An *in-vitro* investigation to determine the neuroinflammatory response of CNS cells to oral bacteria and their virulence factors

by

Rahul Previn

A thesis submitted in partial fulfilment for the requirements for the degree of MSc (by Research) at the University of Central Lancashire

February 2013

ACKNOWLEDGEMENTS

I would like to thank the University of Central Lancashire, UK for the opportunity to undertake my postgraduate research degree. I wish to thank my Principle Investigator (PI) and Director of studies (D0S), Dean, Prof St John Crean for steering me into an interesting, and a hybrid dental-neurosciences project. His inspirational and expert guidance made the challenges of education seem more manageable. I would also like to thank Dr Peter Robinson, my Research Degrees Tutor (RDT), as without his expert help in getting through the various postgraduate degree hurdles would have been impossible. I would like to express my sincere gratitude to my supervisor, Dr Sim Singhrao for the daily guidance, advice, and patience throughout the practical work of the project. I would also like to thank Miss Sophie Poole, currently a PhD student, for ad-hoc assistance in the lab and for guidance in interpreting row data whenever she was nearby. I would like to acknowledge Prof. M. Curtis for the essential reagents I used to investigate my research question without which, my project would be incomplete. Above all, I would like to express my heartfelt gratitude to my family, especially my mother for her undying love, invaluable moral and financial support and encouragement to do well, during my time away from home. Finally I wish to say a huge thank you to all my friends and unmentioned staff members at UCLan for their understanding and providing speedy help when ever needed.

Student Declaration

*****I declare that no material contained in the thesis has been used in any other submission for an academic award and is solely my own work

Signature of Candidate

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Type of Award

MSc (by Research)
.....

School

Postgraduate Medical and Dental Education
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LIST OF ABBREVIATIONS AND SYMBOLS

α	Methylene the first methylene after the hydroxyl in an acyl chain
β	Methylene the second methylene after the hydroxyl in an acyl chain
µg/ml	Microgram (10^{-6}grams) per milliliter
µl	Microliter
µg	Micrograms
µm	Micrometers
ºC	Degrees Celsius; unit of temperature
AD	Alzheimer's disease
AMPA	Alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
APS	Ammoniumpersulphate
ATCC	American Type Culture Collection
BBB	Blood brain barrier
BrdU	Bromodeoxyunidine
BSA	Bovine serum albumin
CA.1-4	Cornisammonis regions 1-4
CD14	Cluster of differentiation 14 protein
$CD4^+$	Cluster of differentiation 4
cm^3	Cubic centimetre
cm^2	Square centimetre
CNS	Central nervous system
CO_2	Carbon dioxide
CR1	Complement receptors 1
CR3	Complement receptors 3
CR4	Complement receptors 4
CSF	Cerebrospinal fluid
dH_20	Distilled water
DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleicacid
DNAse I	Deoxyribonuclease I
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraaceticacid
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimal essential medium
ERK	Extracellular-signal-regulated kinases
FBS	Foetal bovine serum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FISH	Fluorescence *in-situ* hybridisation
g	Gram
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
h	Hour
HCl	Hydrogen chloride
HRP	Horse radish peroxidase
IFN-β	Interferon-β

IFN-γ	Interferon-gamma
IgG	ImmunoglobulinG
IL-1	Interleukin-1
IL-1β	Interleukin-1 beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-13	Interleukin-13
IMR32	Human neuroblastoma cell lines
IU	International units
KCl	Potassium chloride
kDa	Kilodalton (10^3Daltons); unit of mass
KH_2PO_4	Potassium dihydrogen phosphate
L	Liter
LPS	Lipopolysaccharide
LTP	Long term potentiation
M	Molar
mA	Milli amps
MAPK	Mitogen-activated protein kinase
MD-2	Myeloid differentiation protein 2
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation primary response gene 88
mg/ml	Milligrams (10^{-3} grams) per milliliter
ml	Millilitre
mM	Millimolar (10^{-3}moles per liter): unit of concentration
$MgCl_2$	Magnesium chloride
N	Number
nAChR	Nicotinicacetylcholine receptor
NaCl	Sodium chloride
NEAA	Non-essential amino acids
NF-κB	Nuclear factor- kappa B
nm	Nano meter
NMDA	n-methyl-D-aspartate
NO	Nitric oxide
NP40	Nonidet P-40 (octyl phenoxypolyethoxylethanol)
p38 MAPK	Mitogen-activated protein kinase 38
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E2
pH	Percentage hydrogen
PI	Propidium iodide
p moles/ml	Pico moles per millilitres
PRR	Pattern recognition receptors
PVDF	Polyvinylidenedifluoride
RPM	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SVGp12	Human fetal glial cells
TEMED	Tetramethylethylenediamine

TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNF-α	Tumour necrosis factor-α
Tris	Hydroxymethyl
JNK	C-Jun N-terminal kinase

ABSTRACT

Introduction: Innate immune responses are important for brain tissue injury and repair. This study tested the innate responses of the neuroblastoma (IMR32) and the astrocytoma (SGVp12) cell lines and primary mixed glial CNS cells *in vitro* to virulence factors from *Porphyromonas gingivalis* ATCC 33277 and 53978 (W50).

Methods: Following treatment of each cell line, the morphological responses were examined using fluorescein-phalloidin labelling and recorded using the confocal microscope. Binding of two specific endotoxins to cells were determined by immunolabelling with anti-*P. gingivalis* (clones 1B5, and 1A1) antibodies to LPS, and its specific receptor CD14, and gingipains. The Bioplex magnetic bead array analysis from Bio-Rad was performed to measure cytokine release by the human cell lines (IMR32 and SVGp12). In addition a second ELISA assay specifically for TNF-α release was also employed with the same samples.

Results: All cells abundantly expressed the CD14 receptor on their surface membrane and it responded to the *P. gingivalis* endotoxins rapidly by forming cell surface membrane "blebs" at multiple sites. Only prolonged incubation with the virulence factors displayed gingipains localised to perinuclear and lysosomal regions of SGVp12 cells and the primary mixed glial CNS cells. The fluorescein-phalloidin indicated considerable cell detachment and alterations in their actin cytoskeletal filaments from both W50 and 33277. IMR32 confirmed the presence of the CD14 receptor by immunoblotting and reflected changes in its protein levels with endotoxins from W50 and 33277. For example, treatment with *P. gingivalis* 33277 endotoxins, IMR32 cells completely lost their CD14 receptor, but was partially retained after exposure with endotoxins from *P. gingivalis* W50. Similarly, changes were noted with IL-8 chemokine secretion after SGVp12 cells were exposed to *P. gingivalis* W50 and not with *P. gingivalis* 33277. Proinflammatory cytokines IL-6 and TNF-α remained at their physiological levels following treatment of SGVp12 cells to *P. gingivalis* W50 and 33277. Recurrent exposure of SGVp12 cells to endotoxins from *P. gingivalis* 33277 were tested whereby IL-8 acted as a marker of acute phase inflammation. However, the acute phase chemokine did not significantly change over and above the control samples.

Conclusions: All cell types responded to the LPS endotoxin, via the CD14 phagocytic receptor to bind LPS to the surface membrane and gingipains was generally internalised. IMR32 cells completely lost CD14 upon endotoxin treatment possibly cleaved and degraded by gingipains. Only *P. gingivalis* W50 was able to induce the secretion of IL-8 in SVGp12 cells. Literature suggests IL-8 mediates the recruitment and activation of neutrophils in

inflamed tissues. In the same way, IL-8 induction following exposure to *P. gingivalis* W50 in astrocytes would result in the activation and recruitment of microglia to the site of infection/injury in the brain with the appropriate virulence of the microorganism. The results indicate *P. gingivalis* W50 which is more pathogenic in comparison to *P. gingivalis* 33277 use different means of innate immune mechanisms for survival in the host.

Chapter 1: General Introduction

1. General Introduction

1.1 Origins of the concept for research

In 1891, Willoughby D. Miller coined the words “focal infection” in his publication in the Dental Cosmos journal. He implied that a focal infection involved the microbes and their virulence factors from the “foci” of oral microbial infections affecting teeth, mobilise from the mouth and may be responsible for causing infections elsewhere in the body (Miller, 1891). Almost a decade later, the English physician, William Hunter (Hunter, 1900), a strong supporter of the concept observed that the origins of caries, pulpal necrosis and periodontitis were all microbial and proposed they affected the health of remote body organs such as systemic diseases. This phenomenon became accepted as William Hunter’s “focal infection theory” (Hunter, 1900) and his influence had such impact that without the knowledge of the exact connection between oral disease and the development of distant organ pathologies, his proposal for diseased teeth to be extracted in favour of restoration was accepted by dental clinicians worldwide. The contributory factors, as he believed was, surgical intervention during diseased tooth restoration in which oral pathogens were being trapped below the gingival surfaces and entered the vascular channels via “oral sepsis”. However, several decades later the focal infection theory (Hunter, 1900) was re-evaluated and revealed that the pathological importance of extracting teeth had little effect on improving systemic health. As a consequence, the decision to extract teeth in favour of restoration was reversed in the 1950’s.

There is no doubt that the original concept has left an indelible mark on researchers to explore multiple inflammatory pathologies arising from putative immune responses originating from oral infections. The hypothesis is broad and a systematic framework of research was required to provide proof of concept. To this end, researchers began with finding scientific evidence to support the ‘aetiological hypothesis’ beginning with the oral cavity. Dr Socransky, a pioneer in microbiological and immunological aspects of periodontology soon realised that, to answer such a huge scientific question would require overcoming many hurdles some of which involved developing new technology. In time, the pioneer of periodontology was able to overcome the challenges posed by technology to achieve the important milestones such as formulating correct growth culture media and conditions of culture (aerobic/anaerobic) for periodontal pathogens under experimental

conditions. The proof of the concept for Socransky began to bear fruit in the late eighties according to his seminal publication Socransky *et al.,* (1998) and eventually proving the Koch's postulates for the microbial aetiology of periodontal disease. Whilst some important milestones have been achieved to date, others remain elusive. For example, evidence for development of remote body organ inflammatory pathologies as hypothesised by Miller (1891) and Hunter (1900) attract limited support due to the failure of being unable to satisfy the Koch's postulates thus far.

In the meantime, however, scientists began to collect indirect evidence supporting an unhygienic oral environment with chronic periodontitis having a negative affect with the development of a myriad of diseases. These include cardiovascular (Wu *et al.,* 2000; de Oliveira *et al.,* 2010), stroke, aspiration pneumonia (Pihlstrom *et al.,* 2005), renal pathology (Craig, 2008), premature birth events (Offenbacher *et al.,* 1996; Lopez *et al.,* 2002), diabetes (Grossi and Genco, 1998; Cutler *et al.,* 1999; Taylor and Borgnakke, 2008), respiratory diseases (Scannapieco and Mylotte, 1996; Paju and Scannapieco, 2007), rheumatoid arthritis (Gleissner *et al.,* 1998; Bartold *et al.,* 2005; Pischon *et al.,* 2008) osteoporosis (Kuo *et al.,* 2008), pancreatic cancer (Michaud *et al.,* 2007) and cognitive deficit that in some individuals progresses to dementia (Kondo *et al.,* 1994; Riviere *et al.,* 2002; Kim *et al.,* 2007; Noble *et al.,* 2009 Stein *et al.,* 2007; 2010; 2012).

1.1.1 Experimental advances in favour of the Miller and Hunter concept

In 1998, Socransky *et al.,* identified a consortium of the bacterial species from patients with periodontal disease whereby one group of bacteria belonging to the 'red complex' were consistently found. These were *Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola* (Socransky *et al.,* 1998). In the twenty first century, molecular methodologies have identified nuclear material from oral specimens from over 500 different bacterial species (Paster *et al.,* 2006). As per Miller (1891) and Hunter (1900) hypothesis, scientific evidence exists confirming oral bacteria do access the systemic vessels resulting from everyday tasks of the oral cavity such as physical trauma from chewing food, tooth brushing, scaling, root surface debridement, extractions and periodontal surgery (Carroll and Sebor, 1980; Lofthus *et al.,* 1991; Daly *et al.,* 1997; 2001; Kinane *et al.,* 2005; Savarrio *et al.,* 2005; Forner *et al.,* 2006; Tomas *et al.,* 2007). Specific pathogenic oral microflora represents a potential source of infection anywhere in the body.

Indirect evidence for bacterial invasion of the arterial tissues has also been established with sensitive polymerase chain reaction techniques, sequencing and fluorescence *in-situ* hybridisation (FISH). These have identified genetic profiles of two members of the "red complex" periodontal pathogens in the walls of human coronary arterial tissues and atheroma (Cavrini *et al.,* 2005; Kozarov *et al.,* 2005; 2006).

1.1.2. Periodontal disease

Periodontal disease (Figure 1.1) is a multifactorial chronic condition involving oral pathogens, environmental and genetic elements as well as the host's immune system playing a predominant role (Haffagee *et al.,* 1988). Influence of the disease is episodic with periods of remission and relapse. Although the factors contributing to periods of active/inactive disease are not clear, there is consensus amongst clinicians and researchers that the immuno-inflammatory response within the gingivae and the periodontium (periodontal ligament, cementum and alveolar bone) is initiated by a pathogenic bacterial challenge. Gingivitis in some individuals has the potential to progress to periodontal disease. However, it is the host's cellular and molecular interactions initiated by pathogenic bacterial challenge that ultimately destroy periodontal tissues (Page and Beck, 1997). Formation of a deepened gingival sulcus near the point at which the gingiva makes contact with the tooth is described as a periodontal pocket. The depth of the gingival sulcus is determined by inserting a calibrated tool described as a "periodontal probe". Clinically, a probing measurement of more than 3 mm is considered as early periodontitis.

As mentioned earlier, chronic periodontitis (Figure 1.1), in which the individual may/may not experience pain, is a progressive and inflammatory oral disease with complex aetiology showing genetic and immune susceptibility of the host (Armitage, 1999; Flemmig 1999; Haffajee *et al.,* 1988). The "red complex" pathogenic bacteria comprising of *T. denticola, T. forsythia* and *P. gingivalis* (Socransky *et al.,* 1998; Holt and Ebersole, 2005) appear to be the main players. Although much more remains undiscovered concerning periodontal disease, the aetiology of periodontitis in the context of this thesis has been simplified to as being gram negative poly microbial infections of the periodontium (Figures 1.2a and 1.2b).

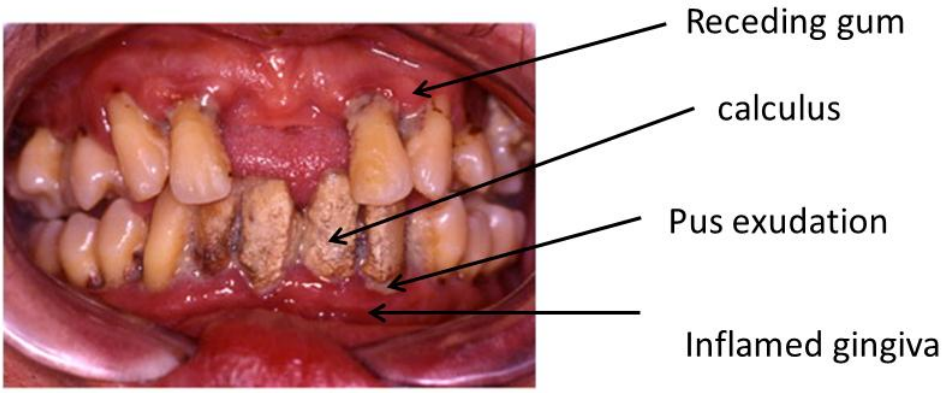

Figure 1.1: Chronic periodontal disease. Image taken from Pizzo G, *et al.,* (2010) Eur J Intern Med. 21(6):496-502 shows chronic periodontal disease in a human subject and tooth loss due to the condition.

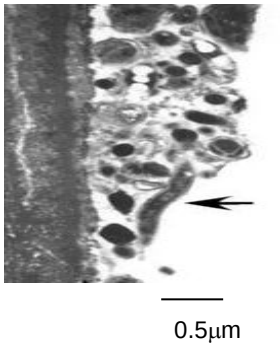

Figure 1.2a: Subgingival pocket with bacteria. Subgingival pocket with typically 10^9 bacteria. Image taken from Noiri Y *et al.,* (2004) J Dent Res 941-5 83(12):941-945.

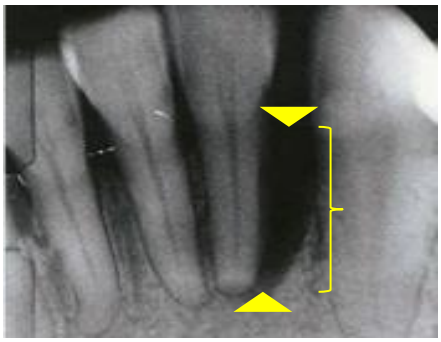

Figure 1.2b: End stage chronic periodontitis. Radiograph of an end stage chronic periodontal pocket as indicated by arrow heads and a bracket

The classical inflammatory response of the gingivae during periodontal disease

The periodontal inflammatory response can be described as an initial acute bacterial challenge stimulating the junctional epithelium to produce a broad range of inflammatory mediators to facilitate both vascular permeability and the influx of neutrophils. The vascular leakage and activation of serum proteins begin to amplify the local inflammatory responses and recruit monocytes/macrophages and T-lymphocytes. Factors produced by activated macrophages can result in the activation of $CD4^+$ T-lymphocytes that aid B-cell differentiation locally in the gingival tissue with antibody production becoming detectable in the blood serum confirming their presence in the body (Haffajee *et al.*, 1995; Kamer *et al.*, 2009; Stein *et al.*, 2012).

All three of the aforementioned red-complex organisms possess known virulence factors of which dentilisin, tannerpains and gingipains respectively are a class of proteolytic enzymes (Curtis *et al.,* 1999; Holt and Ebersole, 2005 Paramonov *et al.,* 2005), which affect the tooth supporting structures (gingiva, periodontal ligament, cementum and alveolar bone). High inflammatory burden can alter the balance between the osteoclast-osteoblasts in favour of bone loss (Assuma *et al.* 1998). Alternatively, bone formation can be down regulated via tumour necrosis factor-α (TNFα) and interleukin-6 (IL-6) expression by the infiltrated macrophages and T lymphocytes (Baker *et al.,* 2000). As bone resorption progresses, the tooth becomes mobile within its socket and eventually falls out.

P. gingivalis is the best-studied (Lotufo *et al.,* 1994) example of periodontal pathogens as it has long been implicated in chronic and severe adult periodontitis (Slots and Listgarten, 1988). It has an armoury of virulence factors (Holt and Bramanti, 1991; Curtis *et al.,* 1999; Paramonov *et al.,* 2005) that destroy matrices anchoring teeth. The trigeminal cranial nerve endings that lie close to the teeth may provide a direct pathway for their access to the brain as is reported for the *Treponema* species (Riviere *et al.,* 2002) and via the vascular channels as originally implied by the founders of the concept (Miller, 1891; Hunter, 1900).

The criterion for investigating responses of central nervous system (CNS) cells to *P. gingivalis* virulence factors was based on an on-going PhD research project which discovered LPS from this pathogen in the brain tissue from a validated diagnoses of Alzheimer's disease (AD) subjects (Poole *et al.,* unpublished data).

1.1.2.1. *Porphyromonas gingivalis*

P. gingivalis is a member of the black pigmented *Bacteroides* genus of bacteria (Figure 1.3) which are anaerobic, gram negative, non-motile, non-spore forming rod shaped that produce dark brown-black colonies on blood agar solid medium (Mayrand and Holt, 1988). The organism was reclassified by Shah and Collins, in 1988, into a new genus termed *Porphyromonas* which includes three different species of bacteria. These are *P. gingivalis, P asaccharolyticus and P. endodontalis*. Of these *P. gingivalis* is associated with chronic periodontal disease (Slots and Genco, 1984).

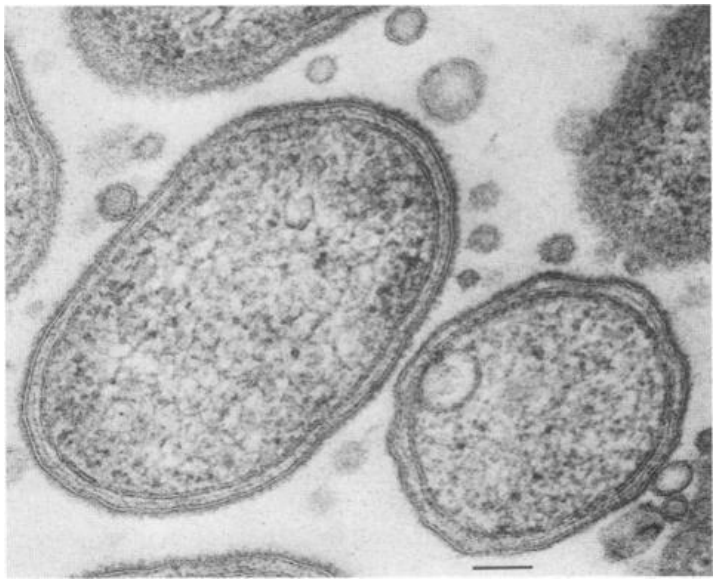

Figure 1.3: Ultrastructure of *P. gingivalis*. An electron microscopy micrograph of *P. gingivalis* ATCC 33277, Bar = 100nm. Image taken from Grenier and Mayrand (1987), Infection and Immunity, 55(1):111-117.

1.1.2.2. Virulence factors of *P. gingivalis*

A number of virulence factors are associated with *P. gingivalis* strains of pathogenic bacteria which help the organism to destroy host's oral surfaces. These include the proteolytic enzyme, gingipains, LPS, outer membrane vesicles, short chain fatty acids (butyric and propionic acids).

Gingipains

Gingipains has long been recognised for its trypsin like enzyme activity (Mayrand *et al.*, 1980; Slots, 1981). It was first purified and characterised from the bacterial capsule (Yoshimura *et al.,* 1984) and subsequently in the outer membrane (Fujimura and Nakamura, 1987). It is now referred to as gingipains (Holt and Ebersole, 2005) and is known to degrade fibronectin and collagen.

Lipopolysaccharide and outer membrane vesicles

LPS is the major component of the outer membrane of all bacteria. The LPS from Gram negative bacteria can exert biological effects by stimulating host cells to produce bioactive inflammatory mediators that destroy the host tissues. The gram negative periodontal pathogens continuously undergo surface membrane modification in which small outer membrane vesicles (Figure 1.4) are released (Grenier and Mayrand, 1987). These are in the nanoparticle size (20-200 nm in diameter) range and also contain gingipains (Duncan *et al.,* 2004). The vesicles contribute towards their biofilm colonisation as well as entering the extracellular milieu due to their small size (Schooling and Beveridge, 2006). They also contain biologically active components that initiate innate immune responses (Manning and Kuehn, 2011). LPS is an example of pathogen-associated molecular patterns (PAMP). PAMP's are not expressed by the host but are found exclusively in bacterial cell walls enabling host cells to distinguish between "self" and "non-self".

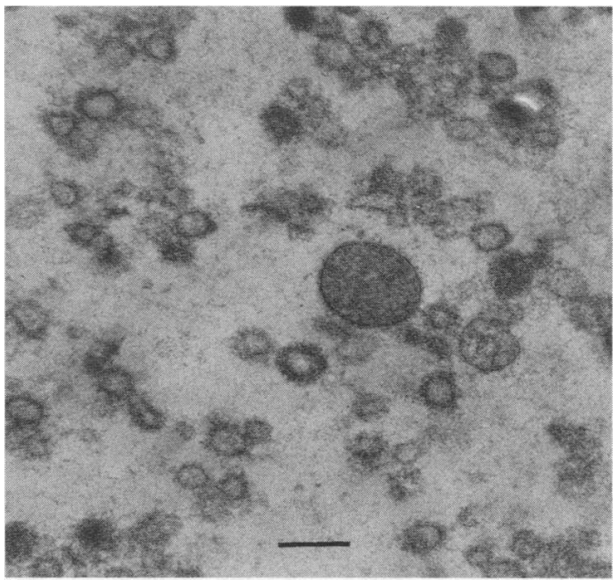

Figure 1.4: High resolution image of the outer membrane vesicles. High resolution transmission electron microscope image of the outer membrane vesicles shed by *P. gingivalis* ATCC 33277. Bar = 100nm. Taken from Grenier and Mayrand (1987), Infection and Immunity, 55(1):111-117

Short chain fatty acids

P. gingivalis also releases by-products of its metabolism such as butyric and propionic acids. Apart from contributing to the noxious smell, they inhibit fibroblast proliferation (Singer and Buckner, 1981) and are toxic to lung fibroblast cells in culture (Van Steenbergen *et al.,* 1982; Touw *et al.,* 1982; Grenier and Mayrand, 1985). All these virulence factors permit greater tissue destruction and disease progression.

One consequence of Hunter's proposal of total tooth extractions may be the result of the discovery by Kondo *et al.,* (1994), linking missing teeth with dementia in a Japanese elderly population. Dementia is a complex condition in which individuals develop poor memory and progressive decline in overall mental function without the loss of consciousness (Lishman, 1978), of which AD is an example. A subsequent longitudinal study by Stein *et al.,* (2006) found a statistically significant association with missing teeth due to periodontal disease and mental health in a group of women belonging to a religious order (Stein *et al.,* 2006). The exact connection between missing teeth due to chronic periodontitis and deteriorating memory is not resolved. However, further reports from Stein *et al.,* (2012) suggest that the titre of circulating antibodies to multiple periodontal pathogens in the elderly individual may represent an indicator of a potential risk factor for developing cognitive deficit during advancing age with potential in some cases to progress to dementia.

Periodontitis and AD, share several important features in that, they are both progressive and have an important inflammatory component (Haffagee *et al.,* 1988; Akiyama *et al.,* 2000). Additionally, both diseases have complex aetiologies including interactions between systemic infections (oral pathogens, bronchopneumonia and urinary tract infections), immunosenescence, aging and genetic factors (D'Aiuto *et al.,* 2004; Engelhart *et al.,* 2004; Noble *et al.,* 2009; Kim *et al.,* 2007; Hajishengallis, 2010). However, unlike periodontal disease, AD does not show signs of remission and relapse as once the memory is lost, it does not return.

Since LPS is the principal initiator of host innate immune responses, it is likely that during life, there is potential for this PAMP to initiate inflammation in the aging CNS. Additionally, literature supports the view that LPS initiated inflammation in the brain can accompany learning and memory impairment (Tanaka *et al.,* 2006; Chen *et al.,* 2008) but, without the knowledge of the exact mechanisms involved. There is only one report having investigated the effect of LPS from *P. gingivalis* on primary rodent astrocytes *in-vitro* (Shapira *et al.,* 2002) in relation to multiple sclerosis (a demyelinating disease). However, unlike multiple sclerosis, neurodegenerative disorders of which aging is a major risk factor

show glial cell activation. Hence this investigation was needed to understand the effects of the virulence factors from periodontal pathogens related to specific glial cell responses. This project utilised the *P. gingivalis,* American Type Culture Collection (ATCC) (ATCC 33277 and ATCC 53978 - W50) whereby both organisms are on the opposite end of the spectrum for their low to high pathogenicity. In addition the all-important biological tools (virulence factors and antibodies) essential for this study were accessible.

1.2 Aging, structure and function of the Central Nervous System and neuro-inflammation

1.2.1 General Introduction

As people grow older, they begin to experience "senior moments" that reflect temporary or benign memory loss. One downside of deteriorating memory is the loss of social values as the benign state can switch in favour of disease. Numerous studies indicate changes taking place in the aging brain. For example the brain weight decreases with age but the cause of reduction remains unclear. The volume of the brain in the 8th decade is reduced by 6%-10% as compared with the third decade. The age related changes are more prominent in the frontal lobe which shows a 10% reduction in volume and 15% reduction in cortical thickness. The corpus callosum is decreased in volume by 12-17%, with accentuation in the anterior 2/5th because of the reduction of fronto-temporal inter-hemisphere fibers.

At the cellular level there are phenotypic changes in the brain such as the activated or the inflammatory status of glia. What is not clear is, if these changes are due to faulty functioning of the innate immune system (immunosenescence) predisposing the senior individuals to microbial infections (Caruso *et al.,* 2009; Hajishengalis, 2010) or if intra-cerebral derived abnormal proteins are contributing to local inflammation. One hypothesis links high inflammatory burden in the elderly to deteriorating memory (Holmes *et al.,* 2003; Dunn *et al.,* 2005; Holmes *et al.,* 2009). In order to understand the impact of inflammation in the aging brain, it is necessary to understand the anatomy and the basic neurobiology of the CNS.

The CNS consists of the brain and the spinal cord which are constantly bathed in the cerebrospinal fluid (CSF) secreted by the choroid plexus. The CSF provides nutrients and cushions the brain from mechanical shock. The CNS consists of gray and white matter. The

gray matter contains cell bodies of neurons, and white matter mainly being, myelinated axons.

1.2.1.1 The Brain

The brain is separated by a deep midline which is known as the longitudinal cerebral fissure into two cerebral hemispheres. In the depth of the fissure, across the midline, the hemispheres are connected to each other by a thick bundle of nerve fibers called the corpus callosum. The brain is highly folded to increase the surface area of the gray matter. These folds are known as gyri and are separated from each other by indentations known as sulci.

Each cerebral hemisphere has four lobes which are defined according to lateral, medial and inferior surfaces of the brain (Figure 1.5).

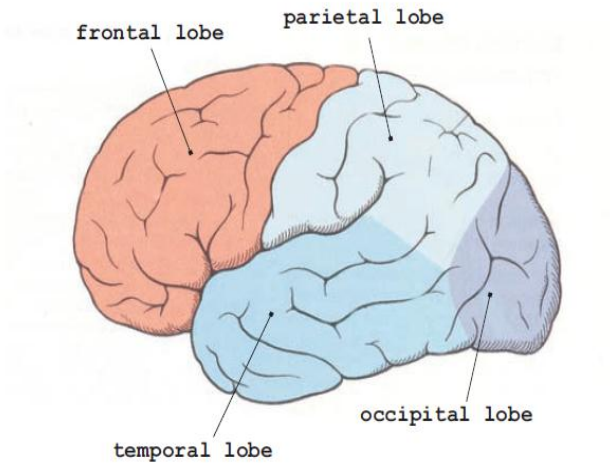

Figure 1.5: Schematic presentation of a human brain hemisphere. The schematic image showing the lateral view of a brain hemisphere whereby the different lobes are colour coded for ease of identification. (Image taken from Kahle and Frotscher, 2003)

The frontal lobe

The frontal lobe is located behind the forehead and is the largest of the four lobes. It occupies the anterior of the cortex and controls the ability to reason, plan, comprehend an idea or action and adapt to new situations.

The parietal Lobe

The parietal lobe is located at the top and rear of the brain. It is concerned with perception of stimuli related to touch, pressure, temperature and pain. Damage to this lobe may lead to a person becoming unfamiliar with parts of their body.

The temporal Lobe

The temporal lobe is just above the ear canal and controls hearing. For this reason, it is sometimes called the auditory cortex. Some functions of the temporal lobe are perception and recognition of auditory stimuli (hearing) and memory (hippocampus). Damage to this area can result in hallucinations, aphasia, and loss of language.

The occipital Lobe

The occipital lobe of the brain is located at the rear of each hemisphere. It is concerned with many aspects of vision. Damage to the occipital lobes can result in blindness.

The cerebral cortex

The hemispheres are completely covered by the cerebral cortex and are composed of gray matter. It is estimated that the brain as a whole contains around 100 billion neurons and at least a 100 trillion synapses. Some neurons make synapses with more than 10 thousand different neurons.

The prefrontal cortex is one of the areas believed to participate in learning and memory (Wickelgren, 1979; Damasio, 1989; Squire, 1992) alongside of the hippocampus. Exactly how the two areas connect the processes involved with learning and memory formation are complex, but anatomically, the hippocampus is joined to rest of the cortex by the subiculum.

Hippocampus

The **hippocampus** belongs to the limbic system (Figure 1.6a) and is also one of the regions believed to play important roles in long-term memory and spatial navigation.

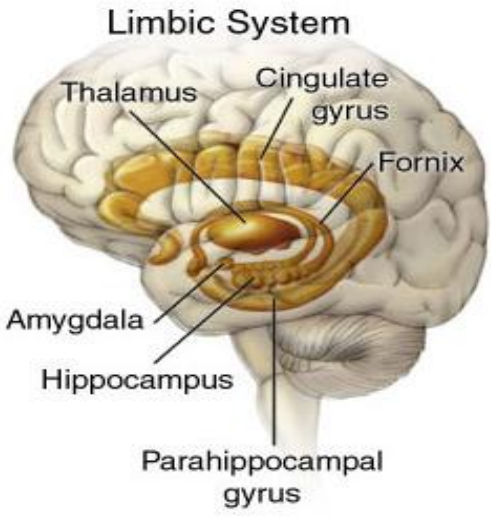

Figure 1.6a: An overview of major limbic system structures in the brain. Location of major limbic system structures in three-dimensional form within the whole brain.

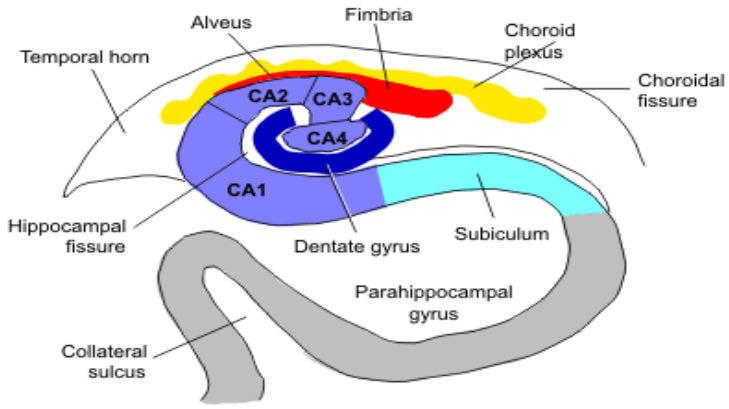

Figure 1.6b: Anatomical regions associated with the hippocampus. Schematic diagram to show the essential anatomical regions associated with the hippocampus

The hippocampus is an elongation of the edge of the cerebral cortex. It can be distinguished as a zone where the cortex narrows into a single layer of very densely packed neurons. The structures that line the edge of the cortex make up the limbic system (Figure 1.6a). These include the hippocampus, cingulate cortex, olfactory cortex, and amygdala. The hippocampus has been likened to a seahorse (*Cornis ammonis*) and the subdivisions CA1 through to CA4 refer to *Cornis ammonis* 1-4 regions containing pyramidal neurons (Figures 1.6b and 1.7).

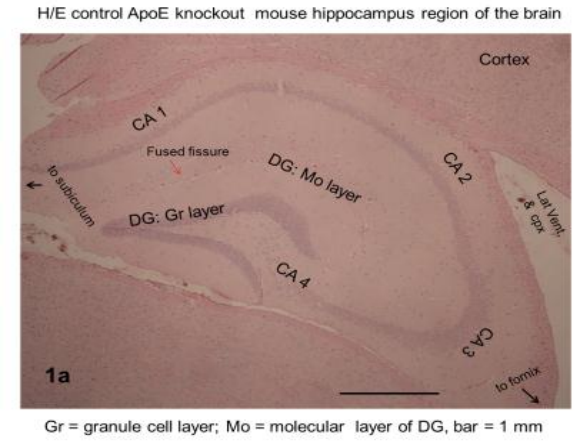

Figure 1.7: Hippocampus: the brain region responsible for memory. Tissue section from a mouse brain showing the hippocampus and its associated histological features which are labelled

An overview of memory

Memory is a functional entity of the brain and its biological measure is "long term potentiation" (Bliss and Lomo, 1973). These scientists first described the process in the hippocampus where neurons "learned" following repetitive electrical stimulations via the perforant pathway of the dentate gyrus granular cells (Bliss and Lomo, 1973). Following high frequency electrical stimulation, Bliss and Lomo (1973) observed an increase in the excitatory post-synaptic potential amplitude of the dentate granule cell population. This increase in the efficacy of synaptic signalling has been related to memory as it can vary in duration from minutes to hours (Abraham *et al.*, 2002).

1.2.2. Cellular neurobiology

1.2.2.1. The nerve cell function and structure

There are approximately 100 billion neurons in the brain (Johnson and Erner, 1972) which originate from the ectoderm during embryogenesis (Allen and Barres, 2009). A neuron consists of a cell body (the soma), processes called dendrites which are the fine extensions. These convey information towards the soma from synapses on the dendritic tree. The primary role of dendrites is to increase the surface area for synapse formation allowing integration of a large number of inputs which are relayed to the cell body. Neurons have one main process called the axon (Figure 1.8) which originates at the axon hillock and is the most excitable part of the neuron due to the high density of sodium channels and is also the part where the action

potential is initiated (Eccles, 1964). The cell membrane is also an important component of the neuron as ion channels, neurotransmitter receptors and ion pumps are located within the lipid bilayer (Klymkowsky and Stroud, 1979; Poulter *et al.*, 1998; Stroud *et al.*, 1990). Neurons are highly specialized for intercellular communication involving the propagation of an action potential down a neuronal axon to a presynaptic terminal. At the presynaptic terminal the signal leads to depolarization of membrane and release of neurotransmitters to receptors on the postsynaptic membrane of another neuron. This step is followed by a depolarization of this second neurons, and further propagation of the signal (Allen and Barres, 2009).

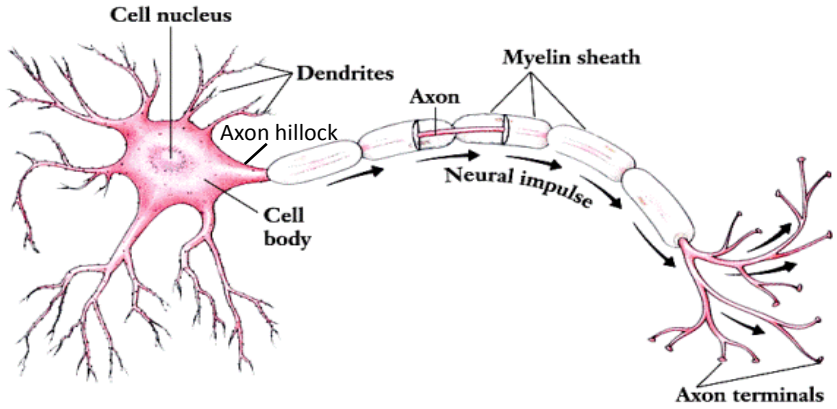

Figure 1.8: A peripheral nerve and its basic anatomical features. Basic structure of a neuron (peripheral nervous system). Image as taken from http://www.google.co.uk/images; re-Notes on biological foundations of behaviour.

Most neurons contain Nissl substance which is mainly composed of ribosomes and polysomes (Singhrao and Nair-Roberts, 2010). Neurofilaments maintain the cytoskeletal architecture of the neuron. Microtubules and microfilaments provide for the flow of the axoplasm and for axonal elongation.

The synapse

The synapse is a highly specialised structure between a presynaptic nerve terminal and a postsynaptic neuron as indicated in figure 1.9. Synapses can vary in size and a typical synapse in the CNS is composed of a presynaptic nerve terminal and a synaptic cleft which physically separates the nerve terminal from the postsynaptic membrane (Figure 1.9). A chemical (neurotransmitter) is released from the presynaptic terminal and is captured by

specific receptors in the post synaptic membrane where it is processed, integrated and propagated by the nerve cell into meaningful information.

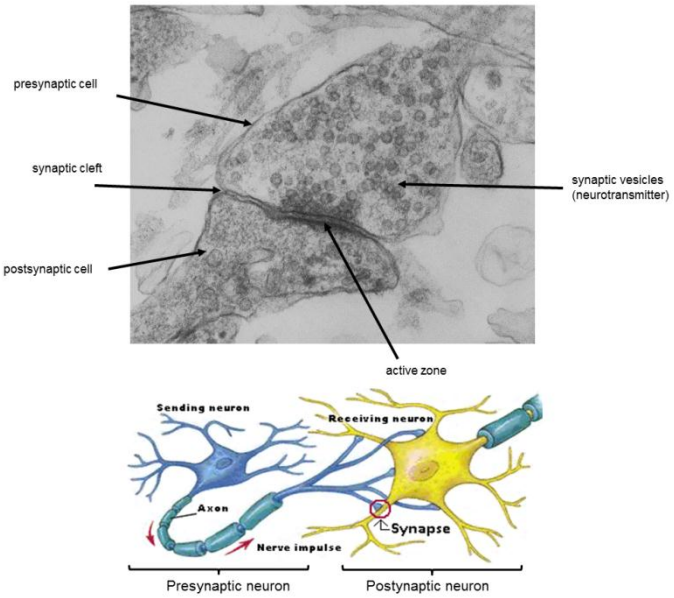

Figure 1.9: A **transmission electron microscope image of a synapse.** Image from http://www.google.co.uk/images. A schematic diagram directly below the electron microscope image shows the synapse in relation to the overall cell anatomy (image from http://www.google.co.uk/images). Some labels added.

Chemical messengers

Neurotransmitters are chemicals released at a synapse by presynaptic neurons that affect the postsynaptic cell in a specific way. They are stored in synaptic vesicles to protect them from enzymatic degradation. There are two types of synaptic vesicles known as small clear-core type which are around 50nm in diameter and these are triggered by single action potentials. The larger dense core vesicles 100nm in diameter are released by burst firing or repetitive stimulation and include the bio-amine and neuropeptide group of neurotransmitters. To prevent neurons from being overloaded, there are inhibitory and excitatory synapses. The most abundantly expressed neurotransmitters in the nervous system are acetylcholine (London *et al.*, 1995; Perry and Kellar, 1995; Perry *et al.*, 1999) glutamate (McGeer and McGeer, 1975; Lindefors *et al.*, 1989; Nair-Roberts *et al.*, 2008), dopamine (Lindefors, 1993; Nair-Roberts *et al.*, 2008), serotonin (Monsma *et al.*, 1993; Plassat *et al.*, 1993; Ruat *et al.*, 1993) and gamma amino butyric acid (GABA) (Balcom *et al.*, 1975; McGeer and McGeer, 1975; Singhrao and Nair-Roberts, 2010).

Glutamate is the most abundant neurotransmitter in the brain and is important in regards to memory and learning. It is the major excitatory neurotransmitter and is toxic to neurons if present in excess. Dopamine is an inhibitory neurotransmitter, meaning it blocks the tendency of a neuron to fire an action potential. Dopamine is associated with motivation and addiction.

GABA is an inhibitory neurotransmitter and individuals lacking GABA in their brain tend to suffer from anxiety disorders and epilepsy. Serotonin is an inhibitory neurotransmitter and is linked to emotions, wakefulness and temperature regulation. Acetylcholine has many functions one of these being related to AD due to lower activity of the enzyme choline acetyltransferase (Bowen *et al.,* 1976; Davies and Maloney, 1976; Perry *et al.,* 1977). All these neurotransmitters appear to be inter-related and have relevance to memory.

Neurotransmitter receptors

When released, the neurotransmitter is captured by a specific receptor in the postsynaptic membrane. Some common examples of receptors are n-methyl-D-aspartate (NMDA) receptors (Decker *et al.,* 2010[a and b]), and alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) (Hsieh *et al.,* 2006) and kainate receptors (Agrawal and Evans, 1986). These receptors respond to the glutamate neurotransmitter (Lacor *et al.,* 2004; 2007). Others, for example, the nicotinic acetylcholine receptor (nAChR) responds to acetylcholine neurotransmitter (Seguela *et al.,* 1993; Wada *et al.,* 1989).

In vivo animal studies provide evidence for the importance of the neurotransmitter receptors in the hippocampus in memory performance and is linked to high densities of nicotinic receptors, particularly α7 and α4β2 subunits (Seguela *et al.,* 1993; Wada *et al.,* 1989). If these receptors are blocked experimentally with the non-selective nicotinic receptor antagonist mecamylamine, memory and long term potentiation become impaired (Kim and Levin, 1996; Welsby *et al.,* 2006; 2009). Similarly either the α7 nicotinic receptor anatagonist methyllycaconite or the α4β2 nicotinic receptor antagonist dihydro-β-erythroidine can have detrimental effects on the working memory (Addy *et al.,* 2003). Conversely, nicotine improves cognitive performance in age-associated memory impairment (White and Levin, 2004). Postsynaptic receptors are trans-membrane proteins that adsorb released neurotransmitters at its binding site. This induces a conformational change and opens an ion channel directly or indirectly. The opening of ion channels on the postsynaptic cell causes a change in the excitation of the cell bringing about brief electrical responses

called “synaptic potentials”. The activated receptor can only return to its resting state once the neurotransmitter has been removed either by enzymatic hydrolysis (e.g. cholinesterase) or re-uptake into the presynaptic nerve terminal or cleared by glial cells (e.g. GABA).

Glia

Astrocytes, microglial cells, oligodendrocytes, ependymal cells are the non-neuronal support cells collectively called glia. Astrocytes and microglia are of significant interest to researchers investigating any aspect of neurodegenerative diseases as they contribute to the developing inflammatory pathology.

Ependymal cells

Ependymal cells are located strategically at the boundary between the CSF and the brain. The ependymal cells express phagocytic receptors, including the mannose receptor and TLRs 2 and 4 (further examples of PRR’s) to detect and clear bacteria (Cserr *et al.,* 1992; Stahl and Ezekowitz, 1998; Laflamme and Rivest, 2001; Husemann *et al.,* 2002).

Differences between astrocytes and microglia

Astrocytes

These are the most common type of glia found throughout the CNS. Deiter (1865) is credited with the earliest description of astrocytes. Astrocytes are derived from ependymoglia of the developing neuronal tube (Abbott *et al.,* 2006). Morphologically astrocytes have oval or irregular nuclei with an open chromatin pattern and stellate morphology with numerous fine processes radiating in all directions. Astrocytes are of two main types, fibrillary and protoplasmic. Fibrillar astrocytes can be demonstrated by immunostaining with glial fibrillary acidic protein (GFAP) (Bignami *et al.,* 1972) and the protoplasmic astrocytes using anti-metallothionine antibody E9 (Neal *et al.,* 1996).

Following injury, activated fibrillary astrocytes become hypertrophic with increased amounts of cytoplasm and express higher levels of GFAP protein. Their increased numbers in the area of injury and hyper-expression of GFAP protein constitutes astrogliosis possibly from cytokines being released by microglia.

Microglia

Microglial cells were first identified by silver impregnation techniques by Rio-Hortega in 1919. These are small cells scattered throughout the CNS. Depending on their functional properties, microglia display different morphologies where the quiescent (ramified) phenotype, is typified by long branching processes attached to a small cell body (Ransohoff and Perry, 2009; Hanisch and Kettenmann, 2007). This phenotype represents a reservoir of non-activated microglia within the CNS. Microglia can become activated by a variety of factors including physical injury, pro-inflammatory cytokines, LPS and inflammatory macromolecules. Once activated microglia undergo several key morphological changes including thickening and shortening of branching processes attached to a hypertrophic cell body to adopt the characteristic morphology of activated microglia (Perry *et al.,* 2010).

Astrocyte cell function

The astrocytes are responsible for the maintenance of homeostasis in the micro-environment of the neurons. For example, they are implicated in the formation and stabilisation of neuronal synapses (Ullian *et al.,* 2001; Christopherson *et al.,* 2005; Allen and Barres, 2009). They also participate in metabolic pathways which modulate the transport of ions, release of transmitters, metabolites. Astrocytes are recognised for their function in maintaining the blood brain barrier (BBB) (Nedergaard *et al.,* 2003) and maintaining tight control of local ion and pH homeostasis, glucose levels and metabolic substrates (Nedergaard *et al.,* 2003; Ransom *et al.,* 2003).

The blood brain barrier and immuno privilege of the CNS

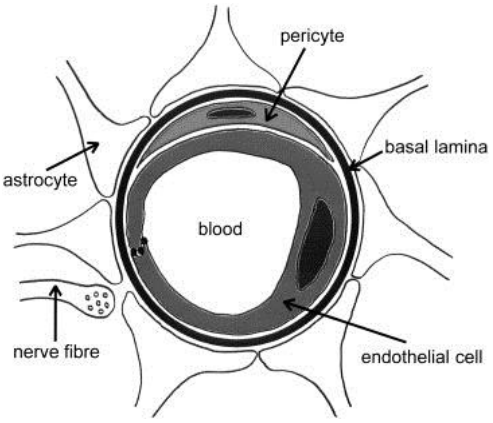

Figure 1.10: Schematic showing the intact blood brain barrier. The section in the transverse plane shows the blood brain barrier is composed of endothelial cells, pericytes and astrocytes. Image is taken from Stam R (2010) published in Br Res Rev 80-97.

In health, the CNS is an immune privileged organ that maintains a physical BBB through the endothelial cells in capillaries (Figure 1.10). The tight endothelial cell junctions offer high electrical resistance and foot processes of astrocytes tightly cover capillary openings to the brain protecting neurons from extrinsic insults. This way the CNS retains its immunosuppressive microenvironment that keeps naive T cells and plasma proteins/toxins out. The intact BBB also prevents microglial cell activation as the result of systemic antigens such as LPS, gaining unrestricted access to the brain parenchyma.

Astrocytes react to injury by adopting the "activated" morphology (Figure 1.11). The reactive fibrillary astrocytes engage in secondary phagocytic activities (al-Ali and al-Hussain, 1996; Nguyen *et al.*, 2011) and can also be considered as antigen presenting cells second in line to microglia.

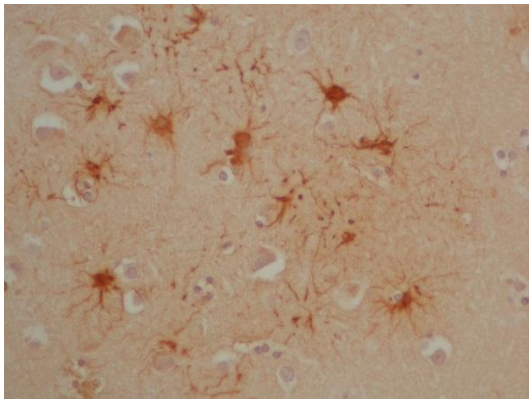

Figure 1.11: Reactive astrocytes in a disease condition. Reactive astrocytes from AD brain stained with anti-GFAP following immunoperoxidase/Diaminobenzidine method. The activated phenotype is whereby the soma of the cell has become enlarged and their fibrillary projections dilate. Image = x20 objective on a light microscope.

The reactive astrocytes (Figure 1.11) can switch on differential expression of multiple genes compared to the non-reactive astrocyte (Eddleston and Mucke, 1993; Eng and Ghirnikar, 2000) in which they synthesize some components of the innate immune system (Levi-Strauss and Mallat, 1987; Morgan and Gasque, 1996). Astroglioma cell lines have proven to be useful models for astrocyte function *in vitro* and have been used to provide evidence for a role in antigen presentation and inflammatory cytokine release in the CNS (Fontana *et al.*, 1987). Using rat astrocytes and human astrocyte-derived tumour cell lines treated with the inflammatory cytokine interferon-gamma (IFN-γ) have shown these cells to secrete all components of the classical and alternative and terminal pathways of the complement system (Levi-Strauss and Mallat, 1987; Walker and McGeer, 1993; Morgan and Gasque, 1996).

Microglial cell function

As mentioned earlier, microglia can become activated (Figure 1.12) by a variety of factors including physical injury, interaction with pro-inflammatory cytokines, LPS and other inflammatory macromolecules.

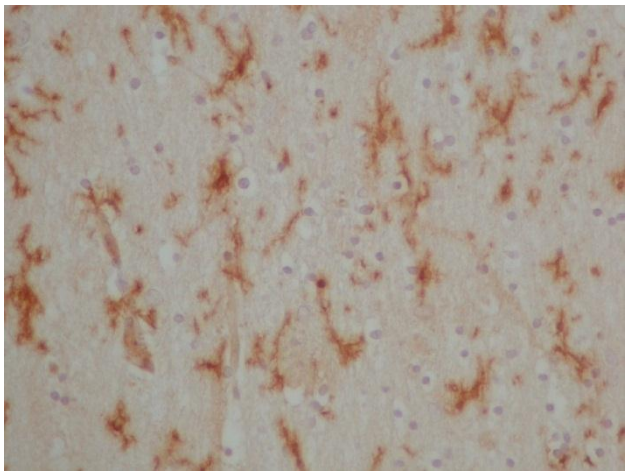

Figure 1.12: Activated microglial cells in a disease condition. Activated microglial cells from AD brain stained with HLA DR (clone CR3/DP, DQ, DR) antibody. The activated phenotype of microglia is in which they have become enlarged and their processes have become thickened as shown. Image = x20 objective on a light microscope.

Brain inflammation, which occurs behind the BBB, therefore differs from inflammation in the periphery by the relative absence of leukocytes (including neutrophils, monocytes, B cells, and T cells) and antibodies. Nevertheless, microglial cells are key cells contributing to brain inflammation. They express a range of proinflammatory interleukin class of cytokines (Hanisch, 2002) and are capable of recognising non-self PAMP's displayed by bacteria and their cellular debris to reduce the inflammatory response.

Importance of glial cells in local innate defence mechanism against pathogen entry

As there is no immune surveillance by cells from the classical adaptive immune system, the resident glial cells provide a local innate defence mechanism capable of defending the CNS against pathogen entry. Most importantly microglia are HLA-class II positive cells capable of

antigen presentation as well as expressing CD14, toll like receptors 2 and 4 (TLR-2 and TLR4) that recognise PAMP's and a range of complement receptors 1, 3 and 4 (CR1, CR3 and CR4) that identify and phagocytise bacteria (Gasque, 2004). The intrinsically activated microglia express *de novo* immune markers including MHC class II proteins complement receptors (CR1, CR3, CR4, TLRs 2 and 4) and pro-inflammatory signalling molecules including the complement anapylatoxins (C4a, C3a, C5a) that recruit more activated microglia to the site of tissue injury (Laflamme and Rivest, 2001; Gasque, 2004) resulting with cytokine synthesis. However, when challenged with systemic LPS, microglial cells again demonstrate an activated phenotype capable of mounting a similar innate immune response to combat the destructive effects of the extrinsically derived endotoxins. Continual exposure of microglia to both, circulating systemic LPS due to concurrent infections such as bronchopneumonia, urinary tract and oral infections, together with intra-cerebral infections result in the continual activation of microglia and the adoption of a hypersensitive 'activated' phenotype. The LPS hyper-sensitized microglia increase synthesis of inflammatory mediators tumour necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) complement factors, TLR-2 and TLR-4 and nitric oxide (NO) that release free radicals and reactive oxygen species (Laflamme and Rivest, 2001; Gasque, 2004; Boje and Arora, 1992; Lodge and Sriram, 1996; Floyd, 1999; Ye and Johnson, 2001; Godbout *et al.,* 2005) increasing the severity of neurodegeneration.

Numerous studies suggest that advancing age is associated with developing inflammatory changes (Letiembre *et al.,* 2007b). It is plausible that the inflammatory status is an adaptive immune mechanism during the aging process or a function of advancing age that makes elderly individuals more susceptible to infections (Caruso *et al.,* 2009; Hajishengalis, 2010). Collectively these factors contribute to the development of poor memory (Dunn *et al,* 2005; Letiembre *et al.,* 2007b). It is reported that the number of innate immune TLR increase in the brain following normal aging in mice (Letiembre *et al.,* 2007a) and similar observations are reported from AD brains (Liu *et al.,* 2005; Letiembre *et al.,* 2007b; Walter *et al.,* 2007) and in AD transgenic animal models (Fassbender *et al.,* 2004; Letiembre *et al.,* 2007b; Walter *et al.,* 2007; Wirths *et al.,* 2010; Frank *et al.,* 2009).

1.2.2.2. Extrinsic factors contributing to neuroinflammation

Numerous studies have shown that LPS from gram negative bacteria either administered directly into the peritoneum, systemic or the brain, all induce neuroinflammation in the form of glial cell activation (Cunningham *et al.,* 2005; Godbout *et al.,* 2005; Chen *et al.,* 2008; Henry *et al.,* 2009) and where measured, the inflammatory response is accompanied by learning and memory impairment (Tanaka *et al.,* 2006; Chen *et al.,* 2008). A different investigation evidenced IL-1β secretion following peripheral challenge with LPS (Henry *et al.,* 2009). The already activated glia synthesise cytokines (IL-1β, tumour necrosis factor-alpha or TNF-α) in response to infection (Cunningham *et al.,* 1996; Henry *et al.,* 2009). Such cytokines are implicated in synaptic loss (Bellinger *et al.,* 1993; Yang *et al.,* 2005; Mishra *et al.,* 2012) and with reduced long term potentiation (Bellinger *et al.,* 1993; Cunningham *et al.,* 1996). Although data on the mechanisms in relation to changes in long term potentiation and inflammatory burden are sparse, only a few *in-vitro* methodological studies suggest the cytokines exert their effect on the NMDA (Yang *et al.,* 2005) and AMPA (Lai *et al.,* 2006) receptors.

Recognition of bacterial lipopolysaccharide (LPS) by innate immune cells

The receptor for LPS is CD14 which is also known as a pattern recognition molecule of the innate immune system (Wright *et al.,* 1990) and is a 55-kD glycosylphosphatidyl inositol (GPI)-anchored surface glycoprotein (Haziot *et al.,* 1988). It lacks the trans-membrane domain and is therefore, unable to initiate intracellular cell signalling. In order to initiate intracellular signalling, CD14 requires a co-receptor with the intracellular domain. A family of type I trans-membrane TLRs has been identified in humans and mice (Poltorak *et al.,* 1998 a and b; Arbour *et al.,* 2000). To date, 10 human TLRs appear to function as innate immune receptors for LPS and other microbial products (Shimazu, 1999). Of these, TLR-2 (LPS from gram positive and TLR-4 from gram negative bacteria) have been reported to function as LPS-signalling receptors.

Following LPS binding to CD14, on myeloid cells, the TLRs initiate intracellular signalling cascades that stimulate release of proinflammatory cytokines (including IL-1, IL-6, and TNF-α), chemokines (IL-8), nitric oxide synthase and other anti-microbial peptides to destroy pathogenic microbes (Han and Ulevitch, 2005). CD14 is abundantly expressed by all CNS cells including neurons, astrocytes and microglia (Griffiths *et al.,* 2010). TLRs recruit

additional adaptor proteins such as myeloid differentiation primary response gene 88 (MyD88) (Kawai and Akira, 2007) MD-2 (Shimazu *et al.*, 1999; Nagai *et al.*, 2002) for optimal intracellular signalling involving the mitogen-activated protein kinase (MAPK) for NF-kB-dependent pro-inflammatory gene expression (Wang *et al.*, 2001; Sanjo *et al.*, 2003; Shim *et al.*, 2005) see figure 1.13.

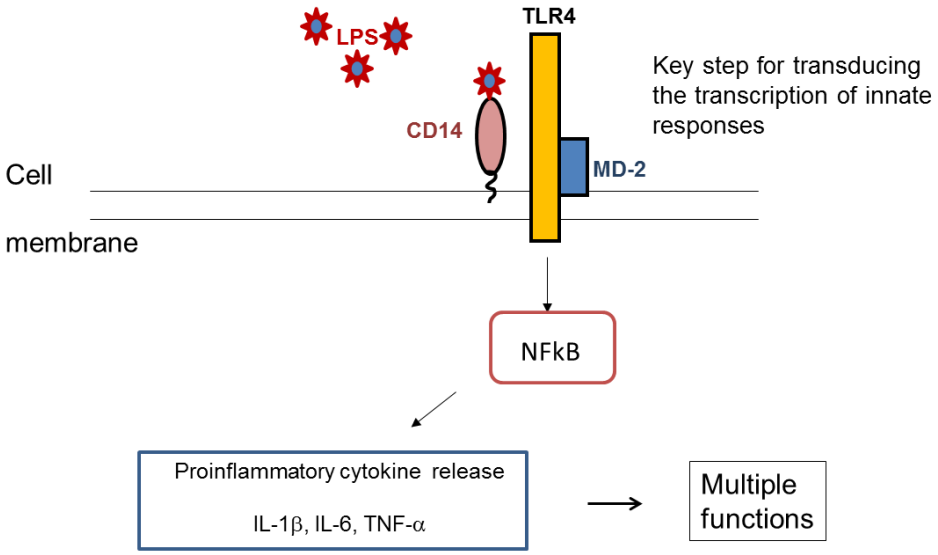

Figure 1.13: A pathway for the recognition of bacterial lipopolysaccharide. LPS from bacterial membranes is transferred to a lipid-binding site on CD14 in the membrane of phagocytes. CD14 promotes the binding of LPS to the TLR4-MD2 complex, which initiates intracellular cell signalling. In the absence of CD14, TLR4-MD2 can still function with some forms of LPS or with much higher LPS concentrations.

The immune cells of the CNS also sense the immune challenges presented via the blood vascular system using the circumventricular organs and the choroid plexus that are devoid of the BBB (Oldfield and Mckinley, 1995). Cells from this region of the brain are fully equipped with the CD14 receptor and the TLR-4 to recognise LPS from the peripheral blood circulation (Lacroix *et al.*, 1998; Laflamme and Rivest, 2001; Beutler *et al.*, 2003). Hence, elements of systemic infections such as those originating from gram negative, oral pathogens, bronchopneumonia and urinary tract infections (Holmes *et al.*, 2003; Dunn *et al.*, 2005, Kamer *et al.*, 2009; Holmes *et al.*, 2009; de Oliveira *et al.*, 2010) reach all organs including the CNS.

1.3. The Rationale

Dental infection may provide a potential pathogenic and inflammatory link during advancing age through its interaction with the CNS innate immune responses. If this is the case, dental intervention will be a priority from an early age.

1.3.1. Aim

The main aim of the study was to determine the responses of CNS cells to direct exposure of endotoxins from periodontal pathogens to incite innate immune responses and activate astrocyte cells to release acute phase cytokines and chemokines following exposure to the culture supernatant of *P. gingivalis* (ATCC 33277 and W50). Where, *P. gingivalis* W50 is more pathogenic than 33277.

1.3.1.1 Objectives

1. To establish brain cell (cell lines and primary cells) cultures.
2. Expose cells in culture to the pathogen and its products from any one/or all of the three red complex pathogens stated (*P. gingivalis* (ATCC 33277 and W50) and *Treponema denticola and Tannerella forsythia*).
3. Measure the cellular responses using immunofluorescence labelling, immunoblotting and ELISA assays for cytokine release.

1.3.1.2 Research approach

The main methodologies used in this investigation are *in vitro* cell culture, immunofluorescence, immunoblotting and commercial Elisa assays which are fully described in the next chapter.

Chapter 2: Investigating responses of CNS cells to *P. gingivalis* virulence factors

2. Investigating responses of CNS cells to *P. gingivalis* virulence factors

2.1 Introduction (in brief)

Aging has an element of neuroinflammation and is viewed in some patients as being a precursor for developing dementia. A number of studies suggest the main differences between the young and the aged brain (age being a major risk factor for AD) is an imbalance in the control of pro-inflammatory mediators especially cytokines (Ye and Johnson, 1999; Godbout *et al.,* 2005; Chen *et al.,* 2008; Henry *et al.,* 2009) perhaps due to inflammation developing quickly on subsequent exposure to pathogens (Holmes *et al.,* 2003; Dunn *et al.,* 2005; Godbout *et al.,* 2005) and/or the organ having diminished ability to cope with the overwhelming inflammatory burden. This study set out to find evidence for inflammatory responses initiated by a periodontal pathogen on CNS cells *in vitro*.

2.1.1. Materials and Methods

2.1.1.1 List and source of reagents

Control and test spent media for *P. gingivalis* (ATCC 33277) and (ATCC 53978 - W50) and the anti-LPS and anti-gingipains (clone 1B5 and clone 1A1) were a kind gift from Prof. M. Curtis and Dr A. Hashim (Centre for Immunology & infectious disease, London, UK). The rabbit anti-human GFAP (gift from Dr J. Newcombe, The MS Society, London, UK), Mouse anti-human CD14 (clone HCD14) and rat anti-mouse CD14 from Bio-Legend, Thermo-Fisher , rabbit anti-PGP 9.5 (UltraClone Ltd, UK), SVGP12 and IMR32 from ATCC, *E.coli* LPS from Sigma Aldrich, UK Normal, goat serum (X0907) and normal rabbit serum (X0902) from DakoCytomation, Germany, Phalloidin-FITC from Molecular probes (UK). Anti-*T. forsythia* antibodies (recombinant bacterial surface protein A (rBspA), gift from Dr Sharma, USA), anti-*T. denticola* antibodies were raised in-house from hybridoma cell lines (TDII (HB-9966) and TDIII (HB-9967) purchased from ATCC as per manufacturer's

instructions. All cell culture media and additives were from Life Technologies Ltd**,** Paisley, UK, encompassing a consortium of companies inclusive of Invitrogen®**.** Applied Biosystems® · Gibco® · Molecular Probes® · Novex® · TaqMan® · Ambion®. IonTorrent®. Bromodeoxyuridine (Boehringer). Blocking solution 0.01 M phosphate buffered saline (PBS) pH 7.3 containing 0.01% normal goat/rabbit serum and 2% tween 20 was prepared in-house. The Bioplex Pro human cytokine group panel kit (cat no. M50-000007A) was purchased from Bio-Rad, UK and TNF-α instant ELISA assay (BMS223INST) from eBioscience.

2.1.1.2 *In vitro* culture

Immortalised cell lines cells

The SV40 immortalised normal human glial cell line SVGp12 was obtained from ATCC ref no. CRL-8621 (Manassas, VA, USA) and cultured in Eagle's minimal essential medium (EMEM) supplemented with heat inactivated 10% foetal calf serum (FBS), 4mM glutamine, 2mM sodium pyruvate and 0.1 mM non-essential amino acids (NEAA) and without the addition of penicillin/streptomycin (Life technologies Ltd., UK).

Use of antibiotics in cell culture: The commonly used antibiotics in cell culture experiments are penicillin and streptomycin (Pen/Strep). Ideal growth conditions (temperature, humidity and growth medium) also encourage the growth of microbial contaminants in incubators. To avoid contamination of cells growing at 37°C, antibiotics are added to growth media. IMR32 cells were very sensitive to infection and hence Pen/Strep was added to their general growth medium during maintenance of these cells. However, there are rare situations when antibiotics can hinder the growth and proliferation of specific cells in culture. The astrocyte cell line SVGp12 is an example of such a cell line as it is generally resistant to infection. ATCC recommended that this cell line should be cultured in their specified growth medium without the addition of antibiotics.

IMR32

The human neuroblastoma cell line IMR32 was obtained from ATCC ref no. CCL-127 (Manassas, VA, USA), and cultured in Dulbecco's modified essential medium (DMEM) supplemented with heat inactivated 10% FBS, 4mM glutamine, 2mM sodium pyruvate and 100IU/ml penicillin/streptomycin (Life technologies Ltd., UK).

Primary mixed CNS cell isolation and culture

Fresh C57/BL6J mice brains from 1 day old pups (N=6, full litter) were collected in Hank's buffer having sacrificed the animals according to the UK Home Office guide lines and on schedule I killing of laboratory animals following approval from the University of Central Lancashire's animal projects committee (reference number RE/11/07/SS). Rest of the procedure was performed under sterile tissue culture conditions as described below.

Using the aseptic technique, under sterile conditions of a class II tissue culture hood, all of the brain tissues were chopped finely using a sterile scalpel blade and collected into a $50cm^3$ Falcon tube containing Hanks buffer (Life Technologies Ltd., UK) and pelleted by centrifugation at 1000 rpm (3 times for 5min). The tissue was digested in 0.05% trypsin (Life Technologies Ltd) in Hank's buffer made with (10% Hanks' balanced salt solution, 0.1M Hepes, 0.15% sodium bicarbonate solution at (Life Technologies Ltd), 100 IU/ml penicillin/streptomycin, 100μg/ml kanamycin, 20 mg/ml gentamycin and incubated at 37°C for 15 min with constant agitation. The mixture was further treated with DNAse I (0.2μg/ml final, Sigma Aldrich) for 1 min. The cells were dissociated periodically using a sterile Pasteur pipette and eventually re-suspended in DMEM supplemented with heat inactivated 10% FBS, 4mM glutamine, 2mM sodium pyruvate and 100IU/ml penicillin/streptomycin (Life technologies Ltd.,). The cells were filtered through a 70μm nylon cell strainer (Becton-Dickinson) and pelleted by centrifugation at 1500rpm for 20 min. The cell pellet was re-suspended in fresh DMEM with additives and seeded at $2x10^6$ cells/10ml/T25 culture flasks coated with 10mg/ml poly-L-lysine (Sigma). Primary mixed cells were also seeded on coated (10mg/ml poly-L-lysine) coverslips. The coverslips were individually placed into 6 well plates and 100μl of the cell suspension at $1x10^5$ cells was transferred onto each coverslip.

Incubation and feeding of cells

All cells were cultured (cell lines, and primary cells) either in flasks (T25) or on sterile uncoated/coated glass coverslips placed in six well plates in the presence of appropriate

culture medium were incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air with regular media changes every two-three days where applicable. Following two weeks growth, the primary CNS cells were fed in DMEM/F12 medium to encourage microglia.

Cell passaging

SVGp12 and IMR32 cell lines were sub-cultured every 4 days in T25 or 75-cm^2 flasks. Each time cells were passaged the medium was removed from the flask and washed x3 with 10 ml of sterile phosphate buffered saline solution (PBS) (8g sodium chloride (NaCl), 0.2g potassium chloride (KCl), 1.44g disodium mono-hydrogen phosphate ($Na_2HPO_412H_20$), 0.24g potassium di-hydrogen phosphate (KH_2PO_4), made up to a litre with water, pH 7.2). After aspirating the PBS, 1ml of x1 solution of trypsin (Life technologies Ltd.,) was used to cover the cells and left to stand for approximately 5 minutes at 37°C. After checking that 100% of the cells had rounded up, 9.5 ml of growth medium was added to the flask and all the liquid was transferred to a 15 ml centrifuge tube and centrifuged at 1000 rpm. The supernatant was aspirated and pellet re-suspended in 10 ml of appropriate growth medium. 0.8µl of the suspension was pipetted onto a haemocytometer to count cell numbers on a microscope. The number of cells in 3 large squares were counted and divided by 3 for the number of cells/ml and multiplied by 10 for the total number of cells collected from the flask. This way the number of population doublings could be counted. Cells were re-plated at around $2.5x10^5$.

Freezing down of cells for stock maintenance

After sub-culturing, any cells that were not re-plated were frozen and stored as viable stocks. The spare cell suspension was re-centrifuges for 5 minutes at 1000 rpm. After removing the supernatant the pellet was re-suspended in 0.5 ml of medium and an equal volume of freezing mix (20% dimethyl sulphoxide (DMSO, Sigma), 80% foetal calf serum). The suspension was transferred to a freezing ampoule and stored at -80°C in a freezing box before being transferred to liquid nitrogen for long-term storage. When recovering cells from liquid nitrogen the ampoule was thawed and suspension pipetted into a 15 ml centrifuge tube. 10 ml of medium was added in a drop-wise fashion and then carefully mixed. The cells were centrifuged at 1000 rpm, supernatant removed and cells re-suspended in 10 ml of medium and re-plated in a T25 flask.

Components in the medium control and their sources

Medium control in this context refers to the sterile liquid medium containing brain heart infusion broth supplemented with haemin (5 mg/l), menadione (1 mg/l), (Sigma-Aldrich, UK) prepared by Dr A. Hashim (Centre for Immunology & infectious disease, London, UK).

Components in the spent medium and their sources

The spent medium is culture supernatant in which *P. gingivalis* (ATCC 33277 and W50) were optimally cultured for 48h. Following growth, each culture was centrifuged at 15,000 rpm at 4°C for 30 min to pellet bacterial cells and the culture supernatant alone was collected. 1ml or 0.5 ml aliquots were prepared in pre labelled sterile Eppendorf® tubes and stored at -80°C until needed. Following addition of protease inhibitors to one of the aliquots from both the culture supernatant and the sterile growth medium (control), they were freeze dried for at least 12h and re-suspended in a 200µl volume of lysis buffer containing 50mM Tris pH 8.0, 1% NP40, 150mM NaCl, 5mM EDTA prior to determining the total protein concentration. The aliquots were stored at -20°C for subsequent analysis

This too was prepared by Dr A. Hashim and was supplied as a gift reagent, courtesy of Professor M. Curtis (Centre for Immunology & infectious disease, London, UK).

Exposure of cells to P. gingivalis virulence factors

Following 95% confluent cell growth, the glass coverslips were exposed to total virulence factors from *P. gingivalis* ATCC 33277 and ATCC 53978 - W50 culture supernatant (gift source) having initially confirmed the presence of LPS and gingipains by immunoblotting using the anti-*P. ginigvalis* (clone 1B5) in EMEM/DMEM as described above. The optimal dilution in growth medium lacking penicillin/streptomycin for all cells determined was ¼, and the time points (8h, 12h, 24h and 48h) for specific needs and according to specific cell types. Following the specified time for treatment of cells, supernatants were collected and stored at -80°C for cytokine analysis using the Bioplex magnetic bead array analysis (Bio-Rad, UK) and by another ELISA assay for TNF-α (eBioScience). Cells from T25 flasks were used to prepare cell lysates for analysis by immunoblotting and cells on coverslips were used for immunofluorescence labeling.

Repeat-exposure of cells to *P. gingivalis* virulence factors

In order to determine the responses of recovered cells to repeat exposure with *P. gingivalis* (ATCC 33277) virulence factors, the detached surviving cells from the first exposure were re-plated in fresh growth medium. Once they had fully recovered and become adherent, they were re-exposed the 2nd and with recovery in between for the 3rd time under the same conditions as the first exposure (see above). The recovered cells were examined for their responses to LPS and differences in the GFAP and CD14 were noted and compared with their

respective controls. The supernatants were collected following each of the treatments as well as at the recovery phase and stored at -80°C for cytokine analysis (see above).

Bromodeoxyunidine incorporation in DNA of dividing cells

Uptake and incorporation of bromodeoxyunidine (BrdU) during DNA synthesis enables the detection of dividing cells in a population. Cells were cultured onto coverslips at $1x10^5$ cells and re-fed with EMEM 2 days before the assay. The coverslips were incubated with bromodeoxyuridine (BrdU; Boehringer) (50 µmol/l) for 4 hour at 37°C.

Cell fixation

Following treatment, the cells (on coverslips) were fixed either in 10% neutral buffered formalin, or 4% paraformaldehyde in PBS at 4°C, ranging from 30 min, 1h to overnight at 4°C. Following a thorough washing of cells in 0.01M phosphate buffered saline, pH 7.3 (PBS), the cells were either used immediately or held in PBS for up to 1 week at 4°C.

2.1.1.3. Immunofluorescence labelling

For all *in vitro* cell cultures

Controls

All cells on coverslips acting as controls included omission of the primary antibody or inclusion of the anti-*P. ginigvalis* (clone 1B5 and/or clone 1A1) antibodies on medium-control challenged cells.

Note: Anti-*P. ginigvalis* (clone 1B5) detects both LPS and gingipains whereas (clone 1A1) is specific for gingipains.

Immunofluorescence localisation of specific cell markers

Immunolabelling was performed with appropriate antibodies (sources as mentioned above). Following fixation (see above) and incubation of cells in the blocking solution containing 0.01% normal goat and/or rabbit serum/or 1% BSA (bovine serum albumin)/PBS/ 0.25%tween 20, for 30 min they were incubated overnight at 4°C in either rabbit anti-human GFAP at 2µg/ml; rabbit anti-PgP 9.5 at 10µg/ml solution, goat anti-Iba-1 at 5µg/ml diluted in blocking solution. Following thorough washes in PBS (3 x for 5 min) in between, secondary detection was performed by using goat anti-rabbit or rabbit anti-goat IgG conjugated to FITC at 5µg/ml in 2% goat serum/PBS/tween20. Following further washes in PBS for 3x 5min, cells on coverslips were mounted onto previously labelled glass microscope slides using the

Vectashield®-mountant containing propidium iodide (PI). The labelling was repeated at least three times for consistency of results.

Immunofluorescence labelling of anti-*P. gingivalis* (gingipains and LPS) and the LPS receptor CD14

Non-specific antibody binding to cells was inhibited with a block solution (see above), as for specific cell markers, the coverslips were subjected to the following primary antibodies mouse anti-human CD14, rat anti-mouse CD14, anti-*P. gingivalis* (clone 1B5 and 1A1) overnight at 4ºC. The detection was performed using the same method as above except the following secondary conjugates were used. These were goat anti-mouse IgG conjugated to FITC, goat anti-rat alexa flour 488 (molecular probes). Following further washes in PBS for 3x 5min, cells on coverslips were mounted onto previously labelled glass microscope slides using the Vectashield®-mountant with PI as before. The procedure was repeated at least three times at each treatment point.

Treponema denticola and *Tannerella forsythia* antibodies on bacterial cell smears

The *Treponema denticola* and *Tannerella forsythia* antibodies were tested on appropriate bacterial cell smears prepared on glass slides obtained from in-house cultures of the periodontal pathogens from another on-going PhD project. Following heat fixation of the bacterial smears, they were equilibrated in PBS and blocked prior to incubation in the anti-*T. forsythia* antibodies (gift from Dr Sharma, USA) and anti-*T. denticola* antibodies raised in-house from hybridoma cell lines (TDII (HB-9966) and TDIII (HB-9967) from ATCC) as per manufacturer's instructions. The procedure was the same as that described for the anti-*P. gingivalis* (clone 1B5) antibody mentioned above.

Fluorescein-phalloidin method for detecting actin cytoskeleton of cells

Following fixation in 4% paraformaldehyde in PBS for 30 min at 4°C. Coverslips were washed in PBS and left to equilibrate in PBS for 10 min. The cells on coverslips were permeablised in 1% triton x-100/PBS for 5min. Non-specific binding sites were blocked in 1% BSA (bovine serum albumin)/PBS for 30 min at room temperature. The stock fluorescein-phalloidin (Molecular Probes) at 200units/ml was diluted in PBS for 5units/ml final concentration and applied to the cells on the coverslip for 30 min in the dark at room temperature. Following washes (3x5min each) the cells were mounted onto glass microscope slides bearing the appropriate identification and examined using the 510 series Zeiss confocal microscope (Carl Zeiss Ltd). The labelling was repeated at least three times for each treatment.

Assessment of BrdU incorporation

Once the protocol was optimised, the assay was repeated just once for each of the repeat treatments. Following labelling (4h), cells were washed in PBS and then fixed in 4% paraformaldehyde/PBS for 30 min at 4°C. Coverslips were washed and left to rehydrate in PBS for 15 min. Cells were permeabilised for 20min in 0.2% triton-100/PBS at room temperature followed by washing in PBS. Endogenous fluorescence was quenched for 15 min in 50mM glycine/PBS. Coverslips were washed and left to rehydrate in PBS for 15 min. Each coverslip was incubated at 37°C, with sheep anti-BrdU polyclonal antibody (Abcam ab1893) diluted 1:100 +2.5µl DNAse I/100µl volume of diluted antibody, in the incubation buffer (66mM Tris, 0.66mM ~MgCl2, 1mM 2-mercaptoethanol, pH 8.2) for no longer than 2h. Following further washing in PBS each coverslip was incubated with rabbit anti-sheep IgG conjugated to FITC (Abcam ab6743) diluted (1/100) in 1% BSA /PBS for 50 min at room temperature. Following further washes with PBS, the coverslip was mounted onto a glass microscope slide using the Vectashield-PI mounting medium as before.

Tunel assay

FragEL™ DNA Fragmentation Detection Kit

To assess if the detached cells were apoptotic cell bodies, they were centrifuged smeared onto a pre-labeled microscope glass slides and fixed in 10% formalin 4°C. They were analysed using the fluorescent version of the FragEL™ DNA Fragmentation Detection Kit according to manufacturer's instructions (Calbiochem). This assay is a non-isotopic system for labelling of DNA breaks in apoptotic nuclei. Detection and analysis of labelling was by fluorescence microscopy at least twice on cells from three different sets of experiments.

2.1.1.4. Biochemistry

Confirming the presence of *P. gingivalis* (ATCC 33277 and W50) supernatants for LPS and gingipains virulence factors

On receipt of the medium control and the spent medium from Dr A Hasim (see above) on ice 1ml or 0.5 ml aliquots were prepared under sterile conditions in pre labelled sterile Eppendorf® tubes and stored at -80°C until needed. Following addition of protease inhibitors (leupeptin, aprotinin, pepstatin) were used to one of the aliquots from medium control and the spent medium from two different strains of bacteria to inhibit the endogenous biological

activity (i.e. cleavage of peptide bonds) which would otherwise lead to false positive results. The contents of the three tubes were freeze dried for 12h and re-suspended in a 200µl volume of lysis buffer containing 50mM Tris pH 8.0, 1% NP40, 150mM NaCl, 5mM EDTA prior to determining the total protein concentration. The aliquots were stored at -20°C for future analyses.

Escherichia coli control LPS

Commercially prepared (phenol extracted) lyophilized powder from *E. coli* LPS was obtained from Sigma Aldrich (UK) and re-suspended (1mg) in 250µl lysis buffer containing protease inhibitors used above and stored at -20°C.

Cell lysate from *in vitro* culture of SVGp12 and IMR32 cells

Following exposure of cells to either the medium-control or to *P. gingivalis* spent media SVGp12 and IMR32 cells were pelleted and washed twice in cold sterile PBS with centrifugation (5 min at 2,500rpm). The cells were lysed in buffer containing 50mM Tris pH 8.0, 1% NP40, 150mM NaCl, 5mM EDTA and protease inhibitors (Roche, UK). Following incubation on ice for 30 min and frequent vortex mixing, the cell homogenate was centrifuged at 12,000 rpm for 20 min at 4°C in a micro-centrifuge (Eppendorf, UK). The supernatant was collected in pre-labelled tubes and stored at -20°C.

Protein assay

Total protein concentration of all cell lysates was determined using the Bradford colorimetric assay (Bradford, 1976). Briefly, protein concentration was obtained from a standard curve prepared using 100-400 mg/ml bovine serum albumin (BSA) diluted in lysis buffer. Following addition of Coomassie blue (Sigma Aldrich, UK) to all standards and test samples; absorbance was measured at 595 nm wavelength using the Jenway 7315 spectrophotometer. The concentration of the unknowns was calculated by comparing absorbance values with the standard curve.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

A mixture of proteins from appropriate samples was separated by SDS-PAGE (Laemmli, 1970). The separation is based on protein size measured in kilo Daltons (kDa). Lower percentage gels (for example, 6%) are better at resolving larger proteins, whereas higher percentage gels (10-15%) are better for smaller proteins. The separated proteins can be visualised by transferring the proteins from the gel matrix onto a nitrocellulose membrane for immunoblotting using the appropriate antibodies to detect proteins present at low concentrations.

Stock buffers for gels and the associated reagents.

Non-reducing Laemmli sample buffer (catalogue no. 161-0737) was purchased from BIO-RAD UK.

BIO-RAD Sample buffer (reducing): 5% β-mercaptoethanol (Sigma Aldrich) added to the non-reducing sample buffer (as above).

Upper buffer or stacking gel buffer: 0.5 Tris base and 0.4% SDS, pH 6.8.

Lower buffer or separating gel buffer: 1.5M Tris base and 0.4% SDS, pH 8.8.

Freshly prepared 10% aqueous ammonium persulphate (APS, Sigma Aldrich).

30% acrylamide/0.8% N, N'-methylene bisacrylamide stock solution and prestained protein markers (GE Healthcare and Lonza).

NNN'N'-tetramethylethylenediamine (TEMED, Sigma Aldrich).

Electrophoresis or running buffer x10 stock: glycine 144g, Tris base 30g/litre of distilled water and 0.1% SDS, pH 8.3.

2.1.1.5. Casting gels and SDS-PAGE

Using the apparatus from the Bio-Rad mini gel electrophoresis system, glass plates (short plate and spacer plate) were cleaned with 70% Ethanol and assembled ready in the stand for receiving the appropriate percentage gel. The required percentage gels were cast using the stock reagents according to the table below enough for making up two gels (Table 2.1).

Table 2.1: Stock reagents for different percentage gels for two gels

Reagents	Resolving Gel		Stacking Gel
	10%	**12.5%**	**4%**
40% bisacrylamide	**3.83 ml**	**4.53 ml**	**575 µl**
1M Tris HCl, pH 8.8	**3.63 ml**	**3.63 ml**	-
1M Tris HCl, pH 6.8	-	-	**1.3 ml**
10% SDS	***100 µl***	*100 µl*	*50 µl*
10% APS	**75 µl**	**75 µl**	**37.5 µl**
dH_20	**7.05 ml**	**6.35 ml**	**4.075 ml**
TEMED	**15 µl**	**15 µl**	**7.5 µl**

The lower or separating gel was poured first to a specified level and overlaid with 70% methanol until set. The methanol was washed away with distilled water prior to pouring the upper or stacking gel. Combs were inserted to create 10 sample loading wells to hold approximately 20µl volume each. Whilst waiting for the gels to set, pre-stained protein markers (GE Healthcare) and all samples (30µg/lane) were prepared in sample (Laemmli) buffer containing 5% β-mercaptoethanol and heated (heat block) for 5 min at 90°C.

Following complete polymerisation, the gels were connected to the Bio-Rad electrophoresis unit as per manufacturer's instructions (Bio-Rad Laboratories Ltd, UK), and 1x electrophoresis buffer was added. The combs were removed and 20µl of each sample was loaded into each of the wells using fine and long tips whilst making sure not to let the sample overflow into the next well. Order of each sample loaded was recorded and electrophoresis was performed at 100 volts until the bromophenol dye front was about 1 cm from the bottom of the gel.

Electro-transfer of proteins onto nitrocellulose membrane

Buffers: Stock transfer buffer (x10: glycine 144g, Tris base 30g/litre of distilled water, pH 8.3) was diluted 1/10 in distilled water to which was added methanol (10% final in the transfer buffer). The nitrocellulose blotting membrane used was immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore UK Ltd.,) and required hydration and equilibration in transfer buffer prior to use. This was accomplished by placing the membrane in methanol for 30 sec and then into transfer buffer for around 2h on a shaking device.

The electrophoretic transfer of the separated proteins in SDS-PGE gels was performed in the Bio-Rad trans-blot transfer cell (Bio-Rad Laboratories Ltd). The following "sandwich" – sponge, moist filter paper (Sigma), moist PVDF membrane, gel, moist filter paper, sponge all held within a plastic cassette were placed into the transfer tank. The electrodes of the cell were connected so that the proteins transferred from the gel (+ve) to the membrane (-ve). The transfer was performed for 2h at a current between 180-200mA.

Immunoblotting

Immunoblotting of all presented results was performed at least three times to confirm the reproducibility of the results. Successful transfer was immediately noted by the presence of pre-stained molecular weight markers on the membrane. Following an initial incubation of the membrane in 5% non-fat milk/PBS for 30min at room temperature using gentle mixing on a Stuart spiramix roller mixer (Fisher Scientific), the membranes were incubated overnight at 4°C in at least 10mls of appropriate dilution of the primary antibody diluted in 5% milk/PBS.

Next day, the membrane was briefly washed in distilled water followed by extensive washings (3x 15 min each) in PBS/0.1% tween20. A further incubation of the blot for 2h at room temperature with horse radish peroxidase (HRP) conjugated goat anti-mouse or rabbit secondary detection antibody (Chemicon) in 5% non-fat milk/PBS as described in Table 2.2. The membrane was again briefly washed in distilled water followed by extensive washings (3x 15 min each) in PBS/0.1% tween20. The blots were developed at varying intervals using the enhanced chemiluminescence (ECL) system (Bio-Rad, Laboratories, UK) under ultra violet light in a gel-doc (Bio-Rad, UK) imaging station using the Molecular Analyst software.

Table 2.2: Shows the conditions and the antibodies and their dilutions used for immunoblotting

Protein	Percentage gel	Primary	secondary
P. gingivalis LPS and gingipains	12.5% Reducing conditions	1B5 (1/20)	Goat anti-mouse-HRP 1/20,000
P. gingivalis gingipains	12.5% Reducing conditions	1A1 (1/50)	Goat anti-mouse-HRP 1/20,000
CD14	12.5% Reducing conditions	CD14 1/1000	Goat anti-mouse-HRP 1/20,000
Rabbit anti-GFAP	12% Reducing conditions	1/250	Goat anti-rabbit-HRP 1/80,000

SVGp12 and IMR32 cells

Electrophoresis of SVGp12 and the IMR32 cell samples was also carried out under reducing conditions as described above at 30µg total protein concentration.

ELISA assays for human cytokines

These were performed only once due to the high cost of the commercial assays. Cytokines which were secreted in response to the virulence factors by the *in vitro* human cell line (SVGp12 and IMR32) cultures were detected by analysing the supernatants collected from SVGP12 cells and IMR32 following treatment with *P. gingivalis* ATCC 33277 and W50 at 24h. Initially the human TNF-α instant ELISA assay (BMS223INST) from eBioscience was performed following manufacturer's instructions. Subsequently, the Bioplex Pro human

cytokine group panel kit (cat no. M50-000007A) was purchased (Bio-Rad). The target cytokines were the following interleukins (IL), IL-2, IL-4, IL-6, IL-8, IL-10, Granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ) and TNF-alpha (TNF-α). The bioplex bead array automated system (Bio-Rad) was used as per manufacturer's instructions.

2.2 Results

2.2.1. SVGp12 a model cell line

The human foetal astrocyte cell line SVGp12 is adherent (Figure 2.1) and in general refractive to bacterial infections and this allows it to be cultured under conditions that do not require antibiotics. SVGp12 cell line was cultured in appropriate growth medium with supplements and was checked by using phase contrast microscopy (Figure 2.1). SVGp12 cells are adherent cells with population doubling approximately every 2.5 days. Cells used for each treatment were at least 95 % confluent.

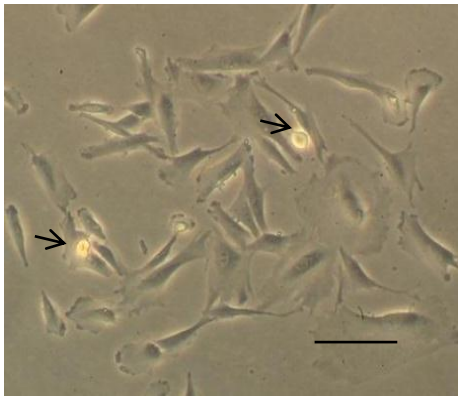

Figure 2.1: The SVGp12 adherent cell monolayer in culture. The cells imaged under phase contrast microscopy. SVGp12 cells were actively dividing (arrows pointing to mitotic bodies), bar =50 microns.

2.2.1.1. Immunofluorescence

If, antibiotics were added, the population doubling time become retarded (data not shown) and cells appeared stressed (Figure 2.2) as demonstrated by fluorescein-phalloidin method for labelling polymerised actin.

Controls

All control cells remained negative wherever the primary antibody was omitted (Figures 2.2a, 2.3a, 2.4a, 2.17a, 2.24a) or when the primary antibodies (anti-*P. gingivalis*, clones 1B5 and 1A1) were applied to medium-control challenged cells or (naïve = completely unchallenged) cells (Figures 2.5a, 2.6a, 2.10a, 2.11, 2.19, 2.20a 2.25a).

The *Treponema denticola* and *Tannerella forsythia* antibodies on bacterial cell smears

Anti-*T. forsythia* antibodies raised to rBspA protein, and the anti-*T. denticola* antibodies only weakly labeled the whole bacterial cells (not shown). Further experiments using these antibodies were terminated at this stage as distinction between the true signal and the background autofluorescence signal was difficult to appreciate when applied to test cells.

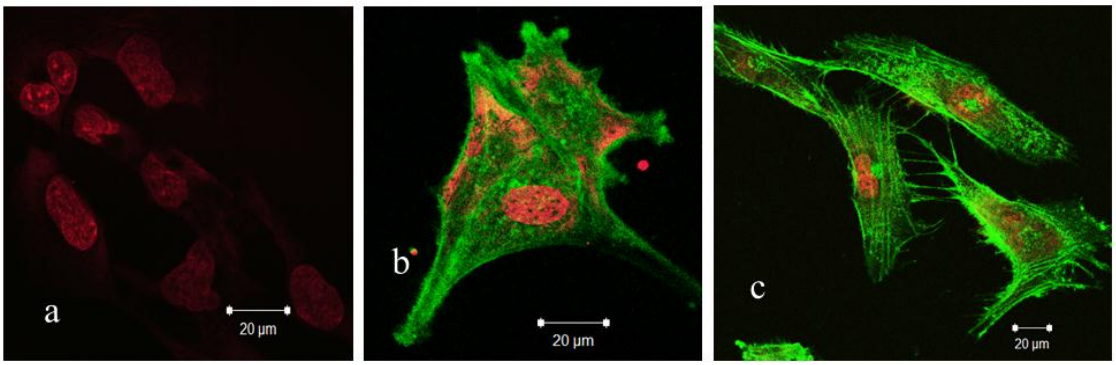

Figure 2.2: Effect of antibiotics (pen/Strep) on SVGp12 cell lines in culture. SVGp12 cells labelled with fluorescein-phalloidin a) control, b) grown under antibiotic free conditions in and c) showing stress filaments when antibiotics were included in their medium.

Ideal growth conditions provided by incubators also encourage the growth of contaminants in culture flasks if sterility is breached during culture. To limit contamination antibiotics are added to growth media. However, there are rare situations when antibiotics can hinder the growth and proliferation of specific cells in culture. The astrocyte cell line SVGp12 is an example of such a cell line. For example, the application of antibiotics on SVGp12 cell line demonstrated stress filaments (Figure 2.2 c) and the population doubling time was much

slower in the antibiotic included medium compared with the medium without antibiotics (5 days to become confluent instead of 2.5 days).

Astrocytes were identified by the cell marker antibody known as the glial fibrillary acidic protein (GFAP). To investigate whether the SVGp12 cell line was from astrocyte origins, anti-GFAP was applied to these cells. Fluorescent staining clearly demonstrated the intracellular staining of GFAP positive intermediate filaments in SVGp12 cells (Figure 2.3).

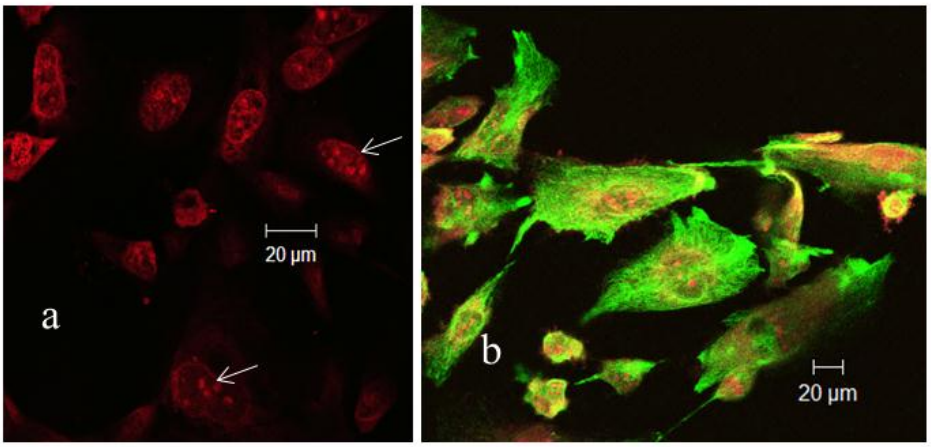

Figure 2.3: SVGp12 cells showing astrocyte cell specific proteins. There is a central nucleus showing several nucleoli (arrows) in a) and b) labelling with mouse anti-GFAP antibody diluted 1/100. GFAP detects intermediate filaments (green). Intermediate filaments form networks that provide support to cells.

Astrocytes express CD14 receptor protein which acts as co- receptor to interact with bacterial LPS. To determine whether the CD14 receptor on SVGp12 cell line is responsive to the effect of bacterial endotoxin, its protein was analysed by immunofluorescence. Fluorescent staining clearly showed SVGp12 cells expressed copious amounts of CD14 receptor protein (Figure 2.4) on the surface membrane as expected.

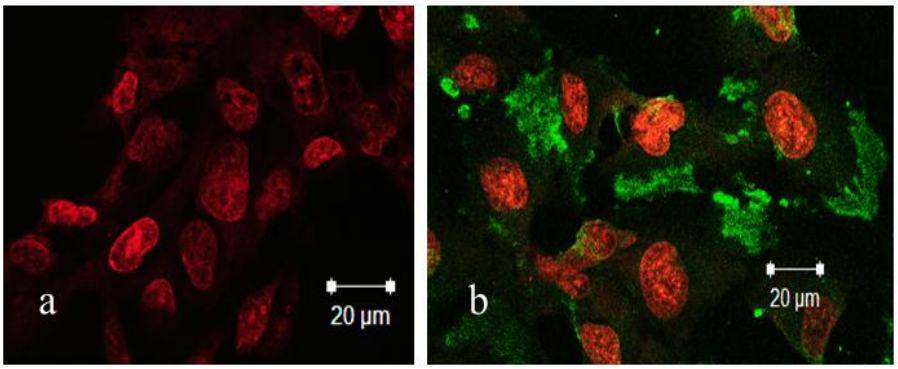

Figure 2.4: SVGp12 cell line expresses the surface membrane receptor, LPS. SVGp12 cells a) control b) labelled with mouse anti-human CD14 (1/100) to show they express the LPS receptor on their surface membrane (green).

To determine the effect of the endotoxins from periodontal pathogen, *P. gingivalis* on different CNS cell types, initially, the SVGp12 cell line was exposed to the endotoxin. One noteworthy feature was the detachment of cells from the plastic on which they were growing. The effect of gingipains was determined from a few cells that remained attached (Figure 2.5). Gingipains in these cells was internalised and compartmentalised into lysosomes as shown in figure 2.5.

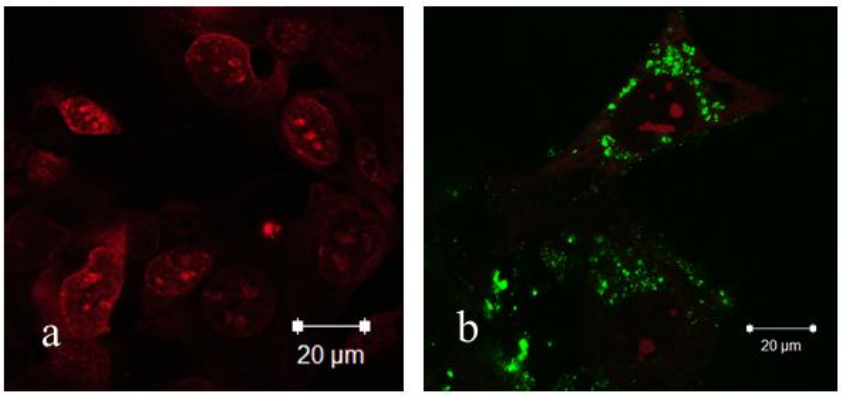

Figure 2.5: Intracellular localisation of gingipains in SVGp12 cell line. SVGp12 cells labelled with mouse anti-1A1 (anti-gingipains), a) no green labelling was observed when anti-*P. gingivalis* antibodies (clones 1A1) was applied to SVGP12 control medium treated cells. Following treatment with *P. gingivalis* 33277, 32h cells b) labelled with mouse anti-1A1 and the labelling was mainly peri-nuclear and appeared within lysosomes.

The effect of the endotoxins on the cell morphology and mechanism of self-modification from the toxic effect was investigated on SVGp12 cells. The results demonstrated LPS binding caused perturbation of the cell membrane in the form of "blebs" as immunolabelling was largely observed in ruffled membrane surfaces (Figures. 2.6 and 2.7).

The anti-*P. gingivalis* antibody clone 1B5 supported the localisation of two different virulence factors and these were due to the shared epitope between LPS and gingipains recognised by this antibody. Figure 2.6 demonstrates that if cells were exposed to the

virulence factors for over 24h, both LPS and gingipains were detected as surface and intracellular antigens.

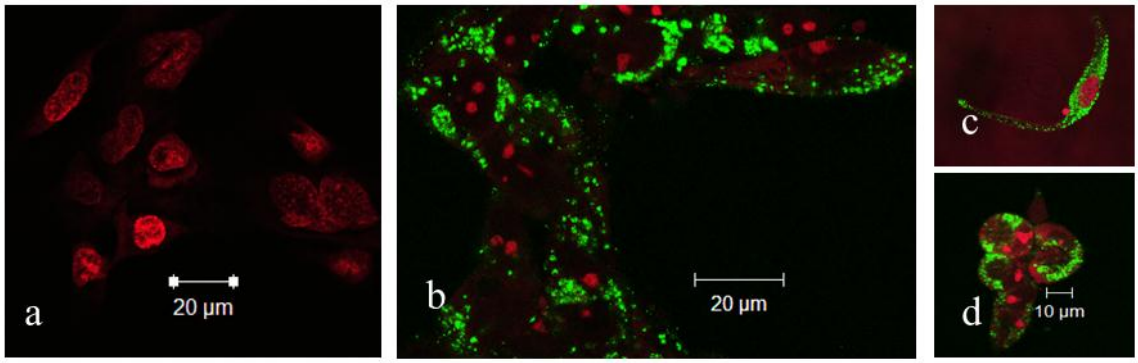

Figure 2.6: Specific response of SVGp12 cells to endotoxins (LPS). SVGp12 cells a) control whereby no green labelling was observed when anti-*P. gingivalis* antibody (clone 1B5) was applied to SVGP12 control medium treated cells, b) labelled with mouse antibody anti-*P. gingivalis* clone 1B5 that detects epitopes for LPS and gingipains. Following treatment with *P. gingivalis* W50, and b-c) 33277 for 24h, various morphological changes were observed.

Compared with control cells, the effect of the endotoxin on actin protein in SVGp12 cells was rounding of cells with enhanced phalloidin labelling and thickened filaments which passed through the nucleus of the detached cells (Figure 2.7c). The thickened filaments are an indication that cellular stress is experienced by cells during exposure to the endotoxins.

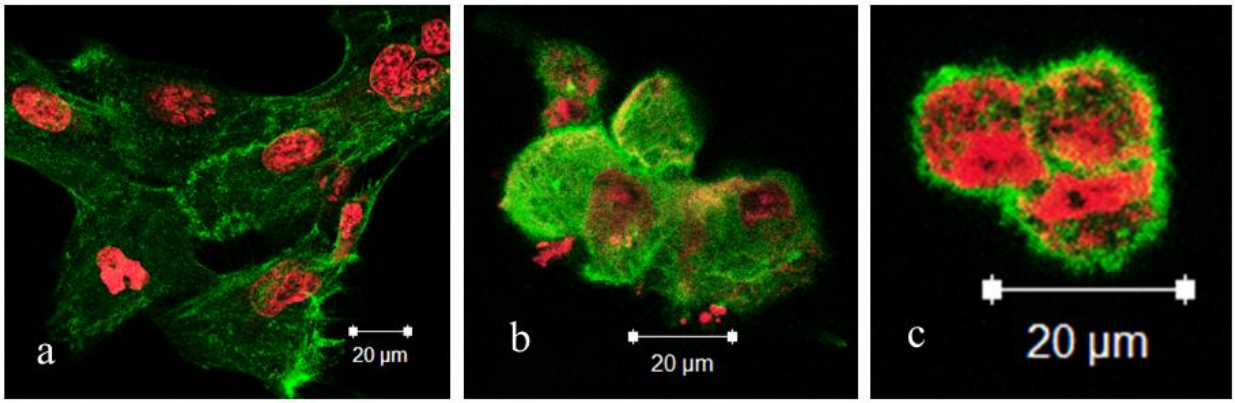

Figure 2.7: Morphological responses of SVGp12 cells to endotoxins. The effect of the LPS from *P. gingivalis* 33277 on the actin cytoskeleton was observed using the fluorescein-phalloidin marker (green). SVGp12 cells a) medium control showed the normal flattened cell morphology and normal distribution of actin filaments. Spent medium from P. gingivalis 33277 1:4, over 24h showed b-c) detached cells which had rounded up. Image c) also shows actin filaments passing through the nucleus of the detached cells.

Following treatment of SVGp12 cells with *P. gingivalis* 33277, 24h, CD14 protein synthesis appeared to be much reduced as demonstrated by immunofluorescence labelling (Figure 2.8).

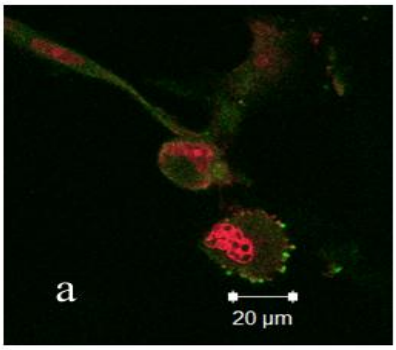

Figure 2.8: CD14 receptor protein on SGVp12 cell following challenge with endotoxins. SVGp12 cells labelled with mouse anti-CD14 following treatment with *P. gingivalis* 33277, 24h. The nuclei appear atypical, being pushed to one side of the rounded cell and CD14 protein has decreased compared with their initial culture shown in figure 2.4b.

Assessment of BrdU incorporation following repeat endotoxin challenge

Following multiple challenges with the endotoxins, one out of three sets of different experiments revealed that the SVGp12 cells were proliferating faster and their population doubling increased at the 2nd challenge to 1 population doubling/day (Figure 2.9). This observation was further supported by BrdU labelling whereby more cells (56%) were labelled by the antibody (proliferating) in the treated cells at the second challenge than the controls (48%). Following the third challenge, the cells appeared more and more like fibroblasts and BrdU labelling was not performed as the population doubling time indicated no further changes taking place in the rate of proliferation.

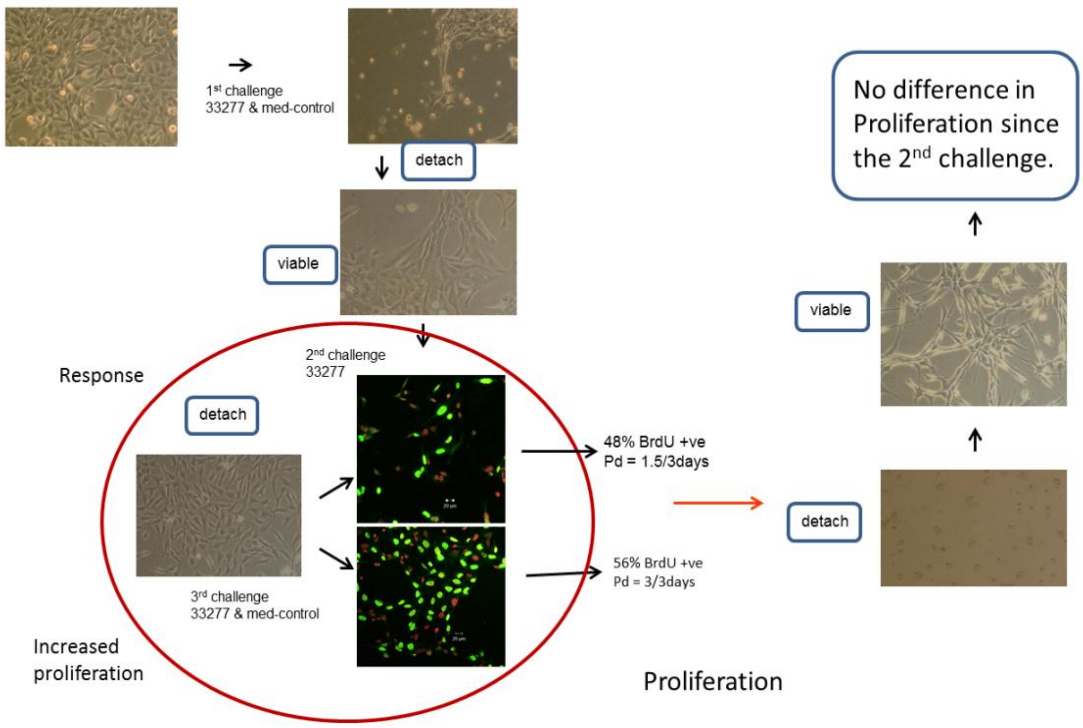

Figure 2.9: Monitoring proliferation following endotoxin repeat-exposures on SVGp12 cells. SVGp12 cells 2nd challenge with Pg33277 initiated proliferation, labelled with BrdU following 3rd consecutive treatment with P. gingivalis 33277, 24h with recovery in between shows little change in proliferation since the 2nd challenge, but morphology appears to be of more slender, fibroblast like cells.

Following multiple challenges with the endotoxins, the SVGp12 cells (not quantified) appeared smaller in size and slender in shape (figures 2.10 and 2.11). GFAP protein levels were much reduced compared with their untreated controls following recovery (Fig 2.10).

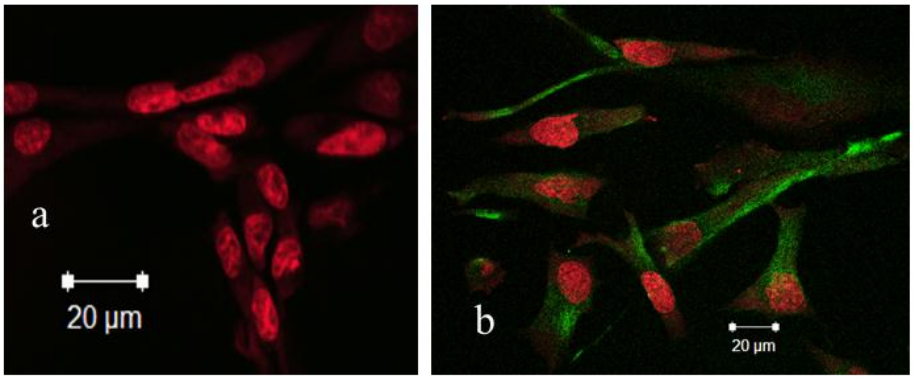

Figure 2.10: Change in GFAP protein on SVGp12 cells in between endotoxin challenges. SVGp12 cells 2nd challenge with Pg33277 showed GFAP levels decreased markedly. A) control with primary antibody omitted. However, recovery of cells (2 passages later) b) returned GFAP levels. The cells generally appeared smaller and slender.

Similarly, multiple challenges of the same cells to the endotoxins also affected the cell size and CD14 protein levels compared with their untreated controls (Figure 2.11).

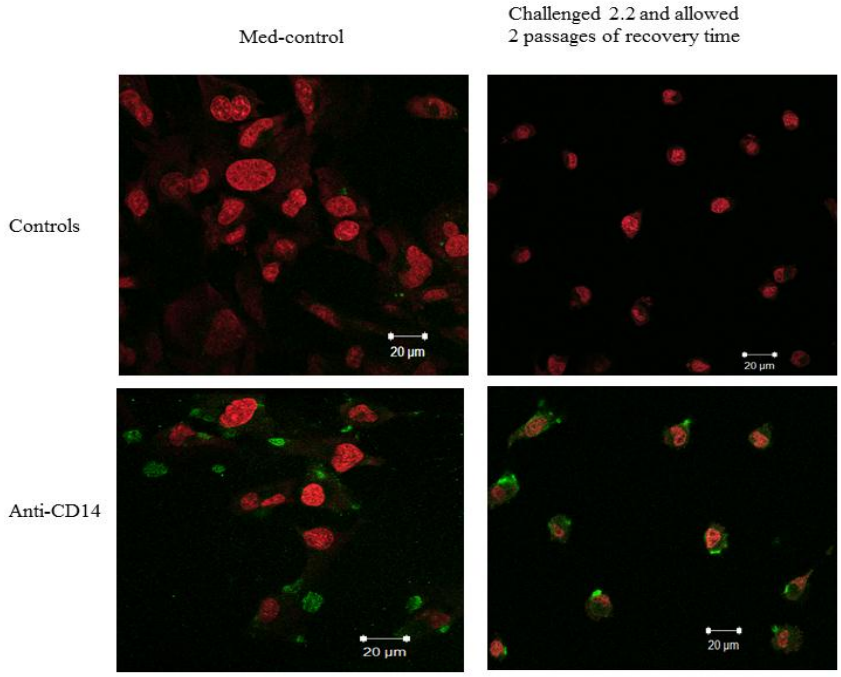

Figure 2.11: CD14 receptor on SVGp12 cell surface membrane in between exposures to endotoxins. Repeated insult with the endotoxin followed by recovery in between showed these cells were able to re-express CD14.

Immunoblotting of SVGp12 cells

The many different virulence factors present in the *P. gingivalis* spent medium that was used to challenge the cells was initially separated by electrophoreses on the SDS-PAGE gels (12.5%). Once the proteins were transferred onto the PVDF membrane, anti-*P. gingivalis* (clone 1B5) detected LPS and gingipains. As mentioned earlier, the antibody is unique in detecting two different proteins that share the same epitope. The lane 1 shows no band as it contains control medium, number of bands are present in lane 2 where, the darker band corresponds to gingipains and laddering bands corresponds to LPS, lane 3 shows no band as it contains LPS from *E. coli*, this demonstrates the specificity of the anti-*P. gingivalis* antibody across different bacterial species. Lane 4 shows no bands as it contains SVGp12 cells that were untreated and hence the neoepitope for LPS and gingipains is absent. Lane 5 shows the upper band corresponds to gingipains and the lower bands correspond to LPS adsorbed by the cells in figure 2.12.

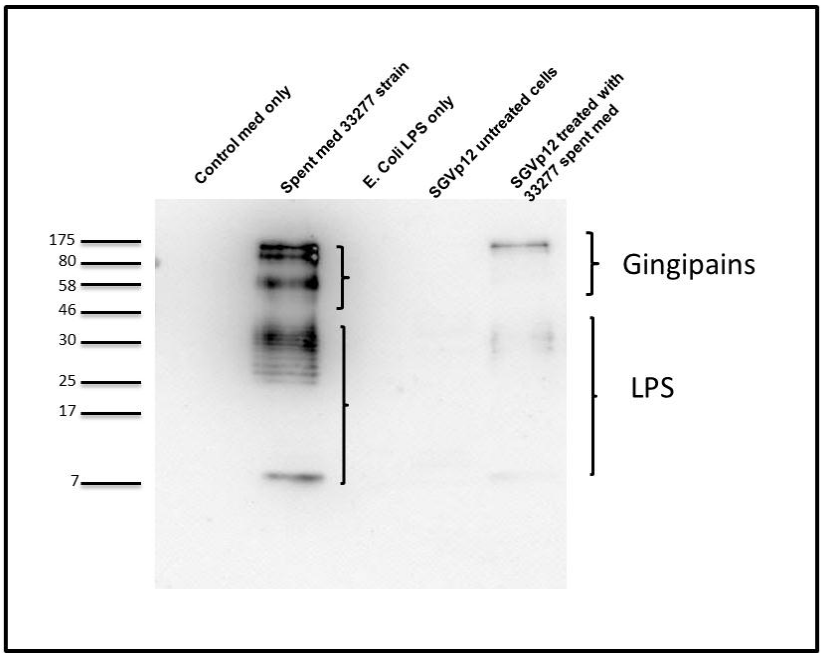

Figure 2.12: SVGp12 cells adsorb LPS. Immunoblot 20 μg of total protein/lane was loaded on a 12.5% SDS-PAGE gel and transferred onto the PVDF membrane. The membrane was incubated overnight at 4 °C in primary antibody anti-*P. gingivalis* (1B5, diluted 1/20) followed by secondary antibody (goat anti-mouse conjugated with HRP 1/20,000). Note the anti-*P. gingivalis* (clone 1B5) has detected both gingipains and LPS from the treated cells.

The medium control (lane 1), *E. coli* LPS (lane 3) and the cells treated with medium control (lane 4) failed to produce any bands whereas the positive control culture supernatant (lane 2) shows a number of bands. The darker bands above 52 kDa size correspond to gingipains (lane 2) and all the bands in a laddering pattern (34-12 kDa sizes) correspond to LPS as confirmed in a). The result in lane 5 confirmed the de-novo antigen detected by the aforementioned antibody was due to LPS in the culture supernatant having an effect on SVGp12 cells.

Western blotting to detect gingipains on challenged SVGp12 cells.

Anti-*P. gingivalis* (clone 1A1) detects gingipains only (Figure 2.13). Lanes 1-5 are the same as those shown in figure 2.12 except only a few bands were observed in the positive control (lane 2) and in the test cells (lane 5). These bands correspond to gingipains. The reasons for the lower position of the band in lane 5 as compared with the higher band in lane 2 may be due to gingipains having been degraded by the living cells.

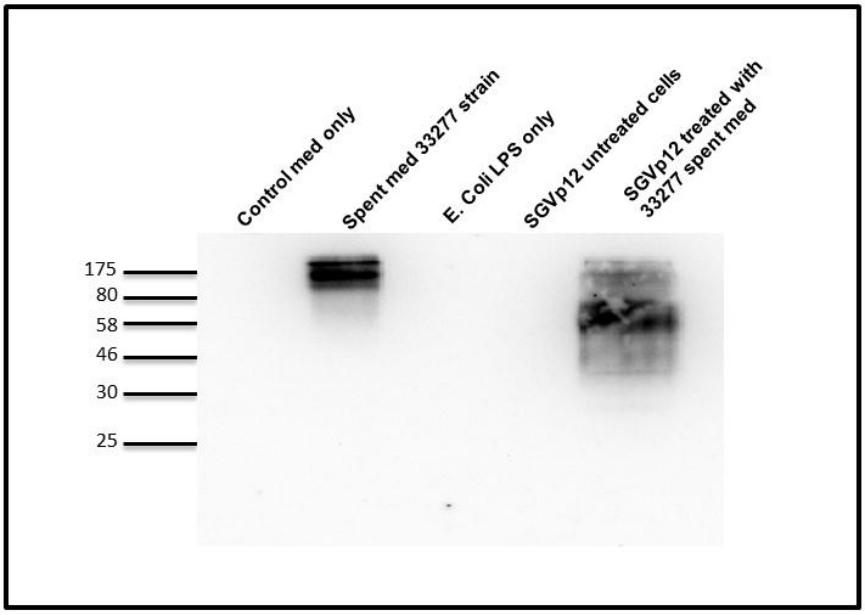

Figure 2.13: SVGp12 cells internalise gingipains. Immunoblot 20 µg of total protein/lane was loaded on a 12.5% SDS-PAGE gel and transferred onto the PVDF membrane. The membrane was incubated overnight at 4 °C in primary antibody (1A1, diluted 1/50) followed by secondary antibody (goat anti-mouse conjugated with HRP 1/20,000). The medium control (lane 1), *E. coli* LPS (lane 3) and the cells treated with medium control (lane 4) failed to produce any bands whereas the positive control culture supernatant (lane 2) shows at least 2 bright bands representing gingipains from the spent medium. The darker bands around the 58 kDa size correspond to gingipains (lane 5) from SVGp12 cells. The reasons for the lower band in lane 5 as compared with the higher band in lane 2 may be due to gingipains having been degraded by the living cells.

A western blot was also performed with anti-GFAP antibody (Figure 2.14) which demonstrated the protein syntheses was perturbed by the endotoxin treatment of SGVp12 cells as compared with the control treated cells.

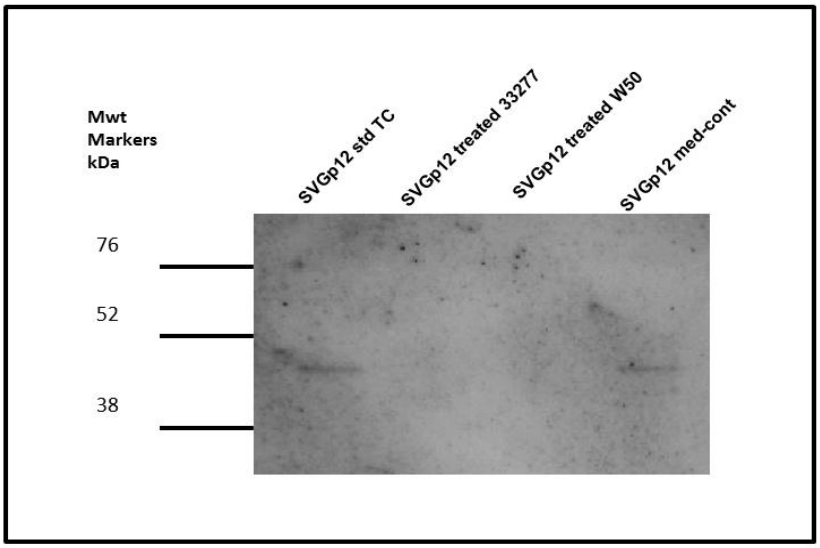

Figure 2.14: Intermediate filaments are perturbed in SVGp12 cells following an endotoxin challenge. SVGp12 cells showing immunoblotting with anti-GFAP following treatment with 33277 and W50 supernatants. GFAP is a monomeric molecule with a molecular mass between 40 and 53 kDa

Immunoblot 20 μg of total protein/lane was loaded on a 12.5% SDS-PAGE gel and transferred onto the PVDF membrane. The membrane was incubated overnight at 4 °C in primary antibody (GFAP diluted 1/250) followed by secondary antibody (goat anti-mouse conjugated with HRP 1/20,000). A weak band was detected (lane 1) in the lysate prepared from SVGp12 cells growing in culture. No bands were detected (lanes 2 and 3) and treated with spent medium from *P. gingivalis* 33277 and W50. A weak band was detected from the cell lysate prepared from SVGp12 cells treated with medium control.

Cytokine release analyses

ELISA assays were performed some of which required plotting a standard curve from a set of standards with known concentration that were supplied in the kit (The eBioscience instant ELISA kit).

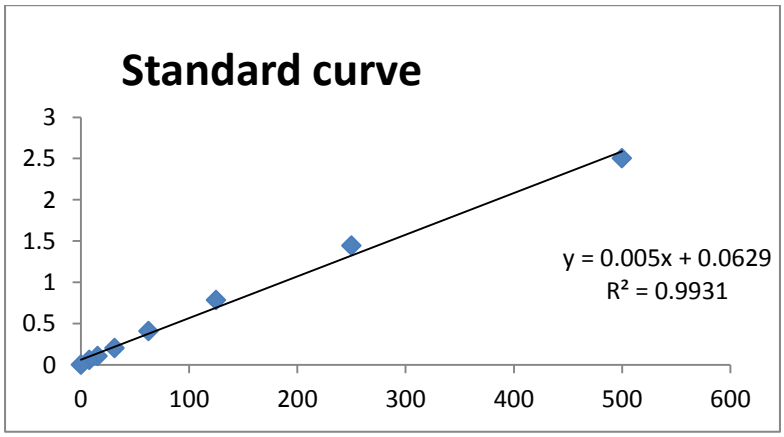

Figure 2.15: Standard curve for the TNF-α instant ELISA. Standard curve was plotted to calculate the concentration of the cytokine TNF-α released by SVGp12 cells under specified culture conditions in the eBioscience instant ELISA.

Concentrations of the TNF-α cytokine were calculated from the standard curve shown in figure 2.15. The results from the eBioscience instant ELISA for TNF-α, detected the cytokine at physiological levels in the SVGp12 cells are presented in Table 2.3.

Table 2.3: The eBioscience instant ELISA showing concentration of the released TNF-α

eBioscience instant ELISA	**Concentration pM/ml**
Sample ID	**TNF-α**
SVGp12, med-control 1:4, 24h	38.58
SVGp12, W50 1:4, 32h	14.58
SVGp12, 33277 1:4, 24h	53.58

Analysis of cytokines using the Bioplex bead array

Numerous cytokines which were secreted in response to the virulence factors by the SVGp12 and IMR32 cells *in vitro* were detected (Table 2.4) using the bioplex bead array automated system (Bio-Rad). The Bioplex Pro human cytokine group panel kit (cat no. M50-000007A) was purchased just once (Bio-Rad). The target cytokines were the following interleukins (IL), IL-2, IL-4, IL-6, IL-8, IL-10, Granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ) and TNF-alpha (TNF-α).

Table 2.4: The Bioplex bead array ELISA (supernatant following a 24h exposure)

Bioplex bead array (pM/ml					
Sample ID	**IL-6**	**IL-8**	**IL-10**	**IFN-γ**	**TNF-α**
SVGp12, med-cont, 1:4, 24h	521.83	995.87	0	0	0
SVGp12, W50, 1:4, 24h	298.73	1299.5	0	0	0
SVGp12, 33277 1:4, 24h	34.67	160.27	0	0	0

IL-2, IL-4 (not shown), IL-10, IFN-γ, TNF-α, were all undetected by the Bioplex bead array system. GM-CSF was also detected in the medium used as control at equivalent levels (not shown). IL-6 secretion was within the physiological range. An important feature of these cells following endotoxin challenge particularly to the *P. gingivalis* W50 was the clear up-regulation of the chemokine IL-8.

Repeat exposures

The second exposure of the same surviving cells with the *P. gingivalis* 33277 showed they released IL-8 cytokine but was not sufficiently different from the values recorded for control supernatants (Table 2.5). Two different ELISA assays revealed these cells secrete IL-6 at physiological levels and is not sufficiently up-regulated above these levels during the endotoxin challenges on SGVp12 cells.

Table 2.5: The Bioplex bead array ELISA following repeat exposure to endotoxins

Bioplex bead array (pM/ml					
Sample ID	**IL-6**	**IL-8**	**IL-10**	**IFN-γ**	**TNF-α**
SVGp12, med only (or control) 1:4, 24h after 1st passage	24.02	995.87	0	0	0
SVGp12, treated 33277 1:4, 24h	34.67	160.27	0	0	0
SVGp12, med only (or control) after 2nd passage 33277,1:4, 24h	55.1	483.1	0	0	0
SVGp12, 33277 med after 3rd treatment 1:4, 24h	42.65	289.1	0	0	0

Following repeat treatments of SVGp12 cells with the *P. gingivalis* 33277 spent medium, IL-8 was not up-regulated as shown.

2.2.2. IMR32 a model cell line

In sharp contrast to the SVGp12 cell line, the human neuroblastoma cell line IMR32 is semi-adherent with a polygonal shaped morphology under phase contrast microscopy. Antibiotics were essential during *in vitro* culture conditions (Figure 2.16). The cells used for the treatment were at least 95 % confluent.

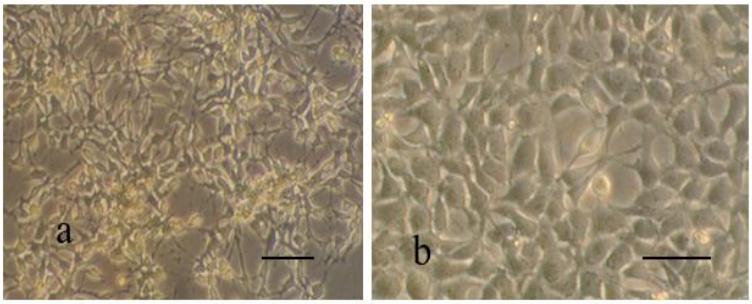

Figure 2.16: Appearance of IMR32 cells under phase contrast. IMR32 Monolayer showed low adhesion to the tissue culture plastics. Bar = 50 microns

2.2.2.1.Immunofluorescence

IMR32 cells expressed neuronal cell marker protein (anti-PgP9.5) as shown in figure 2.17b. This immune marker was mainly intracellular and localised to the soma. These cells also demonstrated the presence of the CD14 receptor by immunolabelling with anti-CD14 (Figure 2.17c).

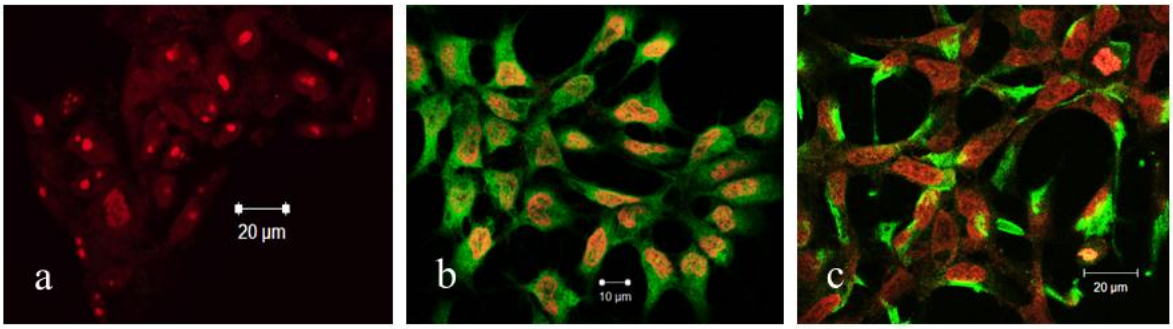

Figure 2.17: Neuronal origins of the IMR32 cells and detection of the LPS receptor. General characteristics of IMR32 cells compared with a) control (no primary antibody included) showed the presence of b) PgP9.5 (1/200) neuron cytoplasmic protein (green), a marker of neurons on a monolayer of 24 h culture c) anti-CD14 1/100 marker detects CD14 phagocytic lipopolysaccharide (LPS) receptor protein (green).

Labelling IMR32 cells from normal growth conditions with phalloidin (Figure 2.18 a) revealed their fine processes that were not detected by immunolabbelling with anti-PgP9.5 (Figure 2.17). Due to their semi-adherent properties, these cells reacted very quickly and withdrew their processes when antibiotics were omitted during the medium control treatment (Figure 2.18 b).

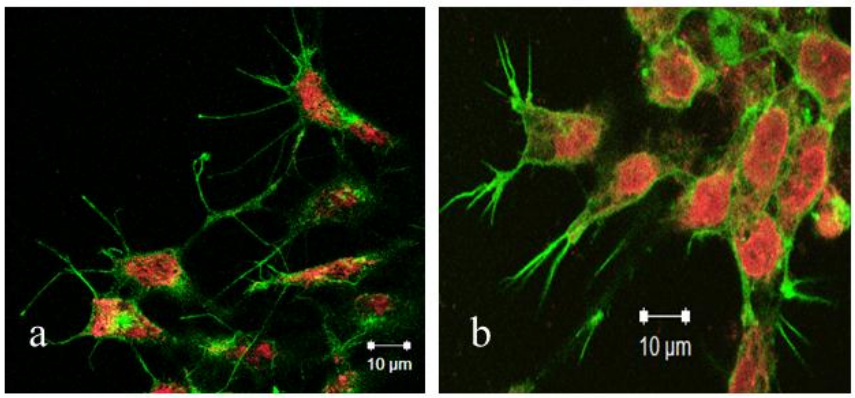

Figure 2.18: IMR32 cell line expresses actin. Fluorescein-phalloidin labelling detected the actin cytoskeletal protein (green) in IMR32 cells a) monolayer in growth medium shows long processes of the cells b) in growth medium without the antibiotics and addition of *P. gingivalis* growth medium (1:4) showed the cells beginning to show stress.

It was not possible to determine the localisation of gingipains on IMR32 cells as they detached instantly following addition of the *P. gingivalis* 33277 endotoxins (Figure 2.19). Therefore, longer exposure of these cells to the endotoxin was not performed.

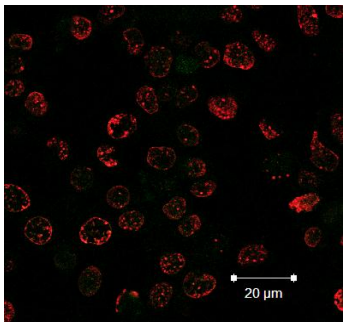

Figure 2.19: Gingipains is not internalised by IMR32 cell line. IMR32 cells labelled with mouse anti-1A1 (anti-gingipains) following treatment with medium control. Cells treated with *P. gingivalis* 33277, showed these cells did not internalise gingipains at early time (8h post exposure).

The effect of IMR32 cells upon short exposure (6h) to endotoxins from *P. gingivalis* 33277 appeared to be related to LPS (Figure. 2.20) as the various morphological changes in these cells were common to those described for SGVp12 cells. Some of the IMR32 cells adsorbed LPS and other cells began to detach from the flask and round up.

FragEL™ DNA Fragmentation assay demonstrated no apoptotic cell death in the detached cells following exposure to the *P. gingivalis* endotoxins. The positive control generated by DNAse I treatment showed the assay was working as all the nuclei were labelled green (data not shown).

Furthermore, the floating cells were collected and re-introduced to culture in their respected medium where they flattened and began to grow as shown in figure 2.20.

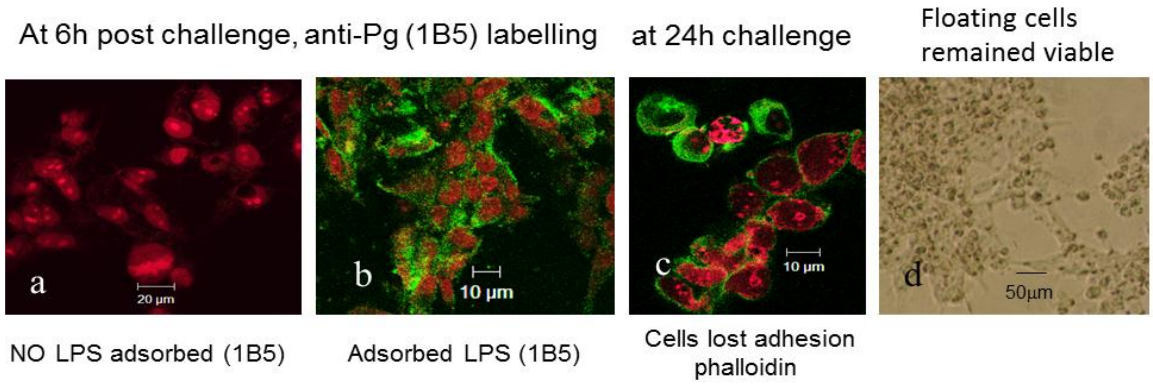

Figure 2.20: Morphological responses of IMR32 cells to endotoxins (LPS). IMR32 Cells challenged for 6hs with a) medium control failed to detect *P. gingivalis* LPS and within 6h IMR32 cell line exposed *in vitro* to virulence factors from periodontal pathogens *P. gingivalis* 33277 demonstrated b) LPS adsorbed at the cell surface. At 24h exposure c) 95% of the cells lost their protoplasmic extensions, rounded up and detached from their plastic/glass substrate as shown by fluorescein-phalloidin labelling. The floating cells d) were collected and re-introduced to culture in their growth medium where they flattened out and began to grow.

Immunoblotting for CD14 receptor

Immunoblotting was performed on cell lysates prepared from IMR32 cells following exposure to control and test endotoxins for 24h. They were separated by SDS-PAGE electrophoresis as described for the anti-*P. gingivalis* antibodies (clones 1B5 and 1A1). The data revealed that the CD14 receptor protein was present on control cells but upon challenge with *P. gingivalis* 33277 the receptor completely diminished. Treatment of the same cells with W50 strain surprisingly, only partially decreased it (Figure 2.21) as compared with the controls.

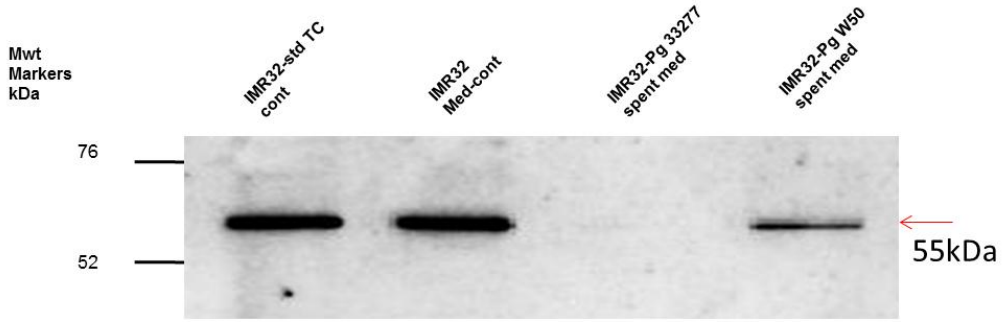

Figure 2.21: IMR32 cells lost CD14 receptor protein following an endotoxin challenge. Immunoblot performed with 20 µg of total protein/lane on a 12.5% SDS-PAGE gel and transferred onto the PVDF membrane. The membrane was incubated overnight at 4 °C in primary antibody (anti-CD14 diluted 1/1000) followed by secondary antibody (goat anti-mouse conjugated with HRP 1/20,000). The IMR32 cells grown under standard tissue culture growth conditions (lane 1), confirmed the abundant expression of the CD14 receptor on these cells. There was no effect on the CD14 expression when the cells were treated with medium control (lane 2). *E. coli* LPS (lane 3) and the cells treated with *P. gingivalis* 33277 supernatant (lane 4) failed to produce any bands whereas the cells treated with *P. gingivalis* W50 (lane 4) showed a reduced band for CD14.

Cytokines release analysis

Any cytokines which may have been secreted in response to the virulence factors by IMR32 cells *in vitro* were analysed using the eBioscience instant ELISA assay and the Bioplex bead array automated system (Bio-Rad). Both assays confirmed that the IMR32 cells did not secrete any of the analysed cytokines (Tables 2.6 and 2.7).

Table 2.6: Data from the eBioscience instant ELISA assay for TNF-α (24h treatment)

Sample ID	**TNF-α (pico moles/ml)**
IMR32, med-control 1:4, 24h	0
IMR32, W50 1:4, 24h	0
IMR32, 33277 1:4, 24h	0

Analysis of TNF-α cytokine using the eBioscience instant ELISA assay shows these cells did not secrete the named cytokine. (All cytokine values = pico moles/ml)

The Bioplex bead array system: cytokine assay

Table 2.7: The Bioplex bead array ELISA assay for IMR32 (24h interval)

Sample ID	**IL-6**	**IL-8**	**IL-10**	**IFN-γ**	**TNF-α**
IMR32, med-cont 1:4, 24h	0	0	0	0	0
IMR32, W50 1:4, 24h	0	0	0	0	0
IMR32, 33277 1:4, 24h	0	0	0	0	0

The Bioplex bead array shows IMR32 neuroblastoma cell line was unable to secrete any of the cytokines in the presence of *P. gingivalis* W50 following primary endotoxin challenge.

Primary CNS mixed cell culture

The primary CNS cells were all adherent cells which lacked any neuronal cell growth as observed under phase contrast microscopy (Figure 2.22).

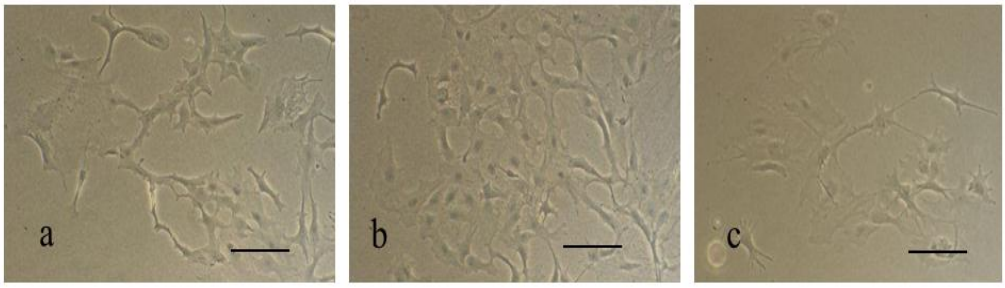

Figure 2.22: Phase contrast images from primary CNS mixed cell culture. These were adherent cells with various morphologies (a-c), bar =50 microns

In order to identify the cell types in the mixed CNS cell culture, glial cell marker antibodies were used. The microglial cell marker Iba-1 failed to demonstrate the presence of these cells in culture although bunches of astrocytes (Figure 2.23) were often present as identified by immunolabelling with the anti-GFAP antibody.

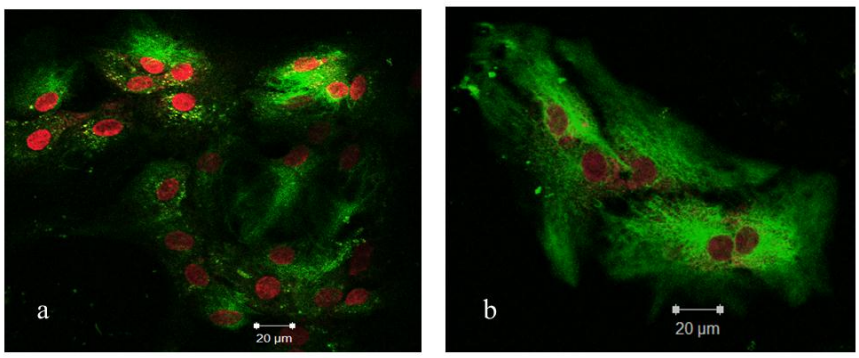

Figure 2.23: Astrocytes were present in the mixed primary cell culture. Primary astrocytes from the CNS mixed culture labelled with rabbit anti-human GFAP as shown in a-b.

To determine whether the CD14 receptor on primary cells was expressed immunolabelling was performed with an antibody raised to the mouse CD14 (Figure 2.24). Although, it identified cells expressing the LPS receptor but their identity was not evaluated by double immunolabelling. The CD14 positive cells were abundant in culture.

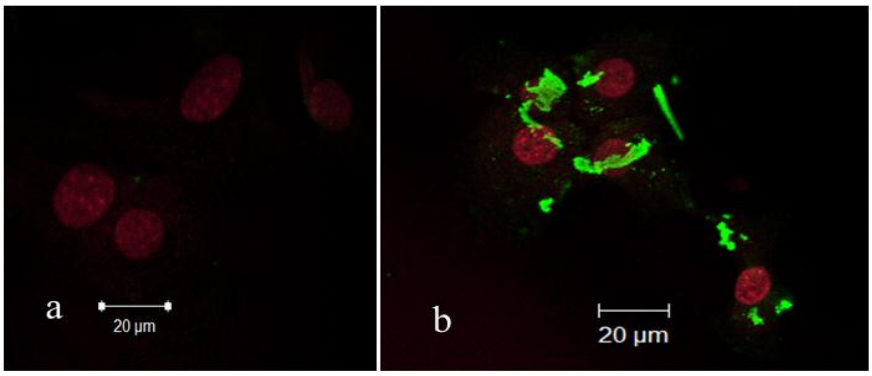

Figure 2.24: Mixed primary cells express the LPS receptor. Primary CNS cells a) control whereby the primary antibody is omitted and b) labelled with rat anti-mouse CD14.

Following addition of the *P. gingvalis* 33277 endotoxins the primary mixed cells reacted in a similar way as the SVGp12 and IMR32 cells described above. Due to the limited supply of these cells and time restraints, further responses were not examined.

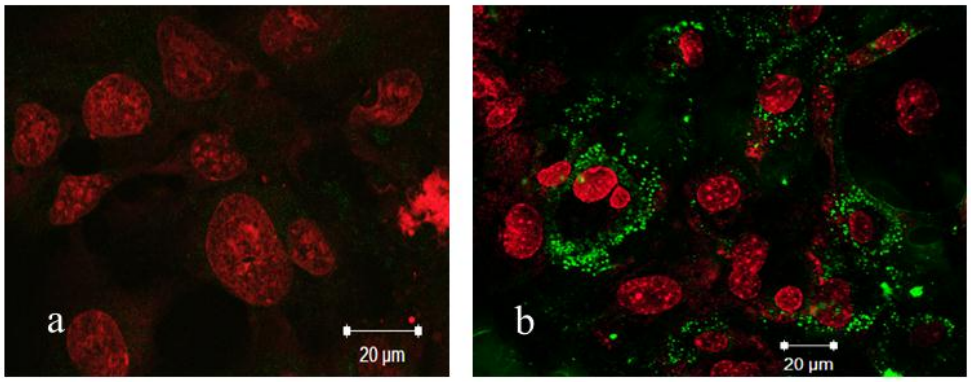

Figure 2.25: The response of primary cells to endotoxins (LPS). Primary CNS cells challenged with a) medium control and b) spent supernatant from 33277, 1:4 for 24h. Both images are labelled with 1B5.

Chapter 3: General Discussion

3. General Discussion

Chronic adult periodontitis has been associated with several conditions and of importance here is deteriorating memory which in some patients progresses to dementia. Chronic periodontitis is a condition caused by numerous periodontal pathogens in which *P.gingivalis* is strongly implicated (Socransky *et al.*, 1989). There is growing evidence that an unhygienic oral environment with chronic periodontitis can negatively affect mental health (Noble *et al.*, 2009). However, knowledge of the exact risk factor giving rise to cognitive deficit that in some individual's progresses to dementia is limited. Around 40% of the population over 85 years are likely to be affected with AD and another 25% in which it is combined with the effects of cardiovascular disease (*www.alz.org/downlo**ads**/facts_figures_2012.pdf - United States)*. Periodontitis is of early onset although its true consequences are felt with advancing age. Around 10-15% of the adult population develops severe, chronic periodontitis (Preshaw *et al.*, 2012).

Neurodegenerative disorders of which aging is a major risk factor show elements of neuroinflammation, meaning the inflammatory response is that of resident brain glial cell activation. There are two main hypotheses to explain how this takes place in the brain. 1) An inter-cerebral stimuli, such as physical trauma, accumulation of abnormal proteins (amyloid beta) and direct infections. 2) Inflammatory stimuli from extra-cerebral sources including all forms of systemic infections once the BBB is breached. Specific pathogenic oral bacteria responsible for periodontitis also infiltrate into the systemic circulation and are a potential source of infection/inflammation in the brain.

In this respect, Miller (1891) and Hunter (1900) hypothesis for oral bacteria being able to participate in distant organ inflammatory pathology by accessing the systemic vessels appears to apply. As everyday tasks of the oral cavity such as physical trauma from chewing food, tooth brushing, scaling, root surface debridement, extractions and periodontal surgery (Carroll and Sebor, 1980; Lofthus *et al.*, 1991; Daly *et al.*, 1997; 2001; Kinane *et al.*, 2005; Savarrio *et al.*, 2005; Forner *et al.*, 2006; Tomas *et al.*, 2007) all contribute to transient bacteraemia.

Previously the brain was considered as an immune-privileged microenvironment due to the existence of a BBB, which denied access of systemic proteins and immune cells into the CNS (Oldfield and Mckinley, 1995). However, it is now recognised that the BBB is

incomplete in the circumventricular organs and the choroid plexus regions (Oldfield and Mickinley, 1995) providing an opportunity for systemic proteins and cells to gain access at these sites of the CNS. Microglial cells in circumventricular organs express CD14 receptor and the TLR-4 and are therefore, capable of detecting the non-self, pathogen associated molecular patterns on bacteria and LPS (Oldfield and Mickinley, 1995; Lacroix *et al.*, 1998; Beutler *et al.*, 2003).

All of the above factors facilitate the inflammatory stimuli to reach the brain and contribute to the activation of astrocytes and microglial cells. The *in vitro* study design described in this thesis was preferred over an *in vivo* animal model approach as experimental conditions and analysis could be performed on large cell numbers of a homogenous astrocyte and neuronal cell populations.

Although, this study intended to test responses of periodontal pathogens directly exposed to cells of interest, logistical reasons and time restraints made this impossible. To understand how a pathogen such as *P. gingivalis* may incite the innate immune system upon access to the CNS, this project investigated the acute phase responses of cells following treatment with a cocktail of virulence factors in the form of spent medium. The rationale for using a cocktail of virulence factors together rather than selected ones on their own was that the whole organisms reside in the body and their crude endotoxin mixtures are likely to contribute to the cumulative pathogenic effect during life. Biochemical methodologies to either synthesize or isolate individual virulence factors to purity are unable to exert their biological effect on cells to the same extent as the additive effect of the crude endotoxin mixtures released by the organism.

The spent medium contained endotoxins including LPS and the outer membrane vesicles, gingipains, propionic and butyric acids, to mention a few from gram-negative anaerobic *P. gingivalis* periodontal pathogen.

3.1. Exposure of cells to *P. gingivalis* virulence factors affect cellular immune responses

Membrane blebbing

Following treatment of cells with the aforementioned virulence factors they initiated a spectrum of cellular responses. Due to the increased resistance to bacterial infections (antibiotic free culture conditions), the SVGp12 glial cell line, provided the most dramatic responses to LPS and gingipains. These included perturbation of the surface membrane in the form of “blebbing” observed following immunolabelling using the anti-*P. gingivalis* (clone

1B5) antibody. The combined microscopy based immunolabelling and biochemistry based immunoblotting data demonstrated this was likely to be due to the LPS binding to the cell membrane as gingipains (anti-gingipains clone 1A1) localised mainly to peri-nuclear sites in the cytoplasm and within lysosomes of the SVGp12 glial cells. Blebbing of the non-CNS cells has also been reported to take place due to reasons other than bacterial endotoxins including exposure of cells to serum, macroglobulin 2 (Dixon *et al.,* 1987) and hyperthermia (Borrelli *et al.,* 1986) and apoptotic cell death (Zierler *et al.,* 2006). LPS is commonly considered as an important virulence factor in gram-negative bacteria. Although not conclusive, our results indicate, the CNS cells are particularly sensitive to LPS.

Loss of cell adhesion

Morphological changes, of which the dominant feature was cell detachment suggests the adhesion of the cells was disturbed. Although not conclusively tested, and it is our view that LPS alone is unlikely to disturb the adhesion of cells. It is reported that the metabolic by products of the pathogens may contribute to disruption of adhesion of cells to their substrates (Scragg *et al.,* 1994). Alternatively, LPS may initiate signalling pathways that lead to other proteases being activated that disturb adhesion of cells.

From the cocktail of endotoxins, it is likely that gingipains is also contributing to detachment of cells (Fujimura and Nakamura, 1987). Gingipains is a protease enzyme which displays commonalities between the activities of trypsin, to degrade matrix proteins (Nakamura *et al.,* 1991) such as fibronectin and collagen. These matrix proteins provide a source of receptors that allow anchorage of cells *in vitro* and link cytoskeletal proteins to regulate cell migration and cell adhesion. If these contacts are disrupted *in vitro* the cells become detached.

Trypsin is used in cell culture methodologies to detach adherent cells for sub-culturing purposes and it is likely that gingipains can also mediate the rounding of cells and detach them from the plastic/glass substrate in a similar way. In support, trypsin hydrolyses lysine-arginine and lysine-arginine-proline bonds to detach adherent cells in culture. Two different forms of gingipains are known to be secreted by the same organism in culture. Both types are known to hydrolyse either lysine or arginine residues on cell membranes (Pike *et al.,* 1994). The reasons for hydrolysis of proteins native to the host by gingipains may be to generate peptides from the host for growth of *P. gingivalis*. Whether cell detachment is cytopathic is not known but, this investigation found that the detached cells recovered the ability to re-attach to the plastic and grow once they were free of the virulence factors.

Anti-proliferative response

The virulence factors also appeared to growth arrest cells especially those having lost their adhesion. It is possible that the inhibitory effect of proliferation is partly due to LPS as Hill and Ebersole (1996) found in their study whereby LPS from *Actinobacillus actinomycetemcomitans* was applied at high concentrations resulting in the reduction of DNA synthesis in quiescent fibroblasts. Layman and Diedrich (1987) also reported that purified LPS from *P. gingivalis* could inhibit gingival fibroblast cell proliferation by 50%. Furthermore, crude preparations of LPS from *P. gingivalis* have similar effect on gingival fibroblasts as that caused by native extract of the whole bacteria (Larjava *et al.,* 1987) indicating the growth inhibition observed in this project was likely to be mediated by the LPS or by gingipains detaching cells.

CD14 mediated LPS- signalling pathway

The SVGp12 cells were very responsive to *P. gingivalis* LPS and other virulence factors present in the spent medium. This study demonstrates that SGVp12 cells challenged with the virulence factors reduced cell surface membrane bound CD14 receptor protein. Gingipains is reported to cleave CD14 from macrophage like cells by direct proteolytic activity resulting in its shedding (Sugawara *et al.,* 2000; Duncan *et al.,* 2004)**.** This mechanism is suggested as an immune evasion strategy employed by *P. gingivalis* bacteria for survival in host tissues (Tada *et al.,* 2002). In addition, CD14 is a substrate for gingipains and can degrade it completely, suggesting that gingipains efficiently digest CD14 to small fragments that are not detected by immunoblotting using anti-CD14 antibody (Sugawara *et al.,* 1998; Tada *et al.,* 2002).

The neuroblastoma cell line IMR32 cells in particular showed complete absence of the CD14 protein following challenge with *P. gingivalis* spent medium despite the abundance of this receptor on these cells. Two main reasons for its complete absence is that either the receptor was shed once the cells were in contact with the LPS from the spent medium or it was down regulated at the gene level by IL-13 cytokine as is reported to occur in human monocytes (Cosentino *et al.,* 1995) or completely degraded by gingipains beyond detection by immunoblotting (Sugawara *et al.,* 2000; Tada *et al.,* 2002). LPS from gram-negative bacteria binds to CD14 which complexes with TLR-4. Although TLRs can bind the endotoxins directly, CD14 is essential to increase the sensitivity of the response to endotoxins by as much as 1000-fold. Briefly the sequence of events in the recognition of LPS by cells may involve a binding protein. The role of the binding protein is to separate LPS into single molecules and transfer them to CD14. The CD14-LPS complex is then presented to TLR4

and its adaptor protein MD-2 via MyD88 or not, initiating intracellular signally cascades as shown in figure 3.1.

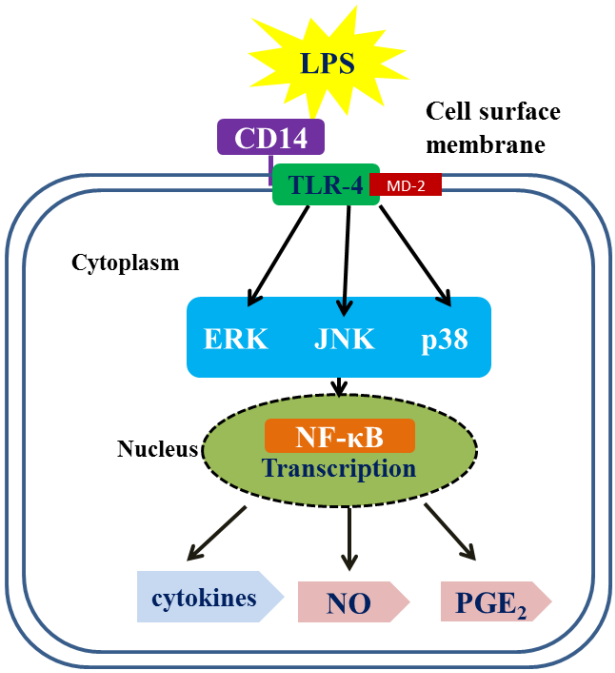

Figure 3.1: **Schematic diagram representing interaction of LPS to CD14 signalling pathways.** Schematic diagram showing the interaction of LPS to the CD14 located in the cell surface membrane initiating signalling pathways by complexing with MD-2 (an adapter protein) independent of MyD88.

The pathways involved are the MyD88 dependent signalling pathway, common to all the TLRs, and the MyD88-independent signalling pathway, is specific to TLR-3 and TLR-4. The MyD88-dependent signalling cascade leads to the activation of NF-κB (Figure 3.1). Activation of NF-κB allows it to enter the nucleus and induce the expression of multiple target genes, such as TNF-α, IL-1, IL-6, IL-8, and others. In addition, the MyD88-dependent signalling pathway leads to the activation of mitogen-activated protein kinases (MAPK), which leads to the production of nitric oxide and additional inflammatory mediators.

Inflammatory cytokine release

A number of reports suggest astrocytes have the capacity to synthesise and secrete cytokines by exposure to proinflammatory stimuli (Aloisi *et al.*, 1992; Choi *et al.*, 2002; Lu *et al.*, 2005; Spooren *et al.*, 2011; Burkert *et al.*, 2012). As a result of LPS interaction with CD14 and the TLR-4, production of proinflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) by human astrocyte SVGp12 cell line was expected. This study revealed that following exposure to *P. gingivalis* (W50) virulence factors, the SVGp12 glial cells up-regulated the secretion mainly

of the chemokine IL-8 (from pico to nanoMoles/ml) whereas IL-6 and TNF-α remained at their physiological concentrations (picomoles/ml). IL-8 is a chemokine that mediates acute phase inflammatory activity and its induction is likely to be due to LPS which has been shown *in vitro* in human umbilical vein endothelial cells (Mao *et al.*, 2002) and *in vivo* (rodents) following administration of bacterial LPS (Feghali and Wright, 1997). IL-8 mediates the recruitment and activation of neutrophils in inflamed tissues (Huber *et al.,* 1991). In the same way, it is proposed here that IL-8 production by astrocytes would result in the activation and recruitment of microglia to the site of infection/injury in the brain.

The suppression of pro-inflammatory cytokines especially IL-6 and TNF-α could be explained by the pathogenicity of the periodontal pathogens used. LPS from *P. gingivalis* (33277 and W50) inhibited the production of the pro-inflammatory mediators tested at 24h time point. Although IL-1β was not analysed, literature suggests that its secretion following challenge with LPS from encapsulated *P. gingivalis* W50 is up-regulated in whole blood (Kunnen *et al.,* 2012). Secretion of IL-1β and TNF-α can mediate the up-regulation of IL-6 (Aloise *et al.,* 1992; Spooren *et al.,* 2011). Both IL-6 and TNF-α release were also suppressed suggesting IL-1β may not have been up-regulated by the *P. gingivalis* virulence factors applied to SVGp12 cells. Alternatively, these results may be genuine as in another investigation, LPS failed to stimulate mRNA or protein expression for IL-1β, TNF-α and IL-6 in pure astrocyte cultures (Lee *et al.,* 1993). One reason may be that astrocytes do not express TLR-4 (Lehnardt *et al.,* 2002) which is necessary to complex with the CD14 receptor to respond to LPS for switching on intracellular signalling.

This study did not investigate if SGVp12 cells expressed TLR-4. However, IL-8 was induced and that indicated some form of intracellular signalling mediated by NF-kB transcription factor was taking place. A study performed following *Burkholderia pseudomallei* infection in human embryonic kidney cells suggests that IL-8 secretion can occur without TLR-4 and this takes place via the NF-kB and a family of stress activated serine/threonine protein kinases such as mitogen-activated protein kinase 38 (p38 MAPK) and the c-jun N-terminal kinase (JNK) pathways (Hill *et al.,* 2008). TLR-independent activation depends on a functional Bsa type III secretion system and requires internalization of the bacterium (Hill *et al.,* 2008). Alternatively, gingipains also induces the synthesis of IL-8 in the human artery endothelial cells involving the TLR signalling and is its synthesis is enhanced in the presence of LPS and gingipains (Deng *et al.,* 2011).

Another report suggests that *P. gingivalis* LPS challenged primary rodent astrocytes

release nitric oxide and prostaglandin E2 (Shapira *et al.,* (2002). This suggests another path of inflammatory mediator release by astrocytes in conjunction with LPS involving the L-arginine-nitric oxide pathway. The activation of this pathway is enhanced in the presence of IL-1β and TNF-α cytokines (Mollace *et al.,* 1998). This study did not attempt to reproduce the Shapira *et al.,* (2002) data as two different ELISA assays revealed TNF-α was not induced above its physiological levels by SVGp12 astrocytes. However, nitric oxide and prostaglandin E2 have a knock on effect on the induction of IL-1β and TNF-α cytokines due to the activation of the cyclooxygenase enzymes by nitric oxide (Mollace *et al.,* 1998; Salvemini *et al.,* 1993). Astrocytes become activated in response to cytokines and may play a neuro-protective role from nitric oxide toxicity (Chen *et al.,* 2001) to induce and stabilize neuronal synapses (Christopherson *et al.,* 2005; Ullian *et al.,* 2001).

LPS mediated signal transduction pathways switch on the p38 MAPK (Obata *et al.,* 2000) and the JNK (Obata *et al.,* 2000; Scheinfeld *et al.,* 2002) can mediate neuronal loss by apoptosis (Nakahara *et al.,* 1998) and are activated in neurodegenerative diseases such as AD (Hensley *et al.,* 1999; Zhu *et al.,* 2004). The neuroblastoma cell line IMR32 was unable to release any of the cytokines tested and this data is in agreement with Lu *et al.,* (2005). It is plausible to suggest that these cells may acquire protection from glial cell synthesised immune effectors or become targets of damage during chronic inflammatory environment. Elevated levels of cytokines have been reported in the AD brains (Akiyama *et al.* 2000) as well as the serum of AD patients during life (Holmes *et al.,* 2009). Periodontal pathogens are also implicated in contributing to the pool of these cytokines in AD patients (Kamer *et al.,* 2009). The paracrine effect of cytokines may contribute to glial cell priming following long term stimulation of trigeminal peripheral nerve fibres which connect to the brain. Several bacteria, including periodontal pathogens, have been found in the aged and the AD brain specimens (Riviere *et al.,* 2002) including our own in house data. Riviere *et al.,* (2002) studies have demonstrated that spirochete species can migrate to the brain via the trigeminal nerve ganglia.

3.2. Repeat-exposure of cells to *P. gingivalis* virulence factors contributed to higher IL-8 chemokine release

Studies in animal models representing the aged brain suggest that a short exposure (4h exposure) to as much as 0.33 mg of bacterial LPS in the systemic circulation is required to illicit an overwhelming inflammatory response (Ye and Johnson, 1999; 2001; Godbout *et al.,* 2005; Chen *et al.,* 2008) which can alter BBB permeability and change resting microglia into their activated phenotype (Ye and Johnson, 1999; 2001; Chen *et al.,* 2008). These studies (Ye and Johnson, 1999; 2001; Godbout *et al.,* 2005; Sheng *et al.,* 2003; Chen *et al.,* 2008) imply that the direct transport of the LPS to brain via antigen presenting cells may not be taking place but instead the brain may be directly exposed to secondary mediators (cytokines). This introduces a new hypothesis that if microglia in the aged brain are in an inflammatory immune-phenotype further stimulations from circulating inflammatory mediators due to severe periodontitis, will cause them to respond by expressing copious amounts of inflammatory cytokines that exacerbate pre-existing neurodegeneration.

This investigation tested the concept by repeat infections of the recovered cells using IL-8 secretion as a guide. Following treatments of SVGp12 cells with the *P. gingivalis* 33277 spent medium, IL-8 levels failed to increase over and above the level observed in controls. However this was only measured once as the assay was expensive. Further studies are necessary to conclude this finding but with *P. gingivalis* W50 spent medium.

3.3. Exposure of cells to *P. gingivalis* virulence factors affects the actin cytoskeleton but, without cell death

Following binding of LPS to SVGp12 cells, the anti-*P. gingivalis* (clone 1B5) antibody demonstrated, marked alteration in cell morphology, reduced cell attachment (rounded cells) and altered F-actin stress in treated cells. All cells including the SVGp12 glial and the IMR32 neuroblastoma cell lines demonstrated these cells have a structural framework of actin which can be dynamically remodelled in response to internal or external signals. Actin protein is central to neurons which have dendrites and an axon that extends over long distances. Several molecules are thought to transmit signals from the plasma membrane to the actin cytoskeleton ranging from small GTPases to phosphoinositol (Majerus *et al.,* 1990). Binding of specific receptors initiates a cascade of events that activates the machinery involved in

movement resulting in the remodelling of the actin cytoskeleton (Sechi and Wehland, 2000). Damage caused by periodontal virulence factors is detrimental to the proper function of these important cells. Microglial cells are not only phagocytic cells but also immune surveyors of the brain. Actin protein facilitates phagocytic activity by extending protoplasmic protrusions of the cell (Carnell and Insall, 2011) to internalise foreign/cellular debris and cellular movement is essential to combat immune responses to a variety of extracellular stimuli (pathogens and their virulence factors) and LPS may play a detrimental role.

In conclusion this investigation has highlighted some important responses of CNS cells caused by *P. gingivalis* virulence factors. Research assessing potential relationships between periodontal pathogens and their impact on early pathological signs contributing to cognitive dysfunction is important for uncovering unique mechanisms that lead to the development of dementia. The long term significance of this research lies in the potential to confirm these results in animal models of periodontal disease and develop interventional clinical trials which may reduce the severity of periodontal diseases in, at risk individuals, implementing relatively cost-effective professional oral hygiene regimes.

Suggestions for future work

This study has highlighted that the various cell surface and cytoskeletal proteins synthesis becomes completely disturbed on the neuroblastoma cell line IMR32 cells and the SGVp12 astrocytes following a challenge with the *P. ginivalis* 33277 and W50 endotoxins. Changes in the CD14 protein on IMR32 cells were interesting and future studies should explore if the CD14 receptor on IMR32 cells was being shed during exposure to the endotoxins or whether the protein was down regulated at the gene level. These tests can be performed using western blotting of the supernatants collected following treatment and real time polymerase chain reaction on total RNA isolated from cells. The effect of a mixture of virulence factors from the red complex periodontal pathogens (*P. gingivalis, T. forsythia and T. denticola*) on CNS cells would be interesting if and when suitable antibodies become available.

To strengthen the relevance of this *in vitro* finding that CD14 receptor protein levels change should be followed in an animal model induced with periodontal disease by the same organism. Our research group has access to the $ApoE^{null}$ mice orally infected (N=12) with 10^9 *P. gingivalis* via an established collaboration with Professor L. Kesavalu (University of Florida).

The Bioplex cytokine assay should be repeated to conclude the described findings as it was only measured once due to high cost of the assay. Further studies are necessary on using a mixed set of virulence factors from the red complex organisms to find out if there is a synergistic effect on cells and especially on microglial cell lines as they are the main cells that respond to immune challenges in the brain.

Chapter 4: References Section

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