The Role of the Alpha 7 Nicotinic Receptor In Modulating the *Porphyromonas gingivalis*-Induced Expression Of Interleukin-8 by Oral Keratinocytes

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Abstract

There is increasing evidence for a role of acetylcholine in modulating the inflammatory response. This has been demonstrated to occur predominantly via the 'cholinergic anti-inflammatory pathway' and mediated by the α7 nicotinic acetylcholine receptor (α7nAChR). Therefore, there is now an abundance of *in vitro* and *in vivo* evidence that suggest pharmacologically target the α7nAChR is a potential anti-inflammatory therapy to treat chronic inflammatory diseases. The role of acetylcholine and the α7nAChR in modulating the periodontal immune response is at present unknown. However, as numerous cells within the periodontium express the α7nAChR it is interesting to speculate that targeting this receptor may modulate the periodontal immune response. Therefore, his study aims to investigate the effects of the α7nAChR on the periodontal pathogen (*P. gingivalis*) induced expression of Interleukin-8 (IL-8) by oral keratinocytes.

Expression of the α7nAChR mRNA was demonstrated to be upregulated in diseased periodontal tissue. In line with previous studies, oral keratinocytes were found to express the α7nAChR using PCR. The acetylcholine mimic; Carbachol when used in high concentrations was found to inhibit dead *P. gingivalis* induced IL-8 expression by OKF6-TERT2 cells *in vitro*. The expression of IL-8 was investigated at both the protein level by ELISA and the transcriptional level by real time PCR. In contrast, the specific α7nAChR agonist (PHA 543613 hydrochloride) when used at pharmacological concentrations potently inhibited expression of IL-8 by OKF6-TERT2 cells cultured with a live *P. gingivalis* biofilm. This was again determined using ELISA and real time PCR. A membrane integrity assay confirmed that the agonist exhibited no toxic effects on the cells. The inhibition of IL-8 expression was found to be mediated at the transcriptional level. Indeed, using a Fast Activated Cell-based ELISA (FACE) NF-κB p65 Profiler Kit activation of the α7nAChR inhibited the phosphorylation of the NF-κB p65 subunit at Serine 468 and serine 536. Therefore, it can be hypothesised that targeting the α7nAChR leads to inhibition of NF-κB p65 subunit translocation into the nucleus and thus downregulated IL-8 transcription.

These data suggest that the α7nAChR plays a role in regulating the pathogen induced immune response at epithelial surfaces. As dysregulated immunity is a hallmark of peri-

odontal disease it is interesting to speculate that pharmacologically targeting the α7nAChR may offer a novel immune-modulatory therapy.

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Author's declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature:……

Print name:……..

Definitions

AC	Adenyl cyclase
ACh	Acetylcholine
AChE	Acetylcholine esterase
AcCoA	Acetyl co-enzyme A
α7nAChR	Alpha7 nicotinic acetylcholine receptor
BChE	Butyrylcholinesterase
BPE	Bovine pituitary extract
cAMK	Ca2+ calmodulin dependent protein kinase
Car	Carbachol
ChAT	choline acetyl transferase
CHT_1	high-affinity choline transporter-1
CIA	collagen-induced arthritis
CREB	cAMP response element-binding protein (CREB)
CTLA-4-Ig	cytotoxic T-lymphocyte antigen 4 immunoglobin
DKSFM	defined serum-free medium
DMEM	Dulbecco's modified eagle medium
E. coli	Escherichia coli
EGF	epithelial growth factor
ELISA	enzyme-linked immunosorbant assay
ERK	extracellular signal regulated mitogen activated protein kinase
FAA	Fastidious anaerobic agar
FACE	Face activated cell-based ELISA
FCS	foetal calf serum
FLS	fibroblast-like synoviocytes
FoxP3	forkhead box protein 3
GAS	Gamma activated sequence
GCF	Gingival crevicular fluid
HMGB1	high mobility group box protein 1
IL	interleukin
IκBα	inhibitor of kappa B alpha
JAK2	janus kinase 2

K-SFM	keratinocyte serum free medium
LDH	Lactate dehydrogenase
LPS	lipopolysaccharide
mAChR	muscarinic receptors
MIP-1α	macrophage inflammatory protein 1 alpha
MMPs	matrix metalloproteinases
nAChR	nicotinic receptors
NAK	NF-κB activating kinase
NF-κB	nuclear factor kappa light chain enhancer of activated B cells
OCTs	organic cation transporters
OPG	osteoprotegerin
PCR	polymerase chain reaction
PD	periodontal disease
Pg	Porphyromonas gingivalis
PI3K	phosphotidyl inositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
OPG- FC	osteoprotegerin-fragment crystallisable region
RANKL	receptor activator of nuclear factor kappa-B ligand
STAT3	signal transducer and activator of transcription 3
TBK1	TANK-binding kinase-1
TLR2	toll-like receptor 2
TNF-α	tumor necrosis factor alpha
VAChT	vesicular acetylcholine transporter

M	Molar
mg	milligram
ml	milliliter
µg	microgram
mM	millimolar
µM	micromolar
nM	nanomolar
MOI	multiplicity of infection
g	gram

CFU	colony forming unit
5HT-3	5-hydroxytryptamine

1 General Introduction

1.1 Periodontal Disease:

Periodontal disease (PD) is a chronic, destructive, inflammatory disease which left untreated leads to tooth mobility, loss of dental function and ultimately tooth loss. PD is an important clinical problem which affects an estimate of 8 - 15% of the UK population (Kelly M, 1998) and represents a significant cost burden to the NHS; where its treatment costs the NHS in Scotland alone at least £20 million annually (www.isdscotland.org). In addition, there is now a multitude of evidence which demonstrate bi-directional links between PD and other chronic inflammatory conditions such as rheumatoid arthritis (Teng, 2003, Rosenstein et al., 2004, Pischon et al., 2008, Zelkha et al., 2010). At present, the pathogenesis of PD has yet to be fully elucidated although key cellular and molecular mechanisms have been identified which contribute to the tissue destruction and bone loss which are the clinical hallmarks of the disease. However, more research is still needed in order to develop better diagnostic tests and novel therapeutic tools to treat the condition.

1.2 Dental Plaque

PD progression results from a complex interplay of bacterial infection and host response and is modified by behavioral and systemic risk factors. In the mid 1960's, dental plaque accumulation was demonstrated to be one of the major etiologic factors in PD pathogenesis (Löe et al., 1965). Clinical studies have demonstrated that regular removal of dental plaque from oral surfaces is a primary prerequisite to prevent disease (Löe et al., 1965, Lindhe et al., 2008).

Dental plaque results from the accumulation and metabolism of bacterial deposits on the tooth surfaces in the oral cavity. Dental plaque may accumulate supragingivally, i.e. on the clinical crown of the tooth, but also subgingivally, or in the subgingival area of the sulcus. Due to differences in local blood supply, pocket depths and oxygen tension, bacterial composition of plaque can vary greatly depending on the site of accumulation (Socransky et al., 1987). Interestingly however, in a 1 mm^3 section of dental plaque weighing approximately 1 mg, more than 10^8 bacteria can be present made up from over

300 different species (Lindhe et al., 2008). This bacterial mass was shown to produce a variety of irritants, such as endotoxins, acids and antigens, which over time lead directly to the destruction of teeth supporting tissues.

Socransky grouped the bacteria found within dental plaque into colour coded complexes according to species co-localisation within a biofilm and their relative pathogenicity (Socransky and Haffajee, 2005). In health, the oral cavity consists mainly of species including: *Actinomyces* (blue complex) and *Veillonella* (Purple complex), all of which associate closely with members of the yellow complex; the streptococcal spp (Socransky and Haffajee, 2005). The orange complexes consist of species such as *Fusobacterium nucleatum*, and *Prevotella intermedium*. The green complex consists of species such as *Campylobacter concisus* and *Aggregatibacter actinomycetemcomitans.* The red complex is usually found around periodontal pockets and consists of bacteria such as *Porphyromonas gingivalis, Tannerella forsythia*, and *Treponema denticola*. The red complexes and the green complexes have been shown to associate closely with members of the orange clusters (Socransky and Haffajee, 2005, Haffajee et al., 2008, Lindhe et al., 2008). Indeed, it is these red complex species that play key roles in the pathogenesis of periodontal disease (Berglundh et al., 1999).

Porphyromonas gingivalis (P. gingivalis) has been proved in many studies to be one of the main causative organisms of chronic periodontitis (Socransky et al., 1998). A broad range of virulence factors are produced by *P. gingivalis*, all of which contribute to the host inflammatory response (Birkedal-Hansen, 1993, Okada and Murakami, 1998).The most potent virulent factors are the gingipains, which consist of three cysteine proteases that are responsible for at least 85 % of the total proteolytic activity of this pathogen (Amano, 2010). Gingipain activity is essential in sequential phases of the disease process: from attachment and colonization, through nutrient acquisition, neutralisation of host defenses and manipulation of inflammatory response, to tissue destruction, invasion and dissemination to systemic sites (Amano, 2010). Other virulence factors include lipopolysaccharide (LPS), which is the major macromolecule found on the outer surface of gram-negative bacteria. The lipopolysaccharide of *P. gingivalis* is unusual in that it performs weakly in endotoxin assays, although it is quite mitogenic. However, this may be because it is structurally different from other gram-negative bacteria (Condorelli et al., 1998, Hamada et al., 1994, Hirose et al., 1997). *P. gingivalis* LPS

provokes an innate immune response when recognized by the human host as a foreign molecule. It is well established that Lipid A, the bioactive region of LPS that is recognized by the innate immune response, engages the Toll-like receptor 4 (TLR-4) complex, triggering the proinflammatory cytokine production. However, *P. gingivalis* LPS can also induce Toll-like receptor 2 (TLR-2) which plays a potent role in initiating a destructive immune response (Jain and Darveau, 2010). Moreover, Darveau et al. have investigated the ability of *P. gingivalis* lipopolysaccharide to stimulate the inflammatory response directly by its interaction with endothelial cells (Darveau et al., 1995). *P. gingivalis* also possess fimbrae (fimA) which have been implicated in the ability to attach and interact with epithelial cells, fibroblasts, and other bacteria. Furthermore, it has been shown that *P. gingivalis* can produce enzymes called chondroitinase and heparitinase that aid in its permeation into the gingival epithelium (Smith et al., 1997). This permeation will result in the ingress of more microbial virulence factors into the periodontal tissues thereby resulting in localised tissue destruction in PD (Smith et al., 1997).

Our understanding of dental plaque structure, growth and the diversity have greatly enhanced by the recent developments in microbiology, molecular biology and microbial genomics (Preshaw and Taylor, 2011). Plaque formation occurs in different phases. It begins with acquired pellicle formation on the tooth surface through molecular adsorption (Lindhe et al., 2008). This thin pellicle is composed of different salivary mucins and antibodies and can act as a substrate for bacterial colonisation. In the first phase of colonisation the first organisms to bind to this pellicle are the streptococcal species (primary colonizers), followed by members of the blue and purple complexes. The primitive film then provides a perfect niche for other bacteria to complex and the assembly of a biofilm begins (Bakaletz, 2004). The orange complexes are the intermediate colonisers that bind primary colonisers via specific surface receptors and adhesion molecules (Socransky and Haffajee, 2005, Haffajee et al., 2008, Lindhe et al., 2008). Once attached, the behavior of the existing bacteria changes leading to active cellular growth of previously starving bacteria and synthesis of new outer membrane components (Lindhe et al., 2008). Members of the green and red complexes then can bind to the intermediate colonisers. These complexes contain the periodontopathic bacteria that are readily found in patients with periodontal disease (Socransky and Haffajee, 2005, Haffajee et al., 2008, Lindhe et al., 2008). During the last phase of biofilm formation (maturation) the bacterial mass increases due to continued multiplication of adhering

microorganisms and adhesion of new bacteria (Lindhe et al., 2008). The plaque biofilm is a perfect ecological niche for periodontopathic bacteria as it facilitates exchange of nutrients and helps the organisms to survive and proliferate.

The dental biofilm provides protection from the host defense mechanisms by firmly adhering to the tooth surface and thereby provide an advantage over free-floating (planktonic) bacteria (Lee and Wang, 2010, Hannig and Hannig, 2009, Fritz, 1999, Fürst et al., 2007, Listgarten, 1994, Munson et al., 2002). In addition, the biofilm provides physical protection from some antimicrobial agents, thus making PD difficult to treat. The extracellular matrix produced by biofilm bacteria encloses the microbial community and protects it from the surrounding environment, including attacks from chemotherapeutic agents such as antibiotics and antiseptics. Furthermore, the extracellular matrix keeps the bacteria banded together, so they are not flushed away by the action of saliva and gingival crevicular fluid. Mechanical methods, including toothbrushing, interdental cleaning, and professional scaling procedures, are required to regularly and effectively disrupt and remove the plaque biofilm. Antiseptics, such as mouthrinses, can help to control the biofilm but must be formulated so as to be able to penetrate the plaque matrix and gain access to the pathogenic bacteria. Therefore the complex nature of the plaque biofilm provides the perfect shelter for periodontal pathogens. These pathogens can express a plethora of virulence determinants that can penetrate the sulcular epithelium and modulate the host immune response (Marsh, 2004).

1.3 Host immune response in Periodontal Disease:

A widely accepted view is that in an attempt to remove the pathogenic microflora of dental plaque the host mounts an immune response which in susceptible individuals becomes dysregulated. Instead of clearing the pathogenic threat the dysregulated immune response leads to bystander damage such as the breakdown of connective tissue and bone loss which are the clinical hallmarks of periodontal disease (Graves, 2008). In response to threat, immune-competent oral keratinocytes and resident macrophages become excessively activated and orchestrate the infiltration of neutrophils and other inflammatory cells into the oral mucosal tissue. As part of this process increased angio-

genesis occurs which helps to support the inflammatory infiltrate. If these inflammatory processes are not effectively regulated it can lead to the creation of a hyper-inflammatory environment characterised by excessive production of immuno-modulatory mediators including; cytokines, chemokines, prostanoids, antimicrobials and enzymes. Many of these mediators, such as the matrix metalloproteinase (MMPs) and antimicrobial peptides play direct roles in localised tissue destruction. In addition, many inflammatory mediators regulate the processes of bone remodeling. Indeed a hyper-inflammatory environment can perturb the balance between bone formation (osteoblastogenesis) and bone resorption (osteoclastogenesis) leading to loss of the alveolar bone which supports the teeth. Therefore the prominent role of the inflammatory response in periodontal disease pathogenesis suggests that host response modulation may provide novel therapeutic interventions for treatment (Preshaw, 2008).

1.4 Interleukin 8

Much research has been focused on the role of the chemokine Interleukin 8 (IL-8) in PD. IL-8 (CXCL8) is a pro-inflammatory chemokine produced by various cells such as monocytes, neutrophils, fibroblasts, lymphocytes, endothelial cells (Gainet et al., 1998, Soehnlein and Lindbom, 2010). The important role of IL-8 in periodontal disease pathogenesis is elucidated by the fact that large amounts can be detected in the gingival crevicular fluid (GCF) of periodontal disease patients (Mathur et al., 1996) and in the gingival tissues in areas exposed to dental plaque (Tonetti et al., 1994). In addition, levels of IL-8 in the gingival crevicular fluid (GCF) have been shown to relate to clinical signs of PD such as the loss of attachment (Jarnbring et al., 2000).

IL-8 has been shown to be expressed by oral keratinocytes (Fitzgerald and Kreutzer, 1995) and its expression is up regulated in these cells by cytokines such as IL-1β and TNF-α (Schluger and Rom, 1997) as well as direct interactions with bacteria (Tonetti, 1997) and bacterial LPS (Taguchi and Imai, 2006). In addition, it has been demonstrated in many studies that infection of oral keratinocytes by *P. gingivalis* can induce IL-8 production in oral epithelial cells (Handfield et al., 2008, Darveau, 2009).

Within the periodontium, the up regulated expression of IL-8 by resident epithelial cells and immune cells initiates a chemotactic IL-8 gradient which is essential for neutrophil migration (Tonetti, 1997). Therefore, the primary function of IL-8 is the recruitment and activation of neutrophils (Gainet et al., 1998, Soehnlein and Lindbom, 2010) and thereby the orchestration of the innate immune response in PD. IL-8 binds to neutrophils through the G-protein coupled receptor CXCR2 (Soehnlein and Lindbom, 2010, Gainet et al., 1998). Once CXCR2 is activated it exerts various effects on neutrophils' including supporting adhesion to endothelial cells, initiating morphological changes to aid transmigration across tissues, exocytosis of primary and secondary granules containing lysosomal enzymes and initiating a respiratory burst in order to aid microbial clearance (Bickel, 1993).

Although IL-8 is required for an effective immune response to periodontal pathogens, it is important that its expression is regulated as excessive levels can be detrimental to the host. Excess IL-8 can induce tissue damage as it is a potent inducer of matrix metalloproteinases (MMP) expression (Heath et al., 1985, Gowen and Mundy, 1986). MMP's represent the most prominent proteinase family associated with PD (Folgueras et al., 2004) and play key roles in remodeling tissue of the periodontium in periodontitis. The 23 MMP's in humans are genetically distinct but structurally similar zinc-dependent metalloendopeptidases. They can be classified into different subgroups based on their primary structures and substrate specifities (Hernández et al., 2011). MMP's can be divided into collagenases (MM-1, -8 and -13), gelatinases (MMP-2 and -9) and stromelysins (MMP-3, -10 and -11) (Bildt et al., 2009). GCF analysis established that during the active stage of PD, neutrophil-derived MMP- 8 and MMP-9 are actively present (Lee et al., 1995). MMPs can synergistically degrade almost all extracellular matrix and basement membrane components and regulate several cellular processes, including inflammatory and immune responses. It has been found in different studies that IL-8 plays an important role in the production of MMP-9 through the degranulation of neutrophil (Sorsa et al., 2006). MMP-1 plays an important role in hydrolyzing type III collagen, and together with MMP-8, it has a potent effect during the initiation of collagen degradation in PD (Birkedal-Hansen, 1993, Ingman et al., 1994, Golub et al., 1997). Moreover, high levels of IL-8 in the periodontium can indirectly stimulate osteoclast differentiation and function. This occurs through enhancement of RANKL (Receptor activator of nuclear factor kappa-B ligand) expression and suppression of osteoprotegerin (OPG)

expression by osteoblasts (Koide et al., 2010, Kajiya et al., 2010). RANKL activates osteoclasts which can ultimately promote bone loss.

Because of its proinflammatory and neutrophil chemoattractant properties, IL-8 has been considered to play a significant role in the early stages of periodontal disease pathogenesis. Therefore, it is interesting to speculate that IL-8 may be a rational target for therapeutic interventions.

1.5 Acetylcholine

ACh is widely distributed in prokaryotic and eukaryotic cells. To date, despite its ubiquitous expression, research into the biological role of ACh has primarily focused on its neurotransmitter function. However, as early as 1963 Whittaker stated that: 'acetylcholine occurs in non-nervous tissues and is widely distributed in nature to suggest a non-nervous function of it ((VP, 1963). Thus, the biological role of ACh in humans has had to be revised to accommodate two roles: neuronal ACh acting as a neurotransmitter to mediate rapid communication between neurons and effector cells and non-neuronal ACh acting as a local signaling molecule involved in the regulation of cellular phenotypes. (Wessler and Kirkpatrick, 2008).

Knowledge of the synthesis, storage, metabolism and actions of ACh has been derived mostly from studies of the mammalian nervous system (Kawashima and Fujii, 2008) and our current understanding is demonstrated in Figure 1. In brief, ACh is synthesized predominantly by choline acetyltransferase (ChAT) from choline and acetyl coenzyme A (AcCoA). AcCoA is the major product of carbohydrate, protein and lipid catabolism in aerobic organisms and is thus present in more or less all cells. Choline on the other hand originates from the intracellular breakdown of choline containing phospholipids or from the uptake of extracellular choline via low- or high- affinity choline transporter 1 (CHT1). At nerve terminals, ACh is taken up by the vesicular acetylcholine transporter (VAChT) and stored in characteristic small, optically clear vesicles. ACh is then released from nerve terminals by exocytosis. Release studies using the human placenta as a model of the non-neuronal cholinergic system, due to the fact it lacks nervous innerva-

tion, have also demonstrated that non-neuronal ACh is actively released despite the tissue being less well endowed with cholinergic vesicles. However, the mechanism of release is hypothesised to be different to neuronal ACh due to the fact that the majority of non-neuronal cells cannot generate action potentials or open voltage regulated calcium channels which trigger exocytosis (Wessler et al., 2001) . Indeed, in the placental model of non-neuronal ACh release it has been found that ACh leaves cells via organic cation transporters (OCTs) (Wessler et al., 2001). OCTs are members of the solute carrier family of transporters responsible for the influx and efflux of organic cations, such as ACh, across cell membranes. There are 37 known members of the OCT family and they are ubiquitously expressed in humans, although the evidence, from the placental model of non-neuronal ACh, suggests that OCT1 and OCT3 are the favoured subtype for involvement in ACh release (Wessler et al., 2001). Unlike a typical hormone, ACh is rapidly hydrolyzed upon its release predominantly by the enzyme acetylcholinesterase (AChE), with a small contribution from butyrylcholinesterase (BChE), into choline and acetate (Wessler and Kirkpatrick, 2008), therefore limiting the efficacies of ACh to cells in close proximity to its site of synthesis and release (Figure 1).

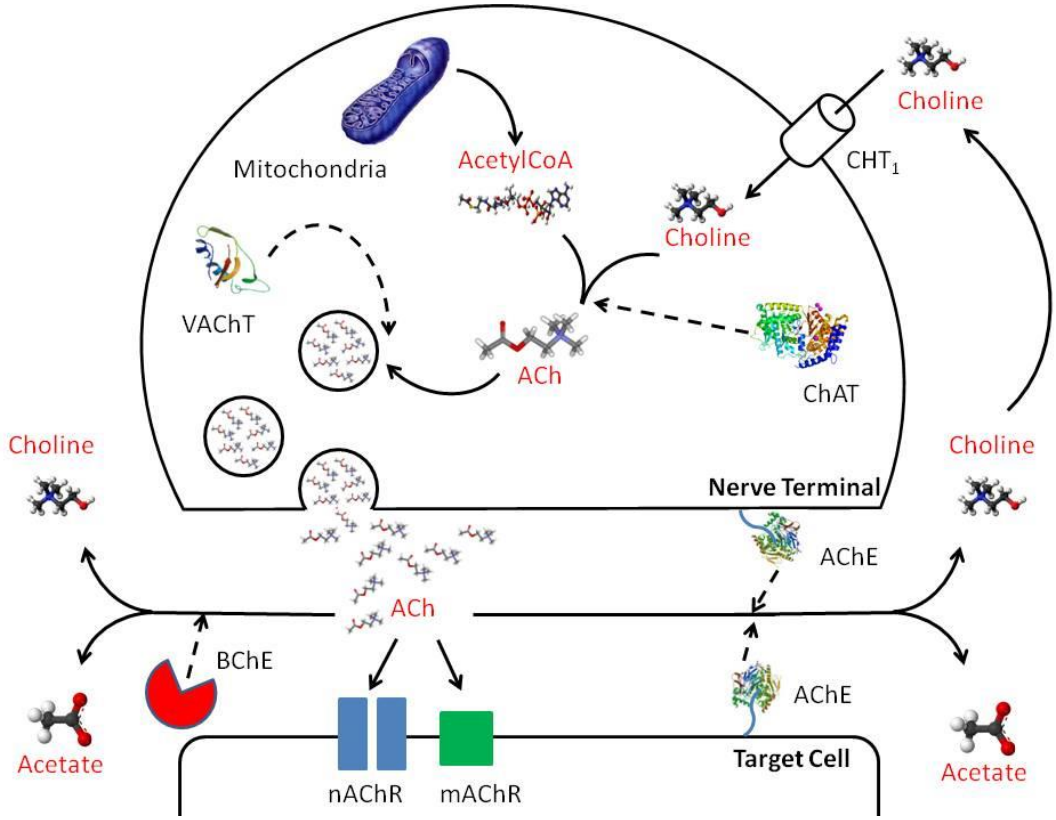

Figure 1

Diagram demonstrating the proposed mechanism of acetylcholine synthesis, release, action and breakdown at a cholinergic nerve terminal. ACh = acetylcholine, ChAT = choline acetyltransferase, CHT_1 = high-affinity choline transporter-1, VAChT = vesicular ACh transporter, AChE = acetylcholinesterase, BChE = butyrylcholinesterase, mAChR = muscarinic receptors and nAChR = nicotinic receptors. (Adapted from (Kummer et al., 2008)).

1.6 Acetylcholine Receptors

Upon release, ACh acts on either muscarinic or nicotinic receptors (mAChR and nAChR, respectively). The metabotropic actions of ACh are mediated by activation of the muscarinic receptor family. These receptors are seven transmembrane glycoproteins encoded by intronless genes; and comprise five distinct subtypes, denoted as muscarinic receptor type 1 - 5 (M1 – M5). Activation of mAChRs is relatively slow and depending

on the subtypes involved causes alterations in cellular levels of phospholipase C, inositol trisphosphate, cyclic AMP (cAMP) and free calcium (Ishii and Kurachi, 2006). Upon activation, mAChRs couple to heterotrimeric guanine nucleotide binding proteins (G proteins) to regulate second messengers and ion channel activities (Wessler and Kirkpatrick, 2008).

Nicotinic acetylcholine receptors (nAChRs) on the other hand are classic representatives of a large superfamily of ligand gated ion channel proteins. The nAChRs are fast ionotropic cationic receptors, which mediate the influx of Na^+ and Ca^{2+} as well as the efflux of K^+ ions. The increase in intracellular Ca^{2+} that arises from nAChR activation can activate calcium sensitive signal transduction protein kinases such as adenylyl cyclase (AC), protein kinase A (PKA), protein kinase C (PKC), Ca^{2+} calmodulin dependent protein kinase (CaMK) and phosphatidylinositol 3-kinase (PI3K). In turn, these phosphorylate downstream targets, such as extracellular signal regulated mitogen activated protein kinase (ERK), which leads to the activation of transcription factors such as the cAMP response element-binding protein (CREB) and *nuclear factor kappa* light chain enhancer of activated B cells (NF-κB). In turn, activation of these transcription factors then modulates gene expression and leads to phenotypical changes in the cell. To date, 13 nAChR subunits have been cloned in humans; seven α like subunits (α2, α3, α4, α5, α6, α7, α9 and α10) and 6 non-α subunits (β2, β3, β4, δ, ε and ϒ). The α2 – α6, β2 – β 4, δ, ε and ϒ subunits can all form hetero-oligomeric receptors, whereas the α7 and α9 subunits can only form homo-oligomeric receptors. To date however the exact signaling mechanism(s) mediating downstream effects of many of the nAChRs are not yet completely understood (Albuquerque et al., 2009).

1.7 The cholinergic system of the periodontium

The influence of neuronally derived ACh on periodontal tissues is currently unknown. However, oral epithelial cells have been demonstrated to express components of a functional non-neuronal cholinergic system. Therefore, epithelially derived ACh can act in both an autocrine and paracrine manner to regulate periodontal tissue function. The epithelial cells lining the human attached gingiva have been shown to express the ACh

synthesising enzyme, ChAT, and two molecular forms of the degrading enzyme, AChE (Nguyen et al., 2000). In addition, although the literature is sparse on the subject there is pharmacological evidence to show that oral epithelial cells express OCTs (Brayton et al., 2002). The demonstration of these components in human oral epithelium is evidence that cells of the periodontium can synthesise and release ACh. Indeed, free ACh has been detected in oral keratinocyte homogenates and culture supernatants (Grando et al., 1993) as well as gingival tissue samples (Rajeswari and Satyanarayana, 1990). In addition, oral keratinocytes and fibroblasts express both nicotinic (nAChR) and muscarinic (mAChR) ACh receptors. The α3, α5, α7, α9, β2 and β4 nAChR receptor subunits (Nguyen et al., 2000) and the muscarinic type 2 (M2), type 3 (M3), type 4 (M4) and type 5 (M5) receptors (Arredondo et al., 2003) are all expressed in human gingival keratinocytes. Furthermore, although the ACh receptor profile of oral fibroblasts has yet to be fully elucidated; periodontal ligament fibroblasts are known to express the α7nAChR (Wang et al., 2010) and pharmacological studies using arecoline and methocramine hemihydrate provide evidence for the expression of muscarinic receptors by gingival fibroblasts (Thangjam et al., 2009, Thangjam and Kondaiah, 2009).

Research into the biology of ACh within the periodontium has mainly centred on its role in the regulation of oral keratinocyte growth and differentiation. Indeed it has been demonstrated that tobacco products act upon nAChRs and can alter cell cycle progression and lead to squamatisation of oral keratinocytes and squamous cell carcinoma (Arredondo et al., 2008a). Interestingly, alterations to oral keratinocyte cell cycle progression and differentiation to the squamous cell phenotype was found to coincide with increased expression of nAChR subunit (Arredondo et al., 2006, Arredondo et al., 2008b). Furthermore activation of the nAChRs was shown to play a central role in the oral keratinocytes response to tobacco products via an intracellular signalling pathway which increased expression and nuclear translocation of both the signal transducer and activator of transcription 3 (STAT-3) and NF-κB transcription factors; which play a role in the control of expression of genes involved in mediating cell growth and apoptosis (Arredondo et al., 2006, Arredondo et al., 2007).

These findings present strong functional evidence for a non-neuronal cholinergic system operating within the periodontium. Although to date the research only suggests an important role for ACh in regulating cellular growth, death and differentiation. Non-

neuronal ACh is however a multifunctional cytotransmitter involved in numerous cellular processes including modulating gene expression, cellular proliferation, cytoskeletal organization, cell-cell contact (tight and gap junctions, desmosomes), locomotion, migration, ciliary activity, electrical activity, secretion and absorption. In addition, non-neuronal ACh also plays a role in the control of unspecific and specific immune functions (Wessler et al., 2003) It is therefore interesting to speculate a similar role for non-neuronal ACh in the periodontal environment.

1.8 The cholinergic anti-inflammatory pathway

Recently the alpha7 nicotinic receptor (α7AChR) has received a great deal of attention from immunologists due its crucial role in regulating the 'cholinergic anti-inflammatory pathway'. The discovery of the 'cholinergic anti-inflammatory pathway' was a major breakthrough and was described as the 'missing link in neuroimmunomodulation' (Pavlov et al., 2003). The pathway was first postulated after pioneering work by the group of Kevin Tracey who demonstrated that systemic, hepatic and splenic Tumour Necrosis Factor alpha (TNF-α) production and symptoms of endotoxemia were exacerbated in rodents which had undergone a vagotomy and reversed in animals subjected to electrical stimulation of the cervical vagal nerve (Borovikova et al., 2000). The pathway is initiated by hallmarks of inflammation activating the afferent vagus nerve and relaying information warning of the occurring inflammation to the brain. Subsequent efferent vagal nerve activity then leads to an increase in ACh release within the inflamed peripheral tissues lying proximal to the nerve. The free ACh then binds to the α7AChRs on immune competent cells and regulates localised inflammatory processes. It is therefore the efferent arm of the vagal nerve reflex which is actually termed the 'cholinergic anti-inflammatory pathway'. The cholinergic anti-inflammatory pathway is therefore important in ensuring the appropriate degree of immune system activation for the perceived threat (Figure 2).

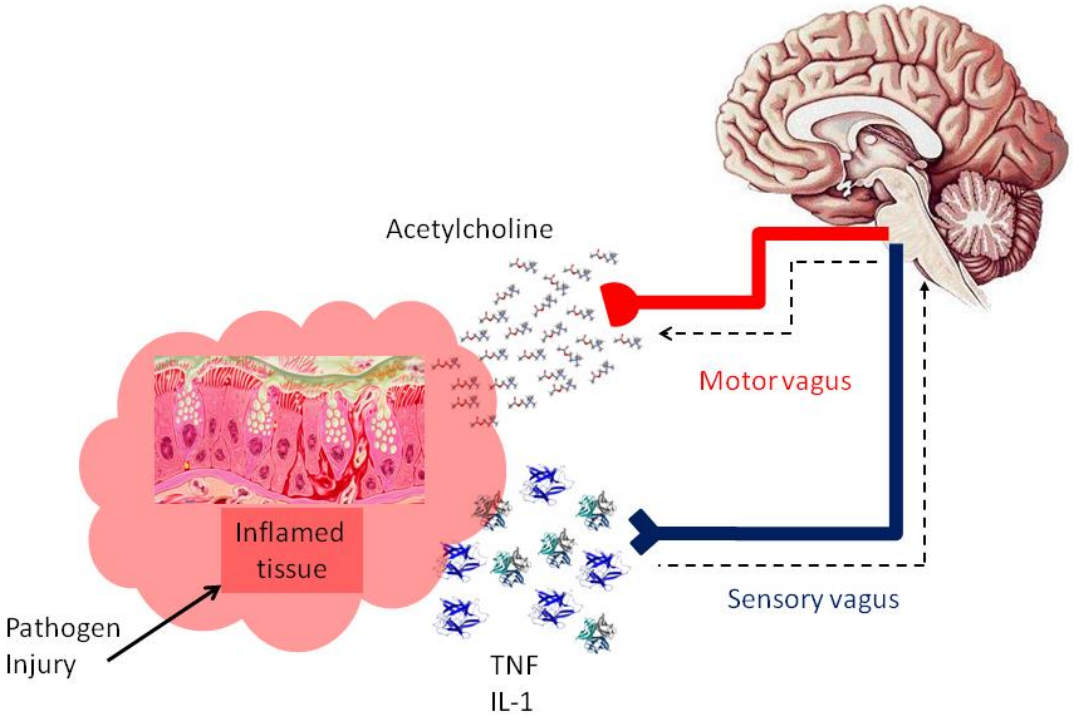

Figure 2

Diagram demonstrating the basic principles of the 'cholinergic anti-inflammatory pathway' Cytokines such as those of the TNF and IL-1 family released from inflamed tissue activate the sensory (afferent) vagus nerve relaying information of the occurring inflammation to the brain. Subsequent (motor) efferent vagal nerve activity then leads to ACh release within the inflamed peripheral tissues lying proximal to the nerve. The free ACh then binds to the α7AChRs on immune competent cells and down regulates local-ised inflammation (Adapted from (Pavlov et al., 2003).

1.9 The α7nAChR and the cholinergic anti-inflammatory pathway

The 'cholinergic anti-inflammatory pathway' has been the subject of extensive research over the last 10 years. Studies have demonstrated that vagus nerve stimulation can attenuate the systemic inflammatory response to endotoxin (Borovikova et al., 2000). Furthermore, studies using α7nAChR-deficient (*α7nAChR*$^{-/-}$) animals have demonstrated the importance of this receptor in modulating the 'cholinergic anti-inflammatory pathway' and controlling unrestrained inflammation in response to pathogens. For example, *α7nAChR*$^{-/-}$ mice were found to be hypersensitive to bacterial lipopolysaccharide (LPS) and exhibited an exaggerated production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Wang et al., 2003). Indeed, using α-bungarotoxin, an inhibitor of the α7nAChR, activation of this receptor was demonstrated to be critical for the suppression of TNF-α release in response to LPS in mice (Wang et al., 2003). In addition, *α7nAChR*$^{-/-}$ mice, upon treatment with LPS or upon infection with *Escherichia coli* (*E. coli*), were shown to develop more severe lung injury and to have more mortality than corresponding *α7nAChR*$^{+/+}$ mice in a model of sepsis induced lung injury (Su et al., 2010).

An anti-inflammatory role for the α7 nAChR in arthritis was suggested using both vagal nerve stimulation and cholinergic agonists. Vagal nerve stimulation was shown to suppress the development of collagen-induced arthritis (CIA) in a rat model and this coincided with a decrease in serum levels of TNF-α and in turns a reduction in osteoclasts within the joint. This suggested an indirect role for cholinergic control of bone destruction (Zhang et al., 2010). Furthermore, mice that were subjected to a cervical vagotomy and induction of arthritis with type II collagen demonstrated that clinical arthritis was exacerbated by vagotomy and ameliorated by nicotine administration (Liu et al., 2011, van Maanen et al., 2009a). However, in a model of CIA, *α7nAChR*$^{-/-}$ mice exhibited significant increases in incidences and severity of arthritis as well as increased synovial inflammation and joint destruction compared to wild type litter mates. This exacerbation was found to coincide with elevated systemic levels of proinflammatory cytokines, an enhanced Th1 profile and elevated TNF-α production from spleen cells (van Maanen et al., 2010). In contrast, using a similar animal model, the lack of α7nAChR was actually found to suppress the development of CIA (Westman et al., 2010). However, this

study was performed over a 14 day period whilst the previous study was performed over a 44 day period which is more representative of the acute phase of the disease. Interestingly though, this second study demonstrated that the α7nAChR plays a role in the adaptive immune response, in addition to localised innate immune responses, in arthritis as in the *α7nAChR*$^{-/-}$ animals a decreased T cell content and proliferation was observed in both the spleen and lymph nodes (Westman et al., 2010).

1.10 Pharmacologically targeting the α7nAChR to treat chronic inflammatory diseases

Targeting the α7AChR has been the subject of investigation for potential therapeutics to treat chronic inflammatory conditions such as, Crohn's disease, psoriasis, rheumatoid arthritis, asthma, sepsis and diabetes (Bencherif et al., 2011). Table 1 shows examples from the literature in which α7AChR agonists have shown promise in animal models for treating a variety of inflammatory conditions.

α7nAChR agonist	Animal model	Inflammatory condition	Outcome	Reference
GTS-21	Mouse	Endotoxemia Sepsis	Improved survival coinciding with decreased serum levels of TNF-α and HMGB1	Pavlov et al., 2007
	Mouse	Pancreatitis	Lower pancreatic inflammation with decreased plasma levels of IL-6 and amylase	van Westerloo et al., 2006
PNU-282987	Mouse	Acute lung injury	Decreased excess lung water and lung vascular permeability with reduced TNF-α and MIP2 in BAL	Su et al., 2007
AR-R17779	Mouse	Rheumatoid Arthritis	Ameliorated clinical arthritis and reduced synovial inflammation with a reduction of TNF-α levels	Van Maanen et al., 2009
	Mouse	Intestinal inflammation	Prevents postoperative ileus characterised by reduced inflammatory cell recruitment	The FO et al., 2007
DMPP	Mouse	Asthma	Reduced airway inflammation with reduced IgE and number of eosinophils in BAL	Blanchet et al., 2005
TC-7020	Mouse	Type 2 Diabetes	Reduced weight gain, glucose and glycated hemoglobin levels and lowered plasma levels of TNF-α	Marrero et al., 2010

Table 1: Specific α7nAChR pharmaceutical agonists investigated in the literature for their *In vivo* therapeutic effects on the pathogenesis of a number of inflammatory conditions.

Indeed, ongoing research has shown that using α7nAChR agonists have been used in regulating cytokine responses and thereby in the treatment of excessive inflammation in preclinical models of disease. An example of an α7nAChR agonist is GTS-21, this compound is tolerated well by human volunteers, and it has been used for mechanistic studies and potential therapeutic development (Pavlov, 2008). GTS-21 has been previously used to counteract deterioration in preclinical studies of Alzheimer's disease (Pavlov et al., 2007). Moreover, this compound has been used to improve survival in murine endotoxemia and severe sepsis. In a study by Pavlov et al., it has been shown

that GTS-21 suppresses dose dependently serum TNF and improves survival during lethal endotoxemia in mice models treated with endotoxin or subjected to cecal ligation and puncture (CLP) (Pavlov et al., 2007). In addition, Van Westerloo et al have demonstrated that treatment with GTS-21 significantly reduces pancreatitis severity and inflammation in mice (van Westerloo et al., 2006). This was indicated by the downregulation of the plasma amylase and lipase levels which protects against pancreatic tissue damage. (van Westerloo et al., 2006). In the same study, GTS-21 has also been shown to suppress neutrophil influx into the pancreas and reduces plasma IL-6 levels (van Westerloo et al., 2006).

Another example of the α7nAChR agonists used *in vivo* in previous studies is PNU-282987, a highly specific α7nAChR agonist. PNU-282987 has been used in an acid-induced acute lung injury mouse model (Su et al., 2007). This compound was found to reduce excess lung water and extravascular plasma equivalents. It also decreased neutrophil accumulation in the airspaces, with the downregulation of TNF-α and MIP-2 levels in the broncho-alveolar lavage (BAL) (Su et al., 2007). In another study by Su et al, PNU 282987 has been found to play an important role in suppressing LPS or live *E. coli*- induced acute lung inflammatory injury in mice (Su et al., 2009). Both specific α7nAChR agonists have downregulated proinflammatory responses (MIP-2 production) and neutrophil migration in the early stage of lung inflammation (Su et al., 2009).

In an arthritis model, mice that were administered nicotine showed inhibited bone degradation and reduced TNF-α expression, while mice that were administered a specific α7nAChR agonist (AR-R17779) demonstrated a total amelioration of clinical arthritis and significantly reduced synovial inflammation accompanied by a reduction of TNF-α levels in both plasma and synovial tissue (van Maanen et al., 2009a). As the agonist AR-R17779 could not pass the blood brain barrier the authors hypothesized that its effects occurred on α7nAChRs proximal to the site of inflammation. Indeed, expression of the α7nAChR was later observed on the intimal lining of the synovium and in cultured fibroblast-like synoviocytes (FLS) (Waldburger et al., 2008, Westman et al., 2010). Moreover, The et al have shown that AR-R17779 abolishes gastric emptying caused by surgical intestinal manipulation and it has been found to prevent post-operative ileus (The et al., 2007). This compound also downregulated pro-inflammatory cytokine release from peritoneal macrophages and suppressed NF-κB activation (The et al., 2007).

DMPP (1, 1-dimethyl-4-phenylpiperzinium), another nicotinic agonist, was found to exhibit anti-inflammatory and bronchopreotective effects through the prevention of airway hyper-responsiveness in a mouse model of asthma (Blanchet et al., 2005). Blanchet et al. have found that DMPP administration intranasally in the asthma mouse model reduced the numbers of lymphocytes and eosinophils in the bronchoalveolar lavage fluid and it also reduced the intracellular calcium release triggered by bradykinin (Blanchet et al., 2005).

TC-7020 binds to α7nAChR with high affinity and exhibits very poor affinity toward other nicotinic subtypes (Mazurov et al., 2011). Oral administration of TC-7020 in a mouse model of type 1 diabetes reduced weight gain and food intake; lowered elevated glucose and glycated hemoglobin levels and reduced elevated plasma levels of triglycerides and TNF-α (Marrero et al., 2010). These effects were reversed when an α7nAChR antagonist (methyllycaconitine) was used, thus confirming the involvement of α7nAChR (Marrero et al., 2010).

Indeed, PHA 543613 proved to be a potent, high affinity agonist of α7nAChR. Due to its excellent in vitro profile, rapid brain penetration, high oral bioavailability in rats and its favorable hERG profile. This highly specific α7nAChR agonist was found to suppress MIP-2 production and neutrophil migration in a live *E. coli*- induced acute lung inflammatory injury in mice (Su et al., 2009). In addition, PHA 543613 has demonstrated efficacy in *in vivo* models for treatment of Alzheimer's disease and Schizophrenia (Wishka et al., 2006).

1.11 Pharmacologically targeting the α7nAChR of specific cells modulates their immune capacity

Numerous *in vitro* studies have demonstrated that activation of the α7nAChR can inhibit the immune response of cells to a variety of stimuli (Table 2).

α7nAChR agonist	Cell Type	Stimulus	Outcome	Reference
Nicotine	Epithelial	TNF-α, LPS	Inhibited IL-8 and TNF-α expression	Li et al., 2011
GTS-21	Macrophages	LPS	Inhibited IL-1β, HMGB1 and TNF-α expression	Rosa-Ballina et al., 2009
Choline	Macrophages	LPS	Inhibited TNF-α expression	Chernyavsky et al., 2010
AR-R17779	Synoviocytes	TNF-α	Inhibited Il-8 and Il-6 expression	Van Maanen et al., 2009
PHA 543613 HCl	Neurons	Intestinal inflammation	Inhibited HMGB1 expression inflammatory cell recruitment	Wang et al., 2012

Table 2: Targeting the α7nAChR on individual cell types inhibits their proinflammatory responses to stimuli.

Indeed, AR-R17779 has been shown to significantly downregulate the expression of IL-6 and several cytokines, including CXCL8 (IL-8) by TNF-α stimulated Fibroblast-like synoviocuyes (FLS) (van Maanen et al., 2010). Furthermore, the pharmacological α7nAChR agonist 3-(2, 4-dimethoxybenzylidene) anabesine (GTS-21) inhibited the Toll-like receptor mediated expression of TNF-α and IL-6 from macrophages (Rosas-Ballina and Tracey, 2009). In airway epithelial cells of cystic fibrosis patients, the activation of the α7nAChR was shown to inhibit Toll like receptor 2 (TLR-2) mediated IL-8 expression (Greene et al., 2010). In addition, activation of the α7nAChR was demon-

strated to inhibit TNF-α induced IL-8 expression in human colon epithelial cells (Summers et al., 2003) and nicotine was also shown to reduce TNF-α expression in HBE16 airway epithelial cells, mainly through the α7nAChR influencing the NF-κB pathway (Li et al., 2011b). Chernyavsky et al have shown that the nicotinic agonist Epibatidine was able to decrease TNF-α in LPS- stimulated macrophage-like U937 cells (Chernyavsky et al., 2010). Moreover, electropuncture pretreatment affected neuronal apoptosis in a recent study by Wang et al, which was reduced by applying PHA 543613 HCl through the inhibition of HMGB1 release into the culture medium (Wang et al., 2012).

Indeed, in vitro studies in macrophages have provided vital clues as to the α7nAChR mediated signaling pathways which result in an anti-inflammatory phenotype. Macrophages/monocytes have also been shown to express the α7nAChR (Toyabe et al., 1997) and its activation demonstrated to mediate signaling pathways leading to down regulated NF-κB nuclear translocation and the suppression of transcription of proinflammatory cytokines. Indeed, treatment of LPS activated human peripheral monocytes with low doses of nicotine caused inhibition of TNF-α and macrophage inflammatory protein 1 alpha (MIP-1α) expression. These effects were mediated through the α7nAChR which led to the suppressed phosphorylation of the Inhibitor of kappa B α (IκBα) and in turn inhibited the transcriptional activity of NF-κB (Yoshikawa et al., 2006) (Figure 3). Furthermore, studies in peritoneal macrophages showed that activation of the α7nAChR can also stimulate the JAK-2/STAT-3 pathway both *in vitro* and *in vivo*. Activation of the STAT-3 cascade by the ligation of nicotine to the α7nAChR led to inhibition of pro-inflammatory cytokine release as STAT-3 is a negative regulator of the inflammatory response (de Jonge et al., 2005) (Figure 3). Interestingly, STAT-3 activation is required for the anti-inflammatory action of IL-10 via the IL-10 receptor and studies have shown that activation of the α7nAChR may only induce specific down regulation of pro-inflammatory cytokines as it did not prevent the constitutive release of the anti-inflammatory; IL-10 (de Jonge et al., 2005).

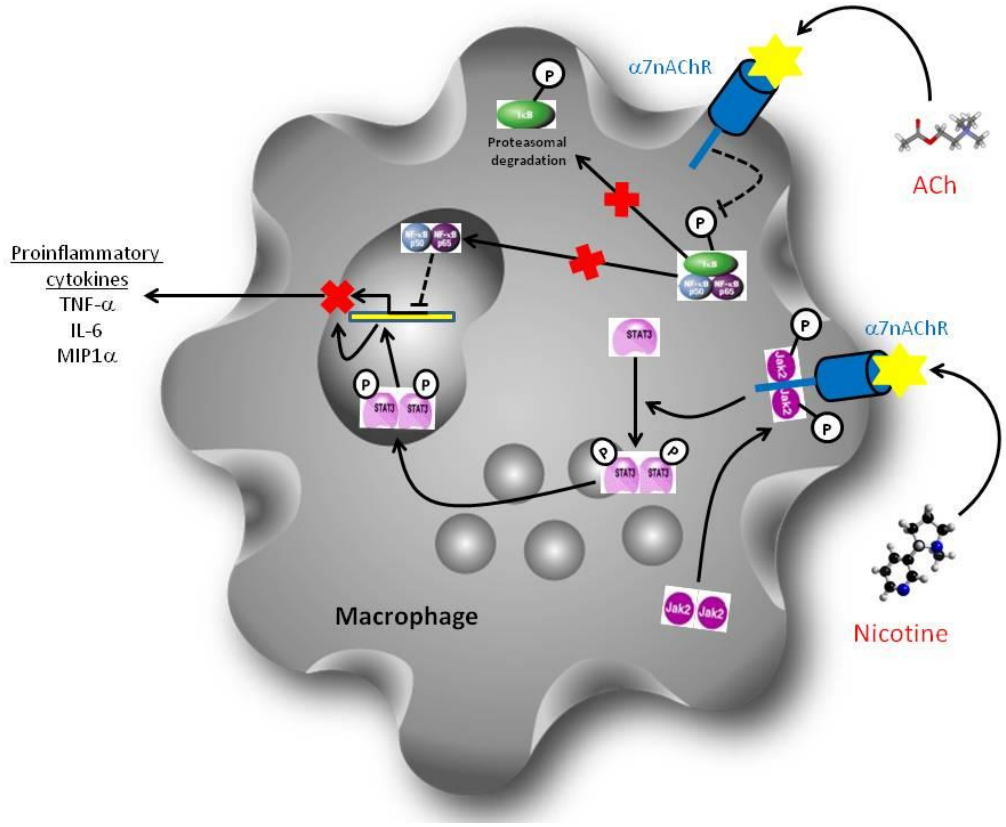

Figure 3

Diagram demonstrating the two major mechanisms by which activation of the α7nAChR can inhibit expression and release of proinflammatory cytokines by macrophages. Activation of the α7nAChR has been demonstrated to inhibit the expression of proinflammatory cytokines. Activation of the α7nAChR inhibits the phosphorylation of Inhibitor of Kappa B (IκB) by the Inhibitor of Kappa B kinase (IKK). This in turn inhibits the dissociation of IκB from the active p50/p65 NF-κB complex and prevents translocation of the active NF-κB transcription factor into the nucleus and therefore inhibits expression of proinflammatory cytokines. Activation of the α7nAChR has also been shown to lead to the formation of a heterodimeric protein complex with phosphorylated Janus Kinase 2 (Jak2). The active JAK2 then activates the signal transducer and activator of transcription 3 (STAT3). The active STAT3 molecule can then translocate to the nucleus where it acts as a negative regulator of proinflammatory cytokine expression.

1.12 Cholinergic regulation of the periodontal immune response?

Evidence suggests that ACh and the α7nAChR can play a key role in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis. It is known that there are remarkable similarities in the pathogenesis of periodontal disease and rheumatoid arthritis; in particular with regard to a poorly modulated inflammatory response resulting in tissue injury and bone loss. Therefore it is interesting to speculate that similar mechanisms may operate within the periodontium to prevent an unrestrained inflammatory response.

To date, the influence of the vagal nerve on the oral cavity is a matter of debate. Indeed whether the vagal nerve can regulate the periodontal immune response is unknown at present. Undoubtedly, the vagal nerve plays an important role as a sensory and nociceptive system to communicate the activation state of the immune system to the brain. However, it is still questionable whether the anti-inflammatory effect on immune cells is mediated directly by vagally released neuronal ACh (Nance and Sanders, 2007). Indeed, it has to be considered that the enzyme responsible for the breakdown of ACh, AChE, is extremely effective and stops neuronal ACh from migrating far from its source. Therefore, for the mechanism to be effective the immune cells need to be in close proximity to the source of neuronal ACh. Although some immune cells do show close membrane apposition with neuronal elements, for example, in the area postrema (Goehler et al., 2006), this is not always the case. Indeed there is little evidence to suggest that there is close apposition of immune cells with neuronal elements within the periodontium. Therefore, the question remains as to how a vagally mediated efferent response on immune cells is migrated through periodontal tissue which are not in close proximity to nerve fibers? Indeed, although a functional relationship between inflammatory disease pathogenesis and the 'cholinergic anti-inflammatory pathway' was confirmed when it was discovered that a decrease in vagal nerve tone (reflecting parasympathetic activity) occurs in patients with rheumatoid arthritis (Stojanovich et al., 2007). The actual response of the isolated inflammatory cells from these patients to cholinergic agonists *ex vivo* was found to be mediated by mechanisms that were independent of va-

gus nerve activity (Bruchfeld et al., 2010). The evidence therefore suggested that non-neuronally derived ACh played a role in regulating localised inflammation.

As described previously (1.6), evidence suggests that functional non-neuronal cholinergic systems operate within the periodontium. Initial studies have demonstrated a role for ACh in oral keratinocyte growth and differentiation (Arredondo et al., 2006, Arredondo et al., 2008). The α7nAChR is widely expressed by cell types found in the periodontium including oral epithelial cells. At present however, it is unknown whether the receptor plays a role in modulating the immune response of these cells to oral pathogens. However, based on substantial evidence from other cell types (section 1.11), it is interesting to hypothesise that pharmacologically targeting the α7nAChR on oral keratinocytes modulates their immune response to periodontal pathogens *in vitro*.

1.13 Objectives:

The aim of this project was to investigate the effects of carbachol and the α7nAChR agonist, PHA543613 HCl, on IL-8 expression by oral keratinocytes in response to the periodontal pathogen; *P. gingivalis*. Evidence in the scientific literature shows that the α7nAChR can regulate the inflammatory response to pathogens of many cells; including immune-competent epithelial cells such as those of the airway (Li et al., 2011b) and colon (Summers et al., 2003). To date, however, there is no direct evidence for a role of α7nAChR in regulating the innate immune response of oral epithelial cells. Existing literature indicates that oral keratinocytes express the α7nAChR (Nguyen et al., 2000, Wang et al., 2010); however its role in the innate immune response against oral pathogens had not been investigated. Interestingly, in various models of sepsis activation of the α7nAChR has been demonstrated to inhibit localized neutrophil recruitment (Saeed et al., 2005). Neutrophil recruitment is one of the key hallmarks of the early stages of periodontal disease pathogenesis. In addition, the chemokine, IL-8, is known to be an important molecule in promoting neutrophil chemotaxis into the periodontal tissues. The α7nAChR has been demonstrated to modulate the expression of TNF-α induced IL8 expression in human colon epithelial cells in response to pathogen (Summers et al., 2003). Therefore it is interesting to speculate that the α7nAChR on oral keratinocytes may modulate their ability to express and release IL-8 and therefore, by modulating neutrophil chemotaxis, play a role in the pathogenesis of periodontal disease.

The specific objectives of the project are:

1- To confirm the presence of α7nAChRs on oral keratinocytes and the OKF6-TERT2 oral keratinocyte cell line to determine their suitability as a model for our *in vitro* studies.

2- To investigate of the direct effects of Carbachol (an ACh mimic) on IL-8 expression by OKF6-TERT2 cells in the presence or absence of dead *P. gingivalis*.

3- To determine the effects of a specific α7nAChR agonist (PHA543613 HCl) on IL-8 expression by OKF6-TERT2 cells in response to a live *P. gingivalis* biofilm.

4- To determine the influence of α7nAChR activation on the NF-κB signaling pathway, and therefore control of IL-8 expression, in OKF6-TERT2 cells.

2 Materials and methods

2.1 Tissue samples

Gingival tissues (n = 15) were obtained from patients 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 probing pocket depths of ≥ 6.0 mm, with clinical attachment loss of ≥ 4.0 mm, which persisted after non-surgical treatment. Patient age ranged from 38 - 64 years with a mean age of 47 years. Healthy control tissues were obtained from patients with clinically healthy periodontal tissues undergoing surgical crown-lengthening procedures (n=10). The tissue samples were immediately submerged in RNA*later* (Qiagen, UK) and stored at - 80 ^{o}C.

2.2 RNA extraction from periodontal tissue samples and preparation of cDNA

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 minutes at 55 ^{o}C in a heat block. The homogenate was then centrifuged at 11000 rpm for 3 minutes 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 11000 rpm. The flow through was then discarded and the membrane column was washed with 350 µl Buffer RW1 (Qiagen, UK) and centrifuged for 15 seconds at 11000 rpm. The flow through was again discarded and on column DNase digestion was performed by incubating the membrane for 15 minutes at room temperature using 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 11000 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.

The cDNA was then synthesised using the high capacity RNA to cDNA kit (Applied biosystems, UK). The mastermix contains random hexamers, dNTP mix (dATP, dGTP, dCTP and dTTP), RNase out, $MgCl_2$ and reverse transcriptase. A NanoDrop 1000 spectrophotometer (Thermo Scientific, UK) was used to assess the quantity and quality of the total RNA. After isolation, mRNA was reverse transcribed to cDNA using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystem, UK). Each reverse transcription reaction consisted of 1000 ng RNA and 4 µl of 5 X complete master mix and made up to a 20 µl total reaction volume with RNase free water. Reverse transcription was performed on a Mycycler Thermal Cycler (Biorad, UK) under the following conditions: 5 minutes at 25 ^{o}C, 30 minutes at 42 ^{o}C and 5 minutes at 85 ^{o}C. The cDNA was stored at 4 ^{o}C for immediate analysis or -20 ^{o}C for long term storage and later analysis.

2.3 TaqMan® Real Time PCR analysis

Real time PCR was carried out using 2 X TaqMan® Gene Expression Master Mix (Applied Biosystems, UK) and 20 X TaqMan® Gene Expression Assay mix for the *α7nAChR* (Hs 01063373_m1) (Applied Biosystems, UK). Expression levels of the *α7nAChR* were corrected for the expression of a house keeping gene; RNA polymerase II; *POL2RA* (Hs_00172187_m1) (Applied Biosystems, UK). Each 20 µl real time PCR reaction consisted of: 10 µl of master mix, 1 µl of gene expression assay mix, 7 µl of

RNase-free water, and 2 µl of cDNA. Analysis of samples was performed in duplicate in a 96 well plate format on a MX3000P^{TM} real time PCR machine (Stratagene, UK) under the following conditions: 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The data was analyzed using MxPro-Mx3000P software, version 4.10 (Stratagene, UK). Relative expression of α7nAChR mRNA was calculated using the $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008). Briefly, the $2^{-\Delta CT}$ value is the CT value of the gene of interest (in this case the *α7nAChR*) for a sample normalized against the CT value for the endogenous housekeeping gene (in this case *POL2RA*).

2.4 Cell culture

OKF6-TERT2 (OKF6) epithelial cells are an immortalized normal oral epithelial cell line originated from keratinocyte cells of the oral mucosa (a kind gift of Dr. James Rheinwald, Brigham and Women's Hospital, Boston, USA). OKF6 cells were maintained at 37 ^{o}C in microaerophilic conditions (5% CO_2) in keratinocyte-serum-free medium (K-SFM, Invitrogen, UK) containing 2 mM L-glutamine, 25 µg/l bovine pituitary extract (BPE) (Invitrogen, UK), 0.2 ng/ml human epithelial growth factor (EGF) (Invitrogen, UK), 100 IU/ml Penicillin (Sigma-Aldrich, UK), 100 mg/ml Streptomycin (Sigma-Aldrich, UK) and 0.2 M calcium chloride in cell culture flasks (Corning Incorporated, USA). The growth media was changed at two-to-three day intervals and cells were passaged when they reached 70 – 80 % confluence.

To passage, cells were first washed with PBS (Sigma, UK). Cells were then removed by addition of 0.05% trypsin and incubation at 37 ^{o}C for around 2 minutes (cells were checked under the microscope to ensure they had completely detached). Dulbecco's Modified Eagles Medium (DMEM) with 10% Foetal Calf Serum (FCS) (Sigma, UK) was then added to the supernatant to inactivate the trypsin. The cells were then centrifuged for 5 minutes at 1000 RPM and washed twice in Hanks Solution (Sigma, UK). Cells were the counted using Trypan blue (Sigma, UK) on a haemocytometer and either resuspended in Defined Serum-Free Medium (DKFSM) to be used for experiments or put back in flasks containing K-SFM at a density 1x 10^5 cells/ml for continuous culture.

2.5 Preparation of *Porphyromonas gingivalis* monospecies biofilm

The monospecies biofilms were prepared using *Porphyromonas gingivalis* (*P. gingivalis)* ATCC 33277. The bacteria were maintained at 37 °C on fastidious anaerobic agar (FAA: Oxoid, Cambridge, UK) with 5% defibrinated horse blood (E&O laboratories, UK), in an anaerobic chamber (Don Whitley Scientific Limited, UK) set at 85 % N_2, 10% CO_2 and 5% H_2 for 3 days. A loopfull of cultured bacteria was then used to inoculate a flask of Schaedler anaerobe broth (Oxoid,, UK) which was then cultured for 2 days at 37 °C in the anaerobic chamber (Don Whitley Scientific Limited, UK). The broth was then centrifuged at 3000 rpm for 5 min to pellet the bacteria that were then washed with PBS (pH 7.4). The bacteria were then resuspended in PBS and diluted to an OD_{550} of 0.2 in a colorimeter (Fisher Scientific, UK) to obtain approximately 1 x 10^8 CFU/ml. The live bacterial counts were confirmed by Miles and Misra plate counting method. The Miles and Misra method is a technique used in microbiology to determine the number of colony forming units (CFU) in a bacterial suspension. Briefly, 5 x 20 µL drops of bacterial suspension were pipetted onto a specific agar plates and incubated under the appropriate culture conditions. After incubation, the number of viable colonies of microorganisms is then determined by counting (Miles *et al*., 1938). *P. gingivalis* suspensions were then standardized to a concentration of 1 x 10^7 CFU/ml in artificial saliva (0.25% porcine stomach mucins, 0.35% sodium chloride, 0.02% potassium chloride, 0.02% calcium chloride dehydrate, 0.2% yeast extract, 0.1% lab lemco powder, 0.5% proteose peptone and 0.05% urea). 500 µl of standardized *P. gingivalis* was then cultured on 13 mm diameter cell culture treated Thermanox® plastic coverslips (NUNC, UK), placed in 24 well cell culture plates (Corning, UK). The cultures were then incubated at 37 °C in an anaerobic environment for 4 days. Every 24 h the spent artificial saliva was replaced with a fresh 500 µl aliquot of artificial saliva. After 4 days of incubation, the artificial saliva was removed and the biofilms were stored at -80 °C to use in cell stimulation experiments.

2.6 Preparation of dead *Porphyromonas gingivalis*

P. gingivalis ATCC 33277 was cultured as described previous (2.5). The bacteria were resuspended in PBS and diluted to an OD_{550} of 0.2 in a colorimeter (Fisher Scientific, UK) to obtain approximately 1 x 10^8 CFU/ml. The bacterial suspension was then heat killed by incubation at 56 °C for 30 minutes.

2.7 Cell stimulation experiments

Oral keratinocytes were prepared by seeding replicate wells of 24 well tissue culture plates with 1 x 10^5 cells/ml and incubating in a humidified atmosphere at 37 ^oC and 5 % CO_2 overnight. To prepare the *P. gingivalis* biofilms; the frozen biofilms (as described in 2.5) were thawed in 500 µl of artificial saliva in an anaerobic chamber for 24 h. On the day of the cell stimulation experiments, the coverslips containing the biofilms were washed 3 times in sterile PBS and then attached to 24 well Millicell® Cell Culture Inserts (Millipore, UK) using sterile Vaseline. To stimulate the previously prepared oral keratinocyte cultures the insert, with biofilm grown on a Thermonex® coverslip attached, was suspended above the cell mono-layers leaving a gap of approximately 0.5 mm. This gap represents the gingival sulcus, which is the space existing between the tooth surface and the surrounding gingival tissues. Therefore, cell growth and function more closely mimic the *in vivo* environment. To determine the effects of Carbachol (Tocris, UK) and PHA 543613 hydrochloride (Tocris, UK) on the *P. gingivalis* biofilm induced IL-8 expresssion by oral keratinocytes; to some wells various concentrations of these compounds were added to the oral keratinocyte cultures 30 min prior to the addition of the *P. gingivalis* biofilm. In addition, some wells were just subjected to incubation with the compounds alone (without biofilm) to ensure they had no effect on IL-8 expression in isolation. For control purposes some wells were just subjected to a change of media (unstimulated and in the absence of any compound). After incubation for 4 and 24 h the supernatant from each well was removed and stored at -20 ^oC for later analysis of IL-8 protein release by ELISA. The remaining adherent cells were then lysed with

RLT lysis buffer (Qiagen, UK) and the lysate stored at -80 ^{o}C for later RNA isolation. All *in vitro* experiments were repeated three times in duplicate wells on different day.

2.8 RNA extraction from *in vitro* cultured cells and cDNA preparation

Total RNA was extracted from cell cultures using an RNeasy kit (Qiagen, UK), using on column DNA digestion with the RNase-free DNase set (Qiagen, UK). Firstly the stimulated cells in the 24-well plate were lysed using 350 µl Buffer RLT (Qiagen, UK) and 1 % ß-Mercaptoethanol. Then, to the lysed cells, equal volume of ethanol (96 – 100 %) was added.

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 11000 rpm. The flow through was then discarded and the membrane column was washed with 350 µl Buffer RW1 (Qiagen, UK) and centrifuged for 15 seconds at 11000 rpm. The flow through was again discarded and on column DNase digestion was performed by incubating the membrane for 15 minutes at room temperature using 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 11000 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.

2.9 Polymerase chain reaction (PCR)

PCR was performed using ReddyMix^{TM} (Thermo Scientific, UK) containing 1.25 units of Thermoprime Plus DNA Polymerase, 75 mM of Tris-HCL (pH 8.8 at 25 ^{o}C), 20 mM $(NH4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2 mM each of dATP, dCTP,

dGTP, and dTTP and a precipitant and red dye for electrophoresis. Each PCR was performed in a 25 µl reaction volume containing 22.5 µl master mix with 0.5 µl of 100 µM of both forward and reverse primers and either cDNA or water as a negative control. The PCR was performed on a Mycycler Thermal Cycler (Biorad, UK) under the following conditions: 94 °C for 2 min, followed by 45 cycles of 95 °C for 25 sec, 60 ^{o}C for 35 sec and 72 °C for 65 sec followed by a final extension at 72 °C for 5 min. The primer sequences used for the *α7nAChR* (5' – 3') were as follows: Fwd = GCA GGA GGC AGA TAT CAG T and Rvs = TGA GGC CAT AGT AGA GCG T. PCR products were subjected to electrophoresis at 110 volts for 45 minutes on a 2% agarose Tris borate gel containing ethidium bromide at 0.5 µg/ml and analysed using the Gel Doc^{TM} XR Imaging System (Bio-Rad Laboratories, UK) with Quantity One Software Version 4.6.7 (Bio-Rad Laboratories, UK).

2.10 SYBR® Green Real Time-PCR analysis

The cDNA obtained from the *in vitro* cultured cells was analysed for IL-8 expression using Quantitative PCR with SYBR Green Real Time PCR technology. The reactions consisted of 12.5 µl of SYBR Green (Invitrogen, UK), 0.5 µl of each of the following primers (5' – 3'), *GAPDH*: Fwd: CAAGGCTGAGAACGGAAG and Rvs: GGTGGTGAAGACGCCAGT or *IL-8*: Fwd: CAGAGACAGCAGAGCACACAA and Rvs: TTAGCACTCCTTGGCAAAAC, 9 µl H_2O and 2.5 µl cDNA (to make a total volume of 25 µl). The real time PCR was performed on a MX3000P^{TM} real time PCR machine (Stratagene, UK) under the following conditions: 94 °C for 10 minutes, followed by 40 cycles of 94 °C for 30 seconds, 60 °C for 60 seconds and 72 °C for 1 minute; then 4 °C as a final hold. Relative expression of IL-8 was calculated using the $2^{-\Delta CT}$ method (Livak & Schmittgan, 2001). Briefly, the $2^{-\Delta CT}$ value for each unknown was calculated as described 2.3. The $2^{-\Delta\Delta CT}$ was then calculated as follows:

$2^{-\Delta\Delta CT}$ = $2^{-\Delta CT}$ of treated sample - $2^{-\Delta CT}$ of untreated control

The method allows you to determine the relative increase (or decrease) in expression (Fold induction) of the gene of interest (IL-8) in cells which are stimulated (*P. gingi-*

valis ± carbachol or PHA543613 HCl) by comparison with the unstimulated control at each time point.

2.11 Enzyme-linked immunosorbent assay (ELISA)

Interleukin 8 (IL-8) concentrations in supernatants from stimulated cells were determined using an ELISA kit as per manufacturer's instructions (Invitrogen, UK). Briefly, monoclonal antibodies specific for IL-8 were pre-coated onto MaxiSorp F96 Immuno-Plates (NUNC, UK) and incubated overnight at 4 °C. The following day the 96-well plate was then blocked with blocking buffer for 2 hours at room temperature. The blocking buffer was phosphate buffered saline (PBS) with 0.5 % (w/v) Bovine Serum Albumin (BSA) (Sigma Aldrich, UK) and 0.1 % (v/v) Tween 20 (Sigma-Aldrich, UK) and tapped dry on absorbent paper to remove excess liquids. 100 µl of standards and samples were pipetted into each well in duplicate along with 50 µl of working detection antibody and the plates were then incubated for two hours at room temperature with continual shaking. The wells were then washed five times using wash buffer and 100 µl of streptavidin-HRP added into each well and plates incubated for 30 minutes with continual shaking. The wells were then aspirated five times using wash buffer and 100 µl of TMB (3, 3', 5, 5'-tetra-methylbenzidine) substrate (R&D Systems, UK) was added to each well and the plates were incubated for a further 30 minutes with continual shaking. Colour development was stopped using 1 M HCl. The intensity of the colour was read at both 650 nm and 450 nm using a Fluostar Omega® microplate reader (BMG Labtech, Germany). Data analysis was done using BMG Analysis Software (BMG Labtech, Germany).

2.12 Cytotoxicity assay

CytoTox-ONE™ Homogenous Membrane Integrity Assay (Promega, UK) was used to measure OKF6 cell viability in the presence of PHA 543613 hydrochloride and car-

bachol. The assay measures the release of lactate dehydrogenase (LDH) from cells with a damaged membrane. PHA 543613 hydrochloride on its own in different concentrations (0.1 nM, 1 nM, 10 nM, 100 nM, 1000 Nm, 10000 nM) or Carbachol in concentrations (1 µM, 10 µM, 100 µM and 1000 µM) were added to the appropriate wells in duplicate and the cells were incubated for 4 and 24 hrs. Control wells contained only media. A volume of CytoTox-ONE™ Reagent equal to the volume of cell culture medium present in each well was added and the plate then shaken for 30 seconds. The plate was then incubated at 22 °C for 10 minutes and fluorescence determined with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The results were calculated as follows: First, the average fluorescence values of the culture medium background were subtracted from all fluorescence values of the experimental wells. Then, the average fluorescence values from experimental maximum LDH release, and culture medium background were used to calculate the percent cytotoxicity as follows:

$$\text{Percent cytotoxicity} = 100 \times \frac{(\text{Experimental - Culture Medium Background})}{(\text{Maximum LDH release - Culture Medium Background})}$$

The data were represented as a percentage of viability (100-cytotoxicity percentage).

2.13 Fast Activated Cell-based ELISA (FACE™) NF-κB p65 profiler Assay:

Fast Activated Cell-based ELISA (FACE) NF–κB p65 Profiler Kits are designed specifically to quantify levels of activated (phosphorylated) forms of the NF-κB p65 subunit and/or total NF-κB p65 subunit. 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 subunit. The kit can be used to determine the levels of the different forms of the NF-κB p65 subunit relative to cell number (cell number corrected). The cell number corrected levels of both the Serine 468 phosphorylated and the Serine 536 phosphorylated NF-κB p65 subunit were then calculated as a percentage of the total cell number corrected NF-κB p65 subunit levels as advised by the manufacturer's protocol.

OKF6-TERT2 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 minutes with either PHA 543613 hydrochloride alone (Tocris, UK), dead *P. gingivalis* alone (200 cells per OKF6-TERT2 cell equivalent), or dead *P. gingivalis* in the presence of PHA 543613 hydrochloride (Tocris, UK). Supernatants were then discarded and the cells were fixed with 100 µl of 4% formaldehyde for 20 minutes. The cells were then washed 3 times with 200 µl wash buffer (0.1% Triton X-100 in PBS) followed by a 20 minute incubation with 100 µl Quenching Buffer (w Buffer containing 1% H_2O_2 and 0.1% Azide) at room temperature. After quenching cells were again washed 2 times 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 supplied primary antibodies are Phospho-NF-κB p65 antibodies (S468 and S536), and Total- NF-κB p65 antibody, diluted 1/500 in Antibody Dilution Buffer (supplied by the manufacturer) (for negative control wells, only Antibody Dilution buffer was added). Plates were then sealed and left overnight at 4 °C. The following day, the cells were again washed 3 times and incubated for 1 hour with 100 µl of diluted secondary antibody (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 minutes. 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 as follows. The cells were washed twice with 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 minutes. After incubation the cells were washed again 3 times with PBS and then 100 µl of 1% SDS solution was added to each well and the plate incubated for 1 hour 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 was calculated as follows:

$$\text{Percentage of the total} = 100 \times \frac{\text{Phosphorylated NF-κB p65 subunit levels}}{\text{Total NF-κB p65 subunit levels}}$$

2.14 Statistical analysis

All experiments were performed in duplicate on three separate occasions. For graphical purposes data is expressed mean ±standard deviation (SD) of the three experiments.

Statistical analysis of real-time PCR, ELISA and cytotoxicity was performed using Graph Pad Prism Version 5 (San Diego, CA, USA). Statistical analysis of the NF-κB assays was performed using SPSS (IBM, Chicago, USA).

Tissue α7nAChR real time PCR data and real time PCR data investigating changes in IL-8 mRNA expression in cultured OKF6-TERT2 cells were analysed by performing a Levene's test on the variance of the natural log transformed $2^{-\Delta CT}$ values followed by an independent *t*-test to compare two means (significance was set at $P<0.05$).

Statistical analysis of ELISA and cytotoxicity experimental data was conducted by firstly performing a Levene's test of homoscedasticity. In all cases the variance of the data was not significantly different and therefore the statistical difference between mean values for treatments and controls in each experiment was determined by a linear ANOVA and a *post hoc* Bonferroni correction.

Statistical analysis of the NF-κB data was carried out by firstly standardising the fluorescence values to the same scale. Next a Levene's test of homoscedasticity was performed. Since the variance of all the data was found not to be significantly different, the statistical difference between mean values for pre-selected treatments and assay controls

was determined by ANOVA and *post hoc* least significant difference test with Holm-Bonferroni (H-B) correction. Significance was set at $p = 0.05$/H-B correction factor.

3 Results

3.1 Expression of the Alpha 7 nicotinic receptor in periodontal tissue and oral keratinocytes

3.1.1 Introduction and Aims

The α7nAChR has previously been shown to be expressed by a variety of cell types found in the periodontium; including oral keratinocytes (Nguyen et al., 2000) and oral fibroblasts (Wang et al., 2010). Before we could begin to explore the possible role of α7nAChR in modulating the oral immune response we first investigated the expression of the α7nAChR in periodontal tissue and oral keratinocytes. Specifically we aimed to:

-Confirm the expression of the α7nAChR in periodontal tissues and determine whether expression levels were elevated in periodontal disease.

-Confirm the expression of α7nAChR by OKF6-TERT2 cells, in order to determine their suitability as a model for oral keratinocytes for subsequent *in vitro* investigations.

3.1.2 Expression of Alpha 7 nicotinic receptor mRNA in healthy and diseased periodontal tissue

Taqman® Real time PCR using a commercially available α7nAChR primer probe set (Applied Biosystems, UK) was employed to confirm the expression of the *α7nAChR* in periodontal tissues and to determine if there were any differences in expression between healthy and diseased tissue. Figure 4 shows that α7nAChR mRNA is expressed in both healthy and diseased periodontal tissue and that there is a significant increase ($*P<0.05$) in α7nAChR mRNA expression in chronic periodontitis tissue samples compared to healthy control tissue samples.

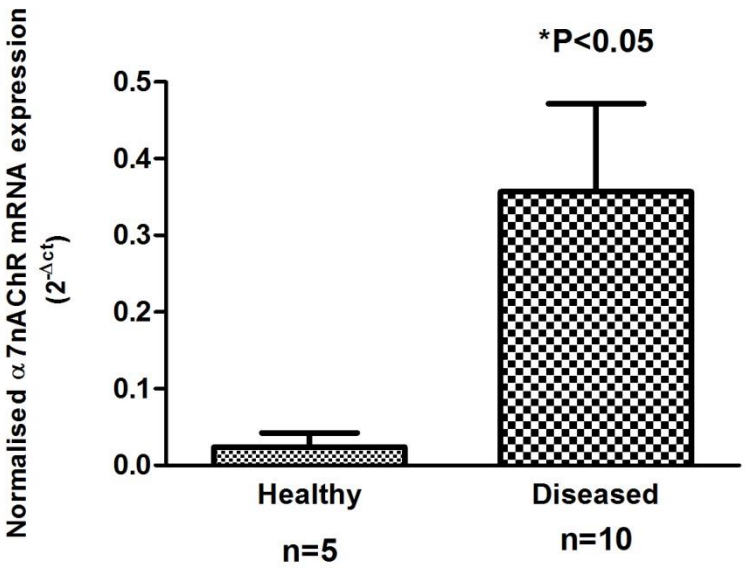

Figure 4: α7nAChR mRNA expression is up regulated in the periodontal tissue of patients with periodontal disease. α7nAChR mRNA expression was normalised against a housekeeping gene; *RNA Polymerase II*. The data presented represents the mean of the normalized α7nAChR mRNA expression for the two cohorts calculated by the $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008). An independent *t*-test was used and the significance level was set at $p = 0.05$.

3.1.3 Expression of the Alpha 7 nicotinic receptor by primary oral cells

The OKF6-TERT2 cell line represents normal oral mucosal keratinocytes which have been immortalized by forced expression of telomerase *via* retroviral transduction (Dickson et al., 2000). To determine their suitability as a model for *in vitro* studies we first confirmed they expressed the receptor in accordance with their primary cell counterparts. Basic PCR analysis revealed that the OKF6-TERT2 cell line, similar to both primary oral keratinocytes and fibroblasts expressed α7nAChR mRNA (Figure 5). These findings were also confirmed using real time PCR (data not shown).

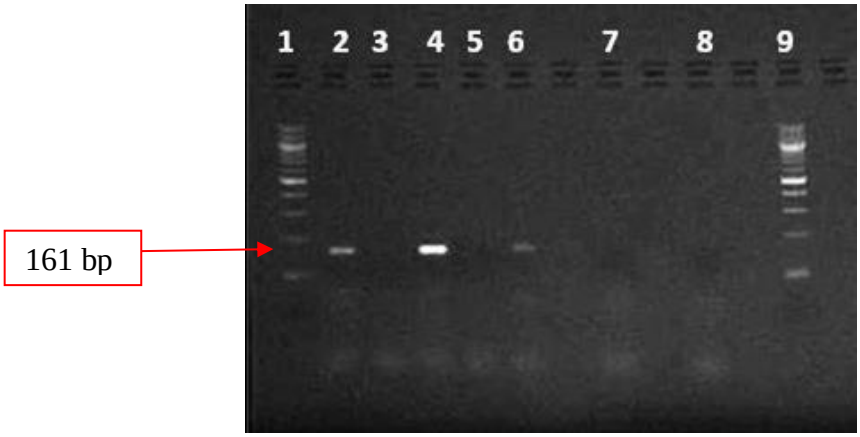

Figure 5: **α7nAChR mRNA is expressed by the OKF6-TERT2 cell line as well as primary oral keratinocytes and fibroblasts.** The primer sequence used in the PCR was unique for the α7nAChR and the PCR yielded products as displayed by gel electrophoresis at the expected size of 161 base pairs (bp). Key: lane 1: 100 bp DNA ladder, Lane 2: cDNA generated from primary oral keratinocytes, Lane 3: no RT control for primary oral keratinocytes, Lane 4: cDNA generated from primary oral fibroblasts, Lane 5: no RT control for primary oral fibroblasts, Lane 6: cDNA generated from OKF6-TERT2 cells, Lane7: no RT control for OKF6-TERT2 cells, Lane 8: RNase free water PCR control, Lane 9: 100 bp DNA ladder.

3.1.4 Conclusion

The mRNA expression data shows that the α7nAChR is expressed in periodontal tissues. Indeed α7nAChR mRNA is expressed by oral keratinocytes and oral fibroblasts. These findings are in agreement with previous studies (Nguyen et al., 2000, Wang et al., 2010). Moreover, expression was also observed in the OKF6-TERT2 cell line. This finding indicates the suitability of the OKF6-TERT2 cell line as a model for oral keratinocytes for use in *in vitro* studies to explore the immuno-regulatory function of the α7nAChR.

3.2 The effect of the acetylcholine mimic Carbamyl chloride (Carbachol) on *P. gingivalis* induced Interleukin-8 expression by OKF6-TERT2 cells

3.2.1 Introduction and aims

To date, there is no direct evidence for a role of the α7nAChR in regulating the immune response in periodontal disease. However, the α7nAChR has been demonstrated to play a role in modulating the immune response of other chronic inflammatory conditions which are similar in their pathogenesis with PD, such as rheumatoid arthritis. Indeed, studies using α7nAChR-deficient (*α7nAChR*$^{-/-}$) (Wang et al., 2003, Su et al., 2010) animals and pharmacological α7nAChR agonists/antagonists (Wang et al., 2010) have demonstrated the importance of this receptor in modulating the 'cholinergic anti-inflammatory pathway' and controlling unrestrained inflammation in such conditions. Therefore, cholinergic strategies have been suggested as potential treatments for multiple inflammatory diseases (Scott and Martin, 2006).

Research utilizing antisense and knockout methodologies have identified an anti-inflammatory role for the α7nAChR expressed on macrophages (Wang et al., 2003) and other various immune-competent epithelial cells (Greene et al., 2010). For example, in airway epithelial cells of cystic fibrosis, the activation of the α7nAChR was shown to inhibit Toll like receptor 2 (TLR-2) mediated IL-8 expression (Greene et al., 2010). In addition, activation of the α7nAChR was demonstrated to inhibit TNF-α induced IL-8 expression in a human colon epithelial cell line (Summers et al., 2003). As confirmed in the previous section oral keratinocytes express the α7nAChR and therefore it could be hypothesised that a similar immunomodulatory phenomenon occurs in these cells.

ACh is chemically unstable *in vivo* and *in vitro* and is easily degraded by acetylcholinesterases. For this reason ACh was not used in these *in vitro* studies. Neither was nicotine; as it is highly toxic and known to be teratogenic and carcinogenic. Therefore, Carbachol (Carbamyl Chloride), a stable ACh mimic, was initially used instead. Carbachol has been used in other *in vitro* studies with no evidence of toxicity and is also used clinically to dilate blood vessels, stimulate intestinal peristalsis and or provoke urinary

bladder muscle activity (Zhou et al., 2011). More importantly, Carbachol is tolerant to acetylcholinesterase and therefore has a prolonged effect *in vitro* and *in vivo*. Indeed, studies have shown that Carbachol has an anti-inflammatory effect via the α7nAChR mediated cholinergic anti-inflammatory pathway. For example, it has been shown that Carbachol inhibits the release of proinflammatory cytokines (TNF-α and IL-6) by LPS-stimulated rat peritoneal macrophages and human blood cells (Lu et al., 2005). Therefore, we used Carbachol in order to explore the effects of ACh in modulating the immune response of oral keratinocytes.

In numerous *in vitro* studies investigating periodontal disease pathogenesis *P. gingivalis* LPS has been the stimulant of choice as *P. gingivalis* has been found to be the main causative organism of chronic periodontitis (Socransky et al., 1998). A broad range of virulence factors are produced by *P. gingivalis*, all of which contribute to the host inflammatory response (Birkedal-Hansen, 1993, Okada and Murakami, 1998). However, LPS is preferred because it is a major component of the outer membrane of *P. gingivalis* and detected by TLR-2 and TLR-4. However, despite its widespread use, *P. gingivalis* LPS has been found to have little biological potency (Dixon and Darveau, 2005). Indeed, LPS from *P. gingivalis* has been shown to elicit a strikingly weak immune response (Jain & Darveau, 2010) as it is capable of synthesizing a heterogeneous population of lipid A molecules, which are subtly different from each other in structure (Jain and Darveau, 2010). For this reason, some studies have employed heat-killed *P. gingivalis* or whole cell extracts instead of *P. gingivalis* LPS alone (Taylor, 2010). Studies using whole *P. gingivalis* have been shown to potently elicit the expression of IL-8 from a number of cell types within the periodontium (Sandros et al., 2000, Handfield et al., 2008). This therefore suggests that using whole bacteria rather than LPS alone elicits *in vitro* a more *in vivo* relevant immune response in cultured cells. Data in the previous section confirmed that the OKF6-TERT2 cell line can be used as a suitable model for *in vitro* studies to explore the immuno-regulatory function of the keratinocyte α7nAChR. Therefore, the aim of this section was to:

- Determine any cytotoxic effects of Carbachol on OKF6-TERT2 cells

- Determine the effects of Carbachol *in vitro* on the dead *P. gingivalis* induced expression of IL-8 by the OKF6- TERT2 cell line.

3.2.2 The effect of Carbachol on OKF6-TERT2 cell viability

Carbamyl chloride (Carbachol) is a stable acetylcholine mimic. To determine whether Carbachol could be used in our *in vitro* experiments to test the effect of targeting the α7nAChR on the IL-8 release from oral keratinocytes we first determined its effects on OKF6-TERT2 cell viability using the Cytotox-one membrane integrity assay (Promega, UK). Figure 6 shows that exposure to 1 – 1000 nM Carbachol had no significant effect on OKF6-TERT2 cell viability over a 24 hour incubation period.

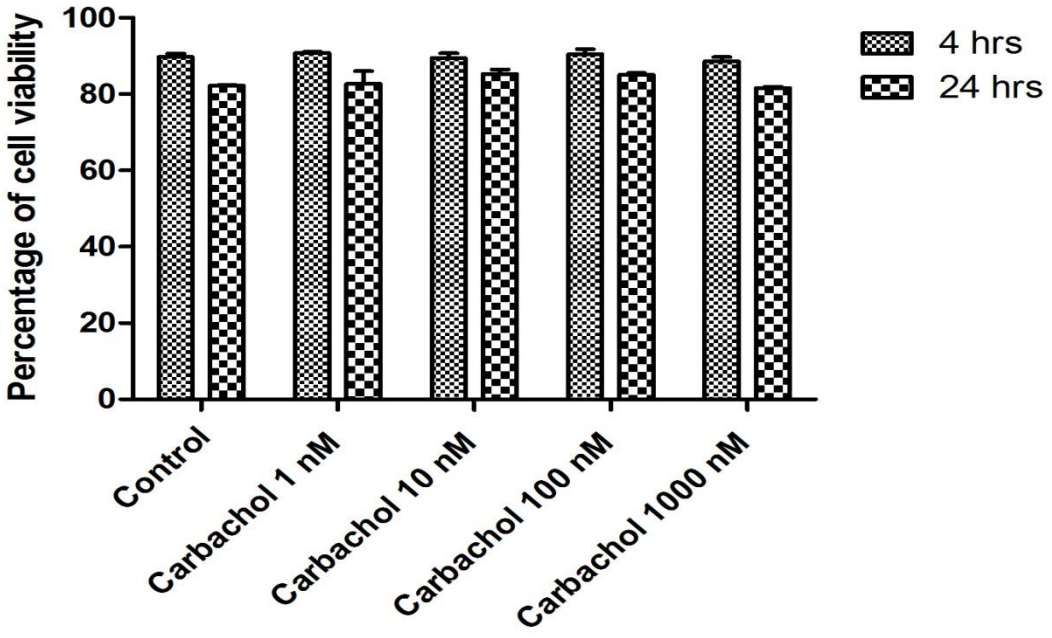

Figure 6: Viability of OKF6-TERT2 cells after exposure to varying concentrations of Carbachol. OKF6-TERT2 cells were cultured in a 96 well plate format at a density of 1 x 10^5 cells/ml cells. The cells were treated with varying concentrations of Carbachol (1 nM – 1000 nM) and membrane integrity, as a marker of viability, was measured after 4 and 24 h using the CytoTox-ONE Homogenous Membrane Integrity Assay kit (Promega, UK). The data represents the mean ±standard deviation (SD) of duplicate wells from three independent experiments. (n=3).

3.2.3 Stimulation of OKF6-TERT2 cells with varying concentrations of dead *P. gingivalis* to determine optimum stimulation for *in vitro* studies

For the *in vitro* studies heat killed *P. gingivalis* strain ATCC 33277 was used. We first determined the optimal concentration of bacteria required for stimulation of IL-8 expression; in terms of number of heat killed *P. gingivalis* cells per OKF6-TERT2 cell (multiplicity of infection or MOI). Figure 7 shows the levels of IL-8 release from OKF6-TERT2 cells stimulated with dead *P. gingivalis* at 100, 200 and 300 MOI. IL-8 release increased in a dose dependent manner.

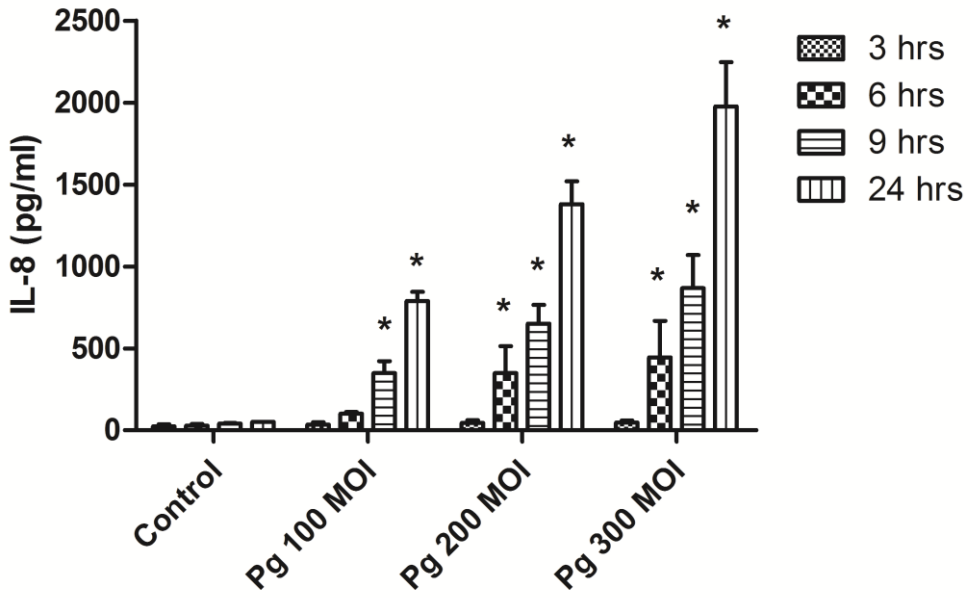

Figure 7: Stimulation of OKF6-TERT2 cells with varying multiplicities of infection of dead *P. gingivalis* and determination of Interleukin-8 release. In order to determine the optimal *P. gingivalis* MOI for the *in vitro* studies, OKF6-TERT2 cells were stimulated with 100, 200 and 300 MOI for 3, 6, 9 and 24 hrs. The data shows a significant increase (* $P<0.05$) in IL-8 protein production by OKF6-TERT2 cells with an MOI of 100 at 9 and 24 h and at 6, 9 and 24 h with an MOI of 200 and 300. The data shown is the mean expression ±standard deviation (SD) from duplicate wells of three independent experiments (n=3). Data were tested for homoscedasticity using Levene's test. Statistical differences between means were then assessed using linear ANOVA and a *post hoc* Bonferroni correction.

3.2.4 The effect of Carbachol on IL-8 release from OKF6-TERT2 cells stimulated with heat killed *P. gingivalis*

After optimising the MOI of dead *P. gingivalis* required to stimulate IL-8 release from OKF6-TERT2 cells experiments were then repeated, however on this occasion Carbachol was introduced into our *in vitro* model system. Cells were either left unstimulated, treated with 1000 nM Carbachol alone, stimulated with dead *P. gingivalis* alone (200 MOI) or first treated with varying concentrations of Carbachol for 30 min prior to stimulation with dead *P. gingivalis* (200 MOI).

Figure 8 shows no effect of Carbachol (1000 nM) alone on IL-8 release. In contrast, a dead *P. gingivalis* preparation (200 cells/OKF6-TERT2 cell) caused a significant up-regulation of IL-8 release (as seen previous: Figure 7). In the presence of varying concentrations of Carbachol, a slight dose dependent decrease in IL-8 release was observed. However, there was only a statistically significant decrease (*$P<0.05$) in IL-8 expression by OKF6-TERT2 cells when pre-exposed to 1000 nM Carbachol at both 6 and 9 h, but not 24 h.

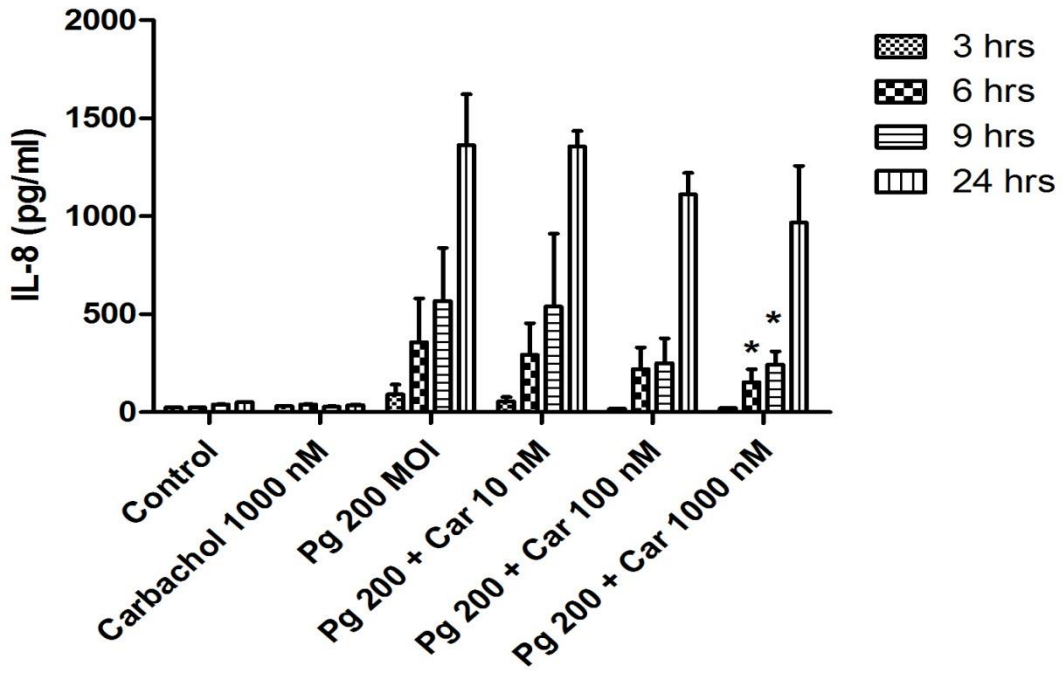

Figure 8: Carbachol inhibits IL-8 protein release from *P. gingivalis* stimulated OKF6-TERT2 cells. Cells were exposed to 3 different concentrations of Carbachol (10 nM, 100 nM and 1000 nM) prior to stimulation with dead *P. gingivalis*. Cells were exposed to dead *P. gingivalis* (200 cells/OKF6-TERT2 cell) alone or Carbachol (1000 nM) alone for control purposes. The cells were stimulated for 3, 6, 9, 24 hrs. The data shown is the mean release of IL-8 ±standard deviation (SD) from duplicate wells of three independent experiments (n=3). Data were tested for homoscedasticity using Levene's test. Statistical differences between means were then assessed using ANOVA, followed by a *post hoc* Bonferroni correction.

To determine if any of the observed decreases in *P. gingivalis* induced IL-8 release from oral keratinocytes seen in figure 8 were due to regulation of IL-8 mRNA transcription, real time PCR was also employed on the mRNA isolated from each well. Figure 9 shows that at 6, 9 and 24 h with 100 and 1000 nM Carbachol there was a significant decrease (*$P<0.05$) in IL-8 mRNA expression by OKF6-TERT2 cells when compared to cells stimulated with *P. gingivalis* alone.

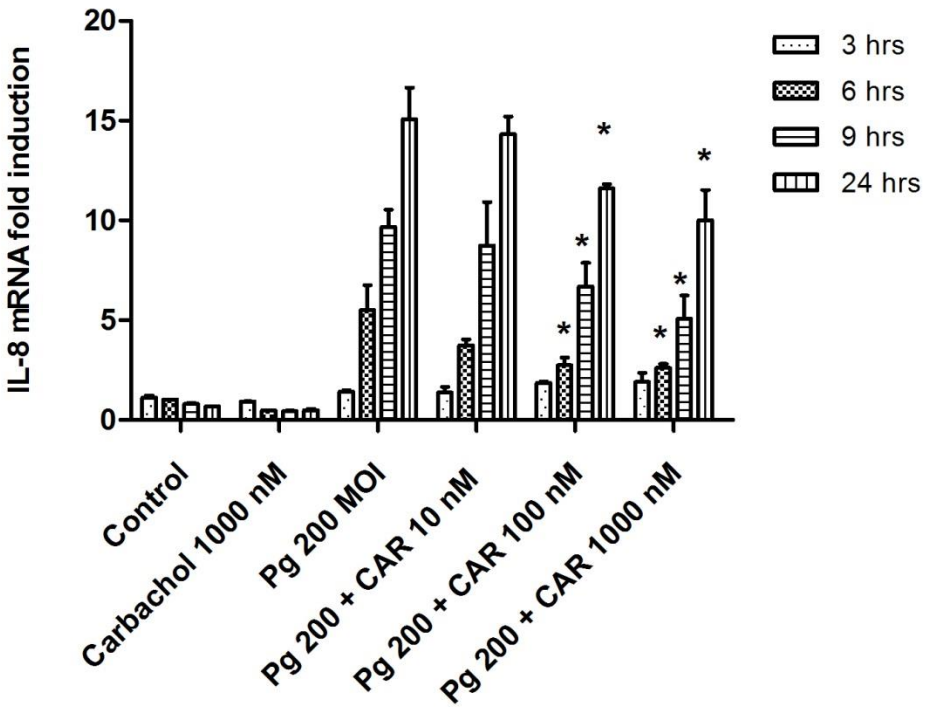

Figure 9: Carbachol inhibits IL-8 mRNA expression by *P. gingivalis* stimulated OKF6-TERT2 cells. OKF6-TERT2 cells were seeded into 24 well plates at a density of 1 x10^5 cells/ml and were exposed to 3 different concentrations of Carbachol (10 nM, 100 nM and 1000 nM) prior to stimulation with dead *P. gingivalis*. Cells were exposed to dead *P. gingivalis* (200 cells/OKF6 cells) alone or Carbachol (1000 nM) alone for control purposes. The cells were stimulated for 3, 6, 9, 24 hrs. SYBR Green Real Time PCR analysis was then used to analyse the expression of IL-8 mRNA. The data shown is the mean IL-8 expression ±standard deviation (SD) from duplicate wells of three independent experiments (n=3). Statistical analysis was performed on the $2^{-\Delta\Delta CT}$ values. Data was analysed by performing a Levene's test on the variance of the natural log transformed $2^{-\Delta\Delta CT}$ values followed by an independent t-test to compare two means (significance was set at $P<0.05$).

3.2.5 Conclusions

Several studies have suggested that the anti-inflammatory role of acetylcholine is mediated by the homomeric α7 subunit of nAChRs (Tracey, 2002, Wang et al., 2003, Ulloa, 2011, de Jonge and Ulloa, 2007). These data demonstrate, using an *in vitro* model system, that IL-8 expression from dead *P. gingivalis* stimulated OKF6-TERT2 cells was significantly downregulated by 1000 nM Carbachol at 6 and 9 h but not 24 h post stimulation. In addition, at the mRNA level significant decreases in IL-8 mRNA expression were observed at 6, 9 and 24 h with 100 and 1000 nM Carbachol. This therefore suggested that Carbachol can inhibit *P. gingivalis* induced IL-8 expression by OKF6-TERT2 cells.

Although significant differences were observed this data must be treated with caution. Indeed, although significant downregulated IL-8 release was observed at 6 and 9 hours with 1000 nM Carbachol, by 24 h expression levels seemed to recover. In addition, the real time PCR data, analyzing IL-8 mRNA expression, did not agree with the protein release data. Furthermore, there was massive variability between experiments when both IL-8 protein release and mRNA expression was measured. This could be due to experimental error. However, it is important to remember that Carbachol exerts promiscuity for a multitude of nicotinic and muscarinic receptors (Koelle, 1971, Miyamoto and Volle, 1974). Furthermore, there is evidence to suggest that different AChRs play differing roles in regulating expression of inflammatory mediators (Koval et al., 2011). A host of evidence suggests that mAChRs can play an opposing role to the α7nAChR and induce expression of proinflammatory cytokines and chemokines (Razani-Boroujerdi et al., 2008). Therefore as oral keratinocytes express a repertoire of both nAChRs (Nguyen et al., 2000) and mAChRs (Arredondo et al., 2003) it is reasonable to hypothesise that using pharmacologically nonspecific compounds, such as Carbachol, may elicit varying effects on IL-8 expression due. This may therefore go some way to explain the variability of the results observed in our studies. Therefore, we cannot conclude from this that a functional α7nAChR mediated anti-inflammatory pathway operates in OKF6-TERT2 cells.

3.3. The effect of the Alpha 7 nAChR specific agonist (PHA 543613 hydrochloride) on *P. gingivalis* induced Interleukin-8 expression by OKF6-TERT2 cells

3.3.1 Introduction and Aims

Data in the previous chapter did not conclusively show that activation of the α7nAChR, can downregulate the expression of IL-8 by OKF6-TERT2 cells stimulated by dead *P. gingivalis*. Nor did the data show that a functional α7nAChR mediated anti-inflammatory mechanism operates in oral keratinocytes. However, as discussed previously, Carbachol is pharmacologically non-specific, as it can act on both nAChRs and mAChRs. When considering potential therapeutics to target the cholinergic anti-inflammatory pathway it is important that they exhibit specificity for the α7nAChR. In recent years a number of α7nAChR selective agonist compounds have been investigated in the literature as potential therapeutics for treatment of inflammatory diseases (Rosas-Ballina and Tracey, 2009). These compounds are capable of eliciting the cholinergic anti-inflammatory pathway (via activation of the α7nAChR) and have shown successful outcomes when applied to *in vivo* animal models with few unwanted side effects. These compounds include PHA 543613 hydrochloride, which is a potent and selective α7nAChR agonist. PHA 543613 hydrochloride has been identified as a potential treatment of cognitive deficits in schizophrenia with an excellent *in vitro* profile. This compound is characterized by rapid brain penetration and high oral bioavailability in rats and robust *in vivo* efficacy in a rat auditory sensory gating model (Wishka et al., 2006, Acker et al., 2008, Ramin et al., 2007). PHA 543613 hydrochloride has also been implicated in *in vivo* studies investigating the anti-inflammatory role of α7nAChR in mediating neuroprotective effects through the inhibition of high mobility group box chromosomal protein 1 (HMGB1) (Wang et al., 2012).

The mechanism by which α7nAChR can exert its anti-inflammatory effects has been reported to include targeting the NF-κB intracellular signaling pathway. This transcription factor signalling pathways plays a regulatory role in inflammation. NF-κB is a key family of transcription factors that are widely used by cytokines to modulate gene ex-

pression. Upon stimulation, NF-κB becomes activated in the cytoplasm, and then translocates into the nucleus where it can modulate transcription of important inflammatory cytokines, including IL-8 (Baeuerle and Henkel, 1994, Williams et al., 2004, Matsusaka et al., 1993) .

Nuclear factor-Kappa B (NF-κB) is a key regulator of proinflammatory gene expression in lymphocytes, macrophages and epithelial cells (Baeuerle and Henkel, 1994, Tak and Firestein, 2001). NF-κB is composed of two subunits, p50 and p65, and is retained in the cytoplasm of cells by a non-covalent interaction with the inhibitory molecule; Inhibitor of Kappa B (IkB). Upon stimulation of cells, IkB is degraded and NF-κB is released and translocated to the nucleus to regulate inflammatory gene expression (Lasar et al., 2004). The key regulatory event in NF-κB activation is the phosphorylation of IkB proteins by the IkB kinase (IKK) complex, which leads to IkB protein ubiquitination and subsequent degradation (Ghosh and Hayden, 2008). This leads to an unmasking of the p65 nuclear localization sequence and the released NF-κB dimers translocate to the nucleus and bind to κB sites in gene promoter regions (Ghosh and Hayden, 2008). Once activated, inducible post-translational events allow the regulation of NF-κB transcriptional activity (Chen and Greene, 2004, Schmitz et al., 2004). To date; eight different phosphorylation sites have been mapped for the strongly activating NF-κB p65 subunit. Three sites are contained in the N-terminal Rel homology domain, whereas five sites including (Ser468 and Ser536) are contained within both C-terminal TADs (Geng, 2008). Basal phosphorylation of Ser468 is exerted by GSK3β (Buss et al., 2004). The same site can also be phosphorylated by IKKβ in response to TNFα or IL-1 stimulation (Schwabe and Sakurai, 2005). Moreover, phosphorylation of p65 Ser468 can be induced by T cell costimulation (Mattioli et al., 2004), but the responsible kinase(s) is/are not yet known (Mattioli et al., 2006). In nuclear translocation of p65, the phosphorylation at serine 536 seems to have an interesting role by defining an IκBα-independent NF-κB pathway (Sasaki et al., 2005). Phosphorylation of p65 NF-κB at Ser536 couples p65 to TAFII31-mediated transcription and is mediated, dependent on the stimulus, by various kinases, including IKKα/β, RSK1, TBK1 (TANK-binding kinase-1)/NAK (NF-κB-activating kinase)/T2K (TRAF2-associated kinase), and IKKϵ (also called IKKi) (Buss et al., 2004, Lawrence et al., 2005). Translocation of NF-κB dimers to the nucleus results in the transcription of a myriad of proinflammatory genes, including IL-8. Most of them overlap with the target genes of STAT3. This is because the promoter region of

many of those genes contains GAS (Gamma activated sequence) or ISRE (STAT-binding elements) in addition to κB (NF-κB binding element) sites (Bollrath and Greten, 2009).

In response to pro-inflammatory stimuli, IL-8 production is dependent on NF-κB in epithelial cells (Oudin and Pugin, 2002). Indeed, stimulation of oral epithelial cells by periodontal bacteria is known to activate several signal transduction cascades, including NF-κB (Huang et al., 2004). Recent analysis of the localization of the p50 and p65 transcription factor components of the NF-κB complex demonstrated significantly increased activity beneath periodontal lesions (Ambili et al., 2005).

In the previous chapter we used dead *P. gingivalis* cells in our *in vitro* systems instead of *P. gingivalis* LPS to be more representative of the *in vivo* situation. However, dental plaque is a polymicrobial biofilm. Planktonic cells still remain a poor substitute for the real challenge that host cells face from the subgingival microbial plaque biofilm. For this reason, in this section we applied a *P. gingivalis* monospecies biofilm into our *in vitro* system. This biofilm was grown on Thermanox™ coverslips, which was then attached to 24 well Millicell® Cell Culture Inserts (Millipore, UK) using sterile Vaseline. To stimulate the previously prepared oral keratinocyte cultures the insert, with biofilm grown on a Thermonex® coverslip attached, was suspended above the cell mono-layers leaving a gap of approximately 0.5 mm. This gap represents the gingival sulcus, which is the space existing between the tooth surface and the surrounding gingival tissues. Therefore, cell growth and function more closely mimic the *in vivo* environment.

The aim of this chapter was to:

- determine the cytoxicity of PHA 543613 hydrochloride, a highly selective α7nAChR agonist, when cultured with OKF6-TERT2 cells

- determine the specific effects of PHA 543613 hydrochloride on the *P. gingivalis* monospecies biofilm induced expression of IL-8 by OKF6-TERT2 cells

- begin to investigate the the effects of PHA 543613 hydrochloride on NF-κB signaling as a mechanism for its ability to downregulate transcription of IL-8 in OKF6-TERT2 cells

3.3.2 The effect of PHA 543613 hydrochloride on the viability of OKF6-TERT2 cells

PHA 543613 hydrochloride is a selective α7nAChR agonist. To determine whether PHA 543613 hydrochloride could be used in our *in vitro* experiments to test the effect of α7nAChR on IL-8 expression by OKF6-TERT2 cells we first determined its effects on cell viability using the Cytotox-one membrane integrity assay (Promega, UK). Figure 10 shows that exposure to 0.1 – 10,000 nM PHA 543613 hydrochloride had no significant effect on OKF6-TERT2 cell viability over a 24 hour incubation period.

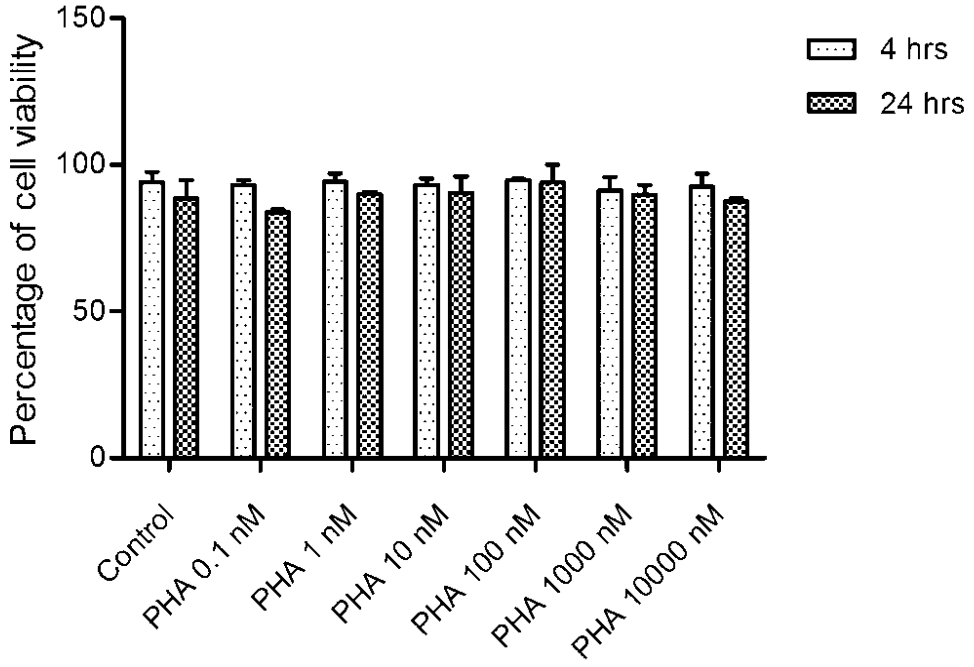

Figure 10: Viability of OKF6-TERT2 cells after exposure to varying concentrations of PHA 543613 hydrochloride. OKF6-TERT2 cells were cultured in a 96 well plate format at a density of 1 x 10^5 cells/ml cells. The cells were treated with varying concentrations of PHA 543613 hydrochloride (0.1 nM – 10000 nM) and membrane integrity, as a marker of viability, was measured after 4 and 24 h using the CytoTox-ONE Homogenous Membrane Integrity Assay kit (Promega, UK). The data represents the mean ±standard deviation (SD) of duplicate wells from three independent experiments. (n=3).

3.3.4 The effect of PHA 543613 hydrochloride on IL-8 expression by OKF6-TERT2 cells stimulated with a live *P. gingivalis* monospecies biofilm

After confirming that PHA 543613 hydrochloride was not toxic to OKF6-TERT2 cells, the experiments in section 3.2 were then repeated however on this occasion PHA 543613 hydrochloride was introduced into our *in vitro* monospecies biofilm system. Cells were either left unstimulated, stimulated with a *P. gingivalis* monospecies biofilm alone, treated with 1000 nM PHA 543613 hydrochloride alone or first treated with varying concentrations of PHA 543613 hydrochloride for 30 min prior to stimulation with a *P. gingivalis* biofilm. The concentrations of PHA 543613 hydrochloride used in this investigation were determined based on the methodology of (Wang et al., 2012).

Figure 11 shows that there is a significant decrease (*P<0.05) in IL-8 release by OKF6-TERT2 cells pre-exposed to 10 nM PHA 543613 hydrochloride at 4 h and 100 nM and 1000 nM PHA 543613 hydrochloride at 4 and 24 h.

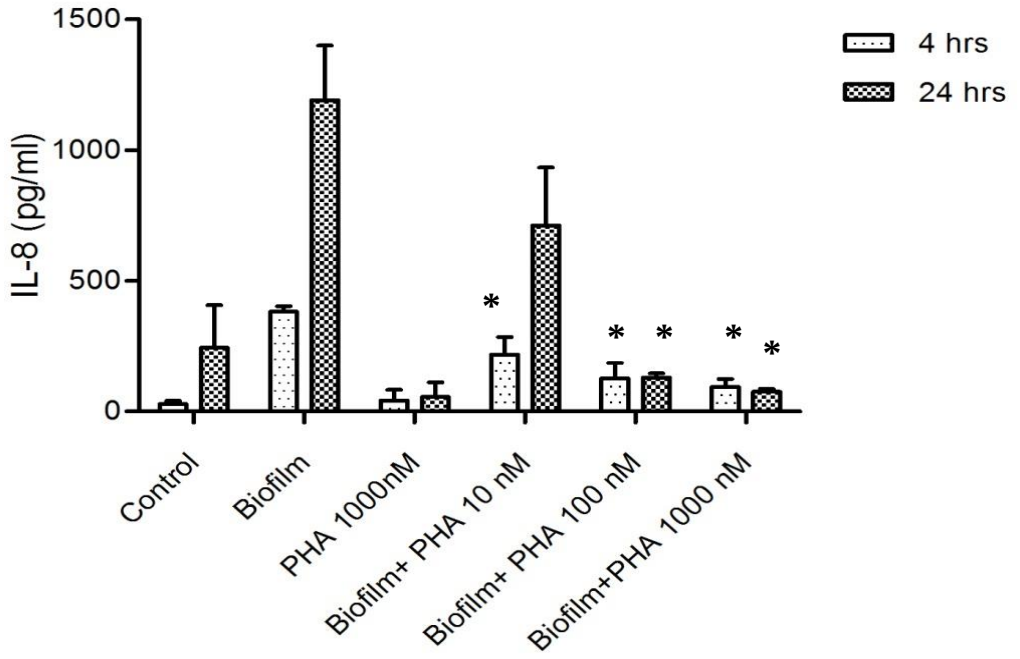

Figure 11: PHA 543613 hydrochloride inhibits IL-8 protein release from *P. gingivalis* stimulated OKF6-TERT2 cells. Cells were exposed to 3 different concentrations of PHA 543613 hydrochloride (10 nM, 100 nM and 1000 nM) prior to stimulation with a *P. gingivalis* biofilm. Cells were exposed to a *P. gingivalis* monospecies biofilm alone or PHA 543613 hydrochloride (1000 nM) alone for control purposes. The cells were stimulated for 4 and 24 hrs. The data shown is the mean release of IL-8 ±standard deviation (SD) from duplicate wells of three independent experiments (n=3). Data were tested for homoscedasticity using Levene's test. Statistical differences between means were then assessed using ANOVA, followed by a *post hoc* Bonferroni correction.

To determine if any of the observed decreases in *P. gingivalis* induced IL-8 release from oral keratinocytes seen in figure 11 were due to regulation of IL-8 mRNA transcription, real time PCR was also employed on the mRNA isolated from each well. Figure 12 shows that there is a significant decrease (*$P<0.05$) in IL-8 mRNA expression by OKF6-TERT2 cells pre-exposed to 10 nM PHA 543613 hydrochloride at 4 h and 100 nM and 1000 nM PHA 543613 hydrochloride at 4 and 24 h.

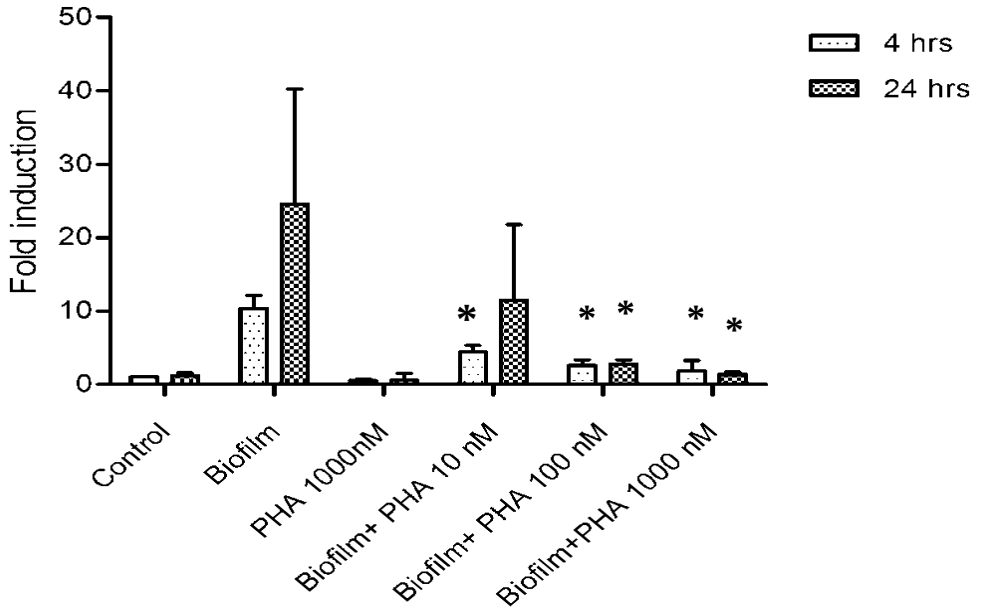

Figure 12: PHA 543613 hydrochloride inhibits IL-8 mRNA expression by OKF6-TERT2 cells stimulated with a *P. gingivalis* monospecies biofilm. OKF6-TERT2 cells were seeded into 24 well plates at a density of 1 x 10^5 cells/ml and were exposed to 3 different concentrations of PHA 543613 hydrochloride (10 nM, 100 nM and 1000 nM) prior to stimulation. Cells were exposed to *P. gingivalis* monospecies biofilm alone or PHA 543613 hydrochloride (1000 nM) alone for control purposes. The cells were stimulated *for* 4 and 24 hrs. SYBR Green Real Time PCR analysis was then used to analyse the expression of IL-8 mRNA. The data shown is the mean IL-8 expression ±standard deviation (SD) from duplicate wells of three independent experiments (n=3). Statistical analysis was performed on the $2^{-\Delta\Delta CT}$ values. Data were analysed by performing a Levene's test on the variance of the natural log transformed $2^{-\Delta\Delta CT}$ values followed by an independent t-test to compare two means (significance was set at $P<0.05$).

3.3.4 The effect of the Alpha 7 nicotinic receptor on the transcriptional activity of the Nuclear factor-kappa B (NF-κB) p65 subunit:

We have demonstrated that PHA 543613 hydrochloride has a potent downregulatory effect on the expression of IL-8 by OKF6-TERT2 cells and that this effect is mediated at the transcriptional level. Therefore, to determine the pathway mediating this phenomenon we investigated the role of NF-κB using the FACE NF-κB p65 Profiler Kit (Active Motif, UK). This profiler kit specifically determines intracellular levels of phospho-NFκB p65 (S468), phospho-NFκB p65 (S536) and the levels of total NF-κB p65 subunit. The kit allows us to specifically determine the levels of the NF-κB P65 subunit phosphorylated at serine 468 and serine 536 in OKF6-TERT2 cells in response to stimuli and in the presence and absence of PHA 543613 hydrochloride.

Stimulation of OKF6-TERT2 cells with *P. gingivalis* caused significantly elevated levels of serine 468 phosphorylated NF-κB p65 subunit. However, in the presence of the PHA 543613 hydrochloride a concentration dependent decrease in serine 468 phosphorylated NF-κB p65 subunit was determined by a linear ANOVA with a significant difference determined observed with 1000 nM of the compound (*P=0.031) (figure 13).

In addition, similar findings were observed when the levels of serine 536 phosphorylated NF-κB p65 subunit were investigated. Indeed, again a concentration dependent decrease in serine 536 phosphorylated NF-κB p65 subunit was determined by a linear ANOVA with a significant difference observed with 1000 nM of the compound (*P=0.031) (figure 14).

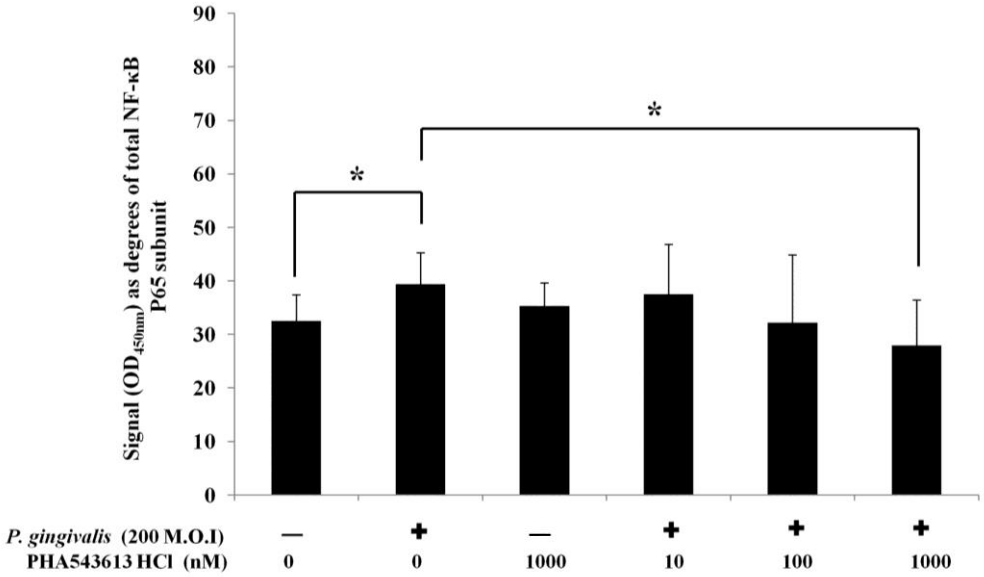

Figure 13: Comparison of intracellular levels of the NF-κB p65 subunit phosphorylated at serine residue 468 in relation to total intracellular levels of the NF-κB p65 subunit. Cells were seeded into 96 well plates at a density of 1 x 10^5 cells/well and left to adhere overnight at 37 ^oC with 5 % CO_2. The following day, cells were stimulated for 30 minutes with either: 1000 nM PHA-543613 HCl alone, dead (heat killed) *P. gingivalis* alone (M.O.I = 200) or in the presence of varying concentrations (10, 100, 1000 nM) of PHA-543613 HCl. The effect of PHA-543613 HCl on the phosphorylation of the NF-κB p65 subunit at serine 468 was then investigated using the FACE^{TM} NF-κB p65 Profiler (Active Motif, UK). The % of the total NF-κB p65 subunit which was phosphorylated at serine 468 was then calculated in accordance with the manufacturer's instructions. The % data was subjected to an angular transformation to parametric data for graphical representation and statistical analysis. The angular transformed data was subjected to Levene's test of homoscedasticity. Since the variance of all the data was not significantly different the statistical difference between mean values for treatments and controls in each experiment was determined by linear ANOVA and a *post hoc* least significant difference test with Holm-Bonferroni (H-B) correction (significance was set at P=<0.05/H-B correction factor).

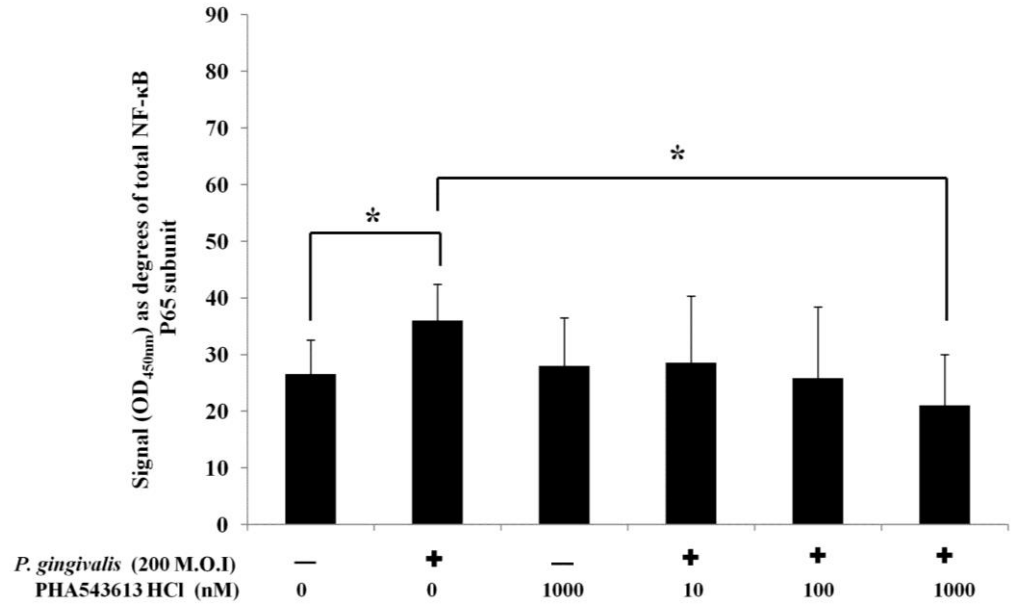

Figure 14: Comparison of intracellular levels of the NF-κB p65 subunit phosphorylated at serine residue 536 in relation to total intracellular levels of the NF-κB p65 subunit. Cells were seeded into 96 well plates at a density of 1 x 10^5 cells/well and left to adhere overnight at 37 ^oC with 5 % CO_2. The following day, cells were stimulated for 30 minutes with either: 1000 nM PHA-543613 HCl alone, dead (heat killed) *P. gingivalis* alone (M.O.I = 200) or in the presence of varying concentrations (10, 100, 1000 nM) of PHA-543613 HCl. The effect of PHA-543613 HCl on the phosphorylation of the NF-κB p65 subunit at serine 536 was then investigated using the FACE^{TM} NF-κB p65 Profiler (Active Motif, UK). The % of the total NF-κB p65 subunit which was phosphorylated at serine 536 was then calculated in accordance with the manufacturer's instructions. The % data was subjected to an angular transformation to parametric data for graphical representation and statistical analysis. The angular transformed data was subjected to Levene's test of homoscedasticity. Since the variance of all the data was not significantly different the statistical difference between mean values for treatments and controls in each experiment was determined by linear ANOVA and a *post hoc* least significant difference test with Holm-Bonferroni (H-B) correction (significance was set at P=<0.05/H-B correction factor).

3.3.5 Conclusions

In recent years a number of α7nAChR selective agonist compounds have been investigated in the literature as potential therapeutics for treatment of inflammatory diseases due to their fewer unwanted side effects (Rosas-Ballina and Tracey, 2009). These compounds are capable of eliciting the cholinergic anti-inflammatory pathway and have shown successful outcomes when applied to *in vivo* animal models (Wishka et al., 2006, Acker et al., 2008, Ramin et al., 2007). In this section, using an *in vitro P. gingivalis* monospecies biofilm model and PHA 543613 hydrochloride, which is a highly selective α7nAChR agonist, we demonstrated that activation of the α7nAChR has a potent downregulatory effect on the expression of IL-8 protein by OKF6-TERT2 cells and that this effect is mediated at the transcriptional level.

In several studies, it has been shown that α7nAChR exert its effects on immune cells (de Jonge et al., 2005), and macrophages (Yoshikawa et al., 2006) via triggering signaling mechanisms that influence the NF-κB signaling pathway. Moreover, NF-κB has been described as the most sensitive nAChR targets in oral keratinocytes (Arredondo et al., 2005). Therefore, in order to determine the pathway mediating α7nAChR signaling in oral keratinocytes we investigated the role of NF-κB using the FACE NF-κB p65 Profiler Kit (Active Motif, UK). These results demonstrate the existence a signaling mechanism mediating the downregulation of NF-κB phosphorylated subunits at serine residue 468 and serine residue 536 through the α7nAChR selective agonist, PHA 543613 hydrochloride. This suggests the involvement of NF-κB pathway in α7nAChR signaling in oral keratinocytes, thus supporting the possible therapeutic implications of α7nAChR in treating periodontal disease.

4 DISCUSSION

There has been extensive research in the last 10 years into the role of the α7nAChR in modulating the inflammatory response. ACh released by the Vagus nerve, as well as non-neuronal ACh, can induce an anti-inflammatory effect via the α7nAChR in various models of inflammatory diseases through a pathway called 'the cholinergic anti-inflammatory pathway' (Pavlov and Tracey, 2005, de Jonge and Ulloa, 2007). Indeed, oral epithelial cells have been demonstrated to express all the components required to synthesise, release and respond to ACh (Nguyen et al., 2000, Grando et al., 1993, Rajeswari and Satyanarayana, 1990). This suggests the possibility of a role of a non-neuronal cholinergic mechanism operating within periodontal tissue. Whether this non-neuronal cholinergic mechanism plays a role in regulating the periodontal immune response is unknown. However, it has previously been shown that the α7nAChR is widely expressed by cells found within the periodontium; including oral keratinocytes (Nguyen et al., 2000) and oral fibroblasts (Wang et al., 2010). In addition, the α7nAChR has also been shown to be expressed by infiltrating immune cells with specific roles in the pathogenesis of periodontal disease; including neutrophils, macrophages, B cells and T cells (Arredondo et al., 2006). Furthermore, there is tentative evidence to suggest that levels of ACh are upregulated in inflamed gingival tissues (Rajeswari and Satyanarayana, 1990). These data therefore suggest that oral ACh and the α7nAChR may play roles in modulating the periodontal immune response.

The data described in this thesis show that pharmacologically targeting the alpha 7 nicotinic receptor of oral keratinocytes can modulate their ability to express IL-8 in the presence of a known oral pathogen; *P. gingivalis*. *P. gingivalis* is thought to be a key pathogen driving periodontal disease pathogenesis (Darveau et al., 2012). Periodontal disease is chronic inflammatory disease affecting the supporting tissues of the teeth. It results from a dysregulated immune response to periodontal pathogens present in a multi-species biofilm that form on the tooth surface; dental plaque (Preshaw and Taylor, 2011). One of the early events in the pathogenesis of the disease is the influx of neutrophils to the site of infection. Interleukin-8 is a chemokine which plays a key role in promoting neutrophil migration (Bickel, 1993). It is well established that oral keratinocytes respond to periodontal pathogens by inducing expression of IL-8 (Huang et al., 1998). However, for the first time the data presented here show that pharmacologically targeting the α7nAChR can inhibit the expression of IL-8 by oral keratinocytes induced

by *P. gingivalis*. Therefore it may be interesting to speculate that the α7nAChR is a potential therapeutic target to treat PD.

It is well established that oral keratinocytes express a functional α7nAChR. This was partially confirmed in this thesis using both conventional and real time PCR. In addition, we have confirmed that an oral keratinocyte cell line (OKF6-TERT2 cells) also express α7nAChR mRNA. Furthermore we have demonstrated that levels of α7nAChR mRNA are up regulated in tissues from patients with periodontal disease in comparison to healthy controls. We therefore attempted immunohistochemical analysis using a commercially available α7nAChR antibody (Santa Cruz, UK). However, despite observed staining we could not be sure of the specificity of this antibody due to unsuccessful attempts to block the staining using a commercially available blocking peptide (Santa Cruz, UK) (data not shown). Therefore, at present we cannot be sure which cell types are responsible for the up regulated α7nAChR mRNA levels observed in diseased periodontal tissue. However, it is well established that a number of professional immune cells such as macrophages, neutrophils, T cells and B cells express the α7nAChR (Arredondo et al., 2006). Therefore, as diseased periodontal tissue is characterised by infiltration of immune cells it is interesting to speculate that these cells are responsible for the observed increase in α7nAChR mRNA in diseased tissue. To confirm this further immunoshitochemical studies would be required performing double staining with a validated specific α7nAChR antibody and antibodies against known markers of each immune cell subset; such as CD68/CD14 for macrophages, CD3 for T cells or neutrophil elastase for neutrophils. In addition, although our PCR analysis confirmed both primary oral keratinocytes and the OKF6-TERT2 cell line express the α7nAChR at the mRNA level, our data does not confirm whether this translates to a functional cell surface protein receptor. This would require immunocytochemical studies also using a validated α7nAChR antibody.

Previous studies have demonstrated that a functional α7nAChR is expressed by oral keratinocytes (Arredondo et al., 2007). In recent years, it has been found that α7nAChR signaling has anti-inflammatory effects on epithelial cells (Summers et al., 2003, van Maanen et al., 2009b, Greene et al., 2010, Li et al., 2011a, Toyabe et al., 1997). Therefore it is interesting to speculate that the α7nAChR plays similar roles in oral keratinocytes. Carbachol is a cholinomimetic drug that is often used as a replacement for the

labile acetylcholine in *in vitro* and *in vivo* studies. In a rat model of endotoxemia, Carbachol was shown to reduce serum levels of IL-6 and TNF-α, but not levels of the anti-inflammatory cytokine IL-10 (Zhou et al., 2011). In addition, *in vitro* studies using rat peritoneal macrophages showed that Carbachol inhibited bacterial lipopolysaccharide (LPS) induced IL-6 and TNF-α *in vitro* (Zhou et al., 2011). Therefore, based on these findings we decided to use Carbachol in our *in vitro* model of oral keratinocyte stimulation by *P. gingivalis*. The studies described in this thesis show that IL-8 expression is decreased from OKF6-TERT2 cells stimulated with dead *P. gingivalis* in the presence of Carbachol. However, a significant effect on IL-8 release at both the protein and mRNA level was only observed with 100 nM and 1000 nM at 6 and 9 hours and not 24 h. One reason for this was possibly the large inter-experimental variability between repeats due to the initial inexperience of the researcher. This could possibly have been reduced by carrying out more than 3 repeat experiments to give a better indication of the true data distribution. This may have indicated that the use of distribution free statistical tests is more appropriate. Alternatively it may have determined whether transformation of the data would be more appropriate prior to the use of parametric tests.

When pharmacologically targeting a specific receptor for therapeutic purposes it is important to consider off target effects of the compounds employed. In addition to the alpha 7 nicotinic receptor, oral keratinocytes express an array of nicotinic (nAChR) and muscarinic (mAChR) receptors. These include the α3, α5, α9, β2 and β4 nAChR receptor subunits (Nguyen et al., 2000) and the muscarinic type 2 (M2), type 3 (M3), type 4 (M4) and type 5 (M5) receptors (Arredondo et al., 2003). Carbachol is a nonspecific ACh mimic which has been demonstrated to elicit its effects on an array of nicotinic (nAChR) and muscarinic (mAChR) receptors. Table 3 shows the reported EC_{50} (concentration of a Carbachol which induces a response halfway between the baseline and maximum after a specified exposure time) and K_i values (The affinity of a ligand for a receptor measured using a competition binding assay) for Carbachol for a host of muscarinic and nicotinic receptors as reported in the literature.

	Muscarinic Receptors			Nicotinic Receptors		
Carbachol	M1	M2	M3	α7	α4β2	α3β4
EC_{50}	116 ± 9.9μM (McKinney et al., 1988)	1.12 ± 0.10μM (McKinney et al., 1988)	8.3 μM (Harris et al., 1995)	580 μM (Anand et al., 1993)	17-34 μM (Eaton et al, 2003)	
K_i	90 ±11 μM (McKinney et al., 1988)	8.5±3.1 μM (McKinney et al., 1988)	5.1 ± 0.4 μM (Jurgen Wess et al., 1993)	12,000 nM (Gopalakrish-nan et al., 1995)	68.24± 1.73 nM (Murali et al., 1995)	3839-4700 nM (Xiao&Kellar, 2004) (Xiao et al., 1998)

Table 3: EC_{50} and K_i values for the action of carbachol against a range of muscarinic and nicotinic receptors.

Due to the concentrations of Carbachol used in this thesis and the EC_{50} and K_i values described in table 3 there is the possibility that the promiscuity of Carbachol for both nAChRs and mAChRs (Koelle, 1971, Miyamoto and Volle, 1974) may be another reason for the large inter-experimental variation observed in our studies. The role of muscarinic and other nicotinic receptors in inflammation is to date not well characterized. However, there is evidence that suggests both types of receptors play differing roles in inflammation (Koval et al., 2011). Indeed, there is evidence to support a pro inflammatory role for muscarinic receptors (Razani-Boroujerdi et al., 2008). Oral keratinocytes are known to express M2, M3, M4 and M5 receptors (Arredondo et al., 2003). The maximal concentration of Carbachol used in this study was 1000 nM (1 μM). Based on the EC_{50} and K_i values for the M3 receptor this concentration can be hypothesised to have little effect on the activation of the oral keratinocyte M3 receptor. In contrast the EC_{50} value of Carbachol for the M2 receptor is much lower showing it has greater affinity for this receptor. Based on the concentrations of Carbachol used in this study therefore we cannot discount the possibility that in the in vitro studies reported here Carbachol is activating M2 controlled signaling pathways. At present the role of the M2 receptor in oral keratinocytes is not well defined. Studies have demonstrated a potential role in oral keratinocytes growth and differentiation (Arredondo et al., 2003). However,

to date no studies have investigated the role of the M2 receptor in the innate immune responses of oral keratinocytes to periodontal pathogens.

In contrast to the muscarinic receptors, the reported EC_{50} value for Carbachol activation of the α7nAChR is 580 μM (580 times) higher than that reported for the M2 receptor). However, EC_{50} values in the literature have been calculated by varying methodologies from stably transfected cell lines to radioligand binding assays. In addition, the literature reporting the EC_{50} values for Carbachol against the α7nAChR are determined using transfected constructs derived from rat or mice species and not human. Therefore, at present the there are no rigorous studies which calculate EC_{50} of carbachol for the human α7nAChR. Despite this it could be concluded that in our studies we in fact used a concentrations of Carbachol (≤1 μM) which are significantly lower than the reported EC_{50} value in other species. However, similar studies have reported the effect of Carbachol in inhibiting bacterial lipopolysaccharide (LPS) induced cytokine expression in peritoneal macrophages macrophages (Zhou et al., 2011). Despite this fact, at present, based on the evidence described above we cannot conclude definitively whether the concentrations of Carbachol used are having a direct effect on the α7nAChR and modulating the innate immune response of oral keratinocytes accordingly.

To overcome the problem of receptor promiscuity, in further studies we used a potent and highly selective α7nAChR agonist; PHA 543613 HCl. The studies described in this thesis show that PHA 543613 HCl has a potent inhibitory effect on IL-8 expression by OKF6-TERT2 cells stimulated with a live biofilm of *P. gingivalis in vitro*. Cell viability studies demonstrated that these inhibitory effects were not due to toxic effects of the compound on the cells. In this study PHA 543613 HCl was used in concentrations ranging from 10 - 1000 nM based on previous literature (Wang et al., 2012, Di Angelantonio et al., 2011). Significant inhibition of *P. gingivalis* induced IL-8 release and mRNA expression by oral keratinocytes was significantly inhibited in the presence of 10, 100 and 1000 nM PHA543613 HCl. However, despite being reported to be highly selective for the α7nAChR, Table 4 shows that PHA 543613 HCl does have some effect on other nicotinic receptors; mainly the α3β4 and α1β1γδ receptor. It is currently unknown if oral keratinocytes express the α1, β1, γ and δ nicotinic receptor subunits or if they possess a functional α1β1γδ receptor. In contrast, it has been reported that oral keratino-

cytes express the α3 and β4 receptor subunits (Nguyen et al., 2000), although whether these cells poses a functional α3β4 receptor is yet to be proven.

PHA 543613 HCl	α7	α3β4	α1β1γδ
EC_{50}	24 nM	> 100 nM (Acker et al., 2008)	> 100 nM (Acker et al., 2008)
K_i	7 nM (Acker et al., 2008)		

Table 4: EC_{50} and K_i values for the action of PHA 543613 HCl against a range of nicotinic receptors.

The EC_{50} value for PHA 543613 HCl against the α3β4 and α1β1γδ receptor has been reported to be > 100 nM (Acker et al., 2008). Therefore in the studies reported in this thesis, if these receptors are present on oral keratinocytes, it can be inferred that the highest concentration of the compound used (1000 nM) may only have minimal effects on these receptors. In addition to the α3β4 and α1β1γδ receptors, PHA 543613 HCl has also been demonstrated to activate the 5-hydroxytryptamine (5-HT_3:serotonin) receptor (Wishka et al., 2006). 5-HT_3 receptors are usually found in both the peripheral (PNS) and central (CNS) nervous systems. Activation of these receptors in the PNS suggests that they play a role in a variety of sympathetic and parasympathetic sensory functions (Jackson and Yakel, 1995, Morales and Wang, 2002, Tecott et al., 1993). In addition, 5-HT_3 receptors are involved in information transfer in the gastrointestinal tract and in the enteric nervous system they regulate gut motility and peristalsis (Galligan, 2002). The 5-HT_3 receptor also play a key role in certain pathologies related to increased serotonin release. For example, in an *in vivo* mouse model persistent activation of 5-HT_3 was shown to cause excitotoxic neuronal cell death and functional changes in the urinary bladder, resulting in bladder hyperdistension, urinary retention, and overflow incontinence (Bhattacharya et al., 2004).

The homology between orthosteric sites in α7nAChR and the 5 HT-3 receptor (Wishka et al., 2006), has been shown to lead to the existence of functional chimeras (Eisele et

al., 1993). PHA 543613 HCl has affinity for the 5-HT_3 receptors with a Ki value of 628 nM (Acker et al., 2008). However, *in vitro* selectivity tests have demonstrated that PHA 543613 HCl has got at least 25-fold binding selectivity and a functional preference for the α7nAChR. Therefore, the relative risk of antagonism of the 5-HT_3 receptor was considered to be low (Wishka et al., 2006). Moreover, there is no evidence in the literature demonstrating the presence of 5-HT_3 receptors in the human oral tissues or on oral keratinocytes. Therefore, it can be tentatively be hypothesized that in our *in vitro* model system the concentrations used were selective for the α7nAChR. In order to confirm this, however, further studies would need to be performed. There are a number of highly selective α7nAChR antagonists commercially available such as methyllycaconitine citrate (Abcam Biochemicals, UK) and α-bungarotoxin (Tocris, UK), many of which have been used in previous experimental protocols to confirm specificity of a compound for the α7nAChR (Waldburger et al., 2008, Beckel et al., 2006, Shytle et al., 2006). These could be applied to the *in vitro* model systems described in this thesis and if the anti-inflammatory response of PHA 543613 HCl is depleted in the presence of antagonists then specificity for the α7nAChR is confirmed. Furthermore, studies have reported the use of Small interfering RNA (siRNA) technology to deplete the expression of the α7nAChR in specific cells *in vitro*. Again, expression of the α7nAChR by oral keratinocytes could be knocked down with predesigned and validated small interfering RNA (siRNA) (Waldburger et al., 2008) prior to use in the *in vitro* model systems. Once again if the anti-inflammatory response of PHA 543613 HCl is reduced when the expression of the α7nAChR is knocked down in oral keratinocytes then specificity for the α7nAChR can be confirmed.

In this thesis data shows that one of the main mechanisms which PHA modulates the immune response of OKF6-TERT2 cells is through inhibited NF-κB activation. As activation of the α7nAChR has previously been shown to inhibit the immune response of monocytes via inhibited NF-κB activation (Yoshikawa et al., 2006) it is interesting to speculate that similar events occur in oral keratinocytes. NF-κB exists in cells in an inactivated form and IL-8 gene transcription is reliant on NF-κB activation; which occurs through phosphorylation of the NF-κB p65 subunit and degradation of the inhibitory protein IκB (Matsusaka et al., 1993, Baldwin and Sharp, 1988). The data presented here suggests that the inhibition of IL-8 expression occurred at the level of transcription and activation of the α7nAChR inhibited *P. gingivalis* induced phosphorylation of the NF-

κB p65 subunit at serine 468 and 536 in oral keratinocytes. Phosphorylation of the NF-κB p65 at serine 563 has been shown to have a role in NF-κB activation (Mattioli et al., 2004, Moreno et al., 2010). In contrast, phosphorylation at serine 468 has been shown to have an important role for NF-κB ubiquitination and degradation (Geng et al., 2009). Despite their opposing functions, phosphorylation at serine 563 and 468 occurs simultaneously (Moreno et al., 2010). Therefore, NF-κB activation and regulation may go hand in hand. Indeed, phosphorylation of serine 468 is suggested to be a hallmark of constitutively activated NF-κB in chronic inflammatory diseases (Buss et al., 2004). Phosphorylation of serine 563 of the NF-κB p65 subunit has been demonstrated to be important in transcription of the IL-8 gene (Buss et al., 2004). However, at present, the role phosphorylation of serine 468 plays in IL-8 transcription is unknown.

The data described in this thesis tentatively indicate that the α7nAChR may play a role in PD pathogenesis. Previous studies have demonstrated that direct activation of nAChRs play a role in the pathogenesis of nicotine-related periodontitis. Indeed, nicotine activation of nAChRs was found to enhance both IL-1β and *P. gingivalis* LPS induced IL-8 release from gingival epithelial cell lines (Kashiwagi et al., 2012). This therefore suggests a direct pro-inflammatory role for nicotine in periodontal disease pathogenesis. Indeed, in a ligature induced rat model of periodontal disease nicotine was shown to up-regulate expression of IL-1β in periodontal ligament cells. In addition, α-bungarotoxin was shown to partially inhibit this phenomenon (Wang et al., 2010). This therefore suggests a role for the α7nAChR in promoting inflammation; which is contradictory to the literature in other inflammatory diseases. However, it is important to stress that the cellular effects of nicotine in the human periodontium or in animal models of periodontal disease have not been fully elucidated at present. Indeed, nicotine is pharmacologically non-specific and also has toxic side effects. As described previously, many cells, including oral keratinocytes, express a plethora of both nAChRs and mAChRs (Nguyen et al., 2000, Arredondo et al., 2003) whose roles in modulating inflammation have yet to be fully elucidated. Therefore, the use of nicotine in these reported studies may have led to the contradictory results obtained in these studies.

In contrast to the study by Kashiwagi *et al*, activation of nAChRs has actually been shown to have a suppressive effect on periodontal immunity. Indeed, although Breivik *et al* (2009) showed that nicotine enhanced bone loss in a rat model of ligature induced

periodontitis, the authors did observe a smaller increase in circulating levels of pro-inflammatory cytokines after intra-peritoneal injection of LPS in the animals treated with nicotine (Breivik et al., 2009). This led the authors to therefore hypothesise that nicotine enhances susceptibility to periodontitis through suppression of protective immune responses via the 'cholinergic anti-inflammatory pathway'. Therefore, as the immune response was inhibited in these animals they failed to clear the pathogenic threat efficiently which led to the progression of disease. This study is therefore in agreement with the current accepted view of nicotine-induced periodontal disease pathogen (Arredondo et al., 2008b).

Whether the α7nAChR is a rational target for PD therapeutic development is still a matter of debate and requires extensive research. What is clear, however, is that for targeting the α7nAChR to treat periodontal disease then careful consideration must be given in the choice of compounds utilized. This is demonstrated by the untoward effects of compound such as nicotine and the promiscuity of compounds such as Carbachol. As many resident and transient cells of the periodontium express a repertoire of both nAChRs and mAChRs it can be hypothesised using pharmacologically non-specific and toxic compounds may elicit complex and detrimental effects *in vivo*. Therefore when considering potential therapeutics to target the cholinergic anti-inflammatory pathway then it is important that they exhibit specificity for the α7nAChR. In addition, although host response modulation offers exciting potential for the development of therapeutics to treat periodontal disease we must still consider that the pathogenic bacteria of plaque are major players in disease pathogenesis. Therefore, when considering any immunomodulatory strategy to treat periodontal disease the need for good oral hygiene and antimicrobial strategies must not be neglected. Therefore, it may be interesting to speculate that the use of specific α7nAChR agonists as a complement to a good oral hygiene regime and in conjunction with current antimicrobial therapies, rather than a standalone treatment, might be more efficacious in the treatment of periodontal disease.

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