (Demuth, Duan et al. 1996) e.g. in *S. gordonii*, SspA and SspB bind mucin-like salivary agglutinin glycoproteins (Jenkinson and Demuth 1997). *Actinomyces naeslundii*, another significant early coloniser, possess type I fimbriae, which bind to salivary acidic proline-rich proteins and to statherin in salivary pellicles (Yeung 1999). Once initial adhesion is established, oral microorganisms will live in a self-produced matrix of extracellular polymeric substances (EPS) (Liu, Liu et al. 2004) which continues its transformation throughout biofilm maturation. EPS mainly consists of polysaccharides, protein, nucleic acid and lipids and provides the mechanical stability of the biofilm but more importantly, immobilizes biofilm cells and keeps them in close proximity, thus allowing for interactions including cell–cell communication and the formation of synergistic micro-consortia (Flemming and Wingender 2010).
Adhesion of bacteria to salivary pellicles and production of EPS forms the foundation for biofilm maturation, allowing subsequent species to aggregate to an already formed layer of bacteria and their products, rather than the acquired pellicle. Secondary colonisers include *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Aggregatibacter actinomycetemcomitans* (Kuboniwa and Lamont 2010). Figure 1-2 shows a schematic representation of bacterial colonisation of the tooth surface. Mechanisms of bacterial co-aggregation involve polysaccharide recognition between bacteria; more specifically, a lectin-like adhesin protein, usually carried on fimbriae or fibrils, and a carbohydrate receptor (Rickard, Gilbert et al. 2003). The majority of co-aggregation adhesins identified have been found on *Streptococcus* spp., *Actinomyces* spp. and *Fusobacterium* spp. The early coloniser *S. gordonii* carries several distinct co-aggregation adhesins able to bind components of saliva, polymorphonuclear cells and species of oral bacteria (Takahashi, Sandberg et al. 1997, Clemans, Kolenbrander et al. 1999, Takahashi, Konishi et al. 2002). Of these, CshA, a fibrillar protein, facilitates co-aggregation with *A. naeslundii*. 

**Figure 1-2. Bacterial colonisation of the tooth surface.** Early colonisers adhere to the salivary pellicle, allowing for secondary colonisers to bind to an already formed bacterial layer. Intermediate coloniser *F. nucleatum* is able to adhere to both early and late colonisers, as well as the salivary pellicle.
(McNab, Forbes et al. 1999) and SspA and SspB polypeptides, which mediate co-aggregation with both *A. naeslundii* and *P. gingivalis* (Love, McMillan et al. 2000, Egland, Du et al. 2001). *A. naeslundii* also carries a co-aggregation adhesin on the tip of its type II fimbriae, which facilitates adhesion to streptococci, polymorphonuclear cells and epithelial cells (Sandberg, Ruhl et al. 1995, Klier, Roble et al. 1998). *F. nucleatum*, described as a bridging organism, exhibits more partnerships than any other species and is able to co-aggregate with both early and late colonisers (Kolenbrander and London 1993). *F. nucleatum* possesses galactose- and arginine-inhibitable adhesins that facilitate interactions with *Streptococcus* spp., *Actinomyces* spp., *Veillonella atypica*, *P. gingivalis* and *A. actinomycetemcomitans* (Kolenbrander and Andersen 1989, Shanitzki, Hurwitz et al. 1997). Co-aggregation of *F. nucleatum* to *P. gingivalis* is mediated via a galactoside moiety on the *P. gingivalis* surface and a lectin on *F. nucleatum* (Rosen and Sela 2006), which can be inhibited by lactose, galactose and other related sugars (Kolenbrander and Andersen 1989, Shanitzki, Hurwitz et al. 1997). Moreover, the same galactose-binding adhesin has been shown to facilitate *F. nucleatum* binding to eukaryotic cells, including human buccal epithelial cells, gingival and periodontal ligament fibroblasts, HeLa cells, murine lymphocytes and macrophages, and murine polymorphonuclear leukocytes (Weiss, Shanitzki et al. 2000). The arginine-inhibitable adhesin of *F. nucleatum*, is also known as RadD, a 350 kDa putative autotransporter protein essential for inter-species adherence as seen by the inability of RadD mutants to form dual-species biofilms in vitro (Kaplan, Lux et al. 2009).

Bacterial aggregation is a very complex process. Aggregation studies of over 300 oral bacterial species indicated that 90% undergo co-aggregation (Kolenbrander 1988). However, these are not random and specific partnerships have been described. An early in vivo study showed that introducing *P. gingivalis* into the mouths of human volunteers resulted in the organism locating almost exclusively on pre-formed, streptococcal-rich supra- gingival plaque (Slots and Gibbons 1978). Subsequent studies have demonstrated strong binding capacity between *P. gingivalis* and *S. gordonii* (Lamont, El-Sabaeny et al. 2002) but not *S. mutans* (Bradshaw D. J. 1998). Other examples of co-aggregation among sub- gingival organisms include binding of *F. nucleatum*, *S. mitis* and *P. gingivalis*, where the presence of *F. nucleatum* promotes
adherence of *P. gingivalis* (Nagayama, Sato et al. 2001). *T. denticola* and *P. gingivalis* have been shown to accumulate in dual-species biofilms, an example of synergy in biofilm formation which can enhance growth of *P. gingivalis* (Grenier 1992, Yamada, Ikegami et al. 2005). Synergy is also seen between *Tannerella forsythia* and *F. nucleatum* in biofilm formation (Sharma, Inagaki et al. 2005) and the three species *P. gingivalis*, *T. denticola* and *T. forsythia* have been observed forming a complex associated with periodontal pockets *in vivo* (Socransky, Haffajee et al. 1998).

While co-aggregation mechanisms are paramount for the physical growth of the oral biofilm, the interactions between bacteria also alter gene regulation. A well-documented example is the co-aggregation pair of *S. gordonii* and *Actinomyces oris*. Following co-aggregation, *S. gordonii* exhibited a threefold change in expression of 23 genes of which 9 were involved in arginine biosynthesis and transport (Jakubovics, Gill et al. 2008). Arginine biosynthesis of *S. gordonii* in monoculture was inefficient, as it was in co-cultures with *A. oris* (without co-aggregation), resulting in *S. gordonii* hindered growth. In dual-species cultures with aggregation, however, *S. gordonii* grew at high cell density in low arginine concentrations; thus *A. oris* was shown to stabilise *S. gordonii* expression of arginine biosynthesis genes and enable its growth when exogenous arginine was limited. In *P. gingivalis* co-aggregation with *S. gordonii*, 30 *P. gingivalis* genes were up-regulated, most of which were related to metabolism and energy production (Simionato, Tucker et al. 2006), and in single-species biofilms, more than 81 *P. gingivalis* cell envelope proteins were either up- or down-regulated (Ang, Veith et al. 2008). Moreover, in three species biofilms of *P. gingivalis*, *F. nucleatum* and *S. gordonii*, 1156 *P. gingivalis* proteins were up- or down-regulated. Of those, HmuR, an outer membrane receptor was up-regulated and its importance in the development of multispecies communities was highlighted when a *P. gingivalis* HmuR mutant was unable to form co-aggregates with *F. nucleatum* and *S. gordonii* (Kuboniwa, Hendrickson et al. 2009).

Apart from co-aggregation, oral bacteria also auto-aggregate and it is reasonable to assume that auto-aggregation could also lead to changes in transcriptional responses. *F. nucleatum*, notorious for its co-aggregation capabilities, also has high auto-aggregation potential (Shen, Samaranayake et al. 2005, Khemaleelakul, Baumgartner
et al. 2006). A study by Merritt and colleagues found rapid auto-aggregation of *F. nucleatum* cells in the presence of saliva, which impacted the expression of a large number of genes (Merritt, Niu et al. 2009). Transcription of genes associated with carbon source acquisition and catabolism, genes able to provide alternative glutamate sources and genes related to sodium homeostasis were up-regulated. These responses were not seen in the absence of auto-aggregation. Interestingly, auto-aggregation also induced the FN1584 operon associated with β-lactamase production, an enzyme that provides resistance to β-lactam antibiotics. This study highlights probably the earliest transcriptional events occurring as *F. nucleatum* transitions from planktonic to biofilm growth, and it is interesting that they include genes offering antibiotic resistance that can impact not only on itself but also on its numerous co-aggregates.

1.5 Microbial aetiology of periodontitis

Microbial analyses over the past two centuries have resulted in multiple hypotheses describing the nature of the pathogenesis of periodontal disease. Although in most cases the tooth-associated biofilm is recognised as the principle agent in driving the host inflammatory responses leading to loss of periodontal tissues, the appreciation of the underlying mechanisms has undergone significant revision.

1.5.1 The Specific and Non-specific plaque hypotheses

The controversy between the ‘Non-specific plaque hypothesis’ and the ‘Specific plaque hypothesis’, lasted over several decades. The ‘Non-specific plaque hypothesis’ suggested that the amount of dental plaque, regardless of its composition, was enough to cause inflammation and inevitable destruction of periodontal tissues (Schultz-Haudt, Bruce et al. 1954). Disregarding individual levels of bacterial virulence, this hypothesis described a threshold capacity of the host to neutralise bacterial products and surpassing this threshold would lead to destructive disease (Theilade 1986). The ‘Specific plaque hypothesis’ proposed that the growth of specific organisms drove destructive periodontal disease and was initially extrapolated from the idea that antibiotics against specific bacterial species, e.g. *Streptococcus* spp., could cure and prevent caries (Loesche, Rowan et al. 1975, Loesche 1976, Loesche, Bradbury et al. 1977).
1.5.2 Microbial shift and the ‘red complex’

With improvement in anaerobic microbial culture techniques, more studies focused on the hunt for disease related organisms. The view of periodontal microbial pathogenesis had left behind the ‘Non-specific plaque hypothesis’ which no longer fitted with the known fact that not all individuals with a large accumulation of dental plaque developed disease. Furthermore, studies had revealed compositional changes in the microbiota during disease progression. Thus, like other polymicrobial diseases, gingivitis was described as a microbial shift disease, owing to a well characterised shift from mostly Gram-positive to mostly Gram-negative species (Slots 1977, Slots 1977, Socransky 1977, Tanner, Haffer et al. 1979). However, in periodontitis, the microbial shift, also termed dysbiosis, was later associated with the formation of distinct microbial complexes consisting predominantly of Gram-negative bacteria. In a landmark study, Socransky and colleagues undertook a cluster analysis of the microorganisms associated with various periodontal disease states and described a colour-coded system. The two groups of clusters of microorganisms most strongly associated with severe periodontitis were the so called ‘red complex’ consisting of \(P.\ gingivalis\), \(T.\ forsythia\) and \(T.\ denticola\) (Socransky, Haffajee et al. 1998, Holt and Ebersole 2005), and the less strongly associated ‘orange complex’ including \(P.\ intermedia\) and \(F.\ nucleatum\) (Socransky, Haffajee et al. 1998, Socransky and Haffajee 2005). However, these studies do not suggest a clear succession in the addition of species but more the association between plaque constituents and the severity of disease. Despite strong association of the ‘red-complex’ species with periodontitis, periodontal pathogens such as \(P.\ gingivalis\) and \(T.\ forsythia\) have also been isolated from healthy individuals (Ximenez-Fyvie, Haffajee et al. 2000, Ximenez-Fyvie, Haffajee et al. 2000, Mayanagi, Sato et al. 2004, Diaz, Chalmers et al. 2006, Riep, Edesi-Neuss et al. 2009). Likewise, Gram-negative \(Veillonella\) spp. as well as \(Moraxella osloensis\) have been associated with health, while Gram-positive \(Filifactor alocis\) and \(Peptostreptococcus stomatis\) have been associated with disease (Kumar, Leys et al. 2006, Griffen, Beall et al. 2012). Thus, defining the shift in the content of oral microbiota, which leads to disease, remains difficult.
1.5.3 The keystone-pathogen hypothesis and the polymicrobial synergy and dysbiosis (PSD) model

Following the identification of the ‘red complex’ (*P. gingivalis*, *T. forsythia*, *T. denticola*), research focused on understanding the virulence factors and pathogenic mechanisms of the three bacteria in line with the concept of a single infective agent responsible for disease pathogenesis (Holt and Ebersole 2005). However, these studies were insufficient in explaining how these ubiquitous microorganisms trigger the initiation of tissue breakdown. The irreversible damage to the periodontium is primarily caused by an exaggerated host inflammatory response, which is triggered by a dysbiotic microflora, but how this homeostatic imbalance in the biofilm occurred remained elusive. The keystone-pathogen hypothesis implies that a specific low-abundance microorganism can support and stabilise a dysbiotic growth of the biofilm, leading to a disruption of host responses (Hajishengallis, Darveau et al. 2012). This hypothesis was supported by a study in a mouse model where the ‘red complex’ bacterium *P. gingivalis*, present at < 0.01% of the total bacterial count, was shown to induce significant alterations in the commensal microbiota, which subsequently resulted in inflammation and bone loss (Hajishengallis, Liang et al. 2011). A notable observation was that *P. gingivalis* did not cause periodontitis in germ-free mice, despite colonisation, demonstrating that the oral commensal microbiota is necessary for tissue and bone destruction (Hajishengallis, Liang et al. 2011). Further, consistent with its role as a keystone pathogen, *P. gingivalis* has been shown to promote a dysbiotic growth of the biofilm in both non-human primates and rabbit models of periodontitis (Hasturk, Kantarci et al. 2007, Page, Lantz et al. 2007). *P. gingivalis* has become the most widely studied periodontal pathogen and the most well characterised keystone pathogen in periodontal disease. Its ability to modulate inflammatory responses using an array of virulence factors will be discussed in detail in the next section.

The evolving view on the pathogenesis of periodontitis combines previous concepts in a model where the initiation of disease is driven by a synergistic and dysbiotic microbial community rather than single species or even micro-consortia such as the ‘red complex’ (Hajishengallis and Lamont 2012). The activity of keystone pathogens is required for the development and successive stability of a disease-provoking
microbiota, which together induce strong inflammatory responses but which also withstand the host innate and adaptive immune mechanisms.

1.6 Porphyromonas gingivalis – a keystone pathogen

*P. gingivalis*, a Gram-negative, anaerobic and asaccharolytic rod, has long been associated with periodontal disease. Decades of research have identified a multitude of virulence factors that *P. gingivalis* utilises and these have been extensively reviewed (Yilmaz 2008, Bostanci and Belibasakis 2012, Mysak, Podzimek et al. 2014). The main virulence factors of *P. gingivalis* are summarised in Table 1-1; however, for the purpose of this study, a selection of these factors will be discussed with the intent of highlighting the keystone pathogen attributes of this bacterium.

The PSD model of periodontal disease suggests that the host immune response is subverted by keystone pathogens, which are aided by accessory pathogens (commensal bacteria that can enhance the virulence of keystone pathogens). The ensuing impaired immune surveillance will favour growth of the whole biofilm, leading to a dysbiotic microbial community composed mainly of pathobionts (commensal microorganisms with the potential to induce pathology under conditions of disrupted homeostasis). This will elevate the overall virulence of the biofilm and cause non-resolving exacerbated inflammatory responses leading to tissue destruction (Hajishengallis and Lamont 2012, Lamont and Hajishengallis 2015).
<table>
<thead>
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<th>Bacterial component</th>
<th>Activity</th>
<th>Reference</th>
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<tr>
<td><strong>Capsule</strong></td>
<td>Presence and type influence initial adhesion to epithelial cells</td>
<td>(Laine, Appelmelk et al. 1997)</td>
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<tr>
<td>Capsular polysaccharide</td>
<td>Co-aggregation with <em>F. nucleatum</em></td>
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<tr>
<td>At least six serotypes of capsular antigen, K1-K6</td>
<td>Resistance to phagocytosis, evasion of immune responses</td>
<td>(Dierickx, Pauwels et al. 2003)</td>
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<td></td>
<td>Reduction of IL-1β, IL-6 and IL-8 transcripts from gingival fibroblasts</td>
<td>(Rosen and Sela 2006)</td>
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<td></td>
<td>Lower invasion efficiency of gingival fibroblasts</td>
<td>(Singh, Wyant et al. 2011)</td>
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<td></td>
<td>K1 serotype induces cell migration chemokines (MCP-1, KC, MIP-2, RANTES) in murine macrophages</td>
<td>(Brunner, Scheres et al. 2010)</td>
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</table>

| **Ltp1**            | Negative regulator of exopolysaccharide production and biofilm formation with *S. gordonii* | (Maeda, Tribble et al. 2008) |
| Tyrosine phosphatase | Down-regulates expression of Mfa1 fimbriae and biofilm development | (Chawla, Hirano et al. 2010) |

| **LtpO**            | O-deacylation of LPS and secretion of gingipains to cell surface | (Chen, Peng et al. 2011) |
| Outer membrane protein | | |

Table 1-1. *Porphyromonas gingivalis* virulence factors
<table>
<thead>
<tr>
<th><strong>RagA / RagB</strong></th>
<th>Involved in virulence of <em>P. gingivalis</em> and uptake of macromolecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane transporter complex</td>
<td>RagB is a TLR agonist (recognised by human monocytic TLR2 and 4), induces</td>
</tr>
<tr>
<td>RagA – outer membrane protein</td>
<td>STAT4 / NF-κB and pro-inflammatory cytokines</td>
</tr>
<tr>
<td>with features of a TonB membrane receptor</td>
<td></td>
</tr>
<tr>
<td>RagB – immunodominant antigen</td>
<td></td>
</tr>
<tr>
<td><strong>Fimbriae</strong></td>
<td>Adhesion to host and other bacteria, invasion of host cells, induction of</td>
</tr>
<tr>
<td>FimA – Major fimbriae</td>
<td>inflammatory responses leading to alveolar bone loss</td>
</tr>
<tr>
<td>Mfa1 – minor fimbriae</td>
<td>Invasion of osteoblasts and subsequent activation of signalling</td>
</tr>
<tr>
<td>53K protein – novel major fimbrilin</td>
<td>Regulator of gene expression in aortic smooth muscle cells – implicated in</td>
</tr>
<tr>
<td>of Mfa1 fimbriae, activity unknown</td>
<td>atherosclerosis</td>
</tr>
<tr>
<td></td>
<td>Production and pathogenicity of outer membrane vesicles</td>
</tr>
<tr>
<td></td>
<td>Potential role in Treg dysregulation in atherosclerosis</td>
</tr>
<tr>
<td><strong>LPS</strong></td>
<td>Generally weak inducer of inflammatory mediators</td>
</tr>
<tr>
<td>Lipopolysaccharide with four Lipid A structural subgroups identified</td>
<td>Different Lipid A moieties have difference immunostimulatory abilities and can</td>
</tr>
<tr>
<td></td>
<td>be agonistic, inert or antagonistic with respect to TLR4 activation</td>
</tr>
<tr>
<td></td>
<td>Controversy surrounding engagement of TLR2</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reviewed by (Amano 2010)</td>
</tr>
<tr>
<td></td>
<td>(Zhang, Ju et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>(Zhang, Ju et al. 2013)</td>
</tr>
<tr>
<td></td>
<td>(Zhang, Sirsjo et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>(Nagano, Hasegawa et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>(Mantri, Chen et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>(Yang, Wu et al. 2014)</td>
</tr>
<tr>
<td></td>
<td>(Jain and Darveau 2010)</td>
</tr>
<tr>
<td></td>
<td>(Herath, Darveau et al. 2013)</td>
</tr>
<tr>
<td></td>
<td>(Kato, Taguchi et al. 2014)</td>
</tr>
</tbody>
</table>
Lipid A structural modifications can lend resistance to antimicrobial peptides (e.g. polymyxin B)

Penta-acylated LPS is a stronger activator of human gingival fibroblasts than tetra-acylated LPS (signalling mediated through TLR4, NF-κB axis)

LPS inhibits osteoblastic differentiation and induces pro-inflammatory cytokines in periodontal ligament stem cells – involvement in periodontal tissue regeneration

LPS induces thrombospondin-1 (TSP-1; major regulator of immune responses) expression from THP-1 cells via TLR2 / NF-κB

Differences in *P. gingivalis* LPS from healthy and periodontal subjects affect colony morphology and polymyxin B resistance – possible involvement of O-antigen moieties

Stimulation of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin in human umbilical vein endothelial cells

<table>
<thead>
<tr>
<th>Gingipains</th>
<th>Rgps responsible for collagenase activity</th>
<th>Required for co-aggregation with other bacteria</th>
<th>Adhesion to host surfaces</th>
<th>Rgps involved in maturation of fimbriae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine proteases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RgpA and RgpB (arginine-specific)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kgp (lysine-specific)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Gokyu, Kobayashi et al. 2014)
(Diaz, Hoare et al. 2015)
(Andrukhov, Steiner et al. 2015)

Reviewed by (Guo, Nguyen et al. 2010)
RgpA and Kgp involved in haemolysis of erythrocytes, haemoglobin release and haem acquisition
Degradation of antimicrobial peptides, components of complement, receptors and cytokines essential for phagocyte functions and appropriate immune response, extracellular matrix proteins, signalling molecules
Induction of apoptosis through degradation of cell adhesion molecules

<table>
<thead>
<tr>
<th>HagA, HagB, HagC</th>
<th>HagA involved in adhesion and invasion of host cells</th>
<th>HagA involved in adhesion and invasion of host cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemaglutininins</td>
<td>HagB induces cytokine and chemokine responses in dendritic cells</td>
<td>HagB and HagC required for adherence to epithelial cells and multi-species biofilm growth</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HbR</th>
<th>Binds haemoglobin and captures released haem</th>
<th>Binds haemoglobin and captures released haem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin receptor conserved in RgpA, Kgp, HagA</td>
<td>Inhibits RANKL-induced osteoclastogenesis in bone marrow macrophages</td>
<td>Inhibits RANKL-induced osteoclastogenesis in bone marrow macrophages</td>
</tr>
<tr>
<td></td>
<td>Promotes IL-8 production in gingival epithelial cells via p38 / ERK 1/2 and ATF-2 / CREB / NF-κB</td>
<td>Promotes IL-8 production in gingival epithelial cells via p38 / ERK 1/2 and ATF-2 / CREB / NF-κB</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HmuR / HmuY</th>
<th>Hmu-mediated haem/iron system</th>
<th>Hmu-mediated haem/iron system</th>
</tr>
</thead>
<tbody>
<tr>
<td>HmuR – haemoglobin receptor</td>
<td>HmuY resistant to gingipains and host proteases</td>
<td>HmuY resistant to gingipains and host proteases</td>
</tr>
<tr>
<td>HmuY – outer membrane protein</td>
<td>HmuY important for growth and viability in haem-limited environments and in</td>
<td>HmuY important for growth and viability in haem-limited environments and in</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme Name</th>
<th>Function</th>
<th>Involved in Pathology</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DPP4, DPP5, DPP7, DPP11</strong>&lt;br&gt;Dipeptidyl peptidases</td>
<td>Amino acid metabolism into di- and tripeptides</td>
<td>Invasion of macrophages</td>
<td>(Olczak, Sosicka et al. 2015)</td>
</tr>
<tr>
<td><strong>PTP-A</strong>&lt;br&gt;Prolyl tripeptidylpeptidase</td>
<td>AOP&lt;br&gt;Novel acylpeptidyl oligopeptidase</td>
<td>Amino acid metabolism into di- and tripeptides</td>
<td>(Ohara-Nemoto, Rouf et al. 2014)</td>
</tr>
<tr>
<td><strong>PepO</strong>&lt;br&gt;Endothelin converting-like enzyme</td>
<td>Suggested that it acts on invasion/lysis of mammalian cell membrane</td>
<td>Amino acid metabolism into di- and tripeptides</td>
<td>(Ansai, Yu et al. 2003)</td>
</tr>
<tr>
<td><strong>SerB</strong>&lt;br&gt;Serine phosphatase</td>
<td>Invasion and intracellular survival&lt;br&gt;Actin re-organisation and suppression of IL-8</td>
<td>Amino acid metabolism into di- and tripeptides</td>
<td>(Tribble, Mao et al. 2006)</td>
</tr>
<tr>
<td><strong>PPAD</strong>&lt;br&gt;<em>P. gingivalis</em> peptidylarginine deiminase</td>
<td>Inactivation of EGF&lt;br&gt;Induction of prostaglandin E$_2$-signalling pathway&lt;br&gt;Citrullination of fibrinogen and α-enolase – involvement in pathogenesis of rheumatoid arthritis</td>
<td>Amino acid metabolism into di- and tripeptides</td>
<td>(Pyrc, Milewska et al. 2013)</td>
</tr>
</tbody>
</table>

Amino acid metabolism into di- and tripeptides:
- (Ito, Nakajima et al. 2006)
- (Nemoto, Ohara-Nemoto et al. 2016)

Citrullination of fibrinogen and α-enolase – involvement in pathogenesis of rheumatoid arthritis:
- (Pyrc, Milewska et al. 2013)
- (Gawron, Bereta et al. 2014)
- (Wegner, Wait et al. 2010)
1.6.1 Subversion of host immune responses

*P. gingivalis* elegantly fulfils the roles of a true keystone pathogen. During colonisation of periodontal tissues with a symbiotic microflora, there is a constant inflammatory state characterised by secretion of antimicrobial peptides, cytokines and a continual influx of circulating neutrophils in the junctional epithelium (Tonetti, Imboden et al. 1998). This inflammatory state controls overgrowth and overt pathogenicity and maintains local tissue homeostasis while protecting the host from infections spreading into the tissues and septicemia. *P. gingivalis* is able to disrupt this through manipulation of Toll-like receptors (TLR) responses, local chemokine paralysis and corruption of the complement system (Darveau, Belton et al. 1998, Darveau 2010, Hajishengallis and Lambris 2011).

The importance of neutrophils in the management of dental plaque is undisputed, as seen in individuals with congenital deficiencies in neutrophil numbers or recruitment, who develop severe periodontitis (Carrassi, Abati et al. 1989, Hart, Shapira et al. 1994, Hajishengallis and Hajishengallis 2014, Moutsopoulos, Konkel et al. 2014). *P. gingivalis* can impair recruitment of neutrophils by down-regulating expression of chemokines and cell adhesion molecules such as IL-8, intercellular adhesion molecule 1 (ICAM-1) and E-selectin (Darveau, Cunningham et al. 1995, Huang, Kim et al. 2001). *P. gingivalis* proteases, or gingipains, particularly Kgp (lysine gingipain), directly degrade IL-8 and IL-6 proteins secreted by human gingival epithelial cells (Stathopoulou, Benakanakere et al. 2009). Moreover, *P. gingivalis* influences IL-8 release from gingival epithelial cells via its SerB phosphatase. SerB binds to and dephosphorylates serine 536 of the RelA/p65 subunit of NF-κB, which prevents nuclear translocation and transcription of the IL-8 gene (Bainbridge, Verma et al. 2010, Takeuchi, Hirano et al. 2013).

Although IL-8 is a powerful neutrophil chemoattractant, it does not function in isolation. Neutrophils are also guided towards inflamed tissues by the activation of complement (Kolaczkowska and Kubes 2013). The complement system is activated by three pathways (classical, alternative and lectin), with its basic functions being opsonisation (C3b main effector molecule), recruitment of macrophages and neutrophils (C5a interaction with C5a receptor (C5aR)), direct killing of organisms through the formation of the membrane attack complex (mediated by C5b-C9) and
further downstream activation of immune responses (Ricklin, Hajishengallis et al. 2010). The complement system is therefore crucially involved in inflammation and immunity. All three complement pathways converge at the C3 component and *P. gingivalis* gingipains can degrade C3 (Popadiak, Potempa et al. 2007), thus inhibiting the complement cascade regardless of the pathway initiated. In addition, *P. gingivalis* RgpA binds the complement inhibitor C4b-binding protein, contributing to serum resistance by decreasing complement activation (Potempa, Potempa et al. 2008). A remarkable ability of *P. gingivalis* Rgps is to generate C5a, the most potent pro-inflammatory effector of complement. While this may seem counterproductive, *P. gingivalis* uses C5a to manipulate C5aR and its crosstalk with TLR2. Through this hijacking mechanism of C5aR-TLR2, *P. gingivalis* has been shown to selectively inhibit TLR2-induced clearance by innate leukocytes while promoting inflammation both *in vitro* and *in vivo* (Wang, Krauss et al. 2010, Liang, Krauss et al. 2011, Maekawa, Krauss et al. 2014). Interestingly, while this exploitation has been shown in neutrophils and macrophages, a recent study showed that this was not the case in dendritic cells (Hajishengallis, Krauss et al. 2016).

Additional immune-subversive mechanisms of *P. gingivalis* include the manipulation of TLR4 through its different LPS lipid A structures. LPS is composed of three domains: lipid A, a core oligosaccharide and an O-antigen (Raetz and Whitfield 2002), with lipid A known to be responsible for the toxic effects of LPS (Raetz, Reynolds et al. 2007). *P. gingivalis* lipid A is unique compared with lipid A structures of other Gram-negative bacteria due to its structural heterogeneity (Kumada, Haishima et al. 1995, Darveau, Pham et al. 2004, Coats, Jones et al. 2009). What is intriguing about *P. gingivalis* lipid A is that it can be stimulatory, inert or antagonistic with respect to TLR4 activation, this being attributed to differences in the acyl chains and phosphate groups (Jain and Darveau 2010). In an inflammatory environment, the TLR4 antagonist lipid A form (tetra-acylated, monophosphorylated) dominates, leading to decreased β-defensin expression and inhibition of epidermal growth factor (EGF)-mediated signalling (Darveau 2010). *P. gingivalis* has also been shown to suppress inflammasome activation by inhibiting the endocytosis of *F. nucleatum* by macrophages (Taxman, Swanson et al. 2012) (for a more detailed description of inflammasome activation see Section 1.8.2.1). This not only adds to its low immune-stimulatory activity but may also
promote synergy between the two species. *P. gingivalis* is also the only bacterium known to express a peptidylarginine deiminase (PPAD), which is able to modify proteins by converting C-terminal arginine residues into citrulline (Pyrc, Milewska et al. 2013). While most research has focused on the link between *P. gingivalis* PPAD-generated immunogenic peptides and rheumatoid arthritis, PPAD has been shown to citrullinate and subsequently inactivate EGF and EGF-EGFR signalling (Pyrc, Milewska et al. 2013), as well as abolish C5a activity (Bielecka, Scavenius et al. 2014). These mechanisms contribute to tissue damage and delayed healing and it is reasonable to assume that PPAD may act on other substrates within the periodontium, favouring disease progression, as seen in the development of experimental periodontitis which was significantly reduced in the presence of a *P. gingivalis* PPAD mutant (Gully, Bright et al. 2014).

*P. gingivalis* is a master manipulator of innate immune responses and the mechanisms it employs are very complex. By intercepting host immune function, *P. gingivalis* protects itself as well as ‘bystanders’ from phagocytic clearance, allowing uncontrolled growth of the biofilm, while simultaneously promoting inflammation. The dysbiotic growth of the biofilm further enhances the tissue’s potentially destructive inflammatory responses, which overpower the host’s immunity. Through these attributes, *P. gingivalis* is able to orchestrate local dysbiosis in both biofilm growth and inflammation, leading to periodontal tissue destruction.

### 1.7 *Fusobacterium nucleatum*: an accessory pathogen?

*F. nucleatum* is a Gram-negative, anaerobic bacterium, one of the most abundant species of the oral cavity, isolated from both healthy and diseased individuals (Griffen, Beall et al. 2012). It is also frequently isolated from extra-oral sites and has been implicated in an array of diseases; however, non-oral afflictions will not be discussed here (reviewed by (Han 2015)).

*F. nucleatum* has been described as a bridging organism, important in biofilm maturation, linking health to the diseased state. This is largely due to its ability to form more partnerships than any other species, with both early and late colonisers, especially obligate anaerobes (Sbordone and Bortolaia 2003, Kolenbrander, Palmer et
...al. 2010). While *F. nucleatum* is not known to harbour many virulence factors (summarised in Table 1-2), it is suggested that its adhesion and aggregation properties are its main virulence attributes. While it is established that *F. nucleatum* plays a critical role in the physical development of the biofilm, for *F. nucleatum* to be a true accessory pathogen it must also enhance the growth and virulence of keystone pathogens and the overall virulence of the biofilm (Hajishengallis and Lamont 2012).
Table 1-2. *Fusobacterium nucleatum* virulence factors

<table>
<thead>
<tr>
<th>Bacterial component</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RadD</strong></td>
<td>Arginine-inhibitable adhesin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Required for co-aggregation with other bacteria</td>
<td>(Kaplan, Lux et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>Cell death of human lymphocytes</td>
<td>(Kaplan, Ma et al. 2010)</td>
</tr>
<tr>
<td><strong>Fap2</strong></td>
<td>Galactose-inhibitable adhesin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell death of human lymphocytes</td>
<td>(Kaplan, Ma et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Haemagglutination, co-aggregation with <em>P. gingivalis</em>, host cell binding and placental colonisation</td>
<td>(Coppenhagen-Glazer, Sol et al. 2015)</td>
</tr>
<tr>
<td><strong>Aim1</strong></td>
<td>Apoptosis Inducing Membrane protein 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Apoptosis of human lymphocytes</td>
<td>(Kaplan, Lux et al. 2005)</td>
</tr>
<tr>
<td><strong>Aid1</strong></td>
<td>Adherence Inducing Determinant protein 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Required for co-aggregation with <em>Streptococcus</em> spp. and <em>Enterococcus faecalis</em></td>
<td>(Kaplan, Kaplan et al. 2014)</td>
</tr>
<tr>
<td><strong>FadA</strong></td>
<td>Fusobacterium adhesin A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Required for binding and invasion of various cell types</td>
<td>Reviewed by (Han 2015)</td>
</tr>
<tr>
<td></td>
<td>Increases endothelium permeability</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Facilitates both intra- and inter-cellular invasion of <em>F. nucleatum</em> and other species</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Function</td>
<td>Induced Effects</td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>-----------------</td>
</tr>
<tr>
<td><strong>FAD-I</strong>&lt;br&gt;Fusobacterium-associated defensin inducer</td>
<td>Required for induction of hBD-2 expression and secretion in oral epithelial cells</td>
<td></td>
</tr>
<tr>
<td><strong>FomA porin</strong>&lt;br&gt;Outer membrane protein</td>
<td>TLR2 dependent signalling <em>in vitro</em> and <em>in vivo</em>, activation of NF-κB, secretion of IL-6, IL-8, IL-10</td>
<td></td>
</tr>
<tr>
<td><strong>LPS</strong>&lt;br&gt;Lipopolysaccharide</td>
<td>IL-8 production in human oral fibroblasts&lt;br&gt;IL-1β and TNF-α production from macrophages</td>
<td></td>
</tr>
<tr>
<td><strong>Fusolisin</strong>&lt;br&gt;Serine protease</td>
<td>Exhibits weak proteolytic activity compared to proteases of other periodontal bacteria&lt;br&gt;Degradation of extracellular matrix proteins (fibronectin, collagen I/IV) and immunoglobulin A&lt;br&gt;Nutritional role in growth of <em>F. nucleatum</em></td>
<td></td>
</tr>
</tbody>
</table>
*F. nucleatum* enhances invasion of gingival epithelial and aortic endothelial cells by *P. gingivalis* (Saito, Inagaki et al. 2008). Moreover, invasion by *P. gingivalis* was significantly reduced by cycloheximide, an inhibitor of *F. nucleatum* invasion but not of *P. gingivalis*, suggesting that interactions between *F. nucleatum* and host cells facilitate *P. gingivalis* invasion (Saito, Inagaki et al. 2008; Saito, Kokubu et al. 2012). A similar effect was observed in human fibroblasts (Metzger, Blasbalg et al. 2009). A recent study has shown activation of NOX1 and NOX2 (involved in reactive oxygen species (ROS) generation) by *F. nucleatum* as a mechanism for facilitating *P. gingivalis* attachment to gingival fibroblasts, leading to their subsequent apoptosis (Ahn, Song et al. 2016). The authors speculate that activation of NOX1/2 provided an oxygen-limited environment which is more favourable to strict anaerobes such as *P. gingivalis*. It has been shown that *F. nucleatum* contributes to the reducing conditions necessary for the growth of *P. gingivalis* (Diaz, Zilm et al. 2002); this of course may benefit other oral pathogenic obligate anaerobes. Moreover, at low oxygen levels, epithelial cells become hypersensitive to pro-inflammatory stimuli and a greater pro-inflammatory and cell signalling response is observed in anaerobic conditions (Grant, Kolamunne et al. 2010). *F. nucleatum* and *P. gingivalis* are often found together at sites of infection and a mouse study showed that the minimal dose of *P. gingivalis* needed for infection was reduced by 1000-fold when *F. nucleatum* was introduced (Metzger, Lin et al. 2009). Furthermore, LPS extracted from *P. gingivalis* which had been co-cultured with *F. nucleatum*, exhibited higher induction of IL-1β, IL-6 and IL-8 in THP-1 cells than single-cultured *P. gingivalis* LPS (Lee and Baek 2013).

In an *in vivo* murine orofacial infection model, *F. nucleatum* enhanced the virulence of *Streptococcus constellatus*; however, the reverse was not true (Kuriyama, Nakagawa et al. 2000). A more recent study investigating synergy in biofilm formation showed that co-aggregation between *F. nucleatum* and *Prevotella* strains enhanced *Prevotella* biofilm formation but not *F. nucleatum* biofilm growth (Okuda, Kokubu et al. 2012). Likewise, adhesion to and invasion of epithelial cells by *P. gingivalis* and *A. actinomycetemcomitans* was greater when the two strains were co-incubated with *F. nucleatum* (Li, Guo et al. 2015). Moreover, a study in a rat model of polymicrobial infections showed that IgG antibody levels directed at *P. gingivalis*, *T. denticola* and *T. forsythia* in a polymicrobial infection with the three strains were lower when *F.*
F. nucleatum was introduced (Kesavalu, Sathishkumar et al. 2007). This could suggest a ‘protective’ role for F. nucleatum with regard to the three periodontal pathogens and an ability to modulate the adaptive host response in favour of disease progression.

Butyric acid is a major by-product of F. nucleatum metabolism, able to down-regulate proliferation and cytokine production in T-cells as well as induce apoptosis in various cell types (Kurita-Ochiai, Fukushima et al. 1995, Kurita-Ochiai, Ochiai et al. 2000, Kurita-Ochiai, Amano et al. 2003). Significant in the progression of periodontitis is butyric acid-induced apoptosis of gingival fibroblasts, which is linked with loss of periodontal attachment (Kurita-Ochiai, Seto et al. 2008). This presents a mechanism by which F. nucleatum and other periodontal bacteria promote the destruction of gingival tissues and with loss of periodontal attachment, F. nucleatum can promote the formation of deep periodontal pockets, providing an ideal environment for growth and invasion of pathogenic species.

These studies highlight the importance and impact of F. nucleatum in periodontitis but it is often difficult to determine whether this bacterium is commensal or pathogenic. However, in light of the work presented, F. nucleatum can be described as an accessory pathogen, contributing to the overall virulence of the biofilm as well as the perseverance of potential keystone pathogens.

1.8 Oral mucosal immunity

1.8.1 The immune cell network at the gingival barrier

The oral cavity harbours a rich and diverse community of commensal and pathogenic microorganisms. This constant exposure challenges the maintenance of immunological tolerance and tissue homeostasis, which rely on the oral epithelium and specialised immune cells and their immune-inflammatory mediators (Belkaid and Artis 2013). A recent study has elegantly characterised the active cell network at the gingival crevice, the site most exposed to the oral biofilm and most susceptible to periodontal disease (Dutzan, Konkel et al. 2016). In healthy subjects, the study revealed an abundance of neutrophils and T cells, a diverse network of antigen presenting cells (APCs) and a small population of innate lymphoid cells. Investigation of the periodontitis group showed T cells as the dominant cell subset, with increased numbers in B cells and the
greatest increase seen in neutrophils (Dutzan, Konkel et al. 2016). Their observations are in agreement with previous studies (Lappin, Koulouri et al. 1999, Jotwani, Palucka et al. 2001, Hajishengallis and Hajishengallis 2014), and the in depth characterisation provides a comprehensive view of the complex cell network patrolling gingival tissues and their role in maintaining homeostasis and/or driving disease.

1.8.2 Innate immune responses

Innate immunity is the host’s primary defence system, playing a key role in the early stages of infection. The oral epithelium, together with cells of the innate immune arm (e.g. neutrophils, macrophages, dendritic cells), use a set of germline encoded pattern recognition receptors (PRRs) to recognise pathogen-associated molecular patterns (PAMPs) expressed by microorganisms (Suresh and Mosser 2013). Ligation of PAMPs to PRRs has two functions: i) the initiation of a cascade of responses, mostly in the form of cytokines and antimicrobial peptides, which result in the direct killing/clearance of microorganisms, and ii) the initiation of an antigen-specific adaptive response when the innate response is ineffectual (Hoebe, Janssen et al. 2004).

PRRs sense the presence of microorganisms by recognising structures conserved among microbial species. Bacterial PAMPs recognised by PRRs include peptidoglycan (PGN), lipoproteins, LPS and lipoteichoic acid (LTA). While all bacteria have peptidoglycan and lipoproteins, LPS is mostly associated with Gram-negative bacteria while LTA is associated with Gram-positive bacteria; this is due to the differences in cell wall formation (Medzhitov 2007). The differences in cell wall components between Gram-positive and Gram-negative bacteria are illustrated in Figure 1-3. Other bacterial factors recognised by PRRs include flagella and CpG oligodeoxynucleotides (Akira, Uematsu et al. 2006).
1.8.2.1 PRR-mediated signalling

Currently, four main PRR classes have been identified and these include the transmembrane Toll-like receptors (TLRs), C-type lectin receptors (CLRs), cytosolic Nod-like receptors (NLRs), and retinoic-acid-inducible gene 1 receptors (RIGs) (Takeuchi and Akira 2010). Expression of PRRs differs among cell types, with PRRs recognising different PAMPs. The multitude of detection systems observed in innate immune responses is attributed to the complex signalling through these PRRs, which involves interplay between families (e.g. integration of TLR- and NLR- signalling pathways occurs for stimulation of IL-1β production (Martinon and Tschopp 2005), as well as the ability of different PRR families to recognise the same ligands (e.g. bacterial flagellin can be recognised by members of both TLR- and NLR- families (Broz and Monack 2013)). Nevertheless, TLRs and NLRs are the main families of PRRs involved in bacterial recognition (Akira, Uematsu et al. 2006). Signalling through these PRRs culminates in the activation of intracellular signalling pathways and specific transcription factors, resulting in the release of inflammatory mediators. For a schematic representation of the TLR and NLR signalling pathways involved in bacterial recognition, see Figure 1-4.
Figure 1-4. Mammalian Toll-like receptor and Nod-like receptor signalling involved in bacterial recognition. TLRs and NLRs recognise various bacterial PAMPs, leading to the induction of intracellular signalling cascades culminating in the activation of the pro-inflammatory signalling pathways MAPK and NF-κB. Signalling through MAPK and NF-κB leads to down-stream transcription factor activation (AP-1, NF-κB) and induction of inflammatory responses. TRIF-dependent signalling leads to production of type I interferons. Additionally, inflammasome activation results in secretion of biologically active IL-1β and IL-18.
The TLR family consists of 10 identified members, with TLR1, 2, 4, 5, 6 and 9 shown to be involved in bacterial recognition. Of those, TLR1, 2, 4, 5 and 6 are expressed on the cell surface, while TLR9 is expressed in the endocytic compartment (Kimble and Beutler 2001). TLR2 generally forms heterodimers with TLR1 or TLR6. The TLR2/TLR1 heterodimer recognises triacylated lipoproteins from Gram-negative bacteria, while the TLR2/TLR6 heterodimer recognises diacylated lipoproteins from Gram-positive bacteria (Jin, Kim et al. 2007, Kang, Nan et al. 2009). TLR4 has been identified as the receptor that interacts with bacterial LPS. LPS is a component of the outer membrane of Gram-negative bacteria and is a complex glycolipid containing a lipid A component responsible for its bioactivity. TLR5 recognises flagellin, a protein compound of bacterial flagella, from both Gram-positive and Gram-negative bacteria (Hayashi et al., 2001). TLR9 is activated by bacterial DNA, specifically unmethylated CpG dinucleotides, which are frequently present in bacteria (Kawai and Akira, 2010).

Following ligand binding, the TLR cytoplasmic Toll/IL-1 receptor (TIR) domain interacts with several adaptor molecules including myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), MyD88-adaptor-like (MAL), TRIF-related adaptor molecule (TRAM) and sterile-alpha and armadillo-motif-containing protein (SARM) (O’Neill and Bowie 2007). Generally, signalling through these adaptors leads to activation of two pathways: a MyD88-dependent signalling pathway, which predominantly leads to production of inflammatory cytokines, and a TRIF-dependent signalling pathway which predominantly leads to production of type I interferons (IFNs) (De Nardo 2015). All TLRs except TLR3 require MyD88 for activation of downstream signalling. Briefly, MyD88 interacts with IRAK1, 2, and 4 followed by ubiquitination of TRAF6. This process activates the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) pathway and the mitogen-activated protein kinase (MAPK) pathway. The MAPK pathway constitutes three arms: p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase 1/2 (ERK1/2). Signalling through these pathways leads to downstream transcriptional events such as activation of the activator protein – 1 (AP-1) heterodimer (Yang et al., 2013). Both the NF-κB and MAPK pathways play critical roles in cytokine gene expression. Although mainly associated with cytokine production, MyD88-dependent signalling can also lead to the expression of IFNs via
activation of interferon regulatory factors (IRFs) (Takaoka, Yanai et al. 2005). TRIF-dependent signalling activation is utilised by TLR3 and TLR4. Initial activation of TLR4 results in MyD88 signalling; however, in the presence of CD14, TLR4 is endocytosed and subsequently utilises TRIF signalling (Figure 1-4) (Kagan, Su et al. 2008, Zanoni, Ostuni et al. 2011). Engagement of TRIF by TLR4 also requires TRAM, leading to recruitment of TRAF3 and TRAF6 which mediate the ubiquitination of RIP1 kinase and initiation of NF-κB, MAPK and IRF3 signalling (De Nardo 2015).

The intracellular NLRs are categorised into four subfamilies depending on the variable N-terminal effector region: caspase recruitment domain (CARD), pyrin domain (PYD), acidic domain or baculovirus inhibitor repeats (BIRs) (Franchi, Warner et al. 2009). Nod1 and Nod2 are NLRs consisting of CARD domains that sense bacterial peptidoglycan (Girardin, Travassos et al. 2003). Following microbial sensing, Nod1 and Nod2 activate NF-κB and MAPK signalling via an adaptor, RIP2/RICK (Hayden and Ghosh 2004). PYR domain-containing NLRs (NLRPs), CARD-domain containing NLRs (NLRCs) and non-NLR absent in melanoma 2 (AIM2) receptors are involved in inflammasome activation and importantly in the induction of inflammatory responses mediated by the IL-1 family of cytokines (IL-1β and IL-18) (Meylan, Tschopp et al. 2006). Upon sensing PAMPs and damage-associated molecular patterns (DAMPs), relevant NLRs and/or AIM2 form multiprotein complexes leading to cleavage of pro-caspase-1 into active caspase-1. Under caspase-1 activated conditions, the precursor cytokines pro-IL-1β and pro-IL-18 are cleaved into their mature, active forms IL-1β and IL-18 (Guo, Callaway et al. 2015). Activated inflammasomes may also be accompanied by pyroptosis, a form of cell death distinct from apoptosis (Miao, Rajan et al. 2011). In the oral mucosa, inflammasomes are important in mediating inflammatory responses in gingival epithelial cells. One of the best characterised inflammasomes is the NLRP3 inflammasome, which can be activated by extracellular ATP (danger signal/DAMP) through purigenic receptors such as ligand-gated ion channels (P2X) (Said-Sadier and Ojcius 2012). It has been shown that depletion of NLRP3 by siRNA abrogated the ability of ATP to induce IL-1β secretion in gingival epithelial cells (Yilmaz, Sater et al. 2010). Furthermore, it was shown that in gingival epithelial cells, NLRP3 and caspase-1 activation was mediated via ATP-induced ROS production through a complex consisting of P2X4/P2X7 (Hung, Choi et al. 2013). Depletion of either P2X4 or P2X7 resulted in
decreased ROS production, caspase-1 activation and IL-1β secretion. However, it was shown that stimulation of P2X₄ was not sufficient for caspase-1 activation, which required ATP-induced P2X₇ signalling; suggesting that in the P2X₄/P2X₇ complex, P2X₄ modulates P2X₇ activity (Hung, Choi et al. 2013). It is important to note that secretion of biologically active IL-1β and IL-18 requires two separate signals, involving both TLRs and NLRs. Expression and intracellular accumulation of pro-IL-1β and pro-IL-18 is TLR-induced, while activation of caspase-1 and subsequent secretion of biologically active IL-1β and IL-18 is NLR-induced – an example of PRR unity. Through their common recognition of PAMPs and simultaneous stimulation, NLRs and TLRs cooperate in shaping the host response to bacterial infections by synergistically activating inflammatory responses (Zasloff 2002).

Crosstalk not only occurs at receptor level but also in downstream signal transduction leading to effector responses. The NF-κB and MAPK signalling pathways mediate complex cellular responses and are key regulators of inflammation and immune responses, as well as cell growth, differentiation and survival (Hayden and Ghosh 2012, Yang, Sharrocks et al. 2013). Other pathways important in regulating cellular responses are the phosphoinositide 3-kinase/protein kinase B (PI3K)/(Akt) pathway and the mammalian target of rapamycin (mTOR) pathway. Activation of these pathways is not a linear event and involves crosstalk, cooperation and feedback loops. For example, Akt is activated by both PI3K and mTOR complex mTORC2, with the mTORC2-Akt pathway regulating downstream Forkhead box protein (FOXO) transcription factors. In a negative feedback loop, prolonged mTORC1 stimulation diminishes the activation of PI3K, which normally promotes the activation of both mTORC1 and mTORC2 (Xie and Proud 2014). PI3K induced Akt, as well as the p38 and JNK MAPK pathways have been shown to regulate NF-κB in both stimulatory and inhibitory ways (Vermeulen, De Wilde et al. 2003, Papa, Zazzeroni et al. 2004, Dan, Cooper et al. 2008, Hoesel and Schmid 2013). The MAPK pathways also exhibit dynamic feedback and crosstalk. For example, JNK activation can be modulated by ERK, p38 and Akt in a series of feedback loops that can dictate transient or sustained JNK phosphorylation (Fey, Croucher et al. 2012). Signalling though these pathways occurs as a complex network with a multitude of overlapping mechanisms which ultimately impact on nuclear substrates that regulate gene expression and synthesis of proteins.
1.8.3 PRR-mediated signalling by oral bacteria

The literature surrounding bacterially induced cell signalling is diverse, with many studies being performed using periodontal ligament (PDL) cells, myeloid or lymphoid cells. Varying results arise from the multitude of stimuli used; i.e. bacterial LPS, purified proteins, bacterial DNA, heat-killed bacteria, live bacteria, etc., as well as different multiplicities of infection (MOIs) and serum conditions. \textit{P. gingivalis} is by far the most widely studied oral bacteria associated with periodontal disease. Therefore, some of the most recent studies investigating \textit{P. gingivalis} induced signalling in various cell types will be discussed.

\textit{P. gingivalis} LPS has been shown to induce activation of NF-κB and MAPK pathways via TLR4, with IL-6 and IL-8 secretion in primary gingival fibroblasts (Jian, Li et al. 2015). In PDL cells, \textit{P. gingivalis} LPS activated the NF-κB and HIF-1α transcription factors, followed by up-regulation of IL-1β and MMP1 (Golz, Memmert et al. 2015), mildly up-regulated the Nrf-2 transcription factor and its targets HO-1 and NQO1 (involved in cellular protective mechanisms against oxidative stress) (Bin, Huangqin et al. 2015), as well as activated the C/EBP β transcription factor followed by production of IL-6, IL-8, MMP8 and MMP9 (Bai, Wei et al. 2016). Additionally, \textit{P. gingivalis} LPS has been shown to stimulate bone resorption by activating RANKL via TLR2-NF-κB mechanisms in osteoblasts and thus promoting osteoclastogenesis (Kassem, Henning et al. 2015). In THP-1 monocytes, a single treatment with \textit{P. gingivalis} LPS resulted in increased TLR2, TNF-α and IL-1β expression, which were all significantly decreased following a second LPS treatment, while IL-10 levels were increased (Sun, Li et al. 2014); this suggests that \textit{P. gingivalis} LPS-induced tolerance can limit inflammation. A recent study has further highlighted mechanisms of endotoxin tolerance. Monocytes pre-stimulated with \textit{P. gingivalis} outer membrane vesicles (OMV) containing LPS failed to secrete TNF-α in response to re-stimulation with live \textit{P. gingivalis} (Waller, Kesper et al. 2016). OMV-induced TNF-α tolerance was shown to be dependent on TLR4 but not TLR2, and regulated by mTOR signalling and IL-10 secretion. Inhibition of TLR4 and mTOR restored TNF-α activity; however, IL-10 levels were not reduced following treatment with rapamycin, suggesting that IL-10 secretion is regulated via different mechanisms (Waller, Kesper et al. 2016).
Neutrophils are the most common leukocytes present in the periodontium and are indispensable for the maintenance of tissue homeostasis. *P. gingivalis* employs many processes in the subversion of neutrophil functions e.g. impaired recruitment via down-regulation of chemokines and cell adhesion molecules, resistance to killing via inactivation of antimicrobial peptides, and hijacking of C5aR-TLR2. It does, however, simultaneously promote inflammation e.g. by delaying neutrophil apoptosis. *P. gingivalis* targets specific pathways that promote clearance while leaving other pro-inflammatory functions intact to mediate tissue damage. A recent review article summarises *P. gingivalis* subversion of neutrophil functions described above (Olsen and Hajishengallis 2016). A subsequent study further highlights the selective nature of this phenomenon. Stimulation of neutrophils with live *P. gingivalis* resulted in release of TNF-α, IL-6, CXCL8 and CCL2 and intracellular ROS production. Addition of red blood cells (RBCs), which *P. gingivalis* binds to, resulted in inhibition of intracellular ROS, increase in TNF-α, IL-6 and CCL2 secretion, with no change in CXCL8 levels (Damgaard, Kantarci et al. 2016). The study presents yet another mechanism by which *P. gingivalis* reduces intracellular killing while promoting extracellular tissue damage.

### 1.8.4 The oral epithelium

Mucosal epithelial cells form both a physical and biological barrier against resident microorganisms. In the oral cavity, gingival epithelial cells, particularly those lining the gingival crevice, are the most exposed to dental plaque (Dutzan, Konkel et al. 2016). Epithelial cells are the primary cells of the oral mucosal barrier that interact with both commensal and pathogenic species, thus their ability to discriminate between the two and mount appropriate responses is essential. Oral epithelial cells are invaded by many bacterial species; however, they are equipped with both membrane bound and cytosolic PRRs, which are able to sense multiple PAMPs. Importantly, epithelial cells possess immune mechanisms that function independently of myeloid and lymphoid cells of the innate and adaptive immune systems (Ross and Herzberg 2016). Signalling through epithelial cells results in the release of cytokines, chemokines and antimicrobial peptides, which not only have direct effects on epithelial cells and microbes (protective and destructive, respectively), but also alert and recruit specialised immune cells – thus moulding the pathology of periodontal disease. Apart from impacting on immune cells, a recent study has shown that epithelial cells also
possess mechanisms of promoting inflammation in fibroblasts (Bi, Koivisto et al. 2016). Microvesicles secreted by epithelial cells upon biofilm stimulation induced a pro-inflammatory phenotype in fibroblasts with activation of the MAPK pathway and secretion of MMP1, MMP3, IL-6 and IL-8 (Bi, Koivisto et al. 2016).

Oral epithelial cells initiate the cascade of immune responses involved in the progression of all types of periodontal diseases. Therefore, understanding how they respond to constitutive bacteria of varying pathogenic potential is important in establishing the mode of host-pathogen interactions at this interface. Classically, the progression of chronic periodontitis is thought of as a three-stage process: health – gingivitis – periodontitis (however gingivitis does not always lead to periodontitis). Considering these parameters, the next section will review the literature on oral epithelial cell responses to oral bacteria, with particular attention to the three bacterial strains that are the focus of this thesis and which are associated with the above mentioned different disease stages: namely A. naeslundii (health), F. nucleatum (gingivitis) and P. gingivalis (periodontitis).

1.8.5 Oral epithelial cell responses to A. naeslundii

Actinomyces spp. are important early colonisers in the oral cavity, possessing the ability to form close metabolic relationships with Streptococcus spp. and bind F. nucleatum and its co-aggregates (Kolenbrander and London 1993, Kolenbrander, Palmer et al. 2006). A. naeslundii is one of the most prevalent Actinomyces spp. isolated from both healthy and periodontitis patients (Vielkind, Jentsch et al. 2015). Although it has been suggested that relative proportions of A. naeslundii decrease with disease severity and with development of deep periodontal pockets (Moore and Moore 1994, Faveri, Figueiredo et al. 2009), other studies have shown persistence during biofilm maturation, involvement in gingivitis as well as dental caries (Brailsford 1998, Tanner, Maiden et al. 1998, Zijnge, van Leeuwen et al. 2010).

There is currently limited information on the interaction of A. naeslundii with oral epithelial cells. Stimulation of primary gingival epithelial cells with A. naeslundii cell wall extracts induced human beta-defensin 2 (hBD-2) and hBD-3 secretion (Yin, Chino et al. 2010). Infections of immortalised epithelial cells (OKF4) with live A. naeslundii
resulted in secretion of pro-inflammatory cytokines and chemokines IL-6, IL-8, TGF-α, MIP-1α and IP-10 (Peyyala, Kirakodu et al. 2012). These results are in accord with studies showing that Gram-positive cell wall components can induce inflammatory responses (Tanabe, Bodet et al. 2007) and that Gram-positive bacteria-induced immune-stimulation utilises TLR2 (possibly via LTA or lipoproteins) (Tietze, Dalpke et al. 2006). The main cell wall component in Gram-positive bacteria is peptidoglycan, which has been shown to enhance pro-inflammatory responses (Gupta, Jin et al. 1995, Kumar, Zhang et al. 2004, Hessle, Andersson et al. 2005). Recently, the peptidoglycan of A. naeslundii was shown to promote bone resorption through activation of osteoclasts and stimulation of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in murine peritoneal macrophages (Sato, Watanabe et al. 2012). Nevertheless, the correlation between A. naeslundii and periodontal disease remains tentative and requires further evaluation.

1.8.6 Oral epithelial cell responses to F. nucleatum

As previously mentioned (Section 1.7), it is increasingly difficult to categorise F. nucleatum as a commensal or pathogenic coloniser of the oral cavity. This difficulty is further demonstrated from studies investigating epithelial cell responses to this bacterium, which reveal both protective and destructive effects. Despite varying experimental conditions, such as using primary cells or established cell lines, different strains of live or heat-killed F. nucleatum, or purified cell wall extracts, overall responses remain comparable.

The pro-inflammatory nature of F. nucleatum is characterised by the bacterium’s ability to activate signalling within epithelial cells, followed by up-regulation of cytokines and chemokines. The protective nature of F. nucleatum is seen from its ability to induce antimicrobial peptides, particularly β-defensins. An early study by Krisanaprakornkit and colleagues showed that exposure of primary gingival epithelial cells to live F. nucleatum resulted in rapid activation of p65 and p50 subunits of NF-κB, suggesting NF-κB pathway activation (Krisanaprakornkit, Kimball et al. 2002). Activation of the MAPK pathways was also assessed, with their potential role in hBD-2 regulation following F. nucleatum infection. F. nucleatum preferentially activated both p38 and JNK MAP kinase pathways, while it had little effect on ERK1/2
Live *F. nucleatum* has been shown to up-regulate IL-8 mRNA at early time points, which was predominantly controlled by NF-κB activation and to a lesser extent p38 and ERK1/2 (Huang, Zhang et al. 2004). In a study using expression microarrays, live *F. nucleatum* activated TLR and MAPK signalling pathways, with induction of IL-6 and IL-8 (Hasegawa, Mans et al. 2007). In another study using gingival epithelial cell multilayers, infections with live *F. nucleatum* induced IL-1β and TNF-α (Dickinson, Moffatt et al. 2011). Furthermore, induction of IL-1α, IL-1β and IL-8 gene expression was shown to be regulated by the NF-κB, p38 and JNK MAPK pathways in *F. nucleatum* infections (Zhang and Rudney 2011).

*F. nucleatum* is a strong inducer of inflammation in epithelial cells, as shown by the up-regulation of both IL-1α and IL-6 cytokines and IL-8, IP-10 and MIP-1α chemokines (Peyyala, Kirakodu et al. 2012). *F. nucleatum*-stimulated IL-8 (at both mRNA and protein level) is also dependent on signalling via TLR9 (Kim, Jo et al. 2012). Several studies have shown secretion of IL-1β by oral epithelial cells following *F. nucleatum* infection, which suggests inflammasome activation. A recent paper has described inflammasome activation by *F. nucleatum* in gingival epithelial cells. Bui and colleagues showed that *F. nucleatum* induced early NF-κB activation and IL-1β gene transcription. Processing of pro-IL-1β was dependent on NLRP3 activation. Moreover, activation of the NLRP3 inflammasome by *F. nucleatum* resulted in the secretion of high-mobility group box 1 (HMGB1) and apoptosis-associated speck-like protein (ASC) danger signals (Bui, Johnson et al. 2015). HMGB1 is a nuclear protein that can bind DNA and increase transcription, and when secreted, can act as a chemokine as well as promote inflammation. HMGB1 utilises several receptors, including TLRs, and can signal via NF-κB and MAPK pathways to induce pro-inflammatory cytokine release (Lotze and Tracey 2005). The ASC adaptor protein was shown to be critical in caspase-1 activation and regulation of inflammasome activation and IL-1β secretion (Hara, Tsuchiya et al. 2013).

It has been shown that ROS production leads to NLRP3 inflammasome activation (Schroder and Tschopp 2010). Two possible sources for ROS have been described: through activation of nicotinamide adenine dinucleotide phosphate (NADPH) and/or mitochondrial ROS (mROS) generation (Dostert, Petrilli et al. 2008, Zhou, Yazdi et al. 2011). NLRX1, an NLR family member, localises to the mitochondria and triggers the generation of mROS (Tattoli, Carneiro et al. 2008). It has been proposed that *F.
F. nucleatum-induced NLRP3 activation in gingival epithelial cells is positively regulated by NLRX1, as depletion of NLRX1 attenuated ATP-induced mROS generation and inhibited F. nucleatum-induced activated caspase-1 (Hung 2016). Collectively, these studies underline the pro-inflammatory potential of F. nucleatum in oral epithelial cells as seen through its ability to activate multiple pathways (NF-κB, MAPK), inflammasomes, and stimulating the secretion of inflammatory cytokines.

Stimulation of oral epithelial cells with heat-killed or live F. nucleatum induces signalling mechanisms, suggesting that responses are more likely mediated via conserved structural motifs (PAMPs). Up-regulation of TLR4 and TLR9 was observed using heat-killed F. nucleatum, as well as activation of NF-κB (p65 transcription factor) and induction of IL-1β, IL-8, GM-CSF, TNF-α, CCL2 and CCL20 (Milward, Chapple et al. 2007). A study using both bacterial treatments showed that live and heat-killed F. nucleatum induced IL-1β and IL-8, whereas only live bacteria induced IL-6. However, heat-killed F. nucleatum induced IL-1β and IL-8 to a lesser extent than live F. nucleatum (Stathopoulou, Benakanakere et al. 2010). In accordance with these studies, a recent investigation has noted that heat-killed F. nucleatum utilises TLR4, activates NF-κB and induces expression of IL-1β, IL-8, GM-CSF, TNF-α, CCL2 and CCL20, as well as S100A8 and S100A9 (genes involved in cellular protection, neutrophil recruitment and activation) (Milward, Chapple et al. 2013).

The impact of F. nucleatum on defensin production has been investigated and live F. nucleatum has been shown to utilise TLR2 and NLRP2 in the induction of hBD-2 and hBD-3 (Ji, Shin et al. 2009). A more recent study confirms these findings, as live F. nucleatum up-regulated hBD-2 in gingival epithelial cells (Li, Guo et al. 2015). However, despite its ability to activate the NF-κB pathway, F. nucleatum may not induce hBD-2 via NF-κB (canonical or non-canonical) (Chung and Dale 2008). F. nucleatum cell wall extracts have been shown to induced hBD-2 and hBD-3, but not hBD-1 in primary gingival epithelial cells (Yin, Chino et al. 2010). Further characterisation has identified FAD-I (Fusobacterium-associated defensin inducer) as the principle F. nucleatum protein responsible for hBD-2 induction, which is mediated via both TLR1/2 and TLR2/6 heterodimers (Gupta, Ghosh et al. 2010, Bhattacharyya, Ghosh et al. 2016).
1.8.7 Oral epithelial cell responses to *P. gingivalis*

Unlike the relatively consistent findings regarding *F. nucleatum*-induced epithelial cell responses, *P. gingivalis*-induced oral epithelial cell signalling can vary significantly. Different experimental designs, particularly those employing live or inactivated bacteria, purified LPS, different expressed proteins (e.g. fimbriae, gingipains, haemoglobin receptor protein (HbR)), MOIs, cell lines and serum conditions elicit varying cell signalling transductions of both pro- and anti-inflammatory/apoptotic potential. The *P. gingivalis* ATCC 33277 type strain is the most widely used along with W50, W83 and 381 strains. These are the predominant strains used in signal transduction studies. For the purposes of the next section, unless otherwise stated, epithelial cell responses are to *P. gingivalis* 33277. *P. gingivalis* 33277 is among the most commonly isolated strains from individuals and, along with 381, is described as being less virulent as compared with W50 and W83 (Igboin, Griffen et al. 2009).

Watanabe and colleagues showed that live *P. gingivalis* induced down-regulation of ERK1/2 in a dose dependent manner (MOI range 10-1000), mild activation of JNK and no effect on the p38 MAPK pathway (Watanabe, Yilmaz et al. 2001). This MAPK pathway regulation was further confirmed when *P. gingivalis* infections (MOI 500) were shown to down-regulate ERK as well as IL-8 mRNA levels (Huang, Zhang et al. 2004). Interestingly, in the same study, heat-, formalin- and protease- treatment of the bacteria attenuated this effect, suggesting that *P. gingivalis* protein factors were mediating down-regulation (Huang, Zhang et al. 2004). A different study also using both live and heat-killed *P. gingivalis* reported a similar effect. Infections of oral epithelial cells with live *P. gingivalis* induced IL-1β secretion, while IL-6 and IL-8 were completely abrogated (Stathopoulou, Benakanakere et al. 2010). Heat-killed *P. gingivalis* completely reversed the response, with no IL-1β secretion but significant IL-6 and IL-8 levels being induced (this was observed with MOIs of 100 but not with MOIs of 10) (Stathopoulou, Benakanakere et al. 2010). The discrepancy between live and heat-killed *P. gingivalis*-induced responses is evident from other studies, in that heat-killed *P. gingivalis* generally elicits a pro-inflammatory response, whereas live *P. gingivalis* tends to be more subversive. It has been shown that heat-killed *P. gingivalis* induces mild activation of NF-κB and its p65 subunit, with up-regulation of GM-CSF, CCL2, CCL20 and the HMOX1 gene (Milward, Chapple et al. 2007). A subsequent study
by the same group confirmed the pro-inflammatory response, showing that heat-killed *P. gingivalis* utilised TLR4, activated NF-κB, and induced secretion of IL-1β, IL-8, GM-CSF, TNF-α, CCL2 and CCL20 from oral epithelial cells (Milward, Chapple et al. 2013). Heat-inactivated *P. gingivalis* loses its proteolytic capabilities, which have been described as essential for the bacteria’s ability to neutralise host responses (Guo, Nguyen et al. 2010). This is also evident from a study showing gingipain mediated degradation of Akt and mTOR following internalisation of *P. gingivalis* W50 and NCTC 11834 (Stafford, Higham et al. 2013). These results present conflicting observations depending on experimental design, particularly the effect of heat-treatment of *P. gingivalis*. While the conflicting responses can be attributed to the loss of biologically active proteins, LPS, which is heat-resistant and PGN, which can be exposed after heat-treatment, may also account for the up-regulation of pro-inflammatory responses.

Various investigations indicate that *P. gingivalis* expressed molecules can be pro-inflammatory. *P. gingivalis* LPS has been shown to stimulate IL-1α, IL-6, IL-10, TNF-α, IFN-γ, IP-10 and MIP-1 proteins via TLR2 in oral epithelial cells (Kocgozlu, Elkaim et al. 2009). Furthermore, *P. gingivalis* LPS has also been shown to activate NF-κB signalling and the p65 transcription factor, as well as the p38 and JNK MAPK pathways (Ding, Wang et al. 2013). In a recent study, *P. gingivalis* HbR induced IL-8 secretion via activation of p38 and ERK1/2 and subsequent activation of ATF-2, CREB and NF-κB transcription factors (Fujita, Nakayama et al. 2014). In a study using *P. gingivalis* 381, the purified fimbriae of this bacterium induced IL-8 via TLR2 and NF-κB in epithelial cells; this response was dependent on soluble CD14 and lipopolysaccharide binding protein (LBP) in serum (Asai, Ohyama et al. 2001). Interestingly, stimulation of epithelial cells with live *P. gingivalis* 381 grown as a mono-species biofilm, inhibited secretion of IL-8 and several other cytokines and chemokines (IL-1α, TGF-α, Gro-1α, MIP-1α and IP-10) (Peyyala, Kirakodu et al. 2012). The same effect was observed in a study where *P. gingivalis* DNA induced IL-8 secretion whereas live *P. gingivalis* did not (Dickinson, Moffatt et al. 2011).

Despite the apparent pro-inflammatory profile of heat-killed *P. gingivalis* and anti-inflammatory profile of live *P. gingivalis*, some studies using live *P. gingivalis* demonstrate both phenotypes, thus highlighting this bacterium’s capability of
manipulating host responses. A study using *P. gingivalis*, 33277, W83 and 381 has shown hBD-2 and hBD-3 induction, as well as IL-8 gene expression in primary gingival epithelial cells (Chung, Hansen et al. 2004). Induction of hBD-2 and IL-8 but not hBD-3 was regulated via protease activated receptor 2 (PAR2) and *P. gingivalis* proteases (Chung, Hansen et al. 2004). It was further shown that *P. gingivalis* preferentially uses the non-canonical NF-κB pathway, through IKKα/TRAf3 for hBD-2 induction (Chung and Dale 2008). Signalling through PAR2/NF-κB by *P. gingivalis* has also been observed in infections of oral carcinoma cell lines (Inaba, Sugita et al. 2014). Stimulation with *P. gingivalis* resulted in pro-MMP9 induction via multiple signalling cascades, including p38, ERK1/2 and PAR2/NF-κB. Signalling through the PAR2 receptor did not lead to the observed activation of the MAPK pathways, suggesting that PAR2/NF-κB forms a separate axis. Up-regulation of pro-MMP9 via the MAPK pathways occurred through activation of the Ets1 transcription factor and heat shock protein 27 (HSP27), downstream of ERK1/2 and p38, respectively, and the cleavage and activation of pro-MMP9 to its active form was gingipain dependent. In contrast, Bondy-Carey and colleagues showed gingipain dependent degradation of MMP9 and TIMP-1 (Bondy-Carey, Galicia et al. 2013). This could be attributed to the use of different cell lines and bacterial MOIs i.e. oral carcinoma cell line (Inaba, Sugita et al. 2014) vs primary oral cells (Bondy-Carey, Galicia et al. 2013) and MOI of 1 (Inaba, Sugita et al. 2014) vs MOI of 100 (Bondy-Carey, Galicia et al. 2013).

Indeed, the study by Inaba and colleagues demonstrates a pro-inflammatory profile of *P. gingivalis*; however, it also suggests a link between *P. gingivalis* and oral squamous cell carcinoma (OSCC). MMPs degrade extracellular matrix and basement membrane components, which promotes carcinoma cell migration and invasion, thus impacting on local tissue destruction as well as promoting metastatic growth in distant sites (Sorsa, Tjaderhane et al. 2004). Infections were carried out in two OSCC cell lines, the highly invasive SAS cells and low invasive Ca9-22 cells (Inaba, Sugita et al. 2014). Interestingly, the activation of signalling pathways and induction of MMP9 was only observed in the SAS cells (Inaba, Sugita et al. 2014), suggesting that molecular actions of *P. gingivalis* can be highly cell-specific. This observation was also confirmed by a study using two OSCC cell lines, the highly invasive SCC-25 cells and the low invasive BHY cells for the assessment of two B7 receptors expression (B7-H1 and B7-DC).
These receptors regulate immune responses against cancer and B7-H1 and B7-DC are inhibitory members of the B7 family, often up-regulated in tumour micro-environments and associated with poor prognosis (Zou and Chen 2008). Groeger and colleagues demonstrated significant up-regulation of B7-H1 and B7-DC following *P. gingivalis* 33277 and W83 challenge in the SCC-25 cells but found inconsistent results in the BHY cells (Groeger, Domann et al. 2011). These results further link *P. gingivalis* to OSCC and highlight its cell specific responses. In both studies, *P. gingivalis* exhibits its pro-inflammatory potential in highly invasive/metastatic cells and it has been suggested that in most cases, the relationship between tumours and bacteria is opportunistic rather than causative (Cummins and Tangney 2013). The use of carcinoma cell lines in *P. gingivalis* studies investigating periodontal diseases is frequent (including in this thesis), and in light of these findings, the choice of a pre-malignant carcinoma cell line like Ca9-22 and H400 could be more relevant.

Inconsistencies also exist in *P. gingivalis*’s pro- and anti-apoptotic effect in oral epithelial cell lines. Exposure of primary gingival epithelial cells to heat-killed *P. gingivalis* resulted in Fas ligand (FasL) –dependent apoptotic cell death (Brozovic, Sahoo et al. 2006). *P. gingivalis* induced up-regulation of FasL gene expression via activation of NF-κB and up-regulation of TLR2 mRNA but not TLR4, resulting in activation of caspase-3 and caspase-8. The pro-apoptotic epithelial phenotype was shown to be caspase-8 dependant and furthermore, infection with the heat-killed bacterium resulted in decreased levels of the anti-apoptotic proteins c-IAP-1/c-IAP-2 (Brozovic, Sahoo et al. 2006). Contrarily, a study using both live and heat-killed *P. gingivalis* demonstrated that live but not heat-killed *P. gingivalis* 33277 induces apoptosis in primary gingival epithelial cells as determined by caspase-3 activation and detection of DNA fragmentation (Stathopoulou, Galicia et al. 2009). Through the use of several gingipain mutants, filtered bacterial supernatants as well as purified gingipains, the authors showed that *P. gingivalis*-induced apoptosis did not require invasion and was gingipain dependent with either Arg- or Lys-gingipain being sufficient (Stathopoulou, Galicia et al. 2009). Finally, the study compared apoptosis induction between *P. gingivalis* 33277 and W50 by Annexin-V staining and showed similar kinetics in the response to both strains (Stathopoulou, Galicia et al. 2009). In a more
recent study, *P. gingivalis* was shown to induce apoptosis in primary gingival epithelial cells through activation of FOXO1 and FOXO3 transcription factors which increased mRNA levels of apoptotic genes BID and TRADD (Li, Dong et al. 2013). It was further shown that activation of these transcription factors by *P. gingivalis* down-regulated TLR2 and TLR4 mRNA levels; however, expression of these receptors was not assessed and although it may suggest attenuation of inflammatory responses, these were not investigated (Li, Dong et al. 2013). The observed down-regulation of TLR2 mRNA is in contrast with the observed up-regulation of TLR2 by Brozovic and colleagues. This suggests that while both live and heat-killed *P. gingivalis* can induce apoptosis in epithelial cells, the mechanisms are different. Moreover, Li and colleagues observed TLR2 and TLR4 mRNA down-regulation in monolayer cultures of primary gingival epithelial cells but only TLR4 down-regulation in multi-layer cultures (Li, Dong et al. 2013). This further highlights the relevance of experimental conditions when investigating *P. gingivalis* responses.

While the studies presented above have shown *P. gingivalis*-induced epithelial cell apoptosis, several others demonstrate *P. gingivalis*-induced inhibition of programmed cell death. *P. gingivalis* was shown to block apoptosis in primary gingival epithelial cells through the phosphorylation of Akt, which is mediated by the PI3K pathway (Yilmaz, Jungas et al. 2004). Inhibition of PI3K resulted in suppression of Akt activation, which inhibited the *P. gingivalis*-anti-apoptotic effect as seen by cytochrome c release (involved in initiation of apoptosis) and DNA fragmentation (Yilmaz, Jungas et al. 2004). It was later shown that through Akt activation, *P. gingivalis* infection causes phosphorylation of Bad thus blocking its pro-apoptotic action (through elevation of anti-apoptotic Bcl-2) (Yao, Jermanus et al. 2010). In an Akt-independent manner, *P. gingivalis* also inhibited caspase-9 activation (Yao, Jermanus et al. 2010). Caspase-9 is an initiator caspase, which once activated, can further activate executioner caspases such as caspase-3 (McIlwain, Berger et al. 2013). *P. gingivalis* can block caspase-dependent apoptotic pathways through inhibition of caspase-3 (Mao, Park et al. 2007). Although this was not investigated, inhibition of caspase-9 by *P. gingivalis* could precede the observed inhibition of caspase-3. Furthermore, the inhibition of caspase-3 by *P. gingivalis* was mediated via activation of JAK1 and the STAT3 transcription factor,
as well as Akt (Mao, Park et al. 2007); thus highlighting two distinct pathways, JAK/STAT and Akt, in *P. gingivalis* pro-survival induction.

Through manipulation of the JAK pathway, *P. gingivalis* not only coordinates anti-apoptotic mechanisms but also modulates inflammatory responses. Phosphorylation of JAK2 and increased levels of IL-6 and IL-1β secretion were reported in gingival epithelial cells following *P. gingivalis*-induced ROS production (Wang, Zhou et al. 2014). Additionally, ROS-mediated phosphorylation of JAK2 induced JNK phosphorylation and activation of the c-Jun transcription factor. Inhibition of JAK2 reduced JNK and c-Jun activation, as well as IL-6 and IL-1β levels (Wang, Zhou et al. 2014). *P. gingivalis*-induced ROS also activates FOXO transcription factors through JNK signalling, resulting in up-regulation of genes encoding various antioxidant components, the anti-apoptotic Bcl-6 protein and IL-1β (mRNA and protein) (Wang, Sztukowska et al. 2015). Silencing of FOXO1 by siRNA reduced mRNA levels of all the observed up-regulated proteins, and IL-1β secreted levels, and resulted in increased *P. gingivalis*-induced apoptosis (Wang, Sztukowska et al. 2015). These findings contradict the earlier described study by Li and colleagues (Li, Dong et al. 2013) where *P. gingivalis*-activated FOXO favoured a pro-apoptotic phenotype. The induction of ROS and secretion of IL-1β in these *P. gingivalis* infections suggests inflammasome activation by the bacterium. However, studies investigating *P. gingivalis*-induced inflammasomes in gingival epithelial cells show an ability of the bacteria to modulate such responses. ATP ligation to P2X7 receptors produces a variety of cellular effects, including inflammasome activation, secretion of IL-1β and induction of cell death (Lister, Sharkey et al. 2007). *P. gingivalis* can inhibit gingival epithelial cell apoptosis induced by ATP ligation of P2X7 through secretion of a nucleoside diphosphate kinase (NDK) homologue, which can hydrolyse extracellular ATP (eATP) (Yilmaz, Yao et al. 2008). An ndk-deficient mutant was no longer able to inhibit ATP-induced apoptosis (Yilmaz, Yao et al. 2008). This effect also impacts on inflammasome activation, as it was demonstrated that gingival epithelial cells infected with *P. gingivalis* transcribe the gene encoding IL-1β but do not secrete the protein unless they are subsequently treated with ATP (Yilmaz, Sater et al. 2010). It was further shown that *P. gingivalis* NDK can modulate eATP/ P2X7-induced ROS production, thus manipulating both inflammatory and cell death pathways (Choi, Spooner et al. 2013). These mechanisms promote the bacterium’s survival, replication
and persistence in gingival epithelial cells. These studies are in contradiction with those described above which show secretion of IL-1β following *P. gingivalis* challenge. Alternative mechanisms for IL-1β processing have been described, implicating proteases derived from other cell types (Netea, Simon et al. 2010), but also a *C. albicans* protease that can efficiently cleave pro-IL-1β (Beausejour, Grenier et al. 1998). Considering *P. gingivalis*’s ability to produce multiple proteases, it could be possible that different IL-1β activation mechanisms are involved in *P. gingivalis* infections.

While adaptive immunity and Th effector responses are not the focus of this thesis, the following section will briefly address how the interaction of *P. gingivalis* with epithelial cells may also manipulate adaptive Th effector responses. Recent studies have shown *P. gingivalis* to enhance the expression of IL-33 in oral epithelial cells (Tada, Matsuyama et al. 2016) and IL-36γ via activated IRF6 transcription factor (Huynh, Scholz et al. 2016). IL-33 augments Th2 cytokine-mediated inflammation in response to bacterial components (Saenz, Taylor et al. 2008), whereas IL-36γ can stimulate production of neutrophil chemokines IL-8 and CXCL1, as well as the Th17 chemokine CCL20 from oral epithelial cells, dendritic cells and macrophages (Huynh, Scholz et al. 2016). IRF6 is able to modulate TLR2 induced inflammatory responses and it is suggested that IRF6 can also modulate *P. gingivalis*-induced inflammation via regulation of IL-36γ (Huynh, Scholz et al. 2016).

While earlier studies have suggested that with the continued presence of bacteria, B cells outnumber T cells in the inflammatory infiltrate (Gemmell, Yamazaki et al. 2002), others have suggested almost equal numbers of B and T cells in diseased gingival tissues (Kawai, Matsuyama et al. 2006), with more recent papers suggesting that progress into the chronic stage of periodontal inflammation is dominated by T lymphocytes (Cardoso, Garlet et al. 2008) (Dutzan, Konkel et al. 2016). Th cells can be subdivided into lineages on the basis of cytokine profiles and it is now recognised that Th1, Th2 and Th17-derived cytokines are present in periodontal tissues (Gaffen and Hajishengallis 2008, Souto, Queiroz-Junior et al. 2014). The more recently discovered Th17 subset is suggested to have a major impact on immune-regulation of host response in tissue destruction with evidence of IL-17 (the prototypical Th17 cytokine) being involved in osteoclastogenesis (Beklen, Ainola et al. 2007), neutrophil and
macrophage recruitment (Yu, Ruddy et al. 2007, Shen and Gaffen 2008, Allam, Duan et al. 2011) and stimulation of pro-inflammatory cytokines (IL-1, IL-6, IL-8) from resident cells (Ouyang, Kolls et al. 2008). Nevertheless, the exact role of Th17 cytokines in either protective or destructive inflammation remains debatable. Importantly, recent studies indicate that *P. gingivalis* can induce a cytokine pattern favouring Th17 polarisation at the expense of Th1 in myeloid APCs (Moutsopoulos, Kling et al. 2012), gingival epithelial cells (Jauregui, Wang et al. 2013) and in monocyte/CD4⁺ T cell co-cultures (Cheng, van Asten et al. 2016). This could favour *P. gingivalis* persistence, as it has been suggested that control of *P. gingivalis* infection may be attributed to Th1 cells (Hajishengallis, Shakhatreh et al. 2007). For further information on aspects of the adaptive host response in periodontitis and the induction of adaptive immunity by *P. gingivalis*, the reader is guided to the following excellent reviews (Gaffen and Hajishengallis 2008, Khader, Gaffen et al. 2009, Cheng, Hughes et al. 2014, Hajishengallis 2014, Hajishengallis and Lamont 2014, Hajishengallis and Sahingur 2014).

1.8.8 Summary of epithelial cell responses to live infections with *A. naeslundii, F. nucleatum* and *P. gingivalis*

A schematic representation of the observed signalling events regulating inflammation in oral epithelial cells following infection with live *A. naeslundii, F. nucleatum* and *P. gingivalis* are presented in Figure 1-5. Most work in this field has focused on characterising the immuno-regulatory and pro-inflammatory cytokines that are produced by epithelial cells following challenge by commensal and periodontal bacteria. However, the characterisation of signal transduction events that precede the production of these immune molecules is presently lacking and, therefore, represents an attractive target for research.
Figure 1-5. Epithelial cell responses to live infections with *A. naeslundii*, *F. nucleatum* and *P. gingivalis*

- **Up-regulation**
- **Down-regulation**
- **Up- and down-regulation**
1.9 Project aims

Essential information is still missing from our understanding of how gingival epithelial cells respond to oral bacteria. Investigating the signalling pathways and downstream mechanisms activated in epithelial cells in response to oral bacteria may identify novel mechanisms of commensal/pathogen recognition and control. Therefore, the main aim of this project was to characterise the epithelial signalling cascades and transcription factors that are activated by oral bacteria associated with health, gingivitis and periodontitis.

Specific objectives were to:

- Determine which signalling pathways and transcription factors are activated in gingival epithelial cells in response to A. naeslundii (commensal-associated), F. nucleatum (gingivitis-associated) and P. gingivalis (periodontitis-associated).

- Characterise the pro-inflammatory cytokine response of gingival epithelial cells to A. naeslundii, F. nucleatum and P. gingivalis.

- Investigate the involvement of P. gingivalis virulence factors in the observed gingival epithelial cell responses.

Given the multiple comparisons between different bacteria, to undertake this work we used the H400 cell line, which was established from a human squamous cell carcinoma of the alveolar process (Prime, Nixon et al. 1990). While the utilisation of primary gingival cells was considered, this approach is thwarted with delays in epithelial cell collection and growth. Furthermore, humans are notoriously dissimilar in terms of their diet, immune status and salivary function, which together with racial, ethnic, and cross-cultural variations in human demographics, can provide a confounding matrix of factors that may give rise to significant heterogeneity with regard to epithelial responses against oral microbes. The use of a cell line provides a standardised tool that can be controlled and manipulated to permit thorough investigations and to derive universally comparable data with regard to epithelial responses.
Chapter 2: Materials and Methods

2.1 General materials and equipment

**H400 cell line growth media:**

H400 cells were grown in Dulbecco’s Modified Eagle Medium Nutrient Mixture F-12 HAM (DMEM F-12; Sigma) supplemented with 10\% v/v foetal bovine serum (FBS; PAA), 4 mM L-Glutamine (PAA), 25 µg/mL hydrocortisone (Sigma), 10 µM sodium pyruvate (Sigma) and 1\% v/v penicillin/streptomycin (Sigma).

Bacterial strains growth media:

**Fastidious Anaerobe Agar**

23 g of Fastidious Anaerobe Agar (FAA) (Bioconnections) was re-suspended in 0.5 L of deionised water and autoclaved at 121°C for 15 min. The agar solution was cooled to 50°C and supplemented with 5\% v/v defibrinated horse blood (TCS). The agar was poured into Petri dishes in a laminar flow cabinet and left to dry. The plates were stored at 4°C until required.

**Blood Agar**

19.8 g of Blood Agar Base No. 2 (BA) (Lab M) was dissolved in 0.5 L of deionised water and autoclaved at 121°C for 15 min. The agar was supplemented with 5\% v/v defibrinated horse blood (TCS) after cooling to 50°C. The agar was poured into Petri dishes in a laminar flow cabinet and left to dry. The plates were stored at 4°C until required.

**Brain Heart Infusion Broth**

3.7 g of Brain Heart Infusion (BHI) broth powder (Lab M) was re-suspended in 100 mL of deionised water and supplemented with 0.25 g/L of L-cysteine (Sigma). The medium was autoclaved at 121°C for 15 min and pre-reduced (to keep the medium oxygen-free) by leaving in an anaerobic cabinet for at least 24 h prior to inoculation.

*P. gingivalis* cultures were grown in BHI broth in the absence of haemin, and thus in haemin-limited conditions. Studies investigating the effects of haemin limitation on *P.*
*P. gingivalis* have identified alterations in the expression of various surface molecules (Bramanti and Holt 1990, Bramanti, Holt et al. 1993, Champagne, Holt et al. 1996). Nevertheless, *P. gingivalis* has been shown to have the ability to accumulate iron in excess *in vitro*, and then utilise iron ‘stores’ during growth under iron-deprivation (Kesavalu, Holt et al. 2003). While the effects of the haemin-limited BHI broth on protein expression were not assessed in this study, *P. gingivalis* cells used in cell culture infections after growth in liquid broth were viable at all time points (as determined by a viability test, data not shown). This could be attributed to *P. gingivalis* accumulating necessary iron stores during growth on FAA plates.

**General chemicals:**
Dulbecco’s phosphate-buffered saline (PBS; Fisher Scientific) consisted of 1.4 M NaCl, 27 mM KCl, 80 mM Na$_2$HPO$_4$ x H$_2$O, 15 mM KH$_2$PO$_4$. Radio Immuno-Precipitation Assay buffer (RIPA) was made by mixing (50 mM Tris-HCl pH 7.5 (Sigma), 150 mM NaCl (Sigma), 1% v/v Triton X-100 (Sigma), 1% w/v Sodium Deoxycholate (Sigma), 0.1% w/v sodium dodecyl sulphate (SDS; Severn Biochem Ltd) and 20 mM ethylenediaminetetraacetic acid (EDTA; Sigma). Cell freezing media was made of 90% v/v FBS and 10% v/v dimethyl sulfoxide (DMSO; Sigma).

**General equipment:**
Tissue culture flasks (T175) and tissue culture plates (24 well, 12 well and 6 well) and black 96-well plates were purchased from Greiner Bio-One. 10 cm tissue culture dishes and 15 mL Falcons were purchased from Jet Biofil. 50 mL Falcons were purchased from Corning. Pipettes (25 mL, 10 mL and 5 mL) were purchased from Fisher Scientific. Pipette tips (1000 µL, 200 µL, 10 µL), 1.5 mL Eppendorf tubes and 10 µL sterile disposable loops were purchased from StarLab. 5 µL sterile disposable loops were purchased from Microspec. Immulon 96 well plates were purchased from Thermo Scientific. 90 mm Petri dishes were purchased from Sterilin. FastRead 102 disposable cell counting slides were purchased from Immune Systems. Sterile Nalgene Cryogenic vials were purchased from Nalge Nunc International. Microbank Cryovial beads were purchased from Pro-Lab Diagnostics. Sterile tissue culture was performed in a Scanlaf MARS Class II safety cabinet. Infections were carried out in a NUAIRE Class II biological safety cabinet. Epithelial cell cultures were incubated in a Binder incubator at 37°C: 5%
CO₂. Bacterial cultures were maintained in a Don Whitley MACS-MG-1000-Anaerobic Workstation. The anaerobic gas consisted of 80% Nitrogen, 10% Carbon Dioxide and 10% Hydrogen and the temperature inside the cabinet was 37°C. Centrifugation at 25°C was carried out in an Eppendorf Centrifuge 5415C (up to 1.5 mL) and a MSE Mistral 3000 Centrifuge (up to 25 mL). Centrifugation at 4°C was carried out in a Thermo Scientific Heraeus Fresco 17 Centrifuge (up to 1.5 mL) and a Thermo Scientific Labofuge 400R Centrifuge (up to 15 mL). Centrifugation at 20°C was carried out in a MSE Mistral 1000 Centrifuge (MSE Scientific Instruments). The microplate reader used was Tecan Infinite F50. The spectrophotometer used was a Cecil Aquarius CE9200 Spectrophotometer (Cecil Instruments Ltd).

2.2 Cell Culture

2.2.1 Cell lines growth and maintenance
The oral epithelial cell line H400 was kindly gifted by Professor Mahvash Tavassoli (Mucosal and Salivary Biology Division, King’s College London, UK). H400 cells were established from a human squamous cell carcinoma of the alveolar process (Prime, Nixon et al. 1990) and were the cell line used in this study. The alveolar process is the ridge of bone that contains the tooth sockets and is attached to the cementum of the roots by the periodontal ligament. To revive cells from frozen stock, cells were quickly thawed at 37°C in a water bath. The cells were then re-suspended in 10 mL of their appropriate culture medium, centrifuged at 25°C, 1200 rpm for 5 min and re-suspended in fresh medium. Cells were then transferred into a T175 flask with a final volume of medium adjusted to 25 mL and incubated at 37°C:5% CO₂. H400 cells were passaged when they reached 70-80% confluency. Cells were washed with 5 mL PBS and incubated with 4 mL of 0.05% Trypsin–EDTA (PAA) for 20 min, 37°C:5% CO₂. After cell detachment, they were re-suspended in 8 mL of medium and split 1:2, 1:3 or 1:6 according to their further use.

2.2.2 Cell counting
Cell counts were determined using a disposable cell counting slide with cells counted in the four outer chambers. The following formula was applied to determine the count of cells/mL in a given harvest.
\[ \text{cells/mL} = \frac{\text{counted cells}}{4} \times 10^4 \]

Knowing the number of cells/mL allows for the adjustment of cell densities according to need.

### 2.2.3 Cell freezing

Cells were collected by trypsination and centrifuged at 25°C, 1200 rpm for 5 min. The cell pellet was re-suspended in freezing medium and 1 mL was transferred to each cryovial containing at least 1 x 10^6 cells/mL. These were slowly frozen at -80°C in an isopropanol bath (Nalgene) for 48 h before transfer to a liquid nitrogen tank for long term storage.

### 2.3 Bacterial culture

#### 2.3.1 Bacterial strains growth and maintenance

*Actinomyces naeslundii*, *Fusobacterium nucleatum* 10953 and *Porphyromonas gingivalis* 33277 were a gift from Professor William Wade (Centre for Immunobiology, Blizard Institute, Barts and The London School of Medicine and Dentistry). *P. gingivalis* W50, *P. gingivalis ΔrgpA/B*, *P. gingivalis Δkgp* and *P. gingivalis ΔserB* were a gift from Professor Mike Curtis (Dean of Dentistry, Barts and The London School of Medicine and Dentistry). *P. gingivalis* W83 and *P. gingivalis ΔPPAD* were a gift from Professor Jan Potempa (Department of Oral Immunology and Infectious Diseases, University of Louisville). All bacterial stocks were kept at -80°C.

**Table 2-1. Bacterial strains used**

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Genotype</th>
<th>Description</th>
<th>Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinomyces naeslundii</em> NCTC 10301</td>
<td>Wild-type</td>
<td>Clinical isolate (Thompson and Lovestedt 1951)</td>
<td>BA</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum s.s polymorphum</em> ATCC 10953</td>
<td>Wild-type</td>
<td>Clinical isolate (Dzink, Sheenan et al. 1990)</td>
<td>FAA</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>Wild-type</td>
<td>Clinical isolate (Coykendall, Kaczmarek et al.)</td>
<td>FAA</td>
</tr>
<tr>
<td><strong>Porphyromonas gingivalis</strong></td>
<td><strong>W50</strong></td>
<td>Wild-type</td>
<td>Clinical isolate (Coykendall, Kaczmarek et al. 1980)</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>---------</td>
<td>-----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Parent strain of the <em>rgpA/B, kgp</em> and <em>serB</em> mutants</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>ΔrgpA/B</strong></th>
<th><strong>rgpA::tetQ</strong></th>
<th><strong>rgpB::erm</strong></th>
<th><strong>rgpA rgpB double deletion mutant</strong> (Aduse-Opoku, Davies et al. 2000)</th>
<th>FAA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E8</strong></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Δkgp</strong></th>
<th><strong>kgp::erm</strong></th>
<th><strong>kgp deletion mutant</strong> (Aduse-Opoku, Davies et al. 2000)</th>
<th>FAA</th>
</tr>
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<tbody>
<tr>
<td><strong>K1A</strong></td>
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</table>

<table>
<thead>
<tr>
<th><strong>ΔserB</strong></th>
<th><strong>serB::erm</strong></th>
<th><strong>serB deletion mutant</strong> (not published)</th>
<th>FAA</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><strong>Porphyromonas gingivalis</strong></th>
<th><strong>W83</strong></th>
<th>Wild-type</th>
<th>Clinical isolate (Coykendall, Kaczmarek et al. 1980)</th>
<th>FAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent strain of the <em>PPAD</em> mutant</td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>ΔPPAD</strong></th>
<th><strong>ppad::erm</strong></th>
<th><strong>ppad deletion mutant</strong> (Wegner, Wait et al. 2010)</th>
<th>FAA</th>
</tr>
</thead>
</table>

BA: Blood Agar  
FAA: Fastidious Anaerobic Agar  
<sup>a</sup> *P. gingivalis* 33277 Δ*serB*, not used in this study, was published by (Tribble, Mao et al. 2006)

*A. naeslundii, F. nucleatum* 10953 and *P. gingivalis* 33277 were revived anaerobically from glycerol stocks by swirling a 10 µL plastic loop into the stock vial and spreading it onto an appropriate agar plate. The remainder of bacterial species and strains were revived from cryogenic bead stocks by steaking one bead of frozen stock onto an appropriate agar plate. Cultures were maintained by sub-culturing once a week. A 5 µL plastic loop was used to streak a single colony onto a fresh agar plate. Three or four day old cultures were used to inoculate BHI broth for infections. A 10 µL loop was used to collect several colonies from an agar plate which was then inoculated into 5 – 45 mL of BHI broth. Inoculated broth was incubated anaerobically for 72 h after which 1 mL
of the culture was used to measure absorbance at 660 nm using a spectrophotometer. This was used to estimate the colony forming units (CFU)/mL using previously established growth curves. Bacteria were harvested by centrifugation at 20°C, 4000 rpm for 20 min and re-suspended in filtered PBS to a cell density of $1 \times 10^9$ cells/mL. From there on, suspensions were kept on ice.

2.4 Epithelial cell infection assays

2.4.1 Cell seeding

Table 2-2. Cell seeding conditions

<table>
<thead>
<tr>
<th>Assay</th>
<th>Plate</th>
<th>Volume</th>
<th>Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Damage and Cytokines</td>
<td>24 well</td>
<td>500 µL</td>
<td>$5 \times 10^5$ cells/mL</td>
</tr>
<tr>
<td>Signalling proteins</td>
<td>6 well</td>
<td>2 mL</td>
<td>$5 \times 10^5$ cells/mL</td>
</tr>
<tr>
<td>Transcription factor DNA</td>
<td>10 cm</td>
<td>10 mL</td>
<td>$4 \times 10^5$ cells/mL</td>
</tr>
</tbody>
</table>

For different assays cells were seeded according to Table 2-2 and all plates incubated for 48 h at 37°C:5% CO$_2$, after which they were serum-starved and further incubated for 24 h. Bacterial suspensions were prepared to a desired concentration which, when added to the cell monolayer, achieved the required Multiplicity of Infection (MOI).

**Damage and Cytokines:** Infected plates were incubated for 24 h and the exhausted culture medium collected. These samples were either stored at 4°C for quantification of LDH activity (within 24 h) or stored at -20°C for quantification of cytokines.

**Signalling proteins:** Time points of 5, 15, 30, 60 and 120 min were assessed. Following incubation, protein lysates were extracted (see Section 2.6.1).

**Transcription factor DNA binding:** Time points of 30, 60 and 120 min were assessed. Following incubation, nuclear proteins were isolated (see Section 2.7.1).

2.4.2 Inhibition of signalling pathways

The MAP kinase pathways JNK, ERK and p38 as well as the NF-κB pathway were inhibited 1 h prior to infection. Inhibitors were diluted in DMSO to a 1000X concentration then further diluted to the final working concentration. The 1000X stock was added to appropriate plates at a 1:1000 dilution to achieve the desired 1X final concentration.
Table 2-3. Signalling pathways inhibitors

<table>
<thead>
<tr>
<th>Pathway inhibited</th>
<th>Inhibitor</th>
<th>Final Concentration</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>JNK (Primary target: JNK1, JNK2, JNK3)</td>
<td>SP600125</td>
<td>10 µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>ERK (Primary target: ERK1 and ERK2)</td>
<td>FR180204</td>
<td>750 nm</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>p38 (Primary target: p38 MAP kinase)</td>
<td>SB203580</td>
<td>10 µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>NF-κB (Primary target: IκB-α)</td>
<td>BAY11-7082</td>
<td>2 µM</td>
<td>Calbiochem</td>
</tr>
</tbody>
</table>

2.5 Cell response assays

2.5.1 Epithelial cell damage assay

Epithelial cell damage was determined using the Cytox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega) according to the manufacturer’s protocol. The assay quantitatively measures the enzymatic activity of lactate dehydrogenase (LDH) in exhausted culture medium. Being a non-secreted cytosolic enzyme, LDH is released upon cell lysis. Released LDH is measured with a coupled enzymatic assay, which results in conversion of a tetrazolium salt (INT) into a red formazan product. The amount of colour formed is proportional to the number of lysed cells. A recombinant porcine LDH (Sigma) was used as a standard to create a calibration curve with a concentration range from 878 to 13.7 mU/mL. Absorbance was measured at 490 nm.

2.5.2 Cytokine Quantification by Luminex assay

IL-1α, IL-1β, IL-6, IL-8, G-CSF and GM-CSF cytokine levels were determined using a Magnetic Luminex Performance Assay (R&D Systems). Cytokine-specific antibodies are used, which are pre-coated onto color-coded magnetic microparticles. The microparticles, standards and samples were all pipetted into wells and the immobilised antibodies bind the cytokines of interest. A biotinylated antibody cocktail specific to the cytokines of interest was added, followed by streptavidin-phycoerythrin conjugate (SA-PE), which binds to the biotinylated antibody. Microparticles were read using a Bio-Plex 200 System (Bio-Rad) machine according to the manufacturer's protocol. Data was analysed using Bioplex Manager 6.1 software.
2.6 Protein assays

2.6.1 Protein extraction
Cells were washed with 1 mL of ice cold PBS and then lysed with 250 µL of RIPA buffer supplemented with 1X protease inhibitors (Thermo Scientific) and 1X phosphatase inhibitors (Sigma). Plates were kept on ice and scraped using a sterile cell scraper. Extracts were transferred to Eppendorf tubes and incubated on ice for 30 min followed by centrifugation at 4°C, 13000 rpm for 10 min. The supernatants were collected and stored at -20°C.

2.6.2 Bicinchoninic Acid (BCA) assay
Total protein concentrations were determined using a BCA Protein Assay kit (Thermo Scientific) according to the manufacturer’s protocol. This biochemical assay is based on a reduction of Cu$^{2+}$ ions to Cu$^{1+}$ in the BCA reagent. The amount of Cu$^{2+}$ reduced is proportional to the amount of protein present. This is depicted by a colour change of the sample solution. BSA was used as the standard with a concentration range of 2000 to 31.25 µg/mL. Absorbance was measured at 562 nm.

2.6.3 Cell signalling by Luminex assay
Detection of phosphorylated signalling proteins and total target proteins in cell lysates was performed using a Bio-Plex Pro cell signalling assay kit (Bio-Rad) according to the manufacture’s protocol. This is a magnetic bead-based immunoassay that utilises tailored antibodies bound to microspheres. Beads and samples are pipetted into wells and after a series of washes to remove unbound protein, a biotinylated detection antibody cocktail is added. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate, which serves as a fluorescent indicator. Fluorescence was measured using a Bio-Plex 200 System (Bio-Rad) machine according to the manufacturer’s protocol. Data was analysed using Bioplex Manager 6.1 software.
2.7 Gene regulation assays

2.7.1 Nuclear cell extract

Nuclear extracts were prepared using a Nuclear Extract Kit (Active Motif) according to the manufacturer’s protocol. Cells were washed with 5 mL of ice cold PBS and scraped into 3 mL of ice cold PBS supplemented with 5% v/v phosphatase inhibitors. This suspension was centrifuged at 4°C, 500 rpm for 5 min and the pellet re-suspended in 250 µL 1 X hypotonic buffer to swell the cell membrane. 25 µL of detergent was then added to release cytoplasmic proteins into the supernatant. After centrifugation, the nuclear pellet was re-suspended in 50 µL of lysis buffer supplemented with 1% v/v protease inhibitors. Extracts were stored at -20°C for further use.

2.7.2 Transcription factor activity

DNA-binding of transcription factors was detected and quantified using a TransAM Transcription Factor ELISA kit (Active Motif) according to the manufacturer’s protocol. The assay was undertaken in a 96-stripwell plate on which oligonucleotides specific for the transcription factors of interest had been immobilised. Primary antibodies were added, which recognise accessible epitopes on bound transcription factors, followed by a secondary HRP-conjugated antibody. Following plate development, absorbance was measured at 450 nm.

2.8 Statistical analysis of data

Data was tested for normality using a Shapiro-Wilks test and found to be normally distributed. Data was therefore analysed by one-way ANOVA using Sigma Plot 12.0 software and Holm-Sidak test performed to establish the location of the statistically significant differences. In all cases, p < 0.05 was taken to be significant.
Chapter 3: Human oral epithelial cell responses to *A. naeslundii*, *F. nucleatum* and *P. gingivalis*

3.1 Introduction

Various host-pathogen interactions in the oral mucosa are able to influence tissue homeostasis. During bacterial colonisation of oral surfaces, the interaction between epithelial cells and resident commensal and pathogenic species leading to specific immune-regulatory responses, dictates the pathological outcome of periodontal diseases (Dale 2002). The microbial shift in oral plaque composition from mostly Gram-positive to mostly Gram-negative species has been well documented in the development of inflammation, and specific bacteria have been described in their association with different disease stages (Socransky, Haffajee et al. 1998, Holt and Ebersole 2005, Socransky and Haffajee 2005). The irreversible damage of the periodontium seen in periodontitis is primarily caused by the host inflammatory response; therefore, investigating epithelial cell responses to bacteria of differing pathogenic potential will contribute to the understanding of the changes in immune surveillance with disease progression.

The aim of this chapter was to characterise gingival epithelial cell responses to *A. naeslundii* (health-associated), *F. nucleatum* (gingivitis-associated) and *P. gingivalis* (periodontitis-associated), by investigating intracellular activation of signalling pathways and transcription factors, as well as the secreted pro-inflammatory cytokine profiles.

The H400 oral epithelial cell line (Prime, Nixon et al. 1990) was chosen to study epithelial responses to *A. naeslundii*, *F. nucleatum* and *P. gingivalis*. H400 cells originate from the alveolar process, a site of growth for bacteria associated with health, gingivitis and periodontitis. To confirm that the H400 cell line was responsive to immune stimulation, a panel of TLR agonists was used. H400 cells released the highest levels of cytokines (IL-1α, IL-1β, IL-6, G-CSF and GM-CSF) in response to Poly (I:C), a TLR3 agonist (data not shown). TLR3 is a double-stranded (ds)RNA receptor and is generally thought to be protective during viral infections; however, it has also been
shown to be up-regulated in response to bacterial LPS (Cario and Podolsky 2000). The H400 cells also released cytokines (IL-1β, G-CSF, GM-CSF) in response to stimulation by synthetic lipoproteins Pam3CSK4 and FSL-1, which are TLR2/TLR1 and TLR2/TLR6 heterodimers agonists (data not shown). Signalling through TLR2 heterodimers has also been shown in response to bacterial components such as lipoteichoic acid (LTA) (Tawaratsumida, Furuyashiki et al. 2009) and lipoproteins (Gerold, Abu Ajaj et al. 2008). These preliminary experiments showed that the H400 cell line responded to TLR (microbial) stimulation and in parallel induced a pro-inflammatory response, making them a suitable cell line to use in this study.

3.2 Methods

3.2.1 Detection of phosphorylated signalling proteins
Activation of signalling pathways in oral epithelial cells was determined using a magnetic bead-based immunoassay detecting intracellular phosphorylated proteins in cell lysates (see Section 2.6.3). H400 monolayers were incubated with the respective bacterial strains (Table 3.1) at an MOI of 100 for up to 2 h (see Section 2.4.1).

Table 3-1. Bacterial strains used in Chapter 3

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Genotype</th>
<th>Description</th>
<th>Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinomyces naeslundii</em> NCTC 10301</td>
<td>Wild-type</td>
<td>Clinical isolate (Thompson and Lovestedt 1951)</td>
<td>BA</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum s.s polymorphum</em> ATCC 10953</td>
<td>Wild-type</td>
<td>Clinical isolate (Dzink, Sheenan et al. 1990)</td>
<td>FAA</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em> ATCC 33277</td>
<td>Wild-type</td>
<td>Clinical isolate (Coykendall, Kaczmarek et al. 1980)</td>
<td>FAA</td>
</tr>
</tbody>
</table>

BA: Blood Agar
FAA: Fastidious Anaerobic Agar

3.2.2 Transcription factors
Nuclear extracts were tested using a TransAM DNA binding assay, which is an ELISA-based system using oligo-dT as the capture molecule to assess DNA binding of
transcription factors (see Sections 2.7.1, 2.7.2). H400 monolayers were incubated with the respective bacterial strains (Table 3.1) at an MOI of 100 for up to 2 h (see Section 2.4.1).

### 3.2.3 Cytokine release
Cytokine levels were determined using a magnetic bead-based assay (Luminex) detecting protein levels in cell supernatants (see Section 2.5.2). H400 monolayers were incubated with the respective bacterial strains (Table 3.1) at MOIs of 0.001, 0.01, 0.1, 1, 10 and 100 for 24 h (see Section 2.4.1).

### 3.2.4 Cell damage assay
Epithelial cell damage was determined by measuring LDH activity in cell culture supernatants (see Section 2.5.1). H400 monolayers were incubated with the respective bacterial strains (Table 3.1) at MOIs of 0.001, 0.01, 0.1, 1, 10 and 100 for 24 h (see Section 2.4.1).

### 3.3 Results
#### 3.3.1 Signalling pathways involved in epithelial cell responses to oral bacteria
The mitogen-activated protein kinase (MAPK) family mediates cellular responses to various stimuli and is a key regulator of immune responses (Yang, Sharrocks et al. 2013). Similarly, the NF-κB pathway plays an important role in the development and maintenance of the immune system by regulating expression of cytokines and growth factors in response to signalling through many receptors (Hayden and Ghosh 2012). The PI3K/AKT/mTOR pathway can regulate many cellular functions, including cell growth, survival, and cell metabolism. Up-regulation of AKT phosphorylation in particular, has been associated with epithelial wound healing (Castilho, Squarize et al. 2013). These signalling pathways are essential components of innate immunity and, therefore, their activation in response to A. naeslundii, F. nucleatum and P. gingivalis was investigated.
3.3.1.1 Activation of the MAPK pathway

*A. naeslundii* did not significantly activate the three MAPK pathways p38, JNK and ERK1/2 (Figure 3-1). However, a ~5-fold increase in p-JNK induction was observed in response to *A. naeslundii* at 60 min post-infection. Contrarily, *F. nucleatum* significantly activated all three MAPK pathways (Figure 3-1). *F. nucleatum* up-regulated phosphorylation of p38 by 2.6-fold at 30 min ($P < 0.001$), with high levels of p38 maintained at 60 and 120 min post-infection (2.4-fold and 2.3-fold respectively) ($P < 0.01$). *F. nucleatum* also up-regulated p-JNK at 30 and 60 min post-infection by 11.6 and 14.4-fold respectively ($P < 0.001$), with phosphorylation reducing to resting levels by 120 min. p-ERK was induced as early as 15 min post-infection (2.9-fold) ($P < 0.001$) in response to *F. nucleatum*, and was maintained at 30 min (2.5-fold; $P < 0.05$) but reduced to resting levels by 120 min (Figure 3-1). *P. gingivalis* did not significantly activate any of the three MAPK pathways; however, a ~4-fold up-regulation of p-JNK was observed at 30 and 60 min post-infection (Figure 3-1). In summary, *F. nucleatum* strongly activated MAPK signalling in H400 epithelial cells, whereas *A. naeslundii* and *P. gingivalis* were less stimulatory, not reaching statistically significant levels of activity.
Figure 3-1. Activation of MAPK signalling pathway components in response to bacterial stimulation. Phosphorylation of p38, JNK and ERK1/2 at different time points post-infection with *A. naeslundii*, *F. nucleatum* and *P. gingivalis* using the H400 oral epithelial cell line. Samples were analysed by phospho-Luminex. An MOI of 100 was used for infections. Data are expressed in relation to un-stimulated resting cells (0, black bar) and are the mean fold change of four to six biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; *P < 0.05; **P < 0.01; ***P < 0.001.
3.3.1.2 Activation of NF-κB and PI3K pathways

A key event in the activation of the NF-κB pathway is the phosphorylation and subsequent degradation of IκB-α. Once p-IκB-α is degraded, the NF-κB complex is free to enter the nucleus to activate gene expression. Therefore, phosphorylation of IκB-α was used as a marker of NF-κB activation. IκB-α phosphorylation was significantly induced in response to *A. naeslundii* at 15 and 30 min post-infection (up 2.2-fold) (*P* < 0.05), with levels returning steadily back to baseline by 120 min (Figure 3-2). *F. nucleatum* induced IκB-α phosphorylation at 15 min post-infection (up 3.3-fold) (*P* <0.001), with the response persisting (between 3.4 and 2.8-fold) up to 120 min (*P* < 0.001) (Figure 3-2). Stimulation with *P. gingivalis* did not activate NF-κB signalling (Figure 3-2). Notably, none of the three bacterial strains induced phosphorylation of AKT (Figure 3-2) indicating that the PI3K/AKT pathway is not induced. These results further support the conclusion that *F. nucleatum* is the stronger activator of signalling pathways in H400 epithelial cells, with *A. naeslundii* and *P. gingivalis* being less stimulatory.

![Figure 3-2. Activation of NF-κB and PI3K / AKT signalling pathways in response to bacterial stimulation.](image)

Phosphorylation of IκB-α and AKT at different time points post-infection with *A. naeslundii*, *F. nucleatum* and *P. gingivalis* using the H400 oral epithelial cell line. Samples were analysed by phospho-Luminex. An MOI of 100 was used for infections. Data are expressed in relation to un-stimulated resting cells (0, black bar) and are the mean fold change of four to six biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; *P* < 0.05; ***P* < 0.001.
3.3.2 Activation of transcription factors in epithelial cell responses to oral bacteria

Activation of MAPK cascades initiates activation of multiple downstream transcription factors including ATF-2, MEF-2, c-Myc, and STAT-1α which can act as stress signals and drive transcription of inflammatory mediators (Yang, Sharrocks et al. 2013). MAPK signalling is also associated with activation of the AP-1 transcription factor family members. AP-1 refers to a group of structurally and functionally related members of the Jun family (c-Jun,Jun B and Jun D) and Fos family (c-Fos, Fos B and Fra 1) which form homo- and heterodimers (Hess, Angel et al. 2004). AP-1 complexes mediate regulation of processes such as differentiation and cell cycle progression, as well as immune responses (Hess, Angel et al. 2004) (Moyes, Runglall et al. 2010). Therefore, activation of these transcription factors was assessed.

3.3.2.1 General MAPK-induced transcription factors

A. naeslundii and F. nucleatum both failed to activate DNA binding of the general MAPK-induced transcription factors ATF-2, MEF-2, c-Myc and STAT-1α (Figure 3-3). P. gingivalis however, up-regulated ATF-2 DNA binding by 2.3-fold ($P < 0.05$) at 30 min post-infection, with levels decreasing back to baseline by 120 min (Figure 3-3). Thus, a differential transcription factor binding profile for ATF-2 was observed between the three oral bacterial strains.
Figure 3. DNA binding activity of general MAPK-induced transcription factors in response to bacterial stimulation. DNA binding activity of ATF-2, MEF-2, c-Myc, and STAT-1α at different time points post-infection with A. naeslundii, F. nucleatum and P. gingivalis using the H400 oral epithelial cell line. Samples were analysed by TransAM ELISA. An MOI of 100 was used for infections. Data are expressed in relation to unstimulated resting cells (0, black bar) and are the mean fold change of three to four biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; *$P < 0.05$. 

Annotated Diagram:

- ATF-2
- MEF-2
- c-Myc
- STAT-1α

Bars represent fold change over time (0, 30, 60, 120 minutes) post-infection with A. naeslundii, F. nucleatum, and P. gingivalis.
3.3.2.2 Activation of c-Fos and c-Jun AP-1 transcription factors

Contrarily, *P. gingivalis* had no effect on the binding capacity of these transcription factors. Of the six AP-1 transcription factor family members tested, only c-Fos and c-Jun DNA binding was significantly altered. While *A. naeslundii* did not significantly activate c-Fos or c-Jun DNA binding (Figure 3-4), an increase in c-Fos binding activity was observed in response to *A. naeslundii* at 60 min (8-fold) and 120 min (10-fold) post-infection (Figure 3-4). In contrast, *F. nucleatum* significantly induced DNA binding of both c-Fos and c-Jun (Figure 3-4). *F. nucleatum* up-regulated c-Fos binding at 60 min (18.3-fold) (*P* < 0.05), with the response increasing at 120 min (26.6-fold) (*P* < 0.001) post-infection. *F. nucleatum* also up-regulated c-Jun DNA binding at an earlier time point of 30 min (5.3-fold) (*P* < 0.05), with levels increasing at 60 min (6.4-fold) (*P* < 0.01) and then decreasing at 120 min post-infection (Figure 3-4). Notably, *P. gingivalis* did not significantly activate c-Fos or c-Jun DNA binding (Figure 3-4). These results demonstrate that *F. nucleatum* is the most potent activator of c-Fos and c-Jun, while *A. naeslundii* and *P. gingivalis* are far less stimulatory.

![Figure 3-4. DNA binding activity of AP-1 family transcription factors c-Fos and c-Jun in response to bacterial stimulation.](image)

*Figure 3-4. DNA binding activity of AP-1 family transcription factors c-Fos and c-Jun in response to bacterial stimulation.* DNA binding activity of Fos family c-Fos and Jun family c-Jun at different time points post-infection with *A. naeslundii*, *F. nucleatum* and *P. gingivalis* using the H400 oral epithelial cell line. Samples were analysed by TransAM ELISA. An MOI of 100 was used for infections. Data are expressed in relation to un-stimulated resting cells (0, black bar) and are the mean fold change of three biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; *P* < 0.05, **P** < 0.01, ***P** < 0.001.
3.3.2.3 Fos B, Fra 1, Jun B and Jun D AP-1 transcription factors activation

*A. naeslundii, F. nucleatum* and *P. gingivalis* all failed to significantly alter the DNA binding activity of other Fos family (Fos B and Fra 1) or Jun family (Jun B and Jun D) transcription factors (Figure 3-5). However, a non-significant suppression of Fos B DNA binding activity was suggested for all three bacteria at all time points (Figure 3-5). In addition, a non-significant suppression of Jun B and Jun D DNA binding was also observed with *P. gingivalis* at 60 and 120 min post-infection for Jun B and at all time points for Jun D (Figure 3-5). *A. naeslundii* and *F. nucleatum* had no effect on Jun B and Jun D binding and none of the bacteria had an effect on Fra 1 DNA binding (Figure 3-5).

In summary, all three bacteria appeared to reduce the DNA binding activity of Fos B, Jun B and Jun D, particularly *P. gingivalis*. While this was not statistically significant, it is possible that these apparently suppressive effects are biologically relevant in the context of infection.
**Figure 3-5. DNA binding activity of AP-1 family transcription factors Fos B, Fra 1, Jun B and Jun D in response to bacterial stimulation.** DNA binding activity of Fos family Fos B and Fra 1 and Jun family Jun B and Jun D at different time points post-infection with *A. naeslundii, F. nucleatum* and *P. gingivalis* using the H400 oral epithelial cell line. Samples were analysed by TransAM ELISA. An MOI of 100 was used for infections. Data are expressed in relation to un-stimulated resting cells (0, black bar) and are the mean fold change of three to four biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA and no statistical significance was found.
3.3.2.4 p65 NF-κB transcription factor activation

Activation of the NF-κB pathway initiates downstream activation of a family of five transcription factor proteins, p65, p50, RelB, c-Rel and p52 which form homo- and heterodimer complexes (Hayden and Ghosh 2012). The canonical p65/p50 heterodimer is primarily associated with IκB-α which is the most thoroughly studied member of the IκB family and plays a role in many key cellular processes such as cell survival, proliferation and immunity (Gilmore 2006). Therefore, activation of the p65 transcription factor was assessed. However, A. naeslundii, F. nucleatum and P. gingivalis all failed to activate DNA binding of p65 (Figure 3-6).

![p65](image)

**Figure 3-6. DNA binding activity of NF-κB family transcription factor p65 in response to bacterial stimulation.** DNA binding activity of p65 (NF-κB) at different time points post-infection with A. naeslundii, F. nucleatum and P. gingivalis using the H400 oral epithelial cell line. Samples were analysed by TransAM ELISA. An MOI of 100 was used for infections. Data are expressed in relation to un-stimulated resting cells (0, black bar) and are the mean fold change of three biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA and no statistical significance was found.
3.3.3 Cytokine release in epithelial cell responses to oral bacteria

Epithelial cells produce a wide range of cytokines in response to injury and infection, and periodontal bacteria are known to induce a variety of pro-inflammatory cytokines in oral epithelial cells (Stadnyk 1994, Graves 2008). Therefore, the pro-inflammatory cytokine response to A. naeslundii, F. nucleatum and P. gingivalis was characterised.

In general, cytokine induction was only significantly altered at an MOI of 100 but, interestingly, differences were observed between the three bacteria. A. naeslundii strongly induced IL-6, GM-CSF and G-CSF release at an MOI of 100 (P < 0.001) (Figure 3-7). F. nucleatum strongly induced IL-1α, IL-1β and GM-CSF at MOIs of 10 and 100 (P < 0.001) and G-CSF at an MOI of 100 (P < 0.001) (Figure 3-7). In contrast, P. gingivalis did not induce cytokine secretion but, notably, IL-8 protein levels were significantly reduced at MOIs of 10 and 100 compared to un-stimulated resting cells (P < 0.001) (Figure 3-7). In summary, F. nucleatum induced the strongest pro-inflammatory cytokine response while P. gingivalis was non-stimulatory or potentially inhibitory.
Figure 3-7. Cytokine release by oral epithelial cells in response to bacterial stimulation. Secretion of IL-1α, IL-1β, IL-6, IL-8, GM-CSF and G-CSF 24 h post-infection with different MOIs of *A. naeslundii*, *F. nucleatum* and *P. gingivalis* using the H400 oral epithelial cell line. Samples were analysed by Luminex. Data are compared with un-stimulated resting cells (0, black bar) and are the mean of at least three (MOIs 0.001 – 10) or ten (MOI 100) biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; ***$P < 0.001$. 

---
3.3.4 Epithelial damage induction by oral bacteria

Induction of inflammatory mediators from epithelial cells, in particular secretion of IL-1α, is widely regarded as being damage-associated. In order to investigate whether the pro-inflammatory response observed was due to induction of epithelial damage, the same 24 h post-infection culture supernatants that were used for cytokine analysis were tested for LDH release (a surrogate marker of cell membrane damage) (see Section 2.4.1).

Interestingly, despite being a strong activator of epithelial cells, F. nucleatum did not cause any detectable damage to H400 cells as measured by LDH assay. Similarly, neither did A. naeslundii nor P. gingivalis (Figure 3-8). However, notably, infection with P. gingivalis led to a reduction in detectable LDH activity in the supernatants at an MOI of 100 (P < 0.01) (Figure 3-8).

![LDH Activity](image)

**Figure 3-8. Damage induction in oral epithelial cells in response to bacterial stimulation.** LDH activity 24 h post-infection with different MOIs of A. naeslundii, F. nucleatum and P. gingivalis using the H400 oral epithelial cell line. Samples were analysed by LDH assay. Data are compared with un-stimulated resting cells (0, black bar) and are the mean of four biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; **P < 0.01.
3.3.5 LDH protein degradation by *P. gingivalis*

The observed decrease in LDH activity following *P. gingivalis* infection (Figure 3-8) could be due to either increased epithelial cell membrane integrity, suggesting a protective role for *P. gingivalis*, or the degradation of LDH by *P. gingivalis* after normal release from the epithelial cell. In order to investigate this, porcine LDH was incubated for 24 h, with and without *P. gingivalis* and LDH protein activity measured following the same LDH assay protocol (see Section 3.2.4). Incubation of the LDH protein with *P. gingivalis* for 24 h resulted in a total abolishment of detectable LDH activity (Figure 3-9), suggesting that LDH is degraded by *P. gingivalis* after release.

**Figure 3-9.** LDH protein degradation (Abolition of LDH activity) following incubation with *P. gingivalis*. Quantification of porcine LDH activity 24 h post-incubation with *P. gingivalis*. Samples were analysed by LDH assay. PBS (No bacteria) served as a negative control. Data are the mean of two biological replicates ± SEM.
3.4 Discussion

The human oral cavity is a complex ecosystem containing microorganisms with differing pathogenic potential. It is now recognized that the host innate immune defense system is active in healthy periodontal tissues and an imbalance or disruption in the expression of inflammatory mediators contributes greatly to disease advancement (Graves, Li et al. 2011). The modulation of intracellular pathways by commensal and periodontal pathogens leading to this disruption, remain unclear. Therefore, this chapter investigated which pathways are activated in oral epithelial cells in response to three oral bacteria: *A. naeslundii*, *F. nucleatum* and *P. gingivalis*, which are associated with health, gingivitis and periodontitis, respectively.

Infection of oral epithelial cells with *A. naeslundii* resulted in significant activation of the NF-κB pathway, as seen by the phosphorylation of IkB-α at early time points (15 and 30 min). The MAPK and PI3K pathways were not significantly induced by this bacterium; however, a clear up-regulation of p-JNK (MAPK) was observed, particularly at 60 min (5-fold increase). This is the first report of *A. naeslundii*-induced modulation of epithelial cell signalling. These properties are similar to another commensal bacterium, *S. gordonii*, which was shown to activate JNK, ERK1/2 and NF-κB (Watanabe, Yilmaz et al. 2001) and alter the expression of the MAPK and NF-κB pathways in a microarray study investigating transcriptional responses in human immortalized gingival keratinocytes (Hasegawa, Mans et al. 2007).

Infection of oral epithelial cells with *F. nucleatum* significantly activated all three MAPK pathways and the NF-κB pathway, while having no effect on the PI3K pathway. Of the MAPK pathways, up-regulation of p-p38 was sustained from 30 to 120 min, while activation of p-JNK and p-ERK1/2 was transient (up-regulation of JNK was observed at 30 and 60 min, up-regulation of ERK1/2 was observed at 15 and 30 min). NF-κB activation by *F. nucleatum* was induced at 15 min, with the response persisting up to 120 min. These findings are in accordance with others that have shown *F. nucleatum* to activate the NF-κB pathway (Krisanaprakornkit, Kimball et al. 2002, Milward, Chapple et al. 2007, Milward, Chapple et al. 2013), and preferentially activate p38 and JNK, while having little effect on ERK1/2 (Krisanaprakornkit, Kimball et al. 2002).
Following *P. gingivalis* infection, none of the investigated pathways (MAPK, NF-κB and PI3K), were significantly up-regulated; however, a 4-fold induction of JNK was suggested at 30 and 60 min post-infection. Studies investigating activation of MAPK pathways in response to *P. gingivalis* have also shown activation of JNK in primary gingival epithelial cells (Watanabe, Yilmaz et al. 2001), in telomerase-immortalized gingival epithelial cells (TIGKs) (Wang, Zhou et al. 2014) and in the highly invasive SAS carcinoma cell line (Inaba, Sugita et al. 2014). Inaba and colleagues also reported phosphorylation of p38, ERK and IκB-α in SAS cells in response to *P. gingivalis*; however, p-p38 and p-IκB-α were induced between 6 and 24 h post-infection, perhaps indicating differences in the kinetic response (Inaba, Sugita et al. 2014). Moreover, in the same study, *P. gingivalis* failed to activate any of the pathways in Ca9-22 cells, which are a less invasive carcinoma cell line. H400 cells, also derived from a squamous cell carcinoma, are node-negative (Prime, Nixon et al. 1990) and thus probably more comparable to the Ca9-22 cell line. The lack *P. gingivalis*-induced p38 phosphorylation observed in this study parallels findings by Watanabe et al., but also contradicts them, as down-regulation of ERK1/2 was not observed. This is probably because down-regulation of p-ERK1/2 was evident at an MOI of 1000 whereas at an MOI of 100 the effect was much milder (Watanabe, Yilmaz et al. 2001). Investigation of PI3K activation by *P. gingivalis* showed no effect on Akt phosphorylation in the H400 cell line over the 2 h infection. *P. gingivalis* has been shown to degrade Akt in a gingipain-dependent manner (Stafford, Higham et al. 2013); however, this effect was seen at 4 h post-infection. Contrarily, other studies have shown induction of p-Akt by *P. gingivalis* in primary gingival epithelial cells (Yilmaz, Jungas et al. 2004) and depletion of Akt by siRNA has highlighted the involvement of this pathway in *P. gingivalis*-induced anti-apoptotic effects (Mao, Park et al. 2007, Yao, Jermanus et al. 2010). However, these results were observed in primary gingival epithelial cells, between 6 and 24 h post-infection and in the study by Mao, et al., at an MOI of 50. Overall, *P. gingivalis* results from this study both parallel and contradict those observed elsewhere, underlying the diversity of responses that this bacterium is able to induce depending on cell type, infection time-points and MOI. Taken together, the data demonstrate that all three bacteria are capable of selectively activating certain signalling pathways and support the notion that *F. nucleatum* is a more potent activator of signalling pathways in oral epithelial cells compared to both *A. naeslundii* and *P. gingivalis*. **95**
Phosphorylation of JNK in response to all three bacterial species decreased at 120 min post-infection, as did *F. nucleatum*-induced p-ERK1/2 levels and *A. naeslundii*-induced p-IκB-α at 60 and 120 min post-infection. One possible explanation for the observed decrease in MAPK pathways could be the up-regulation of dual-specificity protein phosphatases (DUSPs or MKPs). DUSPs have been reported to mediate both positive and negative regulation of MAPK pathways (Patterson, Brummer et al. 2009). An example of a MAP kinase negative regulator is MKP-1, which can interact with p38, JNK and ERK (Caunt and Keyse 2013) and has been shown to de-phosphorylate p-p38 and p-JNK during infection of oral epithelial cells with *C. albicans* (Moyes, Runglall et al. 2010). The down-regulation of p-ERK1/2 could be mediated by MKP-3 as it has been shown to specifically bind and de-phosphorylate ERK1/2 (Caunt and Keyse 2013). IκB-α levels stimulated by *A. naeslundii* significantly decreased at 60 min post-infection and in the case of *F. nucleatum*, although p-IκB-α levels were significantly higher over the 120 min infection, levels at 60 and 120 min were lower than those seen at 15 and 30 min post-infection. This could be attributed to the association of IκB-α with transient NF-κB activation, whereas IκB-β for example, is involved in sustained activation (Tak and Firestein 2001).

The dynamics of transcription factor expression in epithelial cells following oral bacterial infections remain an unexplored area of research. Therefore, this study also analysed the MAPK- and AP-1-associated transcription factor activation profiles, as well as the NF-κB p65 transcription factor. Activation of PI3K-associated transcription factors was not undertaken since none of the tested bacteria activated this pathway.

*A. naeslundii* and *F. nucleatum* both failed to activate DNA binding of the general MAPK-induced transcription factors ATF-2, MEF-2, c-Myc and STAT-1α, whereas *P. gingivalis* transiently up-regulated ATF-2 DNA binding. One study has reported activation of ATF-2 in response to *P. gingivalis* purified haemoglobin receptor (HbR), via activation of the ERK1/2 MAPK pathways (Fujita, Nakayama et al. 2014). While it may be possible that the observed ATF-2 activation seen in this study is induced by *P. gingivalis* HbR, it is likely to be via a different mechanism. *P. gingivalis* did not up-regulate p-ERK1/2 but an induction in p-JNK was observed. Accordingly, experiments are needed to determine if *P. gingivalis*-induced ATF-2 in this system occurs via JNK
activation. Additionally, Fujita and colleagues assessed ATF-2 activation at 12 h post-infection whereas in this study, *P. gingivalis* activated ATF-2 at 30 min post-infection with levels decreasing back to baseline by 2 h, supporting the notion that live *P. gingivalis* elicits a milder inflammatory phenotype. ATF-2 is also associated with the AP-1 complex and once phosphorylated can form homo- or hetero-dimers with Fos/Jun family members and influence many cell processes including inflammation (Yu, Li et al. 2014). Activated ATF-2 complexes can stimulate transcription of genes encoding for pro-inflammatory cytokines, chemokines and cell adhesion molecules (Yu, Li et al. 2014); it is therefore possible that down-regulation of ATF-2 by *P. gingivalis*, following the initial stimulatory cellular response, is a mechanism of dampening the inflammatory immune response.

Of the AP-1 family of transcription factors, only c-Fos and c-Jun DNA binding activity was up-regulated. *A. naeslundii* did not have a statistically significant effect on the activation of c-Fos or c-Jun; however, an increase in c-Fos DNA binding activity was observed at 60 and 120 min post-infection (8- and 10-fold). This is the first time that activation of c-Fos and c-Jun has been assessed during *A. naeslundii* infections. AP-1 transcription factors are primarily activated by MAPK pathways and the up-regulation of c-Fos could be driven by *A. naeslundii* up-regulation of p-JNK. *A. naeslundii*, however, also induced p-IκB-α and the MAPK and NF-κB pathways are often activated simultaneously, such as during the infection of oral epithelial cells by *C. albicans* (Moyes, Runglall et al. 2010). Therefore, further experiments are needed to assess the mechanisms of activation in *A. naeslundii* infections.

*F. nucleatum* strongly induced c-Fos DNA binding at 60 and 120 min post-infection, as well c-Jun at 15 and 30 min post-infection. This is the first report showing activation of AP-1 transcription factors by *F. nucleatum* and the up-regulation of c-Fos and c-Jun mirrors results from signal pathway level, where all three MAPK pathways, as well as the NF-κB pathway were activated by this bacterium. Similar to *A. naeslundii*, further experiments are necessary to identify the signalling axes activated during *F. nucleatum* infections. Contrary to *A. naeslundii* and *F. nucleatum, P. gingivalis* had no effect on the binding capacity of c-Fos and c-Jun, despite it mildly inducing p-JNK. Upon stimulation, activated JNK is known to phosphorylate transcription factors such as c-
Jun (Shaulian and Karin 2002) and *P. gingivalis* has been shown to phosphorylate c-Jun (Wang, Zhou et al. 2014). The JNK subgroup consists of JNK1, JNK2 and JNK3, with JNK3 being predominantly expressed in the brain, while JNK1 and JNK2 are expressed ubiquitously (Davis 2000). It has been shown that JNK1 and JNK2 exert distinct roles in the regulation of c-Jun activity, with JNK1 being the major form that phosphorylates c-Jun, thus contributing to its transcriptional activity, whereas JNK2 mainly contributes to c-Jun degradation (Sabapathy, Hochedlinger et al. 2004). The method used in the current study to detect JNK phosphorylation does not discriminate between the different JNK subgroups. It would be interesting to investigate which JNK subgroup *P. gingivalis* is inducing, and if it were JNK2, this could be an explanation for the lack of c-Jun induction. AP-1 complexes have been shown to be crucial modulators of inflammation (Eferl and Wagner 2003, Zenz, Eferl et al. 2008), with c-Fos playing the dominant role in mediating pro-inflammatory responses to *C. albicans* (Moyes, Run glall et al. 2010) and the Fos-Jun complex regulating inflammatory mediators during *Chlamydia pneumoniae* infections (Wang, Al-Kuhlani et al. 2013). It is therefore possible that c-Fos and c-Jun are the primary epithelial transcription factors controlling immune regulation in response to mucosal-associated microbes.

DNA binding of the remaining AP-1 transcription factors (Fos B, Jun B, Jun D, Fra 1) was not significantly altered by *A. naeslundii, F. nucleatum* or *P. gingivalis*. However, Fos B binding activity was suppressed by all three bacteria, and in addition, *P. gingivalis* also suppressed DNA binding of Jun B and Jun D. The effect of oral bacteria on the binding activity of these transcription factors in epithelial cells has not been previously reported. While c-Fos, c-Jun and Fos B are considered strong transactivators, Jun B and Jun D exhibit weak transactivational potential (Hess, Angel et al. 2004). Fos and Jun family members are differentially expressed and regulated in different cell types, enabling several possible AP-1 dimer combinations to assemble (Eferl and Wagner 2003) and control physiological functions including inflammation, cell differentiation, proliferation and apoptosis (Hess, Angel et al. 2004). Under certain conditions, Jun B and Jun D can act as repressors of AP-1 activity (Hess, Angel et al. 2004). Accordingly, down-regulation of Jun B and Jun D by *P. gingivalis* may provide a mechanism of modulating AP-1-induced responses. The role of Jun B and Jun D in epithelial cells remains unclear. However, in lymphoid cells, these transcription factors have been
shown to be negative regulators of proliferation (Passegue and Wagner 2000, Meixner, Karreth et al. 2004). By promoting progression through the cell cycle through inhibition of Jun B and JunD, *P. gingivalis* may favour anti-apoptotic mechanisms; this regulation has been shown in gastric epithelial cells infected with *Helicobacter pylori* (Tavares and Pathak 2015). Additionally, Jun B is required for efficient osteoclast differentiation in vivo (Kenner, Hoenertz et al. 2004) and has been shown to promote cell death during acute inflammation by regulating IFN-γ production in immune cells (Thomsen, Bakiri et al. 2013). Down-regulation of Jun B could thus impact on bone remodelling (directly relevant to periodontitis pathology) and dampen the pro-inflammatory response while promoting cell survival; these characteristics would correlate with previously described *P. gingivalis* immune suppressive and anti-apoptotic responses. In this study, Fos B DNA binding was down-regulated by all three bacteria. Information regarding the biological functions of Fos B remains sparse. However, down-regulation of Fos B has been associated with epithelial cell dedifferentiation and tumour progression (Milde-Langosh K 2003), as well as rescue from apoptotic cell death in T cells (Baumann, Hess et al. 2003). While there are no reports linking *A. naeslundii* to cancer, both *F. nucleatum* and *P. gingivalis* have been associated with cancers and the progression of tumorigenesis (Rubinstein, Wang et al. 2013, Hajishengallis 2015, Flynn, Baxter et al. 2016); therefore, suppression of Fos B in this context is not surprising.

Changes in DNA binding of the NF-κB transcription factor p65 were also assessed and interestingly, neither *A. naeslundii*, *F. nucleatum* nor *P. gingivalis* activated DNA binding by p65. Both *A. naeslundii* and *F. nucleatum* induced phosphorylation of IκB-α, and while the lack of p65 activation is surprising, it is possible that other NF-κB transcription factors (p50, c-Rel, RelB, p52) are activated or involved in responses to these bacteria. Activation of the p65 and p50 transcription factors has been shown in response to *F. nucleatum* cell wall extracts (Krisanaprakornkit, Kimball et al. 2002) and NF-κB translocation into the nucleus has also been observed in response to *F. nucleatum* infections (Bui, Johnson et al. 2015). Differences in experimental design between these two studies and the work described here include the use of purified cell wall extracts rather than live bacteria, primary gingival epithelial cells rather than a carcinoma cell line and the *F. nucleatum* strain 25586 rather than 10953, all of which may account for the contradicting responses. Nevertheless, while up-regulation of p65
DNA binding was not observed, activation of p-IκB-α was clear. Notably, phosphorylation of IκB-α is not sufficient for activation of NF-κB (Lin, Brown et al. 1995). Following phosphorylation, ubiquitination and proteolytic degradation of IκB-α proceeds and is necessary for NF-κB nuclear translocation (Karin and Ben-Neriah 2000). Therefore, there are various aspects of the NF-κB signal transduction pathway that can be altered and interference in the ubiquitination step has been shown in intestinal epithelial cells infected with non-pathogenic *Salmonella* strains (Neish, Gewirtz et al. 2000). Neish and colleagues highlight a possible mechanism of gastrointestinal tolerance where infection with non-virulent *Salmonella* strains results in IκB-α phosphorylation; however, the subsequent polyubiquitination required for IκB-α degradation is completely abrogated (Neish, Gewirtz et al. 2000). Moreover, this result was observed in two other human epithelial cell lines but not in monocytic cells or human umbilical vein endothelial cells (Neish, Gewirtz et al. 2000), suggesting that this may be an epithelial cell-specific response. Further experiments would have to determine if a similar phenomenon is occurring in oral epithelial cells in response to oral bacteria. Both *A. naeslundii* and *F. nucleatum* are considered commensal colonisers of the oral cavity and both also induced phosphorylation of MAPK pathways and downstream transcription factors. The response to AP-1 for example is enhanced by the presence of NF-κB subunits and vice versa (Stein, Baldwin et al. 1993), thus manipulation of NF-κB activation as described by Neish et al. could contribute to an attenuated inflammatory response and homeostasis at the oral mucosal barrier.

Cytokine and LDH release were also assessed in response to infection with *A. naeslundii*, *F. nucleatum* and *P. gingivalis*. *A. naeslundii* stimulated secretion of IL-6, GM-CSF and G-CSF, *F. nucleatum* induced IL-1α, IL-1β, GM-CSF and G-CSF, while *P. gingivalis* did not induce cytokine secretion but, moreover, detectable IL-8 protein levels were significantly reduced. Despite the pro-inflammatory response observed following infection with *A. naeslundii* and *F. nucleatum*, neither of these bacteria caused damage to the H400 cells. IL-1α, which was secreted following *F. nucleatum* infection, is widely regarded as being damage-associated (Bianchi 2007). However, the lack of detectable LDH activity suggested that IL-1α release by *F. nucleatum* is damage-independent. *P. gingivalis* also did not damage H400 cells; however, it was demonstrated that *P. gingivalis* is able to degrade the LDH protein after its release.
Protein degradation by *P. gingivalis* is well documented, and is mainly conferred through the activity of gingipains, particularly the lysine gingipain Kgp (Guo, Nguyen et al. 2010). Kgp can degrade both IL-8 and IL-6 (Stathopoulou, Benakanakere et al. 2009) and the observed strong reduction of IL-8 levels is in agreement with several other studies (Darveau, Belton et al. 1998, Mikolajczyk-Pawlinska, Travis et al. 1998, Sandros, Karlsson et al. 2000). It is therefore likely that both IL-8 and LDH proteins are degraded following release from epithelial cells in a gingipain-dependent manner and future experiments using *P. gingivalis* gingipain mutants will elucidate this. The pro-inflammatory response observed following *A. naeslundii* and *F. nucleatum* infections is also in agreement with other studies (Stathopoulou, Benakanakere et al. 2010, Dickinson, Moffatt et al. 2011, Peyyala, Kirakodu et al. 2012). The observation that some cytokine responses to *A. naeslundii* and *F. nucleatum* are similar while others are unique, may support the idea that *F. nucleatum* is an intermediate coloniser, bridging commensals with periodontal pathogens.

A summary of the signalling events observed in the H400 epithelial cell line following infection with *A. naeslundii*, *F. nucleatum* and *P. gingivalis* are presented in Figure 3-10.
In summary, the differential transcription factor activity in response to *A. naeslundii*, *F. nucleatum* and *P. gingivalis* parallels the MAPK response seen at the signal pathway level. *A. naeslundii* and *F. nucleatum* exhibited a similar profile of transcription factor activation, with both bacteria activating c-Fos and *F. nucleatum* also activating c-Jun. Notably, *F. nucleatum* was the most potent activator. Both *A. naeslundii* and *F. nucleatum* induced a pro-inflammatory response, which further correlates with the up-stream activation of signalling proteins and transcription factors. The transcription factor profile induced by *P. gingivalis* contrasted with those of *A. naeslundii* and *F. nucleatum* in that c-Fos and c-Jun binding was unaffected but ATF-2 was activated. This
is an interesting observation that correlated with the lack of activation of signalling proteins, which may support *P. gingivalis*’s characterisation as a ‘stealth-like’ pathogen. This notion is further supported by the lack of cytokine production and IL-8 down-regulation observed in response to *P. gingivalis* infection. These opposing effects strongly suggest that epithelial cells recognise and respond differently to the periodontitis associated pathogen *P. gingivalis* as compared with *A. naeslundii* and *F. nucleatum*. It is unclear why this is the case and which of these pathways are driving the observed responses in epithelial cells following challenge with *A. naeslundii*, *F. nucleatum* and *P. gingivalis*. This will be addressed in Chapter 4.
Chapter 4: Effect of signalling pathway inhibition on human oral epithelial cell responses to *A. naeslundii, F. nucleatum* and *P. gingivalis*

4.1 Introduction

Periodontitis is a chronic inflammatory disease that leads to the destruction of tissues surrounding and supporting teeth. While the primary aetiological agent is the bacterial biofilm, it is not sufficient for disease progression. Bacteria may cause direct damage (e.g. breakdown of tissues by proteolytic enzymes); however, tissue destruction relies heavily on a hyper-inflammatory host response (Di Benedetto, Gigante et al. 2013). Many cellular processes precede the production of inflammatory mediators and it is therefore important to identify the specific molecules that contribute to the inflammatory phenotype. For example, while multiple signalling pathways can be activated in a cell following bacterial stimulation, not all are necessarily relevant to disease pathology. Through the use of chemical inhibitors, key pathways contributing to disease progression can be identified. This approach is not only important in the understanding of disease pathogenesis but is also imperative for the identification of appropriate targets for therapeutic intervention.

Recognition of oral bacteria by gingival epithelial cells, which are the first cells to come into contact with the biofilm, is mediated via PRR recognition of PAMPs, resulting in activation of intracellular signalling pathways and secretion of inflammatory compounds such as cytokines and chemokines. Chapter 3 of this study identified the epithelial signal transduction initiated by *A. naeslundii, F. nucleatum* and *P. gingivalis* in the oral epithelial cell line H400. *A. naeslundii* activated the JNK MAPK pathway and the NF-κB pathway, the downstream AP-1 transcription factor c-Fos and induced secretion of IL-6, GM-CSF and G-CSF. *F. nucleatum* was the most potent activator, up-regulating all three MAPK pathways (p38, JNK, ERK1/2) as well as the NF-κB pathway, downstream AP-1 transcription factors c-Fos and c-Jun, and induced secretion of IL-1α, IL-1β, GM-CSF and G-CSF. *P. gingivalis* was the least stimulatory of the three bacteria, with mild up-regulation of JNK and the ATF-2 transcription factor, but no increased cytokine production; in fact IL-8 levels were diminished. While this work has identified
activated (or inhibited) targets of these bacteria, this is not sufficient for knowing the key regulators of these responses. Therefore, the aim of this chapter was to establish, with the use of chemical inhibitors, which of the signalling pathways detected are controlling the downstream activation of transcription factors and secretion of pro-inflammatory mediators, in the H400 cell line model system.

4.2 Methods

4.2.1 Inhibition of signalling pathways

Prior to bacterial infection, the MAP kinase pathways JNK, ERK and p38 as well as the NF-κB pathway were inhibited (see section 2.4.2). Epithelial cells were incubated with the respective inhibitors 1 h prior to infection (Table 4.1). Optimal concentrations for the signal pathway inhibitors were determined in a previous study (Moyes, Runglall et al. 2010).

Table 4-1. Signalling pathway inhibitors used in Chapter 4

<table>
<thead>
<tr>
<th>Pathway inhibited</th>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>JNK</td>
<td>SP600125</td>
<td>10 µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>(Primary target: JNK1, JNK2, JNK3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td>FR180204</td>
<td>750 nm</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>(Primary target: ERK1 and ERK2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p38</td>
<td>SB203580</td>
<td>10 µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>(Primary target: p38 MAP kinase)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF-κB</td>
<td>BAY11-7082</td>
<td>2 µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>(Primary target: IκB-α)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.2.2 Transcription factors

Nuclear extracts were tested using a TransAM DNA binding assay, which is an ELISA-based system using oligo-dT as the capture molecule to assess DNA binding of transcription factors (see Sections 2.7.1, 2.7.2). H400 monolayers were incubated with the respective bacterial strains (Table 4.2) at an MOI of 100 for up to 2 h (see Section 2.4.1).
Table 4-2. Bacterial strains used in Chapter 4

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Genotype</th>
<th>Description</th>
<th>Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinomyces naeslundii</em> NCTC 10301</td>
<td>Wild-type</td>
<td>Clinical isolate (Thompson and Lovestedt 1951)</td>
<td>BA</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em> s.s polymorphum ATCC 10953</td>
<td>Wild-type</td>
<td>Clinical isolate (Dzink, Sheenan et al. 1990)</td>
<td>FAA</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em> ATCC 33277</td>
<td>Wild-type</td>
<td>Clinical isolate (Coykendall, Kaczmarek et al. 1980)</td>
<td>FAA</td>
</tr>
</tbody>
</table>

BA: Blood Agar
FAA: Fastidious Anaerobic Agar

4.2.3 Cytokine release

Cytokine levels were determined using a magnetic bead-based assay (Luminex) detecting protein levels in cell culture supernatants (see Section 2.5.2). H400 monolayers were incubated with the respective bacterial strains (Table 4.2) at an MOI of 100 for 24 h (see Section 2.4.1).

4.2.4 Cell damage assay

Epithelial cell damage was determined by measuring LDH activity in cell culture supernatants (see Section 2.5.1). H400 monolayers were incubated with the respective bacterial strains (Table 4.2) at an MOI of 100 for 24 h (see Section 2.4.1).

4.3 Results

4.3.1 Effect of signalling pathway inhibition on transcription factor activation in epithelial cells following challenge with oral bacteria

Infection of oral epithelial cells with *A. naeslundii*, *F. nucleatum* and *P. gingivalis* resulted in the activation of distinct signalling profiles as discussed in Chapter 3. Activation of the three MAPK pathways p38, JNK and ERK was observed, as well as activation of the NF-κB pathway. Assessment of downstream transcription factors identified the AP-1 (c-Fos and c-Jun) and MAPK (ATF-2) transcription factors as being activated by these bacteria. Therefore, to investigate which signalling pathways are
regulating the activation of these transcription factors, p38, JNK and ERK 1/2 MAPK pathways were inhibited and DNA binding of c-Fos, c-Jun and ATF-2 was evaluated. While the NF-κB associated regulating factor IκB-α was phosphorylated, the NF-κB associated transcription factor p65 was not up-regulated in response to any of the three bacterial species. Therefore, the NF-κB pathway was not inhibited for the assessment of transcription factors activation. Likewise, the PI3K pathway was not activated in response to any of the three bacteria and was also not investigated.

4.3.1.1 Effect of p38 MAPK pathway inhibition on c-Fos, c-Jun and ATF-2 binding activity
Inhibition of the p38 MAPK pathway had a significant effect on c-Fos and ATF-2 transcription factor DNA binding activity but not on c-Jun binding (Figure 4.1). In A. naeslundii infections, inhibition of p38 led to an almost 50% decrease in c-Fos activity at 60 min post-infection ($P < 0.05$) but a 1.4 and 1.5-fold increase in ATF-2 transcription factor binding at 30 and 60 min post-infection respectively ($P < 0.05$). Following F. nucleatum infections, inhibition of the p38 pathway resulted in a 60% decrease in c-Fos DNA binding at 60 min post-infection ($P < 0.05$). Similar to A. naeslundii infections, ATF-2 binding was increased following F. nucleatum infections, particularly at 30 min (1.4-fold) and 120 min (1.5-fold) post-infection, although this was not statistically significant. When cells were challenged with P. gingivalis following p38 inhibition, a similar trend was observed, where ATF-2 binding activity was increased 2-fold at 30 min post-infection, although this was not statistically significant. These results highlight the role of p38 MAPK signalling in the regulation of DNA binding of both c-Fos and ATF-2 transcription factors.
Figure 4-1. DNA binding activity of c-Fos, c-Jun and ATF-2 transcription factors in response to bacterial stimulation after p38 MAPK pathway inhibition. DNA binding activity of c-Fos, c-Jun and ATF-2 at different time points 2 h post-infection with *A. naeslundii*, *F. nucleatum* and *P. gingivalis* using the H400 oral epithelial cell line. Samples were analysed by TransAM ELISA. An MOI of 100 was used for infections. Data are expressed in relation to bacterially stimulated cells without signal pathway inhibition (vehicle, black bar) and are the mean fold change of three biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; *P < 0.05.
4.3.1.2 Effect of JNK MAPK pathway inhibition on c-Fos, c-Jun and ATF-2 binding activity

Inhibition of the JNK MAPK pathway did not have a statistically significant effect on c-Fos, c-Jun or ATF-2 DNA binding activity (Figure 4-2). However, in infections with *A. naeslundii* (at 30 min), *F. nucleatum* (all time points) and *P. gingivalis* (30 and 120 min) JNK inhibition resulted in an increasing trend in c-Fos binding and notably, a decreasing trend in c-Jun binding was observed with *P. gingivalis* infections at all time points. Nevertheless, these results did not reach statistical significance. Therefore, while these results suggest that the JNK MAPK pathway is not involved in the regulation of ATF-2 DNA binding, it may play a minor role in the regulation of c-Fos and c-Jun (*P. gingivalis* specific).
Figure 4-2. DNA binding activity of c-Fos, c-Jun and ATF-2 transcription factors in response to bacterial stimulation after JNK MAPK pathway inhibition. DNA binding activity of c-Fos, c-Jun and ATF-2 at different time points post-infection with *A. naeslundii*, *F. nucleatum* and *P. gingivalis* using the H400 oral epithelial cell line. Samples were analysed by TransAM ELISA. An MOI of 100 was used for infections. Data are expressed in relation to bacterially stimulated cells without signal pathway inhibition (vehicle, black bar) and are the mean fold change of three biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA and no statistical significance was found.
4.3.1.3 Effect of ERK MAPK pathway inhibition on c-Fos, c-Jun and ATF-2 binding activity

Inhibition of the ERK MAPK pathway did not have an effect on DNA binding of c-Fos, c-Jun or ATF-2 transcription factors (Figure 4-3). These results suggest that the ERK MAPK pathway does not play a role in the regulation of c-Fos, c-Jun or ATF-2 in response to *A. naeslundii, F. nucleatum* and *P. gingivalis*, in H400 cells.
Figure 4-3. DNA binding activity of c-Fos, c-Jun and ATF-2 transcription factors in response to bacterial stimulation after ERK MAPK pathway inhibition. DNA binding activity of c-Fos, c-Jun and ATF-2 at different time points post-infection with A. naeslundii, F. nucleatum and P. gingivalis using the H400 oral epithelial cell line. Samples were analysed by TransAM ELISA. An MOI of 100 was used for infections. Data are expressed in relation to bacterially stimulated cells without signal pathway inhibition (vehicle, black bar) and are the mean fold change of three biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA and no statistical significance was found.
4.3.2 Effect of signalling pathway inhibition on cytokine release in epithelial cells following challenge with oral bacteria

Infection of oral epithelial cells with *A. naeslundii*, *F. nucleatum* and *P. gingivalis* resulted in activation of signalling pathways that subsequently led to secretion of cytokines (Chapter 3). To evaluate which signalling pathways are regulating downstream cytokine production, p38, JNK and ERK 1/2 MAPK pathways as well as the NF-κB pathway were inhibited and secretion of IL-1α, IL-1β, IL-8, IL-6, GM-CSF and G-CSF was assessed. Unlike for transcription factor analysis (p65), the NF-κB pathway was assessed for the regulation of cytokine production as IκB-α was phosphorylated (activated) in response to *A. naeslundii* and *F. nucleatum* (Chapter 3), and may be regulating cytokine production in a p65-independent manner. Since the PI3K pathway was not activated in response to any of the three bacteria, this pathway was not investigated for its role in cytokine production.

4.3.2.1 Effect of signalling pathway inhibition on cytokine release following *A. naeslundii* infection

Cytokine release from oral epithelial cells was affected by inhibition of all MAPK pathways and the NF-κB pathway following *A. naeslundii* infection (Figure 4-4). Inhibition of JNK resulted in a 30% decrease in IL-1α and IL-1β levels ($P < 0.05$) and a 1.1-fold increase in GM-CSF levels ($P < 0.05$). ERK inhibition had the mildest effect, resulting in a 20% decrease in GM-CSF levels ($P < 0.05$). Inhibition of the p38 MAPK pathway had the largest effect, resulting in a 90% decrease in IL-6 and GM-CSF levels and a 70% decrease in G-CSF levels ($P < 0.001$). Lastly, inhibition of the NF-κB pathway diminished GM-CSF protein levels by 60% ($P < 0.001$). These results indicate that the MAPK and NF-κB pathways are involved in cytokine release following *A. naeslundii* infection and identify the p38 MAPK pathway as being the major MAPK regulator.
Figure 4-4. Cytokine release by oral epithelial cells in response to *A. naeslundii* following inhibition of JNK, ERK and p38 MAPK pathways and NF-κB pathway. Secretion of IL-1α, IL-1β, IL-8, IL-6, GM-CSF and G-CSF 24 h post-infection with *A. naeslundii* using the H400 oral epithelial cell line. Samples were analysed by Luminex. An MOI of 100 was used for infections. Data are expressed in relation to *A. naeslundii* stimulated cells without signal pathway inhibition (vehicle, black bar) and are the mean fold change of three to four biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; *P* < 0.05, ***P* < 0.001.
4.3.2.2 Effect of signalling pathway inhibition on cytokine release following *F. nucleatum* infections

*F. nucleatum* induced cytokine release was affected by all MAPK pathways as well as the NF-κB pathway (Figure 4-5). Inhibition of JNK resulted in a significant decrease in IL-1α (60%) and IL-1β (70%) levels (*P* < 0.001). ERK inhibition had a mild effect on cytokine release, resulting in a mild increase in IL-6 (1.3-fold) (*P* < 0.05). Inhibition of the p38 MAPK pathway had the biggest effect on cytokine release; levels of IL-1α, IL-1β, IL-6, GM-CSF and G-CSF were reduced by 60%, 80%, 95%, 90% (*P* < 0.001) and 60%- (P < 0.05), respectively. Additionally, IL-1β secretion was also down-regulated by 50% following NF-κB inhibition (*P* < 0.001). These results indicate that MAPK and NF-κB pathways regulate cytokine release during *F. nucleatum* infections, with the p38 MAPK pathway acting as the major MAPK regulator.

**Figure 4-5. Cytokine release by oral epithelial cells in response to *F. nucleatum* following inhibition of JNK, ERK and p38 MAPK pathways and NF-κB pathway.** Secretion of IL-1α, IL-1β, IL-8, IL-6, GM-CSF and G-CSF 24 h post-infection with *F. nucleatum* using the H400 oral epithelial cell line. Samples were analysed by Luminex. An MOI of 100 was used for infections. Data are expressed in relation to *F. nucleatum* stimulated cells without signal pathway inhibition (vehicle, black bar) and are the mean fold change of three to four biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; *P* < 0.05, **P** < 0.001.
4.3.2.3 Effect of signalling pathway inhibition on cytokine release following *P. gingivalis* infections

Cytokine release following *P. gingivalis* infection was affected by the inhibition of JNK and ERK MAPK pathways and the NF-κB pathway (Figure 4-6). Inhibition of JNK displayed an interesting dichotomy in the response: a 60%, 72% and 70% decrease in IL-1β (*P* < 0.05), IL-8 and GM-CSF (*P* < 0.001), respectively, and a 1.6 and 1.9-fold increase in IL-6 (*P* < 0.05) and G-CSF (*P* < 0.01), respectively. Following inhibition of ERK, there was a 1.5-fold increase in GM-CSF levels (*P* < 0.05). Lastly, inhibition of the NF-κB pathway led to a decrease in IL-8 and GM-CSF of 50% and 90%, respectively, (*P* < 0.001). Interestingly, while the p38 pathway appeared to be the most important MAPK pathway for cytokine regulation during *A. naeslundii* and *F. nucleatum* infections, the p38 pathway has no apparent cytokine regulatory role during *P. gingivalis* infections.

**Figure 4-6.** Cytokine release by oral epithelial cells in response to *P. gingivalis* following inhibition of JNK, ERK and p38 MAPK pathways and NF-κB pathway. Secretion of IL-1α, IL-1β, IL-8, IL-6, GM-CSF and G-CSF 24 h post-infection with *P. gingivalis* using the H400 oral epithelial cell line. Samples were analysed by Luminex. An MOI of 100 was used for infections. Data are expressed in relation to *P. gingivalis* stimulated cells without signal pathway inhibition (vehicle, black bar) and are the mean fold change of three to four biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; *P* < 0.05, **P** < 0.01, ***P*** < 0.001.
4.3.3 Effect of signalling pathway inhibition on epithelial cell damage following *A. naeslundii, F. nucleatum* and *P. gingivalis* infections

LDH release from the H400 oral epithelial cells was generally un-affected by the inhibition of signalling pathways, although in infections with *F. nucleatum*, inhibition of the ERK pathway resulted in a non-significant 1.5-fold increase in LDH release (Figure 4-7).

![LDH graph](image)

**Figure 4-7.** Damage induction in oral epithelial cells in response to bacterial stimulation following inhibition of JNK, ERK and p38 MAPK pathways and NF-κB pathway. LDH activity 24 h post-infection with *A. naeslundii, F. nucleatum* and *P. gingivalis* using the H400 oral epithelial cell line. Samples were analysed by LDH assay. An MOI of 100 was used for infections. Data are expressed in relation to bacterially stimulated cells without signal pathway inhibition (vehicle, black bar) and are the mean fold change of three biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA and no statistical significance was found.
4.4 Discussion

Infection of gingival epithelial cells with *A. naeslundii, F. nucleatum* and *P. gingivalis* resulted in the activation of distinct signal transduction pathways. The signalling pathways identified were the three MAPK pathways p38, JNK and ERK1/2, as well as the NF-kB pathway. Downstream of these pathways, activation of c-Fos, c-Jun and ATF-2 was observed as well as secretion of cytokines (Chapter 3). This chapter aimed to identify which signalling pathways are regulating the activation of transcription factors and induction of cytokines.

The three MAPK pathways p38, JNK and ERK1/2 were inhibited and DNA binding of c-Fos, c-Jun and ATF-2 was assessed following infection with *A. naeslundii, F. nucleatum* and *P. gingivalis*. The NF-kB pathway was not inhibited for the assessment of transcription factors because the NF-kB-p65 transcription factor was not up-regulated in response to any of the three bacteria (Chapter 3). Following inhibition of p38, an increase in ATF-2 DNA binding was observed in infections with all three bacteria and a decrease in c-Fos DNA binding was observed in infections with *A. naeslundii* and *F. nucleatum*, while c-Jun DNA binding was unaffected. Inhibition of JNK had no effect on ATF-2 but resulted in an increase in c-Fos DNA binding with all three bacteria and a decrease in c-Jun following *P. gingivalis* infection. Inhibition of the ERK1/2 pathway had no effect on these transcription factors. Therefore, these results suggest a dual regulatory role of the p38 and JNK MAPK pathways: activation of p38 negatively regulates ATF-2 but positively regulates c-Fos, whereas activation of JNK negatively regulates c-Fos and positively regulates c-Jun. The apparent lack of involvement of the ERK MAPK pathway in the regulation of these transcription factors could be attributed to the transient rather than sustained activation observed in Chapter 3. The duration of intracellular signalling dictates distinct biological responses (Marshall 1995). For example, when ERK activation is transient, c-Fos proteins do not accumulate and are not stable (Murphy, Smith et al. 2002). However, when ERK signalling is sustained, c-Fos is phosphorylated, allowing for DNA binding and initiation of transcription (Murphy, Smith et al. 2002).

Negative regulation of ATF-2 by the p38 MAPK pathway has been previously reported in the Ca9-22 epithelial cell line following infection with *P. gingivalis* HbR (Fujita,
Nakayama et al. 2014). Interestingly, however, ATF-2 is generally considered to be positively regulated by p38 (Zarubin and Han 2005). The general control of c-Fos by p38 has also been documented (Whitmarsh and Davis 1996, Tanos, Marinissen et al. 2005), and in epithelial cell infections with the fungus C. albicans, p38 was shown to promote c-Fos transcriptional activity (Moyes, Runglall et al. 2010). Upon activation, the JNK MAPK pathway is known to activate c-Jun and ATF-2 (Wisdom 1999, Shaulian and Karin 2002), but only JNK control of c-Jun activity was observed in this study. Transcriptional regulation by the MAPK pathways is complex, involving simultaneous activation and crossover in the control of AP-1 homo- and hetero-dimers of varying combinations, transactivational potential and gene targets (Shaulian and Karin 2002, Eferl and Wagner 2003). For example, all three MAPK pathways can target ATF-2 phosphorylation (Hazzalin and Mahadevan 2002) and c-Fos can be regulated by ERK (Murphy, Smith et al. 2002, Yang, Sharrocks et al. 2013), as well as JNK and p38 (Silvers, Bachelor et al. 2003). Further complexity has been demonstrated in the activating kinetics of the p38 MAPK pathway where stimulation with different activating-insults resulted in different transcription factor gene expression profiles specific for a given stress, with only 20% overlap in up-regulated genes (Ferreiro, Joaquin et al. 2010). Hence, the control of transcription factor expression by the MAPK pathways will likely differ depending on the stimulus, the duration and the cell type; it is therefore important to interpret signal transduction events according to the specific modes of infection. The results of this study suggest that during infection of oral epithelial cells with oral bacteria, the p38 and JNK MAPK pathways are differentially involved in the regulation of ATF-2, c-Fos and c-Jun DNA binding, while the ERK MAPK has no apparent effect on binding of these transcription factors.

Cytokine release was also assessed following inhibition of p38, JNK, ERK, and the NF-κB pathway. The NF-κB pathway was included because although p65 DNA binding was not observed (Chapter 3), other NF-κB transcription factors (p50, c-Rel, RelB, p52) could be driving pro-inflammatory cytokine responses. Inhibition of both the MAPK and NF-κB pathways affected cytokine release from H400 epithelial cells, with p38 and JNK MAPK pathway inhibition having the most pronounced effect. Inhibition of the p38 pathway resulted in significant decreases in all cytokines quantified with the exception of IL-8. Interestingly, this effect was only observed in response to A. naeslundii and F.
nucleatum infections but not P. gingivalis. The JNK pathway regulated all of the cytokines but in an opposing manner; following JNK inhibition, IL-1α, IL-1β and IL-8 levels were decreased, IL-6 and G-CSF levels were increased, while GM-CSF levels were up-regulated following A. naeslundii infections and down-regulated following P. gingivalis infections. This suggests that the JNK pathway controls different genes depending on the stimulus (e.g. LTA of A. naeslundii and LPS of P. gingivalis), which correlates with p38 activating mechanisms (Ferriero 2010). However, the observed 1.1 fold increase in GM-CSF secretion following A. naeslundii infection may not be biologically significant. Of the three MAPK pathways, ERK had the least effect on cytokine release. Inhibition of ERK resulted in IL-6 up-regulation and both down- and up-regulation of GM-CSF following A. naeslundii and P. gingivalis infections, respectively. This observation parallels JNK responses and thus the same mechanisms may apply. Nevertheless, changes in both IL-6 and GM-CSF levels were of less than ±0.5 fold in all instances, which again may not be biologically significant. These observations correlate with the general lack of transcription factor regulation by ERK in these infections. Lastly, the NF-κB pathway also contributed to cytokine induction, as inhibition resulted in a down-regulation of IL-1α, IL-1β, IL-8 and GM-CSF. However, NF-κB control of cytokine induction was far less potent than the p38 MAPK pathway, particularly for F. nucleatum.

There are few studies that describe the control of cytokine expression by signalling pathways in oral epithelial cells infected with oral bacteria. A study by Huang and colleagues investigated IL-8 mRNA levels following F. nucleatum and P. gingivalis infections in the presence of p38, ERK and NF-κB inhibitors (Huang, Zhang et al. 2004). All three pathways were involved in IL-8 induction, with NF-κB being the main regulator. Interestingly, the ERK pathway participated in both up- and down-regulation of IL-8 following infections with heat-killed and live P. gingivalis respectively (Huang, Zhang et al. 2004); this observation further enforces the idea that signalling pathways employ different regulatory mechanisms depending on the stimulus. The observation that the NF-κB pathway regulates IL-8 expression was also observed in this thesis; however, in the H400 cell line, p38 and ERK did not appear to regulate IL-8 secretion. Nonetheless, mRNA levels do not always correlate with levels of protein expression or cytokine secretion and, therefore, the data are not directly comparable. A more
recent study has shown IL-1β and IL-6 positive regulation by the JNK pathway in *P. gingivalis* infections (Wang, Zhou et al. 2014). In this thesis, during *P. gingivalis* infection, inhibition of JNK resulted in IL-1β down-regulation, which parallels the results by Wang et al.; however, IL-6 levels were increased. This difference could be attributed to the use of different cell lines, MOI and infection time points (i.e. carcinoma cell line vs telomerase-immortalised gingival epithelial cells, MOI of 100 vs 10, and 24 h infection vs 4 h infection). These results demonstrate that the MAPK and NF-κB pathways are regulating pro-inflammatory responses in H400 oral epithelial cells challenged with oral bacteria. The p38 MAPK pathway appears to be the key regulator, while the ERK MAPK pathway is the least involved. Interestingly, the regulation of cytokine production by these pathways was similar in *A. naeslundii* and *F. nucleatum* infections with opposing effects seen in *P. gingivalis* infections. This suggests more than a simple difference in response due to the Gram state of these bacteria. The differential modulation of intracellular signalling by *P. gingivalis* characterised in Chapter 3 is further supported by results in the present chapter and may be due to this bacterium’s unique virulence factors (i.e. gingipains, PPAD, etc.) and their ability to fine-tune immune responses.

p38 and JNK were the only pathways to control DNA binding of transcription factors in H400 epithelial cell infections. Inhibition of p38 down-regulated c-Fos and it is therefore likely that the control of cytokine gene expression is mediated via c-Fos during p38 activation. Inhibition of JNK up-regulated c-Fos and down-regulated c-Jun binding; this could account for the observed dichotomy in cytokine regulation by JNK. Blocking c-Fos and c-Jun expression by siRNA in these infections would demonstrate the precise control of cytokine release by these transcription factors. DNA binding of the p65 NF-κB transcription factor was not detected and it is therefore unlikely that p65 is regulating cytokine gene expression in this system. However, the down-regulation of cytokine release observed following NF-κB (IκB-α) inhibition could therefore be mediated via other NF-κB transcription factors. The two main NF-κB activating pathways, canonical and non-canonical, primarily consist of p65-containing heterodimers and p52-RelB complexes, respectively (Oeckinghaus, Hayden et al. 2011). Most of what is known regarding NF-κB activation and regulation of transcription is from studies focusing on signalling through IκB-α-p65 (Hayden and Ghosh 2008);
functions of the non-canonical NF-κB pathway are currently limited (Sun 2011). Nevertheless, bacterial pathogens have been shown to target non-canonical NF-κB. For example, Gram-negative *H. pylori* induces activation of NF-κB through the non-canonical pathway in an LPS-dependent manner (Ohmae, Hirata et al. 2005). Additionally, *Legionella pneumophila*, also a Gram-negative bacterium, utilises both canonical and non-canonical NF-κB signalling to modulate macrophage responses during infection (Ge, Xu et al. 2009). It is therefore possible that the non-canonical NF-κB pathway was also activated in response to the oral bacteria tested in this thesis. Thus, a more in depth analysis of NF-κB activation, including all transcription factors, would provide a better understanding of NF-κB-regulated responses in oral epithelial cells following bacterial stimulation.

It should be noted that the NF-κB inhibitor BAY11-7082 is described as inhibiting IκB-α phosphorylation, thus impacting only the canonical pathway. This would suggest that even if the non-canonical pathway were involved in the observed responses, it would not have been inhibited and thus would not account for the down-regulation of cytokines following NF-κB inhibition in these infections. However, a recent study has indicated that BAY11-7082 is not a direct inhibitor of the canonical IKK complex, but prevents activation by targeting the ubiquitin system (Strickson, Campbell et al. 2013). Ubiquitination is necessary for the processing of both canonical IκB-α and non-canonical NIK-induced p100 (p100 functions as an IκB-like molecule, inhibiting the nuclear translocation of p52-RelB complexes) (Sun 2011). Thus, it is possible that BAY11-7082 might indeed be inhibiting both canonical and non-canonical NF-κB pathways. Moreover, BAY11-7082 has also been shown to inhibit activation of the NLRP3 inflammasome independent of its inhibitory effect on NF-κB activation (Juliana, Fernandes-Alnemri et al. 2010). This is particularly important as inflammasomes are required for cleavage of pro-IL-1β and pro-IL-18 into their mature active forms (Guo, Callaway et al. 2015). This secondary effect of the BAY11-7082 inhibitor could account for the decreased IL-1β levels observed in *F. nucleatum* infections.

A surprising observation was that while pathway signal inhibition had a clear effect on cytokine responses (this Chapter), these bacteria did not originally induce some of these cytokines (Chapter 3). For example, inhibition of p38 resulted in decreased levels
of IL-1α, IL-1β, IL-6, GM-CSF and G-CSF in both A. naeslundii and F. nucleatum infections; however, in the initial characterisation of responses (Chapter 3), A. naeslundii did not induce IL-1α or IL-1β secretion, and F. nucleatum did not induce IL-6 secretion. This phenomenon was particularly evident following p38 inhibition, but also following inhibition of the other pathways. This presents a limitation of inhibiting signalling pathways when assessing effector responses. The p38 MAPK pathway is recognised as the key regulator of inflammatory responses, and activation of p38 controls expression of various cytokine genes at both transcriptional and post-transcriptional stages (Cuenda and Rousseau 2007). Importantly, p38 is recognised as being a major contributor to mRNA stability during cytokine/stress-induced gene expression (Winzen, Kracht et al. 1999, Thapar and Denmon 2013, Degese, Tanos et al. 2015). Activation of p38 regulates the stability of many inflammatory mediators whose mRNAs have AU-rich (ARE) elements in their 3’ untranslated regions (UTRs) and undergo ARE-mediated decay (Thapar and Denmon 2013). A role for the JNK pathway in mRNA stabilisation has also been described, acting via a JNK response element (JRE) in the 5’ UTR (Chen, Gherzi et al. 2000). Moreover, the NF-κB pathway has also been shown to regulate the stability of cytokine-encoding mRNA by controlling degradation of regnase-1; a regulatory protein which can associate with ARE elements and destabilise mRNA (Iwasaki, Takeuchi et al. 2011). It is likely that these post-transcriptional control mechanisms account for these unexpected results in this chapter.

A further caveat of using small chemical inhibitors targeting specific pathways is that the loss of function of a particular signalling pathway can be complemented by the activation of another. For example, a p38-JNK crosstalk has been described where in p38-deficient myoblasts, JNK activation was enhanced (Perdiguero, Ruiz-Bonilla et al. 2007), and in JNK1−/− and JNK2−/− fibroblasts, increased p38 activity has been detected (Hochedlinger, Wagner et al. 2002). Moreover, inhibition of the p38 MAPK pathway (using the SB203580 inhibitor), resulted in enhanced NF-κB transcriptional activity, driven by the activation of ERK (Birkenkamp, Tuyt et al. 2000). These adverse effects must be kept in mind when interpreting results from biological infections using pathway inhibitors.
In summary, the p38 MAPK pathway appears to be the key regulator of inflammatory responses in oral epithelial cell responses following infection with *A. naeslundii* and *F. nucleatum*, while following infection with *P. gingivalis*, the JNK MAPK pathway was found to be the major regulator of these responses.
Chapter 5: Identification of \textit{P. gingivalis} virulence factors that affect human oral epithelial cell responses

5.1 Introduction

Periodontal disease is a complex multifactorial disorder, driven by a synergistic but dysbiotic microbial community. Certain bacterial species have been associated with the progression of periodontitis, of which \textit{P. gingivalis} is considered prominent due to its keystone pathogen attributes (see Chapter 1). This Gram-negative anaerobic rod can produce an array of virulence factors (Table 1-1), including cysteine proteases (RgpA/B, Kgp) that can modulate the host immune response and cause degradation or cleavage of host cell proteins and surface receptors (Guo, Nguyen et al. 2010), a serine phosphatase (SerB) involved in successful invasion of host tissues (Tribble, Mao et al. 2006), and a peptidylarginine deiminase (PPAD) capable of protein citrullination (Pyrc, Milewska et al. 2013).

In the gingival compartment, epithelial cells are the first host cells encountered by \textit{P. gingivalis}. Adhesion to, and invasion of epithelial cells by \textit{P. gingivalis} has been well documented and reviewed (Andrian, Grenier et al. 2006, Yilmaz 2008). Binding of \textit{P. gingivalis} to epithelial cells induces endocytosis of the bacterium, a process that occurs within 20 min, and results in \textit{P. gingivalis} localisation in the cytosol (Andrian, Grenier et al. 2006). Once inside, \textit{P. gingivalis} is capable of multiplying and maintaining viability for prolonged periods, without inducing apoptotic or necrotic death (Yilmaz 2008). This is due to \textit{P. gingivalis}'s ability to modulate intracellular signalling pathways to promote anti-apoptotic mechanisms and subversion of the host immune response (see Chapter 1). The characterisation of gingival epithelial cell responses to \textit{P. gingivalis} 33277 carried out in Chapter 3 showed a lack of activation of signalling proteins, with only mild up-regulation of phosphorylated JNK and induction of ATF-2 DNA binding observed. The present chapter aimed to determine whether the evasive phenotype of \textit{P. gingivalis} 33277 is common to other strains of \textit{P. gingivalis} and to determine whether known \textit{P. gingivalis} virulence factors, namely RgpA/B, Kgp, SerB or PPAD, are responsible for the evasive phenotype of this bacterium.
5.2 Methods

5.2.1 Detection of phosphorylated signalling proteins

Activation of signalling pathways in gingival epithelial cells was determined using a magnetic bead-based immunoassay detecting intracellular phosphorylated proteins in cell lysates (see Section 2.6.3). H400 monolayers were incubated with the respective bacterial strains (Table 5.1) at an MOI of 100 for up to 2 h (see Section 2.4.1).

Table 5-1. Bacterial strains used in Chapter 5

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Genotype</th>
<th>Description</th>
<th>Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porphyromonas gingivalis</td>
<td>Wild-type</td>
<td>Clinical isolate (Coykendall, Kaczmarek et al. 1980)</td>
<td>FAA</td>
</tr>
<tr>
<td>ATCC 33277</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porphyromonas gingivalis</td>
<td>Wild-type</td>
<td>Clinical isolate (Coykendall, Kaczmarek et al. 1980)</td>
<td>FAA</td>
</tr>
<tr>
<td>W50</td>
<td></td>
<td>Parent strain of the rgpA/B, kgp and serB mutants</td>
<td></td>
</tr>
<tr>
<td>ΔrgpA/B</td>
<td>rgpA::tetQ</td>
<td>rgpA rgpB double deletion mutant (Aduse-Opoku, Davies et al. 2000)</td>
<td>FAA</td>
</tr>
<tr>
<td>E8</td>
<td>rgpB::erm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δkgp</td>
<td>kgp::erm</td>
<td>kgp deletion mutant (Aduse-Opoku, Davies et al. 2000)</td>
<td>FAA</td>
</tr>
<tr>
<td>K1A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔserB</td>
<td>serB::erm</td>
<td>serB deletion mutant (not published)</td>
<td>FAA</td>
</tr>
<tr>
<td>Porphyromonas gingivalis</td>
<td>Wild-type</td>
<td>Clinical isolate (Coykendall, Kaczmarek et al. 1980)</td>
<td>FAA</td>
</tr>
<tr>
<td>W83</td>
<td></td>
<td>Parent strain of the PPAD mutant</td>
<td></td>
</tr>
<tr>
<td>ΔPPAD</td>
<td>ppad::erm</td>
<td>ppad deletion mutant (Wegner, Wait et al. 2010)</td>
<td>FAA</td>
</tr>
</tbody>
</table>

FAA: Fastidious Anaerobic Agar

\(^a\) *P. gingivalis* 33277 ΔserB, not used in this study, was published by (Tribble, Mao et al. 2006)
5.2.2 Transcription factors
Nuclear extracts were tested using a TransAM DNA binding assay, which is an ELISA-based system using oligo-dT as the capture molecule to assess DNA binding of transcription factors (see Sections 2.7.1, 2.7.2). H400 monolayers were incubated with the respective bacterial strains (Table 5.1) at an MOI of 100 for up to 2 h (see Section 2.4.1).

5.2.3 Cytokine release
Cytokine levels were determined using a magnetic bead-based assay detecting protein levels in cell supernatants (see Section 2.5.2). H400 monolayers were incubated with the respective bacterial strains (Table 5.1) at an MOI of 100 for 24 h (see Section 2.4.1).

5.2.4 Cell damage assay
Epithelial cell damage was determined by measuring LDH activity in cell supernatants (see Section 2.5.1). H400 monolayers were incubated with the respective bacterial strains (Table 5.1) at an MOI of 100 for 24 h (see Section 2.4.1).
5.3 Results

5.3.1 Epithelial signalling pathway activation in response to P. gingivalis

The characterisation of H400 epithelial cell responses to P. gingivalis was performed previously using the P. gingivalis strain 33277 (see Chapter 3). For a more in depth investigation of P. gingivalis responses, two additional wild type strains, P. gingivalis W50 and P. gingivalis W83 were analysed. To ensure consistency and comparability between data sets, the characterisation of H400 oral epithelial cell responses was repeated using all three P. gingivalis wild type strains in parallel.

5.3.1.1 Activation of the MAPK pathway

P. gingivalis wild type strains 33277, W50 and W83 failed to significantly activate each of the MAPK pathways. Generally, phosphorylation of p38 and ERK 1/2 remained at consistently low levels over the 2 h infection period and no difference in phosphorylation was observed between P. gingivalis 33277, W50 and W83 (Figure 5-1). However, while not statistically significant, induction of p-JNK was observed in response to P. gingivalis 33277 between 30 and 120 min post-infection (~3.3 - 3.9 fold) (Figure 5-1), which is consistent with the data in Chapter 3 where a ~4-fold up-regulation of p-JNK was observed at 30 and 60 min post-infection with P. gingivalis 33277. In summary, none of the P. gingivalis wild type strains tested appeared to activate MAPK responses in H400 gingival epithelial cells, with the possible exception of P. gingivalis 33277 which mildly induced p-JNK.
Figure 5-1. Activation of MAPK signalling pathway components in response to bacterial stimulation. Phosphorylation of p38, JNK and ERK 1/2 at different time points post-infection with *P. gingivalis* wild type strains using the H400 oral epithelial cell line. Samples were analysed by phospho-Luminex. An MOI of 100 was used for infections. Data are expressed in relation to un-stimulated resting cells (0, black bar) and are the mean fold change of three to six biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA and no statistical significance was found.
5.3.1.2 Activation of NF-κB and PI3K pathways

Phosphorylation of IκB-α and AKT was not significantly induced by *P. gingivalis* 33277, W50 or W83 at any time point examined (Figure 5-2). These results are consistent with the data in Chapter 3 and the argument that *P. gingivalis* is a weak inducer of NF-κB and PI3K signalling.

![Graph of phosphorylation of IκB-α and AKT](image)

**Figure 5-2. Activation of NF-κB and PI3K / AKT signalling pathways in response to bacterial stimulation.** Phosphorylation of IκB-α and AKT at different time points post-infection with *P. gingivalis* wild type strains using the H400 oral epithelial cell line. Samples were analysed by phospho-Luminex. An MOI of 100 was used for infections. Data are expressed in relation to un-stimulated resting cells (0, black bar) and are the mean fold change of three to six biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA and no statistical significance was found.
5.3.2 Activation of epithelial transcription factors in response to *P. gingivalis*

The characterisation of H400 gingival epithelial cell responses to *P. gingivalis* 33277 in Chapter 3 identified ATF-2 as the only transcription factor that was activated following challenge with this bacterium (Figure 3-3). However, two transcription factors that were not originally included in the transcription factor screen performed in Chapter 3 are FOXO-1 and NRF-2. FOXO-1 has been shown to be induced by *P. gingivalis* 33277 in primary gingival epithelial cells (Li, Dong et al. 2013, Wang, Sztukowska et al. 2015). Involvement of NRF-2 in *P. gingivalis* signalling remains unclear; however, NRF-2 has been briefly discussed in the context of periodontal disease (Park, Park da et al. 2011, Dias, Chapple et al. 2013). Therefore, activation of FOXO-1 and NRF-2 was assessed in addition to ATF-2.

Infection with *P. gingivalis* wild type strains 33277, W50 and W83 resulted in activation of ATF-2 DNA binding. *P. gingivalis* 33277 and W83 both significantly up-regulated ATF-2 DNA binding at 30 min post-infection (~2.2 (*P* < 0.05) and ~1.8 fold (*P* < 0.01), respectively), which then diminished by 60 min (Figure 5-3). Stimulation with *P. gingivalis* W50 led to a more sustained activation of ATF-2 DNA binding. At 30 and 60 min post-infection, ATF-2 DNA binding activity was up-regulated 1.9-fold (*P* < 0.01), followed by a slight decline which was nevertheless maintained 1.7-fold above resting levels (*P* < 0.05) (Figure 5-3). Notably, none of the *P. gingivalis* strains tested increased DNA binding activity of FOXO-1 or NRF-2 (Figure 5-3). These data support the initial findings of Chapter 3 and further suggest that FOXO-1 and NRF-2 are not involved in H400 epithelial cell responses to *P. gingivalis*.
Figure 5-3. DNA binding activity of ATF-2, FOXO-1 and NRF-2 transcription factors in response to bacterial stimulation. DNA binding activity of ATF-2, FOXO-1 and NRF-2 at different time points with *P. gingivalis* wild type strains using the H400 oral epithelial cell line. Samples were analysed by TransAM ELISA. An MOI of 100 was used for infections. Data are expressed in relation to un-stimulated resting cells (0, black bar) and are the mean fold change of three biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; *P < 0.05, **P < 0.01.
5.3.3 Epithelial cytokine release in response to *P. gingivalis*

As in Chapter 3, the production of six cytokines/chemokines (IL-1α, IL-1β, IL-6, IL-8, GM-CSF and G-CSF,) was assessed in response to *P. gingivalis* 33277, W50 and W83. Secretion of IL-1α, IL-1β and IL-6 was not altered following infection with *P. gingivalis* 33277, W50 or W83 (Figure 5-4). Notably, IL-8 levels were significantly decreased following challenge with each of the *P. gingivalis* strains (*P* < 0.001), and a similar effect was observed for G-CSF but this did not reach statistical significance (Figure 5-4). In contrast, GM-CSF secretion was significantly induced by *P. gingivalis* 33277 in comparison to *P. gingivalis* W50 (*P* < 0.05) and un-stimulated resting cells (*P* < 0.01) (Figure 5-4). Overall, these results confirm that *P. gingivalis* strains generally do not induce inflammatory cytokines from H400 oral epithelial cells but rather inhibit the release of and/or degrade specific cytokines, namely IL-8 and G-CSF.
**Figure 5-4. Cytokine release by oral epithelial cells in response to bacterial stimulation.**

Secretion of IL-1α, IL-1β, IL-8, IL-6, GM-CSF and G-CSF 24 h post-infection with *P. gingivalis* wild type strains using the H400 oral epithelial cell line. Samples were analysed by Luminex. An MOI of 100 was used for infections. Data are compared with un-stimulated resting cells (vehicle, black bar) and are the mean of eight biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; *P < 0.05, **P < 0.01, ***P < 0.001.
5.3.4 Epithelial damage induced by *P. gingivalis*

LDH activity following infection of H400 cells with *P. gingivalis* 33277, W50 and W83 was significantly lower when compared with un-stimulated resting cells (P < 0.001) (Figure 5-5). Notably, no significant difference in LDH activity was observed between *P. gingivalis* strains.

**Figure 5-5. Damage induction in oral epithelial cells in response to bacterial stimulation.** LDH activity 24 h post-infection with *P. gingivalis* wild type strains using the H400 oral epithelial cell line. Samples were analysed by LDH assay. An MOI of 100 was used for infections. Data are compared with un-stimulated resting cells (vehicle, black bar) and are the mean of three biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; ***P < 0.001.
5.3.5 *P. gingivalis* virulence factors affecting activation of epithelial signalling pathways

5.3.5.1 Activation of the MAPK pathway

Like their respective wild type strains, infection of oral epithelial cells with *P. gingivalis* ΔrgpA/B, ΔserB and ΔPPAD did not activate the p38, JNK or ERK1/2 MAPK pathways (Figure 5-6). Interestingly, infection with the *P. gingivalis* Δkgp mutant significantly induced phosphorylation of p38 (~1.4-fold; *P* < 0.01) and p-JNK (~2.1-fold; *P* < 0.001) but only at 60 min post-infection. p-ERK 1/2 was also induced at the same time point (~1.3-fold) but this did not reach statistical significance. These results suggest that the *P. gingivalis* Kgp is involved in the microbes’ ability to prevent the activation of signalling events in H400 oral epithelial cells.
Figure 5-6. Activation of MAPK signalling pathway components in response to bacterial stimulation. Phosphorylation of p38, JNK and ERK 1/2 at different time points post-infection with *P. gingivalis* mutant strains using the H400 oral epithelial cell line. Samples were analysed by phospho-Luminex. An MOI of 100 was used for infections. Data are expressed in relation to respective parent strains of each mutant (0, black bar) and are the mean fold change of three to four biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; **P < 0.01, ***P < 0.001.
5.3.5.2 Activation of NF-κB and PI3K pathways

Phosphorylation of IκB-α was not significantly induced by *P. gingivalis* ΔrgpA/B, ΔserB or ΔPPAD mutants; however, a non-significant 1.6-fold increase in levels of p-ΙκB-α was observed in response to *P. gingivalis* ΔrgpA/B at 120 min post-infection (Figure 5-7). In contrast, *P. gingivalis* Δkgp significantly induced phosphorylation of IκB-α ~2 - 2.5 fold at 30, 60 and 120 min post-infection (*P* < 0.001) (Figure 5-7). None of the *P. gingivalis* mutant strains induced phosphorylation of AKT (Figure 5-7). These results further support the conclusion that the lysine gingipain plays a role in the ability of *P. gingivalis* to circumvent epithelial cell signalling in the H400 cell line.

![p-IκB-α](image1)

**Figure 5-7. Activation of NF-κB and PI3K / AKT signalling pathways in response to bacterial stimulation.** Phosphorylation of IκB-α and AKT at different time points post-infection with *P. gingivalis* mutant strains using the H400 oral epithelial cell line. Samples were analysed by phospho-Luminex. An MOI of 100 was used for infections. Data are expressed in relation to respective parent strains of each mutant (0, black bar) and are the mean fold change of three to four biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; ***P* < 0.001.
5.3.6 *P. gingivalis* virulence factors affecting activation of epithelial transcription factors

5.3.6.1 ATF-2, FOXO-1 and NRF-2 transcription factor activation

The signal pathway data obtained in this study indicated that *P. gingivalis* Kgp might be important in regulating epithelial cell signalling events. Therefore, subsequent experiments investigating the activation of transcription factors were performed solely with the *P. gingivalis* Δkgp mutant and its wild-type parental control strain W50.

As demonstrated previously (Figure 5-3), *P. gingivalis* W50 transiently activated ATF-2 DNA binding activity, with a significant 1.6-fold increase at 30 min post-infection (*P* < 0.05) (Figure 5-8). Notably, the *P. gingivalis* Δkgp mutant failed to activate ATF-2 binding at 30 min. Neither FOXO-1 nor NRF-2 DNA binding was induced by *P. gingivalis* W50 or the Δkgp mutant, although a non-significant 1.5-fold up-regulation of FOXO-1 DNA binding was observed following stimulation with the *P. gingivalis* Δkgp mutant at 60 min post-infection (Figure 5-8). These results suggest that early activation of ATF-2 in oral epithelial cells might be influenced by *P. gingivalis* Kgp but that FOXO-1 and NRF-2 transcription factors are unaffected.
Figure 5-8. DNA binding activity of ATF-2, FOXO-1 and NRF-2 transcription factors in response to bacterial stimulation. DNA binding activity of ATF-2, FOXO-1 and NRF-2 at different time points post-infection with *P. gingivalis* W50 and *P. gingivalis* Δkgp mutant using the H400 oral epithelial cell line. Samples were analysed by TransAM ELISA. An MOI of 100 was used for infections. Data are expressed in relation to un-stimulated resting cells (0, black bar) and are the mean fold change of three biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; *P < 0.05.
5.3.6.2 Activation of c-Fos and c-Jun AP-1 transcription factors

Activation of the MAPK pathway leads to downstream activation of multiple transcription factors, including the AP-1 associated c-Fos and c-Jun transcription factors (Whitmarsh and Davis 1996). Chapters 3 and 4 identified c-Fos and c-Jun as the main transcription factors regulating H400 oral epithelial responses to A. naeslundii, F. nucleatum and P. gingivalis. Moreover, the p38 MAPK pathway appeared to be the main regulator of pro-inflammatory responses. Given that the P. gingivalis Δkgp mutant significantly activated the p38 and JNK MAPK pathways (Fig 5-6), the downstream activation of c-Fos and c-Jun transcription factors was assessed.

The P. gingivalis Δkgp mutant strain strongly induced DNA binding activity of c-Fos at 60 and 120 min post-infection by ~3.5-fold as compared to un-stimulated resting cells and the P. gingivalis W50 parent strain (P < 0.001) (Figure 5-9). Interestingly, both P. gingivalis W50 and P. gingivalis Δkgp failed to up-regulate c-Jun DNA binding (Figure 5-9). These observations indicate that P. gingivalis Kgp prevents the activation of epithelial signalling pathways that specifically lead to the activation of c-Fos.

Figure 5-9. DNA binding activity of c-Fos and c-Jun transcription factors in response to bacterial stimulation. DNA binding activity of c-Fos and c-Jun at different time points with P. gingivalis W50 and P. gingivalis Δkgp mutant using the H400 oral epithelial cell line. Samples were analysed by TransAM ELISA. An MOI of 100 was used for infections. Data are expressed in relation to un-stimulated resting cells (0, black bar) and are the mean fold change of three biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; ***P < 0.001.
5.3.7 P. gingivalis virulence factors affecting epithelial cytokine release

Secretion of IL-1α was unaffected by P. gingivalis mutant strains ΔrgpA/B, Δkgp, ΔserB and ΔPPAD (Figure 5-10). However, infection with P. gingivalis ΔrgpA/B, Δkgp or ΔserB resulted in a significant decrease in IL-1β and IL-6 secretion. Secreted levels of IL-1β were reduced by 70% following P. gingivalis ΔrgpA/B challenge (P < 0.001), by 60% following P. gingivalis Δkgp challenge (P < 0.01) and by 50% following P. gingivalis ΔserB challenge (P < 0.05) (Figure 5-10). Similarly, IL-6 protein levels were reduced by 60% following challenge with ΔrgpA/B, Δkgp and ΔserB mutants (P < 0.01) (Figure 5-10). In contrast, IL-8, GM-CSF and G-CSF protein levels were significantly increased following infection with P. gingivalis Δkgp. Compared to wild type P. gingivalis W50, secretion of IL-8 was increased 8.8-fold (P < 0.001), GM-CSF 2-fold (P < 0.05) and G-CSF 1.8-fold (P < 0.05) (Figure 5-10). Additionally, challenge with the P. gingivalis ΔrgpA/B mutant also resulted in a non-significant 3.6-fold increase in IL-8 secretion. These results indicate that Rgp, SerB, and notably Kgp can manipulate cytokine secretion from H400 epithelial cells while PPAD has no effect.
Figure 5-10. Cytokine release by oral epithelial cells in response to bacterial stimulation. Secretion of IL-1α, IL-1β, IL-8, IL-6, GM-CSF and G-CSF 24 h post-infection with *P. gingivalis* mutant strains using the H400 oral epithelial cell line. Samples were analysed by Luminex. An MOI of 100 was used for infections. Data are expressed in relation to respective parent strains of each mutant (vehicle, black bar) and are the mean fold change of six to nine biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA; *P < 0.05, **P < 0.01, ***P < 0.001.
5.3.8 *P. gingivalis* virulence factors affecting epithelial damage induction

Detectable LDH levels were significantly reduced following infections with wild type *P. gingivalis* W50 (*P* < 0.001) and W83 (*P* < 0.001), as well as *P. gingivalis* mutant strains ΔrgpA/B (*P* < 0.01), ΔserB (*P* < 0.001) and ΔPPAD (*P* < 0.001) (Figure 5-11). Notably, following *P. gingivalis* Δkgp infections, LDH levels were restored back to baseline and this was significantly different compared to LDH levels detected following *P. gingivalis* W50 challenge (*P* < 0.01) (Figure 5-11).

**Figure 5-11. Damage induction in oral epithelial cells in response to bacterial stimulation.** LDH activity 24 h post-infection with *P. gingivalis* wild type and mutant strains using the H400 epithelial cell line. Samples were analysed by LDH assay. An MOI of 100 was used for infections. Data are compared with unstimulated resting cells (vehicle, black bar) and are the mean of six biological replicates ± SEM. Statistical significance calculated by one-way ANOVA; **P* < 0.01, ***P* < 0.001.
5.3.9 LDH protein degradation by 

Reduced LDH levels in supernatants following 

was previously observed in Chapter 3 (Figure 3-8) and above (Figure 5-5, 5-11). Additionally, incubation of LDH protein with 

resulted in a decrease in detectable LDH, suggesting an ability of 

to degrade LDH proteins (Figure 3-9). To confirm that this effect is attributed to the Kgp gingipain, porcine LDH was incubated with wild type 

and the 

mutant.

Figure 5-12 shows that following incubation with 

, detectable LDH levels return to baseline when compared with LDH levels following incubation with 

(W50) (Figure 5-12). These results demonstrate that the 

is responsible for the degradation of LDH proteins in cell supernatants.

![LDH degradation graph]

**Figure 5-12.** LDH protein degradation (Abolition of LDH activity) following incubation with 

and 

. Quantification of porcine LDH activity 24 h post-incubation with 

and 

mutant. Samples were analysed by LDH assay. PBS (no bacteria) served as a negative control. Data are the mean of two biological replicates ± SEM.
5.4 Discussion

Oral epithelial cell responses to *P. gingivalis* strains 33277, W50 and W83 were comparable, with all three strains failing to significantly activate the MAPK, NF-κB or PI3K pathways. A mild induction of p-JNK was observed following *P. gingivalis* 33277 infections, which was also observed in Chapter 3, indicating that while transient, it is nevertheless a reproducible effect. Downstream up-regulation of ATF-2 was observed in response to all three *P. gingivalis* strains, with W50 being a more potent activator of this transcription factor. Inhibition studies (Chapter 4) did not identify JNK as regulating ATF-2 binding in *P. gingivalis* 33277 infections. This observation is further supported in this Chapter, as while all three *P. gingivalis* strains induced ATF-2 DNA binding, only *P. gingivalis* 33277 up-regulated p-JNK. This suggests that JNK is not acting upstream of ATF-2.

DNA binding of FOXO-1 and NRF-2 was also assessed, and all three *P. gingivalis* strains (33277, W50 and W83) failed to activate these transcription factors. Activation of FOXO-1 by *P. gingivalis* 33277 has been previously shown in primary gingival epithelial cells (MOI 50) (Li, Dong et al. 2013) and in telomerase immortalised gingival epithelial cells (MOI 10) (Wang, Sztukowska et al. 2015). The difference in cell type and MOI used could account for the lack of activation observed in the current study. The NRF-2 transcription factor is a master regulator of antioxidant responses and is up-regulated following oxidative stress (Zhang and Hannink 2003, Jaiswal 2004). NRF-2-induced gene expression increases intracellular glutathione, which is the main antioxidant system utilised by eukaryotic cells and can reduce the effects of cellular ROS (Fernandez-Checa, Garcia-Ruiz et al. 1998). A recent paper has demonstrated a disrupted NRF-2 pathway in neutrophils isolated from periodontitis patients, seen by the lack NRF-2 DNA binding, which was associated with a hyperactive neutrophil phenotype (Dias, Chapple et al. 2013). Additionally, analysis of glutathione levels in gingival crevicular fluid from periodontitis patients demonstrated lower concentrations relative to healthy controls (Chapple, Brock et al. 2002); thus providing a potential mechanism of NRF-2/glutathione disruption in periodontitis. The lack of NRF-2 activation by *P. gingivalis* strains may be surprising given that the epithelial cells are infected and probably stressed. However, *P. gingivalis* can not only inhibit eATP-induced cytosolic and mitochondrial ROS generation in oral epithelial cells, it has also
been shown to increase intracellular glutathione levels, thus further inhibiting oxidative stress (Choi, Spooner et al. 2013). It may, therefore, not be surprising that DNA binding of NRF-2 is not up-regulated following *P. gingivalis* challenge in oral epithelial cells.

The assessment of cytokine release following infection with *P. gingivalis* showed consistent results among the three strains with the exception of GM-CSF levels which were up-regulated by *P. gingivalis* 33277. This result mirrors observations in Chapter 3 where *P. gingivalis* 33277 stimulated a mild increase in GM-CSF, although this was not statistically significant. It is likely that *P. gingivalis* 33277-induced GM-CSF is via the JNK pathway and this is supported by two observations: i) *P. gingivalis* 33277 was the only strain to up-regulate p-JNK and GM-CSF and ii) results in Chapter 4 identified JNK as being the pathway positively regulating GM-CSF secretion in *P. gingivalis* infections. While IL-1α, IL-1β and IL-6 levels were unaltered in *P. gingivalis* infections, the downregulation of IL-8 and G-CSF is likely due to degradation by gingipains, which was observed following infection with *P. gingivalis* strains 33277, W50 and W83. In summary, *P. gingivalis* strain diversity in H400 epithelial cell responses has a minor effect, with *P. gingivalis* 33277 inducing a slight pro-inflammatory phenotype while W50 and W83 were both non-stimulatory.

The data presented here are in agreement with several studies that have investigated *P. gingivalis* strain diversity and variability in periodontitis. An early study examined the distribution of *P. gingivalis* strains in periodontitis and healthy patients and found that *P. gingivalis* W83 was clearly associated with periodontitis whereas *P. gingivalis* 381 (closely related to 33277) was not (Griffen, Lyons et al. 1999). Genome comparison studies later demonstrated that *P. gingivalis* clinical strains had similar open reading frames (ORFs) to W83 (and W50), whereas 5.1% of the ORFs (102 genes) were modified in *P. gingivalis* 33277 (Igboin, Griffen et al. 2009), which may account for this discrepancy. Animal studies have also confirmed clinical observations and demonstrated differences in pathogenic potential between *P. gingivalis* strains (Laine and van Winkelhoff 1998, Marchesan, Morelli et al. 2012). Thus, the results in this chapter support previous studies showing a close correlation of epithelial responses in response to *P. gingivalis* W50 and W83, with responses to *P. gingivalis* 33277 being
more divergent. Likewise, the previous classification of W50 and W83 as being virulent strains, with 33277 being avirulent, may also be supported as increased virulence could be attributed to total evasion of epithelial cell activation by the two strains (W50 and W83).

Following the characterisation of epithelial cell responses to *P. gingivalis* strains 33277, W50 and W83, the possible involvement of Arg-gingipains (RgpA/B), Lys-gingipain (Kgp), SerB and PPAD in *P. gingivalis*’s ability to prevent epithelial cell activation was assessed. Of the four mutants tested, infection of the H400 oral epithelial cell line with *P. gingivalis* Δkgp resulted in activation of the p38 and JNK MAPK pathways, as well as the NF-κB pathway. This is the first report suggesting that the lysine gingipain of *P. gingivalis* plays a role in limiting activation of the MAPK and NF-κB pathways in gingival epithelial cells. The mechanism(s) by which Kgp prevents epithelial cell activation by *P. gingivalis* remain unknown, although several possibilities can be speculated. The observed up-regulation of p-p38, p-JNK and p-IκB-α suggests that Kgp is acting upstream of these molecules, possibly dysregulating kinases or receptors involved in bacterial recognition. Gingipain-dependent degradation of total and p-Akt, as well as mTOR has previously been documented in immortalised human oral keratinocytes (OKF6/Tert2) cells; however, these pathways are not to act upstream of p38, JNK or NF-κB. Moreover, we observed no changes in p-Akt levels following *P. gingivalis* infection, suggesting that this is not a mechanism by which Kgp is preventing epithelial cell activation. A study by Madrigal *et al.* has recently demonstrated that *P. gingivalis* infection of human aortic endothelial cells results in the cleavage of the RIP kinase 1 protein. Moreover, it was also demonstrated that the proteolysis of RIP 1 was induced by *P. gingivalis* Kgp (Madrigal, Barth *et al.* 2012). To investigate whether a similar mechanism exists in oral epithelial cells, total RIP 1 protein levels were assessed following incubation with *P. gingivalis* W50 or *P. gingivalis* Δkgp; however, no difference was observed between the parent and mutant strain (data not shown). Degradation of other intracellular kinases by *P. gingivalis* Kgp has not been reported but given the multitude of gingipain substrates, it is likely that more will be discovered. The proteolytic activities of gingipains have also been investigated for their ability to cleave innate immune receptors. Bacterial LPS is recognised by a receptor complex composed of TLR4 and CD14 (Fitzgerald, Rowe *et al.* 2004). Gingipain-dependent
degradation of the co-receptor CD14 but not TLR4 has been reported in human monocytes (Sugawara, Nemoto et al. 2000), gingival fibroblasts (Tada, Sugawara et al. 2002) and mouse macrophages (Wilensky, Tzach-Nahman et al. 2015), with CD14 expression being more efficiently reduced in murine macrophages by Lys-gingipain (Wilensky, Tzach-Nahman et al. 2015). The reduction in CD14 expression was shown to inhibit LPS-induced TNF-α production from human monocytes (Sugawara, Nemoto et al. 2000) and correlated with a reduced macrophage response to \textit{P. gingivalis} (Wilensky, Tzach-Nahman et al. 2015). Whether or not this mechanism applies to gingival epithelial cells is unknown. Assessing the expression of CD14 on oral epithelial cells following infection with wild-type \textit{P. gingivalis} and gingipain mutants would elucidate this. While gingipains clearly have an effect on host immunomodulatory molecules, it is also reasonable to suspect that they can have effects on bacterial cell surface molecules. For example, Arg-gingipains are indispensable for the maturation of bacterial fimbriae (Guo, Nguyen et al. 2010, Kristoffersen, Solli et al. 2015). Chinese hamster ovary (CHO) cells lacking both TLR2 and TLR4 were activated by gingipain-deficient \textit{P. gingivalis} but not by wild-type \textit{P. gingivalis} (Kishimoto, Yoshimura et al. 2006). The reported unresponsiveness of CHO cells to the wild-type strain was due to the inactivation of an unidentified cell surface component of \textit{P. gingivalis} by gingipains rather than the degradation of a receptor expressed on CHO cells. Furthermore, it was demonstrated that a Δkgp mutant induced higher levels of NF-κB-induced CD25 expression when compared to a Δrgp mutant (Kishimoto, Yoshimura et al. 2006). This observation suggests an interesting mechanism where \textit{P. gingivalis} gingipains inactivate its own cell surface components capable of inducing intracellular pathway activation.

Investigation of the effect of Kgp on activation of epithelial transcription factors showed differences between the wild-type and gingipain mutant in the induction of ATF-2 and c-Fos. The mild up-regulation of FOXO-1 by the Δkgp mutant is unlikely to be biologically relevant given the high SEM between replicates. \textit{P. gingivalis} W50 transiently activated ATF-2 DNA binding whereas \textit{P. gingivalis} Δkgp did not. Chapter 4 identified the p38 MAPK pathway as a possible negative regulator of ATF-2 DNA binding. Given that the parent \textit{P. gingivalis} W50 strain did not activate p38 but the Δkgp mutant did, it is likely that the loss of ATF-2 activation during \textit{P. gingivalis} Δkgp
infection is driven by the activation of the p38 MAPK pathway. Significant activation of c-Fos was observed following *P. gingivalis Δkgp* challenge but not *P. gingivalis W50*. This is the first report suggesting a role for *P. gingivalis* Kgp in preventing up-regulation of c-Fos DNA binding. This observation correlates with the kinetics of activation previously described in Chapter 4. The p38 MAPK pathway, but not JNK, was shown to positively regulate c-Fos DNA binding in oral epithelial cells. Therefore, it is likely that *P. gingivalis Δkgp*-induced c-Fos levels are driven by p38 activation. This is an interesting finding that highlights a novel immune subversion mechanism by *P. gingivalis* in oral epithelial cells, driven by its Lys-gingipain. c-Fos, which is part of the AP-1 complex, could be important in initiating immune responses that promote clearance of the infection. The function of AP-1 as a regulator of inflammatory responses has been well documented (Angel and Karin 1991, Zenz, Eferl et al. 2008), and in oral epithelial cell responses to *C. albicans*, knockdown of c-Fos resulted in significant reduction in pro-inflammatory cytokine secretion (Moyes, Runglall et al. 2010). By preventing c-Fos activation, *P. gingivalis* may promote its survival and persistence in oral epithelial cells.

Secretion of cytokines was also assessed following infection with *P. gingivalis ΔrgpA/B, Δkgp, ΔserB* and ΔPPAD. IL-1α was the only cytokine to be unaffected by all four mutants, which was not unexpected since IL-1α is usually damage-associated and *P. gingivalis* did not damage H400 epithelial cells. Interestingly, IL-1β and IL-6 levels were decreased following infection with *P. gingivalis ΔrgpA/B, Δkgp* and ΔserB. This could suggest that RgpA/B, Kgp and SerB induce the release of these cytokines; however, this was not observed following infection with wild-type *P. gingivalis*. The induction of cytokines by *P. gingivalis* gingipains is probably mediated via cleavage of protease-activated receptors (PARs). PARs are G protein-coupled receptors, uniquely activated by proteolysis, and consist of four described members: PAR1-4 (Soh, Dores et al. 2010). *P. gingivalis* gingipains can cleave and activate PARs expressed on neutrophils (Lourbakos, Chini et al. 1998) and activation of PAR2 has been associated with inflammation and alveolar bone loss in periodontitis (Holzhausen, Spolidorio et al. 2005, Holzhausen, Spolidorio et al. 2006). In oral epithelial cells, both *P. gingivalis* Rgp and Kgp have been shown to cleave PAR1 and PAR2, resulting in release of cytokines, including IL-1β and IL-6 (Lourbakos, Potempa et al. 2001, Giacaman, Asrani et al. 2009).
Reduction of IL-1β and IL-6 following infection with P. gingivalis ΔserB is unexpected, as this serine phosphatase has not been shown to induce cytokine release. It is possible that in the absence of this P. gingivalis virulence factor, epithelial cells may be subjected to a comparative reduction in cellular stress, which may possibly account for the decrease in cytokine secretion observed.

Contrary to IL-1β and IL-6, IL-8, GM-CSF and G-CSF levels were increased following infection with P. gingivalis ΔrgpA/B and Δkgp, suggesting that RgpA/B and Kgp are preventing expression/secretion or are degrading these cytokines. The ability of P. gingivalis gingipains to degrade host cell proteins, particularly the cytokine network, has been well documented (Guo, Nguyen et al. 2010, How, Song et al. 2016). Thus, with regard to the results of this thesis, it is likely that in the H400 gingival epithelial cell line, P. gingivalis Kgp can degrade GM-CSF and G-CSF, and both P. gingivalis Kgp and Rgp can degrade IL-8. Interestingly, Kgp-induced degradation of GM-CSF is observed in P. gingivalis W50 infections, whereas P. gingivalis 33277 induced secretion of GM-CSF. This observation suggests that there may be a difference in the Lys-tingipain between the two wild-type strains. The kgp gene codes for a protein composed of five domains, of which a variable region exists – Kgp44 (Nadkarni, Nguyen et al. 2004). The Kgp proteins of P. gingivalis W50 and W83 are identical in sequence but differ from the Kgp of P. gingivalis 381 (same kgp biotype as 33277) in the Kgp44 region (Nadkarni, Nguyen et al. 2004). This difference may be relevant to the proteolytic capacity of Kgp and virulence of P. gingivalis strains. Lastly, epithelial damage was assessed and LDH activity was rescued back to resting cell levels following infection with the P. gingivalis Δkgp mutant. This was due to the Lys-tingipain degrading LDH in cell supernatant. This novel finding identifies LDH as an additional Kgp substrate.

In summary, the work in this chapter has identified the lysine gingipain (Kgp) of P. gingivalis as playing a role in the limitation of inflammatory responses in H400 oral epithelial cells.
Chapter 6: General discussion

Periodontal disease, one of the most prevalent diseases in the world, develops as a consequence of the interaction of environmental, genetic, host and microbial factors. Gingivitis, the superficial, reversible form of periodontal disease, manifests with a resolving inflammation limited to the gingiva. In disease-susceptible individuals, gingivitis can progress to periodontitis, which is a chronic inflammatory disease, defined by the destruction of connective tissue as well as alveolar bone, ultimately leading to tooth loss. The destruction of periodontal tissues is widely accepted as being a result of the shift from protective immune inflammatory responses to destructive immune mechanisms against periodontal microorganisms. While the host response has been traditionally thought to be mediated by lymphocytes, monocytes/macrophages and neutrophils, increasing evidence has highlighted the important contribution of resident epithelial and PDL cells to the inflammatory response. The epithelial compartment not only provides a physical barrier to infection, but also plays an active role in innate immune defense. Gingival epithelial cells are in constant contact with a biologically complex biofilm consisting of both commensal and pathogenic bacterial species. Through this interaction, epithelial cell responses can impact on local homeostasis as well as the recruitment of specialised immune cells. Therefore, understanding how gingival epithelial cells respond to oral bacteria of varying pathogenic potential has been the focus of this thesis.

6.1 Epithelial cell inflammatory responses differ across dental plaque bacterial species

As the first line of defense between the host and the outside environment, gingival epithelial cells utilise a number of PRRs in the activation of signal transduction pathways leading to the production of innate immune responses (Chung 2015). TLRs, NLRs and PARs have all been implicated in the recognition of and response to oral bacteria (Sugawara, Uehara et al. 2006, Chung, An et al. 2010) and ligation of these PRRs results in activation of intracellular signalling pathways. The activation of intracellular signalling was investigated in H400 epithelial cells in response to commensal A. naeslundii, gingivitis-associated F. nucleatum and periodontitis-associated P. gingivalis. A. naeslundii activated the NF-κB pathway, mildly up-regulated
JNK activation and c-Fos DNA binding, and induced secretion of IL-6, GM-CSF and G-CSF. It is now recognised that the host immune response is active in healthy periodontal tissues and that in the absence of excellent oral hygiene inflammation is the natural state (Graves, Li et al. 2011). It is therefore expected that a commensal oral coloniser such as A. naeslundii would induce an inflammatory response, albeit a mild one. If the inflammatory state induced by A. naeslundii is considered ‘appropriate’ for health in this system, then epithelial responses to F. nucleatum present an exacerbated inflammatory response. F. nucleatum strongly activated the three MAPK pathways p38, JNK and ERK, the NF-κB pathway, up-regulated c-Fos and c-Jun DNA binding and stimulated secretion of IL-1α, IL-1β, GM-CSF and G-CSF. This increased inflammatory state would correlate with symptoms of clinical gingivitis: redness, swelling and significant inflammatory infiltrate (Theilade, Wright et al. 1966, Kinane 2001). In individuals where this inflammation persists, the destructive process may expand, resulting in loss of periodontal ligament and alveolar bone. This might suggest that inflammatory responses to periodontal pathogens, such as P. gingivalis, should be even greater than those induced by F. nucleatum. Interestingly, P. gingivalis was the least stimulatory bacterium, inducing mild JNK activation, transient ATF-2 DNA binding and no pro-inflammatory cytokine secretion but significant IL-8 down-regulation. The ability of P. gingivalis to dampen and evade immune responses not only benefits its own survival and persistence, but also of other opportunistic pathogens/accessory pathogens such as F. nucleatum, which are more effective in inducing inflammation. For this reason, P. gingivalis has been described as a keystone pathogen, able to promote the dysbiotic growth of the oral biofilm, the hallmark of periodontitis pathogenesis (Hajishengallis, Darveau et al. 2012, Hajishengallis and Lamont 2012).

The present study provides evidence that oral epithelial cells respond differently to different dental plaque bacteria. The novelty in this work lies in the thorough screening of intracellular signalling events, particularly transcription factors, and the identification of previously unreported epithelial cell responses to live A. naeslundii, F. nucleatum and P. gingivalis. Although the work presented was done in vitro, using a carcinoma cell line, it provides a robust and reproducible model for the investigation of such responses. The use of primary gingival epithelial cells was not feasible for this project given the length of time required to grow and culture them. Now, following the
identification of key cellular responses to these bacteria, experiments should be carried out in primary cells for confirmation. This is particularly important for *P. gingivalis*, as this bacterium has been shown to elicit varying responses dependent on cell type (Yee, Kim et al. 2014). Likewise, conclusions on cell responses should not be based on studies with a single strain. While Chapter 5 of this study investigated H400 epithelial cell responses to three *P. gingivalis* wild-type strains, the same approach should be taken with *A. naeslundii* and *F. nucleatum*, as strain diversity has also been reported between *F. nucleatum* strains (Elerson Gaetti-Jardim Júnior 2000, Bhattacharyya, Ghosh et al. 2016).

Further studies should also expand on the knowledge gained from epithelial cell monolayers and investigate interactions between epithelial cells and the underlying connective tissues during bacterial challenge. Using a 3-D mucosal model would help gain a more comprehensive view of the regulation of local immune mechanisms as well as have the potential for bridging the gap between cell-based research and *in vivo* research (Barrila, Radtke et al. 2010). Another interesting investigation would be that of polymicrobial infections. Gingival epithelial cells *in vivo* are in contact and become infected with multiple bacterial species. Having thoroughly characterised epithelial cell responses to *A. naeslundii*, *F. nucleatum* and *P. gingivalis* individually, it would be biologically relevant to investigate responses to combinations of two or all three of these bacteria. For example, *F. nucleatum* and *P. gingivalis* are often found together at sites of infection; what would the overall response be when epithelial cells are infected with the immune evasive bacterium *P. gingivalis* and the highly stimulatory *F. nucleatum*? Further studies involving polymicrobial infections and mucosal 3-D cell-culture models will lead to a better understanding of the pathogenic process of mixed infections such as periodontitis.

6.2 Epithelial intracellular signalling pathways differentially regulate pro-inflammatory responses

The interaction of oral bacteria with gingival epithelial cells is mediated by PRR recognition, resulting in activation of intracellular pathways and secretion of cytokines/chemokines. The production of these effector responses is the intermediate mechanism that lies between bacterial stimulation and tissue destruction in
periodontitis. The bacteria-stimulated local inflammatory reaction leads to the recruitment of phagocytes and lymphocytes. In turn, activated lymphocytes also secrete pro-inflammatory cytokines and chemokines, leading to the amplification of the initial local host response, which ultimately leads to the destruction of periodontal tissues (Di Benedetto, Gigante et al. 2013). Therefore, the activation of intracellular signalling in resident periodontal cells, including epithelial cells, is key in establishing complex cytokine and signalling networks involved in loss of connective tissues and alveolar bone.

All cytokines investigated in this study (IL-1α, IL-1β, IL-6, IL-8, GM-CSF and G-CSF) are involved in the pathogenesis of periodontitis. The prominent roles of IL-1 and IL-6 in bone resorption have been extensively reviewed (Graves 2008, Yucel-Lindberg and Bage 2013), IL-8 is a powerful neutrophil chemoattractant (Bickel 1993) and GM-CSF and G-CSF play central roles in the local activation, recruitment and survival of macrophage lineage cells (Hamilton 2002, Lam, O'Brien-Simpson et al. 2014). The signalling pathways controlling secretion of these cytokines from H400 oral epithelial cells were identified and the p38 MAPK pathway was found to be the major regulator. Identifying signalling pathways that trigger the release of pro-inflammatory cytokines, such as p38, is important not only for understanding how inflammation is initiated but also for identifying potential therapeutic targets. The implication of the p38 MAPK pathway in human periodontal inflammation and severity has been described (Travan, Li et al. 2013) and its inhibition as a possible target in the treatment of periodontal disease has been discussed (Kirkwood and Rossa 2009). Targeting pathways that regulate the expression of multiple inflammatory mediators would be more effective than targeting a single cytokine, given the possibility of compensation by other pro-inflammatory cytokines. However, a potential downside of inhibiting signalling pathways is that they elicit diverse effects depending on the nature of the stimulation, duration, intensity and cell type.

A further limitation arose in the use of chemical inhibitors for the assessment of cytokine release following *P. gingivalis* infections; this was because *P. gingivalis* had no stimulatory effect of cytokine production in H400 cells. Figure 6-1 shows secretion of IL-8 from H400 oral epithelial cells in response to *P. gingivalis*, following pathway
inhibit. The data, extrapolated from Figure 4-6, does not show a statistically significant difference between *P. gingivalis* stimulated cells, with or without pathway inhibition (Figure 6-1). Because *P. gingivalis* did not induce IL-8 secretion, the use of pathway inhibitors on their own does that not answer the question of whether *P. gingivalis* is able to actually suppress the inflammatory response. Further experiments should include an alternative agonist known to cause a significant increase in secretion of IL-8 (as well as the other cytokines investigated).

![Graph](image)

**Figure 6-1. IL-8 release by oral epithelial cells in response to *P. gingivalis* following inhibition of JNK, ERK and p38 MAPK pathways and NF-κB pathway.** Secretion of IL-8, 24 h post-infection with *P. gingivalis* using the H400 oral epithelial cell line. Samples were analysed by Luminex. An MOI of 100 was used for infections. Data are compared with *P. gingivalis* stimulated cells without signal pathway inhibition (PG only, blue bar) and are the mean of three biological replicates ± SEM. Statistical significance was calculated by one-way ANOVA and no statistical significance was found.

Additionally to identifying the regulation of cytokine secretion by intracellular pathways, this study also investigated the regulation of c-Fos, c-Jun and ATF-2 transcription factors by the MAPK pathways. The p38 pathway, which was found to be the major regulator of cytokine release, also regulated c-Fos and ATF-2 DNA binding. The second major pathway in regulating cytokine release was the JNK pathway, which was found to regulate c-Fos and c-Jun DNA binding. All three of these transcription factors are part of the AP-1 complex, which is recognised as a crucial modulator of inflammation (Eferl and Wagner 2003, Zenz, Eferl et al. 2008). Future studies should...
focus on identifying gene targets of these transcription factors. Through siRNA knockdown or chemical inhibition of c-Fos, c-Jun and ATF-2, the pro-inflammatory cytokine regulation by these transcription factors can be assessed. Activation of c-Fos has been shown to be crucial in the induction of pro-inflammatory mediators in oral epithelial cells following infection with *C. albicans* (Moyes, Runglall et al. 2010). If c-Fos is found to play the same important role in epithelial cell infections with oral bacteria, this transcription factor could become a target for immunotherapy, offering a more specific control of pro-inflammatory cytokine release. Equally important would be a detailed investigation of the NF-κB pathway, including all transcription factors, of both canonical and non-canonical activation. More so than the AP-1 complex, NF-κB has been considered the archetypal pro-inflammatory pathway (Lawrence 2009). This study has identified cytokine targets of the NF-κB pathway but has not investigated the precise regulation of inflammatory mediators by NF-κB transcription factors. Further studies isolating transcription factors involved in the pathogenesis of periodontitis could provide increased therapeutic selectivity.

6.3 The *P. gingivalis* Lys-gingipain (Kgp) is involved in the inhibition of epithelial cell signalling

*P. gingivalis* has long been implicated in periodontal disease and has become the best described keystone-pathogen in periodontitis. Despite usually being a quantitatively minor constituent of periodontitis-associated biofilms (Kumar, Leys et al. 2006, Abusleme, Dupuy et al. 2013), in a mouse model of periodontitis, *P. gingivalis* was shown to induce significant alterations in the commensal microbiota leading to inflammatory bone loss but remarkably, was unable to do so in germ-free mice, despite colonisation (Hajishengallis, Liang et al. 2011). While it remains uncertain if *P. gingivalis* acts as a keystone pathogen in human periodontitis, evidence from non-human primates strongly supports this hypothesis (Page, Lantz et al. 2007).

Harbouring an array of virulence factors, *P. gingivalis*’s ability to orchestrate inflammatory responses is undisputed. While mechanisms of immune subversion in leukocytes has been reviewed (Zenobia and Hajishengallis 2015), immune subversion in oral epithelial cells by *P. gingivalis* is less clear. Following the observation in Chapter 3 of this study that *P. gingivalis* is a poor stimulant of oral epithelial cells, the possible
involvement of some of its main virulence factors in the suppression of epithelial cell responses was investigated. This resulted in a novel and exciting finding, demonstrating that *P. gingivalis* Kgp plays a role in suppressing activation of the p38 and JNK MAPK pathways, the NF-κB pathway and c-Fos DNA binding. Interestingly, the inflammatory response induced by the non-Kgp-producing *P. gingivalis Δkgp* mutant strongly overlaps that of *A. naeslundii* but less so that of *F. nucleatum*. If in this context *A. naeslundii* and *F. nucleatum*-induced epithelial responses are considered representative of health and gingivitis, then in the spectrum of periodontal disease, the *P. gingivalis Δkgp*-induced epithelial cell phenotype might be more health associated. In this regard, strategies aimed as ‘disarming’ *P. gingivalis* of Kgp may offer promising novel options for treatment of periodontitis.

Further studies should focus on the elucidation of mechanisms by which *P. gingivalis* Kgp is suppressing oral epithelial cell activation. A first step would be to identify the possible epithelial cell surface receptors that activate the observed pathways and see if Kgp can degrade or interfere with their expression. Given that Kgp is targeting intracellular pathways, it would be interesting to conduct expression profiling experiments, such as RNA-Seq, followed by gene set enrichment. This would identify significantly enriched or depleted sets of genes in oral epithelial cells infected with *P. gingivalis* W50 or *P. gingivalis Δkgp*. These results would then be compared to identify differences between the two infections that could be associated with specific pathways. A limitation to this technique is that it would not identify Kgp degraded proteins. To address this limitation, further analysis should be carried out using mass spectrometry to identify differences in proteins expressed during *P. gingivalis* W50 and *P. gingivalis Δkgp* infection of oral epithelial cells. Moreover, the same approach could be used to investigate if Kgp is inactivating *P. gingivalis* cell surface components capable of inducing oral epithelial cell signalling. Lastly, given that *P. gingivalis* 33277 induced a slightly different response in oral epithelial cells compared with W50 and that the Kgp protein of *P. gingivalis* W50 differs from that of 33277, it would be interesting to also include a comparison between *P. gingivalis* 33277 Δkgp and *P. gingivalis* W50 Δkgp.
6.4 Conclusion

Continued research is providing many advances in the understanding of complex, interrelated biological responses involved in maintaining health or promoting disease in periodontal tissues. It is evident that the oral epithelium has an active role in responding to bacterial colonisation and mounting an effective innate immune response. This study has demonstrated that oral epithelial cells respond differently to dental plaque bacterial species and has identified novel signalling pathways and transcription factors activated in response to \textit{A. naeslundii}, \textit{F. nucleatum} and \textit{P. gingivalis}. The association of these three bacterial species with different disease states offers a basal comparison of the dynamics of oral epithelial cell responses during periodontitis pathogenesis. Nevertheless, oral epithelial cells are seldom colonised by single bacterial species, thus it remains difficult to fully understand these complex host-pathogen interactions in the oral mucosa. The identification of a \textit{P. gingivalis} virulence factor involved in the suppression of oral epithelial cell responses opens an avenue of research in the understanding of this bacterium’s immune evasion capabilities. Distinguishing key signalling pathways and bacterial virulence factors involved in periodontal disease progression could lead to the development of novel therapeutics aimed at controlling the destructive inflammatory process leading to periodontitis.
References


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Appendix A: Published papers