A study of the invasion and the cellular response of an *in vitro* 3D oral mucosal model by *Porphyromonas gingivalis*

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Abstract

Periodontitis is a leading cause of tooth loss worldwide. The Gram-negative anaerobe, *Porphyromonas gingivalis*, has been implicated in the initiation and cyclical progression of this inflammatory disease, which may be associated with its ability to invade oral epithelial cells. The majority of studies investigating *P. gingivalis* invasion have utilised monolayer cultures of epithelial cells. However, these do not represent the oral mucosa due to the lack of a multi-layered epithelium and fibroblast-embedded connective tissue. Therefore, a fibroblast-containing, connective-tissue collagen scaffold was used to create three-dimensional oral mucosal models (OMM). These were constructed using oral fibroblasts and either the oral keratinocyte cell line (H357) or normal oral keratinocytes (NOK) isolated from healthy patients. OMM were raised to the air-to-liquid interface allowing keratinocyte stratification and differentiation (gingival/buccal OMM) or completely submerged resulting in epithelium consisting of 2-3 cell layers (junctional epithelial OMM). Both models resembled normal oral tissue in terms of immunohistochemical staining for several cytokeratin markers, laminin 5 and E-cadherin.

A standard antibiotic protection assay was optimised for OMM and percentage invasion was shown to be similar to that of monolayer cultures. The optimal method was an incubation period of 3-6 hours of OMM with *P. gingivalis* in an aerobic atmosphere and release of intracellular *P. gingivalis* by homogenisation. Using these optimised conditions, a range of parameters of *P. gingivalis* invasion were investigated.

At diseased periodontal sites there is an increase in the level of haemin and pocket temperature due to inflammation. The culture of *P. gingivalis* in both a haemin-rich and high temperature environment resulted in an increase in invasion, suggesting that active periodontal sites may preferentially support bacterial internalisation. Additionally, it was shown that following invasion, *P. gingivalis* can leave epithelial cells after as little as three hours, which may contribute to the periods of progression and remission commonly observed with this disease. Furthermore, the concentration of environmental haemin has previously been shown to influence the expression of *P. gingivalis* gingipains and it was thought that this may also influence invasion. Indeed, percentage invasion was shown to increase with loss of gingipain activity, particularly Arg-gingipain. This suggested that the degradation of epithelial cell receptors by gingipains may contribute to a decrease in the ability of this bacterium to invade. Candidate host receptors were the complement receptor CD46, tetraspanin family members and the integrin α5β1. These receptors were blocked using antibodies or cells transfected with siRNA to inhibit their function. A small effect on invasion was seen using anti-α5β1 but the

antibodies to other molecules did not influence the invasion of *P. gingivalis* suggesting that there may be some redundancy in the uptake system exploited by the bacteria.

Finally, the response of epithelial cells to invasion by *P. gingivalis* in terms of cytokine release and expression was determined. Using a semi-quantitative cytokine array, there was a decrease in the majority of cytokines tested in the presence of *P. gingivalis* when compared with TNFα-stimulated control cells which was assumed to be due to the proteolytic action of *P. gingivalis* gingipains. Due to the conflicting nature of the literature regarding the modulation of CXCL8 by *P. gingivalis*, this chemokine was selected for further quantification using monolayer cultures. ELISA and quantitative PCR indicated that, in the presence of *P. gingivalis*, CXCL8 protein concentration decreased in a gingipain-dependent manner, whereas mRNA expression of CXCL8 increased following stimulation by *P. gingivalis*, suggesting post-transcriptional and/or post-translational modification of CXCL8 by gingipains. No change in protein concentration or mRNA expression was observed following stimulation of OMM which may reflect the multi-layered nature of this model. Differences between monolayer and OMM indicate a role for OMM to investigate bacterial invasion and resultant cytokine release due to its comparability with the oral mucosa.

The work presented in this thesis has described the development, characterisation and optimisation of OMM to investigate invasion by *P. gingivalis*. Invasion was shown to be influenced by environmental changes and *P. gingivalis* protease expression. Although *P. gingivalis* degrades key surface molecules including CD46, tetraspanins and α5β1, blocking experiments with antibodies could not explain the protease-dependent effects on invasion. Modulation of cytokine production, particularly CXCL8, by *P. gingivalis* gingipains, may contribute to a disruption in leukocyte recruitment resulting in a dysregulated inflammatory response. Future development of OMM in terms of including an immune cell element and endothelial component to extend the study of *P. gingivalis*-host cell interactions will add value to this model. The data presented here indicate that *P. gingivalis* invasion of the epithelium is likely to be an important contributor to periodontal disease progression.

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Abbreviations

®	Trade mark
α	Alpha
β	Beta
γ	Gamma
μ	Micro
APS	Anionic polysaccharide
Arg	Arginine
ATCC	American Type Culture Collection
B_2M	Beta-2-Microglobulin
BApNA	*N*-α-benzoyl-L-Arg-*p*-nitroanilide
BHI	Brain heart infusion
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CD	Cluster of Differentiation
cDNA	complementary DNA
CFU	Colony forming units
CK	Cytokeratin
C_T	Cycle Threshold
DAPI	4',6-diamidino-2-phenylindole, dilactate
DED	De-epidermalised dermis
DMEM	Dulbecco's Modified Eagles Medium
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
ERK	Extracellular signal regulated kinase
ELISA	Enzyme-Linked Immunosorbent Assay
FA	Fastidious anaerobe
FACs	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluorescein-isothiocyanate
GEC	Gingival epithelial cells
H_2SO_4	Sulphuric Acid
H&E	Haematoxylin and eosin
HA	Haemagglutinin/Adhesin
Ham's F-12	Nutient Mixture F-12
HBSS	Hanks Balanced Salt Solution
HCl	Hydrocloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
i3T3	irradiated murine 3T3 fibroblasts
Ig	Immunoglobulin
IL	Interleukin
JNK	c-Jun N-terminal kinase
kDa	Kilo Dalton
Kgp	Lysine-specific proteinase activity

LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
Lys	Lysine
M	Molar
MAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCP	Monocyte Chemoattractant Protein
MEK	MAPK/ERK kinase
Mg^{2+}	Magnesium ion
MIP-1α	Macrophage Inflammatory Protein-1α
MMP	Matrix metalloprotease
MOI	Multiplicity of Infection
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium Chloride
NCTC`	National Collection of Type Cultures
NOK	Normal Oral Keratinocytes
OD	Optical Density
OMM	Oral Mucosal Model
OPG	Osteoprotegerin
PAR	Protease Activated Receptor
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide 3-Kinase
PMN	Polymorphonuclear
qPCR	Quantitative PCR
RANKL	Receptor activator of nuclear factor-kappa B ligand
RANTES	Regulated upon Activation, Normal T cell Expressed and Secreted
Rgp	Lysine-specific proteinases activity
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT	Reverse Transcription
SFM	Serum Free Medium
TIMP	Tissue inhibitor of MMP
TNF	Tumour Necrosis Factor
Tosyl-Gly-Pro-Lys*p*NA	Toluenesulfonyl-glycyl-L-prolyl-L-lysine *p*-nitroanilide
v/v	Volume per volume
w/v	Weight per volume

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Appendices

Chapter 1 Introduction

1.1 Structure of the oral mucosa

The tissue that lines the oral cavity is known as the oral mucosa. This mucosa consists primarily of three layers: the epithelium, basement membrane and connective tissue (fig 1.1). The epithelial layer provides a relatively impermeable barrier, protecting underlying tissues. The epithelium and connective tissue are separated by the basement membrane which aids in the attachment of these two layers. The connective tissue provides structural support and a matrix within which cells such as fibroblasts and immune cells reside (fig 1.1).

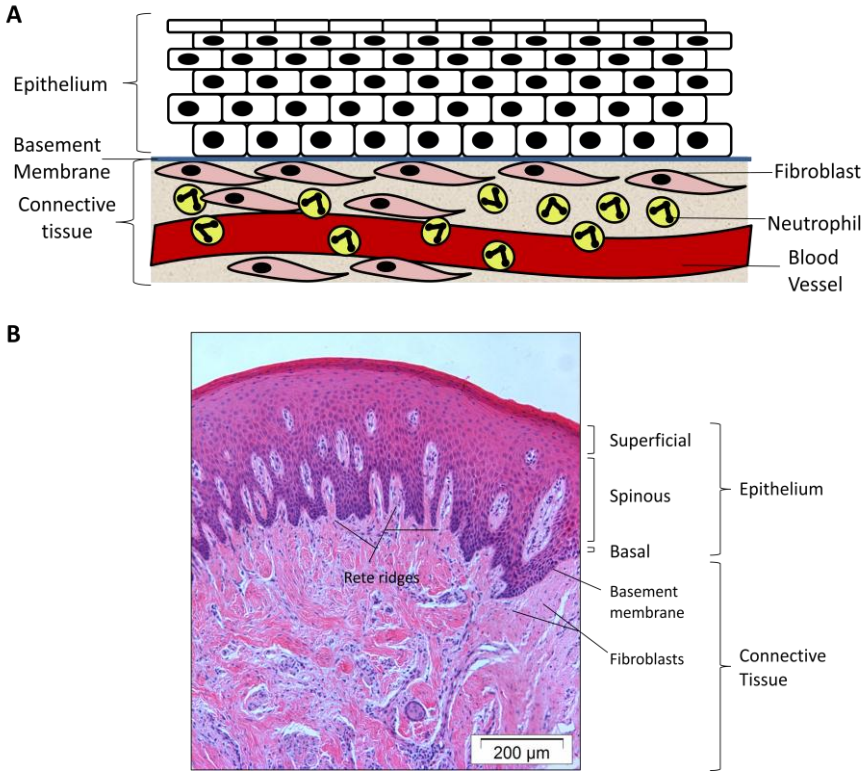

Figure 1.1 Structure of oral mucosa. The oral mucosa is separated into three distinct layers: the epithelium, basement membrane and connective tissue. The epithelium may be differentiated or non-differentiated, keratinised or non-keratinised, depending on its location within the oral cavity. The three layers within the epithelium are designated basal, spinous and superficial. The basement membrane connects the epithelial layer with the connective tissue. Cells such as fibroblasts, neutrophils and blood vessels, held together within an extracellular matrix, form the connective tissue layer. A diagrammatical representation (A) and a haematoxylin and eosin stained section (B) of oral mucosa indicate the location of major structures.

1.1.1 Epithelium

The major cells within the epithelium are epithelial cells, and together these form a dense structure joined by cell-cell junctions (section 1.1.1.2) and cell adhesion receptors, including integrins (section 1.1.1.3). Oral epithelium is a stratified, squamous epithelium, which may or may not be keratinised. There are three distinct layers within the epithelium. These are known as the basal layer, spinous layers (prickle cell layers) and superficial layers (fig 1.1). The basal

layer is a single layer of cuboidal epithelial cells. Mitosis occurs at the basal layer and following each cell division the cells undergo maturational changes resulting in cell detachment and renewing of the epithelial surface. The spinous layers can be distinguished by the presence of several layers of spiky cells following histological fixation, and are located between the basal and superficial layers (fig 1.1). The superficial layers of oral epithelium differ in appearance depending on their location within the oral cavity. These layers may or may not be keratinised. Keratinisation is the result of keratohyaline granules being deposited within the cells resulting in an impermeable layer (orthokeratinised epithelia, e.g. gingival mucosa). The nuclei in non-keratinised epithelia (e.g. buccal mucosa) become enlarged and intracellular vacuoles form (Moss-Salentijn and Hendricks-Klyvert, 1990). The cytokeratin expression within the epithelia of mucosal tissues can be an indicator of its origin (Moll *et al.*, 1982; Chapple and Gilbert, 2002) (section 1.1.1.1.1).

Additional cells commonly found within healthy oral epithelium include melanocytes and merkel cells which are found in the basal layer. In addition, Langerhans cells can be found throughout the whole epithelium, and contribute to the host immune response by acting as antigen presenting cells (section 1.1.3.2). Other immune cells, such as neutrophils, may migrate into the epithelium when required (Liu *et al.*, 2010).

1.1.1.1 Epithelial cytoskeleton

The cytoskeleton of epithelial cells, and indeed all eukaryotic cells, functions as a cellular scaffold. The dynamic nature of the cytoskeleton aids in intracellular trafficking, mitosis and cellular migration (Windoffer *et al.*, 2011). Three types of protein filaments form the cytoskeleton, and these are known as intermediate filaments, microtubules and actin filaments.

1.1.1.1.1 Intermediate filaments

The main function of intermediate filaments is to provide support against extracellular forces (Reichelt, 2007). They also play a role in the transport of membrane organelles (Minin and Moldaver, 2008). Intermediate filaments anchor cells to each other at desmosomal junctions (section 1.1.1.2) and to the extracellular matrix at hemidesmosomes (section 1.1.2.1). There are four main types of intermediate filaments which are, type I (acidic keratins), type II (non-acidic keratins), type III (desmin (muscle cells), vimentin (fibroblasts, endothelial cells, leukocytes, mesenchymal cells), peripherin (peripheral neurons) and glial filament acidic protein (glia)), type IV (neurofilament proteins, largely expressed in neurons), type V (nuclear lamins, which provide structure to the cell nucleus) and type VI (located within the eye) (Minin and Moldaver, 2008).

Type I and type II keratins form heteropolymers, of which there are two types: epithelial keratins (described as cytokeratins) and trichocytic keratins (which are found in hair, wool, nails and horns). Cytokeratins are intracellular intermediate filament proteins only found within epithelial cells. There are over 20 different cytokeratins and the cytokeratin (CK) profile of a cell is an indicator of where the cell has originated (Moll *et al.*, 1982; Chapple and Gilbert, 2002). As epithelia differentiate or become dysplastic/cancerous, the expression of cytokeratins change (Moll *et al.*, 1982). For example, simple epithelia (one cell thick) express CK8/CK18 and CK7/CK19, whereas stratified epithelia express CK5/CK14, CK15 and CK6/CK16 (Bragulla and Homberger, 2009). In addition, the basal and suprabasal expression of cytokeratins differs between keratinised and non-keratinised stratified epithelium (Bragulla and Homberger, 2009). For example, CK13 is expressed in the suprabasal layers of non-keratinised stratified epithelia (Waseem *et al.*, 1998), but not in keratinised epithelia.

1.1.1.1.2 Microtubules

Microtubules have the largest diameter of all of the cytoskeletal proteins and are composed of a protein called tubulin. Microtubules polymerise and depolymerise, lengthening and shortening in length due to the requirements of the cell (Henderson *et al.*, 1999). The main role of microtubules is in the intracellular trafficking of cellular components, e.g. chromosomes, aiding in mitosis (Henderson *et al.*, 1999).

1.1.1.1.3 Actin filaments

Actin filaments play a major role in maintaining the shape of the cell. Actin filaments form linear bundles and are primarily found at the periphery of cells. This location aids in the maintenance of cellular form and may play a role in cellular migration (Henderson *et al.*, 1999), and bacterial invasion (section 1.3.4.2). Actin filaments form part of adherins and tight junctions, which are important in cell-cell contact (section 1.1.1.2), and focal adhesions maintaining cell-extracellular matrix contact (section 1.1.2.1).

1.1.1.2 Cell-cell junctions

The major cell-cell junctions include tight, gap, adherin and desmosomal junctions (fig 1.2). Tight junctions involve integral membrane proteins (claudins and occludins), which are attached to the intracellular actin cytoskeleton and hold the cell membranes of two adjacent cells close together preventing the intercellular passage of molecules (Silverthorn, 2004). Gap junctions consist of a channel, which is formed from transmembrane connexin molecules and connects the cytoplasm of one cell with the cytoplasm of an adjacent cell, allowing the passage of small (<1kDa) molecules (Goodenough and Paul, 2009). Adherin junctions associate with the actin cytoskeleton and are involved in the adhesion of two adjacent cells. Cadherins are calcium-dependent adherins. Homophilic adhesion of cadherins, mediated by calcium, specifies adhesion

between like cell types and hence these junctions are key to holding specific tissues together (Farquhar and Palade, 1963). The nomenclature for cadherins implies their site of origin. For example, E-cadherin is mainly found within epithelial tissue and N-cadherin in neuronal and endothelial tissue (Gumbiner, 2005). The desmosomal cadherins, desmogleins and desmocollins, are another type of calcium-dependent adherins which form heterotypic interactions in association with intermediate filaments within epithelial cells to form desmosomal junctions (also termed desmosomes) (Jamora and Fuchs, 2002). Desmosomes function in the resistance of shearing forces and are found in simple and stratified squamous epithelia (Mattey and Garrod, 1986). Cell-cell junctions not only function in the joining of individual epithelial cells but also play a role in the maintenance of cellular polarity and differentiation (Bryant and Mostov, 2008).

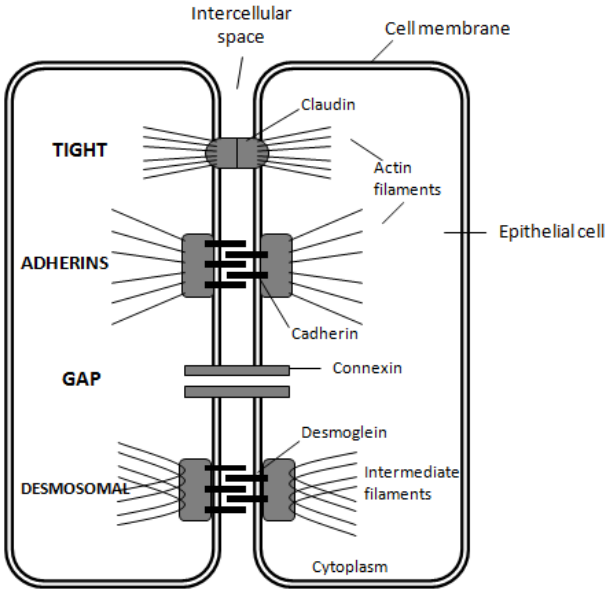

Figure 1.2 Diagram of the major epithelial cell-cell junctions. Epithelial cells are connected by tight, gap, adherin and desmosomal junctions. Tight and adherin junctions involving integral membrane proteins, e.g. claudin and cadherin, respectively, are involved in cell adhesion via association with the actin cytoskeleton. Similarly desmosomes associate with intracellular intermediate filaments and involve the integral membrane protein, desmoglein. Gap junctions are cytoplasmic bridges created by connexin proteins allowing the passage of small molecules between adjacent cells. Adapted from Silverthorn (2004).

1.1.1.3 Integrins

Integrins are cell adhesion receptors found in all nucleated cells and are involved in cell-cell and cell-extracellular matrix (ECM) interactions. They are cell surface heterodimers composed of one α and one β subunit (fig 1.3). Within mammals, there are 18 α and 8 β subunits, comprising 24 different combinations (Takada *et al.*, 2007). Integrins are activated via inside-out signalling (Moser *et al.*, 2009; Shattil *et al.*, 2010). Activated integrins then bind extracellular ligands including ECM proteins (e.g. fibronectin, laminin and collagen) and other cell surface molecules, including intercellular cell adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) aiding in epithelial adhesion to the ECM and adjacent epithelial cells

(Silverthorn, 2004). Integrins are closely associated with intracellular signalling mechanisms, and ligand binding results in the control of a number of cell functions including cell proliferation, cell survival, cytoskeletal rearrangement, cell migration and gene transcription (Lodish *et al.*, 2008). The integrins expressed by gingival epithelial cells include α2β1, α3β1, α5β1, α6β1, αvβ6 and α6β4 (Andrian *et al.*, 2006).

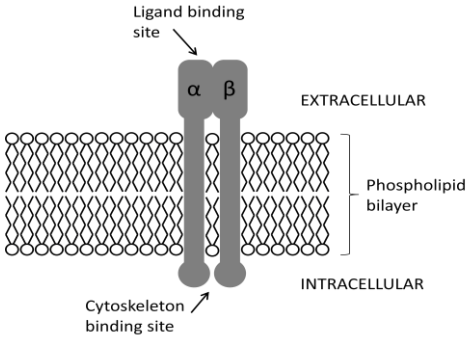

Figure 1.3 Diagrammatical representation of an integrin heterodimer. Integrins are transmembrane-spanning heterodimeric proteins, composed of an α and a β subunit. There is a ligand binding site within the extracellular domain and a cytoskeletal binding site located intracellularly.

In association with integrins, are tetraspanins (Berditchevski, 2001). These 4-transmembrane spanning proteins have two extracellular domains, one small (EC1) and one large (EC2) (fig 1.4). Tetraspanins are important in clustering of integrins at the cell surface (Yang *et al.*, 2004; Singethan and Schneider-Schaulies, 2008) and the recruitment of other molecules, including additional adhesion molecules and intracellular signalling proteins to form a network, known as a 'tetraspanin web', which plays a role in cellular processes including cell migration, differentiation and intracellular signalling (Charrin *et al.*, 2009).

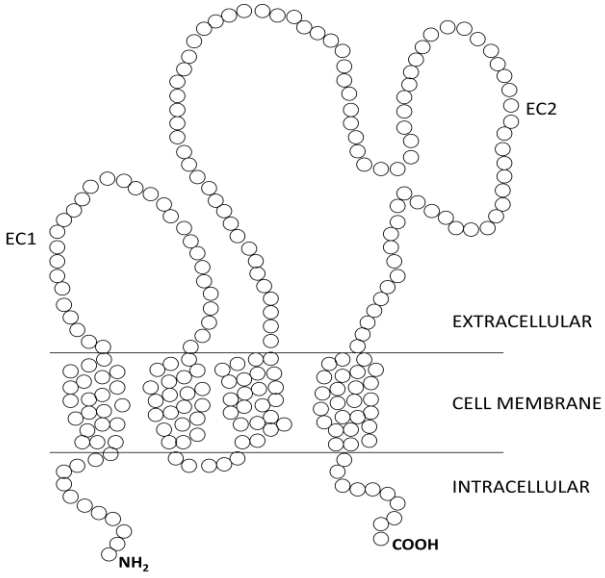

Figure 1.4 Diagram to show the localisation of amino acid residues within a tetraspanin molecule. Tetraspanins are transmembrane spanning proteins with two extracellular loops, one small (EC1) and one large (EC2). Tetraspanins have intracellular -NH_2 and -COOH domains. Each circle represents one amino acid residue. (Adapted from Levy *et al*. (1998)).

1.1.2 Basement membrane

At the interface between epithelium and connective tissue is the basement membrane which consists of three distinct layers, the lamina lucida, lamina densa and lamina reticularis. The lamina lucida resides at the cellular interface and is composed primarily of collagen type XVII, laminins 5, 6 and 10, and integrins (including α6β4). The lamina densa is located at the cellular-matrix interface and is composed of collagen type IV, laminin 1, nidogen, proteoglycans (e.g. perlecan, bamacan, collagen XVIII) and stored growth factors. The lamina reticularis forms the basal portion of the basement membrane, located at the matrix interface and is composed of collagen types I, III and V, proteoglycans and stored growth factors (Evans *et al.*, 2010). Primarily the basement membrane functions in the attachment of the epithelium to the underlying connective tissue via cell-ECM junctions (section 1.1.2.1). In addition, the basement membrane is an important regulator in the movement of cytokines, chemokines, growth factors and metabolites between the epithelium and cells within the ECM, and vice versa (Iozzo, 1998; Evans *et al.*, 2010).

1.1.2.1 Cell-extracellular matrix junctions

The major cell-extracellular matrix junctions are focal adhesions and hemidesmosomes. Focal adhesions involve epithelial cell integrins which anchor the actin cytoskeleton via binding to fibronectin within the ECM, resulting in the direct attachment of cells to the ECM (Wehrle-Haller, 2011). Hemidesmosomes aid adhesion of epithelial cells to underlying basement membrane molecules and are important in epithelial stability (Koh *et al.*, 2008). Hemidesmosomes consist of multi-protein complexes involving anchoring fibrils (collagen type VII) in the ECM, laminin in the lamina densa, integrin α6β4 in the lamina lucida, and intermediate filaments within the cell, functioning as adhesion molecules at the cell-ECM interface (Borradori and Sonnenberg, 1999).

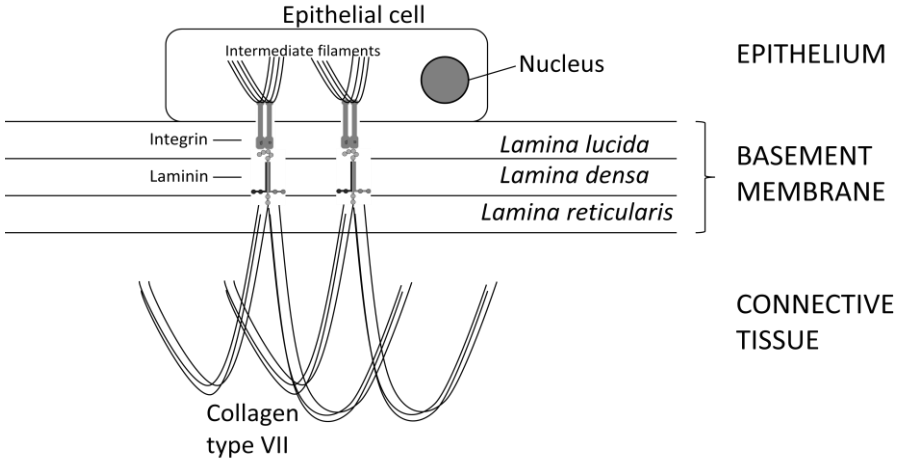

Figure 1.5 Diagrammatical representation of a hemidesmosme. Hemidesmosomes act as a linker between the connective tissue and the epithelium. Integrins and laminin within the basement membrane connect collagen type VII fibres within the connective tissue with intermediate filaments within the epithelial cells. Adapted from Borradori and Sonnenberg (1999).

1.1.2.2 Laminins

Laminins are glycoproteins expressed abundantly throughout the basement membrane. Laminins are heterotrimers consisting of one α subunit, one β subunit and one γ subunit (Burgeson *et al.*, 1994). Together these subunits form a cross-like structure (fig 1.6) held together by disulphide bonds. To date, there have been 18 laminins described in the literature (Durbeej, 2010), which are distributed throughout diverse tissues such as skin, lung, kidney, central nervous system, mammary gland, vascular smooth muscle and oral mucosa (Nguyen and Senior, 2006; Colognato *et al.*, 2007; Gerthoffer, 2007; Rebustini *et al.*, 2007; Sugawara *et al.*, 2008; Goldberg *et al.*, 2010; Peña *et al.*, 2010; Polyak and Kalluri, 2010). Laminins play an important role in the maintenance of tissue architecture, most significantly during embryogenesis (Dziadek, 1995, Schéele *et al.*, 2005). Laminins bind matrix proteins, such as collagen, to maintain a sturdy matrix structure, and transmembrane receptors such as integrins, resulting in cellular-matrix interactions (fig 1.5), which are important in cell migration (Gerthoffer, 2007), proliferation (Koh *et al.*, 2008), adhesion (Moore and Winder, 2010) and epithelial morphogenesis (Rebustini *et al.*, 2007).

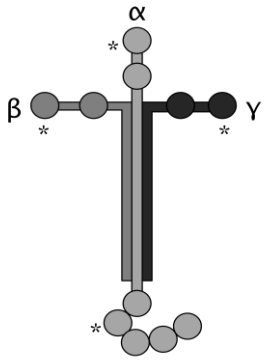

Figure 1.6 Diagram of a laminin protein. Laminins are heterotrimers made up of an α, β and γ subunit. Laminins play important roles in cell migration, proliferation and adhesion via binding to integrins. Asterisks (*) represent the location of major integrin binding sites. Adapted from Belkin and Stepp (2000).

1.1.3 Connective tissue

Connective tissue consists of ground substance, fibrous proteins and cells, which include fibroblasts (section 1.1.3.1), endothelial, neuronal and immune cells (section 1.1.3.2). Ground substance is composed primarily of proteoglycans, glycoproteins, phosophoproteins and water, forming a gelatinous substance, known as the ECM, which provides mechanical support to epithelia, including the oral mucosa (Silverthorn, 2004). Of the four types of fibrous proteins found within the connective tissue, long, unbranched collagen fibres are the most abundant. Within the connective tissue of oral mucosa, type I and type III collagen fibres are the most common. Type I collagen is the principal collagen found in the connective tissue layer, forming thick fibres. Collagen type III forms delicate fibres and is more glycosylated than collagen type I (Moss-Salentijn and Hendricks-Klyvert, 1990). The three additional fibrous proteins are elastin, fimbrillin and fibronectin. Elastin and fimbrillin combine to form filaments, adding

strength and elasticity to the connective tissue. Fibronectin is important in connecting the ECM to the epithelium at focal adhesions via its association with cellular integrins (Barczyk *et al.*, 2010) (section 1.1.2.1).

In addition to a structural support, the connective tissue allows the diffusion of metabolites into epithelial cells from the vast capillary networks within the ECM, and vice versa. Furthermore, the extravasation of neutrophils from the circulatory system, migration through the connective tissue and into the epithelium aids in defence against pathogenic attack. Also present within the connective tissue of oral mucosa are lymphatic vessels, nerves and salivary glands (Moss-Salentijn and Hendricks-Klyvert, 1990).

1.1.3.1 Fibroblasts

As the most abundant cell type within the connective tissue, fibroblasts are responsible for synthesising precursors of ECM including the four types of protein fibres and ground substance, playing a critical role in wound healing. In addition, fibroblasts influence epithelial differentiation and keratinocyte adhesion by secreting numerous cytokines and growth factors, including keratinocyte growth factor (KGF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and fibroblast growth factor 10 (FGF-10) (Saintigny *et al.*, 1993; Werner and Smola, 2001; Marchese *et al.*, 2001). These factors stimulate keratinocytes to secrete cytokines including interleukin 1 (IL-1) which acts as part of a positive feedback loop to increase the secretion of KGF by fibroblasts thus contributing to the regulation of keratinocyte growth and differentiation (Boxman *et al.*, 1993; Maas-Szabowski *et al.*, 1999; Wong *et al.*, 2007b). This is an example of fibroblast-epithelial cross-talk, which is important in maintaining epithelial integrity.

1.1.3.2 Immune cells

The major resident immune cells (leukocytes) within the connective tissue of oral mucosa are macrophages, mast cells and lymphocytes (Moss-Salentijn and Hendricks-Klyvert, 1990). Neutrophils migrate through the oral mucosa in response to a chemotactic stimulus. Immune cells are crucially important for the control of infection by bacteria, viruses, fungi or other microorganisms/particles/dead cells. The mouth, which is a warm and moist environment with a continuous supply of nutrients, provides optimum conditions for the survival and colonisation by such pathogens. Therefore the primary role of immune cells within the oral mucosa is to recognise ‘non-infectious self’ from ‘infectious non-self’ resulting in a targeted response culminating in the removal of pathogenic organisms and damaged tissue/cells (Janeway Jr, 1992), maintaining oral health. There are two arms of the immune response: innate and adaptive immunity. Innate immunity is the initial, non-specific activation of immune cells such as macrophages and neutrophils, resulting in the rapid removal of invading pathogens. Adaptive

immunity occurs after hours to days following the first infection with a pathogen. This arm of the immune response involves the presentation of pathogenic antigens to cells including T and B lymphocytes by antigen-presenting cells, resulting in the increased killing of the pathogen, production of antibodies aiding the recognition of pathogens by macrophages, and the production of memory cells which are important for a rapid, specific immune clearance should the pathogen be encountered again (Silverthorn, 2004). Leukocytes are classified according to morphological and/or functional characteristics.

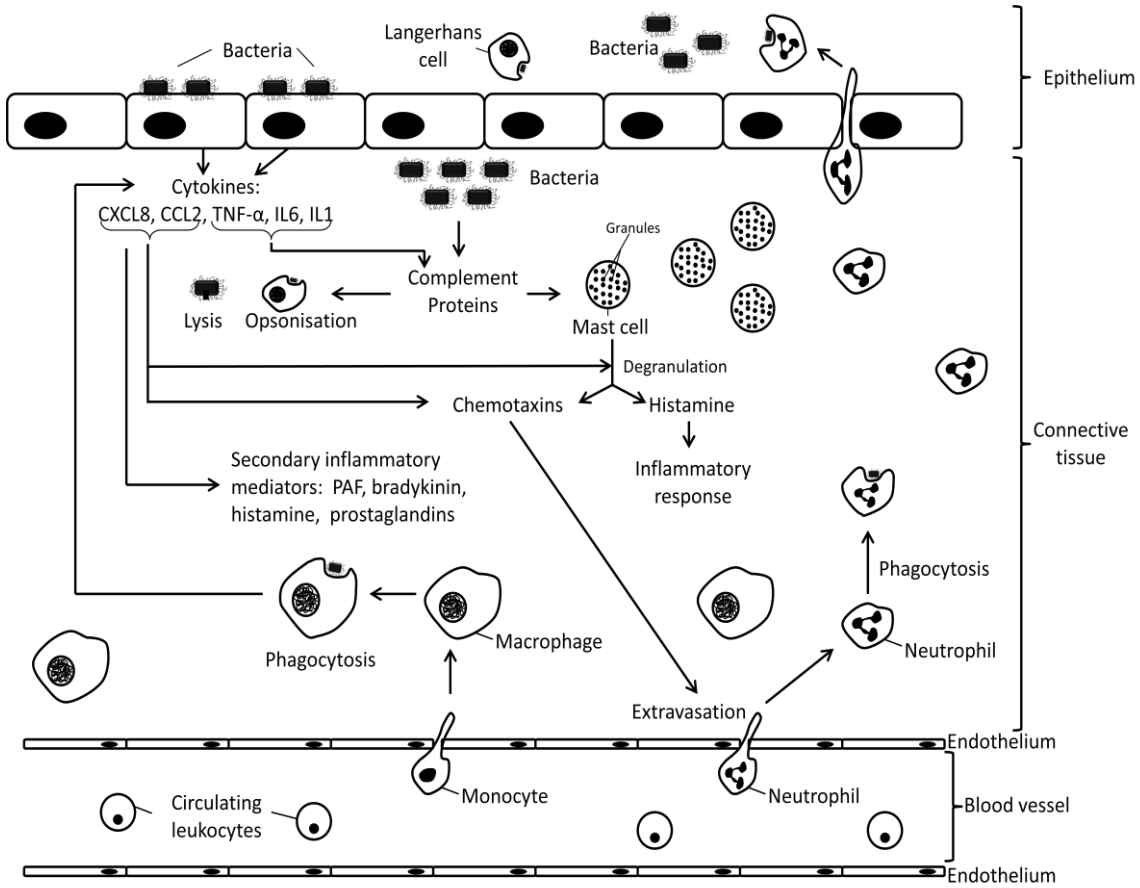

Figure 1.7 A diagram to show the host cells and inflammatory mediators important in innate immunity. The epithelium, connective tissue and blood vessel forming part of the oral mucosa are depicted showing the major resident and infiltrating immune cells important in the phagocytosis of bacteria/bacterial products, chemotaxis resulting in extravasation of additional immune cells, and inflammation. The release of cytokines due to the association of bacteria with epithelial cells contributes to chemotaxis, release of secondary inflammatory mediators and mast cell degranulation. **Abbreviations:** CCL2 (monocyte chemoattractant protein 1), CXCL8 (interleukin 8), TNF-α (tumour necrosis factor alpha), IL-1 (interleukin 1), PAF (platelet-activating factor).

Mast cells and neutrophils are known as granulocytes because within their cytoplasm there are many granules, which when activated by chemical stimuli (e.g. bacterial degradation products), degranulate, releasing their contents which include, heparin and cytokines that are involved in inflammation. Inflammation is characterised by redness, warmth and swelling, which is contributed in part by an increase in vascular permeability and blood vessel dilation (Silverthorn, 2004). Mast cell degranulation is triggered by cytokines, including interleukin 1

and complement proteins (fig 1.7). Neutrophils rapidly leave the surrounding blood vessels by extravasation, where they act as phagocytes.

Macrophages and neutrophils are known as phagocytes. Phagocytes ingest and kill 'foreign' particles, which are recognised by the host as 'non-self' (Janeway Jr, 1992). Ingested particles such as whole bacterial cells, cell fragments and/or other particles, are engulfed by the phagocyte and enter a cytoplasmic vesicle, known as a phagosome. The fusion of phagosomes with intracellular lyosomes (which contain enzymes and oxidising agents) kills ingested pathogens. The precursors of macrophages are monocytes and these are commonly found within the circulation until they enter the connective tissue and differentiate into larger macrophages where they spend the rest of their lives (Silverthorn, 2004) (fig 1.7).

Langerhans cells (dendritic cells) are also resident within the connective tissue and epithelia of oral mucosa and, along with macrophages, act as antigen-presenting cells, forming a link between the innate and adaptive immune responses. These immune cells ingest pathogens and present microbial antigens, complexed to major histocompatibility complex (MHC) class II, on the surface of the cell. These cells then migrate to local lymph nodes where naïve T-helper cells are activated and undergo clonal expansion. B-cells, also within local lymph nodes, recognise the antigen-MHC II complex, endocytose and process the antigen, presenting the antigen-MHC II complex on its surface. This aids in further activation of T-cells. In addition, B cells differentiate into plasma cells, which secrete antibodies specifically directed against the infecting pathogen, resulting in opsonisation (enhanced phagocytosis) of the antigen and an increase in phagocytic clearance (Janeway Jr, 1992; Silverthorn, 2004). T and B cells are found within connective tissue during chronic infection aiding in a more targeted removal of pathogens compared with innate immune cells such as macrophages and neutrophils.

1.2 Periodontal disease

1.2.1 Structure of the periodontium

The periodontium includes the tissues that surround and support the teeth. The major components of the periodontium are the gingivae (i.e. soft tissue surrounding the teeth (oral mucosa)), periodontal ligament, root cementum and alveolar bone (fig 1.8).

The structure of the epithelium that surround the teeth is related to its function in its primary role as part of the innate defence. As dental plaque builds up on the tooth surface, the epithelium (particularly the sulcular and junctional epithelium that faces the tooth surface) is exposed to challenge by bacteria and bacterial products. Therefore, the orthokeratinisation of gingival epithelium forms a partially impermeable barrier to bacteria/bacterial products. In addition, exfoliation of gingival surfaces aims to prevent the colonisation of bacteria on epithelial

surfaces (Walker, 2004). Furthermore, gingival crevicular fluid (GCF) bathes subgingival tissues and comprises a mixture of antimicrobial agents, including lysozyme, immunoglubulins and antimicrobial peptides (Walker, 2004). The presence of these agents and other extracellular proteins within the GCF and local environment may select for the colonisation and propogation of bacterial species that may contribute to periodontal disease progression (Marsh *et al*., 1994) (section 1.2.3).Sulcular epithelium faces the tooth enamel (fig 1.8) and is characterised by the absence of rete ridges, which are present in gingival epithelium. Cells of the sulcular epithelium are para-keratinised and there is a high turnover rate because it is highly prone to damage. As the epithelium progresses towards the cemento-enamel junction, it becomes junctional epithelium which is highly specialised. Junctional epithelium rapidly divides with a 2-6 day turnover (Chapple and Gilbert, 2002) and this high turnover rate prevents the accumulation of keratin resulting in non-keratinised epithelium (Heyden *et al.*, 1992). Junctional epithelium tapers apically from approximately 20 cell layers thick to 1-2 cell layers finishing with a single cell at the cemento-enamel junction, which is in contact with the tooth surface (Hatakeyama *et al.*, 2006).This epithelium possesses large intercellular gaps (Soames and Davies, 1977; Bosshardt and Lang, 2005) that forms an imperfect barrier to bacteria but enables the easy migration of polymorphonuclear (PMNs) cells and GCF to counteract pathogenic attack. Indeed, approximately 30,000 PMNs migrate per minute into the gingival sulcus to maintain periodontal health (Schiött and Löe, 1970). Oral epithelial cells secrete numerous cytokines and chemokines, including IL-1β, IL-6, TNF-α and CXCL8, particularly in response to bacteria and other microbes, which act as important inflammatory mediators and immune cell activators (Walker, 2004) (section 1.3.4.4).

The periodontal ligament consists of periodontal ligament fibres, neurovascular channels, ground substance and cellular elements such as fibroblasts, osteoblasts and undifferentiated mesenchymal cells. Periodontal ligament fibres are surrounded by tissue fluid providing an environment in which the tooth is able to withstand mechanical forces (Chapple and Gilbert, 2002).

The root cementum anchors periodontal ligament fibres to the root of the tooth. There are two types of cementum: acellular and cellular. Acellular cementum forms next to the tooth dentine and cellular cementum, consisting of cementoblasts, attaches to acellular cementum and provides an anchor for periodontal ligament fibres (Chapple and Gilbert, 2002).

Alveolar bone is connected to the cementum via periodontal ligament fibres (fig 1.8). Alveolar bone is a mineralised tissue involving the dynamic balance between bone-forming osteoblasts and bone-resorbing osteoclasts to maintain skeletal homeostasis (Sato and Takayanagi, 2006;

Hernández *et al.*, 2011). An imbalance in favour of osteoclastic activity results in bone resorption (section 1.2.3.2) (Vernal *et al.*, 2004).

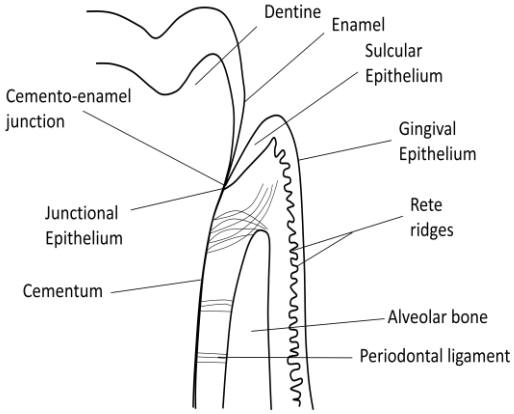

Figure 1.8 Diagram of the periodontium. Features of the periodontium are depicted here, including the gingival, sulcular and junctional epithelium, periodontal ligament, cementum and alveolar bone. These structures maintain the position and integrity of the tooth within its socket.

1.2.2 Clinical features

Periodontitis is characterised by an inflammation of the periodontium (fig 1.8), leading to progressive loss of tooth supporting structures including the periodontal ligament, gingival tissue, cementum and alveolar bone. In some cases tooth loss may occur (Caton and Lowenguth, 1993; Priestland, 1994; Weinmann and Geron, 2011). Periodontitis is always preceded by gingivitis (gingival inflammation) (Schätzle *et al.*, 2003). It is thought that periodontitis is the leading cause of tooth loss, within the adult population, worldwide (Choi and Seymour, 2010). The first sign of periodontal attachment loss occurs when there is an apical migration of the junctional epithelium leading to the formation of a periodontal pocket (Chapple and Gilbert, 2002) (fig 1.9). The depth of the pocket is an indicator of the level of attachment loss and is measured using a periodontal probe. Therefore, an increase in pocket probing depth (>3mm) indicates an increase in the level of attachment loss, and progression of disease.

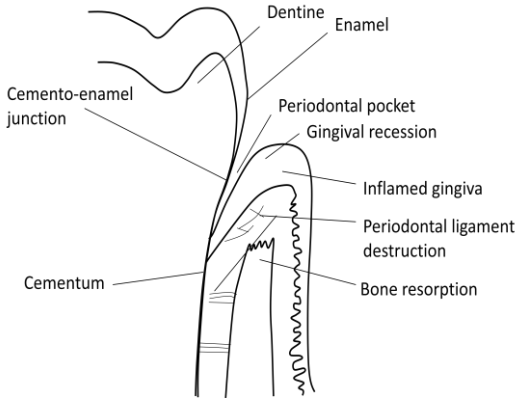

Figure 1.9 Formation of a periodontal pocket. Periodontitis is characterised by inflammation of the gingivae, destruction of periodontal ligament, alveolar bone resporption and loss of cementum. Progression of disease may result in tooth loss as destruction of the periodontium continues.

Periodontitis can be separated into two distinct categories: chronic and aggressive. Aggressive periodontitis may be further categorised as localised or generalised. Chronic and aggressive periodontitis share similar clinical features such as the presence of dental plaque/calculus, gingival redness, oedema, bleeding upon probing, increased probing depth, decreased periodontal attachment and alveolar bone loss (Offenbacher *et al.*, 2008). Differences between chronic and aggressive periodontitis include the age of onset, rate of progression, pattern of destruction, clinical signs of inflammation and the amount of plaque and calculus present (table 1.1). Chronic periodontitis is characterised by a late onset of disease, typically after 35 years of age, where periodontal detachment shows no defined pattern. Aggressive periodontitis is most often seen in individuals at an early age, and commonly localised aggressive periodontitis is diagnosed when less than 30% of sites are affected and generalised aggressive periodontitis diagnosed when more than 30% of sites are affected (Stabholz *et al.*, 2010). In terms of the rate of progression, it has been reported that aggressive periodontitis has a greater rate of progression compared with chronic periodontitis, hence the name designation (Frydman and Simonian, 2011). In the early stages of disease there are minimal signs of clinical inflammation associated with a minimal biofilm in aggressive periodontitis compared with chronic periodontitis (Stabholz *et al.*, 2010). However, as the diseases progress, differences occur between localised and generalised aggressive periodontitis in that with generalised aggressive periodontitis more teeth are affected and there is a larger plaque biofilm present, compared with localised aggressive periodontitis.

Table 1.1 Classification of periodontitis. Chronic and aggressive (localised and generalised) periodontitis may be categorised in terms of disease characteristics including age of onset, rate of progression, pattern of destruction, clinical signs of inflammation and the presence of dental plaque and/or calculus. Adapted from Stabholz *et al* (2010).

Characteristic	**Chronic**	**Aggressive**	
		Localised	**Generalised**
Age of onset	>35 years	<35 years	<35 years
Rate of progression	Slow/cyclical	Rapid	Rapid
Pattern of destruction	No defined pattern	<30% sites affected (primarily localised to first molars or incisors)	>30% sites affected
Clinical signs of inflammation	High	Low	Medium
Amount of dental plaque/calculus	Considerable	Minimal	Average

As diagnosis is relatively subjective, there has recently been a move towards the development of rapid, objective screening tests based on individual biomarkers within the GCF or saliva,

(Chapple, 2009). This would allow more accurate, early diagnosis and improved, customised treatment plans (Offenbacher *et al.*, 2008). Biomarkers such as bacterial plaque composition, tissue breakdown products, host proteolytic enzymes and inflammatory mediators are all important in the pathogenesis of disease (section 1.2.3) and are currently being investigated as potential biomarkers for diagnosis (Khiste *et al.*, 2011). Biomarkers such as beta glucuronidase (Lamster *et al.*, 1994), cathepsin B (Eley and Cox, 1996) and the RANKL/OPG ratio (Belibasakis and Bostanci, 2012) show the most promise in terms of accuracy of diagnosis (Chapple, 2009; Buduneli and Kinane, 2011). However, as yet, there is not one distinct biomarker that is capable of objectively diagnosing periodontal disease. This is probably due to the multi-factorial nature of this disease, which is affected not only by a range of genetic (section 1.2.2.1) and environmental factors (section 1.2.2.2), but also inter-individual differences in the microbial composition of the plaque biofilm and the specific immune responses to bacterial challenge (section 1.2.3.1) (Stabholz *et al.*, 2010; Khiste *et al.*, 2011; Laine *et al.*, 2012).

1.2.2.1 Genetic factors

There has been shown to be an association of periodontitis within families (Petit *et al.*, 1994) suggesting that there is a genetic link for the initiation of periodontitis. Numerous candidate gene-association studies have been performed in an attempt to identify genetic polymorphisms associated with aggressive and chronic periodontitis. These have recently been extensively reviewed (Stabholz *et al.*, 2010; Vijayalakshmi *et al.*, 2010; Laine *et al.*, 2012). Reports have indicated a wide range of polymorphisms within genes encoding cytokines (Kinane *et al.*, 1999; Sumer *et al.*, 2007; Reichert *et al.*, 2008), host-derived proteases (Ustun *et al.*, 2008), receptors involved in metabolic processes (de Brito Jr *et al.*, 2004), immune activation (Sugita *et al.*, 1999) and antigen recognition (Bonfil *et al.*, 1999), suggesting that changes in these processes may act as specific risk factors for disease initiation. However, determining which polymorphisms are the most crucial in disease development is difficult due to the multi-factorial nature of disease, inter-individual variation, and the limitations of individual gene association studies. As these studies are commonly performed using limited numbers of individuals, this may lead to false-positive or false-negative results, therefore genome wide association studies may be more informative in the future (Laine *et al.*, 2012).

1.2.2.2 Environmental factors

The environmental factors that contribute to periodontitis include the state of oral hygiene/amount of dental plaque, smoking, stress and systemic factors (Stabholz *et al.*, 2010).

The positive correlation between the quantity of plaque and gingivitis is well known (Marsh, 1994). All forms of periodontitis are preceded by gingival inflammation/gingivitis (Schätzle *et*

al., 2003), further implicating microbial aetiology in the initiation of this disease (section 1.2.3.1). Therefore individuals with poor oral hygiene are more susceptible to developing gingival inflammation, which may progress to periodontitis if left untreated (Syed and Loesche, 1978).

Throughout the literature there are reports of the association of smoking with an increased risk of periodontitis (Preber *et al.*, 1980; Laxman and Annaji, 2008; Heikkinen *et al.*, 2008). This may be because smoking affects the oral microflora (Zambon *et al.*, 1996), inflammatory responses (Bergstrom and Preber, 1986), and healing potential of periodontal connective tissues (Stabholz *et al.*, 2010).

Psychological stress has also been indicated as a risk factor for the development of periodontitis (Moss *et al.*, 1996; Genco *et al.*, 1999). The effect of stress may influence two factors, which could contribute to the development of disease. These are 'health impairing behaviours' and 'pathophysiological factors' (Stabholz *et al.*, 2010). 'Health impairing behaviours' include changes in behaviour due to a negative mental health status, e.g. depression, leading to a decrease in self welfare and oral hygiene, increased smoking and poor nutrition (Monteiro da Silva *et al.*, 1996). 'Pathophysiological factors' include increased levels of glucocorticoid and catecholamine levels which may affect inflammatory and immunological responses contributing to an increase in the loss of tooth supporting structures (Boyapati and Wang, 2007). However, as 'stress' is difficult to quantify, the contribution to the pathogenesis of periodontitis is still debatable (Stabholz *et al.*, 2010).

Systemic diseases which have been shown to be associated with an increase in periodontitis include diseases that are related to the function of the immune response, in particular leukocyte deficiencies and immunosuppression, e.g. secondary to HIV infection (Lamster *et al.*, 1998). Leukocyte disorders include neutropenia (Baehni *et al.*, 1983; Stabholz *et al.*, 1990), Chediak-Higashi syndrome (Bailleul-Forestier *et al.*, 2008), chronic granulomas (Buduneli *et al.*, 2001) and histocytosis syndromes (Deas *et al.*, 2003). These deficiencies in leukocyte function are commonly inherited genetic conditions and contribute to a decreased removal of pathogenic bacteria leading to an exacerbation in bacterial-related diseases, such as periodontitis.

Periodontitis is also associated with a number of other diseases that have been shown to influence the host response to bacterial challenge or contribute to the clinical features of periodontitis. These include diabetes mellitus (Gurav and Jadhav, 2011), obesity (Suvan *et al.*, 2011), osteoporosis (Jeffcoat, 1998) and rheumatoid arthritis (Saini, 2011).

1.2.3 Pathogenesis

A pre-requisite for the initiation of inflammation observed in periodontitis, is the presence of a polymicrobial plaque biofilm, which accumulates on the tooth surface (Kinane and Attström, 2005). In terms of disease pathogenesis, the ecological plaque hypothesis has been proposed (Marsh et al., 1994). This hypothesis suggests that an excessive bacterial load, causing stress to the oral environment results in a change in the local environment, e.g. increase GCF flow, increased inflammation, which in turn allows growth of key organisms that contribute to local tissue destruction, directly or indirectly. Such 'pathogenic organisms' associated with destructive periodontitis are *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* (Marsh, 1994; Ximenez-Fyvie *et al.*, 2000; see section 1.2.3.1). However, the identification of a specific contributing factor and/or individual/group of microbes, leading to the initiation of disease, or the mechanisms behind this 'microbial shift', have not yet been elucidated (Darveau, 2010). This is due to the large number of different species described as associated with the disease (section 1.2.2), and inter-subject variation. The immune system and inflammatory response play a crucial role in the maintenance of periodontal health as shown by studies in which individuals with neutropenia or other leukocyte disorders have a greater incidence of periodontitis (section 1.2.2.1). Therefore, the balance between bacterial load and the host response is crucial for maintaining periodontal health. A disruption in this dynamic balance may result in disease progression (Darveau, 2010). It has been suggested that there are different patterns of disease progression, including linear and cyclical progression (Socransky *et al.*, 1984). Linear progression involves the slow, continuous increase in periodontal detachment over time, whereas cyclical progression is recognised by intermittent bursts of periodontal detachment with longer periods of remission (Socransky *et al.*, 1984). However, recently, Gilthorpe *et al*. (2003) evaluated the literature regarding periodontal disease progression and suggested that the linear and cyclical patterns of disease progression may be a manifestation of the same phenomenon, by which at some periodontal sites disease progressively worsens, and at other sites disease progression lessens and health improves (Gilthorpe *et al.*, 2003). However, it still remains that disruptions in the fine balance between host-pathogen interactions may result in the progression of disease (Darveau, 2010).

1.2.3.1 Microbial aspects

It is well known that poor oral hygiene contributes to the development of diseases of the oral cavity. Poor oral hygiene leads to the build-up of dental plaque, which has been positively associated with the severity of periodontal disease (Grenier and Mayrand, 1986; Genco *et al*., 1988). More than 600 different types of oral bacteria have been described (Chen *et al*., 2010). This wide variety of bacterial species adhere to teeth to form a biofilm. The establishment of a biofilm provides microorganisms with a more stable environment within which to multiply and propagate (Grenier and Mayrand, 1986; Dalwai *et al.*, 2006). Early colonisers of oral structures

include Gram-positive cocci (Marsh, 1994; Rosan and Lamont, 2000), which generally contribute to the normal microbial flora of a healthy mouth (Marsh, 1994). As dental plaque accumulates, there is a shift from a predominantly supragingival Gram-positive aerobic/facultative population to an increased number of anaerobic Gram-negative bacilli. These migrate apically and so are particularly found within subgingival plaque (Listgarten, 1988; Marsh, 1994). As secondary colonisers of the oral cavity, the Gram-negative microbes, e.g. *P. gingivalis*, *T. forsythia* and *T. denticola*, adhere to these early colonisers, such as *Streptoccocus gordonii* and *Actinomyces naeslundii* (Syed and Loesche, 1978; Boyd and McBride, 1984; Mayrand and Holt, 1988).

As understanding of the oral microbial ecology has developed, so have the theories behind the role dental plaque plays in the pathogenesis of periodontitis. Initially two hypotheses were proposed to describe the relationship between microbial load and disease initiation. These were the non-specific (Theilade, 1986) and the specific (Loesche, 1976) plaque hypotheses.

The non-specific plaque hypothesis suggested that all microorganisms within dental plaque contribute to tissue inflammation and destruction. Therefore removal of a thick plaque biofilm would re-establish a healthy supra- and sub-gingival environment due to the decrease in microbial load. The specific plaque hypothesis suggested that specific organisms within dental plaque contribute to disease, such as the association of localised aggressive periodontitis and *Aggregatibacter actinomycetemcomitans* (Fine *et al*., 2007). Therefore, eliminating individual bacterial species may lead to the resolution of the disease.

However, since those earlier hypotheses, the ecological plaque hypothesis has been proposed byMarsh (1994). This hypothesis suggests that an increase in microbial load, e.g. due to a decrease in oral hygiene, leads to an increase in inflammation (section 1.2.3.2) resulting in a change in the local environment, e.g. increase in GCF flow, decrease in pH. This change in environmental factors, such as an increase in environmental proteins, may result in the proliferation of proteolytic bacteria (e.g. Gram negative anaerobic bacteria), contributing to an imbalance in the resident microflora that is likely to increase inflammation. Prevention or resolution of disease therefore can be achieved by the targeting of 'disease-causing' organisms directly, as well as interfering with the environmental conditions responsible for their selection (Marsh, 2003). This importance of a change in environment for the enrichment of such organisms was determined through numerous pure culture (McDermid *et al*., 1988) and mixed culture (Bradshaw *et al*., 1998) studies.

The ecological plaque hypothesis highlights the importance of determining the specific organisms within dental plaque and the environmental cues that result in a change in the

microbial flora. The Human Oral Microbiome Project (Chen *et al*., 2010) is currently sequencing the full and partial genomes of the approximately 600 prokaryotic organisms within the human oral cavity. A more comprehensive view of microbial ecology will contribute to a better understanding of the contribution of microorganisms to disease, and which specific organisms to target for the prevention/resolution of disease.

Due to the close proximity of the microbial biofilm and periodontal tissues, and the few intercellular junctions within junctional epithelium (section 1.2.1), the exposure of the host to bacterial challenge is particularly high. Indeed, it has been reported that the most commonly detected bacterial species associated with gingival crevicular epithelial cells, were *P. gingivalis*, *T. denticola*, *Prevotella intermedia*, *Streptococcus intermedius*, *Campylobacter rectus*, *Streptococcus sanguinis* and *Streptococcus oralis* (Colombo *et al.*, 2007). These microorganisms possess numerous virulence features that may contribute to the tissue destruction observed in periodontitis. For example, Gram-negative anaerobic bacteria secrete numerous proteolytic enzymes that are capable of degrading components of the extracellular matrix (Al-Shibani and Windsor, 2008; Guo *et al.*, 2010), and preventing host immune responses (Potempa *et al.*, 2009). As metabolic end-products, some anaerobic bacteria produce volatile sulphur compounds, such as hydrogen sulphide (H_2S), methyl mercaptan, and dimethyl sulphide. These compounds have been shown to be cytotoxic to epithelial cells and other cellular components of the periodontium (Yoshimura *et al.*, 2000; Murata *et al.*, 2008; Zhang *et al.*, 2010). These metabolic end-products, in addition to other end-products including lactate, succinate, formate and ethanol may enter periodontal tissues resulting in an increase in tissue damage, activation of immune responses and/or dysregulation of host defences (Bartold *et al.*, 1991; Kurita-Ochiai *et al.*, 1995; Dashper *et al.*, 2011). Furthermore, these bacteria are capable of disrupting the host blood coagulation system, which may result in an increase in bleeding at sites of infection, contributing to an increase in the inflammatory response and an inhibition of tissue repair (Bamford *et al.*, 2007).

1.2.3.2 Host aspects

In an attempt to prevent the deleterious effects of individual bacterial species, the host initiates an inflammatory and immune response to eliminate mucosal colonisation by bacteria. As it has been described previously (section 1.1.3.2), the oral mucosa is host to numerous immune cells that are capable of eliciting a non-specific, as well as a targeted immune response to bacterial colonisation, contributing to tissue inflammation. Inflammation and activation of immune cells is important in maintaining periodontal health. However, a disruption in the fine balance between bacterial load and host defence mechanisms plays a role in the pathogenesis of disease. As the bacterial load is usually too small to cause the high levels of periodontal destruction observed, it is thought that the exacerbated contribution (or dysregulation) of the host defences

causes the most damage. For example, excessive inflammatory responses (Preshaw and Taylor, 2011), hyper-responsive neutrophils (Kantarci *et al.*, 2003), defective immune activation (Sugita *et al.*, 1999), and/or increased activation of host-derived proteases (Guo *et al.*, 2010) may play a role in periodontal tissue destruction.

The innate immune response is an important first line of defence against infiltrating pathogens. Initial innate defence barriers, include structural (epithelial membranes, cellular junctions), mechanical (mastication), and chemical (antimicrobial peptides (Gorr and Abdolhosseini, 2011), human β-defensins (Lu *et al.*, 2004), soluble CD14 (Jin and Darveau, 2001) and lipopolysaccharide binding protein (LBP) (Ren *et al.*, 2004; Choi *et al.*, 2011)). These barriers are the initial attempts at controlling pathogenic colonisation.

Once a pathogen or its secreted/released products, have gained access to the epithelium, epithelial cells secrete cytokines and chemokines, which act as chemoattractants for immune cells, including neutrophils from the blood supply and macrophages, dendritic cells and mast cells resident within the connective tissue. These cells target non-self molecules (section 1.1.3.2), removing them from the site of infection.

The favoured host response to bacterial colonisation is the rapid removal of the contaminating microbes with a quick inflammatory response, immediate immune cell activation and rapid resolution of inflammatory processes, once the threat has subsided. However, in some individuals unfavourable host responses are observed, including abscess formation and chronic inflammation (Van Dyke, 2011).

The activation of the host cellular immune and inflammatory processes by bacteria and other pathogens occurs via pathogen associated molecular patterns (PAMPs), which include bacterial lipopolysaccharide (LPS) (section 1.3.3.1), capsule (section 1.3.3.7), flagellin, fimbrillin (section 1.3.3.2), peptidoglycan and bacterial DNA (Kawai and Akira, 2005). These conserved structures activate pattern recognition receptors (PRRs), including membrane bound Toll-like receptors, which are expressed intracellularly and on the surface of host cells including epithelial cells, fibroblasts and leukocytes (Janeway Jr, 1992; Kawai and Akira, 2005; Yoshioka *et al.*, 2008). Activation of Toll-like receptors results in the secretion of chemokines and cytokines including, IL-6, IL-1β TNF-α and CXCL8 (fig 1.7). It has been reported that there is an increase in the detection of these cytokines within the gingival crevicular fluid of patients exhibiting periodontitis (Teles *et al.*, 2010a; Teles *et al.*, 2010b; Andrukhov *et al.*, 2011), suggesting a role for these mediators in disease pathogenesis. These cytokines are known as pro-inflammatory cytokines and are important in the activation of secondary inflammatory mediators, including platelet activation factor (PAF), prostaglandins and histamine (fig 1.7)

(Bascones-Martínez *et al.*, 2009). These secondary factors play important roles in vasodilation and increasing vascular permeability resulting in inflammation. The inflammatory response is also initiated via activation of complement proteins, which are small proteins within the serum. Complement proteins form a cascade, which culminates in the opsonisation of bacteria and increased bacterial lysis (Bascones-Martínez *et al.*, 2009) (fig 1.7). The release of chemokines such as CXCL8 and CCL2 results in the recruitment of neutrophils and monocytes to the site of infection via a chemotactic gradient (Nussbaum and Shapira, 2011) (fig 1.7). This neutrophil response is crucial for the maintenance of periodontal health as it has been shown that individuals with neutropenia exhibit an increased susceptibility to periodontitis (section 1.2.2.2). Neutrophils release serine proteases including elastase and cathepsin G and metalloproteases 8 and 9 via their activation and/or cell death (Figueredo *et al.*, 2005). An increased number of neutrophils and/or hyper-responsive neutrophils at diseased sites (Kantarci *et al.*, 2003; Guentsch *et al.*, 2009) may contribute to tissue degradation by these host-derived proteases. However, in contrast, an aberrant expression of these proteins from leukocytes may also contribute to disease initiation, as it has been reported that patients exhibiting mutations within the genes encoding these enzymes may have an increased chance of developing periodontal disease (de Haar *et al.*, 2004; de Haar *et al.*, 2006). Impaired neutrophil chemotaxis to the site of infection may also play a role in disease pathogenesis. For example it has been reported that in some patients exhibiting localised periodontitis there is a dysregulation in neutrophil chemotaxis (Mizuno *et al.*, 2011). Furthermore, an impaired chemotactic signal caused by the dysregulation of cytokine release due to the action of bacterial proteases (Van Dyke *et al.*, 1982) may also result in disease progression due to the failure to remove bacterial load by 'mis-informed' immune cells, thus resulting in a prolonged/chronic inflammatory response.

Chemokines and cytokines have been implicated in a number of processes contributing to disease progression (Preshaw and Taylor, 2011). Periodontal ligament fibroblasts from patients with periodontitis have been shown to over-express cytokines, including IL-6, which may result in the increased activation of immune cells and so contribute to chronic inflammation (El-Awady *et al.*, 2010). The location of these fibroblasts, deep within the periodontium, may be crucial in the degradation of more essential tooth supporting structures. Furthermore, it has been suggested that bone homeostasis is regulated by cytokines and an increase in pro-inflammatory cytokines may result in an increase in bone resorption (Cochran, 2008; Darveau, 2010). Bone resorption is a characteristic of periodontitis, which is thought to be due to an imbalance in the ratio of receptor-activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) (Boyle *et al.*, 2003; Nagasawa *et al.*, 2007). RANKL is a ligand for RANK, which is present on osteoclast precursors. Binding of RANKL to RANK results in the differentiation of these osteoclast precursors into macrophage-like cells, which results in bone degradation. OPG is a soluble receptor of RANKL preventing the binding of RANKL to RANK, thus inhibiting bone

resorption (Darveau, 2010; Belibasakis *et al.*, 2010). The regulation of RANKL and OPG is achieved by cytokines, e.g. RANKL is induced by the pro-inflammatory cytokines TNF-α and IL-1β, whereas OPG is induced by transforming growth factor (TGF) β (Cochran, 2008; Mormann *et al.*, 2008). Due to the presence of pro-inflammatory cytokines, such as TNF-α and IL-1β, at inflamed periodontal sites, it is not a surprise that there is also an upregulation of RANKL in patients exhibiting disease compared with healthy controls (Belibasakis and Bostanci, 2012). Therefore, suggesting a mechanism by which bone resorption occurs in patients exhibiting periodontal disease. With regards to the inflammatory role of fibroblasts within the oral mucosa, these cells also secrete many pro-inflammatory cytokines including CXCL8, IL-6, IL-1β and TNF-α. The activation of these fibroblasts may occur indirectly via stimulation by inflammatory cytokines initially secreted by epithelial cells, or directly once the epithelial barrier has been breached.

In addition to cells of the innate immune response, cells of the adaptive immune system, e.g. T and B lymphocytes have also been implicated as effectors in the pathogenesis of periodontitis (Berglundh *et al.*, 2007). Macrophages and dendritic cells secrete cytokines that aid in migration and activation of these lymphocytes. Upon activation by antigen presenting cells, T lymphocytes proliferate and differentiate into subsets, including T helper (Th) cells, cytotoxic T cells and regulatory T cells (Treg). Th cells can be further separated into Th1, which secrete interferon gamma (IFN-γ), IL-2, IL-12, TNF-α and TNF-β leading to the eradication of intracellular pathogens; Th2 which secrete IL-4, IL-5, IL-6, IL-9 and IL-13 stimulating antibody production by B cells and contributing to the eradication of extracellular pathogens (Mosmann and Coffman, 1989) and T17 cells which are pro-inflammatory and pro-resorptive. The secretion of IL-17 and RANKL by T17 cells has been shown to be upregulated in periodontitis, contributing to bone resorption and disease pathogenesis (Dutzan *et al.*, 2009). Treg cells secrete IL-10 and TGF-β, which are anti-inflammatory cytokines (Vernal and Garcia-Sanz, 2008). Alterations in the Th cell population subsets may lead to disease progression. For example, a Th1 response has been reported to result in 'stable' periodontitis and a Th2 response may result in disease progression, possibly due to the activation of B cells (Gemmell *et al.*, 2007). B cells and their differentiated subtypes, plasma cells, which secrete antibodies, have been found to be the most prominent immune cell type within periodontal lesions (Berglundh and Donati, 2005). Plasma cells have been implicated in tissue destruction via the secretion of cytokines, including IL-6, IL-1β and TNF-α. These cytokines induce the release matrix metalloproteases (MMPs), such as MMP-8 and MMP-13 from host cells (Berglundh *et al.*, 2007). MMP-13 is capable of degrading numerous matrix proteins including fibronectin, proteoglycans, laminin and collagen type IV (Wahlgren *et al.*, 2002) and over-expression of MMP-8 is important in the degradation of collagen type I, II and III (Danielsen *et al.*, 2011).

During normal tissue turnover and wound healing, host MMPs and their inhibitors, i.e., tissue inhibitors of MMPs (TIMPs) act in concert. These proteins are mainly secreted by matrix derived cells such as fibroblasts, but are also secreted by keratinocytes and leukocytes. MMPs degrade unwanted matrix proteins and TIMPs prevent excessive and uncontrolled tissue degradation (Amâlinei *et al.*, 2010). However, when there is an imbalance in this relationship, unwanted tissue degradation occurs and this may explain the majority of damage observed in periodontitis (Offenbacher, 1996). In the gingival crevicular fluid of patients exhibiting periodontitis there has been shown to be an increase in the collagen degrading MMPs, such as MMP-8, MMP-1, and MMP-13 (Hayakawa *et al.*, 1994; Kinane *et al.*, 2003; Hernandez *et al.*, 2006). These MMPs are known as collagenases and are capable of degrading collagen, which is present in the ECM (Reynolds and Meikle, 1997). In addition, MMP-9 has been found at increased concentrations in periodontitis patients compared with healthy controls (Skurska *et al.*, 2010). MMP-9 is known as a gelatinase and as such degrades collagen type IV, V, VII, X, XI and XIV, gelatine, elastin, proteoglycans and fibronectin (Reynolds and Meikle, 1997), all of which are found within the connective tissue of oral mucosa (section 1.1.3). The expression of TIMP-1 has also been shown to be decreased in patients with periodontitis (Hayakawa *et al.*, 1994), suggesting that disruptions in the balance between MMPs and TIMPs may result in the increased tissue loss observed in periodontitis.

Recently it has been suggested that the failure of the resolution of inflammatory processes may be just as crucial in progression as the maintenance of the inflammatory process in the pathogenesis of periodontitis (Van Dyke, 2011). Pro-resolving lipid mediators therefore have been investigated for their potential in treating periodontitis and these are beginning to be of some use within the clinic (Van Dyke, 2011) (section 1.2.4).

There seems to be a circular relationship between the host and microbial community, in which there is continuous host-pathogen cross-talk (Chapple, 2009). Maintenance of periodontal health may be initiated and prolonged via low level PRR stimulation (e.g. by commensal bacteria), however, it is not known whether it is the increase in microbial load, presence of specific microbial species, immune suppression, or other factors that trigger disease initiation and maintain progression. A confounding issue is also that the primary importance of each of these may differ between subjects, or even sites.

1.2.4 Treatment

As the clinical features of chronic and aggressive periodontitis are similar, so is the treatment regime. Chapple (2009) suggests that the treatment of periodontitis should include four steps: mechanical, antimicrobial, tissue regeneration and behavioural and economic.

The best strategy for preventing periodontal disease is the maintenance of good oral hygiene. Regular tooth-brushing and flossing prevents the build-up of bacterial biofilms which contribute to disease. If left to accumulate, gingival inflammation may progress to periodontitis, by which the irreversible loss of periodontal tissues surrounding the tooth may eventually result in tooth loss. The primary treatment of periodontal disease involves the mechanical removal of disease-causing plaque and calculus from the teeth and surrounding structures by scaling and root planing, soft tissue curettage and, depending on the severity of disease, gingivoplasty, to reduce the periodontal pocket and so enable adequate removal of plaque by brushing (von Troil-Linden *et al.*, 1995).

However, mechanical therapies do not completely remove all bacteria from the periodontal tissues and may lead to the re-colonisation of treated sites, particularly by host cell internalised microbes that have escaped initial immune recognition. In the past the use of systemic antibiotic therapy was considered acceptable particularly for patients exhibiting active periodontal disease or for patients that failed to respond to surgical intervention (Walker *et al.*, 1981; Slots and Rams, 1990). However, the problem with systemic antibiotics is that they do not reach the periodontal pocket in high enough concentrations (Mombelli and Samaranayake, 2004). Therefore, there has been a strategy of targeted application of antibiotics at the site of the disease (i.e. the periodontal pocket) to achieve therapeutic doses. The periodontal pocket is able to retain antibiotic-soaked devices inserted into it. Commonly used topical antimicrobials include metronidazole, chlorhexidine and doxycycline, and also systemically delivered antibiotics including amoxycillin and most recently, azithromycin (Wang, 2010; Hirsch *et al.*, 2011).

One problem with the increasing use of antibiotics has been a rise in the incidence of bacterial resistance to once effective drugs. Consequently, alternative antimicrobial agents have been sought recently, and in particular there has been renewed interest in plant-derived antimicrobials (Takarada *et al.*, 2004; Okamoto *et al.*, 2004), and an increase in the research into other anti-microbial treatments, including, light therapy (Silva *et al.*, 2012), synthetic peptides (Daep *et al.*, 2006), probiotics (Bosch *et al.*, 2011) and prebiotics (Al-Hebshi *et al.*, 2010).

As research continues regarding the specific mechanisms involved in the initiation and progression of periodontal disease, the direct targeting of bactericidal therapy against individual bacterial species may be of use in treating disease, when the major causative organisms are known or suspected. For example, vaccines directed against virulence factors of pathogenic organisms and the stimulation of T cells towards a Treg subset may prove more useful in the future (Gibson III and Genco, 2001; Choi and Seymour, 2010).

As mentioned previously (section 1.2.3.2), the resolution of inflammation may be just as important as the initiation of inflammation in the progression of disease, therefore pro-resolving lipid mediators of inflammation such as lipoxins, resolvins and protectins have been investigated recently as potential treatments for periodontitis (Van Dyke, 2008; Serhan, 2008).

The progression of periodontitis results in the irreversible loss of periodontal tissues, therefore the replacement of these hard and soft tissues has been quite extensively researched in recent years, as a way of restoring tooth supporting structures. The majority of these studies are currently in the initial stages of development, whereas some are already established periodontal therapies within the clinical setting (McClain and Schallhorn, 2000; Chen and Jin, 2010). Regeneration of individual tissues, including gingival soft tissue, periodontal ligament and alveolar bone is being developed and tested (Yang *et al.*, 2006). However, due to the complexity of the periodontium, (i.e. including cementum, periodontal ligament, alveolar bone and oral mucosa), the regeneration of a complete mucosal structure is not yet possible. More recently there has been a move towards the use of stem cells which have the ability to differentiate into any cell type and show promise in the future of tissue regeneration (Yen and Sharpe, 2008; Grimm *et al.*, 2011).

Finally, behavioural and economic issues play an important role in the initiation and progression of periodontitis. Therefore, there may be a psychological basis for behavioural change, requiring additional mental health service intervention (Tonetti, 1998). In addition, changing individuals perceptions of oral hygiene and motivating them to take control of their own health are important strategies in preventing and controlling periodontitis (Chapple, 2009).

1.3 *Porphyromonas gingivalis*

Originally classified within the *Bacteroides* genus, *Porphyromonas gingivalis* has undergone significant reclassification since its initial isolation in 1921, by Oliver & Wherry, in which anaerobic Gram-negative rods were shown to produce black-pigmented colonies when grown on blood agar (Mayrand and Holt, 1988) (fig 1.10). After numerous changes of nomenclature and re-classification, the *Bacteroides* were taxonomically divided into 3 genera: *Bacteroides* – saccharolytic, non-pigmenting species, *Prevotella* – moderately saccharolytic, pigmenting and non-pigmenting species, and *Porphyromonas* – asaccharolytic, black-pigmenting species (Shah and Collins, 1988; Paster *et al.*, 1994).

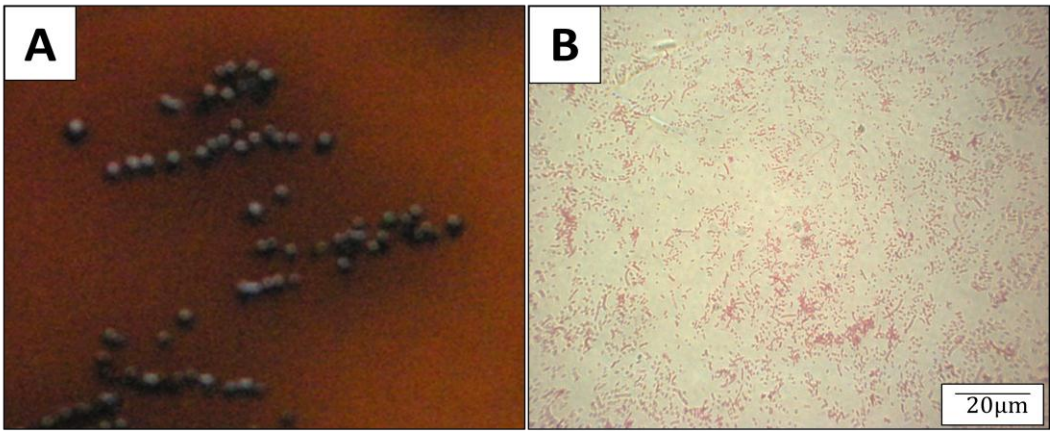

Figure 1.10 Black-pigmented *Porphyromonas gingivalis* colonies and Gram-stained *P. gingivalis* cells. On blood agar, *P. gingivalis* forms black-pigmented colonies (A). Cells appear as cocco-baccili on Gram-staining (B).

Some reports have indicated that *P. gingivalis* plays a significant role in the initiation and progression of periodontitis (Zambon *et al.*, 1981; Rautemaa *et al.*, 2004; Dalwai *et al.*, 2006). Supporting evidence comes from the observation that although *P. gingivalis* has been isolated from healthy individuals, the subgingival microflora, in severe forms of periodontitis, contains significantly higher numbers of *P. gingivalis* (Slots and Genco, 1984). Furthermore, Tanner *et al*. (2007) reported an association of *P. gingivalis* with periodontitis and attachment loss, as determined by 16S rDNA probe analysis and, in a hamster periodontitis model when *P. gingivalis* was introduced intra-orally an increase in alveolar bone resorption at the ligature site was observed (Hojo *et al.*, 2008).

In addition, *P. gingivalis* possesses numerous virulence features including fimbriae, proteases and LPS that are capable of activating the host immune system resulting in inflammation and disease progression (Darveau *et al*., 2004). Direct proteolytic activity of gingipains and collagenases may also contribute to loss of periodontal supporting structures leading to the phenotypic characteristics of periodontitis. Furthermore, immunisation with *P. gingivalis* fimbriae has been shown to protect against periodontal destruction in a rat model (Evans *et al*., 1992), suggesting that *P. gingivalis* fimbriae may be responsible for periodontal destruction. Similarly this has been shown for immunisation with *P. gingivalis* gingipains (Genco *et al*., 1999). Immune evasion through cellular invasion (Lamont *et al*., 1995) and cleavage of complement component C3 preventing serum opsonisation (Cutler *et al*., 1993), may be a strategy exhibited by *P. gingivalis* that may be necessary for its prolonged survival within the oral cavity where, should there be a change in the local environment, selection for the enrichment of *P. gingivalis* may occur. This may allow this organism, with its specific virulence features, to breach host defences, contributing to disease progression.

Recently, *P. gingivalis* has been described as a 'keystone pathogen' (Hajishengallis *et al*., 2011). Working with murine models, the authors inoculated specific pathogen-free (SPF; harbour commensal oral bacteria) and germ-free (GF; raised aseptically) mice with low levels of *P. gingivalis*. It was found that only the SPF mice exhibited bone loss, GF mice did not. In addition, it was shown that there was an increase in the amount of the oral microflora and a change in its composition. Furthermore, engineered mice lacking the C5a receptor, inoculated with *P. gingivalis* did not develop bone loss (Hajishengallis *et al*., 2011). These results suggested that the presence of commensal bacteria and complement are crucial in *P. gingivalis* induced bone loss, which may be important in periodontitis. Therefore, *P. gingivalis* may transform the commensal microflora into a "dysbiotic state" compromising the host-pathogen relationship contributing to disease initiation and progression (Hajishengallis *et al*., 2012)

1.3.1 Structure

P. gingivalis is a Gram-negative, assacharolytic, short, anaerobic rod (fig 1.10). Within some human hosts, *P. gingivalis* colonises the periodontal pocket (see section 1.2.1) as part of a subgingival microbial biofilm. Particularly within this environment, there is a limited nutrient supply, low oxygen tension and a higher than physiological temperature (Mettraux *et al.*, 1984; Haffajee *et al.*, 1992). Therefore, *P. gingivalis* must be physiologically adapted to survive within this environment.

As a Gram-negative bacterium, *P. gingivalis* possesses both an inner (cytoplasmic) and an outer membrane separated by a thin layer of peptidoglycan. The inner membrane is a phospholipid bilayer and the outer membrane is an asymmetric bilayer consisting of an inner phospholipid layer and an outer lipopolysaccharide layer (Yoshimura *et al.*, 2009). Within the outer membrane, proteins are situated, designated as outer membrane proteins, including RagA and RagB proteins (section 1.3.3.6), fimbriae (section 1.3.3.2) and membrane bound cysteine proteinase-adhesin complexes, known as gingipains (section 1.3.3.3). Gingipains are also an important soluble enzyme secreted by *P. gingivalis* which have been shown to be important in a number of functions, including nutrient acquisition and immune cell evasion. *P. gingivalis* also possesses a capsule, which has been shown to be an important virulence feature of this organism (Singh *et al.*, 2011). To survive within the oral cavity, *P. gingivalis* must first adhere to oral structures, i.e. epithelium, tooth surfaces, or most commonly primary bacterial colonisers. It does this via adhesins present on its cell surface, which include fimbriae (section 1.3.3.2) and haemagglutinins (section 1.3.3.4). After the initial adherence, *P. gingivalis* must colonise and survive within the subgingival environment. It achieves this via expression of molecules important for resisting oxidative stress (Capestany *et al.*, 2008), nutrient acquisition (Smalley *et al.*, 2011), immune evasion (Darveau *et al.*, 1998), and it is thought, cellular internalisation (Lamont *et al.*, 1995).

1.3.2 Growth requirements

P. gingivalis has a number of specific growth requirements, including nutritional (iron), atmospheric (anaerobic) and environmental (including pH and temperature) factors.

P. gingivalis is an asaccharolytic microorganism that obtains energy from the fermentation of nitrogenous substrates such as peptides containing glutamic acid and/or aspartic acid (Takahashi *et al.*, 2000; Takahashi and Sato, 2001; Takahashi and Sato, 2002; Goulet *et al.*, 2004). These substrates can be obtained from dental plaque (Hyatt and Hayes, 1975; Singer and Kleinberg, 1983), GCF and through bacterial degradation of larger peptides (section 1.3.3.3) (Takahashi, 2003). The fermentation of these substrates into organic acids, including butyrate, acetate, propionate, succinate, and ammonia (Takahashi, 2003) may contribute to maintaining a suitable environmental pH (Takahashi, 2003). As *P. gingivalis* is acid-sensitive and has shown maximal growth under the strict conditions of pH 6.5-7 (McDermid *et al.*, 1988; Takahashi and Schachtele, 1990), this modulation of environmental pH may aid in the survival of *P. gingivalis* within the oral cavity. In addition, these end-products have been suggested to play a role in the pathogenesis of periodontitis via their contribution to tissue damage (Dashper *et al.*, 2011) (section 1.2.3.1).

P. gingivalis also requires iron for growth. It obtains this from the environment via haemophore and protease systems (Smalley *et al.*, 2011), in the form of haemin (FeII-protoporphyrin IX). *P. gingivalis* stores haemin on its cell surface, giving it a black-pigmentation (Gibbons and Macdonald, 1960; Kuboniwa *et al.*, 1998). This ability to store haemin may be a requirement for *P. gingivalis* survival, as haemin levels within the periodontal pocket are variable and *P. gingivalis* requires a constant supply for growth (Genco, 1995). Numerous sources of iron are available within human hosts, e.g. haemoglobin, *cytochrome c*, methaemoglobin, myoglobin (Bramanti and Holt, 1991) and non-porphyrin containing compounds, such as transferrin (Inoshita *et al.*, 1991), lactoferrin and ferric and ferrous inorganic iron (Bramanti and Holt, 1991). To promote survival and propagation, *P. gingivalis* accesses free haemin from these iron sources via proteolytic and haemolytic activity (Bramanti and Holt, 1991; Genco *et al.*, 1994).

P. gingivalis has been described as a strict anaerobe (Shah and Collins, 1988) but it can tolerate oxygen for a short time (Dashper *et al.*, 2004). Under increased oxygen tension, *P. gingivalis* upregulates a number of genes, including those encoding bacterioferritin co-migratory protein (bcp) (Johnson *et al.*, 2011), OxyR (Meuric *et al.*, 2008), genes involved in the Clp system (Capestany *et al.*, 2008), rubrerythrin (Mydel *et al.*, 2006) and HtrA (Roy *et al.*, 2006), which are important in bacterial tolerance to oxidative stress. *P. gingivalis* also possesses mechanisms for DNA repair following oxidative stress-induced mispairing (Robles *et al.*, 2011).

Physiological temperature is typically around 37°C. However, elevated temperatures are observed within periodontal pockets exhibiting active periodontal disease, where the temperature can increase approximately 2°C above baseline (Haffajee *et al.*, 1992; Niederman *et al.*, 1995). At elevated temperatures it has been reported that there is a decrease in the expression of *P. gingivalis* fimbriae (Amano *et al.*, 2001), a reduction in gingipain activity (Percival *et al.*, 1999), and an increased resistance to oxidative stress (Vanterpool *et al.*, 2010). Furthermore, there is a modification of LPS (section 1.3.3.1) at elevated temperature, increasing the proportion of expressed Lipid A towards a mono-phosphorylated, penta-acylated form, which acts as a TLR4 agonist (Curtis *et al.*, 2011) (section 1.3.3.1). These factors may be important in the virulence and survival of this bacterium under the conditions of elevated temperatures observed in periodontitis.

1.3.3 Virulence features

As mentioned, *P. gingivalis* has been implicated in the pathogenesis of periodontitis (section 1.2.3.1). As such, there are a number of features of this bacterium that have been suggested to play a role in its virulence, contributing to the severity and/or progression of disease (Hernández *et al.*, 2011). Molecules, such as gingipains, haemagluttinins, other outer membrane proteins, lipopolysaccharide (LPS) and the bacterial capsule, which have direct contact with host tissues, have been implicated in the pathogenesis of disease. These virulence features will be introduced here and discussed in relation to their direct role in periodontitis.

1.3.3.1 Lipopolysaccharide

As a Gram-negative bacterium, *P. gingivalis* possesses LPS as a major macromolecule on the outer membrane. LPS consists of three domains. These are lipid A, which is the most conserved between bacterial species (Raetz *et al.*, 2007) and the inner-most domain; a short oligosaccharide core and an O-antigen, which is a long polysaccharide that interacts with the external environment (Jain and Darveau, 2010). LPS has been shown to induce a strong innate immune response, via binding to receptors present on numerous cell types. Peripheral blood leukocytes have a particularly high expression of these receptors, which aid in recognition of this Gram-negative bacterium (Zarember and Godowski, 2002). The lipid A portion of LPS acts as a PAMP, which in turn activates PRRs. LBP initially binds to LPS, converting oligomeric LPS into a monomeric form. This is then recognised by soluble or membrane-bound CD14, which binds to the MD-2-TLR4 complex resulting in an intracellular signalling cascade culminating in the release of pro-inflammatory chemokines and cytokines, including TNF-α, IL-1β, IL-6 and CXCL8 (Takeuchi and Akira, 2001). This pro-inflammatory response aids in the recruitment of leukocytes to the site of infection and removal of bacteria/bacterial products resulting in a return to periodontal homeostasis. However, if this immune response becomes

over-active or uncontrolled an increase in damage to host tissues may be observed (Jain and Darveau, 2010).

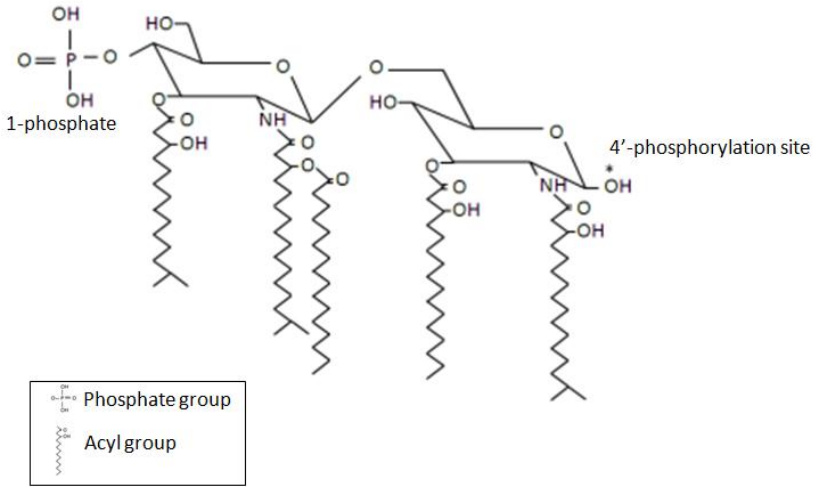

Figure 1.11 The structure of mono-phosphorylated, penta-acylated lipid A from *P. gingivalis*. Five acyl groups can be seen attached to a disaccharide backbone. This lipid A structure acts as a TLR4 agonist (table 1.2) but may also be di-phosphorylated via addition of a phosphate group to the 4'-phosphorylation site (*). Tetra-acylated species also exist. These are predominantly found at high haemin concentrations (table 1.2) (adapted from Coats *et al*., 2009).

Lipid A is a β-1,6-linked D-glucosamine disaccharide which may be phosphorylated at the 1' and 4' positions and N- and/or O-acylated (fig 1.11). *P. gingivalis* lipid A is differentially modified in the presence of excess haemin and high temperatures typically observed in sub gingival periodontal pockets. Although there is a heterogeneous expression of lipid A, under conditions of excess haemin (10µg ml^{-1}) the major lipid A species is tetra-acylated, mono-phosphorylated, whereas at low haemin concentrations (e.g. 1µg ml^{-1}) the major lipid A species is tetra-acylated, non-phosphorylated (Al-Qutub *et al.*, 2006; Coats *et al*., 2009). In addition, when there is a small temperature increase of 2°C, from 37°C to 39°C, there is a shift from a predominantly tetra-acylated lipid A structure, which may be mono- or non-phosphorylated, to an increase in the proportions of mono-phosphorylated, penta-acylated lipid A structure (Curtis *et al.*, 2011a). It has been shown that these lipid A structures differentially activate TLR4 (table 1.2). For example, the penta-acylated mono-phosphorylated lipid A has been shown to be a TLR4 agonist, which is more susceptible to killing by human β-defensins (h-BDs) (Curtis *et al.*, 2011). At high haemin concentrations, an increase in the tetra-acylated, mono-phosphorylated lipid A acts as a TLR4 antagonist resulting in immune suppression (Coats *et al.*, 2005; Reife *et al.*, 2006). At low haemin concentrations, the predominant non-phophorylated lipid A structure, a result of the action of 1- and 4'-phosphatases (PG1773 and PG1587, respectively) (Coats *et al.*, 2009), does not activate TLR4 resulting in immune evasion. These differential responses of *P. gingivalis* lipid A to environmental cues may contribute to the pathogenesis of disease through the suppression and evasion of the host immune response. However, high haemin and high temperature would be expected to be present together at diseased periodontal sites, so the effects on LPS structure *in vivo* are unclear.

1.3.3.2 Fimbriae

P. gingivalis possesses fimbriae, which are short hair-like projections that protrude from the outer membrane of this organism. These proteinaceous filaments play important roles in host adhesion (Hamada *et al.*, 1998; Sojar *et al.*, 2002), cellular invasion of the bacterium (Isogai *et al.*, 1988; Njoroge *et al.*, 1997), auto-aggregation (Lin *et al.*, 2006), colonisation of the oral cavity (Maeda *et al.*, 2004), and stimulation of the host inflammatory response (Vaahtoniemi *et al.*, 1993; Hajishengallis *et al.*, 2009), contributing to the pathogenesis of disease. *P. gingivalis* possesses two fimbrial types, a major (FimA) type and a minor (Mfa1) type.

The major fimbriae (FimA), encoded by the *fimA* gene, are composed of filaments of a repeating 43kDa protein, fimbrillin, and were originally isolated from *P. gingivalis* strain 381 (Yoshimura *et al.*, 1984). The *fimA* gene has been classified into 6 types: I, II, III, IV, V and Ib, based on their nucleotide sequences (Nakagawa *et al.*, 2002). Much of the amino-terminal sequence of fimbrillin is conserved (Lee *et al.*, 1991), however, molecular cloning and sequencing of the gene has shown little homology with other Gram-negative bacteria, suggesting the major fimbriae is specific for *P. gingivalis* (Dickinson *et al.*, 1988). *P. gingivalis* expressing type II fimbriae have been shown to be the most prevalent strains isolated from patients exhibiting periodontitis (Amano *et al.*, 1999), have an increased ability to adhere to and invade oral epithelial cells (Kato *et al.*, 2007), and have been shown to be more virulent in animal models of periodontitis (Nakano *et al.*, 2004). FimA fimbriae bind eukaryotic proteins such as collagen type I, laminin and fibronectin (Hamada *et al.*, 1998), and prokaryotic proteins such as glyceraldehyde-3-phosphate dehydrogenase (Maeda *et al.*, 2004), playing a role in the initial colonisation of the oral cavity.

Table 1.2 The differential expression of *P. gingivalis* lipid A. The mass ions, determined by matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry, of various lipid A structures isolated from *P. gingivalis* LPS are shown. The acylation and phosphorylatin of these species are described in the presence of low (1ug ml^{-1}) and high haemin (10ug ml^{-1}) (Al-Qutub *et al*., 2006), and at physiological (37°C) and high (39-41°C) temperatures (Curtis *et al*., 2011). The TLR4 activation of these species is shown (Reife *et al*., 2006; Coats *et al*., 2005) and the prominent immune response exhibited in response to host cell challenge.

m/z	**Acylation**	**Phosphorylation**	**TLR4 activation**	**Predominant species in presence of haemin**	**Predominant species at 37°C and 39-41°C**	**Prominent immune response**
1,693	Penta-	Mono-	Agonist	Low (1ug ml^{-1}) haemin- not predominant	High temp (39-41°C)	More susceptible to killing by β-defensins 2 and 3
1,449	Tetra-	Mono-	Antagonist	High (10ug ml^{-1}) hamein-predominant	37°C	Immune suppression
1,368	Tetra-	Non-	No activation	Low (1ug ml^{-1}) haemin-predominant	37°C	Immune evasion

The minor fimbriae (Mfa1) are shorter (Hamada *et al.*, 1996), and as such are hidden beneath the longer and more abundant FimA fimbriae (Yoshimura *et al.*, 2009). These minor fimbriae are important in co-adhesion with early colonisers of the oral cavity such as *Streptococcus gordonii* (Park *et al.*, 2005), therefore establishing colonisation, and auto-aggregation (Lin *et al.*, 2006), contributing to the stability of this bacterium within the microbial biofilm.

1.3.3.3 Gingipains

P. gingivalis possesses cell surface-associated and extracellular cysteine proteinases (gingipains), which comprise two arginine (Arg)-specific proteinases (RgpA and RgpB) and one lysine (Lys)-specific proteinase (Kgp) (Potempa *et al.*, 1995). Gingipains play a major role in degradation of host cell proteins (table 1.3), and may also contribute to host cell adhesion of *P. gingivalis* (Chen and Duncan, 2004) (section 1.3.4.1). Arg-specific gingipains cleave at Arg-X dipeptide bonds, and in all cases, Lys-gingipain cleaves at the C-terminal side of lysine residues within polypeptide chains (Pike *et al.*, 1994). Arg- and Lys- specific gingipains are encoded by three genes: *rgpA*, *rgpB* (Arg-specific proteinases) and *kgp* (Lys-specific proteinase). RgpA and Kgp exist as multi-domain proteins consisting of a prepropeptide, a catalytic domain and associated adhesin domains (Potempa and Travis, 1996) (fig 1.12). The adhesin/hemagglutinin (HA) domains of both gingipains are virtually identical, particularly the HA2 domain, which have been shown to be identical (Slakeski *et al.*, 1999).

The different isoforms of RgpA include: i) the non-covalent association of the catalytic domain with haemagglutinin domains (HRgpA), ii) soluble/secreted monomeric catalytic domains ($RgpA_{cat}$), or iii) monomeric, highly glycosylated membrane bound catalytic domains (mt-$RgpA_{cat}$). RgpB is not associated with an adhesin domain (fig 1.12), therefore exists only as a soluble (RgpB) and glycosylated membrane bound (mt-RgpB) catalytic domain. Similar to RgpA, Kgp isoforms include, catalytic-adhesin associated domains, soluble monomeric catalytic domains and highly glycosylated membrane bound catalytic domains (Curtis *et al.*, 2001).

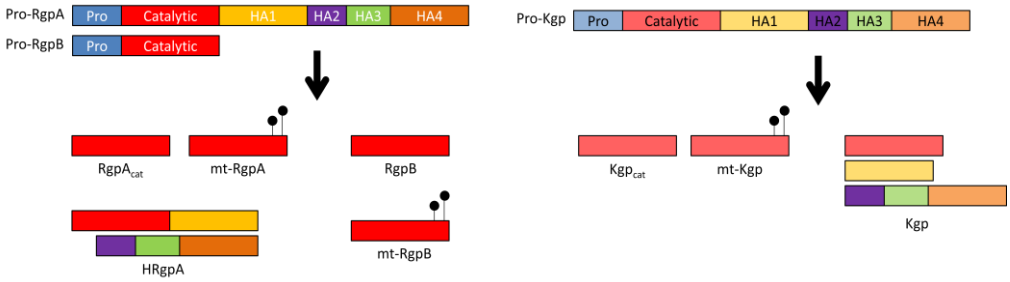

Figure 1.12 Catalytic processing and assembly of *P. gingivalis* gingipains. The major isoforms of RgpA, RgpB and Kgp are shown. All gingipains begin as a pro-peptide and catalytic processing removes the 'pro' fragment leaving catalytic domains (Rgp_{cat}, RgpB, Kgp_{cat}), which may be secreted into the extracellular domain, membrane bound domains which are glycosylated (🏌), and non-covalently associated haemagglutinin-adhesin (HA) domains (HRgpA, Kgp). RgpB does not have associated HA domains.

Haemagglutinin domains are designated HA1, HA2, HA3 and HA4 (fig 1.12). Post-translational processing of gingipains is achieved primarily by Arg-specific cleavage, but also via Lys-specific cleavage, resulting in membrane bound and soluble gingipain components (Potempa and Travis, 1996). In addition to self-processing, gingipains, particularly Rgps, are important for maturation of fimbriae indicated by the failure of a *P. gingivalis* Rgp knockout mutant to develop fimbriae, as analysed by Western blot and electron microscopy (Nakayama *et al.*, 1996; Kadowaki *et al.*, 1998).

Gingipains have been shown to be important virulence features of this bacterium due to the large necrotising abscesses that are formed following introduction of wild-type *P. gingivalis* into animal models, when compared with gingipain-null mutant strains (Yoneda *et al.*, 2003). The functions of gingipains are commonly distinguished via the use of gingipain knockout mutants, which have indicated roles of gingipains in adherence, nutrient acquisition, disruption of host defences and tissue destruction through the direct proteolytic degradation of ECM proteins (Baba *et al.*, 2001) or the indirect activation of host MMPs (Andrian *et al.*, 2007) (table 1.3).

The adherence of *P. gingivalis* to other microorganisms (co-aggregation), within the polymicrobial biofilm is important in the initial colonisation and survival of this bacterium. In addition, *P. gingivalis* gingipains are involved in adhesion of the organism to eukaryotic proteins, including ECM proteins (O'Brien-Simpson *et al.*, 2005) and oral epithelial cells (Chen and Duncan, 2004). Adhesion is a prerequisite for the invasion of host cells, which is thought to aid in evasion of the host immune response. In particular, the haemagglutinin/adhesin domains of gingipain proteins are the most important domains contributing to the adherence of *P. gingivalis* (Chen and Duncan, 2004). Gingipains also work in concert with fimbriae (section 1.3.3.2) enabling adhesion of *P. gingivalis* to host cells and molecules of the ECM. An example of this is the exposure of cryptic ligand binding sites by gingipains, in particular Rgps, within the host, exposing binding sites for subsequent fimbrial interactions (Kontani *et al.*, 1997) (table 1.3).

For its continued survival, *P. gingivalis* must obtain nutrients from the environment. Gingipains are important proteolytic enzymes that are capable of degrading host polypeptides (Oda *et al.*, 2009), thereby processing environmental products into a nutritional source that can be metabolised. Due to the requirement of this microorganism for haem (section 1.3.2), gingipains are also important molecules in haemagglutination, haemoglobin binding, haemolysis and haem/iron uptake from haem-containing molecules present within inflamed periodontal pockets (Smalley *et al.*, 2008) (table 1.3). Gingipains also manipulate blood coagulation pathways,

which may be important in the induction of inflammation (Inomata *et al.*, 2009), and may provide a supply of nutrients via red blood cell leakage into the periodontal pocket.

Gingipains are additionally capable of disrupting components of innate and adaptive immunity contributing to chaotic signalling events and so playing a role in the pathogenesis of disease. The majority of this disruption is due to the degradation of host proteins such as antimicrobial peptides (Carlisle *et al.*, 2010), complement proteins (Schenkein, 1988), host cell surface receptors (Tada *et al.*, 2002) and cytokines (Banbula *et al.*, 1999).

Gingipains have been shown to cleave protease activated receptors (PARs), e.g. PAR-1 to PAR-4. These receptors are present on the surfaces of host cells, including epithelial cells, neutrophils and platelets. RgpB and HRgpA have been shown to activate PAR-1 and PAR-4 on stably transfected myofibroblasts (Lourbakos *et al.*, 2001). Furthermore, activation of PAR-2 on the surface of neutrophils and cleavage of PAR-1 on oral epithelial cells, by Rgp-specific gingipains, results in receptor activation and release of proinflammatory cytokines including, IL1β, CXCL8 and TNF-a (Giacaman *et al.*, 2009). This upregulation of pro-inflammatory cytokines may lead to the induction of RANKL (section 1.2.3.2), and in combination with the proteolytic degradation of OPG by the Kgp-specific gingipain (Yasuhara *et al.*, 2009), may cause a disruption in bone homeostasis resulting in bone resorption. Activation of T cells also occurs via PAR-2, which may exacerbate the host immune and inflammatory responses (Belibasakis *et al.*, 2010). Usually the tightly regulated activation of platelet PARs is achieved by thrombin. The uncontrolled activation of PARs in the presence of gingipains may therefore result in disruption of the host blood coagulation pathway (Lourbakos *et al.*, 2001) (table 1.3) (fig 1.13).

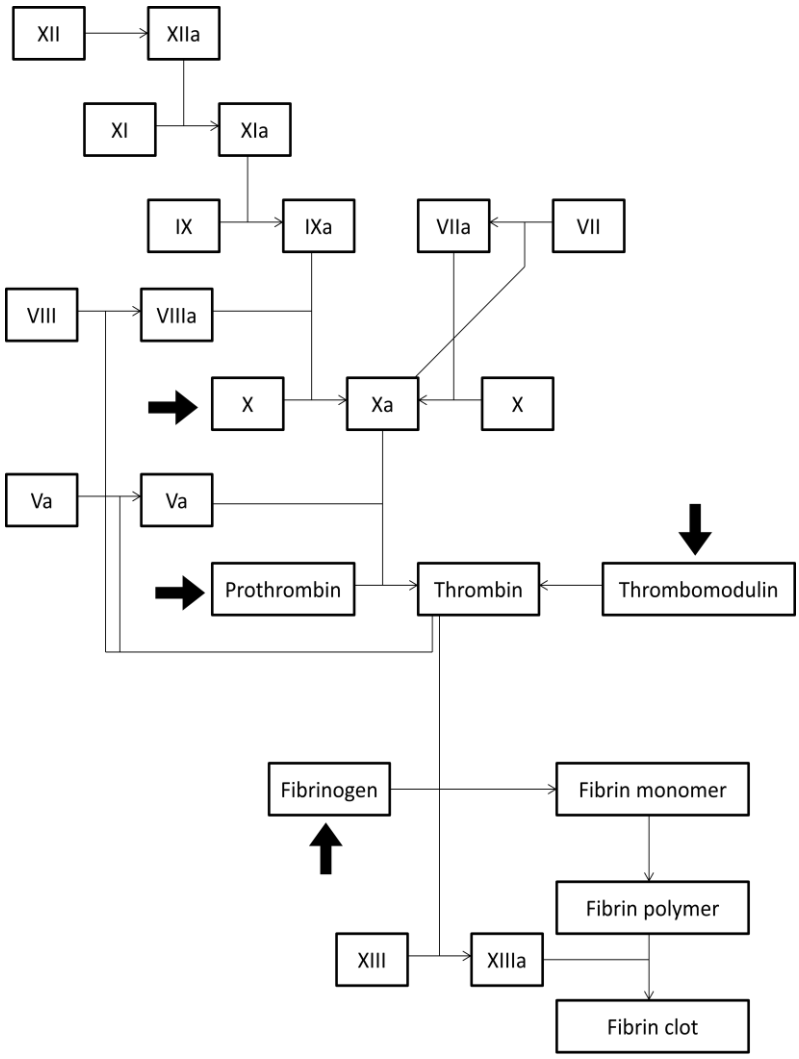

Figure 1.13 Modulation of blood coagulation pathway by *P. gingivalis* gingipains. The clotting factors involved in the blood coagulation pathway, of which prothrombin, thrombomodulin, fibrinogen and factor XI are modulated by *P. gingivalis* gingipains. Block arrows indicate the factors affected by gingipains (see table 1.3).

Table 1.3 Contribution of *P. gingivalis* gingipains and haemagglutinin (HagA) to the pathogenesis of periodontitis. The gingipain domains which are the major contributors within each category of nutrient acquisition, adherence, tissue damage, disruption of blood coagulation pathways and host defences are shown. Rgp=arginine-specific gingipains A and B (RgpA and RgpB), Kgp=lysine-specific gingipain, cat=catalytic domain, H/HA=haemagglutinin domains, HagA=haemagglutinin A, encoded by the *hagA* gene.

Category	Action	Major contributing gingipains	Reference
Adherence	Co-aggregation	RgpB HRgpA, Kgp, HagA (HA1)	Yamada *et al*., 2005 Ito *et al*., 2010
	Adherence to ECM proteins: fibronectin, collagen type IV,	$RgpA_{cat}$, $RgpB_{cat}$, Kgp	O'Brien-Simpson *et al*., 2005, McAlister *et al*., 2009
	Adherence to host cells (epithelial, endothelial cells)	HRgpA (epithelial) HagA (epithelial and endothelial)	Chen and Duncan, 2004, Pathirana *et al*., 2007b Belanger *et al*., 2011, Song *et al*., 2005
	Maturation of fimbriae	$RgpA_{cat}$, $RgpB_{cat}$	Nakayama *et al*., 1996, Kato *et al*., 2007
	Exposure of cryptic ligands	Rgp_{cat} Kgp_{cat}	Kontani *et al*., 1997 Pathirana *et al*., 2007b
Nutrient Acquisition	Haemagglutination	HRgpA, RgpB, Kgp, HagA	Shi *et al*., 1999, Grenier *et al*., 2003
	Haemolysis	Kgp (HA2/HA3 domain) RgpB Kgp, HRgpA	Smalley *et al*., 2008, Dashper *et al*., 2004 Li *et al*., 2010 Simpson *et al*., 2004
	Haemoglobin binding	RgpA, HagA (HA2 domain) Kgp	Nakayama *et al*., 1998 Kuboniwa *et al*., 1998
	Haem/iron uptake	Kgp, HRgpA	Simpson *et al*., 2004, Olczak *et al*., 2001
	Degradation of haem containing proteins: haptoglobin, transferrin, haemopexin	Kgp Rgp, Kgp	Sroka *et al*., 2001 Brochu *et al*., 2001

	Degradation of serum proteins: albumin, immunoglobulin	Rgp, Kgp	Oda *et al*., 2007, 2009
Disruption of blood coagulation system	Disruption of fibrinogen	Kgp	McAlister *et al*., 2009
	Activation of prothrombin	HRgpA	Imamura *et al*., 2001
	Activation of factor IX	HRgpA	Imamura *et al*., 2001
	Degradation of thrombomodulin	Rgps, Kgp	Inomata *et al*., 2009
Disruption of host defences	Degradation of antimicrobial peptides	Kgp, RgpA, RgpB	Carlisle *et al*., 2010 Gutner *et al*., 2009
	Degradation of complement proteins: C3, C4, C5	HRgpA, RgpB, Kgp	Schenkein, 1988, Grenier *et al*., 1992, 2003, Popadiak *et al*., 2007
	Binding complement protein C4b	HRgpA	Potempa *et al*., 2008
	Cleavage of host cell surface receptors: CD14 (monocytes, fibroblasts), CD4 and CD8 (T cells), ICAM-1 (epithelial cells)	HRgpA (CD14 fibroblasts) Rgp (CD14 macrophage-like cells) RgpA, RgpB, Kgp (CD4, CD8 T cells) Rgps (ICAM-1, epithelial cells)	Tada *et al*., 2002 Duncan *et al*., 2004 Kitamura *et al*., 2002 Tada *et al*., 2003
	Cytokine degradation: IL1β, IL-6, CXCL8, TNF-α (see section 1.3.4.4)	Rgp, Kgp HRgpA	Banbula *et al*., 1999 Uehara *et al*., 2008 Mikolajczyk-Pawlinska *et al*., 1998 Calkins *et al*., 1998 Stathopoulou *et al*., 2009b

	Cleavage of protease activated receptors: PAR-1 (epithelial cells, myofibroblasts), PAR-2 (epithelial cells, fibroblasts, neutrophils, osteoblasts, T cells)	RgpB, HRgpA (myofibroblasts) Rgp (PAR-1), Kgp (PAR-2) (epithelial cells) HRgpA, Kgp (epithelial cells) Rgp (osteoblasts) Rgp (neutrophils) Rgp (Jurkat T cells)	Lourbakos *et al*., 2001 Giacaman *et al*., 2009 Uehara *et al*., 2008 Abraham *et al*., 2000 Lourbakos *et al*., 1998 Belibasakis *et al*., 2010)
Tissue damage	Degradation of ECM proteins: fibrinogen, fibronectin, collagen type IV	$RgpA_{cat}$	Baba *et al*., 2001
	Activation of host MMPs (MMP-8, MMP-9, MMP-2)	Rgps	Andrian *et al*., 2007, Grayson *et al*., 2003
	Degradation of cellular adhesion molecules: ICAM-1, E-cadherin	Rgp_{cat} Kgp	Baba *et al*., 2001 Katz *et al*., 2002
	Induction of host cell apoptosis	Rgp, Kgp	Grenier *et al*., 2003 Stathopoulou *et al*., 2009a Urnowey *et al*., 2006

1.3.3.4 Other haemagglutinins (HagA and HagB)

P. gingivalis possesses additional genes, aside from gingipain genes, which encode haemagglutinin domains. There are five haemagglutinin genes: *hagA*, *hagB*, *hagC*, *hagD* and *hagE*, of which *hagA* shares 90% homology with the adhesin domains of *rgpA* and *kgp* (Han *et al.*, 1996). The major haemagglutinins are HagA and HagB (Lépine *et al.*, 1995) and are important in adhesion to and invasion of oral epithelial cells and endothelial cells by *P. gingivalis* (Song *et al.*, 2005; Belanger *et al.*, 2011). HagA is also important in co-aggregation of *P. gingivalis* with other bacterial species within the plaque biofilm (Ito *et al.*, 2010) and haem acquisition via haemagglutination (Grenier *et al.*, 2003) (table 1.3).

1.3.3.5 Other proteases

P. gingivalis also possesses other proteolytic enzymes, which may play a role in the virulence of this organism. These include SerB-phosphatase (Bainbridge *et al.*, 2010), periodontain (Nelson *et al.*, 2003), collagenase (PrtC; Kato *et al*., 1992, Wittstock *et al*., 2000), endothelin-like converting enzyme (PepO; Awano *et al*., 1999), dipeptidyl and tripeptidyl aminopeptidases (Oda *et al.*, 2009), sialidase and sialoglycoproteases (Aruni *et al.*, 2011).

Although of all the proteases encoded by *P. gingivalis*, gingipains are the most predominant, these other proteases may also contribute to the pathogenesis of periodontitis via nutrient acquisition, (dipeptidyl and tripeptidyl aminopeptidases), immune cell evasion (sialidases and sialoglycoproteases), disruption of blood coagulation (endothelin-like converting enzyme), ECM degradation (collagenase), immune modulation (SerB phosphatase) and modulation of host proteases (periodontain).

1.3.3.6 Other outer membrane proteins

The surface of *P. gingivalis* is the structure which comes directly into contact with host tissue and hence outer membrane proteins are important in bacterial virulence. The most abundant outer membrane proteins are OmpA-like proteins and porins (Iwami *et al.*, 2007; Yoshimura *et al.*, 2009). *P. gingivalis* possesses an OmpA-like protein, which shares homology with the E-coli OmpA protein (Nagano *et al.*, 2005). This protein plays a role in membrane stability, host cell adhesion, invasion, biofilm formation and immune stimulation (Iwami *et al.*, 2007; Smith *et al.*, 2007). Additionally, OmpA proteins form a pore through which solutes can pass contributing to metabolic processes of this organism (Sugawara and Nikaido, 1992).

RagA and RagB are also important outer membrane proteins that play a role in the virulence of this bacterium. RagA shows homology to Ton-B dependent outer membrane receptors. These receptors traverse the periplasmic space and are important in the recognition and active transport of extracellular ligands across the outer membrane (Postle and Kadner, 2003). RagB is

a lipoprotein (Curtis *et al.*, 1999) and together with RagA serves in the uptake of extracellular macromolecules such as glycoproteins (Yoshimura *et al.*, 2009), acting as a nutrient acquisition system important in the survival of this bacterium at the site of infection.

Other nutrient acquisition outer membrane proteins include haem uptake systems performed by proteins such as HmuR (Olczak *et al.*, 2008), IhtB (Dashper *et al.*, 2000) and Tlr (Aduse-Opoku *et al.*, 1997) and haemin-binding protein 35 (Shoji *et al.*, 2011).

1.3.3.7 Capsule

Some strains of *P. gingivalis* possess a polysaccharide capsule. This surrounds the outer membrane of the bacterium, acting as a shield against host immune attack. Evading the host immune response may be an important mechanism in the survival of *P. gingivalis* at sites of infection, possibly contributing to prolonged inflammation. Indeed, the phagocytosis of encapsulated strains was significantly lower than non-encapsulated strains (Singh *et al.*, 2011). In addition, it has been reported that encapsulated strains of *P. gingivalis* are more resistant to host-derived defensins (Igboin *et al.*, 2011). This capsule has also been shown to be an important virulence determinant. This was confirmed using the murine abscess model (section 1.4.3), in which encapsulated strains were shown to induce a greater abscess than non-encapsulated strains, suggesting a role of the capsule in disease progression. Furthermore, different serotypes of the *P. gingivalis* capsule can induce differential activation of the host immune response. For example, serotypes K1 and K2 induced a higher cytokine response following stimulation of dendritic cells compared with serotypes K3-6 (Vernal *et al.*, 2009), which may influence the host inflammatory response. Taken together, the capsule of *P. gingivalis* may be an important feature of this organism contributing to disease initiation and progression.

1.3.3.8 Outer membrane vesicles

P. gingivalis releases outer membrane vesicles into the extracellular environment. These may play a role in virulence because as components of these vesicles include LPS, gingipains (mt-RgpA, HRgpA and Kgp) and other outer membrane proteins (Grenier and Mayrand, 1987).

1.3.4 Interaction of *P. gingivalis* with host cells

1.3.4.1 Adhesion

Adherence is an essential first step in the colonisation of the oral cavity by *P. gingivalis*. Adhesion of microorganisms to oral structures and/or other microorganisms prevents the ever present risk of being ‘washed away’ by GCF (Gibbons, 1984). Adherence to oral structures is facilitated by numerous bacterial cell surface-associated components, including fimbriae, proteinases, haemagglutinins and LPS (Cutler *et al.*, 1995; Njoroge *et al.*, 1997). The major

feature of *P. gingivalis* which is important in adhesion to host cells is thought to be fimbriae. Colonisation of subgingival areas requires suitable conditions for growth and proliferation, such as, epithelial attachment sites, decreased oxygen tension required for growth of this anaerobic microbe and availability of haemin and other nutrients (Nelson *et al.*, 2003).

In addition to adhering to early bacterial oral colonisers, *P. gingivalis* has the ability to adhere to and invade a variety of eukaryotic cells *in vivo* and *in vitro*, including various oral cell lines (Duncan *et al.*, 1993; Madianos *et al.*, 1996; Njoroge *et al.*, 1997), fibroblasts (Pathirana *et al.*, 2007b), endothelial cells (Dorn *et al.*, 2000), and oral epithelial cells (Lamont *et al.*, 1995; Rautemaa *et al.*, 2004). Invasion of host cells could provide protection against the host immune response and other local defences. Epithelial cells are one of the initial host cells that are exposed to *P. gingivalis*, therefore *P. gingivalis* association with epithelial cells will be focussed upon.

There are a number of mechanisms exhibited by the host in which epithelial colonisation by bacterial cells may be hindered. Epithelial cell desquamation results in the reduced ability of bacteria to replicate to a high number on gingival and other oral cell surfaces (Gibbons and Houte, 1975). However, exfoliation of epithelial cells may also explain how *P. gingivalis* is transferred to other sites within the oral cavity (Rudney *et al.*, 2001). In contrast, teeth do not desquamate, therefore colonisation of these structures, by early Gram-positive colonisers, may provide an anchor for the colonisation of later Gram-negative colonisers, providing stability and hence a greater opportunity for the invasion of oral epithelial cells. Whilst within an epithelial cell the bacterium is protected against higher oxygen tensions in the surrounding oral cavity and the detrimental effects of host serum components. Components of saliva such as histatins, cystatins, lactoferrin, mucin glycoprotein MG2 and fibronectin have the ability to inhibit *P. gingivalis* adherence to supragingival mucosa and viability (Lamont and Jenkinson, 2000).

Adhesion of *P. gingivalis* to epithelial cells and subsequent cellular internalisation is not yet fully understood. Adhesion is thought to be via the association of fimbriae with epithelial cells, which is a two-way process, i.e., to adhere, bacterial fimbriae must bind to some receptor(s) located on the epithelial cell surface. It has been suggested that this is the fibronectin-binding integrin, α5β1 (Nakagawa *et al.*, 2002). In the presence of antibodies directed against the α5 integrin subunit, Nakagawa *et al.* (2002) reported inhibition of adherence and invasion of the human oral epithelial cell line, HEp-2, by type II recombinant FimA coupled to microspheres. Therefore, suggesting an importance of this integrin in adhesion of *P. gingivalis* to oral epithelial cells. In addition, Yilmaz *et al*. (2002) showed that the binding of *P. gingivalis* (ATCC 33277) to gingival epithelial cells in the presence of β1 integrin antibodies was inhibited in a dose-dependent manner. Although this suggests that there is a role for integrins in the

adherence of *P. gingivalis* to epithelial cells, this mechanism has not yet been fully elucidated and does not rule out the possible involvement of other mechanisms.

This initial adherence of *P. gingivalis* to oral surfaces is a pre-requisite to, but the major rate-limiting step in, host cell internalisation (Winkler *et al.*, 1987). In response to adhesion of *P. gingivalis* to oral epithelial cells, numerous host cell changes occur that may aid bacterial internalisation. These include: intracellular Ca^{2+} fluxes, cytoskeleton rearrangement, stimulation of intracellular signalling pathways such as mitogen-activated protein kinases (MAPK) and other protein phosphorylation (Andrian *et al.*, 2006).

1.3.4.2 Invasion

P. gingivalis invasion of oral epithelial cells correlates with the severity of inflammation of gingival tissue (O'Brien-Simpson *et al.*, 2000), suggesting that *P. gingivalis* invasion is important in the pathogenesis of disease.

Scanning electron micrographs show that *P. gingivalis* invades primary cultures of gingival epithelial cells (Lamont *et al.*, 1995; Belton *et al.*, 1999) and epithelial cell lines (Duncan *et al.*, 1993; Madianos *et al.*, 1996). Invasion is a rapid process, shown by the internalisation of fluorescently labelled *P. gingivalis* within primary cultures of gingival epithelial cells. *P. gingivalis* cells located within the perinuclear region of the epithelial cells, after approximately 10-15 minutes (Belton *et al.*, 1999). *P. gingivalis* invasion of epithelial cells aids in *P. gingivalis* viability and it has been reported that *P. gingivalis* has the ability to replicate within epithelial cells (Lamont *et al.*, 1995; Madianos *et al.*, 1996). In addition, it has been reported that *P. gingivalis* can spread inter- and intra-cellularly (Hintermann *et al.*, 2002; Yilmaz *et al.*, 2006).

The invasion of non-phagocytic host cells (such as epithelial cells) by bacteria has been reported throughout the literature, particularly for gastrointestinal bacteria. Two main mechanisms of cellular invasion have been described; these are the trigger and zipper mechanisms. The trigger mechanism involves the delivery of bacterial virulence factors, upon host cell contact, delivered into the host cell via bacterial type III secretion systems. This results in the direct activation of cytoskeletal proteins that can cause ruffling of the host cell membrane (fig 1.14) (Ofek *et al.*, 2003) and lead to bacterial entry. *Salmonella typhimurium* and *Shigella flexneri* utilise this mechanism for cellular invasion. The zipper mechanism is initiated via bacterial ligands binding to host-cell surface receptors. This results in receptor clustering and the formation of a 'phagocytic cup'. Invasion occurs following intracellular signalling and actin remodelling resulting in the engulfment of bacteria (Cossart and Sansonetti, 2004). *Listeria monocytogenes* and *Yersinia pseudotuberculosis* are thought to invade host cells via this mechanism.

P. gingivalis does not possess a type III secretion system (Lamont and Yilmaz, 2002), hence it is thought that the invasion of *P. gingivalis* into host cells is unlikely to occur via the trigger mechanism. As mentioned, the epithelial adhesion and invasion of *P. gingivalis* is thought to be via the α5β1 integrin and bacterial fimbriae (section 1.3.3.2). Invasion of primary gingival epithelial cells by *P. gingivalis* can be inhibited in the presence of cytochalasin D, which inhibits actin polymerisation, and nocodazole, which depolymerises microtubules, suggesting a significant role for cytoskeleton rearrangement in host cell internalisation (Lamont *et al.*, 1995). Furthermore, following cellular invasion by *P. gingivalis*, Yilmaz *et al*., (2003) showed that the tyrosine kinase focal adhesion kinase (FAK), and its adapter protein paxillin, which are important for cystoskeletal remodelling, migrated from the cytosol and nuclear region to the cell periphery. This redistribution was reversed after 24 hour incubation, where paxillin and FAK co-localized with *P. gingivalis* in the perinuclear region (Yilmaz *et al.*, 2003), thus further implicating cytoskeletal rearrangement in the cellular internalisation of *P. gingivalis*. The epithelial receptor-mediated entry of *P. gingivalis* and the rearrangement of the filamentous actin and microtubule networks, suggests a 'zipping' mechanism of invasion. However, there may be more than one mechanism of invasion operating due to the variety of virulence features expressed and different *P. gingivalis* strains may preferentially utilise a particular mechanism. These mechanisms may include clathrin-mediated endocytosis (Boisvert and Duncan, 2008) (section 1.3.4.2.1) or lipid rafts (Tsuda *et al.*, 2008) (section 1.3.4.2.2), both of which have been implicated in *P. gingivalis* invasion.

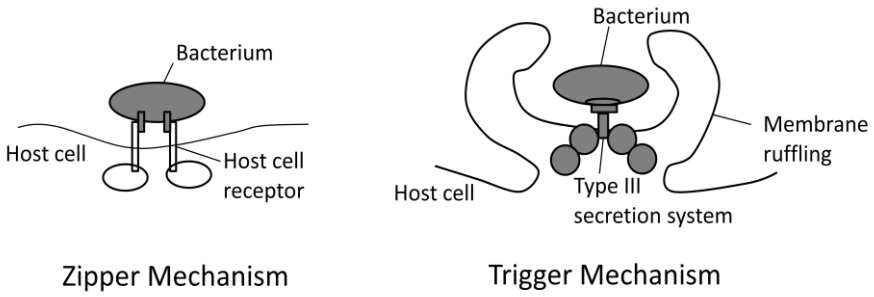

Figure 1.14 Mechanisms of host cell internalisation by invasive bacteria. The zipper mechanism involves the binding of bacteria to host cell receptors and formation of a phagocytic cup resulting in bacterial invasion. The trigger mechanism involves major cytoskeletal rearrangements, producing membrane ruffling, upon an influx of virulence factors secreted by bacteria via their type III secretion system, ultimately resulting in cellular internalisation. (Adapted from Cossart and Sansonetti, 2004).

The modulation of intracellular signalling pathways by *P. gingivalis* may aid in the cellular internalisation of this bacterium, via cytoskeletal rearrangements, and may contribute to the pathogenesis of periodontal disease by inducing the secretion of pro-inflammatory cytokines (section 1.3.4.4). The association of mitogen-activated protein kinase (MAPK) pathways with *P. gingivalis* internalisation of oral epithelial cells has been reported (Watanabe *et al.*, 2001). MAPK pathways involve numerous serine-threonine protein kinases which are sequentially

phosphorylated and dephosphorylated in response to external stimuli, ultimately regulating gene expression through activation of transcription factors, including NFκB. The MAPK superfamily includes c-Jun N-terminal (JNK), extracellular signal regulated kinase (ERK1/2), MEK (MAPK/ERK kinase), which is an upstream regulator of ERK1/2, and p38 MAPK (Robinson and Cobb, 1997) (fig 1.15). In response to *P. gingivalis*, the down-regulation of ERK1/2 activity after 15 minute infection has been shown, correlating with the previously observed time period of host cell invasion (Belton *et al.*, 1999). However, *P. gingivalis* also activated JNK but had no effect on p38 or NFκB, and therefore shows selectivity towards activation of one MAP kinase pathway over another (Watanabe *et al.*, 2001). Stimulation of oral epithelial cells with protein kinase inhibitor, staurosporine and tyrosine-specific protein kinase inhibitor, genistein, significantly reduced the ability of *P. gingivalis* to invade, suggesting an importance of signal transduction mechanisms, and more specifically JNK, in epithelial cell internalisation of *P. gingivalis* (Sandros *et al.*, 1996; Watanabe *et al.*, 2001). However, the application of toxin B from *Clostridium difficile*, a specific inhibitor of Rho family GTPases (Rho, Rac, and Cdc42), which are known to regulate signalling pathways culminating in cytoskeletal rearrangements (fig 1.15), did not inhibit phosphorylation of JNK by *P. gingivalis*, or retard invasion (Watanabe *et al.*, 2001). A specific inhibitor of JNK would provide conclusive evidence regarding the involvement of this protein kinase in host cell internalisation. The association of JNK with cytoskeletal re-organisation further implicates JNK in *P. gingivalis* invasion.

The role of phosphoinositide 3-kinase (PI3K) in bacterial-host cell internalisation is not fully understood, but it may correlate with rearrangements of the actin cytoskeleton through cellular signalling pathways resulting in the closure of the phagocytic cup (Cossart and Sansonetti, 2004). The internalisation of fluorescent beads, coated with *P. gingivalis* membrane vesicles, was shown to decrease in the presence of PI3K inhibitors (wortmannin and LY294002), suggesting that there may be a role of PI3K signalling in the cellular internalisation of *P. gingivalis*. However, more work is required to elucidate the role, if any, of PI3K in the cellular internalisation of *P. gingivalis*.

Calcium ion fluxes have been reported to occur following contact of *P. gingivalis* with epithelial cells (Izutsu *et al.*, 1996; Belton *et al.*, 2004). These transient increases in calcium ion concentrations may contribute to intracellular signalling pathways resulting in cytoskeletal rearrangement (Andrian *et al.*, 2006).

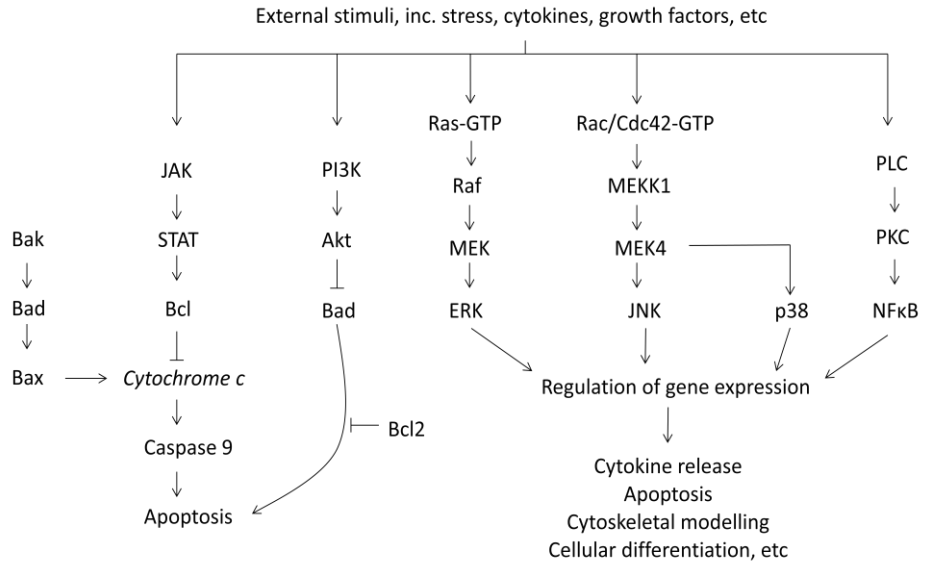

Figure 1.15 Intracellular signalling pathways. Simplified signalling pathways culminating in the regulation of apoptosis and gene expression, which may influence cytokine release, apoptosis, cytoskeletal remodelling and cellular differentiation. **Abbreviations:** Janus Kinase (JAK), Signal Transducer and Activator of Transcription (STAT), apoptosis regulators (Bcl, Bax, Bak, Akt, Bad), phosphoinositide 3-kinase (PI3K), guanosine-5'-triphosphate (GTP), mitogen-activated protein kinase (MEK), c-Jun N-terminal kinase (JNK), phospholipase C (PLC), protein kinase C (PKC), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), family of GTP-ases (Rac/Cdc42, Ras). Adapted from Ruwhof *et al*. (2000), Robinson and Cobb (1997).

1.3.4.2.1 Clathrin-mediated endocytosis

Bacterial internalisation may occur via vesicular trafficking. This involves a dynamic system where vesicles bind at the cell membrane resulting in the carriage of extracellular molecules into the intracellular environment. Clathrin-coated vesicles have been implicated in the epithelial internalisation of *P. gingivalis* (Sandros *et al.*, 1993; Boisvert and Duncan, 2008). Clathrin-coated vesicles consist of the protein, clathrin, which forms a 'basket-like' structure, stabilised by proteins called adaptins. Dynamin, which is a GTP-binding protein, forms a ring around the neck of the vesicle resulting in scission of a ligand-bound vesicle, in a GTPase dependent manner (Lamaze *et al.*, 2001), which may then be transported intracellularly with help from the actin cytoskeleton (Henderson *et al.*, 1999). These vesicles may then be delivered to early or late endosomes. Early endosomes are involved in ligand dissociation and vesicular recycling, whereas late endosomes are involved in the delivery of vesicles to lysosomes, where they are hydrolysed and breakdown products delivered into the cell cytoplasm for recycling (Henderson *et al.*, 1999). It has been proposed that the trafficking of *P. gingivalis* within early endosomes may present a mechanism of bacterial exit from the cell via endosomal recycling to the cell membrane (Takeuchi *et al.*, 2011). *P. gingivalis* cells have been observed free within the cytoplasm and also surrounded by endosomal membranes (Sandros *et al.*, 1994; Takeuchi *et al.*, 2011). It has been suggested that shortly after invasion, *P. gingivalis* traffics to autophagosomes (Dorn *et al.*, 2001), which are multi-membranous vacuoles important in the recycling of cellular

organelles. Autophagosomes mature into autolysosomes where degradation of the vacuole load occurs (Dunn, 1994). Dorn *et al*. (2001) showed the localisation of *P. gingivalis* within autophagosomes, which lack hydrolytic enzymes. The authors proposed that the bacteria are able to replicate within this vacuole, until released to cause re-infection (Dorn *et al.*, 2001). In addition, this vacuole may provide a niche in which this organism can increase the concentration of free amino acids required for survival (Sinai and Joiner, 1997).

The study reported by Boisvert and Duncan (2008), described evidence for clathrin in the association of fluorescent beads coated with the adhesin domain of Arg-gingipain (HA1). By fluorescence microscopy they showed that these fluorescent beads were surrounded by clusters of clathrin. Furthermore, gene silencing of clathrin resulted in the decreased association of HA1 with epithelial cells (Boisvert and Duncan, 2008). However, conflicting data within the literature has indicated that *P. gingivalis* does not require clathrin for internalisation. For example, Tsuda *et al*. (2008) reported that an Eps15 mutant lacking EH-domains, of which clathrin is composed, did not inhibit the internalisation of fluorescent beads coated with *P. gingivalis* membrane vesicles (Tsuda *et al.*, 2008). Therefore, these authors suggested a different mechanism of *P. gingivalis* invasion, i.e. via lipid rafts.

1.3.4.2.2 Internalisation via lipid rafts

Membrane subdomains rich in cholesterol, sphingolipids and glycosylphosphatidylinositol-anchored proteins (GPI-APs), known as lipid rafts are thought to be utilised by bacteria to adhere to and invade host cells (Lafont and Van Der Goot, 2005). Tsuda *et al*. (2008) used fluorescence microscopy to show that the internalisation of fluorescent beads coated with bacterial membrane vesicles, which have surface components of *P. gingivalis*, was inhibited following treatment of epithelial cells with cholesterol-binding agents. Another type of lipid raft is thought to be rich in the protein caveolin, with which the ganglioside GM1 clusters. Caveolae form invaginations in the cell membrane and are thought to be involved in cholesterol homeostasis, endocytosis and cell signalling (Simons and Ikonen, 1997; Parton and Richards, 2003). It has been shown that the fluorescent beads coated with *P. gingivalis* vesicles were shown to co-localise with GM1 and caveolin-1, further suggesting a role of lipid rafts in the host cell association of *P. gingivalis* (Tsuda *et al.*, 2008). In addition, compounds used to disrupt lipid raft composition, such as filipin, nystatin and methyl-β-cyclodextrin (MβCD) were shown to decrease fluorescent bead internalisation as observed by microscopy (Tsuda *et al.*, 2008). However, there is controversy regarding the existence of lipid rafts (Munro, 2003), and more research is required into the characterisation of these cholesterol and sphingolipid enriched microdomains before an understanding of the association of lipid rafts with bacterial pathogenesis is complete (Lafont and Van Der Goot, 2005).

Nevertheless, the mechanism of invasion of oral epithelial cells by *P. gingivalis* is not yet fully understood and as such requires more research.

1.3.4.3 Intracellular lifestyle of *P. gingivalis*

Intracellular *P. gingivalis* cells have been shown to up-regulate and down-regulate specific subsets of genes/proteins, compared with those bacteria that remain extracellular (Xia *et al.*, 2007; Suwannakul *et al.*, 2010). Those factors that are down-regulated upon internalisation include FimA, gingipains (RgpA, RgpB, Kgp), and haemin acquisition proteins (HmuR, FetB, IhtB) (Xia *et al.*, 2007). The decreased expression of FimA is thought to aid in evasion of intracellular microbial recognition systems (Kufer *et al.*, 2006). Modulation of gingipains following internalisation is suggested to be important for the continued survival of the host cell. As the cytoplasm is rich in nutrients, it is thought that the expression of gingipains is not as important as within the extracellular environment, where nutrient acquisition within nutrient poor environments is key to survival (Xia *et al.*, 2007). This has also been suggested as the reason for the decreased expression of haemin acquisition proteins, and an increase in the expression of haemin storage proteins by intracellular *P. gingivalis*, i.e. due to the high intracellular haemin concentrations (Xia *et al.*, 2007).

P. gingivalis is equipped with a cohort of stress-protector proteins including alkyl hydroxyperoxide reductase subunit C (Johnson *et al.*, 2004), rubrerythrin (Mydel *et al.*, 2006), superoxide dismutase (Ohara *et al.*, 2006) and transcriptional activator *oxyR* (Diaz *et al.*, 2006). As an anaerobic organism, *P. gingivalis* is unable to survive for prolonged periods in environments of increased oxygen tension (Madianos *et al.*, 1996), for example during initial colonisation of the oral cavity and colonisation of additional sites, where there is a high probability of exposure to air, or increased oxidative stress as the result of host defences, e.g. neutrophils (Mydel *et al.*, 2006; Diaz *et al.*, 2006). However, the above 'stress-protection' systems appear to allow the organism sufficient time to become established in an anaerobic environment for successful colonisation. These systems are especially important in intracellular survival, as it has been shown that intracellular *P. gingivalis* show an enhanced expression of oxidative stress proteins, such as alkyl reductases, thiol peroxidise, rubrerethrin and members of the Clp family (Xia *et al.*, 2007).

In animal models of periodontitis, the formation of an abscess following infection with *P. gingivalis* is a sign of the pathogenic potential of this organism. As such, it could be assumed that this organism, in humans, may have the same effect, i.e. cause cell death. It has been reported in the literature that *P. gingivalis* induces apoptosis of oral epithelial cells (Sheets *et al.*, 2006; O'Brien-Simpson *et al.*, 2009). This induction of apoptosis may occur via up-regulation of pro-apoptotic molecules, including Bax and caspase-9, which may be gingipain-

dependent (Stathopoulou *et al.*, 2009a). However, there is conflicting evidence in the literature. Some studies have suggested that intracellular *P. gingivalis* may protect the host from apoptotic cell death, possibly through the inactivation of pro-apoptotic Bad and inhibition of caspase-9 activation (Yao *et al.*, 2010), and/or through manipulation of the JAK/STAT signalling pathway (Mao *et al.*, 2007) (fig 1.15). This suggests that *P. gingivalis* may be adapted to live in concert with the host and as such its definition as a 'pathogenic' organism may require revision (Xia *et al.*, 2007). Whether *P. gingivalis* acts to prevent or induce apoptosis may be due to the experimental conditions of each study, such as the cells used, strains of *P. gingivalis* and length of incubation times. For example, over time, intracellular *P. gingivalis* has been shown to down-regulate gingipains (Xia *et al.*, 2007), which may contribute to prolonged cellular survival (Boisvert and Duncan, 2010), via preventing the activation of pro-apoptotic molecules (Stathopoulou *et al.*, 2009a).

1.3.4.4 Host response to *P. gingivalis*

Epithelial cells secrete chemokines, including macrophage inflammatory protein-1α (MIP-1α/CCL3), monocyte chemoattractant protein-1 (MCP-1/CCL2) and interleukin 8 (IL-8/CXCL8), which aid leukocyte migration, regulating inflammatory responses (Huang *et al.*, 2001). In addition, the increased expression of pro-inflammatory cytokines, including the interleukins IL-1β, IL-6 and tumour necrosis factor-α (TNF-α) has been shown in patients exhibiting periodontitis, compared with healthy controls (Andrukhov *et al.*, 2011). In relation to oral epithelial cell invasion by *P. gingivalis* it has been suggested that the expression of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6, is positively correlated with the adhesive/invasive ability of *P. gingivalis* (Sandros *et al.*, 2000), suggesting an importance of cellular invasion in the pathogenesis of disease.

Up-regulation of CXCL8 and ICAM-1 in gingival epithelial cells in response to challenge by oral pathogens, such as *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum*, has been reported to be the result of the host immune response culminating in the migration of leukocytes to the site of inflammation (Huang *et al.*, 1998; Han *et al.*, 2000). However, challenge by *P. gingivalis* has been shown to cause a down-regulation in the expression of CXCL8 and ICAM-1 (Madianos *et al.*, 1997; Huang *et al.*, 1998; Darveau *et al.*, 1998). Interestingly, the transcription of CXCL8 and ICAM-1 genes was increased in the presence of all three bacteria, including *P. gingivalis*, suggesting that translational and/or post-translational regulatory mechanisms are in place resulting in the decrease in secretion of CXCL8 and ICAM-1 by gingival epithelial cells in the presence of *P. gingivalis* (Huang *et al.*, 2001) (table 1.4). This down-regulation of protein secretion by *P. gingivalis* has been termed local chemokine paralysis (Darveau *et al.*, 1998). However, conflicting data remains in the literature as to the specific cause of this down-regulation, and, whether there is a decrease in cytokine expression

or not. Table 1.4 highlights specific studies using live, whole cell *P. gingivalis* incubated with oral epithelial cells, and indicates the wide variety, and often conflicting sets, of data reported in the literature regarding the up-regulation/down-regulation of pro-inflammatory cytokines. These differences may be due to the differences in experimental profile between studies, the epithelial cells and the strains of *P. gingivalis* used. Therefore, further work is required in this area to elucidate the specific mechanism and ramifications of the modulation of cytokine/chemokine protein expression by epithelial cells in the presence of live, whole cell *P. gingivalis*.

The *P. gingivalis* gingipain cysteine proteinases have been shown to degrade purified CXCL8 (Mikolajczyk-Pawlinska *et al.*, 1998) and Huang *et al*. (2001) suggested that this may be the mechanism of decreased CXCL8 secretion, with the arginine-specific gingipain playing the greatest role (Giacaman *et al.*, 2009; Stathopoulou *et al.*, 2009b). However, it has been shown that *P. gingivalis* does not activate NF-κB due to down-regulation of ERK1/2 (Watanabe *et al.*, 2001), therefore this could explain the decreased secretion of CXCL8 in the presence of *P. gingivalis*. In addition, a *P. gingivalis* SerB knockout mutant has been shown to inhibit the suppression of CXCL8 secretion by epithelial cells (Hasegawa *et al.*, 2008), suggesting that the presence of SerB expressed by wild-type *P. gingivalis* acts to suppress CXCL8 release. In contrast, *P. gingivalis* has been shown to inhibit the production of IL-6 (Moffatt and Lamont, 2011). *P. gingivalis* has been shown to induce microRNA-203, which binds to suppressor of cytokine signalling 3 (SOCS3) in gingival epithelial cells. This protein targets STAT3, decreasing its activation and inhibiting IL-6 release. However, in the presence of *P. gingivalis*, the induction of microRNA-203 inhibits the action of SOCS3, resulting in an increase in the release of IL-6, which may contribute to bone resorption and further pro-inflammatory effects (Moffatt and Lamont, 2011). Despite these in depth studies, the precise mechanism of cytokine induction/down-regulation needs to be further investigated.

The down-regulation of CXCL8 may play a role in tissue damage in periodontal disease due to the resulting decreased recruitment of neutrophils to aid in the host immune response against bacterial antigens (Madianos *et al.*, 1997; Huang *et al.*, 2001). Nakayama *et al*. (1995) presented data to suggest that secreted Arg-gingipain also contributes directly to the inhibition of PMNs. Using *rgpA*, *rgpB* and *rgpArgpB* knock-out mutants, the authors reported a decrease in chemiluminescence response of guinea-pig PMNs in the presence of the wild-type strain ATCC 33277 compared to the mutants. The *rgpArgpB* mutant showed almost no inhibition of PMNs. This suggests the significance of gingipains in the inhibition of PMNs, which may result in progression of periodontal destruction due to decreased immune response to *P. gingivalis* challenge (Nakayama *et al.*, 1995).

Arg-gingipain and, to a lesser extent, Lys-gingipain, have the ability to degrade tumour necrosis factor-alpha (TNF-α) (Mezyk-Kopec *et al.*, 2005). TNF-α exists in the soluble, excreted form and membrane bound form and is an important part of the inflammatory process involving immune cell activation. Release of the soluble form results from the proteolytic cleavage of membrane bound TNF-α by metalloprotease TNF-α converting enzyme (ADAM-17) (Black *et al.*, 1997). TNF-α is secreted in response to bacterial LPS and IL-1. TNF-α activation of its specific receptor results in signal transduction via activation of NF-κB and MAPK pathways and induction of apoptosis.

It has been reported previously that gingipains have the ability to degrade the major LPS receptor, CD14 (Sugawara *et al.*, 2000; Duncan *et al.*, 2004), leading to suppression of activation by LPS, thus reducing the secretion of TNF-α. Mężyk-Kopeć *et al*. (2005) showed that gingipains are able to degrade both soluble and membrane-bound TNF-α. However, an increase in TNF-α has been recorded in relation to periodontal disease (Górska *et al*., 2003). Nevertheless, an initial increase in TNF-α combined with the effects of other released cytokines at the site of inflammation aid the host in preventing bacterial invasion, whereas the degradation of such cytokines by *P. gingivalis* may contribute to disease progression.

P. gingivalis LPS and fimbriae mediate cytokine release from gingival epithelial cell lines (Asai *et al.*, 2001). Wang and Ohura (2002) proposed that *P. gingivalis* LPS binds to CD14 expressed on the surface of human gingival fibroblasts stimulating cytokine production (IL-1 and IL-6) via activation of NF-κB and activating protein (AP-1). However, this is not consistent with the other findings above and so further characterisation of LPS-mediated cytokine release is required to increase understanding of the mechanism(s) involved (Andrian *et al.*, 2006).

Gingipains are a major factor in the virulence of *P. gingivalis* (Katz *et al.*, 2002) and it has been suggested that they contribute to tissue destruction and may also play a role in degradation of the basement membrane (Andrian *et al.*, 2004). Stimulation of gingival epithelial cells by *P. gingivalis* induces the release of MMPs, specifically MMP-1, MMP-2, MMP-7, MMP-8, MMP-9 and MMP-13, which have been shown to be associated with the severity of periodontal disease (Tervahartiala *et al.*, 2000; Andrian *et al.*, 2006) (section 1.2.3.2). These MMPs are specific collagenases and gelatinases, which degrade connective tissue components and basement membrane type IV collagen and so are likely to contribute to periodontal tissue destruction (Andrian *et al.*, 2007).

Table 1.4 The effect of *P. gingivalis* on the secretion and mRNA expression of chemokines and cytokines by oral epithelial cells. This table shows selected references that outline the protein secretion and/or mRNA expression of chemokines and/or cytokines released in response to *P. gingivalis*. The cytokine(s) described in each article, the experimental profile, how the cytokine/chemokine was detected and the result of the experiment is shown. Only whole live bacterial cells are reported in the table, unless otherwise stated. **Cytokines/chemokines:** Interleukin 1 receptor-associated receptor-M (IRAK-M), monocyte chemoattractant protein (MCP-1/CCL2), interleukin (IL), interleukin 8 (CXCL8), tumour necrosis factor alpha (TNFα), macrophage inflammatory protein 3a (MIP3a/CCL20), granulocyte-macrophage colony-stimulating factor (GM-CSF), soluble intercellular adhesion molecule-1 (sICAM1). ***P. gingivalis* strains (Pg):** ATCC33277, 381, W83, W50, MS-1-2 (clinical strain); gingipain mutant strains MT10 (Δ*rgpA*), G-102 (Δ*rgpB*), KDP133/E8 (Δ*rgpArgpB*), KDP129/V2296/K1A (Δ*kgp*), KDP136/KDP128 (Δ*rgpArgpBkgp*); multiplicity of infection (MOI). **Epithelial cells (EC):** epithelial cell lines (KB, OKF6/TERT2, epi4); primary human gingival epithelial cells (HGEC); cultured as monolayers unless otherwise stated. **Experimental profile:** 'extracellularly' (*P. gingivalis* incubated with epithelial cells for the specified time points→conditioned media analysed or mRNA expression tested); statistically significant increase/decrease/no change in secretion/expression relative to unstimulated cells is shown. **Additional abbreviations:** Polymerase chain reaction (PCR), protease inhibitor (PI), Tosyl-L-lysyl-chloromethane hydrochloride (TLCK).

Cytokine(s) of interest	**Experimental profile**	**Assessed by**	**Increase/Decrease/no change?**	**Reference**
CXCL8, ICAM1	EC: KB Pg: 381 MOI: 150 Profile: Pg 90min→wash →antibiotic 8hrs	ELISA (CXCL8) Immunofluorescent staining (ICAM1)	CXCL8, ICAM1 decrease	Madianos *et al*., 1997
		mRNA expression not analysed		
CXCL8, ICAM1	EC: HGEC Pg: ATCC33277 (live and heat-killed) MOI: 100-2000 Profile: Pg 2hr→wash→24hr metronidazole	ELISA (CXCL8) Flow cytometry (ICAM1)	CXCL8 and ICAM1 decrease (MOI100-2000) Heat-killed: CXCL8 increase (MOI500-2000)	Huang *et al*., 1998
		mRNA expression not analysed		

CXCL8	EC: HGEC Pg: ATCC33277, 381 MOI: 10^8/well Profile: extracellularly 18 hours	Cytoscreen CXCL8 Immunoassay kit (Biosource International, Camarillo, CA)	CXCL8 decrease	Darveau *et al*., 1998
		Real-time PCR	CXCL8 decrease	
CXCL8	EC: KB Pg: 381+/-PI (TLCK) MOI: 100 Profile: extracellularly 30min	ELISA	CXCL8 decrease (no change with PI)	Zhang *et al*., 1999
		Northern Maxikit (Ambion, Inc)	CXCL8 increase	
CXCL8, ICAM1	EC: immortalised GECs Pg: ATCC33277; V2296 (Δ*kgp*), parent W83; MT10 (Δ*rgpA*), G-102 (Δ*rgpB*), parent 381 MOI: 500-2000 Profile: Pg 2hr→wash→2-20hr metronidazole	ELISA	All strains: CXCL8 and ICAM1 decrease after 12 hours	Huang *et al*., 2001
		Northern blot analysis	ATCC33277: CXCL8 and ICAM1 increase up to 4 hours, decrease after 8 hours	

IL1β, IL-6, CXCL8, TNFα	EC: KB, pocket EC Pg: 381 MOI: 150 Profile: extracellularly 4 hours	Immunohistochemistry	All cytokines increase	Sandros *et al*., 2000
		In situ hybridisation	All cytokines increase	
IL1β, IL-6, CXCL8, TNFα	EC: Palatal cell organotypic model Pg: ATCC33277 MOI: 10^5/model Profile: extracellularly 24 hours	ELISA	All cytokines increase	Andrian *et al*., 2004
		mRNA expression not analysed		
IL1β, IL-6, CXCL8, TNFα	EC: KB Pg: ATCC33277 MOI: 20 Profile: 1hr Pg→wash→ metronidazole 1.5hr→wash →6hr-3days	ELISA	IL1β, IL-6 TNFα not detectable CXCL8 increase after 6 hours, up to 3 days	Eick *et al*., 2006
		PCR→ gel electrophoresis→ densitometry	CXCL8 increase after 3 days IL1β, TNFα, IL-6 initially increase but decrease after 3 days	
CXCL8, TNFα, IL-6, GM-CSF	EC: HGEC Pg: ATCC33277 MOI: 100 Profile: extracellularly 4 hours	Luminex 100 technology	CXCL8, TNFα, IL-6, GM-CSF increase	Eskan *et al*., 2007
		mRNA expression not analysed		

CXCL8	EC: Immortalised GECs Pg: ATCC33277 MOI: 100 Profile: extracellularly 4 hours	ELISA	No change	Hasegawa *et al*., 2008
		mRNA expression not analysed		
IL1α, IL1β, IL-6, TNFα	EC: OKF6/TERT2 Pg: ATCC33277 (parent), KDP133Δ*rgpArgpB*, KDP129Δ*kgp*, KDP136 Δ*rgpArgpBkgp* MOI: 10, 100, 1000 Profile: Pg 3hr→wash→0-48hr	Protein secretion not analysed		Giacaman *et al*., 2009
		Real-time PCR	IL1α: up to 6, 12hr (parent increase) up to 6hr (Δ*kgp* increase) IL-1β: up to 48hr (parent increase) up to 12hr (Δ*kgp* increase) TNFα: up to 6hr (parent and Δ*kgp* increase) IL-6: up to 12hr (parent and Δ*kgp* increase) All cytokines: after 48hr (Δ*rgpArgpB*, Δ*rgpArgpBkgp* no change)	
IL1α, IL1β, IL-6, CXCL8, TNFα, CCL20, sICAM1, CCL2	EC: KB Pg: W50 MOI: 10, 50, 100, 500, 1000, 10000 Profile: Pg 90min→wash→16hr	ELISA	sICAM1, CXCL8, IL-6 increase up to MOI100 (MOI500 and 1000 decrease) CCL2 increase (MOI10), no change (MOI>10) CCL20 increase at MOI500-1000 IL1α increase at MOI100-1000 IL1β, TNFα not detected	O'Brien-Simpson *et al*., 2009
		mRNA expression not analysed		

<table>
<tr>
<td>IL1β, IL-6, IL8</td>
<td>EC: HGEC
Pg: W50 (parent), E8 (ΔrgpArgpB), K1A (Δkgp), KDP128 (ΔrgpArgpBkgp), ATCC33277
MOI: 100
Profile: extracellularly 4 and 24 hours</td>
<td>ELISA, Western blot</td>
<td>ATCC33277: IL1β decrease (4hr), IL1β increase (24hr), IL-6 decrease (4 and 24 hr), CXCL8 decrease (4 and 24 hr)
<table>
<tr><th>Cytokine</th><th>W50</th><th>E8</th><th>K1A</th><th>KDP128</th></tr>
<tr><td>IL1b</td><td>Dcrease after 4 hr</td><td>Decrease 4 hr</td><td>No change</td><td>No change</td></tr>
<tr><td>IL-6</td><td>Decrease after 30 min</td><td>Decrease after 30 min</td><td>No change</td><td>No change</td></tr>
<tr><td>IL-8</td><td>Decrease after 30 min</td><td>Decrease after 1hr</td><td>No change</td><td>No change</td></tr>
</table>
</td>
<td>Stathopoulou et al., 2009, 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td colspan="2">mRNA expression not analysed</td>
<td></td>
</tr>
<tr>
<td>IL1β, IL10, IL 12, IL-6, CXCL8, CCL2, TNFα</td>
<td>EC: HGEC multilayer
Pg: ATCC33277
MOI: 100
Profile: extracellularly 2&24 hours</td>
<td>Millipore MILLIPLEX Map kit Human Cytokine/Chemokine custom 7-Plex Multi-Cytokine Detection System</td>
<td>No change in all tested cytokines</td>
<td>Dickinson et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td colspan="2">mRNA expression not analysed</td>
<td></td>
</tr>
<tr>
<td>IL-6, CXCL8</td>
<td>EC: KB
Pg: ATCC33277, MS-1-2
MOI: not specified
Profile: extracellularly 18 hours</td>
<td>ELISA</td>
<td>ATCC33277: IL-6 increase, CXCL8 no change
MS-1-2: IL-6 and IL8 decrease</td>
<td>Kirschbaum et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Real time-PCR</td>
<td>IL-6 and CXCL8 increase</td>
<td></td>
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</table>

IRAK-M, CXCL8, CCL2	EC: HGEC, epi4 Pg: 381 (live and heat-killed) MOI: 50 Profile: extracellularly 12 hours	ELISA	CXCL8 no change (live), CXCL8 increase (killed), CCL2 no change	Takahashi *et al*., 2010
		Real time PCR	CCL2 no change, IRAK-M increase, CXCL8 increase (live and killed)	
CCL20	EC: GEC Pg: ATCC33277 MOI: 50 Profile: extracellularly 16 hours	Protein concentration not analysed		Dommisch *et al*., 2010
		Real time PCR	CCL20 increase	

1.4 Experimental models

1.4.1 Monolayer

To investigate *P. gingivalis* invasion of oral mucosa, the most commonly implemented method is the antibiotic protection assay, using monolayer cultures of primary oral epithelial cells (Lamont *et al.*, 1995; Weinberg *et al.*, 1997) or oral epithelial cell lines (Duncan *et al.*, 1993; Madianos *et al.*, 1996; Umeda *et al.*, 2006). The antibiotic protection assay involves the incubation of a known number of bacterial cells, commonly at a multiplicity of infection (MOI) of 100, i.e. 100 bacterial cells for each epithelial cell, incubated with a monolayer culture of cells. After incubation, non-adherent bacteria are washed away and adherent non-internalised bacteria are killed using antibiotics. Cells are lysed and a viable count of internalised bacterial cells can be determined.

Monolayer cultures of oral derived cell lines are reproducible and easily obtainable. However, the use of cell lines has reduced value compared to primary cultures of oral epithelial cells as these virally transformed or tumour-derived cells almost certainly express different cell surface markers and have been shown to exhibit a lower percentage adherence and invasion by *P. gingivalis* (Duncan *et al.*, 1993; Lamont *et al.*, 1995). These differences were discussed in a review by Andrian *et al.* (2004), in which the authors suggest that the decreased number of receptors for *P. gingivalis* on KB cells, down-regulation of the MAPK/ERK1/2 signalling pathway in gingival epithelial cells in comparison to KB cells, and greater incidence of envelopment by endosomal vacuole of *P. gingivalis* within KB cells plays a role in the differences in percentage invasion noted between the two cell types. However, it has been shown that although originally isolated as an epidermal carcinoma of the mouth, there is now contamination of the KB cell line with the cervical carcinoma cell line, HeLa (Catalogue of Cell Lines and Hybridomas, ATCC). Therefore, comparison of *P. gingivalis* invasion of specific oral epithelial cell lines may help identify a suitable transformed/tumour-derived monolayer candidate to use in the event of limited primary cell availability.

In 1975, Rheinwald and Green were the first to propose the *in vitro* culture of primary human keratinocytes using an irradiated mouse fibroblast 3T3 feeder layer. The feeder layer helped to promote cell survival, adhesion and proliferation of keratinocytes (Wang *et al*., 2001). However, the presence of mouse fibroblasts within cultured epithelial sheets is a disadvantage as there may be contamination with mycoplasma, viruses and prions, and as an animal-derived product, cannot be used in patient treatment/transplantation. Therefore, researchers have developed keratinocyte monolayer cultures using collagen-coated flasks, in the absence of a feeder layer (Riva *et al.*, 2007). This resulted in pure keratinocyte cultures, in which data from subsequent *in vitro* studies may not be skewed by the presence of contaminating fibroblasts.

Monolayer cultures are useful because they are relatively easy to maintain and reproduce. However, the absence of a multi-layer, stratified squamous epithelium, fibroblast-infiltrated lamina propria and associated epithelial cell interactions and other features of normal oral mucosa indicate that *P. gingivalis* invasion of monolayer cell cultures will not provide a complete understanding of the complexity involved in bacterial invasion of periodontal tissues. Therefore, the engineering of a suitable model to represent normal oral mucosa may be a step towards a greater understanding of the mechanisms surrounding oral mucosal challenge by *P. gingivalis*.

1.4.2 Tissue-engineered oral mucosa

The development of full-thickness, multi-layer, engineered oral mucosa which resembles normal oral mucosa has a number of uses (Moharamzadeh *et al.*, 2007a), including clinical applications involving grafting in reconstructive surgery (Izumi *et al.*, 2004), and *in vitro* applications including biocompatibility testing and as models of disease, e.g. to assess the invasive capability of *P. gingivalis* (Andrian *et al.*, 2004).

Full-thickness engineered oral mucosa requires a connective tissue layer (lamina propria) with infiltrating fibroblasts, a continuous basement membrane and stratified squamous oral epithelial cell layers (Moharamzadeh *et al.*, 2007a). The connective tissue layer may consist of a suitable scaffold, by which fibroblasts may be introduced, to provide sufficient support for the epithelial cell layer. Fibroblasts are commonly introduced by seeding onto the scaffold surface after which they infiltrate the scaffold synthesising extracellular matrix and collagen, providing multifactorial signalling between the connective tissue and epithelial cell layer, inducing cytokine and MMP release and facilitating differentiation and proliferation of epithelial cells (Atula *et al.*, 1997; Andrian *et al.*, 2004; Moharamzadeh *et al.*, 2007a). Primary oral epithelial cells or oral cell lines may be introduced onto the scaffold surface (fig 1.16). Cells are then raised to the air-to-liquid interface after a few days to form a differentiated epithelial layer; cell lines form multilayered epithelium but show less differentiation than primary cultures. A number of scaffolds have been used to engineer full-thickness oral mucosa. These include naturally-derived scaffolds (acellular cadaveric dermis (AlloDerm™) and de-epidermalised dermis (DED)), collagen-based scaffolds (collagen gel) and synthetic scaffolds (polycarbonate-permeable membranes) (Moharamzadeh *et al.*, 2007a). A number of *in vitro* three-dimensional oral mucosal models are commercially available and have been developed by SkinEthic Laboratories (Nice, France) and MatTek Corp. (Ashland, MA, USA). These constructs are expensive but show little batch-to-batch variability and are commonly used for biocompatibility/safety testing and drug discovery/development.

Increased expression of proliferation marker Ki-67 and cytokeratins CK14, CK19 and secretion of metalloproteases, including, gelatinase A-type IV collagenase and gelatinase B-type IV collagenase, have been reported for an engineered oral mucosa consisting of a fibroblast-populated collagen scaffold seeded with primary oral epithelial cells (Rouabhia and Deslauriers, 2002). Analysis of the culture supernatant from this model indicated the constitutive presence of secreted cytokines, including IL-1β, IL-8 and TNF-α (Rouabhia and Deslauriers, 2002). The presence of fibroblasts within the scaffold is required in order to produce an engineered three-dimensional construct that most closely represents oral mucosa. In a DED construct, the presence of fibroblasts within the collagen matrix showed an improved expression of differentiation markers, such as involucrin, loricrin and filaggrin, by seeded keratinocytes (Lee *et al.*, 2000). The authors suggest that the presence of fibroblasts is essential in the formation of differentiated epithelium. The use of naturally-derived scaffolds, e.g. DED or AlloDerm™, provides a natural, highly durable collagen matrix with a high-tensile strength, in which one side consists of an intact basal lamina for the attachment of epithelial cells and the opposing side, which is more porous, allowing infiltration by seeded fibroblasts (Izumi *et al.*, 1999).

For the investigation of *P. gingivalis* invasion, there has only been one study using tissue-engineered oral mucosa. Andrian *et al.* (2004) used a collagen-based scaffold of bovine skin type III collagen mixed with fibroblasts, supported with an anchor to prevent collagen contraction. Epithelial cells were seeded onto the engineered lamina propria and after 5 days were raised to the air-liquid interface, forming a three-dimensional construct consisting of stratified epithelium. Interaction of fibroblasts with epithelial cells induced the expression of β1 and α2β1 integrins and secretion of basement membrane proteins, including laminin. *P. gingivalis* invasion of this model was visualised by transmission electron microscopy showing that *P. gingivalis* penetrated below the superficial layers and a few *P. gingivalis* cells reached the underlying connective tissue. This engineered oral mucosa was used by the same research group to show that *P. gingivalis* was able to regulate the production of MMPs and TIMPs by oral epithelial cells (Andrian *et al.*, 2007).

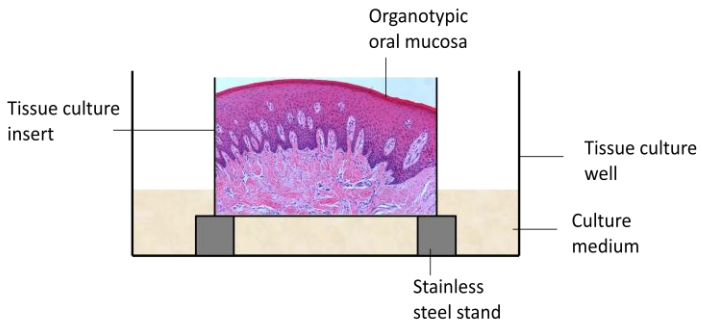


Figure 1.16 Culture of an organotypic model. Organotypic oral mucosal models are cultured within tissue culture inserts, within a tissue culture well, and then raised to the air-to-liquid interface using stainless steel stands to enable keratinocyte stratification and differentiation.

1.4.3 Animal models

The most commonly used animal model to investigate the invasive ability/virulence of *P. gingivalis* is the murine abscess model (Chen *et al.*, 1987; Chen *et al.*, 1990; O'Brien-Simpson *et al.*, 2001; Nakano *et al.*, 2004; Kesavalu *et al.*, 2007). A similar model exists for the use of guinea pigs instead of mice (Sundqvist *et al.*, 1979; Mayrand and McBride, 1980). After subcutaneous injection of *P. gingivalis* the following signs are indicative of virulence: phlegmonous abscesses with pus or lesions and/or necrosis, ulcerative spreading lesions, secondary lesions, erythema, ruffling, septicaemia and death. The severity of disease presentation is indicative of the virulence of the bacterial strain (Steenbergen *et al.*, 1987). Genco *et al.* (1991) modified this model creating a murine/guinea-pig chamber model which involves subcutaneous implantation of Teflon cages, allowing continual access to chamber contents (Genco *et al.*, 1991), including secreted host factors and virulence factors from injected bacteria. This model increases the scope of investigating the virulence of *P. gingivalis* but is relatively expensive to establish and requires specialist husbandry facilities.

Models simulating periodontal disease of the oral cavity in animals have been developed. Kesavalu *et al*. (2007) introduced bacterial species into a rat model by oral gavage to investigate polymicrobial infections and associated alveolar bone resorption. Similarly, bone resorption and gingipain proteolytic activity was reported following the induction of experimental periodontitis in a hamster model and murine model respectively, via the introduction of *P. gingivalis* at the gingival margins of maxillary molar teeth (Pathirana *et al.*, 2007a; Hojo *et al.*, 2008). These models provide data to explain the *in vivo* virulence of *P. gingivalis*, however, the study of the specific mechanisms involved in *P. gingivalis* virulence are limited.

1.4.4 Summary of experimental models

Monolayer cultures of tumour-derived or virally transformed cell lines show good reproducibility, little batch-to-batch variability and are readily available a day or two after sub-culturing. Monolayer cultures of primary oral keratinocytes have lower availability compared to cell lines because there may be periods when biopsies may not be obtainable, the length of time taken to reach confluence is longer than that of a highly proliferative cell-line and the number of sub-cultures is vastly limited in comparison. However, primary cell cultures more closely resemble normal oral epithelial cells *in vivo*, which is important when investigating specific cell responses. Nevertheless, monolayer cultures are a quick and easy method to investigate a wide variety of invasive mechanisms of *P. gingivalis*, from cytoskeletal rearrangements (Yilmaz *et al.*, 2003) and cytokine release (Huang *et al.*, 2001) to the effects of knockout mutants on invasive ability (Pathirana *et al.*, 2008). However, these *in vitro* methods only partly mimic the *in vivo* situation. There is incomplete differentiation of epithelial cells and there are no interactions between the epithelium and the extracellular matrix (Lee *et al.*, 2000). Simulation of

the disease process *in vivo* by means of an animal model serves to provide a greater understanding of the mechanisms involved in invasion as cell-extracellular matrix interactions are present and host cell interactions/immune responses exist. These models assess the clinical features of periodontal disease, including bone resorption, cytokine release (Hojo *et al.*, 2008) and tissue destruction (O'Brien-Simpson *et al.*, 2001) but there is limited information from animal models about the mechanisms of invasion of *P. gingivalis*. Therefore, the development of multi-layered engineered three-dimensional oral mucosa, which resembles normal oral mucosa, may provide a suitable model for the investigation of *P. gingivalis* invasion. The absence of the 'whole body' still limits this *in vitro* system but the presence of epithelial-extracellular matrix communication provides a better model than a single layered epithelial cell system. In addition, cells of the immune system can be introduced to the model (Schaller *et al.*, 2004) to better represent the *in vivo* situation, where host immune cells contribute to the host response in the presence of *P. gingivalis*.

1.5 Summary

The oral mucosa is a complex structure consisting of a multi-layered, differentiated and occasionally keratinised epithelium connected to an underlying connective tissue by means of a basement membrane. This connective tissue layer is home to fibroblasts, mast cells, macrophages and infiltrating neutrophils. The oral mucosa forms part of the periodontium, along with the periodontal ligament, root cementum and alveolar bone. Periodontitis is an inflammatory disease that has been shown to have a bacterial aetiology and *P. gingivalis* has been implicated in disease progression.

The majority of studies on cell interaction with *P. gingivalis* utilise a simple monolayer culture of various types of epithelial cells, either primary cells obtained from a biopsy or carcinoma cell lines. However, this model is limited in terms of the absence of an epithelial multilayer and a fibroblast-embedded connective tissue scaffold, and is therefore not as representative of the normal oral mucosa as tissue-engineered mucosal equivalents. Such models, therefore, need to be used to gain more relevant information on how *P. gingivalis* is likely to interact with the periodontium.

P. gingivalis is an invasive bacterium and has been shown to multiply intra-cellularly and to propagate inter- and intra-cellularly. However, the mechanism of cellular invasion by *P. gingivalis* is not yet fully understood. It is thought to occur via fimbrial interaction with the α5β1 integrin and subsequent cytoskeletal rearrangements, although other mechanisms have been proposed such as clathrin-mediated endocytosis and internalisation via lipid rafts. Also, gingipains have been suggested to contribute to this process in both a promotional and

inhibitory way. Therefore, the role of gingipains, α5β1 and accessory molecules on *P. gingivalis* invasion needs further study.

One aspect on which there are conflicting data within the literature is the epithelial cytokine response to *P. gingivalis*. Both epithelial- and fibroblast-derived cytokines contribute to the pathogenesis of disease, e.g. resulting in bone resorption and/or dysregulated neutrophil chemotaxis. However, there are very few data on the cytokine responses of 3D tissue engineered constructs to *P. gingivalis* and its internalisation. Therefore, for a more representative picture of the cellular responses to *P. gingivalis*, studies should be conducted in mixed cell models.

As well as the cellular features of the periodontium, the environment of the periodontal pocket, within which *P. gingivalis* resides, is quite unique with regards to the high levels of haemin available and higher than physiological temperatures. These factors have been shown to influence the expression of *P. gingivalis* virulence features including LPS and gingipains. Since *P. gingivalis* may utilise the host intracellular environment to evade immune attack, the effect of these environmental factors on cellular internalisation needs to be understood.

1.6 Aims and Objectives

The aims of this study were, therefore to:-

- Develop full-thickness three-dimensional organotypic oral mucosal models (OMM) to resemble normal oral mucosa, including junctional epithelium
    - Compare two fibroblast-embedded connective tissue scaffolds, rat-tail type I collagen and human de-epidermalised dermis and two types of epithelial cell: the oral squamous cell carcinoma of the tongue (H357) and normal oral keratinocytes (NOK) isolated from healthy patients
    - Characterise OMM comparing with normal oral mucosa, in terms of cytokeratin, laminin and E-cadherin expression
    - Optimise OMM for use in an antibiotic protection assay with *P. gingivalis* investigating the period of incubation, atmospheric conditions and methods of OMM lysis
- Compare the percentage invasion of H357 and NOK monolayer and OMM cultures by *P. gingivalis*
    - Evaluate the advantages and disadvantages of each model system
    - Investigate the bacterial survival, intracellular multiplication and release of internalised *P. gingivalis* from monolayer and OMM cultures
    - Determine the effect bacterial culture conditions, including haemin concentration and culture temperature, have on *P. gingivalis* invasion

- Investigate the effect of gingipains on epithelial cell invasion by *P. gingivalis* and expression of cellular receptors
    - Compare the percentage invasion of *P. gingivalis* gingipain mutants
    - Observe changes in α5β1, CD46 and the tetraspanins CD9, CD63, CD81, CD82, CD151 following incubation with *P. gingivalis* gingipain mutants
    - Determine whether disruption of α5β1, CD46 or tetraspanin function influences epithelial internalisation by *P. gingivalis*
- Investigate the cytokine/chemokine response of epithelial monolayers and OMM to *P. gingivalis* and its cellular internalisation
    - Using a cytokine antibody array, determine important epithelial pro-inflammatory cytokines released in response to internalised *P. gingivalis*
    - Quantify levels of secreted individual cytokines (e.g. CXCL8) and their gene expression and how these responses may be modified by *P. gingivalis* gingipains

Chapter 2 Materials and Methods

2.1 Materials

All materials were purchased from Sigma, Poole, UK unless otherwise stated.

2.2 Cell culture conditions

The oral epithelial cell line, H357 (originally isolated from squamous cell carcinoma of the tongue, Health Protection Agency Culture Collections, Porton Down, Salisbury, UK), and primary oral keratinocytes (see section 2.3) were cultured in Green's Medium (Rheinwald and Green, 1975) at 37°C in a humidified and 5% CO_2/95% air atmosphere. Table 2.1 shows the supplements for Green's Medium added in a 3:1 ratio of Dulbecco's Modified Eagles Medium (DMEM) + GlutaMAX™-1 (Gibco®, UK) and Nutrient Mixture F-12 (Ham's F-12).

Primary oral fibroblasts (see section 2.4) were cultured in a humidified atmosphere at 37°C and 5%CO_2/95% air in complete medium (CDMEM), which consisted of DMEM + GlutaMAX™-1 supplemented with foetal calf serum (FCS), penicillin/streptomycin and amphotericin B (table 2.1). Medium was changed every 3-4 days and cells were passaged when 80-90% confluent.

Cells were passaged by washing twice with phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+} and incubating with 0.05% (v/v) porcine trypsin/0.02% (v/v) ethylene diamine tetra-acetic acid (EDTA) at 37°C for 5-10 minutes (dependent on the cell type), until cells lifted from the surface of the flask. Medium containing 10% (v/v) FCS was added to the cell suspension to inhibit the enzymatic activity of the trypsin/EDTA, and cells were centrifuged at 200g for 5 minutes. The supernatant was removed, the cell pellet resuspended in medium and re-seeded at 0.5-1x10^6 cells/T75 flask or 1-2x10^6cells/T175 flask.

All cells were maintained as frozen stocks and regularly tested for mycoplasma infection.

Table 2.1 The constituents of Green's Medium and complete medium. All the following supplements were added in a 3:1 ratio of DMEM + GlutaMAX™-1:Ham's F-12 for Green's medium, to culture the H357 cell line and normal oral keratinocytes (NOK). Asterisked supplements alone were added to DMEM+GlutaMAX™-1 to achieve complete medium (CDMEM) to support the growth of primary oral fibroblasts.

Supplement	Final Concentration
Foetal calf serum*	10% (v/v)
Amphotericin B*	625ng ml^{-1}
Penicillin*	50U ml^{-1}
Streptomycin*	50U ml^{-1}
Adenine	0.1mM
Insulin	5µg ml^{-1}
Transferrin	5µg ml^{-1}
Triiodothyronine	5µg ml^{-1}
Hydrocortisone	0.4µM
Epidermal growth factor	10ng ml^{-1}

2.3 Isolation and culture of normal oral keratinocytes

Primary normal oral keratinocytes were isolated from oral biopsies as previously described (Bhargava *et al*., 2004). Briefly, buccal or gingival biopsies were obtained with written, informed consent from patients undergoing dental surgery at Charles Clifford Dental Hospital, Sheffield, or from healthy donors, under ethical approval granted by the Sheffield Research Ethics Committee (04/Q2305/78, STH Research Department: STH13793). Biopsies were washed with PBS supplemented with 50U ml^{-1} penicillin, 50U ml^{-1} streptomycin and 625ng ml^{-1} amphotericin B, to remove debris, and incubated in 0.1% (w/v) trypsin overnight at 4°C. The epithelium was separated from the connective tissue by gentle scraping and seeded into a tissue culture flask (T-75/biopsy (dependent on the size of the biopsy)) with approximately $5x10^5$ lethally irradiated murine 3T3 fibroblasts (i3T3) (XCELLentis, Gent, Belgium, irradiated with 60 Grays using a cobalt-60 source irradiator). Keratinocytes were cultured in Green's Medium and passaged a maximum of three times due to a lack of epithelial integrity after this time. At each passage NOK were seeded with a feeder layer of i3T3 fibroblasts. The culture purity was confirmed by cytokeratin staining (see Appendix 1) prior to seeding for 2D or 3D culture.

2.4 Isolation and culture of normal oral fibroblasts

Primary normal oral fibroblasts (NOF) were isolated from the connective tissue of oral biopsies by incubating in 0.05% (w/v) collagenase type I (GibcoBRL, Paisley, Scotland) in CDMEM overnight at 37°C. Digested tissue was centrifuged at 1000rpm for 5 minutes. The pellet was resuspended in CDMEM and fibroblasts were expanded. Human fibroblasts were not used after passage 9 due to decreased proliferation rates and signs of cell death at later passages.

2.5 Bacterial strains and growth conditions

P. gingivalis strains (table 2.2) were cultured anaerobically (80% N_2, 10% H_2, 10% CO_2) at 37°C (miniMACS Anaerobic Workstation, Don Whitley Scientific, UK). Plate-cultured laboratory strains were grown on fastidious anaerobe agar (FA; LabM Limited, Lancashire, UK), supplemented with 10% (v/v) defibrinated horse blood (Oxoid, Hampshire, UK). Mutant strains were cultured on FA-blood agar plus the appropriate antibiotic for selection. *P. gingivalis* strains were stored at -80°C as frozen glycerol stocks and streaked onto FA-blood agar weekly. For use in experiments, *P. gingivalis* was sub-cultured from a stock FA-blood agar plate and used at 2 days old. Planktonic cultures were grown in brain heart infusion (BHI) broth (Oxoid, Hampshire UK), supplemented with yeast extract, haemin, vitamin K and cysteine (table 2.3). Overnight broth cultures were used in experiments. Culture purity was tested by Gram-staining and microscopic examination.

Table 2.2 *P. gingivalis* strains used in this study. The table below shows the designation of the *P. gingivalis* strain, the gene that has been mutated, the antibiotic selection marker, where the strain was obtained from and a reference to the original literature.

Strain	Mutation	Antibiotic resistance	Origin	Reference
NCTC 11834	Laboratory strain	None	National Collection of Type Cultures http://www.hpacultures.org.uk/products/bacteria/search.jsp?searchtext=11834&dosearch=true accessed 27/07/11	(Curtis *et al*., 1991)
W50	Laboratory strain	None	Liquid nitrogen stocks within the School of Clinical Dentistry, Sheffield, UK	(Smalley and Birss, 1987, Sojar *et al*., 1997)
E8	Δ*rgpArgpB* (parent strain W50)	10µg ml^{-1} erythromycin	M. Curtis, Barts and The London School of Medicine and Dentistry, London, UK	(Aduse-Opoku *et al*., 2000)
K1A	Δ*kgp* (parent strain W50)	10µg ml^{-1} erythromycin	M. Curtis, Barts and The London School of Medicine and Dentistry, London, UK	(Aduse-Opoku *et al*., 2000)
EK18	Δ*rgpArgpB*Δ *kgp* (parent strain W50)	10µg ml^{-1} erythromycin, 20µg ml^{-1} chloramphenicol	J. Higham, University of Sheffield, UK	Manuscript in preparation. See Appendix 2 for verification of gene knockout.

Table 2.3 The supplements added to brain heart infusion (BHI) broth for the culture of *P. gingivalis* strains. Yeast extract and brain heart infusion broth were autoclaved, and the additional filter sterilised supplements added prior to bacterial culture.

Supplement	Company	Concentration
Brain heart infusion broth	Oxoid, Hampshire, UK	37g L^{-1}
Yeast extract	Oxoid, Hampshire, UK	5mg ml^{-1}
Haemin	Sigma, UK	5µg ml^{-1}
Cysteine-hydrochloride	ICN Biomedicals Inc, Basingstoke, UK	0.5mg ml^{-1}
Vitamin K	Sigma, UK	1µg ml^{-1}

2.6 Characterisation of *P. gingivalis* mutants using the BA*p*NA and tosyl-Gly-Pro-Lys*p*NA hydrolysing assay

Overnight planktonic cultures of *P. gingivalis* W50, E8, K1A and EK18 were analysed using *N*-α-benzoyl-L-Arg-*p*-nitroanilide (BA*p*NA) and toluenesulfonyl-glycyl-L-prolyl-L-lysine *p*-nitroanilide (tosyl-Gly-Pro-Lys*p*NA) as substrates for arginine and lysine gingipains respectively. An increase in absorbance due to the cleavage of nitroaniline indicated the presence of Arg- or Lys-gingipain activity. BHI without bacteria was used as a negative control.

2.6.1 BA*p*NA Assay

The assay was performed as described previously (O'Brien-Simpson *et al*., 2001). Briefly, 2mM BA*p*NA in propan-2-ol (Fisher-Scientific, UK) was diluted 3:10 in enzyme buffer (pH8.0, table 2.4) to form the substrate buffer. Overnight planktonic *P. gingivalis* cultures were adjusted to OD0.5 at 600nm (approximately equal to $1.7x10^9$ cells), with supplemented BHI broth, to ensure the same number of cells were present in each reaction, and added to a 96-well plate with PG buffer (table 2.5), fresh 100mM cysteine (pH8.0) and substrate buffer (table 2.6 shows the volumes of the buffers in each 231µl reaction). The 96-well plate was incubated at 37°C for 1 hour and the absorbance read at 405nm using a microplate reader (POLARstar Galaxy, BMG Labtechnologies, Buckinghamshire, UK). All buffers were stored at 4°C except the 100mM cysteine, which was freshly prepared prior to each assay.

Table 2.4 Enzyme buffer. Enzyme buffer was prepared by dissolving the following reagents in distilled water and adjusting to pH 8.0.

Reagent	Company	Concentration
Tris-HCl	Sigma, UK	400mM
Sodium Chloride (NaCl)	Sigma, UK	100mM
Cysteine	BDH Ltd, Poole, UK	20mM

Table 2.5 PG buffer. The following reagents were dissolved in distilled water to produce PG buffer, pH8.0.

Reagent	Company	Concentration
Tris-HCl	Sigma, UK	50mM
NaCl	Sigma, UK	150mM
Calcium Chloride ($CaCl_2$)	Sigma, UK	5mM
Cysteine	BDH Ltd, Poole, UK	5mM

Table 2.6 BApNA reaction mixture. The volumes of each buffer added to a 96-well plate to assay arg-gingipain activity of *P. gingivalis* strains.

Reagent	**Volume (µl)**
Broth cultured *P. gingivalis* (OD0.5)	8.9
PG Buffer	91.1
Fresh 100mM cysteine	20
Substrate buffer (3:10 2mM BApNA:enzyme buffer)	111

2.6.2 Tosyl-Gly-Pro-Lys-*p*NA Assay

Similar to the BA*p*NA assay, whole cells of *P. gingivalis* wild-type and gingipain knockout mutants (OD0.5 at 600nm) were incubated with PG buffer (table 2.5) and 2µg ml^{-1} Tosyl-Gly-Pro-Lys-*p*NA in 10mM dithiotreitol (DTT) for 2 hours and the absorbance read at 405nm using a microplate reader. The volumes of each component of the assay are shown in table 2.7 to give a total volume of 100µl.

Table 2.7 Tosyl-Gly-Pro-Lys-pNA assay reaction mixture. The volumes of each buffer added to a 96-well plate to assay lys-gingipain activity of *P. gingivalis* strains.

Reagent	**Volume (µl)**
Broth cultured *P. gingivalis* (OD0.5)	10
PG Buffer	40
Substrate buffer (Tosyl-Gly-Pro-Lys-*p*NA in 10mM DTT)	50

2.7 Isolation of human neutrophils from whole blood

Human whole blood from healthy volunteers was collected with written informed consent (University of Sheffield Ethics Committee) and immediately added to 3.8% (w/v) sodium citrate (9:1 blood:sodium citrate) to prevent coagulation. Whole blood was centrifuged at 400g for 20minutes and the upper serum layer was removed. Hanks Balanced Salt Solution (HBSS) without Ca^{2+} or Mg^{2+} was added at a 50:50 ratio. The blood HBSS mixture was delicately added to the surface of the density gradient medium Ficoll-Paque Plus (GE Healthcare) and then centrifuged at room temperature at 400g for 40 minutes (no brake). Following centrifugation the mononuclear leukocyte layer and the Ficoll-Paque Plus layers were removed leaving the red blood cell layer containing neutrophils. Red blood cells were lysed by adding sterile lipopolysaccharide-free water (Baxter, Berkshire, UK) and incubating for 30 seconds. The cells were then brought back to an isotonic solution by addition of an equal volume of 1.8% (w/v) NaCland centrifuged at 400g for 10 minutes. This lysis was repeated approximately 3-4 times

until all red blood cells were lysed leaving intact neutrophils. Neutrophils were counted using a haemocytometer and added directly to oral mucosal models (see section 2.9).

2.8 Culture of Oral Mucosal Models (OMM)

2.8.1 OMM based on de-epidermalised dermis (DED)

2.8.1.1 Preparation of DED from glycerol-preserved skin

Sterile, glycerol-preserved allografts of human skin were obtained from the Euro Skin Bank (Beverwijk, The Netherlands). The skin was washed 5 times in PBS and placed on a rolling shaker overnight at 4°C to remove the glycerol. Skin was then incubated with 1M NaCl overnight at 37°C, to allow complete de-cellularisation. The epidermis was removed by gentle scraping of the surface of the skin and washing in PBS. The sterility of the DED was confirmed by overnight incubation of DED in CDMEM at 37°C.

2.8.1.2 Culture of DED OMM

DED was used as a scaffold for keratinocyte culture in the construction of DED-OMM. DED was cut using a 12mm diameter sterile cork borer. Discs of DED were placed, basement membrane side up, into Costar® Snapwell™ tissue culture inserts (Fisher Scientific, Loughborough, UK) with a pore size of 0.4µm. NOF ($5x10^5$/model) and H357 ($5x10^5$/model) or normal oral keratinocytes ($5x10^5$/model) were mixed and resuspended in Green's Medium. Cells were added as a mixture to the surface of the DED and fully submerged in culture medium. Medium was added to the well surrounding the insert so that the level of medium was equal inside and out. Models were kept fully submerged for 3-4 days, after which the level of the medium was gradually reduced until they were completely at an air-to-liquid interface. Models were incubated in a humidified atmosphere at 37°C in 5% CO_2 for 7-10 days, with medium changes every 2-3 days, to achieve a differentiated and stratified epithelium.

2.8.2 OMM based on collagen

2.8.2.1 Isolation of rat-tail type 1 collagen

Rat-tail type I collagen was isolated from the tails of Wistar rats as previously described (Rajan *et al*., 2007). Briefly, redundant rat tails were kindly donated by Mrs Christine Freeman, University of Sheffield at the end of a licensed study and stored at -20°C. Prior to collagen isolation, tails were thawed overnight at 4°C. Under a sterile class II laminar flow hood, tails were folded and twisted approximately 4-5cm from the base of the tail and the bone removed to expose the tendons. Tendons were removed from the tails, cut, washed in PBS and dissolved for 7 days in 0.1M sterile acetic acid at 4°C. The collagen solution was freeze dried (VirTis Benchtop K Manifold freeze drier, SP Scientific, Suffolk, UK), re-dissolved in 0.1M acetic acid to a stock concentration of 8mg ml^{-1} and stored at 4°C for use in culturing OMM.

2.8.2.2 Culture of collagen OMM (air-exposed and submerged)

As an alternative to DED, collagen-based OMM models were also prepared and studied. Collagen models were constructed using the protocol adapted from Dongari-Bagtzoglou and Kashleva (2006). Keeping everything on ice, human buccal fibroblasts at a concentration of $1x10^6$/model, in complete medium, were added to a solution of DMEM, reconstitution buffer (sodium bicarbonate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and sodium hydroxide), FCS, L-glutamine and rat-tail type I collagen (table 2.8). The solution was neutralised by addition of 1M sodium hydroxide until the solution turned light pink in colour, distributed into tissue culture inserts (0.4µm pore size or 8µm for neutrophil experiments, ThermoScientific, Northumberland, UK) and incubated in a humidified atmosphere at 37°C for 2 hours. Inserts were then bathed in CDMEM for 2 days, after which $1x10^6$ oral keratinocytes (H357 or NOK) per model were seeded onto the surface of the collagen in Green's Medium. After 2 days, models were raised to an air-to-liquid interface or left completely submerged for 0, 4, 7, 10, 13, 16 days in Green's Medium.

Table 2.8 The concentration of each component used to prepare collagen-OMM cultures.

Component	Final Concentration
DMEM	13.8mg ml^{-1}
Sodium Bicarbonate	2.25mg ml^{-1}
HEPES	2mM
1M NaOH	6.3mM
Foetal calf serum	8.5% (v/v)
L-Glutamine	2.1mM
Rat-tail type I collagen	5.28mg ml^{-1}
Human Fibroblasts	$1.4x10^6$/ml CDMEM

2.9 Incorporation of neutrophils into OMM

Neutrophils were isolated from whole blood as previously described (section 2.7). Air-exposed H357-OMM based on collagen were stimulated with 10ng ml^{-1} IL-1β for 4 hours aerobically. OMM were inverted and approximately $7x10^6$ neutrophils per model added to the underside of the insert membrane (8µm pore size) and incubated at 37°C for 2 hours to allow the neutrophils to adhere. Inserts were returned to their original orientation and incubated overnight at 37°C and 5%CO_2/95% air allowing for migration of neutrophils through the connective tissue of the OMM.

2.10 Histology of OMM

OMM were removed from the inserts before or after infection, fixed in 10% (v/v) PBS-buffered formalin for a minimum of 24 hours, processed overnight using a Leica TP1020 benchtop tissue processor (Leica Microsystems, Milton Keynes, UK) (table 2.9) and embedded in paraffin wax using a Leica EG1160 embedding centre (Leica Microsystems). Sections (4µm) were prepared using a Leica RM2235 microtome (Leica Microsystems), floated onto a paraffin section mounting bath (Barnstead Electrothermal, UK), mounted onto glass slides and placed in an oven at 55°C for 2 hours. Sections were stained with haematoxylin and eosin using a Leica ST4040 Shandon Linear Stainer (Leica Microsystems) (for staining protocol see table 2.10) to analyse the histology of the samples. In some experiments, immunohistochemical staining was performed (section 2.14).

Table 2.9 Dehydration and embedding schedule for paraffin embedded tissue. The length of time the tissue was exposed to the following solutions using a Leica benchtop tissue processor is shown.

Solution	Length of time (hours)
10 % (v/v) neutral buffered formalin	1
70% ethanol	1
70% ethanol	1
90% ethanol	1
90% ethanol	1
100% ethanol	1
100% ethanol	1
100% ethanol	1
Xylene	1.5
Xylene	1.5
Paraffin wax	2
Paraffin wax	2

Table 2.10 Haematoxylin and eosin staining protocol using a Shandon linear stainer. The following protocol was performed to H&E stain paraffin embedded sections. Each step lasted for 1 minute.

Step	Solution
1	Xylene (x3)
2	100% ethanol (x2)
3	95% ethanol (x2)
4	Running tap water (x2)
5	Shandon Harris Haematoxylin (Thermo Scientific) (x5)
6	Running tap water
7	Acid alcohol (1% HCl in 70% ethanol)
8	Running tap water
9	Eosin Y (Thermo Scientific) (x2)
10	Running tap water
11	95% ethanol (x3)
12	100% ethanol (x3)
13	Xylene (x3)

2.11 *Porphyromonas gingivalis* cell invasion

2.11.1 Invasion of oral epithelial cell monolayers

An antibiotic protection assay, based on a previously described method (Lamont *et al*., 1995), was employed to assess the invasion and intracellular survival of *P. gingivalis* strains in oral epithelial cells (H357 or NOK). In the following section, the term invasion refers to bacteria internalised by mammalian cells. H357 and NOK were seeded at a density of $5x10^4$ cells/well in a 24 well plate and incubated in a humidified atmosphere at 37°C and 5% CO_2 for 2 days. Prior to commencing the assay, epithelial cells were washed 3 times in PBS (Oxoid, Hampshire, UK) and incubated in serum-free medium (3:1 ratio of DMEM:Ham's F-12, without antibiotics (SFM)) for 1 hour at 37°C, 5% CO_2. To prevent non-specific binding of bacteria, 2% (w/v) bovine serum albumin (BSA) in SFM was added to the cells and incubated at 37°C, 5% CO_2 for 1 hour. A minimum of 3 wells containing cell monolayers were trypsinised (0.05% trypsin-0.02% EDTA) and counted using a haemocytometer in order to obtain a mean number of cells per well. Plate-cultured *P. gingivalis* were swabbed from a plate into 1ml PBS and washed 3 times by centrifuging at 13,000rpm for 3 minutes and resuspending in 1ml PBS. Bacteria were counted using a Helber counting chamber (Hawksley, Sussex, UK). The bacterial suspension was diluted in SFM to a multiplicity of infection of 100 (MOI100; i.e. 100 bacterial cells for each epithelial cell), and incubated with the epithelial cells for 90 minutes at 37°C, 5% CO_2/95% air or anaerobically (80% N_2, 10% H_2, 10% CO_2) at 37°C. Cells were then washed 3

times with PBS to remove non-adherent bacteria and 200µg ml^{-1} metronidazole in SFM was added for 1 hour at 37°C, 5% CO_2 to kill adherent bacteria. Cells were washed and lysed in sterile distilled water, using scraping and agitation, and the resultant suspension was serially diluted and plated, in duplicate, on blood agar plates. Viable counts of *P. gingivalis* were made 4-5 days after incubation of plates in an anaerobic atmosphere at 37°C. The number of intracellular bacteria recovered was expressed as a percentage of the original innoculum. Assays were independently repeated three times in triplicate. Statistical analysis employed student's unpaired t-test.

For antibody blocking experiments, H357 epithelial monolayers were pre-blocked with 2% (w/v) BSA as previously described in this section. The monoclonal antibodies against CD9 (20µg/ml), CD63 (20µg ml^{-1}), CD81 (1:10), CD82 (1:10), CD151 (32.5µg ml^{-1}), CD46 (10µg ml^{-1}), alpha-5 (20µg ml^{-1}) or an IgG1 isotype control (20µg ml^{-1}) (see table 2.17 for suppliers) were incubated with the epithelial cells for 30 minutes, after which the cells were washed gently to remove any unbound antibody. *P. gingivalis* cells (strain W50, NCTC or E8; MOI100), with 1x protease inhibitor cocktail (Complete Mini ETDA-free (Roche, UK)), were added for 1.5 hours and metronidazole-treated (200µg ml^{-1}) for 1 hour to kill the external adherent bacteria. Epithelial cells were lysed and intracellular *P. gingivalis* calculated as a percentage of the original bacterial suspension incubated in parallel as described previously in this section.

2.11.2 Invasion of OMM

Oral mucosal models were constructed and cultured at the air-to-liquid interface for 7-10 days or submerged for 3-5 days. The OMM were then washed 3 times in PBS and incubated at 37°C and 5% CO_2 in SFM overnight. Plate-cultured *P. gingivalis* strains were washed 3 times and diluted to a final concentration of $2x10^7$ cells/300µl for each insert in SFM (assuming that a confluent 10mm diameter well contains approximately $2x10^5$ cells as a confluent monolayer, a MOI100 equivalent for the surface of a 10mm tissue culture insert would be approximately $2x10^7$). Models were incubated with bacteria for 1.5, 3, 4, 6, 10 and 24 hours aerobically or anaerobically, metronidazole treated and lysed using mincing and vigorous cutting with a scalpel and pipetting or homogenised using a disperser (Tissue Ruptor, Qiagen, West Sussex, UK). Lysis of models was required to determine the viable intracellular bacteria compared with a viable count of the original bacterial suspension, which was serially diluted and plated during the invasion.

2.11.3 Intracellular bacterial survival and release

To evaluate intracellular bacterial survival and release into the supernatant H357 monolayers were infected with *P. gingivalis* (strain NCTC 11834; MOI1 or MOI100 as previously described (section 2.11.1.)). Metronidazole (200µg/ml) was added for 1 hour to kill the external adherent bacteria. Following invasion and extracellular killing, SFM was added and epithelial cells were either lysed (time point 0 hour) or incubated aerobically at 37°C for 1.5, 3, 6, 24 or 48 hours, lysing with sterile water and scraping after each time point. Viable counts of the intracellular bacteria were made at each time point (section 2.11.1). Additionally, at each time point, viable counts of the supernatant prior to epithelial lysis were performed to investigate the percentage of intracellular bacteria that had been released. To consider whether the presence of intracellular bacteria in the supernatant was due to epithelial cell desquamation or not, desquamated cells were counted using a haemocytometer in triplicate from 3 individual wells at each time point. Experiments were performed in triplicate.

2.12 Epithelial viability

2.12.1 LDH assay

The CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega, UK) was used to detect lactate dehydrogenase (LDH) release as a surrogate marker of epithelial cell death. We assessed the suitability of this assay as a tool to investigate epithelial viability in the presence of *P. gingivalis*. The effect of *P. gingivalis* on lactate dehydrogenase supplied as a positive control in the assay kit was determined. The LDH positive control (bovine heart LDH) was diluted 1:5000 in SFM and incubated with or without *P. gingivalis* NCTC 11834 for 6 hours. Tris-buffered tetrazolium dye (INT-chloride) and Triton X-100 (Assay Buffer) were added to a reconstituted Substrate Mix (lyophilized diaphorase, lactate and NAD^+) and incubated with centrifuged SFM+positive control, with or without *P. gingivalis*, in the dark at room temperature for approximately 20 minutes. The absorbance was measured using a plate reader (POLARstar Galaxy, BMG Labtechnologies) at 490nm. The LDH remaining after exposure to *P. gingivalis* was calculated as a percentage of the absorbance recorded in the absence of *P. gingivalis*.

2.12.2 MTT Assay

To assess the epithelial viability of OMM following *P. gingivalis* invasion MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed. H357-OMM were infected with *P. gingivalis* NCTC 11834 (MOI100) for 4 hours. H357-OMM without bacteria acted as a control. OMM were washed with PBS and incubated with 0.5mg ml^{-1} MTT in PBS at 37°C for 1 hour. During this time the yellow tetrazole MTT was reduced to formazan (purple colour) in living cells. The purple dye was released from the epithelium with 100% propan-2-ol + 2.5mM hydrochloric acid (HCl) overnight at 4°C. The solution was measured

spectrophotometrically at a wavelength of 570nm using a microplate reader. Absorbance values were normalized to acidified propan-2-ol alone.

2.13 Bacterial viability

To assess the viability of *P. gingivalis* following homogenisation, plate cultured NCTC 11834 was washed 3 times as previously described (section 2.11.1) and diluted in PBS to a concentration of $1x10^6$ cells/ml. Cells were subjected to homogenisation for 0, 3, 6, 9, 12, 15, 18, 21 and 24 seconds to elucidate the optimum homogenisation time to release intracellular *P. gingivalis* from OMM without affecting bacterial viability. At each time point bacteria were serially diluted, plated onto blood-FA plates and colonies were counted 4-5 days later.

2.14 Immunohistochemical staining of OMM and monolayer

Gingival or buccal biopsies or OMM were paraffin embedded and 4µm sections cut and placed on SuperFrost® PLUS slides (VWR International, Lutterworth, UK). The sections were deparaffinised in 2 washes of xylene (Fisher Scientific, Leicestershire, UK) for 5 minutes and re-hydrated for 2x5 minutes in 100% ethanol (Fisher Scientific). Peroxidase activity was quenched by incubating slides in 3% (v/v) hydrogen peroxide (Fisher Scientific) in 100% methanol (Fisher Scientific) for 20 minutes. Slides were washed in PBS and, dependent upon the primary antibody, subjected to high temperature antigen retrieval (table 2.11). This involved incubation of slides in 2.95mg ml^{-1} sodium citrate in distilled water (pH6.0) at high power in a microwave (Panasonic NN-E252W) for 8 minutes. Slides were washed twice in PBS and blocked with 100% horse serum for 30 minutes at room temperature. Primary mouse monoclonal antibodies were diluted in horse serum to an optimised concentration (see table 2.11) and sections incubated overnight at 4°C in a humidified atmosphere. Slides were washed in PBS and incubated with mouse biotinylated secondary antibody (VECTASTAIN® Elite ABC-Peroxidase Kits (Vector Laboratories, Peterborough, UK) prepared according to manufacturer's instructions for 30 minutes. After washing, slides were incubated with Avidin Biotinylated enzyme Complex (ABC) reagent (Vector Laboratories) for 30 minutes, enabling binding to the biotinylated secondary antibody. Finally, slides were washed and 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories) substrate was added, which produced a dark brown precipitate, corresponding to the location of the bound primary antibody. Slides were counterstained with haematoxylin (table 2.12), using the Leica ST4020 Small Linear Stainer (Leica Microsystems, Milton Keynes, UK) and mounted using DPX non-aqueous mounting medium (Merck, Nottingham, UK).

Immunohistochemical staining for cytokeratin 14 and E-cadherin was performed by the Histology Department at the Northern General Hospital, Sheffield, UK.

Staining of the epithelial cell surface, as opposed to staining of tissue sections, was performed similarly as described above. Monolayers of H357 cells were cultured on sterile coverslips and infected with *P. gingivalis* NCTC 11834 as in section 2.11.1. Similarly, H357-OMM were infected with *P. gingivalis* NCTC 11834 as described (section 2.11.2). Monolayers and OMM were washed 3 times with PBS to remove any external adherent bacteria. Epithelial cells were fixed and permeabilised with cold 100% methanol for 15 minutes and endogenous peroxidase activity was quenched with 3% (v/v) hydrogen peroxide in methanol. OMM and monolayers were incubated with *P. gingivalis* antibody (table 2.11) overnight at 4°C. Secondary antibody and Vector ABC reagent was added, as described above, and DAB substrate was used to visualise *P. gingivalis* staining. Monolayers cultured on coverslips were mounted onto microscope slides (Menzel-Gläser, ThermoScientific) using aqueous mountant (Farrants medium (Gurr)). Epithelium from OMM was carefully removed from the connective tissue layer using forceps and also mounted onto microscope slides using the same aqueous mountant.

All staining was visualised using the BX51 upright microscope (Olympus, Essex, UK) and cell^D imaging software (Olympus UK Ltd). An isotype mouse IgG1 control (Dako, Copenhagen, Denmark) antibody (1:50) was used during each staining procedure, to stain at least one tissue section, in order to confirm the specificity of the primary antibody under test.

Table 2.11 The primary antibodies used in immunohistochemical analysis of sections of buccal or gingival biopsy or H357-OMM or NOK-OMM. The table shows the commercial supplier of each antibody, the clone of the mouse monoclonal antibody, the expected locality of staining, the optimised concentration at which the antibody was used and the antigen retrieval method used to expose intracellular epitopes prior to immunohistochemical staining.

Antibody	Clone	Company	Predicted locality of staining	Concentration	Antigen retrieval
Pancytokeratin	AE1/AE3	DakoCytomation, Copenhagen, Denmark	All epithelial cells	1:100	Citrate Buffer & high temp
Cytokeratin 13	AE8	AbCam, Cambridge, UK	Non-keratinising epithelium	1:50	Citrate Buffer & high temp
Laminin V	P3H9-2	AbCam, Cambridge, UK	Basal epithelial cells	1:100	Citrate Buffer & high temp
E-cadherin	36B5	Vectorlabs, Peterborough, UK	Intercellular junctions	1:50	High pH target retrieval solution (Dako, Copenhagen, Denmark)
Cytokeratin 14	LL002	Vectorlabs, Peterborough, UK	Basal cells of squamous epithelium	1:20	High pH target retrieval solution (Dako, Copenhagen, Denmark)
Neutrophil Elastase	NP57	DakoCytomation, Copenhagen, Denmark	Neutrophils	1:50	None
P. gingivalis	MAb 1B5	M. Curtis, Barts and The London School of Medicine	*P. gingivalis* $RgpA_{cat}$, mt-$RgpA_{cat}$, mt-RgpB, APS (Curtis *et al*., 1999)	1:50	Citrate Buffer & high temp
P. gingivalis	MAb 1A1	M. Curtis, Barts and The London School of Medicine	*P. gingivalis* adhesin domain of HRgpA (Curtis *et al*., 1996)	1:50	Citrate Buffer & high temp

Table 2.12 Counterstaining protocol using the Small Linear Stainer. The table shows the order in which the slides were processed and stained, each step was for a duration of 30 seconds.

Step	Process
1	Harris's haematoxylin (x2)
2	Running tap water
3	1% (v/v) acid alcohol (1% HCl in 70% isopropanol)
5	Running tap water
4	Scott's tap water substitute (3.5g L^{-1} sodium bicarbonate & 20g L^{-1} magnesium sulphate)
5	Running tap water
6	95% ethanol (x2)
7	100% ethanol (x2)
9	Xylene (x4)

2.15 Embedding procedure for *P. gingivalis*

Plate-cultured *P. gingivalis* NCTC 11834 cells were resuspended in PBS and washed 3 times. Bacterial cells were fixed in 10% (v/v) buffered formalin for 5 minutes. Excess formalin was removed by centrifugation and the pellet was resuspended in equal volumes of human serum and human fibrinogen (kindly donated by Dr Simon Tazzyman, Department of Infection and Immunity, University of Sheffield). A *P. gingivalis* embedded fibrin clot formed after incubation at room temperature for 15 minutes. The clot was inserted into a tissue processing cassette and processed for sectioning and immunohistochemical analysis using MAb1B5 (as described in sections 2.10 & 2.14).

2.16 Immunofluorescence staining of OMM infected with *P. gingivalis*

Plate cultured *P. gingivalis* NCTC 11834 were washed 3 times in PBS and labelled with 5-(6)-carboxyfluorescin succinylester (Invitrogen) in PBS (0.4µg ml^{-1}) (FITC) for 30 minutes in the dark at 4°C. Bacterial cells were washed 4 times with PBS by centrifuging at 13,000rpm for 3 minutes. FITC-labelled *P. gingivalis* NCTC 11834 at an MOI of 100 in SFM was added to H357-OMM and incubated overnight at 5%CO_2/37°C. OMM were fixed in 10% (v/v) buffered formalin and embedded in optimum cutting temperature (OCT) formulation of water-soluble glycols and resins (FisherScientific), at approximately -43°C. Sections (10µm) were prepared using a Microm HM560 cryostat (ThermoScientific), at -20 to -30°C, and mounted on microscope slides. Slides were flooded with 1µg ml^{-1} Hoechst 33342 (ThermoScientific, Northumberland, UK) and mounted using Prolong®Gold antifade reagent (Invitrogen, Paisley,

UK). Staining was visualised using the Zeiss Axiovert 200 inverted fluorescence microscope and the Axiovision imaging software (Zeiss, Ltd).

2.17 Cytokeratin staining of normal oral keratinocytes

Cytokeratin staining of normal oral keratinocytes was performed to confirm that there was a pure culture of epithelial cells for use in invasion assays and the culture of OMM (Appendix 1). Briefly, oral epithelial cells were isolated from biopsies (see section 2.3) and cultured on glass coverslips in 6 well plates until confluent. Cells were washed twice in PBS, fixed and permeabilised with cold 50/50 v/v methanol/acetone. Monoclonal mouse anti-human Cytokeratin, Clone MNF-116 (DakoCytokeratin), at a concentration of 10µg ml^{-1} diluted in PBS and 1% (v/v) normal goat serum (Vector Laboratories Incorporated) was added to the cells and incubated for 1 hour at room temperature. Cells were incubated with 10µg ml^{-1} anti-mouse IgM (µ chain specific)-FITC conjugated antibody (Sigma, UK), for 30-45 minutes at room temperature in the dark. Nuclei were counterstained with 300nM 4', 6-diamidino-2-phenylindole, dilactate (DAPI) (Invitrogen). Staining was visualised using the Zeiss Axiovert 200 inverted fluorescence microscope and the Axiovision imaging software (Zeiss, Ltd).

2.18 Detection of inflammatory cytokines from monolayer and OMM

2.18.1 Antibody array

NOK air-to-liquid interface or submerged oral mucosal models, or NOK monolayers, with or without fibroblasts, were washed three times with PBS and incubated in SFM with or without 25ng ml^{-1} TNF-α (Peprotech, London, UK) at 37°C and 5% CO_2 for 4 hours. Plate-cultured *P. gingivalis* NCTC 11834 were washed 3 times and added at MOI100 for monolayers and $2x10^7$/model for 1.5 hours or 4 hours respectively, in SFM with or without 25ng ml^{-1} TNF-α, at 37°C and 5% CO_2. External, non-adherent *P. gingivalis* were removed with three washes of PBS and monolayers and models were incubated with 200µg ml^{-1} metronidazole in SFM with or without 25ng ml^{-1} TNF-α for 4 hours at 37°C and 5% CO_2. The resultant conditioned media, from above and below the tissue culture inserts, were removed and analysed for the expression of secreted inflammatory cytokines using RayBio® Human Inflammation Antibody Array 3 (Insight Biotechnology Ltd, Middlesex, UK) according to the manufacturer's instructions. Briefly, membranes were blocked with 1x Blocking Buffer (supplied in the kit) for 30 minutes at room temperature with gentle rocking. Neat conditioned media were added individually to membranes and incubated overnight with gentle rocking at 4°C to allow attachment of cytokine proteins to antibodies printed on the membrane. Membranes were washed and incubated with biotin-conjugated antibodies for 2 hours. A 1000-fold dilution of HRP-conjugated streptavidin was added for 2 hours, following which a HRP substrate buffer in combination with a stabilised chemiluminescent luminal (detection solution) was added. The array was exposed to CL-XPosure Film (ThermoScientific) and the chemiluminescence signal detected using the

Compact X4 Automatic X-Ray Film Processor (Xograph Healthcare, Gloucester, UK). The intensities of signals were analysed using Quantity One software (Bio-Rad, UK). Internal positive controls and negative 'background' intensities were used as references to calculate the relative signal intensities of cytokines. The densities of cytokine dots of interest were averaged and the average background density subtracted. This value was divided by the average density of internal positive control dots to give the relative average density of each dot. The relative average density of corresponding cytokine dots on different membranes from OMM or monolayers and for each condition were compared.

2.18.2 Enzyme-Linked Immunosorbent Assay (ELISA)

As described in section 2.18.1, conditioned media from H357 monolayers and H357-OMM, infected with *P. gingivalis* were collected and analysed for interleukin 8 (IL-8/CXCL8) protein by enzyme-linked immunosorbent assay (ELISA). In addition, H357 or NOK monolayers and air-exposed H357-OMM were pre-incubated with or without 25ng ml^{-1} TNF-α or protease inhibitor cocktail (Roche, Hertfordshire, UK) for 4 hours and cells were infected by *P. gingivalis* NCTC 11834, W50, E8, K1A and EK18 overnight, with or without 25ng ml^{-1} TNF-α or protease inhibitor cocktail. Conditioned media or cell lysates (scraping after sterile water treatment) were collected, centrifuged at 13,000rpm for 3 minutes to remove any cell debris and/or bacteria, and stored at -80°C until analysed. Culture supernatants or cell lysates from 4 hour invasion and overnight infection were analysed by ELISA using a kit from R&D Systems (Abingdon, UK) following the manufacturer's instructions. Briefly, 96 well plates were coated with anti-human monoclonal capture antibody directed against CXCL8 overnight at room temperature. The plate was washed three times with wash buffer (R&D Systems) and blocked with reagent diluent (0.1% BSA, 0.05% Tween20 in Tris-buffered saline, pH 7.4 (R&D Systems)) for 1 hour at room temperature. After washing 3 times, standards and samples (with or without diluting 1:10-1:5000 in reagent diluent) were added to the plate for 2 hours at room temperature. After washing 3 times, the plate was incubated with biotinylated anti-human polyclonal detection antibody and was incubated for 2 hours. Following washing, streptavidin-horseradish peroxidase conjugate was added for 20 minutes in the dark. A tetramethylbenzidine/hydrogen peroxide substrate solution was then added for a maximum of 20 minutes and the reaction stopped with 2N sulphuric acid (H_2SO_4). ELISA plate absorbance values were read using a spectrophotometer (Infinite 200 PRO, Tecan, Reading, UK) at 450nm (with wavelength correction set to 570nm) and analysed using DeltaSoft Microplate Analysis Software (BioMetallics, Inc). Student t-tests were performed to assess any statistical differences in the release of CXCL8 from OMM and monolayers infected with different *P. gingivalis* strains.

2.18.3 Real-time Polymerase Chain Reaction

As described in section 2.18.2, H357 monolayer and H357-OMM were infected overnight with the *P. gingivalis* strains W50, E8, K1A and EK18. The epithelial cells were then collected and analysed for interleukin 8 (IL-8/CXCL8), monocyte chemoattractant protein-1 (MCP-1/CCL2), interleukin 6 (IL-6) and regulated upon activation, normal T cell expressed and secreted (RANTES/CCL5) mRNA expression by real-time polymerase chain reaction (real-time PCR). In addition, H357 or NOK monolayers and air-exposed H357-OMM were pre-incubated with or without 25ng ml^{-1} TNF-α for 4 hours and cells were infected with *P. gingivalis* NCTC 11834, W50, E8, K1A and EK18 overnight, with or without 25ng ml^{-1} TNF-α. Treated and untreated cells were lysed using QiaShredder (Qiagen) according to the manufacturer's instructions or lysed with lysis buffer (supplied in the RNeasy Mini Kit (Qiagen)) and drawn through a 21guage hypodermic needle and syringe to mechanically lyse the cells. RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration of RNA was measured using a NanoDrop 1000 Spectrophotometer (ThermoScientific) at 260/280nm. Complementary DNA (cDNA) was synthesised from 1µg RNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Warrington, UK). The reagents shown in table 2.13 were mixed on ice and placed in a Peltier thermal cycler (MJ Research PTC-200 Thermo Cycler, UK). The reverse transcription (RT) reaction consisted of 60 minutes at 37°C followed by 5 minutes at 95°C, after which the sample was stored at -20°C. Real-time PCR was performed using the StepOne Real-time PCR System (Applied Biosystems) or the 7900HT Fast Real-Time PCR System (Applied Biosystems) in a 48 (Invitrogen) or 96 clear well reaction plate (Sarstedt, Leicester, UK), respectively. The thermal cycles consisted of initial exposure of the samples to 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of denaturation and extension steps at 95° for 15 seconds and 60°C for 1 minute, respectively. Quantification of CXCL8 and CD81 gene expression was performed using the TaqMan universal PCR master mix and Assays on Demand™ gene expression reagents for human CXCL8 (Assay ID: Hs00174103_m1, Applied Biosystems) and CD81 (Assay ID: Hs00174717_ml, Applied Biosystems). Reagents for the TaqMan assay are shown in table 2.14. Gene expression of CCL5, CCL2 and IL-6 was quantified using the SYBR Green (Applied Biosystems) method, which increasingly binds to double-stranded DNA as the PCR reaction progresses, intensifying the levels of fluorescence, which is detected in real-time. The reaction mixture contained reagents shown in table 2.15. The primer sequences used in this study are shown in Appendix 3. The housekeeping/endogenous controls for TaqMan and SYBR Green assays were β-2-Microglobulin (B_2M) (VIC reporter, Applied Biosystems) and U6 (Sigma) respectively. The results were analysed using the $2^{-\Delta\Delta CT}$ method. The threshold cycle (C_T) values for each reaction, referring to the number of cycles with which the fluorescent signal passes a pre-selected threshold, were calculated using the RQ Manager Software (Applied Biosystems). The C_T values of the relevant endogenous controls were used to normalise the amount of cDNA

in each sample by subtracting this C_T value from the sample C_T value, giving a ΔC_T value. The $\Delta\Delta C_T$ value was calculated by subtracting the untreated control (without TNF-α) from the ΔC_T values of each sample. Finally, the fold change in gene expression, of each sample, relative to the untreated control was calculated.

Table 2.13 The components used to synthesise cDNA. Reverse transcription was performed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). The following reagents were added to MicroAmp Fast reaction tubes (Applied Biosystems). Nuclease-free water was added to give a total volume of 20µl.

Reagent	Volume (µl)
2x RT buffer	10
20x RT enzyme mix	1.0
RNA (1µg)	Up to 9µl
Total	**20**

Table 2.14 The reagents used in TaqMan real-time PCR. Real-time PCR for CXCL8 and CD81was performed using the volumes of reagents shown in the table. All reagents were mixed on ice. Sample and endogenous control reactions were performed in the same well.

Reagent	Volume (µl)
TaqMan Master Mix	5
Primer (forward and reverse)	0.5
Housekeeping gene (B_2M)	0.5
Nuclease-free water	3
cDNA	1
Total	**10**

Table 2.15 The reagents used in SYBR Green real-time PCR. Real-time PCR for IL-6, CCL2 and CCL5 was performed using the volumes of reagents shown in the table. All reagents were mixed on ice. Sample and endogenous control reactions were performed in separate wells.

Reagent	Volume (µl)
Power SYBR Green PCR Master Mix	10
Forward Primer	7
Reverse Primer	1
Nuclease-free water	1
cDNA	1
Total	**20**

2.19 Two-dimensional agarose gel electrophoresis

To determine the specificity of the real-time PCR primers, two-dimensional gel electrophoresis was performed. A 1% (w/v) agarose (Hi-Res Standard Agarose, GeneFlow Ltd, Staffordshire, UK) gel was prepared in 1x TAE buffer (table 2.16) by heating on full power in a microwave for approximately 2 minutes. The liquid was left to cool for 5-10 minutes and 1µl ethidium bromide added for every 50ml solution. Amplified PCR products for CCL5, IL-6 and CCL2 and a DNA ladder (exACT Gene 100bp, Fisher Scientific, UK) were added to the solidified gel, which was surrounded by 1x TAE buffer, placed in a Bio-Rad Mini-sub®Cell GT system (Hertfordshire, UK), attached to a Bio-Rad powerpack and run at approximately 75Volts. The gel was viewed under a UV transilluminator and images taken using the G:BOX (SynGene) and the GeneSnap software (SynGene).

Table 2.16 Components of TAE buffer. The table shows the amounts of each component required to achieve 50x TAE buffer, diluted to 1L in distilled water.

Component	Company	Mass or Volume
Tris Base	Fisher Scientific	242g
Glacial acetic acid	Sigma	57.1ml
0.5M EDTA (pH 8.0)	Fisher Scientific	100ml

2.20 Separation of epithelial cells associated with *P. gingivalis*

2.20.1 Using Dynabeads to separate epithelial cells

In an attempt to separate epithelial cells that were associated with bacteria, from those that were not following an invasion assay, Dynabeads® M-270 Epoxy (Dynal®, Invitrogen, UK) were used. These magnetic beads are coated with a hydrophilic layer of glycidyl ether (epoxy) functional groups, allowing for the direct binding of proteins, in this instance, on the surface of *P. gingivalis* cells. The Dynabeads® were stored in dimethylformamide (DMF) at 4°C according to the manufacturer's instructions. Prior to their use in experiments, Dynabeads® were washed 4 times in PBS to remove the DMF. This was achieved by applying a magnetic field (EasySep®Magnet, Stem Cell Technologies, Bath, UK), congregating the beads to the side of the tube allowing for the removal of liquid and the ease of washing. Beads ($1x10^8$/ml) were blocked for 30 minutes in 0.1% (w/v) BSA in PBS, and incubated aerobically for 2 hours with or without approximately $2x10^9$/ml *P. gingivalis* NCTC 11834. Following incubation, adhesion of *P. gingivalis* to the beads was assessed microscopically. The beads were then washed 3 times with PBS to remove any unbound bacteria and the beads ± bacteria (MOI100) were incubated with pre-blocked (2% (w/v) BSA in SFM) epithelial cells (H357) for 1.5 hours. Cells were washed 3 times with PBS to remove the non-cell associated beads and 200µg ml^{-1} metronidazole was added for 1 hour. The cells were washed 3 times and dissociated from the bottom of the tissue culture well using trypsin/EDTA. Epithelial cells associated with magnetic

beads (hence those associated with bacteria) were separated, using a magnet, into the pellet and those not associated with beads were removed in the supernatant. These two cell populations were counted using a haemocytometer and plated at a density of $1x10^5$/well in a 24-well plate. These cells were cultured, alongside wild-type H357, in Green's Medium at 37°C/5%CO_2 overnight. The following day, an antibiotic protection assay with *P. gingivalis* NCTC 11834 was performed (as in section 2.11.1).

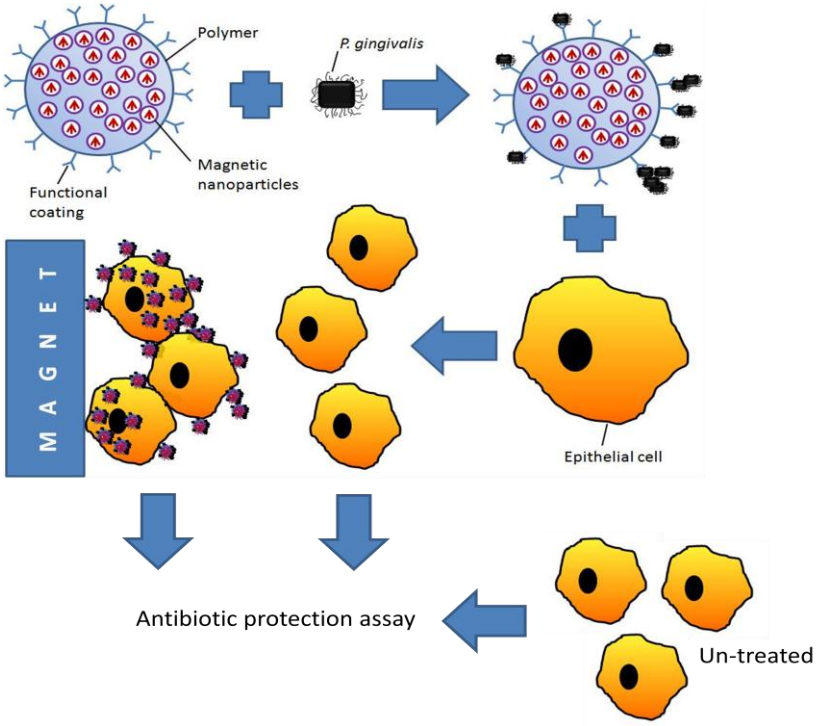


Figure 2.1 Separation of epithelial cells using magnetic beads associated with *P. gingivalis*. Dynabeads® were incubated with *P. gingivalis* NCTC 11834 for 2 hours and unbound bacteria were washed from the beads. *P. gingivalis*-associated beads were incubated with monolayer cultures of H357 epithelial cells for 90 minutes. Loosely adhered *P. gingivalis*-associated beads were washed from the epithelial cells and monolayers were trypsinised and separated using a magnet. Epithelial cells associated with *P. gingivalis*-beads were separated towards the magnet and epithelial cells not associated with *P. gingivalis*-beads were separated from the supernatant. These two cell populations, in addition to un-treated epithelial cells were seeded in a 24-well plate and cultured at 37°C/5% CO_2 overnight. The following day an invasion assay was performed on these epithelial cells.

2.20.2 Identifying cell populations by flow cytometry

P. gingivalis NCTC 11834 were fluorescently labelled using 0.4µg ml^{-1} FITC (as in section 2.16). Pre-blocked H357 cells were incubated for 1.5 hours with MOI100 labelled *P. gingivalis*, fluorescent beads (4.5µm diameter, Fluoresbrite BB Carboxylate Microsphere, Polysciences, Inc, Warrington, UK), or a combination of both. After this incubation period, cells were washed 3 times with PBS, trypsined and kept on ice. Cells were analysed for fluorescence using the LSRII Flow Cytometer (BD Biosciences) at the University of Sheffield Flow Cytometry Core Facility. Briefly, cells were separated according to their fluorescence and hence their association with *P. gingivalis* and/or fluorescent beads. Beads, *P. gingivalis* and H357 cells alone were

analysed in parallel and used as controls to adjust side scatter, fluorescence and particle size parameters.

2.21 Tetraspanin and alpha-5 integrin expression analysed by flow cytometry

H357 monolayers were cultured until approximately 90% confluent, washed 3 times with PBS to remove any serum or antibiotic and incubated in 2% (w/v) BSA in SFM for 1 hour. SFM (unstimulated) or W50, E8, K1A or EK18 (stimulated) was added at MOI100 overnight at 37°C and 5% CO_2. Approximately $6x10^5$ cells were trypsinised (0.05%trypsin/0.02%EDTA) at 37°C and neutralised with Green's Medium (50:50). Cells were pelleted at 6000rpm (Biofuge 13, Heraeus Instruments, Basingstoke, UK) for 2 minutes, resuspended in 10µg ml^{-1} primary antibody for CD9, CD63, CD151 and IgG1 and 1:10 dilution of α5, CD81 and CD82 (see table 2.17) and incubated in FACs buffer (PBS+0.1% (v/v) sodium azide+0.1% (w/v) BSA) at 4°C for 30-40 minutes. Cells were washed with 1ml FACs buffer and incubated with AlexaFluor 488-conjugated anti-human secondary antibody (1:100; Invitrogen, Paisley, UK) for 30-40 minutes at 4°C in the dark. Cells were washed twice with FACs buffer. Prior to cell-surface tetraspanin determination on FACsCalibur (BD Biosciences, Oxford, UK), 5µl (approximately 4µM) TO-PRO®-3 (Invitrogen) was added, which was used as a live/dead stain. TO-PRO®-3 has a high affinity for double-stranded DNA and does not penetrate the cell membrane of intact/live cells. Alexafluor 488-conjugated secondary antibody was viewed under the FL-1 channel and TO-PRO®-3 was viewed under FL-4. The fluorescence of each tetraspanin and alpha-5 was compared for both stimulated and unstimulated samples, gating around the live cells only, preventing false positive results. An overlay plot of IgG1 control (stimulated) and IgG1 control (unstimulated) was overlaid with each primary antibody (stimulated and unstimulated) and the median value was analysed using CellQuestPro Software (BD Biosciences).

2.22 Knockdown of CD81 using siRNA

Transfection was performed using CD81 siRNA (Applied Biosystems, siRNA ID: s2724) or non-target control siRNA (Applied Biosystems, Silencer®Select #1 negative control) and Oligofectamine™ (Invitrogen), according to the manufacturer's instructions. Briefly, 0.5µl of 50µM siRNA and 5µl Oligofectamine per 24well plate were incubated with Opti-MEM® (Invitrogen), to a total volume of 50µl, at room temperature for 30 minutes. An additional 50µl Opti-MEM® was then added to this solution. H357 monolayers, at 60% confluence, were washed twice with Opti-MEM® and 100µl of the transfection mixture added to the wells to a total volume of 250µl per well in Opti-MEM®. After 3 hours, 250µl of Green's Medium (containing 20% serum) was added to the wells and incubated for 48 hours. Transfected cells were used to perform an antibiotic protection assay (section 2.11.1) to compare the percentage invasion of CD81 knockdown cells by *P. gingivalis* W50. The transfection efficiency was

determined by real-time PCR (as in section 2.18.3) using TaqMan primers (Applied Biosystems, Assay ID:Hs00174717_m1, see table 2.14). Knockdown of CD81 was also shown by flow cytometry (section 2.21).

Table 2.17 Anti-tetraspanin, -CD46 and -α5 antibodies. The antibodies that were used in the analysis of tetraspanin, CD46 and alpha 5 integrin expression and antibody blocking prior to a *P. gingivalis* antibiotic protection assay.

Antibody	Clone	Species	Origin
CD9	602.29	Mouse monoclonal	Courtesy of Dr Pete Monk, Department of Infection and Immunity, University of Sheffield Medical School
CD63	H5C6	Mouse monoclonal	Courtesy of Dr Pete Monk, Department of Infection and Immunity, University of Sheffield Medical School
CD81 (TAPA-1)	1D6	Mouse monoclonal	Serotec, Oxford, UK
CD82 (KAI1)	B-L2	Mouse monoclonal	Serotec, Oxford, UK
CD151	14A2	Mouse monoclonal	Courtesy of Dr Pete Monk, Department of Infection and Immunity, University of Sheffield Medical School
α5/CD49e	238307	Mouse monoclonal	R&D Systems, Abingdon, UK
CD46	MEM-258	Mouse monoclonal	Serotec, Oxford, UK
IgG1 (Isotype control)	DAK-GO1	Mouse monoclonal	Dako, Copenhagen, Denmark

2.23 Statistical Analysis

All comparisons were analysed using a students' unpaired, two-tailed t-test with unequal variance. Calculations were performed using Microsoft® Excel, Microsoft® Office, 2007.

Chapter 3 Characterisation and optimisation of oral mucosal models to study *Porphyromonas gingivalis* invasion

3.1 INTRODUCTION

For *in vitro* studies, the oral environment has, for a long time, been modelled using a simple monolayer culture of orally-derived cells (Aruni *et al.*, 2011; Belton *et al.*, 1999; Lamont *et al.*, 1995). However, with the dawn of tissue engineering, the *in vitro* culture of oral mucosal tissues has progressively been used as a more representative model of the oral environment (Andrian *et al.*, 2004; Rouabhia and Deslauriers, 2002; Mackenzie and Fusenig, 1983).

These engineered oral mucosal tissues have been used in the clinical setting as grafts to promote wound healing (MacNeil *et al.*, 2011 Bhargava *et al*., 2008) and within the laboratory setting to study the effects of microbial infection (Andrian *et al.*, 2004; Andrian *et al.*, 2007; Décanis *et al.*, 2009; Dickinson *et al.*, 2011; Mostefaoui *et al.*, 2004; Semlali *et al.*, 2011; Yadev *et al.*, 2011), to model oral dysplasia and cancer invasion (Colley *et al*., 2011; Gaballah *et al.*, 2008) and the biocompatibility of dental materials (Chai *et al.*, 2010; Moharamzadeh *et al.*, 2008a; Moharamzadeh *et al.*, 2008b).

Tissue-engineered oral mucosa may be cultured using a variety of supporting, fibroblast-embedded scaffolds (Moharamzadeh *et al.*, 2007a). Two scaffolds: acellular DED and type I collagen have been utilised previously for the culture of organotypic models (Dongari-Bagtzoglou and Kashleva, 2006, Yadev *et al*., 2011) but have not been fully characterised in terms of histological comparisons with normal oral mucosa. This chapter provides information regarding these issues in an attempt to determine which scaffold is most suited to study bacterial invasion.

To verify the comparability of oral mucosal models with the native tissue, novel models require characterisation in terms of expression of key proteins, comparing with normal oral mucosa for similarities and/or differences (Dongari-Bagtzoglou and Kashleva, 2006; Kinikoglu *et al.*, 2009; Rouabhia and Deslauriers, 2002; Yadev *et al.*, 2011). OMM based on collagen have previously been characterised in terms of the expression of Ki-67, E-cadherin (Dongari-Bagtzoglou and Kashleva, 2006), cytokeratins 14, 19 and 10, the integrin subunits β1, α2β1 (Rouabhia and Deslauriers, 2002), cytokeratin 13 and laminin 5 (Kinikoglu *et al.*, 2009). This chapter provides characterisation of the popular air-exposed stratified epithelial model and also characterisation of a novel model which resembles junctional epithelium. Due to the close proximity of junctional epithelium with the poly-microbial plaque biofilm (Bosshardt and Lang, 2005), this

model was developed in an attempt to represent the oral epithelial-microbial interactions in periodontitis.

There are many pathogenic bacteria that are capable of invading human cells (Bamburg, 2011; Hunstad and Justice, 2010). Therefore, the use of a three-dimensional model designed and optimised for the study of bacterial invasion seems logical in this present day when monolayer cultures are becoming recognised as a deficient model to study the host response adequately. This is due to its flaws, including the lack of a multi-layered epithelium and the incorporation of other contributing cell types (Altmann *et al.*, 2009). However, in terms of microbial infection there are limited data regarding the optimisation of oral mucosal models for invasion by pathogenic bacteria, in particular, *P. gingivalis*. Two recent studies by Dickinson *et al*. (2011) and Andrian *et al.* (2004) describe *P. gingivalis* invasion of multi-layered epithelial cultures in a qualitative/semi-quantitative manner using microscopic analyses. However, throughout the literature there is a lack of quantitative data regarding the invasion of *P. gingivalis* into organotypic mucosal cultures. Therefore, this chapter provides data regarding the optimisation, and modification of the commonly used antibiotic protection assay, to study the invasion and recovery of viable intracellular *P. gingivalis*.

3.1.1 Aims and Objectives

The aim of the work described in this chapter was to develop and assess the use of a range of tissue engineered oral mucosal models for the study of *P. gingivalis* invasion into oral epithelium. To achieve this, the *in vitro* organotypic models were characterised by histological analysis and immunohistochemical staining. Subsequently the models were optimised for bacterial invasion in terms of the assay culture environment, period of infection and the lysis technique used to release intracellular bacteria. The viability of the model following invasion was assessed, as was the depth of penetration of *P. gingivalis* through the model.

3.2 METHODS

The following methods were used in this chapter:

- Isolation and culture of NOK, H357 and human fibroblasts (sections 2.1, 2.2, 2.3 & 2.4)
- *P. gingivalis* culture (section 2.5)
- Culture of collagen and DED OMM (section 2.8)
- Neutrophil isolation and incorporation into OMM (section 2.7 & 2.9)
- Immunohistochemical staining (section 2.14)
- Antibiotic protection assay (section 2.11.1)
- Modified antibiotic protection assay (section 2.11.2)
- Histology of OMM (section 2.10)

- Epithelial viability (LDH, MTT) (section 2.12)
- Bacterial viability (section 2.13)
- Immunofluorescence staining (section 2.16)
- Formation of fibrin clot (section 2.15)
- Statistical analysis (Section 2.22)

3.3 RESULTS

3.3.1 Characterisation of an oral mucosal model

3.3.1.1 Comparison of collagen and DED OMM

Tissue-engineered OMM based on DED and rat-tail type I collagen, previously described by Haddow *et al.* (2003) and Dongari-Bagtzoglou and Kashleva (2006) respectively, were cultured. DED-OMM and collagen-OMM seeded with the oral keratinocyte cell line H357, or NOK were fixed, sectioned and stained with haematoxylin and eosin (H&E), which is the routine histological stain used for examining tissue sections. Figure 3.1 shows H&E stained sections of H357 and NOK cultured on DED (Fig 3.1A and 3.1C respectively) and rat-tail type I collagen (fig 3.1B and 3.1D respectively) at the air-to-liquid interface. In these figures, the nuclei stained blue and the cytoplasm and connective tissue stained pink. In both DED-OMM and collagen-OMM there was significant fibroblast infiltration (black arrows, fig 3.1) and a multi-layered epithelium. Keratinocyte differentiation could be recognised in NOK-OMM based on DED as a flattening of keratinocytes as the number of epithelial layers increased and the increased keratinisation and loss of cell nuclei in the upper-most layers (fig 3.1C). Only slight keratinocyte differentiation was detected in NOK-OMM based on collagen, whereas H357 models did not show epithelial differentiation in either model. Other differences in the histology between DED-OMM and collagen-OMM can clearly be seen, such as the absence of rete ridges in collagen-OMM. For reasons that are unclear, sometimes DED-based models failed to grow properly and the epithelium remained at just two or three cells thick, but this was not apparent until several days of culture. However, models based on collagen were more reproducible, grew reliably, were less costly because the collagen was extracted in-house, were easier to manipulate and gave greater epithelial coverage of approximately 15 cell layers compared with approximately 10 cell layers in DED-OMM. These advantages were felt to outweigh the disadvantage of the lack of a basement membrane (which remains in tact in DED), so models based on collagen were used in all subsequent experiments.

3.3.1.2 Time course of epithelial growth

After raising to an air-to-liquid interface, H357-OMM were cultured for a further 0, 4, 7, 10, 13 and 16 days in order to determine the optimum length of time to culture OMM. Whilst submerged, the keratinocytes covered the collagen matrix with 1-5 cell layers (fig 3.2, day 0). When the models were raised to the air-to-liquid interface, the epithelium began to stratify, and the epithelial layers increased (fig 3.2). The optimal length of time to culture the models was determined to be 7-10 days after raising to the air-to-liquid interface. This time period resulted in maximal epithelial stratification without significant loss of tissue integrity. At later time points, invasion of H357 cells into the collagen was observed and, at day 16, extensive keratin deposits, often termed 'keratin-pearls' (pink), could be seen in the upper epithelial layers.

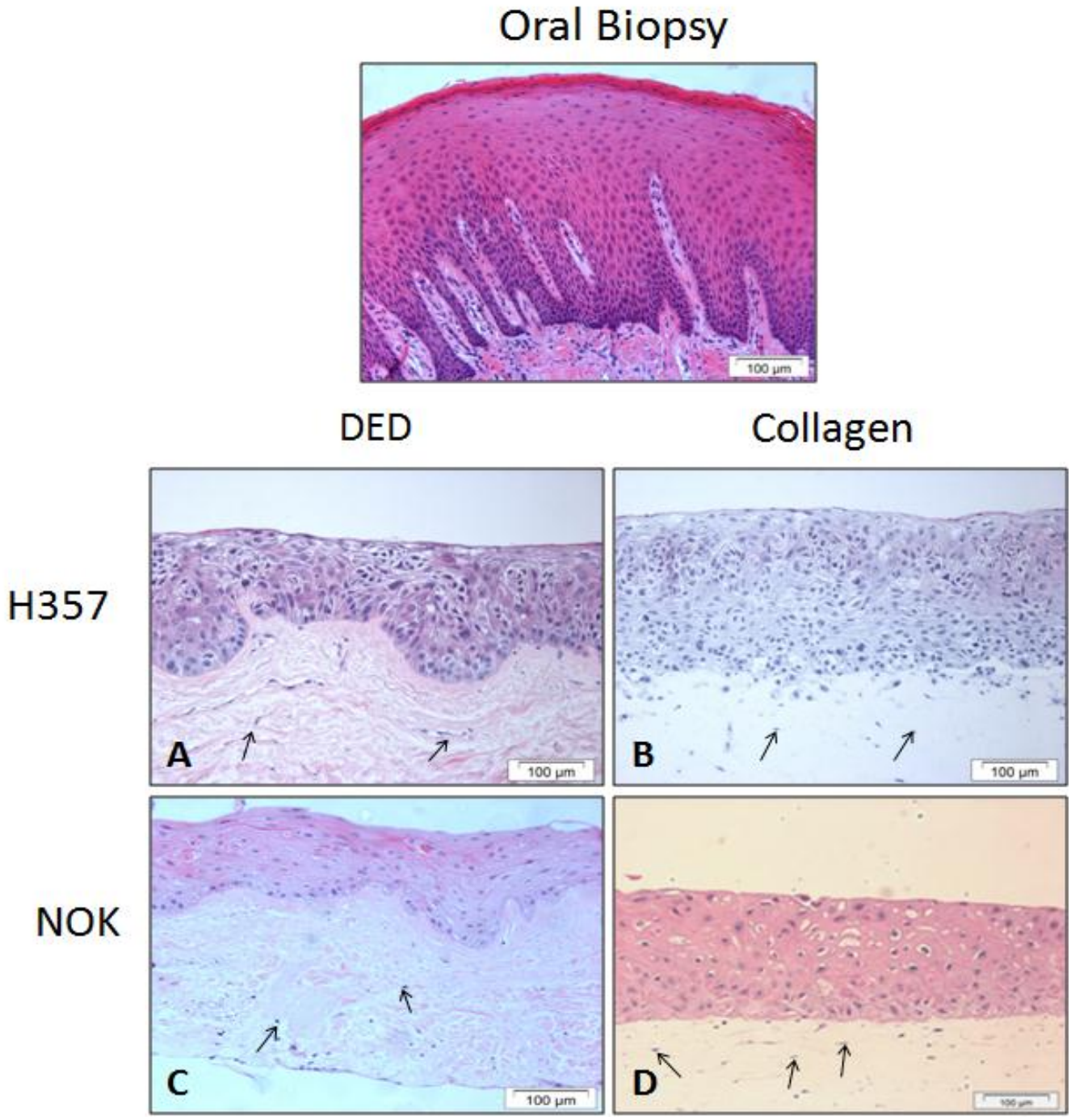


Figure 3.1 Haematoxylin and Eosin (H&E) staining of the three-dimensional oral mucosal model and buccal biopsy. H&E stained buccal biopsy is shown (oral biopsy), in addition to H&E stained sections of organotypic mucosal model (OMM). To culture OMM, fibroblasts were suspended in rat-tail type I collagen or seeded onto the surface of de-epidermalised dermis (DED). After 0-3 days the H357 cell line or normal oral keratinocytes (NOK) were seeded onto the surface and raised to an air-to-liquid interface after 2-3 days. Following 7-12 days in culture, models were fixed in 10% formalin, paraffin embedded, sectioned and H&E stained. The keratinocytes proliferated to form a stratified epithelium and fibroblasts could be seen (black arrows) within H357 DED-OMM (A), H357 collagen-OMM (B), NOK DED-OMM (C) and NOK collagen-OMM (D) (NOK-DED (C) was cultured by Dr Vanessa Hearnden, School of Clinical Dentistry, Sheffield).

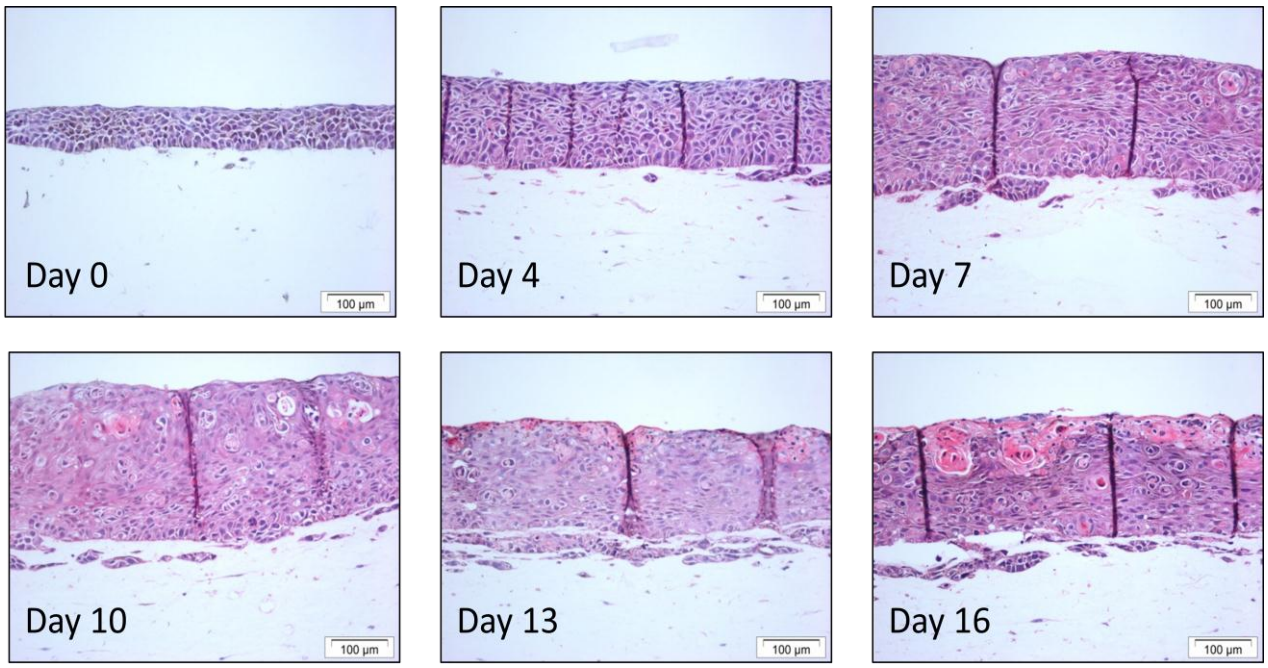


Figure 3.2 Epithelial growth of H357-OMM over time. Fibroblast-embedded type I collagen was seeded with the H357 cell line and submerged in culture medium for 2 days, after which models were raised to the air-to-liquid interface for 4, 7, 10, 13 and 16 days. Models were formalin fixed, paraffin embedded and H&E stained. [N.B. The "block-like" appearance of the epithelium is an artefact due to folding of the tissue during processing and/or sectioning]

3.3.1.3 Histological comparison of submerged and air-exposed OMM

In terms of relevance to periodontitis, *P. gingivalis* is most likely to invade the junctional epithelium (Bosshardt and Lang, 2005), which is deep in the gingival sulcus in close proximity to the plaque-covered tooth surface. The reduced oxygen tension and presence of complex nutritional components provides optimal conditions for the survival and propagation of this anaerobic bacterium. Therefore, in an attempt to more closely resemble junctional epithelium, which is only a few cell layers in depth (Gao and Mackenzie, 1992), models were left completely submerged for 3-5 days, and this produced models that were only 1-3 cell layers thick (fig 3.3).

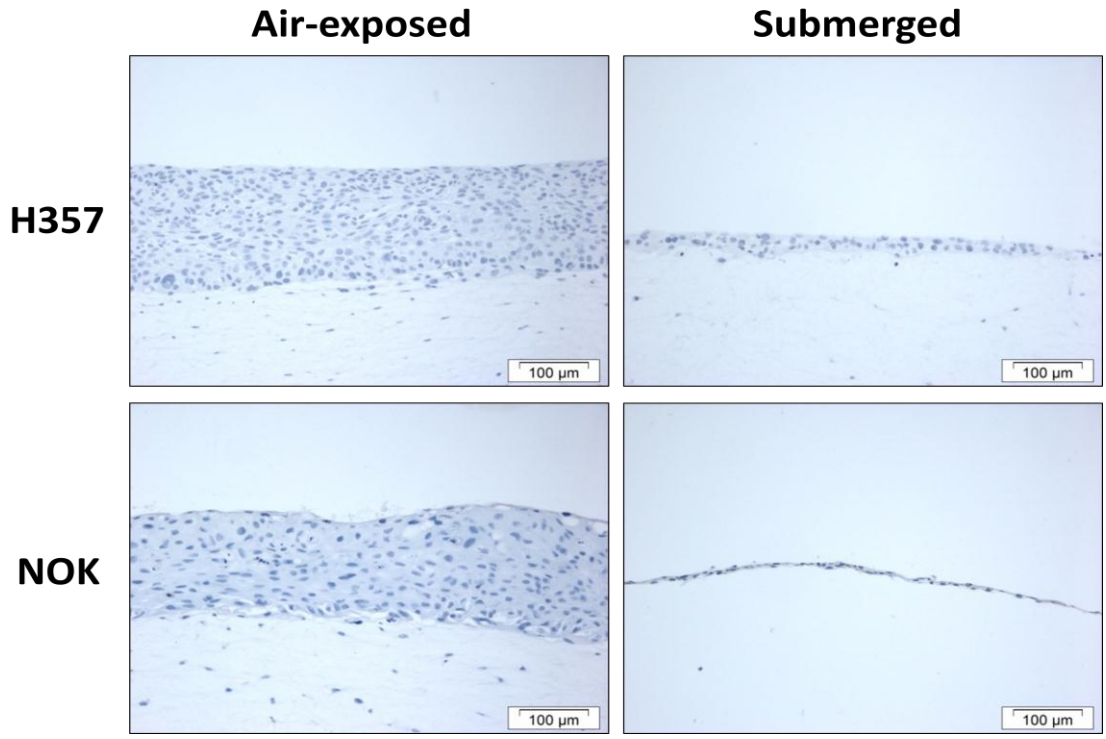


Figure 3.3 Histological comparison of air-exposed and submerged OMM. Fibroblast-embedded rat-tail type I collagen models, seeded with H357 or NOK were raised to the air-to-liquid interface for 7-10 days or left completely submerged in culture medium for 3-5 days. Haematoxylin stained paraffin-embedded sections are shown.

3.3.1.4 Immunohistochemical comparison of OMM with normal oral biopsy

Building on data from Rouabhia & Deslauriers (2002) and Dongari-Bagtzoglou & Kashleva (2006), models were further characterised against oral biopsies isolated from patients and volunteers at the Charles Clifford Dental Hospital, Sheffield, UK. Laboratory-engineered oral mucosa was compared immunohistochemically with gingival and buccal biopsies for similarities and/or differences.

Air-exposed H357 and NOK models were stained for pan-cytokeratin (AE1/AE3), cytokeratin 13, cytokeratin 14, laminin 5 and E-cadherin using specific antibodies. Control slides were stained using a non-immune murine IgG1 antibody as an isotype matched negative control, which did not show any staining (fig 3.4).

Staining for pan-cytokeratin using anti-AE1/AE3 antibodies was positive in both models showing diffuse staining throughout the whole epithelium, which was comparable with the positive staining observed in both gingival and buccal biopsies (fig 3.5).

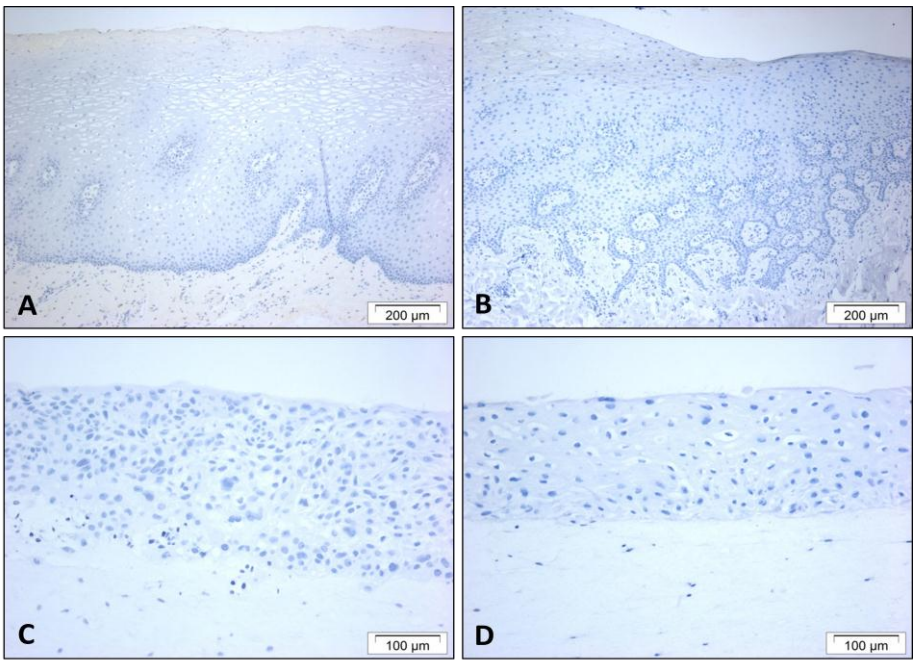


Figure 3.4 Immunohistochemical staining for murine IgG1 isotype control. Tissue sections of buccal mucosa (A), gingival tissue (B), H357-OMM (C) and NOK-OMM (D) were stained immunohistochemically using an IgG1 negative control antibody. After 2-3 days OMM were raised to the air-to-liquid interface for approximately 7-10 days. [N.B. A and B are shown at a lower magnification to include basal and apical epithelial layers and the upper sections of the connective tissue]

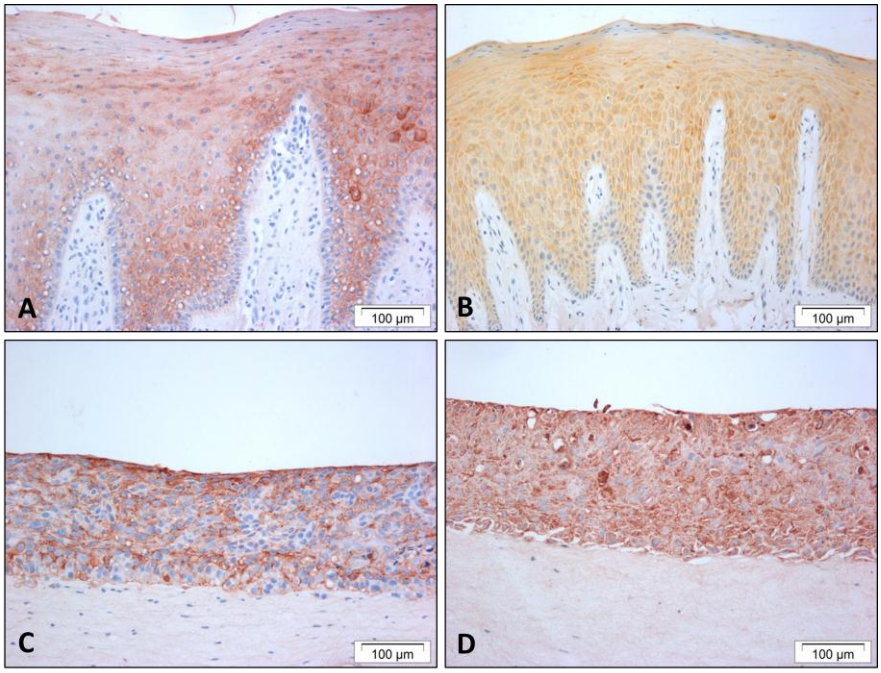


Figure 3.5 Immunohistochemical staining for pancytokeratin (clone AE1/AE3). Tissue sections of buccal mucosa (A), gingival tissue (B), H357-OMM (C) and NOK-OMM (D) were stained immunohistochemically for pancytokeratin. After 2-3 days OMM were raised to the air-to-liquid interface for approximately 7-10 days.

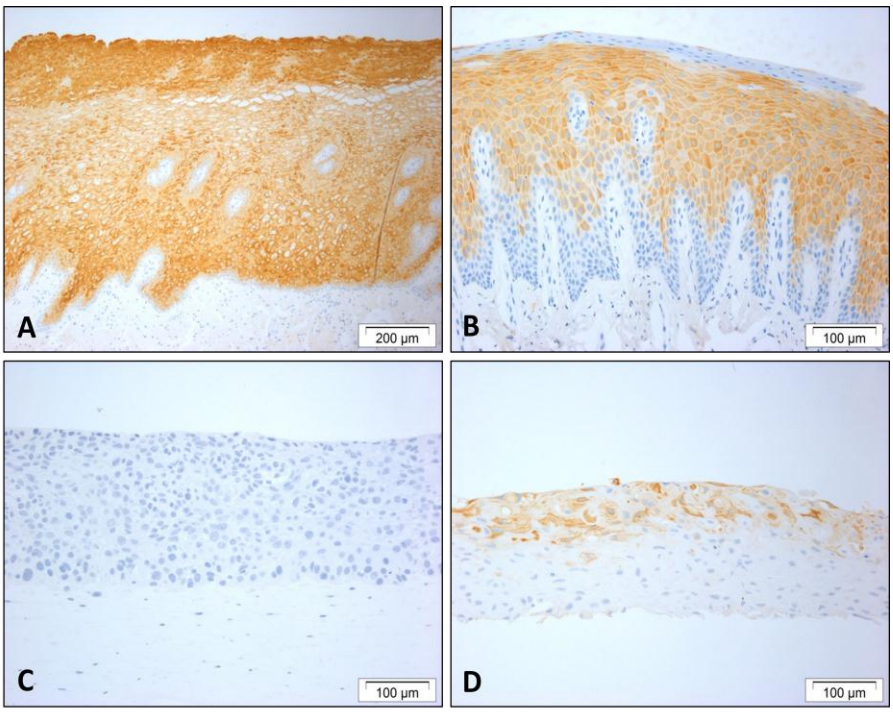


Figure 3.6 Immunohistochemical staining for cytokeratin 13. Tissue sections of buccal mucosa (A), gingival tissue (B), H357-OMM (C) and NOK-OMM (D) were stained immunohistochemically for cytokeratin 13. After 2-3 days OMM were raised to the air-to-liquid interface for approximately 7-10 days. [N.B. Buccal mucosa (A) is shown at a lower magnification to include basal and apical epithelial layers and the upper section of connective tissue]

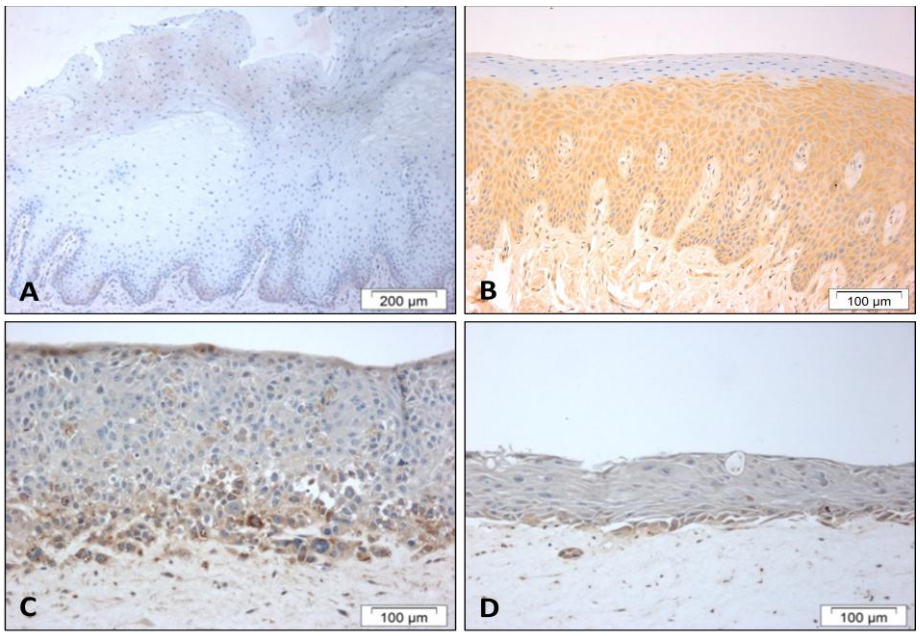


Figure 3.7 Immunohistochemical staining for laminin 5. Tissue sections of buccal mucosa (A), gingival tissue (B), H357-OMM (C) and NOK-OMM (D) were stained immunohistochemically for laminin 5. After 2-3 days OMM were raised to the air-to-liquid interface for approximately 7-10 days. [N.B. Buccal mucosa (A) is shown at a lower magnification to include basal and apical epithelial layers and the upper section of connective tissue]

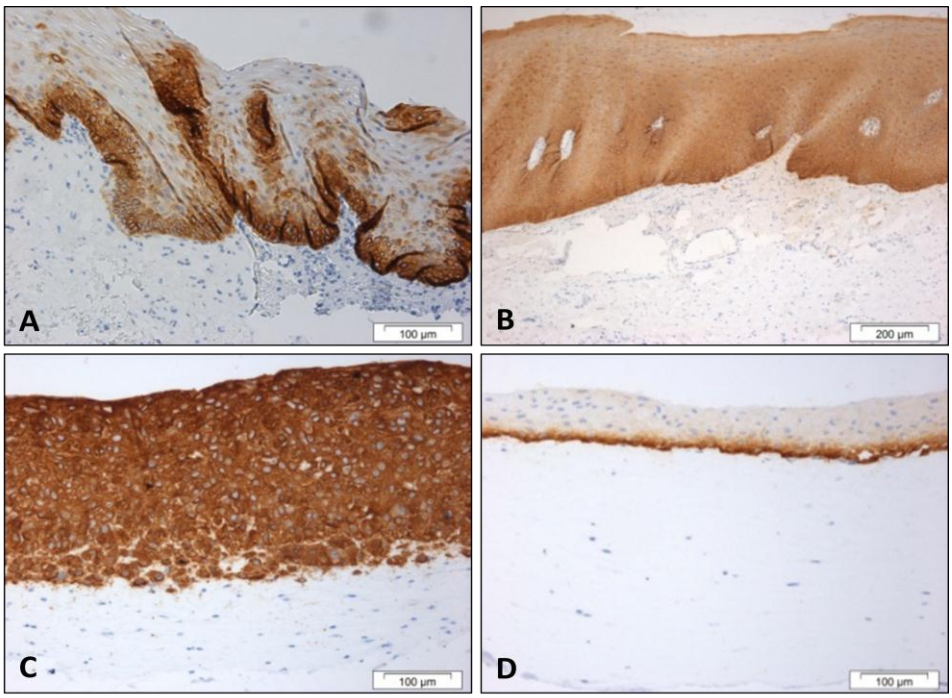


Figure 3.8 Immunohistochemical staining for cytokeratin 14. Tissue sections of buccal mucosa (A), gingival tissue (B), H357-OMM (C) and NOK-OMM (D) were stained immunohistochemically for cytokeratin 14. After 2-3 days OMM were raised to the air-to-liquid interface for approximately 7-10 days. [N.B. Gingival mucosa (B) is shown at a lower magnification to include basal and apical epithelial layers and the upper section of connective tissue]

Differences in cytokeratin 13 staining can be seen between buccal and gingival biopsy. Figure 3.6 shows that the buccal biopsy stained profusely throughout the whole epithelium, whereas the gingival biopsy stained the spinous epithelial layers only, distinctively lacking staining in the basal or keratinised layers. H357-OMM did not stain for cytokeratin 13, whereas NOK-OMM showed staining in the upper-most differentiated epithelial layers (fig 3.6).

Immunohistochemical analysis of the basement membrane protein laminin 5 showed a significant level of background staining. Despite this, staining of H357-OMM and NOK-OMM mirrored the staining observed in the buccal biopsy, showing expression in the basal epithelial layers only (fig 3.7). However, staining of the gingival biopsy showed diffuse epithelial expression, but lacked staining in the superficial keratinised layers (fig 3.7).

Staining for cytokeratin 14 highlighted differences between H357- and NOK-OMM. In the H357-OMM, staining was not restricted to any epithelial layer and diffuse staining of all of the epithelium was seen (fig 3.8). This mirrored the staining observed in the gingival biopsy. NOK models were cultured using keratinocytes isolated from buccal biopsies, and in these, the basal epithelial layers only were stained, which was in line with staining observed in the oral biopsy taken from the buccal mucosa.

E-cadherin staining of intercellular junctions within H357-OMM and NOK-OMM was very similar to that seen in buccal and gingival biopsies (fig 3.9).

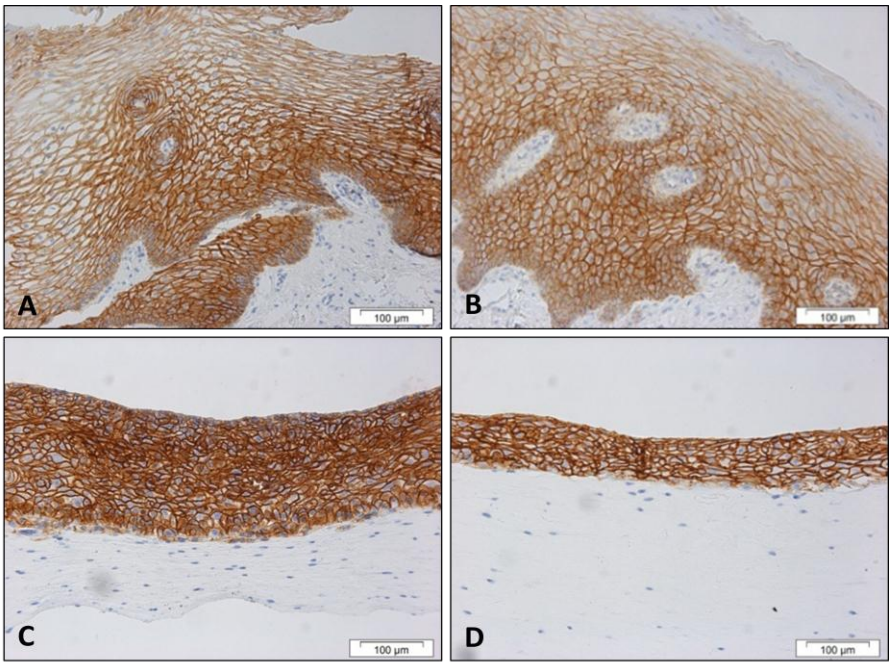


Figure 3.9 Immunohistochemical staining for E-cadherin. Tissue sections of buccal mucosa (A), gingival tissue (B), H357-OMM (C) and NOK-OMM (D) were stained immunohistochemically for E-cadherin. After 2-3 days OMM were raised to the air-to-liquid interface for approximately 7-10 days.

Submerged models were also stained immunohistochemically for the same markers, i.e. pancytokeratin, cytokeratin 13, laminin 5, cytokeratin 14 and E-cadherin (fig 3.10). The same expression profile for all markers was seen with submerged OMM compared with air-exposed OMM. For example, the only negative staining for the submerged OMM was for cytokeratin 13 in the H357 model (fig 3.10), which was comparable to the air-exposed model (fig 3.6). All other submerged OMM stained positively for pancytokeratin, laminin 5, cytokeratin 14 and E-cadherin.

3.3.2 Incorporation of neutrophils into OMM

The host immune response to bacterial challenge involves the recruitment of such immune cells as neutrophils to the site of infection. The oral mucosal model, which was characterised in this chapter, lacked any immune cells, which are important in the removal of bacterial species *in vivo*. Therefore, to try to make the models at least partially immune-competent, neutrophils were isolated from the whole blood of healthy volunteers and added to the basal surface of IL-1β-stimulated H357-OMM until they adhered and then OMM were returned to the culture medium overnight, allowing neutrophil migration through the OMM. IL-1β-stimulated H357-OMM was used in order to generate a chemoattractant gradient to allow neutrophil recruitment. Figure 3.11

shows the presence of neutrophils in the connective tissue layer of stimulated H357-OMM. Multi-lobular nucleated cells were clearly visible in figures 3.11A & 3.11D (*) within the collagen layer. However, no neutrophils were detected in the epithelial layer. This may have been due to the thickness or type of the collagen scaffold or that the chemoattractant gradient was insufficient. Attempts were made to increase neutrophil migration using *P. gingivalis* as the stimulus but similar numbers of neutrophils were seen and again these were confined to the collagen layer.

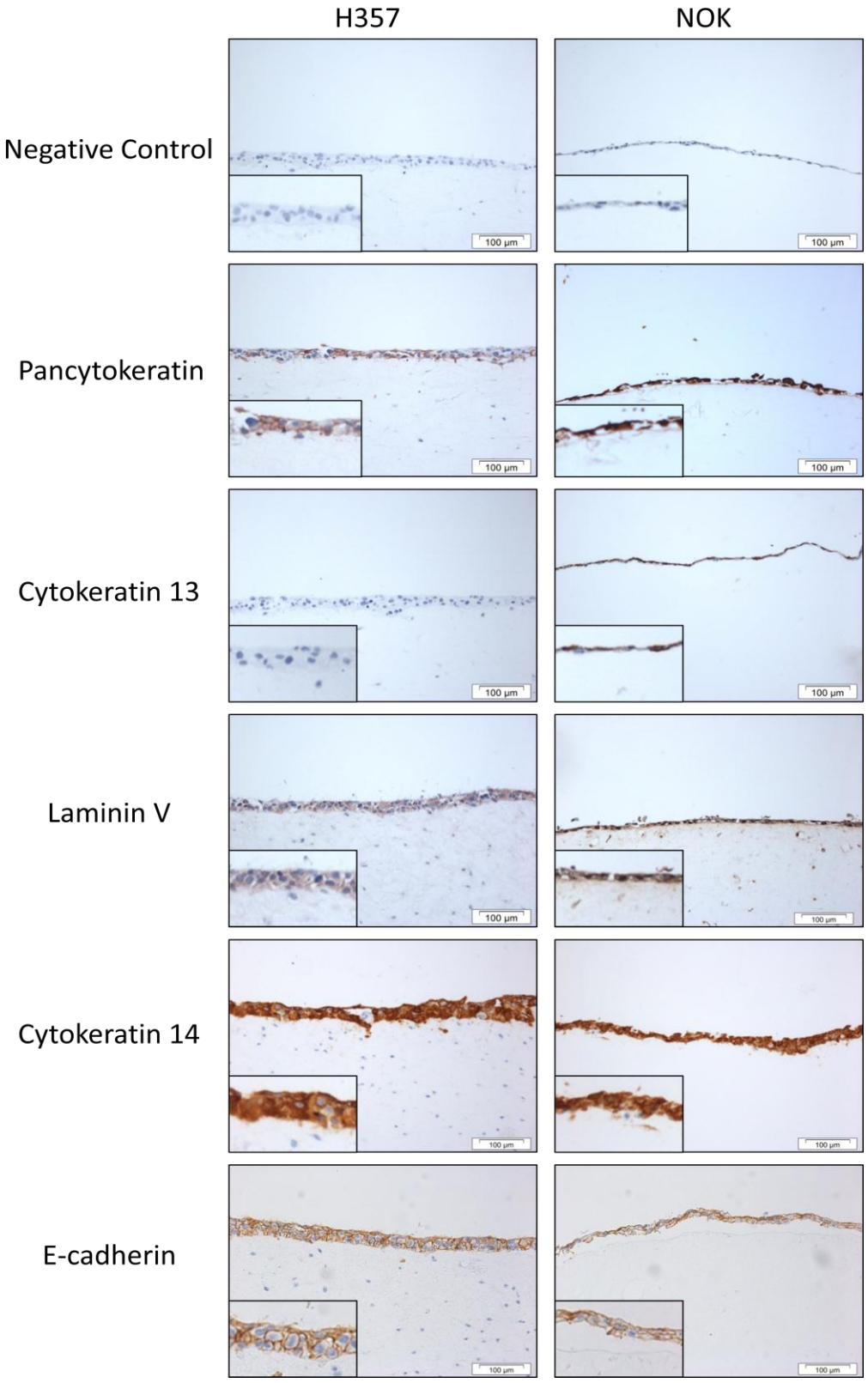


Figure 3.10 Immunohistochemical staining of submerged H357-OMM and NOK-OMM. OMM were completely submerged in culture medium for 3-5 days, after which the models were formalin fixed, paraffin embedded, sectioned and stained immunohistochemically using antibodies directed against pancytokeratin, cytokeratin 13, laminin 5, cytokeratin 14 and E-cadherin. Murine IgG was used as an isotype-matched negative control. Inset boxes are approximately 2.3x magnified.

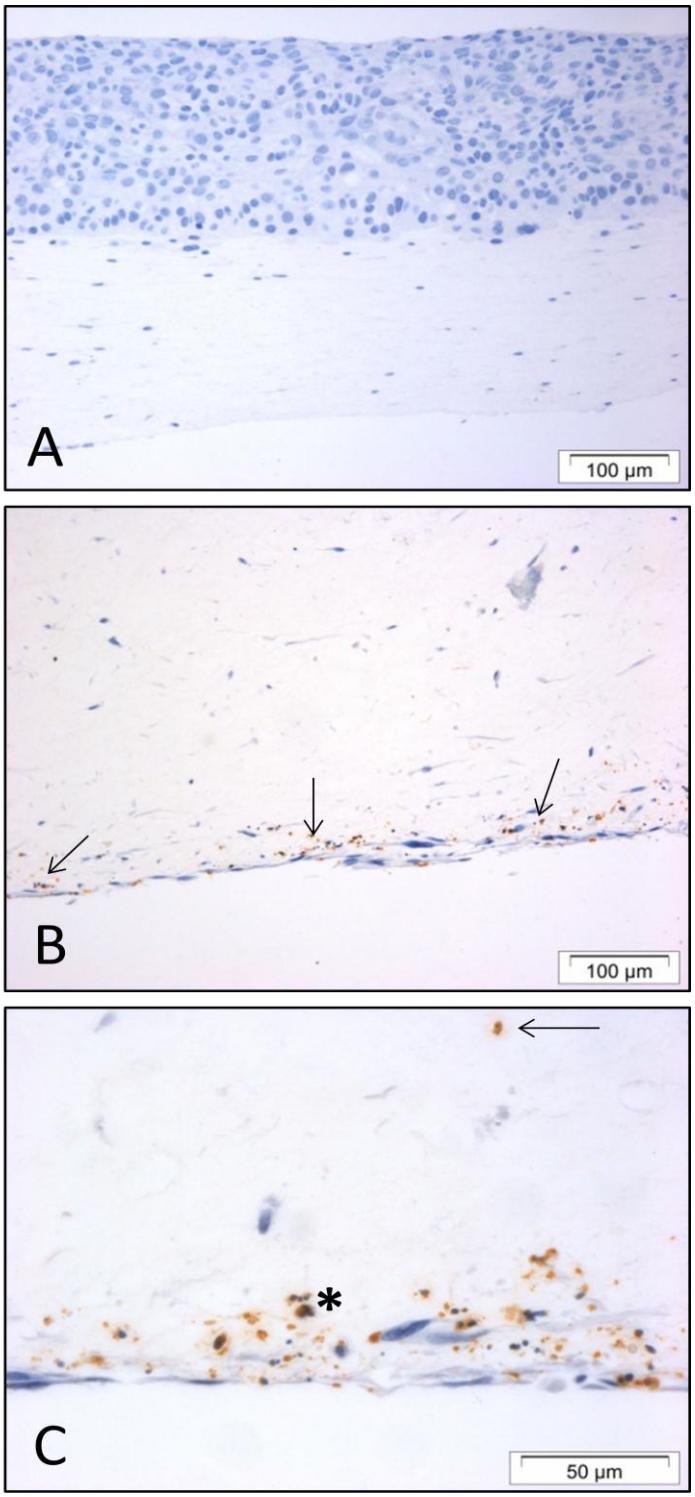


Figure 3.11 Neutrophil migration through H357-OMM. Neutrophils were isolated and incubated with H357-OMM overnight in the presence of IL-1β. Sections of OMM were stained using an anti-neutrophil elastase primary antibody by immunohistochemistry (B & C). Murine IgG was used as an isotype-matched negative control (A). Arrows depict neutrophils. Multi-lobular nuclei can be seen (*).

3.3.3 Optimisation of OMM for *Porphyromonas gingivalis* invasion

Following characterisation of the model, the experimental design of a standard antibiotic protection assay was modified to evaluate *P. gingivalis* invasion of OMM.

The standard antibiotic protection assay, used in many studies of bacterial invasion (Choi *et al.*, 2011; Chu *et al.*, 2010; Lamont *et al.*, 1995), involves numerous steps which had to be optimised and adapted differently in the 3D model compared with 2D culture. The steps that were investigated were the length of time for optimal bacterial invasion, the atmosphere the assay would need to be performed under: anaerobic or aerobic, and the lysis technique used to recover intracellular bacteria. The viability of OMM following invasion was also assessed to ensure any changes to the experimental design did not compromise epithelial viability.

3.3.3.1 Time course of *P. gingivalis* invasion

Previously in the literature, the incubation time used to investigate *P. gingivalis* invasion of a similar organotypic mucosal model was 24 hours anaerobically (Andrian *et al.*, 2004). As this was the only report regarding *P. gingivalis* invasion of a full-thickness organotypic model, initial experiments were performed overnight anaerobically. However, during this incubation period the epithelium showed signs of degradation and surface epithelial cell loss (fig 3.12). In particular, NOK-OMM showed the greatest epithelial disruption compared with H357-OMM. Shown later in this chapter, *P. gingivalis* may only invade the superficial layers of epithelium (fig 3.23) suggesting that, particularly with NOK-OMM, there was likely to be reduced levels of bacterial detection, when analysed using an antibiotic assay, due to the removal of epithelial layers during the washing steps. In particular, it was discovered that extensive washing of the models, over a 1 hour time period, was required to ensure the removal of metronidazole from the tissue.

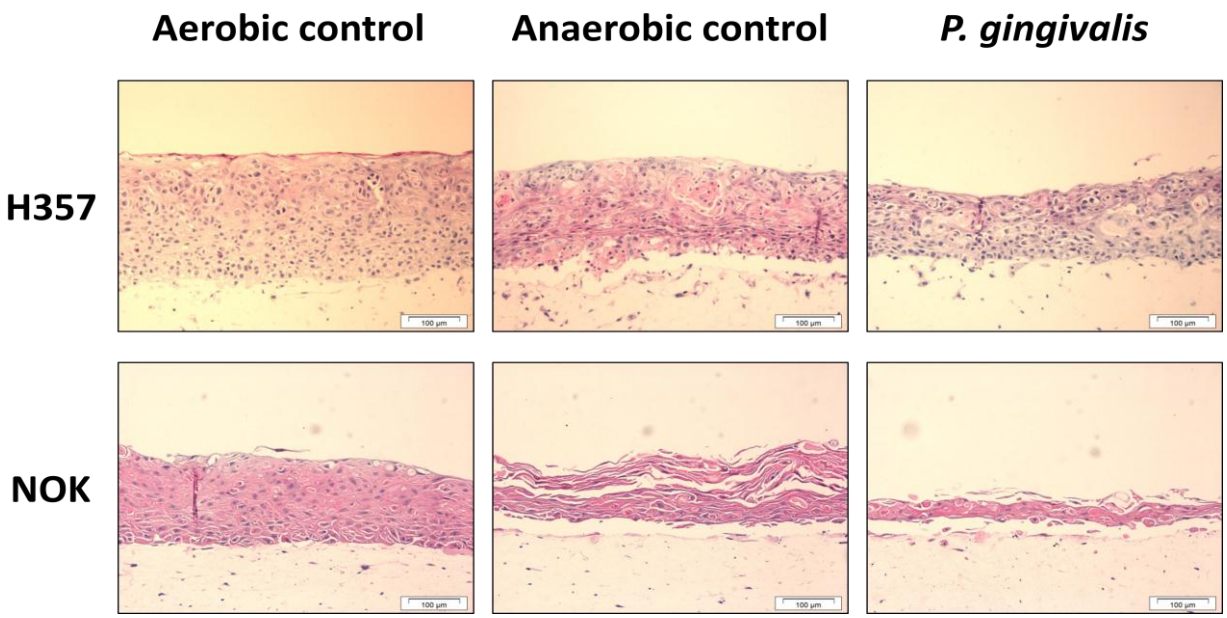


Figure 3.12 Epithelial damage of H357-OMM and NOK-OMM following overnight anaerobic incubation with *P. gingivalis*. H357-OMM and NOK-OMM were cultured at the air-to-liquid interface for 7-10 days following which OMM were exposed to *P. gingivalis* NCTC 11834 (MOI100) overnight (approximately 16 hours) anaerobically. Control images are of representative models sacrificed prior to the experimental procedure.

Therefore, to preserve epithelial integrity, a time course of invasion was performed aerobically, and was used to indicate the length of time with which to incubate *P. gingivalis* with the OMM to give maximal percentage invasion. Figure 3.13 shows the time course of invasion for air-exposed OMM indicating that 3-6 hours incubation was maximal for *P. gingivalis* NCTC 11834, to invade and to subsequently be recovered from the intracellular environment. Figure 3.13 shows that invasion increased gradually, up to a maximum of 4.25±0.84% at 6 hours and decreased after 6 hours resulting in a very low percentage recovery of intracellular bacteria after 24 hours (0.16±0.08%). The reduced percentage recovery of intracellular *P. gingivalis* at 24 hours compared with 6 hours suggested that the viability of intracellular bacteria decreased over time. Indeed, when cultured extracellularly in culture medium alone, the viability of *P. gingivalis* NCTC 11834 decreased to zero after 24 hours (fig 3.14).

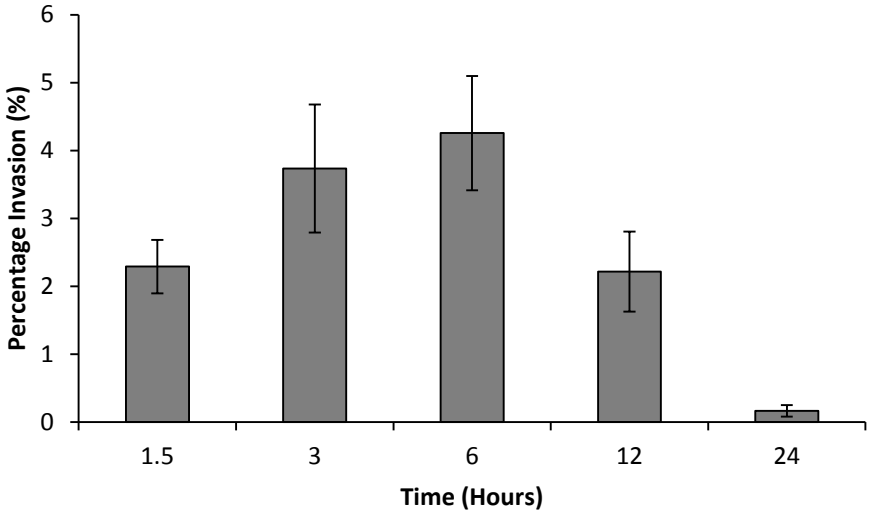


Figure 3.13 A time course for invasion of air-exposed H357-OMM by *P. gingivalis*. Air-exposed H357-OMM were cultured and exposed to $2x10^7$ *P. gingivalis* (being a nominal MOI100 because OMM and 24-well plate monolayer cultures have similar surface areas) for 1.5, 3, 6, 12 or 24 hours. Metronidazole was added to kill external adherent bacteria and the intracellular bacteria were enumerated by colony counting after lysing the OMM by homogenisation. Bars indicate the percentage invasion relative to the original bacterial suspension plated during bacterial incubation with OMM. Percentage invasions are means (±SEM) of 3 independent experiments performed in triplicate.

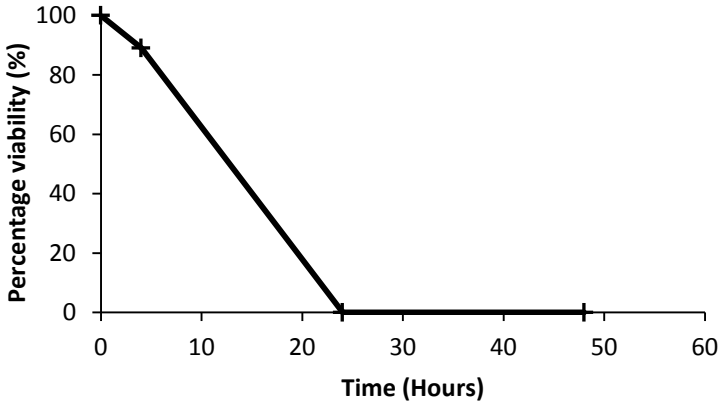


Figure 3.14 Percentage viability of *P. gingivalis* NCTC 11834 cultured aerobically in serum-free culture medium over time. Plate cultured *P. gingivalis* NCTC 11834 was harvested and resuspended in serum-free culture medium at $2x10^7$ (representative of the number of bacterial cells added to epithelial cells in a typical 24-well invasion assay) for 48 hours at 5% CO_2/95% air. Samples of the culture medium were removed at each time point, serially diluted and colonies were counted. Counted colonies were analysed as a percentage of the original bacterial suspension plated prior to aerobic incubation.

Submerged models were also used to investigate the optimal infection time with *P. gingivalis* (fig 3.15). Similarities in the percentage invasion of *P. gingivalis* into submerged models compared with air-exposed models were observed, peaking at 3 hours (3.69±1.37%). Therefore, for subsequent experiments, a 4 hour incubation period with *P. gingivalis* was routine for both submerged and air-exposed OMM, as no significant difference in percentage invasion was observed between 3 and 6 hours.

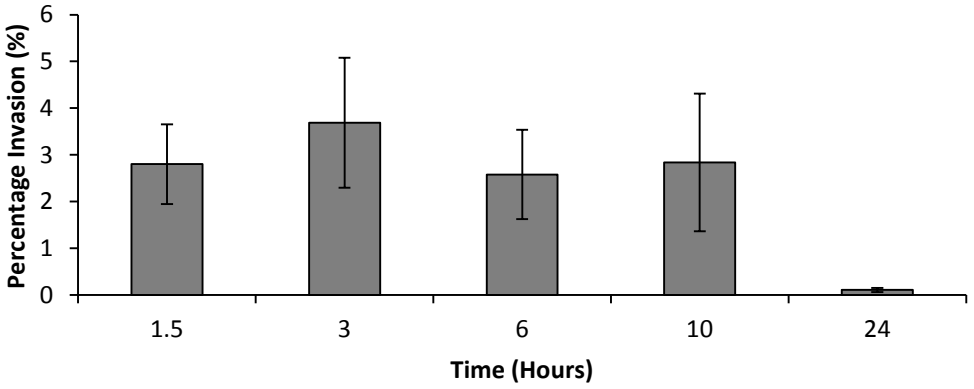


Figure 3.15 A time course for invasion of submerged H357-OMM by *P. gingivalis*. Submerged H357-OMM were cultured and exposed to *P. gingivalis* ($2x10^7$) for 1.5, 3, 6, 10 or 24 hours. Metronidazole was added to kill external adherent bacteria and the intracellular bacteria were enumerated by colony counting after lysing the OMM by homogenisation. Bars indicate the percentage invasion relative to the original bacterial suspension plated during bacterial incubation with OMM. Percentage invasions are means (±SEM) of 3 independent experiments performed in triplicate.

3.3.3.2 Bacterial recovery from OMM: Homogenisation or manual chopping?

In order to release the intracellular bacteria from the model, two methods were investigated. These were mechanically chopping of the model with a scalpel and vigorously pipetting up and down, or homogenisation using a commercially available homogeniser (TissueRuptor, Qiagen). The release of *P. gingivalis* from H357-OMM using these two methods is shown in figure 3.16. No significant difference in percentage recovery was shown (p=0.533). However, experimentally it was found that cutting was more time consuming and did not give a homogeneous suspension, which proved difficult to pipette. Therefore, for subsequent experiments homogenisation was used to release internalised bacteria from OMM.

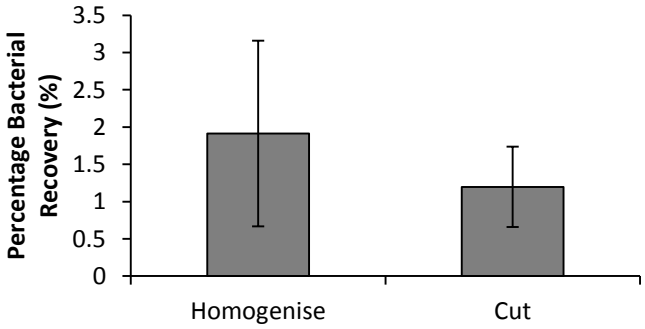


Figure 3.16 Comparison of two lysis techniques to release intracellular *P. gingivalis* from H357-OMM. Air-exposed H357-OMM were infected with *P. gingivalis* NCTC 11834 for 4 hours aerobically. Models were lysed by homogenisation or mechanical cutting with a scalpel. Invasion was calculated as the number of viable colonies counted as a percentage of the viable count of the original bacterial suspension. Data shown are means of three independent experiments performed in triplicate (±SD).

To ensure that the viability of *P. gingivalis* was not affected by homogenisation, a suspension of *P. gingivalis* was homogenised and the bacterial viability assessed by colony counting on blood agar. No significant difference in bacterial viability was found up to 10 seconds, which was the length of time used to release intracellular bacteria from OMM, although it did decline with longer periods of homogenisation (fig 3.17).

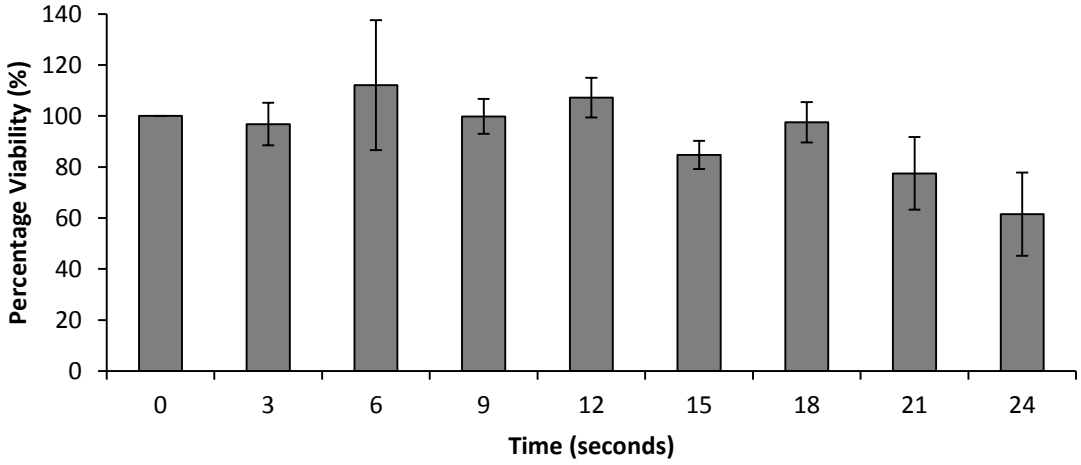


Figure 3.17 The viability of *P. gingivalis* NCTC 11834 after homogenisation. Plate cultured NCTC 11834 was re-suspended in PBS and subjected to homogenisation for 0, 3, 6, 9, 12, 15, 18, 21 and 24 seconds. Viable colony counts were made and bars indicate the mean percentage bacterial viability (± SD) after adjusting the colony counts of 0 second homogenisation to 100%. Results shown are means of triplicate experiments.

3.3.3.3 Anaerobic or aerobic incubation?

As *P. gingivalis* is an anaerobic bacterium and the epithelial model is cultured in an aerobic 5% CO_2 atmosphere, it was difficult to know in which atmosphere the assay should be performed to produce optimal results. It has been shown that incubation of OMM overnight anaerobically resulted in marked epithelial destruction (fig 3.12), but does the culture atmosphere affect bacterial invasion, particularly during the optimal 4 hour incubation period? Therefore, H357-OMM were cultured and infected with *P. gingivalis* NCTC 11834 (MOI100) aerobically or anaerobically for 4 hours and the proportions of the recovered bacteria were compared. The percentage invasions shown in figure 3.18 indicated that when incubated aerobically there was a greater recovery of internalised bacteria, compared with anaerobic incubation (5.08±2.11% and 2.89±0.74%, respectively). However, this was not significantly different ($p=0.166$) suggesting that the atmosphere the assay was performed under, at least over 4 hours, did not affect bacterial viability. Consequently, for subsequent experiments, the aerobic 5% CO_2 condition was chosen for invasion assays as this method was technically easier to perform in terms of entering and re-entering the anaerobic chamber during each incubation period and it was better for the maintenance of epithelial integrity.

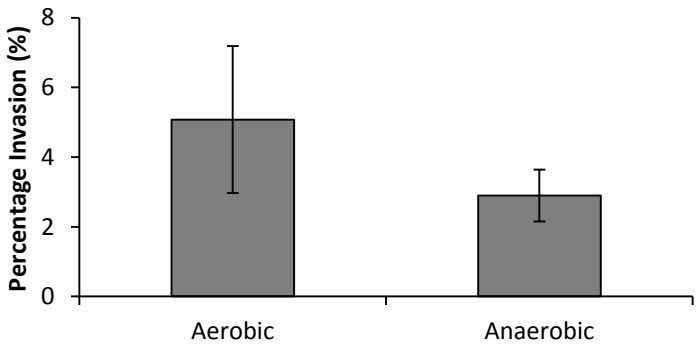


Figure 3.18 Invasion of H357-OMM by *P. gingivalis* under anaerobic and aerobic conditions. Rat-tail type I collagen models seeded with the H357 cells were raised to the air-to-liquid interface for 7-10 days and infected with *P. gingivalis* NCTC 11834 (MOI100) for 4 hours aerobically or anaerobically. Models were lysed by homogenisation and invasion was calculated as the number of viable colonies counted as a percentage of the original bacterial suspension plated during bacterial incubation with OMM. Data shown are means of three independent experiments performed in triplicate (±SD).

3.3.3.4 Viability of OMM following invasion

Data in figure 3.13 shows that over a 4 hour time period, bacterial invasion was at a maximum. Therefore, the viability of H357-OMM was investigated following invasion by *P. gingivalis* over the same period. Epithelial viability was measured using a lactate dehydrogenase or MTT assay, whilst tissue damage was observed by H&E staining.

3.3.3.4.1 H&E

Histological staining of OMM following 4 hour invasion of H357-OMM by *P. gingivalis* NCTC 11834 indicated that there were no macroscopic changes in epithelial morphology or loss of epithelial integrity over this time period (fig 3.19).

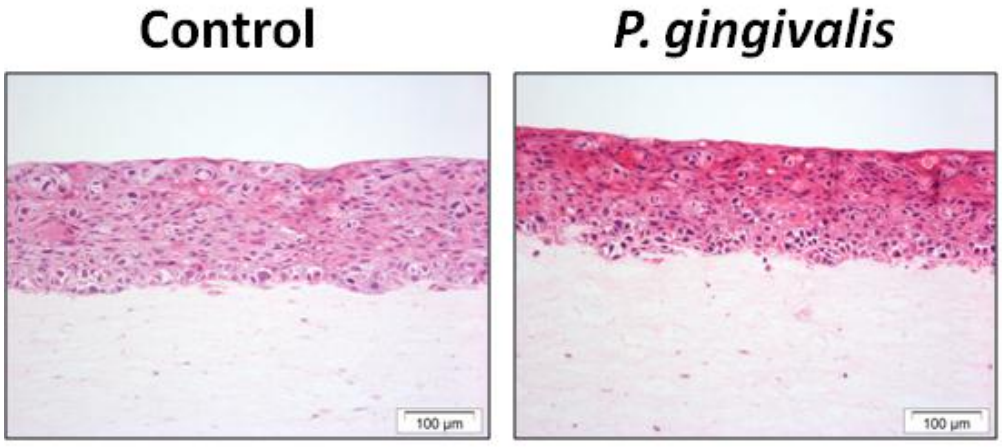


Figure 3.19 H&E stained sections of H357-OMM incubated for 4 hours with *P. gingivalis*. H357-OMM were cultured at an air-to-liquid interface for 7-10 days following which OMM were exposed to *P. gingivalis* NCTC 11834 (MOI100) for 4 hours aerobically. Control image is a representative model sacrificed prior to the experimental procedure.

3.3.3.4.2 LDH

To quantitatively assess the viability of H357-OMM a lactate dehydrogenase assay was chosen. LDH is a soluble cytoplasmic enzyme that is released from cells when they undergo necrosis or apoptosis. The released enzyme is then able to reduce the assay substrate producing a coloured product, the absorbance of which can be measured by a spectrophotometer. Due to their short culture period and relatively low experimental cost, preliminary assays used monolayer cultures of H357. Monolayers were incubated with *P. gingivalis* overnight and an LDH assay was performed. Figure 3.20 shows that the baseline LDH release from uninfected monolayers was 7.44±0.51%, relative to 100% cell lysis using saponin. However, in the presence of *P. gingivalis*, this percentage decreased to 0.68±0.51%, suggesting an increase in epithelial viability compared with the uninfected control. As this was thought to be unlikely, purified LDH (in the form of a positive control supplied in the LDH assay kit) was incubated with live *P. gingivalis* NCTC 11834 for 6 hours. The results showed an 88.2% decrease ($p<0.05$) in the reduction of the assay substrate (fig 3.21), suggesting that LDH was destroyed by *P. gingivalis*. Therefore, this assay could not be used to study epithelial viability in the presence of *P. gingivalis*.

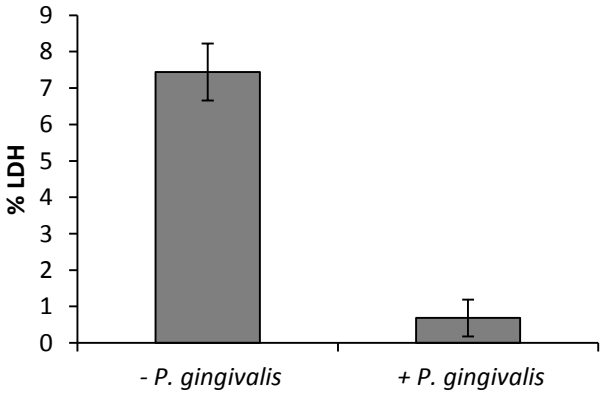


Figure 3.20 A lactate dehydrogenase assay to show lactate dehydrogenase release from epithelial cells treated with *P. gingivalis*. *P. gingivalis* NCTC 11834 was incubated H357 monolayers overnight. A LDH assay was performed on the conditioned medium and the bars show the percentage LDH (±SD) after incubation with or without *P. gingivalis*, relative to H357 monolayers lysed using saponin to indicate 100% cell death. The assay was performed in triplicate, bars are means±SD.

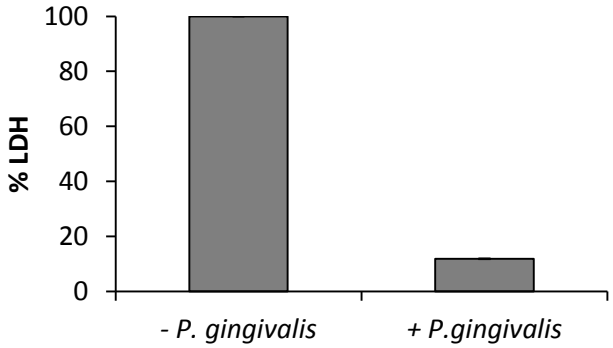


Figure 3.21 A lactate dehydrogenase assay to show destruction of the lactate dehydrogenase positive control by *P. gingivalis*. *P. gingivalis* NCTC 11834 was incubated with a LDH positive control supplied in the assay kit (bovine heart LDH, Promega) for 6 hours. A LDH assay was performed on the resulting solution and the bars show the percentage LDH (±SD) remaining after incubation with or without *P. gingivalis*. The assay was performed in triplicate, bars are means±SD.

3.3.3.4.3 MTT

The sensitivity of the MTT assay to *P. gingivalis* cells was assessed prior to using MTT to study epithelial cell viability. The reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was pre-incubated with live, whole *P. gingivalis* cells to see if the bacterium was capable of reducing the yellow tetrazole MTT to a purple formazan, a process which occurs in living cells. It was found that over the 1 hour incubation period (the manufacturer's recommended time for use of MTT to detect cell viability), no change in colour was detected in the presence of *P. gingivalis* (data not shown) and so this assay was used to evaluate the epithelial viability of H357-OMM following invasion by *P. gingivalis* NCTC 11834 (fig 3.22). No significant difference in the viability of OMM was detected over the 4 hour invasion period when compared to the viability of un-infected OMM (p=0.733) thus confirming 4 hours as an optimal infection time for OMM with *P. gingivalis*.

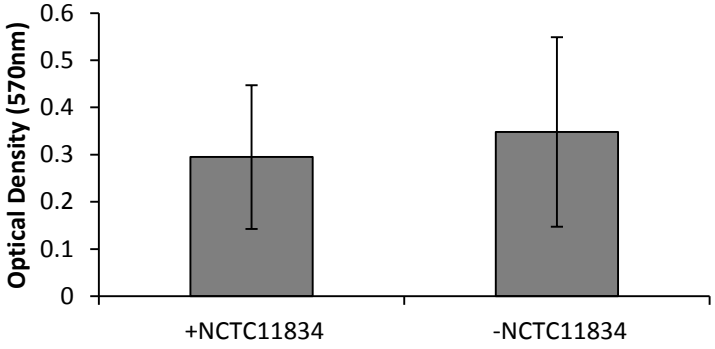


Figure 3.22 Viability of H357-OMM following invasion by *P. gingivalis* NCTC 11834 assessed by MTT. H357-OMM were cultured at the air-to-liquid interface and incubated with or without NCTC 11834 (MOI100) for 4 hours. MTT reagent was added for 1 hour and the insoluble formazan released from the OMM by acidified isopropanol. The absorbance at 570nm was recorded and the histogram shows the mean absorbance values (±SD) from 3 individual experiments repeated in triplicate, normalised to acidified isopropanol alone.

3.3.4 How far does *Porphyromonas gingivalis* penetrate into the model?

3.3.4.1 Immunohistochemical assessment

A monoclonal antibody (MAb 1B5) raised against the *P. gingivalis* catalytic subunit of arginine-gingipain ($RgpA_{cat}$) (Curtis *et al.*, 1999), and also immunoreactive with *P. gingivalis* membrane type $RgpA_{cat}$ (mt-$RgpA_{cat}$), membrane-type RgpB (mt-RgpB) and an anionic cell surface polysaccharide (APS), all of which possess a Manα1-2Manα-1-phosphate epitope (Rangarajan *et al.*, 2008), was kindly provided by Professor Mike Curtis, Barts and The London School of Medicine. The specificity of the MAb 1B5 antibody was confirmed by staining fibrin-embedded whole *P. gingivalis* cells (fig 3.23C). Immunohistochemistry using this antibody was performed on sections of H357- and NOK-OMM infected with *P. gingivalis* NCTC 11834 (fig 3.23). Staining indicated that this bacterium invaded only the superficial layers of epithelium (fig 3.23). However, staining within NOK-OMM was not as conclusive as the staining observed in H357-OMM. This may be due to the possible desquamation of the surface epithelial layers seen in infected NOK-OMM. The penetration of *P. gingivalis* into the connective tissue layer may occur in submerged models where the epithelium is a lot thinner (fig 3.24). Although convincing, it was difficult to be certain that the intracellular staining was specific since the apparent *P. gingivalis* cells were so few, and it was difficult to accurately visualise individual *P. gingivalis* cells using this technique.

Similar staining was observed within *P. gingivalis* infected H357-OMM, using a monoclonal antibody (MAb 1A1) directed against an epitope on the adhesin domain of the cysteine protease-adhesin heterodimer (HRgpA) (Curtis *et al.*, 1996) (fig. 3.25).

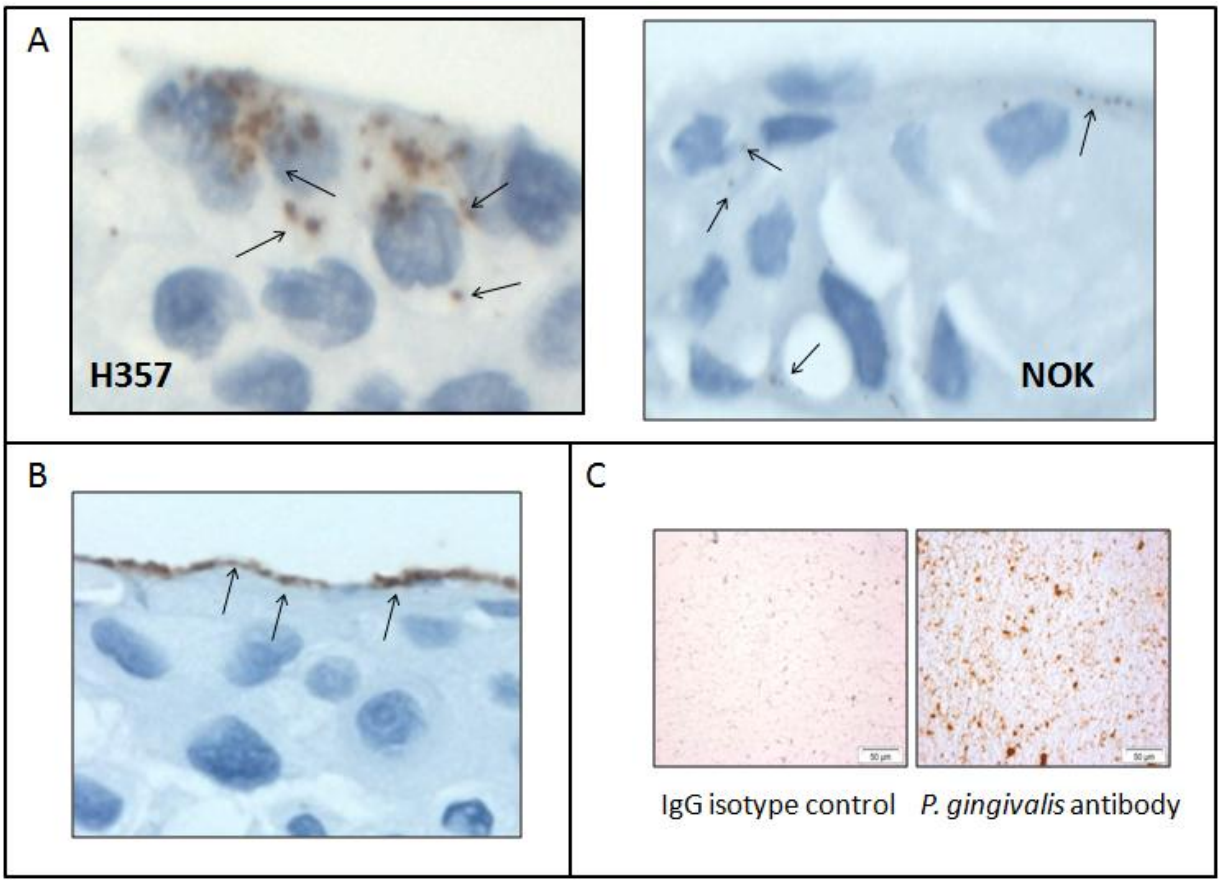


Figure 3.23 Immunohistochemical staining to assess the tissue penetration of air-exposed H357-OMM and NOK-OMM by *P. gingivalis* NCTC 11834. Sections of air-exposed H357-OMM infected with *P. gingivalis* NCTC 11834 overnight, were stained using immunohistochemistry (the primary antibody was MAb 1B5 (Curtis *et al.*, 1999)). Staining was detected in the superficial layers of H357-OMM (mag. 3480x) and possibly within NOK-OMM (mag. 3750x) (A) and adhered to the surface of H357-OMM (mag. 1800x) (B). Arrows depict possible staining of intracellular bacteria in OMM. Staining of a separate tissue section with the negative control antibody IgG2a showed no staining. To confirm the immunoreactivity of the *P. gingivalis* antibody, fibrin clots of *P. gingivalis* whole cells produced positive staining with MAb 1B5 (C). Images are representative staining of at least three independent experiments.

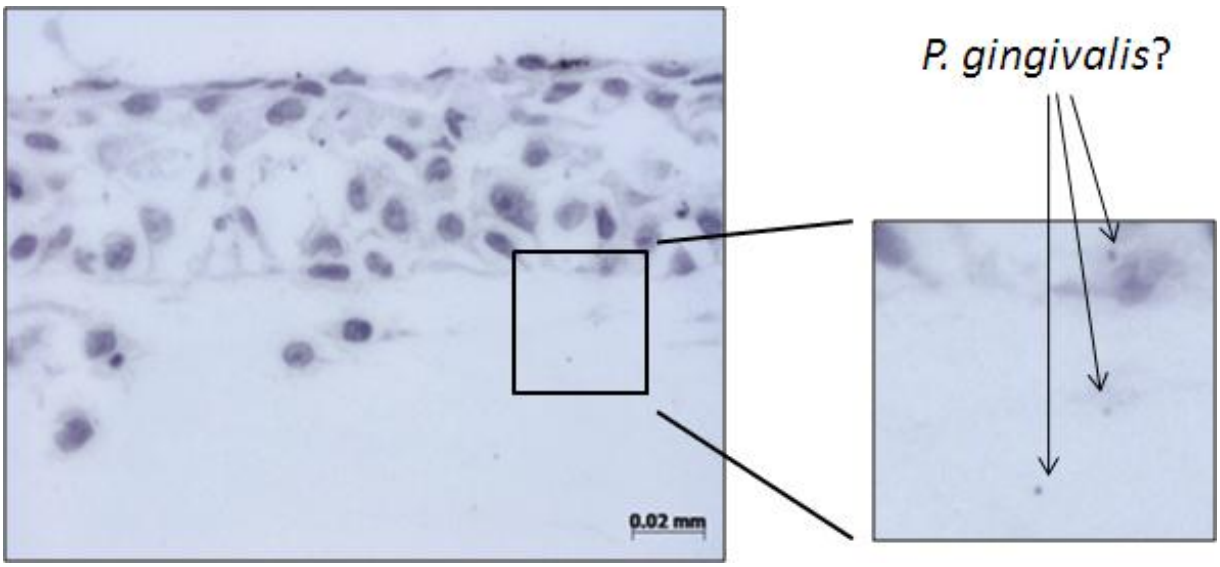


Figure 3.24 Immunohistochemistry to show the possible intracellular localisation of *P. gingivalis* within submerged OMM. Sections of submerged H357-OMM were stained using immunohistochemistry with MAb1B5 primary antibody. Arrows show apparent penetration of *P. gingivalis* within the epithelium and connective tissue layer. Magnified box approximately 2x greater magnification.

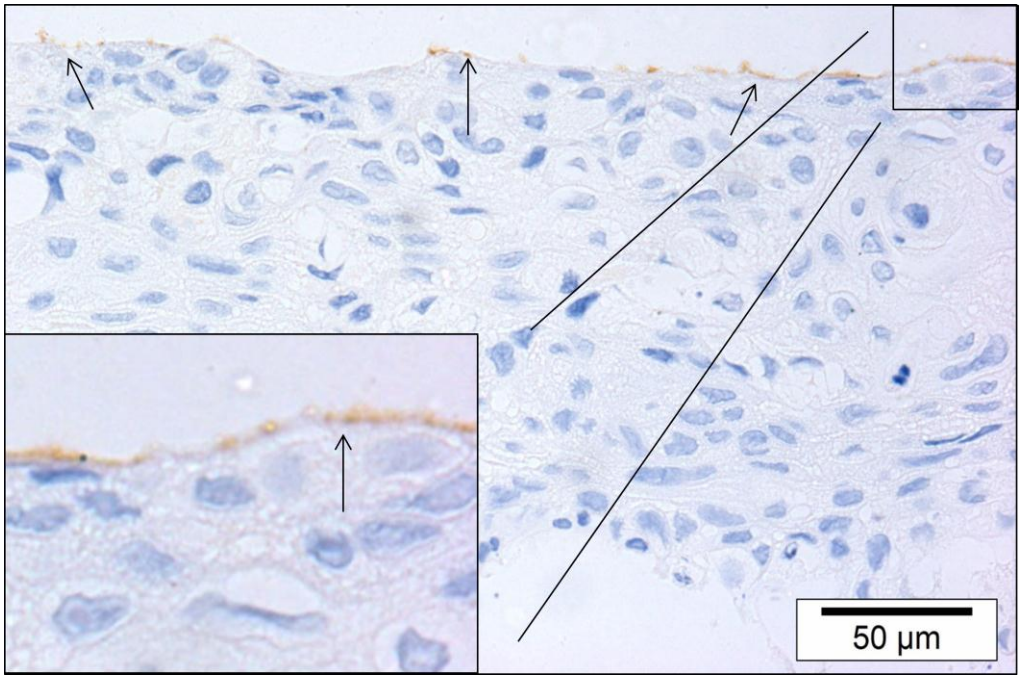


Figure 3.25 Immunohistochemical staining of H357-OMM using MAb 1A1 monoclonal antibody. Sections of air-exposed H357-OMM infected with *P. gingivalis* NCTC 11834 overnight, were stained using immunohistochemistry (with the primary antibody MAb 1A1 (Curtis *et al.*, 1996)). Staining was detected in the superficial layers of H357-OMM. Arrows depict possible staining of intracellular bacteria. Inset box is approximately 2.5x greater magnification.

3.3.4.2 Immunofluorescence assessment

With immunohistochemical staining, determining the depth of epithelial penetration by *P. gingivalis* proved difficult. This was due to the small size of this bacterium and concern that small 'brown specks' may represent, non-specific staining, making it difficult to distinguish actual bacterial cells. Therefore, an immunofluorescence method was used in which *P. gingivalis* was fluorescently (FITC) labelled prior to the addition to the OMM. However, this technique showed some background fluorescence (fig 3.26), though *P. gingivalis* was identified within the superficial layers of epithelium (as with immunohistochemical staining) and small numbers appeared to be present in lower layers of the epithelium (fig 3.26). No staining was observed in the connective tissue layer of an air-exposed model.

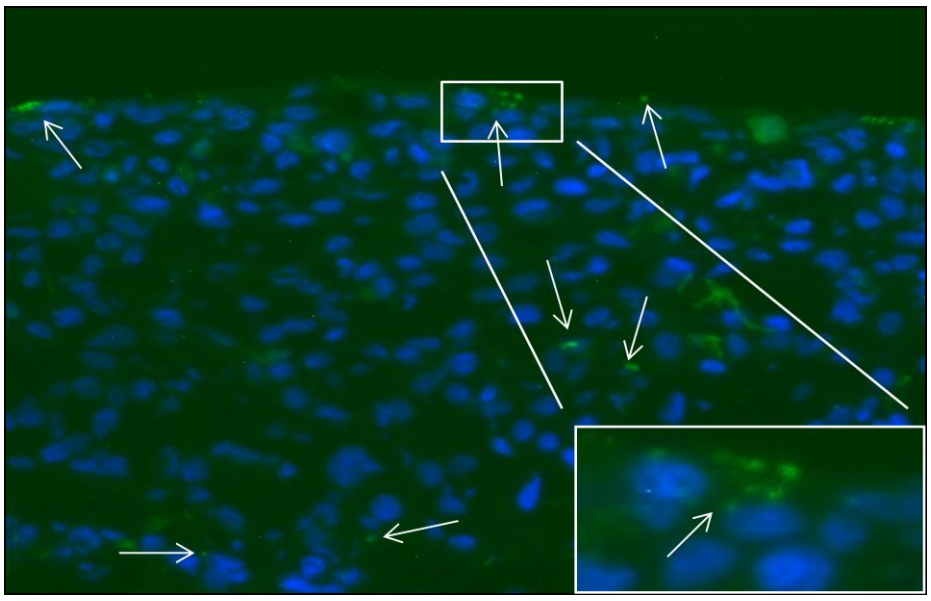


Figure 3.26 Immunofluorescence staining of *P. gingivalis* in H357-OMM. *P. gingivalis* NCTC 11834 was FITC-labelled and incubated overnight with air-exposed OMM. Frozen sections were obtained and epithelial cell nuclei stained using Hoechst. White arrows indicate possible locations of *P. gingivalis* within the epithelium. Magnification 400x, inset box is approximately 2.5x greater magnification.

3.4 DISCUSSION

Until recently there have been few reports in the literature regarding the use of three-dimensional organotypic oral mucosal models to study *P. gingivalis* invasion. Organotypic models are more representative of normal oral mucosa than a simple monolayer and the use of these models to study bacterial invasion may provide further understanding of the pathogenesis of periodontitis.

3.4.1 Characterisation of the oral mucosal model

The use of a variety of connective tissue scaffolds for the culture of oral mucosal models has been evaluated previously (Moharamzadeh *et al.*, 2007a). The comparison of a DED scaffold with a collagen type I scaffold in this study indicated differences in the histological and molecular structure of the cultured organotypic models. Differences such as the absence of rete ridges in collagen-OMM were obvious, but more importantly collagen-OMM did not possess a basement membrane as previously observed (Yadev *et al*., 2011) (fig 3.1). DED maintains components of the basement membrane such as laminin and collagens type IV and VII (Okamoto and Kitano, 1993; Ralston *et al.*, 1999; Yadev *et al*., 2011), and so this is one advantage of using DED as a scaffold. However, when studied immunohistochemically, collagen-OMM weakly expressed laminin 5 (fig 3.7), which was in agreement with Costea *et al* (2003) who also showed staining for laminin 5 in the epithelium of collagen models. Laminin 5 acts as an adhesive protein connecting the connective tissue layer with the epithelium. The presence of laminin 5 in collagen-OMM suggests that keratinocytes and/or fibroblasts play a role in the formation of basement membrane components (Okamoto and Kitano, 1993). In their particular favour though, collagen models showed high reliability and reproducibility in comparison with DED-OMM, which was a significant consideration for future experimental work. Additionally, compared with DED-OMM, collagen-OMM gave a greater epithelial coverage (fig 3.1) and were less costly because the collagen was isolated in-house. In spite of the deficiencies of collagen-OMM, these models were chosen for future work as they represented the *in vivo* situation more closely than monolayer cultures of epithelial cells, as there is the presence of a stratified epithelial layer and the capacity for fibroblast-epithelial cross-talk (Maas-Szabowski *et al.*, 2001; Sanaie *et al.*, 2002).

Keratinocyte differentiation could be recognised in NOK-OMM based on DED as a flattening of cells accompanied by epithelial stratification, and a loss of cell nuclei in the upper most layers. Only slight keratinocyte differentiation was detected in NOK-OMM based on collagen. This may be due to differences in the origin of the oral biopsy. Biopsies obtained from gingivae were more likely to produce differentiated and keratinised epithelia (such as those that were cultured on DED (fig 3.1)), whereas biopsies isolated from buccal mucosa tended to remain

non-keratinised (such as those which were cultured on collagen (fig 3.1)). This has been noted previously where the origin of the oral biopsy influenced the morphology of reconstructed epithelia (Gibbs and Ponec, 2000). Therefore, to remain consistent and because of their availability, collagen OMM cultured throughout this study used NOK isolated from buccal biopsies.

Positive staining for pan-cytokeratin confirmed the presence of an epithelial layer and further highlighted the differences between NOK- and H357-OMM. As a carcinoma cell line, H357 showed epithelial cell invasion into the connective tissue layer as previously observed (Nystrom *et al*., 2005) (fig 3.5). In terms of the use of this model to study bacterial invasion, the infiltration of keratinocytes into the collagen support was considered negligible and not significant in the interpretation of future results.

Organotypic collagen oral mucosal models have previously been shown to stain positively for E-cadherin (Dongari-Bagtzoglou and Kashleva, 2006), which is an important molecule in the maintenance of epithelial structural integrity. Similarly, in this chapter, staining for E-cadherin was positive in all epithelial layers of the buccal biopsy, NOK-OMM and H357-OMM (fig 3.9). Staining was also positive in the basal, suprabasal and prickle cell layers of the gingival epithelium. However, no staining was observed in the superficial keratinising layers (fig 3.9) (Ye *et al.*, 2000), indicating a loss of cell-cell contacts between the more differentiated keratinocytes. In contrast to the study by Gasparoni *et al*. (2004) where a loss of E-cadherin expression in immortalised and cancerous cells was reported, here the intensity of staining seen in H357-OMM was the same as for NOK-OMM (fig 3.9).

Immunohistochemical characterisation of air-exposed collagen H357- and NOK-OMM indicated similarities with normal buccal tissues in the expression of pan-cytokeratin, laminin 5 and E-cadherin (figs 3.5, 3.7 and 3.9 respectively). This suggests that the organotypic models are valid comparators to normal oral mucosa, which has also been reported previously (Dongari-Bagtzoglou and Kashleva, 2006; Horiguchi *et al.*, 1994; Rouabhia and Deslauriers, 2002).

The cytoplasmic expression of laminin 5 in the epithelial layers of the gingival biopsy was not expected. Laminin 5 is a major component of the basement membrane (Kinumatsu *et al.*, 2009), and as such, staining would be expected to be confined to this area. The diffuse staining observed throughout the epithelium may be due to the staining technique or a degree of non-specific staining, despite all efforts to reduce this.

In contrast to oral biopsies, cytokeratin 13 staining of H357-OMM was negative. As H357 originated from a squamous cell carcinoma of the tongue, the absence of staining may be due to the previously reported reduction in expression of this cytokeratin in epithelial cell carcinomas (Yanagawa *et al.*, 2007). NOK-OMM showed positive staining for cytokeratin 13, comparable to positive staining of the suprabasal epithelial layers of buccal tissues, which is in accordance with Costea *et al*. (2003). This suprabasal staining of NOK-OMM indicated that this epithelium was not keratinised, as cytokeratin 13 is specifically expressed in stratified, non-keratinised epithelia (Jacques *et al.*, 2009). An example of this can be seen in figure 3.6 where staining of gingival mucosa was positive in the suprabasal layers but was absent from the upper keratinised layer. As NOK-OMM were cultured using keratinocytes isolated from buccal mucosa, positive staining in the superficial layers not only confirms the non-keratinised origin of these cells but also the maintenance of tissue-specific-expression markers (Gibbs and Ponec, 2000).

Immunohistochemical analysis of cytokeratin 14 showed profuse staining of H357-OMM and gingival mucosa throughout the whole epithelium, whereas staining of NOK-OMM and buccal biopsy was restricted to basal epithelial layers only. The similarity in staining of NOK-OMM and buccal mucosa was to be expected as the NOK-OMM were cultured using fibroblasts and keratinocytes isolated from the buccal mucosa. Therefore, this staining verified the comparability of NOK-OMM with normal buccal mucosa. It is widely accepted that in oral mucosa, cytokeratin 14 is almost exclusively expressed in the basal layers of keratinised and non-keratinised epithelia (Sardella *et al*., 2012; van der Velden *et al*., 1999), regardless of the origin within the oral cavity. However, staining for cytokeratin 14 has also been detected suprabasally (Jacques *et al*., 2009), particularly in inflamed tissue. The gingival biopsy used in this study was obtained from a patient with periodontal disease which may account for the positive staining observed in all epithelial layers. As H357 is derived from a squamous cell carcinoma, this cell line will behave differently to 'normal' epithelial cells. Indeed, Heyden *et al*. (1992) reported that the staining of dysplastic lesions for cytokeratin 14 showed distribution throughout all epithelial layers. This may account for the differences observed in cytokeratin staining between NOK- and H357-OMM. Furthermore, this differential staining (fig 3.8) suggests that NOK-OMM shows sufficient similarity to normal buccal mucosa while H357-OMM is similar to gingival mucosa.

In vivo, the majority of oral tissues are continuously bathed in liquid, i.e. GCF. Multi-layered keratinocyte cultures which were left completely submerged in culture medium have been shown to lack markers of terminal differentiation, such as cytokeratin 10 (Parnigotto *et al.*, 1998; Roguet *et al*., 1994). In addition, junctional epithelium isolated from healthy individuals lacked the expression of many markers of differentiation and was shown to express markers of

simple epithelia (Gao and Mackenzie, 1992). Many researchers have attempted to replicate junctional epithelium *in vitro* (Pan *et al.*, 1995; Papaioannou *et al.*, 1999). Although these models formed a multi-layered epithelium, they lacked a connective tissue element containing fibroblasts, which is present *in vivo*. When totally submerged, OMM produced epithelia of only 1-3 cell layers thick, which is similar to junctional epithelium (Hatakeyama *et al.*, 2006). These submerged OMM stained similarly to their air-exposed counterparts for pan-cytokeratin, cytokeratin 13, laminin 5, cytokeratin 14 and E-cadherin suggesting comparability with normal mucosa. However, without the staining of a biopsy of junctional epithelium it is difficult to confirm full comparability with native tissue. In addition, there are currently no tissue-specific markers for junctional epithelium. In a review by Shimono *et al*. (2003) the authors suggested that cytokeratin 19 may be a differential marker for secondary junctional epithelium derived from gingival epithelium. However, cytokeratin 19 has also been shown to be a marker of simple epithelia and stains positively in the basal layers of stratified epithelia (Hatakeyama *et al.*, 2006), indicating that this may not be a suitable differential marker for junctional epithelium. The absence of desmoglein 1 and 2 (which is expressed at cellular junctions), has been reported to be a feature of junctional epithelium (Hatakeyama *et al.*, 2006). This absence of expression may suggest the origin of the epithelial cells but it is not a positive indicator. Junctional epithelium has been likened to cancerous epithelium (Heyden *et al.*, 1992) in that it is non-keratinised, non-differentiating, has a high cellular turnover rate and expresses markers of specialised epithelia (Heyden *et al.*, 1992; Mackenzie *et al.*, 1989). As such, we suggest that H357 submerged OMM may be a relevant model of the junctional epithelium, particularly as a model with which to compare the full-thickness multi-layered OMM. Obtaining a biopsy of junctional epithelium is extremely difficult. Given the similarities mentioned above and due to the unreliability of tissue-specific markers for junctional epithelium, it can be assumed that the H357-OMM is sufficiently similar to junctional epithelium for it to be a reasonable model for the study of bacterial invasion.

Air-exposed H357-OMM were cultured and maximal epithelial growth with minimal loss of epithelial integrity was confirmed after 7-10 days at the air-to-liquid interface as assessed histologically (fig 3.2). Although H357-OMM does not represent stratified oral mucosa as closely as NOK-OMM, H357-OMM was used in optimisation experiments for *P. gingivalis* invasion due to the submerged model resembling junctional epithelium and the high availability and ease of culture of the cell line. The highly invasive laboratory strain of *P. gingivalis*, NCTC 11834 (Suwannakul *et al.*, 2010), was used in optimisation experiments as small differences in percentage invasion would be more noticeable if invasion was high to begin with.

3.4.2 Optimisation of OMM for *Porphyromonas gingivalis* invasion

Initial invasion assays were performed overnight anaerobically, as suggested in the literature (Andrian *et al*., 2004). However, the recovery of viable *P. gingivalis* was minimal after this time, which was probably due to the loss of epithelial integrity overnight in an anaerobic atmosphere (fig 3.12). It has previously been reported that, in the presence of *P. gingivalis*, there was a decrease in the transepithelial electrical resistance (TER) through a multilayer of immortalised gingival keratinocytes cultured on a polycarbonate membrane, after 24 hour apical introduction of *P. gingivalis*, suggesting that this bacterium is capable of destroying cell-cell contacts (Groeger *et al*., 2010). However, *P. gingivalis* at a MOI of 10^4 was required to record a decrease in the TER. Interestingly, the researchers reported an accelerated reduction in transepithelial resistance when *P. gingivalis* was incubated basolaterally, suggesting that if *P. gingivalis* was to penetrate the connective tissue, the effect on tissue destruction would be greater, possibly due to the activation of host cell-derived MMPs (Andrian *et al*., 2007). However, no difference in TER was reported for *P. gingivalis* at a MOI of 100, even after 48 hours (Groeger *et al*., 2010), which is in accordance with Dickinson *et al*. (2011), using a similar model of infection. Therefore, the loss of epithelial integrity as assessed by histology in this study was probably due to prolonged culture (24 h) in an anaerobic atmosphere (Shrieve *et al*., 1983; Nagaraj *et al*., 2004) and not directly due to the deleterious effects of *P. gingivalis*. Indeed, over 4 hours, no change in epithelial viability was detected (fig 3.19).

The maintenance of an intact epithelial barrier is an essential first step in the host innate immune response (Chapple, 2002). Therefore, a time course of infection of H357-OMM by *P. gingivalis* NCTC 11834 was performed. It was shown that 3-6 hours was optimal (fig 3.13) with no change in epithelial viability over 4 hours (fig 3.22). In monolayer cultures, invasion assays are usually performed over 1-2 hours (Lamont *et al*., 1995; Duncan *et al*., 1993), during which *P. gingivalis* has been shown to maximally invade after 10-12 minutes (Belton *et al*., 1999; Rautemaa *et al*., 2004). The reason for this longer time period for invasion of OMM compared to monolayer culture may be due to: i) the expression of different cell surface receptors, ii) decreased bacterial cell death and/or intracellular bacterial replication/survival, iii) decreased epithelial cell loss or, iv) penetration of the bacteria through the epithelial layers 'freeing up' the upper most epithelial layers for additional invasion by extracellular bacteria. In fact, high numbers of oral bacteria have been detected in the gingival epithelium and adjacent connective tissue of patients with periodontitis, when assessed by Gram staining, although the identity of specific bacteria was unknown (Saglie *et al*., 1986). In addition, the intracellular detection of *P. gingivalis* throughout the majority of gingival and pocket epithelial layers of biopsies obtained from patients exhibiting periodontitis has been reported (Rautemaa *et al*., 2004). Visualisation of *P. gingivalis* was performed using an antibody directed against a membrane-bound thiol

proteinase of *P. gingivalis* (Rautemaa *et al*., 2004). This antibody has been shown to share sequence homology with the secreted form of thiol proteinase, lys-gingipains and haemagglutinins (e.g. HagA) from various *P. gingivalis* strains (DeCarlo and Harber, 1997).

An antibody (MAb 1B5) directed against $RgpA_{cat}$ and shown to be immunoreactive against mt-Rgp_{cat}, mt-RgpB and APS (Rangarajan *et al*., 2008) was used in this study. Immunohistochemistry using this antibody to stain whole *P. gingivalis* cells identified single bacterial cells (fig 3.23), and although the majority of cells were clumped together, suggested that this antibody was suitable for detecting whole cells of *P. gingivalis*. Similar staining was also observed using the MAb 1A1 monoclonal antibody (Curtis *et al*., 1996). As these antibodies have been shown to be primarily active against Manα1-2Manα-1-phosphate (Rangarajan *et al*., 2008) and members of the haemagglutinin family (Curtis *et al*., 1996), respectively, there may be a limit to their usefulness for the detection of certain bacterial strains, particularly gingipain knock-out mutants. However, these antibodies were suitable for the identification of *P. gingivalis* NCTC 11834, in this study, but in the future an antibody directed against whole cell *P. gingivalis* may broaden the detection of this bacterium in tissue sections.

With regards to how far *P. gingivalis* can penetrate the oral mucosa, Rautemaa *et al*. (2004) did not detect thiol proteinase within the connective tissue layer of oral biopsies, in agreement with immunohistochemistry staining in this study, suggesting that, particularly in thick, multi-layered epithelial biopsies, *P. gingivalis* primarily invades the epithelial layers only.

The use of *in vitro* models of multi-layered epithelium has also attempted to answer the question of how far *P. gingivalis* is able to penetrate the oral mucosa. A study by Andrian *et al.* (2004) identified *P. gingivalis* ATCC33277 within the connective tissue scaffold of an organotypic oral mucosal model as analysed by TEM (Andrian *et al*., 2004). The authors used three-dimensional oral mucosal models cultured from cells obtained from palatal biopsies onto a scaffold of bovine type III collagen. The differences between the models used in this study may explain the differences in epithelial penetration observed. In contrast to the report by Andrian *et al*. (2004), but in agreement with results presented in this chapter, Papapanou *et al*. (1994) reported that after 4 hours, *P. gingivalis* was found in the superficial (upper) layer of an *in vitro* culture of multilayered epithelial cells, and after 8 hours had penetrated further than this superficial layer, suggesting the movement of *P. gingivalis* through oral epithelium. The authors commented that such rapidity of movement may counteract the slower act of epithelial desquamation indicating a possible mechanism by which *P. gingivalis* may breach host defences (Papapanou *et al*., 1994). In addition, Dickinson *et al.* (2011) reported that following invasion of a three-layered gingival epithelial multilayer, *P. gingivalis* penetrated the upper 2 layers but was not found

within the bottom third layer, even after 24 hours, suggesting that *P. gingivalis* is unable to gain access deeper within the epithelium over this time period (Dickinson *et al*., 2011).

Of future interest will be the question of how *P. gingivalis* gains access to lower epithelial cell layers. It has been reported that *P. gingivalis* is capable of intracellular spreading (Takeuchi *et al*., 2011; Yilmaz *et al*., 2006) and intercellular movement (Hintermann *et al*., 2002; Katz *et al*., 2002; Balkovetz and Katz, 2003). In addition, it has previously been reported that *P. gingivalis* was able to penetrate a reconstituted basement membrane equivalent (fibroblast-containing bovine type III collagen seeded with primary oral keratinoctyes), after 24 hour incubation (Andrian *et al*., 2004). Although in this present study no bacteria were recovered from the lower chamber of the tissue culture insert after infection of H357-OMM, this does not mean to say that *P. gingivalis* is not able to gain access through a thinner epithelial and connective tissue layer. However, due to the restriction of the depth of collagen required for the culture of OMM in this study, this was not investigated further. Nevertheless, this phenomenon is important to investigate, particularly as *in vivo* the blood vessels are located close to the epithelial layers, and so *P. gingivalis* penetration could result in low level bacteraemia and play a possible role in atherosclerotic disease (Hayashi *et al*., 2011).

Although *P. gingivalis* can be detected microscopically within oral biopsies and mucosal models, the intracellular viability of *P. gingivalis* is important for the continued survival of this bacterium within the oral cavity. Therefore, the invasion of OMM by *P. gingivalis* was determined using a modified antibiotic protection assay. This assay was optimised for invasion by this bacterium and determined that 4 hour incubation aerobically gave the optimal percentage invasion, and lysis of the epithelial cells was more efficient using a homogeniser (figs 3.13, 3.18 & 3.16 respectively).

The percentage with which *P. gingivalis* invaded the oral mucosal model in this study was approximately 4% (fig 3.13). The invasion of *P. gingivalis* reported throughout the literature for both monolayer and organotypic model varies considerably. This is due to the environmental conditions on any particular day of the assay, the strain of *P. gingivalis*, the particular epithelial cell used, whether internalisation was assessed by microscopy or viable colony counting and how the percentage invasion was calculated, i.e. was invasion calculated as a percentage of the original bacterial suspension plated before or after the completion of the assay, or as a percentage of the total cell associated bacteria. Therefore it is difficult to compare percentage invasions between individual studies. Nevertheless, the percentage invasion of epithelial cells by *P. gingivalis* as assessed by colony counting is usually less than or equal to 10% (Lamont *et al*., 1995; Suwannakul *et al*., 2010; Saito *et al*., 2009).

This chapter provides evidence for the intracellular recovery of *P. gingivalis* from an organotypic oral mucosal model, in accordance with Papapanou *et al.* (1999) and Sandros *et al.* (1994), who report the recovery of intracellular *P. gingivalis* at a percentage of no greater than 10% from multilayered cultures of pocket epithelial cells. The minimal recovery of *P. gingivalis* after 24 hour incubation with OMM, in this study (fig 3.13), may be due to the aerobic atmosphere with which the assay was performed, epithelial desquamation or may be due to the culture medium. Indeed, the viability of *P. gingivalis* in the culture medium decreased over time (fig 3.14), suggesting that the atmosphere and the environment may both play a role in the decreased survival and subsequent detection of this bacterium.

The culture of an oral mucosal model which resembles junctional epithelium was investigated for the intracellular recovery of *P. gingivalis*. Certainly junctional epithelium possesses wide intercellular spaces and few desmosomes (Schroeder and Listgarten, 2003). Therefore it would be assumed that invasion of bacteria into the junctional epithelium may be greater than invasion into a stratified epithelial layer with many desmosomes and tight junctions. However, as seen in figures 3.15 and 3.13, the percentage invasion of submerged and air-exposed models were similar, at around 4%. This may be because the submerged models are not a perfect representation of the junctional epithelium in that they still hold tight cellular associations as shown by staining for E-cadherin (fig 3.9). Also, as H357 is a cancer cell and is always undergoing cellular proliferation, the cellular junctions are likely to remain identical throughout the epithelium (Hoteiya *et al*., 2009). *P. gingivalis* is able to degrade E-cadherin (Katz *et al*., 2002; Katz *et al*., 2000), which may be important in the mechanism of invasion. However, this may only be important during increased incubation periods with *P. gingivalis*, and not the 4 hour incubation used for this assay. Therefore, an OMM cultured using cells obtained from a biopsy of junctional epithelium would give more conclusive results as to the depth of penetration, survival and viable recovery of *P. gingivalis* in a more relevant and representative model for *P. gingivalis* invasion.

The incorporation of neutrophils into OMM was successful (fig 3.11), however they did not migrate further than 200µm through the connective tissue layer when introduced basally. Schaller *et al*. (2004) report the transepithelial migration of PMNs through the commercially available mucosal model (SkinEthic Laboratory, Nice, France), in the presence of *Candida albicans*. Sections were stained using 1% toluidine blue and 1% pyronine G, which may have lead to the over identification of PMNs within the model as keratinocytes stained similarly to PMNs. Despite this, PMNs were shown to migrate through the polycarbonate membrane and were found close to the surface of the epithelium when introduced basally (Schaller *et al*.,

2004). However, in this study the multi-layered epithelium was cultured on a polycarbonate membrane and not on a representative connective tissue scaffold which may indicate why the authors identified PMNs close to the epithelial surface. However, in accordance with data presented in this chapter, PMNs are capable of migration through a connective tissue layer, which may become more important as modifications of OMM to reduce the depth of the connective tissue layer become possible.

3.4.3 Conclusion

The organotypic oral mucosal model introduced in this chapter was characterised and shown to resemble normal oral mucosa, particularly NOK-OMM. In addition, OMM which were completely submerged in culture medium showed characteristics of junctional epithelium. These models were optimised to study the invasion of *P. gingivalis* and results presented here suggested that *P. gingivalis* was able to invade the superficial layers of epithelium and may penetrate further, although further work is required. Optimal incubation of OMM with *P. gingivalis* was 3-6 hours when assessed by viable counting. The complete culture of a model, as opposed to obtaining commercial equivalents, gives greater scope in the future for the development of this model to further represent the *in vivo* situation. Modifications may include the incorporation of endothelial cells, immune cells, basement membrane proteins, variation in the thickness of the connective tissue and epithelial components, and modulation of cellular protein expression.

Chapter 4 *Porphyromonas gingivalis* invasion of monolayer and organotypic oral mucosal model

4.1 INTRODUCTION

P. gingivalis has been shown to invade, survive and multiply within oral epithelial cells (Madianos *et al*., 1996), and to propagate intra- and inter-cellularly (Yilmaz *et al*., 2006; Katz *et al*., 2002), which may contribute to the progression of periodontal disease. Therefore, understanding the mechanisms involved in invasion could help the understanding of the pathogenesis of the disease and ultimately in the development of more effective therapeutic agents (Chapple, 2009). As mentioned in Chapter 3, the use of monolayer cultures of epithelial cells to study bacterial invasion has been common place. However, tissue-engineered, multi-layered organotypic oral mucosal models have been recently used to study *P. gingivalis* invasion (Andrian *et al*., 2004; Papaioannou *et al*., 2003; Dickinson *et al*., 2011), and may be more representative of the oral environment.

An organotypic oral mucosal model based on type I collagen was characterised and optimised for *P. gingivalis* invasion in Chapter 3. In this chapter, epithelial cell invasion of the simple, widely reported monolayer with *P. gingivalis* will be compared with organotypic epithelial cultures in an attempt to identify similarities or differences in degree of invasion and to report any advantages or disadvantages of the use of organotypic models over monolayer cultures.

Invasion is reliant on a number of factors including host cell receptors, bacterial receptors/virulence features and the host cell environment. *P. gingivalis* possesses proteases, known as gingipains, which are an important virulence feature of this bacterium previously shown to influence epithelial cell invasion (Park and Lamont, 1998, Chen *et al*., 2001, Suwannakul *et al*. 2010). This study will also compare the contribution of gingipains to the invasion of *P. gingivalis* into monolayer and organotypic epithelial cultures.

Bleeding upon probing and elevated periodontal pocket temperatures are clinical characteristics of periodontitis (Greenstein, 1984; Haffajee *et al*., 1992). Differences in the concentration of haemin and temperature influence the structure of *P. gingivalis* lipid A, a component of LPS (Al-Qutub *et al*., 2006; Curtis *et al*., 2011). For example, at high haemin concentrations the structure of lipid A contains predominantly mono-phosphorylated, tetra-acylated moieties, and as such acts as a TLR4 antagonist (Al-Qutub *et al*., 2006). However, at high temperatures the predominant lipid A species is penta-acylated and mono-phosphorylated, acting as a TLR4 agonist (Curtis *et al*., 2011). Due to differences in the lipid A structure under conditions commonly observed at diseased periodontal sites, it is unclear what role LPS structure plays in

disease. The data presented in this chapter suggests that the concentration of haemin in the bacterial culture medium and the temperature at which *P. gingivalis* is grown, influences invasion of oral epithelial cells by this bacterium.

It has been proposed that the intracellular environment may act as a protective niche for the survival and propagation of *P. gingivalis* (Houalet-Jeanne *et al*., 2011; Madianos *et al*., 1996), suggesting that invasion may be a critical factor in bacterial survival *in vivo*. This chapter will study the intracellular survival of *P. gingivalis*, in monolayer and organotypic cultures. In addition, since periodontitis is a disease that is thought to be cyclical in nature (Socransky *et al*., 1984), it is possible that bursts of activity may be caused by re-infection, at the same site or at a different site within the oral cavity, when *P. gingivalis* 'escapes' from epithelial cells. Therefore, this chapter will assess whether intracellular *P. gingivalis* is released from epithelial cells and what the viability status is of the epithelial cells.

P. gingivalis invasion into oral epithelial cells is not uniform, i.e. not every cell has the same number of intracellular bacteria but there seems to be some cells that have high numbers of intracellular bacteria, whereas others have very few or none. Presented here are preliminary data obtained using two methods to try to separate the two epithelial cell populations; those which have intracellular bacteria and those which do not. Successful isolation of *P. gingivalis*-containing cells will aid future analysis to identify what factors these cells have that makes them preferentially internalise bacteria.

4.1.1 Aims and Objectives

The aims of this part of the study were i) to compare invasion of two models of the oral epithelium: monolayer and organotypic culture by *P. gingivalis* strains, ii) assess the differences or similarities in percentage invasion of these two models, iii) investigate the factors that may influence invasion, including gingipain expression, haemin and temperature culture conditions and iv) assess the viability of both internalised *P. gingivalis* and the invaded epithelial cells.

4.2 METHODS

The following methods were used in this chapter:

- Isolation and culture of NOK, fibroblasts and H357 cell line (sections 2.2, 2.3 & 2.4)
- Culture of *P. gingivalis* (section 2.5)
- Culture of oral mucosal models (section 2.8)
- *P. gingivalis* cell invasion (section 2.11)
- Separation of epithelial cells associated with *P. gingivalis* (section 2.20)
- Statistical analysis (section 2.22)

4.3 RESULTS

4.3.1 *P. gingivalis* invasion of monolayer and OMM

The tissue-engineered OMM, which was characterised and optimised for *P. gingivalis* invasion in Chapter 3, was used in the antibiotic protection assay to compare the epithelial invasion of *P. gingivalis* strains with monolayer cultures of oral epithelial cells.

4.3.1.1 *P. gingivalis* strains: NCTC 11834 and W50

Initially, standard antibiotic protection assays for monolayer cultures of the H357 cell line and NOK were performed using two laboratory strains of *P. gingivalis*, NCTC 11834 and W50. The invasion of H357 monolayers by strain NCTC 11834 (3.19±1.68%) was significantly higher than the percentage invasion by strain W50 (0.27±0.18%, $p<0.05$) (fig 4.1). Similarly, in NOK monolayers, NCTC 11834 invaded at a significantly higher percentage than W50 (2.15±0.90% versus 0.08±0.05% respectively, $p<0.05$) (fig 4.2). The total invasion of NOK was slightly lower than H357 for both *P. gingivalis* strains, which may be due to differences in the expression of cell surface receptors.

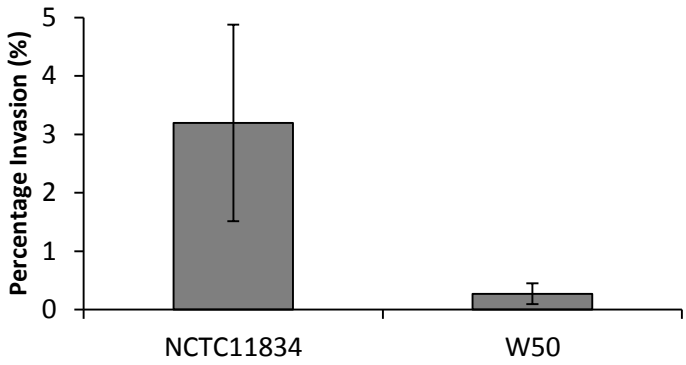


Figure 4.1 Histogram to show the invasion of H357 monolayer by *P. gingivalis* strains NCTC 11834 and W50 using a standard antibiotic protection assay. H357 monolayers were cultured and *P. gingivalis* added at MOI 100 for 1.5 hours, after which metronidazole was added to kill the external, adherent *P. gingivalis*. Intracellular bacteria were released by osmotic lysis using sterile distilled water and scraping. Bacterial colonies were enumerated after serial dilution and plating onto blood-FA plates. The number of intracellular bacteria recovered is expressed as a percentage of the infecting inoculum. Graphs show mean±SD of three independent experiments performed in triplicate.

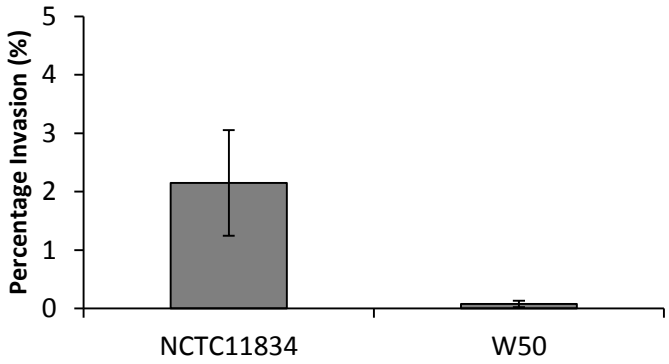


Figure 4.2 Histogram to show the invasion of NOK monolayer by *P. gingivalis* strains NCTC 11834 and W50 using a standard antibiotic protection assay. NOK monolayers were cultured and *P. gingivalis* added at MOI 100 for 1.5 hours, after which metronidazole was added to kill the external adherent *P. gingivalis*. Intracellular bacteria were released by osmotic lysis using sterile distilled water and scraping. Bacterial colonies were enumerated after serial dilution and plating onto blood-FA plates. The number of intracellular bacteria recovered is expressed as a percentage of the infecting inoculum. Graphs show mean±SD of three independent experiments performed in triplicate.

Comparing the invasion of these two strains, NCTC 11834 and W50, in the organotypic model showed a similar trend, with NCTC 11834 invading at a significantly higher percentage than W50 (3.12±0.79% and 0.17±0.31 respectively, $p<0.005$) (fig 4.3). The total invasion of monolayer cultures was similar to the invasion of OMM for both strains of *P. gingivalis*, though the infection period used for OMM was longer (4 hours).

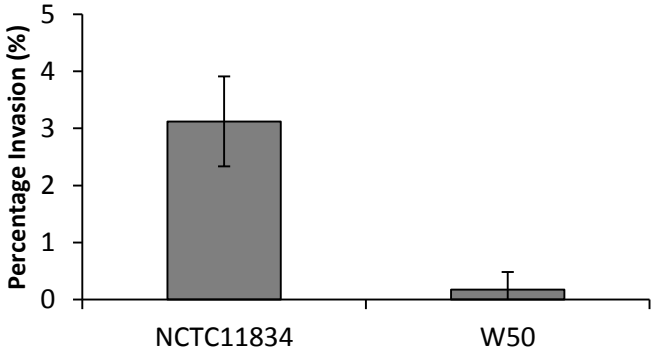


Figure 4.3 Histogram to show the invasion of H357-OMM by *P. gingivalis* strains NCTC 11834 and W50 using a modified antibiotic protection assay. H357-OMM were cultured and *P. gingivalis* was added at MOI 100 for 4 hours, after which metronidazole was added to kill the external adherent *P. gingivalis*. The models were lysed by homogenisation and the intracellular bacteria enumerated by serial dilution and plating onto blood-FA plates. The number of intracellular bacteria recovered is expressed as a percentage of the infecting inoculum. Graphs show mean±SD of three independent experiments performed in triplicate.

4.3.1.2 Epithelial cells: NOK and H357

As shown in figures 4.1 & 4.2, the invasion of monolayer cultures of H357 and NOK were not significantly different for *P. gingivalis* NCTC 11834, e.g. 3.19±1.68% (H357) and 2.15±0.90% (NOK) ($p>0.05$).

However, a significant difference in percentage invasion between H357-OMM and NOK-OMM by *P. gingivalis* NCTC 11834 was observed, i.e. 3.38±0.45% and 0.98±1.02% respectively ($p<0.05$) (fig 4.4). This suggests that when cultured in a multi-layered system, at an air-to-liquid interface on a collagen matrix, NOK are not as susceptible to invasion by *P. gingivalis*, compared with H357 cells. This could be due to differences in the cell surface expression of adhesion molecules, such as integrins (Tsuda *et al*., 2008, Nakagawa *et al*., 2005).

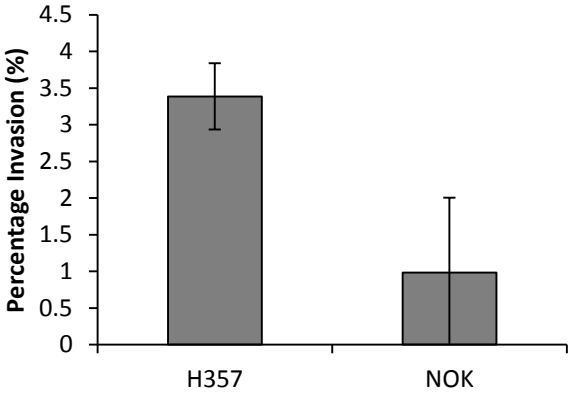


Figure 4.4 Histogram to show the invasion of H357- and NOK-OMM by *P. gingivalis* NCTC 11834 using a modified antibiotic protection assay. H357- and NOK-OMM were cultured and *P. gingivalis* was added at MOI 100 for 4 hours, after which metronidazole was added to kill the external adherent *P. gingivalis*. The models were lysed by homogenisation and the intracellular bacteria enumerated by serial dilution and plating onto blood-FA plates. The number of intracellular bacteria recovered is expressed as a percentage of the infecting inoculum. Graphs show mean±SD of three independent experiments performed in triplicate, $p<0.05$.

4.3.2 Factors influencing *P. gingivalis* invasion

Using the easier to culture and more readily available H357 cell line, preliminary data are presented to assess the influence of *P. gingivalis* gingipains, environmental haemin and temperature on the invasion of oral epithelial cells by *P. gingivalis*.

4.3.2.1 Invasion by *P. gingivalis* gingipain mutants

P. gingivalis possesses virulence features that may be important in influencing invasion of oral epithelial cells. It has been shown previously that a highly invasive *P. gingivalis* subtype possesses reduced Arg-gingipain activity, and that a double arginine knockout mutant (Δ*rgpA*Δ*rgpB*) showed a significantly higher percentage invasion than the wild-type bacterium (Suwannakul *et al*., 2010). Shown in figure 4.5 are similar data indicating that the absence of the arginine-specific gingipain is important for the invasion of oral keratinocytes. This was

shown in the H357 monolayer as a statistically significant (p=0.02) 16-fold increase in invasion by strain E8 (Δ*rgpA*Δ*rgpB*) compared with the wild-type parent strain (W50) (fig 4.5). In addition, the lysine-specific gingipain mutant (Δ*kgp* or K1A) showed a significantly higher percentage invasion than W50 (0.34±0.17% and 0.03±0.03% respectively, p=0.04). K1A invaded at a slightly lower percentage than E8, although this was not significant (p=0.45).

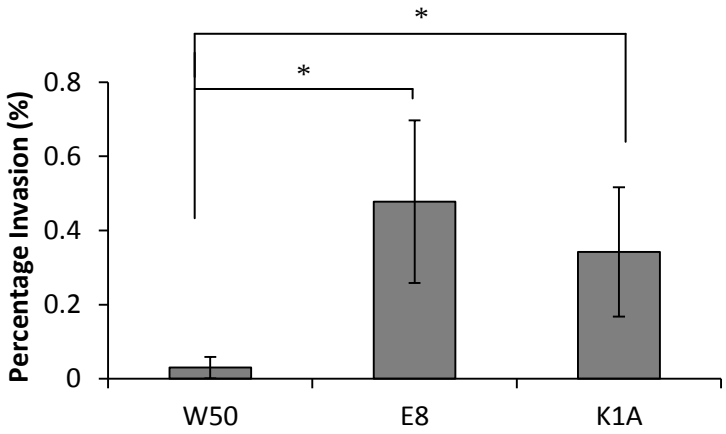


Figure 4.5 Histogram to show the invasion of H357 monolayer by *P. gingivalis* strains W50 (parent strain), E8 (Δ*rgpA*Δ*rgpB*) and K1A (Δ*kgp*) using a standard antibiotic protection assay. H357 monolayers were cultured and *P. gingivalis* added at MOI 100 for 1.5 hours, after which metronidazole was added to kill the external adherent *P. gingivalis*. Intracellular bacteria were released by osmotic lysis using sterile distilled water and scraping. Bacterial colonies were enumerated by serial dilution and plating onto blood-FA plates. Significant differences between W50 and E8, and W50 and K1A, $p<0.05$ (*) were observed. The number of intracellular bacteria recovered is expressed as a percentage of the infecting inoculum. Graphs show mean±SD of three independent experiments performed in triplicate.

Using NOK monolayers, there was a similar 14-fold increase in invasion by strain E8 compared with the wild-type strain. The same trend in data was observed with NOK monolayers compared with H357 monolayers, i.e. E8 invaded at a higher percentage than W50 and K1A. Similarly, K1A invaded at a higher percentage than W50 but not as high as E8.

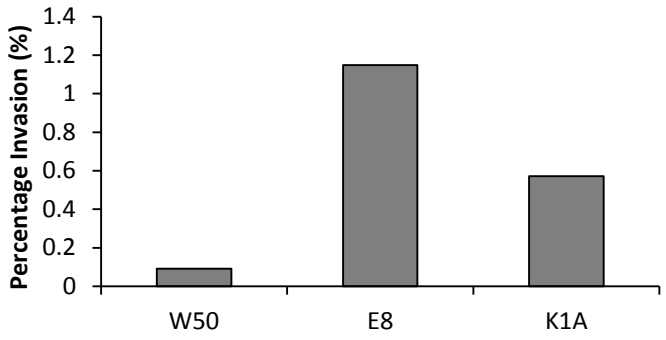


Figure 4.6 Histogram to show the invasion of NOK monolayer by *P. gingivalis* strains W50 (parent strain), E8 (Δ*rgpA*Δ*rgpB*) and K1A (Δ*kgp*) using a standard antibiotic protection assay. NOK monolayers were cultured and *P. gingivalis* added at MOI 100 for 1.5 hours, after which metronidazole was added to kill the external adherent *P. gingivalis*. Intracellular bacteria were released by osmotic lysis using sterile distilled water and scraping. Bacterial colonies were enumerated by serial dilution and plating onto blood-FA plates. The number of intracellular bacteria recovered is expressed as a percentage of the infecting inoculum. Graph is representative of two independent experiments performed in triplicate.

Comparing the data of *P. gingivalis* invasion of monolayer with the invasion of OMM, similar trends in the data was found, although there were differences in the total percentage invasion (fig 4.7). For the invasion of H357-OMM, strain E8 (0.19±0.04%) showed a significantly higher percentage invasion than wild-type W50 (0.04±0.03%, p=0.003). However, although the invasion of strain K1A (0.10±0.07%) was higher than W50, this was not statistically significant (p=0.14). The percentage invasion of H357-OMM by E8 was higher than K1A (as shown for monolayers in figures 4.5 & 4.6), but this was also not significant (p=0.07).

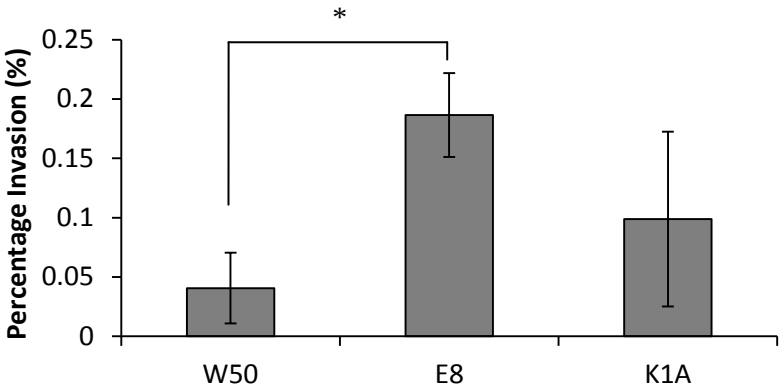


Figure 4.7 Histogram to show the invasion of H357-OMM by *P. gingivalis* strains W50 (parent strain), E8 (Δ*rgpA*Δ*rgpB*) and K1A (Δ*kgp*) using a modified antibiotic protection assay. H357-OMM were cultured and *P. gingivalis* was added at MOI 100 for 4 hours, after which metronidazole was added to kill the external adherent *P. gingivalis*. The models were lysed by homogenisation and the intracellular bacteria enumerated by serial dilution and plating onto blood-FA plates. The number of intracellular bacteria recovered is expressed as a percentage of the infecting inoculum. Graphs show mean±SD of three independent experiments performed in triplicate, $p<0.05$ (*).

It has been shown that invasion of oral epithelial cells by the double arginine- and single lysine-knockout mutants were higher than the wild-type. However, it may be that the presence of either of these gingipains could compensate for the absent one. Therefore, a triple arginine- and lysine-knockout mutant was used in an antibiotic protection assay to further investigate the role of gingipains in *P. gingivalis* invasion. The invasion of the H357 epithelial cell line by the triple gingipain knockout mutant (Δ*rgpA*Δ*rgpB*Δ*kgp*, EK18) was significantly lower than the invasion by the parent W50 strain (0.004±0.003% and 0.14±0.01% respectively, $p<0.001$) (fig 4.8).

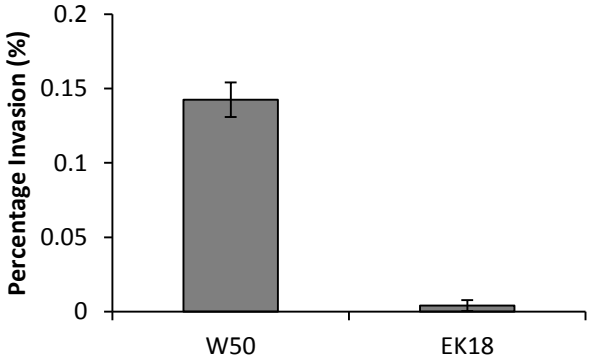


Figure 4.8 Histogram to show the invasion of H357 monolayer by *P. gingivalis* strains W50 (parent strain) and the triple gingipain mutant EK18 (Δ*rgpA*Δ*rgpB*Δ*kgp*) using an antibiotic protection assay. H357 monolayers were cultured and *P. gingivalis* was added at MOI 100 for 1.5 hours, after which metronidazole was added to kill the external adherent *P. gingivalis*. The monolayers were lysed and the intracellular bacteria enumerated by serial dilution and plating onto blood-FA plates. The number of intracellular bacteria recovered is expressed as a percentage of the infecting inoculum. Graphs show mean±SD of three independent experiments performed in triplicate.

4.3.2.2 Effect of haemin concentration and elevated temperature on invasion

As the total percentage invasion of OMM with *P. gingivalis* was not significantly different from that seen in monolayers and experimental trends were maintained, the following experiments were performed using monolayer cultures for ease of maintenance and reproducibility of cell culture.

As gingivitis progresses to periodontitis the environment within the gingival crevice/periodontal pocket changes. The inflammatory response results in an increase in temperature and, as the disease progresses, increasing numbers of red blood cells and iron become available for utilisation by the organism, as bleeding of the gingiva occurs. Therefore, the effect of haemin concentration and elevated temperature on invasion was investigated to observe whether changes in the environment that *P. gingivalis* is exposed to predisposes an increase or decrease in the internalisation of *P. gingivalis*, which may propose a link between disease progression and bacterial invasion.

As shown in figure 4.9, as the concentration of haemin increased, the percentage invasion of H357 monolayers by NCTC 11834 increased in a dose-dependent manner: 2.17±0.89%, 3.25±0.40% and 7.07±1.58% (1µg ml^{-1}, 5µg ml^{-1} and 10µg ml^{-1} haemin in the growth medium of the infecting inoculum, respectively; $p<0.05$). Similarly, when the culture temperature for *P. gingivalis* was raised from 37°C to 41°C, the invasion of the H357 cell line by strain NCTC 11834 increased approximately 4-fold, from 2.87±0.70% to 12.15±2.11% respectively ($p<0.05$) (fig 4.10). These results suggest an importance, not only of the virulence features of *P. gingivalis* in influencing invasion (e.g. gingipains) but also the environment in which *P. gingivalis* is accommodated. Of course, these may not be mutually exclusive, since gingipains have been shown to be important in the acquisition of haemin (Lewis *et al*., 1999). It must be mentioned that the experiment performed at high temperature was only performed once. This was due to difficulties obtaining growth at this high temperature and the lack of suitable equipment to ensure an adequate anaerobic atmosphere throughout culture. Therefore this result must be interpreted with caution, and as such, requires further work.

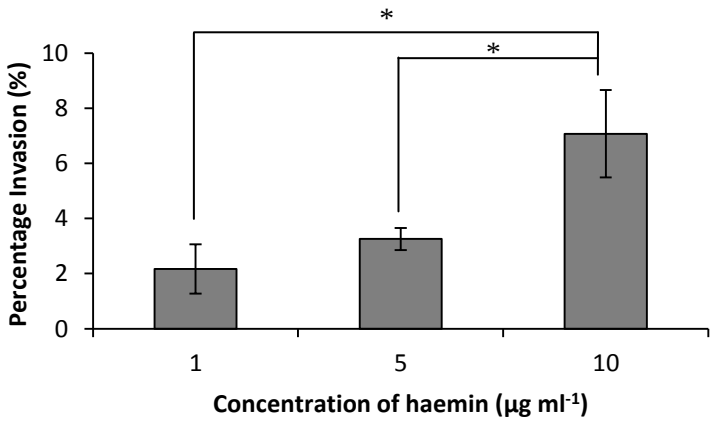


Figure 4.9 Histogram to show the invasion of H357 monolayer by NCTC 11834 cultured in different concentrations of haemin. *P. gingivalis* NCTC 11834 was cultured overnight in BHI broth supplemented with cysteine, vitamin K and 1µg ml^{-1}, 5µg ml^{-1} or 10µg ml^{-1} haemin. NCTC 11834 (MOI100) was incubated with H357 monolayers for 1.5 hours, after which metronidazole was added to kill the external adherent bacteria. Intracellular bacteria were released by osmotic lysis using sterile distilled water and scraping. Bacterial colonies were enumerated by serial dilution and plating onto blood-FA plates. The number of intracellular bacteria recovered is expressed as a percentage of the infecting inoculum. Graphs show mean±SD of three independent experiments performed in triplicate, *$p<0.05$.

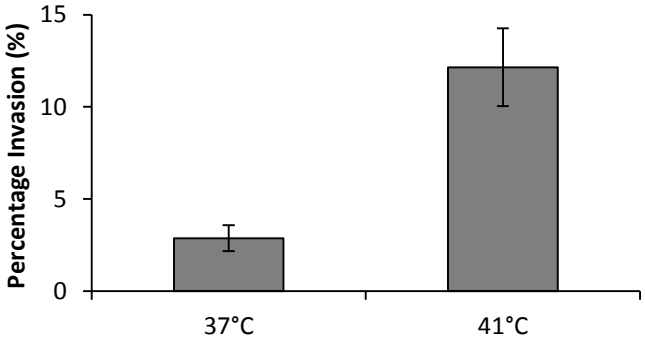


Figure 4.10 Histogram to show the invasion of H357 monolayer by NCTC 11834 cultured at 37°C and 41°C. *P. gingivalis* NCTC 11834 was cultured on blood FA-plates for 2 days at 37°C or 41°C. NCTC 11834 (MOI100) was incubated with H357 monolayers for 1.5 hours, after which metronidazole was added to kill the external adherent bacteria. Intracellular bacteria were released by osmotic lysis using sterile distilled water and scraping. Bacterial colonies were enumerated by serial dilution and plating onto blood-FA plates. The number of intracellular bacteria recovered is expressed as a percentage of the infecting inoculum. Graphs show mean±SD of three internal repeats of a single experiment.

4.3.3 The fate of *P. gingivalis* following epithelial cell invasion

Following epithelial cell invasion by *P. gingivalis*, it has been shown previously that this bacterium is capable of intracellular replication (Madianos *et al*., 1996). As periodontal disease is a cyclical disease in which there are periods of active disease followed by prolonged periods of remission (Socransky *et al*., 1984), survival of *P. gingivalis* and its release from oral epithelial cells was investigated.

Figure 4.11 shows the survival of *P. gingivalis* in the epithelial cell culture medium used to maintain monolayers and OMM (3:1 DMEM:Ham's F-12; SFM with or without supplements). The number of viable bacteria increased in most media but only for around 4 hours and then they declined, suggesting that *P. gingivalis* does not thrive in this medium (the presence of FCS within the medium caused an intial increase in growth probably due to the increased nutrient source). The same was true when intracellular survival of *P .gingivalis* was assessed. In both monolayer and OMM, the persistence of *P. gingivalis* within epithelial cells was limited to 24 hours, and no significant intracellular replication was observed (fig 4.13 & 4.14, respectively). The percentage invasion of H357 monolayer cultures was higher when incubated with *P. gingivalis* MOI100 compared with MOI1, with initial percentage invasion values of 3.20±0.11% and 0.58% respectively. The percentage of viable *P. gingivalis* cultured from the supernatant for MOI100 and MOI1 cultures was maximal at 6 hours with percentages of 23.34±8.52% and 19.00% of the total intracellular+extracellular bacteria respectively. The number of desquamated epithelial cells at 48 hours, recorded as a percentage of the total number of epithelial cells seeded at the start of the experiment (i.e. $2x10^5$) was similar after incubation of H357 monolayers with *P. gingivalis* MOI100 (10.0±2.5%) and MOI1 (13.75%). Similarly, the

invasion of organotypic cultures initially started at 3.84% but diminished to almost zero at 48 hours (0.001%). At this time point there were still some viable colonies in OMM, whereas for monolayer cultures there were none. In contrast to monolayer cultures, the maximal detection of *P. gingivalis* in the culture supernatant was after 24 hours, with a percentage of 24.56%. The percentage of desquamated epithelial cells as a percentage of the estimated number available on the surface of the H357-OMM (i.e. $2x10^5$) increased over time and at 48 hours was similar to the values recorded for the monolayer cultures, i.e. 15.42%.

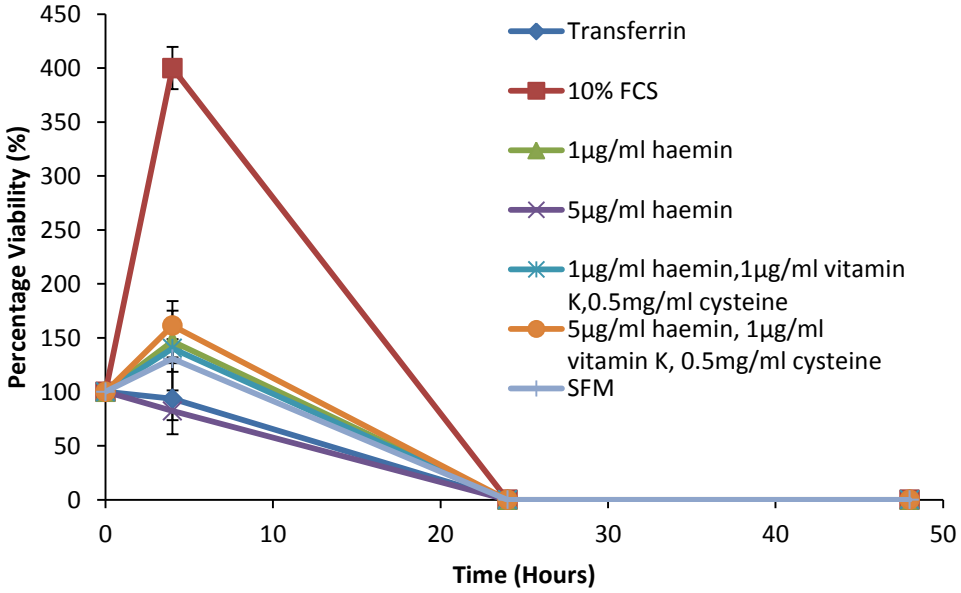


Figure 4.11 Percentage viability of *P. gingivalis* NCTC 11834 cultured aerobically in different media. *P. gingivalis* NCTC 11834 cells were suspended in serum-free culture medium (SFM) at $2x10^7 ml^{-1}$ (representative of the number of bacterial cells added to epithelial cells in a typical 24-well invasion assay) for 48 hours in 5% CO_2/95% air. In addition, SFM was supplemented with 5µg ml^{-1} transferrin, 10% foetal calf serum (FCS), haemin (1µg ml^{-1}, 5µg ml^{-1}) or vitamin K (1µg ml^{-1})+cysteine(0.5m g ml^{-1})+haemin (1µg ml^{-1}, 5µg ml^{-1}). Samples of the culture medium were removed at each time point, serially diluted and colonies were counted. Counted colonies were expressed as a percentage of the original bacterial suspension. Data presented is representative of two experiments performed in triplicate. The histogram shows the mean±SD of a single representative experiment with 3 internal repeats.

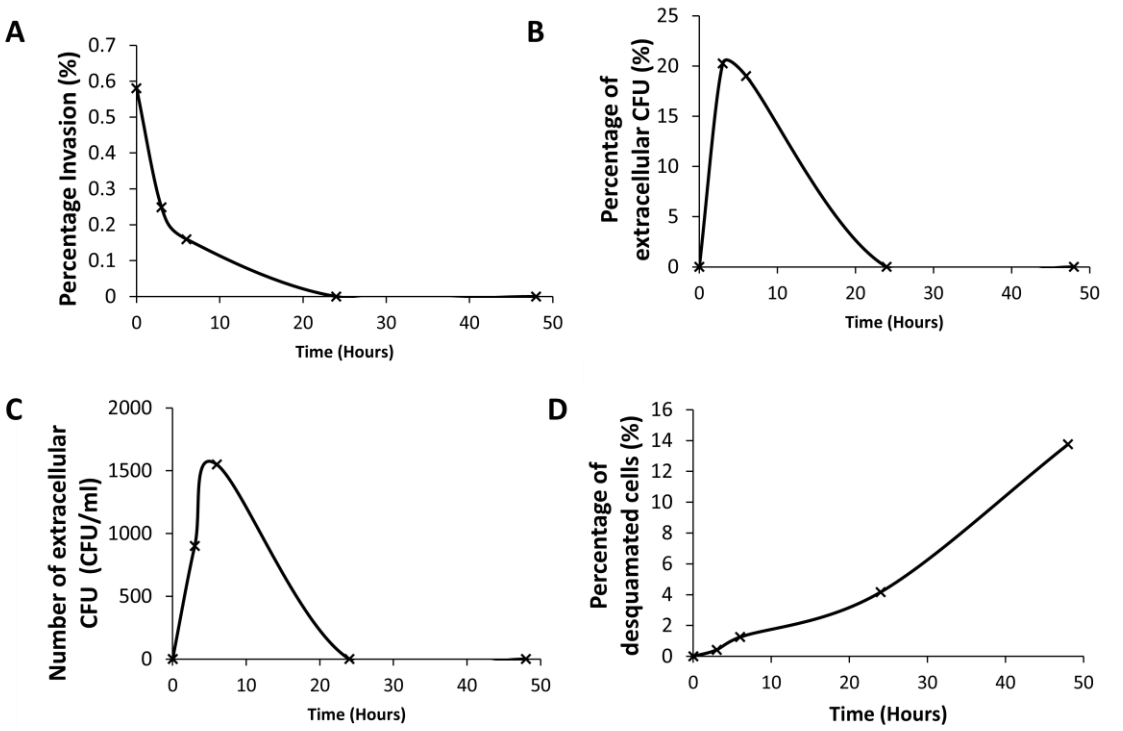


Figure 4.12 Line charts to show the percentage invasion, CFU recovered from supernatant and number of desquamated epithelial cells after invasion of H357 monolayers with *P. gingivalis* (MOI1). H357 monolayers were cultured and exposed to *P. gingivalis* NCTC 11834 (MOI1) for 90 minutes. Following which H357 cells were washed, incubated with metronidazole for 1 hour to kill the external adherent bacteria and washed to remove residual metronidazole. The percentage invasion was then determined as the number of intracellular bacteria expressed as a percentage of the original bacterial suspension. Time 0 hours refers to measurements made immediately after metronidazole treatment. Additional measurements were made at 3, 6, 24 and 48 hours. Measurements included percentage invasion (A) and the number of bacteria released from the epithelial cells which is presented as the number of colony forming units (CFU) after viable counting on blood FA-agar plates (CFU/ml (C)), and extracellular CFU as a percentage of the total viable intracellular and extracellular CFU (B). The number of desquamated cells was also counted using a haemocytometer at each time point (D) and expressed as a percentage of the estimated total number of epithelial cells ($2x10^5$) at the start of the experiment. Graphs shown are representative of two independent experiments repeated in triplicate.

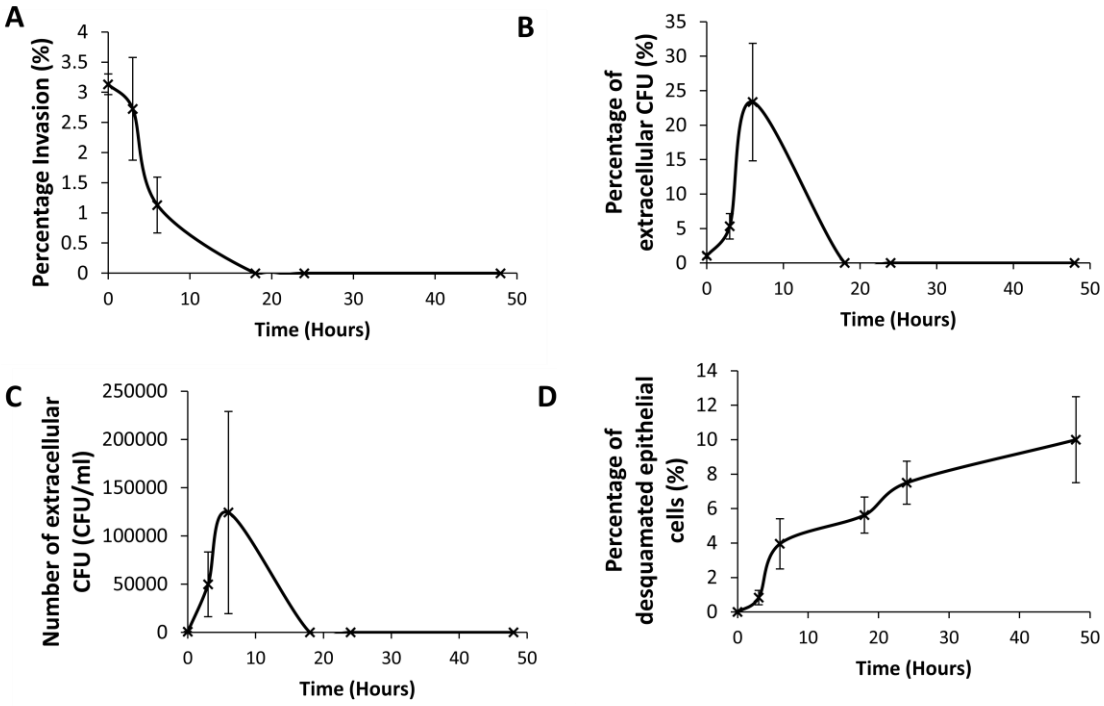


Figure 4.13 Line charts to show the percentage invasion, CFU recovered from supernatant and number of desquamated epithelial cells after invasion of H357 monolayers with *P. gingivalis* (MOI100). H357 monolayers were cultured and exposed to *P. gingivalis* NCTC 11834 (MOI100) for 90 minutes. Following which H357 cells were washed, incubated with metronidazole for 1 hour to kill the external adherent bacteria and washed to remove residual metronidazole. The percentage invasion was then determined as the number of intracellular bacteria expressed as a percentage of the original bacterial suspension. Time 0 hours refers to measurements made immediately after metronidazole treatment. Additional measurements were made at 3, 6, 18, 24 and 48 hours. Measurements included percentage invasion (A) and the number of bacteria released from the epithelial cells which is presented as the number of colony forming units (CFU) after viable counting on blood FA-agar plates (CFU/ml (C)), and extracellular CFU as a percentage of the total viable intracellular and extracellular CFU (B). The number of desquamated cells was also counted using a haemocytometer at each time point (D) and expressed as a percentage of the estimated total number of epithelial cells ($2x10^5$) at the start of the experiment. In all cases error bars indicate means±SEM of three independent experiments, repeated in triplicate.

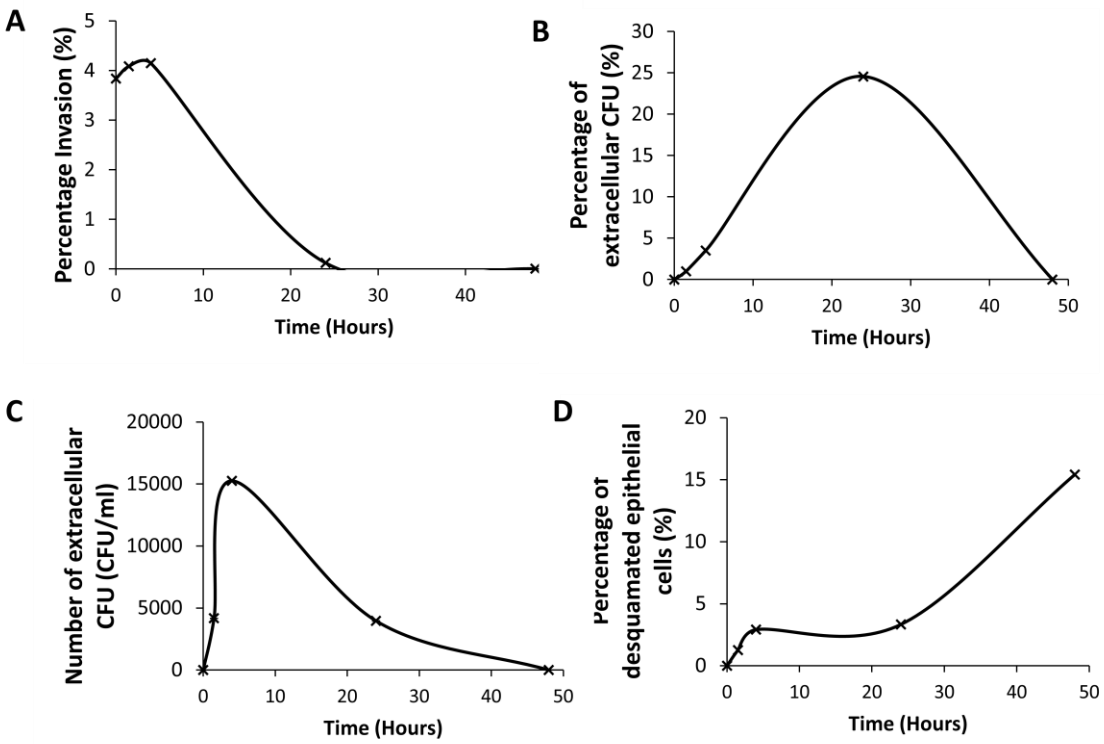


Figure 4.14 Line charts to show the percentage invasion, CFU recovered from supernatant and number of desquamated epithelial cells after invasion of H357-OMM by *P. gingivalis*. H357-OMM were cultured and exposed to *P. gingivalis* NCTC 11834 (MOI100) for 4 hours. Following which H357 were washed, incubated with metronidazole for 1 hour to kill the external adherent bacteria and again washed extensively to remove residual metronidazole. The percentage invasion was then presented as the number of intracellular bacteria calculated as a percentage of the original bacterial suspension incubated with the epithelial cells. Time 0 hours refers to measurements made immediately after metronidazole treatment. Additional wells were then washed and incubated at 37°C/5% CO_2 in SFM and measurements made at 1.5, 4, 24 and 48 hours. Measurements included percentage invasion (A) and the number of bacteria released from epithelial cells presented as the number of colony forming units (CFU) counted by viable counting on blood FA-agar plates (CFU/ml (C)), and extracellular CFU as a percentage of the total viable intracellular and extracellular CFU (B). The number of desquamated epithelial cells were counted using a haemocytometer at each time point (D) and expressed as a percentage of the total number of epithelial cells estimated to be on the surface of OMM ($2x10^5$) to make comparisons with monolayer data. Graphs shown are representative of two independent experiments repeated in duplicate.

4.3.4 Use of methods to separate epithelial cells associated with *P. gingivalis*

4.3.4.1 Rationale

It was observed previously that *P. gingivalis* associates with individual epithelial cells differently (Rudney *et al*., 2005). For example, there seem to be some epithelial cells that have a high bacterial cell load compared with others that may have none or only a few associated bacterial cells. This was observed in both monolayer and OMM cultures (fig 4.15). Therefore, the question was posed as to what the difference is, if any, between cells that 'preferentially' internalise bacteria and those that do not. To investigate this, initially the process involved the separation of epithelial cells with intracellular/associated bacteria and those without. Due to the high adherence of this bacterium externally to epithelial cells it was difficult to separate cells that exclusively contained intracellular bacteria, hence cells associated with bacteria were sought to be separated from those that were not. Two methods are shown below, which show promising signs in the isolation of bacterial-associated epithelial cells from a mixed cell population. *P. gingivalis* NCTC 11834 was used in these experiments because this strain invades at a relatively high level.

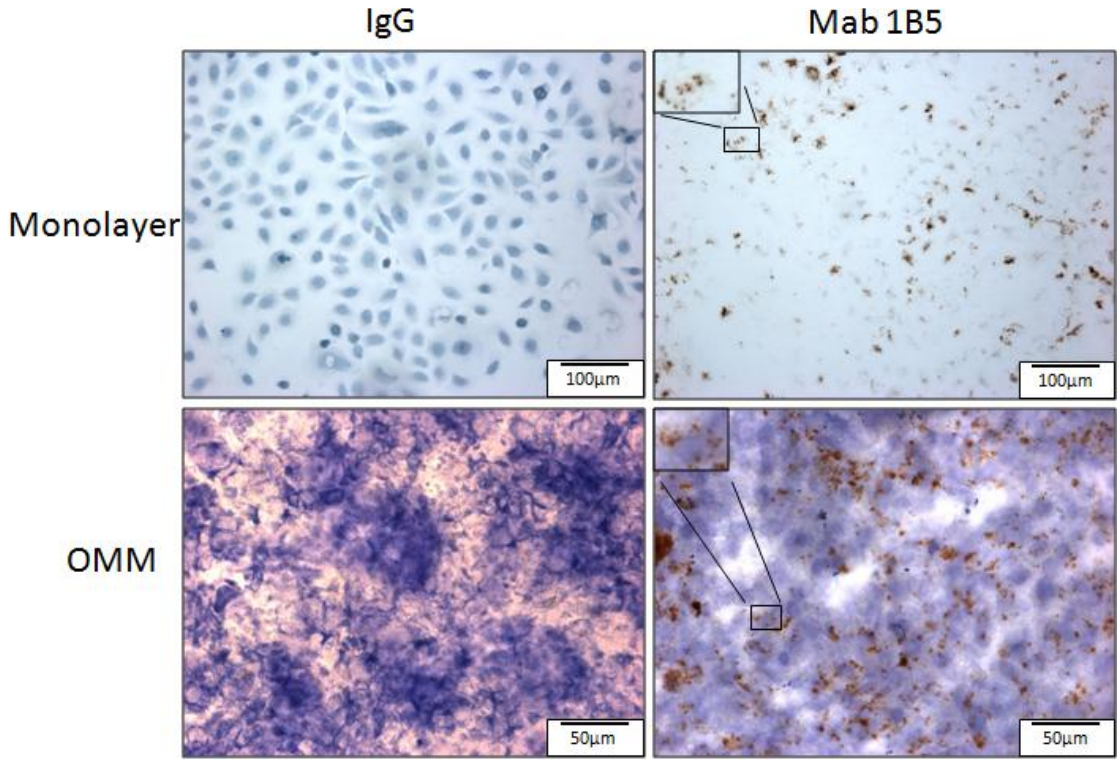


Figure 4.15 Immunohistochemical analysis of H357 monolayer and H357-OMM incubated with *P. gingivalis*. H357 monolayers were cultured on coverslips and H357-OMM cultured in tissue culture inserts. *P. gingivalis* NCTC 11834 was incubated with monolayers for 90 minutes and H357-OMM for 4 hours, following which the epithelial cells were fixed, permeabilised and immunohistochemical analysis performed using either the MAb 1B5 antibody to stain for *P. gingivalis* or an IgG2 antibody negative control. Areas of brown staining indicate localisation of *P. gingivalis*. Haemotoxylin counterstain rendered the epithelial cell nuclei blue. Inset boxes are approximately 2.5x magnified.

4.3.4.2 Magnetic beads

When H357 monolayers were incubated with *P. gingivalis*-coated magnetic beads (Dynabeads®), and then the cells were released into suspension, separation of cell populations was possible using a magnetic field (fig 4.16). Figure 4.16 shows that following incubation of epithelial cells with *P. gingivalis*-coated beads there was approximately 9.24±3.73% cells associated with bacteria/magnetic beads compared with 90.75±3.73% not associated with bacteria/beads. In contrast, when epithelial cells were incubated with beads alone (not bound to *P. gingivalis*) there was a significantly lower proportion of epithelial cells associated with the uncoated beads (4.14±1.29%) (p=0.002), suggesting that *P. gingivalis* attached to the surface of the beads aided uptake of the magnetic beads by H357 cells.

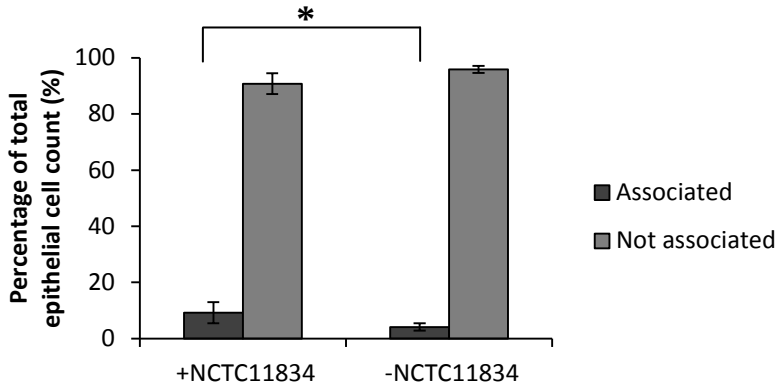


Figure 4.16 Epithelial cell counts of cells associated with magnetic beads and those not associated with magnetic beads after invasion of H357 epithelial cell line with *P. gingivalis*-coated or uncoated beads. Magnetic Dynabeads® were incubated with or without *P. gingivalis* NCTC 11834 to allow attachment of the bacteria to the beads. A standard invasion assay was performed using the H357 epithelial cell line with coated or uncoated beads. Following incubation of H357 with magnetic beads, epithelial cells were trypsinised to allow detachment from the culture plate and were exposed to a magnetic field. The epithelial cells which migrated towards the magnet are referred to as 'associated' and those epithelial cells not associated with magnetic beads referred to as 'not associated'. The separation of epithelial cells after invasion of magnetic beads coated and not coated with *P. gingivalis* is shown. Epithelial cells associated and not associated with beads were counted using a haemocytometer and expressed as a percentage of the total cell count. Bars indicate means±SD of three independent experiments repeated in triplicate. The percentage of epithelial cells associated with beads was significantly higher (*p=0.002) when beads were coated with *P. gingivalis* compared with beads which were not.

In order to investigate whether the epithelial cells that had been invaded by *P. gingivalis* (i.e. 'associated' epithelial cells) were 'different' in some way that favoured bacterial internalisation compared with '*P. gingivalis*-free' epithelial cells (bars in +NCTC 11834 in fig 4.16,), these two cell populations were cultured overnight alongside fresh H357 epithelial cells (untreated) and an invasion assay was performed the next day to investigate whether *P. gingivalis* was capable of invading the previously invaded epithelial cells at a higher percentage than the ones not previously invaded. Figure 4.17 shows there was no significant difference between the invasion of the two cell populations (2.64% (bead-associated) and 2.41% (bead-not associated)

(p>0.05)) and the H357 untreated culture (2.55%, p>0.05), suggesting that the 'invaded phenotype' was not a stable feature in cell culture. However, there was no way of separating cells associated with un-coated beads that might have been present in the suspension from cells-associated with *P. gingivalis*-coated beads. Nonetheless, the data suggest that there was no stable sub-population in the H357 cell culture. Epithelial cells not invaded by *P. gingivalis* were lysed as a control to see if residual *P. gingivalis* was present from the previous invasion assay, however no intracellular *P. gingivalis* was detected by viable counting. This is in agreement with previous data (fig 4.13) indicating that previously invaded *P. gingivalis* did not influence the viable counts.

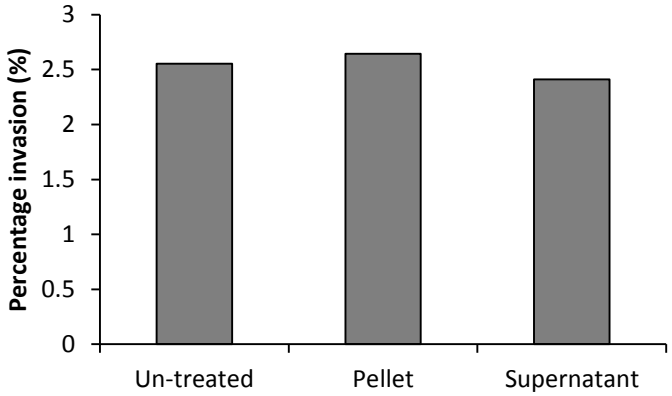
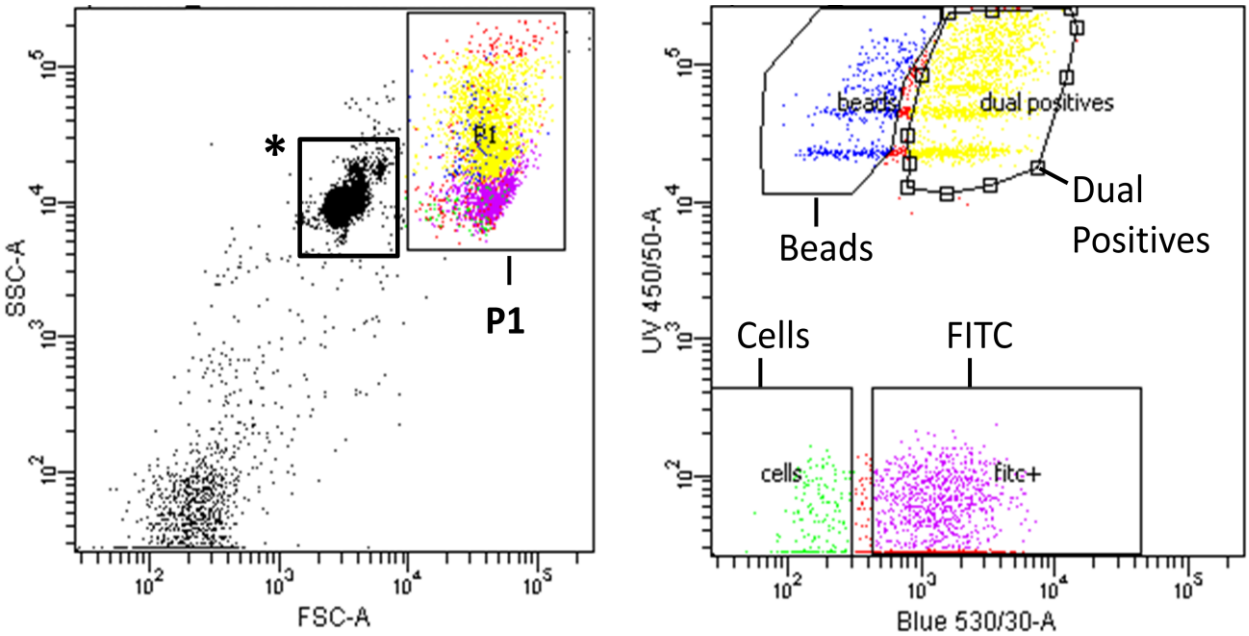


Figure 4.17 Invasion of H357 epithelial cells associated with or without magnetic beads by *P. gingivalis* NCTC 11834. H357 epithelial cells were invaded by *P. gingivalis* NCTC 11834 bound to magnetic beads, trypsinised and separated using a magnet. Epithelial cells associated with the magnetic beads (and *P. gingivalis*) were removed by the magnet, washed and plated for an antibiotic protection assay. Similarly, epithelial cells which were not attracted to the magnet, hence those which were not associated with beads (or *P. gingivalis*), were processed identically. An invasion assay with each of these cell populations, and fresh (untreated) H357 plated simultaneously, was performed with *P. gingivalis* NCTC 11834. After metronidazole treatment the epithelial cells were lysed, the intracellular bacteria plated and colonies counted. Percentage invasion was recorded as the number of colonies counted as a percentage of the original bacterial suspension also plated on blood FA-agar for viable counting. Bar chart is representative of two independent experiments performed in triplicate.

4.3.4.3 Flow cytometry

As an alternative approach, H357 monolayer cultures were exposed to FITC-labelled *P. gingivalis* NCTC 11834 and blue fluorescent beads (Fluoresbrite BB Carboxylate Microspheres, Polysciences Inc) for 1.5 hours, trypsinised and the fluorescence of the epithelial cells analysed by flow cytometry. Data presented in figure 4.18 suggests that there were 4 populations of epithelial cells: cells alone (4.7%), cells associated with bacteria (20.5%), cells associated with beads (11.2%) and cells associated with both beads and bacteria (48.5%). Due to an overlap in fluorescence, 15.1% of epithelial cells were not assigned fluorescence (red dots on histogram, fig. 4.18). Data presented here suggest that epithelial cells associate with (either internally or on the surface) *P. gingivalis* at a higher percentage than fluorescent beads. There were epithelial cells that were associated with beads or bacteria alone, but also in combination. In addition,

some epithelial cells were not associated with either bacteria or beads suggesting that there may be differences between the epithelial surfaces within the same culture plate which preferentially favour attachment and/or invasion of 'particles', be it either bacteria or beads, or a combination. It may be possible by flow cytometric cell sorting to separate the cells associated with bacteria from the cells that have no bacteria.



Population	#Events	%Parent
All Events	30,000	####
P1	3,762	12.5
cells	175	4.7
beads	420	11.2
fitc+	772	20.5
dual positives	1,824	48.5

Figure 4.18 Populations of H357 cells associated with fluorescent beads and/or *P. gingivalis*. H357 monolayers were incubated with FITC-*P. gingivalis* and fluorescent beads (Fluoresbrite BB Carboxylate Microsphere, Polysciences Inc) for 90 minutes, then washed and trypsinised. The forward scatter (FSC)/side scatter (SSC) histogram shows a population of beads alone, not associated with cells (*). These were gated out of the analysis and cells only were analysed (P1). The UV 450/50/Blue 530/30 histogram shows the cell populations of bacterial-associated H357 (FITC/purple), bead-associated H357 (beads/blue), both bacteria- and bead-associated H357 (dual positives/yellow) and H357 alone (cells/green). The table shows the percentage of the cell population for each variable within the P1 region.

4.4 DISCUSSION

4.4.1 *P. gingivalis* invasion of monolayer and OMM

The invasion of oral epithelial cells by *P. gingivalis* has been shown previously to be dependent on bacterial fimbriae, with type II fimbriae rendering the bacterium most highly invasive (Nakagawa *et al*., 2002; Kato *et al*., 2007). It is well known that W50 is a sparsely fimbriated strain of *P. gingivalis* (Sojar *et al*., 1997; Lee *et al*., 1991), and as such contributes to a significantly lower invasive capacity compared with *P. gingivalis* NCTC 11834, which is highly fimbriated (Suwannakul *et al*., 2010). This greater epithelial invasion was observed for both monolayer and organotypic cultures (figs 4.1and 4.3 respectively).

The invasion of monolayer and OMM cultures were performed over different periods of time, i.e. 1.5 hours and 4 hours respectively. This was to achieve the maximal percentage invasion of these cultures by *P. gingivalis*. Therefore, a direct comparison of the levels of invasion between monolayer and OMM was not possible. Despite this, the total percentage invasion of OMM was almost identical to the recorded percentage invasion for monolayer, which may be due to similarities in their surface areas, suggesting that independent of the epithelial culture model there may be a maximal proportion of the original bacterial suspension that is able to invade and/or only a certain proportion of epithelial cells have the capacity to be invaded. Nonetheless, similarities in invasion between these two culture systems suggests that although OMM is more representative of the oral mucosa (Chapter 3), investigating cellular invasion by *P. gingivalis* may be suitable using the simpler monolayer model.

As for the choice of NOK and H357 cells; there was no significant difference in the percentage invasion by *P. gingivalis* between these two cell types when cultured as a monolayer. Since H357 cells were more readily available than NOK, monolayer models with the former have an advantage. However, the invasion of NOK by *P. gingivalis* NCTC 11834 was significantly lower than that seen with the H357 cell line when both were cultured as organotypic models (OMM). This may be due to differences between the NOK-OMM and H357-OMM, in areas such as cellular differentiation, total receptor expression or polarity, and/or intracellular signalling pathways (Ertel, 2006), all of which may influence bacterial invasion. As mentioned, the organotypic model based on NOK has a multi-layered differentiated epithelium that is histologically representative of the oral mucosa (Chapter 3), suggesting that the use of NOK-OMM to study the invasion of oral epithelial cells by *P. gingivalis* may be more relevant than monolayer cultures. However, as previously mentioned (section 3.3.1.4), submerged H357-OMM were shown to resemble junctional epithelium and therefore may be just as appropriate to study *P. gingivalis* invasion as NOK-OMM.

Monolayer and OMM cultures may be used to study other aspects of host-pathogen interactions, rather than limited to invasion, and these include, protein release, gene expression, receptor expression and intracellular signalling. Therefore, considering data obtained from this study, i.e. monolayer (H357/NOK) or OMM (H357/NOK), the question may be posed; which culture system is the most suitable to study these interactions in the future? As a cell line, H357 is easy to obtain, reliable and quick to culture. No noticeable differences were observed between the total percentages of *P. gingivalis* invasion recorded for H357-OMM and H357 monolayer cultures for either strains W50 or NCTC 11834 (figs 4.1 & 4.3) and no significant difference was recorded between NOK and H357 monolayer cultures for the invasion of strain NCTC 11834. Therefore, H357 monolayer cultures may be just as suitable to study trends between experimental variables, where such variables are not reliant upon the cell-type or absolute end-point values. For example, invasion of NOK monolayers showed the same trend as invasion of H357 monolayers, with strain E8 (Δ*rgpArgpB*) invading at a higher percentage than the parent strain (W50) (figs 4.5 & 4.6). However, as mentioned, monolayer cultures are not as representative of the oral mucosa as organotypic cultures. Furthermore, H357-OMM is not as representative of the oral mucosa as NOK-OMM because H357 cells are derived from an oral squamous cell carcinoma and differ from primary epithelial cultures. Data in this study suggests that if primary cells are in short supply or difficult to obtain or culture, then monolayer cultures of cell lines may be used to identify trends between experimental variables. However, NOK-OMM (particularly cultured using isolated juctional epithelial cells) may be more useful in interpreting absolute percentage invasion or other absolute end-point values, which may influence further experimental procedures such as *in vivo* experimentation and/or drug development. In addition, as NOK may express different receptors or other cell surface proteins and intracellular signalling pathways, it should be recommended that NOK-OMM be the model of choice to answer such definitive questions.

As a model system to study bacterial invasion, the mechanisms may differ between monolayer and OMM due to the contributing factors of the multi-layered epithelium and the presence of fibroblasts. However, whilst monolayers may be a useful, highly reproducible model to study invasion *in vitro*, the culture of a reproducible model incorporating primary human cells, a multi-layered epithelium and additional cell types, e.g. fibroblasts, supported by a matrix (as presented in this study), should be recommended as a relevant *in vitro* model of the *in vivo* cellular micro-environment encountered by *P. gingivalis*.

4.4.2 Factors influencing *P. gingivalis* invasion

Gingipains are a major virulence feature of *P. gingivalis* and have been shown to play a role in modulating the oral epithelial cell invasion of this bacterium. The invasion of H357 oral epithelial cells with *P. gingivalis* wild-type (W50) and gingipain knockout mutants (E8

(*ΔrgpArgpB*) and K1A (*Δkgp*)) has previously been reported (Suwannakul *et al*., 2010). The trend in invasion, with E8 invading monolayer cultures of oral epithelial cells at a significantly higher percentage than the wild-type was also shown in this chapter (fig 4.5). K1A invaded at a lower level (Suwannakul *et al*, 2010), not significantly different from the parent strain W50. Moreover, the invasion of H357-OMM with these *P. gingivalis* strains also showed the same trend in invasion, confirming the use of monolayer cultures to assess differences in experimental trends, rather than culturing OMM, which is more time consuming. However, in contrast to this data, it has previously been reported that Arg- and Lys-gingipain mutants of *P. gingivalis* cause smaller abscesses in a murine model of *P. gingivalis* infection (Yoneda *et al*., 2001), decreased invasion *in vitro* of oral epithelial cells when compared with the parent strain (Park and Lamont., 1998), and decreased adhesion of *P. gingivalis* to epithelial cells in the presence of soluble gingipains (Chen *et al*., 2001). However, these particular studies have targeted gingipain knockout in the more highly fimbriated strains of *P. gingivalis* such as ATCC 33277. The parent strain in this study was W50, which is sparsely fimbriated, and this may account for differences in the invasion trends observed.

As mentioned previously, fimbriae have been shown to be a major virulence feature of *P. gingivalis* and are involved in epithelial cell adhesion via host cell integrins (Tsuda *et al*., 2008; Nakagawa *et al*., 2005). *P. gingivalis* gingipains, more specifically Arg-gingipains, have been shown to be important in prefimbrillin processing to fimbrillin that comprise the *P. gingivalis* fimbriae (Lee *et al*., 1991). Indeed, it has been reported that a *P. gingivalis* RgpARgpB-null mutant showed very little fimbrillin expression by Western blot or electron microscopy (Nakayama *et al*., 1996; Kadowaki *et al*., 1998). In addition, a triple gingipain-null mutant treated with exogenous gingipains was highly fimbriated, whereas the untreated mutant strain was sparsely fimbriated (Kato *et al*., 2007). Therefore, it could be assumed that the adhesion to, and subsequent invasion of epithelial cells by gingipain knockout mutants would be lower than the parent strain. However, as the *P. gingivalis* parent strain (W50) used in this study is sparsely fimbriated, gingipain knockout may not further influence fimbrillin expression significantly. Consequently, it could be speculated that invasion of oral epithelial cells by this strain of *P. gingivalis* may be via a mechanism independent of fimbriae, for example via clathrin-mediated endocytosis (Boisvert and Duncan, 2008) or lipid rafts (Tsuda *et al*., 2008).

Using the same strains of *P. gingivalis* as the ones used in this chapter, Suwannakul *et al*. (2010) reported that a highly invasive sub-population of *P. gingivalis* showed a down-regulation in the gene encoding RgpB, and *P. gingivalis* cells with low gingipain activity showed higher epithelial cell invasion than *P. gingivalis* with high gingipain activity. In addition, Chen *et al*., (2001) report the increased adhesion to epithelial cells of a *P. gingivalis* double RgpARgpB knockout mutant compared with the parent strain (ATCC 33277) and single Rgp knockout

mutants (Chen *et al*., 2001). This further confirms the gingipains as important regulators of the invasion process, particularly in less fimbriated, less invasive strains of *P. gingivalis*. It could be speculated that gingipains may affect the epithelial cell surface by degrading host cell receptors thereby inhibiting invasion. Therefore, *P. gingivalis* with a lower gingipain activity may be internalised with greater efficiency due to the availability of an increased number of host cell receptors. This is explored further in Chapter 5.

Also presented in this chapter is the epithelial cell invasion of a triple gingipain knockout mutant (Δ*rgpA*Δ*rgpB*Δ*kgp*, EK18), kindly provided by J. Higham (University of Sheffield). Previous research has shown little tissue penetration and reduced basement membrane destruction by a *P. gingivalis* triple gingipain mutant compared to the wild-type strain in an *in vitro* organotypic model of the oral mucosa (Andrian *et al*., 2004). In this study, the invasion of EK18 was significantly lower than the parent strain, W50 (fig 4.8). This was also shown by Kato *et al*. (2007), where the invasion of a triple gingipain mutant was lower than that of the parent strain. This decrease in invasion may be due to the complete loss of fimbriae because of the loss of prefimbrillin processing by the gingipains or the lack of differential processing by gingipains of other, yet unidentified, cell surface adhesins important for *P. gingivalis* invasion.

The gingipains are not the only modulator of *P. gingivalis* invasion. Numerous mechanisms are involved and the influence of increasing the temperature and concentrations of haemin within the bacterial culture medium was investigated in this study; mechanisms that may not be distinctly separate from the role gingipains play within the oral cavity. A sign of periodontitis is bleeding upon probing. The haemin within blood has been shown to be utilised by *P. gingivalis* as a growth source (Wyss *et al*., 1992). High concentrations of haemin have been shown to influence the structure of lipid A rendering it more of a TLR4 antagonist with mono-phosphorylated, tetra-acylated form of lipid A predominantly expressed (Al-Qutub *et al*., 2006). In contrast, at elevated temperatures, lipid A acts as a TLR4 agonist exhibiting penta-acylated, mono-phosphorylated species (Curtis *et al*., 2011). The invasion of *P. gingivalis* was shown to be significantly higher when cultured at both high temperature (41°C) and high haemin concentrations (10µg ml^{-1}) (fig 4.9 & 4.10 respectively). As it has been shown that different structures of lipid A exist at high temperature and high haemin concentrations, differences in the composition of lipid A may not be sufficient in explaining its role in invasion. More importantly it has previously been reported that *P. gingivalis* grown under haemin-limiting conditions only expressed 50% of the *fimA* promoter activity compared with bacteria grown under normal haemin concentrations (Xie *et al*., 1997). This suggests that the expression of fimbriae, which play an important role in invasion, may be diminished under haemin-limiting conditions and possibly explains why a lower percentage invasion of oral epithelial cells was found when *P. gingivalis* was cultured in 1µg ml^{-1} haemin than in 10µg ml^{-1}. In addition, FimA expression was

shown to be increased in excess haemin conditions (Masuda *et al*., 2006), further suggesting a role for fimbriae in the increase in *P. gingivalis* invasion under increasing haemin concentrations.

In summary, these data add to a hypothesis for the influence of environment on *P.gingivalis* invasion. Under conditions of inflammation, the environment within which *P. gingivalis* resides is rich in haemin and the temperature of the periodontal pocket is raised. The data presented here propose that this environment, in terms of haemin and temperature influence *P. gingivalis* entry within oral epithelial cells, enabling this bacterium to avoid immune cell detection. *P. gingivalis* possesses numerous virulence features, which modulate epithelial internalisation. In this high haemin and high temperature environment it may be that *P. gingivalis* gingipains are down-regulated as they are no longer required for haemin acquisition and/or are denatured at elevated temperatures (Lewis *et al*., 1999, Masuda *et al*., 2006). The consequence would be that cellular receptors may not be destroyed by gingipains. Therefore suggesting a mechanism by which the environment and gingipains act in concert to increase the epithelial colonisation by *P. gingivalis*. This may be particularly important in the pathogenesis of disease where high temperatures and high concentrations of haemin are present, contributing to the cycles of disease exacerbation observed in these individuals (Socransky *et al*., 1984). However, overall there is still limited information and further work is required to confirm or refute this hypothesis.

4.4.3 The fate of *P. gingivalis* following epithelial cell invasion

As a microorganism, it is within the interests of *P. gingivalis* to survive at sites within the mouth that it inhabits. The low oxygen tensions of the periodontal pocket and incorporation into the bacterial biofilm aid in the survival of this anaerobic bacterium. However, there is the constant threat from immune cells rendering extracellular bacteria more susceptible to killing. Therefore to be internalised by oral epithelial cells may aid immune evasion. To remain viable intracellularly, *P. gingivalis* modulates host apoptotic pathways (Mao *et al*., 2007). In addition, the protein expression of *P. gingivalis* dramatically alters following invasion. It has been observed that there is an up-regulation of oxidative stress and heat-shock proteins and a down-regulation of gingipains and fimbriae (Xia *et al*., 2007; Capestany *et al*., 2008), which may contribute to intracellular survival. In addition, Suwannakul *et al*. (2010) showed increased oxidative stress resistance, a down-regulation of *rgpB* and an up-regulation of iron acquisition genes in a more invasive sub-population of *P. gingivalis*.

The data presented in this study indicated that the intracellular viability of *P. gingivalis* NCTC 11834 within the H357 oral epithelial cell line, when cultured as a monolayer or OMM, began to decrease after 3 hours and intracellular persistence was limited to approximately 6 hours (figs

4.12, 4.13 & 4.14). This is in agreement with Li *et al*., (2008) who described a steep decline in the intracellular recovery of viable *P. gingivalis* after 6 hours, diminishing to almost zero 48 hours after invasion of endothelial, KB (HeLa) and smooth muscle cells. However, this is in contrast to other data reported in the literature, which shows the intracellular persistence of *P. gingivalis* for up to approximately 4-8 days (Houalet-Jeanne *et al*., 2001; Madianos *et al*., 1996; Eick *et al*., 2006). Differences between the data reported in the literature and data presented in this chapter are difficult to explain but may be due to the differences in epithelial cells used (H357 oral keratinocytes compared with the HeLa contaminated, KB cell line) and differences in the strains of *P. gingivalis* used. Madianos *et al.* (1996) reported an initial invasion of 60% after incubating KB epithelial cells with *P. gingivalis* at a MOI of 1. This percentage invasion is very high but the authors explain that the low MOI may have prevented epithelial cell stress enabling a higher percentage invasion to be recorded. However, this seems unlikely as invasion has previously been shown to increase as the MOI increases, up to a maximum percentage after incubation of epithelial cells with *P. gingivalis* at MOI100 (Lamont *et al*., 1995; Li *et al*., (2008)). This has been our experience also, as reported in this chapter, where a lower percentage invasion was recorded after *P. gingivalis* invasion with MOI1 compared with MOI100 (fig 4.12 and 4.13 respectively).

There are numerous mechanisms by which the observed decrease in *P. gingivalis* intracellular survival over 24 h reported in this chapter may have occurred. It has been shown that the medium and the aerobic atmosphere, within which the experiment was performed, were not suitable for prolonged survival of *P. gingivalis* in the absence of epithelial cells (fig 4.11). Furthermore, additions of substrates, important in *P. gingivalis* growth, to the culture medium was not sufficient to maintain bacterial survival for longer than 4-6 hours, probably due to the aerobic atmosphere. Therefore, it may be assumed that this medium, over the time period of the experiment, had adverse effects on the survival and detection of viable *P. gingivalis*. The intracellular environment may also have contributed to the death of this bacterium via trafficking to phagolysosomes (Takeuchi *et al*., 2011). In addition, when epithelial cells undergo oxidative phosphorylation, reactive oxygen species are produced, e.g. superoxide species and hydrogen peroxide. Although intracellular *P. gingivalis* has been shown to upregulate proteins essential for combating oxidative stress, such as superoxide dismutase (Ohara *et al*., 2006), alkyl hydroperoxide reductase (Johnson *et al*., 2004) and rubererythrin (Mydel *et al*., 2006), and an invasive sub-population of *P. gingivalis* has been shown to have greater oxidative stress resistance (Suwannakul *et al*., 2010), this up-regulation may not be sufficient over the time periods described here to be protective, particularly as cells continue to respire. It should be mentioned that the H357 cell line was used in all these experiments and it has been reported that some cancer cells up-regulate genes involved in oxidative phosphorylation, suggesting that NOK may be a more suitable cell to use for these experiments. However, the data reported in

the literature regarding the intracellular survival of *P. gingivalis* has primarily been performed using the KB cell line, which has widely been shown to be contaminated with HeLa cells, originally isolated from cervical cancer. Therefore, the H357 cell line used in this study is a more relevant cell type as it was harvested from the oral cavity.

In addition, the decreased detection of intracellular *P. gingivalis* after 6 hours may be the result of latency once internalised. This has been proposed as a mechanism for the intracellular persistence of uropathogenic *E. coli* within bladder epithelial cells (Hunstad and Justice, 2010) and for *P. gingivalis* within oral epithelial cells (Li *et al*., 2008). It has been suggested that unculturable intracellular bacteria within this 'latent state' may become culturable once in contact with fresh host cells (Li *et al*., 2008), reversing the quiescent state and indicating a mechanism by which intracellular bacteria may persist within host tissue, occasionally being released to cause exacerbations in disease (Socransky *et al*., 1984, Takeuchi *et al*., 2011). This aspect has not been explored here but would be useful further work. However, it has been shown here that *P. gingivalis* are found within the supernatant following invasion providing an opportunity for these external bacteria to re-enter previously invaded, or as yet un-invaded, epithelial cells.

In terms of intracellular replication, Madianos *et al*. (1996) and Houalet-Jeanne *et al*. (2001) report the intracellular multiplication of *P. gingivalis* after the first or second day following invasion. This is in contrast to data presented in this chapter and by Li *et al*. (2008), where no bacterial replication was observed. The use of KB cells in those studies is not as relevant as oral keratinocytes, and as previously mentioned this may be the reason why differences in observations exist. Papapanou *et al*. (1994) have reported the possible intracellular replication of *P. gingivalis* within pocket epithelial cells by electron microscopy. However, without investigating this further, the intracellular replication within this more relevant cell type cannot be fully elucidated.

The recolonisation of periodontal sites by intracellular bacteria has been suggested by Muller *et al*. (1996). The authors report that the number of intracellular bacteria is greater than the number of bacteria within subgingival plaque, suggesting that the high numbers within this intracellular store may be the origin for recolonisation (Muller *et al*., 1996). Therefore, the release of intracellular *P. gingivalis* may be an important mechanism in recolonisation. Indeed, it has been reported elsewhere that intracellular *P. gingivalis* is capable of exiting epithelial cells and infecting 'new' host cells, contributing to cell-to-cell transmission (Li *et al*., 2008, Yilmaz *et al*., 2006). Data presented in this study also suggests that *P. gingivalis* is capable of exiting host epithelial cells. In agreement with data presented by Takeuchi *et al*. (2011), there was an increase in the number of extracellular bacteria following invasion, up to 6 hours, after which

there was a dramatic decline in detection (fig 4.13). Intracellular pathogens use a number of strategies to exit host cells, including induction of host cell apoptosis, actin-based protrusion, or extrusion (Hybiske and Stephens, 2008). In a recent study, *P. gingivalis* exit has been shown to be dependent on actin polymerisation, lipid rafts and microtubule assembly, suggesting that *P. gingivalis* release from epithelial cells may be an active process (Takeuchi *et al*., 2011). However, the extracellular *P. gingivalis* detected in this study was probably due, in part, to epithelial desquamation. These desquamated cells contained intracellular bacteria following the antibiotic protection assay and it was shown that the number of desquamated epithelial cells increased over time, particularly in monolayer cultures, compared with OMM. However, the percentage of *P. gingivalis* released into the supernatant showed a greater prolonged release from OMM compared with monolayer. As the number of desquamated epithelial cells detected from OMM at 48 hours was not higher than that from monolayer cultures this suggests that *P. gingivalis* may be released by mechanisms other than epithelial desquamation. However, the sensitivity of the method for detecting epithelial cell desquamation requires improvement, as only the cells that were completely or partially intact were counted, eliminating the detection of completely lysed cells, which may have occurred as a mechanism of *P. gingivalis* release. Indeed, it has been reported that a higher number of *P. gingivalis* cells are associated with dead epithelial cells compared with viable epithelial cells, suggesting that *P. gingivalis* release may also occur during epithelial cell death and desquamation (Dierickx *et al*., 2002).

In terms of comparison of monolayer and OMM cultures, the persistence of *P. gingivalis* within OMM was significantly longer than monolayer, even though the percentage of desquamated epithelial cells was similar. As OMM consists of a multi-layered epithelium, the percentage of desquamated cells was recorded as a percentage of the number of epithelial cells estimated to be on the surface layer of the model and not the total number of epithelial cells. Therefore, as OMM is multi-layered there are other layers of cells that could be involved, which may explain the prolonged survival of this bacterium. However, in all the *in vitro* studies, there was an eventual decline in the recovery of viable *P. gingivalis*, which is consistent with reports in the literature and which may be due to the culture system used. It may not, therefore, be representative of the *in vivo* situation where there is constant desquamation. Indeed, within the mouth, shedding surfaces, i.e. mucosal tissues, are in the close vicinity of non-shedding surfaces, i.e. the tooth surface, which harbours many bacterial species. This may therefore provide a continuous reservoir of bacteria for the re-infection of oral epithelial cells and as such the experimental methods do not directly mimic the *in vivo* situation. Consequently, the data reported here should be interpreted with caution and further modifications to the experimental protocol and the *in vitro* culture model may be required to determine the *in vivo* relevance. Nonetheless, the experimental protocol and culture model used in this study to investigate *P. gingivalis* intracellular survival, suggests that *P. gingivalis* does not replicate intracellularly and

only survives within the epithelium for 3 hours. Following this, there is either bacterial cell death or *P. gingivalis* enters a dormant 'unculturable' state.

4.4.4 Comparison of methods to separate epithelial cells associated with *P. gingivalis*

For bacterial invasion of host cells there seems to be some cells that are 'super-invaded' and some cells not invaded at all. This was shown in figure 4.15, in which anti-*P. gingivalis* staining revealed the extent of invasion in H357 monolayers and H357-OMM. This has been reported previously by Rudney *et al*., (2005). Therefore it seemed reasonable to investigate what differences exist between epithelial cells which have high bacterial loads and those which do not, in an attempt to dissect the epithelial cell contribution to the invasion process. In order to do this, invaded and the non-invaded cell populations needed to be separated.

There are a number of commercial cell separation kits available. Separation of a cell type of interest from a mixed cell population is commonly performed by labelling the required cell type using an antibody against a specific cell surface receptor and separating the mixed cell populations using magnetic or non-magnetic beads, or fluorescent-activated cell sorting (FACS). The major cell separation kits include MACS® Cell Separation (Miltenyi Biotec Ltd, Germany), EasySep® (StemCell™ Technologies, Grenoble, France) and Dynabeads® (Invitrogen, UK), which are based on magnetic beads and pluriBead (pluriSelect, Germany), CEDARLANE cellect™ Immunocolumn and CEDARLANE® Lympholyte PURE (Cedarlane, Canada), which are non-magnetic systems relying on cell separation via density gradient media, a recovery column and sieve system. These magnetic and non-magnetic bead systems are limited to the availability of the specific antibody-coupled bead required for the specified cell type. Dynabeads® have been further developed to allow binding of any antibody of interest to the beads, increasing its applications. In this study, Dynabeads® were chosen as one of the methods to explore separation of invaded from non-invaded epithelial cells. In addition, H357 epithelial cells were incubated with fluorescently-labelled *P. gingivalis* or fluorescent beads and the fluorescence of the epithelial cells was measured by flow cytometry to assess the populations of cells which preferentially took up any particulate matter (beads only) compared with cells that were associated with bacteria. It should be mentioned that neither of these separation systems differentiated adherent, non-internalised from internalised *P. gingivalis*. Therefore, the term 'associated cells' refers to the epithelial cells associated with bacteria, i.e. epithelial cells with *P. gingivalis* internalised as well as adhered to the cell surface.

Data presented here indicate that it is possible to separate *P. gingivalis* associated epithelial cells from non-associated cells. A percentage of epithelial cells were shown to associate with magnetic beads alone, which were not coupled to *P. gingivalis*, suggesting that epithelial cells

are capable of internalising or associating with 'particles', in this case magnetic beads (fig 4.16). This was also observed when performing flow cytometry in which some epithelial cells were associated with fluorescent beads alone (fig 4.18). Using magnetic beads it was possible to separate a higher percentage of epithelial cells which associated with *P. gingivalis*-coated beads compared with un-coated beads. This suggests that there is a population of epithelial cells that preferentially associate with *P. gingivalis*-beads compared with beads alone. What is not clear though is whether those epithelial cells that associate with beads alone are also part of the population of epithelial cells that associate with *P. gingivalis*-beads, or whether they are a distinct population. Indeed, the flow cytometry data indicated that there were 4 distinct populations (fig 4.18), suggesting that there may be epithelial cells that associate with particulate matter regardless of the expression of specific adhesins etc, whereas other epithelial cells specifically 'recognise' *P. gingivalis* adhesins. Using the magnetic beads to separate epithelial cells that associate preferentially with *P. gingivalis* alone will prove difficult, however, there was a distinctly higher percentage of the population that did not associate with magnetic beads at all. However, because the magnetic beads are 2.8μm diameter, and a little larger when coupled with *P. gingivalis*, the entry into/association with epithelial cells may have been inhibited, accounting for the high number of epithelial cells recorded as not associated with magnetic beads. In addition, the intracellular localisation of one or two beads for example may have reduced the efficiency of the magnetic separation process. Indeed, the flow cytometry data suggested that the number of epithelial cells not associated with *P. gingivalis* was 15.9%, which seems a little more likely when compared with the immunohistochemical staining data (fig 4.15). Therefore, further work will be required to examine the percentage of epithelial cells that preferentially internalise/associate with *P. gingivalis* following infection.

Despite these difficulties, it was shown that following separation of the two cell populations the epithelial cells could then be re-seeded and cultured. However, when an invasion assay using *P. gingivalis* was performed with these two cell populations, no significant difference in invasion was observed. This suggests that differences between the two populations in the original cultures were only transient and insufficiently stable to be manifest after re-culture. Actual epithelial cell separation and sorting by FACS was not attempted in this study. However, it was shown that there were distinct populations of cells that associated with fluorescent *P. gingivalis* and those that did not, making such cell sorting a possible way to study this further.

4.4.5 Conclusion

The invasion of oral epithelial cells by *P. gingivalis* is a complex process that relies on host-pathogen-environmental cross-talk. *P. gingivalis* gingipains, environmental haemin and temperature have all been shown to be important modulators of this. In particular, within conditions that mimic the *in vivo* environment found at diseased periodontal sites, i.e. high

temperatures and haemin concentrations, invasion increased. Thus, suggesting a role for these factors in the pathogenesis of disease. However, more work is required to elucidate mechanistic and functional roles of these in the invasion process.

Methods have been proposed to separate bacterial-associated epithelial cells and the future study of these may further scientific knowledge as to the epithelial contribution during *P. gingivalis* invasion.

The fate of intracellular *P. gingivalis* in terms of survival and multiplication reported here was in contrast to some reports previously published as we failed to show bacterial survival for longer than 3 hours, or multiplication over the 48 hours the experiments were performed. This was observed with both monolayer and OMM cultures, although the survival within OMM was more prolonged than monolayer cultures. We proposed a mechanism by which *P. gingivalis* may re-infect periodontal sites, *in vivo*, by epithelial desquamation.

However, additional work is required in order to identify the key step(s) important in the pathogenesis of disease, which may contribute to the development of novel therapeutic agents used in the treatment of periodontitis. Mimicking the periodontal environment *in vitro* is essential, in addition to the continued utilisation and development of organotypic cultures of primary, orally-derived cells as a more representative model of the oral mucosa.

Chapter 5 Modification of epithelial cell surface receptors by *P. gingivalis* and its influence on invasion

5.1 INTRODUCTION

Epithelial cell internalisation by *P. gingivalis* is a complex process. To date, the precise mechanism of invasion and the identification of all crucial epithelial cell receptors has not yet been fully investigated (section 1.3.4.2).

It was shown in chapter 4 that *P. gingivalis* lacking the arginine-specific gingipains invaded keratinocytes at a higher percentage than the wild-type strain (section 4.3.2.1). It was reasoned, that gingipains may affect the epithelial cell membrane in some way, inhibiting invasion, and this may be due to the degradation of epithelial cell receptors important for the cellular internalisation of this bacterium. As it is currently thought that epithelial invasion by *P. gingivalis* occurs via the association of bacterial fimbriae with the integrin α5β1, the α5 integrin subunit was one of the molecules investigated in this chapter. However, *P.gingivalis* gingipains may also affect accessory molecules that interact with membrane integrins disrupting their normal clustering and function (Mahtout *et al*., 2009). Consequently, members of the tetraspanin family of molecules have also been investigated.

Tetraspanins have been implicated in a number of host-pathogen interactions. For example, Human Immunodeficiency Virus (HIV) buds in cell membranes rich in CD9, CD81, CD53 and CD63 (Deneka *et al*., 2007). The Hepatitis C Virus (HCV) envelope glycoprotein, E2, binds to CD81, which is crucial for cellular internalisation of the virus (Pileri *et al*., 1998) and the blockade of *Plasmodium falciparum* and *Plasmodium yoelii* sporozoite internalisation of hepatocytes was achieved using CD81 monoclonal antibodies and CD81 silencing (Silvie *et al*., 2002; Silvie *et al*., 2006). In addition, pre-treatment of epithelial cell lines with anti-CD9, -CD63 and -CD151 antibodies, recombinant EC2 domains and small interfering RNA (siRNA), inhibited the adhesion of *Neisseria meningitidis* to epithelial cells (Green *et al*., 2011).

The entry of *P. gingivalis* into oral epithelial cells has been suggested to involve the integrin α5β1 (Tsuda *et al*., 2008; Nakagawa *et al*., 2005), lipid rafts (Tsuda *et al*., 2008) and/or clathrin-mediated endocytosis (Boisvert and Duncan, 2008). Tetraspanins have been implicated in these processes. HCV invades hepatocytes via clathrin-mediated endocytosis where CD81 oligomerisation may lead to increased HCV internalisation (Meertens *et al*., 2006, Zeisel *et al*., 2011). Also, although shown to be distinct from lipid rafts, cholesterol has been shown to be important in the organisation of tetraspanins required for the cellular invasion of malaria sporozoites. In addition, tetraspanins are involved in clustering of integrins and other cellular

proteins into 'microdomains' at the cell surface (Singethan and Schneider-Schaulies, 2008; Yang *et al*., 2004). Therefore, the question was posed as to whether the α5β1 integrin and/or tetraspanins were important in the invasion of epithelial cells by *P. gingivalis*.

Finally, an epithelial membrane molecule that has been reported to be affected by *P. gingivalis* gingipains is the complement receptor CD46. CD46 acts as a co-factor in the proteolytic inactivation of complement proteins C3b and C4b (Liszewski *et al*., 1991) to prevent prolonged complement activation that can lead to host tissue damage. CD46 has been shown to associate with β1 integrins, including α5β1 (Lozahic *et al*., 2000), which is thought to be important in *P. gingivalis* invasion. In addition, CD46 interacts with the tetraspanins CD9, CD81, CD82 and CD151, as part of the tetraspanin web (Lozahic *et al*., 2000) and Mahtout *et al*. (2009) have reported proteolysis of CD46 by *P. gingivalis* whole cells and by purified Lys-gingipain. Consequently we considered it possible that such proteolysis may influence invasion and so explain the enhanced invasion seen with the gingipain mutants of *P. gingivalis*.

5.1.1 Aims

The aim of this study was to investigate the involvement, if any, of surface effector molecules, in particular the tetraspanins and the integrin α5β1 in the invasion of oral epithelial cells by *P. gingivalis*. Initially, the presence of a range of tetraspanins, including CD9, CD63, CD81, CD82 and CD151, and the α5 integrin subunit on the epithelial cell surface, following incubation with *P. gingivalis* was analysed by flow cytometry. CD81 and α5 were shown to be degraded by *P. gingivalis* in a gingipain-dependent manner. Therefore CD81 was targeted by RNA silencing, and CD81, CD9, CD63, CD82, CD151, CD46 and α5 by blocking antibody, to determine whether these molecules are targets for gingpains and explain the enhanced invasion by gingipain mutants. Such findings should inform our understanding of the molecular process of the invasion of epithelial cells by *P. gingivalis*.

5.2 METHODS

The following methods were used in this chapter:

- Culture of H357 cell line (section 2.2)
- Culture of bacterial strains (section 2.5)
- Tetraspanin and α5β1 integrin detection analysed by flow cytometry (section 2.20)
- Knockdown of CD81 using siRNA (section 2.21)
- mRNA extraction, cDNA synthesis and real-time PCR to confirm CD81 knockdown (section 2.18.3)
- *Porphyromonas gingivalis* invasion of monolayer cultures of H357 (section 2.11.1)
- Statistical analysis (section 2.22)

5.3 RESULTS

5.3.1 *P. gingivalis* affects the presence of CD81 and the α5 integrin subunit on H357 cells

H357 monolayers were treated overnight with or without *P. gingivalis* W50 and probed using monoclonal antibodies for cell surface presence of the tetraspanins CD9, CD63, CD81, CD82 and CD151 and the α5 integrin subunit by flow cytometry. Only live cells were analysed, and were selected for by performing a live-dead stain using TO-PRO®-3 and gating around the live cells using the FACsCalibur software (fig 5.1). Figures 5.2 and 5.3 show the histograms for each tetraspanin and α5 integrin subunit overlaid with an IgG1 negative control, respectively. All tetraspanins and α5 integrin were expressed by the H357 cells as shown by a shift to the right in the recorded fluorescence. The most highly expressed tetraspanin was CD9, with a median fluorescence value of 330.77. CD81 was the next most highly expressed tetraspanin, followed by CD82 (with median fluorescence values of 194.56 and 111.4 respectively). CD63 and CD151 were the least expressed of the tetraspanins investigated with median fluorescence values of 31.06 and 11.76 respectively. Interestingly, the only tetraspanin that was affected by overnight exposure to *P. gingivalis* W50 was CD81, with a 2.33-fold decrease in detection after exposure (fig 5.2). α5 detection also diminished in the presence of *P. gingivalis* W50 overnight, with approximately a 2-fold decrease in detection (fig 5.3).

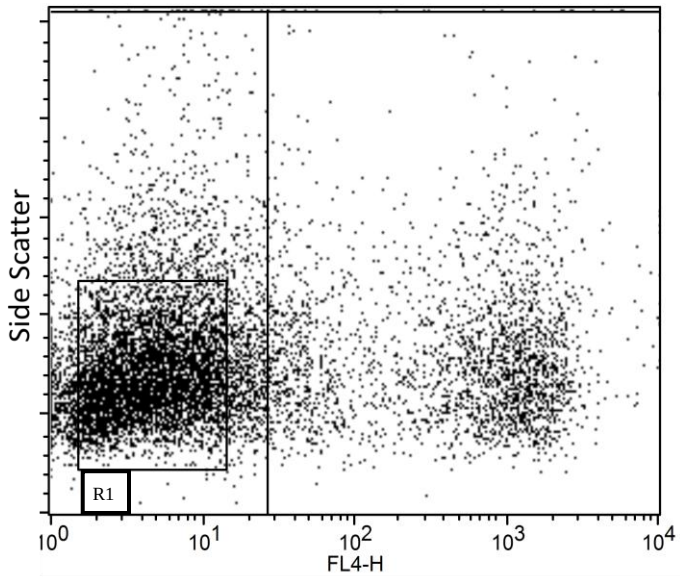


Figure 5.1 Dot plot of live-dead staining of H357 cells using TO-PRO®-3. H357 cells were stained with TO-PRO®-3 which entered non-viable cells. This was detected as an increase in fluorescence on the dot plot, as shown. Live cells were gated (R1) to eliminate dead cells from subsequent analyses. This gating was performed for every flow cytometric analysis performed.

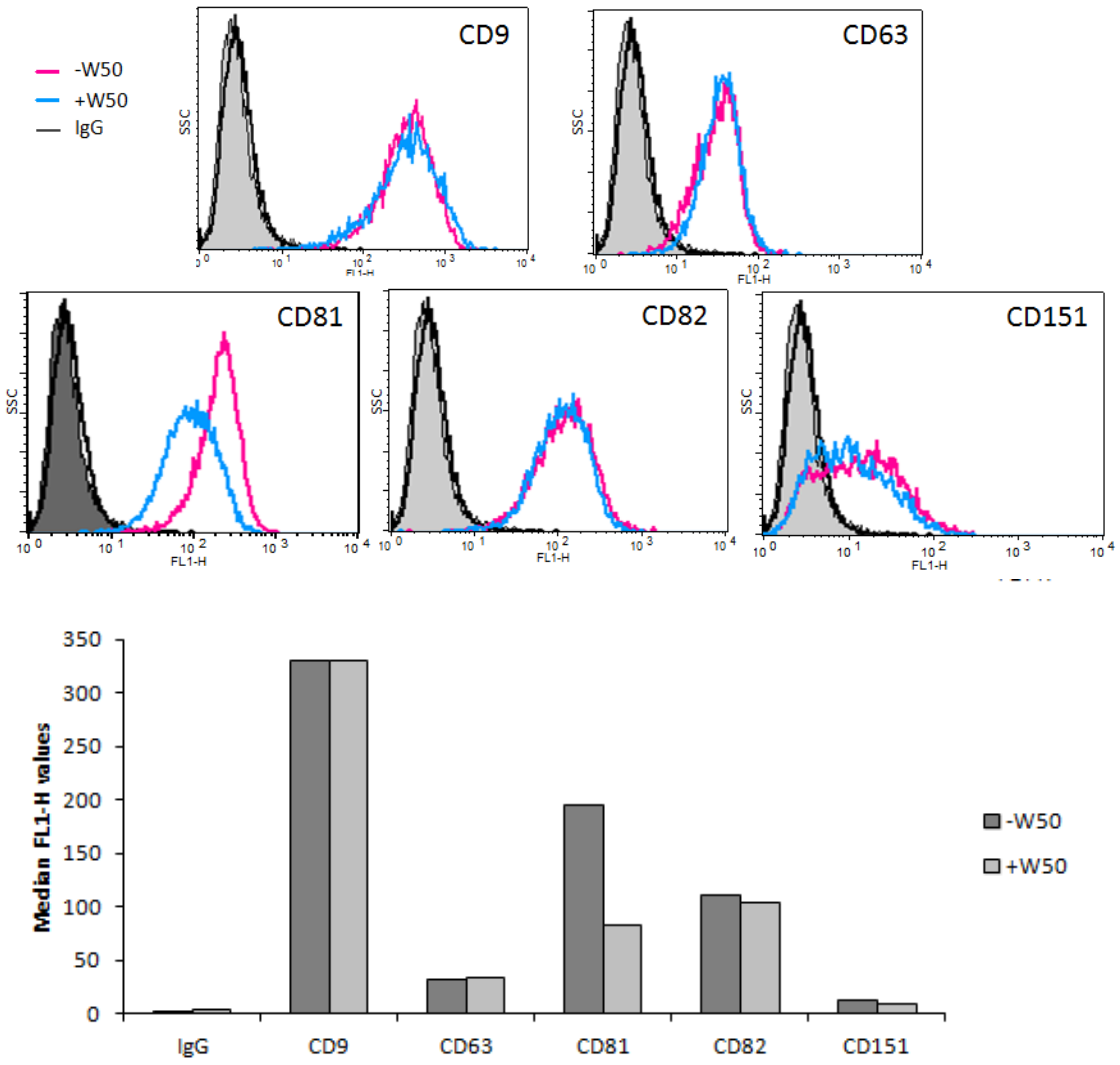


Figure 5.2 The detection of membrane-bound tetraspanins after overnight treatment with *P. gingivalis* W50. H357 monolayers were stimulated with *P. gingivalis* W50 cells overnight at 37°C/5% CO_2. H357 cells without *P. gingivalis* were used as a control. The presence of cell membrane-bound tetraspanins CD9, CD63, CD81, CD82 and CD151 was assessed using flow cytometry. Primary anti-tetraspanin antibodies were incubated with H357 for 30min followed by FITC-conjugated secondary antibody. Cells were washed and analysed using the FACsCalibur cytometer. The dead cells were gated out of analysis (see figure 5.1) and median fluorescence values were plotted for each tetraspanin antibody with or without W50 (one representative experiment shown, repeated in duplicate). The IgG1 isotype control (IgG) for W50-treated H357 (thick black line) and IgG control for untreated H357 (filled peak) did not show any change in fluorescence. FL1-H indicates fluorescence intensity and SCC indicates the number of cells.

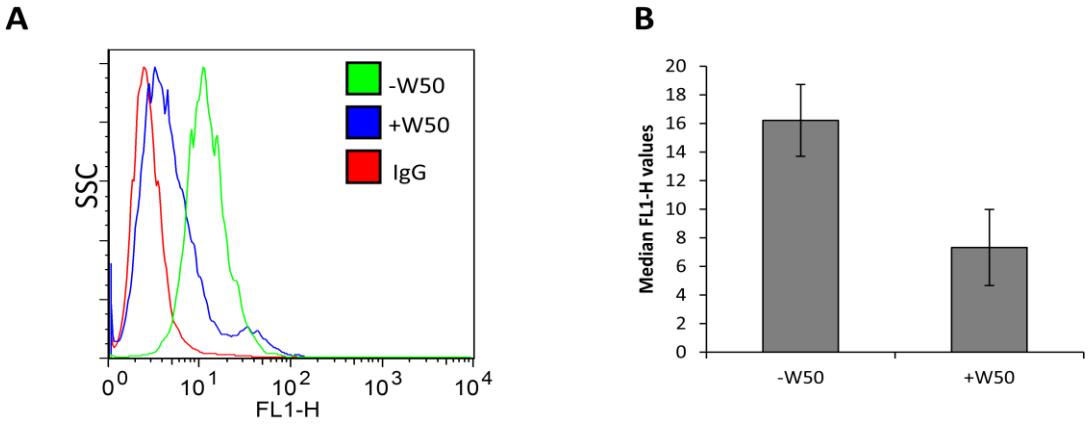


Figure 5.3 The detection of membrane-bound integrin subunit α5 after overnight treatment with *P. gingivalis* W50. H357 monolayers were stimulated with *P. gingivalis* W50 cells overnight at 37°C/5% CO_2. H357 cells without *P. gingivalis* were used as a control. The cell surface presence of α5 subunit was assessed using flow cytometry (A). The median fluorescence values are plotted (B) for the α5 in *P. gingivalis*-treated and-untreated H357 cells. Bars represent means±SEM of three independent experiments. FL1-H indicates fluorescence intensity and SCC indicates the number of cells.

5.3.2 Detection of CD81 and the α5 integrin subunit is affected by *P. gingivalis* gingipains

To investigate whether the decrease in CD81 and α5 cell surface detection was due to *P. gingivalis* proteases, gingipain knockout mutants were incubated with H357 monolayer cultures overnight. Figures 5.4 and 5.5 show that after incubation with W50, as previously shown (fig 5.2 & 5.3), there was reduction in the level of fluorescence for CD81 and α5 respectively, indicating a loss of the cell surface presence of these receptors. In the presence of E8 (Δ*rgpArgpB*) and K1A (Δ*kgp*), at a MOI of 100, there was a slight increase in the fluorescence intensity signal for both CD81 and α5 compared with H357 cells incubated with W50 (with median fluorescence values of 250.29 (E8), 296.93 (K1A) and 205.35 (W50) for CD81 and median fluorescence values of 7.32±2.52 (E8), 11.50±2.56 (K1A) and 2.75±0.15 (W50) for α5). When H357 cells were incubated overnight with the triple gingipain mutant (EK18, Δ*rgpArgpB*Δ*kgp*) there was no reduction in the detection of CD81 or α5 compared with the fluorescent signal from untreated cells (median fluorescent values of 410.47 and 388.91, respectively for CD81 and median fluorescent values of 18.17±2.91 and 16.21±2.66, respectively for α5). These data suggest that, for CD81, the lysine-specific gingipain may be slightly more effective in reducing the cell surface detection of this tetraspanin as the arginine-specific gingipain knockout mutant showed median fluorescent values closer to W50, whereas incubation of H357 cells with the lysine-specific gingipain knockout mutant showed less of a reduction in the median fluorescence value. Following incubation of epithelial cells with the Rgp and Kgp knockout mutants, there was decrease in the detection of the α5 integrin subunit compared with the un-treated control. This reduction was not as marked compared with that

seen in the presence of W50 indicating a partial role for each of the gingipains in modulating the presence of α5 on the surface of oral epithelial cells.

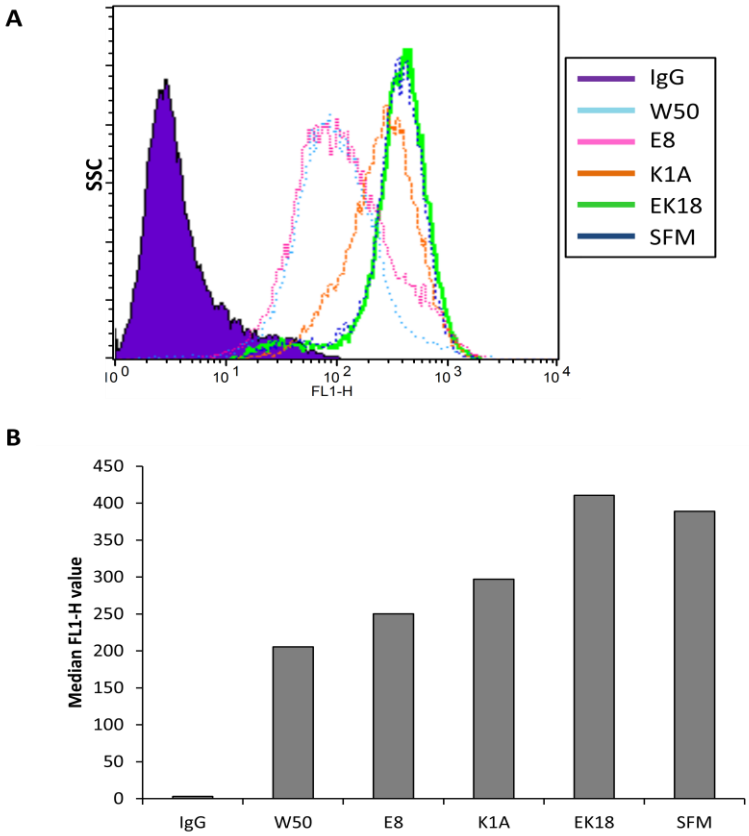


Figure 5.4 CD81 cell surface detection following incubation of *P. gingivalis* strains with H357 monolayers. H357 monolayer cultures were exposed to *P. gingivalis* wild-type (W50) and *P. gingivalis* gingipain knock-out mutants (E8 (Δ*rgpArgpB*), K1A (Δ*kgp*), EK18 (Δ*rgpArgpB*Δ*kgp*)) or serum free medium (SFM) without *P. gingivalis*. Monolayers were trypsinised and incubated with primary antibody directed against CD81 or an IgG1 isotype control. Alexa Fluor-conjugated antibody was incubated with the cells on ice and epithelial cells were analysed for fluorescence using the FACsCalibur flow cytometer. The representative histogram of fluorescence (FL1-H) against SSC (cell number) is shown (A) and the median FL1-H values plotted for epithelial cells treated with W50, E8, K1A, EK18 and medium alone (SFM). The histogram is representative of duplicate experiments. The filled purple peak shown in the FL1-H/SSC histogram (A) represents IgG1 probed H357 cells in medium alone. No deviation in the peak was observed when *P. gingivalis*-treated H357 cells were probed. FL1-H indicates fluorescence intensity and SCC indicates the number of cells.

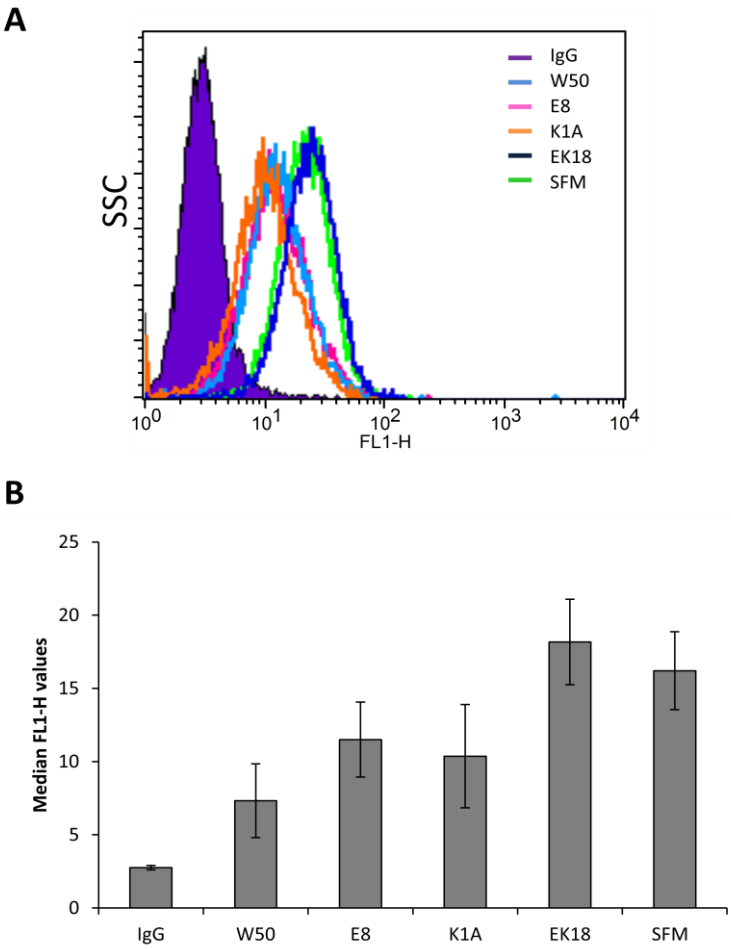


Figure 5.5 α5 cell surface detection following incubation of *P. gingivalis* strains with H357 monolayers. H357 monolayer cultures were exposed to *P. gingivalis* wild-type (W50) and *P. gingivalis* gingipain knock-out mutants (E8 (Δ*rgpArgpB*), K1A (Δ*kgp*), EK18 (Δ*rgpArgpB* Δ*kgp*)) or serum free medium (SFM) without *P. gingivalis*. Monolayers were trypsinised and incubated with primary antibody directed against alpha-5 or an IgG1 isotype control. Alexa Fluor conjugated antibody was incubated with the cells on ice and epithelial cells were analysed for fluorescence using the FACsCalibur Flow Cytometer. A representative histogram of fluorescence (FL1-H) against side scatter (SSC) is shown (A) and the median FL1-H values plotted for epithelial cells treated with W50, E8, K1A, EK18 and medium alone (SFM). Results are means±SEM of three independent experiments. The filled purple peak shown in the FL1-H/SSC histogram (A) represents IgG1 probed H357 cells in medium alone. No deviation in the peak was observed when *P. gingivalis*- treated H357 cells were probed. FL1-H indicates fluorescence intensity and SCC indicates the number of cells.

5.3.3 Antibody blocking of CD9, CD63, CD81, CD151, CD46 or α5 does not influence invasion of epithelial cells by *P. gingivalis*

5.3.3.1 Antibody blocking

It has previously been shown that the pre-treatment of epithelial cells with anti-CD9, -CD63 and -CD151 inhibited the adhesion of *Neisseria meningitidis* as analysed by microscopy (Green *et al.*, 2011). As adhesion is a pre-requisite for epithelial cell invasion by *P. gingivalis* an investigation of whether these antibodies would affect *P. gingivalis* invasion of oral epithelial cells was implemented. In addition, α5 has been suggested to be important in *P. gingivalis*

invasion (Nakagawa *et al*., 2005). As the detection of α5 and CD81 was shown to be affected by *P. gingivalis* gingipains (section 5.3.2), these proteins were also investigated as potential candidates that may modify *P. gingivalis* invasion. H357 monolayers were blocked using antibodies directed against CD9, CD63, CD151 (kindly provided by P. Monk, University of Sheffield), CD81 or α5 and an antibiotic protection assay was performed. Percentage invasion was compared for two strains of *P. gingivalis*, NCTC 11834 and W50. To minimise any degradation of the antibodies by proteases, protease inhibitors were added with the *P. gingivalis* cells. No significant difference was detected in percentage invasion following blocking of CD9, CD63, CD81 or CD151 for either *P. gingivalis* W50 or NCTC 11834, compared with the IgG1 control antibody treated cells (fig 5.6 and 5.7 respectively). The anti-alpha-5 antibody resulted in a reduced invasion of *P. gingivalis* NCTC 11834, but in terms of percent invasion this did not reach significance, however, the value was 35% lower than that seen with the IgG control. In contrast, the reduction in invasion seen with strain W50 was less, at only 10%. Similarly, antibody blocking of either CD81 or α5 did not influence the invasion of *P. gingivalis* E8 (Δ*rgpArgpB*) (data not shown).

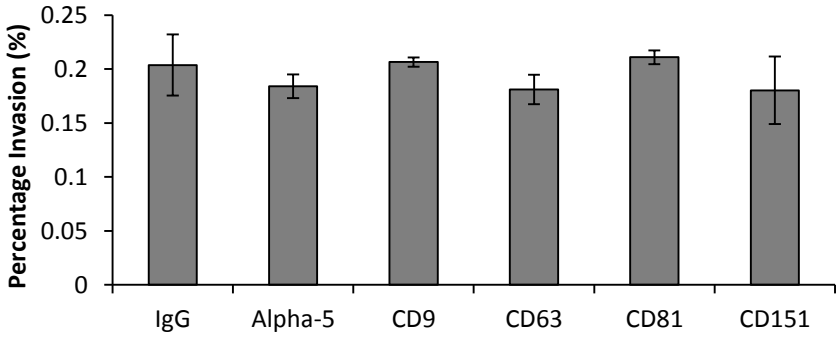


Figure 5.6 Invasion of epithelial cells by *P. gingivalis* W50 in the presence of anti-tetraspanin and α5 antibodies. H357 monolayers were blocked for 30 minutes with anti-α5, -CD9, -CD63, -CD81, and -CD151 antibody or IgG1 isotype control antibody at 37°C/5% CO_2. Invasion of blocked cells by *P. gingivalis* W50 in the presence of protease inhibitor cocktail was performed for 90 minutes, extracellular bacteria were killed with metronidazole and the intracellular bacteria released from the epithelial cells, serially diluted, plated onto blood-FA agar and colonies were counted. Invasion was calculated as the number of intracellular bacteria expressed as a percentage of the original bacterial suspension. Histogram bars indicate mean percentage (±SD) of two independent experiments repeated in triplicate.

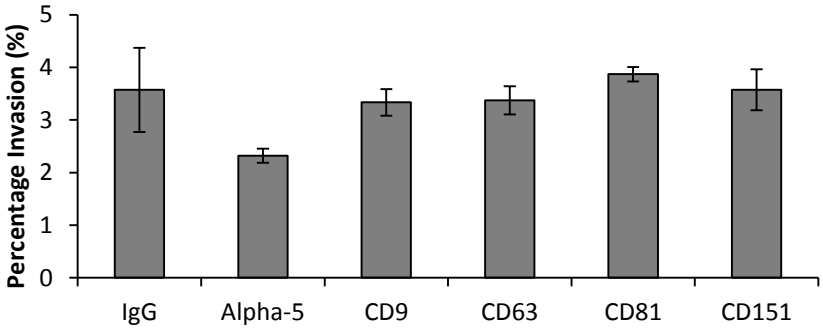


Figure 5.7 Invasion of epithelial cells by *P. gingivalis* NCTC 11834 in the presence of anti-tetraspanin and alpha-5 antibodies. H357 monolayers were blocked for 30 minutes with anti-α5, -CD9, -CD63, -CD81, and -CD151 antibody or IgG1 isotype control antibody at 37°C/5% CO_2. Invasion of blocked cells by *P. gingivalis* W50 in the presence of protease inhibitor cocktail was performed for 90 minutes, extracellular bacteria were killed with metronidazole and the intracellular bacteria released from the epithelial cells, serially diluted, plated onto blood-FA agar and colonies were counted. Invasion was calculated as the number of intracellular bacteria expressed as a percentage of the original bacterial suspension. Histogram bars indicate mean percentage (±SD) of two independent experiments repeated in triplicate.

It has previously been shown that *P. gingivalis* whole cells and purified Lys-gingipain were capable of degrading recombinant CD46 (Mahtout *et al*., 2009). Therefore, in addition to CD81 and α5, it was thought that the degradation of CD46 may influence invasion. However, as with the data reported for CD81 and α5 (fig 5.6), preliminary data suggest that there is also no change in percentage invasion after CD46 antibody blocking, in the presence of protease inhibitors (data not shown).

5.3.3.2 Knockdown of CD81 by transfection with siRNA

The flow cytometry data indicated that detection of CD81 and α5 were diminished in the presence of *P. gingivalis* strain W50. To investigate whether the reduction in the presence of these proteins was important in invasion and to verify the antibody blocking data, H357 cells were transiently transfected with siRNA directed against CD81. Transfection with α5 siRNA was not attempted due to the importance of this molecule in epithelial cell adhesion (Gong *et al*., 1997). As such, reducing the expression of this integrin subunit was deemed impractical in terms of further culture of this cell line and the subsequent antibiotic protection assay, which requires many washing steps. Figure 5.8 shows that expression of CD81 after siRNA transfection was reduced by approximately 60%, as determined by quantitative (q)PCR.

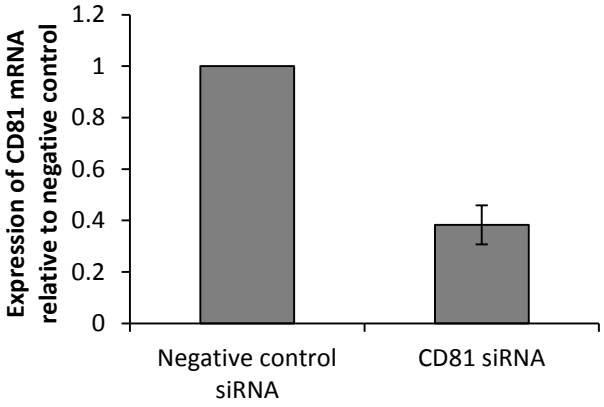


Figure 5.8 Quantitative PCR of CD81 after siRNA transfection. Transient transfection of H357 monolayers was performed as previously described (section 2.22). Epithelial cells were washed, lysed and mRNA extracted. cDNA was synthesised and qPCR was performed for cells transfected with CD81 siRNA or a negative control siRNA (Applied Biosystems). The histogram shows the expression of CD81 mRNA in transfected cells normalised to β2microglobulin and relative to the negative control transfected cells.

In addition to confirming the knockdown of CD81 by qPCR, flow cytometry was performed to assess the cell surface presence of CD81 protein following transfection. Figure 5.9 indicated that 69% of the cell population had CD81 knockdown by approximately 9.39-fold, compared with the siRNA negative control (as shown by the region highlighted as M2 compared to M1). However, this knockdown was not complete and all cells expressed a little CD81, as shown by an incomplete return to the IgG1 negative control median fluorescence value (median fluorescence values of 22.47 (M2) and 2.59 (IgG)).

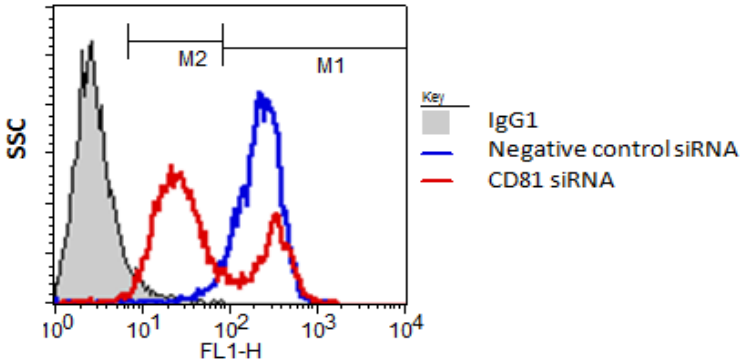


Figure 5.9 CD81 cell surface presence after siRNA transfection assessed by flow cytometry. Transient transfection of H357 monolayers was performed as previously described (section 2.22) using CD81 siRNA or the negative control siRNA. Monolayers were trypsinised and incubated with primary antibody directed against CD81 or an IgG1 isotype control. Alexa Fluor conjugated antibody was incubated with the cells and epithelial cells were analysed for fluorescence using the FACsCalibur flow cytometer. The histogram of fluorescence (FL1-H) against SSC (cell number) is shown. The grey-filled peak represents IgG1-probed H357 transfected with CD81 siRNA, the red and blue pe2ks represent CD81-probed H357 transfected with CD81 siRNA and negative control siRNA respectively. Within the population of cells transfected with CD81 siRNA, M1 indicates a sub-population expressing higher levels of CD81 compared with M2, which shows lower levels of CD81 detection.

H357 epithelial cells, treated with CD81 siRNA to knock down the expression of CD81, were used in an invasion assay with *P. gingivalis* W50. While numerically the difference in percentage invasion failed to reach significance (0.48±0.02% (negative siRNA) and 0.62±0.08% (CD81 siRNA), *P. gingivalis* invasion into the transfected cells was 23% lower than in the control non-transfected cells (5.10).

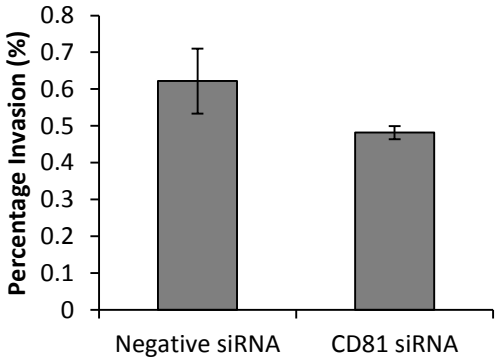


Figure 5.10 Invasion of H357 monolayers by *P. gingivalis* W50 after CD81 siRNA transfection. H357 monolayers were transiently transfected with CD81 siRNA or a negative control scramble siRNA. Invasion of transfected cells with *P. gingivalis* W50 in the presence of protease inhibitor cocktail was performed for 90 minutes, extracellular bacteria were killed with metronidazole and the intracellular bacteria released from the epithelial cells, serially diluted, plated onto blood-FA agar and colonies were counted. Invasion was calculated as the number of intracellular bacteria expressed as a percentage of the original bacterial. Bars indicate mean percentage (±SD) of two independent experiments, performed in triplicate.

5.4 DISCUSSION

All the tetraspanins tested in this study and the α5 integrin subunit, were expressed on the surface of the H357 epithelial cell line as analysed by flow cytometry (fig 5.2 & 5.3). CD9, CD81 and CD82 were the most highly expressed, with CD63 and CD151 showing lower levels. CD63 and CD151 are abundantly expressed intracellularly (Pols and Klumperman, 2009; Penas *et al*., 2000) and, in agreement with the data presented here, Green *et al*. (2011) also showed low epithelial cell surface detection of CD63 and CD151.

In terms of the detectionof tetraspanins, the overnight incubation of H357 monolayers with *P. gingivalis* W50 was shown to only reduce the presence of CD81, whereas the detection of CD9, CD63, CD82 and CD151 remained unchanged after *P. gingivalis* challenge (fig 5.2). The reduction in the detection of CD81 was shown to be gingipain dependent (fig 5.4), where the presence of the lysine-specific gingipain contributed more to its reduction compared with the arginine-specific gingipains (fig 5.4). Extracellularly, CD81 has one lysine and one arginine residue in the EC1 domain and 9 lysine and no arginine residues in the EC2 domain (appendix 6). This may explain the slight increase in reduction in the detection of CD81 protein by flow cytometry in the presence of *P. gingivalis* E8 (Δ*rgpArgpB*) when compared with *P. gingivalis* K1A (Δ*kgp*). However, there was still a reduction in CD81 detection following incubation of H357 with K1A, compared with EK18 (Δ*rgpArgpBkgp*), suggesting that the cleavage of the arginine residue in the EC1 domain decreased affinity of the antibody for this protein contributing to the reduction in protein detection. Antibodies for CD9, CD63 and CD151 were directed against the EC2 domain, however when H357 cells were incubated with *P. gingivalis* W50, there was no reduction in the detection of these proteins even though within the EC2 domain, CD9 possesses 12 lysine and 1 arginine, CD63 possesses 9 lysine and 5 arginine and CD151 possesses 4 lysine and 6 arginine residues. Due to the high number of possible cleavage sites available for gingipains, the lack of reduction in fluorescence after incubation with *P. gingivalis* suggests that folding of the tetraspanins, interaction with other proteins or post-translational modifications may influence access of the gingipains to these tetraspanins. Of interest, CD81 does not possess any potential sites for glycosylation, compared with all the other tetraspanins tested in this study (Levy and Shoham, 2005) and it is possible that glycosylation may protect these tetraspanins from proteolytic modification. This may account for the observed decrease in fluorescence of CD81 alone after incubation of epithelial cells with *P. gingivalis* W50.

Similarly, the presence of *P. gingivalis* W50 was shown to result in a decrease in the cell surface detection of the α5 integrin subunit, in a gingipain-dependent manner (fig 5.5). This receptor in its heterodimer form, α5β1, has been implicated as an important means of *P. gingivalis* entry into epithelial cells (Nakagawa *et al*., 2005, Tsuda *et al*., 2004). However, when

this receptor was blocked by an antibody directed against α5, no significant difference in the invasion of the H357 cell line was observed for strain W50 (fig 5.6). However, preliminary data suggested that blocking the α5 integrin subunit may result in the decreased invasion of H357 by *P. gingivalis* NCTC 11834 (fig 5.7), although this was not significant. This antibody has not been verified as a blocking antibody and as such additional work is required to determine the role of α5 in the invasion of oral epithelial cells by *P. gingivalis*. Differences observed between the two strains of *P. gingivalis* may be a feature of the differences in expression of fimbriae between the two strains. W50 is sparsely fimbriated in comparison with NCTC 11834, suggesting that the mechanisms of invasion may differ between the two strains. For example, data presented here suggest that NCTC 11834 invasion of oral epithelial cells may require α5, and it has been shown previously that there is an association of the major fimbriae FimA with α5β1 that is important for invasion. Therefore, this more fimbriated strain may invade via α5 whilst the sparsely fimbriated strain may invade via a different route, e.g. clathrin-mediated endocytosis or lipid rafts (Tsuda *et al*., 2008, Boisvert and Duncan, 2008).

In terms of the contribution of tetraspanins to bacterial invasion, data in the literature are limited and, to date, no studies have investigated the role of tetraspanins in *P. gingivalis* invasion. It has been reported that there is a requirement for the tetraspanin CD81 in the invasion of HeLa cells by *Listeria monocytogenes in vitro* (Tham *et al*., 2010). However, for the invasion of oral epithelial cells by *P. gingivalis*, the data presented in this chapter suggests that CD81, CD9, CD63 and CD151 are not important, as no significant differences were observed after antibody blocking (fig 5.6) or CD81 silencing (fig 5.10). The data presented for CD81 is in agreement with, but for CD9, CD63 and CD151, is in contrast to Green *et al*. (2011) who observed that the epithelial adhesion of *Neisseria meningitidis* was reduced after treatment of endometrial and pharynx epithelial cells with recombinant EC2 domains and blocking antibodies, respectively, for the tetraspanins CD9, CD63 and CD151, but not for CD81 (Green *et al*., 2011). Similarly, preliminary data indicates that antibody blocking of CD46 does not influence *P. gingivalis* invasion suggesting that the proteolysis of this molecule at the cell surface by *P. gingivalis* gingipains does not affect the cellular internalisation of this bacterium. The limited role of CD46 and the tetraspanins tested here in the invasion of *P. gingivalis* may be due to the total lack of dependency for these molecules in the process of *P. gingivalis* invasion, or may be a result of the recruitment of other tetraspanins or proteins to the cell surface as part of the tetraspanin web, conserving the 'cellular epitope(s)' required for invasion. Indeed, tetraspanins have been shown to accumulate at the cell surface, in association with other tetraspanins and proteins, forming 'adhesion platforms' (Barreiro *et al*., 2008) and it has been proposed that within this 'web' there may be a tetraspanin redundancy where certain tetraspanins may be substituted for one another (Levy and Shoham, 2005; Charrin *et al*., 2009).

There are also experimental factors to take into consideration. Any incomplete protease inhibition of *P. gingivalis* gingipains by protease inhibitors may have lead to cleavage of the blocking antibody, rendering the tetraspanin/integrin 'unblocked' and as such no differences in invasion would be observed. To overcome this possibility, H357 cells were transfected resulting in the transient knock-down in the presence of CD81 at the cell surface. Although there was a small decrease in invasion, this was not significant. However, the knockdown of CD81 was incomplete (fig 5.8). The percentage of cells that showed CD81 knockdown was approximately 70% yet invasion by *P. gingivalis* was unaffected. In order to verify the hypothesis that loss of CD81 prevents/decreases invasion, it would be expected that the transfected cells show a decrease in invasion by up to 70%, assuming no upregulation of additional receptors occurred. The percentage invasion of cells treated with the negative control siRNA was 0.62%, therefore after transfection it would be expected that invasion should be around 70% less, at approximately 0.19%. The experimental data revealed a percentage invasion of 0.48% after transfection with CD81 siRNA, suggesting that the invasion of H357 cells by *P. gingivalis* was marginally but not statistically significantly influenced by CD81. However, because all cells were not completely devoid of CD81, as shown by an incomplete knockdown in fluorescence to the IgG baseline level, it may be that a low level of this tetraspanin is all that is required to facilitate *P. gingivalis* invasion. In addition, the invasion of *P. gingivalis* into epithelial cells is not uniform (section 4.3.4.1) and, although not investigated here, the invasion efficiency may be greater in the non-transfected cells to compensate for the lack of CD81 expression on the rest of the cell population.

In Chapter 4 it was shown that the *P. gingivalis* arginine-specific gingipain knockout mutant, E8, invaded H357 cells at a higher percentage than the wild-type strain (fig 4.6). It was therefore proposed that the degradation of tetraspanin proteins, CD46 or α5 at the cell surface by gingipains may prevent uptake. However, as shown in figures 5.4 and 5.5, the presence of CD81 and α5 was not significantly different after incubation of H357 cells with E8 when compared with the wild-type, suggesting both strains cleave CD81 and α5 to a similar extent, yet the invasion of oral epithelial cells by these two strains was significantly different. These data corroborate the suggestion that CD81 and α5 may not play a role in *P. gingivalis* W50 invasion.

As flow cytometry is a method limited by the binding ability of the chosen antibody to the epitope of interest, the data presented here do not rule out the fact that the extracellular domains of CD9, CD63, CD82 and CD151 may have been affected by *P. gingivalis* gingipains since there are potential sites for cleavage. The antibodies directed against CD9, CD63 and CD151 were specific only for the EC2 domain limiting detection of cleavage to this domain alone. Therefore, there may be a wider role for the tetraspanins in periodontitis, which was not

observed in this study. For example, cleavage of the CD151 tetraspanin within the QRD(194-196) site, by the *P. gingivalis* arginine-specific gingipain, may disrupt binding of the α3 and α6 integrin subunits (Kazarov *et al*., 2002) resulting in altered cell migration, intracellular signalling and a decrease in cellular binding to laminin, suggesting a mechanism of the disruption of epithelial integrity observed *in vitro* (Andrian *et al*., 2004) and *in vivo* (Hernández *et al*., 2011).

Although the results suggest CD81, CD46 and α5 do not influence *P. gingivalis* invasion, the degradation of these proteins may be important in the modulation of other cellular processes that could contribute to the characteristic features of periodontitis. For example, there is a high cellular expression of many tetraspanins, including CD81, on leukocytes, such as B-cells. Paired with CD19, CD81 is important in B-cell differentiation and cell signalling (Bradbury *et al*., 1992; Shoham *et al*., 2003). Possible loss of this tetraspanin may occur in the presence of *P. gingivalis* resulting in an impaired immune response and prolonged bacterial survival. In addition, osteopontin is a ligand for the integrin α4β1 (Bayless *et al*., 1998), which associates with CD81 (Serru *et al*., 1999). Osteopontin is a chemoattractant aiding leukocyte migration (Morimoto *et al*., 2010). Therefore, disruption in the ability of CD81 to associate with other cellular molecules by the action of *P. gingivalis* gingipains may result in the impairment of the immune response promoting immune cell evasion and allowing bacterial survival at sites of infection. Perhaps the most important is the association of CD81 with intracellular signalling pathways, including phosphatidylinositol 4-kinase type II (PI4KII) (Yauch and Hemler, 2000), protein kinase C (PKC) (Zhang *et al*., 2001) and phosphoinositide-3-kinase (PI3K) (Kotha *et al*., 2008). Dysregulation of intracellular signalling events may compromise a wide range of cellular processes including protein secretion (e.g. cytokines, matrix metalloproteases (MMPs), antimicrobial peptides), cell survival, migration, proliferation, differentiation and intracellular trafficking, ultimately contributing to disease. Also, CD81 pairs with claudin-1 (Harris *et al*., 2008), which is a component of tissue tight junctions and disruption in CD81 association may affect epithelial integrity and promote bacterial passage through the oral mucosa. In addition, shedding of membrane-bound CD46 by *P. gingivalis* renders host cells more susceptible to lysis due to impaired inactivation of complement proteins, which may in part, cause the characteristic tissue damage observed in periodontitis. Soluble CD46 has been shown to increase the secretion of CXCL8 from epithelial cells in a dose-dependent manner (Mahtout *et al*., 2009) which, if extrapolated to the *in vivo* situation, may contribute to a prolonged inflammatory response contributing to chronic periodontal inflammation. Furthermore, α5β1 has been shown to play a role in cell survival (Zhang *et al*., 1995), cell adhesion (Gong *et al*., 2007) and intracellular signalling (Howe *et al*., 1998). Therefore, disruption of processes including cell signalling and organisation of tetraspanin-enriched microdomains may occur in the presence of *P. gingivalis*,

suggesting a range of mechanisms that may be explored further to gain more understanding of the role of *P. gingivalis* in the pathogenesis of periodontitis.

The importance of tetraspanins and α5β1 in bacterial invasion, or more specifically in the pathogenesis of disease, remains to be elucidated and further investigations are required. However, as it stands, the current data suggest that there are probably more important receptors/cell surface molecules than tetraspanins involved in the epithelial uptake of *P. gingivalis* and a possible role for α5β1 in strain-specific *P. gingivalis* invasion. The proteolytic activity of gingipains on another, as yet unknown, epithelial surface receptor may influence invasion.

5.4.1 Conclusion

In conclusion, the data presented here indicate differential cleavage of the tetraspanin CD81 and integrin subunit α5 by *P. gingivalis* gingipains. Although no effect of α5 or CD81 blockade or CD81 silencing was observed for *P. gingivalis* invasion of oral epithelial cells, future work to optimise the experimental technique or the utilisation of different techniques such as immunofluorescence may be required. However, the extensive involvement of tetraspanins in the recruitment of important cellular receptors, including CD46 and integrins, into microdomains lends itself to additional future study as to their combined and individual roles in bacterial adhesion/invasion and wider roles in terms of the pathogenesis of disease.

Chapter 6 Cytokine response of oral epithelial monolayer and OMM following *Porphyromonas gingivalis* infection

6.1 INTRODUCTION

Periodontitis is an inflammatory disease in which the presence of subgingival plaque has been implicated as a causative agent (Listgarten, 1988, Socransky *et al*., 1998). The close proximity of dental plaque to gingival tissues exposes the host to a constant challenge, with which the host quickly and initially responds by initiating the release of numerous inflammatory cytokines and chemokines. As such, the levels of serum cytokines, including interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and interleukin 10 (IL-10) have been shown to be significantly increased in patients with periodontitis compared with healthy individuals (Andrukhov *et al*., 2011). In addition, cytokines/chemokines such as interleukin 8 (IL-8/CXCL8), interleukin 6 (IL-6), interleukin 1-beta (IL-1β) and TNF-α have been detected in the gingival crevicular fluid (GCF) of patients exhibiting gingivitis or periodontitis (Teles *et al*., 2010a; Teles *et al*., 2010b) and decreases in such inflammatory markers following treatment such as scaling and root planning have been reported (Thunell *et al*., 2010).

The oral epithelium is the first line of defence against oral pathogens. The expression of pathogen-associated molecular patterns (PAMPs) by bacteria activate pattern recognition receptors (PRRs) on epithelial cell surfaces, initiating signal transduction events, which result in the release of cytokines, contributing to the innate immune response (Stadnyk, 1994). As an oral pathogen, *P. gingivalis* possesses PAMPs which include lipolysaccharide (LPS) (Herath *et al*., 2011), capsular polysaccharide (d'Empaire *et al*., 2006) and fimbriae (Eskan *et al*., 2007, Hajishengallis *et al*., 2004). These differentially activate PRRs including TLR4, TLR2 and RP105 (Kimoto *et al*., 2003, Eskan *et al*., 2008, Eskan *et al*., 2007), modulating the host cytokine response.

There is conflicting data in the literature regarding the cytokine response of oral epithelial cells to live *P. gingivalis*. This may be due to the use of different strains of *P. gingivalis*, differences in epithelial cultures, the time period of incubation or the detection method used to quantify cytokine concentrations. However, there seems to be a consensus, although not thoroughly investigated to date, that *P. gingivalis* induces the transcription of inflammatory cytokines such as CXCL8, IL-6 and TNF-α, whereas, at the protein level, a decrease in detection is common. It is thought that this may be due to degradation by bacterial proteases, more specifically gingipains (Stathopoulou *et al*., 2009; Fletcher *et al*., 1997).

There are few data though on the cytokine responses of 3D culture systems, so this chapter aims to compare these with monolayer cultures, evaluating the effect of *P. gingivalis* invasion on

cytokine release and gene expression. Also, the effect of gingipains on cytokine release will be investigated.

Throughout this chapter the new nomenclature for chemokines (CCL- and CXCL-) will be used (Bacon *et al*., 2002), however for ease of description when dually referring to cytokines or chemokines, the term cytokine will be used interchangeably to refer to chemokines and cytokines alike.

6.1.1 Aims and Objectives

This chapter will examine the secretory cytokine response of monolayer and organotypic cultures of oral epithelial cells in response to *P. gingivalis*, by cytokine array, ELISA and at the transcriptional level by quantitative (q)PCR.

6.2 METHODS

The following methods were used in this chapter:

- Isolation and culture of NOK and human fibroblasts and culture of H357 cell line (sections 2.3 & 2.4)
- *P. gingivalis* culture (section 2.5)
- Culture of air-exposed and submerged collagen OMM (section 2.8)
- Detection of inflammatory cytokines from monolayer and OMM, i.e. antibody array, ELISA, real-time PCR (section 2.18)
- Agarose gel electrophoresis (section 2.19)
- Statistical analysis (section 2.22)

6.3 RESULTS

6.3.1 Detection of multiple cytokine levels from monolayers and OMM in response to invasion by *P. gingivalis*

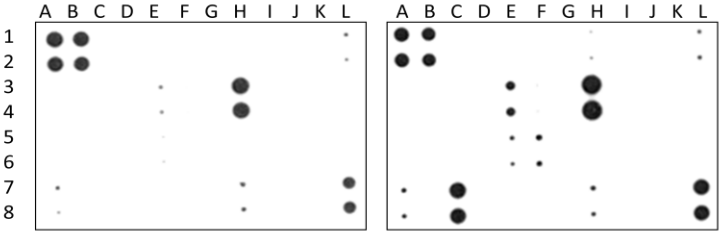
There are few reports in the literature that have investigated the epithelial response to *P. gingivalis* invasion. The majority of these have shown that the invasion of oral epithelial cells by *P. gingivalis* results in a decrease in the cytokine release from epithelial cells (Madianos *et al*., 1997, Huang *et al*., 2001). However, there is conflicting data indicating that there may be an increase in the cytokine response following *P. gingivalis* invasion (Eick *et al*., 2006). Due to the conflicting nature of these data and the limited knowledge within the literature regarding the epithelial response to invasion, this was investigated here. A cytokine array was used (RayBio® Human Inflammation Antibody Array 3), allowing for the simultaneous detection of 40 inflammatory cytokines from the conditioned media of NOK monolayer cultures and NOK-OMM. NOK cultures were used as this cell type secretes a constitutively higher concentration of cytokines than H357 and it was thought that this may aid in the detection of any cytokine modifications in the presence of *P. gingivalis*. The strain of *P. gingivalis* that was used was NCTC 11834 as this strain has been shown to invade epithelial cells at a high level in comparison to other strains of *P. gingivalis* (Chapter 4).

6.3.1.1 Comparison of TNF unstimulated and TNF stimulated monolayer

Due to the previously reported degradation of cytokines by *P. gingivalis* gingipains (Stathopoulou *et al*., 2009), monolayers and OMM cultures were pre-stimulated with TNF-α prior to, and during, incubation with *P. gingivalis*. The concentration of TNF-α used in this study was 25ng ml^{-1}, which is a saturating dose of TNF-α (Turner *et al*., 2010). It was not used to directly replicate the inflammatory response that occurs *in vivo*, rather to stimulate the epithelial cells to achieve their maximal cytokine response and to ensure that there was an adequate concentration of cytokines present in order to better quantify any possible changes in cytokine response.

Initially, conditioned media from TNF-α stimulated NOK monolayers were compared with unstimulated monolayers, to assess the contribution of TNF-α to the detection of multiple cytokines using the cytokine array membranes (fig 6.1A). Densitometry was used to semi-quantify the responses. As expected, in the presence of TNF-α there was an increase in inflammatory cytokines including CXCL8, IL-1α, CCL2, GM-CSF, IL-6, CXCL10, CCL5 and TIMP-2 (fig 6.1). Of these the most increased in detectionfollowing TNF-α stimulation were CCL2 (47.3 fold), IL-6 (8.46 fold) and CXCL10 (4.93 fold), which was not surprising as TNF-α plays an important role in the early inflammatory process (Jackson, 2007; Jönsson *et al*., 2011). No decrease in cytokine response was detected.

A



	A	B	C	D	E	F	G	H	I	J	K	L
1	Pos	Pos	Neg	Neg	Eotaxin	Eotaxin 2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
2	Pos	Pos	Neg	Neg	Eotaxin	Eotaxin 2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
3	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
4	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
5	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
6	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
7	RANTES	TGF-β1	TNF-α	TNF-β	s TNF R1	s TNF RII	PDGF-BB	TIMP-2	Blank	Blank	Neg	Pos
8	RANTES	TGF-β1	TNF-α	TNF-β	s TNF R2	s TNF RII	PDGF-BB	TIMP-2	Blank	Blank	Neg	Pos

B

Cytokine	Also known as:	Fold Change
CXCL8	Interleukin 8 (IL8)	0.35
IL-1α	Interleukin 1α	0.24
GM-CSF	Granulocyte-macrophage colony-stimulating factor	2.95
IL-6	Interleukin 6	8.46
CCL2	Monocyte Chemotactic Protein 1 (MCP-1)	47.30
CXCL10	Interferon-gamma-inducible Protein 10 (IP-10)	4.93
CCL5	Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES)	1.38
TIMP-2	Tissue Inhibitor of MetalloProtease 2	0.08
TNF-α	Tumour Necrosis Factor α	361.54

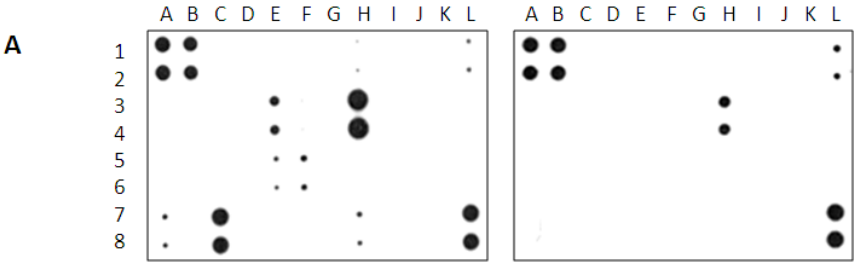
Figure 6.1 Cytokine immunoblot of NOK monolayer cultures stimulated with TNF-α. NOK monolayers were stimulated with 25ng/ml TNF-α in serum free medium (SFM) or non-stimulated (SFM only) for 9.5 hours. Supernatants were incubated with the antibody membrane (A) and the density of each dot was analysed. Using the internal positive control (Pos) of each blot, the relative density was calculated allowing comparisons of fold-changes between blots. The fold changes of important cytokines are shown (B) which compares unstimulated and TNF-α stimulated NOK monolayers. Data are from one representative experiment that was repeated in duplicate.

6.3.1.2 Comparison of un-invaded and *P. gingivalis*-invaded monolayers

The release of multiple cytokines from NOK monolayers (pre-stimulated with TNF-α) was assessed, in the presence of intracellular *P. gingivalis*. NOK monolayers were exposed to TNF-α for 9.5 hours (un-invaded) or pre-stimulated with TNF-α and an antibiotic protection assay performed as follows (invaded).

After stimulating for 4 hours with TNF-α, monolayers were incubated in serum free medium (SFM), with or without *P. gingivalis* NCTC 11834, for 1.5 hours aerobically, during which time the bacteria invaded the oral epithelial cells. In order to only detect the cytokines released into the supernatant in response to intracellular bacteria, metronidazole was added for 4 hours, during which time the extracellular bacteria were killed and the epithelial cells released cytokines into the supernatant. The conditioned medium was collected and incubated with the antibody cytokine array. The resultant arrays are shown in figure 6.2. In the presence of intracellular *P. gingivalis* there was a decrease in the detection of CXCL8, GM-CSF, IL-6, CCL2, CXCL10, CCL5, TIMP-2 and TNF-α (fig 6.2B) with fold-decreases ranging from 0.71 to 0.99. There was only one cytokine that increased in detection following *P. gingivalis* invasion and that was IL-1α (2.33 fold, fig 6.3).

A



	A	B	C	D	E	F	G	H	I	J	K	L
1	Pos	Pos	Neg	Neg	Eotaxin	Eotaxin 2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
2	Pos	Pos	Neg	Neg	Eotaxin	Eotaxin 2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
3	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
4	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
5	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
6	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
7	RANTES	TGF-β1	TNF-α	TNF-β	sTNF R1	sTNF RII	PDGF-BB	TIMP-2	Blank	Blank	Neg	Pos
8	RANTES	TGF-β1	TNF-α	TNF-β	sTNF R2	sTNF RII	PDGF-BB	TIMP-2	Blank	Blank	Neg	Pos

B

Cytokine	Also known as:	Fold Change
CXCL8	Interleukin 8 (IL8)	-0.71
IL-1α	Interleukin 1α	2.33
GM-CSF	Granulocyte-macrophage colony-stimulating factor	-0.80
IL-6	Interleukin 6	-0.99
CCL2	Monocyte Chemotactic Protein 1 (MCP-1)	-0.98
CXCL10	Interferon-gamma-inducible Protein 10 (IP-10)	-0.96
CCL5	Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES)	-0.97
TIMP-2	Tissue Inhibitor of MetalloProtease 2	-0.97
TNF-α	Tumour Necrosis Factor α	-0.99

Figure 6.2 Cytokine immunoblot of NOK monolayer cultures stimulated with TNF-α and *P. gingivalis* NCTC 11834. NOK monolayers were pre-stimulated with 25ng/ml TNF-α in serum free medium (SFM) for 4 hours. After which, cells were exposed to TNF-α with or without NCTC 11834 in SFM, for 1.5 hours. Following washing in PBS, cells were incubated for 4 hours with 200μg/ml metronidazole to kill the external adherent bacteria, during which time cytokines were released into the supernatant. Supernatants were incubated with the antibody membrane and the density of each dot was analysed. Using the internal positive control of each blot (Pos), the relative density was calculated allowing comparisons of fold-changes between blots. The fold changes of important cytokines are shown (B) which compares TNF-α stimulated with TNF-α + NCTC 11834 stimulated NOK monolayers. Negative values represent fold decreases and positive values represent fold increases. Data are from one representative experiment that was repeated in duplicate.

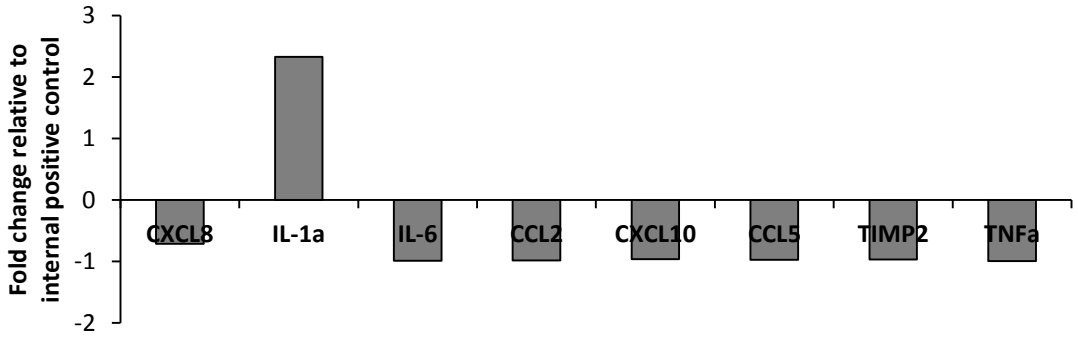


Figure 6.3 Graphical representation of the fold changes in cytokines following the invasion of NOK monolayer by *P. gingivalis* NCTC 11834. NOK monolayers were invaded by *P. gingivalis* NCTC 11834 for 1.5 hours and the external adherent bacteria killed with 200μg/ml metronidazole. Culture supernatants were incubated with the antibody membrane and the density of each dot was analysed. Using the internal positive control of each blot, the relative density was calculated allowing comparisons of fold-changes between infected and non-infected arrays. Only data for positive cytokine spots was used.

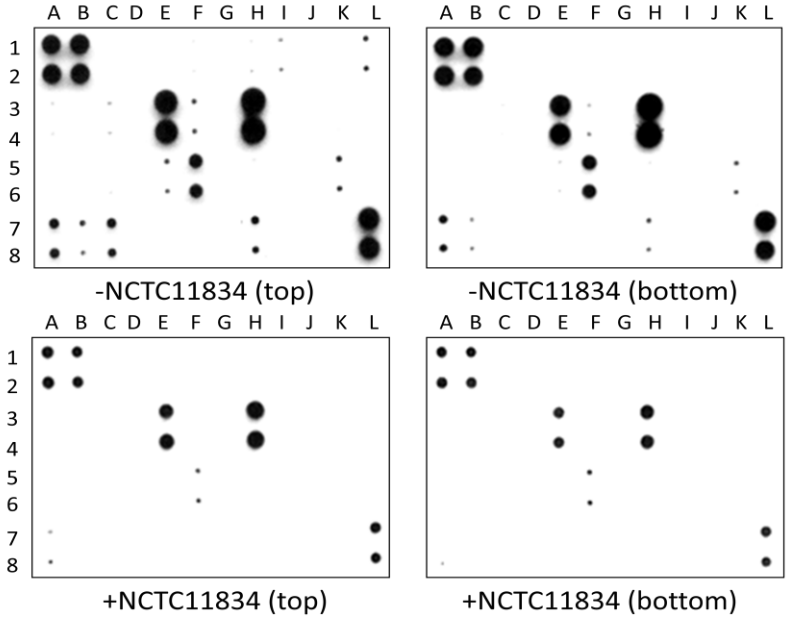
6.3.1.3 Comparison of un-invaded and *P. gingivalis*-invaded OMM

Oral mucosal models (OMM) cultured using normal oral keratinocytes (NOK) were subsequently assessed for the release of inflammatory cytokines into the supernatant following invasion by *P. gingivalis* NCTC 11834, comparing with un-infected NOK-OMM. As previously described, NOK-OMM were pre-stimulated with TNF-α for 4 hours, after which models were incubated in serum free medium with or without *P. gingivalis* NCTC 11834 for 4 hours aerobically. This time period was shown previously to result in the highest percentage invasion as analysed by an antibiotic protection assay (section 3.3.3.1). Metronidazole was added for 4 hours and the medium above and below the culture insert was collected and incubated with the antibody cytokine array.

The culture supernatant analysed directly above OMM following invasion by *P. gingivalis* resulted in a decrease in CCL2, CXCL10, CCL5, TIMP-2 and TNF-α (fig 6.4), showing fold decreases of 0.61, 0.99, 0.61, 0.10 and 0.10 respectively, compared with uninfected NOK-OMM. However, following invasion there was an increase in CXCL8 (2.59 fold) and IL-6 (0.30 fold) (fig 6.5), which differed from the fold decreases of 0.71 fold and 0.99 fold respectively in monolayer cultures (fig 6.3).

The release of cytokines into the bottom of the culture insert was also analysed to see if there was any contribution from fibroblasts that would be missed by only analysing supernatants from above the insert. Figure 6.4 shows similar cytokines in both top and bottom chambers, although a little reduced in the lower chamber, which may be due to the decreased diffusion of cytokines through the collagen layer. In addition, TNF-α was absent from the bottom of the culture insert before infection, compared with supernatant from the top of the insert (fig 6.4), indicating the cytokine applied to the top of the model did not diffuse through the epithelial and connective tissue layers, and as such, indicated that OMM have a good permeability barrier and emphasised the localised effect of cytokines within the oral mucosa. Therefore, in subsequent assays, medium from above the insert only was analysed.

Interestingly, the presence of intracellular *P. gingivalis* in OMM epithelial layers did not result in an increase in IL-1α, which was observed in monolayer (fig 6.3), but a decrease of 0.99 fold was detected (fig 6.5).



	A	B	C	D	E	F	G	H	I	J	K	L
1	Pos	Pos	Neg	Neg	Eotaxin	Eotaxin 2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
2	Pos	Pos	Neg	Neg	Eotaxin	Eotaxin 2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
3	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
4	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70
5	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
6	IL-13	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ
7	RANTES	TGF-β1	TNF-α	TNF-β	sTNF R1	sTNF RII	PDGF-BB	TIMP-2	Blank	Blank	Neg	Pos
8	RANTES	TGF-β1	TNF-α	TNF-β	sTNF R2	sTNF RII	PDGF-BB	TIMP-2	Blank	Blank	Neg	Pos

Figure 6.4 Cytokine immunoblot of NOK-OMM stimulated with TNF-α and *P. gingivalis* NCTC 11834. NOK-OMM were pre-stimulated with 25ng/ml TNF-α in serum free medium (SFM) for 4 hours. After which, cells were exposed to TNF-α with or without NCTC 11834 in SFM, for 4 hours. Following washing in PBS, cells were incubated for 4 hours with 200μg/ml metronidazole to kill the external adherent bacteria, during which time cytokines were released into the supernatant. Supernatants from the top of the insert (top) and from the bottom of the insert (bottom) were incubated with the array membrane and the density of each resultant dot was analysed. Using the internal positive control of each blot (Pos), the relative density was calculated allowing for comparisons of fold-changes between blots.

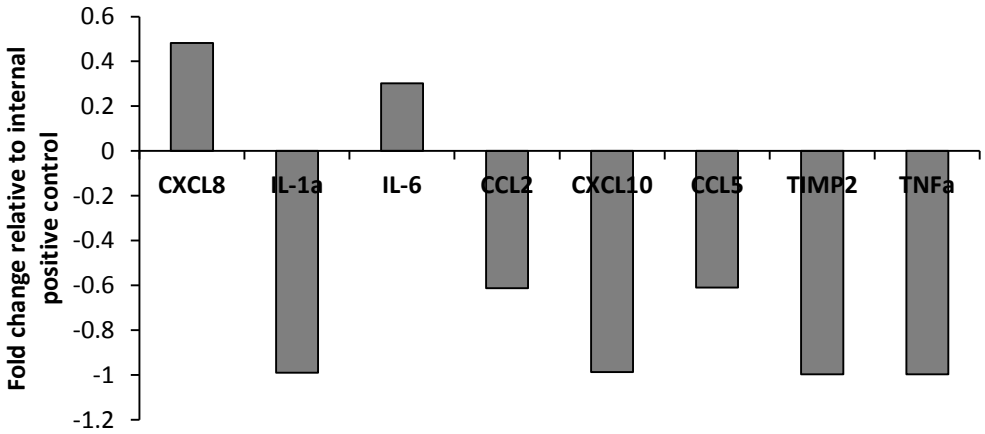


Figure 6.5 Graphical representation of the fold changes in cytokines following the invasion of NOK-OMM by *P. gingivalis* NCTC 11834. NOK-OMM were invaded with or without *P. gingivalis* NCTC 11834 for 4 hours and the external adherent bacteria killed with 200μg/ml metronidazole. Culture supernatants from the top of the insert were incubated with the antibody membrane and the density of each resultant dot was analysed. Using the internal positive control of each blot, the relative density was calculated allowing comparisons of fold-changes between infected and non-infected blots. Only data for positive spots was analysed and presented.

6.3.1.3.1 Comparison of submerged and air-exposed

In an attempt to assess whether the epithelial multi-layer of OMM contributed to the differences observed between monolayer and OMM, NOK-OMM were cultured in the submerged and air-exposed culture conditions (described in section 2.8) and culture supernatant from above the OMM insert was analysed using the cytokine array (fig 6.6). Figure 6.6 shows only a slight difference between air-exposed and submerged models following invasion by *P. gingivalis*. These data suggest that there was an increase in the detection of IL-1α (1.79-fold) and slight increases in the detection of IL-6 (0.11-fold) and CCL2 (0.216-fold) from OMM cultured in the submerged condition compared with OMM cultured at the air-liquid interface. In addition, GM-CSF was prominent in submerged OMM compared with air-exposed OMM. The remaining cytokines were slightly down-regulated in submerged OMM compared with air-exposed OMM, including CCL5, TIMP-2, TNF-α, MIP-1β and TGF-β1 (fig 6.6).

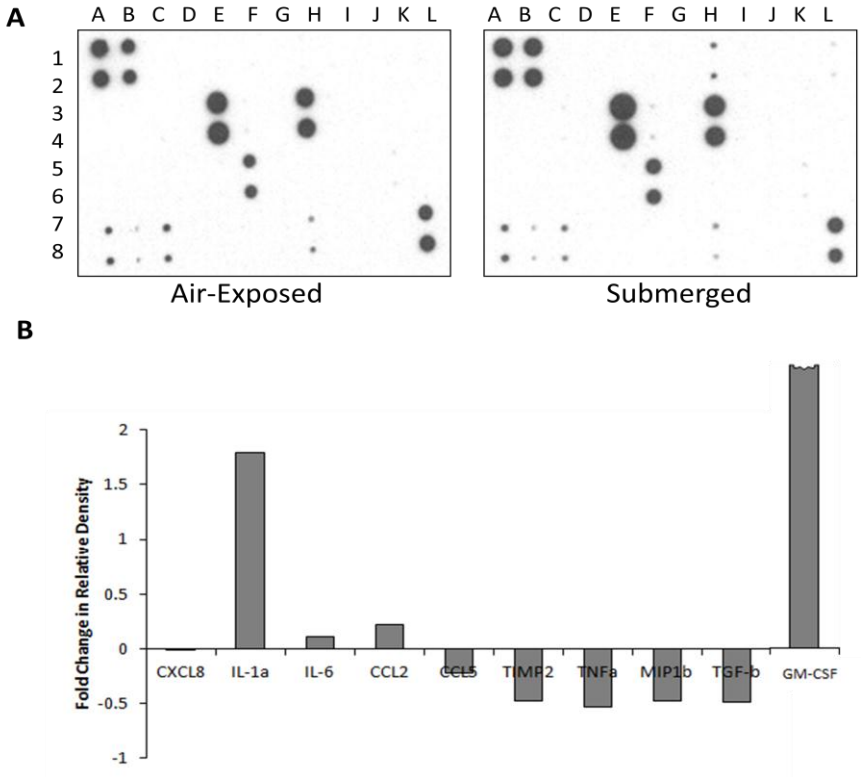


Figure 6.6 Cytokine immunoblot of NOK-OMM cultured at the air-to-liquid interface compared with NOK-OMM cultured completely submerged in culture medium and stimulated with *P. gingivalis* NCTC 11834. NOK-OMM were cultured at the air-to-liquid interface or completely submerged and were pre-stimulated with TNF-α for 4 hours. OMM were then invaded with *P. gingivalis* NCTC 11834 for 4 hours and the external adherent bacteria killed with 200μg/ml metronidazole. Culture supernatants from the top of the insert were incubated with the array membrane (A) and the density of each dot was analysed. Using the internal positive control of each blot, the relative density was calculated allowing comparisons of fold-changes of infected submerged OMM versus air-exposed OMM (B).

Notwithstanding the above relative values of cytokines, it should be mentioned that the sensitivity of each membrane embedded antibody is different. These differences are shown in

appendix 4. Care must be taken when analysing the data and from making direct comparisons of cytokines in relation to the size of their 'dot'. As such the cytokine array was initially used to semi-quantitatively screen a number of pro-inflammatory cytokines in an attempt to determine the cytokines of importance in relation to *P. gingivalis* invasion.

Results from the cytokine array data revealed that CXCL8, IL-6, CCL2 and CCL5 may be important pro-inflammatory cytokines in the oral mucosal response to *P. gingivalis* invasion. This was due to the incomplete reduction in the detection of these cytokines and the differences observed between monolayer and OMM cultures in the presence of intracellular *P. gingivalis*.

6.3.2 Quantitative analysis of CXCL8 release from monolayers and OMM in response to *P. gingivalis*

6.3.2.1 CXCL8 protein release from H357 monolayer following invasion by *P. gingivalis* gingipain mutants

As there was a marked difference between the detection of CXCL8 between monolayer and OMM in the presence of intracellular *P. gingivalis* when analysed by cytokine array (figs 6.3 & 6.5, respectively), the concentration of this cytokine was quantified using an ELISA. In addition, as there is limited data in the literature regarding the contribution from the individual arg- and lys-specific gingipains, on the release of cytokines, gingipain mutants were utilised to investigate this. Due to the lack of gingipain mutants constructed within the NCTC 11834 strain of *P. gingivalis*, the gingipain mutants used were constructed within the less invasive strain, W50, (wild-type). These were strains E8 (Δr*gpArgpB*) and K1A (Δ*kgp*) and a triple gingipain mutant EK18 (Δr*gpArgpB*Δ*kgp*) (table 2.2).

It has previously been shown that there was no difference between the invasion of NOK and H357 monolayer cultures (Chapter 4). Therefore, due to the ease of culture and availability of the H357 cell line, this was used for protein quantification in ELISA studies. Compared with NOK, H357 cells constitutively produce lower concentrations of cytokines, and as ELISA is capable of detecting picogram concentrations of protein, this cell line was deemed suitable to assess the release of CXCL8 from oral epithelial cells.

Initially, to directly compare previous findings from the cytokine array, monolayer cultures of H357 were pre-stimulated with or without TNF-α for 4 hours and exposed to *P. gingivalis* W50, E8 (Δr*gpArgpB*) or K1A (Δ*kgp*) for 1.5 hours. Metronidazole was added for 4 hours, after which the conditioned media were analysed using ELISA for CXCL8.

Following invasion of *P. gingivalis* gingipain mutants there was no significant difference in the cytokine response from that released by un-invaded cells (SFM) (fig 6.7). This was in contrast

to the results reported using the cytokine array, which suggested that following invasion there was a 0.71-fold decrease in CXCL8 release in the presence of *P. gingivalis* NCTC 11834 (fig 6.2). This may be due to the different cell type (H357 compared with NOK) or the use of a different strain of *P. gingivalis* (W50 compared with NCTC 11834).

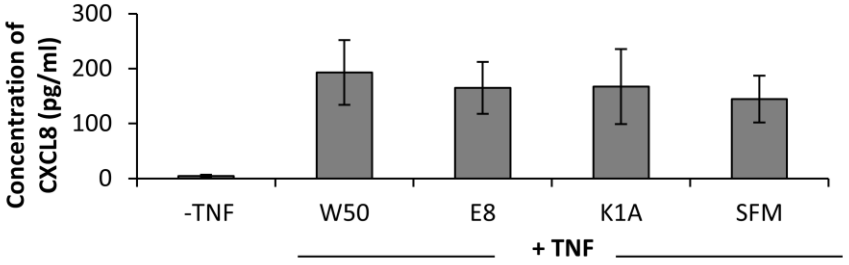


Figure 6.7 The concentration of secreted CXCL8 following invasion of H357 monolayers by *P. gingivalis* strains analysed by ELISA. Confluent monolayer cultures of the H357 cell line were exposed to serum free medium (SFM) +/- TNF-α for 4 hours. *P. gingivalis* wild-type and gingipain mutants (W50, E8 (Δ*rgpArgpB*), K1A (Δ*kgp*)) in the presence of TNF-α, and SFM+/-TNF-α, were then incubated with H357 monolayers for 1.5hours, following which metronidazole (in SFM+/-TNF-α) was added to kill the external adherent bacteria. Subsequently the monolayers were incubated with SFM+/-TNF-α for 4 hours. The conditioned medium following invasion was analysed by ELISA and the concentrations of samples were correlated to a standard curve generated using DeltaSoft Microplate Analysis Software. This histogram shows the mean concentration±SEM of three independent experiments performed in duplicate.

6.3.2.2 CXCL8 protein release from H357 monolayer following overnight incubation with *P. gingivalis* strains

Given the discrepancy between the array data for CXCL8 with NCTC 11834 and the ELISA data for CXCL8 in the presence of strain W50, it seemed possible that this might be due to differences in proteolytic capability and/or the invasiveness of these bacterial strains. Consequently, CXCL8 release was assessed by ELISA after overnight incubation. Again, due to the lack of suitable gingipain-null mutants in the more invasive NCTC 11834 strain, W50 and its gingipain-null mutants were used. H357 monolayers were pre-treated with TNF-α for 4 hours and then infected with *P. gingivalis* wild-type and gingipain mutants. A protease inhibitor cocktail (appendix 5) was used in the presence of *P. gingivalis* wild-type (W50) and medium alone (without *P. gingivalis*), as a control to inhibit any protease activity that may have contributed to a decreased detection of cytokine release. Indeed, figure 6.8 indicates that in the presence of *P. gingivalis* wild-type there was a significant decrease in the detection of CXCL8, compared with culture medium alone (SFM) (8.9±15.5pg/ml and 1169.7±610.9pg/ml respectively, p=0.03). Medium without TNF-α was used as a negative control, which confirmed that H357 constitutively secreted approximately 101.8±44.8pg/ml CXCL8 over the experimental time period. This increased approximately 11.5-fold in the presence of TNF-α (fig 6.8). Incubation of H357 monolayers with *P. gingivalis* gingipain mutants resulted in the

decreased detection of CXCL8, at similar levels, if not slightly higher, than the wild-type, but this was not statistically significant. Results indicated that both the arginine- and lysine-specific gingipains play a role in the reduction of CXCL8 detection. The detection of CXCL8 was slightly lower following stimulation with K1A compared with E8, with 98.2±109.8pg/ml and 132.4±129.2pg/ml CXCL8 respectively, but this was not statistically significant ($p>0.05$).

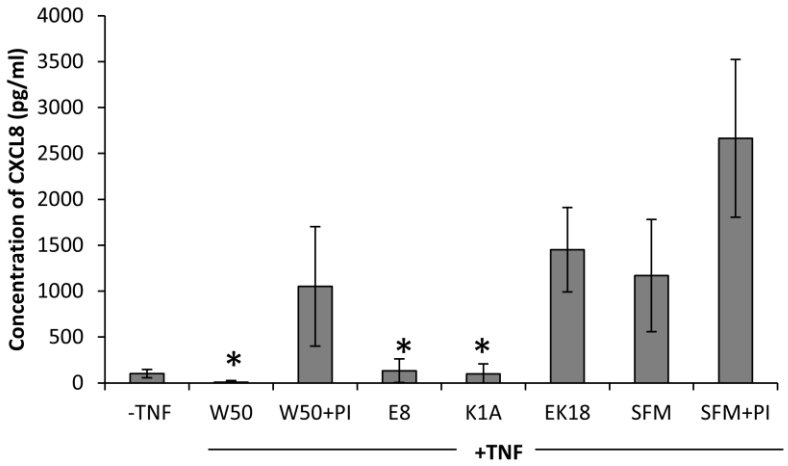


Figure 6.8 The concentration of secreted CXCL8 following overnight incubation of H357 monolayers with *P. gingivalis* strains analysed by ELISA. Confluent monolayer cultures of the H357 cell line were exposed to serum free medium (SFM) +/- TNF-α for 4 hours. *P. gingivalis* wild-type and gingipain mutants (W50, E8 (ΔrgpArgpB), K1A (Δkgp)) in presence of TNF-α, and SFM+/-TNF-α, were then incubated with H357 monolayers overnight. In addition, protease inhibitor (PI) cocktail (Roche) was added to cultures of W50 and SFM in the presence of TNF-α, and also incubated with the H357 cell line overnight at 37°C. The conditioned medium following overnight incubation was analysed by ELISA and the concentrations of samples were correlated to a standard curve generated using DeltaSoft Microplate Analysis Software. This histogram shows the mean concentration±SD of three independent experiments performed in duplicate. * Indicates statistically significant differences from SFM+TNF ($p<0.05$).

Incubation of pre-stimulated H357 monolayers with *P. gingivalis* W50, in the presence of a protease inhibitor cocktail, resulted in the recovery of the detection of CXCL8 up to the level of CXCL8 secretion detected from pre-stimulated culture medium alone (1050.9±650.2pg/ml and 1169.7±610.9 respectively, $p=0.83$) (fig 6.8). Furthermore, the overnight incubation of pre-stimulated H357 monolayers with a *P. gingivalis* triple gingipain mutant (EK18, Δr*gpArgpB*Δ*kgp*) confirmed the importance of gingipains in the modulation and degradation of CXCL8 secreted by oral epithelial cells because there was no change in the concentration of CXCL8 in the conditioned medium following incubation with the triple mutant compared with the control (1450.6±459.2pg/ml, fig 6.8).

6.3.2.3 CXCL8 protein release from H357-OMM following overnight incubation by *P. gingivalis* strains

Assessing the release of CXCL8 from pre-stimulated H357-OMM after incubating overnight with *P. gingivalis* gingipain mutants showed no difference in the detection of CXCL8 in the presence of the parent strain or any of the mutant strains (fig 6.9). In addition, no increase in CXCL8 detection was shown for TNF-α-stimulated positive control over the unstimulated negative control. CXCL8 release from H357-OMM was approximately 100-fold greater than from comparable monolayer cultures, which made assessment of the stimulatory/inhibitory effect of bacteria difficult to determine, even though all samples were diluted extensively to ensure absorbance values fell within the detectable standard curve range of the ELISA. In addition, differences between the invasion of NOK-OMM and H357-OMM cultures were previously shown (section 4.3.1.2), it was deemed that the use of NOK-OMM for determining the CXCL8 release would have been too great due to its higher constitutive release in comparison to H357.

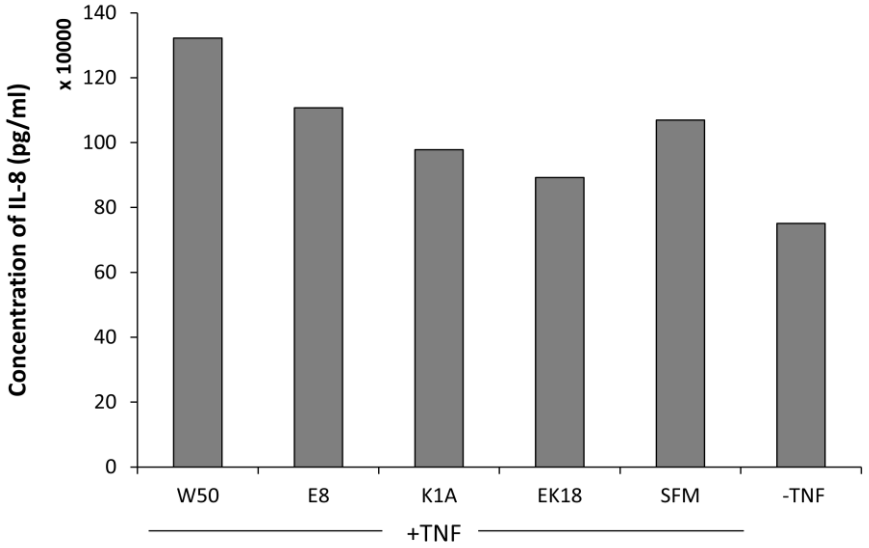


Figure 6.9 The concentration of secreted CXCL8 following overnight incubation of H357-OMM with *P. gingivalis* strains as analysed by ELISA. H357-OMM were exposed to serum free medium (SFM) +/- TNF-α for 4 hours. *P. gingivalis* wild-type and gingipain mutants (W50 (parent), E8 (Δ*rgpArgpB*), K1A (Δ*kgp*) and EK18 (Δ*rgpArgpB*Δ*kgp*)) in the presence of TNF-α, and SFM+/-TNF-α, were then incubated with H357-OMM overnight at 37°C. The conditioned medium following overnight incubation was analysed by ELISA and the concentrations of CXCL8 were correlated to a standard curve generated using DeltaSoft Microplate Analysis Software. This histogram shows the mean concentration of two independent experiments performed in duplicate.

6.3.3 Expression of mRNA following incubation of monolayer and OMM overnight with *P. gingivalis* strains

6.3.3.1 Interleukin 8 (CXCL8)

6.3.3.1.1 Monolayer

As it has been shown that overnight incubation of H357 monolayer cultures with *P. gingivalis* resulted in a reduction in the detection of CXCL8 protein, it is possible that this was due to the destructive effects of gingipains. Therefore, H357 monolayer cultures were pre-stimulated with TNF-α and assessed for the expression of CXCL8 mRNA following overnight incubation with *P. gingivalis* to determine at which point the gingipains exerted their effects. Figure 6.10 indicates that in the presence of *P. gingivalis* wild-type and the two gingipain-null mutants (E8 and K1A), there was a significant increase in the gene transcription of CXCL8 compared with the medium-only control (SFM) ($p<0.05$ in all cases). This suggests a role for the gingipains in the post-translational modification/destruction of CXCL8.

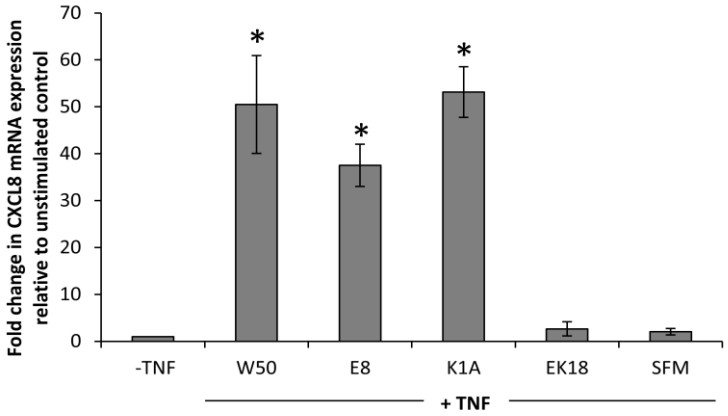


Figure 6.10 The mRNA expression of CXCL8 following overnight incubation of H357 monolayers with *P. gingivalis* gingipain mutants as analysed by qPCR. Confluent monolayer cultures of the H357 cell line were exposed to serum free medium (SFM) +/- TNF-α for 4 hours. *P. gingivalis* wild-type and gingipain mutants (W50, E8 (Δ*rgpArgpB*), K1A (Δ*kgp*)) in the presence of TNF-α, and SFM+/-TNF-α, were then incubated with H357 monolayers overnight at 37°C. The cells were lysed and RNA extracted using a RNeasy Mini Kit (Qiagen). Complementary DNA was synthesised using the high capacity RNA-to-cDNA Kit (Applied Biosystems) and qPCR was performed using TaqMan primers and probes (Applied Biosystems). This histogram shows the mean fold change in CXCL8 expression relative to the unstimulated control (-TNF)±SEM of at least three independent experiments performed in duplicate. * Indicates statistically significant differences from SFM ($p<0.05$).

Following overnight incubation with the *P. gingivalis* triple gingipain knockout mutant (EK18) there was no significant increase in CXCL8 expression compared with the medium-only control (SFM) (2.65±1.6 fold, $p>0.05$) (fig 6.10). This suggests that the presence of gingipains, in combination or separately, (as with the wild-type and both E8 and K1A single and double gingipain knockout mutants), may play a role in the stimulation of CXCL8 mRNA transcription. Furthermore, invasion may also stimulate transcription because the gingipain-null mutant

showed extremely low percentage invasion in comparison to the other strains of *P. gingivalis* tested (fig 4.8).

To ensure the increase in CXCL8 mRNA following incubation of H357 monolayers with W50, E8 and K1A was not due to a synergistic response in combination with the presence of TNF-α, the experiment was repeated in the absence of TNF-α (fig 6.11). As seen previously, there was significant up-regulation of CXCL8 mRNA expression in the presence of W50 (42.2±3.96 fold), E8 (26.03±6.00 fold) and K1A (33.53±8.79 fold), indicating that there was no synergistic response.

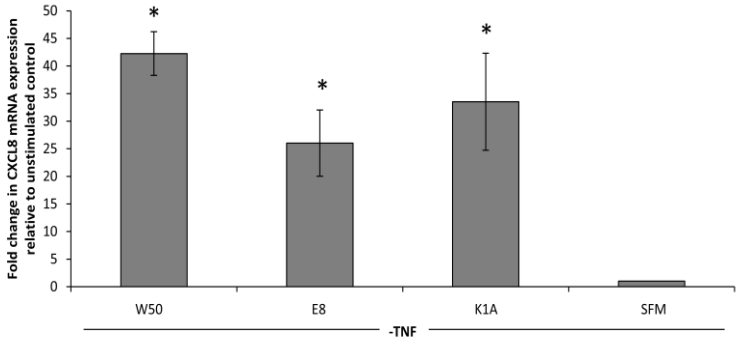


Figure 6.11 The relative levels of CXCL8 mRNA following overnight incubation of unstimulated H357 monolayers with *P. gingivalis* strains as analysed by real-time PCR. Confluent monolayer cultures of the H357 cell line were exposed to serum free medium (SFM) without TNF-α for 4 hours. *P. gingivalis* wild-type and gingipain mutants (W50, E8 (Δ*rgpArgpB*), K1A (Δ*kgp*)) in the absence of TNF-α, and SFM-TNF-α, were then incubated with H357 monolayers overnight at 37°C. The cells were lysed and RNA extracted using a RNeasy Mini Kit (Qiagen). Complementary DNA was synthesised using the high capacity RNA-to-cDNA Kit (Applied Biosystems) and qPCR was performed using TaqMan primers and probes (Applied Biosystems). This histogram shows the mean fold change in CXCL8 expression relative to the unstimulated control (-TNF)±SEM of three independent experiments performed in duplicate. *$p<0.05$ relative to the unstimulated control.

6.3.3.1.2 OMM

The expression of CXCL8 mRNA by H357-OMM following overnight incubation with *P. gingivalis* wild-type (W50) and gingipain-null mutants was assessed (fig 6.12). There was no difference between the mRNA expression of CXCL8 in the presence of *P. gingivalis* and that produced by the TNF-α-stimulated control. Similarly there was no change in the concentration of secreted CXCL8 protein detected (fig 6.10), suggesting a limited response to *P. gingivalis*.

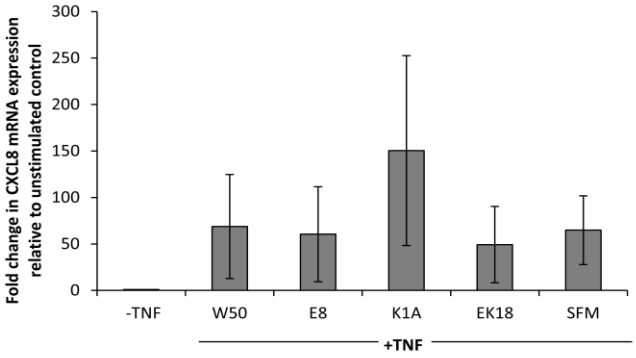


Figure 6.12 The relative levels of CXCL8 mRNA following overnight incubation of H357-OMM with *P. gingivalis* strains as analysed by real-time PCR. H357-OMM were exposed to serum free medium (SFM) +/- TNF-α for 4 hours. *P. gingivalis* wild-type and gingipain mutants (W50, E8 (Δ*rgpArgpB*), K1A (Δ*kgp*), EK18 (Δ*rgpArgpB*Δ*kgp*) in presence of TNF-α, and SFM+/-TNF-α, were then incubated with H357-OMM overnight at 37°C. The cells were lysed and RNA extracted using a Rneasy Mini Kit (Qiagen). Complementary DNA was synthesised using the high capacity RNA-to-cDNA Kit (Applied Biosystems) and qPCR was performed using TaqMan primers and probes (Applied Biosystems). This histogram shows the mean fold change in CXCL8 expression relative to the unstimulated control (-TNF), data shown is representative of three independent experiments performed in duplicate.

The mRNA of IL-6, CCL2 and CCL5 was then analysed following overnight incubation of H357 monolayers and H357-OMM with *P. gingivalis* W50, E8, K1A and EK18 to further elucidate the transcriptional response to *P. gingivalis*. In all circumstances, monolayers or OMM were pre-stimulated with TNF-α for 4 hours and incubated overnight in the presence or absence of TNF-α with or without *P. gingivalis* W50, E8, K1A or EK18. Cells were trypsinised, RNA isolated, cDNA synthesised and the expression of mRNA analysed using SYBR Green labelled primers to IL-6, CCL2 or CCL5 by qPCR. The specificity of the primers was assessed by running amplified PCR products after the qPCR on an agarose gel. The primers amplified a single portion of cDNA, specific for the gene of interest, as shown in figure 6.13. The product lengths of IL-6, CCL2 and CCL5 are shown as 97, 101 and 112 base pairs respectively.

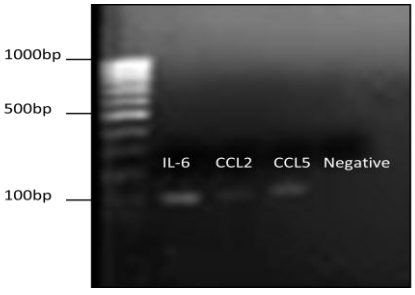


Figure 6.13 The specificity of IL-6, CCL2 and CCL5 primers assessed by gel electrophoresis. Amplified PCR products of qPCR reactions using the primers for IL-6, CCL2 and CCL5 described in appendix 3 were loaded onto a 1% agarose gel and electrophoresis was performed. The single amplified product indicated the specificity of each primer pair to amplify the gene of interest. The negative control lane was the PCR product without cDNA.

6.3.3.2 Interleukin 6 (IL-6)

6.3.3.2.1 Monolayer

The expression of IL-6 following pre-stimulation with TNF-α and overnight incubation of H357 monolayers with *P. gingivalis* W50, E8 and K1A is shown in figure 6.14. Stimulation with TNF-α did not induce a significant increase in transcription of IL-6 (p=0.16). In the presence of *P. gingivalis* wild-type (W50) and the gingipain knockout mutants (E8, K1A and EK18) there was up-regulation of IL-6 mRNA expression with fold increases of 5.04±0.83, 5.43±1.20, 7.23±3.01 and 4.84±1.66 respectively, relative to the unstimulated control. However, the up-regulation of IL-6 expression compared with the TNF-α stimulated control was not significant (p>0.05 for all strains).

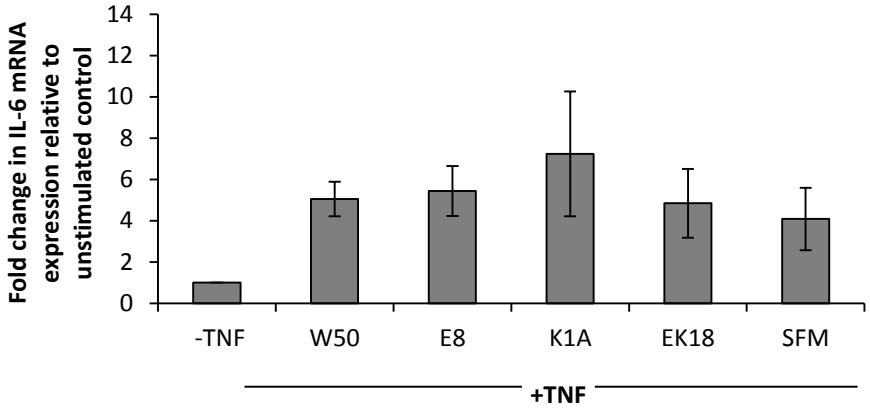


Figure 6.14 The relative levels of IL-6 mRNA following overnight incubation of H357 monolayers with *P. gingivalis* strains. Confluent monolayer cultures of the H357 cell line were exposed to serum free medium (SFM) +/- TNF-α for 4 hours. *P. gingivalis* wild-type and gingipain mutants (W50, E8 (Δ*rgpArgpB*), K1A (Δ*kgp*) and EK18 (Δ*rgpArgpBkgp*) in the presence of TNF-α, and SFM+/-TNF-α, were then incubated with H357 monolayers overnight at 37°C. The cells were lysed and RNA extracted using a Rneasy Mini Kit (Qiagen). Complementary DNA was synthesised using the high capacity RNA-to-cDNA Kit (Applied Biosystems) and qPCR was performed using SYBR Green primers (Applied Biosystems). This histogram shows the mean fold change in IL-6 expression relative to the unstimulated control (-TNF)±SEM of three independent experiments performed in duplicate.

6.3.3.2.2 OMM

For H357-OMM, no fold increase/decrease in IL-6 mRNA expression was detected either after stimulation with TNF-α or *P. gingivalis* (fig 6.15).

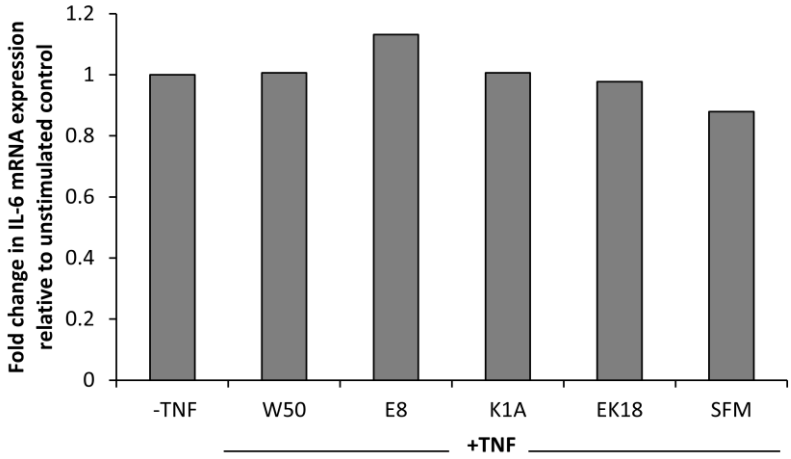


Figure 6.15 The relative levels of IL-6 mRNA following overnight incubation of H357-OMM with *P. gingivalis* strains. H357-OMM were exposed to serum-free medium (SFM) +/- TNF-α for 4 hours. *P. gingivalis* wild-type (W50) and the gingipain knock-out mutants (E8 (Δ*rgpArgpB*), K1A (Δ*kgp*) and EK18 (Δ*rgpArgpBkgp*)) in presence of TNF-α, and SFM+/-TNF-α, were then incubated with H357-OMM overnight at 37°C. The cells were lysed and RNA extracted using a Rneasy Mini Kit (Qiagen). Complementary DNA was synthesised using the high capacity RNA-to-cDNA Kit (Applied Biosystems) and qPCR was performed using SYBR Green primers (Applied Biosystems). This histogram shows the mean fold change in IL-6 expression relative to the unstimulated control (-TNF) of two independent experiments performed in duplicate.

6.3.3.3 CCL2/Monocyte Chemotactic Protein 1

6.3.3.3.1 Monolayer

The expression profile of CCL2 following incubation of H357 monolayers with *P. gingivalis* W50, E8 and K1A is shown in figure 6.16 and partly mirrored the mRNA expression of CXCL8 (shown in fig 6.11). There was an increased expression after incubation with W50, E8 and K1A compared with the unstimulated and TNF-α stimulated control, whereas following incubation with the triple mutant there was no significant fold change in CCL2 mRNA expression compared with the TNF-α stimulated control (2.16 and 2.49 respectively) (fig 6.16). However, although the experimental trends were identical, large differences in expression between experiments indicated no significant differences for any *P. gingivalis* strains, when compared with the unstimulated control.

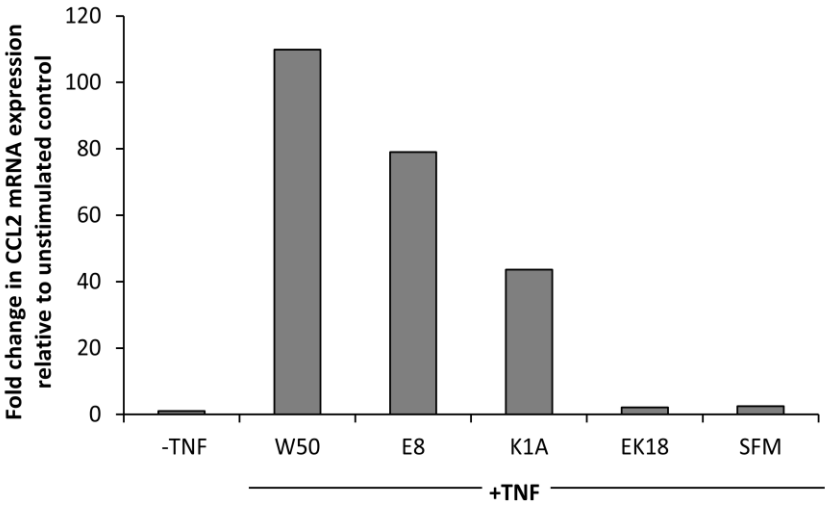


Figure 6.16 The mRNA expression of CCL2 following overnight incubation of H357 monolayers with *P. gingivalis* strains. Confluent monolayer cultures of the H357 cell line were exposed to serum free medium (SFM) +/- TNF-α for 4 hours. *P. gingivalis* wild-type and gingipain mutants (W50, E8 (Δ*rgpArgpB*), K1A (Δ*kgp*)) in presence of TNF-α, and SFM+/-TNF-α, were then incubated with H357 monolayers overnight at 37°C. The cells were lysed and RNA extracted using a Rneasy Mini Kit (Qiagen). Complementary DNA was synthesised using the high capacity RNA-to-cDNA Kit (Applied Biosystems) and qPCR was performed using SYBR Green primers (Applied Biosystems). This histogram shows the mean fold change in CCL2 expression relative to the unstimulated control (-TNF), representative data from two independent experiments performed in duplicate.

6.3.3.3.2 OMM

For H357-OMM, no fold increase/decrease in CCL2 mRNA expression was detected either after stimulation with TNF-α or *P. gingivalis* (fig 6.17).

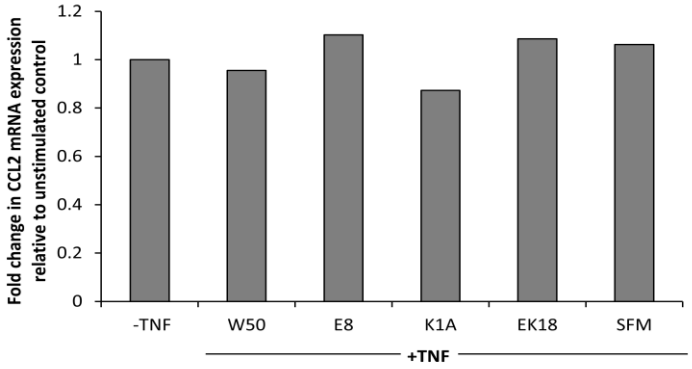


Figure 6.17 The mRNA expression of CCL2 following overnight incubation of H357-OMM with *P. gingivalis* strains. H357-OMM were exposed to serum free medium (SFM) +/- TNF-α for 4 hours. *P. gingivalis* wild-type (W50) and the gingipain knock-out mutants (E8 (Δ*rgpArgpB*), K1A (Δ*kgp*) and EK18 (Δ*rgpArgpBkgp*)) in presence of TNF-α, and SFM+/-TNF-α, were then incubated with H357-OMM overnight at 37°C. The cells were lysed and RNA extracted using a Rneasy Mini Kit (Qiagen). Complementary DNA was synthesised using the high capacity RNA-to-cDNA Kit (Applied Biosystems) and qPCR was performed using SYBR Green primers (Applied Biosystems). This histogram shows the mean fold change in CCL2 expression relative to the unstimulated control (-TNF)±SEM of two independent experiments performed in duplicate.

6.3.3.4 CCL5/Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES)

6.3.3.4.1 Monolayer

There was no significant change in the mRNA expression of CCL5, relative to the unstimulated control, from H357 monolayer cultures after TNF-α stimulation (1.75±0.22 fold), W50 (1.57±0.43 fold), E8 (1.08±0.26 fold), K1A (1.79±0.89 fold) and EK18 (2.05±0.90 fold) (fig 6.18). No fold increases were statistically significant when compared with the TNF-α stimulated control ($p>0.05$).

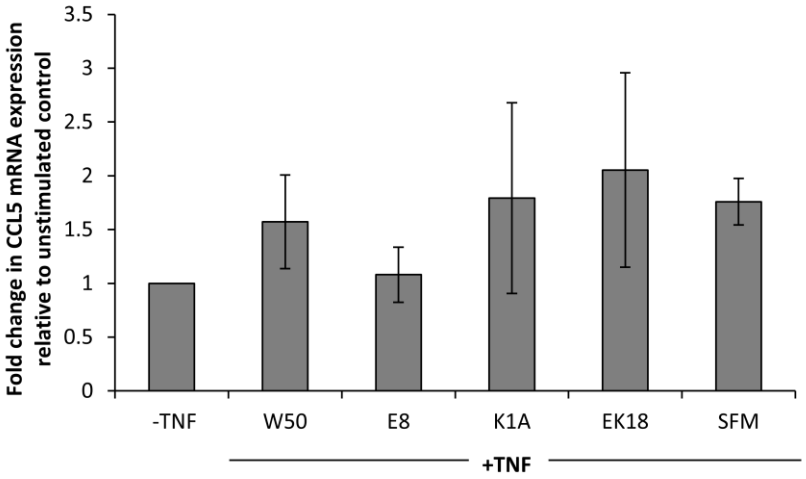


Figure 6.18 The mRNA expression of CCL5 following overnight incubation of H357 monolayers with *P. gingivalis* strains. Confluent monolayer cultures of the H357 cell line were exposed to serum free medium (SFM) +/- TNF-α for 4 hours. *P. gingivalis* wild-type and gingipain mutants (W50, E8 (Δ*rgpArgpB*), K1A (Δ*kgp*)) in presence of TNF-α, and SFM+/-TNF-α, were then incubated with H357 monolayers overnight at 37°C. The cells were lysed and RNA extracted using a Rneasy Mini Kit (Qiagen). Complementary DNA was synthesised using the high capacity RNA-to-cDNA Kit (Applied Biosystems) and qPCR was performed using SYBR Green primers (Applied Biosystems). This histogram shows the mean fold change in CCL5 expression relative to the unstimulated control (-TNF)±SEM of three independent experiments performed in duplicate.

6.3.3.4.2 OMM

For H357-OMM, no change in CCL5 mRNA expression was detected either after stimulation with TNF-α or *P. gingivalis* (fig 6.19).

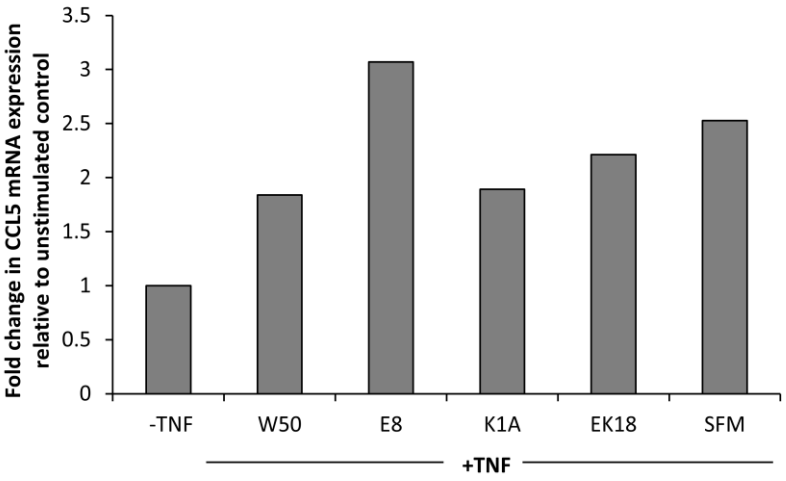


Figure 6.19 The mRNA expression of CCL5 following overnight incubation of H357-OMM with *P. gingivalis* strains. H357-OMM were exposed to serum free medium (SFM) +/- TNF-α for 4 hours. *P. gingivalis* wild-type (W50) and the gingipain knock-out mutants (E8 (Δ*rgpArgpB*), K1A (Δ*kgp*) and EK18 (Δ*rgpArgpBkgp*)) in presence of TNF-α, and SFM+/-TNF-α, were then incubated with H357-OMM overnight at 37°C. The cells were lysed and RNA extracted using a RNeasy Mini Kit (Qiagen). Complementary DNA was synthesised using the high capacity RNA-to-cDNA Kit (Applied Biosystems) and qPCR was performed using SYBR Green primers (Applied Biosystems). This histogram shows the mean fold change in CCL5 expression relative to the unstimulated control (-TNF) of two independent experiments performed in duplicate.

6.4 DISCUSSION

6.4.1 Evaluation of cytokine protein release to *P. gingivalis*

The release of cytokines and chemokines by oral epithelial cells forms part of the first line of defence in response to bacterial challenge (Stadnyk *et al*., 1994). The literature regarding the epithelial cytokine responses to intracellular *P. gingivalis* alone (i.e. the application of an antibiotic to kill the external bacteria) or both internalised and extracellular bacteria (i.e. no addition of antibiotic) is extremely varied (table 1.4). The cytokine response of epithelial cells to either internal or internal and external *P. gingivalis* was investigated in this chapter.

In summary, it was found that the intracellular localisation of a highly invasive strain of *P. gingivalis* NCTC 11834, within monolayer cultures of primary oral epithelial cells, attenuated the epithelial cytokine response when analysed semi-quantitatively using a cytokine array. Due to the lack of gingipain-null mutants in this strain, subsequent quantitative analysis of the selected cytokine, CXCL8, was performed using the strain W50 (wild-type) and its gingipain mutants. However, this strain invades epithelial cells at approximately 20-fold less than strain NCTC 11834. Therefore, it was thought that to achieve the maximal epithelial CXCL8 response, *P. gingivalis* must be incubated with epithelial cells for a longer time period. As there remains inconsistent data within the literature regarding the epithelial cytokine response to *P. gingivalis* (internalised and external), it was deemed that an overnight incubation of epithelial cells with *P. gingivalis* would be sufficient for the internalisation of this strain as well as allowing enough time for the epithelial cells to respond. When experiments were performed overnight, the presence of *P. gingivalis* (intra- and extracellular) also resulted in a decrease in CXCL8 release from monolayer cultures. Further experiments indicated that *P. gingivalis* gingipains play a role in the degradation of CXCL8. Subsequent mRNA expression data revealed CXCL8 was degraded at the protein level and not inhibited at the transcriptional level. Data reported for OMM were less conclusive, suggesting the presence of *P. gingivalis*, either internalised or both internalised and extracellular, may not induce this CXCL8 degradation. This is consistent with a recent study by Hajishengallis *et al*. (2011), in which CXCL8 was demonstrated in junctional epithelium in germ-free animals, suggesting a constitutive release of CXCL8 *in vivo*. However, the methodology employed here requires additional modifications, such as optimisation of the ELISA method to accurately assess small changes in the CXCL8 response of OMM in the presence of *P. gingivalis*, to elucidate the role of OMM in determining the host cytokine response to *P. gingivalis*.

Following TNF-α stimulation, there was secretion of numerous inflammatory cytokines from NOK monolayers into the surrounding medium. Cytokines such as CCL2, IL-6, CXCL10 and CCL5 were detected (fig 6.1). It is well known that TNF-α is an important pro-inflammatory cytokine *in vivo* and *in vitro*, inducing the release of other inflammatory cytokines (Wong *et al*.,

2007a), therefore, experimentally this release was expected. The reason TNF-α stimulation was used as the 'background' against which to judge the effect of *P. gingivalis* stimulation was because it has been shown previously that live *P. gingivalis* results in the inhibition of cytokine accumulation (Stathopolou *et al*., 2009), even so much as to be coined the term 'local chemokine paralysis' (Darveau *et al*., 1998).

In this study, live intracellular *P. gingivalis* were shown to attenuate the cytokine response of NOK monolayer cultures, as well as NOK-OMM cultures (for the majority of cytokines tested), as indicated by cytokine array (figs 6.2 & 6.4 respectively). Even in the presence of a stimulatory concentration of TNF-α, intracellular *P. gingivalis* caused a reduction in the detection of many cytokines, including CCL2, CCL5, CXCL10 and GM-CSF from both monolayer and OMM cultures. Attenuation of cytokine release in response to *P. gingivalis* (internalised or internal+external), has been well described in the literature, with no change or decreases in the concentrations of CXCL8, IL6 and TNF-α following stimulation with live, whole cells of *P. gingivalis*, for both monolayer (Stathopoulou *et al*., 2009, Huang *et al*., 2001) and multi-layered cultures of gingival epithelial cells (Dickinson *et al*., 2011).This attenuation of the host cytokine profile, in response to *P. gingivalis*, has been suggested to be important in the survival of this organism at the site of infection by depressing the host immune response (Darveau *et al*., 1998; Curtis *et al*., 2001), enabling bacterial replication and cell to cell spreading (Tribble and Lamont, 2010). The attenuation of chemokines such as CXCL8 and CCL2 may reduce the chemotactic gradient which is essential for the recruitment of neutrophils and monocytes to the infection site. Indeed, an *in vitro* study reported the reduced migration of neutrophils through a Transwell system in the presence of *P. gingivalis*, which was due to the abrogation of a CXCL8 chemotactic gradient (Madianos *et al*., 1997).

In contrast, some reports in the literature suggest an induction of the pro-inflammatory cytokine response following intracellular or internal+external *P. gingivalis* challenge (Eick *et al*. 2006 and Eskan *et al*. 2007). In addition, Andrian *et al*. (2004) reported the increased detection of CXCL8, IL-6, TNF-α and IL-1β, when analysed by ELISA, following incubation of organotypic oral mucosal models with *P. gingivalis* ATCC33277 for 24 hours. Similarly, Sandros *et al*. (2000) reported the increased detection, by immunohistochemistry, of CXCL8, IL-6 and TNF-α after overnight stimulation of monolayer cultures of KB and pocket/junctional epithelial cells by *P. gingivalis* 381. Differences within the literature may be a result of i) differences between strains of *P. gingivalis* (expressing different PAMPs), ii) the MOI used, iii) the viability of *P. gingivalis* (live *P. gingivalis* have been shown to attenuate the cytokine response, whereas heat-killed *P. gingivalis* have not (Stathopolou *et al*., 2009) and invasive strains (*P. gingivalis* 381) were shown to induce CCL2, whereas less invasive strains (W50) did not (Kang *et al*., 2002), iv) the length of incubation (the longer the time the decreased detection of cytokines

(Stathopolou *et al*., 2009)) v) the type and origin of the oral epithelial cell (e.g. immortalised or primary) vi) the culture method (e.g. monolayer or multi-layer) and, vii) the detection method employed (e.g. immunohistochemistry is not quantitative whereas ELISA is). It is for these reasons that comparison of the literature is difficult. Overall, in terms of the data presented here, there seemed to be an overlap between the induction and attenuation of the cytokine response between the two model systems used. For example, *P. gingivalis* incubation with NOK and H357 monolayer cultures induced a reduction in the cytokine profile (figs 6.3 & 6.7 respectively), supporting the findings of Darveau *et al*. (1998), whereas following invasion of H357- and NOK- OMM (figs 6.5 & 6.9 respectively), this reduction was not observed to the same extent, with levels of IL-6 and CXCL8 remaining unchanged, if not increased, compared with unstimulated control OMM cultures, which would be in keeping with the findings of Andrian *et al*. (2004).

IL-6 and CXCL8 protein showed no change or a slight increase following invasion of H357- and NOK-OMM respectively, by *P. gingivalis* compared with OMM not exposed to *P. gingivalis* (figs 6.5 & 6.9 respectively), when analysed by ELISA and antibody array respectively. This lack of attenuation in the detection of these ligands may be due to the contribution of the additional layers of epithelial cells in the OMM and resulting in the increased autocrine response to pro-inflammatory cytokines and so the accumulation of these secondary cytokines. The contribution of cytokine release from fibroblasts (Ara *et al*., 2009) or the longer incubation time of OMM with *P. gingivalis* may also be factors. Invasion was performed for 1.5 hours in monolayer cultures and for 4 hours in OMM, as these time periods gave approximately similar percentage invasions (Chapter 4). However, it would be expected that the longer *P. gingivalis* was present extracellularly, the longer the opportunity for cytokine degradation to occur. However, this was not the observation made, suggesting intrinsic differences in overall cytokine responses between monolayer and OMM.

Although the differences in parameters make individual comparisons of OMM and monolayer difficult, there was interestingly an up-regulation of IL-1α protein detected from monolayer cultures but decreased in OMM (figs 6.3 & 6.5 respectively). IL-1α is a pro-inflammatory cytokine that stimulates the activation of other important inflammatory cytokines (Okada and Murakami, 1998). This cytokine is initially synthesised as a pre-protein until it is cleaved by calpain intracellularly, resulting in the mature form of IL-1α protein prior to secretion (Kobayashi *et al*., 1990). Calpain is activated by the presence of calcium (Kobayashi *et al*., 1990) and *P. gingivalis* has been reported to activate the influx of, or the release of intracellular stores of calcium (Izutsu *et al*., 1996, Belton *et al*., 2004), suggesting a possible mechanism for the induction of IL-1α secretion observed. The differences in IL-1α secretion between OMM and monolayers may be due to the decreased diffusion of IL-1α across the multi-layered

epithelium or the diffusion of IL-1α in a basal direction that is not available in monolayer cultures. Indeed, immunohistochemical analysis of tissue sections of HIV-positive and negative patients with oropharyngeal candidiasis showed parabasal expression of IL-1α and CXCL8 (Tardif *et al*., 2004), suggesting the possibility of basal release of cytokines in this model system. However, in these patients, cytokine expression may have been immune cell derived, although the authors did not mention whether or not they detected any suprabasal staining of cytokines. Similar to monolayer cultures, there was approximately a 1.7-fold increase in IL-1α following invasion of the submerged model compared with the air-exposed model. Since the primary difference between these two models is the thickness of the epithelium, the multiple layers of epithelium appear to influence the secretion or detection of IL-1α protein. Furthermore, the collagen matrix was of similar depth in both so diffusion of IL-1α basally would be similar in both cases.

Comparing submerged models with air-exposed models there was an increase in IL-1α, GM-CSF, IL-6 and CCL2 in submerged OMM. This may be due to the contribution of the fibroblasts through the thin epithelial layer of the submerged model. Therefore, it may be assumed that the analysis of the conditioned medium above the fibroblast-positive, air-exposed OMM may not have included many cytokines released from the fibroblasts due to incomplete diffusion through the thick epithelial layer or the decreased stimulatory effect by *P. gingivalis*. Interestingly, decreases in the levels of IL1-α, CXCL10 and TIMP2 were detected in the medium above *P. gingivalis* NCTC 11834 infected air-exposed NOK-OMM when analysed using the cytokine array (fig 6.6), indicating that *P. gingivalis* may have the ability to specifically target individual cytokines for destruction, or modulate their release in this model of the oral mucosa. Submerged OMM has been shown to be more representative histologically of the junctional epithelium (Chapter 3). Therefore, the cytokine profile observed after stimulation of submerged OMM is the most interesting in terms of the qualitative analysis of the host response *in vitro*. However, the quantification of cytokines using the antibody array (relative to an in-built control) by densitometry depends upon the development of colour being linear in proportion to the amount of antigen. Since this may not have been the case, this method can only be viewed as semi-quantitative. As such, many changes in pro-inflammatory cytokines, including CXCL8, IL-1α, CCL5, TIMP2, MIP10, TGF-β and GM-CSF really required further quantification in relation to their modulation. Although differences were detected between submerged and air-exposed OMM with intracellular *P. gingivalis*, the majority of quantitative analyses, (i.e. ELISA and quantitative PCR), were performed with the air-exposed model mainly to compare findings with previously reported engineered oral mucosal constructs. Because the submerged model is novel, further investigation of the cytokine release from this model and any fibroblast contribution made, is required.

Results presented in this chapter, for the cytokine array, were specific for invasive bacteria because a bactericidal concentration of metronidazole was used during the secretion of epithelial/fibroblast derived cytokines into the conditioned medium. Although effects from extracellular proteases or other virulence factors, due to the presence of dead adherent *P. gingivalis*, cannot be ruled out, the results suggest that intracellular *P. gingivalis* may play a role in modulating the host cytokine response, which is a concept that has been suggested previously. For example, the most adhesive/invasive strains of *P. gingivalis* resulted in the greatest reduction in PMN migration (Madianos *et al*., 1997) and an increase in CCL2 from Human Umbilical Vein Endothelial Cells (HUVEC) was reported after stimulation with the highly invasive strain 381 but not the less invasive strains, W50 and DPG3 (Kang *et al*., 2002). In addition, the intracellular release of SerB, a phosphoserine phosphatase, has been shown to suppress CXCL8 secretion from gingival epithelial cells (Hasegawa *et al*., 2008) and miR-203 was up-regulated in response to intracellular *P. gingivalis* (Moffatt and Lamont, 2011). This microRNA targets the gene encoding suppressor of cytokine signalling 3 (SOCS3), which may result in the modulation of cytokine release via diminished activation of signal transducer and activator of transcription 3 (Stat3), which is a downstream target of SOCS3.Therefore, if this can be extrapolated to the *in vivo* situation, a critical step in the survival of this bacterium may include its rapid internalisation into oral epithelial cells.

In this study, the antibody array was primarily used to screen changes in a range of cytokines that may play a role in the oral epithelial cell response to *P. gingivalis*. As the cytokine array is based on an antibody-detection system, this method is dependent on whether the cytokines are degraded or not and/or where they are cleaved by *P. gingivalis* and whether these cleaved products are able to bind to the array antibodies. If IL-1α is only partially degraded by *P. gingivalis*, this might explain the high detection of IL-1α from monolayer cultures while the detection of all other cytokines was reduced. In addition, each dot of the array has its own specificity, which is shown in Appendix 4, therefore slight changes in the density of certain dots may or may not indicate large changes in actual protein within the conditioned medium and the fold changes indicated in the results section are only indicative rather than truly quantitative. The gingipains of *P. gingivalis* have been suggested to degrade cytokines *in vivo* (Mydel *et al*., 2006) and *in vitro* (Darveau *et al*., 1998), with the possibility that the lysine-specific gingipain is responsible for the majority of CXCL8 degradation (Stathopoulou *et al*., 2009). Therefore, in this study the chemokine CXCL8 was taken forward for further analysis in order to determine the effects of *P. gingivalis* gingipains on the regulation of this secreted protein and whether or not this occurred at the transcriptional or post-transcriptional level.

To investigate the effect of gingipains, the less invasive W50 strain and its mutants were used because of their availability. In addition, due to the similarities in percentage invasion between

NOK and H357 monolayers, H357 cells were used in these experiments due to their rapid growth rate and ready availability. As a consequence, comparisons with the array data are difficult due to these differences in parameters. However, using identical experimental procedures, H357 monolayer cultures were pre-stimulated with TNF-α and infected with *P. gingivalis* for 90 minutes. The metronidazole-treated conditioned medium was removed after 4 hours and the concentration of CXCL8 determined by ELISA. In contrast to the data obtained from the cytokine array, in which there was a decrease in the CXCL8 response, analysis using ELISA suggested that there was no change in the amount of secreted CXCL8 compared with the TNF-α stimulated control (fig 6.7). This indicates that the induction of approximately 200 pg ml^{-1} CXCL8 by pre-stimulation of epithelial cells with TNF-α was too high a concentration of chemokine for intracellular *P. gingivalis* to degrade over the incubation period of 4 hours. However, when TNF-α pre-stimulated monolayers were exposed to *P. gingivalis* for a longer time, i.e. overnight without metronidazole treatment, there was a decrease in the detection of secreted CXCL8 by H357 monolayer cultures. This confirmed the data obtained from the cytokine array, in which intracellular *P. gingivalis* negatively modulated host cell cytokine release. This suggests that the time period of 4 hour post-invasion may not be long enough for cytokine modulation by this less invasive bacterium, in comparison with NCTC 11834. This decreased detection was firstly shown to be specifically due to the presence of proteases as W50 plus protease inhibitor resulted in the detection of CXCL8 concentration up to the level of the bacteria-free TNF-α stimulated control.

Gingipains have been shown to be important in the degradation of many proteins including transferrin, haemoglobin and defensins (Guo *et al*., 2010). The data presented here indicate that gingipains contribute to the degradation of CXCL8, with both the lysine-specific and the arginine-specific gingipains equally contributing to the abrogation of CXCL8 detection. As such, where one gingipain was present the CXCL8 response from epithelial cells matched the response obtained when challenged with W50 wild-type. These findings are in contrast to a previous study that found the lysine-specific gingipain solely responsible for the attenuation of CXCL8 detection (Stathopoulou *et al*., 2009). The authors used the same gingipain mutants that were used in this study but showed that there was no change in the detection of CXCL8 following incubation with K1A for 4 hours (with overnight pre-stimulation of epithelial cells with heat-killed ATCC33277), whereas after stimulation with E8 and W50 no detection of CXCL8 was reported, suggesting the importance of the lysine-specific gingipain in the reduction of the CXCL8 response. The longer incubation period used in this chapter may explain why there was little detection with K1A as the lysine-specific gingipain may have a longer duration of action, which was not observed after 4 hours. Nonetheless, the suppression of CXCL8 in the presence of *P. gingivalis* was shown to be due to the action of gingipains, as a triple gingipain knock-out mutant reversed the reduction in CXCL8 detection (fig 6.8), which

was also shown by Stathopoulou *et al*. (2009). Therefore, data presented here suggests that all gingipains, are capable of cleaving CXCL8 if incubated with epithelial cells for a longer time period than 4 hours, e.g. for 16 hours.

Using H357-OMM, stimulation with TNF-α alone showed no increase in CXCL8 release (fig 6.9) compared with monolayer cultures (fig 6.8), and no decrease in CXCL8 concentration after incubation with *P. gingivalis* overnight (fig 6.9). This may be due to the very high levels of CXCL8 constitutively expressed by H357-OMM, which was in the region of 100-fold greater than H357 monolayers. The ELISA method employed was probably too sensitive and so not capable of differentiating these high concentrations. Due to the high levels of CXCL8 production in the absence of TNF-α, when TNF-α was added to H357-OMM the levels of secreted CXCL8 were already at a maximum and therefore no further increase in CXCL8 was detected after stimulation. In addition, it may be that this high concentration of CXCL8 was not affected by the presence of *P. gingivalis*, or not enough as to be detected by the methods employed in this chapter.

The modulation of cytokine and chemokine release via signalling pathways involving NFκB (Bagaitkar *et al*., 2010), phospholipase C (PLC) and p38 mitogen-activated kinase (MAPK) (Dommisch *et al*., 2010, Shpacovitch *et al*., 2002) may be important mechanisms by which *P. gingivalis* is able to exploit the host in an attempt to evade host defences, promoting bacterial survival. A chemotactic gradient initiated by CXCL8 release from epithelial cells is crucial in the recruitment of neutrophils to the site of infection and the removal of pathogenic bacteria. The attenuation of CXCL8 and other chemokine secretion by oral epithelial cells may play a protective role in preventing chronic inflammation and tissue damage.

The modulation of cytokine protein release by epithelial cells following exposure to *P. gingivalis* was investigated further. It has been shown previously that gingipains play a role in this modulation. However, the question was whether this was at the protein level or the transcription level. Data presented so far in this chapter suggest that modification occurred post-translationally as the triple gingipain knock-out mutant did not affect the protein detection of CXCL8, compared with the TNF-α stimulated control (fig 6.8). It must be acknowledged however that invasion of cells by the triple mutant (EK18) was lower than the other strains, which may have influenced results. In order to investigate this further, the mRNA expression of CXCL8 was analysed following pre-stimulation with TNF-α and overnight exposure to *P. gingivalis* wild-type (W50) and gingipain mutants (E8, K1A and EK18), as previously described. Data obtained from monolayer cultures indicated that *P. gingivalis* wild-type was a potent inducer of CXCL8 transcription (fig 6.10), even without pre-stimulation with TNF-α (fig 6.11). In addition, the absence of one gingipain, regardless of whether it was arginine-specific

(E8) or lysine-specific (K1A), also resulted in the significant increase in mRNA expression relative to the TNF-α-stimulated and unstimulated control. However, when H357 epithelial monolayers were incubated overnight with the triple gingipain mutant, this increase in CXCL8 mRNA expression was not observed, suggesting either gingipains are essential for initiation of signalling pathways leading to transcription of CXCL8, or a certain minimal level of invasion is required before up-regulation of this cytokine occurs. Indeed, CCL2 was similarly not up-regulated by strain EK18, whereas there was up-regulation of this gene transcript in the presence of W50, E8 and K1A (figs 6.16), although it did not reach statistical significance. Gingipains have been shown to activate PARs expressed on the surface of oral epithelial cells, which are important in regulating CCR5, IL-1β, TNF-α, IL-1α and IL-6 expression (Giacaman *et al*., 2009). In addition, gingipains are important in processing pre-fimbrillin (Kadowaki *et al*., 1998), which is also a major virulence feature of *P. gingivalis*. The absence of this processing may render the *P. gingivalis* triple mutant, either directly or indirectly (via a decreased invasive capacity), incapable of stimulating epithelial cell up-regulation of CXCL8, CCL2, IL-6 and CCL5 (figs 6.8, 6.16, 6.14 & 6.18 respectively), via fimbriae (Bagaitkar *et al*., 2010).

IL-6 is a potent activator of B cells and contributes to the process of bone resorption (Okada and Murakami, 1998). Data from the cytokine array in this chapter indicated that IL-6 was induced in submerged NOK-OMM by *P. gingivalis* more than in air-exposed NOK-OMM (fig 6.6). However, in terms of mRNA expression, IL-6 was up-regulated after TNF-α stimulation, but this was not further induced significantly by *P. gingivalis* or any gingipain mutants (figs 6.14 & 6.15). The reason for the detection of a higher concentration of IL-6 protein from submerged NOK-OMM than air-exposed NOK-OMM may be due to the possible activation of fibroblasts within the submerged model due to the thinner epithelial layer and the closer proximity of *P. gingivalis* to the fibroblast-embedded matrix.

CCL5 is a chemoattractant for basophils, eosinophils, monocytes and T-helper 1 cells. Here we found NOK monolayer cells to release CCL5 protein constitutively, which was enhanced by TNF-α stimulation (fig 6.1). A slight increase in CCL5 expression, though not statistically significant, was observed only with Lys-gingipain mutants. There are a number of possible reasons for this. First, production of CCL5 may be temporal and may decline after an initial stimulation. Second, there may have been variation between cytokine arrays, where an observed increase in CCL5 following TNF-α stimulation in figure 6.1 may be an artefact and quantitative analysis needs to be performed. Third there may be an influence of the different cell types used, for example, the weak transcription of this gene in response to *P. gingivalis* may be due to the lack of receptors for *P. gingivalis* PAMPs on this cell type.

There was no change in the mRNA expression of CXCL8, IL-6, CCL2 and CCL5 after TNF-α stimulation or *P. gingivalis* treatment of H357-OMM (figs 6.12, 6.15, 6.17 and 6.19, respectively). This may be because within the multi-layered epithelium, the surface layers only are exposed to *P. gingivalis*. Overnight stimulation may not have been long enough to induce the transcription of cytokines further down the epithelial layers and therefore as a percentage of the total mRNA, the induced mRNA may have been insignificant. Alternatively, all of these may be under temporal control or that there is a high constitutive expression of these cytokines and this may have masked any subtle differences following stimulation with TNF-α or *P. gingivalis*.

The cytokines studied here are part of a greater network of cytokines which induce and suppress each other via intracellular signalling pathways in response to external stimuli, in an attempt to protect the host from damage. For example, TNF-α stimulates IL-6 release (Yang *et al*., 2003), IL-6 induces CCL2 secretion (Eklassi *et al*., 2008) and the inhibitory/stimulatory effects of TGF-β1 and IL-2 work in concert to regulate Th1 and Th2 responses (Wahl and Chen, 2003). Occasionally, there is overproduction of these inflammatory mediators, or underproduction leading to dysregulation and consequent overgrowth of pathogens. It is the fine balance between these mechanisms that ensures a 'healthy host' and maintenance of tissue homeostasis. When this balance is disrupted by mechanisms including modulation of host cell signalling by pathogens, immunocompromisation or genetic polymorphisms leading to loss of cytokine function, disease takes hold and therefore investigating the host response to pathogenic bacteria is important.

6.4.2 Conclusion

For monolayer cultures of epithelial cells, there was attenuation in the cytokine response following invasion and overnight stimulation with *P. gingivalis*. More specifically for CXCL8, the attenuation in protein was shown to be directly modulated by gingipains, where the absence of all gingipains was required to abrogate this effect. This was confirmed by quantitative PCR, which showed up-regulation of the CXCL8 transcript in the presence of *P. gingivalis*. Thus suggesting that *P. gingivalis* has the ability to modulate the host immune response by dampening the chemotactic gradient essential for the recruitment of leucocytes to the site of infection, thereby preventing the removal of this bacterium from the periodontal pocket. Interestingly, the data presented here suggests that intracellular bacteria also play a role in dampening the host immune response. This is likely to be an important mechanism for the survival, intra- and inter-cellular transmission and invasion of pocket epithelium.

Engineered oral mucosa, which resembles the normal oral mucosa, showed differences in the cytokine responses to *P. gingivalis*, compared with monolayer. In OMM, there seemed to be

limited activation of CXCL8 in response to *P. gingivalis* compared with monolayer cultures and no protein degradation was detected. No change in the transcript of CXCL8 was found suggesting a failure of *P. gingivalis* to stimulate the host tissue.

Comparison of OMM with monolayer epithelial cultures has not been reported before. However, further work may be required to address the questions raised in this chapter regarding the experimental procedure when working with OMM. In particular, data revealed differences between multi-layered OMM (air-exposed) and OMM with fewer cell layers (submerged), suggesting epithelial multi-layers may contribute to the detection/modulation of host cytokine release. As the submerged model more closely resembles junctional epithelium, the epithelium most likely to be encountered by *P. gingivalis* (Chapter 3), further investigations using this model are required. Table 6.1 provides a summary of the findings within this chapter.

Table 6.1 The cytokine response of monolayer and OMM to *P. gingivalis*. This table shows the strain of *P. gingivalis* used (NCTC or W50 (including gingipain mutants Δ*rgpArgpB* and Δ*kgp* and Δ*rgpArgpBkgp*)), the epithelial cell (normal oral keratinocytes (NOK) or the oral epithelial cell line (H357)) cultured as a monolayer or oral mucosal model (OMM), the experimental procedure performed, i.e. incubation of epithelial cells with *P. gingivalis* with or without the addition of antibiotic (internal and internal+external, respectively), the tested cytokines and the result obtained from the cytokine array, ELISA and real-time PCR.

	Strain		**Epithelial cell**		**Culture**		**Procedure**		**Tested cytokines**					**Result**
	NCTC 11834	**W50**	**NOK**	**H357**	**Monolayer**	**OMM**	**Internal**	**Internal + external**	**CXCL8**	**IL6**	**CCL2**	**CCL5**	**Others**	
Array (protein)	✓		✓		✓		✓		✓	✓	✓	✓	✓	All decreased
	✓		✓			✓	✓		✓	✓	✓	✓	✓	All decreased apart from CXCL8 and IL6
ELISA (protein)		✓		✓	✓		✓		✓					No change
		✓		✓	✓			✓	✓					Decrease with Δ*rgpArgpB* and Δ*kgp* but not Δ*rgpArgpBkgp*
		✓		✓		✓		✓	✓					No change
Real-time PCR (mRNA)		✓		✓				✓	✓					All increase except Δ*rgpArgpBkgp*
		✓		✓		✓		✓	✓					No change
		✓		✓	✓			✓		✓				No change
		✓		✓		✓		✓		✓				No change
		✓		✓	✓			✓			✓			All increase except Δ*rgpArgpBkgp*
		✓		✓		✓		✓			✓			No change
		✓		✓	✓			✓				✓		No change
		✓		✓		✓		✓				✓		No change

Chapter 7 General Discussion

7.1 General Discussion

Periodontitis is an inflammatory disease characterised by progressive loss of tooth supporting structures and is the leading cause of tooth loss worldwide (Choi and Seymour, 2010). Periodontitis has been shown to have a complex microbiological aetiology, although *P. gingivalis* is thought to be an organism that is pivotal to the progression of disease (Dalwai *et al.*, 2006; Curtis *et al*., 2011). The initial colonisation of the oral cavity by *P. gingivalis* occurs either via adhesion to primary colonising bacteria (Park *et al*., 2005) and/or directly to the surface of oral structures, including the oral mucosa (Rautemaa *et al*., 2004). One consequence of adhesion to the latter is internalisation into the epithelium and this study has explored the relationships and responses of the epithelium to *P. gingivalis*.

To investigate epithelial-*P. gingivalis* interactions *in vitro*, a model of the oral epithelium was required. The most commonly used model within the vast amount of literature is the epithelial cell monolayer (Lamont *et al*., 1995). While this model is easy to manipulate, gives high reproducibility and is cost-effective, it does not completely resemble the oral mucosa. The oral mucosa consists of a stratified and often differentiated epithelium supported by a connective tissue layer, within which oral fibroblasts and other cells such as macrophages, mast cells and endothelial cells reside (section 1.1.3.2). These two layers are separated by a basement membrane. Investigators have sought to reproduce such mucosa *in vitro*, and research is ongoing in this field (Colley *et al*., 2011, Kinikoglu *et al*., 2011) in an attempt to produce an organotypic model, more suitable than monolayer cultures, for experimental investigations.

The development and characterisation of organotypic oral mucosal models, which resemble normal oral mucosa in terms of structure (by H&E staining), and cytokeratin, laminin and E-cadherin expression were performed in this study. However, the periodontium comprises regions with multilayered epithelium, and the junctional epithelium which is just a few cells thick. Consequently, two models were developed and characterised, these were: i) a full-thickness stratified mucosal model, which was raised to the air-to-liquid interface (air-exposed) during culture to enable keratinocyte stratification and differentiation, and ii) a 'submerged' model, not raised to the air-to-liquid interface, that most resembled junctional epithelium due to the resultant few epithelial layers and a lack of epithelial differentiation. The culture of OMM using two epithelial cell types was also compared. These were the tumour-derived oral epithelial cell line, H357 and NOK isolated from healthy patients. It was found that air-exposed OMM cultured using NOK showed a higher degree of epithelial differentiation when assessed by H&E staining, compared with OMM cultured using H357, whereas submerged OMM cultured using

H357 more closely resembled junctional epithelium due to the high turn-over rate of this epithelial cell line. Therefore, both models were used for invasion studies.

The choice of connective tissue scaffold was also evaluated. The air-to-liquid interface culture of H357 and NOK on two different fibroblast-embedded scaffolds: rat-tail type I collagen and DED were compared. It was shown that models cultured on DED more closely resembled the *in vivo* mucosa due to the presence of an in-tact basement membrane, rete ridges and the fact that it was human in origin. However, we experienced considerable unreliability with models grown on DED and so for most experiments took the pragmatic step of using the collagen scaffolds because they proved to be more reproducible and reliable, had lower cost and were easy to manipulate in downstream procedures. Such procedures included lysis of OMM following invasion and greater manipulation of the depth of the connective tissue layer for the incorporation of neutrophils. Although the use of collagen scaffold was a compromise, it was felt that its use would be suitable for investigating epithelial-*P. gingivalis* interactions since its main function was as a matrix to support the underlying fibroblasts. Future work to improve the reproducibility of DED-OMM cultures may be useful, in particular when considering invasion of *P. gingivalis* into the connective tissue, since a basement membrane is formed in DED models.

It has been widely reported that *P. gingivalis* has the ability to invade oral epithelial cells *in vivo* (Rautemaa *et al*., 2004) and *in vitro* (Lamont *et al*., 1995), which may aid this bacterium to evade host defences and so could contribute to re-infection. The commonly used method to investigate the cellular internalisation of *P. gingivalis* is an antibiotic protection assay (Lamont *et al*., 1995). However, due to the limited literature regarding the use of this assay to study *P. gingivalis* invasion of multi-layered epithelial models, optimisation the experimental conditions was necessary. Results suggested that the highest bacterial recovery occurred following incubation of OMM and bacteria for 3-6 hours in an aerobic atmosphere, and model lysis by homogenisation. Anaerobic conditions over prolonged periods proved too detrimental to the epithelium.

Using the protocol developed, it was found that the invasion of H357-OMM was significantly higher than NOK-OMM by *P. gingivalis* NCTC 11834. Previous experiments had shown that there was no difference between the invasion of H357 and NOK when cultured as monolayers, suggesting that differences between these two cell types become apparent when cultured as part of an organotypic model. Therefore, it was proposed that because H357 are more readily available than NOK, the use of H357 to determine experimental trends may be just as suitable as NOK, particularly in investigating *P. gingivalis* invasion. However, when investigating absolute end-point values, NOK-OMM may be more suitable than H357-OMM due to possible

differences in receptor expression/activation, cellular differentiation and/or intracellular signalling pathways.

P. gingivalis was shown to invade the superficial layers of the oral epithelium of OMM. It was discovered that some intracellular bacteria were capable of leaving these superficial layers, but it was difficult to ascertain whether this was because of active release or desquamation. Desquamation was observed but bacteria were detected free in the supernatant prior to significant desquamation. However, whatever the mechanism, the fact that *P. gingivalis* emerges into the environment provides a mechanism for re-infection. Although intracellular replication was not detected in this study, it has been proposed previously that *P. gingivalis* may enter a dormant uncultivable state until in contact with 'uninfected' epithelial cells (Li *et al*, 2008). This may explain why intracellular *P. gingivalis* were not detected by viable counting after 24 hours but were detected immunohistochemically. This may implicate host cell internalisation by *P. gingivalis* in the pathogenesis of disease, particularly if these bacteria remain in a 'dormant' state (Li *et al*., 2008), preventing host cellular apoptosis (Yao *et al*., 2010) and are subsequently released into the extracellular environment or spread intracellularly (Yilmaz *et al*., 2006) to cause re-infection.

It is thought that penetration through to the connective tissue may result in increased periodontal detachment due to the action of bacterial collagenases (Kato *et al*., 1992) and activation of host MMPs (Tervahartiala *et al.*, 2000). This may be more pronounced in junctional epithelium *in vivo*, where the distance to penetrate to the connective tissue is a lot less compared with the gingival and sulcular epithelium. There is a greater expression of β1 integrin subunits, in particular the fibronectin-binding integrin α5β1, on the underside of the epithelium and *P. gingivalis* may invade via this route (Nakagawa *et al.*, 2002). For example, if *P. gingivalis* was able to penetrate the connective tissue, this may provide an additional mechanism of intra/intercellular spreading via re-infection of the epithelium from below, continuing the survival of this bacterium. However, attempts to assess the level of penetration through OMM by *P. gingivalis* were inconclusive because it was difficult to visualise individual bacteria using immunohistochemistry, and attempts to use immunofluorescence were hampered by high background signals, which could not be overcome. Future work using *in situ* hybridisation may prove more useful.

The percentage invasion was shown to increase with change in the expression of *P. gingivalis* gingipains and with the growth of *P. gingivalis* in haemin-rich and high temperature conditions. Within the periodontal pocket of patients exhibiting active periodontal disease, there may be an increased concentration of haemin, which may originate from the increased level of bleeding at the site of infection (Offenbacher *et al*., 2008) and a higher than physiological temperature due

to inflammation. This increase in temperature and haemin concentration may influence the invasive capacity of this bacterium, possibly via modification of gingipain activity. Although the complete absence of gingipains rendered *P. gingivalis* almost unable to invade oral epithelial cells, possibly due to the lack of fimbrial protein processing, the absence of a single gingipain, in particular the Arg-specific gingipains, resulted in an elevated percentage invasion over the wild-type strain. It was considered that this may be due to receptor degradation by gingipains, resulting in a decrease in receptor-mediated cellular invasion. Although it was clearly demonstrated that gingipains degrade α5 and the accessory molecule tetraspanin CD81, antibody blocking (α5β1 and CD81) and RNA silencing (CD81) did not result in a decrease in *P. gingivalis* W50 invasion. This might suggest that there are other mechanisms of internalisation that need to be explored. In addition, the strain of *P. gingivalis* that was used in these experiments (W50) was only slightly fimbriated and invasion of a more highly fimbriated/invasive strain may, in contrast, preferentially invade via these epithelial receptors. This was indeed indicated by antibody blocking of the integrin subunit α5, which lowered epithelial cell invasion by NCTC 11834, suggesting that there may be differences in invasion strategies between *P. gingivalis* strains.

The response of epithelial cells to challenge by *P. gingivalis*, in terms of cytokine release, is a widely debated subject within the literature. Data presented here suggests that there is a decrease in the detection of extracellular CXCL8 protein following incubation of *P. gingivalis* with epithelial cell monolayers overnight. This reduction in protein detection was shown to occur in a gingipain-dependent manner (either Arg-specific or Lys-specific gingipains) but this was not observed at the mRNA level. Indeed there was a significant increase in CXCL8 gene expression following incubation of epithelial cells with *P. gingivalis* wild-type and single gingipain mutants when compared with the non-infected control, suggesting post-transcriptional and/or post-translational modification of CXCL8 by *P. gingivalis*. Incubation of epithelial cells with the triple (Arg- and Lys-specific gingipain) knockout mutant showed no increase in CXCL8 mRNA expression when compared with the non-infected control, suggesting that both the Arg- or Lys-specific gingipains may be important in initiating intracellular signalling pathways culminating in the initiation of CXCL8 gene transcription. As these experiments were only performed at a single time point of 16 hours, a time course of CXCL8 release may be more informative as to the rate of CXCL8 degradation by *P. gingivalis* gingipains. Due to the lack of availability of gingipain mutants locally in a more invasive strain of *P. gingivalis*, it was difficult to determine the cytokine response of epithelial cells to invasion, although preliminary data using an antibody array revealed a decrease in all inflammatory cytokines tested following epithelial invasion by strain NCTC 11834. Using this array, CCL2, CCL5 and IL-6 were selected as potential pro-inflammatory cytokines/chemokines for future investigations, due to their persistence following challenge by *P. gingivalis*. The lack of significant protein

degradation observed may contribute to their persistence at the site of infection resulting in the recruitment of immune cells, activation of additional pro-inflammatory cytokines and/or the initiation/progression of bone resorption. Determining the cytokine response of OMM requires additional work because results indicated that they produce high constitutive concentrations of pro-inflammatory cytokines, e.g. CXCL8, possibly due to their high cell number. Moreover, investigations into the individual cytokine contribution from fibroblasts and epithelial cells may prove interesting. In addition, determining the quantitative cytokine response from NOK-OMM, in particular submerged NOK-OMM, may give a greater understanding of the modulation of cytokine release by *P. gingivalis* in a more relevant model. NOK-OMM were not used in this study due to the high constitutive cytokine release from this cell type and the additional contribution from increased epithelial layers.

In terms of modelling the epithelial response to *P. gingivalis* and other microorganisms in periodontitis, the epithelium is not the only aspect that needs to be considered. The plaque biofilm is home to approximately 900 species, of which the majority have yet to be identified and/or are as yet unculturable (Jenkinson, 2011). Therefore, the use of a single planktonic species, as was utilised in this thesis, is not representative of the *in vivo* situation (Peyyala *et al*., 2012). The microbial biofilm is a dynamic structure providing nutrients and suitable atmospheric conditions contributing to species survival, and has been shown to contribute to an increase in the cellular invasiveness of oral bacteria such as *Fusobacterium nucleatum* (Gursoy *et al*., 2010), and may increase the virulence factors of *P. gingivalis* (Tenorio *et al*., 2011). In addition, the invasion of *P. gingivalis* has been shown to increase in the presence of other microbes including *Fusobacterium nucleatum* (Saito *et al*., 2008), *Filifactor alocis* (Aruni *et al*., 2011) and *Candida* species (Tamai *et al*., 2011).

As reconstructed oral mucosa becomes more sophisticated (e.g., by incorporation of immune cells (Schaller *et al*., 2004) and endothelial cells (Takei *et al*., 2006)), the contributions from cellular cross-talk (Murakami and Okada, 1997, Egles *et al*., 2010) will add to our understanding of host-microbial interactions. Oral mucosal models can be used for other applications and to investigate a range of other biological aspects, including cancer, therapeutics, toxicity testing, etc. Furthermore, the modification of these models by substituting cell types, e.g. oral epithelial cells for vaginal epithelial cells and/or incorporation of gene-knockout cells, will completely change and multiply the uses of these highly adaptable models.

7.2 Summary

Oral mucosal models are currently not widely used in the literature, particularly to study bacterial infection. However, within recent years they have, and are becoming, a useful tool to investigate host-pathogen interactions, as an alternative to monolayer cultures. We have shown

the development, characterisation and optimisation of oral mucosal models, representative of the normal oral mucosa, to study cellular invasion by *P. gingivalis*. The level of invasion was modified by the abrogation of *P. gingivalis* gingipain expression and culture of this bacterium within a haemin-rich, high temperature environment. Although no effect on invasion was found by blocking CD81 or α5, data presented here, in combination with previously published literature suggests that the process of invasion is highly complex and is a culmination of the environment within which the bacterium and host cells reside, the expression of cellular proteins present on both the bacteria and host cells and is ultimately a fine balance between the host and invading pathogen. The cellular response to *P. gingivalis* reported here suggested that there is a decrease in the release of CXCL8 protein, which is due to protein degradation by *P. gingivalis* gingipains. Additional pro-inflammatory chemokines/cytokines of interest may include CCL2, CCL5 and IL-6, which may contribute to the pathogenesis of disease. The future development of OMM to make them more representative of the normal oral mucosa and the optimisation of experimental techniques may prove invaluable, particularly in investigating the host response to *P. gingivalis* challenge.

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Appendices

Appendix 1

Isolated primary NOKs were stained by immunofluorescence for the epithlelial marker pan-cytokeratin. Positive staining of all cells indicated absence of contaminating fibroblasts and confirmed culture purity.

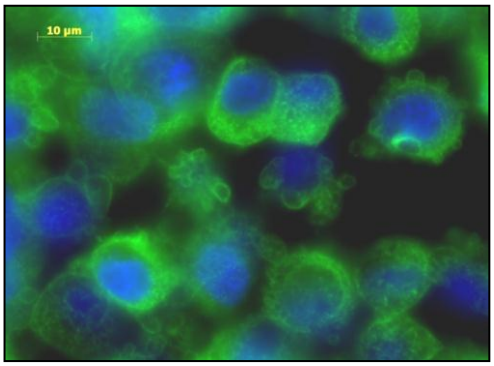

Figure A1.1 Pancytokeratin staining of normal oral keratinocytes (NOK). Briefly, oral epithelial cells were isolated from oral biopsy and cultured on glass coverslips until confluent. Cells were fixed and permeabilised with cold methanol/acetone. Monoclonal mouse anti-human cytokeratin (clone MNF-116) was added to the cells and incubated for 1 hour at room temperature. Cells were incubated with anti-mouse FITC-conjugated secondary antibody and nuclei were counterstained with DAPI.

Appendix 2

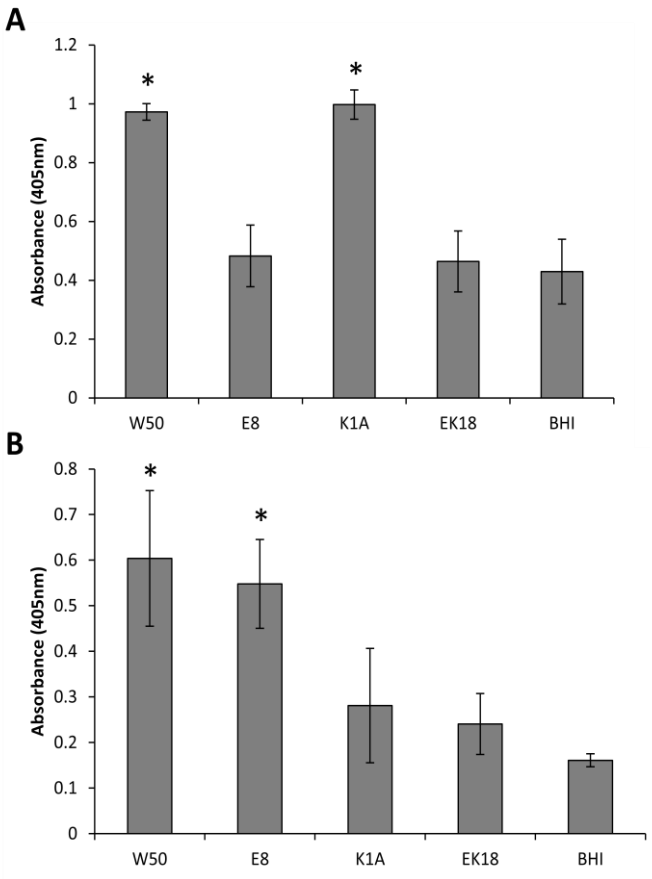

Figure A2.1 Characterisation of *P. gingivalis* strains using the BApNA (A) and tosyl-Gly-Pro-LyspNA (B) hydrolysing assays. The gingipain activity of *P. gingivalis* strains was verified prior to experimentation. The methods are outlined in section 2.6. Briefly, whole cell *P. gingivalis* strains (W50, E8 (Δ*rgpArgpB*, K1A (Δ*kgp*) and EK18 (Δ*rgpArgpB* Δ*kgp*)) were incubated with *N*-α-benzoyl-L-Arg-*p*-nitroanilide (BA*p*NA) or toluenesulfonyl-glycyl-L-prolyl-L-lysine *p*-nitroanilide (tosyl-Gly-Pro-Lys*p*NA). Hydrolysis of these substrates resulted in a colour change that was indicated by an increase in absorbance when compared with the brain heart infusion (BHI) negative control. Hydrolysis of BApNA (A) by W50 and K1A indicated that these strains possessed Arg-specific gingipain activity, whereas hydrolysis of the tosyl-Gly-Pro-Lys*p*NA substrate (B) by W50 and E8 indicated that these strains possessed Lys-specific gingipain activity. Failure of EK18 to hydrolyse either substrate verified the absence of Arg- and Lys-specific gingipain expression. Results presented are means±SD of three independent experiments repeated in triplicate.

Appendix 3

Table A3.1 Sequences of primers used in Chapter 6. The primer sequences for interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1/CCL2), Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES/CCL5) and the housekeeping gene U6 used in real-time PCR experiments.

IL-6	Forward	5' - ACCCCTGACCCAACCACAAAT
	Reverse	5' - AGCTGCGCAGAATGAGATGAGTT
CCL5	Forward	5' - GAGCTTCTGAGGCGCTGCT
	Reverse	5' - TCTAGAGGCATGCTGACTTC
CCL2	Forward	5' - CAAGCAGAAGTGGGTTCAGGA
	Reverse	5' - TTAGCTGCAGATTCTTGGGTTG
U6	Forward	5' - CTCGCTTCGGCAGCACA
	Reverse	5' - AACGCTTCACGAATTTGCGT

Appendix 4

Table A4.1 The sensitivity of cytokine array antibodies (Chapter 6). The sensitivity of the following antibodies (pg ml^{-1}), embedded in the RayBio® Human Inflammation Antibody Array 3 membranes. Sensitivity values were retrieved online: http://www.raybiotech.com/human_array_sensitivity.pdf (accessed 07/03/12). **Abbreviations:** C-C chemokine ligands (CCL), C-X-C chemokine ligands (CXCL), interleukin (IL), granulocyte colony-stimulating factor (GCSF), granulocyte macrophage colony-stimulating factor (GM-CSF), intercellular adhesion molecule-1 (ICAM-1), interleukin 6 soluble receptor (IL-6 sR), (CCL11/Eotaxin-1) (CCL24/Eotaxin-2), interferon gamma-induced protein 10 (IP10/CXCL10), monocyte chemotactic protein 1 (MCP-1/CCL2), monocyte chemotactic protein 2 (MCP-2/CCL8), macrophage colony-stimulating factor (M-CSF), monokine induced by gamma interferon (MIG/CXCL9), regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), soluble tumour necrosis factor receptors I and II (sTNF RII/TNFRS1B, sTNF RI/TNFRS1A), transforming growth factor beta 1 (TGF-β1).

Antibody	pg ml^{-1}	Antibody	pg ml^{-1}	Antibody	pg ml^{-1}	Antibody	pg ml^{-1}
Eotaxin-1	1	**IL-2**	25	**IP-10**	10	**RANTES**	2000
Eotaxin-2	1	**IL-3**	100	**MCP-1**	3	**sTNF RII/TNFRS1B**	10
GCSF	2000	**IL-4**	1	**MCP-2**	100	**sTNF RI/TNFRS1A**	100
GM-CSF	100	**IL-6**	1	**M-CSF**	1	**TGF-β1**	200
ICAM-1	100000	**IL-6 sR**	10	**MIG**	1	**TIMP-2**	1
IL-1α	1000	**IL-7**	100	**MIP-1α**	10	**TNF-α**	10
IL-1β	100	**IL-8**	1	**MIP-1β**	20	**TNF-β**	1000

Appendix 5

Table A5.1 Concentrations of the individual components of the protease inhibitor complex. A combined cocktail of serine and cysteine proteases used to inhibit gingipain activity (complete Mini ETDA-free (Roche, UK)).

Protease	Concentration
Chymotrypsin	1.5 µg ml^{-1}
Thermolysin	0.8 µg ml^{-1}
Papain	1 mg ml^{-1}
Pronase	1.5 µg ml^{-1}
Pancreatic extract	1.5 µg ml^{-1}
Trypsin	0.002 µg ml^{-1}

Appendix 6

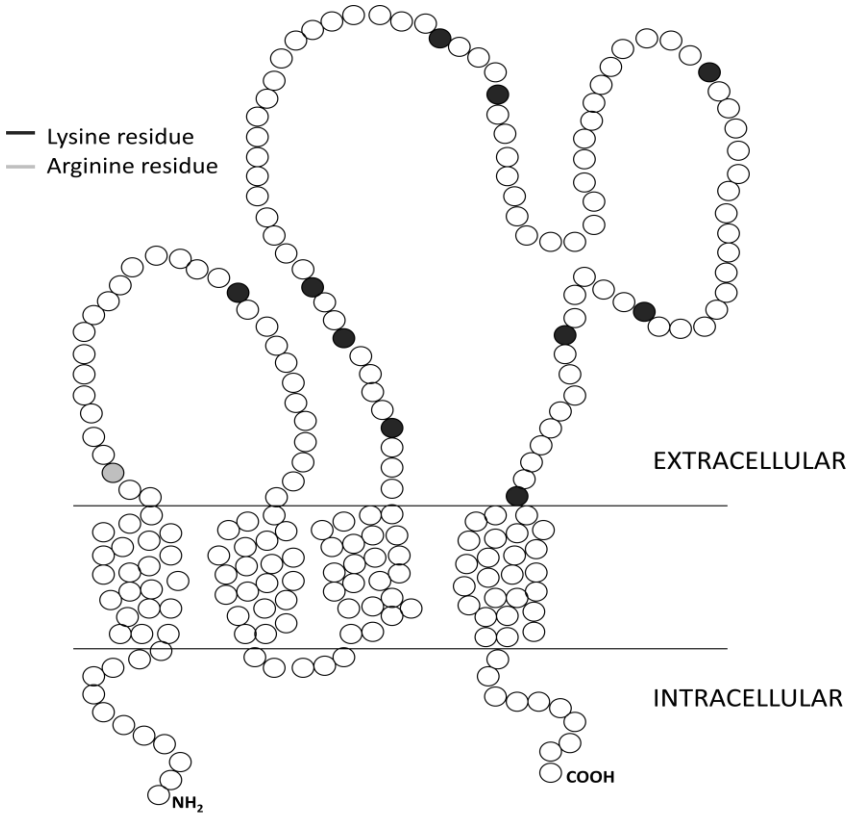

Figure A6.1 Potential cleavage sites of CD81 by *P. gingivalis* gingipains. Arginine (10) and lysine (1) residues are indicated on this schematic of the 4-transmembrane spanning tetraspanin, CD81. Each circle represents an amino acid. Adapted from Levy *et al*. (1998).