The Role of IL-33 and IL-17 Family Cytokines in Periodontal Disease

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Abstract

IL-33 and IL-17 family cytokines (IL-17A – IL-17F) have been shown to play roles in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease. However knowledge of their role in periodontal disease pathogenesis is limited. The aim of this study was therefore to determine clinical associations between IL-33 and IL-17 family cytokines and chronic periodontitis. In addition, to begin to investigate the biological significance of these associations using *in vitro* model systems.

97 patients with chronic periodontitis and 77 healthy volunteers were recruited in Glasgow and Newcastle. Serum, gingival crevicular fluid (GCF) and saliva were analysed for levels of IL-33 and IL-17 family cytokines by ELISA. Periodontal tissues from 17 chronic periodontitis patients and 10 healthy subjects from Glasgow were also investigated for IL-33 and IL-17 family cytokines mRNA expression by real time PCR. Immunohistochemical analysis was also performed on tissue to investigate expression of IL-33 and IL-17E at the protein level. *In vitro* experiments were performed using the OKF6/TERT-2 oral keratinocyte cell line and primary human gingival epithelial (PHGE) cells. The cells were stimulated with either a live *Porphyromonas gingivalis* monospecies biofilm or recombinant cytokines and changes in expression of cytokines, chemokines and their receptors evaluated by real-time PCR, immunocytochemical analysis or ELISA. In addition, transcriptional activity was monitored by analysis of changes in the phosphorylation (activation) of the NF-κB p65 subunit transcription factor using serum, GCF and saliva. IL-17A and IL-17A/F levels were higher in chronic periodontitis patients, but serum IL-17E was lower. IL-17A, IL-17A/F and the serum IL-17A:IL-17E ratio correlated positively with clinical parameters. IL-33, and IL-17 family cytokine (except IL-17B) gene transcripts were higher in tissue of chronic periodontitis patients. In addition, IL-33, ST2, IL-17E and IL-17RB proteins are expressed in periodontal tissues. Furthermore, IL-33 protein expression is upregulated in tissue of chronic periodontitis patients. *In vitro* models showed that IL-33 and its receptors (ST2 and ST2L) are expressed by oral keratinocytes (OKF6/TERT-2 cells and PHGE cells) and IL-33 expression up-regulated in response to *P. gingivalis*. However, IL-33 failed to induce expression of a range of inflammatory mediators and receptors in OKF6/TERT-2 cells. *In vitro*, IL-17E inhibited *P. gingivalis* monospecies biofilm and IL-17A induced

expression of chemokines (IL-8 and/or CXCL5) by OKF6/TERT-2 cells at the transcriptional level by blocking the phosphorylation (activation) of the NF-κB p65 subunit.

This study demonstrates clinical associations between IL-33 and IL-17 family cytokines and chronic periodontitis. The expression of IL-33 by oral keratinocytes and its up regulation upon exposure to *P. gingivalis* suggest it plays a role in the innate immune response to pathogens within the periodontium. However, the role of IL-33 in the periodontal inflammatory response remains to be elucidated. The negative correlations between serum levels of IL-17A and IL-17E and correlations with disease parameters, combined with their differing effects on the induction of expression of key neutrophil chemoattractants (CXCL5 and CXCL8), suggest opposing roles in periodontal immunity. Indeed, it can be hypothesised that the differential regulation of chemokine expression is due to IL-17A having pro- and IL-17E having anti-inflammatory properties. Indeed, as neutrophils play a key role in the early events associated with periodontal disease progression, the data suggests IL-17E is a rational target for therapeutic intervention.

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Declaration

The work presented in this thesis represents original work carried out by the author. This thesis has not been submitted in any form to any other degree at the University of Glasgow or any other institution.

Signature……..

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Abbreviations

AMP	antimicrobial peptide
ATP	adenosine triphosphate
ATTC	American Type Culture Collection
^{o}C	degree Celsius
C	complement component (e.g., C3, C3a and C5a)
CCL	chemokine (C-C motif) ligand (e.g., CCL10)
CCR	chemokine (C-C motif) receptor (e.g., CCR2)
CD	cluster of differentiation (e.g., CD3 and CD4)
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
cm^2	square centimetre
CO_2	carbon dioxide
CXCL	C-X-C motif chemokine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DKSFM	defined keratinocyte serum-free medium
DMEM	dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
E. coli	*Escherichia coli*
e.g.	for example (Latin: *exempli gratia*)
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal regulated kinase
FACE	Fast activated cell-based ELISA
Fc	fragment crystallisable region

G-CSF	granulocyte colony-stimulating factor
GATA	globin transcription factor
GCF	gingival crevicular fluid
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour(s)
H_2	hydrogen gas
HCl	hydrochloric acid
HMGB	high-mobility group box protein
HRP	horseradish peroxidase
hTERT	human telomerase reverse transcriptase
HUVEC	human umbilical vein endothelial cell
i.e.	that is (Latin = *id est*)
ICAM	intercellular adhesion molecules
IFN	interferon
Ig	immunoglobulin (e.g., IgE, IgG and IgM)
IκB	inhibitor of kappa B
IL-	interleukin (e.g., IL-8)
IL-10R	interleukin 10 receptor (e.g., IL-10R1)
IL-17R	interleukin 17 receptor (e.g., IL-17RA)
IL-1RA	IL-1 receptor antagonist
IL-1RAcP	interleukin-1 receptor accessory protein
INT	intensity
IU/ml	international units per millilitre
JNK	c–Jun N–Terminal
JunB	jun B proto-oncogene
kDa	kilodalton
kHz	kilohertz
KSFM	keratinocyte serum-free medium
LPS	lipopolysaccharide
M	molar
M-CSF	macrophage colony-stimulating factor
MAMP	microbe associated molecular pattern
MAP	mitogen activated protein

MCP	monocyte chemotactic protein (e.g., MCP-1)
mg	milligrams
µg/ml	micrograms per millilitre
mg/ml	milligrams per millilitre
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
min	minute(s)
MIP	macrophage inflammatory protein
µl	microliter
ml	millilitre
ml^2	square millilitre
µM	micromolar
mM	millimolar
MMP	matrix metalloproteinase (e.g., MMP8)
mRNA	messenger ribonucleic acid
N_2	nitrogen gas
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng/ml	nanograms per millilitre
nM	nanomolar
NSAIDs	non-steroidal anti-inflammatory drugs
OD	optical density
OKF6/TERT-2 cell	human oral keratinocyte cell line
OPG	osteoprotegerin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween
PCR	polymerase chain reaction
pg/ml	picograms per mililiter
PGE	prostanglanding E (e.g., PGE_2)
pH	logarithmic measure of hydrogen ion
PHA	phytohemagglutinin
PHGE cells	primary human gingival epithelial cells
PMA	phorbol 12-myristate 13-acetate

RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
Real-time PCR	real-time polymerase chain reaction
rhIL	recombinant human interleukin (e.g. rhIL-33)
RNase	ribonuclease
rpm	rounds per minit
RPMI media	Roswell Park Memorial Institution media
RT	reverse transcriptase
SCID mice	severe combined immunodeficient mice
SDD	sub-antimicrobial dose doxycycline
SOCS	suppressor of cytokine signalling (e.g., SOCS3)
sST2	shorter soluble receptor form of the receptor ST2 (IL1RL1)
ST2	interleukin 1 receptor-like 1 (IL1RL1)
ST2L	longer transmembrane form of the receptor for ST2
ST2V	variant soluble receptor form of the receptor ST2
STAT6	signal transducer and activator of transcription 6
TGF	transforming growth factor (e.g., TGF-α)
Th1 cell	T helper type 1 cell
Th2 cell	T helper type 2 cell
Th17 cell	T helper type 17 cell
TIMP	tissue inhibitors of metalloproteinase
TLR	toll-like receptor
TNF	tumour necrosis factor (e.g., TNF-α)
TRAF	TNF receptor-associated factor
UK	United Kingdom
v/v	volume/volume
w/v	weight/volume
x g	times gravity

Chapter 1: Introduction

1.1 Periodontal disease

The periodontium is a term that refers to the specialised periodontal tissues that support the teeth in their positions in the upper and lower jaws. The periodontium consists of four major tissues: alveolar bone, cementum, periodontal ligament and gingiva. Since the main function of periodontium is to support the teeth, maintaining a healthy periodontium is very important in ensuring masticatory function. However, there are many diseases and conditions the pathogenesis of which are known to precipitate damage to the periodontium and may eventually lead to tooth loss (Armitage, 1999).

Plaque induced gingivitis is the most common form of periodontal disease (Ababneh *et al.*, 2012; Albandar & Kingman, 1999; Page, 1985). It is characterised by inflammation of the gingiva and is associated with the presence of bacterial plaque at the gingival margin. However, this results in no observable loss of bone and no loss of tooth attachment. Indeed, the inflammation that is characteristic of gingivitis is reversible upon removal of gingival plaque (Mariotti, 1999).

Without proper oral health care, plaque induced gingivitis can progress to chronic periodontitis. Chronic periodontitis is characterised by destruction of the alveolar bone, cementum, periodontal ligament and gingiva, which results clinically in the formation of a periodontal pocket and/or gingival recession. Periodontal disease affects 60 - 90 % of the population (Bartold *et al.*, 2010). In addition, The World Health Organisation (WHO) reported severe chronic periodontitis in 5 – 20 % of the adult population worldwide (Jin *et al.*, 2011). In the UK, advanced chronic periodontal disease was found to affect 8 – 15 % of the population (Kelly *et al.*, 1998). Furthermore, periodontal disease represents a significant cost burden to the National Health Service; with treatment and its sequelae costing the National Health Service in Scotland alone at least £20 million annually ("Scottish dental practice board: annual report," 2009). In addition, evidence suggests that bi-directional links occur between periodontal disease and other chronic inflammatory conditions such as rheumatoid arthritis, diabetes and cardiovascular disease (Kaur *et al.*, 2013; Pizzo *et al.*, 2010). Therefore, it can be hypothesised that treatment of periodontal disease and

associated conditions places an even larger cost burden on limited National Health Service resources than previously described.

Although gingivitis and chronic periodontitis are initiated and sustained by bacterial plaque, the host defence mechanisms are believed to play an important role in their pathogenesis (Lindhe *et al.*, 1999). In an attempt to remove the plaque microflora the periodontium mounts an immune response. In susceptible individuals this can result in dysregulated production of immuno-modulatory mediators (cytokines, chemokines, prostanoids, and enzymes); which actually fail to clear the pathogens and cause bystander damage (Graves, 2008). In addition, evidence is now emerging that suggests elevated levels of these immune system mediators migrate into the peripheral circulation and influence the aetiology of other diseases or conditions such as rheumatoid arthritis, diabetes and cardiovascular disease (Kaur, *et al.*, 2013; Pihlstrom *et al.*, 2005; Williams *et al.*, 2008). The prominent role of the inflammatory response in the pathogenesis of periodontal disease and associated conditions therefore suggests that host response modulation may provide novel therapeutic interventions (Preshaw, 2008).

1.2 Dental biofilm

Dental biofilm (also known as dental plaque) has similar properties with biofilms found in other parts of body and the environment. Dental biofilm is a complex multi-species biofilm with over 800 bacterial species being isolated by culture methods (Aas *et al.*, 2005; Becker *et al.*, 2002; Paster *et al.*, 2001; Preza *et al.*, 2008). However, this figure is now known to be a gross underestimate as advancements in microbial sequencing technologies have identified numerous un-culturable species in dental biofilm (Dethlefsen *et al.*, 2007; Keijser *et al.*, 2008). The constituent species of dental biofilm varies between individuals and is determined by the oral environment. The oral environment, in turn, is determined by factors such as genetics, age, diet, smoking, alcohol intake and individual oral hygiene practices (Marsh, 1991). These factors have profound effects on the microbial composition of dental biofilm and therefore the onset of oral pathologies such as dental caries and periodontal disease (Baehni & Takeuchi, 2003).

Dental biofilm accumulation on tooth surfaces has long been known to associate with inflammation and destruction of the periodontium (Lovdal *et al.*, 1958; Ramfjord *et al.*, 1968; Waerhaug, 1956, 1967). Initially, the biofilm bacteria themselves were thought to play the major role in the pathogenesis of periodontal disease. Loe and colleagues (1965) were amongst the earliest groups to describe the involvement of specific bacteria in periodontal disease progression. Their studies demonstrated that the composition of dental biofilm associated with a healthy gingiva tissue consists predominantly of Gram-positive bacteria with very few Gram-negative species. In contrast, there was up to a 40 % increase in the number of Gram-negative bacteria in dental biofilm associated with an inflamed gingiva. Therefore, these authors introduced the specific plaque hypothesis (Loesche, 1976). The introduction of this hypothesis led to the quest to find specific pathogenic organisms that may be responsible for the aetiology of periodontal disease. This led in the coming years to the identification of around 20 culturable bacterial species which had associations with periodontal disease (Paster*, et al.*, 2001). Of these species, only a few are well-studied; for example *Porphyromonas (P.) gingivalis*, *Tannerella (T.) forsythus*, *Aggregatibacter (A.) actinomycetemcomitans*, *Campylobacter (C.) rectus*, *Streptococcus (S.) constellatus*, *Fusobacterium (F.) nucleatum*, and *Treponema (T.) denticola* (Estrela *et al.*, 2010; Komiya Ito *et al.*, 2010; Paster*, et al.*, 2001; Slots & Ting, 1999; Socransky *et al.*, 1998; Socransky *et al.*, 1988). However, sequence-based mapping of the oral microbiota has identified the presence of around 1179 taxa in dental biofilm and showed that 68 % of the phylotypes present were known un-culturables (Dewhirst *et al.*, 2010). This therefore raises the possibility that some of those bacterial species we are yet to culture have important roles in the pathogenesis of periodontal disease.

The formation of dental biofilm starts with the establishment of the salivary pellicle on enamel surfaces immediately after tooth brushing. The early colonisers attach to this salivary pellicle. Early colonising species are predominantly (60 – 90 %) *Streptococci*, with the remainder made up of a variety of other species including *Capnocytophaga*, *Actinomyces*, *Eikenella*, *Haemophilus*, *Prevotella*, *Propionibacterium* and *Veillonella* (Kolenbrander *et al.*, 2010; Nyvad & Kilian, 1987). The early colonising species grow laterally and co-aggregate to form a niche environment which propagates their growth and

survival. This leads to an increase in the thickness of the biofilm (vertical growing) (Filoche *et al.*, 2010; Socransky & Haffajee, 2005). Co-aggregation between bacterial species has been demonstrated to be important for bacterial colonisation, metabolic communication, genetic exchange (Hojo *et al.*, 2009) and therefore survival during early biofilm formation (Bradshaw *et al.*, 1998). Without mechanical disruption of early dental biofilm, the colonising species continue to grow and proliferate causing changes in biofilm physiology. The metabolic activity of the aerobic species reduces the oxygen concentration and pH within the biofilm promoting colonisation of the intermediate and subsequent late species (Hojo*, et al.*, 2009). *F. nucleatum* is a prominent intermediate species and has been isolated from dental biofilm associated with periodontal health and disease. Importantly, *F. nucleatum* was demonstrated to co-aggregate with both early and late colonising species in dental biofilm and therefore this species is an important bridging organism that promotes pathogenic biofilm formation (Kolenbrander *et al.*, 2002). The presence of *F. nucleatum*, as well as physiological changes in the biofilm micro-environment, thus provide the perfect conditions for the late colonising pathogenic Gram-negative anaerobes, such as the *Actinobacillus, Prevotella, Porphyromonas* and *Treponema* species (Kolenbrander*, et al.*, 2002).

P. gingivalis is a Gram-negative oral anaerobe and is one of the most studied bacterial species in relation to the pathogenesis of periodontal disease (Estrela*, et al.*, 2010). *P. gingivalis* is present in 85.7 % of biofilm samples from patients with periodontal disease, compared to only 23.1 % of samples from healthy subjects (Yang *et al.*, 2004). The presence of *P. gingivalis* has also been shown to positively correlate with clinical parameters such as the clinical probing depth of the periodontal pocket (Kawada *et al.*, 2004). Furthermore, treatment and healing outcomes have also been shown to associate with decreasing presence of *P. gingivalis* within the subgingival biofilm (Haffajee *et al.*, 1997; Kawada*, et al.*, 2004). Indeed, the importance of this organism in disease pathogenesis has been eloquently demonstrated *in vivo* as oral inoculation of *P. gingivalis* in mice caused significant inflammation, induced bone loss and periodontal tissue destruction (Hajishengallis *et al.*, 2011; Wang *et al.*, 2007a).

Although the presence of *P. gingivalis* in subgingival biofilm has long been associated with periodontal diseases (Curtis *et al.*, 2001; Lamont & Jenkinson,

1998; Van Dyke, 2007); studies have shown that *P. gingivalis* can also present in the biofilm of healthy subjects; and in fact in patients with periodontal disease *P. gingivalis* is actually present at low levels (Kumar *et al.*, 2006) compared to many other species. Therefore in recent years questions have been raised as to whether *P. gingivalis* alone is the sole aetiological agent for periodontal disease. In fact, oral inoculation of *P. gingivalis* into specific pathogen free mice, but not the germ free mice, was shown to induce periodontal bone loss (Hajishengallis, *et al.*, 2011). This therefore demonstrated the contributing role of commensal bacteria in *P. gingivalis*-induced bone loss. In addition, *P. gingivalis* inoculation into specific pathogen free mice led to the increase in bacterial load compared to the sham control. Therefore *P. gingivalis* was found to be important in promoting biofilm formation which was in agreement with previous findings in a rabbit periodontitis model (Hasturk *et al.*, 2007). These studies led to a change in researcher's attitude toward the role of *P. gingivalis* in periodontal disease pathogenesis. Previously, it was thought periodontal diseases were associated with an increased dental biofilm biomass (Loe, *et al.*, 1965; Loesche & Syed, 1978; Moore *et al.*, 1982; Theilade *et al.*, 1966; Zee *et al.*, 1996). However, studies on subgingival biofilm stability showed that a healthy periodontium was associated with 75.5 % conservation of biofilm microbiota whilst diseased or deteriorating periodontal conditions were often associated with < 50 % conservation (Kumar, *et al.*, 2006). In addition, health-associated dental biofilm was shown to be inhabited by a rich diversity of bacterial flora and this diversity was reduced in biofilm associated with periodontal diseases; with putative periodontal pathogens becoming the prominent species (Kistler *et al.*, 2013). Therefore, it is now apparent that the constituent species of dental biofilms is a more important factor than bacterial load. In addition, *P. gingivalis*, even at low levels, can alter the composition of biofilm flora therefore the current concept implicates *P. gingivalis* as being a keystone pathogen shaping the dental biofilm community and disease pathogenesis (Darveau *et al.*, 2012).

Despite *P. gingivalis* having been shown to be associated with the onset and progression of periodontal diseases (Curtis, *et al.*, 2001; Lamont & Jenkinson, 1998; Van Dyke, 2007), the fact still remains that *P. gingivalis* has been reported to be present in biofilm of periodontally healthy individuals (Bik *et al.*, 2010; Ximenez-Fyvie *et al.*, 2000) and subjects are not equally susceptible to *P.*

gingivalis exposure (Johnson *et al.*, 1988; Savitt & Socransky, 1984). This therefore points to a far more complex pathogenesis for periodontal diseases involving not just oral pathogens but other factors such as the host immune response.

1.3 Host immune response and periodontal disease

The host immune response is important in maintaining the health of periodontal tissues. This is particularly highlighted in patients with immunodeficiencies. Patients with functional leukocyte disorders such as Chediak-Higashi syndrome and chronic granulomatous disease, which manifest as compromised neutrophil responses, have been demonstrated to be at greater risk of periodontal disease (Deas *et al.*, 2003; Kinane, 1999; Tempel *et al.*, 1972). In addition, patients with neutropenias (chronic neutropenia, chronic benign neutropenia and cyclic neutropenia), which are granulocyte disorders characterized by an abnormally low number of neutrophils have also been shown to have increased periodontal inflammation and bone loss (Baehni *et al.*, 1983; Deas, *et al.*, 2003; Deasy *et al.*, 1980; Stabholz *et al.*, 1990). Furthermore, patients with human immunodeficiency virus (HIV) infection, which is characterised by decreased numbers of peripheral $CD4^+$ T cells, were found to be susceptible to periodontal disease (Lucht *et al.*, 1991).

The presence of pathogens in periodontal pockets will activate innate and adaptive immune responses in an attempt to clear the pathogenic threat as well as promote tissue homeostasis. However, the persistent presence of pathogens can cause the continuous activation of innate and adaptive immune responses; which in turn causes inappropriate inflammatory mediator (cytokine, chemokine, antimicrobial proteins and enzymes) synthesis and secretion that directly or indirectly lead to periodontal tissue destruction (Monack *et al.*, 2004; Preshaw & Taylor, 2011). These inflammatory mediators, which can be produced by periodontal host cells in response to pathogen, are known to cause degradation of extracellular matrix of periodontal tissue (Liu *et al.*, 2010). In addition, they can play important roles in driving osteoclast activity and therefore promoting loss of alveolar bone (Bartold, *et al.*, 2010).

1.3.1 Innate immunity and periodontal disease

The formation of a dental biofilm usually occurs on tooth surfaces at the occlusal area and gingival margin. Without mechanical disruption, the biofilm will grow into a thick mature biofilm extending into the subgingival area (subgingival biofilm). The subgingival biofilms are comprised of mostly Gram-negative, anaerobic bacteria which lead to the deposition of virulence factors into the gingival crevicular fluid (GCF). These substances can cause injury to host cells directly. However, the host is equipped with an innate defence system which is designed to recognise these substances and protect the tissue from microbial attack.

Cytokines and chemokines play important roles in initiating immune responses through activation of innate immunity (Medzhitov, 2010). In the periodontium, host cells such as epithelial cells, fibroblasts, macrophages and dendritic cells play a key role in the initial sensing of microbial presence through an array of pattern recognition receptors (PRRs) expressed on their surfaces (Andrukhov *et al.*, 2013; Beklen *et al.*, 2008; Jotwani *et al.*, 2010; Mahanonda *et al.*, 2009; Shimada *et al.*, 2012). In health the presence of commensal bacteria in a dental biofilm activate a low level innate immune response. This low level response is important in priming host tissue cells and promoting tissue homeostasis. A shift in the composition of the dental biofilm and the presence of pathogenic organisms however cause an amplification of this immune response by localised cells (Handfield *et al.*, 2008; Taylor, 2010). The greater presence of pathogenic organisms leads to an increase in the number of microbe associated molecular patterns (MAMPs) derived from pathogens which drive tissue inflammation (Hajishengallis, 2009). Activation of PRRs (e.g., Toll-like receptor-2 (TLR-2), TLR-3, TLR-4 and TLR-5) by respective MAMPs induce increased expression of cytokines and chemokines such as interleukin-8 (IL-8), IL-6, IL-1β, interferon gamma (IFN-γ), IL-4, IL-12, tumor necrosis factor alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), C-X-C motif chemokine-10 (CXCL10), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, chemokine (C-C motif) ligand-20 (CCL20), eotaxin and eotaxin-2 (Andrukhov, *et al.*, 2013; Beklen, *et al.*, 2008; Eskan *et al.*, 2007; Hosokawa *et al.*, 2013;

Jotwani, *et al.*, 2010; Kocgozlu *et al.*, 2009; Luo *et al.*, 2012; Mahanonda, *et al.*, 2009; Milward *et al.*, 2013; Shimada, *et al.*, 2012).

Many of the pathogenic organisms found in dental biofilm possess a host of virulence factors. Many of these virulence factors are termed MAMPs. MAMPs are highly conserved structures of microorganisms such as lipopolysaccharide (LPS), peptidoglycan, lipoprotein, bacterial DNA and double stranded RNA (Mahanonda & Pichyangkul, 2007). MAMPs interact with PRRs, such as TLRs, and initiate innate immune responses. Numerous resident and recruited host cells of periodontal tissues express surface TLRs. These include neutrophils, langerhans cells, monocytes/macrophages, osteoblasts, periodontal ligament fibroblasts, gingival fibroblasts and gingival epithelial cells (Mahanonda & Pichyangkul, 2007). Interactions between MAMPs and TLRs leads to information transmission through intracellular signalling pathways that in turn leads to the expression of inflammatory mediators and antimicrobial agents as well as the promotion of immune cell differentiation and activation. Therefore TLRs play a major role in initiating defence mechanisms aimed to eradicate pathogenic threats.

P. gingivalis possesses several inherent MAMPs such as LPS, fimbriae and bacterial DNA, which are capable of invoking innate immune responses (Bostanci & Belibasakis, 2012). LPS is a major component of the outer membrane of Gram-negative bacteria. The main function of LPS is to provide structural integrity and protection to the bacteria. *P. gingivalis* LPS is recognised by TLR-2 and -4 (Darveau *et al.*, 2004). *P. gingivalis* LPS activation of TLR-2 and TLR-4 has been shown to induce monocytes and macrophages to produce pro-inflammatory cytokines and chemokines such as TNF-α, IL-12, IL-1β, IL-7, IL-8, IL-17A, CXCL2, CXCL10, CCL5 and IFN-γ, as well as vascular factors such as vascular cell adhesion molecule 1 (VCAM-1) and vascular endothelial growth factor (Bostanci *et al.*, 2007; Hirschfeld *et al.*, 2001; Zhou *et al.*, 2005). In oral epithelial cells, LPS of *P. gingivalis*, via TLR-2, was also shown to induce increased expression of IL-6, IL-8, IL-1β, IL-1α, TNF-α, GM-CSF, eotaxin, eotaxin 2, CXCL10, MIP-1α and MIP-1β (Kocgozlu, *et al.*, 2009; Luo, *et al.*, 2012; Milward, *et al.*, 2013). Therefore the evidence suggests that TLR-2 plays a key role in driving the oral innate immune response against *P. gingivalis*. Indeed, the persistent activation of TLR-2 by *P. gingivalis* may therefore play a role in periodontal disease pathogenesis. This was elucidated further *in vivo* as TLR-2-deficient mice were

shown resistant to bone loss following oral infection with *P. gingivalis* (Burns *et al.*, 2006).

The fimbriae of *P. gingivalis* is a thin, filamentous, cell surface appendage that is involved in facilitating cellular adherence, and also contributes to host virulence. Through these fimbriae, *P. gingivalis* can adhere to early colonizing bacteria and therefore play a prominent role in the formation of dental biofilms (Bostanci, *et al.*, 2007). Fimbriae of *P. gingivalis* has also been shown to induce production of pro-inflammatory cytokines and chemokines, such as IL-1ß, IL-8, IL-6 and TNF-α, from host cells like dendritic cells, macrophages and endothelial cells via TLR-2 and TLR-4 (Aoki *et al.*, 2010; Davey *et al.*, 2008; Jotwani & Cutler, 2004; Pollreisz *et al.*, 2010; Takahashi *et al.*, 2006; Zhou, *et al.*, 2005). In addition, the fimbriae of *P. gingivalis* has also shown to induce production of IL-1ß, IL-6 and IL-8 by gingival epithelial cells; again via TLR-2 (Asai *et al.*, 2001; Gao *et al.*, 2012).

The deoxyribonucleic acid (DNA) of bacteria is known to be involved in activation of immune responses. The un-methylated CpG (-C-phosphate-G-) dinucleotide component of bacterial DNA is known to be recognised by host cells via TLR-9 (Dalpke *et al.*, 2006). In monocytes, DNA of *P. gingivalis* was shown to induce increased expression of IL-1ß, IL-6, IL-8 and TNF-α via TLR-9 (Sahingur *et al.*, 2010; Sahingur *et al.*, 2012). In addition, *P. gingivalis* and *A. actinomycetemcomitans* DNA induced increased expression of TNF-α and IL-6 in macrophages, gingival fibroblasts and HEK293 cells (human embryonic kidney 293 cell line) which had been transfected with TLR-9 (Nonnenmacher *et al.*, 2003). However, study also showed immunosuppression effect of bacterial DNA. For example, DNA of *P. gingivalis* was shown to upregulate the expression of the suppressor of cytokine signalling (SOCS), including SOCS1 and SOCS5 and downregulate the expression of IL-10 by cultured splenocytes (Taubman *et al.*, 2007).

As well as inducing the release of cytokines and chemokines, activation of TLRs can also induce the increased expression and release of host antimicrobial agents. Once such family of molecules are the antimicrobial peptides (AMPs); which includes the α-defensins, β-defensins, cathelicidins (LL-37) and calprotectin. AMPs are also defined as host defense peptides because of their

essential role in innate immunity. AMPs are generally comprised of less than 50 amino acids and characterized by their cationic and amphipathic properties. In general, when AMPs are folded in membrane mimetic environments, one side of the AMP is positively charged (mainly due to lysine and arginine residues) and the other side contains a considerable proportion of hydrophobic residues (Shai, 1999). The microbiocidal activity of AMPs is related to this hydrophobic and cationic structure. These properties facilitate their attraction and attachment to the anionic membranes of bacteria, viruses and fungi. This amphipathic structure leads to the creation of pores in microbial membranes which increase membrane permeability and ultimately leads to disruptions in ion gradients and energy dissipation and hence cell lysis (Izadpanah & Gallo, 2005). In addition to their microbiocidal function many AMPs also play a role in dictating immune responses in a cytokine/chemokine-like fashion. For example, cathelicidin (LL-37) is a chemoattractant of neutrophils, monocytes and T cells through the formyl peptide receptor-like 1 (FPRL1) (De *et al.*, 2000). In addition, human β-defensin-2 was shown to induce mast cells to release histamine and produce prostaglandin D2 (Befus *et al.*, 1999).

The complement system consists of small protein networks which are involved in innate and adaptive immune responses to microorganisms (Dunkelberger & Song, 2010). The complement system consists of three different converging pathways: the classical pathway, the lectin pathway and the alternative pathway. Activation of the classical pathway and lectin pathway require binding of antibody and its antigen, and binding of mannose binding lectin (MBL) to a pathogen's carbohydrate moieties respectively. The activation of the alternative pathway depends on the spontaneous formation of C3b (from C3) which binds to carbohydrates, lipids and proteins on the surface of foreign objects; including bacteria (Sarma & Ward, 2011). Activation of the complement system leads to the production of anaphylatoxins C3a and C5a and vasoactive amines. Vasoactive amines cause an increase in vascular permeability, an important stage in the acute inflammatory response. In addition, C3a and C5a activate resident mast cells inducing the release of cytokines such as TNF-α, which increases the expression of adhesion molecules that further promote migration of polymorphonuclear leukocytes to sites of inflammation (Ohlrich *et al.*, 2009). *In vitro* and *in vivo*, C3a and especially C5a are also found to be powerful

chemoattractants that attract neutrophils, monocytes and macrophages to the site of inflammation upon activation (Ohlrich, *et al.*, 2009; Toews & Vial, 1984; Toews *et al.*, 1985; van Lookeren Campagne *et al.*, 2007). Activation of C5a promotes inflammation through C5a-induced vasodilation, increased vascular permeability and flow of inflammatory exudate that encourage migration of polymorphonuclear leukocytes and monocytes/macrophages to the site of inflammation (Krauss *et al.*, 2010; Snyderman, 1972). The bacterial killing by the complement system is achieved by promotion of phagocytosis (e.g., through the 3b opsonin), and also by direct killing of bacteria through the C5b-9 membrane attack complex (Ricklin *et al.*, 2010). Levels of cleaved C3 have been shown to be higher in the GCF of the gingivitis patients (Attstrom *et al.*, 1975; Niekrash & Patters, 1986; Patters *et al.*, 1989). In addition, even higher levels of cleaved C3 are found in the GCF of patients with chronic periodontitis (Monefeldt *et al.*, 1995; Niekrash & Patters, 1985; Niekrash *et al.*, 1984). Similarly, GCF levels of C5 were shown to be higher in chronic periodontitis (Attstrom, *et al.*, 1975) and C5 was highly expressed in gingival tissue explant cultures from chronic periodontitis patients (Lally *et al.*, 1982).

The resident cells of periodontal tissues include epithelial cells, gingival and periodontal ligament fibroblasts, endothelial cells, dendritic cells, osteoblasts, osteoclasts and cementoblasts (Hans & Hans, 2011). In the presence of pathogens, chemokines such as IL-8 and CXCL10 are released by these resident cells and function to induce the migration of other immune cells such as polymorphonuclear leukocytes, monocytes and T lymphocytes into tissues (Larsen *et al.*, 1989; Modi *et al.*, 1990; Taub *et al.*, 1993). The migrating immune cells, in conjunction with resident cells, serve to regulate periodontal innate immunity. GCF contains approximately 95 % polymorphonuclear leukocytes, 1-3 % monocytes/macrophages and 1-2 % lymphocytes (Ebersole, 2003); and activation of these cells, especially polymorphonuclear leukocytes and monocytes/macrophages plays a key role in the early defence of periodontal tissues by recognising, engulfing and killing microorganisms. Complement activation by periodontal pathogens, such as *P. gingivalis*, induces an acute inflammatory response which is characterised by vasodilation, increased vascular permeability and increased flow of inflammatory exudate to the site of inflammation. Cell migration is aided by the increased expression of a number of

chemokines (e.g., IL-8, CXCL10 and CCL20) by oral keratinocytes in response to *P. gingivalis* (Dommisch *et al.*, 2010; Eskan *et al.*, 2008b; Kinane *et al.*, 2006). IL-8 is a known chemoattractant for polymorphonuclear leukocytes and T lymphocytes (Larsen, *et al.*, 1989; Modi, *et al.*, 1990) and CXCL10 is known as a chemoattractant for monocytes and T lymphocytes (Taub, *et al.*, 1993). At sites of infection/inflammation, polymorphonuclear leukocytes identify bacteria through opsonins (e.g., IgG and C3b); host-derived molecules that adhere to bacterial surfaces and target the organisms for engulfment and phagocytosis (Nussbaum & Shapira, 2011). Polymorphonuclear leukocytes also kill bacteria directly through the release of oxidative and enzymatic molecules (Nussbaum & Shapira, 2011; Scott & Krauss, 2012). Like polymorphonuclear leukocytes, macrophages also identify bacteria through opsonins (e.g., IgG and C3b) and also destroy them by phagocytosis (Stuart & Ezekowitz, 2005; van Lookeren Campagne, *et al.*, 2007). Through surface receptors such as TLRs, cluster of differentiation 14 (CD14) and CD36 macrophages can recognise microbial pathogens by their MAMPs. Activation of macrophage TLRs then promote their antimicrobial action, leading to phagocytosis and the further expression of cytokines and chemokines, which in turn promote further migration and activation of phagocytes and therefore propagate the inflammatory response (Taylor *et al.*, 2005).

Dendritic cells are the most important antigen presenting cells (Steinman, 1991). Langerhans cells, a unique epithelial subset of dendritic cells were found in high number in the sulcular epithelium, and their presence was found to be positively associated with dental biofilm formation (Wilensky *et al.*, 2013). Dendritic cells are known for their capability to phagocytose and endocytose pathogens or antigens. Once internally processed, dendritic cells generate a major histocompatibility complex (MHC)-peptide complex and migrate to secondary lymphoid organs to interact with and activate T lymphocytes (Thery & Amigorena, 2001). Although not as competent as dendritic cells, macrophages have also been shown to have the capacity to act as an antigen presenting cells (Barker *et al.*, 2002; Unanue, 1984). Therefore dendritic cells and macrophages act as important cells that link innate and adaptive immunity within the periodontium.

1.3.2 Adaptive immunity and periodontal disease

There have been numerous studies which indicate an important role for adaptive immunity in the pathogenesis of periodontal disease. Anti *P. gingivalis* antibodies were found in serum of patients with chronic periodontitis but not in healthy subjects (Kojima *et al.*, 1997; Maeda *et al.*, 1994; Tabeta *et al.*, 2000; Whitney *et al.*, 1992). In addition, the antibody levels were found to be positively associated with the levels of *P. gingivalis* in dental biofilm (Kojima, *et al.*, 1997). The anti *P. gingivalis* antibody titre was also found to be elevated in GCF of patients with periodontal disease (Mooney & Kinane, 1997; Reinhardt *et al.*, 1989; Tew *et al.*, 1985) and the levels in GCF were found to be higher compared to the levels in serum (Reinhardt, *et al.*, 1989; Tew, *et al.*, 1985). These indicate the involvement of antibody producing cells and therefore adaptive immunity in periodontal disease.

The number of T cells and B cells is elevated in gingival tissue of patients with periodontal disease. For example, immunohistochemistry and flow cytometry showed increased numbers of T cells and B cells were present in gingival biopsies from advanced chronic periodontitis patients compared to healthy subjects (Berglundh *et al.*, 1998). Lappin and colleagues (1999) showed immunohistochemically that numbers of B cells and T cell were increased in periodontal tissue samples compared to healthy subjects and that there were more B cells than T cells in the diseased periodontal tissue. Furthermore T helper type 17 (Th17) cells have been found within the periodontium in periodontal disease patients and are implicated to play an important osteoclastogenic role (Sato *et al.*, 2006). Berglundh and Donati (2005) reviewed studies investigating the presence of immune cells in periodontal samples (biopsies, GCF and blood) and found that plasma cells are the most common cells (50 %), followed by B cells (about 18 %) and that total T cells combined contributed only 10 % of the total immune cell population.

Animal models have shown that lymphocytes are involved either directly or indirectly in periodontal disease pathogenesis. For example, Baker and colleagues (1999) studied the severe combined immunodeficient (SCID) mice, which are lacking in B and T lymphocytes. SCID mice challenged with *P. gingivalis* exhibited less bone loss compared to their immune-competent wild

type counterparts, suggesting that the B and T lymphocytes are involved in bone resorption. In addition, studies using non-obese diabetic (NOD)/SCID mice, engrafted with human peripheral blood lymphocytes ($CD4^+$ T cells) from a patient with localized juvenile periodontitis, then challenged with *A. actinomycetemcomitans*, exhibited greater bone loss than wild type control mice (Teng *et al.*, 2000). Furthermore, adoptive transfer of *A. actinomycetemcomitans*-responsive B cells to athymic (without thymus) rats caused an increase in bone resorption when the rats were challenged with *A. actinomycetemcomitans* compared to rats immunized with non-antigen specific cells (Han *et al.*, 2006). Collectively these studies demonstrate that lymphocytes have a contributing role in periodontal disease pathogenesis.

In vitro, oral pathogens were shown to induce cytokine release from oral epithelial cells, which in turn induced human monocyte-derived dendritic cells (MDDCs) to mediate polarisation of T helper type 2 (Th2) cells from $CD4^+$ T cells (Rimoldi *et al.*, 2005). Conversely, oral pathogens could also directly induce MDDCs to mediate polarization of T helper type 1 (Th1) cells from $CD4^+$ T cells. Human MDDCs in response to the periodontal pathogen *P. gingivalis* were shown to induce maturation and polarization of $CD4^+$ T cells towards both Th1 and Th2 cells (Jotwani *et al.*, 2003). In addition, the importance of T cells in protecting periodontal tissues was shown *in vivo* as T cell deficient rats were found to suffer greater periodontal bone loss compared to control wild type rats (Yoshie *et al.*, 1985). Additionally, temporarily B lymphocyte deficient rats inoculated with a mixture of periodontal pathogens were also shown to present with greater periodontal bone loss compared to controls (Klausen *et al.*, 1989). Together, *in vivo* evidence indicates a potential role for adaptive immunity in the pathogenesis of periodontal disease. However, to date, our understanding of this role is still limited.

1.3.3 The role of the host immune response in soft tissue destruction

One of the major clinical hallmarks of periodontal disease is the destruction of the soft tissues which support the teeth. The destruction of periodontal soft tissues can be mediated both by bacterially derived factors as well as host response molecules.

P. gingivalis possesses several inherent virulence factors which are capable of invoking damaging effects on host cells (Bostanci & Belibasakis, 2012). Gingipains are a group of cysteine proteinases secreted by *P. gingivalis*. Up to 85 % of the total proteolytic activity of *P. gingivalis* is mediated by gingipains (Potempa *et al.*, 1997). Gingipains have various effects on the immune system. They have been shown to be capable of disrupting the function of T cells by cleaving surface receptors such as CD2, CD4 and CD8 (Kitamura *et al.*, 2002). They are also capable of inactivating cytokines such as IL-4, IL-5 and IL-12 by their proteolytic activity (Tam *et al.*, 2009; Yun *et al.*, 2001) and therefore disrupting immune regulation. In addition, gingipains are also known to encourage adhesion of *P. gingivalis* to host epithelial cells and fibroblasts (Andrian *et al.*, 2004; Chen *et al.*, 2001) and directly degrade extracellular matrix components such as laminin, fibronectin, collagen type III, IV and V (Potempa *et al.*, 2000).

In addition to gingipains, *P. gingivalis* secrete enzymes such as chondroitinase and heparitinase, which are capable of degrading the proteoglycans within the human gingiva (Smith *et al.*, 1997). In addition, *P. gingivalis* is also known to produce proteases such as collagenase, fibrinolysin and phospholipase A, which directly degrade periodontal tissues (Schenkein *et al.*, 1999). The activity of these enzymes promotes the permeation of *P. gingivalis* into the gingival epithelium and can provide a gateway for other organisms to invade. In addition, these enzymes play a direct role in localised tissue destruction.

Under normal physiological conditions, periodontal tissues achieve homeostasis by continuous remodelling of connective tissues. This is achieved by the degradation of the old, injured or infected extracellular matrix (ECM). The ECM is comprised of interstitial and basement membrane which in turn are held together by a variety of proteins: collagen, fibronectin, laminin and proteoglycans. These proteins can be degraded by endopeptidases, for example, the matrix metalloproteinases (MMPs); metal-dependant endopeptidases which play important roles in remodelling by degradation of the ECM (Birkedal-Hansen, 1993). Fibroblasts play a very important role in restoring the degraded ECM by synthesising and secreting collagen (Midwood *et al.*, 2004). The processes of ECM synthesis and degradation occurs throughout life and are finely balanced in order to maintain tissue homeostasis. However, in diseases such as arthritis and

cancer degradation of the ECM is not balanced by synthesis, which in part is due to inappropriate regulation of endopeptidase activity (Reynolds *et al.*, 1994).

There are four major groups of MMPs; collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10 and MMP-11) and membrane-type (MMP-14, MMP-15, MMP-16, MMP- 17) (Sorsa *et al.*, 2004). Collectively, members of the MMP family are able to degrade most of the ECM macromolecules (Birkedal-Hansen, 1993). MMPs are secreted in the form of a pro-enzyme by host cells such as fibroblasts, keratinocytes, endothelial cells and monocytes/macrophages. The release of MMPs by these cells is regulated by cytokines and growth factors such as IL-1, TNF-α, platelet-derived growth factor (PDGF), transforming growth factor alpha (TGF- α) and epidermal growth factor (EGF) which are mostly released by host cells after tissue injury or during inflammation (Birkedal-Hansen, 1993; Reynolds, *et al.*, 1994). The activities of MMPs are controlled by tissue inhibitors of metalloproteinases (TIMPs) whose expression is also regulated by host cells such as keratinocytes, fibroblasts and monocytes/macrophages (Birkedal-Hansen, 1993; Reynolds, *et al.*, 1994). The balanced activity between MMPs and TIMPs plays an important role in tissue homeostasis. Therefore, conditions which lead to increased MMP activity over TIMP activity are characterized by tissue destruction (Birkedal-Hansen, 1993; Reynolds, *et al.*, 1994).

Like other diseases that involve soft tissue destruction, such as arthritis and cancer, periodontal disease is associated with increased MPP activity (Reynolds, *et al.*, 1994). Among all MMPs, MMP-8, MMP-9 and MMP13 were identified as potential important contributors in pathologic soft tissue destruction in periodontal disease (Sorsa, *et al.*, 2004). Immunohistochemical analysis of periodontal tissue samples showed that MMP-1, MMP-3, MMP-8 and MMP-13 were highly expressed in gingival samples from periodontal disease patients; but not expressed in healthy subjects (Hernandez *et al.*, 2006; Ingman *et al.*, 1994; Sorsa *et al.*, 2011; Tervahartiala *et al.*, 2000). In addition, MMP evaluation of GCF samples showed elevated levels of MMP-2, MMP-8, MMP-9 and MMP-13 in periodontal disease patients compared to healthy subjects (Hernandez *et al.*, 2010; Hernandez Rios *et al.*, 2009; Sorsa *et al.*, 2010; Sorsa, *et al.*, 2011). Additionally, periodontal treatment was also shown to reduce the GCF level of MMP-8 (Hernandez, *et al.*, 2010; Mantyla *et al.*, 2006) as well as the plasma

level of MMP-9, and a reduction in levels was associated with periodontal healing (Marcaccini *et al.*, 2009). The pathologic soft tissue destruction in periodontal disease was also seen to associate with increased expression of MMPs over TIMPs (Bildt *et al.*, 2008; Garlet *et al.*, 2006; Hernandez Rios, *et al.*, 2009; Pozo *et al.*, 2005).

At the cellular level, MMPs such as MMP-1, MMP-3, MMP-8 and MMP-9 were found to be expressed by oral keratinocytes, fibroblasts, endothelial cells, macrophages and polymorphonuclear leukocytes (Birkedal-Hansen, 1993; Hannas *et al.*, 2007; Ingman, *et al.*, 1994). Periodontal pathogens and cytokines were shown to regulate expression and release of the MMPs. For instance, *P. gingivalis* and *A. actinomycetemcomitans* were shown to induce gingival epithelial cells and periodontal fibroblasts to express MMP-1, MMP-2, MMP-3 and MMP-9 (Andrian *et al.*, 2007; Chang *et al.*, 2002; DeCarlo *et al.*, 1997). In addition, IL-1α, IL-1ß, TNF-α and IL-17A were shown to induce periodontal fibroblasts to express MMP-1, MMP-2, MMP-3, MMP-8, MMP-10, MMP-13 and MMP-14 (Ahn *et al.*, 2013; Beklen *et al.*, 2007; Chang, *et al.*, 2002; Cox *et al.*, 2006). Immunohistochemical analysis revealed immune cells in the periodontium such as neutrophils and macrophages, also express MMPs; such as MMP-7, MMP-8 and MMP-13 (Kiili *et al.*, 2002; Tervahartiala, *et al.*, 2000). Once released, MMPs are capable of mediating the degradation of the extracellular matrix, including the interstitial and basement membranes of the periodontium (Birkedal-Hansen, 1993). In addition, MMPs are also capable of processing the degradation of the bioactive substrates such cytokines, chemokines, growth factors, and immune modulators thereby mediating the inflammatory response that contributes to the pathogenesis of periodontal disease (Kuula *et al.*, 2009; Sorsa *et al.*, 2006).

1.3.4 The role of the host immune response in hard tissue destruction

Like the soft tissues, hard tissue (bone) undergoes life-long remodelling to maintain homeostasis. In bone remodelling, the matured and injured bone tissue is removed and replaced with new bone tissue. Bone remodelling occurs in response to a functional demand such as mechanical loading, where the bone tissue is removed when not required and added to in response to an increased load. There are two cell types that are directly responsible for bone tissue

remodelling: osteoblasts and osteoclasts. Osteoblast cells are responsible for bone tissue deposition while osteoclast cells are responsible for resorption of old or injured bone tissue. The imbalance of osteoclast cell activity over osteoblast cell activity leads to increased osteoclastogenesis (bone destruction) over osteblastogenesis (bone formation) and therefore bone resorption. This phenomenon is seen in chronic inflammatory diseases such as arthritis and periodontal disease (Figure 1-1).

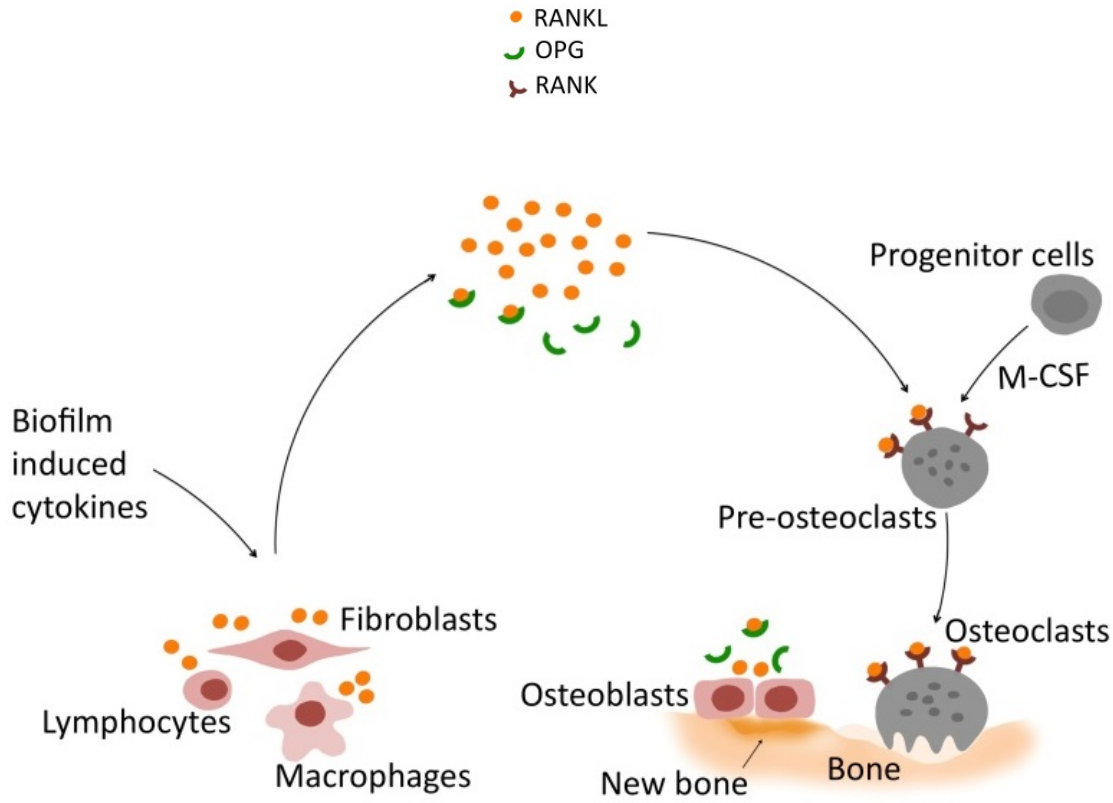

Figure 1-1: Bone remodelling during chronic inflammation

In health, bone resorption by osteoclasts and bone deposition by osteoblasts occurs in balance to maintain alveolar bone homeostasis. Homeostasis is controlled by balanced expression of RANKL and OPG within the extracellular area. However, chronic inflammation leads to an increase in RANKL production by host cells such as fibroblasts, osteoblasts, macrophages and lymphocytes. This overproduction of RANKL increases the RANKL:OPG ratio allowing increased RANKL to bind to RANK on pre-osteoclast cells surfaces. The RANKL/RANK binding complex signals osteoclast formation and leads to increased bone resorption. RANKL = receptor activator of nuclear factor-κB ligand; RANK = receptor activator of nuclear factor-κB; OPG = osteoprotegerin; and M-CSF = macrophage colony-stimulating factor.

Osteoclasts are multinucleated cells that are derived from the monocyte/macrophage lineage (Boyle *et al.*, 2003; Lerner, 2000). Osteoclast cells require cytokines to regulate proliferation and activation. One of the earliest cytokines identified in mediating their activation is M-CSF, which is produced by many cell types including osteoblasts, fibroblasts, endothelial cells, macrophages and lymphocytes (Rajavashisth *et al.*, 1990; Rambaldi *et al.*, 1987; Sieff *et al.*, 1988; Yamashita *et al.*, 2012). The role of M-CSF is to promote the proliferation and survival of pre-osteoclasts as well as mature osteoclasts (Chambers, 2000). Binding of M-CSF to macrophage colony-stimulating factor receptor (M-CSFR; a member of the tyrosine kinase receptor superfamily) results in activation of several transcription factors including c-Fos (a part of a bigger Fos family of transcription factors such as c-Fos and FosB), which leads to the initiation of osteoclastogenesis (Bartold, *et al.*, 2010). M-CSF is also involved in inducing expression of receptor activator of nuclear factor kappa-B ligand (RANKL), another important mediator for osteoclast formation (Ji *et al.*, 2009). This membrane bound protein is expressed by a variety of cells, including activated T cells, B cells, osteoblasts, fibroblasts and bone marrow stromal cells (Bartold, *et al.*, 2010; Collin-Osdoby *et al.*, 2001; Katagiri & Takahashi, 2002; Quinn & Saleh, 2009). The binding of RANKL to its receptor (RANK) on pre-osteoclast cells results in osteoclast formation (Bartold, *et al.*, 2010). Therefore, *in vivo*, RANKL has been demonstrated to play a crucial role in bone resorption (Lacey *et al.*, 1998; Pettit *et al.*, 2001). The activity of RANKL is however mediated by osteoprotegerin (OPG), a soluble tumour necrosis factor receptor-like molecule that acts as decoy and blocks the RANKL–RANK binding and thus prevents osteoclastogenesis (Bartold, *et al.*, 2010). OPG is produced by endothelial cells, smooth muscle cells, osteoblasts and bone marrow stromal cells (Venuraju *et al.*, 2010). The expression of OPG is also regulated by inflammatory cytokines. For instance, an *in vitro* study showed that IL-1β, and TNF-α were capable of inducing OPG release from human umbilical vein endothelial cells (HUVECs) (Zannettino *et al.*, 2005).

The ratio of RANKL/OPG is an important and decisive factor in mediating inflammation induced bone destruction. The role of the host immune response in modulating osteoclastogenesis has been well defined. Lymphocytes, especially T cells, can exert both stimulatory and inhibitory effect on osteoclasts through

expression of mediators such as RANKL and M-CSF that are directly involved in osteoclastogenesis. In addition, lymphocytes produce pro-inflammatory cytokines such as IL-1, IL-6, TNF-α and IL-17A that induce local cells to produce RANKL (Quinn & Saleh, 2009). In contrast, lymphocytes also produce OPG, GM-CSF, IFN-γ, IFN-ß, IL-4, IL-10 and IL-13 that can inhibit osteoclastogenesis (Quinn & Saleh, 2009).

In periodontal disease, bone tissue destruction has been associated with an elevated RANKL/OPG ratio. Semi quantitative real-time polymerase chain reaction analysis showed that gingival tissue sample of chronic periodontitis patients expressed high levels of RANKL but low levels of OPG compared to samples of healthy gingival tissue (Liu *et al.*, 2003). Indeed, immunohistochemical analysis confirmed these findings at the protein level (Crotti *et al.*, 2003). The serum and GCF RANKL/OPG ratios are also elevated in patients with chronic periodontitis compared with healthy subjects (Baltacioglu *et al.*, 2014; Mogi *et al.*, 2004).

At the cellular level, RANKL was found to be highly expressed by lymphocytes and macrophages within diseased gingival tissue (Crotti, *et al.*, 2003; Kawai *et al.*, 2006; Liu, *et al.*, 2003). In contrast, OPG was found to be equally expressed by endothelial cells in tissues of both chronic periodontitis patients and healthy subjects (Crotti, *et al.*, 2003). In addition, *in vitro* analysis has showed that RANKL and OPG can also be expressed by cells of mesenchymal origin such as gingival fibroblasts and periodontal ligament cells (Kajiya *et al.*, 2010). *In vitro,* activated T cells and B cells have also been found to express high levels of RANKL. However, the expression of OPG by these cells was either below the level of detection or at most in moderate levels (Choi *et al.*, 2001; Kawai, *et al.*, 2006). The periodontal pathogen, *A. actinomycetemcomitans,* was shown to activate polarization of peripheral blood lymphocytes to become RANKL producing T cells and B cells (Kawai, *et al.*, 2006). In addition, co-culture of RANKL-producing activated $CD8^+$ T cells with osteoclast cells showed that blockage of OPG by a monoclonal antibody lead to increased osteoclastic activity (Choi, *et al.*, 2001). *In vitro*, connective tissue cells such as gingival fibroblasts and periodontal ligament fibroblasts were also found to express RANKL and OPG upon stimulation with either cytokines or pathogens. For instance, IL-1α stimulation was shown to induce increased expression of OPG (Hormdee *et al.*,

2005). In addition, *P. gingivalis* was shown to induce increased expression of RANKL and decreased expression of OPG from gingival fibroblasts and periodontal ligament fibroblasts (Belibasakis *et al.*, 2007). Therefore, the evidence suggests that periodontal pathogens can modulate the RANKL/OPG ratio and drive the hard tissue destruction associated with periodontal disease.

1.4 IL-17 family cytokines

1.4.1 Introduction

The IL-17 family cytokines is one of the most recently described. To date, the family consists of six members (IL-17A - IL-17F) (Iwakura *et al.*, 2011). IL-17 family cytokines signal through complexes of five known receptors (IL-17RA - IL-17RE) (Zhang *et al.*, 2011b). IL-17A, which is also known as IL-17, is the founding member of the IL-17 family cytokines. This cytokine was discovered in 1995 by Yao and colleagues as being expressed by a specific subset of $CD4^+$ T cells, termed Th17 cells (Yao *et al.*, 1995a; Yao *et al.*, 1995b). Since its discovery, Th17 cells have been found to play central roles in the defence of mucosal surfaces against extracellular pathogens and contribute to human and experimental autoimmunity (Gaffen, 2009a). The discovery of IL-17A and Th17 cells stimulated great interest among immunologists which eventually led to the discovery of additional members of the IL-17 family; IL-17B, IL-17C, IL-17D, IL-17E and IL-17F by a sequence homology search of IL-17A (Iwakura, *et al.*, 2011). At present our knowledge with regard to the role of the IL-17 family cytokines play in health and disease, how these cytokines can interact to co-ordinate immune responses and the exact signalling mechanisms of some family members is lacking.

1.4.2 IL-17A, IL-17F and IL-17A/F

The human IL-17A cytokine is a glycoprotein expressed as a 155 amino acid precursor protein, which is released as a mature 136 amino acid protein after cleavage to remove the signal peptide (Fossiez *et al.*, 1996). Human IL-17A is secreted as a 35 kDa homodimer which can either be glycosylated or unglycosylated (Fossiez, *et al.*, 1996; Yao, *et al.*, 1995b). The IL-17F protein

consists of 163 amino acids and shares 40 % homology with IL-17A and 26 % homology with IL-17C (Starnes *et al.*, 2001). Like IL-17A, IL-17F is expressed as a disulphide-linked homodimer glycoprotein containing a cysteine-knot motif (Fossiez, *et al.*, 1996; Hymowitz *et al.*, 2001). In addition to the formation of IL-17A and IL-17F homodimers, *in vitro* a heterodimeric IL-17A/F molecule has been shown to be produced by HEK293 cells (a human embryonic kidney cell line) and activated human $CD4^+$ T cells (Wright *et al.*, 2007).

IL-17A, IL-17F and IL-17A/F are known to be produced by immune (innate and adaptive) cells such as Th17 cells, $CD8^+$ T cells, γδ T cells, natural killer (NK) cells, natural killer T (NKT) cells, macrophages, neutrophils and mast cells; as well as stromal cells (Table 1-1). Interestingly, studies have shown that IL-17A and IL-17F are not always co-expressed. For example, evaluation of colonic epithelial cell lines (CMT93 and Colon26) revealed expression of the messenger ribonucleic acid (mRNA) encoding IL-17F and not IL-17A (Ishigame *et al.*, 2009).

Studies have demonstrated that IL-23 is important in promoting Th17 cell development from memory and naïve $CD4^+$ T cells (Aggarwal *et al.*, 2003; Harrington *et al.*, 2005; Oppmann *et al.*, 2000). In addition, stimulation of Th17 cells with IL-23 alone or in combination with phorbol 12-myristate 13-acetate (PMA) and ionomycin has been shown to increase or augment the expression of IL-17A and IL-17F (Aggarwal, *et al.*, 2003; Harrington, *et al.*, 2005; Park *et al.*, 2005). Similarly, γδ T cells, IL-17A and IL-17F were found constitutively expressed but expression was increased in response to either IL-23, PMA, ionomycin or pathogens (Do *et al.*, 2010; Haas *et al.*, 2009; Lockhart *et al.*, 2006; Martin *et al.*, 2009; Sutton *et al.*, 2009). In addition, purified mouse γδ T cells stimulated with heat-killed *Mycobacterium tuberculosis*, heat-killed Escherichia (*E.) coli* and LPS from *Salmonella minnesota* showed increased expression of IL-17A *in vitro*. Furthermore, addition of IL-23 to the stimulating culture led to further augmented expression of IL-17A (Martin, *et al.*, 2009). Table 1-1 details further the variety of stimulants which induce IL-17A, IL-17F and IL-17A/F expression in a variety of cell types.

Cell type	Cytokine expressed	Stimulators or conditions	References
Th17 cells	IL-17A IL-17F IL-17A/F	MOG peptide, IL-23, combinations of PMA & ionomycin, anti-CD3, anti-CD28 & rhIL-2	(Aggarwal, *et al.*, 2003; Chang & Dong, 2007; Harrington, *et al.*, 2005; Liang *et al.*, 2007; Park, *et al.*, 2005; Wright, *et al.*, 2007)
γδ T cells	IL-17A IL-17F	Combinations of PMA & ionomycin, heat-killed *Mycobacterium tuberculosis*, *E. coli* (heat-killed), *Candida (C.) albicans* (live), IL-23 and IL-1β	(Do, *et al.*, 2010; Haas, *et al.*, 2009; Lockhart, *et al.*, 2006; Martin, *et al.*, 2009; Sutton, *et al.*, 2009)
$CD8^+$ T cells	IL-17A	Cells derived from lesional psoriatic skin (unstimulated)	(Teunissen *et al.*, 1998)
NK cells	IL-17A	Combinations of PMA & ionomycin, and soluble toxoplasma antigen (STAg)	(Luci *et al.*, 2009; Passos *et al.*, 2010)
NKT cells	IL-17A	Combinations of IL-23 & anti-CD3 antibody	(Rachitskaya *et al.*, 2008)
Monocytes/ macrophages	IL-17A IL-17F	LPS (*E. coli*), chitin, *Bordetella pertussis* toxin, macrophages isolated from BALF of asthmatic patients and macrophages isolated from colon of IBD patients	(Andreasen *et al.*, 2009; Bosmann *et al.*, 2013; Da Silva *et al.*, 2008; Fujino *et al.*, 2003; Michel *et al.*, 2007; Song *et al.*, 2008; Starnes, *et al.*, 2001)
Mast cells	IL-17A IL-17F	TNF-α, IgG complexes, C5a, LPS (not specify), combinations of LTA (not specify) & MALP-2, IgE cross-linking and mast cells from synovial tissue of RA patients	(Hueber *et al.*, 2010; Ikeda *et al.*, 2003; Mrabet-Dahbi *et al.*, 2009)
Neutrophils	IL-17A	*Bordetella pertussis* (live), Neutrophils from kidney following acute kidney ischaemic-reperfusion injury	(Andreasen, *et al.*, 2009; Li *et al.*, 2010)
Epithelial cells	IL-17A IL-17F	*Citrobacter rodentium*, lung epithelial cells in response to I.P ovalbumin injection	(Ishigame, *et al.*, 2009; Suzuki *et al.*, 2007)

Table 1-1: Cellular distribution of IL-17A, IL-17F and IL-17A/F

MOG = myelin oligodendrocyte glycoprotein; PMA = phorbol 12-myristate 13-acetate; LTA = lipoteichoic acid; MALP-2 = macrophage-activated lipopeptide 2 ; Ig = immunoglobulin (e.g., IgG, IgE and IgE); NK cells = natural killer cells; NKT cells = natural killer T cells; RA = rheumatoid arthritis; IBD = inflammatory bowel disease; BALF = bronchoalveolar lavage fluid; and I.P = intraperitoneal.

1.4.3 Receptors for IL-17A, IL-17F and IL-17A/F

IL-17F and IL-17A have the highest homology among members of IL-17 family cytokines and they are known to bind to the same receptor. IL-17A receptor (IL-17RA) was found to be a pivotal receptor for IL-17A signalling as stimulation of fibroblast cells isolated from IL-17RA deficient mice showed no expression of the IL-17A induced chemokine CXCL1 (Toy *et al.*, 2006). Although IL-17RA also binds IL-17F and IL-17A/F, studies have shown that IL-17RA binds with high affinity to IL-17A, medium affinity to IL-17A/F and low affinity to IL-17F (Kuestner *et al.*, 2007; Wright, *et al.*, 2007). In addition, studies have shown that IL-17RC is also required for IL-17A signalling. This was demonstrated using small interfering RNA (siRNA) knockout of IL-17RA and IL-17RC in synoviocytes isolated from rheumatoid arthritis patients where deficiency of either receptor failed to induce IL-17A mediated expression of IL-6 and IL-8 (Zrioual *et al.*, 2008). In addition, IL-17RC has been shown to have similar affinities for IL-17A, IL-17F and IL-17A/F (Kuestner, *et al.*, 2007; Wright, *et al.*, 2007). Furthermore, immunoprecipitation studies also showed that IL-17RA and IL-17RC are capable of assembling *in vitro*, supporting a model in which IL-17A, IL-17F and IL-17A/F signalling are mediated by a heterodimeric receptor complex containing at least one IL-17RA and IL-17RC protein (Toy, *et al.*, 2006). Since IL-17A, IL-17F and IL-17A/F showed different binding affinity to IL-17RA it was hypothesised that they induce differential intracellular signalling strength. This was confirmed in an *in vitro* study that showed IL-17A had the greatest capability of inducing mouse lung epithelial cells to produce CXCL1, followed by IL-17A/F and IL-17F (Liang, *et al.*, 2007).

Numerous cell types, such as T cells, B cells, macrophages, dendritic cells, and colonic mucosal epithelial cells have been shown to co-express IL-17RA and IL-17RC (Ishigame, *et al.*, 2009). In addition, human foreskin fibroblast, synovial fibroblasts, brain endothelial cells and bronchial epithelial cells have been shown to be responsive to IL-17A, IL-17F and IL-17A/F stimulation. This therefore suggests a variety of cell types are capable of responding to IL-17 signalling.

1.4.4 Effect of IL-17A, IL-17F and IL-17A/F on target cells

IL-17A, IL-17F and IL-17A/F are known to be involved in inflammation and host defence against infection. Activation of signalling pathways through IL-17RA by IL-17A, IL-17F or IL-17A/F induces the release of pro-inflammatory and antimicrobial proteins, which play a major role in recruiting neutrophils, fighting pathogens and if expressed in an unbalanced fashion promote tissue destruction. IL-17A, IL-17F and IL-17A/F are known to induce expression of cytokines (e.g., IL-6, G-CSF and GM-CSF), chemokines (e.g., CXCL1, CXCL2 and CXCL8), matrix metalloproteinases (e.g., MMP1, MMP3 and MMP9), and antimicrobial peptides (e.g., β defensin-2, S100A7 and S100A8) from a variety of cells (Table 1-2). Indeed, despite being originally identified as a T cell associated cytokine the majority of IL-17A released during an inflammatory response is now known to be produced by, and act upon, innate immune cells (Cua & Tato, 2010).

Stimulant	Target cells	Effects	References
IL-17A	Macrophages	Increase expression of IL-1ß, IL-3, IL-6, IL-9, TNF-α, CCL5, MMP-3 and PGE_3	(Barin *et al.*, 2012; Ishigame, *et al.*, 2009; Jovanovic *et al.*, 1998)
IL-17A	Neutrophils	Increase expression of MMP-9 and myeloperoxidase	(Zelante *et al.*, 2007)
IL-17A	Dendritic cells	Promotes development of dendritic cells from bone marrow-derived cells and increases expression of CD11c, CD40, CD80, CD86, MHC class II	(Antonysamy *et al.*, 1999; Schnyder-Candrian *et al.*, 2006)
IL-17A IL-17F IL-17A/F	Keratinocytes	Increase expression of antimicrobial peptides (B-defensins, S100A7, S100A8 and S100A9), ICAM-1, IL-6, IL-8, CXCL1 and GM-CSF.	(Albanesi *et al.*, 1999; Albanesi *et al.*, 2000; Kawaguchi *et al.*, 2006; Liang, *et al.*, 2007; Liang *et al.*, 2006; Teunissen, *et al.*, 1998; Wright, *et al.*, 2007)
IL-17A IL-17F IL-17A/F	Fibroblasts	Increase cells proliferation and migration; and Increase expression for IL-8, IL-6, PGE_2, G-CSF, ICAM-1, MMP-1, MMP-3, MMP-13, CCL2, CCL7, CXCL1 and CXCL20	(Chang & Dong, 2007; Fossiez, *et al.*, 1996; Hu *et al.*, 2010; Park, *et al.*, 2005; Valente *et al.*, 2012; Yagi *et al.*, 2007; Yao, *et al.*, 1995b)
IL-17A IL-17F	Endothelial cells	Increase expression of IL-6, G-CSF GM-CSF, TGF-B1, TGF-B2, MCP-1, Lymphotoxin-B and IL-2	(Fossiez, *et al.*, 1996; Numasaki *et al.*, 2004a; Numasaki *et al.*, 2004b)
IL-17A IL-17F	Synoviocytes	Increased expression of IL-6, IL-8, G-CSF, GM-CSF, CXCL1, CXCL2, CXCL5, CCL20, VEGF and PGE_2	(Chabaud *et al.*, 1998; Zrioual, *et al.*, 2008)
IL-17A	Osteoblasts	Increased expression of CXCL1, CXCL2, CXCL5, CCL2, PGE_2, and RANKL	(Ruddy *et al.*, 2004; Shen *et al.*, 2005)

Table 1-2: Effect of IL-17A, IL-17F and IL-17A/F on target cells

1.4.5 Role of IL-17A, IL-17F and IL-17A/F in inflammation and infection

IL-17A, IL-17F and IL-17A/F play a number of immuno-regulatory roles during inflammation and infection. In a rheumatoid arthritis model, IL-17A was found localised to the T cell infiltrate in rheumatoid synovium samples (Chabaud *et al.*, 1999), indicating the possible role of IL-17A in the pathogenesis of rheumatoid arthritis. In addition, supernatants from synovial tissue of rheumatoid arthritis patients, cultured *ex vivo*, was found to contain biologically active IL-17A that induced *in vitro* synoviocyte cultures to produce IL-6 and leukaemia inhibitory factor (LIF) (Chabaud, *et al.*, 1998).

In a collagen induced arthritis (CIA) model, the contribution of IL-17A in the pathogenesis of arthritis was determined when the disease was found to be markedly suppressed in IL-17-deficient mice (Nakae *et al.*, 2003a). The authors also showed that IL-17A was responsible for the priming of collagen-specific T cells as well as increasing production of collagen-specific IgG2a, which suggest that IL-17A plays role in the development of CIA by activating autoantigen specific cellular and humoral responses. In addition, IL-1 receptor antagonist (IL-1RA) deficient mice showed spontaneous arthritis development; which was hypothesised to be due to unopposed activity of IL-1. However, the spontaneous development of arthritis was not observed in IL-1RA deficient mice which were also deficient in IL-17A (Nakae *et al.*, 2003b); suggesting that IL-17A plays a pivotal role in disease pathogenesis. Furthermore, elevated IL-17A expression was shown in T cells isolated from the lymph nodes of IL-1RA deficient mice.

The role of IL17F in the pathogenesis of rheumatoid arthritis is not as well characterised as IL-17A. Using the IL-1 receptor antagonist-deficient mice, the spontaneous development of arthritis was found to be only partially suppressed in mice that were also IL-17F deficient (Ishigame, *et al.*, 2009). The contribution of the IL-17A/F heterodimer to the pathogenesis of arthritis remains to be determined.

In an experimental autoimmune encephalomyelitis (EAE) study, IL-17A deficient mice exhibited significantly delayed onset and progression of disease compared to wild type mice. In addition, IL-17F deficient mice only showed a slight delay

in disease onset and progression compared to wild type mice (Yang *et al.*, 2008). This study also showed that $CD4^+$ T cell infiltration into the central nervous system tissues was greatly reduced in IL-17A deficient and IL-17F deficient mice. However, mRNA expression of the chemokines, CCL2 and CCL7 were reduced in the central nervous system of IL-17F deficient mice; but this reduction in expression was more profound in IL-17A deficient mice. In addition, CXCL1 expression in the central nervous system was severely impaired in IL-17A deficient mice but not in IL-17F deficient. This suggested that IL-17A is playing a more pivotal role than IL-17F in the initiation of inflammation in EAE. Indeed intraperitoneal injection of an anti-IL17A antibody was shown to delay the development of EAE when compared to injection with control IgG (Park, *et al.*, 2005). Furthermore, mice administered with recombinant human IL-17A (rhIL-17A) developed signs of EAE (Park, *et al.*, 2005). Similarly to arthritis, the contribution of the IL-17A/F heterodimer to the pathogenesis of EAE remain to be determined.

The roles of IL-17A and IL-17F have also been studied in inflammatory bowel diseases; such as ulcerative colitis and Crohn's disease. Immunohistochemical analysis showed that IL-17A was expressed in colonic samples of patients with ulcerative colitis and Crohn's disease, but not in healthy subjects. In addition, double-staining with anti-CD3 and anti-CD86 antibodies showed that those IL-$17A^+$ cells in the colonic samples were T cells and monocytes/macrophages (Fujino, *et al.*, 2003). IL-17A mRNA expression was also found to be significantly increased in tissue of patients with ulcerative colitis and Crohn's disease which also co-incided with increased circulating levels of IL-17A (Fujino, *et al.*, 2003). As for IL-17F, mRNA expression was found to be significantly increased in inflamed colonic lesions (Seiderer *et al.*, 2008). In an acute trinitrobenzenesulfonic acid-induced colitis mouse model, IL-17A was found to be increased in colonic tissue samples and accompanied with colonic inflammation and increased expression of IL-6 and MIP-2. However, the trinitrobenzenesulfonic acid-induced colitis was found to be suppressed in IL-17R deficient mice (Zhang *et al.*, 2006). Furthermore, in a dextran sulphate sodium (DSS) acute colitis model, symptoms such as diarrhoea, bloody faeces and increased weight loss were found to be more severe in IL-17A deficient mice, whereas milder symptoms were observed in IL-17F deficient mice as

compared to the wild type controls (Yang, *et al.*, 2008). Similarly to arthritis and EAE the contribution of the IL-17A/F heterodimer to the pathogenesis of inflammatory bowel diseases remain to be determined.

IL-17A, IL-17F and IL-17A/F were found to play important roles in infection. *In vivo*, mice intraperitoneally injected with *E. coli* LPS were shown to have increased serum levels of IL-17A, IL-17F and IL-17A/F (Bosmann, *et al.*, 2013). In a *Pneumocystis carinii* mouse infection model, IL-17A expression was found to be increased in the lung of the infected mice. In addition, if the mice were intra-nasally administered with an anti-IL17A neutralising antibody, the *Pneumocystis carinii* burden was found to increase 10,000-fold at four weeks post-infection when compared to the control mice (Rudner *et al.*, 2007). In the *Klebsiella pneumonia* lung infection model, the lung production of IL-17A and IL-17F was found to be significantly reduced in the IL-23 p19 deficient mice and these mice exhibited substantial mortality from a sub-lethal dose of *Klebsiella pneumonia* (10^3 CFU). In contrast, administration of IL-17A was shown to restore the bacterial control in the lung (Happel *et al.*, 2005). IL-17R deficient mice infected with the *Klebsiella pneumonia* also showed a 100 % mortality rate after 48 h, compared with only a 40 % mortality rate in the controls. The IL-17R deficient mice also presented with delayed neutrophil migration and greater dissemination of *Klebsiella pneumonia* compared with control mice (Ye *et al.*, 2001). The role of IL-17A, IL-17F and IL-17A/F in the recruitment of neutrophils was therefore investigated *in vivo*. In mice which were intranasally challenged with IL-17A, IL-17F and IL-17A/F, the IL-17A/F challenged mice demonstrated a significant increase in bronchoalveolar lavage fluid neutrophils, CXCL1 and CXCL5 (Liang, *et al.*, 2007). Although the induction of neutrophil recruitment was similar in mice challenged with IL-17A and IL-17A/F, the bronchoalveolar lavage fluid expression of CXCL1 and CXCL5 was significantly less in mice treated with IL-17A/F compared to those challenged with IL-17A. In addition, mice treated with IL-17F were shown to have lower levels of neutrophil recruitment and expression of CXCL1 and CXCL5. These data indicate that IL-17A, IL-17F and IL-17A/F were required in the protection against infection. However, overproduction of IL-17A, IL-17F and IL-17A/F could induce increased migration of neutrophils to the site of inflammation.

1.4.6 IL-17B, IL-17C and IL-17D

IL-17B, IL-17C and IL-17D remain poorly characterized. Homology sequence analysis showed that IL-17A, IL-17B and IL-17C shared 26 – 28 % amino acid identity (Li *et al.*, 2000a). The predicted precursor proteins of IL-17B and IL-17C were found to be 180 and 197 amino acids respectively (Li, *et al.*, 2000a). IL-17D, shares 27 % homology with IL-17B and is 202 amino acids in length, making IL-17D the largest member of the IL-17 family (Starnes *et al.*, 2002). IL-17B, but not IL-17C, was found to be expressed in the human spinal cord, pancreas, small intestine and stomach (Li, *et al.*, 2000a; Shi *et al.*, 2000). IL-17B was also found to be expressed in chondrocytes of normal bovine articular cartilage (Moseley *et al.*, 2003) and in inflamed cartilage from collagen induced arthritis mice (Yamaguchi *et al.*, 2007). IL-17B was also found to be expressed in the neuron cell body and axons (Moore *et al.*, 2002). IL-17C was found to expressed in the broad range of cells such as $CD4^+$ T cells, $CD11b^+$ MHC class II^+ macrophages, and $CD11c^+$ MHC class II^+ dendritic cells (Yamaguchi, *et al.*, 2007).

Of all the IL-17 family cytokines IL-17D is the least well studied. IL-17D has been found to be highly expressed in skeletal muscle, brain, adipose tissue, heart, lung and pancreas and lowly expressed in bone marrow, the fetal liver and kidney (Starnes, *et al.*, 2002). IL-17D was also found to be lowly expressed in resting $CD4^+$ T cells and resting $CD19^+$ B cells, and even lower expression was found in activated $CD4^+$ T cells, resting and activated $CD8^+$ T cells, resting and activated $CD14^+$ monocytes and activated $CD19^+$ B cells (Starnes, *et al.*, 2002).

1.4.7 Receptors for IL-17B, IL-17C and IL-17D

In comparison to IL-17A, IL-17F and IL-17A/F our knowledge of the receptor complexes for IL-17B, IL-17C and IL-17D is not as complete. Kinetic binding analysis showed that IL-17B binds to IL-17RB with relatively high affinity, which was confirmed *in vitro* by co-immunoprecipitation (Shi, *et al.*, 2000). IL-17RB has been shown to be expressed in the brain, skeletal muscle, lung, stomach, pancreas, liver, colon, small intestine, kidney and testis (Lee *et al.*, 2001; Shi, *et al.*, 2000). IL-17RB has also been shown to be expressed by a number of cell lines such as WRL-68 human embryonic liver cells, Colo587 pancreas

adenocarcinoma-mesothelioma cells, PANC-1 pancreatic epithelioid carcinoma cells, HeLa cancer cells, K562 leukemia cells, Raji Burkitts lymphoma cells and colorectal adenocarcinoma cell lines (Shi, *et al.*, 2000). Despite its affinity for IL-17RB, to date there is no conclusive proof that binding initiates IL-17B signalling and therefore the role of IL-17B in the immune system is not presently understood (Chang & Dong, 2011). Recently, it has been found that IL-17C is a ligand for the orphan receptor IL-17RE, and the IL-17C/IL-17RE signalling complex mediates host defence and autoimmune inflammation (Chang *et al.*, 2011; Ramirez-Carrozzi *et al.*, 2011; Song *et al.*, 2011). However, the receptor(s) for IL-17D at present remain unknown.

1.4.8 Role of IL-17B, IL-17C and IL-17D in inflammation and infection

The role of IL-17B, IL-17C and IL-17D in both health and disease remain poorly characterised. However, initial findings suggest that IL-17B, IL-17C and IL-17D have pro-inflammatory functions and play a role in the pathogenesis of chronic inflammatory diseases. For instance, in the collagen induced arthritis mouse model, levels of IL-17B and IL-17C were found to be highly elevated in the arthritic paws of mice (Yamaguchi, *et al.*, 2007). In addition, IL-17B and IL-17C bone marrow chimeric mice, prepared by intravenous injection of bone marrow precursor cells that had been retrovirally transduced with IL-17B and IL-17C, were clearly shown to exacerbate arthritis, which was accompanied with increased serum level of TNF-α (Yamaguchi, *et al.*, 2007). Intraperitoneal injection of rhIL-17B was also shown to cause a dose-dependent influx of polymorphonuclear leukocytes into the peritoneal cavity within 4 h (Shi, *et al.*, 2000). In addition, intranasal administration of adenovirus expressed IL-17C was shown to cause bronchoalveolar lavage neutrophilia (Hurst *et al.*, 2002). Additionally, *in vitro* analysis showed that IL-17B and IL-17C were capable of inducing increased expression of IL-1β from a mouse fibroblast cell line (3T3), and both IL-17B and IL-17C were also shown to induce increased expression of IL-1β, IL-6 and IL-23 from mouse macrophages (Yamaguchi, *et al.*, 2007). Furthermore, *Mycoplasma pneumoniae* and a TLR-5 agonist (flagellin) were also shown to induce IL-17C expression in lung and gut tissues (Van Maele *et al.*, 2010; Wu *et al.*, 2007).

IL-17D mRNA expression has been detected in rheumatoid nodules (Stamp *et al.*, 2008) indicating a role in joint inflammation. However, the role of IL-17D in disease pathogenesis is little studied *in vivo*. *In vitro*, IL-17D has been shown to have an inhibitory effect on myeloid progenitor cell populations (Starnes, *et al.*, 2002) and also inhibits hematopoietic progenitor colony formation (Broxmeyer *et al.*, 2006). In addition, IL-17D was also reported to induce endothelial cells to produce IL-6, IL-8 and GM-CSF via NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) dependent pathways (Starnes, *et al.*, 2002).

1.4.9 IL-17E

IL-17E is a 177 amino acid protein with 16 – 20 % homology to IL-17A, IL-17B and IL-17C (Lee, *et al.*, 2001). IL-17E mRNA has been detected in brain, spinal cord, skeletal muscle, heart, lung, salivary gland, thymus, thyroid gland, trachea, liver, spleen, small intestine, stomach, colon, uterus, placenta, kidney, prostate and testis tissue (Fort *et al.*, 2001; Kim *et al.*, 2002; Lee, *et al.*, 2001; Pan *et al.*, 2001). Studies have also shown that a number of cells are capable of expressing IL-17E including; polarized Th2 cells (Fort, *et al.*, 2001), primary bone marrow-derived mast cells (Ikeda, *et al.*, 2003), alveolar macrophages (Kang *et al.*, 2005), eosinophils (Dolgachev *et al.*, 2009), basophils (Wang *et al.*, 2007b), lung epithelial cells (Angkasekwinai *et al.*, 2007), intestinal epithelial cells (Zaph *et al.*, 2008) and brain capillary endothelial cells (Sonobe *et al.*, 2009).

IL-17RB is the known receptor for IL-17E (Lee, *et al.*, 2001; Shi, *et al.*, 2000). In addition, although *in vitro* binding assays showed IL-17E does not bind to IL-17RA directly, IL-17RA forms a complex with IL-17RB for effective IL-17E signalling (Ely *et al.*, 2009; Hymowitz, *et al.*, 2001). *In vitro*, naïve T cells isolated from wild type mice cultured in the presence of IL-17E, TGF-β and IL-4 exhibited significantly enhanced IL-9 secretion. However, no similar effect was observed with naïve T cells isolated from IL-17RA-deficient mice (Angkasekwinai *et al.*, 2010). Furthermore, splenocytes isolated from either IL-17RB knockout or IL-17RA knockout mice did not release IL-5 or IL-13 in response to IL-17E stimulation (Rickel *et al.*, 2008). In addition, *in vivo*, both IL-17RB and IL-17RA knockout mice did not respond to intranasal administration of IL-17E compared to wild type mice which exhibited increased bronchoalveolar lavage fluid

cellularity, increased bronchoalveolar lavage fluid IL-5 and IL-13 levels and also increased expression of lung CCL2, CCL11, IL-5, IL-13, IL-9 and IL-10 mRNA (Rickel, *et al.*, 2008). These studies therefore confirmed that both IL-17RA and IL-17RB are required for effective IL-17E signalling.

1.4.10 Effect of IL-17E on target cells

IL-17E is a unique member of the IL-17 family cytokines. It differs from other IL-17 family members in its ability to promote a Th2-biased inflammatory response. In addition, IL-17E is a 'double edged sword' as it also has the ability to down regulate localised destructive inflammatory responses; including the ability to inactivate the function of Th17 cells. As stated above, a number of cells express IL-17RB such as Th2 cells, mast cells, macrophages, eosinophils, basophils, epithelial cells and endothelial cells thus make those cells possible targets for IL-17E signalling.

IL-17E is known to regulate Th2 cell differentiation, which is associated with sustained expression of transcription factors JunB (Jun B proto-oncogene) and GATA-3 (GATA binding protein 3) (Angkasekwinai, *et al.*, 2007; Wang, *et al.*, 2007b). *In vitro* studies showed that IL-17E treatment of Th2 cells resulted in increased expression of the prototypic Th2 cytokine, IL-4 (Angkasekwinai, *et al.*, 2007; Wang, *et al.*, 2007b). In addition, IL-17E regulates IL-4 expression through induction of the NFATc1 (nuclear factor of activated T cells, cytoplasmic 1) and JunB transcription factors, which in turn leads to the IL-4-dependent upregulation of GATA-3 expression (Angkasekwinai, *et al.*, 2007). Interestingly, IL-17E can regulate Th2 memory cell differentiation and the production of IL-5 and IL-13 in an IL-4-independent manner, by sustained expression of transcription factors MAF, JunB and GATA-3 (Wang, *et al.*, 2007b). IL-17E was also demonstrated to promote the differentiation of the recently discovered innate cell populations; such as natural helper cells (NHCs), multipotent progenitor type2 (MMP^{type2}) cells, nuocytes, and innate type 2 helper (Ih2) cells (Moro *et al.*, 2010; Neill *et al.*, 2010; Price *et al.*, 2010; Saenz *et al.*, 2010b). In addition, stimulation of these cells with IL-17E has been shown to induce expression of Th2 related cytokines such IL-4, IL-5 and IL-13 (Saenz, *et al.*,

2010b). These finding suggest that IL-17E could also induce a Th2-biased immunity through interactions with innate immune cell populations.

IL-17E has been found to have a key role in negatively regulating potentially damaging inflammatory responses. IL-17E has been demonstrated to block the function of Th17 cells. It is known that the differentiation of Th17 cells is promoted by IL-6 and TGF-ß1, whereas IL-23 is required for the subsequent expansion or survival of Th17 cells (Weaver *et al.*, 2007). *In vitro,* addition of IL-23 to a culture of naïve $CD4^+$ T cells and LPS-activated $CD11c^+$ dendritic cells, in the presence of neutralizing antibodies against IFN-γ and IL-4, had been shown to induce IL-17-producing cells (Kleinschek *et al.*, 2007). Interestingly, addition of IL-17E to the culture caused the population of Th17 cells induced by IL-23 to reduce by half, accompanied by reduced expression of IL-17A and increased expression of IL-13. Furthermore, this study also showed that addition of an anti-IL-13 antibody to the co-culture caused an increase in the population of Th17 cells accompanied by increased expression of IL-17A (Kleinschek, *et al.*, 2007). IL-17E is known to be capable of inducing IL-13 release from immune cells such as Th2 cells, mast cells and macrophages (Angkasekwinai, *et al.*, 2010; Ikeda, *et al.*, 2003; Kang, *et al.*, 2005). It has therefore been hypothesised that IL-17E induced expression of IL-13 is the mechanism by which it regulates Th17 responses. In addition to IL-13, IL-4 has also been shown to inhibit proliferation and activation of Th17 cells by a mechanism which is dependent on STAT6 (signal transducer and activator of transcription 6) (Cooney *et al.*, 2011; Harrington, *et al.*, 2005; Park, *et al.*, 2005). Since IL-17E was also shown to induce Th2 cells to produce IL-4 (Angkasekwinai, *et al.*, 2007; Wang, *et al.*, 2007b) it has also been hypothesised that IL-17E inhibits Th17 cell proliferation via IL-4.

IL-17E can inhibit cytokine and chemokine production from cells stimulated with pathogenic bacteria. IL-17E, together with other cytokines such as IL-4, IL-13 and IL-10, has been shown to inhibit production of chemokines (e.g., CCL2, CCL3, IL-8 and CXCL9) by naive $CD4^+$ T cells stimulated with LPS or IFN-γ (Stolfi *et al.*, 2011). In addition, IL-17E was also shown to inhibit production of IL-23 and TNF-α by Th1 cells stimulated by bacterial LPS or PNG (Caruso *et al.*, 2009a; Caruso *et al.*, 2009b). Furthermore, IL-17E has been shown to directly inhibit

LPS-induced IL-23 expression by macrophages, independent of the pathway related to IL-4, IL-13 and STAT6 (Zaph, *et al.*, 2008).

Contrary to the studies demonstrating an anti-inflammatory role for IL-17E there are also some *in vitro* studies that support the role of IL-17E as a pro-inflammatory cytokine. An *in vitro* study on a human renal cell line (TK-10) showed that stimulation with IL-17E increased expression of NF-kB-responsive luciferase reporter gene activity in addition to inducing release of IL-8 (Lee, *et al.*, 2001). In addition, studies on an embryonic fibroblast cell line (NIH-3T3) (Pan, *et al.*, 2001) and primary human lung fibroblasts (Letuve *et al.*, 2006) found that IL-17E could induce the expression of CCL5, CCL11, CXCL8 (IL-8), GM-CSF and G-CSF.

1.4.11 Role of IL-17E in inflammation and infection

IL-17E is known to play a major role in Th2 driven pathologies. Elevated expression of IL-17E and IL-17RB was observed in asthmatic lung tissues, atopic dermatitis skin lesions, as well as in the parasite infected lung and gut (Hurst, *et al.*, 2002; Wang, *et al.*, 2007b). Transgenic overexpression of IL-17E in wild type mice has been shown to cause a Th2 driven inflammatory response, characterised by a mixed infiltration of neutrophils, eosinophils, lymphocytes, plasma cells and macrophages, in multiple tissues such as the liver, heart, lung, lymph nodes, kidney, spleen, and urinary bladder (Pan, *et al.*, 2001). In addition, IL-17E overexpression in lung epithelial cells was shown to associate with increased inflammation, mucus production and airway infiltration by macrophages and eosinophils (Angkasekwinai, *et al.*, 2007). Studies suggested that the IL-17E induced inflammation *in vivo* is caused by a Th2-biased response, characterised by increased expression of Th2 cytokines such as IL-4, IL-5 and IL-13. For instance, mice injected with IL-17E show signs of splenomegaly and spleen eosinophilia, which is accompanied by increased level of serum immunoglobulin (IgE and IgG1), as well as increased mRNA expression of IL-4, IL-5 and IL-13 in various organ tissues such as the spleen, stomach, small intestine, kidney, liver, lung and colon (Fort, *et al.*, 2001; Pan, *et al.*, 2001). Intranasal administration of IL-17E into naïve mice was shown to induce inflammation similar to that seen in asthmatic patients, which included increased levels of

eosinophils, increased IL-5 and IL-13 levels in BAF, goblet cell hyperplasia, and increased airway hyperresponsiveness. (Rickel, *et al.*, 2008). In addition, in an allergen induced experimental asthma model, mice which were intranasally sensitized with allergen from *Aspergillus (A.) oryzae* and chicken ovalbumin protein were shown to develop a Th2-biased response with increased eosinophilia as well as increased bronchoalveolar lavage fluid IL-4, IL-5 and IL-13 levels. However, blockage of IL-17E by intraperitoneal injection of an anti-IL-17E antibody was shown to reduce these responses (Angkasekwinai, *et al.*, 2007).

IL-17E is known *in vitro* to inhibit the Th17 cell response. Th17 cells are characterised by the production of IL-17A, IL-17F, IL-21 and IL-22 (Ouyang *et al.*, 2008). Th17 cells and their effector cytokines are involved in regulating the expression of various other cytokines and chemokines by keratinocytes and fibroblast to mediate host defence against pathogens, as well as to mediate pathogenesis of many autoimmune diseases such as rheumatoid arthritis and encephalomyelitis (Kurebayashi *et al.*, 2013; Ouyang, *et al.*, 2008). The ability of IL-17E to inhibit Th17 cells suggests it may play an important role in regulating potentially destructive localised inflammatory responses. Indeed, *in vivo*, IL-17E deficient mice were found to be susceptible to EAE; which was associated with increased peripheral expression of IL-23 as well as increased recruitment of Th17 cells into the central nervous system. The role of IL-17A in driving EAE was demonstrated by the fact that neutralisation of IL-17A with an anti-IL-17A monoclonal antibody prevented the development of disease. In addition, treatment of mice with recombinant IL-17E prevented development of EAE demonstrating the importance of this cytokine in regulating the IL-17A/Th17 response (Kleinschek, *et al.*, 2007). IL-17E deficient mice, chronically infected with the parasite *Trichuris (T.) muris* were also shown to develop severe intestinal inflammation characterised by lymphocyte infiltration, crypt elongation and an absence of goblet cells. This observation was found to be associated with increased level of pro-inflammatory cytokines such as IL-17A and IFN-γ in the mesenteric lymph node and caecum, which is consistent with the hypothesis that IL-17E could act to limit localised inflammation (Owyang *et al.*, 2006).

The role of IL-17E in microbial immunity has been a subject of study over the last 10 years. Evaluation of the large intestine of germ free mice found increased expression of IL-23, as well as an increase in the population of Th17 cells, compared to the conventionally reared mice (Zaph, *et al.*, 2008). However, the presence of commensal bacteria in the gut induced increased expression of IL-17E by intestinal epithelial cells and intraperitoneal administration of IL-17E to germ free mice reduced expression of IL-23 and the population of Th17 cells in the large intestine (Zaph, *et al.*, 2008). Consistent with a role for IL-17E in limiting IL-23 and Th17 cells in the large intestine, neutralization of IL-23 also resulted in a decreased Th17 cell response. In line with this data, IL-17E mRNA expression was found to be decreased in the ileum of germ free mice compared to the specific pathogen free mice, and expression of IL-17E mRNA in specific pathogen free mice continued to increase with age (Sawa *et al.*, 2011) indicating a possible association with microbial colonisation of the intestine. IL-17E also repressed the RORγt^{+} (RAR-related orphan receptor gamma, thymus) innate lymphoid cell production of IL-22 which was demonstrated to be important for bacterial clearance *in vivo* (Aujla *et al.*, 2008; Rubino *et al.*, 2012; Zheng *et al.*, 2008). In addition, mice fed by parental nutrition (intravenous feeding, bypassing the usual process of eating and digestion) showed decreased luminal levels of molecules of innate immunity; the Paneth cell antimicrobial molecule secretory phospholipase - A2 (sPLA2) and the goblet cell glycoprotein mucin – 2 (MUC-2). However, addition of exogenous IL-17E into diet was shown to increase luminal levels of sPLA2 and MUC-2 (Heneghan *et al.*, 2013). Additionally, the authors showed that *ex vivo* incubation of intestinal tissue segments isolated from parental feeding mice with combinations of *E. coli* and exogenous IL-17E was shown to increase tissue levels of sPLA2 and decrease *E. coli* entero-invasion. This study suggests that IL-17E could induce increased expression of MUC-2 and sPLA2 by Paneth cells which is important in clearance of pathogenic bacteria in digestive system.

In the endotoxemia mouse model, where mice were intraperitoneally injected with LPS and peptidoglycan, mice pre-treated with IL-17E (intraperitoneal injection) exhibit decreased serum levels of pro-inflammatory cytokines including IL-12/p70, TNF-α and IL-6; and this finding was found to coincide with decreased survival rate of LPS injected mice pre-treated with IL-17E (Caruso, *et*

al., 2009b). In addition, in the peptidoglycan-induced colitis mouse model, where mice were intravenously administered with peptidoglycan, mice pre-treated with IL-17E (intraperitoneal injection) were shown to have lesser severity of the colitis as shown by: the absence of diarrhoea and less weight loss; and lesser histopathological evidence of colonic inflammation, which was found to associate with decreased colonic protein levels of IL-12 and INF-γ (Caruso, *et al.*, 2009a).

1.4.12 IL-17 family cytokines and periodontal disease

The role of the IL-17 family cytokines in the pathogenesis of periodontal disease is poorly understood. In human studies, IL-17A has been shown to be associated with periodontal disease pathogenesis. There are Th17 cells within the periodontium (Adibrad *et al.*, 2012; Cardoso *et al.*, 2009) and IL-17A levels are elevated in solubilized tissue (Behfarnia *et al.*, 2013; Honda *et al.*, 2008; Ohyama *et al.*, 2009; Takahashi *et al.*, 2005), serum (Duarte *et al.*, 2010; Schenkein *et al.*, 2010) and GCF (Buduneli *et al.*, 2009; Vernal *et al.*, 2005) of chronic periodontitis patients.

Serum levels of IL-17A and IL-17A/F correlate with clinical parameters of periodontal disease such as clinical probing depth (CPD) and clinical attachment loss (CAL) (Ozcaka *et al.*, 2013; Schenkein, *et al.*, 2010). In addition, GCF levels of IL-17A in patients with chronic periodontitis (Buduneli, *et al.*, 2009) and serum levels of IL-17A in patients with aggressive periodontitis (Duarte, *et al.*, 2010) are reduced after non-surgical therapies. Additionally, in an *ex vivo* study, IL-17A levels in the bathing supernatant of gingival tissues from periodontal disease patients were found to be significantly higher compared to tissue from healthy sites (Vernal, *et al.*, 2005). The levels of IL-17A (serum and GCF) in periodontal disease patients with rheumatoid arthritis were also found to be higher compared to systemically healthy chronic periodontitis patients and patients with only rheumatoid arthritis (Gumus *et al.*, 2013), indicating a possible contributing link between periodontal disease and rheumatoid arthritis.

Polymorphisms in the IL-17A gene have been shown to have an association with periodontal disease. Studies of peripheral blood showed that a single nucleotide

polymorphism (rs10484879) correlated with incidences of chronic periodontitis and peri-implantitis (Kadkhodazadeh *et al.*, 2013). In addition, Correa and colleagues (2012) reported a higher distribution of a single nucleotide polymorphism (rs2275913) in chronic periodontitis patients compared to healthy subjects. Furthermore, carriers of this single nucleotide polymorphism (rs2275913) were shown to have higher serum levels of IL-17A, which was accompanied by increased myeloperoxidase activity and IL-8 expression in periodontal tissues (Correa*, et al.*, 2012)

In vivo models have suggested that IL-17A plays a protective role in periodontal disease. Indeed, IL-17RA deficient mice show exacerbated bone loss in a *P. gingivalis* induced model (Yu *et al.*, 2007). In this IL-17R deficient animal model, IL-17 conferred protection by promoting neutrophil migration into the gingival tissues. The absence of IL-17R signalling significantly compromised the antimicrobial effects of infiltrating neutrophils, rendering the animals more susceptible to periodontal disease. However, IL-17A has been implicated in the pathogenesis of many chronic inflammatory diseases, such as rheumatoid arthritis, psoriasis, Crohn's disease and multiple sclerosis (Korn *et al.*, 2009). Numerous studies of models of arthritis, a diseases process with notable parallels to periodontal disease, demonstrated that IL-17A may contribute to initiation and perpetuation of chronic destructive inflammation (Miossec *et al.*, 2009). In addition, neutralising IL-17A is therapeutically beneficial and a neutralising anti-IL17A monoclonal antibody has demonstrated promise as a treatment for rheumatoid arthritis in human trials (Genovese *et al.*, 2010). In this respect, Liang and colleagues (2010) demonstrated that old mice had significantly increased spontaneous periodontal bone loss compared to young mice, which was associated with increased expression of IL-17A. In addition, young Del-1 (an endogenous leukocyte-endothelial adhesion inhibitor) deficient mice were found to develop spontaneous periodontal disease, which was characterised by excessive neutrophil migration into periodontal tissues and increased expression of IL-17A (Eskan *et al.*, 2012). Interestingly, the authors also demonstrated that Del-1 expression in periodontal tissues diminished with age. Furthermore, Eskan and colleagues (2012) showed periodontal disease was prevented if the Del-1 deficient mice were crossed with IL-17A receptor deficient mice. Additionally, the IL-17A deficient mice which were orally infected with *P. gingivalis* exhibited

significant bone loss which was accompanied with a decrease in neutrophil migration into the gingivae (Yu, *et al.*, 2007). Together these *in vivo* studies suggest IL-17A plays a potentially double-edged role in periodontal disease, with the ability to both protect against infection and perpetuate inflammation. Therefore, an effective and balanced IL-17A response is required for the protection of the oral mucosa against pathogens.

The effect of IL-17A on periodontal cells has also been evaluated *in vitro.* The rhIL-17A was shown to induce human gingival fibroblast cells to produce IL-6 (Takahashi, *et al.*, 2005). IL-17A was also shown to inhibit the expression of Del-1 by endothelial cells which could contribute to the continuous and excessive migration of neutrophils into inflamed periodontal tissues (Eskan, *et al.*, 2012).

Apart from IL-17A, the role of other IL-17 family members in periodontal disease is less well studied. Knowledge of the role of IL-17F in periodontal disease has not been explored in detail. Analysis of peripheral blood samples showed that there was no difference in IL-17F gene expression between healthy subjects and chronic periodontitis patients (Jain *et al.*, 2013). Furthermore, mRNA evaluation of periodontal tissue samples also showed no difference in IL-17F expression between tissues from healthy subjects and chronic periodontitis patients (Honda, *et al.*, 2008). In contrast, levels of IL-17F were found to be significantly increased in serum, GCF and saliva samples of patients who suffer both polycystic ovarian syndrome and gingivitis, as compared to systemically healthy individuals (Ozcaka, *et al.*, 2013). Similarly, studies on the association between IL-17E and chronic periodontitis are limited. However, IL-17E was detected in GCF and serum of periodontal disease patients and levels negatively correlated with periodontal clinical probing depth (Ozcaka, *et al.*, 2013). To date there are no studies investigating the role of IL-17B, IL-17C and IL-17D in the pathogenesis of periodontal disease.

1.5 IL-10

1.5.1 Introduction

Interelukin-10 (IL-10) is the founding member of the IL-10 family cytokines, which also includes IL-19, IL-20, IL-22, IL-24, IL-26 and also more distantly related members such as IL-28A, IL-28B, and IL-29 (Ouyang *et al.*, 2011). IL-10, was originally found to be secreted by Th2 cells upon stimulation with mitogen or antigen (Fiorentino *et al.*, 1989). In fact, IL-10 was originally termed cytokine synthesis inhibitory factor (CSIF) as it was shown to inhibit production of IL-2, IL-3, lymphotoxin, IFN-γ and granulocyte macrophage colony-stimulating factor (GM-CSF) by Th1 clone cells stimulated with a combination of antigen and antigen presenting cells (Fiorentino, *et al.*, 1989). Since then, many studies have followed investigating the anti-inflammatory effect of IL-10 and it is now well characterised as an anti-inflammatory cytokine (Moore *et al.*, 2001; Sabat *et al.*, 2010).

IL-10 is a 178 amino acid polypeptide (Vieira *et al.*, 1991) and formation of a homodimer has been shown to be required to produce the active IL-10 cytokine (Tan *et al.*, 1993; Zdanov *et al.*, 1995). IL-10 has been shown to be expressed by various immune cells such as Th2 cells, Th1 cells, B cells, dendritic cells, macrophages, mast cells, eosinophils, neutrophils, $CD4^+$ T cells and $CD8^+$ T cells (Couper *et al.*, 2008; Sabat, *et al.*, 2010). *In vitro*, the release of IL-10 by these cells was found to be elicited by exposure to various endogenous and exogenous mediators such as LPS and catecholamines (Sabat, *et al.*, 2010). IL-10 requires two receptor subunits (IL-10R1 and IL-10R2) to mediate effective intracellular signalling. The human IL-10R1 was identified by expression cloning from a human Burkitt's lymphoma cell line and was found to have 70 % genetic sequence homology and 60 % amino acid sequence homology to the previously identified mouse IL-10R1 (Ho *et al.*, 1993; Liu *et al.*, 1994). The structure of human IL-10R1 was found to be similar to those of the class II cytokine receptor family which includes the interferon receptor (IFNR) (Liu, *et al.*, 1994). Indeed, IL-10 exhibits structural similarities with IFN-γ (Zdanov, *et al.*, 1995) and hence it has been demonstrated that both these cytokines share the same class II cytokine receptors (Zdanov, *et al.*, 1995). IL-10R2 was previously known as CRFB4, and is

a transmembrane protein of unknown function belonging to the class II cytokine receptor family. *In vitro* studies have shown that IL-10 did not induce signal transduction in cells which only express IL-10R1. Furthermore, IL-10R1 and IL10R2 can be co-precipitated in the presence of IL-10 (Kotenko *et al.*, 1997). Additionally macrophages and splenocytes isolated from IL-10R2 deficient mice did not respond to exogenous IL-10 stimulation *in vivo* (Spencer *et al.*, 1998). These studies therefore indicate the importance of IL-10R2 for functional IL-10 signalling.

1.5.2 Effect of IL-10 on target cells

IL-10 is known for its ability to act as an anti-inflammatory and immunosuppressive cytokine. It is well established that monocytes/macrophages are a key target for IL-10. In studies of primary human monocytes stimulated with IFN-γ, LPS, and combinations of IFN-γ and LPS, IL-10 was shown to strongly inhibit the expression of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, TNF-α, GM-CSF, and G-CSF (de Waal Malefyt *et al.*, 1991). A similar pattern of inhibition was also observed in studies using a macrophage cell line (Fiorentino *et al.*, 1991). In addition, IL-10 was also shown to induce increased IL-1RA mRNA expression by LPS-stimulated monocytes (Jenkins *et al.*, 1994). IL-1RA is known for its role in antagonising the pro-inflammatory effect of IL-1α and IL-1β (Perrier *et al.*, 2006). Furthermore, bone marrow derived macrophages isolated from IL-10 deficient mice were found to secrete significantly lower amounts of IL-23 compared to wild type controls (Schuetze *et al.*, 2005). Since most of the pro-inflammatory action of IL-23 is mediated through IL-17A, TNF-α and IL-6; inhibition of IL-23 by IL-10 could down regulate Th17 driven responses (Stetsko & Sauder, 2008). IL-10 was also found to inhibit the production of IL-12 by LPS-activated monocytes (D'Andrea *et al.*, 1993). As IL-12 is important for Th1 cell proliferation it was concluded that IL-10 can also inhibit the Th1 response (Stetsko & Sauder, 2008). A direct effect of IL-10 on T cell responses has also been demonstrated. Human IL-10 was shown to inhibit the antigen and phytohemagglutinin (PHA) proliferative responses of Th1 and Th2 cells (Del Prete *et al.*, 1993). Furthermore, IL-10 inhibits the release of IFN-γ by Th1 cells and the release of IL-4 and IL-5 by Th2 cells in response to antigen-specific

stimulation. Together these data indicate that IL-10 could directly or indirectly inhibit the response of Th1, Th2 and Th17 cells to various inflammatory stimuli.

IL-10 was also shown to have an inhibitory effect on polymorphonuclear leukocyte function. Studies have shown that IL-10 inhibits the production of pro-inflammatory cytokines such as TNF-α and IL-1ß by polymorphonuclear leukocytes stimulated with LPS (Cassatella *et al.*, 1993). In addition, IL-10 was also shown to inhibit LPS-stimulated polymorphonuclear leukocytes production of chemokines such as MIP-1α, MIP-1ß and IL-8 as well as the production of PGE_2 (Kasama *et al.*, 1994; Niiro *et al.*, 1997). Cytokine and chemokine production by other immune cells in response to antigen such as eosinophils and mast cells were also shown to be inhibited by IL-10 (Arock *et al.*, 1996; Takanaski *et al.*, 1994).

The anti-inflammatory effect of IL-10 on epithelial cells has also been investigated. Denning and colleagues (2000) showed that both receptors for IL-10, IL-10R1 and IL-10R2 were expressed by colonic epithelial cells, and IL-10 was shown to block the IFN-γ-induced expression of Class II MHC molecules on cultured intestinal epithelial cells. IL-10 was also shown to inhibit the *Chlamydia trachomatis* induced expression of IL-6, IL-8 and TNF-α from Hela cells (cervical cancer cell line). In addition, IL-10 was also shown to inhibit the release of TNF-α and IL-6 from primary human keratinocytes (isolated from neonate foreskins) stimulated with combinations of LPS and IFN-γ (Becherel *et al.*, 1995).

1.5.3 Role of IL-10 in inflammation and infection

Elevated expression of IL-10 has been found in tissues derived from studies of numerous chronic inflammatory diseases such as inflammatory bowel diseases, atopic dermatitis and rheumatoid arthritis (Asadullah *et al.*, 1998; Katsikis *et al.*, 1994). IL-10 levels were elevated in serum and synovial fluid samples from patients with rheumatoid arthritis (al-Janadi *et al.*, 1996; Cush *et al.*, 1995). In line with the above data, monocytes purified from the synovial fluid of rheumatoid arthritis patients were found to express higher levels of IL-10 than monocytes derived from healthy subjects (Alanara *et al.*, 2010). In addition,

serum IL-10 was also increased in patients with active ulcerative colitis (Mitsuyama *et al.*, 2006).

In vivo, the role of IL-10 as an anti-inflammatory cytokine has been eloquently demonstrated. In a collagen induced arthritis mouse model, IL-10 deficient mice were found to be prone to higher incidence and increased severity of arthritis (Finnegan *et al.*, 2003; Johansson *et al.*, 2001), which was found to associate with decreased serum levels of anti-collagen antibodies (IgG1 and IgG2a) as well as increased proliferation of macrophages (Finnegan, *et al.*, 2003). In addition, intraperitoneal injection of recombinant IL-10 into the collagen induced arthritis mouse model supressed the severity of symptoms (Tanaka *et al.*, 1996). Similarly, in an EAE mouse model, where encephalomyelitic mice were induced by flank injection of encephalitogenic peptide, IL-10 deficient mice were shown to develop a more severe EAE compared to sham controls (Bettelli *et al.*, 1998). In addition, mice injected with IL-10 transgenic T cells, which had been engineered to secrete IL-10 were more resistant to EAE. IL-10 has also been shown to have anti-inflammatory properties in colitis disease models. Dextran sulphate sodium-induced colitis mice exhibited increased expression of TNF-α and IL-1ß in colonic tissue of IL-10 deficient mice compared to sham controls (Tomoyose *et al.*, 1998), In addition, IL-10 deficient mice were shown to develop spontaneous bowel inflammation showing an important immuno-regulatory function of IL-10 within the gastrointestinal tract (Kuhn *et al.*, 1993; Murai *et al.*, 2009).

Studies have shown that IL-10 plays a role in fungal and bacterial induced pathologies. In a mouse model of *C. albicans infection,* IL-10 deficient mice were shown to be resistant to renal candidiasis when compared to the wild type mice (Vazquez-Torres *et al.*, 1999). This resistance was thought to be achieved through neutrophil activity as neutrophils isolated from the peritoneum of IL-10 deficient mice were found to have greater ability to kill *C. albicans* blastoconidia and hyphae when compared to those isolated from wild type mice (Vazquez-Torres, *et al.*, 1999). This finding is in line with other studies that showed IL-10 plays a role in negatively regulating neutrophil function. Similarly, intraperitoneal injection of *E. coli* into IL-10 deficient mice resulted in reduced recovery (CFU/ml) of *E. coli* from peritoneal fluid, blood and lung when

compared to wild type mice. This indicates endogenous IL-10 impaired bacterial clearance. However, although IL-10 impairs the clearance of *E. coli* and can facilitate the outgrowth and dissemination of bacteria during peritonitis, it in turn was shown to protect mice from lethality by attenuating the development of a systemic inflammatory response syndrome by a mechanism that involves inhibition of TNF-α (Sewnath *et al.*, 2001).

In summary, IL-10 was found to be elevated in tissue samples as well as in clinical samples of patients with chronic inflammatory diseases such as rheumatoid arthritis and ulcerative colitis. These findings were in agreement with the *in vivo* findings that demonstrated disease severity in animal models (EAE, arthritis and colitis) was greater in IL-10 deficient mice. However, in microbial infection models, IL-10 was found to negatively impact on microbial clearance from tissues/organs. However, despite hindering clearance of pathogenic organisms, IL-10 was found to protect mice from associated excessive inflammation induced pathologies. Therefore these studies clearly demonstrate a role for IL-10 in negatively regulating immune responses.

1.5.4 IL-10 and periodontal disease

The role of IL-10 in the pathogenesis of periodontal disease has been studied previously. Using quantitative real-time polymerase chain reaction (real-time PCR) and enzyme-linked immunosorbent assay (ELISA), IL-10 (mRNA and protein) expression were found to increase in gingival tissue samples of chronic periodontitis patients when compared to healthy subjects (Napimoga *et al.*, 2011). Furthermore, analysis of GCF has shown that IL-10 is only detected in samples of chronic periodontitis patients but not healthy subjects (Gamonal *et al.*, 2000). In contrast, IL-10 levels in saliva and serum were found to be greater in healthy subjects compared to chronic periodontitis patients (Gumus *et al.*, 2014). Furthermore, Gumus and colleagues (2014) showed that IL-10 levels in saliva and serum negatively correlated with clinical parameters such as periodontal probing depth, clinical attachment loss and bleeding index.

In vivo, IL-10 deficient mice orally inoculated with *P. gingivalis* were shown to suffer severe alveolar bone loss compared to wild type controls (Sasaki *et al.*,

2004). In addition, IL-10 deficient mice also exhibited accelerated spontaneous bone loss compared to wild type controls (Al-Rasheed *et al.*, 2003). *In vitro*, primary human gingival fibroblasts were found to upregulate expression of IL-10 in response to *P. gingivalis* (LPS) (Almasri *et al.*, 2007). In addition, gingival mononuclear cells isolated from *P. gingivalis* infected mice showed increased spontaneous production of IL-10 compared to mononuclear cells isolated from sham infected mice (Kobayashi *et al.*, 2011). These studies therefore suggest that IL-10 plays an anti-inflammatory role in the oral cavity and can inhibit the pathogenesis of periodontal disease.

1.6 IL-33

1.6.1 Introduction

The discovery of IL-33 has an interesting history. It was previously reported in 1991 as DVS27 and its expression was found to be highly upregulated in canine vasospastic cerebral arteries (Onda *et al.*, 1999). Later in 2003 it was discovered as a nuclear factor from high endothelial venules (NF-HEV) (Baekkevold *et al.*, 2003). Even though the cytokine properties of this molecule were not apparent at the time, these studies reported at least two important features: (a) nuclear localization and (b) a molecular structure that contained a structural homology with DNA-binding domains. Therefore it was initially thought to play a role as a transcription factor. In 2005, however, it was discovered as the latest IL-1 family member and shown to bind to the orphan IL-1 receptor, ST2 (also known as IL-1RL1, interleukin 1 receptor-like 1), hence it was renamed IL-33 (Schmitz *et al.*, 2005). IL-33 is the eleventh member of the IL-1 family cytokines that also includes IL-1α, IL-1β, IL-1RA, IL-18, IL-1F5, IL-1F6, IL-1F7, IL-1F8, IL-1F9 and IL-1F10 (Barksby *et al.*, 2007; Sims *et al.*, 2001). Like other members of the IL-1 family, IL-33 was found to be involved in regulating inflammatory and immune processes. Indeed, in its role as a cytokine, IL-33 was found to induce Th2 associated cytokine production both *in vitro* and *in vivo*.

1.6.2 Molecular structure

The human IL-33 gene was found to be located on the short arm of chromosome 9 at location 9p24.1 (Baekkevold, *et al.*, 2003; Schmitz, *et al.*, 2005), while its mouse counterpart was found located on the syntenic chromosome region 19qC1 (Schmitz, *et al.*, 2005). Human and mouse IL-33 share 55 % amino acid sequence homology. Analyses of the cDNA (complementary deoxyribonucleic acid) sequences of the human and mouse IL-33 gene showed they encoded a 270 and 266 amino acids protein respectively, with reported molecular weights of 30 kDa for human IL-33 and 29.9 kDa for mouse IL-33 (Schmitz, *et al.*, 2005). Schmitz and colleagues (2005) reported that IL-33 was produced as a 30 kDa propeptide that required cleavage by caspase-1 for generation of the mature form. Caspase-1 was shown to cleave IL-33 at Ser_{111}, transforming the propeptide into the 18 kDa mature form (amino acids 112 – 270). This post-translational processing is common for other IL-1 family members such as IL-1α, IL-1ß and IL-18. For instance, pro-IL-1 and pro-IL-18 were found to require peptide cleavage by caspase-1 and/or caspase-8 for maturation (Arend *et al.*, 2008; Dinarello, 1998) and calpain was reported as a required factor for the processing of mature IL-1α (Arend, *et al.*, 2008; Dinarello, 1997).

Despite these findings more recent data has found caspase-1 may have contradictory effects on the biological activity of IL-33. Cayrol and Girard (2009) reported that the full length IL-33 protein is itself biologically active and that processing by caspase-1 results in IL-33 inactivation. They further demonstrated that caspase-1 cleavage does not occur at the site initially proposed (Ser_{111}), but after residue Asp_{178} (aspartic acid 178) between the fourth and fifth predicted beta-strands of the IL-1-like domain. In addition, they also found that caspase-3, the prototypic apoptotic caspase shares a similar cleavage site with caspase-1 which is at $DGVD_{178}G$, and both caspase-1 and caspase-3 activation leads to the production of an inactive 20 – 22 kDa fragment of IL-33. Therefore the authors concluded that IL-33 is inactivated by endogenous caspases during cell apoptosis.

Alignment of IL-33 protein sequences revealed two evolutionarily conserved regions: the predicted homeodomain-like helix–turn–helix (HTH) motif (amino acids 1 – 65) within the N-terminal portion (Baekkevold, *et al.*, 2003) and the C-

terminal IL-1-like domain (amino acids 112 – 270) (Schmitz, *et al.*, 2005). The cytokine activity is expected to reside in the carboxyl (c-) terminal of IL-33, which is predicted to form a β-trefoil structure, similar to IL-1 and IL-18. These similar conserved features likely reflect common evolutionary roots (Arend, *et al.*, 2008).

1.6.3 Functions of IL-33

Schmitz and colleagues (2005) were the first to report the cytokine activity of IL-33 when they observed that the protein mediates its biological effects via the ST2 receptor to activate NF-κB and MAP (mitogen activated protein) kinases. They also observed that IL-33 was able to drive the production of Th2-associated cytokines (i.e., IL-5 and IL-13) from *in vitro* polarized Th2 cells. In addition, *in vivo*, they found IL-33 was capable of inducing the expression of IL-4, IL-5, and IL-13 and promoting severe pathological changes in mucosal organs. Studies have shown positive associations between IL-33 expression and inflammatory disease activity in tissues such as the synovial membrane, lung and colon. This indicated a direct or indirect role of IL-33 as a cytokine driving disease pathogenesis. For example, IL-33 was abundantly expressed in the synovial membrane from patients with rheumatoid arthritis (Xu *et al.*, 2008). IL-33 was also abundantly expressed in the lungs of mice that were exposed to pulmonary challenge with either LPS or house dust mite extracts (Hammad *et al.*, 2009). In addition, IL-33 mRNA levels were markedly increased in the colon of mice that were infected with the nematode parasite *T. muris* (Humphreys *et al.*, 2008).

Even though the evidence for the function of IL-33 as a cytokine is clear, researchers are still in debate as to how IL-33 is released from cells. Some studies report that cells do not release IL-33 unless they become injured or undergo necrosis (Cayrol & Girard, 2009; Nile *et al.*, 2010). For example, Cayrol and Girard (2009) showed that mechanical endothelial cell damage induced the release of full-length IL-33 and high-mobility group box protein 1 (HMGB1) from endothelial cells. However, neither IL-33 nor HMGB1 were found in the supernatants of undamaged control cells. In addition Carol and Girard (2009) also showed both IL-33 and HMGB1 were released from cells subjected to necrosis by exposure to several cycles of freeze/thawing. In addition, IL-33 has

been detected in the culture supernatants from damaged fibroblast-like synoviocytes but not activated peripheral blood mononuclear cells (PBMC) and non-damaged fibroblast-like synoviocytes (Matsuyama *et al.*, 2010). These findings demonstrated that IL-33 had similarity with the 'alarmin' family of proteins which are also only released in response to cell damage. This family includes HMGB1, defensins, heat shock proteins, lactoferrin, cathelicidin and IL-1α (Cayrol & Girard, 2009; de la Rosa *et al.*, 2008; Haraldsen *et al.*, 2009; Moussion *et al.*, 2008; Oppenheim *et al.*, 2007). However, despite being characterised as an 'alarmin', studies have found that under certain conditions IL-33 is released by viable cells such as monocytes, macrophages, fibroblasts and glial cells (Hudson *et al.*, 2008; Li *et al.*, 2008; Sanada *et al.*, 2007; Talabot-Ayer *et al.*, 2009). *In vitro*, THP-1 cells (human monocyte cell line) forced to express pro-IL-33 and subjected to LPS or PMA stimulation did secrete pro-IL-33 (Talabot-Ayer, *et al.*, 2009). In addition, IL-33 was reported to be secreted from PMA-stimulated rat cardiac fibroblasts (Sanada, *et al.*, 2007) and from adenosine triphosphate (ATP) stimulated mixed glial cell cultures and astrocyte-enriched cultures (Hudson, *et al.*, 2008). A combination of LPS and aluminium hydroxide adjuvant (alum) treated human macrophages were also shown to secrete pro-IL-33 which was found to be dependent on NLRP3 (nucleotide-binding domain and leucine rich repeat containing pyrin domain 3) and its adaptor molecule ASC (apoptosis-associated speck-like protein containing CARD) (Li, *et al.*, 2008).

Immunohistochemical studies revealed that IL-33 is predominantly localized to the nucleus in numerous cells, indicative of a role for IL-33 as a nuclear protein (Baekkevold, *et al.*, 2003; Carriere *et al.*, 2007; Kuchler *et al.*, 2008; Moussion, *et al.*, 2008; Onda, *et al.*, 1999). Database searches revealed significant structural homology between the first 65 amino N-terminal residues of IL-33 and the DNA-binding homeodomains of several drosophila and vertebrate transcription factors, indicating that IL-33 may function as a transcription factor (Baekkevold, *et al.*, 2003). Furthermore, Carriere and colleagues (2007) showed that the evolutionarily conserved homeodomain-like helix-turn-helix motif of IL-33 mediated nuclear localization, heterochromatin-association, and targeting of IL-33 to mitotic chromosomes. In addition, IL-33 was found to have transcriptional repressor properties which were mediated by the homeodomain-like helix-turn-helix motif. Furthermore, Roussel and colleagues (2008) showed

that IL-33 binds to chromatin by docking into the acidic pocket formed by the histone H2A–H2B dimer at the surface of the nucleosome. Based on the current understanding, IL-33 has similarity to IL-1α and HMGB1 which also have been shown to have transcriptional regulatory properties (Lotze & Tracey, 2005; Maier *et al.*, 1994; Scaffidi *et al.*, 2002; Werman *et al.*, 2004). Therefore, like IL-1α and HMGB1, IL-33 is a dual function protein acting as a pro-inflammatory cytokine extracellularly and as nuclear factor with transcriptional regulatory properties intracellularly.

1.6.4 IL-33 expression in cells and tissues

IL-33 is constitutively expressed in a variety of cells and expression increased in response to pro-inflammatory mediators. The following cells have been described to constitutively express IL-33: High endothelial venules (HEV) and endothelial cells (HEV ECs) from human tonsils, (Baekkevold, *et al.*, 2003; Carriere, *et al.*, 2007), endothelial cells of large and small blood vessel (Matsuda *et al.*, 2009; Moussion, *et al.*, 2008), fibroblastic reticular cells of lymphoid tissues (Moussion, *et al.*, 2008), smooth muscle cells of bronchial, pulmonary and coronary arteries (Prefontaine *et al.*, 2009; Schmitz, *et al.*, 2005), epithelial cells of skin (keratinocytes), stomach, colonic mucosa, tonsillar crypts and salivary gland (Beltran *et al.*, 2010; Moussion, *et al.*, 2008; Schmitz, *et al.*, 2005) and glial cells of the central nervous system (Hudson, *et al.*, 2008). In addition, IL-33 expression studies showed that skin, gut, lung, brain and spinal cord are prominent sites of IL-33 expression (Hudson, *et al.*, 2008; Moussion, *et al.*, 2008; Schmitz, *et al.*, 2005).

IL-33 was found to be highly expressed in inflamed or diseased tissues (Beltran, *et al.*, 2010; Matsuda, *et al.*, 2009; Matsuyama, *et al.*, 2010; Xu, *et al.*, 2008). However, IL-33 expression was not detected in resting immune cells such as monocytes, monocyte-derived dendritic cells, natural killer (NK) cells, B cells and T cells (Schmitz, *et al.*, 2005). However, Schmitz and colleagues (2005) did report a modest amount of IL-33 expression in monocytes and dendritic cells upon stimulation with LPS. In addition, a later study showed that IL-33 was modestly expressed in resting monocytes but this expression was significantly upregulated upon LPS stimulation (Nile, *et al.*, 2010). In fact, a further study

showed that the IL-33 expression in murine bone marrow derived macrophages is increased by up to 300 fold when stimulated with LPS (Goh *et al.*, 2009).

Mouse primary macrophages stimulated with TLR-3 (Poly I:C) and TLR-4 (LPS) agonists exhibited upregulated IL-33 mRNA expression and using a LPS-stimulated TANK-binding kinase 1 (TBK-1)$^{-/-}$ MEF (mouse embryonic fribroblasts) IL-33 expression was found to be TBK-1-dependent (Polumuri *et al.*, 2012). In addition, human corneal epithelial cells required stimulation by TLR-3 (Polyl:C) and TLR-5 (Flagelin) agonists to express IL-33, and this IL-33 expression was found to be suppressed after blocking nuclear translocation of the NF-κB p65 subunit by a IκBα (inhibitor of kappa B, alpha) inhibitor (BAY11-7082) and NF-κB inhibitor (quinazoline) (Zhang *et al.*, 2011a). Cytokines such as IL-1ß, TNF-α, IFN-γ and IL-17A were shown to induce IL-33 expression by keratinocytes, fibroblasts, macrophages and endothelial cells (Kamekura *et al.*, 2012; Meephansan *et al.*, 2012; Nomura *et al.*, 2012; Palmer *et al.*, 2009; Savinko *et al.*, 2012; Xu, *et al.*, 2008). Nomura and colleagues (2012) showed that TNF-α induced expression of IL-33 by nasal fibroblasts was mediated by phosphoinositide 3-kinase (PI3K), c-Jun N-Terminal (JNK) and NF-κB pathways. In addition, EGFR, ERK (extracellular-signal-regulated kinase), p38 and JAK (Janus kinase)/STAT1 pathway were found to mediate the IL-17A induced expression of IL-33 by normal human epidermal keratinocytes (Meephansan, *et al.*, 2012).

1.6.5 IL-33 receptors

ST2 was originally discovered by Yanagisawa and colleagues (1993) as an orphan receptor. The specific ligand for ST2 was demonstrated to be IL-33 in 2005 (Schmitz, *et al.*, 2005). ST2 belongs to the IL-1 receptor family, which is a subfamily of the IL-1R/TLR superfamily and therefore ST2 is also known as IL-1 receptor-like-1 (IL1RL-1). The other members of this subfamily are interleukin-1 receptor accessory protein (IL-1RAcP), IL-18R, IL-18RAcP, IL-1Rrp2 and IL-1RAPL (Dunne & O'Neill, 2003; O'Neill & Dinarello, 2000; Schmitz, *et al.*, 2005). Similarly to other family members, ST2 has an extracellular three-immunoglobulin (Ig) domain (IL-33 binding domain) and also an intracellular TIR (toll/interleukin-1 receptor) domain that functions to activate intracellular signalling pathways (O'Neill & Dinarello, 2000; Schmitz, *et al.*, 2005). There are

three variants of the IL-33 receptor described by researchers: a longer transmembrane form of the receptor (ST2L), a shorter soluble receptor (sST2) and a variant soluble receptor (ST2V) (Iwahana *et al.*, 2004; Iwahana *et al.*, 1999; Schmitz, *et al.*, 2005; Turnquist *et al.*, 2008; Yanagisawa, *et al.*, 1993). Analysis of the exon-intron structure of the ST2 gene family revealed that the ST2L, sST2 and ST2V are generated by alternative splicing which is controlled by two distinct promoters, a proximal promoter and a distal promoter (Bergers *et al.*, 1994; Iwahana, *et al.*, 1999; Li *et al.*, 2000b; Tominaga *et al.*, 1999).

IL-33 has been shown to specifically bind to ST2 (Palmer *et al.*, 2008; Schmitz, *et al.*, 2005). However, for active signal transduction to occur IL-1RAcP is required. Therefore the active IL-33 receptor (IL-33R) is a heterodimeric molecule consisting of ST2 and IL-1RAcP (Ali *et al.*, 2007; Chackerian *et al.*, 2007). The importance of this heterodimer was demonstrated by Palmer and colleagues (2008) who showed that IL-1RAcP increased the affinity of murine IL-33 for ST2 four fold. Even though sST2 has the same affinity for IL-33 as ST2, it does not possess the intracellular component and therefore the binding complex formed between sST2, IL-33 and IL-1RAcP does not lead to active signal transduction. Therefore, sST2 merely functions as a decoy that competes with ST2 for IL-33 (Hayakawa *et al.*, 2007; O'Neill, 2008; Schmitz, *et al.*, 2005).

ST2 is constitutively expressed by immune cells such as mature mast cells and mast cell precursors, dendritic cells, macrophages, B cells and Th2 cells but not Th1 cells (Allakhverdi *et al.*, 2007; Espinassous *et al.*, 2009; Joshi *et al.*, 2010; Komai-Koma *et al.*, 2011; Lecart *et al.*, 2002; Matsuda, *et al.*, 2009; Moritz *et al.*, 1998; Turnquist, *et al.*, 2008; Xu *et al.*, 1998; Yanagisawa *et al.*, 1997). ST2 has also found to be abundantly expressed by epithelial cells in the colonic mucosa, cornea and lung (Beltran, *et al.*, 2010; Lin *et al.*, 2013; Yagami *et al.*, 2010) as well as keratinocytes of skin (Hueber *et al.*, 2011).

1.6.6 Effects of IL-33 on target cells

Mast cells were found to constitutively express ST2 (Allakhverdi, *et al.*, 2007; Matsuda, *et al.*, 2009; Moritz, *et al.*, 1998; Schmitz, *et al.*, 2005), which make them a target cell for IL-33. *In vitro*, mast cells directly respond to IL-33 and

activate protein kinase signalling cascades involving NF-κB, ERK1/2 and p38 (Matsuda, *et al.*, 2009; Schmitz, *et al.*, 2005). In the case of p38, this was found to be inactivated by the presence of sST2 (Matsuda, *et al.*, 2009). Activation of protein kinase signalling cascades have been shown in mast cells to result in the expression of inflammatory mediators such as IL-1β, IL-5, IL-6, IL-10, IL-13, TNF-α, GM-CSF, CXCL8/IL-8, CCL1, monocyte chemotactic protein 1 (MCP-1) and MIP-1α (Allakhverdi, *et al.*, 2007; Ho *et al.*, 2007; Palmer, *et al.*, 2008; Xu, *et al.*, 2008).

ST2 was also abundantly expressed by Th2 cells, but not any other T lymphocyte subsets, such as naïve $CD4^+$ T cells, Th1 cells, Th17 cells and regulatory T cells (Lecart, *et al.*, 2002; Lohning *et al.*, 1998; Nakae *et al.*, 2007; Xu, *et al.*, 1998), Therefore IL-33 plays a role in regulating Th2 responses. Naïve $CD4^+$ T cells stimulated with PHA or *Staphylococcal enterotoxin B* (SEB) were shown to differentiate into effector Th2 cells in the presence of IL-33 (Komai-Koma *et al.*, 2007; Kurowska-Stolarska *et al.*, 2008). IL-33 was also shown to enhance the production of Th2 mediators such as IL-5 and IL-13 (Guo *et al.*, 2009; Kurowska-Stolarska, *et al.*, 2008; Schmitz, *et al.*, 2005). In fact, IL-33 was also shown to induce Th2 cells to express IL-33 (Guo, *et al.*, 2009). *In vitro*, Komai-koma and colleagues (2007) showed that IL-33 acts as a chemoattractant for Th2 cells that have high levels of ST2 expression. In addition, the authors showed injection of recombinant IL-33 into the footpad of ST2-knockout mice, which had been adoptively transferred with polarized Th2 cells, led to the localised accumulation of transferred Th2 cells at the injection site.

Macrophages have been shown to express ST2 indicating that these cells could respond to IL-33 signalling (Espinassous, *et al.*, 2009; Joshi, *et al.*, 2010). Indeed, Espionassous and colleagues (2009) showed that the release of TNF-α by murine peritoneal macrophages cells in response to LPS was increased in the presence of IL-33. Furthermore, the authors showed the potentiating effect of IL-33 was abolished when macrophages are treated with an anti-ST2 antibody. IL-33 also stimulated mouse bone marrow derived macrophages to express the Th2 cytokines: IL-5 and IL-13 (Yang *et al.*, 2013). In addition, IL-33 also stimulated IL-13-primed peripheral blood derived macrophages to express CCL17 and CCL24 (Kurowska-Stolarska *et al.*, 2009). IL-33 stimulation also increased the

expression of MD-2 (myeloid differentiation protein 2) and TLR-4 in macrophages, therefore increasing their sensitivity to bacterial LPS (Espinassous, *et al.*, 2009). IL-33 was also shown to induce naïve macrophage to express CCL3, a marker for classically activated macrophages (M1). However, when IL-33 was added to polarised M1 macrophages, an increase in expression of CCL18, a marker for alternatively activated macrophages (M2) was observed (Joshi, *et al.*, 2010). This indicates that IL-33 stimulation of naïve macrophages favours M1 chemokine generation whilst addition to polarised macrophages promotes and amplifies M2 chemokine expression. Interestingly, Mun and colleagues (2010) observed that IL-33 also stimulates the formation of tartrate-resistance acid phosphate $(TRAP)^+$ osteoclasts from human monocytes via activation of signalling molecules that are critical for osteoclast cell development such as Syk (spleen tyrosine kinase), phospholipase Cγ2, Gab2, MAP kinases, MAP3K7 (mitogen-activated protein kinase kinase kinase 7), and NF-κB. Furthermore, using an osteogenic disk model, IL-33 was able to induce bone resorption by $CD14^+$ monocyte-derived osteoclasts. However, in contrast, ST2 deficient mice presented with normal bone formation but increased bone resorption and IL-33 abolished the generation of tartrate-resistance acid phosphate $(TRAP)^+$ osteoclasts even in the presence of RANKL and M-CSF (Schulze *et al.*, 2010).

B cells have been demonstrated to express ST2 (Komai-Koma, *et al.*, 2011; Yanagisawa, *et al.*, 1997) and IL-33 demonstrated to activate B1 cell proliferation and induce production of IgM, IL-5 and IL-13 (Komai-Koma, *et al.*, 2011). In addition, IL-33 was shown to require IL-4 to induce B cells proliferation and production of IgE (Komai-Koma *et al.*, 2012). Thus these studies indicate a role for IL-33 in modulating B cell function in a Th2 dependant manner. IL-33 was also shown to augment dendritic cells expansion from mouse bone marrow in a time and dose dependent manner (Mayuzumi *et al.*, 2009). This role was found to be dependent on GM-CSF as IL-33-induced expression of dendritic cells was completely blocked by an anti-GM-CSF antibody. IL-33 was also found to induce dendritic cells to express IL-6, IL-1β, TNF-α and CCL17 (Besnard *et al.*, 2011). IL-33 was also shown to activate mouse bone marrow derived dendritic cells to express high level of MHC class II molecules and CD86 (Rank *et al.*, 2009). This study also showed that IL-33-activated dendritic cells were capable of priming naïve $CD4^+$ T cells to produce IL-5 and IL-13. The capability of IL-33-

activated dendritic cells in priming naïve $CD4^+$ T cells to produce IL-5 and IL-13 was also confirmed by Besnard and colleagues (2011). Therefore, IL-33 can influence dendritic cells function and promote Th2 responses.

In terms of granulocytes, IL-33 has been shown to induce the production of IL-13, CCL17 and TGF-ß from eosinophils (Stolarski *et al.*, 2010). In addition, IL-33 exacerbated eosinophil-mediated airway inflammation by increasing the numbers of eosinophils, macrophages, lymphocytes and levels of IL-13, TGF-ß, CCL3, CCL17, and CCL24 in the lung. Schneider and colleagues (2009) also showed that basophils produce pro-inflammatory mediators, such as histamine, IL-4 and IL-6 upon treatment with IL-33. In addition, IL-33 activation of neutrophils was shown to increase expression of the surface complement receptor 3 (CR3) which in turn increased phagocytosis of opsonised *C. albicans* (Le *et al.*, 2012).

Numerous epithelial cells express ST2L and are therefore a key target for IL-33. Yagami and colleagues (2010) evaluated the effect of IL-33 on normal human bronchial epithelial cells and found that IL-33 increased the expression of IL-8 in a dose and time dependant manner. The IL-33 induced expression of IL-8 was found to be mediated by an ST2 dependant pathway. IL-33 stimulation led to the increased phosphorylation of the MAPK, ERK but not p38. In addition, in the presence of an ERK inhibitor (PD98059), a significant reduction in IL-33 mediated IL-8 expression by normal human bronchial epithelial cells was observed. In contrast, the authors showed that IL-33 had no effect on the expression of IL-1ß, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-α, MCP-1 and CXCL10. Furthermore, Fujita and colleagues (2012) demonstrated that IL-33 induced the expression of IL-17F by bronchial epithelial cells in a dose dependant manner. In agreement with previous studies (Yagami, *et al.*, 2010), the authors showed that IL-33 signalling led to the increased phosphorylation of ERK but not p38 and JNK. However, the authors also demonstrated the induction of increased phosphorylation of mitogen and stress-activated protein kinase 1 (MSK1). MSK1 has been shown to involved in the phosphorylation of NF-κB *in vitro* and *in vivo* (Vermeulen *et al.*, 2003). Although IL-17A shares high homology with IL-17F, the authors also found that even though there is basal release of IL-17A from

bronchial epithelial cells, IL-33 stimulation did not induce increased expression (Fujita, *et al.*, 2012).

Kamekura and colleagues (2012) evaluated the effect of IL-33 on human nasal epithelial cells (HNEC). Stimulation of HNECs with IL-33 (100 ng/ml) for 6 h induced expression of IL-8 and GM-CSF. The expression of IL-8 and GM-CSF were found to be significantly decreased if the HNECs were pre-incubated with an anti-IL-33 or anti-ST2 antibody, indicating the direct role of IL-33 signalling. In analysing signalling pathways involved in the IL-33 mediated inflammatory response in HNECs, cells were pre-treated with inhibitors of ERK, p38, JNK, NF-κB and EGFR prior to treatment with IL-33. The authors found that IL-33-mediated expression of IL-8 was dose-dependently reduced by inhibitors of ERK (PD98059) and EGFR (AG1478), which is consistent with other studies (Fujita, *et al.*, 2012; Yagami, *et al.*, 2010). In addition, they also found that IL-33-mediated expression of GM-CSF was dose-dependently reduced by treatment with the inhibitor of JNK (SP600125), NF-κB (IMD-0354) and EGFR (AG1478).

Meephanson and colleagues (2012) studied the effect of IL-33 on normal human epidermal keratinocytes. The authors found that after 48 h of stimulation with IL-33 (100 ng/ml) a significant increase in IL-8 expression was observed from normal human epidermal keratinocytes. On the other hand, Balato and colleagues (2012) demonstrated that IL-33 alone could not induce the expression of IL-6, VEGF, MCP-1 and IL-20 by normal human epidermal keratinocytes. However, IL-33 could increase the TNF-α induced expression of IL-6, VEGF, MCP-1 and IL-20. Parallel results were also found using the immortalised human keratinocyte cell line (HaCaT). In addition, HaCaT cells require co-stimulation with IL-33 for IL-17A to induce expression of IL-20 and VEGF. Interestingly, however, intracellular IL-33 has been found to actually suppress NF-κB (Ali *et al.*, 2011). Therefore, as intracellular IL-33 suppresses NF-κB function, it was hypothesised that IL-33 knockdown would enhance the TNF-α-induced expression of IL-8. Indeed, as expected, knock down of IL-33 with small interfering RNA (siRNA) enhanced the TNF-α-mediated expression of IL-8 (Oliveira *et al.*, 1994).

Studies on human and mouse fibroblasts isolated from the heart (Zhu & Carver, 2012), skin (Wong *et al.*, 2012) lung (Kurokawa *et al.*, 2011) and embryo

(Funakoshi-Tago *et al.*, 2008) showed that IL-33 could induce expression of various cytokines and chemokines such as IL-6, IL-13, MCP-1, MCP-3, CXCL1, CXCL10, CCL2 and CCL11, as well as TGF-β. While IL-33 could induce increased expression of CCL11 by mouse lung fibroblasts, co-stimulation with IL-33 and IL-13 has been shown to synergistically augment CCL11 expression (Kurokawa, *et al.*, 2011). In rat cardiac fibroblasts, IL-33 stimulation could induce translocation of the phosphorylated p65 subunit of NF-kB into the nucleus in a dose dependent manner. In addition, in murine embryonic fibroblasts of TRAF6-deficient mice, IL-33 failed to induce MCP-1, MCP-3 and IL-6 expression; indicating that TRAF6 is important for IL-33-induced signal transduction (Funakoshi-Tago, *et al.*, 2008). The study also showed that IL-33-induced activation of p38 and JNK was completely impaired in TRAF6-deficient murine embryonic fibroblasts. In addition, IL-33 has also been shown to induce phosphorylation of STAT3 by primary human dermal fibroblasts (Wong, *et al.*, 2012). In human fibroblasts isolated from rheumatoid arthritis synovial tissue, Kunisch and colleagues (2012) showed that stimulation with IL-33 did not significantly induce increased expression of IL-6, MCP-1, MMP-1 and MMP-3 and no significant increases in the phosphorylation of p38, ERK or JNK were observed. However, using a lentiviral expression system, fibroblasts that were induced to overexpress intracellular IL-33 and stimulated with TNF-α exhibited increased expression of IL-6, IL-8, MCP-1 and increased PGE_2 secretion.

1.6.7 Role of IL-33 in inflammation and infection

IL-33 and ST2 expression was found to be elevated in the synovial tissue, synovial fluid and serum of rheumatoid arthritis patients (Carriere, *et al.*, 2007; Matsuyama, *et al.*, 2010; Palmer, *et al.*, 2009; Xu, *et al.*, 2008). This therefore implicated a role for IL-33 in the pathogenesis of rheumatoid arthritis. Numerous *in vivo* studies have investigated the role of IL-33 in the pathogenesis of rheumatoid arthritis using a collagen induced arthritis mouse model. In collagen induced arthritis mice, an increase in IL-33 expression was detected during the early phases of inflammation in the joint. Furthermore, intraperitoneal injection with a monoclonal anti-ST2 antibody decreased the severity of disease (Palmer, *et al.*, 2009). Intraperitoneal injection with sST2-Fc (engineered fusion protein) into collagen induced arthritis mice was also shown to attenuate the mean

arthritic index and significantly lower the serum levels of IL-12, TNF-α and IFN-γ (Leung *et al.*, 2004). In addition, when CIA was attempted in ST2-knock out mice there was markedly attenuated levels of IL-17, IFN-γ, and TNF-α in the draining lymph nodes and lower infiltration of mononuclear and polymorphonuclear leukocytes cells into synovial tissues (Xu, *et al.*, 2008). Furthermore, collagen induced arthritis mice intraperitoneally injected with recombinant IL-33 exhibited increased disease severity and significantly increased levels of IL-5, IL-17, IL-12, IFN-γ and TNF-α in the draining lymph nodes (Xu, *et al.*, 2008).

In inflammatory bowel diseases, IL-33 was found to be highly expressed in diseased tissue samples (Beltran, *et al.*, 2010; Carriere, *et al.*, 2007; Sponheim *et al.*, 2010). Carriere and colleagues (2007) also showed that serum levels of IL-33 were significantly increased in inflammatory bowel diseases patients compared to healthy subjects. This indicated a possible role for IL-33 in the pathogenesis of inflammatory bowel diseases. Wild type mice intraperitoneally injected with recombinant IL-33 presented with marked epithelial cell hyperplasia in the gastrointestinal tract (GIT), accompanied with GIT eosinophilia and mononuclear cell infiltration (Schmitz, *et al.*, 2005). However, IL-33 deficient mice exhibited increased severity of symptoms, inflammation and myeloperoxidase activity in a dextran induce colitis mouse model (Oboki *et al.*, 2010). In contrast, in a trinitrobenzene sulfonic acid-induced experimental colitis model, induction of colitis in mice treated with recombinant IL-33 resulted in reduced inflammatory cell migration and weight loss compared to the mice treatment with saline or control IgG. In addition, mice treated with recombinant IL-33 exhibited decreased serum levels of IFN-γ and increased serum level of Th2 cytokines (IL-5 and IL-13) (Duan *et al.*, 2012). These therefore suggested that IL-33 had a protective role in inflammatory bowel diseases.

IL-33 has been demonstrated to play a role in airway inflammation. IL-33 was highly expressed in smooth muscle and epithelial cells of bronchial tissue from asthma patients (Prefontaine, *et al.*, 2009; Prefontaine *et al.*, 2010). In an ovalbumin-induced airway inflammation mouse model, the expression of IL-33 and ST2 in lungs was increased (Hayakawa, *et al.*, 2007; Louten *et al.*, 2011). In addition, when the ovalbumin-induced airway inflammation model was run in

ST2 deficient mice, decreased severity of airway inflammation was observed which was characterised by a reduction in inflammatory cell numbers (eosinophils and macrophages) and reduced levels of IL-5 in the bronchoalveolar lavage fluid (Kurowska-Stolarska*, et al.*, 2008). In addition, IL-33 deficient mice also showed decreased pulmonary inflammation characterised by decreased cell migration (eosinophils and lymphocytes) as well as decreased Th2 cytokines (IL-5 and IL-13) in the bronchoalveolar lavage fluid (Louten*, et al.*, 2011; Oboki*, et al.*, 2010). Intranasal administration of recombinant IL-33 was also shown to induce immediate allergic responses as evidenced by increased mucous production by the airway epithelium and increased levels of IL-5 and IL-13 in lung tissue as well as in bronchoalveolar lavage fluid (Louten*, et al.*, 2011).

A role for IL-33 in infection was first suggested when levels of the soluble receptor for IL-33 (sST2) were found to be elevated in patients with sepsis and trauma (Brunner *et al.*, 2004). Indeed, IL-33 has been demonstrated to play a role in infection in a number of mouse models. In a *Pseudomonas aeruginosa* keratitis mouse model, sub-conjunctival injection with recombinant IL-33 was shown to induce decreased severity of inflammation and decreased bacterial load as well as a decrease in polymorphonuclear leukocytes infiltration. This was accompanied by decreased expression of TNF-α and MIP-2, and increased expression of Th2 cytokines (IL-4 and IL-5) (Hazlett *et al.*, 2010). In a mouse model of sepsis, where the systemic inflammation was induced by cecal ligation and puncture (CLP), intravenous injection of IL-33 was shown to reduce mortality rate and this was associated with increased neutrophil influx into the peritoneal cavity and increased bacterial clearance (Alves-Filho *et al.*, 2010). Furthermore, IL-33 plays a role in protection against fungal pathogens as in an acute *C. albicans* peritoneal infection model. Pre-treatment with IL-33 (intraperitoneal injection) prior to *C. albicans* infection (intraperitoneal injection) was shown to induce rapid fungal clearance and reduced *C. albicans* associated mortality rate. This was associated with a rapid recruitment of neutrophils to the site of infection site in the IL-33 pre-treated mice (Le*, et al.*, 2012).

1.6.8 IL-33 and periodontal diseases

IL-1 family cytokines have been shown to have important roles in periodontal disease (Kinane *et al.*, 1992; Masada *et al.*, 1990; Orozco *et al.*, 2006; Pradeep *et al.*, 2009). Since IL-33 is a member of the IL-1 family, a role in periodontal disease pathogenesis is anticipated. However, although the role of IL-33 has been massively studied in other chronic inflammatory diseases, little is known about its role in periodontal disease.

There is tentative evidence that IL-33 may play a role in periodontal disease. Nile and colleagues (2010) have shown that LPS from the periodontal pathogen *P. gingivalis* can induce upregulated IL-33 expression in human monocytes (THP-1 cell line and primary monocytes). In addition, primary human gingival fibroblasts were shown to secrete IL-33 after stimulation with TNF-α (Beklen & Tsaous Memet, 2014). However, reports are varied with regard to the association between levels of IL-33 in biological samples and periodontal disease. IL-33 was found to be below detection levels in GCF samples of chronic periodontitis and healthy subjects when evaluated using a multiplex bead immunoassay (Papathanasiou *et al.*, 2014). However, Buduneli and colleagues (2012) showed that IL-33 levels in GCF were lower in chronic periodontitis patients compared to healthy samples, whilst no difference in IL-33 levels was observed in saliva and serum levels between the two groups.

1.7 Background and aims of study

Chronic periodontitis is a chronic inflammatory disease initiated by periodontal pathogens (e.g., *P. gingivalis*) that exist in dental biofilms (Armitage, 1999). Although initiated by pathogens the real damage to periodontal tissues is caused by an excessive host immune response to these pathogens (Lindhe, *et al.*, 1999). Therefore a greater understanding of the host oral immune response is required for us to fully delineate the pathogenesis of periodontal disease.

Cytokines play an important role in mediating the host immune response. IL-1 family cytokines such as IL-1α, IL-1β and IL-18 have been shown to have important roles in periodontal diseases (Jandinski *et al.*, 1991; Kinane, *et al.*,

1992; Masada, *et al.*, 1990; Orozco, *et al.*, 2006). However, the role of IL-33, another member of the IL-1 family of cytokines, in periodontal disease has yet to be fully investigated. IL-33 has been shown to be expressed in many tissues such as skin, gut, lung, brain and spinal cord (Hudson, *et al.*, 2008; Moussion, *et al.*, 2008; Schmitz, *et al.*, 2005) and expression found to be higher in inflammatory conditions (Beltran, *et al.*, 2010; Matsuda, *et al.*, 2009; Matsuyama, *et al.*, 2010; Xu, *et al.*, 2008). This pattern of IL-33 expression in tissues exposed to the environment indicates that IL-33 may be expressed in the oral epithelium. Furthermore, periodontal pathogens such as *P. gingivalis* have been demonstrated to stimulate upregulated expression of IL-33 in human monocytes (THP-1 cell line and primary monocytes) (Nile, *et al.*, 2010). Therefore it can be hypothesised that similar regulatory events may occur in oral epithelial cells.

The cytokine IL-17A has received considerable attention since the discovery of the Th17 cell subset (Aggarwal, *et al.*, 2003). Th17 cells have been demonstrated to be present in the periodontium (Schenkein, *et al.*, 2010), however analysis of diseased tissue suggests there is limited association of Th17 cells with periodontal disease progression (Culshaw *et al*, unpublished). In fact, it is now known that despite IL-17A being described as a T cell-secreted cytokine, much of the IL-17A released during an inflammatory response is produced by innate immune cells (Cua & Tato, 2010). Indeed, IL-17A has been shown to be associated with periodontal disease pathogenesis (Behfarnia, *et al.*, 2013; Honda, *et al.*, 2008; Ohyama, *et al.*, 2009; Takahashi, *et al.*, 2005). However, since the discovery of IL-17A, genomic sequencing efforts have led to the discovery of several putative IL-17(A) homologues; IL-17B - IL-17F. To date, little research has been conducted investigating the association of these other IL-17 family members with periodontal disease. In addition, evidence suggests that unlike other IL-17 family members, which have pro-inflammatory functions, IL-17E can down regulate localized destructive inflammatory responses in conditions including rheumatoid arthritis (Gaffen, 2009a). Therefore, it is interesting to speculate that IL-17E plays differing roles to IL-17A in the pathogenesis of periodontal disease. However, to date this hypothesis has not been investigated.

Based on these proposed hypotheses the aims of this study therefore were:

1. To evaluate levels of IL-10, IL-33 and IL-17 family cytokines in serum, saliva and GCF from patients with chronic periodontitis and healthy subjects.

2. To evaluate the expression of IL-33 and its receptors (ST2L and sST2) in periodontal tissue samples from patients with chronic periodontitis and healthy subjects.

3. To evaluate the mRNA expression of IL-10 and IL-17 family cytokines in periodontal tissue samples from patients with chronic periodontitis and healthy subjects.

4. To evaluate the expression of IL-33 and its receptors (ST2L and sST2) in oral keratinocyte cells using an *in vitro* live *P. gingivalis* monospecies biofilm model.

5. To evaluate the effect of IL-33 on the expression of inflammatory mediators by oral keratinocyte cells *in vitro*.

6. To evaluate the expression of IL-17E and its receptor (IL-17RB) in periodontal tissue samples from patients with chronic periodontitis and healthy subjects.

7. To evaluate the role of IL-17E in modulating the expression of chemokines (IL-8 and CXCL5) by oral keratinocytes in response to stimulation with *P. gingivalis* and IL-17A.

Chapter 2: Materials and methods

2.1 Study samples

Serum, gingival crevicular fluid (GCF) and saliva samples from healthy and chronic periodontitis patients were used in this study. Samples were selected from independent studies conducted at Glasgow Dental Hospital and School and Newcastle School of Dental Sciences and were from subjects that were systemically healthy non smokers. The sample collection and ethical approvals for the Glasgow sample cohort were as previously described (Lappin *et al.*, 2009; Pathiyal *et al.*, 2005). The sample collection and ethical approvals for the Newcastle cohort are as previously described (Davies *et al.*, 2011; Jaedicke *et al.*, 2012; Preshaw & Heasman, 2002).

The age range of the subjects in this study was 22 – 55 years. To be included in the study subjects had to be systemically healthy with a minimum of 16 teeth and at least four molars in different quadrants. Subjects were excluded if they previously had surgical periodontal therapy and if they were currently taking or had previously taken antibiotics or any other medication during the past six months. In addition, all smokers or former smokers and females who were pregnant at the time of the study or within the previous year were also excluded.

All subjects had a clinical periodontal examination carried out by a single calibrated clinician (Kappa scores for clinical probing depths and clinical attachment levels were 0.63 ($p < 0.001$) and 0.59 ($p < 0.001$) respectively). The following data were obtained: age, gender and clinical parameters for periodontal conditions (clinical probing depth (CPD), clinical attachment loss (CAL) and bleeding on probing (BOP)).

Full mouth periodontal chartings were recorded using a University of North Carolina PCP 15 manual probe at six sites around every tooth: mesiobuccal, distobuccal, mesiolingual, distolingual, midlingual and midbuccal. The gingival recession was recorded, which is the distance between the cemento-enamel junction and the gingival margin. A negative value was recorded if the gingival margin was coronal to the cemento-enamel junction. The probe was gently inserted into the gingival crevice or pocket along the long axis of the tooth until

resistance was felt. CPD was defined as the distance between the gingival margin and the base of the crevice or pocket. The CAL is a combined value of gingival recession and CPD. BOP was also recorded 30 seconds after placing the probe into the crevice or pocket.

Subjects were grouped as chronic periodontitis patients if they demonstrated a minimum of two sites with a CPD and CAL of ≥ 5 mm. The healthy subjects had no history of chronic periodontitis and no sites with a CPD or CAL of ≥ 2 mm.

2.2 Serum, gingival crevicular fluid and saliva samples

2.2.1 Serum samples

Blood was collected between 0900 and 1100 h (to minimize diurnal variations in biochemical parameters) from a peripheral vein in a coagulant tube. After clotting the sample was centrifuged (200 x g) and the serum isolated, snap frozen in aliquots and stored at –80 ^{o}C.

2.2.2 Gingival crevicular fluid samples

Gingival crevicular fluid (GCF) samples were obtained from the buccal aspects of two interproximal sites in single rooted teeth from each individual participating in the study. Except for the controls, GCF samples were obtained from sites with obvious dental biofilm accumulation and visible signs of inflammation such as hyperaemia. GCF samples were collected with filter paper strips (Oraflow Inc, New York). Prior to GCF sampling, supragingival biofilm was removed carefully by sterile curettes and the surfaces were dried and isolated by cotton rolls. Filter paper strips were placed in the gingival sulcus/pocket for 30 seconds. Care was taken to avoid mechanical trauma and strips visually contaminated with blood were discarded. Two strips from each patient were placed into one polypropylene tube before freezing at -80 ^{o}C. The strips were eluted prior to assay by the following method. The strips were placed into a microcentrifuge tube containing 50 µl of phosphate buffered saline (PBS) containing 0.05 % Tween® 20 (PBST) and centrifuged at 13,000 x g for 2 min. The supernatant (50

µl) was collected and transferred to a new microcentrifuge tube. The centrifugation was repeated three times with new PBST to give a total volume of 150 µl.

2.2.3 Saliva samples

Saliva was collected according to a modification of the method described by Navazesh and Christensen (1982). Prior to saliva sample collection, the subject was instructed to refrain from intake of any food or beverage for 1 h. Smoking, chewing gum and intake of coffee were prohibited during this hour. The subject was instructed to minimise all facial movements, particularly movements of the mouth. To begin saliva collection the subject was asked to void the mouth of saliva by swallowing. The subject was then asked to lean slightly forward over the tube and funnel. The subject was then instructed to keep his/her mouth slightly open and to allow saliva to drain into the funnel. At the end of the 5 min collection period, the subject was asked to collect any remaining saliva in his/her mouth and expectorate into the funnel. The saliva volume was measured using micropipettes (Finnipipette, Lab systems). Saliva samples were aliquoted and stored at -80 ^{o}C.

2.3 Tissue samples

Periodontal tissue samples were obtained from 26 subjects: 9 healthy and 17 chronic periodontitis. Healthy tissue samples were taken from patients undergoing non-periodontal disease related procedures such as crown lengthening and tooth extraction. Disease tissue samples were taken from patients suffering from chronic periodontitis, who required surgical periodontal therapy as part of their periodontal treatment regime. Gingival tissues were obtained from subjects with written consent, undergoing open flap debridement in the Unit of Periodontics at Glasgow Dental Hospital. Ethical review and approval was provided by the West of Scotland Research Ethics Committee. Patients undergoing open flap debridement had clinical probing depths of ≥ 5.0 mm with clinical attachment loss of ≥ 5.0 mm, which persisted after non-surgical treatment. Subject age ranged from 38 - 64 years with a mean age of 47 years.

The tissue samples were immediately submerged in RNA*later* (Qiagen, UK) and stored at -80 ^{o}C.

2.4 Cell culture

2.4.1 OKF6/TERT-2 cells

OKF6/TERT-2 cells were a kind gift from the Rheinwald laboratory (Brigham and Women's Hospital, Boston). The cells were originated from keratinocyte cells of the oral mucosa, which have been immortalized by forced ectopic expression of the telomerase catalytic subunit, hTERT (Dickson *et al.*, 2000). The cells resemble primary oral keratinocyte cells and are regarded as a valuable and reproducible model for normal oral epithelial cell studies (Dongari-Bagtzoglou & Kashleva, 2006).

OKF6/TERT-2 cells were grown in keratinocyte serum-free medium (KSFM) (Invitrogen, UK) supplemented with 25 µg/ml bovine pituitary extract (Invitrogen, UK), 0.2 ng/ml epidermal growth factor (Invitrogen, UK), 2 mM L-glutamine (Sigma-Aldrich, UK), 100 IU/ml penicillin (Sigma-Aldrich, UK), 100 mg/ml streptomycin (Sigma-Aldrich, UK), and 0.4 mM calcium chloride. The OKF6/TERT-2 cells were allowed to grow in vented tissue culture flasks (Corning, UK) in a humidified atmosphere with 5 % CO_2 at 37 ^{o}C. The growth media was changed at two or three day intervals until the cells reach 70 % confluence at which point they were subcultured.

To subculture, the spent media was removed from the flask and cells were washed with pre-warmed PBS (Invitrogen, UK). Appropriate volumes (e.g., 4 ml for a 75 cm^2 flask) of 0.05 % trypsin/EDTA (ethylenediaminetetraacetic acid) (Invitrogen, UK) were then added to the flask and incubated at 37 ^{o}C until the cells detached (2 to 5 min). The trypsin was then inactivated by adding double the volume of dulbecco's modified eagle medium (DMEM) with 10 % (v/v) fetal bovine serum (Invitrogen, UK) to the flask. The cell suspension was then removed from the flask and placed in a sterile 25 ml centrifuge tube and centrifuged at 1000 rpm for 5 min. The supernatant was again removed and cells

were resuspended in 4 ml Hank's solution (Sigma-Aldrich®, UK) and then centrifuged at 1000 rpm for 5 min. The cells were finally resuspended in 4 ml KSFM, and viable cell counts were performed using a haemocytometer. A 1-2 x 10^5 cell suspension was then prepared in KSFM and cells seeded into a vented 75 cm^2 cell culture flask (Corning, UK). The cells were then incubated in a humidified atmosphere with 5 % CO_2 at 37 ^oC.

2.4.2 Primary human gingival epithelial cells

Commercially available primary human gingival epithelial (PHGE) cells were bought from CELLnTEC advanced cell systems (Switzerland). This is a pooled suspension of primary cells isolated from healthy adult gingival tissues of three or more donors. The PHGE cells were grown and subcultured as described for the OKF6/TERT-2 cells (Section 2.4.1). Only cells at passage two to five were used experimentally.

2.4.3 Cryopreservation of cells

Cryopreservation of cells (OKF6/TERT-2 cells and PHGE cells) was carried out using dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK) as a freezing medium. DMSO (20 %) was prepared by adding 4 ml of DMSO to 16 ml fetal bovine serum (Invitrogen, UK) and the solution was filter-sterilized (0.22 µm). For the purpose of cryopreservation, the cells were harvested during exponential growth. The cells were pelleted and suspended in a KSFM (Invitrogen, UK) at a concentration of 2 x 10^6 cells/ml in a sterile universal tube. In a dropwise manner, an equal volume of cold 20 % DMSO was then added to the cell suspension and 1 ml of the cell solution was transferred to a labelled cryovial. The cryovial was then chilled on ice for 30 min, and then placed in a pre-chilled insulation box to be kept at -80 ^oC for 6 to 16 h. The cryovial was finally transferred into liquid nitrogen for long term preservation.

2.4.4 Thawing of cryopreserved cells

The cryopreserved cells (OKF6/TERT-2 cells and PHGE cells) were removed from liquid nitrogen and quickly thawed at 37 ^{o}C. The cryovial outer surface was disinfected with 70 % ethanol, and carefully the cells were transferred into a 25 cm^2 cell culture flask (Corning, UK) pre-filled with 9 ml warm KSFM (Invitrogen, UK). The cells were then incubated overnight in a humidified atmosphere with 5 % CO_2 at 37 ^{o}C. The media were changed the next day. Growth and subculture was performed as described previously (Section 2.4.1 and Section 2.4.2).

2.5 *Porphyromonas gingivalis* monospecies biofilm

2.5.1 Bacterial growth conditions

The monospecies biofilms were prepared using *P. gingivalis* ATCC 33277, which was obtained from the American Type Culture Collection (ATCC, USA). The bacteria were maintained at 37 ^{o}C on fastidious anaerobic agar (Oxoid, Cambridge, UK) with 5 % defibrinated horse blood (E&O laboratories, UK) in an anaerobic chamber (Don Whitley Scientific Limited, UK) for 3 days. The chamber was set at 85 % N_2, 10 % CO_2 and 5 % H_2. Prior to biofilm preparations, the bacteria were grown at 37 ^{o}C in schaedler anaerobe broth (Oxoid, UK) for 2 days. After 2 days the broth was centrifuged at 3000 rpm for 5 min to obtain a pellet of bacteria, which was then washed three times with PBS (pH 7.4). The bacteria were then resuspended in PBS and standardized at 0.2 OD_{550} in a colorimeter (Fisher Scientific, UK) to obtain approximately 1×10^8 CFU (colony forming unit)/ml. Live bacterial counts were confirmed as described in Section 2.5.2.

2.5.2 Standard plate counting method

The method was adapted from the Miles and Misra method (1938) and was used to determine the number of colony forming units (CFU) in a bacterial suspension. The bacterial suspension was serially diluted (1:10 dilutions) up to 10^{-5} with PBS. Fastidious anaerobic agar (Oxoid, UK) plates with 5 % defibrinated horse blood (E & O laboratories, UK) were prepared in advance.

The surfaces of the fastidious anaerobic agar plates were first sufficiently dried to allow a 20 µl drop of dilution to be absorbed onto the plate in 15 - 20 min. Each plate was then divided into four equal sectors. In each sector, three drops of the appropriate dilution were placed onto the surface of the agar and the drops were allowed to spread naturally over an area of 1.5 to 2.0 cm. The plates were left upright to dry on the bench before inversion and incubation in an anaerobic chamber (Don Whitley Scientific Limited, UK) for 24 to 48 h with continuous observation. The chamber setting was 85 % N_2, 10 % CO_2 and 5 % H_2. Colonies were then counted by eye. The following equation was used to calculate the number of CFU per ml from the original sample dilution: CFU/ml = Average number of colonies for a dilution x 50 x dilution factor.

2.5.3 Artificial saliva

Artificial saliva was used to prepare a *P. gingivalis* monospecies biofilms. The use of artificial saliva was to simulate the biofilm growth conditions of the oral cavity. Artificial saliva base was prepared by mixing the following substances in distilled water: 0.25 % (w/v) porcine stomach mucin (Sigma-Aldrich, UK), 0.35 % (w/v) sodium chloride (VWR International, Belgium), 0.02 % (w/v) potassium chloride (VWR International, Belgium), 0.02 % (w/v) calcium chloride dihydrate (VWR International, Belgium), 0.2 % (w/v) yeast extracts (Sigma-Aldrich, UK), 0.1 % (w/v) lab lemco powder (Oxoid, UK) and 0.5 % (w/v) proteose peptone (Sigma-Aldrich, UK). The artificial saliva base was autoclaved at 121 ^{o}C for 15 min and left to cool. Finally, an autoclaved solution of 0.05 % (v/v) urea (Oxoid, UK) was added to the base to produce the final artificial saliva solution which was kept at 4 ^{o}C prior to use.

2.5.4 Preparation of *Porphyromonas* gingivalis monospecies biofilms

P. gingivalis cultures (Section 2.5.1) were standardized to 1×10^7 CFU/ml in artificial saliva (Section 2.5.3). Next, 500 µl of the standardized *P. gingivalis* solution was cultured onto 13 mm diameter cell culture treated Thermanox® plastic coverslips (Nalge Nunc International, UK) placed in 24-well cell culture plates (Corning, UK). The cultures were incubated at 37 ^{o}C in an anaerobic

chamber (Don Whitley Scientific Limited, UK) for 4 days. The chamber setting was at 85 % N_2, 10 % CO_2 and 5 % H_2. Daily, the spent artificial saliva was replaced with 500 µl new artificial saliva. After the final incubation, the artificial saliva was removed and the biofilms were kept at -80 ^{o}C prior to use for cell stimulation experiments.

2.5.5 Validation of the *Porphyromonas* gingivalis monospecies biofilms

2.5.5.1 Viability test

The *P. gingivalis* monospecies biofilms were revived by thawing in 500 µl artificial saliva and incubating overnight in an anaerobic chamber. The biofilms were removed from the artificial saliva the next day and washed in PBS. The excess PBS was removed by wiping the coverslip (biofilm free side) on sterile paper. Each biofilm was then placed in 1 ml PBS in a 5 ml universal tube. To re-suspend the *P. gingivalis*, the universal tube was subjected to sonication at 35 kHz for 5 min in an ultrasonic bath (Fisherbrand® FB 11021; Fisher Scientific, UK). The CFU of the *P. gingivalis* suspension was determined using a standard plate counting method (Section 2.5.2).

2.5.5.2 Gram staining

Gram staining is a bacteriological laboratory technique used to differentiate Gram-positive and Gram-negative bacterial species based on the biochemical properties of their cell walls. The *P. gingivalis* monospecies biofilms were revived by thawing in 500 µl artificial saliva and incubating overnight in an anaerobic chamber. The biofilms (coverslip) were removed from the artificial saliva the next day and washed in PBS. The excess PBS was removed by wiping the coverslip (biofilm free side) on sterile paper, then the biofilms were left to dry. The biofilms were subjected to Gram staining procedure, using a Gram staining kit (Pro-Lab Diagnostics Inc., UK) which involved the following steps. The primary stain (crystal violet) were added to the coverslip (biofilm) and incubated for 1 min. The coverslip was rinsed with a gentle stream of water for a maximum of five seconds to remove unbound crystal violet. Then the Gram's iodine (an agent that fixes crystal violet to bacterial cell walls) was added for 1

min, followed by a gentle rinse with acetone for about three seconds and a rinse with a gentle stream of water. Then safranin, a secondary stain was added to the coverslip for 1 min, followed by rinsing with a gentle stream of water for a maximum of five seconds. The coverslip was then placed on a glass slide for viewing under a microscope. The Gram-positive bacteria will retain the primary stain (crystal violet) and not uptake the secondary stain (safranin) causing it to look purple under a microscope. While Gram-negative bacteria will lose the primary stain and uptake the secondary stain causing it to appear red under a microscope. The gram stain process was used to confirm visually the presence of an abundant monospecies biofilm of the Gram-negative organism (*P. gingivalis)* had grown on the coverslips.

2.6 Cell stimulation studies

2.6.1 Stimulation of cells with a live *Porphyromonas gingivalis* monospecies biofilm

A day prior to the stimulation experiments, OKF6/TERT-2 cells (Section 2.4.1) or PHGE cells (Section 2.4.2) were seeded in duplicate at 2 x 10^5 cells/ml in 24-well cell culture plates (Corning, UK), and incubated overnight in a humidified atmosphere at 37 ºC with 5 % CO_2. At the same time, the *P. gingivalis* monospecies biofilm (Section 2.5.4) were thawed in 500 µl artificial saliva and incubated overnight in an anaerobic chamber.

After an overnight incubation, the spent media was removed from the cell monolayer which was subsequently washed once with pre-warmed PBS (Invitrogen, UK). The cells were then placed in 1 ml of defined keratinocyte serum-free medium (DKSFM) (Invitrogen, UK).

The *P. gingivalis* monospecies biofilms were taken out of artificial saliva and washed by dipping three times in PBS. The excess PBS was removed by wiping the coverslip (on the biofilm-free side) on sterile paper. Each biofilm was then attached to a 24-well Millicell® cell culture insert (Millipore, UK) using sterile commercially available Vaseline®. The biofilm was then suspended above the cell monolayer in the wells of a 24-well plate as shown in Figure 2-1.

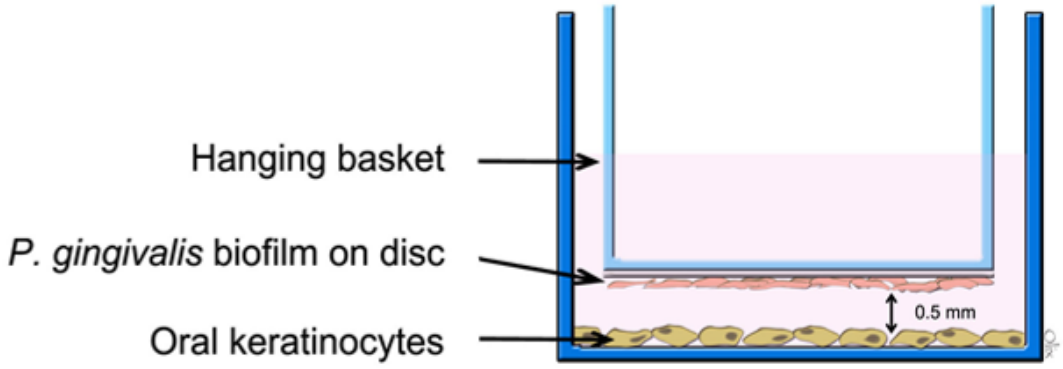

Figure 2-1: Diagrammatic representation of the *P. gingivalis* monospecies biofilm model

A live *P. gingivalis* monospecies biofilm is cultured on cell culture treated Thermanox® plastic coverslips which is then secured to the base of a hanging basket with sterile Vaseline® (Sherry *et al.*, 2013). The biofilm is then suspended 0.5 mm above a monolayer of cultured oral keratinocytes. The 0.5 mm is a physiologically relevant space representing the gap between the tooth surface and gum tissue.

The experimental protocols used in this thesis are summarised in Table 2-1. The co-cultures were incubated for between 3, 6, 9 and 24 h in a humidified atmosphere with 5 % CO_2 at 37 ^{o}C. A duplicate unstimulated control for each incubation time point was also performed. After completion of the respective incubation period, the culture supernatant was removed and stored at -20 ^{o}C for analysis by ELISA. The remaining adherent cells were lysed with 350 µl of RLT lysis buffer (Qiagen, UK) with 1 % ß-Mercaptoethanol. The lysate was stored at -80 ^{o}C for future RNA isolation.

Experiment design	Stimulation conditions	Incubation Hour (h)	Evaluation
Effect of *P. gingivalis* biofilm stimulation on OKF6/TERT-2 cells or PHGE cells	**(a)** Control; and (**b**) Biofilm[a]	3, 6, 9 & 24 h	a) IL-8 protein release (ELISA) b) mRNA expression and protein release of IL-33 and its receptors (real-time PCR and ELISA respectively) c) mRNA expression of IL-17 family (real-time PCR; OKF6/TERT-2 cells only)
		9 h	a) IL-8 protein release (ELISA) b) IL-33 and ST2 protein expression (immunocytochemistry)
Effect of IL-17E on OKF6/TERT-2 cells stimulated by a *P. gingivalis* biofilm	**(a)** Control; (**b**) rhIL-17E (50 ng/ml); (**c**) Biofilm[a]; and (**d**)Treat with rhIL-17E (50 ng/ml) for 30 min, followed by biofilm[a]	4 & 24 h	a) mRNA expression and protein release of IL-8 and CXCL5 (real-time PCR and ELISA respectively)
Effect of IL-17E on OKF6/TERT-2 cells stimulated by IL-17A	**(a)** Control; (**b**) rhIL-17E (400 ng/ml); (**c**) rhIL-17A (10 ng/ml); (**d**) Treat with combination of rhIL-17A (10 ng/ml) and rhIL-17E (10, 50, 100, 200 or 400 ng/ml); and (**e**) Treat with rhIL-17E (10, 50, 100, 200 or 400 ng/ml) for 30 min, followed rhIL-17A (10 ng/ml)	24 h	a) mRNA expression and protein release of IL-8 (real-time PCR and ELISA)
Effect of IL-33 on OKF6/TERT-2 cells	**(a)** Control; (**b**) PMA (10 ng/ml); and (**c**) rhIL-33 (10, 50 or 100 ng/ml)	4, 24 & 48 h	a) mRNA expression of IL-8, IL-1RA, G-CSF, TLR-2 and TLR-4 (real-time PCR) b) Protein release of IL-8, IL-1RA and G-CSF (ELISA) c) Proteome profiler analysis (24 h only)

Table 2-1: Oral keratinocyte stimulation experimental protocols

See Section 2.5.4 for biofilm preparation ([a]). Detail descriptions of the materials used are as follow: rhIL-17A = recombinant human IL-17A (Cell Signalling, USA); rhIL-17E = recombinant human IL-17E (PeproTech, USA); PMA = phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich®, UK); and rhIL-33 = recombinant human IL-33 (PeproTech®, UK).

2.6.2 Effect of IL-17E on OKF6/TERT-2 cells stimulated by *Porphyromonas gingivalis* monospecies biofilm

OKF6/TERT-2 cells were prepared for stimulation experiments as described previously (Section 2.4.1). *P. gingivalis* monospecies biofilms were prepared for stimulation experiment as described previously (Section 2.5.4). The experimental protocol is summarised in Table 2-1. Briefly, the cells were either stimulated with rhIL-17E (50 ng/ml) alone, biofilm alone or 30 min pre-incubation with rhIL-17E (50 ng/ml) followed by biofilm stimulation. The stimulations were carried out in DKSFM (Invitrogen, UK). The cultures were incubated for 4 and 24 h in humidified atmosphere at 37 ^{o}C and 5 % CO_2. Duplicate unstimulated controls for each incubation time point was also included in the experiment. After completion of the respective incubation period, the culture supernatant was removed and stored at -20 ^{o}C for analysis by ELISA. The remaining adherent cells were lysed with 350 µl of RLT lysis buffer (Qiagen, UK) with 1 % ß-Mercaptoethanol. The lysate was stored at -80 ^{o}C for future RNA isolation.

2.6.3 Effect of IL-17E on OKF6/TERT-2 cells stimulated by IL-17A

OKF6/TERT-2 cells were prepared for stimulation experiments as described previously (Section 2.4.1). The experimental protocol is summarised in Table 2-1. Briefly, the cells were either incubated with rhIL-17E (400 ng/ml) alone, stimulated with rhIL-17A (10 ng/ml) alone, stimulated with combination of rhIL-17A (10 ng/ml) and rhIL-17E (10, 50, 100, 200 or 400 ng/ml) or 30 min pre-incubation with rhIL-17E (10, 50, 100, 200 or 400 ng/ml) followed by stimulation with rhIL-17A (10 ng/ml). The stimulations were carried out in DKSFM (Invitrogen, UK). The cultures were then incubated for 24 h in a humidified atmosphere with 5 % CO_2 at 37 ^{o}C. A duplicate of unstimulated control for each incubation time point was also included in the experiment. After completion of the respective incubation period, the culture supernatant was removed and stored at -20 ^{o}C for analysis by ELISA. The remaining adherent cells were lysed with 350 µl of RLT lysis buffer (Qiagen, UK) with 1 % ß-Mercaptoethanol. The lysate was stored at -80 ^{o}C for future RNA isolation.

2.6.4 Effect of IL-33 on OKF6/TERT-2 cells

OKF6/TERT-2 cells were prepared for stimulation experiments as described previously (Section 2.4.1). The experimental protocol is summarised in Table 2-1. Briefly, the cells were incubated in duplicate with either PMA (10 ng/ml) (Sigma-Aldrich®, UK) as a positive control or rhIL-33 (10, 50 or 100 ng/ml) (PeproTech®, UK). The culture was then incubated for 4, 24 and 48 h in a humidified atmosphere with 5 % CO_2 at 37 ^{o}C. A duplicate unstimulated control for each incubation time point was also included in the experiment. After completion of the respective incubation period, the culture supernatant was removed and stored at -20 ^{o}C for analysis by ELISA. The remaining adherent cells were lysed with 350 µl of RLT lysis buffer (Qiagen, UK) with 1 % ß-Mercaptoethanol. The lysate was stored at -80 ^{o}C for future RNA isolation.

2.6.5 Validating the bioactivity of recombinant human IL-33

Naïve $CD4^{+}$ T cells isolated from cord blood produced enhanced levels of IL-5 when cultured with an anti-CD3 antibody and IL-33, compared with anti-CD3 antibody alone (Kurowska-Stolarska, *et al.*, 2008). Since peripheral blood mononuclear cells (PBMCs) were also known to compose naïve $CD4^{+}$ T cells (Pflanz *et al.*, 2002), we used easily accessible PBMCs instead of cord blood cells for validation of the bioactivity of rhIL-33 (PeproTech®, UK) prior to the IL-33 stimulation study (Section 2.6.4). The study was carried out using PBMCs isolated from human venous blood. Based on the finding by Kurowska-Stolarska and colleagues (2008), the bioactivity of rhIL-33 in this study was evaluated by comparing levels of IL-5 produced by PBMCs when cultured with an anti-CD3 antibody and rhIL-33, and anti-CD3 antibody alone.

Human venous blood was collected in an EDTA tube (final concentration of 1.5 – 2.0 mM) and used within 2 h of drawing. PBMCs were isolated from venous whole blood using Polymorphprep™ (Axis-Shield, UK) as previously devised by Boyum (1968). Briefly, a Pasteur pipette was used to layer 5 ml blood over 5 ml Polymorphprep™ in 15 ml centrifuge tubes. The tubes were centrifuged at 500 x g for 30 – 35 min at room temperature (20 ^{o}C). The rotor was allowed to decelerate without the brake. Using a Pasteur pipette, the upper plasma layer was then drawn off, leaving the PBMC layer. The PBMC layer was aspirated using

a Pasteur pipette, and transferred into a fresh 15 ml centrifuge tube containing 10 ml RPMI (Roswell Park Memorial Institution) media (Sigma-Aldrich, UK). The RPMI was supplemented with 10 % fetal bovine serum (Sigma-Aldrich®, UK), 2 mM L-glutamine (Sigma-Aldrich®, UK) and 1 % penicillin/streptomycin solution (Sigma-Aldrich®, UK). The tube was then centrifuged at 400 x g to pellet the cells. The cells were then washed twice in 10 ml PBS by centrifugation at 400 x g for 10 min. Cells were finally resuspended in 5 – 10 ml RPMI and counted.

For the bioactivity assay, the cells were seeded in 6-well plates at 1 x 10^6 cells/ml (3 ml in each well) and stimulated in duplicate with combinations as follow: anti-CD3 (3 µg/ml) alone; anti-CD3 (3 µg/ml) and rhIL-33 (50 ng/ml); anti-CD3 (3 µg/ml) and rhIL-33 (100 ng/ml). The cells were incubated for 3 days in a humidified atmosphere at 37 ^oC and 5 % CO_2. The supernatants were collected and centrifuged at 10000 x g for 15 min at 4 ^oC. The supernatants were transferred into a new centrifuge tubes and stored at -20 ^oC prior to analysis for IL-5 by ELISA (PeproTech®, UK). The experiment was repeated with three separate donors.

2.7 Protein analyses

2.7.1 Enzyme-linked Immunosorbent Assay

Cytokine concentrations in biological fluids and cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA). Although various kits from various sources were used in this study (Table 2-2), the general assay principal was mainly the same. The general ELISA procedure is described as below.

Nunc-Immuno® MaxiSorp® 96-well Plates (Nalge Nunc International, UK) were coated with coating antibody diluted in coating buffer, and incubated overnight (12 – 18 h). The plate was washed between 1 and 5 times with wash buffer PBS with 0.05 % Tween® 20 (Sigma-Aldrich, UK). The plate was then blocked with blocking buffer for 2 h at room temperature. The plate was washed again between 1 and 5 times with wash buffer. Subsequently, duplicates of sample or pre-prepared standards were added to the wells of the 96-well plate and the

plate incubated for 2 h at room temperature. The plate was washed between 1 and 5 times with wash buffer. After washing, the detection antibody was added to each well and the plate incubated for 1 h at room temperature. The plate was washed again between 1 and 5 times with wash buffer. The plate was then incubated with Streptavidin-HRP for 30 min at room temperature. Finally, substrate solution e.g., 3,3′,5,5’-tetra-methylbenzidine (TMB) substrate (KPL, Gaithersburg, USA) was added to each well and the plate incubated for 5 – 30 min to allow colour development. Colour development was then stopped using 1 M HCl and the absorbance was measured at 450 nm with wavelength correction set at 650 nm using a microplate reader (FLUOstar Omega; BMG Labtech, Germany). For some assays 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or ABTS (Sigma-Aldrich, UK) was used as a substitute substrate solution. On these occasions colour development was measured directly without addition of a stop solution at 405 nm with wavelength correction set at 650 nm. The plate was monitored at 5-min intervals for approximately 30 min. Data analysis was performed using Labtech data analysis software (BMG Labtech, Germany). In all cases, data was analysed using a log-log or 4-parameter curve fit.

Although the general assay principal was the same; there were some subtle differences in assay procedure depending on assay source. These are detailed in Table 2-2. For each individual ELISA the assay was optimized and validated prior to use. Table 2-3 shows the concentration of each antibody used in each assay. In addition, the sensitivity limits of each assay were determined as two mean standard deviations higher than the mean baseline from 6 replicates standard curves (Table 2-3) (Chaloner-Larsson *et al.*, 1997).

Manufacturer	Coating antibody (ab)	Blocking	Detection antibody (ab)	Peroxidase	Substrate	Solutions
Invitrogen, UK	Dilute in coating buffer, incubate overnight (4°C)	Use diluent, block for 1hr (RT)	Dilute in diluent, incubate with samples for 2 h (RT)	Streptavidin-HRP 30 min (RT)	TMB	Coating buffer: 0.1 M $NaHCO_3$ (pH 8.2) Diluent: PBS + 0.5% BSA + 0.1% + Tween® 20 (pH 7.4)
PeproTech, USA	Dilute in PBS, incubate overnight (RT)	Use diluent, block for 1hr (RT)	Dilute in diluent, incubate for 2 hr (RT)	Streptavidin-HRP 30 min (RT)	ABTS	Diluent: 0.1% BSA, 0.05% Tween® 20 in PBS
R&D System, UK (DuoSet®)	Dilute in PBS, incubate overnight (RT)	Use diluent, block for 1hr (RT)	Dilute in diluent, incubate for 2hr (RT)	Streptavidin-HRP 30 min (RT)	TMB	Diluent: 1% BSA in PBS (0.2 µm filtered)
R&D System, UK (Quantikine® ST2)	Ab-coated plate is supplied	Use diluent, incubate with sample for 2hr (RT)	Use as supplied, incubate for 2 hr (RT)	HRP conjugated to detection ab	TMB	Diluent & detection Ab: supplied by manufacturer
Life Science, UK	Dilute in PBS, incubate overnight (4°C)	Use diluent, block for 2hr (RT)	Dilute in diluent, incubate for 1hr (RT)	Streptavidin-HRP 30 min (RT)	TMB	Diluent: 2% BSA in PBS (0.2 µm filtered)

Table 2-2: Manufacturer variations in ELISA procedure

BSA = Bovine serum albumin; TMB = 3, 3′, 5, 5'-tetra-methylbenzidine substrate (KPL, Gaithersburg, USA); ABTS = 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (Sigma-Aldrich, UK)); $NaHCO_3$ = sodium hydrogen carbonate; PBS = phosphate-buffered saline; RT = room temperature; HRP = horseradish peroxidase; and Ab = antibody.

ELISA	Suppliers	Capture antibody (µg/ml)	Detection antibody (µg/ml)	Sensitivity limit (pg/ml)	Range (pg/ml)
Human IL-1RA	PeproTech®, USA	0.5	0.5	15.1	7.8 – 1000
Human IL-5	PeproTech®, USA	0.25	0.5	64	31.2 - 2000
Human CXCL5	R&D Systems, UK	2.0	0.10	7.8	7.8 -1000
Human IL-8	Invitrogen, UK	1.0	0.04	12.5	12.5 – 800
Human IL-10	PeproTech®, USA	1.0	0.5	39	7.8 - 1000
Human IL-33	Life Science, UK	5	1	15.5	3.9 - 500
Human ST2	R&D Systems, UK	UN	UN	5.6	3.9 - 1000
Human G-CSF	PeproTech®, USA	1	0.25	16	7.8 - 1000
Human IL-17A	PeproTech®, USA	0.5	0.25	1.9	3.9 – 1000
Human IL-17B	R&D System, UK	2.0	2.00	>7.8 – <15.6	15.6 – 2000
Human IL-17C	R&D Systems, UK	2.0	0.20	>7.8 – <15.6	15.6 – 2000
Human IL-17D	PeproTech®, USA	1.0	0.50	>19.6 – <39	39.1 – 5000
Human IL-17E	PeproTech®, USA	1.0	0.25	>2 – <3.9	3.9 – 1000
Human IL-17F	PeproTech®, USA	1.0	1.00	>2 – <3.9	3.9 – 1000
Human IL-17A/F	R&D Systems, UK	0.8	4.00	>4.9 – <9.8	9.8 – 2500

Table 2-3: ELISA antibody concentrations and sensitivities

The sensitivity limits of each assay were determined as two mean standard deviations higher than the mean baseline from six replicate standard curves (Chaloner-Larsson, *et al.*, 1997). UN = undisclosed concentration (readily prepared by manufacturer).

2.7.2 Immunocytochemistry

OKF6/TERT-2 cells (Section 2.4.1) were seeded at 2 x 10^5 cells/ml on top of glass disc (13 mm^2) in 24-well cell culture plates (Corning, UK) and incubated overnight in a humidified atmosphere at 37 ^oC and 5 % CO_2. After overnight incubation, the cells were stimulated with *P. gingivalis* monospecies biofilm (Section 2.5.4) for 9 h. After completion of the incubation period, the culture supernatant was removed and IL-8 release analysed by ELISA to confirm cells had been stimulated before further analysis was undertaken.

Immunocytochemical analyses of IL-33 and ST2 expression by OKF6/TERT-2 cells were performed using the ImmPRESS™ detection system kit (Vector Laboratories, USA). After the cell supernatants were removed, the cells on discs were washed twice for 10 min each with Tris-buffered saline (with 0.05 % Tween®) (TBST). The cells were then fixed in cold methanol (-20 ^oC) for 15 min. After aspiration of the methanol the discs were air dried for 10 min. The cells were then permeabilized for 10 min at room temperature using 0.2 % Triton X-100 (Sigma-Aldrich, UK) diluted in PBS. The discs were then washed twice for 10 min each with TBST. To avoid non-specific binding, the discs were blocked for 30 min at room temperature with 2.5 % normal horse serum. The serum was then aspirated and replaced with the appropriate primary antibody, pre-absorbed primary antibody or isotype control diluted in 2.5 % horse serum in TBST (Table 2-4). The discs were then incubated overnight on a shaker at 4 ^oC. The discs were brought up to room temperature the next day and antibody solutions were aspirated from the wells prior to washing twice for 10 min each with TBST. The discs were then incubated for 30 min at room temperature with the appropriate ready to use secondary antibody (ImmPRESS™ kit; Vector, USA) (Table 2-4). Then the discs were washed twice for 10 min each with TBST. Finally, the peroxidase activity was visualised by addition of ImmPACT™ DAB (3, 3'-diaminobenzidine) peroxidase substrate (Vector Laboratories, USA). Discs were incubated with DAB peroxidase substrate until the desired stain intensity developed. The discs were then rinsed with tap water for 5 min. The discs were then counter stained with filtered Harris Haematoxylin (Sigma-Aldrich, UK), and the excess washed off with running tap water. The discs were mounted on glass slides using mounting medium (Dako, UK). Photomicrographs were obtained using the Olympus® BX40 microscope system and Cell^B Olympus® Soft Imaging

software. The number of IL-33 and ST2 expressing cells were determined by counting the protein immunoreactive cells on photomicrographs obtained from at least five random high-power microscopic fields (400 x magnifications) as described in Section 2.7.4.

Pre-absorbing antibody experiments were only carried out on the mouse monoclonal anti-IL-33 antibody (Enzo® Life Science, UK). Pre-absorption was carried out by adding 5 µg/ml mouse monoclonal anti-IL-33 antibody to a double concentration of rhIL-33 (PeproTech®, UK) in 2.5 % horse serum in TBST and incubating for 1 h on a shaker at 37 ^{o}C. For control purposes, the antibody alone was subjected to the same protocol prior to use.

	Primary antibody	Isotype control	Secondary antibody ImmPRESS™ detection system (Vector Laboratories, USA)
IL-33	Mouse monoclonal anti-IL-33 antibody (5 µg/ml) (Nessy-1, Enzo® Life Science, UK)	Mouse monoclonal IgG1 (5 µg/ml) (Thermo Scientific, UK)	anti-mouse IgG
ST2	Rabbit anti-IL1RL1 antibody (0.2 µg/ml) (Sigma-Aldrich®, UK)	Rabbit IgG (0.2 µg/ml) (Abcam®, UK)	anti-rabbit IgG
IL-17E	Mouse monoclonal anti-IL-17E antibody (1 µg/ml) (Abcam®, UK)	Mouse monoclonal IgG1 (1 µg/ml) (Thermo Scientific, UK)	anti-mouse IgG
IL-17RB	Rabbit anti-IL17RB antibody (0.2 µg/ml) (Sigma-Aldrich®, UK)	Rabbit IgG (0.2 µg/ml) (Abcam®, UK)	anti-rabbit IgG

Table 2-4: Antibodies used for immunocyto- and immunohisto- chemistry

2.7.3 Immunohistochemistry

Diseased tissue samples were taken from patients suffering from chronic periodontitis, who required surgical periodontal therapy as part of their periodontal treatment regime. Whereas, healthy tissue samples were taken from patients undergoing non-periodontal disease related procedures such as crown lengthening and tooth extraction (Section 2.3).

Tissue samples were fixed with 4 % formaldehyde and embedded in paraffin. The immunohistochemistry for IL-33, ST2, IL-17E and IL-17RB was performed on 5 µm thick paraffin embedded sections from periodontal tissues and from tonsil (used as a positive control). The immunohistochemistry was performed using the ImmPRESS™ detection system kit (Vector Laboratories, USA).

Briefly, 5 mm paraffin sections were deparaffinised by heating in an oven at 60 ^{o}C for 35 min, and rehydrated in graded alcohol. The sections were incubated with 0.5 % hydrogen peroxidase in methanol to block any endogenous peroxidase activity. For antigen retrieval, sections were pre-treated by boiling in 0.1 M citrate buffer (pH 6.0) in a microwave (700 W, 5 min). Nonspecific reactions were blocked for 30 min at room temperature using ready to use 2.5 % normal horse serum. Then, specimens were incubated overnight in a humidified chamber at 4 ^{o}C with the appropriate concentrations of primary antibodies diluted in 2.5 % horse serum in TBST (Table 2-4). Next day, the specimens were washed twice for 5 min in TBST and the sections incubated for 30 min at room temperature with the appropriate secondary antibody (Table 2-4). After washing twice for 5 min in TBST, the peroxidase activity was visualised using ImmPACT™ DAB (3, 3'-diaminobenzidine) peroxidase substrate solution (Vector Laboratories, USA). The sections were then lightly counterstained with Harris Haematoxylin (Sigma-Aldrich®, UK). The sections were mounted on glass slides using glycerol mounting medium (Dako, UK).

Photomicrographs were obtained using the Olympus® BX40 microscope system and Cell^{B} Olympus® Soft Imaging software. The number of IL-33, ST2 and IL-17RB expressing cells were determined by counting the protein immunoreactive cells on photomicrographs obtained from at least five random high-power microscopic fields (400 x magnifications) as described in Section 2.7.4.

2.7.4 Quantification of immunostained cells

Quantification of immunostained cells in the immunohistochemical or immunocytochemical analysis was performed using a manual cell counting grid as described by Bologna-Molina and colleagues (2011). This method uses only a digital camera adapted to a microscope and a personal computer. Briefly, photomicrographs of 400 x magnification were captured using the Olympus® BX40 microscope system, and stored as jpeg files. At least five random photomicrographs were captured for each sample. This equates to between 300 – 500 cells for immunocytochemical analysis, and a range of 300 – 1000 cells for immunohistochemical analysis. Each jpeg file was opened using Microsoft Office PowerPoint™ (Microsoft Corporation,USA). The 6 x 6 grid (Figure 2-2) was placed over the entire image completely. For immunohistochemical analysis, areas not containing the tissue of interest were visually excluded. For each image, cell counting was started in the top left corner and finished in the top right corner (Figure 2-2). The number of both negative and positive stained cells was counted manually in each image. This image counting procedure was carried out by three independent individuals, and the average percentage of positive stained cells calculated by all individuals was used. The percentage of positive stained cells was calculated as follows: Percentage of positive cells = (positive cells / total cells) x 100.

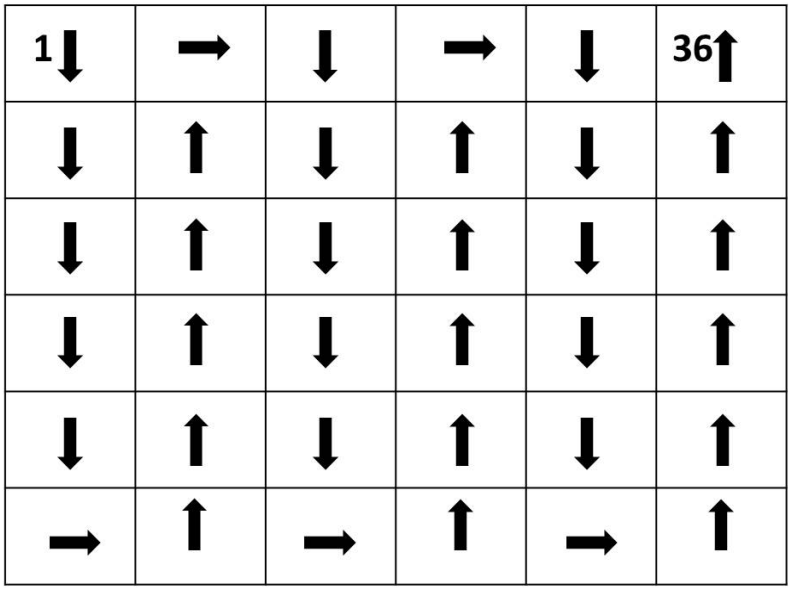

Figure 2-2: Schematic figure of the grid used

The counting starts at box 1, and continuous as shown by arrows until box 36. Numbers of positive and negative cells were counted for each box.

2.7.5 FACE™ NF-κB p65 profiler assay

Fast Activated Cell-based ELISA (FACE™) kits are highly sensitive 96-well assays designed for detecting activated protein within mammalian cells without the requirement for preparation of cell extracts, electrophoresis or membrane blotting. FACE™ NFκB p65 profiler kits are designed to quantify levels of activated (phosphorylated) forms of NFκB p65 subunits and/or total NFκB p65. The antibodies used in this FACE™ kit were specific for total (regardless of phosphorylation state) NF-κB p65 subunit, Serine 468 phosphorylated NF-κB p65 subunit and the Serine 536 phosphorylated NF-κB p65 subunit. The kit can be used to determine the levels of the different forms of the NF-κB p65 subunit relative to cell number or it can also be used to determine NF-κB p65 subunit phosphorylation relative to total NF-κB p65 subunit levels found in the cells.

OKF6/TERT-2 cells were seeded into 96 well plates at a density of 1 x 10^5 cells/ml and left to adhere overnight in a humidified atmosphere with 5 % CO_2 at 37 ^oC. The following day, cells were stimulated for 30 min with either rhIL-17E (50 ng/ml) alone, rhIL-17A (10 ng/ml) alone, or 30 min pre-incubation with rhIL-17E (50 ng/ml) followed by stimulation with rhIL-17A (10 ng/ml). Supernatants were then removed and the cells were fixed with 100 µl of 4 % formaldehyde in PBS for 20 min. The cells were then washed 3 times with 200 µl wash buffer (0.1 % Triton X-100 in PBS), followed by a 20 min incubation with 100 µl quenching buffer (wash buffer with addition of 1 % hydrogen peroxide and 0.1 % Azide) at room temperature. After quenching, cells were again washed twice and 100 µl antibody blocking buffer (supplied by the manufacturer) was added to each well and the plate incubated for 1 h at room temperature. Cells were then washed twice as previous and 40 µl of diluted primary antibody was added to the cells. The primary antibodies used in the study were Phospho-NF-κB p65 antibodies (Serine 468 and Serine 536) and Total-NF-κB p65 antibody, which were diluted 1/500 in antibody dilution buffer. Antibody dilution buffer only was used as a negative control (All antibodies and antibody dilution buffers were supplied by manufacturer). The plates were then sealed and left overnight at 4 ^oC. The following day, the cells were again washed 3 times and incubated for 1 h with 100 µl of diluted secondary antibody (horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, diluted 1/2000 in antibody dilution buffer). Cells were then washed with wash buffer 3 times and 200 µl of PBS twice, followed by the

addition of 100 µl of developing solution (supplied by the manufacturer) for 2 - 20 min. The absorbance of each well was then measured at 450 nm with a reference wavelength of 655 nm using a Fluostar Omega® microplate reader (BMG Labtech, Germany). Data analysis was done using BMG Analysis Software (BMG Labtech, Germany).

To determine whether any differences observed in activated NF-κB p65 or total NF-κB p65 were due to differences in cell number; after the plate was read a crystal violet assay was performed. The cells were washed twice with wash buffer and then twice with PBS and then 100 µl of crystal violet solution was added to each well and the plate incubated for 30 min. After incubation the cells were washed again 3 times with PBS and then 100 µl of 1 % sodium dodecyl sulphate solution was added to each well and the plate incubated for 1 h at room temperature. Finally, the absorbance was read again at 595 nm. The measured OD_{450} readings are corrected for cell number by dividing the OD_{450} for a given well by the OD_{595} reading for that well. Then the percentage of the total NF-κB p65 subunit phosphorylated at a certain residue was calculated as follows:

Percentage of phosphorylation at a specific residue =

$$100 \times \frac{\text{Phospho-NF-κB p65 subunit levels}}{\text{Total NF-κB p65 subunit levels}}$$

2.7.6 Proteome profiler array

A human cytokine array (panel A) (Proteome Profiler™ Array; R&D Systems, UK) was used to quantify the cytokines released by OKF6/TERT-2 cells in response to 24 h stimulation with 100 ng/ml rhIL-33 (PeproTech®, UK) and 10 ng/ml PMA (Sigma-Aldrich®, UK) as described in Section 2.6.4.

The kit contains a nitrocellulose membrane which has been blotted with 36 selected capture antibodies (Figure 2-3). Cell culture supernatants are mixed with a cocktail of biotinylated detection antibodies and incubated with the nitrocellulose membrane. Any cytokine/detection antibody complex present is bound by its cognate immobilized capture antibody on the membrane. Following

a wash to remove unbound material, the bound cytokine/detection antibody complex was labelled with streptavidin-HRP, an enzyme reporter. Then chemiluminescent substrate was applied to the membrane. Luminol is oxidized in the presence of HRP and hydrogen peroxide to form an exited state product that emits light. The light produced at each spot is in proportion to the amount of cytokine bound.

The kit was used in accordance with the manufacturer's instructions and all incubations performed at room temperature (20 ^{o}C). All buffers were supplied by the manufacturer (formulation undisclosed). Briefly, the membranes were blocked in 2 ml array buffer 4 for 1 h on a rocking platform. Supernatants were centrifuged at 10000 rpm for 10 min to remove particulates. Then 1 ml of each sample was added to 0.5 ml of Array Buffer 4 and 15 µl of detection antibody cocktail and incubated for 1 h. After removal of the buffer from the membranes, the sample/antibody mixtures were added to the membranes and incubated overnight at 4 ^{o}C on a rocking platform. The next day, the membranes were washed 3 times for 10 min in 20 ml wash buffer. Next, 2 ml of streptavidin-HRP (1/2000 dilution) was added to the membranes and incubated for 30 min on a rocking platform. This was followed by three 10 min washes in 20 ml wash buffer. Finally, 1000 ml chemiluminescent detection reagent, Chemi Reagent 1 and 2 (R&D Systems, UK) was placed onto the membrane, carefully covering the whole surface, and incubated for 1 min. Excess solution was drained off the membrane which was then wrapped in cling film and placed in an autoradiography film cassette with identification numbers facing up. The membranes were exposed to X-ray film (Fujifilm Super RX, UK) for 20 min. The films were then developed using the KODAK MIN-R Mammography processor (Kodak, UK). Digital images were taken of the developed films using a Gel Doc^{TM} XR Imaging System (Bio-Rad Laboratories, UK) and Quantity One® Software Version 4.6.7 (Bio-Rad Laboratories, UK).

Cytokine array data on digital images were then quantified using Quantity One® Software Version 4.6.7 (Bio-Rad Laboratories, UK). First, digital image data were inverted prior to analysis procedure. This has to be done as the original digital images has light spots on the dark background (i.e., the signal intensity of the background is greater than the signal intensity of the sample spots). Then spot intensity (INT/mm^2) was measured for all visible spots on cytokine array images.

The designated circular area (mm^2) for intensity measurement was maintained unchanged for each spot measured (6.37 mm^2). At every spot, the intensity was measured at least three times and the highest intensity value was recorded. The background signal (signal from the media only control) was subtracted from each spot. The average intensity from duplicate spots was calculated for each cytokine or chemokine.

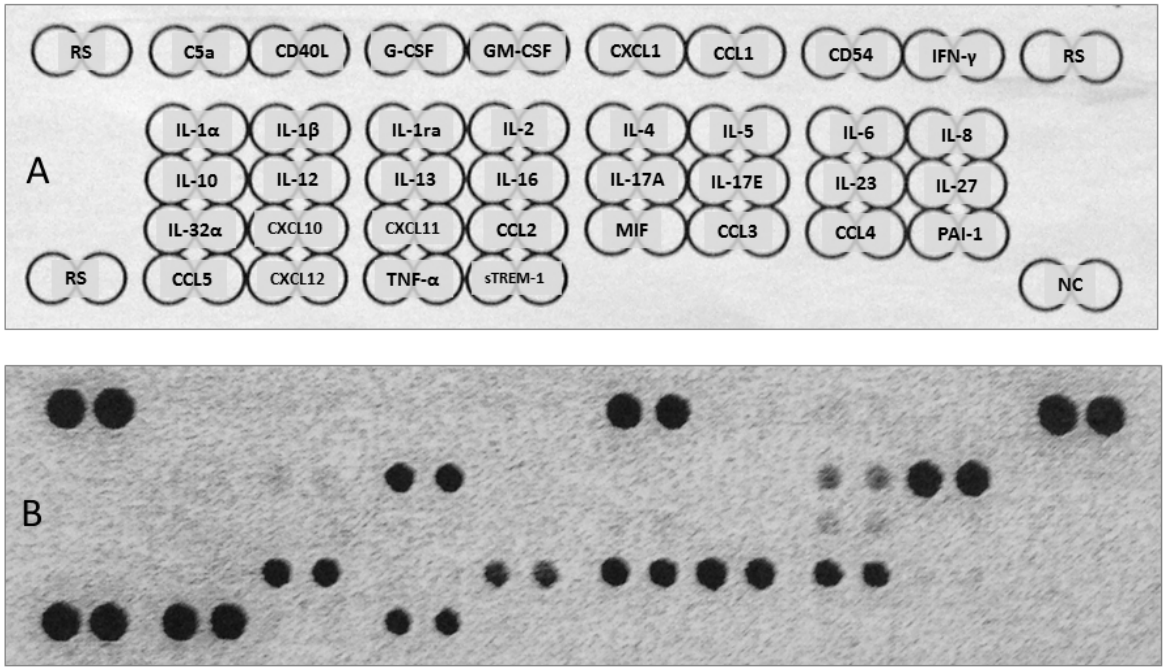

Figure 2-3: Cytokine array membrane of proteome profiler system

A, represents a schematic membrane layout and **B**, represents example of processed membrane. RS = reference spot and NC = negative control.

2.8 Molecular biology

2.8.1 RNA extraction and purification from periodontal tissue samples

Extraction and purification of total RNA from periodontal tissues samples was carried out using the RNeasy® fibrous tissue kit (Qiagen, UK). In brief, a maximum of 30 mg of periodontal tissue was placed in an eppendorf tube containing 300 µl of 1 % ß-Mercaptoethanol in Buffer RLT (Qiagen, UK). The mixture was then homogenized using a disposable rotor-stator tissue homogenizer for 20 – 40 seconds. Then, 590 µl of RNase-free water and 10 µl of Proteinase K (Qiagen, UK) were added to the homogenous lysate and the mixture was incubated for 10 min at 55 ^{o}C in a heat block. The homogenate was then centrifuged at 10000 rpm for 3 min at room temperature to remove the tissue remnant. Next, the supernatant (700 – 900 µl) was transferred into a 1.5 ml microcentrifuge tube, followed by addition of an equal volume of ethanol (96 – 100 %). The mixture was gently mixed by pipetting up and down before being transferred onto an RNeasy spin column (Qiagen, UK) placed in a 2 ml collection tube and centrifuged for 15 seconds at 10,000 rpm. The flow through was then discarded and the membrane was washed with 350 µl Buffer RW1 (Qiagen, UK) and centrifuged for 15 seconds at 10000 rpm. The flow through was again discarded and on column DNase (deoxyribonuclease) digestion was performed by incubating the membrane for 15 min at room temperature with a mixture of 10 µl DNase I stock solution (Qiagen, UK) and 70 µl Buffer RDD (Qiagen, UK). Afterwards, the column was washed again using Buffer RW1 (Qiagen, UK). Next, the column was washed twice with 500 µl Buffer RPE (Qiagen, UK). Finally, RNase-free water (30 µl) was used to elute the RNA from the column by centrifugation at 10,000 rpm for 1 min. This final procedure was repeated to get a final elution volume of 60 µl. A NanoDrop 1000 spectrophotometer (Thermo Scientific, UK) was used to assess the quantity and quality of the extracted total RNA.

2.8.2 RNA extraction and purification from *in vitro* cultured cells

Total RNA was extracted from cell cultures using the RNeasy kit (Qiagen, UK) according to the manufacturer's instructions. Firstly, cells in a 24-well plate were lysed using 350 µl Buffer RLT (Qiagen, UK) containing 1 % ß-Mercaptoethanol. The lysate was homogenized by passing at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to a RNase-free syringe. Then, an equal volume of ethanol (96 – 100 %) was added to the homogenised lysate. The mixture was gently mixed by pipetting up and down before being transferred onto an RNeasy spin column (Qiagen, UK) placed in a 2 ml collection tube. The tube was then centrifuged for 15 seconds at 10,000 rpm. The flow through was then discarded and the membrane was washed with 350 µl Buffer RW1 (Qiagen, UK) and centrifuged for 15 seconds at 10,000 rpm. The flow through was again discarded and on column DNase digestion was performed by incubating the membrane for 15 min at room temperature with a mixture of 10 µl DNase I stock solution (Qiagen, UK) and 70 µl Buffer RDD (Qiagen, UK). Afterwards, the column was washed again using Buffer RW1 (Qiagen, UK). Next, the column was washed twice with 500 µl Buffer RPE (Qiagen, UK). Finally, RNase-free water (30 µl) was used to elute the RNA from the column by centrifugation at 10,000 rpm for 1 min. This final procedure was repeated to get a final elution volume of 60 µl. A NanoDrop 1000 spectrophotometer (Thermo Scientific, UK) was used to assess the quantity and quality of the extracted total RNA.

2.8.3 Reverse transcription

High Capacity RNA-to-cDNA Master Mix (Applied Biosystems®, UK) was used to reverse transcribe mRNA into cDNA. The master mix contains manufacturer optimised concentration of $MgCl_2$, dNTPs (dATP, dCTP, dGTP and dTTP), recombinant RNase inhibitor protein, reverse transcriptase, random primers, oligo-dT (deoxythymine nucleotides) primer and stabilizers. The 20 µl reverse transcription reactions were prepared by mixing total RNA prepared in 16 µl of RNase free water (500 – 1000 ng) with 4 µl of master mix. For no-RT (reverse transcriptase) control, similar 20 µl reverse transcription reactions were prepared but without the enzyme component, reverse transcriptase. The tubes

were sealed and air bubbles were eliminated by brief centrifugation. The tubes were loaded into a thermal cycler (MyCycler™ Thermal Cycler; Bio-Rad Laboratories, UK), and reverse transcription performed under the following conditions: 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C and held at 4 °C. The cDNA was stored at -20 °C prior to use.

2.8.4 Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out using the ReddyMix™ PCR Master Mix (Thermo Scientific, UK). A ready-to-use master mix comes in 1.1 x concentration. The final 1 x concentration of master mix contains: 0.625 units ThermoPrime Taq DNA polymerase, 75 mM Tris-HCL (pH 8.8 at 25 °C), 20 mM ammonium sulphate ($(NH_4)_2SO_4$), 1.5 mM $MgCl_2$, 0.01 % (v/v) Tween® 20, 0.2 mM each of dATP, dCTP, dGTP and dTTP, and also a precipitant and red dye for electrophoresis. Amplification reactions were performed in a 25 µl reaction volume which consisted of 22.5 µl master mix, 0.5 µM of each primer (forward and reverse), 0.5 – 125 ng of cDNA and PCR grade water. The PCR reactions were performed under the following conditions: 95 °C for 2 min, followed by 35 to 45 cycles of 95 °C for 25 seconds, 48 - 63 °C (depending on primer annealing temperature) for 35 seconds and 72 °C for 65 seconds and finally 72 °C for 5 min. The primers and annealing temperatures used in this study are listed in Table 2-5.

PCR products were analysed using agarose gel electrophoresis. The agarose gel was prepared by adding 2 % agarose (w/v) (Invitrogen, UK) and 0.5 µg/ml ethidium bromide to 0.5 x Tris-Borate-EDTA buffer (Sigma-Aldrich®, UK) and heating gently before pouring into a pre-made cast and allowing to set . The ladder loading was prepared by mixing 1 µl of 100 base pair DNA Ladder (New England Biolabs, UK) with 1 µl 6 x Blue Loading Dye (New England Biolabs, UK) and 4 µl of distilled water. As most reactions were performed using ReddyMix™ PCR Master Mix, there was no requirement to add a loading dye. The samples and ladder were loaded onto the agarose gel which was then ran at 95 volts for 45 min. Images of electrophoresis gels were captured under ultraviolet lighting using the Gel Doc™ XR Imaging System (Bio-Rad Laboratories, UK) and Quantity One® Software, version 4.6.7 (Bio-Rad Laboratories, UK).

Gene	Primer	Forward (F) and Reverse (R) primer 5'→ 3'	T_a (°C)	bp
IL-33	F	TGTCAACAGCAGTCTACTGTGG	63	158
	R	GCAAAAGTAATGGATTGATCATTGTATGTGCT		
ST2 (total)	F	TAGTGTGACGGCGACCAGG	60	151
	R	GCCTTTTCCAAAACAAGCAG		
IL-17A	F	GGAATCTCCACCGCAATGAG	60	201
	R	ACACCAGTATCTTCTCCAGCC		
IL-17B	F	CTGGGGCTACAGCATCAACC	60	231
	R	GTGCAGCCCACAGCGATGGT		
IL-17C	F	CCGTTCAGTGTGACCGCCGA	60	339
	R	GTTGGGAAGAGGCAGCCTGC		
IL-17D	F	GCCAAAGAGATAGGGACGCA	60	288
	R	TTCATCAGTCAGCCATCGGT		
IL-17E	F	CCAGGTGGTTGCATTCTTGG	62	78
	R	TGGCTGTAGGTGTGGGTTCC		
IL-17F	F	TGAAGCTTGACATTGGCATC	60	174
	R	TTCCTTGAGCATTGATGCAG		
IL-17RA	F	GTCATCCTGCTCATCGTCTG	60	74
	R	TTGGTGTCATCACTGTATTTTTCAC		
IL-17RB	F	CCATCCCTCCAGATGACAAC	60	168
	R	TGCTCCTTCCTTGCCTCCAAGTTA		
IL-17RC	F	ACCAGAACCTCTGGCAAGC	60	232
	R	GAGCTGTTCACCTGAACACA		
IL-17RD	F	AAGTCAGGCCGGTCCCTATAC	60	66
	R	GTCGGGCTCCTCGTCAATAA		
IL-17RE	F	GCTGGAAGCTGCCCTCTGCC	60	74
	R	CTCGAGCTGTGGCATTCGGG		

Table 2-5: Primers used in basic PCR

All PCR primers were obtained from Invitrogen, UK. T_a = annealing temperature; and bp = base pair.

2.8.5 Taqman® real-time PCR

Taqman® real-time PCR was carried out using 2 x TaqMan® Gene Expression Master Mix (Applied Biosystems®, UK), and most of the primers and fluorescent probe assays were obtained from Applied Biosystems®, UK (Table 2-6). The 20 x TaqMan® Gene Expression Assay master mix contains 5 µM TaqMan® MGB (minor

groove binder) probes and 18 µM of each forward and reverse primer. The 20 µl real-time PCR reactions were prepared in a 96-well plate format, and each reaction contained 10 µl of master mix, 1 µl of gene expression assay mix, 7 µl of RNase-free water and 2 µl of cDNA (1 to 100 ng is recommended for each 20 µl). The plate was briefly centrifuged to remove any air bubbles. Gene amplification steps were carried out using a MX3000P™ real-time PCR instrument (Stratagene®, UK). Running conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 min. The data was analysed using MxPro-Mx3000P software, version 4.10 (Stratagene®, UK).

Real-time PCR data for the periodontal tissue study was analysed using the $2^{-\Delta CT}$ method (Schmittgen & Livak, 2008), whereas real-time PCR data for cell culture studies was analysed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Gene	Species	Primers and fluorescent probes (Assay ID / primer sequence)	Supplier
IL-33	Human	Hs00369211_m1	AB
ST2 (total)	Human	Hs00545033_m1	AB
IL-1RA	Human	Hs00174099_m1	AB
IL-10	Human	Hs00961622_m1	AB
G-CSF	Human	Hs00236884_m1	AB
IL-17A	Human	Hs00174383_m1	AB
IL-17B	Human	Hs00975262_m1	AB
IL-17C	Human	Hs00171163_m1	AB
IL-17D	Human	Hs00370528_m1	AB
IL-17E	Human	Hs00224471_m1	AB
IL-17F	Human	Hs00369400_m1	AB
IL-17RA	Human	Hs01064648_m1	AB
IL-17RB	Human	Hs00218889_m1	AB
POLR2A	Human	Hs00172187_m1	AB
ST2L	Human	Forward: 5'-GCACTTTGTTCACCAGATTCT-3' Reverse: 5'-CCAGGTAGCATATCTCTCCCA-3'	PD
sST2	Human	Forward: 5'-TTGTTTGCTGTCTGATCTTTGTAG-3' Reverse: 5'-ACCAACGATAGGAGGGAGTG-3'	PD

Table 2-6: Primer and fluorescent probes used in Taqman® real-time PCR

POLR2A = RNA polymerase II; AB = Applied Biosystems®, UK; and PD = Primerdesign Ltd, UK.

2.8.6 SYBR® Green real-time PCR

SYBR® Green real-time PCR was carried out using a ready to use 2 x concentration of SYBR® GreenER™ PCR master mix (Invitrogen, UK). The master mix contains hot-start *Taq* DNA polymerase, SYBR® GreenER™ fluorescent dye, $MgCl_2$, dNTPs (with dUTP instead of dTTP), UDG, and stabilizers. The forward and reverse primers were used at final concentration of 200 nM (Table 2-7). The 25 µl real-time PCR reactions were prepared in a 96-well plate format and each

reaction contained 12.5 µl of master mix, 0.5 µl of each forward and reverse primer, and up to 5 µl cDNA template (cDNA was generated from up to 1 µg of total RNA and used at a maximum of 10 % (v/v) of the real-time PCR reaction volume). The RNase free water was used to complete the 25 µl reaction volume. The 96-well plate was briefly centrifuged to remove any air bubbles. Amplification of target gene sequence was carried out using a MX3000P™ real-time PCR instrument (Stratagene®, UK). Running conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 min. For melt curve analysis, all products generated during the PCR amplification reaction are melted at 95 °C for 60 seconds and then annealed by gradual increases in temperature every 30 seconds from 55 °C to 95 °C. The data was analysed using MxPro-Mx3000P software, version 4.10 (Stratagene®, UK).

Real-time PCR data for the periodontal tissue study was analysed using the $2^{-\Delta CT}$ method (Schmittgen & Livak, 2008), whereas real-time PCR data for cell culture studies was analysed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Gene	Species	Primer (5'→ 3')	Supplier
MMP-2	Human	Forward: TGATCTTGACCAGAATACCATCGA Reverse: GGCTTGCGAGGGAAGAAGTT	Invitrogen, UK
MMP-9	Human	Forward: CCCTGGAGACCTGAGAACCA Reverse: CCCGAGTGTAACCATAGCGG	Invitrogen, UK
CXCL5	Human	Forward: CCCTGGGTTCAGAGACCTCCA Reverse: CCAGAAAATTTTGGACGGTGGAAACA	Invitrogen, UK
IL-8	Human	Forward: CAGAGACAGGAGAGCACACAA Reverse: TTAGCACTCCTTGGCAAAAC	Invitrogen, UK
TLR-2	Human	Forward: TGCTTTCCTGCTGGAGATTT Reverse: TGTAACGCAACAGCTTCAGG	Invitrogen, UK
TLR-4	Human	Forward: TTCAAGACCAAGCCTTTCAG Reverse: CATAGTCCT TCCATGATAGA	Invitrogen, UK
GAPDH	Human	Forward: CAAGGCTGAGAACGGGAAG Reverse: GGTGGTGAAGACGCCAGT	Invitrogen, UK

Table 2-7: Primers used in SYBR® Green real-time PCR

2.9 Statistical analysis

In the case of comparing more than two categorical data (e.g., gender), statistical analysis was carried out using the Chi-square test.

As for numerical data, Q-Q plot analysis was carried out to check normality of distribution. In certain circumstances, natural log transformation was used to facilitate representation of normally distributed data. On the other hand, for proportional data (e.g., %s), the data was first angular transformed prior to analysis for normal distribution.

When comparing two numerical variables, statistical analysis for the normally distributed data was carried out using the independent *t*-test. However, when comparing more than two numerical variables, statistical analysis for the normally distributed data was analysed using the ANOVA with a Bonferroni correction. Whereas, when data were not normally distributed, statistical analysis for comparing two numerical variables was carried out using the Mann-Whitney test; and a Bonferroni correction was carried out if more than two variables were compared.

When measuring correlations between two variables, data were first analysed for the bivariate distribution. Natural log transformations of the data were used to facilitate representation of linear and normally distributed data. When data were linear and normally distributed, the statistical analysis was carried out using the Pearson correlation coefficient test. Whenever necessary, adjustment for possible confounding factors was carried out using the Partial correlation test.

The significance level for all statistical analyses were set at $p = 0.05$. All statistical analyses were carried out using SPSS (Version 18).

Chapter 3: IL-33 and periodontal disease

3.1 Introduction

Depending on the disease model, IL-33 has been shown to have both exacerbatory and protective roles. IL-33 is known to aggravate inflammation in many chronic inflammatory conditions such as rheumatoid arthritis and allergy (Beltran, *et al.*, 2010; Louten, *et al.*, 2011; Xu, *et al.*, 2008). However, in conditions such as atherosclerosis and Type 2 diabetes it has been shown to have a protective role and can promote the resolution of potentially damaging inflammatory responses (Miller, 2011). To date the role of IL-33 in chronic periodontitis is not known. IL-33 levels in plasma, GCF and saliva were found not to differentiate between healthy and diseased individuals (Buduneli, *et al.*, 2012). However, there are studies which implicate this cytokine in pathogenesis of the disease. LPS derived from the periodontal pathogen (*P. gingivalis*) has been shown to induce increased expression of IL-33 by human monocytes (Nile, *et al.*, 2010). In addition, TNF-α induces IL-33 expression in human gingival fibroblasts (Beklen & Tsaous Memet, 2014).

At present, the expression of IL-33 and its regulation has yet to be investigated in oral keratinocytes. Oral keratinocytes are known to express an array of TLRs and release a variety of inflammatory mediators in response to stimuli, and *in vivo* occupy the space adjacent to the dental biofilm on the tooth surface. Due to their proximity, oral keratinocytes will therefore be one of the first cells to encounter periodontal pathogens and co-ordinate host defence mechanisms accordingly. It is therefore interesting to speculate that oral keratinocytes express IL-33 and that expression is upregulated in response to periodontal pathogens. Epithelial cells (intestinal, bronchial and corneal) have been shown to express IL-33 (Beltran, *et al.*, 2010; Schmitz, *et al.*, 2005). Indeed, signalling through TLR-3 and TLR-5 induces increased IL-33 expression in human corneal epithelial cells (Zhang, *et al.*, 2011a). Likewise, TLR-4 signalling in bronchial epithelial cells and TLR-9 signalling in sinonasal epithelial cells has also been found to induce the expression of IL-33 (Reh *et al.*, 2010; Willart *et al.*, 2012).

Despite studies reporting increased expression of IL-33 by a variety of cells in response to bacteria, the mechanisms which promote its extracellular release are still a matter of debate. However, a variety of studies have demonstrated that epithelial cells express the ST2 receptor and can respond to IL-33 signalling.

Indeed, IL-33 has been shown to induce increased release a variety of cytokines and chemokines from bronchial epithelial cells, nasal epithelial cells and epidermal keratinocytes (Balato, *et al.*, 2012; Fujita, *et al.*, 2012; Kamekura, *et al.*, 2012; Meephansan, *et al.*, 2012; Yagami, *et al.*, 2010). However, whether oral keratinocytes express the ST2 receptor and can respond to IL-33 signalling is to date unknown.

The aim of this study was to investigate the role of IL-33 in periodontal disease pathogenesis in further detail. Firstly, to determine associations between levels of IL-33 in biological fluids and clinical parameters of periodontal disease. Secondly, using *in vitro* model systems, to determine the role IL-33 plays in the oral epithelial immune response to periodontal pathogens. Based on the previous literature it was hypothesised that:

1. IL-33 levels in serum, GCF and saliva in periodontal tissue cannot be correlated with periodontal disease.

2. IL-33 and its receptors are expressed by periodontal epithelial cells and its expression is modulated by the periodontal pathogen; *P. gingivalis*.

3. Periodontal epithelial cells express the membrane bound form of the IL-33 receptor (ST2L) and can respond to IL-33 signalling by initiating the expression of inflammatory mediators

In order to investigate these hypotheses; the specific aims of the study were as follows:

1. To evaluate IL-33 levels in serum, GCF and saliva of chronic periodontitis patients and healthy subjects. This was to be achieved using samples collected as part of previous studies at Glasgow University Dental School (Lappin, *et al.*, 2009; Pathiyal, *et al.*, 2005) and Newcastle University School of Dental Sciences (Davies, *et al.*, 2011; Jaedicke, *et al.*, 2012; Preshaw & Heasman, 2002)

2. To evaluate the expression of IL-33 and its receptors (ST2L and sST2) in periodontal tissue samples from chronic periodontitis patients and healthy subjects.

3. To evaluate the expression of IL-33 and its receptors (ST2L and sST2) in OKF6/TERT-2 cells and PHGE cells using an *in vitro* live *P. gingivalis* monospecies biofilm model.

4. To evaluate the effect of rhIL-33 on the expression of inflammatory mediators by OKF6/TERT-2 cells *in vitro*.

3.2 Results

3.2.1 Analysis of IL-33 levels in clinical samples

3.2.1.1 Clinical and demographic parameters of subject participants

Serum, GCF and saliva samples from 77 healthy subjects and 97 chronic periodontitis patients were used in this study. Samples were selected from independent studies conducted at Glasgow Dental Hospital and School and Newcastle School of Dental Sciences and were from subjects/patients that were systemically healthy non smokers. The sample collection and ethical approvals for the Glasgow sample cohort were as previously described (Lappin*, et al.*, 2009; Pathiyal*, et al.*, 2005). The sample collection and ethical approvals for the Newcastle cohort are as previously described (Davies*, et al.*, 2011; Jaedicke*, et al.*, 2012; Preshaw & Heasman, 2002)

Table 3-1 describes comparisons of demographic (gender and age) and clinical parameters between the healthy and chronic periodontitis cohort. There was no significant difference in the incidence of chronic periodontitis between males and females ($p > 0.05$). However, the median age of the chronic periodontitis patients was significantly higher than the healthy subjects ($p < 0.001$). In addition, as expected, all the clinical parameters measured were significantly higher in the chronic periodontitis patients in comparison to the healthy subjects ($p < 0.001$).

		Healthy (n = 77)	CP (n = 97)	*p* value
Gender	Male, n (%)	43 (55.84)	39 (40.20)	0.082[a]
	Female, n (%)	34 (44.16)	58 (59.80)	
Age (years)		28 (27.00 – 49.00)	47.00 (42.00 – 53.00)	<0.001[b]
Clinical parameters				
	CPD (mm)	1.48 (1.21 – 1.69)	2.87 (2.69 – 3.27)	<0.001[b]
	CAL (mm)	1.40 (1.10 – 1.85)	3.33 (3.03 – 3.94)	<0.001[b]
	BOP (%)	2.70 (0.00 – 9.00)	36.00 (25.00 – 48.80)	<0.001[b]

Table 3-1: Patient demographics and clinical periodontal measurements of study groups

The demographic variable of gender was presented as number of subjects (n) and percentage (%); and the demographic variable of age and clinical parameters were presented as medians (interquartile range). Statistical analyses were carried out using the Chi-square test ([a]) and the Mann-Whitney test with a Bonferroni correction ([b]) (IBM SPSS Statistics, version 19). The significance level was set at $p = 0.05$. CP = chronic periodontitis, n = number of subjects, CPD = clinical probing depth, CAL = clinical attachment loss and BOP = bleeding on probing.

3.2.1.2 Serum, gingival crevicular fluid and saliva levels of IL-33

Serum, GCF and saliva levels of IL-33 were measured by a commercially available ELISA (Life Science, UK). Table 3-2 shows levels of IL-33 were below the detection limit of the assay in serum, GCF and saliva samples of healthy subjects and chronic periodontitis patients (Limit of sensitivity = 15.5 pg/ml; determined as two mean standard deviations higher than the mean baseline from 6 replicate standard curves (Chaloner-Larsson, *et al.*, 1997)).

	Status	n	Median (pg/ml)	IQR	Z statistic	*p* value
Serum IL-33	Healthy	17	1.90	1.30 – 2.25	-0.466	0.641
	CP	23	1.40	1.00 – 2.60		
GCF IL-33	Healthy	17	0.00	-	-	-
	CP	23	0.00	-		
Saliva IL-33	Healthy	7	0.00	-	-	-
	CP	23	0.00	-		

Table 3-2: Levels of IL-33 in serum, gingival crevicular fluid and saliva

IL-33 was measured in biological samples using the human IL-33 Elisa Kit (Life Science, UK). The table represents median levels of IL-33 in serum, GCF and saliva samples of the healthy and chronic periodontitis (CP) cohort. Statistical analyses were carried out using the Mann-Whitney test (IBM SPSS Statistics, version 19). Significance level was set at $p = 0.05$. n = number of subjects.

3.2.1.3 Expression of IL-33 mRNA in periodontal tissues

The failure to detect any IL-33 in serum, GCF or saliva was hypothesised to be due to the ELISA used in this study. Therefore expression of IL-33 mRNA in periodontal tissues was investigated; firstly by basic PCR and then quantitatively by real-time PCR.

Periodontal tissue samples were obtained from 27 subjects. Healthy tissue samples were taken from 10 patients undergoing non-periodontitis related procedures such as crown lengthening and tooth extraction. Seventeen tissue samples were taken from patients suffering from chronic periodontitis, who required surgical periodontal therapy as part of their periodontal treatment regime. Patients undergoing surgical periodontal therapy had clinical probing depths of ≥ 5.0 mm, with clinical attachment loss of ≥ 5.0 mm, which persisted after non-surgical treatment.

Figure 3-1 shows that mRNA for IL-33 was detected in periodontal tissue samples from 3 healthy individuals and 3 chronic periodontitis patients.

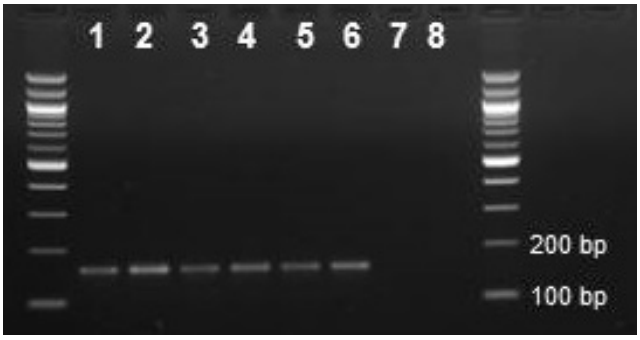

Figure 3-1: IL-33 mRNA expression in healthy and diseased periodontal tissue

Expression of IL-33 mRNA in periodontal tissues was investigated by basic PCR. IL-33 mRNA is expressed in healthy (Lanes 1, 2 and 3) and diseased (Lanes 4, 5 and 6) periodontal tissue samples. No-RT (reverse transcriptase) reactions and RNase free water (Lanes 7 and 8 respectively) were used as controls. The 100 base pair DNA ladder was used as reference. The expected band size for IL-33 was 158 base pairs (bp).

Real-time PCR was employed to quantitate levels of IL-33 mRNA in healthy and diseased periodontal tissue. Figure 3-2 shows that IL-33 mRNA was significantly upregulated in diseased periodontal tissue samples when compared to healthy control tissues ($p < 0.05$).

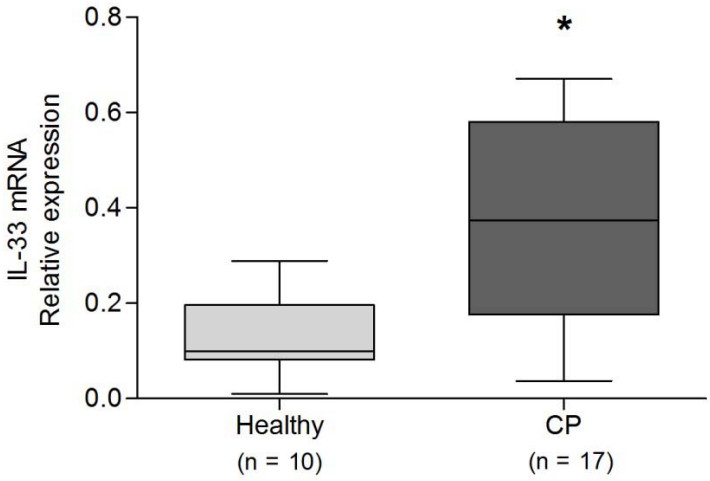

Figure 3-2: Real-time PCR analysis of IL-33 mRNA expression in healthy and diseased periodontal tissues

Quantification of IL-33 mRNA expression in periodontal tissues was performed by real-time PCR. RNA polymerase II was used as a reference gene. The box-and-whiskers plot represents the median relative expression ($2^{-\Delta CT}$) of IL-33 mRNA from tissues of healthy subjects and chronic periodontitis (CP) patients. Statistical analysis was carried out using the Mann-Whitney test (IBM SPSS Statistics, version 19). * = $p < 0.05$. Whiskers were determined by the Tukey method.

3.2.1.4 Expression of IL-33 protein in periodontal tissues

Since IL-33 mRNA was upregulated in diseased periodontal tissue (Figure 3-2), levels of IL-33 protein in periodontal tissue samples was evaluated using immunohistochemistry.

IL-33 was found to be expressed in the epithelial layer (Figure 3-3) and connective tissue (Figure 3-4) of healthy periodontal tissue and visually expression seemed to be elevated in tissue of chronic periodontitis patients. IL-33 expression in periodontal tissue samples was quantified by calculating the number of IL-33 positive cells in the epithelial and connective tissue layers (Bologna-Molina, *et al.*, 2011). Elevated expression was confirmed as the percentage of IL-33 positive cells was significantly higher in the epithelial and connective tissue layers of diseased periodontal tissue in comparison to healthy periodontal tissue (Figure 3-5).

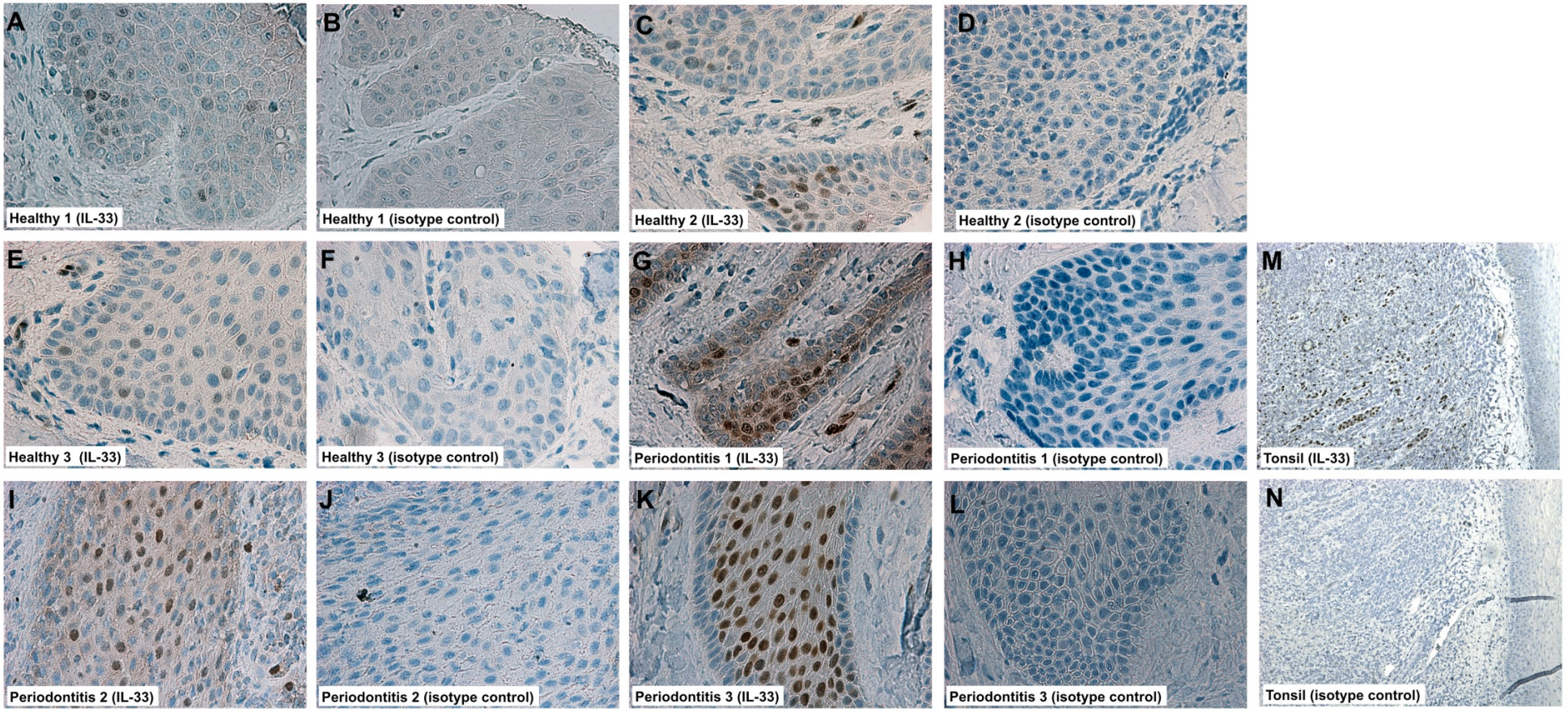

Figure 3-3: IL-33 expression in the epithelial layer of healthy and diseased periodontal tissue

Expression of IL-33 in periodontal tissues was determined by immunohistochemical analysis using a mouse monoclonal anti-IL-33 antibody (Nessy -1, Enzo® Life Science, UK). The panels show representative photomicrographs of 5 µm-thick paraffin-embedded sections of periodontal tissue specimens obtained from healthy subjects (**A** - **F**) and chronic periodontitis patients (**G** - **L**). An isotype control antibody was used to estimate non-specific binding. Tonsil tissue specimens were stained for IL-33 as a positive control (**M-N**). Original magnification x 400.

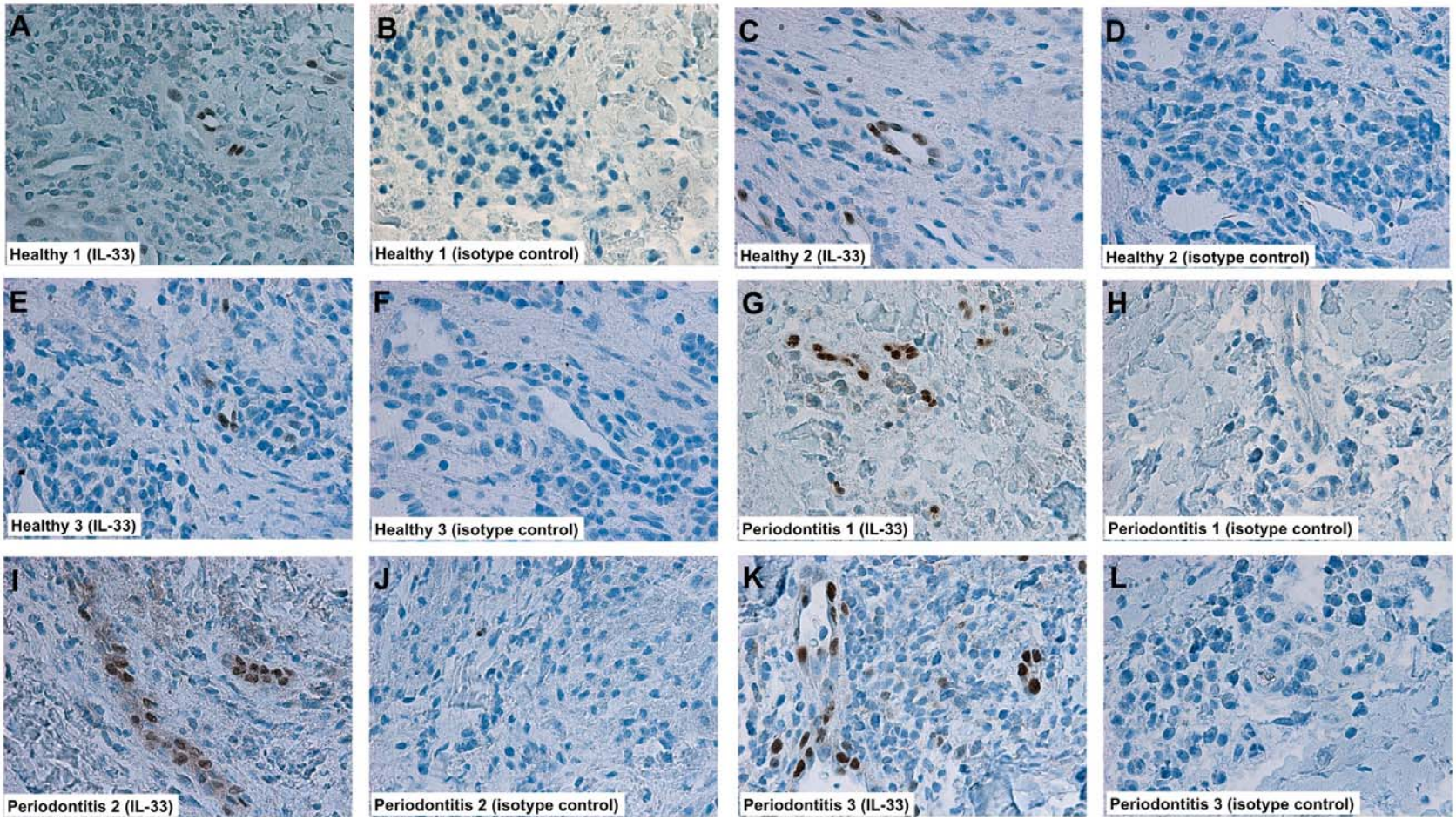

Figure 3-4: IL-33 expression in the connective tissue of healthy and diseased periodontal tissue

Expression of IL-33 in periodontal tissues was determined by immunohistochemical analysis using a mouse monoclonal anti-IL-33 antibody (Nessy -1, Enzo® Life Science, UK). The panels show representative photomicrographs of 5 µm-thick paraffin-embedded sections of periodontal tissue specimens obtained from healthy subjects (**A** - **F**) and chronic periodontitis patients (**G** - **L**). An isotype control antibody was used to estimate non-specific binding. Original magnification x 400.

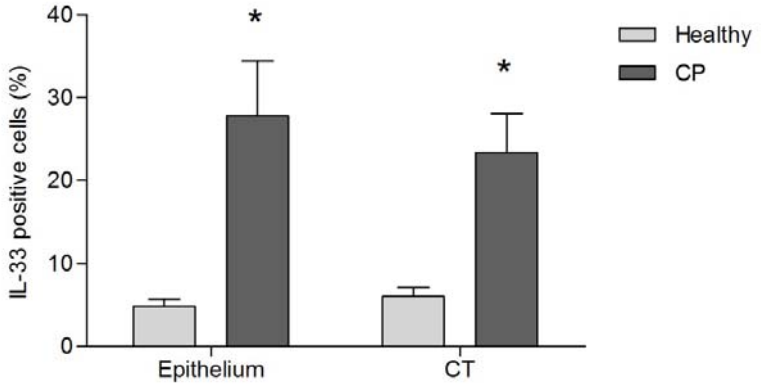

Figure 3-5: Percentage of IL-33 positive cells in the epithelial layer and connective tissue of healthy and diseased periodontal tissues

The % of IL-33 positive cells was determined from photomicrographs using the methodology described by Bologna-Molina and colleagues (2011). Bars show the percentage of IL-33 positive cells in the epithelial layer (epithelium) and connective tissue (CT) of healthy and chronic periodontitis (CP) tissue. Data was derived from 5 micrographs depicting different fields from 3 different tissue samples. Prior to statistical analyses the % data was angular transformed. Statistical analysis was carried out using the independent *t*-test (IBM SPSS Statistics, version 19). * = $p < 0.05$. Error bars indicate the standard error of the mean (SEM).

3.2.1.5 Expression of ST2 mRNA in periodontal tissues

The expression of the IL-33 receptor (ST2) in periodontal tissue was first investigated by basic PCR and then quantitatively by real-time PCR.

Figure 3-6 shows that mRNA for ST2 was detected in periodontal tissue samples from 3 healthy subjects and 3 chronic periodontitis patients.

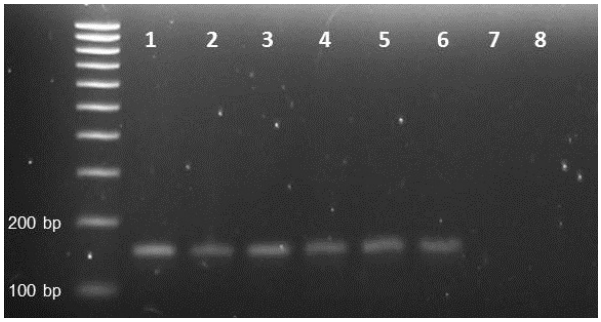

Figure 3-6: ST2 mRNA expression in healthy and diseased periodontal tissue.

Expression of ST2 mRNA in periodontal tissues was investigated by basic PCR. ST2 mRNA is expressed in healthy (Lanes 1, 2 and 3) and diseased (Lanes 4, 5 and 6) periodontal tissue samples. No-RT (reverse transcriptase) reactions and RNase free water (Lanes 7 and 8 respectively) were used as controls. The 100 base pair DNA ladder was used as reference. The expected band size for ST2 was 151 base pairs (bp).

Real-time PCR was employed to quantitate levels of ST2 mRNA in healthy and diseased periodontal tissue. Figure 3-7 shows that there was statistically significant increase in ST2 mRNA levels in diseased periodontal tissue samples compared to healthy control ($p < 0.05$).

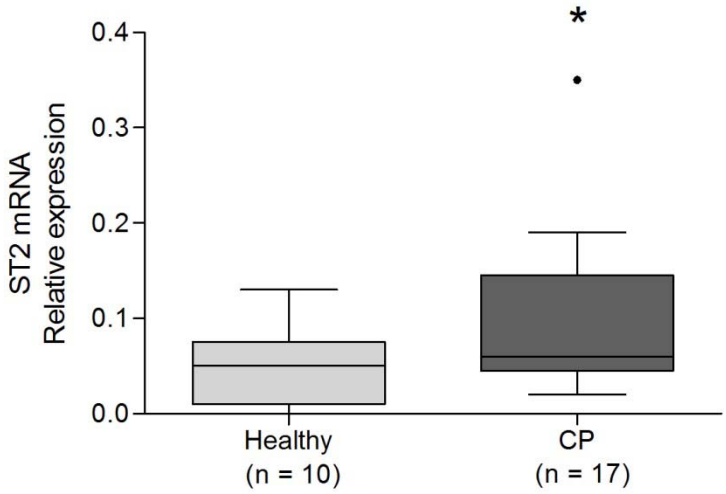

Figure 3-7: Real-time PCR analysis of ST2 mRNA expression in healthy and diseased periodontal tissues

Quantification of ST2 mRNA expression in periodontal tissues was performed by real-time PCR. RNA polymerase II was used as a reference gene. The box-and-whiskers plot represents the median relative expression ($2^{-\Delta CT}$) of ST2 mRNA from tissues of healthy subjects and chronic periodontitis (CP) patients. Statistical analyses were carried out using the Mann-Whitney test (IBM SPSS Statistics, version 19). * = $p < 0.05$. Whiskers were determined by the Tukey method and a value of 1.5 x IQR (interquartile range) was set as a demarcation line for outliers (.)

Although the data showed that ST2 was expressed in periodontal tissues the primer/probe sets used in the PCR analysis could not differentiate between mRNA encoding the membrane bound IL-33 receptor (ST2L) and the soluble decoy receptor (sST2). Therefore, the study was repeated using custom designed primer/probe sets for real-time PCR to specifically quantify levels of ST2L and sST2 mRNA in healthy and diseased periodontal tissue. Figure 3-8 shows that mRNA for sST2, not ST2L was significantly upregulated in diseased periodontal tissue samples when compared to healthy control tissues ($p < 0.05$).

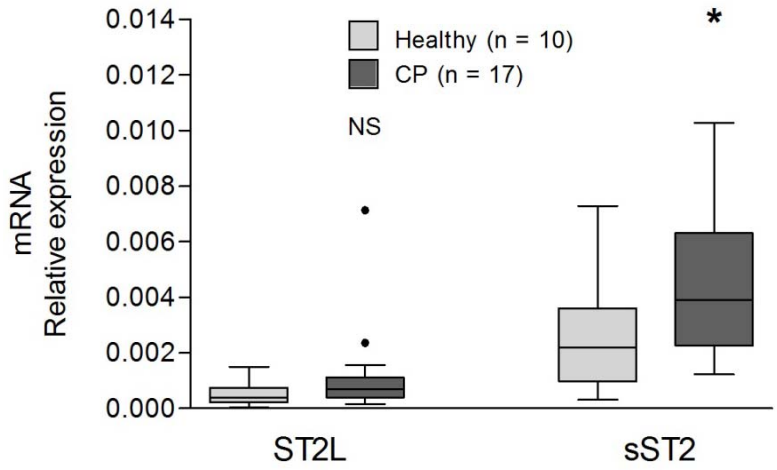

Figure 3-8: Real-time PCR analysis of ST2L and sST2 mRNA expression in healthy and diseased periodontal tissues

Quantification of ST2L and sST2 mRNA expression in periodontal tissues was performed by real-time PCR. RNA polymerase II was used as a reference gene. The box-and-whiskers plot represents the median relative expression ($2^{-\Delta CT}$) of ST2L and sST2 mRNA from tissues of healthy subjects and chronic periodontitis (CP) patients. Statistical analyses were carried out using the Mann-Whitney test with a Bonferroni correction (IBM SPSS Statistics, version 19). * = $p < 0.05$. Whiskers were determined by the Tukey method and a value of 1.5 x IQR (interquartile range) was set as a demarcation line for outliers (·).

3.2.1.6 Expression of ST2 protein in periodontal tissues

Since ST2 mRNA was upregulated in diseased periodontal tissue (Figure 3-7), levels of ST2 protein in periodontal tissue samples were evaluated using immunohistochemistry. At present, no antibodies are commercially available which can distinguish between the two different ST2 isoforms, therefore analysis of only total ST2 protein is reported.

ST2 was found to be expressed in the epithelial layer (Figure 3-9) and connective tissue (Figure 3-10) of healthy periodontal tissue. However no elevated expression in diseased periodontal tissue could be observed. In addition, when the number of ST2 positive cells in the epithelial layer and connective tissue were quantified no significant difference between healthy and diseased periodontal tissue was determined (Figure 3-11).

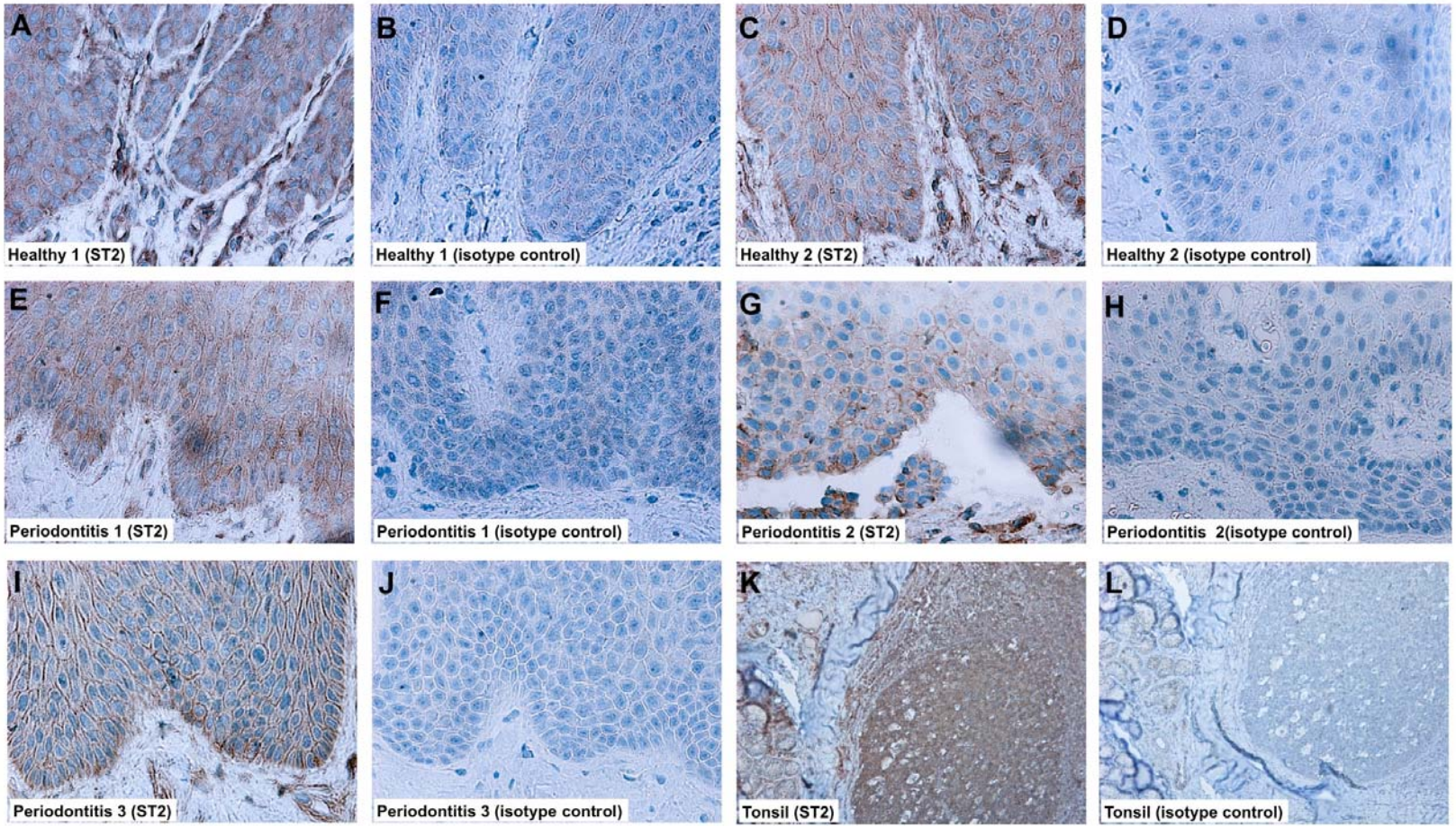

Figure 3-9: ST2 expression in the epithelial layer of healthy and diseased periodontal tissue

Expression of ST2 in periodontal tissues was determined by immunohistochemical analysis using a rabbit anti-IL1RL1 antibody (Sigma-Aldrich®, UK). The panels show representative photomicrographs of 5 µm-thick paraffin-embedded sections of periodontal tissue specimens obtained from healthy subjects (**A** - **D**) and chronic periodontitis patients (**E** - **J**). An isotype control antibody was used to estimate non-specific binding. Tonsil tissue specimens were stained for ST2 as a positive control (**K-L**). Original magnification x 400.

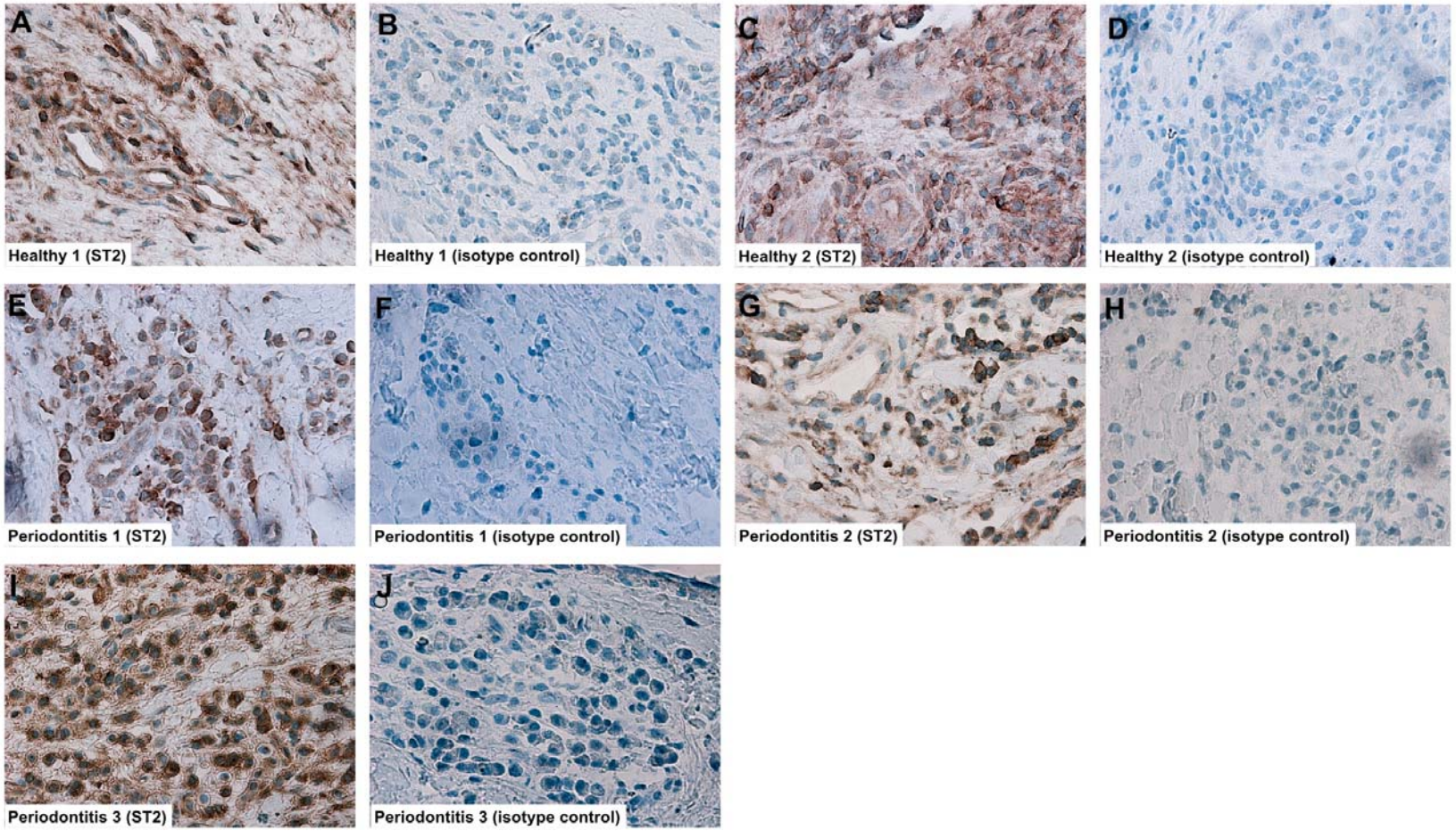

Figure 3-10: ST2 expression in the connective tissue of healthy and diseased periodontal tissue

Expression of ST2 in periodontal tissues was determined by immunohistochemical analysis using a rabbit anti-IL1RL1 antibody (Sigma-Aldrich®, UK). The panels show representative photomicrographs of 5 µm-thick paraffin-embedded sections of periodontal tissue specimens obtained from healthy subjects (**A** - **D**) and chronic periodontitis patients (**E** - **J**). An isotype control antibody was used to estimate non-specific binding. Original magnification x 400.

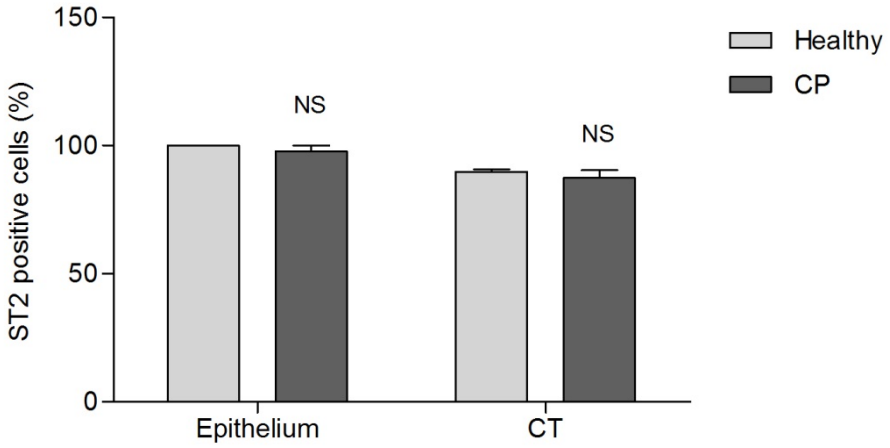

Figure 3-11: Percentage of ST2 positive cells in the epithelial layer and connective tissue of healthy and diseased periodontal tissues

The % of ST2 positive cells was determined from photomicrographs using the methodology described by Bologna-Molina and colleagues (2011). Bars show the percentage of ST2 positive cells in the epithelial layer (epithelium) and connective tissue (CT) of healthy and chronic periodontitis (CP) tissue. Data was derived from 5 micrographs depicting different fields from 3 different tissue samples. Prior to statistical analyses the % data was angular transformed. Statistical analysis was carried out using the independent *t*-test (IBM SPSS Statistics, version 19). NS = not significant ($p > 0.05$). Error bars indicate the standard error of the mean (SEM).

3.2.2 Expression of IL-33 by oral epithelial cells in response to *Porphyromonas gingivalis*

3.2.2.1 Validation of the *in vitro* live *Porphyromonas gingivalis* monospecies biofilm model

To investigate whether oral epithelial cells express IL-33 and if expression was modulated by the periodontal pathogen (*P. gingivalis*); a live biofilm model system was employed. This model had been previously developed by colleagues in our laboratory (Sherry, *et al.*, 2013) and is designed to be more *in vivo* relevant. In this *in vitro* model, the live *P. gingivalis* monospecies biofilm is separated from the oral keratinocytes by a 0.5 mm space. This space represents the distance between dental biofilm and epithelial cells in the gingival sulcus.

For this model system, a live *P. gingivalis* monospecies biofilm is cultured on Thermanox™ coverslips (Nunc Nalgene, UK). These biofilms are made in batches and can be used immediately or stored at –70 °C long term for later use. However, before the biofilms were used in the investigations reported in this thesis, it was important to determine whether there was any batch to batch

variability in biofilms which may affect the results of further investigations. In addition, it was important to ensure that storage at -70 °C had no significant effects on biofilm viability.

Figure 3-12 shows there were no significant differences in viability (CFU/ml) between batches of *P. gingivalis* monospecies biofilms cultured on Thermanox™ coverslips before and after storage at –70 °C for 14 days.

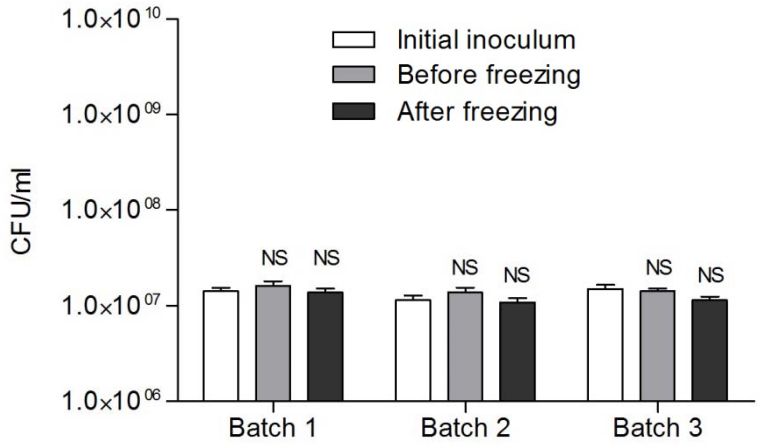

Figure 3-12: The effect of freezing on *P. gingivalis* monospecies biofilms

Bars show colony forming unit (CFU)/ml of *P. gingivalis* recovered from monospecies biofilms. Initial inoculums = CFU/ml of *P. gingivalis* used to seed onto Thermanox™ coverslips to prepare biofilms; before freezing = CFU/ml recovered from Thermanox™ coverslips 4 days after seeding; and after freezing = CFU/ml recovered from Thermanox™ coverslips after storage at –70 °C for 14 days. The data was generated from duplicate wells of three independent experiments. Statistical analysis of the data was performed on the natural log transformed CFU/ml values using the ANOVA with *a* Bonferroni correction. * = $p < 0.05$; NS = not significant. Error bars indicate the standard error of the mean (SEM).

In addition to determining CFUs, the viability of the biofilms was assessed visually using a simple Gram stain. Figure 3-13 shows representative images of biofilms from the 3 batches both before and after freezing. Visually, no differences in biofilm structure were observed.

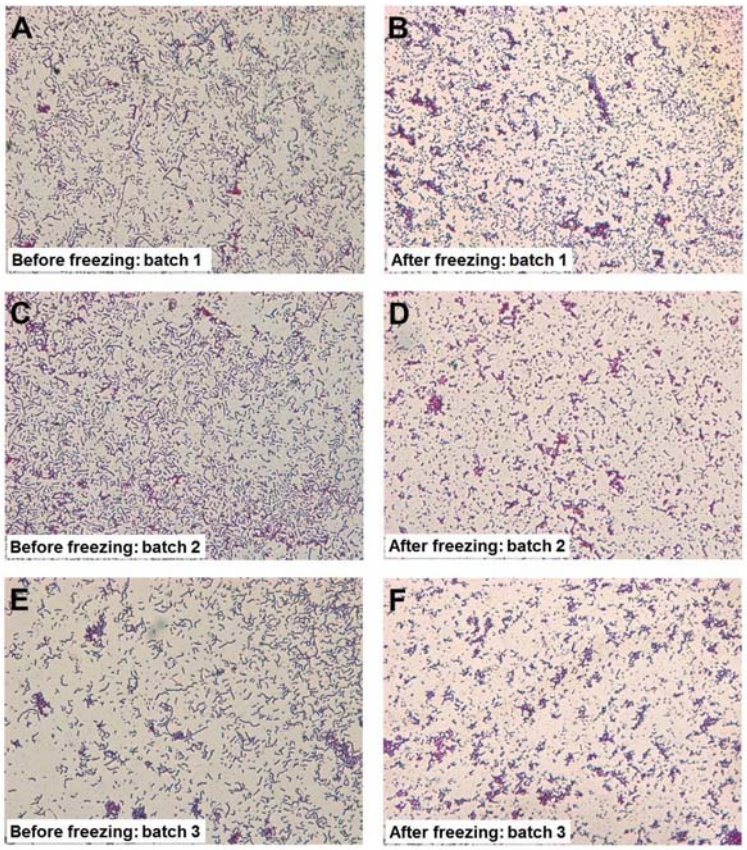

Figure 3-13: Gram stained *P. gingivalis* monospecies biofilms before and after freezing

Figure shows images of three batches of Gram stained *P. gingivalis* monospecies biofilms before and after freezing at –70 °C for 14 days. *P. gingivalis* monospecies biofilms appear pink-red in colour after staining. Each panel is a representative image from one of three independent experiments carried out in triplicate. Original magnification x 400.

These data showed that there was no significant variability between batches of live *P. gingivalis* monospecies biofilms. In addition, there was no significant loss of viability when biofilms were frozen for long term storage and revived 24 h prior to use in the *in vitro* model system.

3.2.2.2 IL-33 expression by OKF6/TERT-2 cells in response to *Porphyromonas gingivalis*

Previously, immunohistochemical analysis revealed that IL-33 was expressed in the epithelial layer of periodontal tissues. Therefore we investigated whether oral keratinocytes express IL-33 and whether expression was modulated by the periodontal pathogen; *P. gingivalis*.

For the initial studies an oral keratinocyte cell line, OKF6/TERT-2, was used. OKF6/TERT-2 cells were cultured *in vitro* and exposed to a live *P. gingivalis* monospecies biofilms for 3, 6, 9 and 24 h as previously described (Section 2.6.1). Unstimulated cells acted as controls at each time point. To confirm the cells had been stimulated, the release of the chemokine IL-8 (CXCL8) into the bathing supernatant was measured by ELISA. Figure 3-14 shows that there was a significant increase in IL-8 release from OKF6/TERT-2 cells in response to *P. gingivalis* stimulation at all time points.

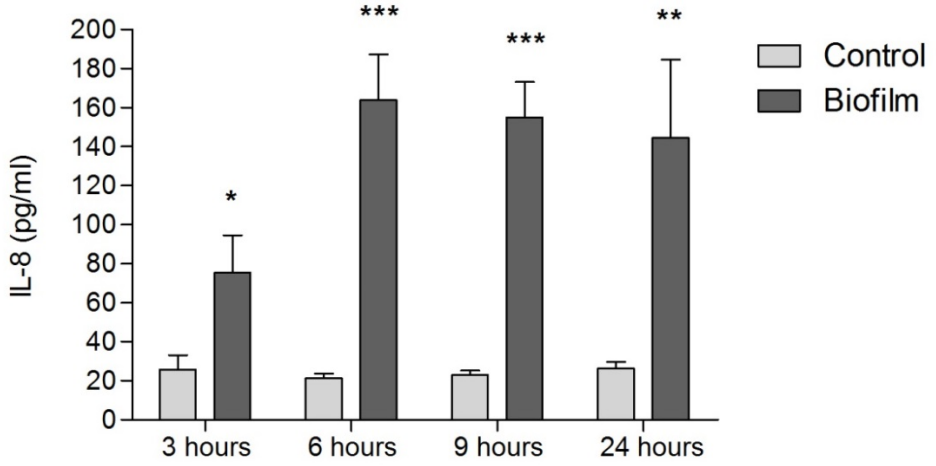

Figure 3-14: Release of IL-8 (CXCL8) from OKF6/TERT-2 cells in response to a live *P. gingivalis* monospecies biofilm

IL-8 release into bathing supernatants was measured by ELISA. The bars represent the mean supernatant concentrations of IL-8 in unstimulated (Control) and a live *P. gingivalis* monospecies biofilm (Biofilm) stimulated OKF6/TERT-2 cells at 3, 6, 9 and 24 h. The data was generated from duplicate wells of three independent experiments. Statistical analyses were carried out using the independent *t*-test (IBM SPSS Statistics, version 19) on the natural log transformed IL-8 levels (pg/ml). * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. Error bars indicate the standard error of the mean (SEM).

Once stimulation of the cells had been confirmed, the expression of IL-33 mRNA was investigated by real-time PCR. Figure 3-15 shows that IL-33 mRNA was expressed by OKF6/TERT-2 cells and levels were significantly upregulated in response to *P. gingivalis*.

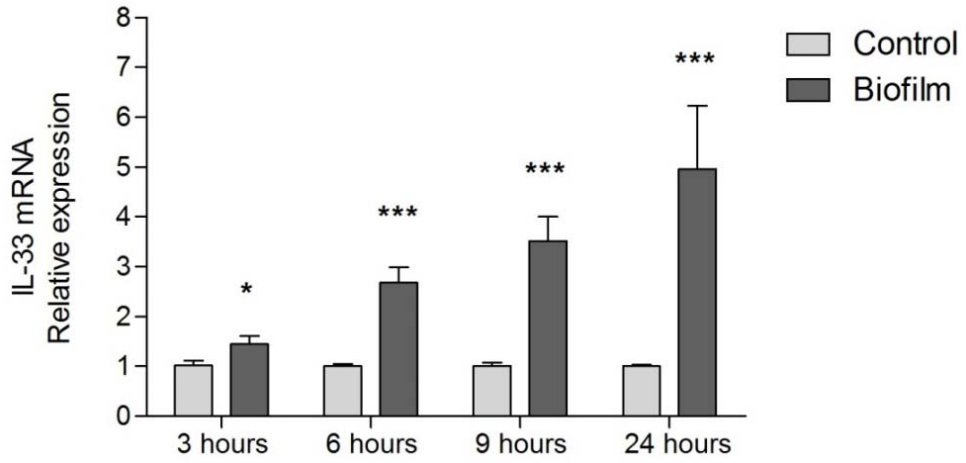

Figure 3-15: The effect of a live *P. gingivalis* monospecies biofilm on IL-33 mRNA expression by OKF6/TERT-2 cells

Quantification of IL-33 mRNA expression in OKF6/TERT-2 cells was performed by real-time PCR. The bars represent mean relative expression ($2^{-\Delta\Delta CT}$) of IL-33 mRNA in unstimulated (Control) and a live *P. gingivalis* monospecies biofilm (Biofilm) stimulated OKF6/TERT-2 cells at 3, 6, 9 and 24 h. RNA polymerase II was used as a reference gene. The data was generated from duplicate wells of three independent experiments. Statistical analyses were carried out using the independent *t*-test (IBM SPSS Statistics, version 19) on the natural log transformed IL-33 mRNA relative expression ($2^{-\Delta\Delta CT}$) values. * = $p < 0.05$ and *** = $p < 0.001$. Error bars indicate the standard error of the mean (SEM).

Having established that IL-33 mRNA expression is upregulated in response to stimulation with *P. gingivalis,* we next investigated whether IL-33 protein was released from stimulated OKF6/TERT-2 cells. Figure 3-16 shows that using a commercially available ELISA kit (Enzo® Life Science, USA) no IL-33 release was detected in the bathing supernatants of unstimulated or *P. gingivalis* monospecies biofilm stimulated OKF6/TERT-2 cells.

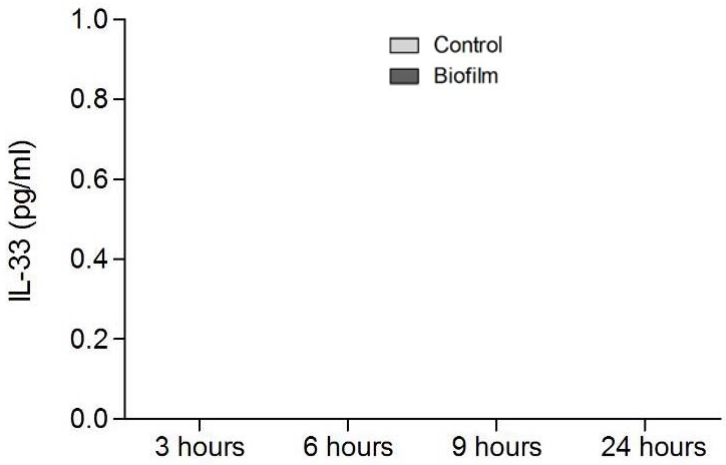

Figure 3-16: Release of IL-33 from OKF6/TERT-2 cells in response to a live *P. gingivalis* monospecies biofilm

IL-33 release into bathing supernatants was measured by ELISA. No IL-33 was detected in any of the bathing supernatants analysed from unstimulated (Control) and live *P. gingivalis* monospecies biofilm (Biofilm) stimulated OKF6/TERT-2 cells at 3, 6, 9 and 24 h. The data was generated from duplicate wells of three independent experiments.

In monocytes, IL-33 is not released from cells in response to *P. gingivalis* LPS. However, increased protein expression can be observed intracellularly (Nile, *et al.*, 2010). To determine if similar responses occurred in OKF6/TERT-2 cells; immunocytochemical analysis was employed. OKF6/TERT-2 cells were cultured on glass coverslips in the presence and absence of a live *P. gingivalis* monospecies biofilm for 9 h. Prior to immunocytochemistry, stimulation was confirmed by measurement of IL-8 release (Figure 3-17).

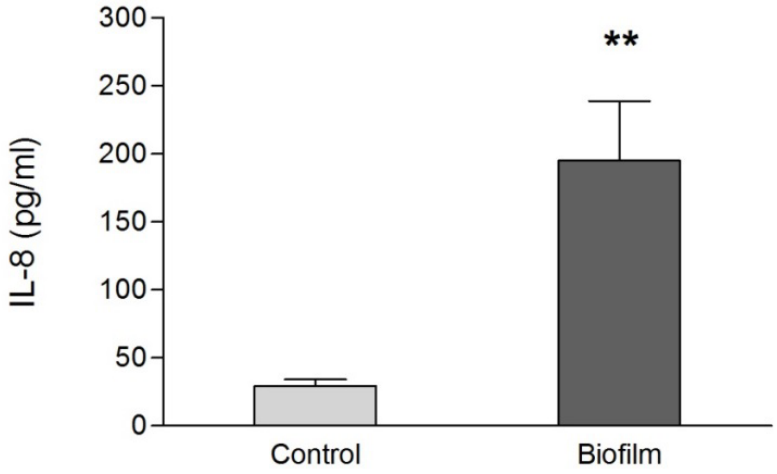

Figure 3-17: Release of IL-8 (CXCL8) from OKF6/TERT-2 cells cultured on glass coverslips and stimulated with a live *P. gingivalis* monospecies biofilm for 9 h

IL-8 release into bathing supernatants was measured by ELISA. The bars represent the mean supernatant concentrations of IL-8 in unstimulated (Control) and a live *P. gingivalis* monospecies biofilm (Biofilm) stimulated OKF6/TERT-2 cells at 9 h. The data was generated from duplicate wells of three independent experiments. Statistical analyses were carried out using the independent *t*-test (IBM SPSS Statistics, version 19) on the natural log transformed IL-8 levels (pg/ml). ** = $p < 0.01$. Error bars indicate the standard error of the mean (SEM)

Once stimulation had been confirmed, immunohistochemistry analysis of fixed cells was conducted using a mouse monoclonal anti-IL-33 antibody (Nessy -1, Enzo® Life Science, UK). IL-33 was found to be expressed in OKF6/TERT-2 cells and an increase in intracellular expression could be observed after stimulation for 9 h with a live *P. gingivalis* monospecies biofilm (Figure 3-18). To confirm the specificity of the antibody, HUVECs (which are known to be positive for IL-33) were used as a positive control (Baekkevold*, et al.*, 2003; Moussion*, et al.*, 2008). In addition, for negative control purposes, stimulated OKF6/TERT-2 cells were also exposed to a mouse monoclonal anti-IL-33 antibody (Nessy -1, Enzo® Life Science, UK) which had previously been pre-absorbed with an excess of rhIL-33 (PeproTech®, UK) (Figure 3-18).

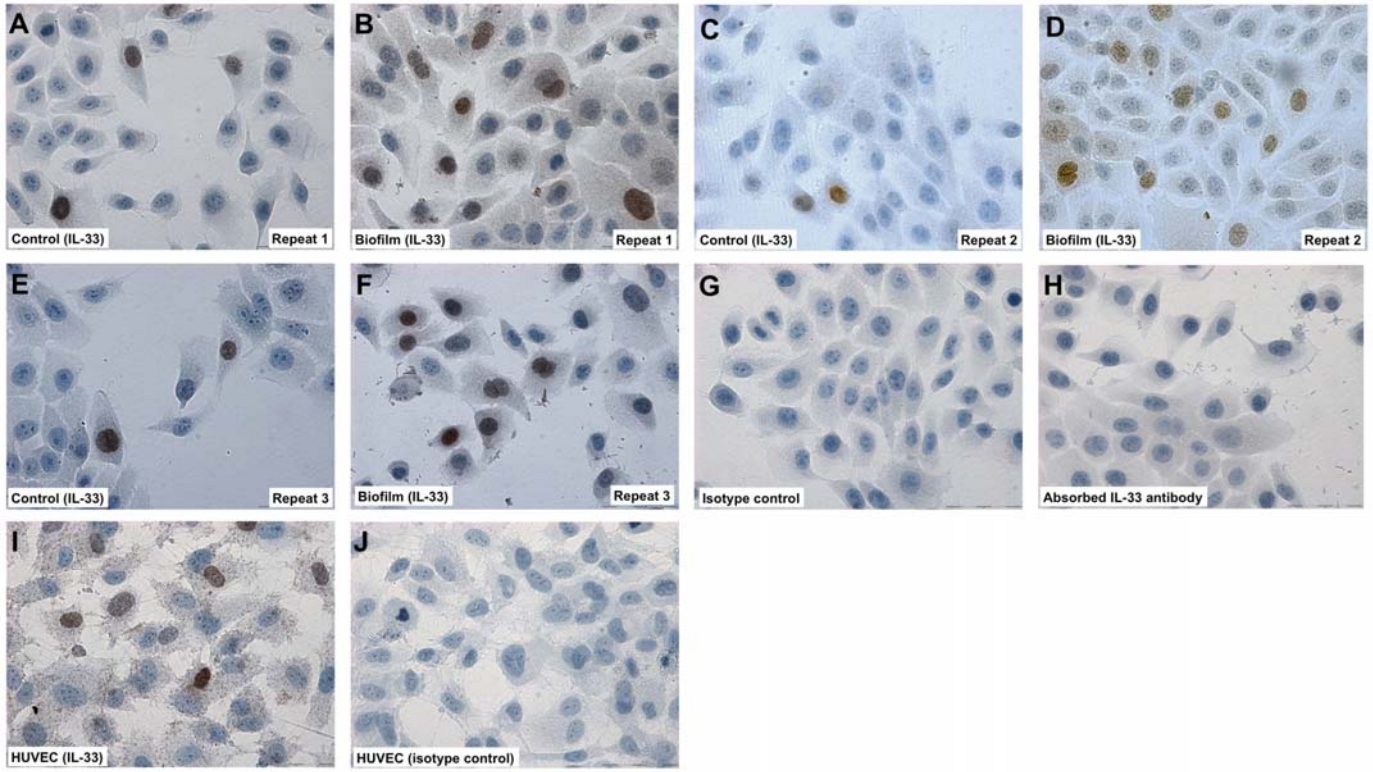

Figure 3-18: Intracellular IL-33 expression by OKF6/TERT-2 cells cultured on glass coverslips and stimulated with a *P. gingivalis* monospecies biofilm for 9 h

Intracellular expression of IL-33 was determined using a mouse monoclonal anti-IL-33 antibody (Nessy-1, Enzo® Life Science, UK). The panels show representative photomicrographs of methanol fixed OKF6/TERT-2 cells on glass coverslips after 9 h incubation either with media only as a control (**A, C** and **E**) or a live *P. gingivalis* monospecies biofilm (**B, D** and **F**). An isotype control antibody was used to determine non-specific binding (**G**). Specificity of the IL-33 antibody was confirmed by pre-absorption of the mouse monoclonal anti-IL-33 antibody with an excess of rhIL-33 (PeproTech®, UK) (**H**). HUVEC cells were also exposed to a mouse monoclonal anti-IL-33 antibody (Nessy-1, Enzo® Life Science, UK) (**I**) and an isotype control (**J**) to further confirm antibody specificity. Original magnification x 400.

The use of an isotype control, pre-absorption studies and a cell line known to express IL-33 confirmed the specificity of the mouse monoclonal anti-IL-33 antibody (Nessy-1, Enzo® Life Science, UK). Therefore, to quantify increases in intracellular IL-33 expression the counting method described by Bologna-Molina and colleagues (2011) was employed. Figure 3-19 shows that the % of IL-33 positive OKF6/TERT-2 cells was significantly higher after stimulation with a live *P. gingivalis* monospecies biofilm for 9 h.

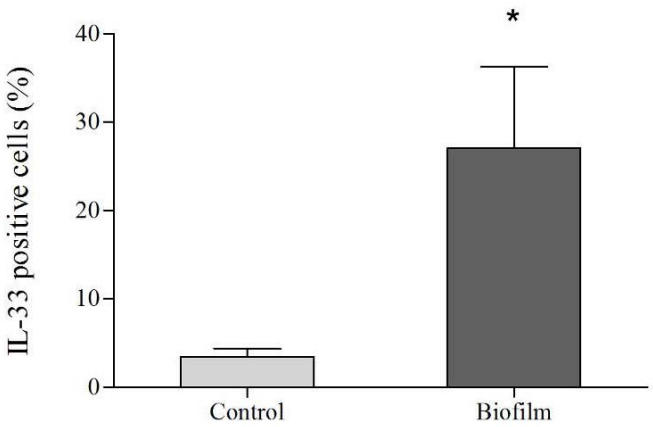

Figure 3-19: Percentage of IL-33 positive OKF6/TERT-2 cells on glass coverslips after incubation with media alone or a live *P. gingivalis* monospecies biofilm for 9 h

The % of IL-33 positive cells was determined from photomicrographs using the methodology described by Bologna-Molina and colleagues (2011). Bars show the percentage of IL-33 positive OKF6/TERT-2 cells after 9 h incubation with media alone (Control) or with a live *P. gingivalis* monospecies biofilm (Biofilm). Data was derived from 5 micrographs depicting different fields from 3 different coverslips. Prior to statistical analyses the % data was angular transformed. Statistical analysis was carried out using the independent *t*-test (IBM SPSS Statistics, version 19). * = $p < 0.05$. Error bars indicate the standard error of the mean (SEM).

3.2.2.3 ST2 expression by OKF6/TERT-2 cells in response to *Porphyromonas gingivalis*

Previous data established that IL-33 expression by OKF6/TERT-2 cells is modulated by a live *P. gingivalis* monospecies biofilm. Therefore, the effect of a live *P. gingivalis* monospecies biofilm on expression of both sST2 and ST2L was also investigated.

Real-time PCR analysis showed that levels of sST2 mRNA, but not ST2L mRNA were significantly upregulated in *P. gingivalis* monospecies biofilm stimulated OKF6/TERT-2 cells after 6, 9 and 24 h (Figure 3-20).

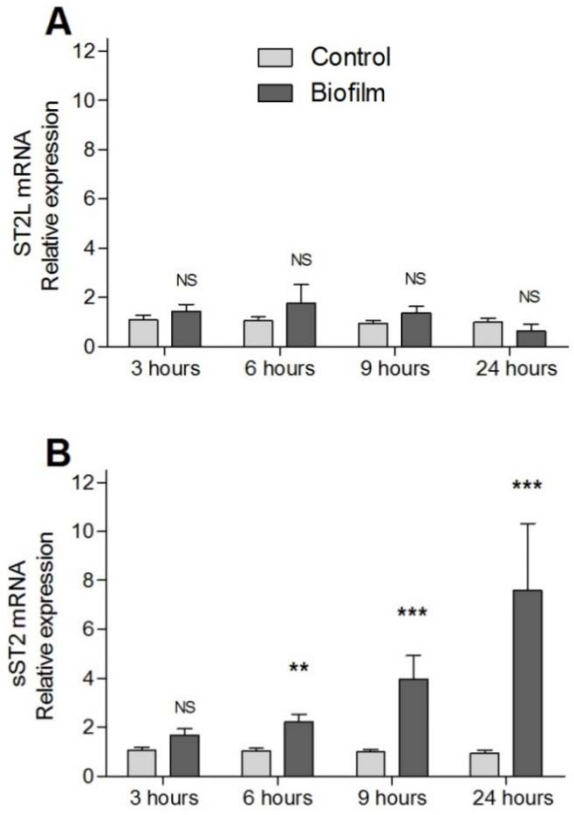

Figure 3-20: The effect of a live *P. gingivalis* monospecies biofilm on sST2 and ST2L mRNA expression by OKF6/TERT-2 cells

Quantification of ST2L (**A**) and sST2 (**B**) mRNA expression in OKF6/TERT-2 cells was performed by real-time PCR. The bars represent mean relative expression ($2^{-\Delta\Delta CT}$) of ST2L and sST2 mRNA in unstimulated (Control) and a live *P. gingivalis* monospecies biofilm (Biofilm) stimulated OKF6/TERT-2 cells at 3, 6, 9 and 24 h. RNA polymerase II was used as a reference gene. The data was generated from duplicate wells of three independent experiments. Statistical analyses were carried out using the independent *t*-test (IBM SPSS Statistics, version 19) on the natural log transformed sST2 and ST2L mRNA relative expression ($2^{-\Delta\Delta CT}$) values. ** = $p < 0.01$; *** = $p < 0.001$.* = $p < 0.05$ and NS = not significant. Error bars indicate the standard error of the mean (SEM).

Having established that sST2 mRNA expression is upregulated in response to stimulation with *P. gingivalis* we next investigated whether sST2 protein was also released from stimulated OKF6/TERT-2 cells. Figure 3-21 shows that using a commercially available ELISA kit (Quantikine® ST2; R&D System, UK), sST2 protein was detected at low levels in the cell supernatant and there was no difference in sST2 levels between *P. gingivalis* monospecies biofilm stimulated OKF6/TERT-2 cells and non stimulated controls at all the time points investigated.

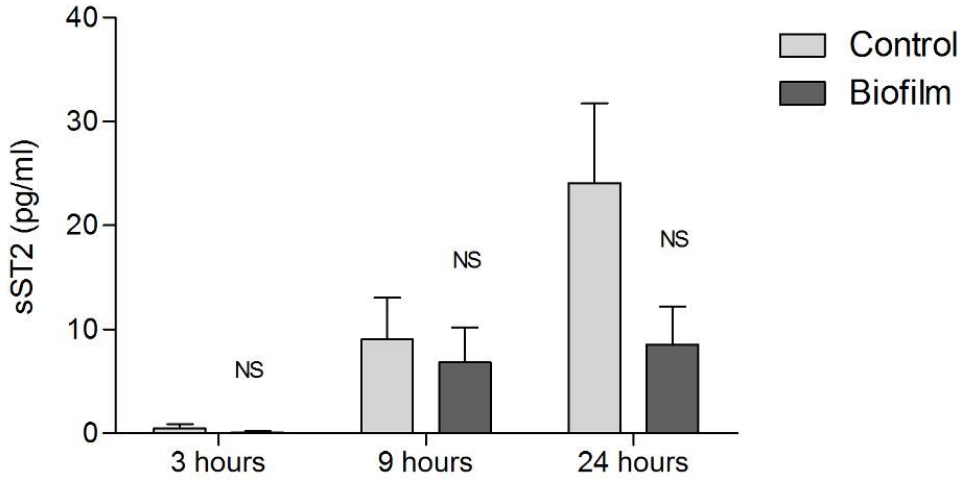

Figure 3-21: Release of sST2 from OKF6/TERT-2 cells in response to stimulation with a live *P. gingivalis* monospecies biofilm

sST2 release into bathing supernatants was measured by ELISA. The bars represent the mean supernatant concentrations of sST2 in unstimulated (Control) and a live *P. gingivalis* monospecies biofilm (Biofilm) stimulated OKF6/TERT-2 cells at 3, 9 and 24 h. The data was generated from duplicate wells of three independent experiments. Statistical analyses were carried out using the independent *t*-test (IBM SPSS Statistics, version 19) on the natural log transformed sST2 levels (pg/ml). NS = not significant ($p > 0.05$). Error bars indicate the standard error of the mean (SEM).

The ELISA analysis revealed there was no significant change in sST2 levels in bathing supernatants from *P. gingivalis* stimulated OKF6/TERT-2 cells. We therefore used immunocytochemistry to confirm there was no change in intracellular or membrane bound (ST2L) levels under the same conditions. OKF6/TERT-2 cells were cultured on glass coverslips in the presence and absence of a live *P. gingivalis* monospecies biofilm for 9 h. Prior to immunocytochemistry, stimulation was again confirmed by measurement of IL-8 release. As investigations were conducted alongside IL-33 immunocytochemistry

investigations, the data confirming that all cells cultured on coverslips were in fact stimulated by a live *P. gingivalis* monospecies biofilm is shown Figure 3-17.

Immunocytochemistry investigations of ST2 expression were performed using a rabbit anti-IL1RL1 antibody (Sigma-Aldrich®, UK). This antibody was not specific for any of the ST2 isoforms (sST2 or ST2L) and stained only total ST2. Figure 3-22 shows that ST2 was detected in/on the majority of OKF6/TERT-2 cells exposed to fresh media only (control) and a live *P. gingivalis* monospecies biofilm for 9 h.

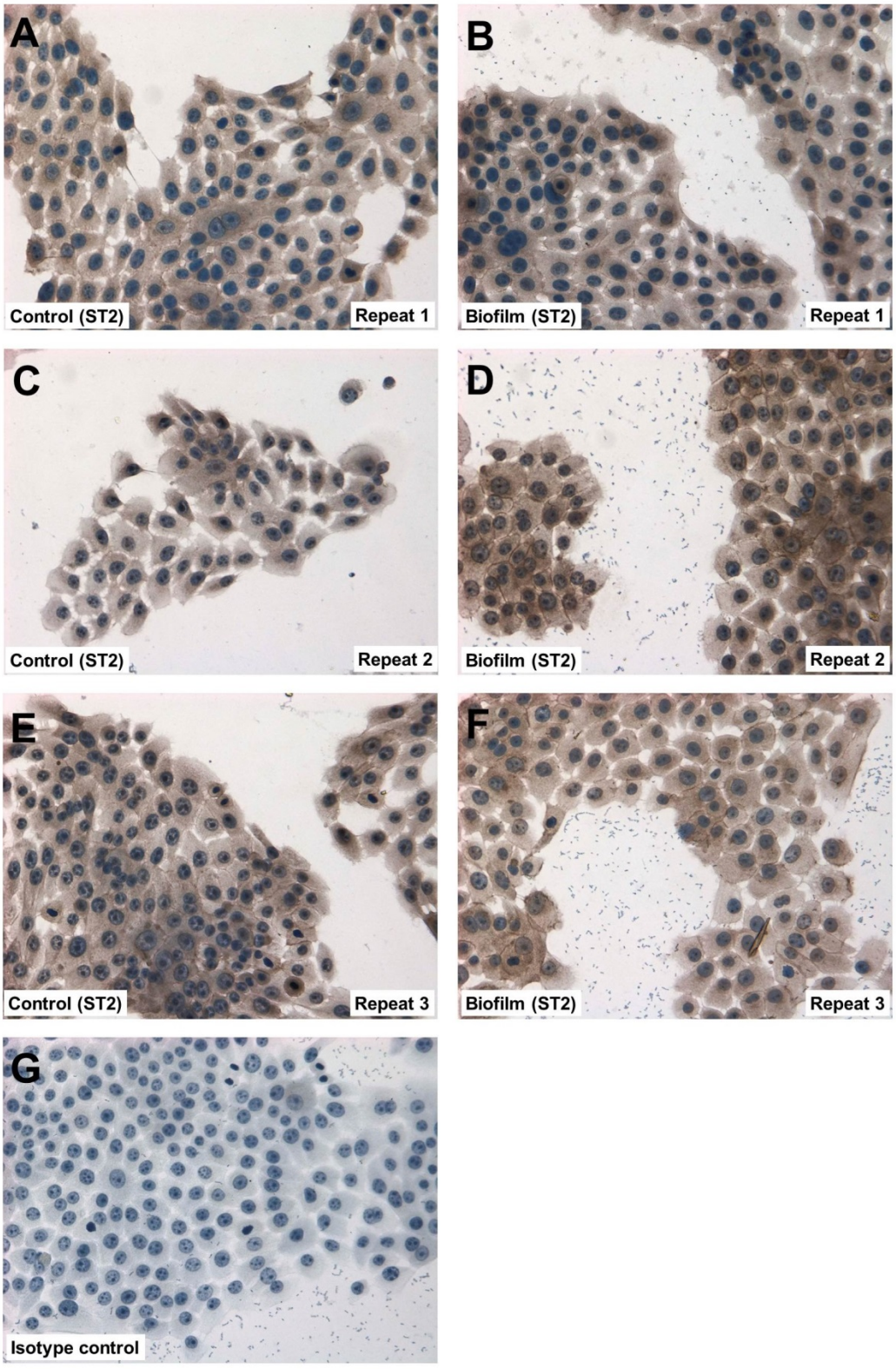

Figure 3-22: ST2 expression by OKF6/TERT-2 cells cultured on glass coverslips and stimulated with a live *P. gingivalis* monospecies biofilm for 9 h

Expression of ST2 protein was determined by immunocytochemical analysis using a rabbit anti-IL1RL1 antibody (Sigma-Aldrich®, UK). The panels show representative photomicrographs of methanol fixed OKF6/TERT-2 cells on glass coverslips after 9 h incubation either with media only as a control (**A, C** and **E**) or a live *P. gingivalis* monospecies biofilm (**B, D** and **F**). An isotype control antibody was used to determine non-specific binding (**G**). Original magnification x 400.

Despite the abundance of staining, the % of ST2 positive OKF6/TERT-2 cells was still quantified by the counting method described by Bologna-Molina and colleagues (2011). Figure 3-23 shows that, as expected, there was no significant difference in the % of ST2 positive cells after stimulation with a live *P. gingivalis* monospecies biofilm for 9 h.

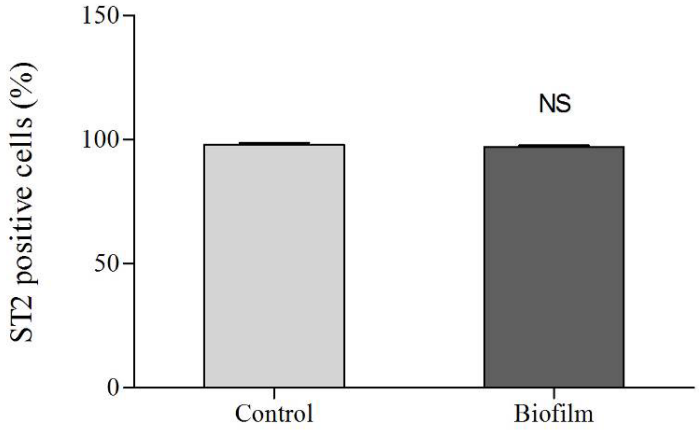

Figure 3-23: Percentage of ST2 positive OKF6/TERT-2 cells on glass coverslips after incubation with media alone or a live *P. gingivalis* monospecies biofilm for 9 h

The % of ST2 positive cells was determined from photomicrographs using the methodology described by Bologna-Molina and colleagues (2011). Bars show the percentage of ST2 positive OKF6/TERT-2 cells after 9 h incubation with media alone (Control) or with a live *P. gingivalis* monospecies biofilm (Biofilm). Data was derived from 5 micrographs depicting different fields from 3 different coverslips. Prior to statistical analyses the % data was angular transformed. Statistical analysis was carried out using the independent *t*-test (IBM SPSS Statistics, version 19). * = $p < 0.05$. Error bars indicate the standard error of the mean (SEM).

3.2.2.4 IL-33 expression by primary human gingival epithelial cells in response to *Porphyromonas gingivalis*

Previously the data demonstrated that IL-33 is expressed by the OKF6/TERT-2 oral keratinocyte cell line and expression was upregulated in response to a live *P. gingivalis* monospecies biofilm. However, in order to determine the *in vivo* relevance of these findings, the studies were repeated using primary human gingival epithelial (PHGE) cells (CELLnTEC advanced cell systems, Switzerland).

PHGE cells were cultured *in vitro* and exposed to a live *P. gingivalis* monospecies biofilm for 3, 9 and 24 h as previously described (Section 2.6.1). Unstimulated cells acted as controls at each time point. Stimulation was once again confirmed by measurement of IL-8 release into bathing supernatants. Figure 3-24 shows that there was a significant increase in IL-8 release from PHGE cells in response to *P. gingivalis* stimulation at all time points.

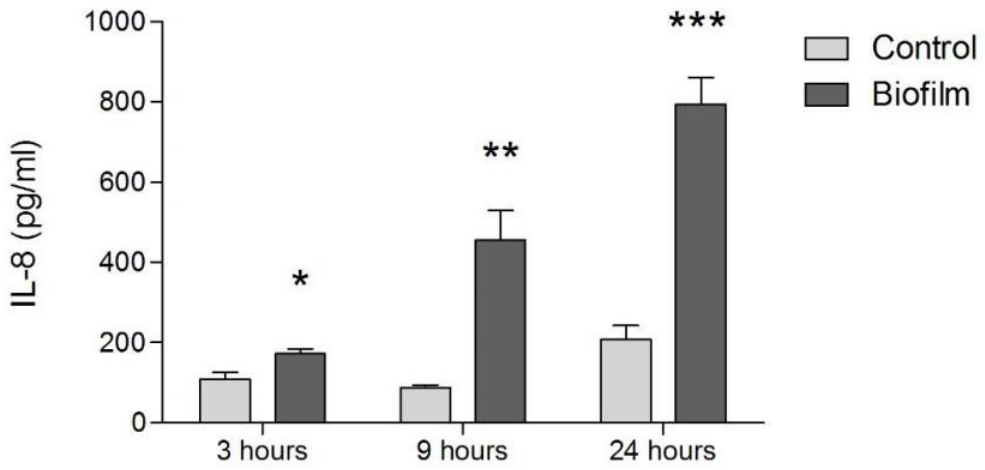

Figure 3-24: Release of IL-8 (CXCL8) from primary human gingival epithelial cells in response to a live *P. gingivalis* monospecies biofilm

IL-8 release into bathing supernatants was measured by ELISA. The bars represent the mean supernatant concentrations of IL-8 in unstimulated (Control) and a live *P. gingivalis* monospecies biofilm (Biofilm) stimulated PHGE cells at 3, 9 and 24 h. The data was generated from duplicate wells of three independent experiments. Statistical analyses were carried out using the independent *t*-test (IBM SPSS Statistics, version 19) on the natural log transformed IL-8 levels (pg/ml). * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. Error bars indicate the standard error of the mean (SEM).

Once stimulation of the PHGE cells had been confirmed, real-time PCR was employed to quantitate levels of IL-33 mRNA. Figure 3-25 shows that levels of IL-33 mRNA were significantly upregulated in *P. gingivalis* stimulated PHGE cells after 9 h stimulation. However, expression returned to basal levels after 24 h stimulation.

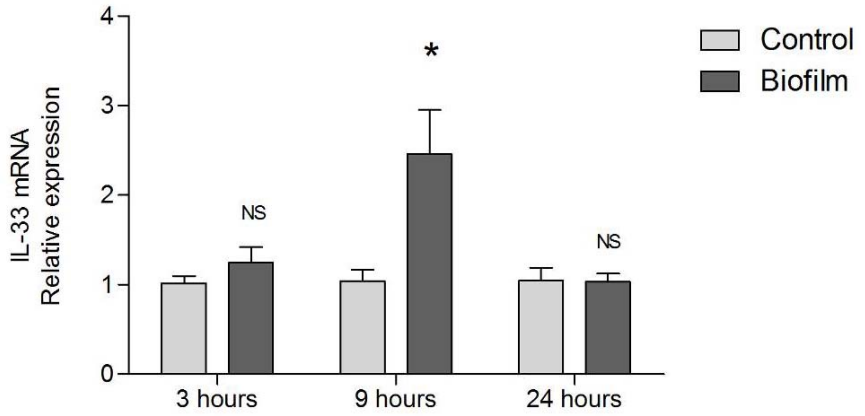

Figure 3-25: Effect of a live *P. gingivalis* monospecies biofilm on IL-33 mRNA expression by primary human gingival epithelial cells

Quantification of IL-33 mRNA expression in PHGE cells was performed by real-time PCR. The bars represent mean relative expression ($2^{-\Delta\Delta CT}$) of IL-33 mRNA in non stimulated (Control) and a live *P. gingivalis* monospecies biofilm (Biofilm) stimulated PHGE cells at 3, 9 and 24 h. RNA polymerase II was used as a reference gene. The data was generated from duplicate wells of three independent experiments. Statistical analyses were carried out using the independent *t*-test (IBM SPSS Statistics, version 19) on the natural log transformed IL-33 mRNA relative expression ($2^{-\Delta\Delta CT}$) values. * = $p < 0.05$ and Ns= not significant. Error bars indicate the standard error of the mean (SEM).

Having established that IL-33 mRNA expression is upregulated in PHGE cells in response to stimulation with *P. gingivalis*; we next investigated whether IL-33 protein was released from stimulated cells. Figure 3-26 shows that using a commercially available ELISA kit (Enzo® Life Science, USA) no IL-33 release was detected in the bathing supernatants of unstimulated or *P. gingivalis* monospecies biofilm stimulated PHGE cells.

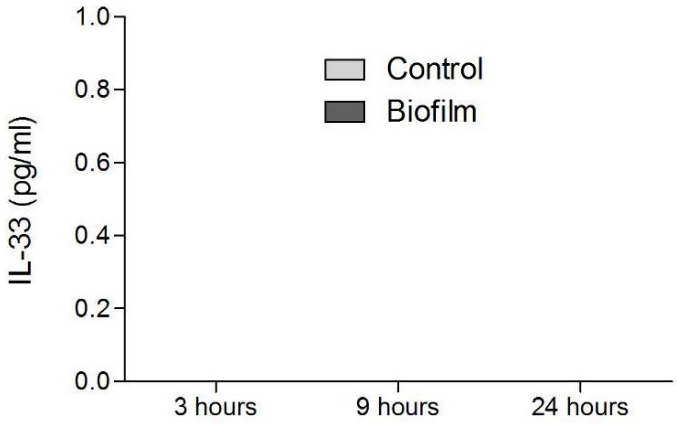

Figure 3-26: Release of IL-33 from primary human gingival epithelial cells in response to a live *P. gingivalis* monospecies biofilm

IL-33 release into bathing supernatants was measured by ELISA. No IL-33 was detected in any of the bathing supernatants analysed in unstimulated (Control) and a live *P. gingivalis* monospecies biofilm (Biofilm) stimulated PHGE cells at 3, 9 and 24 h. The data was generated from duplicate wells of three independent experiments.

The ELISA failed to demonstrate the release of IL-33 from *P. gingivalis* stimulated PHGE cells. Therefore, immunocytochemistry analysis was employed to detect intracellular levels of IL-33. PHGE cells were cultured on glass coverslips in the presence and absence of a live *P. gingivalis* monospecies biofilm for 9 h. Prior to immunocytochemistry, stimulation was confirmed by measurement of IL-8 release (Figure 3-27).

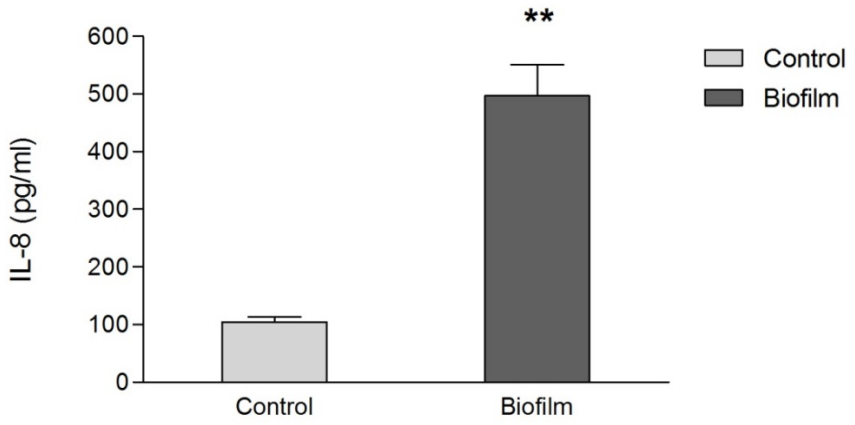

Figure 3-27: Release of IL-8 (CXCL8) from primary human gingival epithelial cells cultured on glass coverslips and stimulated with a *P. gingivalis* monospecies biofilm for 9 h

IL-8 release into bathing supernatants was measured by ELISA. The bars represent the mean supernatant concentrations of IL-8 in unstimulated (Control) and a live *P. gingivalis* monospecies biofilm (Biofilm) stimulated at 9 h. The data was generated from duplicate wells of three independent experiments. Statistical analyses were carried out using the independent *t*-test (IBM SPSS Statistics, version 19) on the natural log transformed IL-8 levels (pg/ml). ** = $p < 0.01$. Error bars indicate the standard error of the mean (SEM).

Once stimulation had been confirmed, immunocytochemistry analysis of fixed cells was conducted using a mouse monoclonal anti-IL-33 antibody (Nessy-1, Enzo® Life Science, UK). The specificity of this antibody had been determined previously (Figure 3-18). IL-33 was found to be expressed in PHGE cells and an increase in intracellular expression could be observed after stimulation for 9 h with a live *P. gingivalis* monospecies biofilm (Figure 3-28).

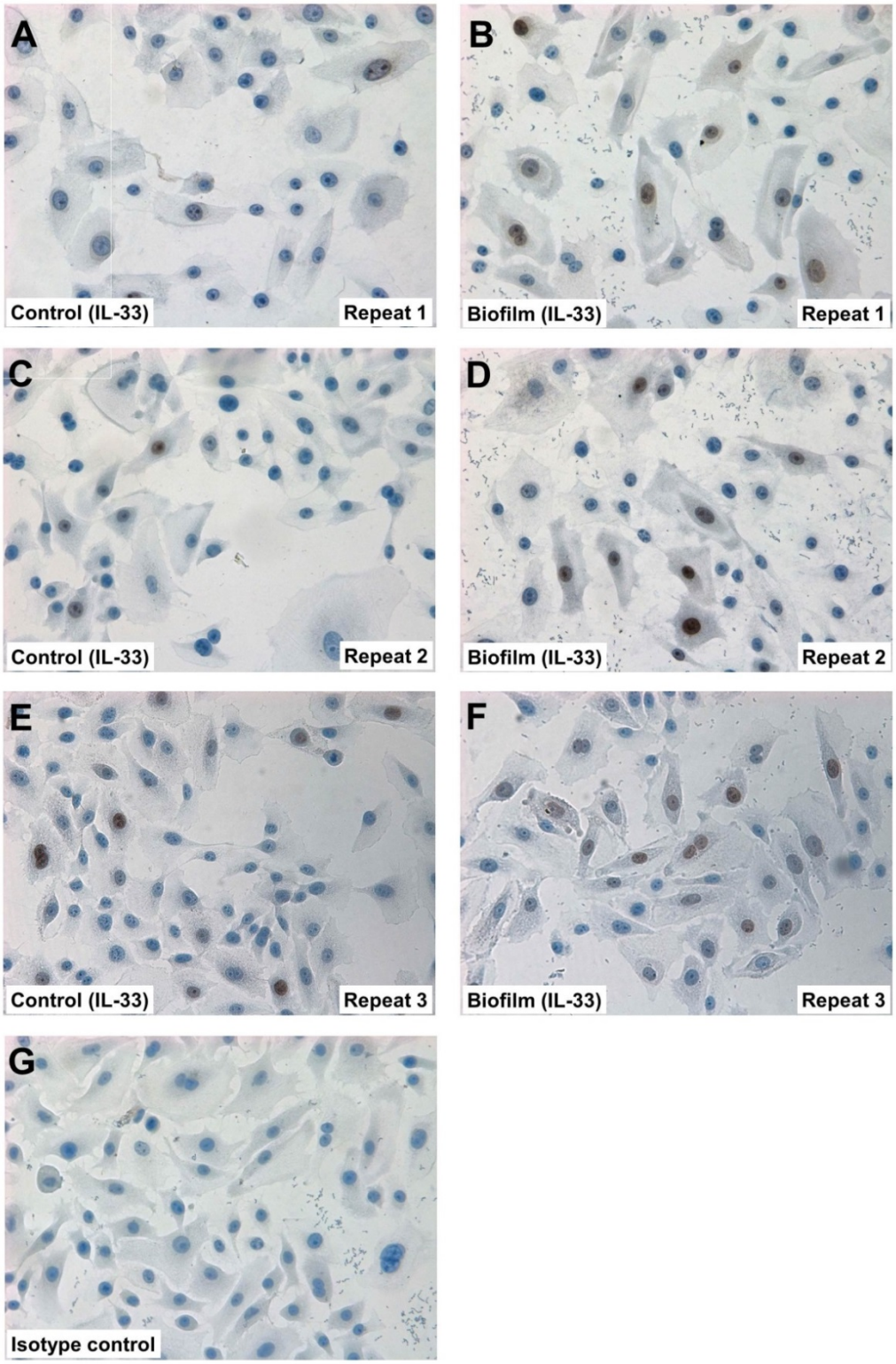

Figure 3-28: Intracellular IL-33 expression by primary human gingival epithelial cells cultured on glass coverslips and stimulated with a live *P. gingivalis* monospecies biofilm for 9 h

Intracellular expression of IL-33 was determined using a mouse monoclonal anti-IL-33 antibody (Nessy-1, Enzo® Life Science, UK). The panels show representative photomicrographs of methanol fixed PHGE cells on glass coverslips after 9 h incubation either with media only as a control (**A, C** and **E**) or a live *P. gingivalis* monospecies biofilm (**B, D** and **F**). An isotype control antibody was used to determine non-specific binding (**G**). Original magnification x 400.

To quantify increases in intracellular IL-33 expression, the counting method described by Bologna-Molina *et al* (2011) was employed. Figure 3-29 shows that the % of IL-33 positive PHGE cells was significantly higher after stimulation with a live *P. gingivalis* monospecies biofilm for 9 h.

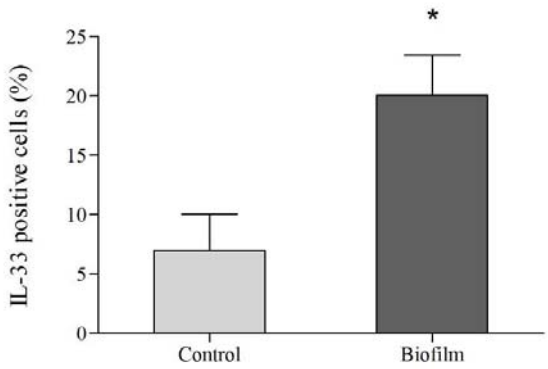

Figure 3-29: Percentage of IL-33 positive primary human gingival epithelial cells on glass coverslips after incubation with media alone or a live *P. gingivalis* monospecies biofilm for 9 h

The % of IL-33 positive cells was determined from photomicrographs using the methodology described by Bologna-Molina and colleagues (2011). Bars show the percentage of IL-33 positive PHGE cells after 9 h incubation with media alone (Control) or with a live *P. gingivalis* monospecies biofilm (Biofilm). Data was derived from 5 micrographs depicting different fields from 3 different coverslips. Prior to statistical analyses the % data was angular transformed. Statistical analysis was carried out using the independent *t*-test (IBM SPSS Statistics, version 19). * = $p < 0.05$. Error bars indicate the standard error of the mean (SEM).

3.2.2.5 ST2 expression by primary human gingival epithelial cell in response to *Porphyromonas gingivalis*

Having established that IL-33 is expressed by PHGE cells; and demonstrating that its expression is upregulated in response to a live *P. gingivalis* monospecies biofilm; it was logical to also investigate whether there were similar effects on ST2 expression.

Real-time PCR was employed to quantitate levels of ST2L and sST2 mRNA in non stimulated and *P. gingivalis* monospecies biofilm stimulated PHGE cells. Figure 3-30 shows that neither sST2 nor ST2L mRNA was significantly upregulated by PHGE cells after *P. gingivalis* monospecies biofilm stimulation.

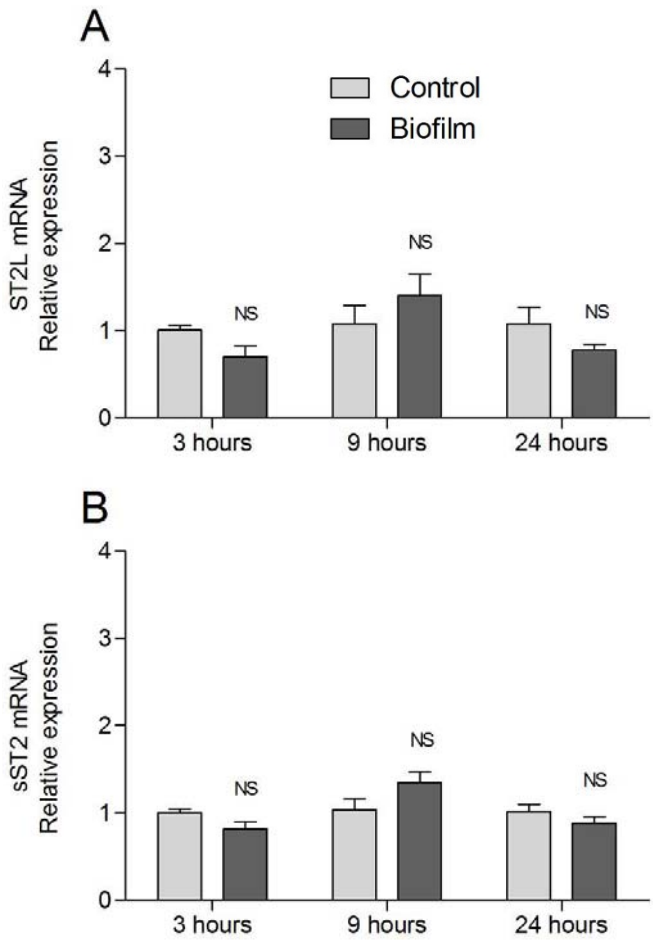

Figure 3-30: Effect of a live *P. gingivalis* monospecies biofilm on sST2 and ST2L mRNA expression by primary human gingival epithelial cells

Quantification of ST2L (**A**) and sST2 (**B**) mRNA expression in PHGE cells was performed by real-time PCR. The bars represent mean relative expression ($2^{-\Delta\Delta CT}$) of ST2L and sST2 mRNA in non stimulated (Control) and a live *P. gingivalis* monospecies biofilm (Biofilm) stimulated PHGE cells at 3, 9 and 24 h. RNA polymerase II was used as a reference gene. The data was generated from duplicate wells of three independent experiments. Statistical analyses were carried out using the independent *t*-test (IBM SPSS Statistics, version 19) on the natural log transformed sST2 and ST2L mRNA relative expression ($2^{-\Delta\Delta CT}$) values. NS = not significant ($p > 0.05$). Error bars indicate the standard error of the mean (SEM).

Having established that sST2 mRNA is expressed by PHGE cells but not regulated by a live *P. gingivalis* monospecies biofilm; we next confirmed this finding at the protein level by investigating the release of sST2 protein from these cells. Figure 3-31 shows that using a commercially available ELISA kit (Quantikine® ST2; R&D System, UK) sST2 protein was detected at low levels in the cell supernatant and there was no difference in sST2 levels between *P. gingivalis* monospecies biofilm stimulated PHGE cells and unstimulated controls.

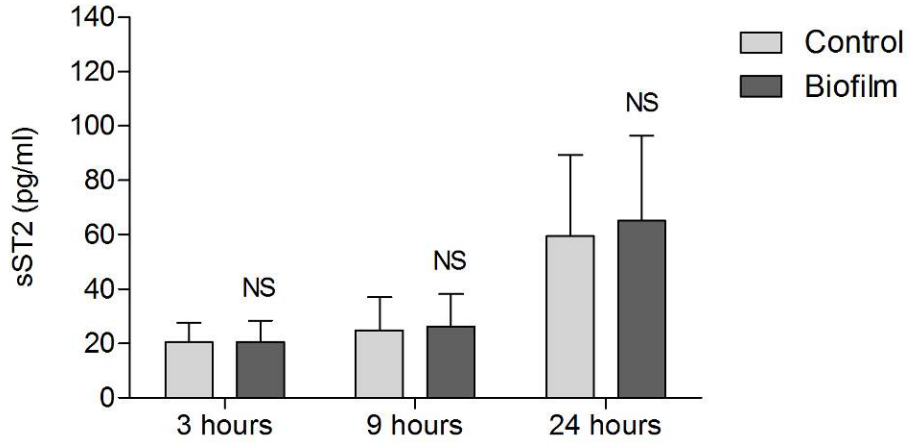

Figure 3-31: Release of sST2 from primary human gingival epithelial cells in response to stimulation with a live *P. gingivalis* monospecies biofilm

sST2 release into bathing supernatants was measured by ELISA. The bars represent the mean supernatant concentrations of sST2 in unstimulated (Control) and a live *P. gingivalis* monospecies biofilm (Biofilm) stimulated PHGE cells at 3, 9 and 24 h. The data was generated from duplicate wells of three independent experiments. Statistical analyses were carried out using the independent *t*-test (IBM SPSS Statistics, version 19) on the natural log transformed sST2 levels (pg/ml). NS = not significant. Error bars indicate the standard error of the mean (SEM)

The data shown in Figure 3-31 confirmed at the protein level the findings of the mRNA for sST2 shown in Figure 3-30. To confirm the findings of Figure 3-31 at the protein level for ST2L however, immunocytochemistry analysis was employed. PHGE cells were cultured on glass coverslips in the presence and absence of a live *P. gingivalis* monospecies biofilm for 9 h. Prior to immunocytochemistry, stimulation was again confirmed by measurement of IL-8 release. As investigations were conducted alongside IL-33 immunocytochemistry investigations, the data confirming that all cells cultured on coverslips were in fact stimulated by a live *P. gingivalis* monospecies biofilm is shown in Figure 3-27.

Once stimulation had been confirmed, immunocytochemistry analysis of fixed cells was conducted using a rabbit anti-IL1RL1 (anti-ST2) antibody (Sigma-Aldrich®, UK). This antibody was not specific for any of the ST2 isoforms (sST2 or ST2L) and stained only total ST2. Figure 3-32 shows that protein for ST2 was found expressed in unstimulated PHGE cells and no visual increase in surface receptor ST2L protein expression was observed in *P. gingivalis* monospecies biofilm stimulated cells.

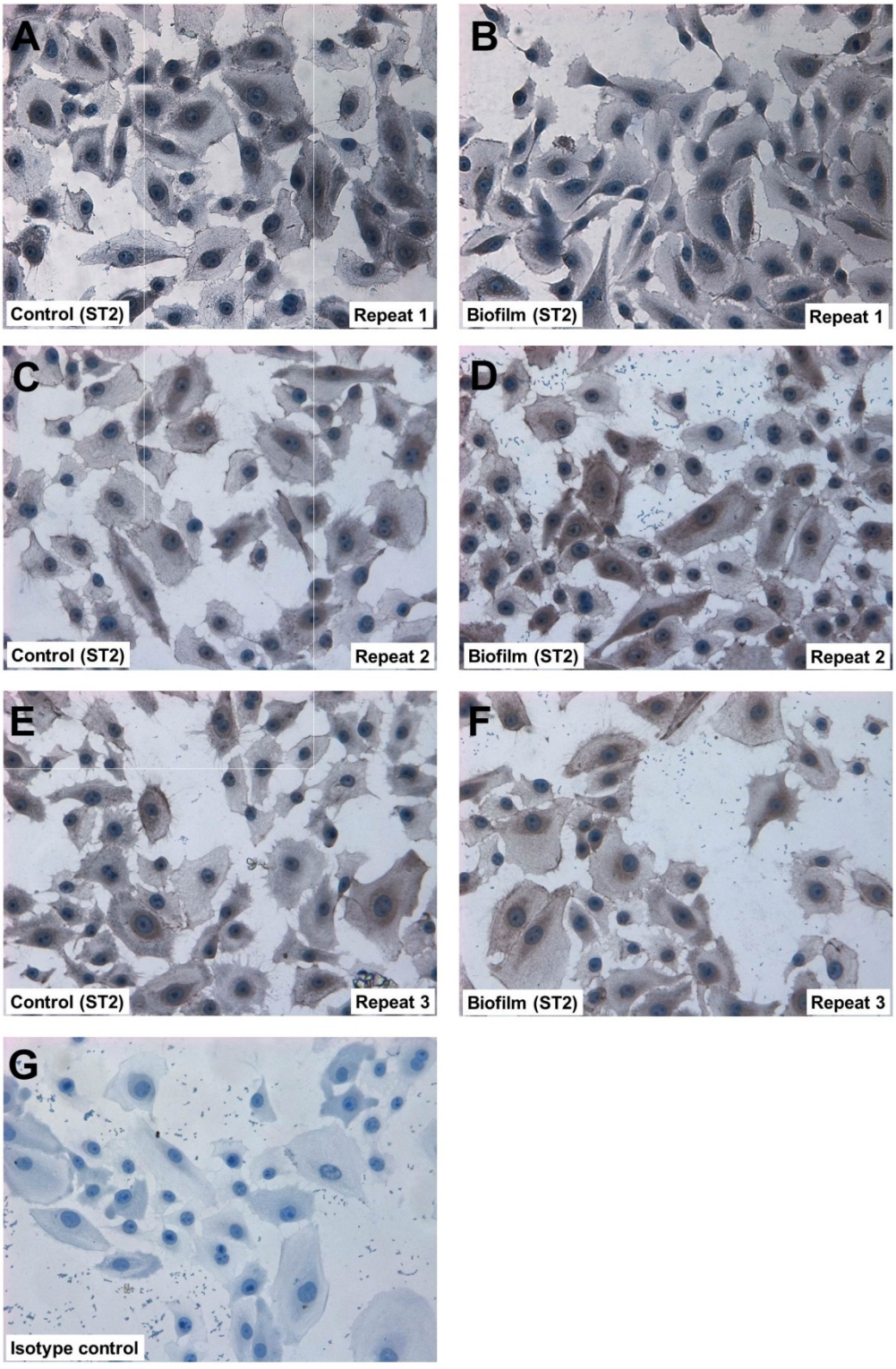

Figure 3-32: ST2 expression by primary human gingival epithelial cells cultured on glass coverslips and stimulated with a live *P. gingivalis* monospecies biofilm for 9 h

Expression of ST2 protein was determined by immunocytochemical analysis using a rabbit anti-IL1RL1 antibody (Sigma-Aldrich®, UK). The panels show representative photomicrographs of methanol fixed from PHGE cells on glass coverslips after 9 h incubation either with media only as a control (**A, C** and **E**) or a live *P. gingivalis* monospecies biofilm (**B, D** and **F**). An isotype control antibody was used to determine non-specific binding (**G**). Original magnification x 400.

Despite the abundance of staining, the % of ST2 positive from PHGE cells was still quantified by the counting method described by Bologna-Molina and colleagues (2011). Figure 3-33 shows that, as expected, there was no significant difference in the % of ST2 positive cells after stimulation with a live *P. gingivalis* monospecies biofilm for 9 h.

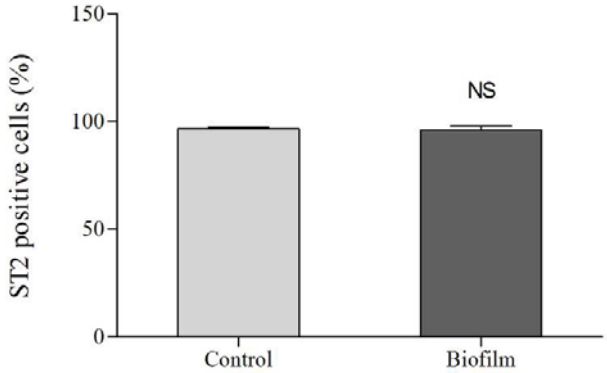

Figure 3-33: Percentage of ST2 positive primary human gingival epithelial cells on glass coverslips after incubation with media alone or a live *P. gingivalis* monospecies biofilm for 9 h

The % of ST2 positive cells was determined from photomicrographs using the methodology described by Bologna-Molina and colleagues (2011). Bars show the percentage of ST2 positive PHGE cells after 9 h incubation with media alone (Control) or with a live *P. gingivalis* monospecies biofilm (Biofilm). Data was derived from 5 micrographs depicting different fields from 3 different coverslips. Prior to statistical analyses the % data was angular transformed. Statistical analysis was carried out using the independent *t*-test (IBM SPSS Statistics, version 19). * = $p < 0.05$. Error bars indicate the standard error of the mean (SEM).

3.2.2.6 Effect of IL-33 on OKF6/TERT-2 cells

The previous data confirmed that oral keratinocytes express the membrane bound form of the IL-33 receptor (ST2L). Therefore, it is rational to assume that these cells can mediate IL-33 signalling. Therefore, the effect of rhIL-33 on the innate immune response of OKF6/TERT-2 cells was investigated *in vitro*.

Prior to investigations the bioactivity of the commercially obtained rhIL-33 (PeproTech®, UK) was confirmed. This was achieved using the methodology of Kurowska-Stolarska and colleagues (2008). The authors showed that IL-33 enhanced production of IL-5 by anti-CD3 antibody activated naïve $CD4^+$ T cells isolated from cord blood. As cord blood cells are difficult to obtain; PBMCs,

which are known to contain naïve $CD4^+$ T cells (Pflanz, *et al.*, 2002), were used instead.

Figure 3-34 shows that recombinant human IL-33 significantly augmented IL-5 release from anti-CD3 antibody activated PBMCs in a dose dependant manner.

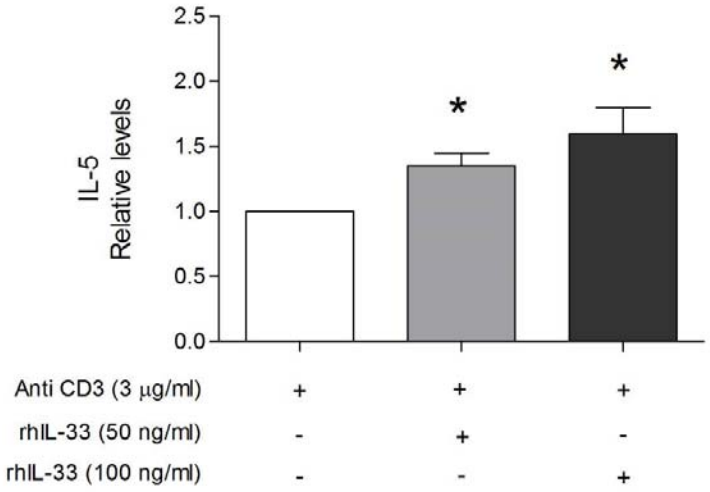

Figure 3-34: Effect of recombinant human IL-33 on IL-5 release from anti-CD3 antibody activated PBMCs

IL-5 release into bathing supernatants was measured by ELISA. The figure represents mean relative levels (relative to control) of IL-5 in bathing supernatant of peripheral blood mononuclear cells (PBMCs) after 3 days. The data is derived from three independent experiments, carried out in duplicate on PBMCs isolated from three separate donors. Statistical analyses were carried out using the ANOVA with a Bonferroni correction (IBM SPSS Statistics, version 19) on the natural log transformed IL-5 relative levels. * = $p < 0.05$. Error bars indicate the standard error of the mean (SEM).

Once the bioactivity of the rhIL-33 had been confirmed, OKF6/TERT-2 cells were stimulated *in vitro* with 10, 50 and 100 ng/ml rhIL-33 for 4, 24 and 48 h; as described in Section 2.6.4. For control purposes, cells were either stimulated with 10 ng/ml PMA or left unstimulated.

Initial investigations were performed to determine the effect of rhIL-33 on the release of IL-8 (CXCL8) from OKF6/TERT-2 cells. Figure 3-35 shows that PMA (10 ng/ml) induced a significant increase in IL-8 release from OKF6/TERT-2 cells at all time points when compared to unstimulated cells. However, no effect on IL-8 release was observed when cells were stimulated with various concentrations of rhIL-33 at any of the time points investigated.

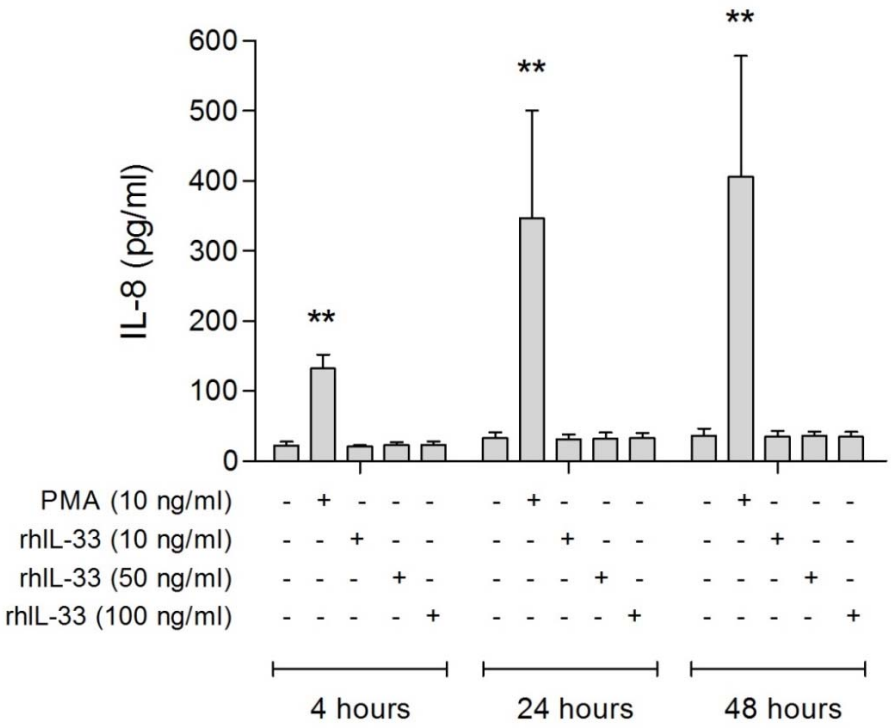

Figure 3-35: The effect of phorbol 12-myristate 13-acetate and recombinant human IL-33 on IL-8 expression by OKF6/TERT-2 cells

IL-8 released into bathing supernatants was measured by ELISA. The figure represents mean supernatant levels of IL-8 in PMA and rhIL-33 stimulated OKF6/TERT-2 cells. Unstimulated cells acted as controls. Figure represents three independent experiments, carried out in duplicate. Incubation periods were 4, 24 and 48 h. Statistical analysis was performed on the natural log transformed IL-8 levels (pg/ml) using the ANOVA with a Bonferroni correction (IBM SPSS Statistics, version 19). ** = $p < 0.01$. Error bars indicate the standard error of the mean (SEM).

The failure of IL-33 to induce IL-8 expression from OKF6/TERT-2 cells was confirmed at the mRNA level. Figure 3-36 shows that PMA significantly upregulated the expression of IL-8 mRNA in OKF6/TERT-2 cells. However, no significant changes in IL-8 mRNA expression were observed in cells stimulated with 100 ng/ml rhIL-33.

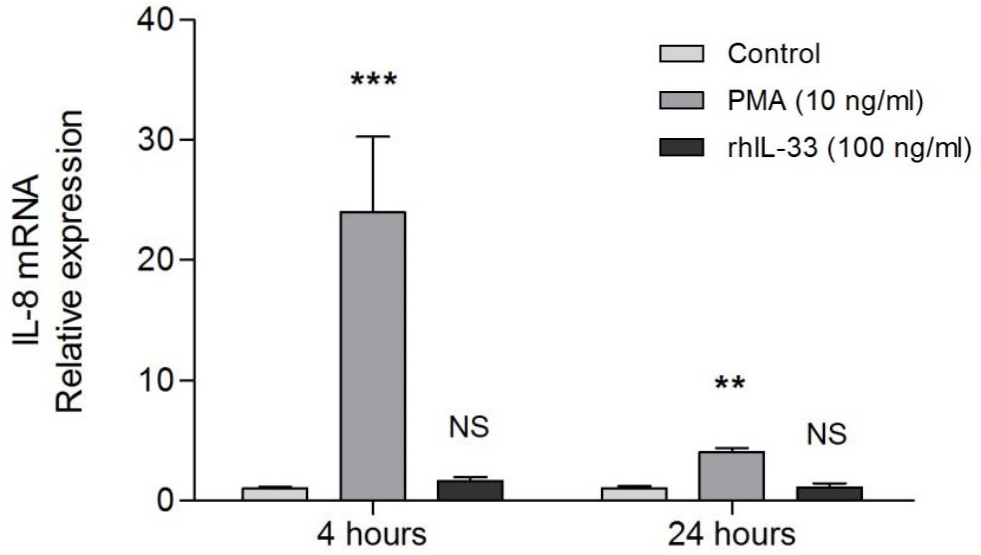

Figure 3- 36: Effect of phorbol 12-myristate 13-acetate and recombinant human IL-33 on IL-8 mRNA expression by OKF6/TERT-2 cells

Quantification of IL-8 mRNA expression in OKF6/TERT-2 cells was performed by real-time PCR. The figure represents mean relative expression ($2^{-\Delta\Delta CT}$) of IL-8 mRNA in PMA and rhIL-33 stimulated OKF6/TERT-2 cells. Unstimulated cells acted as controls. Incubation periods were 4 and 24 h. GAPDH was used as a reference gene. Figure represents three independent experiments carried out in duplicate. Statistical analysis was performed on the natural log transformed IL-8 mRNA relative expression ($2^{-\Delta\Delta CT}$) values using the ANOVA with a Bonferroni correction (IBM SPSS Statistics, version 19). ** = $p < 0.01$ and *** = $p < 0.001$. Error bars indicate the standard error of the mean (SEM).

To further investigate whether rhIL-33 had any effect on the innate immune responses of OKF6/TERT-2 cells a holistic approach was undertaken. The use of the Proteome Profiler™ Array (R&D Systems, UK) allowed us to rapidly and economically detect changes in expression of 36 different cytokines and chemokines without performing numerous immunoassays. Therefore the supernatants collected from OKF6/TERT-2 cells stimulated with PMA and rhIL-33 (Section 2.6.4) were exposed to the array (Section 2.7.6).

Figure 3-37 visually suggested that PMA (10 ng/ml) induced upregulated release of CD40L, G-CSF, GM-CSF, CXCL1, IL-1RA, IL-6 and IL-8 from OKF6/TERT-2 cells. However, no changes in release of any cytokines and chemokines could be observed from OKF6/TERT-2 cells stimulated by rhIL-33.

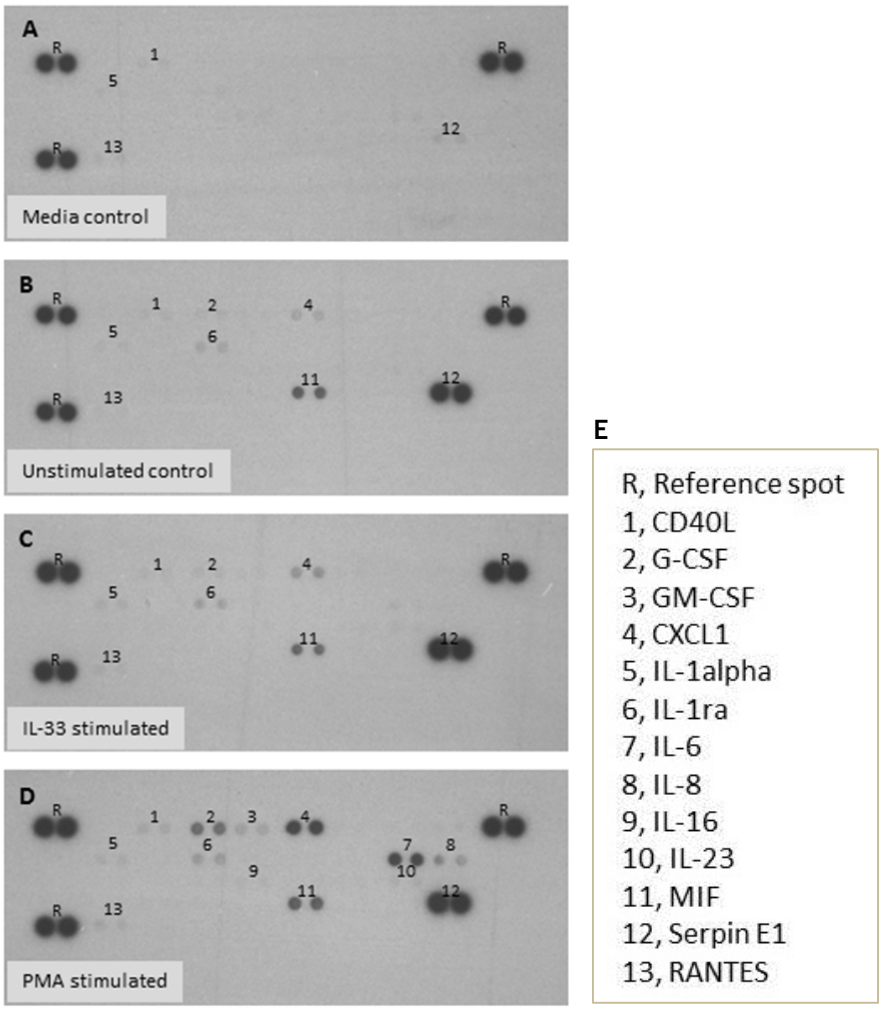

Figure 3-37: Proteome profiler analysis of phorbol 12-myristate 13-acetate and recombinant human IL-33 stimulated OKF6/TERT-2 cells

Cytokines and chemokines released into bathing supernatants were measured using the Proteome Profiler™ Array (R&D Systems, UK). Images show membranes incubated with culture media only (**A**), supernatants from unstimulated cells (**B**), rhIL-33 (100 ng/ml) stimulated cells (**C**), and PMA (10 ng/ml) stimulated cells (**D**). Panel (**E**) shows the key for the membranes. For membranes incubated with bathing supernatants; these were pooled samples derived from 24 h time points of three independent experiments. The X-ray films were exposed for 20 min prior to imaging.

The membranes only give a visual indication to changes in cytokine and chemokine release from stimulated cells. Therefore, in order to quantify these changes the pixel densities on the developed X-ray film were analyzed using a Gel Doc™ XR Imaging System (Bio-Rad Laboratories, UK) and Quantity One® Software Version 4.6.7 (Bio-Rad Laboratories, UK).

Pixel density analysis (Figure 3-38) showed that PMA (10 ng/ml) induced upregulated release of CD40L, G-CSF, GM-CSF, CXCL1, IL-1α, IL-1RA, IL-6, IL-8

and RANTES from OKF6/TERT-2 cells. However, no changes in release of any cytokines and chemokines could be observed from OKF6/TERT-2 cells stimulated by rhIL-33.

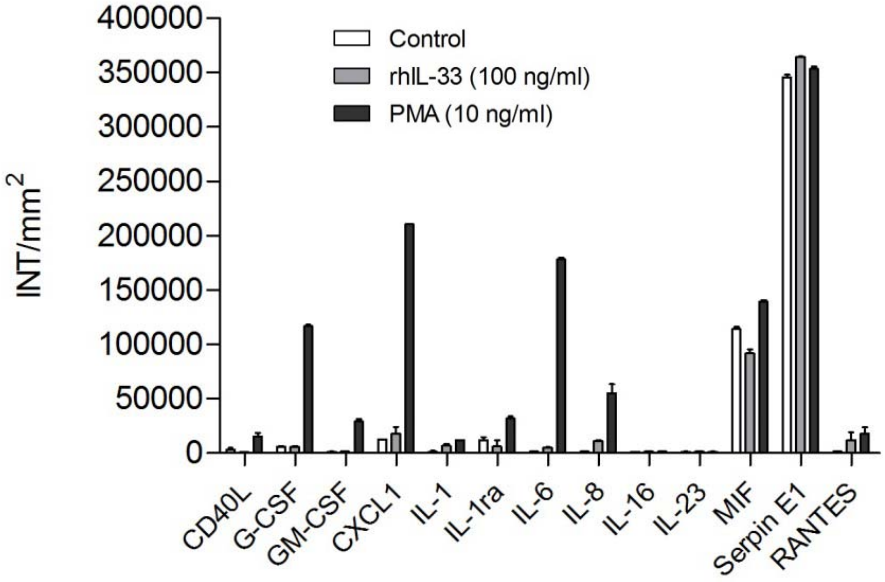

Figure 3-38: Pixel density analysis to determine changes in cytokine and chemokine expression by OKF6/TER-2 cells stimulated by recombinant human IL-33 and phorbol 12-myristate 13-acetate

Pixel densities for each cytokine and chemokine were measured on the membranes shown in Figure 3-37 using a Gel Doc™ XR Imaging System (Bio-Rad Laboratories, UK) and Quantity One® Software Version 4.6.7 (Bio-Rad Laboratories, UK). Each bar represents the average intensity on membranes which were probed with supernatants from unstimulated cells (Control), cells stimulated with rhIL-33 (100 ng/ml) and cells stimulated with PMA (10 ng/ml). The pixel intensity was corrected for the media control. Error bars indicate the standard error of the mean (SEM).

To confirm the findings of the Proteome Profiler™ Array (R&D Systems, UK), expression of selected genes (G-CSF and IL-1RA) was investigated further using standard real-time PCR and ELISA technologies.

Figure 3-39A shows there were significant increase in G-CSF mRNA by OKF6/TERT-2 cells after PMA (10 ng/ml) stimulation at 4 h ($p < 0.001$) and 24 h ($p < 0.01$); when compared to the unstimulated control. However, no change in expression of G-CSF mRNA expression could be observed from OKF6/TERT-2 cells stimulated by rhIL-33 after either 4 or 24 h. Figure 3-39B shows no changes in IL-1RA mRNA expression by OKF6/TERT-2 cells after PMA (10 ng/ml) stimulation at 4 h when compared to unstimulated control ($p > 0.05$). However, IL-1RA mRNA

expression was significantly decreased 24 h after stimulation ($p < 0.01$). Once again, no changes in expression of IL-1RA mRNA could be observed from OKF6/TERT-2 cells stimulated by rhIL-33 after either 4 or 24 h. Figure 3-39C shows there was significant increase in G-CSF protein levels in supernatants of OKF6/TERT-2 cells stimulated by PMA (10 ng/ml) after 24 h stimulation ($p < 0.01$); when compared to the unstimulated control. However, there was no change in levels of G-CSF after stimulation with rhIL-33 ($p > 0.05$). Figure 3-39D shows no changes in supernatant levels of IL-1RA protein observed from OKF6/TERT-2 cells stimulated for 24 h by either PMA (10 ng/ml) or rhIL-33 when compared to the unstimulated control ($p > 0.05$).

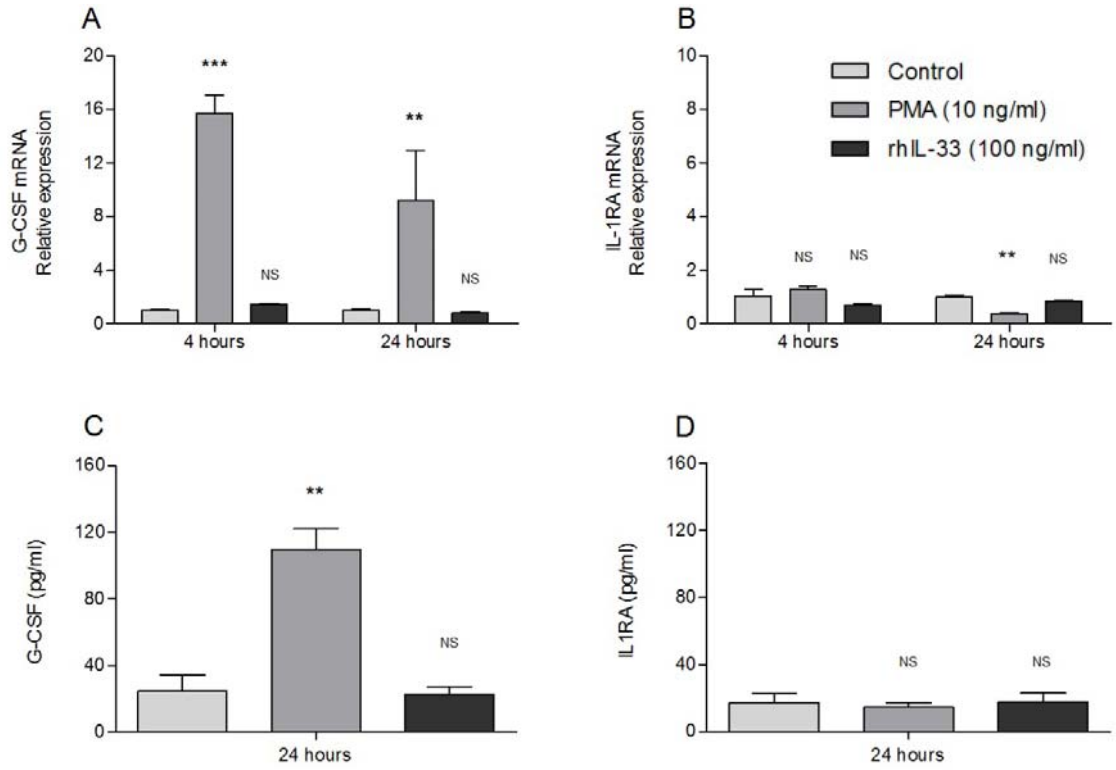

Figure 3-39: The effect of phorbol 12-myristate 13-acetate and recombinant human IL-33 on G-CSF and IL-1RA expression by OKF6/TERT-2 cells

Quantification of mRNA expression and supernatant protein levels were performed by real-time PCR and ELISA respectively. The bars represent mean relative mRNA expression ($2^{-\Delta\Delta CT}$) of G-CSF and IL-1RA (**A** and **B**), as well as mean supernatant levels of G-CSF and IL-1RA (**C** and **D**) by OKF6/TERT-2 cells stimulated by either PMA (10 ng/nl) or rhIL-33 (100 ng/ml). Unstimulated cells acted as controls. For real-time PCR analysis, RNA polymerase II was used as a reference gene. The data was generated from duplicate wells of three independent experiments. Statistical analysis of real-time PCR and ELISA data was performed on the natural log transformed values using the ANOVA with a Bonferroni correction (IBM SPSS Statistics, version 19). ** = $p < 0.01$; *** = $p < 0.001$ and NS = not significant ($p > 0.05$). Error bars indicate the standard error of the mean (SEM).

The data suggested that rhIL-33 had no direct effect on the innate immune responses of OKF6/TERT-2 cells. However, IL-33 has been suggested to augment bacterially induced innate immune responses by promoting increased expression of TLRs (Joshi, *et al.*, 2010). Therefore, changes in expression of TLR-2 and TLR-4 mRNA in OKF6/TERT-2 cells after stimulation with PMA (10 ng/ml) and rhIL-33 (100 ng/ml) were investigated by real-time PCR.

Figure 3-40A shows significant increase in TLR-2 mRNA by OKF6/TERT-2 cells after PMA (10 ng/ml) stimulation at 4 h and 24 h ($p < 0.01$) when compared to the unstimulated control. However, no change in expression of TLR-2 mRNA expression could be observed from OKF6/TERT-2 cells stimulated with rhIL-33 (100 ng/ml) at both time points. Figure 3-40B shows significant increase in TLR-4 mRNA by OKF6/TERT-2 cells after PMA (10 ng/ml) stimulation at 4 h ($p < 0.01$), but not at 24 h ($p > 0.05$) when compared to the unstimulated control. However, no change in expression of TLR-4 mRNA could be observed from OKF6/TERT-2 cells stimulated by rhIL-33 at both time points.

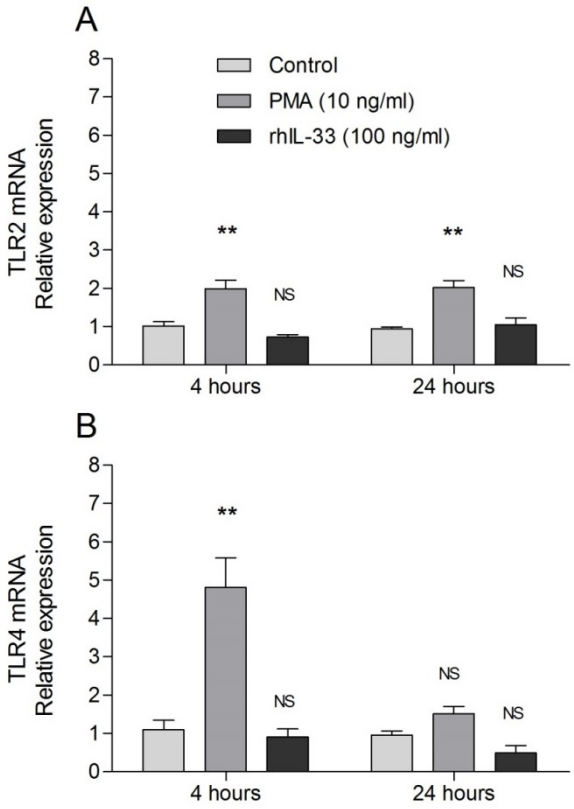

Figure 3-40: The effect of phorbol 12-myristate 13-acetate and recombinant human IL-33 on TLR-2 and TLR-4 mRNA expression by OKF6/TERT-2 cells

Quantification of TLR-2 and TLR-4 mRNA expression by OKF6/TERT-2 cells was performed by real-time PCR. The bars represent mean relative expression ($2^{-\Delta\Delta CT}$) of TLR-2 (**A**) and TLR-4 (**B**) in unstimulated (Control), PMA (10 ng/ml) and rhIL-33 (100 ng/ml) stimulated cells. The data was generated from duplicate wells of three independent experiments. RNA polymerase II was used as a reference gene. Statistical analysis was performed on the natural log transformed $2^{-\Delta\Delta CT}$ values using the ANOVA with a Bonferroni correction (IBM SPSS Statistics, version 19). ** = $p < 0.01$; NS = not significant ($p > 0.05$). Error bars indicate the standard error of the mean (SEM).

3.3 Discussion

The data, for the first time, showed that IL-33 (mRNA and protein) was expressed in periodontal tissues. Furthermore, expression was significantly elevated in tissue of patients with chronic periodontitis. These findings are in agreement with previous data. IL-33 has been shown to be expressed in numerous epithelial tissues; including bronchial, colonic, nasal, skin and giant papillae (Kamekura, *et al.*, 2012; Manetti *et al.*, 2010; Matsuda, *et al.*, 2009; Prefontaine, *et al.*, 2009; Seidelin *et al.*, 2010). In addition, in various chronic inflammatory disease models, pathogenesis has been associated with elevated expression of IL-33 (Miller, 2011). Furthermore, levels of IL-33 have been found to be upregulated within the chronically inflamed tissue of patients with rheumatoid arthritis, inflammatory bowel diseases, asthma and chronic allergic conjunctivitis (Beltran, *et al.*, 2010; Matsuda, *et al.*, 2009; Matsuyama, *et al.*, 2010; Prefontaine, *et al.*, 2009; Xu, *et al.*, 2008). In agreement with these findings, our data demonstrate that elevated epithelial expression of IL-33 is associated with periodontal disease. This therefore was suggestive of a role for IL-33 in periodontal disease pathogenesis.

The data presented in this thesis also showed that both the soluble form (sST2) and membrane bound form (ST2L) of the IL-33 receptor (ST2 or IL-1RL1) were expressed in periodontal tissue. Interestingly, at the mRNA level, expression of total ST2 was significantly increased in tissue of patients with chronic periodontitis. Furthermore, using primers to distinguish between ST2L and sST2 mRNA, it was found that expression of sST2, but not ST2L mRNA was elevated in tissue from patients with chronic periodontitis. These data are similar to findings in other chronic inflammatory diseases. mRNA expression of total ST2 and sST2, not ST2L were found to be increased in colonic tissue samples of patients with ulcerative colitis as compared to healthy subjects (Beltran, *et al.*, 2010; Pastorelli *et al.*, 2010). Similarly, total ST2 mRNA was also found to be increased in human nasal mucosa samples of patients with allergic rhinitis when compared to healthy subjects (Kamekura, *et al.*, 2012). In addition, no difference in ST2L mRNA expression was also reported between synovial tissue samples of rheumatoid arthritis and osteoarthritis patients (Talabot-Ayer *et al.*, 2012).

Despite these interesting findings at the mRNA level, confirmation at the protein level proved problematic in our studies. For IL-33, attempts to quantify levels in clinical samples (serum, GCF and saliva) from healthy volunteers and patients with periodontal disease proved unsuccessful. The ELISA analyses showed that in all samples IL-33 levels were below the detection limits of the ELISA kit used (Human IL-33 ELISA; Life Science, UK). The lowest standard for the Human IL-33 ELISA from Enzo Life sciences was 3.9 pg/ml. However, the actual limits of sensitivity in our hands was calculated as 15.5 pg/ml based on the concept of determining two mean standard deviations higher than the mean baseline from 6 replicate standard curves (Chaloner-Larsson, *et al.*, 1997).

In our investigations, serum IL-33 levels ranged from between 4.4 - 12.6 pg/ml; below the limits of detection. In addition, no IL-33 was detected in any of the saliva or GCF samples. This was found to be in contrast to some previous findings (Table 3-3). For analysis of levels of IL-33 in saliva and GCF only a few studies are reported (Table 3-3). However, these studies highlight the problems faced when using commercially available ELISA kits from different sources. Using an IL-33 ELISA assay from GenWay Biotech (USA), with reported detection limits of between 700 – 500000 pg/ml, Nizam *et al* (2014) and Buduneli *et al* (2012) reported high IL-33 levels in saliva and GCF (>1000 pg/ml) in healthy subjects ranging from 1100 – 14000 pg/ml (Table 3-3). Conclusions from these findings must however be drawn with care. Indeed, the literature consistently shows that the most abundant cytokines and chemokines in GCF such as IL-1ß, IL-6, IL-8, CXCL10 are reported to be presented in ranges of between 5 - 400 pg/ml using multiplex immunoassays as well as commercially available ELISA kits (Becerik *et al.*, 2012; de Lima Oliveira *et al.*, 2012; Shimada *et al.*, 2013). Similarly, the most abundant cytokines present in saliva, such as IL-1ß, IL-6 and TNF-α, were reported to be presented at ranges between 2 -700 pg/ml as measured by multiplex immunoassays as well as commercially available ELISA kits. Indeed, only levels of IL-8 in saliva have been reported to be higher (1800 – 2400 pg/ml) (Gursoy *et al.*, 2009; Teles *et al.*, 2009; Tobon-Arroyave *et al.*, 2008). Therefore, it is hard to believe that IL-33 levels in these fluids can be in the multiple thousand pg/ml ranges. Indeed, Papathanasiou and colleagues (2014) using a multiplex immunoassay (Millipore Corporation, USA) which has a sensitivity of between 0.4 – 55.8 pg/ml (company statement) reported that IL-33

was below the detection limit in the GCF of healthy subjects and chronic periodontitis patients. These were more in agreement with the findings reported in this thesis.

Biological Fluid	Disease	Healthy IL-33 (pg/ml)	Disease IL-33 (pg/ml)	LDL	ELISA source	Reference
Serum	RA	42.8 (±3.0)	138.6* (±16.5)	23.0	A1	Xiangyang *et al* (2012)
Serum	pSS	24.1 (13.4-31.6)	54.5* (26.2-169.5)	23.0	A1	Zhao *et al* (2013)
Serum	AS	146 (63-167)	450#* (217-730)	32.0	A2	Han *et al* (2011)
Serum	RA	Below detection	392.0* (±889.4)	23.0	A1	Mu *et al* (2010)
Saliva	CP	7700 (1100-14000)	13300#* (2200-27700)	700	A3	Nizam *et al* (2014)
Saliva	CP	1100 (±500)	1200 (±600)	700	A3	Buduneli *et al* (2012)
GCF	CP	3823 (±1970)	8300* (±4857)	700	A3	Buduneli *et al* (2012)
GCF	CP	Below detection	Below detection	0.4	A4	Papathanasiou *et al* (2014)

Table 3-3: Comparison of published studies measuring levels of IL-33 by ELISA in biological fluids of healthy subjects and patients with chronic inflammatory disease

RA = rheumatoid arthritis, pSS = primary Sjogren's Syndrome, AS = ankylosing spondylitis, CP = chronic periodontitis, A1 = ELISA (R&D System, USA), A2 = ELISA (Peprotech®, USA), A3 = ELISA (GenAway Biotech, USA), A4 = Multiplex immunoassay (Millipore Corporation, USA), LDL = lower detection limit, # = estimation of values from figures, and * = significant difference between health and disease ($p < 0.05$).

Based on our data and the available literature, it seems that evaluation of IL-33 levels in biological fluids can be variable depending upon the source of the ELISA used in the study. Therefore, perhaps a different approach could have been taken in our investigations; for example the use of western blot analysis or immunoprecipitation analysis to evaluate levels of IL-33 in biological fluids. The reasons for the problems encountered in our studies using ELISA are unknown. However, it is possible that in biological fluids there may be other mediators which bind to IL-33 and mask the epitope on the protein to which the capture antibody in the commercially available ELISA binds. Indeed, one such molecule may be sST2 which is well known to act as a decoy receptor and bind to IL-33

(Hayakawa, *et al.*, 2007; O'Neill, 2008; Schmitz, *et al.*, 2005). In addition, the lack of success with this ELISA kit could be due to numerous other factors including; sensitivity of the assay, sample preparation and long term storage and repeated freeze/thawing of samples. The shortcoming in our study was we did not have a suitable positive control sample to assess the efficiency of the ELISA kit used. Due to the problems that arose during the investigations into levels of IL-33 in biological fluids and sample limitations we therefore did not evaluate serum levels of sST2 in these samples.

Despite the failure to detect IL-33 at the protein level in biological fluids; using an immunohistochemical approach IL-33 protein was detected in periodontal tissue. Sections of periodontal tissue were stained using a mouse monoclonal anti-IL-33 antibody (Nessy-1, Enzo® Life Science, UK). This antibody was used extensively throughout this thesis for both the immunohistochemistry and immunocytochemistry. Although this antibody had routinely been used by other researchers in the field of IL-33 biology (Kamekura, *et al.*, 2012; Manetti, *et al.*, 2010); it was important that its specificity was determined in our hands before any inferences from results could be made. In this thesis we have confirmed specificity in a number of ways. Firstly, an appropriate isotype control (mouse monoclonal IgG1; Thermo Scientific, UK) was used to exclude non-specific binding. Secondly, staining of human tonsil tissue (Figure 3-3) which is known to contain IL-33 expressing immune cells (Baekkevold, *et al.*, 2003; Moussion, *et al.*, 2008) was performed as a positive control. Thirdly, staining of HUVEC cells, which are also known to express IL-33 (Baekkevold, *et al.*, 2003; Hayakawa *et al.*, 2009; Savinko, *et al.*, 2012) was also performed (Figure 3-18). Finally, pre-absorption studies using an excess of rhIL-33 (PeproTech®, UK) (Section 2.7.2) were also conducted (Figure 3-18). In isolation, these steps do not confirm specificity. However, when the evidence is combined we can be fairly sure that the antibody used in our investigations is specific for IL-33. In addition, our methodological approach for determining specificity is in agreement with the literature which suggests: (1) the use of appropriate diluent control, isotype control and pre-absorbed antibody in replace of primary antibody, (2) the use of sections of tissue with cells that are known to express the protein of interest and (3) the use of several antibodies that are directed against the same protein

(Burry, 2000, 2011). However, due to budget restriction, we only used one type of primary antibody in our study.

A specific and reliable IL-33 antibody was important to our immunohistocehmical analysis as it allowed us to quantify expression of IL-33 in tissues using the methodology of Bologna-Molina and colleagues (2011). This is a highly reproducible cell/nuclei counting technique that can be applied to both immunohistochemistry and immunocytochemistry, which uses only a digital camera attached to a microscope and a personal computer (Bologna-Molina *et al.*, 2008; Bologna-Molina *et al.*, 2009; Gonzalez-Ramirez *et al.*, 2011; Ramirez-Amador *et al.*, 2009). This quantification technique was employed as it is easy, cheap and highly reproducible. As an alternative, quantification can also be conducted using an advanced image scanner and image analysis software specifically designed for cell counting. However, we did not have access to the necessary equipment and in addition this automated method has been associated with issues of specificity and reproducibility (Persohn *et al.*, 2007).

The immunohistochemical analysis of periodontal tissues confirmed the findings at the mRNA level, showing that IL-33 protein levels are elevated in the epithelial and connective tissue layer of periodontal tissue from patients with chronic periodontitis. The tissue for this study was derived from patients undergoing surgery for chronic periodontitis and had clinical probing depths of ≥ 5.0 mm and clinical attachment loss of ≥ 5.0 mm. These data are similar to findings in other chronic inflammatory diseases, where upregulation of IL-33 was observed in tissues of patients with rheumatoid arthritis, inflammatory bowel diseases and chronic allergic conjunctivitis (Beltran, *et al.*, 2010; Matsuda, *et al.*, 2009; Xu, *et al.*, 2008). In addition, IL-33 levels were found to be upregulated in nasal epithelium of chronic allergic rhinitis patients, bronchial epithelium of asthma patients, colonocytes of ulcerative colitis patients, skin of systemic sclerosis patients and giant papillae of allergic conjunctivitis patients (Kamekura, *et al.*, 2012; Manetti, *et al.*, 2010; Matsuda, *et al.*, 2009; Prefontaine, *et al.*, 2009; Seidelin, *et al.*, 2010).

To confirm the findings for ST2 at the mRNA level a similar approach to that undertook for IL-33 was taken. In this instance immunohistochemical analysis of tissues was conducted using a rabbit anti-IL1RL1 (ST2) antibody (Sigma-Aldrich®,

UK). However, this antibody is only able to detect total ST2 and can not distinguish between sST2 and ST2L. In addition, the specificity of this antibody was not investigated in as greater detail as the IL-33 antibody. Firstly, an appropriate isotype control (rabbit IgG; Abcam®, UK) was used to exclude non-specific binding. Then, the tonsil tissues, which were known to contain ST2 expressing cells (Ciccia *et al.*, 2013) were used as a positive control. However, due to budget and time restrictions, we were unable to do pre-absorption studies. Therefore, in future studies, further steps to determine the specificity of this antibody in line with measures reported in the literature (Burry, 2000, 2011) need to be performed to confirm fully specificity. Therefore, we can only conclude that the specificity of this antibody had been partially confirmed. Nonetheless, the pattern of ST2 staining in periodontal tissue was comparable with ST2 expression in other epithelial tissues such as skin and corneal epithelium ("The human protein atlas (ST2)," 2013). The human protein atlas is a publicly available database of comprehensive antibody-based protein expression profiles of various normal and cancer tissues developed by certified pathologists (Uhlen *et al.*, 2005; Uhlen *et al.*, 2010). Interestingly, ST2 staining was abundant throughout periodontal tissue in both health and disease. This however made quantification by the methodology of Bologna-Molina and colleagues (2011) difficult. This abundance in staining may again be due to issues of antibody specificity. However, this abundance of ST2 staining has been observed in other epithelial tissues. For example, immunohistochemical analysis revealed an abundance of ST2 staining in epithelial tissue of skin, oral mucosa and cornea (Hueber*, et al.*, 2011; "The human protein atlas (ST2)," 2013; Lin*, et al.*, 2013; Meephansan *et al.*, 2013).

Despite the proposed abundance of ST2 staining in epithelial tissues, there are studies that report increased ST2 immunostaining in cells from the skin of psoriatic disease and colonic epithelial cells of ulcerative colitis (Manetti*, et al.*, 2010; Pastorelli*, et al.*, 2010). To our knowledge there are no antibodies commercially available to distinguish between the two isoforms of ST2 protein (ST2L and sST2). Since many studies, including our own, report that at the mRNA level; sST2 and not ST2L is upregulated in diseased tissue samples compared to healthy control, the availability of an antibody that can differentiate the two ST2 isoforms would benefit immunohistochemical investigations.

Our analysis of gingival tissue samples confirmed that IL-33 levels are upregulated in tissues of patients with chronic periodontitis. In addition, our mRNA data suggests that tissue levels of sST2, but not ST2L are upregulated in periodontal disease. IL-33 is well known to promote cellular immune responses by signalling through its membrane receptor ST2L. Formation of the IL-33/ST2L complex induces a conformational change in the extracellular domain of the ST2L that facilitates recruitment of IL-1RAcP (Schmitz, *et al.*, 2005). Together; IL-33, ST2 and IL-1RAcP form an active receptor complex and the TIR (toll/interleukin-1 receptor) domain of the receptor rapidly assembles the adaptor and kinase signaling proteins such as myeloid differentiation primary-response protein 88 (myD88), IL-1R-associated kinase 1 (IRAK1), IRAK4 and (TNF receptor-associated factor 6) (TRAF6) (Ali, *et al.*, 2007; Chackerian, *et al.*, 2007; Palmer, *et al.*, 2008). Studies have shown that IL-33 induced protein-kinase cascades can activate NF-κB, ERK1/2, p38 and JNK1 (Funakoshi-Tago, *et al.*, 2008; Iikura *et al.*, 2007; Kurowska-Stolarska, *et al.*, 2008; Pushparaj *et al.*, 2009; Schmitz, *et al.*, 2005) and eventually lead to the induction of expression of numerous inflammatory mediators. This IL-33 signalling was shown to be inhibited by the decoy receptor sST2 that competes with ST2L for IL-33 binding. *In vitro*, soluble ST2 directly bound to IL-33 and suppressed activation of NF-κB in a thymoma cell line stably expressing ST2L, suggesting that the complex of soluble ST2 and IL-33 fails to bind to ST2L (Hayakawa, *et al.*, 2007). *In vivo*, mice administered an intra-articular irritant (bovine serum albumin) exhibited dose dependent hypernociception, which was found to be IL-33 mediated. The response was inhibited by administration of sST2 (Verri *et al.*, 2008). Additionally, in a murine model of asthma, pre-treatment with sST2 reduced the expression of IL-4, IL-5, and IL-13 from IL-33-stimulated splenocytes (Hayakawa, *et al.*, 2007). These studies demonstrate the inhibitory effect of sST2 on IL-33 signalling. Hence, dysregulation of IL-33 and sST2 expression could contribute to pathogenesis of chronic inflammatory diseases. Indeed, IL-33 and sST2 were shown to be involved in the pathogenesis of chronic inflammatory diseases such as asthma, rheumatoid arthritis and inflammatory bowel diseases (Kakkar & Lee, 2008; Miller, 2011).

Since IL-33 and sST2 were suggested to be upregulated in the gingival tissue of chronic periodontitis patients, we needed to further explore the cell types

within the periodontium that contribute to their expression. In addition, we aimed to determine if initiating factors of periodontal disease, such as the periodontal pathogen *P. gingivalis*, could regulate expression of this cytokine and its receptors. The immunohistochemical analysis suggested that both IL-33 and ST2 (total) were expressed in the epithelial layer of the periodontium (Figure 3-3 and Figure 3-9). Epithelial cells, particularly within the sulcular/pocket epithelium, are located adjacent to the tooth surface which harbours the dental biofilm and represent the first line of defence against periodontal pathogens. It was therefore interesting to investigate whether these cells express IL-33 and ST2, and whether expression was modulated by the periodontal pathogen; *P. gingivalis*.

In this study we originally chose to use the OKF6/TERT-2 cell line. OKF6/TERT-2 cells were a kind gift from the Rheinwald laboratory (Brigham and Women's Hospital, Boston). The cells were originated from keratinocyte cells of the oral mucosa, which have been immortalized by forced ectopic expression of the telomerase catalytic subunit, hTERT (Dickson*, et al.*, 2000). The cells resemble primary oral keratinocyte cells and are regarded as a valuable and reproducible model for normal oral epithelial cell studies (Dongari-Bagtzoglou & Kashleva, 2006). We chose to use a cell line in the first instance due to the fact that the OKF6/TERT-2 cells were readily available at the time. In addition, these cells are easy to maintain *in vitro* and are well characterised. The fact that these cells were immortal was advantageous as it provided a constant supply of cells which was required for method optimisation at the beginning of the study. However, after initial studies using a cell line we confirmed our findings using commercially available primary human gingival epithelial cells (PHGE) (CELLnTEC advanced cell systems, Switzerland). These cells were isolated from healthy gingiva and are pooled from at least 3 healthy donors.

For stimulation studies we used the periodontal pathogen *P. gingivalis* (ATTCC 33277 strain). *P. gingivalis* was chosen as this species was shown to highly associate with periodontal diseases (Bostanci & Belibasakis, 2012). In addition, *P. gingivalis* is also known for one of the most studied periodontal pathogens (Wang *et al.*, 2013). The ATTCC 33277 strain was used in this study as it has been widely used by other laboratories and associates with periodontal diseases (Guyodo *et al.*, 2012; Naito *et al.*, 2008). *P. gingivalis* has been used extensively

for *in vitro* cell stimulation studies. However, researchers use a variety of different strains, live or dead forms, planktonic or biofilm cultured forms and a plethora of specific antigens such as LPS, fimbriae and bacterial DNA. Research has shown that these variables can have differential effects on cell responses *in vitro*. Indeed, human and mouse macrophages stimulated with different forms of *P. gingivalis* including the live organism, LPS or fimbriae were found to exhibit differential patterns of gene expression (Yu *et al.*, 2010; Zhou & Amar, 2006; Zhou, *et al.*, 2005). The findings indicate host immune cells sense live *P. gingivalis* and its components differently, which translate into the expression of different inflammatory cytokine profiles. *In vitro*, *P. gingivalis* exists in a biofilm containing both live and dead bacteria as well as a plethora of specific antigens (LPS, fimbriae and bacteria DNA). Therefore, to model the *in vivo* situation *in vitro*, culturing organisms as a biofilm for stimulation studies may be more appropriate. Indeed, in chronic inflammatory conditions such as chronic wound infection, dermatitis and periodontal disease the majority of organisms are found present in biofilm form (Bjarnsholt *et al.*, 2008; Pihlstrom, *et al.*, 2005; Vlassova *et al.*, 2011). Furthermore, gene expression profile analysis has showed difference in the gene expression profiles of bacteria grown planktonically and bacteria grown as a biofilm; including genes encoding antigens known to activate immune responses (Southey-Pillig *et al.*, 2005; Waite *et al.*, 2005). In addition, studies on the acellular filtrate of planktonic verses biofilm cultured *Staphylococcus aureus* showed that the acellular filtrate from the biofilm cultured organisms had stronger cytokine stimulatory activity on leukocytes than that from planktonic cultured organisms (Sadowska *et al.*, 2013). Monospecies biofilms of *F. nucleatum, P. gingivalis* and *Actinomyces naeslundii* also induced distinct patterns of cytokine expression in oral keratinocytes when compared to their respective planktonic bacteria species (Peyyala *et al.*, 2012). Therefore it was decided in our studies that in order to mimic the *in vivo* situation more accurately a live *P. gingivalis* monospecies biofilm was to be used in stimulation studies. In addition, to further mimic the *in vivo* situation we used a live *P. gingivalis* monospecies biofilm model in which oral epithelial cells were stimulated with the biofilm attached to a cell culture insert suspended 0.5 mm above the cells (Sherry, *et al.*, 2013). This mimics the relationship between the dental biofilm and epithelium of the gingival crevice/pocket which is separated by GCF. In this model, some of the bacteria were observed to detach and fall

from the biofilm to directly contact the oral epithelial cells, which further resembles the events in the gingival sulcus/pocket epithelium *in vivo*.

The data presented in this manuscript shows for the first time that IL-33 is expressed by oral epithelial cells at both the mRNA and protein level. This is in line with literature that shows IL-33 is expressed by various other epithelial cells of the body, including bronchial, colon, nasal, skin and giant papillae (Kamekura, *et al.*, 2012; Manetti, *et al.*, 2010; Matsuda, *et al.*, 2009; Prefontaine, *et al.*, 2009; Seidelin, *et al.*, 2010). In addition, the data demonstrates for the first time significant upregulation of IL-33 mRNA expression by oral epithelial cells in response to the periodontal pathogen; *P. gingivalis*. This is in agreement with literature that shows stimulation of TLRs such as TLR-2, TLR-3, TLR-4 and TLR-5 by their respective agonist increases expression of IL-33 in corneal epithelial cells, bronchial epithelial cells and sinunasal epithelial cells, as well as monocytes (Reh, *et al.*, 2010; Willart, *et al.*, 2012; Zhang, *et al.*, 2011a). In addition, it has specifically been shown that LPS of *P. gingivalis* can induce upregulated IL-33 expression in human monocytes (Nile, *et al.*, 2010).

Although *P. gingivalis* induced increased expression of IL-33 mRNA in oral epithelial cells we found that IL-33 was not released into bathing supernatants using the *in vitro* model system. As the same ELISA kit was used in this investigation as was used for analysis of clinical samples this finding could be explained by the inability of the commercial kit to accurately measure IL-33 levels as discussed previously. However, a similar phenomenon has been reported in the literature. In monocytes, *P. gingivalis* induced elevated intracellular expression of IL-33, however the protein was not released from cells unless subjected to necrosis (Nile, *et al.*, 2010). In addition, cells isolated from rheumatoid arthritis patients, including fibroblast-like synoviocytes and PBMCs were found not to release IL-33 after stimulation with combinations of IL-1ß, TNF-α, and anti-CD3/CD28 respectively (Matsuyama, *et al.*, 2010). Likewise, TNF-α stimulated intestinal epithelial cells showed increased intracellular IL-33 expression but no extracellular release was detected (Pastorelli, *et al.*, 2010). In contrast to these findings, however, *in vitro* studies using rat cardiac fibroblasts, mixed glial cell and astrocyte-enriched cultures and human macrophages have been shown to secrete IL-33 upon stimulation with combinations of LPS and PMA,

PMA only, ATP, and combinations of LPS and aluminium hydroxide adjuvant (alum) (Hudson, *et al.*, 2008; Li, *et al.*, 2008; Sanada, *et al.*, 2007; Talabot-Ayer, *et al.*, 2009). Literature also shows that IL-33 is released by cells into the extracellular environment when physically injured or subjected to mechanical stress (Cayrol & Girard, 2009; Kakkar *et al.*, 2012). In addition, IL-33 release was found to be ATP dependant in bronchial epithelial cells and corneal epithelial cells (Kakkar, *et al.*, 2012; Kouzaki *et al.*, 2011; Zhang, *et al.*, 2011a). Based on these findings, therefore, a limitation of our study was that potential mechanisms of IL-33 release from oral epithelial cells were not investigated further.

To determine if IL-33 protein mirrored mRNA and was upregulated intracellularly by stimulated oral epithelial cells; an immunocytochemical approach was undertaken. This once again employed the specific mouse monoclonal anti-IL-33 antibody (Nessy-1, Enzo® Life Science, UK) and the quantitative approach of Bologna-Molina and colleagues (2011). This analysis confirmed the findings at the mRNA level and showed that elevated IL-33 protein expression occurs intracellularly in oral epithelial cells stimulated with *P. gingivalis*. This finding casts doubts on the hypothesis that IL-33 is released from epithelial cells in response simply to stimulation with whole pathogens or associated antigens. However, further research investigating IL-33 release mechansisms by oral epithelial cells is required to confirm this.

In addition to IL-33, expression of ST2 by oral epithelial cells was also investigated *in vitro*. Oral epithelial cells were found to express mRNA encoding both ST2L and sST2. This is unsurprising given the fact that numerous other epithelial cells of the colonic mucosa, cornea and lung (Beltran, *et al.*, 2010; Lin, *et al.*, 2013; Yagami, *et al.*, 2010) as well as keratinocytes of skin (Hueber, *et al.*, 2011) have all been demonstrated to express these receptors. These studies also demonstrate that expression of sST2 and not ST2L mRNA is upregulated in OKF6/TERT-2 cells that come into contact with the periodontal pathogen; *P. gingivalis*. Indeed, in other cell types expression of sST2 has been found to be inducible whilst ST2L is found to be constitutively expressed. In murine alveolar macrophages, stimulation with LPS or pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) has been shown to induce increased expression of sST2 mRNA but not ST2L mRNA (Oshikawa *et al.*, 2002). In addition,

stimulation of alveolar epithelial cells with a combination of IL-1β, TNF-α and IL-4 and stimulation of intestinal epithelial cells with TNF-α upregulated sST2 protein release with no effect on ST2L (Pastorelli, *et al.*, 2010; Tajima *et al.*, 2007).

Although we showed increased expression of sST2 mRNA in oral keratinocytes in response to *P. gingivalis*, we found that *P. gingivalis* does not induce increased release of sST2 into bathing supernatant as measured by ELISA. Interestingly, Mildner and colleagues (2010) showed LPS did not induce increased expression of sST2 by PBMCs. This was despite the fact that in response to LPS, PBMCs expressed pro-inflammatory cytokines including IL-1α, IL-1β, IL-6 and TNF-α which were expected to act in an autocrine manner and subsequently induce sST2 expression. It is therefore possible that *P. gingivalis* does not induce increased release of sST2 by oral keratinocytes. Again, this would be despite the fact that *P. gingivalis* can induce release of IL-1α, IL-1β and TNF-α from oral keratinocytes (Eskan *et al.*, 2008a; Kraus *et al.*, 2012; Peyyala *et al.*, 2013). However, at present the reason for these sST2 ELISA results in our study are not known. sST2 was measured in bathing supernatants at levels of between 10 – 80 pg/ml; which is above the lowest detection limit (5.6 pg/ml) of the ELISA. This is in line with previous finding that show spontaneous sST2 secretion occurs in lung epithelial cells (Mildner, *et al.*, 2010). However, no upregulation of sST2 protein release was measured in samples stimulated by *P. gingivalis* despite the findings at the mRNA level. There is also possibility that sST2 is bound to IL-33, hence masking the sST2 epitopes and hindering ELISA analysis of bathing supernatants as previous for the analysis of biological samples. The shortcoming in our study was that we did not use suitable positive control samples to assess the efficiency of the ELISA kit used. Hence, if given time and budget our plan was to confirm the findings by evaluating the efficiency of ELISA further or by measuring sST2 levels in bathing supernatants by alternative means such as western blot. Instead, we investigated expression of ST2 (total) in oral keratinocytes by immunohistochemical analysis. This revealed no observable differences in total ST2 expression between unstimulated and *P. gingivalis* stimulated OKF6/TERT-2 cells and PHGE cells.

Interestingly, although levels of sST2 mRNA were upregulated by OKF6/TERT-2 cells in response to *P. gingivalis*, a similar phenomenon was not observed in

PHGE cells. In addition, in contrast to OKF6/TERT-2 cells, IL-33 mRNA levels did not remain significantly upregulated after 24 h stimulation with *P. gingivalis* in PHGE cells. The difference in expression pattern may relate to differences between the use of a cell line and primary cells. Indeed, studies have shown there is both a qualitative and quantitative expression profile differences between cell lines and primary cells (Pan *et al.*, 2009). In addition, the basal layer of the oral epithelium is known to consist of a heterogeneous mixture of primary cells with varying proliferation capacity (Jones & Klein, 2013). This *in vitro* phenotypic-distinct subpopulation of primary cells was also observed in retina pigment epithelium (Chaloner-Larsson, *et al.*, 1997). Therefore, there could be phenotypical differences between the epithelial cell population from which OKF6/TERT-2 cells are derived and the population of epithelial cells that predominate the PHGE cells (CELLnTEC advanced cell systems, Switzerland). This therefore may explain the differences in regulation of IL-33 expression.

Evidence from our *in vitro* studies thus far suggested that oral keratinocytes express the transmembrane form of the IL-33 receptor (ST2L) and therefore can respond to IL-33 signalling. Therefore, this was investigated further *in vitro* using rhIL-33 (PeproTech®, UK). However, in our investigations rhIL-33 did not induce OKF6/TERT-2 cells to upregulate the expression of a panel of cytokines and chemokines as well as TLR-2 and TLR-4. This was in contrast to literature describing studies performed on other epithelial cells as well as macrophages (Table 3-4).

Cells	Stimulation (Concentration)	Stimulation time	Increased expression	References
NHBE	IL-33 (10 & 50ng/ml)	24h	IL-8	Yagami *et al* (2010)
NHBE BEAS-2A	IL-33 (100ng/ml)	12, 24 & 48h	IL-17F	Fujita *et al* (2012)
HNEC	IL-33 (100ng/ml)	6h	IL-8 & GM-CSF	Kamekura *et al* (2012)
NHEK	IL-33 (100ng/ml)	48h	IL-8	Meephanson *et al* (2012)
HaCaT	IL-33 (100ng/ml) & TNF-α (20ng/ml)	24h	IL-6, VEGF, MCP-1 & IL-20	Balato *et al* (2012)
HaCaT	IL-33 (100ng/ml) & IL-17A (10ng/ml)	24h	IL-20 & VEGF	Balato *et al* (2012)
HMVEC	IL-33 10 & 50ng/ml	24h	IL-6, IL-8 & MCP-1	Yagami *et al* (2010)
HMC-1	IL-33 (100ng/ml)	24h	VEGF & MCP-1	Balato *et al* (2012)
Macrophages	IL-33 (50ng/ml)	6, 8 & 10h	MD-2 & TLR-4	Espinassous *et al* (2009)
Macrophages	IL-33 (20ng/ml) & IL-13 (10ng/ml)	48h	CCL17 & CCL24	Kurowska-Stolarska *et al* (2009)

Table 3-4: Effect of IL-33 on cells

NHBE = normal human bronchial epithelial, BEAS-2A = bronchial epithelial cell line, HNEC = human nasal epithelial cells, NHEK = normal human epidermal keratinocytes, HaCaT = immortal human keratinocyte cell line, HMVEC = human microvascular endothelial cells, TNF-α = tumour necrosis factors-α, GM-CSF = granulocyte-macrophage colony-stimulating factor, VEGF = vascular endothelial growth factor, MCP-1 = monocyte chemoattractant protein-1, MD-2 = myeloid differentiation protein 2, and TLR-4 = toll-like receptor 4.

One explanation for the findings in this thesis may have been the concentration of rhIL-33 used in our studies. The rhIL-33 (PeproTech®, UK) exhibited bioactivity at 50 and 100 ng/ml in a T cell stimulation assay; where IL-5 was measured as an output. However, we observed no response of OKF6/TERT-2 cells to 10, 50 and 100 ng/ml rhIL-33, despite the fact that studies showed the membrane bound receptor (ST2L) is expressed by these cells. Studies have shown that expression of ST2L is most prevalent in hematopoietic cells, such as T cells and mast cell, compared to other cell types such as epithelial cells and fibroblasts (Bergers, *et al.*, 1994; Gachter *et al.*, 1996). Therefore, although 100 ng/ml is an effective concentration for activating T-cells, it may not be sufficient to elicit a response in cells where active membrane associated ST2L is less abundant such as

OKF6/TERT-2 cells. Therefore, it may be pertinent to repeat these experiments using a higher concentration of rhIL-33. The literature shows that *in vitro* studies with IL-33 have utilised a variety of concentrations and stimulation times to induce expression of mediators in a variety of cell types (Table 3-4). This variation could be due to the cell type and culture conditions, the source of rhIL-33 and the sample preparation for analysis of phenotypic changes. In our studies we explored a limited number of concentrations of rhIL-33 and stimulation times. Therefore, it may be pertinent to repeat these experiments using different concentrations and sources of rhIL-33 as well as variations in simulation time and culture conditions. Indeed, the proteome profiler analysis was only performed on the 24h stimulated samples (100 ng/ml only), which means earlier events may have been missed. Furthermore, although the profiler analyses a variety of mediators, it is possible that the right panel of cytokines and chemokines were not investigated in our studies. Finally, the recruitment of the IL-1RAcP to the IL-33/ST2L is important for mediation of IL-133 signalling (Ali*, et al.*, 2007; Chackerian*, et al.*, 2007; Palmer*, et al.*, 2008; Schmitz*, et al.*, 2005). Although, IL-1RAcP was found to be constitutively expressed in epithelial cells such as the A549 cell line and primary bronchial epithelial cells (Coulter *et al.*, 1999) there is no evidence to confirm its expression in oral keratinocytes. Therefore we should have investigated IL-1RAcP expression in oral keratinocytes as this may explain why in our *in vitro* system we saw no genotypic or phenotypic changes in oral keratinocytes stimulated with rhIL-33.

In conclusion, the data in this chapter support a role for IL-33 in the pathogenesis of periodontal disease. In addition, it shows that oral keratinocytes express IL-33 and that the periodontal pathogen, *P. gingivalis*, can induce increased intracellular expression of this cytokine. Furthermore, oral keratinocytes also express the receptor for IL-33 suggesting that mechanisms of autocrine and paracrine IL-33 signalling occur within the periodontium. However, at present the exact means by which IL-33 is released from cells, its intracellular role in oral keratinocytes and its overall contribution to the early oral mucosal immune responses to periodontal pathogens remain to be elucidated.

Chapter 4: IL-17 family cytokines and periodontal disease

4.1 Introduction

The IL-17 family cytokines consists of six members (IL-17A - IL-17F) (Gaffen, 2009b). IL-17A is the founding member and has been associated with periodontal disease pathogenesis (Buduneli, *et al.*, 2009; Correa, *et al.*, 2012; Duarte, *et al.*, 2010; Gumus, *et al.*, 2013; Honda, *et al.*, 2008; Kadkhodazadeh, *et al.*, 2013; Lester *et al.*, 2007; Ozcaka, *et al.*, 2013; Ozcaka *et al.*, 2011; Saraiva *et al.*, 2013; Schenkein, *et al.*, 2010; Silva *et al.*, 2012; Vernal, *et al.*, 2005). *In vivo* models have suggested that IL-17A plays a protective role in periodontal disease. Indeed, IL-17RA deficient mice show exacerbated bone loss in a *P. gingivalis* induced model of periodontal disease (Yu, *et al.*, 2007). However, an excessive IL-17A response promotes inflammatory alveolar bone loss in an ageing mouse model of periodontal disease (Eskan, *et al.*, 2012). Indeed, IL-17A signalling activates NF-κB which in turn induces expression of a variety of pro-inflammatory mediators (Koenders *et al.*, 2005). Furthermore, IL-17A can act in synergy with TNF-α and IL-1ß and augment the expression of pro-inflammatory mediators from keratinocytes and fibroblasts (Iyoda *et al.*, 2010; Koenders *et al.*, 2011). In addition, IL-17A can modulate bone remodelling through induction of RANKL both *in vivo* and *in vitro* (Kotake *et al.*, 1999; Kotake *et al.*, 2012).

Like IL-17A, IL-17F can be secreted as a homodimer. However a heterodimeric species consisting of a disulphide linked IL-17A and IL-17F molecule can also be formed (Wright, *et al.*, 2007). Similarly to IL-17A, both the IL-17F homodimer and IL-17A/F heterodimer have pro-inflammatory properties (Fujie *et al.*, 2012; Liang, *et al.*, 2006). Indeed, elevated levels of IL-17F and IL-17A/F have been associated with chronic inflammatory diseases such as rheumatoid arthritis (Kotake, *et al.*, 1999). However, the role of IL-17F and IL-17A/F in the pathogenesis of periodontal disease is currently unknown.

In contrast to IL-17A and IL-17F, the biology of IL-17B, IL-17C and IL-17D are poorly understood at present and their cellular sources have still to be fully elucidated. Levels of IL-17B, IL-17C and IL-17D are elevated in inflammation driven pathologies such as rheumatoid arthritis; and *in vivo* IL-17B, IL-17C and IL-17D can induce pro-inflammatory cytokine release from a variety of epithelial and myeloid cells (Pappu *et al.*, 2010). In relation to the periodontium, IL-17C has been demonstrated to be expressed by oral epithelial cells and is known to

upregulate expression of a number of pro-inflammatory mediators (Ramirez-Carrozzi, *et al.*, 2011). However, little is known about the expression and functions of IL-17B, and IL-17D. Indeed, in terms of periodontal disease pathogenesis the roles of IL-17B, IL-17C and IL-17D remain to be elucidated.

IL-17E has been described as a 'double edged sword' and has been shown to down-regulate localised destructive inflammation and promote Th2 driven pathologies (Monteleone *et al.*, 2010). Indeed, IL-17E plays a major role in the clearance of parasitic infections (Fallon *et al.*, 2006) and the pathogenesis of allergic disease (Angkasekwinai, *et al.*, 2007; Ballantyne *et al.*, 2007). IL-17E induces activation and differentiation of Th2 cells and regulates their recruitment to sites of inflammation (Tamachi *et al.*, 2006; Wang, *et al.*, 2007b). IL-17E also regulates Th9 cells (Angkasekwinai, *et al.*, 2010) and activates multi-potent progenitor type 2 cells (MPP^{type2}) (Saenz, *et al.*, 2010b), innate type 2 helper cells (Ih2) (Price, *et al.*, 2010), natural helper cells (NHCs) (Moro, *et al.*, 2010) and neuocytes (Neill, *et al.*, 2010). In contrast, IL-17E down-regulates Th1/Th17 and IL-17A responses (Emamaullee *et al.*, 2009; Kleinschek, *et al.*, 2007; Owyang, *et al.*, 2006) and can directly inhibit toll-like receptor ligand driven expression of pro-inflammatory cytokines via p38 MAP kinase-driven SOCS3 activation in human blood monocytes and intestinal $CD14^+$ cells (Caruso, *et al.*, 2009a; Caruso, *et al.*, 2009b). However, despite extensive literature on the immunological functions of IL-17E, to date very little research has been undertaken to determine whether it plays a role in the pathogenesis of periodontal disease.

IL-10 is a prototypic anti-inflammatory cytokine. In periodontal disease IL-10 was shown to play an anti-inflammatory role as evidenced by the data from *in vitro*, *in vivo* and clinical studies (Al-Rasheed, *et al.*, 2003; Sasaki, *et al.*, 2004). In other chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease the role of IL-10 as an anti-inflammatory cytokine has been confirmed (Finnegan, *et al.*, 2003; Kuhn, *et al.*, 1993; Murai, *et al.*, 2009; Tanaka, *et al.*, 1996). As IL-10 is a well characterised anti-inflammatory cytokine, the relationship between fluid levels of IL-17A and IL-10 was investigated and similarities between the IL-17A and IL-17E relationship determined to provide more evidence for an anti-inflammatory role of IL-17E in periodontitis pathogenesis.

The specific aims of this study were to:

1. Determine levels of IL-17 family cytokines and IL-10 in clinical samples and correlate levels with both clinical and demographic parameters.

2. Determine levels of IL-17 family cytokines and IL-10 mRNA expression in gingival tissue of patients with chronic periodontitis and healthy volunteer control.

4.2 Results

4.2.1 Clinical and demographic parameters of subject participants

Serum, gingival crevicular fluid (GCF) and saliva samples from 77 healthy subjects and 97 chronic periodontitis patients were used in this study. The clinical and demographic parameters of the subjects/patients cohort are described in Section 3.2.1.1.

4.2.2 Serum levels of IL-17 family cytokines

Serum levels of IL-17 family cytokines were measured by ELISA. Table 4-1 shows serum levels of IL-17A and IL-17A/F were significantly increased ($p < 0.001$) in the patients with chronic periodontitis (47.5 and 31.4 pg/ml respectively), compared to healthy subjects (15.5 and 8.0 pg/ml respectively). In contrast, the serum level of IL-17E was significantly decreased ($p = 0.007$) in the chronic periodontitis patients (17.2 pg/ml) compared to healthy subjects (35.3 pg/ml). Serum levels of the other members of IL-17 family cytokines: IL-17B, IL-17C, IL-17D and IL-17F were not significantly different between the two groups. The serum IL-17A:IL-17E ratio was also significantly increased ($P < 0.001$) in patients with chronic periodontitis compared to healthy subjects.

	Status	n	Median (pg/ml)	IQR	Z statistic	*p* value
IL-17A	Healthy	77	15.5	5.3 – 30.1	-7.568	< 0.001*
	CP	97	47.5	38.7 – 98.0		
IL-17B	Healthy	40	38.8	30.8 – 48.1	-0.257	0.798
	CP	55	33.8	18.2 – 59.3		
IL-17C	Healthy	40	40.9	2.9 – 49.8	-1.581	0.114
	CP	55	31.6	12.0 – 51.7		
IL-17D	Healthy	38	22.1	20.1 – 30.6	-0.785	0.432
	CP	41	23.8	21.0 – 28.8		
IL-17E	Healthy	77	35.3	23.1 – 44.0	-2.676	0.007*
	CP	97	17.2	15.9 – 50.3		
IL-17F	Healthy	77	14.3	4.0 – 32.2	-0.106	0.916
	CP	97	14.2	7.2 – 20.0		
IL-17A/F	Healthy	77	8.0	0.0 – 26.0	-6.950	< 0.001*
	CP	97	31.4	24.8 – 53.7		
IL-17A: IL-17E	Healthy	77	0.5$^{\#}$	0.1 – 1.0	-6.867	< 0.001*
	CP	97	2.6$^{\#}$	0.8 – 4.5		

Table 4-1: Levels of IL-17 family cytokines and the IL-17A:IL-17E ratio in serum

IL-17 family cytokines were measured in serum samples using the appropriate ELISA kit (Table 2-3). The table represents median levels of IL-17 family cytokines and the IL-17A:IL-17E ratio in serum of the healthy subjects and chronic periodontitis (CP) patients. Statistical analyses were carried out using the Mann-Whitney test with a Bonferroni correction (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects; IQR = interquartile range; IL-17A:IL-17E = the serum ratio of IL-17A to IL-17E and $^{\#}$ = ratio (no units).

4.2.3 Correlations between serum levels of IL-17 family cytokines and clinical parameters

The serum IL-17 family data was seen to approximate a normal distribution following natural log transformations. Therefore, correlations were carried out using bivariate analysis using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19).

Table 4-2 shows the serum level of IL-17A significantly positively correlated with all clinical parameters measured: CPD (r = +0.429, p < 0.001), CAL (r = +0.464, p < 0.001) and BOP (r = +0.331, p < 0.001). Serum level of IL-17A/F also significantly positively correlated with all clinical parameters measured: CPD (r = +0.544, p < 0.001), CAL (r = +0.436, p < 0.001) and BOP (r = +0.375, p < 0.001). In contrast, the serum level of IL-17E was found to significantly negatively

correlate with CPD (r = -0.265, p < 0.001) and CAL (r = -0.291, p < 0.001). Serum levels of the other members of the IL-17 family (IL-17B, IL-17C, IL-17D and IL-17F) were found not to significantly correlate with any of the clinical parameters. As expected, all the clinical parameters measured significantly positively correlated to each other (r > +0.700, p < 0.001).

		CPD	**CAL**	**BOP**
IL-17A	n	174	174	174
	r	+0.429	+0.464	+0.331
	p	<0.001*****	<0.001*****	<0.001*****
IL-17B	n	97	97	97
	r	-0.005	-0.048	-0.002
	p	0.958	0.641	0.986
IL-17C	n	97	97	97
	r	-0.024	-0.054	+0.135
	p	0.817	0.598	0.237
IL-17D	n	79	79	79
	r	+0.135	+0.117	+0.168
	p	0.237	0.307	0.139
IL-17E	n	174	174	174
	r	-0.265	-0.291	-0.060
	p	<0.001*****	<0.001*****	0.433
IL-17F	n	169	169	169
	r	-0.025	-0.002	+0.024
	p	0.746	0.978	0.753
IL-17A/F	n	169	169	169
	r	+0.544	+0.436	+0.375
	p	<0.001*****	<0.001*****	<0.001*****
BOP	n	203	203	
	r	+0.796	+0.743	
	p	<0.001*****	<0.001*****	
CAL	n	203		
	r	+0.876		
	p	<0.001*****		

Table 4-2: Correlation between serum levels of IL-17 family cytokines and clinical parameters

The table represents the correlations between serum levels of IL-17 family cytokines and clinical parameters for periodontal disease (CPD, CAL and BOP). The serum level values were subjected to a natural log transformation. The correlations were analysed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Significance = p<0.05 (*). n = number of subjects; r = the Pearson correlation coefficient; CPD = clinical probing depth; CAL = clinical attachment loss and BOP = bleeding on probing.

4.2.4 Correlations between serum levels of IL-17 cytokine family members

In addition to associations between circulating levels of IL-17 family cytokines and clinical parameters; associations between IL-17 family members were also determined. Once again these associations were determined using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19).

Table 4-3 shows that serum level of IL-17A positively correlated with serum level of IL-17A/F (r = +0.600, p < 0.001). The serum level of IL-17A was also found to positively correlate with the serum level of IL-17F (r = +0.172, p = 0.026) although this correlation was weak. The serum level of IL-17C was positively correlated with the serum levels of IL-17A (r = +0.201, p = 0.048) and IL-17F (r = +0.326, p = 0.001). In addition, a weak but significant positive correlation was also found between serum levels of IL-17A/F and IL-17B (r = +0.201, p = 0.049). Interestingly, the serum level of IL-17A significantly negatively correlated with the serum level of IL-17E (r = -0.227, p = 0.003). There was no correlation between serum levels of IL-17F and IL-17A/F.

		IL-17A/F	IL-17F	IL-17E	IL-17D	IL-17C	IL-17B
IL-17A	n	168	168	173	79	97	97
	r	+0.600	+0.172	-0.227	+0.092	+0.201	+0.152
	p	<0.001*	0.026*	0.003*	0.419	0.048*	0.128
IL-17B	n	96	96	97	79	97	
	r	+0.201	+0.089	-0.031	+0.012	+0.167	
	p	0.049*	0.387	0.767	0.912	0.107	
IL-17C	n	96	96	97	79		
	r	+0.008	+0.326	+0.036	-0.050		
	p	0.936	0.001*	0.725	0.660		
IL-17D	n	79	79	79			
	r	+0.031	+0.138	+0.045			
	p	0.789	0.226	0.693			
IL-17E	n	169	169				
	r	+0.131	-0.062				
	p	0.090	0.420				
IL-17F	n	169					
	r	+0.057					
	p	0.464					

Table 4-3: Correlations between serum levels of IL-17 family cytokines

The table represents correlations between serum levels of each IL-17 family cytokine. The serum level values were subjected to a natural log transformation. The correlations were analysed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects and *r* = the Pearson correlation coefficient.

4.2.5 Correlations between serum IL-17A:IL-17E ratio and clinical parameters

The literature suggests that IL-17A and IL-17E have opposing roles in the inflammatory response. The serum analysis demonstrated a significant negative correlation between circulating levels of IL-17A and IL-17E in peridontitis (table 4-3). Therefore it was hypothesised that the circulating ratio of IL-17A:IL-17E could be a determinant of periodontal disease. Therefore, associations between individuals serum IL-17A:IL-17E ratio and clinical parameters were determined. Associations were determined using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Serum IL-17A:IL-17E ratios were subjected to natural log transformations to facilitate graphical representation.

Figure 4-1 shows that the serum IL-17A:IL-17E ratio has a weak but significant positive correlation with CPD (r = +0.335, p < 0.001) and BOP (r = +0.286, p < 0.001). In addition, the serum IL-17A:IL-17E ratio has a significant moderate positive correlation with CAL (r = +0.480, p < 0.001).

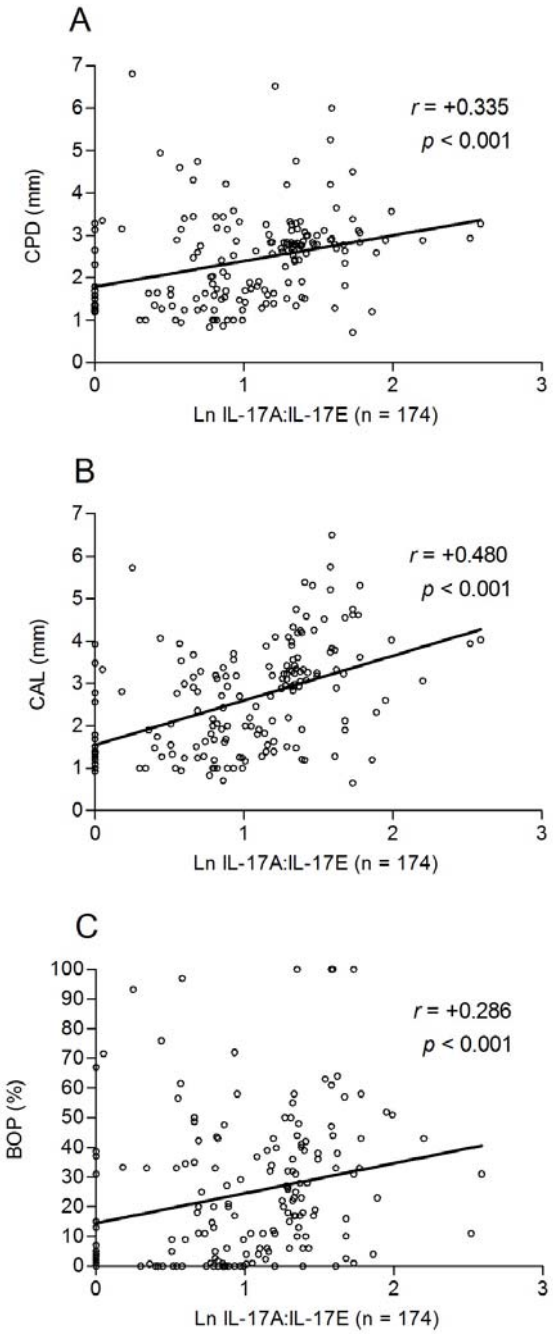

Figure 4-1: Correlations between the serum IL-17A:IL17E ratio and clinical parameters

The serum IL-17A:IL-17E ratio of all subjects (regardless of disease state) was calculated. To facilitate graphical representation the IL-17A:IL-17E ratio's were subjected to a natural log transformation (Ln). Correlation analysis against CPD (**A**), CAL (**B**) and BOP (**C**) was then performed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Significance = p<0.05. r = the Pearson correlation coefficient.

4.2.6 Correlations between serum levels of IL-17 family cytokines and age

Immunosenescence is a term that refers to the gradual deterioration of the immune system due to increase in age. Development and maintenance of peripheral immune responses are postulated to be affected by aging (Miller, 1991). Evidence also shows that immunosenescence could play a role in the pathogenesis of periodontal disease (Rajendran *et al.*, 2013).

Since there was a large spread of subjects in terms of age enrolled in our study (22 – 55 years) we determined the association between serum levels of IL-17 family cytokines and age. Once again, correlations were determined using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19).

Table 4-4 shows serum levels of IL-17A and IL-17A/F positively correlated with age (r = +0.338, p < 0.001; and r = +0.598, p < 0.001 respectively) regardless of disease state. Interestingly, although the serum IL-17A:IL-17E ratio was also found to significantly positively correlate with age (r = +0.259, p = 0.001), the correlations are found not significant after correction for disease (clinical probing depth).

		IL-17A	IL-17B	IL-17C	IL-17D	IL-17E	IL-17F	IL-17A/F	IL-17A: IL-17E
Age	n	174	97	97	79	174	169	169	172
	r	+0.338	+0.157	-0.092	+0.107	+0.031	-0.080	+0.598	+0.259
	p	<0.001*****	0.124	0.372	0.347	0.684	0.303	<0.001*****	0.001*****
	$r_{partial}$	+0.160	+0.173	+0.179	-0.142	+0.001	+0.109	+0.381	+0.142
	p	0.036*****	0.092	0.081	0.217	0.994	0.085	<0.001*****	0.064

Table 4-4: Correlations between serum levels of IL-17 family cytokines and age

The table represents correlations between levels of IL-17 family cytokines and age. The values were subjected to a natural log transformation. The correlations were analysed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Adjustment for disease severity was carried out using the Partial correlation test controlling for clinical probing depth (CPD) (IBM SPSS Statistics, version 19). Significance = p<0.05 (*). n = number of subjects; r = the Pearson correlation coefficient; $r_{partial}$ = the Partial correlation and IL-17A:IL-17E = the serum ratio of IL-17A to IL-17E.

4.2.7 Relationship between serum levels of IL-17 family cytokines and gender

Incidence of humoral and cell mediated autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and systemic erythematosus were found to be higher in females compared to males (Beeson, 1994; Whitacre *et al.*, 1999), indicating possible differences in immune regulation between genders. However, epidemiological studies have demonstrated a higher prevalence and severity of periodontal disease in men than women (Shiau & Reynolds, 2010).

Since our study involved both male and female subjects, we compared the serum levels of IL-17 family cytokines between genders. Our Q-Q plot analysis showed the data was not normally distributed, and hence statistical analysis was carried out using the Mann-Whitney test with a Bonferroni correction (IBM SPSS Statistics, version 19).

Table 4-5 shows analysis demonstrating that serum levels of IL-17 family cytokines were found not to be significantly different between males and females.

	Gender	n	Median (pg/ml)	IQR	Z statistic	*p* value
IL-17A	Male	72	42.20	13.40 – 50.30	-2.354	0.133
	Female	102	37.65	14.53 – 47.15		
IL-17B	Male	42	39.70	25.00 – 57.10	-2.141	0.224
	Female	55	31.00	16.83 – 42.93		
IL-17C	Male	42	31.70	9.80 – 50.90	-0.284	1.000
	Female	55	39.05	16.20 – 50.75		
IL-17D	Male	35	24.30	20.40 – 31.10	-1.032	1.000
	Female	44	23.05	20.55 – 25.15		
IL-17E	Male	73	16.90	15.90 – 21.10	-0.994	1.000
	Female	101	17.00	16.00 – 34.43		
IL-17F	Male	69	14.80	13.20 – 20.10	-0.383	1.000
	Female	100	15.60	13.75 – 26.70		
IL-17A/F	Male	69	23.10	0.00 – 26.50	-0.210	1.000
	Female	100	23.00	8.00 – 25.50		

Table 4-5: Comparison of serum levels of IL-17 family cytokines between males and females

The table represents median levels of IL-17 family cytokines in the serum of male and female subjects. Statistical analyses were carried out using the Mann-Whitney test with a Bonferroni correction (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects; and IQR = interquartile range.

4.2.8 Gingival crevicular fluid levels of IL-17A, IL-17E, IL-17F and IL-17A/F

GCF levels of IL-17 family cytokines were measured by ELISA. Due to sample limitations, only GCF levels of IL-17A, IL-17E, IL-17F and IL-17A/F were measured. Table 4-6 shows GCF levels of IL-17A, IL-17F and IL-17A/F were significantly increased in the patients with chronic periodontitis (94.9 pg/ml, 6.4 pg/ml and 19.0 pg/ml respectively) as compared to healthy subjects (44.4 pg/ml, 3.9 pg/ml and 5.5 pg/ml respectively). GCF levels of IL-17E were not significantly different between the two groups. The GCF IL-17A:IL-17E ratio was also not significantly different between the two groups.

	Status	n	Median (pg/ml)	IQR	Z statistic	*p* value
IL-17A	Healthy	37	44.4	5.0 – 117.2	-3.585	<0.001*
	CP	41	94.9	76.6 – 119.6		
IL-17E	Healthy	37	86.6	2.0 – 127.0	-0.996	0.319
	CP	41	70.6	40.6 – 145.3		
IL-17F	Healthy	21	3.9	1.9 – 7.8	-2.138	0.023*
	CP	13	6.4	5.8 – 12.5		
IL-17A/F	Healthy	21	5.5	4.9 – 15.0	-4.235	<0.001*
	CP	13	19.0	15.0 – 35.0		
IL-17A: IL-17E	Healthy	37	0.5#	0.2 – 2.4	-0.980	0.320
	CP	41	0.6#	0.2 – 1.5		

Table 4-6: Levels of IL-17A, IL-17E, IL-17F, IL-17A/F and the IL-17A:IL-17E ratio in gingival crevicular fluid

The table represents median levels of IL-17A, IL-17E, IL-17F, IL-17A/F and IL-17:IL-17E ratio in GCF of the healthy subjects and chronic periodontitis (CP) patients. Statistical analyses were carried out using the Mann-Whitney test with a Bonferroni correction (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects; IQR = interquartile range; IL-17A:IL-17E = the serum ratio of IL-17A to IL-17E and # = ratio value (no units).

4.2.9 Correlations between gingival crevicular fluid levels of IL-17A, IL-17E, IL-17F, IL-17A/F and clinical parameters

To determine any associations between GCF levels of IL-17 family cytokines and clinical parameters, as the data was not normally distributed natural log transformation were performed prior to analysis. The correlations were carried out using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19).

Table 4-7 shows the GCF level of IL-17A positively correlated with all clinical parameters measured: CPD (r = +0.611, p < 0.001), CAL (r = +0.543, p < 0.001) and BOP (r = +0.273, p = 0.016). As did GCF levels of IL-17E: CPD (r = +0.415, p = 0.001), CAL (r = +0.463, p < 0.001) and BOP (r = +0.355, p = 0.002). In addition, the GCF level of IL-17F positively correlated with CPD (r = +0.481, p = 0.008), CAL (r = +0.449, p = 0.005) and BOP (r = +0.349, p = 0.043). Furthermore, the GCF level of IL-17A/F positively correlated with CPD (r = +0.435, p = 0.010), CAL (r = +0.444, p = 0.008) and BOP (r = +0.368, p = 0.032).

		CPD	CAL	BOP
IL-17A	n	77	77	77
	r	+0.611	+0.543	+0.273
	p	<0.001*	<0.001*	0.016*
IL-17E	n	77	77	77
	r	+0.415	+0.463	+0.355
	p	0.001*	<0.001*	0.002*
IL-17F	n	34	34	34
	r	+0.481	+0.449	+0.349
	p	0.008*	0.005*	0.043*
IL-17A/F	n	34	34	34
	r	+0.435	+0.444	+0.368
	p	0.010*	0.008*	0.032*

Table 4-7: Correlation between gingival crevicular fluid levels of IL-17A, IL-17E, IL-17F, IL-17A/F and clinical parameters

The table represents the correlations between GCF levels of IL-17A, IL-17E, IL-17F, IL-17A/F and clinical parameters for periodontal disease (CPD, CAL and BOP). The serum level values were subjected to a natural log transformation. The correlations were analysed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Significance= $p<0.05$ (*). n = number of subjects; *r* = the Pearson correlation coefficient; CPD = clinical probing depth; CAL = clinical attachment loss and BOP = bleeding on probing.

4.2.10 Correlations between gingival crevicular fluid levels of IL-17A, IL-17E, IL-17F and IL-17A/F

In addition to associations between GCF levels of IL-17 family cytokines and clinical parameters; associations between each IL-17 family member were also determined. Once again these associations were determined using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19).

Table 4-8 shows that the GCF level of IL-17A positively correlated with the GCF level of IL-17E (r = +0.384, p = 0.001), IL-17F (r = +0.638, p < 0.001) and IL-17A/F (r = +0.867, p < 0.001). In addition, the GCF level of IL-17E positively correlated with the GCF levels of IL-17F (r = +0.422, p = 0.013) and IL-17A/F (r = +0.684, p < 0.001). Furthermore, the GCF level of IL-17F positively correlated with the GCF level of IL-17A/F (r = +0.677, p < 0.001).

		IL-17A/F	IL-17F	IL-17E
IL-17A	n	34	34	78
	r	+0.867	+0.638	+0.384
	p	<0.001*	<0.001*	0.001*
IL-17E	n	34	34	
	r	+0.684	+0.422	
	p	<0.001*	0.013*	
IL-17F	n	34		
	r	+0.677		
	p	<0.001*		

Table 4-8: Correlations between gingival crevicular fluid levels of IL-17A, IL-17E, IL-17F and IL-17A/F

The table represents correlations between levels of each IL-17A, IL-17E, IL-17F and IL-17A/F. The serum level values were subjected to a natural log transformation. The correlations were analysed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects and *r* = the Pearson correlation coefficient.

4.2.11 Correlations between gingival crevicular fluid levels of IL-17A:IL-17E ratio and clinical parameters

As was performed for serum (Section 4.2.5), the associations between individuals GCF IL-17A:IL-17E ratio and clinical parameters were determined. Associations were determined using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). GCF IL-17A:IL-17E ratios were subjected to natural log transformations to facilitate graphical representation.

Figure 4-2 shows that the GCF IL-17A:IL-17E ratio showed a weak but significant positive correlation with CPD (r = +0.344, p = 0.002) and CAL (r = +0.250, p = 0.029), but no significant correlation with BOP (r = +0.116, p = 0.318).

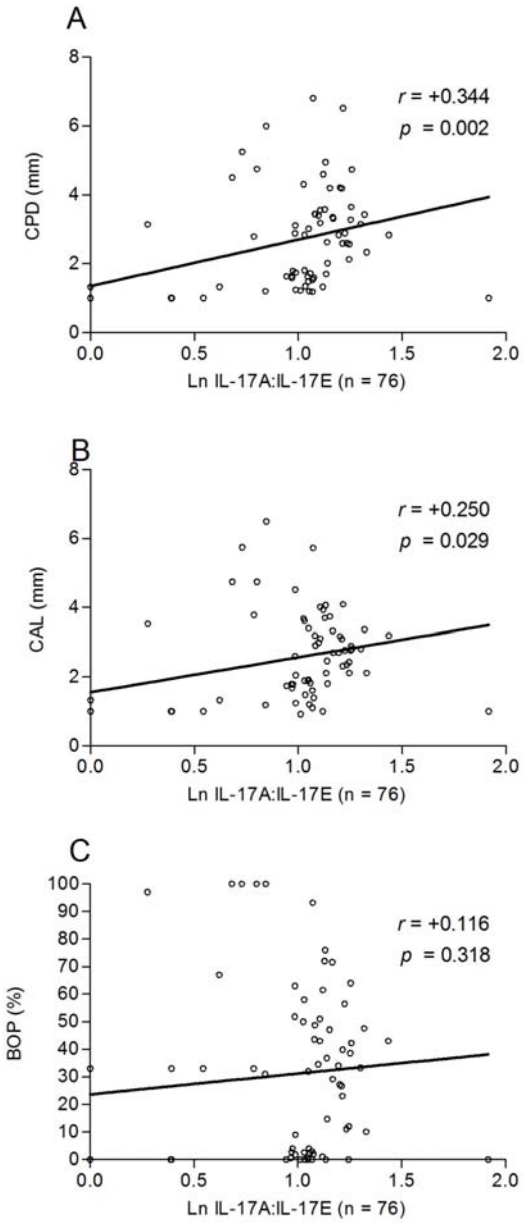

Figure 4-2: Correlations between the GCF IL-17A:IL17E ratio and clinical parameters

The GCF IL-17A:IL-17E ratio of all subjects (regardless of disease state) was calculated. To facilitate graphical representation the IL-17A:IL-17E ratio's were subjected to a natural log transformation (Ln). Correlation analysis against CPD (**A**), CAL (**B**) and BOP (**C**) was then performed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Significance = $p<0.05$. *r* = the Pearson correlation coefficient.

4.2.12 Correlations between gingival crevicular fluid levels of IL-17A, IL-17E, IL-17F, IL-17A/F and age

Correlations between GCF levels of IL-17 family cytokines and age were determined using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19).

Table 4-9 shows GCF levels of IL-17A, IL-17E, IL-17F and IL-17A/F positively correlated with age (r = +0.498, p < 0.001; r = +0.357, p = 0.001; r = +0.481, p = 0.004; and r = +0.531, p = 0.001 respectively). However, only GCF levels of IL-17A, IL-17E and IL-17F show significant positive correlation after corrected for disease severity parameter (clinical probing depth). In addition, no correlation was determined between the GCF IL-17A:IL-17E ratio and age after correction for disease state (CPD).

		IL-17A	**IL-17E**	**IL-17F**	**IL-17A/F**	**IL-17A: IL-17E**
Age	n	78	78	34	34	77
	r	+0.498	+0.357	+0.481	+0.531	+0.202
	p	<0.001*****	0.001*****	0.004*****	0.001*****	0.078
	$r_{partial}$	+0.474	+0.303	+0.405	+0.236	+0.144
	p	<0.001*****	0.008*****	0.019*****	0.186	0.218

Table 4-9: Correlations between gingival crevicular fluid levels of IL-17A, IL-17E, IL-17F, IL-17A/F, IL-17A:IL-17E ratio and age

The table represents correlations between levels of IL-17A, IL-17E, IL-17F, IL-17A/F, IL-17A:IL-17E ratio and age. The GCF level values were subjected to a natural log transformation. The correlations were analysed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Adjustment for disease severity was carried out using the Partial correlation test controlling for clinical probing depth (CPD) (IBM SPSS Statistics, version 19). Significance = p<0.05 (*). n = number of subjects; r = the Pearson correlation coefficient; $r_{partial}$ = the Partial correlation and IL-17A:IL-17E = the serum ratio of IL-17A to IL-17E.

4.2.13 Relationship between gingival crevicular fluid levels of IL-17A, IL-17E, IL-17F, IL-17A/F and gender

Statistical comparisons of GCF levels of IL-17 family cytokines between male and female subjects were carried out using the Mann-Whitney test with a Bonferroni correction (IBM SPSS Statistics, version 19).

Table 4-10 shows the GCF levels of IL-17 family cytokines were not significantly different between males and females.

	Gender	n	Median	IQR	Z statistic	*p* value
IL-17A	Male	35	89.70	69.07 – 118.90	-2.365	0.072
	Female	42	69.83	5.00 – 119.83		
IL-17E	Male	35	89.20	54.50 – 161.77	-2.144	0.128
	Female	42	56.70	5.38 – 126.20		
IL-17F	Male	13	7.20	6.10 – 11.38	-2.116	0.136
	Female	21	3.90	1.90 – 7.20		
IL-17A/F	Male	13	13.56	9.45 – 29.05	-2.428	0.060
	Female	21	6.48	3.56 – 13.71		

Table 4-10: Comparison of gingival crevicular fluid levels of IL-17A, IL-17E, IL-17F and IL-17A/F between males and females

The table represents median levels of IL-17A, IL-17E, IL-17F and IL-17A/F in the GCF of male and female subjects. Statistical analyses were carried out using the Mann-Whitney test with a Bonferroni correction (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects; and IQR = interquartile range.

4.2.14 Saliva levels of IL-17A, IL-17E, IL-17F and IL-17A/F

Saliva levels of IL-17 family cytokines were measured by ELISA. Due to sample limitation, only saliva levels of IL-17A, IL-17E, IL-17F and IL-17A/F were measured. Table 4-11 shows saliva levels of IL-17A, IL-17E and IL-17F were significantly increased in patients with chronic periodontitis (21.8 pg/ml, 56.3 pg/ml and 30.7 pg/ml respectively) as compared to healthy subjects (12.7 pg/ml, 22.4 pg/ml and 9.6 pg/ml respectively). The saliva levels of IL-17A/F were not significantly different between the two groups. Also, the saliva IL-17A:IL-17E ratio was not significantly different between the two groups.

	Status	n	Median (pg/ml)	IQR	Z statistic	*p* value
IL-17A	Healthy	37	12.73	8.13 – 21.16	-3.337	0.001*
	CP	65	21.84	13.14 – 51.37		
IL-17E	Healthy	37	22.40	4.00 – 60.60	-3.655	<0.001*
	CP	65	56.63	27.56 – 112.55		
IL-17F	Healthy	37	9.60	8.00 – 19.02	-6.219	<0.001*
	CP	65	30.70	19.91 – 55.37		
IL-17A/F	Healthy	37	9.40	1.90 – 17.60	-1.438	0.151
	CP	65	11.02	7.46 – 37.17		
IL-17A: IL-17E	Healthy	37	0.8#	0.3 – 1.28	-0.867	0.386
	CP	65	0.8#	0.5 – 1.1		

Table 4-11: Levels of IL-17A, IL-17E, IL-17F, IL-17A/F and the IL-17A:IL-17E ratio in saliva

The table represents median levels of IL-17A, IL-17E, IL-17F, IL-17A/F and the IL-17A:IL-17E ratio in saliva of the healthy subjects and chronic periodontitis (CP) patients. Statistical analyses were carried out using the Mann-Whitney test with a Bonferroni correction (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects; IQR = interquartile range; IL-17A:IL-17E = the serum ratio of IL-17A to IL-17E and # = ratio value (no unit).

4.2.15 Correlations between saliva levels of IL-17A, IL-17E, IL-17F, IL-17A/F and clinical parameters

To determine any associations between saliva levels of IL-17 family cytokines and clinical parameters, as the data was not normally distributed natural log transformation were performed prior to analysis. Once again, the correlations were carried out using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19).

Table 4-12 shows the saliva level of IL-17A positively correlated with CPD (r = +0.585, p = 0.001) and CAL (r = +0.520, p = 0.002). The saliva level of IL-17E positively correlated with all clinical parameters measured: CPD (r = +0.673, p < 0.001), CAL (r = +0.761, p < 0.001) and BOP (r = +0.480, p = 0.004). The Saliva level of IL-17F also positively correlated with CPD (r = +0.462, p < 0.001) and CAL (r = +0.404, p = 0.020). The Saliva level of IL-17A/F did not correlate with any of the clinical parameters.

		CPD	CAL	BOP
IL-17A	n	102	102	102
	r	+0.585	+0.520	+0.275
	p	0.001*	0.002*	0.115
IL-17E	n	102	102	102
	r	+0.673	+0.761	+0.480
	p	<0.001*	<0.001*	0.004*
IL-17F	n	102	102	102
	r	+0.462	+0.404	+0.254
	p	<0.001*	0.020*	0.167
IL-17A/F	n	102	102	102
	r	+0.199	+0.212	+0.050
	p	0.555	0.457	0.856

Table 4-12: Correlations between saliva levels of IL-17A, IL-17E, IL-17F, IL-17A/F and clinical parameters

The table represents the correlations between saliva levels of IL-17 family cytokines and clinical parameters for periodontal disease (CPD, CAL and BOP). The serum level values were subjected to a natural log transformation. The correlations were analysed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects; r = the Pearson correlation coefficient; CPD = clinical probing depth; CAL = clinical attachment loss and BOP = bleeding on probing.

4.2.16 Correlations between saliva levels of IL-17A, IL-17E, IL-17F and IL-17A/F

In addition to associations between saliva levels of IL-17 family cytokines and clinical parameters; associations between each IL-17 family member were also determined. Once again these associations were determined using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19).

Table 4-13 shows that the saliva level of IL-17A positively correlated with the saliva levels of IL-17E (r = +0.267, p = 0.001), IL-17F (r = +0.408, p < 0.001) and IL-17A/F (r = +0.442, p < 0.001). The saliva level of IL-17E positively correlated with the saliva levels of IL-17F (r = +0.411, p < 0.001) and IL-17A/F (r = +0.360, p < 0.001). The Saliva level of IL-17F positively correlated with the saliva level of IL-17A/F (r = +0.338, p = 0.001).

		IL-17A/F	IL-17F	IL-17E
IL-17A	n	102	102	102
	r	+0.442	+0.408	+0.267
	p	<0.001*	<0.001*	0.001*
IL-17E	n	102	102	
	r	+0.360	+0.411	
	p	<0.001*	<0.001*	
IL-17F	n	102		
	r	+0.338		
	p	0.001*		

Table 4-13: Correlations between saliva levels of IL-17A, IL-17E, IL-17F and IL-17A/F

IL-17A, IL-17E, IL-17F and IL-17A/F were measured in saliva samples using the appropriate ELISA kit (Table 2-3). The table represents correlations between levels of each IL-17 family cytokine. The serum level values were subjected to a natural log transformation. The correlations were analysed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects and r = the Pearson correlation coefficient.

4.2.17 Correlations between saliva levels of IL-17A:IL-17E ratio and clinical parameters

As was performed for serum and GCF (4.2.5 and 4.2.11), the associations between individuals saliva IL-17A:IL-17E ratio and clinical parameters were determined. Associations were determined using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Saliva IL-17A:IL-17E ratios were subjected to natural log transformations to facilitate graphical representation.

Figure 4-3 shows that the saliva IL-17A:IL-17E ratio did not to significantly correlate with any of the clinical parameters.

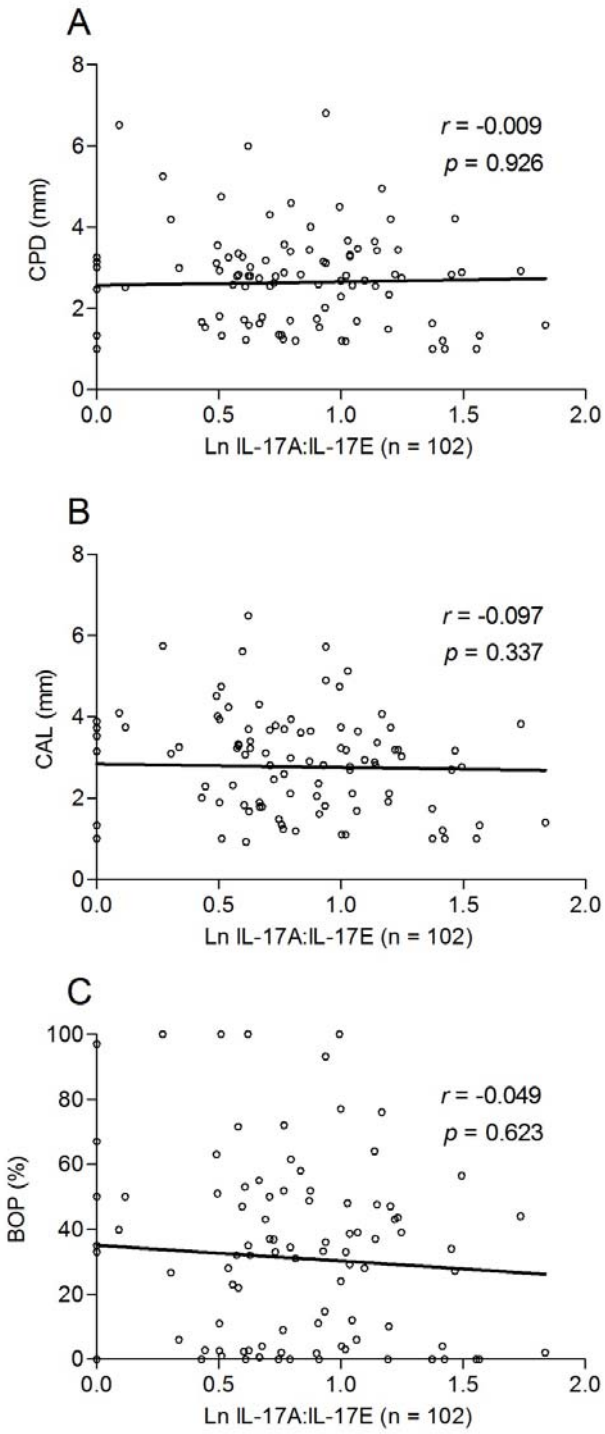

Figure 4-3: Correlations between the saliva IL-17A:IL17E ratio and clinical parameters

The saliva IL-17A:IL-17E ratio of all subjects (regardless of disease state) was calculated. To facilitate graphical representation the IL-17A:IL-17E ratio's were subjected to a natural log transformation (Ln). Correlation analysis against CPD (**A**), CAL (**B**) and BOP (**C**) was then performed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Significance = $p<0.05$. *r* = the Pearson correlation coefficient.

4.2.18 Correlations between saliva levels of IL-17A, IL-17E, IL-17F, IL-17A/F and age

Correlations between saliva levels of IL-17 family cytokines and age were determined using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19).

Table 4-14 shows saliva levels of IL-17E, IL-17F and IL-17A/F positively correlated with age (r = +0.495, p < 0.001; r = +0.525, p < 0.001; and r = +0.356, p < 0.001 respectively), regardless of disease state. Although saliva levels of IL-17A also show significant positive correlation with age (r = +0.235, p = 0.017), this correlation is found not to significant after correction for disease severity (clinical probing depth). In addition, the saliva IL-17A:IL-17E ratio did not significantly correlate with age after correction for disease state.

		IL-17A	IL-17E	IL-17F	IL-17A/F	IL-17A: IL-17E
Age	n	102	102	102	102	102
	r	+0.235	+0.495	+0.525	+0.356	-0.117
	p	0.017*	<0.001*	<0.001*	<0.001*	0.243
	$r_{partial}$	+0.168	+0.389	+0.415	+0.343	-0.120
	p	0.092	<0.001*	<0.001*	<0.001*	0.032

Table 4-14: Correlations between saliva levels of IL-17A, IL-17E, IL-17F, IL-17A/F, IL-17A:IL-17E ratio and age

The table represents correlations between levels of IL-17 family cytokines and age. The saliva level values were subjected to a natural log transformation. The correlations were analysed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Adjustment for disease severity was carried out using the Partial correlation test controlling for clinical probing depth (CPD) (IBM SPSS Statistics, version 19). Significance = p<0.05 (*). n = number of subjects; r = the Pearson correlation coefficient; $r_{partial}$ = the Partial correlation and IL-17A:IL-17E = the serum ratio of IL-17A to IL-17E.

4.2.19 Relationship between saliva levels of IL-17A, IL-17E, IL-17F, IL-17A/F and gender

Statistical comparisons of saliva levels of IL-17 family cytokines between male and female participants were carried out using the Mann-Whitney test with a Bonferroni correction (IBM SPSS Statistics, version 19).

Table 4-15 shows saliva levels of IL-17 family cytokines were not significantly different between males and females.

	Gender	n	Median	IQR	Z statistic	*p* value
IL-17A	Male	48	21.72	11.32 – 51.00	-1.552	0.484
	Female	54	15.01	9.60 – 34.08		
IL-17E	Male	48	52.22	25.39 – 108.77	-2.371	0.072
	Female	54	32.39	5.33 – 75.30		
IL-17F	Male	48	24.12	17.57 – 36.18	-0.727	1.000
	Female	54	23.50	9.70 – 41.08		
IL-17A/F	Male	48	9.45	5.51 – 32.59	-0.443	1.000
	Female	54	11.03	7.18 – 21.07		

Table 4-15: Comparison of saliva levels of IL-17A, IL-17E, IL-17F and IL-17A/F between males and females

The table represents median levels of IL-17A, IL-17E, IL-17F and IL-17A/F in the saliva of male and female subjects. Statistical analyses were carried out using the Mann-Whitney test with a Bonferroni correction (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects; and IQR = interquartile range.

4.2.20 mRNA expression of IL-17 family cytokines in periodontal tissues

Analyses of IL-17 family cytokine expression in clinical samples (serum, GCF and saliva) indicated a possible association between IL-17A, IL-17E, IL-17F, IL-17A/F and chronic periodontitis. To further confirm this finding, quantitative real-time PCR was used to investigate expression of these cytokines at the mRNA level in periodontal tissue samples from chronic periodontitis patients and healthy subjects undergoing non-periodontal related surgery.

Figure 4-4 shows that mRNA for IL-17 family cytokines (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F) were all expressed in periodontal tissue samples. In addition, apart from IL-17B, mRNA species for the remaining IL-17 family

cytokines were significantly upregulated in tissue from patients with chronic periodontitis compared to healthy subjects (all $p < 0.05$).

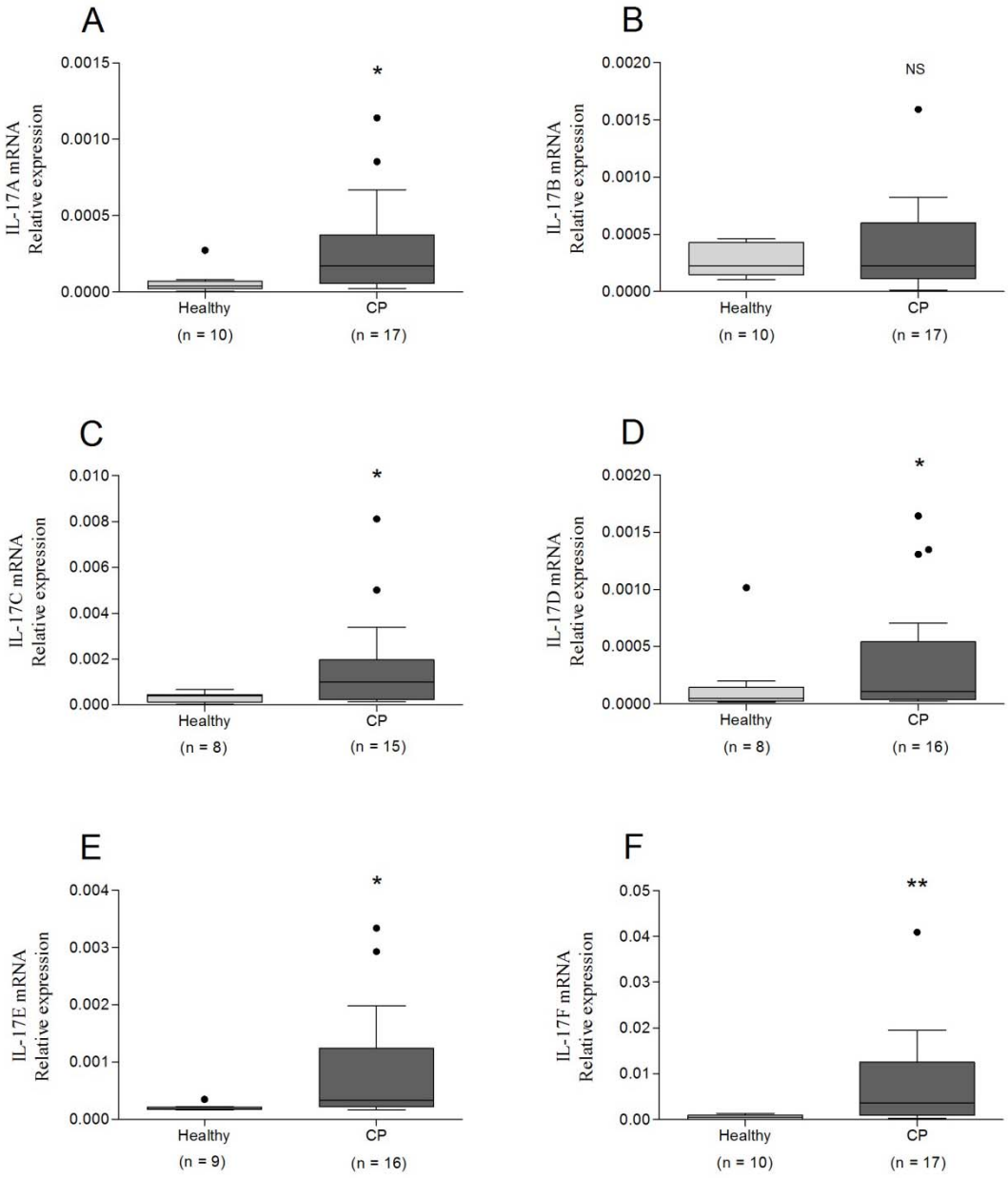

Figure 4-4: Real-time PCR analysis of IL-17 family cytokines mRNA expression in healthy and diseased periodontal tissues

Quantification of IL-17 family cytokine mRNA expression in periodontal tissues was performed by real-time PCR. Box-and-whiskers plot represent the median relative expression ($2^{-\Delta CT}$) IL-17 family mRNA levels in tissues from healthy subjects and chronic periodontitis (CP) patients. RNA polymerase II was used as a reference gene. Statistical analyses were carried out using the Mann-Whitney test with a Bonferroni correction (IBM SPSS Statistics, version 19). * = $p < 0.05$, ** = $p < 0.01$, and NS = $p > 0.05$. Whiskers were determined by the Tukey method. Outliers were determined as 1.5 x IQR (interquartile range) above or below the median value and shown as black circles.

4.2.21 Serum levels of IL-10

The opposing associations between the clinical parameters of periodontal disease and serum levels of IL-17E suggested that IL-17E may play a role as a negative regulator of periodontal immunity. Therefore, we investigated whether the relationship between biological levels of IL-17E and clinical parameters was similar to that of the archetypical anti-inflammatory cytokine IL-10.

The serum level of IL-10 was measured by ELISA. Table 4-16 shows that the serum level of IL-10 was significantly decreased in patients with chronic periodontitis (69.6 pg/ml) compared to healthy subjects (107.8 pg/ml).

	Status	n	Median (pg/ml)	IQR	Z statistic	*p* value
IL-10	Healthy	45	107.80	30.50 – 200.40	-2.209	0.027*
	CP	57	69.60	52.15 – 90.40		

Table 4-16: Levels of IL-10 in serum

IL-10 were measured in serum samples using the appropriate ELISA kit (Table 2-3). The table represents median levels of IL-10 in serum of the healthy and chronic periodontitis (CP) patients. Statistical analyses were carried out using the Mann-Whitney test (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects and IQR = interquartile range.

4.2.22 Correlations between serum levels of IL-10 and clinical parameters

To determine any associations between the serum level of IL-10 and clinical parameters, as the data was not normally distributed natural log transformation were performed prior to analysis. The correlations were carried out using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19).

Table 4-17 shows that the serum level of IL-10 negatively correlated with all clinical parameters but only statistically significantly with CPD (r = -0.208, p = 0.036) and BOP (r = -0.204, p < 0.040).

		CPD	CAL	BOP
IL-10	n	102	102	102
	r	-0.208	-0.141	-0.204
	p	0.036*	0.157	0.040*

Table 4-17: Correlation between serum levels of IL-10 and clinical parameters

The table represents the correlations between IL-10 and clinical parameters for periodontal disease (CPD, CAL and BOP). The serum level value was subjected to a natural log transformation. The correlations were analysed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects; *r* = the Pearson correlation coefficient; CPD = clinical probing depth; CAL = clinical attachment loss and BOP = bleeding on probing.

4.2.23 Correlations between serum levels of IL-10 and IL-17 family cytokines

The association between circulating levels of IL-10 and IL-17 family cytokines was determined using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19).

Table 4-18 shows that the serum level of IL-10 positively correlated with the serum levels of IL-17C (r = +0.240, p = 0.025), IL-17E (r = +0.212, p = 0.033) and IL-17F (r = +0.250, p = 0.011). There is no correlation between IL-10 and other members of the IL-17 family cytokines (IL-17A, IL-17B, IL-17D and IL-17A/F).

		IL-17A	IL-17B	IL-17C	IL-17D	IL-17E	IL-17F	IL-17A/F
IL-10	n	102	88	87	74	102	102	102
	r	-0.187	-0.140	+0.240	-0.120	+0.212	+0.250	-0.149
	p	0.060	0.194	0.025*	0.310	0.033*	0.011*	0.134

Table 4-18: Correlations between serum levels of IL-10 and IL-17 family cytokines

The table represents correlations between levels of each cytokine. The serum level values were subjected to a natural log transformation. The correlations were analysed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects and *r* = the Pearson correlation coefficient.

4.2.24 Correlations between serum IL-17A:IL-10 ratio and clinical parameters

The literature suggests that IL-17A and IL-10 have opposing roles in the inflammatory response. Therefore, as was previously performed for IL-17E, the circulating IL-17A:IL-10 ratio for each individual subject was calculated. Associations between the IL-17A:IL-10 ratio and clinical parameters were then determined using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19).

Figure 4-5 shows that the serum IL-17A:IL-10 had strong significant positive correlations with all clinical parameters measured: CPD (r = +0.559, p < 0.001), CAL (r = +0.536, p < 0.001) and BOP (r = +0.455, p < 0.001).

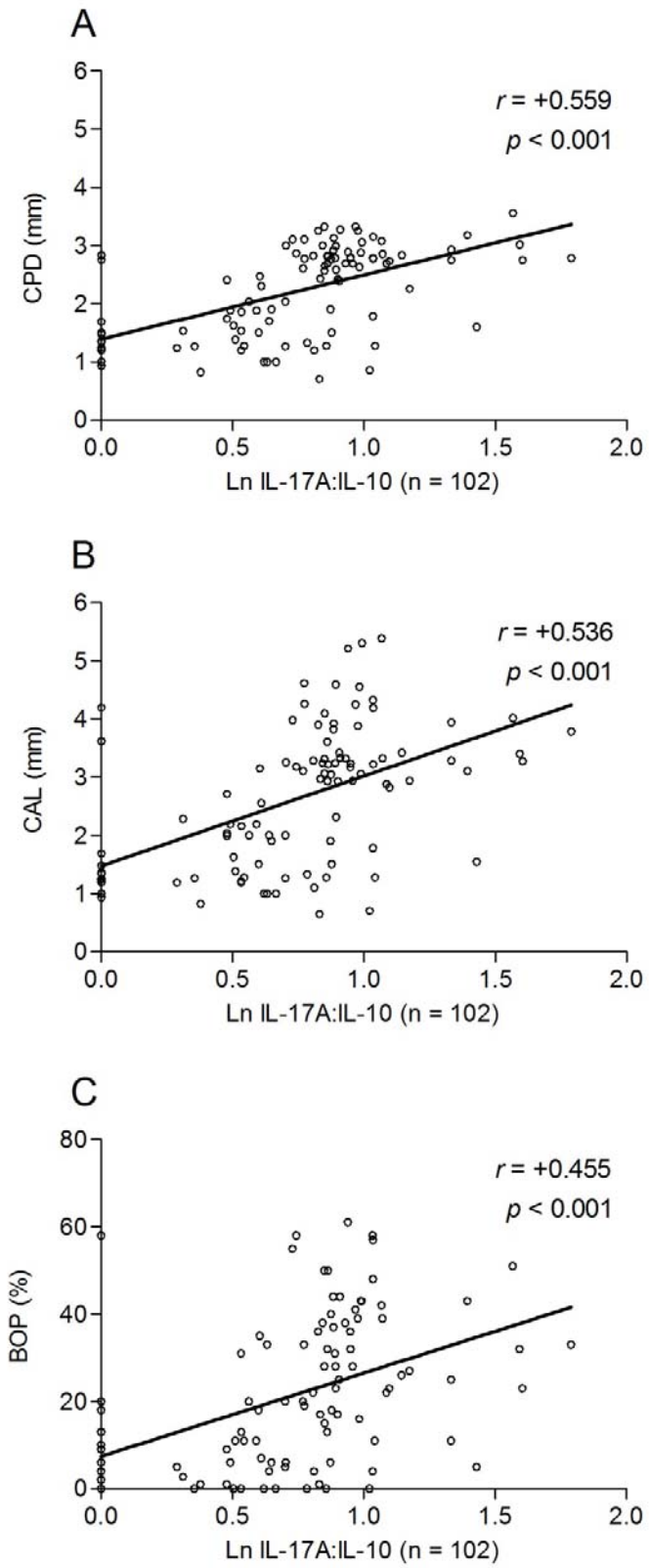

Figure 4-5: Correlations between the serum IL-17A:IL10 ratio and clinical parameters

The serum IL-17A:IL-17E ratio of all subjects (regardless of disease state) was calculated. To facilitate graphical representation the IL-17A:IL-10 ratio's were subjected to a natural log transformation (Ln). Correlation analysis against CPD (**A**), CAL (**B**) and BOP (**C**) was then performed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Significance = $p<0.05$. r = the Pearson correlation coefficient.

4.2.25 Correlations between serum levels of IL-10 and age

Correlations between the serum level of IL-10 and age were determined using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19).

Table 4-19 shows serum levels of IL-10 was found not to significantly correlate with age regardless of disease state. Although, the serum IL-17A:IL-10 ratio was found to significantly positively correlate with age (r = +0.402, p < 0.001), the correlations are found not significant after correction for the disease severity (clinical probing depth).

		IL-10	IL-17A:IL-10
Age	n	102	102
	r	-0.167	+0.402
	p	0.093	<0.001*****
	$r_{partial}$	-0.153	0.154
	p	0.106	0.125

Table 4-19: Correlations between serum levels of IL-10, IL-17A:IL-10 ratio and age

The table represents correlations between serum levels of IL-10 and IL-17A:IL-10 ratio and age. The serum level values were subjected to a natural log transformation. The correlations were analysed using the Pearson correlation coefficient test (IBM SPSS Statistics, version 19). Adjustment for disease severity was carried out using the Partial correlation test controlling for clinical probing depth (CPD) (IBM SPSS Statistics, version 19). Significance = p<0.05 (*). n = number of subjects; r = the Pearson correlation coefficient; $r_{partial}$ = the Partial correlation and IL-17A:IL-10 = the serum ratio of IL-17A to IL-10.

4.2.26 Relationship between serum levels of IL-10 and gender

Statistical comparisons of the serum IL-10 level between male and female subjects were carried out using the Mann-Whitney test (IBM SPSS Statistics, version 19).

Table 4-20 shows the serum level of IL-10 was not significantly different between males and females.

	Gender	n	Median	IQR	Z statistic	*p* value
IL-10	Male	42	69.60	31.75 – 106.00	-1.823	0.068
	Female	60	83.85	57.15 – 164.68		

Table 4-20: Comparison of serum levels of IL-10 between males and females

The table represents median levels IL-10 in serum of male and female subjects. Statistical analyses were carried out using the Mann-Whitney test (IBM SPSS Statistics, version 19). Significance = $p<0.05$ (*). n = number of subjects; and IQR = interquartile range.

4.2.27 mRNA expression of IL-10 cytokine in periodontal tissues

Previous data has shown that IL-10 levels are significantly elevated in periodontal tissue of patients with periodontal disease (Napimoga, *et al.*, 2011). To confirm this finding quantitative real-time PCR method was used to evaluate expression of IL-10 mRNA in periodontal tissue samples from chronic periodontitis patients and healthy subjects undergoing non-periodontal related surgery.

Figure 4-6 shows that mRNA for IL-10 was found expressed in healthy periodontal tissue samples and expression was significantly upregulated in tissue isolated from chronic periodontitis patients ($p < 0.05$).

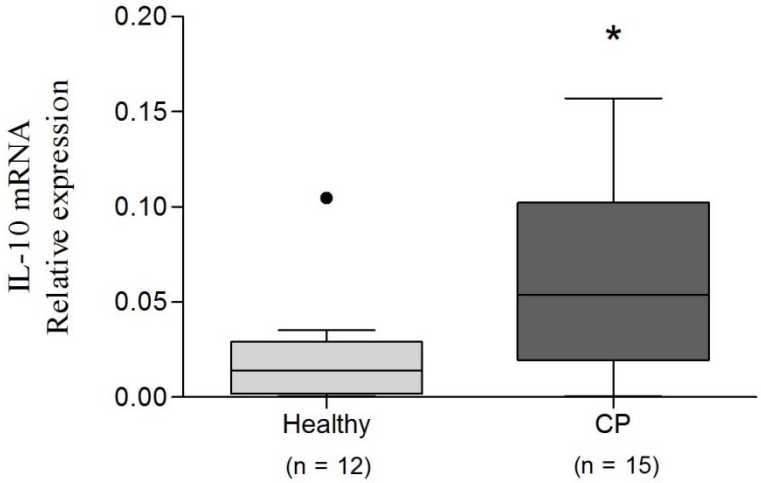

Figure 4-6: Real-time PCR analysis of IL-10 mRNA expression in healthy and diseased periodontal tissues

Quantification of IL-10 mRNA expression in periodontal tissues was performed by real-time PCR. Box-and-whiskers plot represent the median relative expression ($2^{-\Delta CT}$) of IL-10 mRNA in tissue from healthy subjects and chronic periodontitis (CP) patients. RNA polymerase II was used as a reference gene. Statistical analyses were carried out using the Mann-Whitney test (IBM SPSS Statistics, version 19). * = $p < 0.05$. Whiskers were determined by the Tukey method. Outliers were determined as 1.5 x IQR (interquartile range) above or below the median and shown as black circles.

4.3 Discussion

In agreement with previous studies these findings confirm that serum, GCF, and saliva levels of IL-17A are elevated in chronic periodontitis patients and correlate with clinical parameters (Buduneli, *et al.*, 2009; Duarte, *et al.*, 2010; Ozcaka, *et al.*, 2011; Schenkein, *et al.*, 2010; Vernal, *et al.*, 2005). Our real-time PCR analysis also confirmed that the IL-17A mRNA levels are upregulated in the gingival tissue of chronic periodontitis patients compared to healthy subjects (Behfarnia, *et al.*, 2013; Honda, *et al.*, 2008; Ohyama, *et al.*, 2009).

In addition to confirming previous findings; this study further advances our understanding of the associations between IL-17F and the IL-17A/F heterodimer and chronic periodontitis. This study demonstrated significant increased serum levels of the IL-17A/F heterodimer in chronic periodontitis patients that correlated with clinical parameters (Table 4-1 and 4-2). However, serum levels of IL-17F were not significantly different between the 2 groups and did not correlate with clinical parameters (Table 4-1 and 4-2). The serum levels of IL-17A, IL-17F and IL-17A/F did positively correlate to one another (Table 4-3). Additionally, significant increases in GCF levels of IL-17F and the IL-17A/F heterodimer were demonstrated in chronic periodontitis patients and these correlated with clinical parameters (Table 4-6 and 4-7). In saliva, significant differences in IL-17F levels were observed between chronic periodontitis patients and healthy subjects; which correlated with clinical parameters (Table 4-11 and 4-12). In contrast, saliva levels of IL-17A/F were found not to significantly differ and did not correlate with any clinical parameters (Table 4-11 and 4-12). The GCF and saliva levels of IL-17A, IL-17F and IL-17A/F are found to positively correlate to one and other.

Associations between serum levels of IL-17A and IL-17A/F and chronic inflammatory diseases has been observed previously. Serum levels of IL-17A and IL-17A/F were elevated in patients with rheumatoid arthritis, lymphoproliferation syndrome (autoimmune syndrome), systemic sclerosis, systemic lupus erythematous and polycystic ovarian syndrome (Boggio *et al.*, 2014; Nakashima *et al.*, 2012; Ozcaka, *et al.*, 2013; Shimamoto *et al.*, 2013; Tanasescu *et al.*, 2010). However, serum levels of IL-17F were also found to be elevated in chronic inflammatory disease such as rheumatoid arthritis and

systemic lupus erythematous (Shimamoto, *et al.*, 2013; Tanasescu, *et al.*, 2010). This is in contrast to our findings which showed no difference in serum IL-17F levels (Table 4-1). However, the literature on IL-17F is contradictory with some studies on systemic sclerosis and rheumatoid arthritis reporting no differences in serum IL-17F levels between health and disease (Gumus, *et al.*, 2013; Nakashima, *et al.*, 2012). In fact, serum levels of IL-17F were reported to be lower in patients with chronic hepatitis C virus infection as well as severe helminthic infection (*Echinococcus multilocularis*) compared to healthy subjects (Lechner *et al.*, 2012; Sousa *et al.*, 2012). These data suggest that the role of IL-17F in pathogenesis is disease specific.

Apart from investigating IL-17A, few studies have evaluated the levels of other IL-17 family members in GCF. In line with our findings (Table 4-6), Gumus and colleagues (2013) showed increased GCF levels of IL-17A and IL-17F in chronic periodontitis patients with rheumatoid arthritis compared to chronic periodontitis patients who are systemically healthy. In addition, GCF levels of 17A, IL-17F and IL-17A/F were positively correlated to one and other (Table 4-7). In contrast to our findings (Table 4-6), however, Gumus and colleagues (2013) showed no difference in the GCF levels of IL-17A/F between both groups. Also in line with our data, Ozcaka and colleagues (2013) showed that GCF levels of IL-17A and IL-17F were increased in gingivitis patients who suffered polycystic ovarian syndrome compared to healthy subjects. However, they found that GCF levels of IL-17A and IL-17F were not correlated with any clinical parameters for periodontal disease (CPD and BOP).

Similar to GCF, there are not many studies that evaluated the levels of IL-17 family cytokines in saliva, with the exception of IL-17A. In line with our findings (Table 4-11), Ozcaka and colleagues (2013) showed increased saliva levels of IL-17A and IL-17F in gingivitis patients who suffered polycystic ovarian syndrome compared to healthy subjects. In addition, saliva levels of IL-17A positively correlated with clinical parameters such as CPD and CAL (Table 4-12). However, saliva levels of IL-17A/F did not correlate with any clinical parameters and no difference in saliva levels of IL-17A/F was determined between the two groups (Table 4-11 and 4-12).

Real-time PCR analyses revealed for the first time that mRNA expression of IL-17F is elevated in gingival tissue samples of chronic periodontitis patients compared to healthy subjects (Figure 4-4). This is in line with studies on other inflammatory diseases such ulcerative colitis, psoriasis and lichen planus which also demonstrated elevated IL-17F mRNA levels in diseased mucosa/epithelia (Fujino*, et al.*, 2003; Im *et al.*, 2012; Johansen *et al.*, 2009; Johnston *et al.*, 2013; Piccinni *et al.*, 2014; Seiderer*, et al.*, 2008).

This study is first to investigate associations between serum levels of IL-17B, IL-17C and IL-17D and chronic periodontitis. However, no significant differences between serum levels of these family members were observed between chronic periodontitis patients and healthy subjects (Table 4-1). Furthermore, serum levels of IL-17B, IL-17C and IL-17D did not significantly correlate with clinical parameters (Table 4-2). Due to sample limitation, levels of IL-17B, IL-17C and IL-17D were not evaluated in GCF and saliva. The Literature revealed a limited number of studies evaluating associations between serum levels of IL-17B, IL-17C and IL-17D with disease. Serum levels of IL-17B were shown to be elevated in patients with severe helminthic infection (*Echinococcus multilocularis*) and systemic lupus erythematous (Lechner*, et al.*, 2012; Robak *et al.*, 2013). Serum levels of IL-17C did not differ between healthy subjects and patients with hepatitis B (He *et al.*, 2013). In addition, serum levels of IL-17B, IL-17C and IL-17D were actually shown to be lower in patients with acute exacerbated chronic pulmonary heart disease compared to healthy subjects (Chen *et al.*, 2012). Therefore, although research is at an early stage, the roles of IL-17B, IL-17C and IL-17D in pathogenesis may also be disease specific.

Real-time PCR analyses demonstrated for the first time increased mRNA expression of IL-17C and IL-17D in the gingival tissue samples of chronic periodontitis patients compared to healthy subjects (Figure 4-4). However, no difference in IL-17B mRNA expression was observed. In line with literature, IL-17C mRNA levels were also shown to be increased in psoriatic skin as well as in the colonic mucosa of ulcerative colitis patients (Im*, et al.*, 2012; Johansen*, et al.*, 2009; Johnston*, et al.*, 2013). In contrast, Im and colleagues (2012) showed no difference in the mRNA expression of IL-17B and IL-17D in colonic mucosal tissue of ulcerative colitis patients and IL-17B and IL-17D mRNA levels were also

found to be decreased in the skin of psoriasis patients (Johansen, *et al.*, 2009; Johnston, *et al.*, 2013).

The major novel finding of interest in this study is the association between IL-17E and chronic periodontitis. Chronic periodontitis patients presented with decreased serum levels of IL-17E, which negatively correlated with CPD and CAL (Table 4-1 and 4-2). Serum levels of IL-17E also negatively correlated with IL-17A (Table 4-3). In GCF and saliva, however, the findings were slightly different. GCF levels of IL-17E were not significantly different between chronic periodontitis patients and healthy subjects; however levels did positively correlate with all clinical parameters (Table 4-6 and 4-7). Furthermore, saliva levels of IL-17E were significantly higher in chronic periodontitis patients and levels again correlated with all clinical parameters (Table 4-11 and 4-12). The decreased serum level of IL-17E in chronic periodontitis patients has been suggested previously as serum levels of IL-17E were shown to be lower in patients with a combination of gingivitis and polycystic ovarian syndrome (Ozcaka, *et al.*, 2013). In addition, in patients with inflammatory bowel diseases, serum levels of IL-17E are also lower compared to their respective healthy subjects (Ozcaka, *et al.*, 2013; Su *et al.*, 2013). In contrast, studies of IL-17E levels in GCF and saliva are limited for comparison. In line with our findings Gumus and colleagues (2013) showed no difference in GCF levels of IL-17E in chronic periodontitis patients who suffered rheumatoid arthritis compared to chronic periodontitis patients who are systemically healthy. In addition, Ozcaka and colleagues (2013) also showed that GCF levels of IL-17E in gingivitis patients who suffered polycystic ovarian syndrome were no different to healthy subjects. Additionally, Ozcaka and colleagues (2013) showed that GCF levels of IL-17E positively correlated with CPD and BOP. GCF levels of IL-17E were also shown to have positive correlations with IL-17A, IL-17F and IL-17A/F (Gumus, *et al.*, 2013). In addition, in line with our data, saliva levels of IL-17E were found to be increased in gingivitis patients who suffered polycystic ovarian syndrome compared to healthy subjects, and these were found to positively correlate with clinical parameters (Ozcaka, *et al.*, 2013). At the tissue level, real-time PCR analyses demonstrated increased mRNA expression of IL-17E in gingival tissue samples of chronic periodontitis patients compared to healthy subjects (Figure 4-4). In line with our findings, IL-17E mRNA expression was shown to be increased in sinus

mucosal tissue samples of chronic rhinosinusitis patients (Lam *et al.*, 2013). In addition, IL-17E mRNA levels were also found to be increased in the sputum of patients with chronic rhinitis and asthma (Kwon *et al.*, 2012; Seys *et al.*, 2013). Furthermore, in a mouse model, IL-17E mRNA expression was elevated in lung tissues of ovalbumin induced lung inflammation (Kawashima *et al.*, 2013) as well as in the skin of formaldehyde induced atopic dermatitis (Kim *et al.*, 2013b).

Chronic periodontitis patients presented with decreased serum levels of IL-17E, which negatively correlate with CPD and CAL, and serum levels of IL-17E are also negatively correlated with IL-17A (Table 4-1, 4-2 and 4-3). These findings suggest that the circulating IL-17A:IL-17E ratio may be a predictive marker of disease. Indeed, our data show serum IL-17A:IL-17E ratios are significantly higher in chronic periodontitis patients compared to healthy subjects (Table 4-1) and the serum IL-17A:IL-17E ratios have positive moderate correlations with both CAL and CPD (Figure 4-1). In contrast to the serum analysis, however, the GCF and saliva IL-17A:IL-17E ratio are not significantly increased in chronic periodontitis patients (Table 4-6 and 4-11); possibly because GCF and saliva levels of IL-17E tended to be greater. However, the GCF IL-17A:IL-17E ratio had a weak but positive correlation with CPD and CAL (Figure 4-2). No correlations were observed between the saliva IL-17A:IL-17E ratio and clinical parameters (Figure 4-3). Differences in levels of IL-17 family cytokines in serum, saliva and GCF could possibly be explained by the anatomical location from which these biological fluids are derived (Buduneli & Kinane, 2011). However, this is a matter of conjecture until we have a complete picture of IL-17 family cytokine biology. In line with these findings, serum levels of IL-17A and IL-17E were shown to be negatively correlated in patients with gingivitis and polycystic ovarian syndrome (Ozcaka*, et al.*, 2013). Furthermore the serum IL-17A:IL-17E ratio was shown to be higher in patients with rheumatoid arthritis than healthy subjects (Gumus*, et al.*, 2013). There were few studies that describe the associations between serum levels of IL-17A and IL-17E and chronic inflammatory disease. The GCF IL-17A:IL-17E ratio was compared in chronic periodontitis patients who suffered from rheumatoid arthritis to chronic periodontitis patients who were systemically healthy and no significant differences found (Gumus*, et al.*, 2013).

Interestingly, our data show that serum levels of IL-17A and IL-17A/F have significant positive correlations with age regardless of disease state (Table 4-4).

However, although serum ratio of IL-17A:IL-17E and IL-17A:IL10 also have significant positive correlations with age, the correlations are found not significant after correction for disease severity (clinical probing depth) (Table 4-4 and Table 4-19). In addition GCF levels of IL-17A, IL-17E, IL-17F and IL-17A/F have significant positive correlations with age, however only GCF levels of IL-17A, IL-17E and IL-17F shows significant positive correlation after correction for disease severity (clinical probing depth) (Table 4-9). Additionally, saliva levels of IL-17E, IL-17F and IL-17A/F have significant positive correlation with age regardless of disease severity (Table 4-14). Although saliva levels of IL-17A also show significant positive correlation with age, this correlation is found not significant after correction for disease severity (clinical probing depth) (Table 4-14).

Studies show gradual deterioration of immune system occurs with aging (immunosenescence). For instance, although peripheral neutrophils numbers were found to increase with age, the migration ability of the aged neutrophils upon GM-CSF stimulation *in vitro* was found to be defective (Butcher *et al.*, 2000). In addition, age related products such as advanced glycation end products (AGE), were shown to induce increased expression of pro-inflammatory cytokines by phagocytic cells such as monocytes and macrophages (Thornalley, 1998). The population of T lymphocytes are also found to be altered by age, with age being associated with a decreased population of naïve T cells in healthy adults due to increase in apoptotic activity (Chakravarti & Abraham, 1999; Herndon *et al.*, 1997). These studies show aging can alter the balance of the immune system, which can lead to disturbances in tissue homeostasis as well as responses to infection. Clinical data has demonstrated that aging patients have a reduced capacity for fighting infection compared to young patients (Schneider, 1983). In periodontal disease, age has also been shown to associate with disease severity (Brown *et al.*, 1996; Haas *et al.*, 2014; Loe *et al.*, 1992). In addition, autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis and Weagner's granulomatosis have been shown to have some features of immunosenescence (Peters *et al.*, 2009).

In line with our data, serum levels of IL-17A from chronic periodontitis patients have been found to associate with age (Schenkein, *et al.*, 2010). However, serum levels of IL-17A were found not to correlate with age of patients with

psoriasis and rheumatoid arthritis (Arican *et al.*, 2005; Metawi *et al.*, 2011; Oh *et al.*, 2011). In addition, serum levels of IL-10 did not correlate with age in patients with hepatitis C virus infection and *Plasmodium falciparum* infection (malaria) (Lyke *et al.*, 2004; Reiser *et al.*, 1997). Serum levels of IL-17A and IL-10 in healthy subjects were also found not to correlate with age (Kim *et al.*, 2011; Sivro *et al.*, 2013).

In this study, we show that serum levels of all members of IL-17 family cytokines as well as IL-10 are not significantly different between males and females (Table 4-5). In addition, GCF and saliva levels of IL-17A, IL-17E, IL-17F and IL-17A/F are also not significantly different between males and females (Table 4-10 and 4-15). Epidemiological studies showed the prevalence of chronic periodontitis is greater in males than females (Shiau & Reynolds, 2010). However, this finding is probably due to differences in lifestyle choices between males and females; for example alcohol consumption, smoking, oral hygiene practices and frequency of dental visit (Burt, 2005). Although, gender-specific biological factors cannot be ruled out. Indeed, IL-17RA deficient male mice were found to be much more susceptible to periodontal disease than male mice (Yu *et al.*, 2008). However, in agreement with our findings, serum levels of IL-17A were found not to differ between genders in systemically healthy subjects (Gourh *et al.*, 2009). In addition, although serum levels of IL-17A were found to positively associate with chronic diseases such as psoriasis, systemic lupus erythematous and chronic liver disease, no difference between males and females was observed (Arican, *et al.*, 2005; Kakumu *et al.*, 1997; Vincent *et al.*, 2013). Similarly, although serum levels of IL-10 were found to be increased in chronic liver disease and traumatic injury, the serum levels in these subjects were not different between males and females (Kakumu, *et al.*, 1997; Sperry *et al.*, 2008).

IL-17A, IL-17F and IL-17A/F have been implicated to play roles in various chronic inflammatory disease including rheumatoid arthritis, multiple sclerosis, psoriasis and inflammatory bowel diseases (Chabaud, *et al.*, 1999) (Fujino, *et al.*, 2003; Ishigame, *et al.*, 2009; Malakouti *et al.*, 2014; Pappu *et al.*, 2011; Seiderer, *et al.*, 2008). Structurally, the IL-17A, IL-17F and IL-17A/F heterodimer are closely related. The IL-17A and IL-17F monomers have a high degree of homology (40 %) (Starnes, *et al.*, 2001), and they both present with a conserved structure of four cysteines that form a knot motif in their tertiary structure (Fossiez, *et al.*, 1996;

Hymowitz, *et al.*, 2001). In addition, IL-17A and IL-17F monomers can be linked at the cysteine knot motif to form the IL-17A/IL-17F heterodimer (Wright, *et al.*, 2007). IL-17A, IL-17F and IL-17A/F are known to require similar receptors to mediate cellular responses; IL-17RA and IL-17RC (Kuestner, *et al.*, 2007; Wright, *et al.*, 2007). The similarities in structure and function between these 3 family members may explain why they have similar roles in the pathogenesis of chronic inflammatory diseases.

IL-17A is a known pro-inflammatory cytokine involved in the pathogenesis of periodontal disease (Behfarnia, *et al.*, 2013; Buduneli, *et al.*, 2009; Liang, *et al.*, 2010; Takahashi, *et al.*, 2005). However, studies on the roles of IL-17F and IL-17A/F in periodontal disease are limited. In this study we confirmed the association of IL-17A in the pathogenesis of periodontal disease. In addition, we show elevated levels of IL-17F and IL-17A/F protein in clinical samples (serum, GCF and saliva) as well as elevated levels of mRNA expression in diseased periodontal tissue. These data indicate that IL-17F and IL-17A/F may have a role in pathogenesis of periodontal disease. Since IL-17F and IL-17A/F are known to be produced by Th17 cells (Wright, *et al.*, 2007) and Th17 cells are known to be present in the tissue of periodontal disease (Adibrad, *et al.*, 2012; Cardoso, *et al.*, 2009) the findings in our study are perhaps unsurprising. However, we cannot be sure of the cellular source of these cytokines within the oral cavity at present. Studies have shown that epithelial cells derived from the bronchus and lung activated by IL-17A, IL-17F and IL-17A/F increase expression of GRO-α, IL-8 and CXCL1 (Kawaguchi, *et al.*, 2006; Liang, *et al.*, 2007; Wright, *et al.*, 2007). Hence, similar to other types of tissue, IL-17F and IL-17A/F probably play an important role as part of cytokine network in regulating immune responses in periodontal tissues. However, more studies are required to confirm the role of IL-17A, IL-17F and IL-17A/F in the pathogenesis of periodontal disease.

IL-17E is described as an anti-inflammatory cytokine with functions opposing to those of IL-17A (Monteleone, *et al.*, 2010; Tosello Boari *et al.*, 2012). IL-17E inhibits Th17 cell proliferation via IL-4 and IL-13 (Cooney, *et al.*, 2011; Kleinschek, *et al.*, 2007). IL-17E induces expression of IL-4 and IL-13 by many cell types including; Th2 cells, mast cells and macrophages (Angkasekwinai, *et al.*, 2010; Angkasekwinai, *et al.*, 2007; Ikeda, *et al.*, 2003; Kang, *et al.*, 2005; Wang, *et al.*, 2007b). *In vitro*, IL-17E was shown to inhibit IL-17A release from

$CD4^+$ T cells isolated from the colon of patients with inflammatory bowel diseases (Su, *et al.*, 2013). In addition, the ability of IL-17E to supress the Th17/IL-17A inflammatory response in EAE was also shown *in vivo* (Kleinschek, *et al.*, 2007). Since IL-17A and IL-17E have opposing functions, it is interesting to speculate that the ratio between these family members could become an important determinant for chronic periodontitis. However, further studies into the biology of these cytokines in relation to periodontal disease are required before this can be confirmed.

To determine whether the relationship between IL-17A and IL-17E was similar to that of a prototypic anti-inflammatory cytokine; levels of IL-10 in biological samples were also investigated. Th17 cells express the receptor for IL-10 (IL-10R1) and stimulation with IL-10 directly inhibits Th17 cell proliferation and secretion of IL-17A (Heo *et al.*, 2010; Huber *et al.*, 2011). IL-10 is also known to indirectly down regulate Th17 driven responses (through IL-17A, TNF- α and IL-6) via inhibition of IL-23 (Stetsko & Sauder, 2008). In addition, IL-10 was shown to suppress the proliferation of Th17 cells in mice with established colitis (Huber, *et al.*, 2011). These studies suggest that IL-10 may play an important role in IL-17A biology. In addition, IL-17E was also shown to inhibit $CD4^+$ T cells isolated from colon of inflammatory bowel diseases patients in an IL-10 dependent manner (Su, *et al.*, 2013), suggesting a further complexity in the relationship between IL-17A and IL-17E. This study (Table 4-16 and 4-17) suggests a similar complexity occurs in patients with chronic periodontitis as serum levels of IL-10 were significantly elevated and that this increase correlated with clinical parameters (Andrukhov *et al.*, 2011; Enwonwu *et al.*, 2005; Havemose-Poulsen *et al.*, 2005). This study also shows week positive correlations between serum levels of IL-10 and IL-17C, IL-17E and IL-17F, and quite interestingly IL-10 also showed a negative correlation with serum IL-17A (Table 4-18). However, this correlation did not quite reach significance (r = -0.187, p = 0.060). However, the serum IL-17A:IL-10 ratio did have a positive moderate correlation with CPD, CAL and BOP (Figure 4-5).

Real-time PCR analysis (Figure 4-6) confirmed previous findings demonstrating elevated levels of IL-10 mRNA expression from tissues from chronic periodontitis patients compared to healthy subjects (Garlet *et al.*, 2004; Napimoga, *et al.*, 2011). This is in line with the findings in the other chronic inflammatory diseases

such as rheumatoid arthritis, ulcerative colitis and atopic dermatitis that show increased IL-10 expression in diseased compared to healthy control tissue (Furuzawa-Carballeda & Alcocer-Varela, 1999; Gambichler *et al.*, 2008; Olsen *et al.*, 2007).

In chronically inflamed periodontal tissue, inflammatory mediators including cytokines and chemokines are produced by innate and adaptive immune cells. Some of these chemical mediators leave the tissue and enter the circulatory system and have endocrine-like effects (Engleberg *et al.*, 2012). In addition, these mediators accumulate in the GCF found in the gingival sulcus/pockets and can also enter the oral cavity to become part of mixed saliva (whole saliva) (Kaufman & Lamster, 2002). Although the mediators in serum, GCF and saliva are derived from the same source, in different biological mediums they are subjected to diluting processes and protein degradation. Thus, it is not surprising that quantification of these mediators in the clinical samples is varied. Hence, differences in levels of IL-17 family cytokines in serum, GCF and saliva in our study could possibly be explained by the anatomical location from which these biological fluids are derived (Buduneli & Kinane, 2011).

GCF collection can be performed in three ways: absorbent paper strips or paper points; capillary tubes and gingival crevicular washes. The use of a capillary tube is necessary when large amounts of GCF are required (Skaleric *et al.*, 1987). However, this method can cause irritation to the gingival sulcus. Gingival crevicular washes are beneficial for collection of cellular components of GCF (Adonogianaki *et al.*, 1993). However, this method requires a trained and experienced investigator. In our study, we used the paper strip collection method as this is probably the most commonly used methodology (Adonogianaki *et al.*, 1994; Guentsch *et al.*, 2011; Ozkavaf *et al.*, 2001). The GCF volume from a paper strip can be quantify using a Periotron® (Tozum *et al.*, 2004). However, the measurement is only accurate over a limited volume range (Griffiths, 2003). This can be problematic as minimal inaccuracies in GCF volume measurement can have profound effects on calculated concentrations of protein mediators. Therefore, in our study we presented data as total amounts in GCF and did not correct for volume.

For isolation of serum, the method used is well established. However, variations in the sample collection process could have an impact on the analytical outcome. These variations may include: types of additive used in the collection tube; sample processing time and temperature; sample storage conditions; hemolysis of the sample; and the number of freeze-thaw cycles prior to analysis (Tuck *et al.*, 2009). It is difficult, if not impossible, to make sure all studies carried out in exactly similar conditions. Hence variation in findings could occur in serum sample analysis between studies.

Whole saliva is actually a mixture of oral fluids; including major and minor salivary gland secretions, as well as other additional components derived from GCF, expectorated bronchial secretions, serum and blood cells from oral wounds (Kaufman & Lamster, 2000). The use of whole saliva for evaluating markers for soft tissue inflammation and hard tissue destruction in periodontal disease has been the subject of considerable research activity (Giannobile *et al.*, 2009). Since inflammatory mediators in GCF can make their way into saliva, the use of saliva as a diagnostic marker has advantages over serum and GCF as collection is non-invasive and only requires minimal training. In our study, we used whole unstimulated saliva collected by the method of Navazesh and Christensen (1982). The use of unstimulated saliva in investigating cytokine levels has been previously reported (Javed *et al.*, 2013; Liu *et al.*, 2014). However, there are draw backs in the use of saliva to evaluate levels of protein markers. Bacteria derived from dental biofilm, including anaerobic species, can survive in the saliva medium (Bowden, 1997; de Jong *et al.*, 1984; De Jong *et al.*, 1986). These bacteria can then produce proteolytic enzymes that in combination with proteolytic enzymes derived from the host which are also present can break down proteinacious markers which will effect analysis (Chauncey, 1961). In our study, the serum, GCF and saliva samples were collected as part of previous studies (Davies*, et al.*, 2011; Jaedicke*, et al.*, 2012; Pathiyal*, et al.*, 2005; Preshaw & Heasman, 2002). The samples had been subjected to long term storage and had possibly undergone multiple freeze-thaw episodes. Therefore, it is impossible to discount the fact that these factors may have affected the results of our protein analysis.

Our findings indicate that IL-10 and IL-17 family cytokines; especially IL-17A, IL-17E, IL-17F and IL-17A/F could have a potential role in the pathogenesis of

periodontal disease. Of greatest interest was the relationship between IL-17A and IL-17E, which may play opposing roles. The negative correlations between IL-17A and IL-17E were similar to that of the prototypic anti-inflammatory cytokine; IL-10. Indeed, the serum ratio of IL-17A:IL-17E and IL-17A:IL-10 both negatively associated with clinical parameters of periodontal disease. However, the biological significance of these relationships require further investigation.

Chapter 5: IL-17E and periodontal disease

5.1 Introduction

Although our understanding of the IL-17E biology is increasing, how IL-17E expression is regulated and its target cell populations are still poorly understood. The evidence to date however suggests that even though IL-17E and IL-17A are characterised as being members of the same cytokine family, they play vastly opposing roles in the pathogenesis of inflammatory diseases. This therefore makes dissemination of the role IL-17E plays in the pathogenesis of periodontal disease an exciting target for further investigation.

IL-17E has been detected in GCF and serum of periodontal patients and levels negatively correlated with periodontal clinical probing depth (CPD) (Ozcaka, *et al.*, 2013). In the previous chapter, the data confirmed that IL-17E can be detected in serum and GCF of patients with chronic periodontitis. In addition, IL-17E can also be detected in saliva. Interestingly, serum levels of IL-17E negatively correlated with CAL and CPD. However, levels in GCF were actually elevated in chronic periodontitis patients and furthermore at the mRNA level IL-17E expression was elevated in tissue of chronic periodontitis patients. The biological significance of these findings are at present unknown; but may be related to the functional role IL-17E plays in the pathogenesis of periodontal disease. Therefore, in this chapter the expression of IL-17E in periodontal tissues was investigated further and its potential role in pathogenesis of periodontal disease evaluated using *in vitro* model systems.

Based on the previous literature and the findings in Chapter 4, IL-17E was hypothesised to act as an anti-inflammatory cytokine down regulating potentially damaging localised pro-inflammatory responses in the periodontium; including those driven by IL-17A

In order to investigate this hypothesis; the specific aims of this chapter were as follows:

1. To further evaluate the expression of IL-17E and its receptors in periodontal tissue samples from patients with chronic periodontitis patients and healthy subjects.

2. To evaluate the role of IL-17E in modulating the expression of cytokine expression by oral keratinocytes in response to stimulation with *P. gingivalis* and IL-17A.

5.2 Results

5.2.1 Analysis of IL-17E expression in periodontal tissues

5.2.1.1 Expression of IL-17E in periodontal tissues

Previously, the expression of IL-17E mRNA in periodontal tissues was confirmed. In addition, expression levels were found to be elevated in chronic periodontitis patients (Figure 4-4). However, the cell types responsible for IL-17E expression had not been determined. Therefore, immunohistochemical analysis using a mouse monoclonal anti-IL-17E antibody (Abcam® UK) was employed to investigate further.

Figure 5-1 shows IL-17E expression associated with blood vessels as well as invading leukocytes in tissues derived from chronic periodontitis patients. There was no evidence of IL-17E staining in the epithelium or connective tissue layers. This suggested that IL-17E is not expressed by oral keratinocytes or fibroblasts.

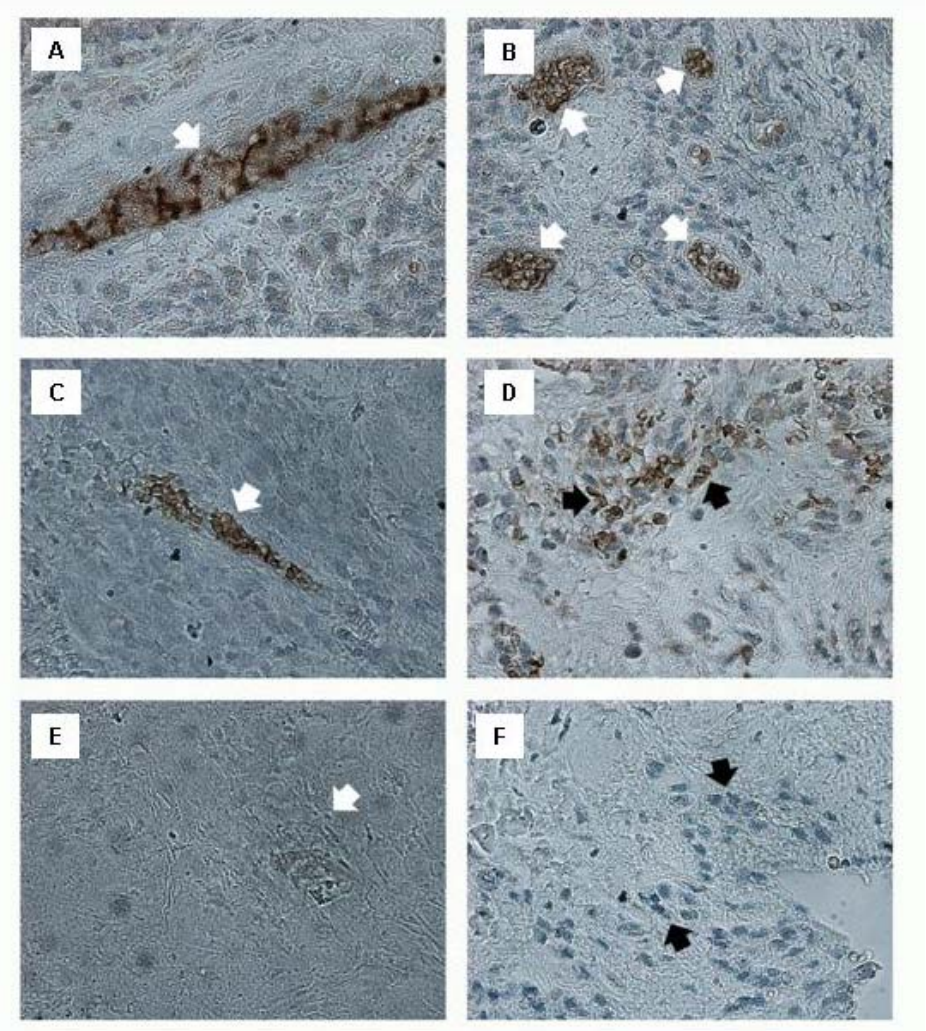

Figure 5-1: IL-17E expression associated with blood vessels and inflammatory cell infiltrates in diseased periodontal tissues

Expression of IL-17E was determined by immunohistochemical analysis using a mouse monoclonal anti-IL-17E antibody (Abcam® UK). The panels show representative photomicrographs of 5-µm-thick paraffin-embedded sections of periodontal tissue specimens obtained from chronic periodontitis patients. Positive staining was associated with blood vessels in tissue derived from 3 individual donors (white arrows) (**A** – **C**), as well as in invading leukocytes (black arrows) (**D**) (a representative image of findings observed in all 3 diseased tissue samples). An isotype control antibody was used to estimate non-specific binding (**E** and **F**). Original magnification x 100.

5.2.1.2 Expression of IL-17RB in periodontal tissues

Figure 5-1 showed that IL-17E was expressed in diseased periodontal tissues. Therefore to determine its target cell populations; the expression of its known receptor, IL-17RB, was also investigated. Again, immunohistochemical analysis a using a rabbit anti-IL-17RB antibody (Sigma-Aldrich®, UK) was employed.

Faint expression of IL-17RB was found in the epithelial layer of diseased periodontal tissue (Figure 5-2). In addition, strong IL-17RB staining was associated with immune cells found in diseased periodontal tissue (Figure 5-3).

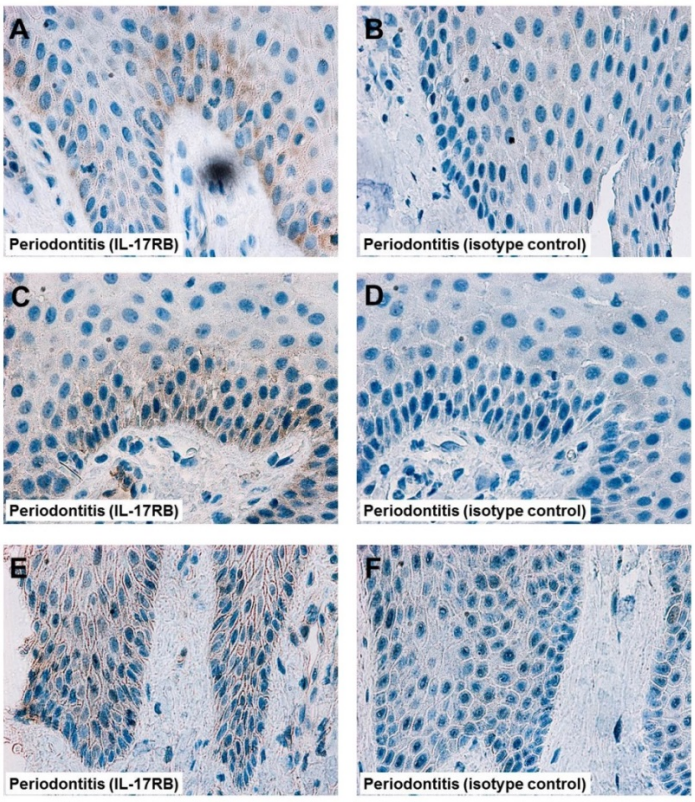

Figure 5-2: IL-17RB expression in the epithelial layer of diseased periodontal tissues

Expression of IL-17RB in periodontal tissues was determined by immunohistochemical analysis using a rabbit anti-IL-17RB antibody (Sigma-Aldrich®, UK). The panels show representative photomicrographs of 5 µm-thick paraffin-embedded sections of periodontal tissue specimens obtained from chronic periodontitis patients. Positive staining was associated with epithelial cells (**A**, **C** and **E**). An isotype control antibody was used to estimate non-specific binding (**B**, **D** and **F**). Original magnification x 400.

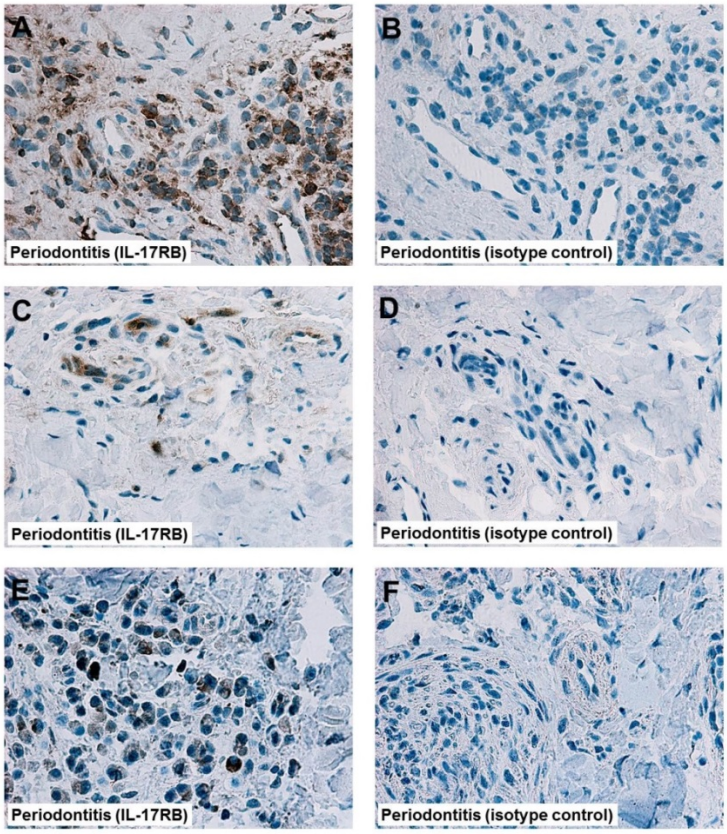

Figure 5-3: IL-17RB expression associated with immune cells in diseased periodontal tissues

Expression of IL-17RB in periodontal tissues was determined by immunohistochemical analysis using a rabbit anti-IL-17RB antibody (Sigma-Aldrich®, UK). Panels show representative photomicrographs of 5 µm-thick paraffin-embedded sections of periodontal tissue specimens obtained from chronic periodontitis patients. Positive staining was associated with invading leukocytes (**A**, **C** and **E**). An isotype control antibody was used to estimate non-specific binding (**B**, **D** and **F**). Original magnification x 400.

5.2.2 Analysis of IL-17 family cytokines in oral keratinocytes

5.2.2.1 Expression of IL-17 family cytokines mRNA in oral keratinocytes

The proposed expression of IL-17RB in the epithelial cell layer was indicative of oral keratinocytes being a target cell population for this cytokine. Therefore the role of oral keratinocytes in IL-17 family cytokine biology was investigated further. Basic PCR analysis investigating the expression of IL-17 family cytokine receptors in OKF6-TERT-2 cells (an oral keratinocyte cell line) revealed that all known IL-17 family receptors; including those purported to mediate IL-17E signalling; IL-17RB are expressed (Figure 5-4A). Interestingly, basic PCR analysis also indicated that oral keratinocytes may express IL-17B, C and D but do not express IL-17A, IL-17E and IL-17F (Figure 5-4B).

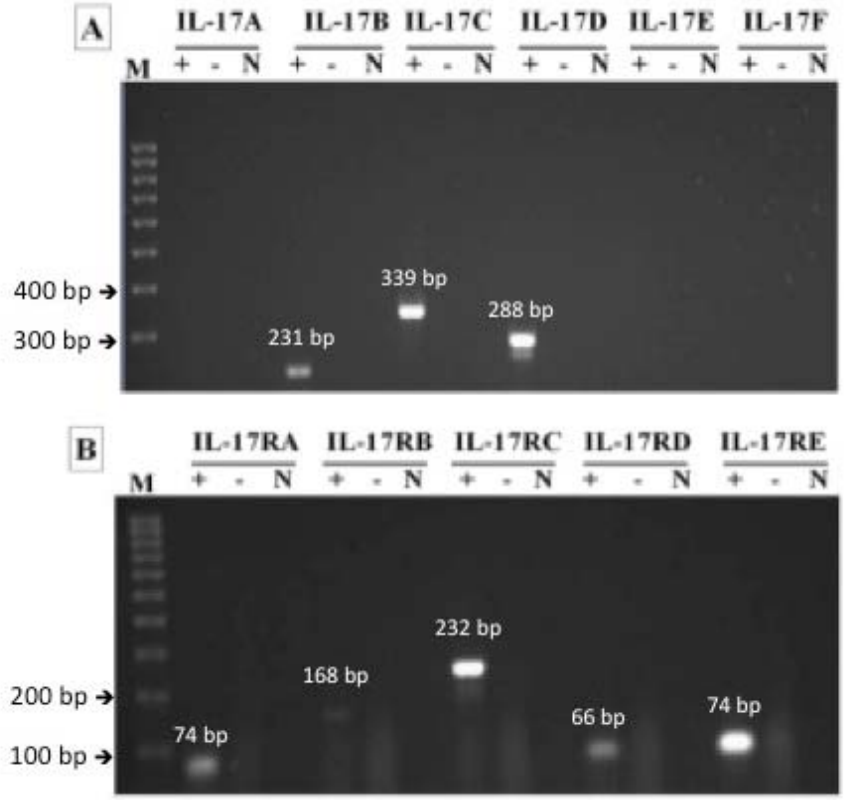

Figure 5-4: Expression of mRNA for IL-17 family cytokines and their receptors in OKF6/TERT-2 cells

Expression of IL-17 family cytokines and their receptors mRNA in OKF6/TERT-2 cells was investigated by basic PCR. The figures represent mRNA expression of IL-17 cytokine receptor family (**A**) and mRNA expression of IL-17 family cytokines (**B**). The 100 base pair DNA ladder was used as reference. All bands ran at reported sizes for genes of interest (Table 2-5). M = lane containing 100 base pair DNA ladder; + = PCR samples with cDNA; - = PCR samples with water in place of cDNA; and N = PCR with samples from no-RT (reverse transcriptase) controls.

Figure 5-4B showed that IL-17E mRNA was not expressed by OKF6/TERT-2 oral keratinocytes. However, these cells were unstimulated. Therefore to determine whether bacterial challenge with a known periodontal pathogen would induce expression of IL-17E and other IL-17 family cytokines the *in vitro* model of *P. gingivalis* infection described previously was employed.

Figure 5-5 shows that IL-17A, IL-17E and IL-17F mRNA are not expressed by OKF6/TERT-2 oral keratinocytes; even after stimulation with a live *P. gingivalis* monospecies biofilm. In addition, the real-time analysis confirmed that IL-17B, IL-17C and IL-17D are expressed by OKF6/TERT-2 oral keratinocytes. However, expression of none of these cytokines was upregulated in response to stimulation by a live *P. gingivalis* monospecies biofilm.

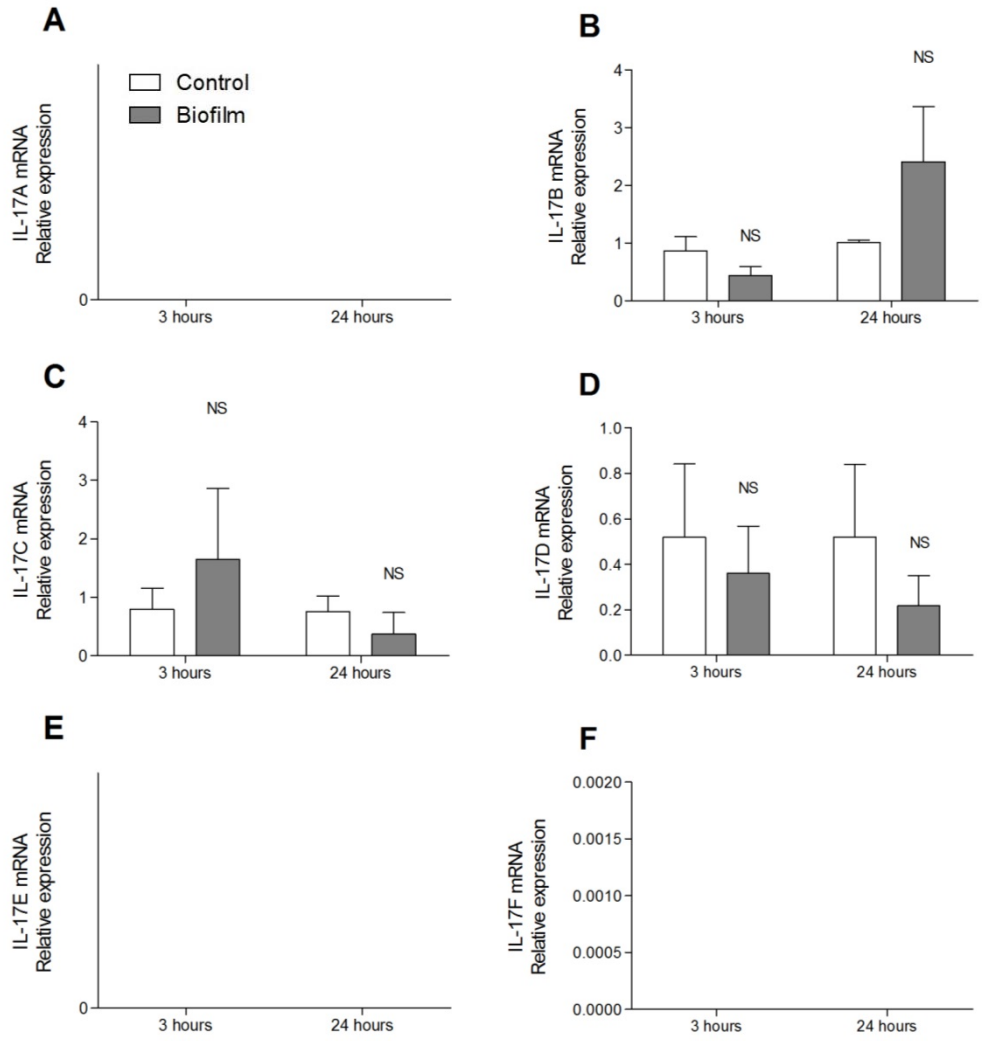

Figure 5-5: The effect of a live *P. gingivalis* monospecies biofilm on IL-17 family cytokine mRNA expression by OKF6/TERT-2 cells

Quantification of IL-17 family cytokines mRNA expression (**A** – **F**) in OKF6/TERT-2 cells was performed by real-time PCR. The bars represent mean relative expression ($2^{-\Delta\Delta CT}$) of IL-17 family cytokine mRNA in unstimulated (Control) and a live *P. gingivalis* monospecies biofilm (Biofilm) stimulated OKF6/TERT-2 cells at 3 and 24 h. RNA polymerase II was used as a reference gene. The data was generated from duplicate wells of three independent experiments. Statistical analyses were carried out using the independent *t*-test (IBM SPSS Statistics, version 19) on the natural log transformed IL-17 family cytokines mRNA relative expression ($2^{-\Delta\Delta CT}$) values. NS = not significant ($p > 0.05$). Error bars indicate the standard error of the mean (SEM).

As the basic PCR analysis suggested that oral keratinocytes express both IL-17RA and IL-17RB (reported to be required for IL-17E signalling) we also investigated if *P. gingivalis* induced elevated expression of these receptors. Figure 5-6 shows that OKF6/TERT-2 cells constitutively express both IL-17RA and IL-17RB mRNA and *P. gingivalis* does not induce changes in mRNA expression.

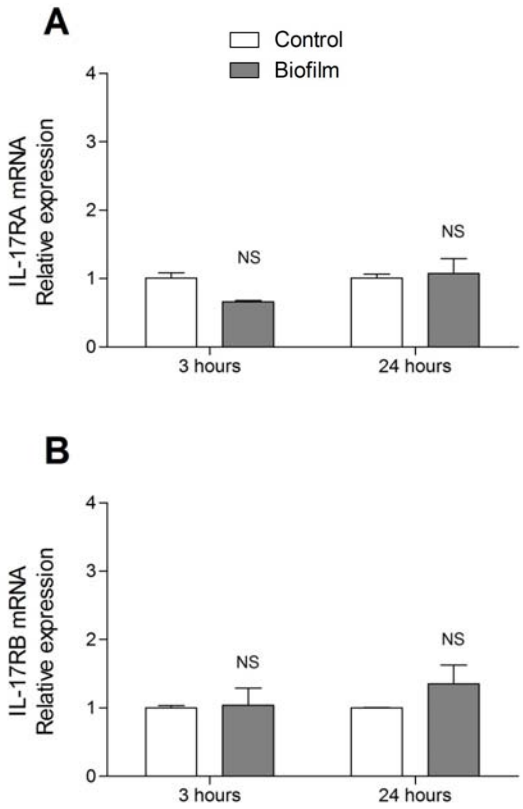

Figure 5-6: The effect of a live *P. gingivalis* monospecies biofilm on IL-17RA and IL-17RB mRNA expression by OKF6/TERT-2 cells

Quantification of IL-17RA and IL-17B mRNA expression in OKF6/TERT-2 cells was performed by real-time PCR. The bars represent mean relative expression ($2^{-\Delta\Delta CT}$) of IL-17RA (**A**) and IL-17RB (**B**) mRNAs in unstimulated (Control) and a live *P. gingivalis* monospecies biofilm (Biofilm) stimulated OKF6/TERT-2 cells at 3 and 24 h. RNA polymerase II was used as a reference gene. The data was generated from duplicate wells of three independent experiments. Statistical analyses were carried out using the independent *t*-test (IBM SPSS Statistics, version 19) on the natural log transformed IL-17 family cytokines mRNA relative expression ($2^{-\Delta\Delta CT}$) values. NS = not significant ($p > 0.05$). Error bars indicate the standard error of the mean (SEM).

5.2.2.2 IL-17E negatively regulates *P. gingivalis* induced chemokine expression by oral keratinocytes

The fact that oral keratinocytes express IL-17RA and IL-17RB indicated that these cells were a target for IL-17E signalling. Literature suggests that IL-17E can act as a negative regulator of bacterially induced inflammatory mediator expression by numerous cell types including $CD4^+$ T cells and macrophages (Stolfi, *et al.*, 2011; Zaph, *et al.*, 2008). Therefore, the effect of IL-17E on *P. gingivalis* induced expression of chemokines by oral keratinocytes was investigated *in vitro*.

Figure 5-7 showed that rhIL-17E alone had no effect on OKF6/TERT-2 cells expression of IL-8 and CXCL5. In contrast, stimulation with a live *P. gingivalis* monospecies biofilm induced increased expression of both chemokines at the mRNA and protein level. Interestingly, however, exposure of OKF6/TERT-2 cells to rhIL-17E 30 min prior to stimulation with a live *P. gingivalis* monospecies biofilm led to a significant decrease in the *P. gingivalis* induced expression of both chemokines.

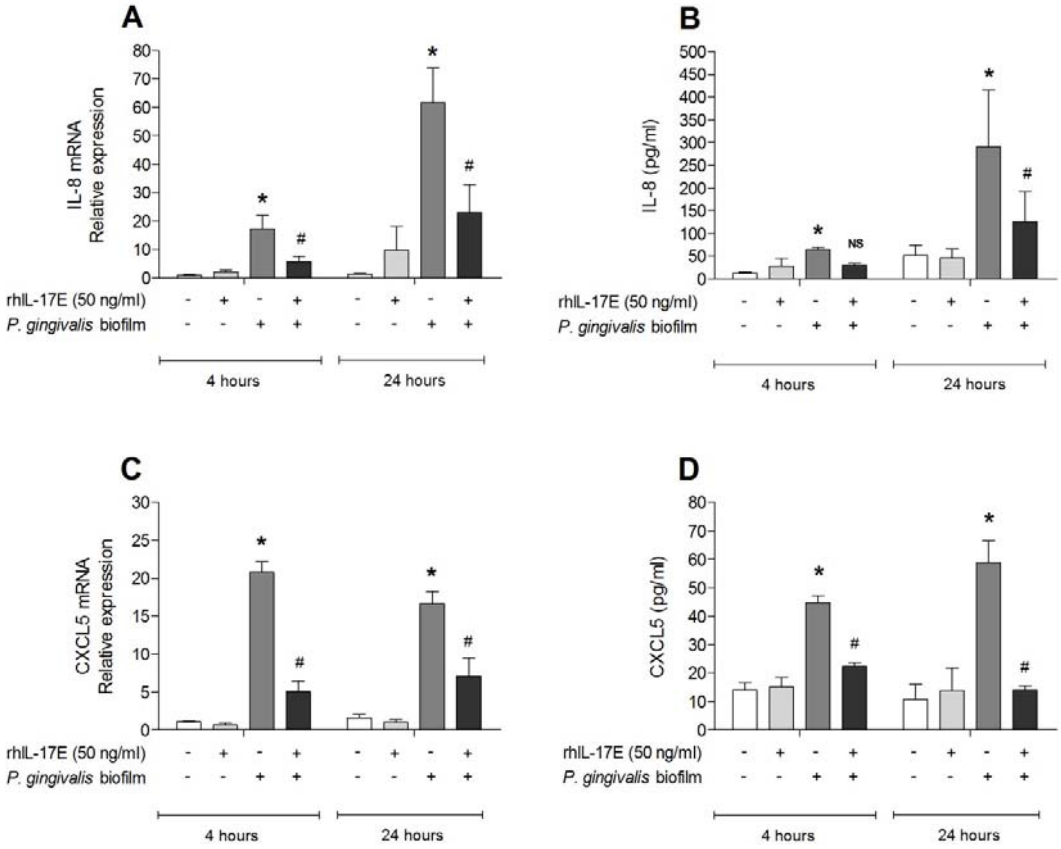

Figure 5-7: Effect of IL-17E on *P. gingivalis* induced expression of CXCL8 (IL-8) and CXCL5 by OKF6/TERT-2 cells

Quantification of IL-8 and CXCL5 mRNA expression and supernatant protein levels were performed by real-time PCR and ELISA respectively. The bars represent mean relative mRNA expression ($2^{-\Delta\Delta CT}$) of IL-8 (**A**) and CXCL5 (**C**), and mean supernatant level of IL-8 (**B**) and CXCL5 (**D**) in three stimulation conditions of OKF6/TERT-2 cells at 4 and 24 h: rhIL-17E (50 ng/ml), biofilm, or combination of rhIL-17E (50 ng/ml) and biofilm. Unstimulated wells acted as a control. For real-time PCR analysis GAPDH was used as a reference gene. The data was generated from duplicate wells of three independent experiments. Statistical analysis of ELISA and real- time PCR data were performed on the natural log transformed values using the ANOVA with a Bonferroni correction (IBM SPSS Statistics, version 19). * = compare to control ($p < 0.05$); $^{\#}$ = compare to biofilm only ($p < 0.05$); and NS = not significant. Error bars indicate the standard error of the mean (SEM).

5.2.2.3 IL-17E negatively regulates IL-17A induced IL-8 expression by oral keratinocytes

Previous studies from our laboratory have demonstrated that IL-17A induces a pro-inflammatory expression profile in OKF6/TERT-2 cells; including promotion of IL-8 and CXCL5 expression (Culshaw *et al*, unpublished). The negative correlations between levels of IL-17A and IL-17E and clinical parameters of periodontal disease described previously along with the findings in Figure 5-7 led to the hypothesis that IL-17E may also negatively regulate the IL-17A induced expression of chemokines by oral keratinocytes. This was therefore investigated *in vitro*.

In the first instance OKF6/TERT-2 cells were stimulated concurrently with rhIL-17A and rhIL-17E. Figure 5-8A shows stimulation of OKF6/TERT-2 cells with rhIL-17A alone for 24 h induced significant release of IL-8 ($p < 0.01$). In contrast, stimulation with rhIL-17E alone had no significant effect on IL-8 release. A linear analysis showed there was a dose-dependent decrease in rhIL-17A-induced IL-8 release from OKF6/TERT-2 cells when stimulated concomitantly with varying concentrations of rhIL-17E. However, post-test analysis revealed a significant inhibitory effect only with 400 ng/ml rhIL-17E ($p < 0.01$).

In the second instance OKF6-TERT-2 cells were stimulated with varying concentrations of rhIL-17E for 30 min prior to stimulation with rhIL-17A. Figure 5-8B shows stimulation with rhIL-17A alone for 24 h induced significant release of IL-8 ($p < 0.01$). The ANOVA analysis showed there was a dose-dependent decrease in rhIL-17A-induced IL-8 release from OKF6/TERT-2 cells when stimulated with varying concentrations of rhIL-17E 30 min prior to rhIL-17A ($p < 0.01$). On this occasion, a significant inhibition of rhIL-17A induced IL-8 release was observed with all concentrations of rhIL-17E. Real-time PCR analysis was employed to determine whether the inhibitory effect of IL-17E was mediated at the transcriptional level. Figure 5-8C shows that stimulation of OKF6/TERT-2 cells with rhIL-17A alone induced a significant increase in IL-8 mRNA expression in comparison to control unstimulated cells. Furthermore, 30 min pre-stimulation with 10 and 50 ng/ml rhIL-17E reduced the rhIL-17A induced upregulation of IL-8 mRNA expression. However, this reduction was only significant when cells were pre-stimulated for 30 min with 50 ng/ml rhIL-17E.

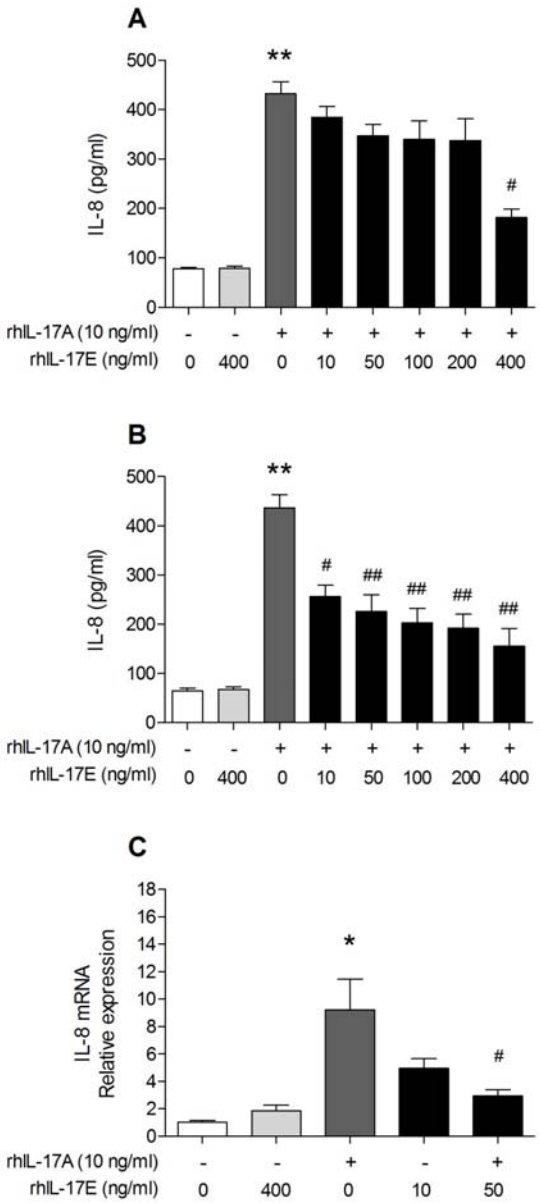

Figure 5-8: Effect of IL-17E on IL-17A induced expression of CXCL8 (IL-8) by OKF6/TERT-2 cells

Quantification of IL-8 mRNA expression and IL-8 bathing supernatant protein levels were performed by real-time PCR and ELISA respectively. (**A)** The bars represent mean supernatant level of IL-8 release from OKF6/TERT-2 cells stimulated concomitantly with combination of rhIL-17A and rhIL-17E (see x axis for concentration). (**B)** The bars represent mean supernatant level of IL-8 release from OKF6/TERT-2 cells pre-incubated with rhIL-17E for 30 min prior to stimulation with rhIL-17A (see x axis for concentrations). (**C)** The bars represent mean relative mRNA expression ($2^{-\Delta\Delta CT}$) of IL-8 from OKF6/TERT-2 cells pre-incubated with rhIL-17E for 30 min prior to stimulation with rhIL-17A (see x axis for concentrations). Unstimulated wells acted as a control. The data was generated from duplicate wells of three independent experiments. For real-time PCR analysis GAPDH was used as a reference gene. Statistical analysis of ELISA and real-time PCR data were performed on the natural log transformed values using the ANOVA with a Bonferroni correction (IBM SPSS Statistics, version 19). * = compare to control (* = $p < 0.05$, ** = $p < 0.01$); and $^{\#}$ = compare to IL-17A (10 ng/ml) only ($^{\#}$ = $p < 0.05$, $^{\#\#}$= $p < 0.01$). Error bars indicate the standard error of the mean (SEM).

5.2.2.4 IL-17E negatively regulates the IL-17A induced response of oral keratinocytes through NF-κB mediated pathways

Previous research has demonstrated that IL-17E negatively regulates expression of cytokines (i.e., IL-1ß, IL-6, IL-12 and TNF-α) by LPS and peptidoglycan stimulated $CD4^+$ T cells. This inhibition of expression occurs through induction of SOCS3 (Suppressor of cytokine signalling 3) via p38 MAP kinase activation (Caruso*, et al.*, 2009b). Evidence suggests that SOCS3 expression limits the activation of NF-κB in granulocytes (Chhabra *et al.*, 2014). We therefore investigated the effect of IL-17A/IL-17E on intracellular levels of two phosphorylated forms of the NF-κB p65 subunit implicated in the process of nuclear translocation, and therefore NF-κB p65 subunit activation, using the FACE™ NF-κB p65 Profiler (Active Motif, UK).

Figure 5-9A shows stimulation of OKF6/TERT-2 cells with 10 ng/ml rhIL-17A caused significantly elevated phosphorylation of the NF-κB p65 subunit at serine 468 ($p < 0.05$). Furthermore, there was a significant decrease in rhIL-17A induced NF-κB P65 serine 468 subunit phosphorylation when 50 ng/ml rhIL-17E was added to the cultures 30 min prior ($p < 0.05$). Likewise, stimulation of OKF6/TERT-2 cells with 10 ng/ml rhIL-17A caused significantly elevated phosphorylation of the NF-κB p65 subunit at serine 536 ($p < 0.05$) (Figure 5-9B). In addition, there was a significant decrease in rhIL-17A induced NF-κB P65 serine 536 subunit phosphorylation when 50 ng/ml rhIL-17E was added to the cultures 30 min prior ($p < 0.05$) (Figure 5-9B).

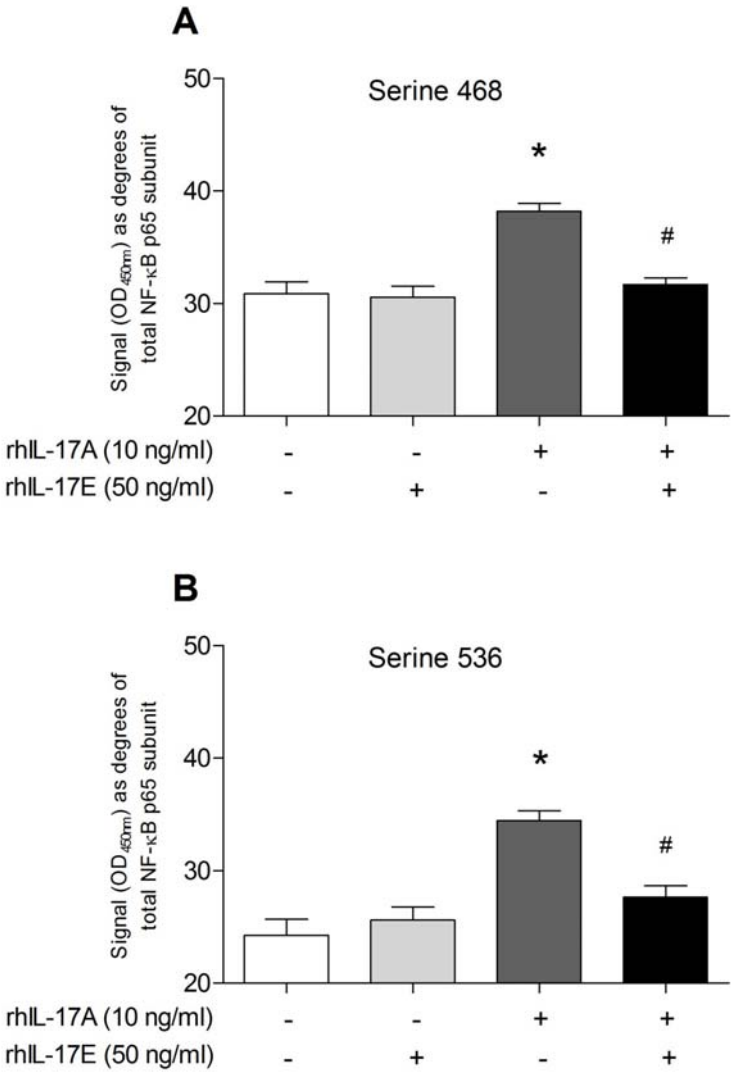

Figure 5-9: Effect of IL-17E on IL-17A induced phosphorylation of the NF-κB p65 subunit at serine 468 and serine 536 by OKF6/TERT-2 cells

Quantification of NF-κB p65 subunit phosphorylation was carried out using the FACE™ NF-κB p65 Profiler (Active Motif, UK). (**A**) The bars represent the mean angular transformed % of NF-κB p65 subunit phosphorylated at serine 468 pre-incubated with IL-17E for 30 min prior to stimulation with IL-17A (see x axis for concentrations). (**B**) The bar represent mean angular transformed % of NF-κB p65 subunit phosphorylated at serine 536 pre-incubated with IL-17E for 30 min prior to stimulation with IL-17A (see x axis for concentrations). Unstimulated wells acted as a control. The data was generated from duplicate wells of three independent experiments. Statistical analysis was performed on angular transformed data using the ANOVA with a Bonferroni correction (IBM SPSS Statistics, version 19). * = compare to control (* = $p < 0.05$); and $^{\#}$ = compare to IL-17A (10 ng/ml) only ($^{\#}$ = $p < 0.05$). Error bars indicate the standard error of the mean (SEM).

5.3 Discussion

Immunohistochemical analysis revealed for the first time expression of IL-17E and IL-17RB in gingival tissue samples of chronic periodontitis patients (Figure 5-1 and 5-2). These IL-17E positive stained cells were found to associate with blood vessels as well as invading leukocytes, but not epithelial cells. In contrast, IL-17RB was observed to be expressed by invading leukocytes in gingival tissue samples of chronic periodontitis patients and at lower levels in epithelial cells. The expression of IL-17E in the periodontium was confirmed at the mRNA level in gingival tissue samples (Figure 4-4). In addition, both real-time and basic PCR analysis, using different primer sets, demonstrated a lack of IL-17E mRNA expression by oral keratinocytes (Figure 5-4 and 5-5), which was in agreement with the immunohistochemical findings (Figure 5-1). In contrast, both real-time and basic PCR analysis, again using different primer sets, showed that IL-17RB mRNA was expressed by oral keratinocytes (Figure 5-4 and 5-6); which was in agreement with the immunohistochemical findings (Figure 5-2). The findings presented in this thesis are in line with previous findings that showed immunochemical staining of IL-17E in endothelial cells and invading inflammatory cells in chronic inflammatory disease tissue samples from the cerebrum of multiple sclerosis patients, bronchus of asthma patients, coronary artery of atherosclerosis patients, skin of atopic dermatitis patients and colon of ulcerative colitis patients (Corrigan *et al.*, 2011; de Boer *et al.*, 2010; Hvid *et al.*, 2011; Letuve*, et al.*, 2006; Sonobe*, et al.*, 2009). However, in contrast to our findings, immunohistochemical staining of IL-17E was observed in epithelial cells of atopic dermatitis patients and colon of ulcerative colitis patients (Caruso*, et al.*, 2009b; Hvid*, et al.*, 2011). Immunohistochemical staining of IL-17RB has also been observed in endothelial cells, inflammatory cells and epithelial cells in tissue from the artery of atherosclerosis patients, skin of atopic dermatitis patients and skin of psoriasis patients (Corrigan*, et al.*, 2011; de Boer*, et al.*, 2010; Hvid*, et al.*, 2011; Kim *et al.*, 2013a).

In this study IL-17E and IL-17RB protein was detected immunohistochemically using a mouse monoclonal anti-IL-17E antibody (Abcam®, UK) and a rabbit anti-IL17RB antibody (Sigma-Aldrich®, UK), respectively. Use of the mouse monoclonal anti-IL-17E antibody (Abcam®, UK) has not been previously reported in the literature. In contrast, use of the rabbit anti-IL17RB antibody (Sigma-

Aldrich®, UK) has been reported in a previous immunohistochemical study (de Boer, *et al.*, 2010). Due to the limited number of publications using these antibodies, a limitation of this study is that specificity was not investigated in as greater detail as the IL-33 antibody (Section 3.3). An appropriate isotype control for each antibody was used to exclude the non-specific binding. However, we did not have appropriate tissue samples to use as a positive control in this study. In addition, due to budget and time restrictions, we were also unable to undertake pre-absorption studies. Therefore, the specificity of these antibodies has only been partially confirmed. Further studies using a suitable positive control, pre-absorption with recombinant protein and use of several antibodies against IL-17E are required to confirm fully specificity (Burry, 2000, 2011). Nonetheless, the pattern of IL-17E staining in periodontal tissue samples is comparable with IL-17E staining in other diseased tissue samples (Corrigan, *et al.*, 2011; de Boer, *et al.*, 2010; Letuve, *et al.*, 2006). In addition, the pattern of IL-17RB cell staining in epithelium and connective tissue layer of periodontal tissue samples is comparable with the archived staining in normal oral mucosa catalogued in the human protein atlas ("The human protein atlas (IL17RB)," 2013). The human protein atlas is a publicly available database of comprehensive antibody-based protein expression profiles in normal and cancer tissues certified by pathologists (Uhlen, *et al.*, 2005; Uhlen, *et al.*, 2010). In this study, we observed IL-17E staining in invading leukocytes (determined morphologically). At present, the subset(s) of lymphocytes which express IL-17E in diseased periodontal tissue remain undetermined. Future studies are required to delineate in detail the lymphocyte populations that express IL-17E using double staining with known markers of lymphocyte subsets; e.g., CD3 (T cell marker) and CD20 (B cell marker) (Batran *et al.*, 2013).

Our data show increased mRNA expression of IL-17 family cytokines (except IL-17B) in tissue derived from patients with chronic periodontitis (Section 4.2.2). For many of these cytokines the cell types responsible for expression have yet to be delineated. As oral keratinocytes are one of the first cell types within the periodontium to encounter periodontal pathogens, expression of these cytokines by these cells was investigated further. Our basic PCR analysis shows the pattern of mRNA expression of IL-17 family receptors and cytokines by unstimulated OKF6/TERT-2 cells. mRNA encoding all IL-17 family receptors were found to be

expressed by unstimulated OKF6/TERT-2 cells. However only mRNA encoding IL-17B, IL-17C and IL-17D was expressed by unstimulated OKF6/TERT-2 cells. These findings were confirmed by real-time PCR analysis. However, expression of IL-17B, IL-17C, IL-17D, IL-17RA and IL-17RB mRNA was not upregulated in response to *P. gingivalis* stimulation. In line with our findings, IL-17A mRNA was found not to be expressed by human oral keratinocytes (Al-Samadi *et al.*, 2014). However, IL-17A, IL-17E and IL-17F were found to be expressed in other epithelial cells, including corneal epithelial cells, lung epithelial cells and intestinal/colonic epithelial cells (Angkasekwinai, *et al.*, 2007; Arranz-Valsero *et al.*, 2013; Ishigame, *et al.*, 2009; Suzuki, *et al.*, 2007; Zaph, *et al.*, 2008). In agreement with our findings, IL-17B and IL-17C are also expressed in other epithelial cells, including mammary epithelial cells and intestinal epithelial cells (Huang *et al.*, 2013; Song *et al.*, 2014). However, no studies have previously reported expression of IL-17D in any epithelial cell type. As for the IL-17 receptor family, our findings are in line with literature that showed expression of IL-17RA, IL-17RB, IL-17RC and IL-17E in various types of epithelial cells, including gingival epithelial cells, oral keratinocytes, mammary epithelial cells, corneal epithelial cells, and intestinal epithelial cells (Al-Samadi, *et al.*, 2014; Arranz-Valsero, *et al.*, 2013; Huang, *et al.*, 2013; Song, *et al.*, 2014; Takahashi *et al.*, 2011). In contrast, no specific reference was found on the expression of IL-17RD on epithelial cells.

Our study shows IL-17E is present in gingival tissue samples of chronic periodontitis patients. Although oral keratinocytes were found not to express IL-17E, they do express IL-17RA and IL-17RB, the receptors required for IL-17E signalling (Ely, *et al.*, 2009; Hymowitz, *et al.*, 2001; Lee, *et al.*, 2001; Shi, *et al.*, 2000). This indicates that oral keratinocytes are a target for IL-17E signalling. Therefore, the role of IL-17E in oral keratinocyte mediated innate immune responses was investigated *in vitro*. Previous literature had demonstrated that IL-17E could regulate the innate immune response of bacterial LPS stimulated myeloid cells (Caruso, *et al.*, 2009b). Therefore, similar *in vitro* studies were replicated using the OKF6/TERT-2 cell line. In our investigations *P. gingivalis* upregulated the expression of IL-8 and CXCL5 in accordance with previous studies (Barksby *et al.*, 2009; Huang *et al.*, 1998). In contrast, IL-17E alone had no effect on IL-8 expression. However, IL-17E

inhibited the *P. gingivalis* induced expression of both these chemokines and this inhibition was evident at the transcriptional level (Figure 5-7). In addition, IL-17E also inhibited IL-17A-induced expression of IL-8 by oral keratinocytes, which was again mediated at the transcriptional level (Figure 5-8). This therefore implies that IL-17E can directly negatively regulate the pro-inflammatory functions of IL-17A.

IL-17A promotes recruitment of neutrophils and monocytes by inducing chemokine expression from a variety of epithelial cells (Shahrara *et al.*, 2009). Commensurate with these findings IL-17A induced oral keratinocytes to express IL-8. However, the notable inhibition of IL-17A and *P gingivalis* induced expression of IL-8 by IL-17E suggests that IL-17E can regulate neutrophil responses in early inflammation. It is known that effective neutrophil responses are important for periodontal health (Kinane *et al.*, 2011). Although the principle role of neutrophils is protection, these cells can also release a variety of factors that can cause tissue damage, including reactive oxygen species, collagenases and other proteases (Nathan, 2006; Scott & Krauss, 2012). Chronic and persistent activation of neutrophils by a microbial biofilm in the periodontium can therefore lead to gingival tissue damage and periodontal disease (Nussbaum & Shapira, 2011). Therefore it is important the neutrophil migration and activation are tightly controlled. The literature shows increased activities of neutrophils are associated with periodontal disease (Kinane*, et al.*, 2011; Liu *et al.*, 2001). It is known that IL-8 is important chemoattractant for neutrophils and IL-8 expression by host cells, such as oral keratinocytes and fibroblasts is induced by IL-17A and *P. gingivalis* (Luo*, et al.*, 2012; Macpherson *et al.*, 2014; Mahanonda *et al.*, 2008; Wang & Ohura, 2002). Since our data shows IL-17E could negatively regulate the expression of IL-8 by oral keratinocytes in response to stimulation with IL-17A and *P. gingivalis*, the presence of IL-17E in periodontal tissue may be important in regulating neutrophil migration and therefore be an important immunoregulatory control mechanism during early inflammation.

In our studies we further investigated the potential mechanism by which IL-17E can inhibit IL-17A induced expression of IL-8 by oral keratinocytes. IL-17A was shown to promote IL-8 expression via intracellular signalling pathways leading to the phosphorylation of the NF-κB p65 subunit at serine 536 and serine 468

(Figure 5-9). Phosphorylation of serine 536 of the NF-κB p65 subunit has been demonstrated to be important in transcription of the IL-8 gene (Buss *et al.*, 2004), while phosphorylation at serine 468 has been shown to have an important role for NF-κB ubiquitination and degradation (Geng *et al.*, 2009). However, despite their opposing functions, phosphorylation at serine 536 and 468 occurs simultaneously (Mattioli *et al.*, 2004).

NF-κB is a complex of proteins responsible for cytokine production and cell survival through control over DNA transcription (Memet, 2006). The NF-κB family of proteins has 5 members including; p50, p52, RelA (p65), c-Rel and RelB (Hoffmann *et al.*, 2006; Moynagh, 2005). These proteins exist in homo- or hetero-dimeric forms and remain inactive in the cell cytoplasm by binding to IκBs. IκBs are inhibitory molecules that prevent translocation of NF-κB into the nucleus (Memet, 2006). Activation of either the classical or alternative pathway leads to ubiquitination and degradation of IκBs through phosphorylation events that free NF-κB for nuclear translocation (Lawrence, 2009; Memet, 2006). It is known that the classical pathway is stimulated by microorganisms and cytokines (including IL-17A) which results in activation of the NF-κB p65 subunit (RelA) complex (Karin & Ben-Neriah, 2000; Olsson Akefeldt *et al.*, 2013; Xie *et al.*, 2010). In contrast, proteins such as CD40 ligand, RANKL and BAFF (B-cell activating factor) are known to stimulate the alternative pathway which results in activation of the NF-κB RelB complex (Lawrence, 2009).

In line with literature, our study shows increased phosphorylation of the NF-κB p65 subunit at serine 536 and serine 468 by oral keratinocytes occurs upon stimulation with IL-17A (Figure 5-9). Interestingly, the data shows that when oral keratinocytes are primed with IL-17E prior to IL-17A stimulation, the phosphorylation of the NF-κB p65 subunit at serine 536 and serine 468 is decreased. There is no evidence in the literature demonstrating that IL-17E can directly inhibit NF-κB phosphorylation. The mechanism by which IL-17E promotes this inhibitory effect is currently unknown. It could be due to the result of competing for the same receptor as both IL-17A and IL-17E require IL-17RA for intracellular signalling (Ely, *et al.*, 2009; Hymowitz, *et al.*, 2001; Toy, *et al.*, 2006). However, this remains a matter of conjecture and further study is required to understand in detail the pathway(s) involved.

Based on the findings in this study, and the current literature, it could be argued that increased expression of IL-17E in periodontal tissue occurs as an attempt to down-regulate the damaging localised periodontal inflammatory response. Th17/IL-17A responses have been shown to be associated with inflammatory tissue damage in chronic inflammatory diseases, including periodontal disease, rheumatoid arthritis and inflammatory bowel diseases (Adibrad, *et al.*, 2012; Feng *et al.*, 2011; Leipe *et al.*, 2010). Since *in vitro* data show that IL-17E is capable of inhibiting Th17 responses via IL-4 and IL-13 (Angkasekwinai, *et al.*, 2007; Cooney, *et al.*, 2011; Harrington, *et al.*, 2005; Kleinschek, *et al.*, 2007; Park, *et al.*, 2005; Wang, *et al.*, 2007b), it is possible that one role of IL-17E in inflammatory disease pathogenesis is to inhibit Th17/IL-17A pro-inflammatory responses. In addition, *in vivo* studies using a collagen-induced arthritis model showed that increased expression of IL-17A occurs during early stage disease, whereas increased expression of IL-17E occurs during the later stages and is associated with enhanced production of IL-4 (Kaiwen *et al.*, 2012). Additionally, *in vivo*, using a *Chlamydia muridarum* lung infection mouse model, elevated IL-17E expression in the lung was shown to occur during the latter stages of infection, whilst IL-17A expression occurred during the early stages (Mosolygo *et al.*, 2013). These studies add weight to the hypothesis that IL-17E expression occurs as a mechanism to regulate Th17/IL-17A responses.

In summary, our data show expression of IL-17E and it receptors (IL-17RA and IL-17RB) in periodontal tissues. This indicates possible involvement of IL-17E in pathogenesis of periodontal disease. *In vitro* analysis shows that IL-17E is capable of negatively regulating the IL-17A and *P. gingivalis* induced expression of chemokines (i.e., IL-8 and CXCL5) by oral keratinocytes. In addition, this negative regulation occurs at the transcriptional level and is mediated by inhibition of phosphorylation and therefore activation of the NF-κB p65 subunit. The finding that IL-17E acts as an anti-inflammatory cytokine in periodontal tissues may be of major importance for studies into IL-17A/Th17 induced pathologies. However, further studies are required to look in detail at the mechanisms by which IL-17E perpetrates its anti-inflammatory effects.

Chapter 6: General discussion

The major findings of this study are as follows:

1. IL-33 and its receptors (ST2L and sST2) are expressed in gingival tissue samples and the expression of IL-33 (mRNA and protein), as well as sST2 (mRNA) is up-regulated in gingival tissue samples of chronic periodontitis patients.

2. IL-33 and its receptors (ST2L and sST2) are expressed in oral keratinocytes. In addition, the periodontal pathogen, *P. gingivalis,* is capable of inducing oral keratinocytes to upregulate the expression of IL-33.

3. There are associations between serum, GCF and saliva levels of IL-17 family cytokines and IL-10 with clinical outcomes of chronic periodontitis.

4. The clinical association studies suggest that IL-17A, IL-17F and the IL-17A/F heterodimer may have pro-inflammatory functions, whilst IL-17E may have anti-inflammatory functions in pathogenesis of chronic periodontitis.

5. IL-17E is capable of negatively regulating the IL-17A and *P. gingivalis* induced expression of chemokines (i.e., IL-8 and CXCL5) by oral keratinocytes.

6. IL-17E mediates its anti-inflammatory function at the transcriptional level by inhibiting activation and therefore nuclear translocation of the NF-κB p65 subunit in oral keratinocytes.

Cytokines are known to play an important role in maintaining tissue homeostasis. They are also shown to play roles in defence against pathogens by co-ordinating both innate and adaptive immune mechanisms. For examples, cytokines can induce increased expression of pattern recognition receptors (e.g., TLR's); promote chemotaxis of phagocytes (polymorphonuclear leukocytes and macrophages) and also promote activation and proliferation of T cell populations and antibody producing B lymphocytes (Espinassous, *et al.*, 2009; Modi, *et al.*, 1990; Takatsu, 1997; Taub, *et al.*, 1993). In addition, cytokines also play

important roles in regulating tissue repair. Indeed, cytokines can co-ordinate tissue re-modelling by mechanisms such as inducing extracellular matrix production by fibroblasts (Appleton, 1994).

Despite the important role cytokines play in tissue homeostasis and protection against pathogens, it is important that their activity occurs in a controlled 'balanced' manner. Cytokines are known to interact and function in networks (Balkwill & Burke, 1989; Nathan & Sporn, 1991). In simplistic terms, cytokines can be grouped into two classes: pro-inflammatory and anti-inflammatory. The biological activity of these two classes plays an important role in regulating cytokine networks and ensuring a 'balanced' response. The balanced activities between the two groups of cytokines are important in initiating the appropriate immune response against pathogenic threats whilst also maintaining the integrity of host tissues. However, numerous chronic inflammatory pathologies such as periodontal disease and rheumatoid arthritis are characterised by an excessive inflammatory responses. This dysregulated chronic inflammation is characterised by an unbalanced cytokine response (Graves, 2008; Kinane*, et al.*, 2011; McInnes & Liew, 2005; McInnes & Schett, 2007). In periodontal tissue, pro-inflammatory cytokines such as IL-1 and TNF-α have been shown to drive soft tissue destruction via promotion of MMP expression and activation by host cells (Birkedal-Hansen, 1993; Reynolds*, et al.*, 1994). In addition, cytokines such as IL-1, IL-6, IL-17A and TNF-α have also been shown to drive destruction of hard tissues by promoting excessive production of RANKL and inhibiting production of OPG, and thus promoting osteoclastogenesis (Quinn & Saleh, 2009). Therefore, long standing uncontrolled activity of pro-inflammatory cytokines leads to the excessive tissue destruction that manifests clinically in periodontal disease. Therefore, a dysregulated cytokine response plays an important role in the pathogenesis of periodontal disease.

The pathogenesis of periodontal disease is characterised by an excessive inflammatory response to pathogenic bacteria within dental plaque. This inflammation is coordinated by cytokines and chemokines. Indeed, dysregulated expression of cytokines is associated with inflammation of periodontal tissue. Periodontal pathogens such as *P. gingivalis* induce expression of numerous cytokines and chemokines by cells of the periodontium (Kinane*, et al.*, 2011).

The presence of *P. gingivalis* in dental plaque has been demonstrated to induce increased expression of IL-1α, IL-1ß, IL-8, IL-17A and TNF-α by various cell types such as epithelial cells and macrophages (Bostanci, *et al.*, 2007; Hirschfeld, *et al.*, 2001; Kocgozlu, *et al.*, 2009; Luo, *et al.*, 2012; Milward, *et al.*, 2013; Zhou, *et al.*, 2005). Furthermore, elevated levels of IL-1α, IL-1ß, IL-6, IL-8, IL-17A and TNF-α are associated with periodontal disease (Graves, 2008). Hence excessive levels of pro-inflammatory cytokines and chemokines are associated with disease outcome. However, the biological role of many of these mediators in the pathogenesis of periodontal disease remains unclear. Furthermore, it is now known that anti-inflammatory cytokines can act to regulate the inflammatory response within tissues and therefore it is now hypothesised that cytokines and chemokines act in 'networks' to ensure an appropriate and coordinated immune response. In terms of periodontal tissue we do not fully understand the complexity of these networks, how they are regulated in health and the factors that lead to a dysregulated response as observed in periodontal disease (Figure 6-1). Therefore, key cytokine networks still remain to be studied which will aid our understanding of the pathogenesis of periodontal diseases.

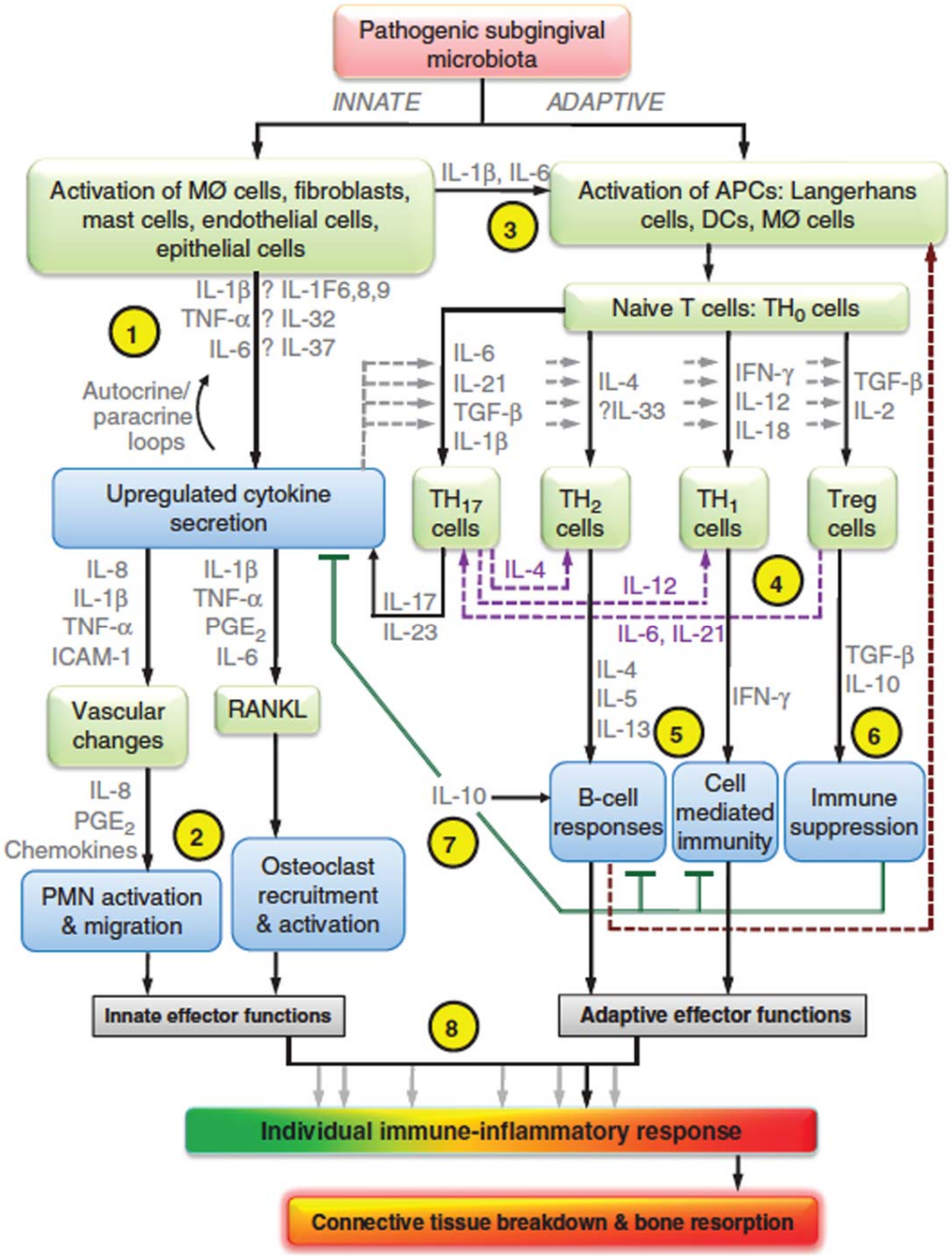

Figure 6-1: Proposed cytokine networks involved in co-ordinating the innate and adaptive arms of the periodontal immune response and their role in transition from periodontal health to disease

The presence of periodontal pathogens in the subgingiva activates innate and adaptive immune responses. These immune responses are regulated by networks of cytokines and chemokines and are tightly regulated. In certain individuals however, these networks are pushed toward pro-inflammatory pathways that are over-excessive for the perceived threat. Therefore, periodontal tissue destruction and bone resorption occur. The figure is taken from the Journal of Clinical Periodontology (Kinane, *et al.*, 2011). Permission to reproduce this figure has been granted by John Wiley & Sons, Inc.

As mentioned previously, the role of numerous individual cytokines in the pathogenesis of periodontal disease is still a matter of debate. Since IL-33 was shown to play a role in pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease and atopic dermatitis (Miller, 2011), we anticipated that IL-33 would play a role in periodontal disease pathogenesis. In our study, we found that IL-33 and its receptors (ST2L and sST2) are expressed in periodontal tissue. In addition, the expression of IL-33 (mRNA and protein) and sST2 (mRNA) are increased in diseased tissue. Furthermore, *in vitro* we showed that oral keratinocytes express IL-33, ST2L and sST2; and expression of IL-33 (protein and mRNA) is increased in these cells after stimulation with a live *P. gingivalis* monospecies biofilm. These findings suggested that IL-33 may have a pro-inflammatory role in periodontal disease. Indeed, previous studies have demonstrated that IL-33 can mediate tissue destruction and bone loss. For example, IL-33 was shown to promote bone resorption in a collagen induced arthritis mouse model (Palmer*, et al.*, 2009). In addition, serum levels of IL-33 and MMP-3 were positively correlated in rheumatoid arthritis patients, and the serum levels of IL-33 was found to associate with arthritic bone resorption (Xiangyang*, et al.*, 2012). However, whether IL-33 plays a similar role in destruction of periodontal tissues and alveolar bone remains to be elucidated. Indeed, despite evidence suggesting IL-33 mediates tissue destruction and bone loss in rheumatoid arthritis (Beltran*, et al.*, 2010; Louten*, et al.*, 2011; Xu*, et al.*, 2008), studies have also demonstrated that IL-33 may actually play a protective anti-inflammatory role in atherosclerosis, hepatitis, obesity and type 2 diabetes (Miller, 2011; Miller *et al.*, 2010; Miller *et al.*, 2008; Sanada*, et al.*, 2007; Volarevic *et al.*, 2012). The differences in the role of IL-33 in varying pathologies are currently unknown. However, it could be due to differential regulation in tissues. Furthermore, as cytokines operate in 'networks' the function of IL-33 may not also be dependent on the tissue in which it is expressed but also its interactions with other cytokines and chemokines. Therefore, an understanding of the role of a cytokine in a 'network' may be more important than understanding their roles in isolation.

The importance of investigating the relationship between networks of cytokines and disease was emphasised by our IL-17 family cytokine study. This study

showed that levels of IL-17A, IL-17F and IL-17A/F heterodimer are found increased in clinical samples (i.e., serum, GCF and saliva) of chronic periodontitis patients compared to healthy subjects, and levels of these cytokines were positively associated with clinical parameters for periodontal disease (CPD, CAL and BOP). In addition, levels of these cytokines positively correlated to each other. These findings suggested that IL-17A, IL-17F and the IL-17A/F heterodimer play a pro-inflammatory role in periodontal disease pathogenesis as has been demonstrated for other chronic inflammatory diseases (Iwakura, *et al.*, 2011; Kolls & Linden, 2004). For example, IL-17A and IL-17F were shown to induce increased expression of MMPs (e.g., MMP-1, MMP-3, MMP-8 and MMP-13) by chondrocytes and synovial cells (Tanigawa *et al.*, 2011; Toh *et al.*, 2010). MMPs are known to associate with destruction of soft tissue and cartilage in inflammatory conditions. *Ex vivo*, IL-17A was shown to induce released of MMP-1 and increased collagenase activity by synovium explant of rheumatoid arthritis (Chabaud *et al.*, 2000). In addition, injection of murine IL-17A into mouse knee joint was shown to degrade the joint cartilage (Chabaud *et al.*, 2001). IL-17A, via RANKL was also shown to stimulate osteoclastogenesis and bone erosion in arthritis mouse model (Lubberts *et al.*, 2003).

Interestingly, the study shows serum levels of IL-17E and IL-10 are decreased in patients with chronic periodontitis, and serum levels negatively correlate with clinical parameters (CPD, CAL and BOP). Furthermore, the study shows serum levels of IL-17E and IL-10 are negatively correlated with serum levels of IL-17A. Indeed, the serum IL-17A:IL-17E and IL-17A:IL-10 ratio both negatively correlated with all clinical parameters (CPD, CAL and BOP). These findings indicate that IL-17E and IL-10 play opposing roles to IL-17A in periodontal immunity. Indeed, it could be hypothesised that IL-17E and IL-10 act as anti-inflammatory cytokines as suggested by the literature (Caruso *et al.*, 2011; Moore, *et al.*, 2001; Sabat, *et al.*, 2010). Therefore, as IL-17A is a pro-inflammatory cytokine, it could be said that periodontal disease is characterised by increased levels of pro-inflammatory cytokines in conjunction with decreased levels of anti-inflammatory cytokines. The role of IL-10 as an anti-inflammatory cytokine in periodontal disease is well established (Al-Rasheed, *et al.*, 2003; Sasaki, *et al.*, 2004). The literature on IL-17E and periodontal disease is however

in its infancy. However, studies in other disease models have suggested an anti-inflammatory role for IL-17E (Caruso, *et al.*, 2011).

Studies investigating clinical associations between levels of mediators in biological fluids and disease state are useful for determining a role in disease pathogenesis. However, in order to fully understand the biological significance of such findings more detailed studies are required (i.e., *in vitro* and *in vivo*). Among the interesting findings in our clinical association study was the positive correlation between the serum IL-17A:IL-17E ratio and clinical parameters (CPD, CAL and BOP). Further investigations began to delineate the biological significance of this finding and *in vitro* it was shown that IL-17E negatively regulates NF-κB p65 mediated increased expression of IL-8 by oral keratinocytes stimulated with IL-17A. Furthermore, IL-17E also negatively regulated the *P. gingivalis* induced expression of IL-8 by oral keratinocytes. These findings indicate that IL-17A and IL-17E are true pro- and anti-inflammatory cytokines respectively. Previous studies similarly demonstrated that IL-17A induces increased release of IL-8 via activation of the NF-κB transcription factor in fibroblast-like synoviocytes (Hwang *et al.*, 2004). However, how IL-17E negatively regulates NF-κB p65 activation is not known. It is possible that IL-17E may have an indirect effect on NF-κB p65 activation as IL-17A and IL-17E share the same receptor (IL-17RA). Therefore, inhibition of activation could be simply due to the fact that IL-17E competes with IL-17A for the same receptor. However, further studies are required to delineate the mechanisms involved in more detail. Indeed, competition for receptors suggests that there is even more complex interplay between IL-17 family cytokines which we are yet to discover. Studies suggest that although IL-17A, IL-17F and IL-17A/F all have pro-inflammatory cytokine activity (Hu *et al.*, 2011), their ability to activate an innate immune response varies subtly due to differential affinities for IL-17RA (Kuestner, *et al.*, 2007; Liang, *et al.*, 2007; Wright, *et al.*, 2007). Hence, competition for the same receptor and differential binding affinities may be a further mechanism by which activity of IL-17 family cytokines are regulated. However, there is still a lot more to understand about individual members of the IL-17 family of cytokines and their receptors before we can begin to speculate how this family operates in a network to co-ordinate immune responses.

Recently a link between IL-33 and IL-17E has been discovered. Both IL-17E and IL-33 are known to induce a Th2 immune response (Eiwegger & Akdis, 2011). In addition, it was shown that elevated expression of IL-33 in the lung tissues of house dust mite allergy is IL-17E dependent (Gregory *et al.*, 2013). IL-17E and/or IL-33 are known to be capable of activating innate immune cells including natural helper cells (NHCs), multi-potent progenitor type 2 (MPP^{type2}) cells, nuocytes, and innate type 2 helper (Ih2) cells, and also promote a Th2 response by stimulating the release of IL-4, IL-5 and IL-13 (Saenz *et al.*, 2010a; Saenz, *et al.*, 2010b; Salimi *et al.*, 2013). Since IL-4 and IL-13 are known to inhibit Th17 cells response (Cooney, *et al.*, 2011; Harrington, *et al.*, 2005; Park, *et al.*, 2005), there is also a possibility that IL-33 and IL-17E can also inhibit the Th17 response. These findings suggest that the association between IL-17A, IL-17E and IL-33 in periodontal disease pathogenesis is an exciting area for further study. Indeed, *in vivo* studies using a mouse model of periodontal disease would be beneficial in determining the role of these cytokines individually and collectively in the pathogenesis of periodontal disease.

The Immune system is in dynamic equilibrium, regulated by networks of cytokines. Under normal physiologic conditions, the activities of these cytokine networks are balanced between activities of pro- and anti-inflammatory cytokines. However in chronic inflammatory diseases such as chronic periodontitis and rheumatoid arthritis, the balance of these cytokine network activities are found to tip towards pro-inflammatory (Preshaw & Taylor, 2011; Xu *et al.*, 2013). A complete understanding of this un-balanced cytokine network activity in periodontal disease is important if immune modulation therapy is to be considered for treatment of the disease. Immune modulation therapy could be used to potentially restore the balance between the pro- and anti-inflammatory network activities. Usually, immune modulators in the form of either recombinant, synthetic or natural preparations of protein mediator are used. There are already many immune modulators that are licenced for human use, for example: anakinra, recombinant IL-1RA (Kineret, Amgen Inc., CA) and infliximab, a chimeric monoclonal antibody against TNF-α (Remicade, Centocor, Inc., Malvem, PA). Immune modulation therapy has been used in the treatment of a variety of chronic inflammatory diseases. For example immune modulation targeting pro-inflammatory cytokines has been used in treating rheumatoid

arthritis. Anakinra (Kineret, Amgen Inc., CA) which is recombinant IL-1RA blocks the activity of IL-1 cytokines. In clinical trials Anakinra has been successful in treating rheumatoid arthritis and acute gout (Fleischmann *et al.*, 2006; So *et al.*, 2007). In addition, the TNFRp75-Fc fusion protein (Enbrel®, Enbrel, Immunex, Seattle), which is a fusion of soluble TNFRp75 (TNF receptor p75) and Fc portion of IgG1, and targets TNF-α has been developed. In clinical trial, subcutaneous injection of TNFRp75-Fc was shown to significantly reduce the severity of rheumatoid arthritis (Moreland *et al.*, 1997). However, using immune modulators to treat periodontal disease has not been as successful as other chronic inflammatory disease. In terms of periodontal disease studies looking at patients with both arthritis and periodontal disease seemed to show potential for immunomodulation therapies. Indeed, for rheumatoid arthritis patients who also had chronic periodontitis, intravenous infusion treatment with infliximab (anti-TNF-α) (Remicade, Centocor, Inc., Malvem, PA) reduced GCF levels of TNF-α and improved periodontal status (CPD, CAL and BOP) (Mayer *et al.*, 2009). Similarly, Kobayashi and colleagues (2014) showed tocilizumab (a monoclonal anti-IL-6 receptor antibody) treatment of patients with a combination of rheumatoid arthritis and chronic periodontitis also showed an improvement in periodontal disease status (CPD, CAL and BOP). However, it could be argued that these were indirect effects due to treatment of rheumatoid arthritis and not direct therapeutic effects on periodontal disease.

Treatment of periodontal disease in isolation using immunomodulation therapies has been a subject of investigation. In animal studies, anti-cytokine modulators were investigated using the *Macaca fascicularis* primate model of experimental periodontitis. Functional-blocking soluble receptors for IL-1 and TNF were injected to sites of periodontal destruction and revealed that injection of these anti-cytokine modulators reduced periodontitis related alveolar bone loss by 50 – 60 % (Assuma *et al.*, 1998; Delima *et al.*, 2001). In another studies, OPG (a soluble decoy receptor for RANKL) was used as an anti-cytokine modulator. A preparation of OPG-Fc fusion protein was intraperitoneally injected to the *A. actinomycetemcomitans* induced (oral inoculation) periodontitis mouse model. These studies showed treatment with OPG significantly inhibit the RANKL associated alveolar bone loss (Mahamed *et al.*, 2005; Teng, *et al.*, 2000). A study was also performed using a C-C motif chemokine receptor 2 (CCR2) antagonist to

treat periodontitis using a *P. gingivalis*-induced periodontitis mouse model. The study found that mice treated with CCR2 antagonist exhibited reduced alveolar bone loss compared to control mice (Barros *et al.*, 2011).

Despite successful studies investigating immune modulation therapies for periodontal disease in animal model, very few have advanced to human clinical trials. Doxycycline, a synthetic non-specific MMP inhibitor, has been investigated in human cohorts. Emingil and colleagues (2004) showed 6-month treatment with a sub-antimicrobial dose of doxycycline (SDD) (20 mg, twice a day) plus scaling and root planing resulted in significant improvement in clinical outcomes and this was associated with a reduction of MMP-8 levels in GCF. Similarly, Caton and colleagues (2000) showed significant improvement in clinical outcomes (CPD and CAL) of chronic periodontitis in patients treated for 9 months with SDD (20 mg, twice a day) plus scaling and root planing compared to a parallel placebo group. In addition to SDD, therapies targeting arachidonic acid metabolites (e.g., thromboxane, prostacyclins and prostaglandins) for treatment of periodontal disease have been investigated in human cohorts. Arachidonic acid metabolites are pro-inflammatory mediators known to associate with bone resorptive activity in periodontal disease (Williams *et al.*, 1989). Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and aspirin are cyclooxygenase inhibitors that block the formation of arachidonic acid metabolites. Chronic periodontitis patients treated for 6 months with flurbiprofen (NSAID) (50 mg, twice a day) plus dental prophylaxis (teeth cleaning) every three months presented with significant less alveolar bone loss compared to the parallel placebo group (Jeffcoat *et al.*, 1995). However the use of NSAIDs is associated with increased bleeding time, incidence of peptic ulcers and gastrointestinal bleeding (Lanza *et al.*, 2009). Hence, the use of these drugs to treat periodontitis has to be approached with care.

Immune modulation therapies may offer therapeutic strategies for treatment of periodontal diseases. However, at present our understanding of the role of cytokines in periodontal immunity and disease pathogenesis is still lacking compared to other diseases. Therefore, before anti-cytokine therapies can begin to be considered, studies, such as described in this thesis, are required to advance our basic knowledge of periodontal cytokine biology and provide a

platform for further research to identify rational targets for therapeutic interventions.

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