INTERACTIONS BETWEEN PORPHYROMONAS GINGIVALIS AND MACROPHAGES IN ORAL PATHOLOGY

Belfield, Louise Alicia

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Abstract

INTERACTIONS BETWEEN PATHOGENIC BACTERIA AND MACROPHAGES IN ORAL PATHOLOGY

Louise Alicia Belfield

Macrophages play a fundamental role in driving both inflammatory and immunosuppressive conditions of the oral mucosa. Periodontitis, a chronic inflammatory condition affecting the supporting structures of the teeth, is widely prevalent, affecting a large proportion of the global population, and has been linked to the development of systemic inflammatory diseases. Oral squamous cell carcinoma (OSCC) is placed sixth in the WHO rankings of cancer incidence worldwide, and despite continuing research into underlying mechanisms, incidence is on the rise. Aberrant macrophage function has been implicated in the pathogenesis of both diseases. On recruitment to sites of inflammation, macrophages become polarised within a spectrum of effector phenotypes depending on the factors they encounter in their microenvironment. These cells are highly plastic and continuously adapt their effector functions in response to locally derived stimuli. Mechanisms have been developed by pathogenic bacteria and transformed host tissues to exploit this plasticity and manipulate macrophage phenotype to facilitate disease progression. However, this plasticity is also available for therapeutic manipulation. The main objectives of this study therefore were to investigate the interactions between macrophages and pathogenic stimuli in the context of oral pathology with a view to identifying novel therapeutic targets.

Firstly, a reproducible model of M1 and M2 macrophage polarisation using the THP-1 cell line was established to study their interactions with pathogenic stimuli. Treating the cells with combinations of PMA plus IFNγ or IL24 for 24 hours led to two distinct populations of cells: PMA + IFNγ treated cells expressed higher levels of pro-inflammatory cytokines TNFα, IL21β and IL26, but lower levels of IL210 and TGF2β, characteristic of M1 macrophages. PMA + IL24 treated cells expressed lower levels of TNFα, IL21β and IL26 and higher levels of IL210 and TGF2β, characteristic of M2 macrophages.

As P. gingivalis LPS is present in the developing periodontal lesion, cytokine expression from macrophages exposed to LPS during polarisation was investigated. Exposure of macrophages to 1 µg/ml Pg LPS during polarisation led to a statistically significant down-regulation of inflammatory cytokines TNFα (10-, 4- and 5.5 -fold decrease in PMA, M1 and M2 cells, respectively) and IL-1β (1.9-, 2.0- and 1.5 -fold decrease in PMA, M1 and M2 macrophages, respectively) in response to subsequent stimulation with LPS. IL-6 production was not affected. The same pattern of cytokine down regulation was observed regardless of...
LPS species used, and in most cases, at a lower dose of 1 ng/ml LPS during polarisation. Finally, as macrophages recruited to the tumour environment will be influenced by tumour-secreted factors, the response of macrophages to LPS stimulation in the presence of OSCC conditioned media was examined. Contrariwise to polarisation with LPS, exposure of macrophages to OSCC produced factors during polarisation led to an amplification of IL-1β (13.8-, 2.3- and 8.8 –fold increase in PMA, M1 and M2 cells, respectively), and IL-6 (16.8-, 17.3- and 44.9 –fold increase in PMA, M1 and M2 cells, respectively), but not TNFα in response to LPS. Counter intuitively, these findings suggest that LPS manipulation of macrophage polarisation might result in a more M2 –like population of cells, whereas OSCC produced factors may result in a more M1- like population of cells. Viewed therapeutically, one short, single exposure of macrophages to LPS would up-regulate pro-inflammatory cytokines, whereas prolonged or chronic exposure would lead to the down-regulation of pro-inflammatory cytokines, therefore, LPS as a therapeutic modulator of macrophage function in an immunosuppressive (M2) environment to an inflammatory environment (M1) would only be viable as a single dose. For chronic inflammatory disease however, a repasted or prolonged exposure of macrophages to LPS skews macrophages to display a more M2-like cytokine profile and could dampen down detrimental pro-inflammatory cytokine production. The continued study of macrophage/ P. gingivalis interactions may shed light on pathogenic mechanisms not only in oral pathological conditions, but in a range of diseases.
Table of Contents

Chapter 1 General introduction .............................................................................................................. 17
  Overview of oral mucosal immunity ....................................................................................................... 17
  The innate immune response in the oral mucosa .................................................................................. 17
  The adaptive immune response in the oral mucosa ............................................................................. 20
  Oral Mucosal Tolerance ....................................................................................................................... 23
  Aberrant innate cell function leads to pathology in the oral mucosa – key role of macrophages .......... 25
  Immune function in periodontitis .......................................................................................................... 26
    *P. gingivalis* and oral epithelial cell interactions .............................................................................. 26
  *Porphyromonas Gingivalis* LPS and Toll like receptor interactions ................................................ 27
  Periodontal disease and its systemic implications .................................................................................. 30
  Immune function in oral cancer ............................................................................................................ 31
    Macrophages in oral cancer ................................................................................................................. 31

Chapter 2 – Materials and methods ........................................................................................................ 35
  Materials and reagents .......................................................................................................................... 35
  General cell culture: Cell lines and growth conditions ........................................................................ 38
    THP-1 ............................................................................................................................................... 38
    H357 ................................................................................................................................................. 38
  Primary tissue collection and culture .................................................................................................... 38
    Collection of control (non-tumour) mucosa: .................................................................................. 39
    Collection of oral squamous cell carcinoma tissue: ...................................................................... 39
  Generation of M1- and M2- like THP-1 macrophages ......................................................................... 39
    H357 conditioned media .................................................................................................................. 40
  LPS biological activity assay: Limulus Amebocyte Assay (LAL) .......................................................... 40
  H357 and THP-1 co-culture experiments ............................................................................................ 41
  Cell viability - Trypan blue exclusion test ............................................................................................ 42
    THP-1 responses to *P. gingivalis* LPS ............................................................................................. 42
    H357 responses to *P. gingivalis* LPS ............................................................................................. 43
  Enzyme Linked Immunosorbent Assay (ELISA) .................................................................................. 44
  Flow cytometry ..................................................................................................................................... 46
  Total RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction from
  THP-1 cell line ...................................................................................................................................... 48
Chapter 3 - Optimisation of an M1/M2 macrophage polarisation model using the cell line THP-1

Macrophages in the oral mucosa ........................................................................................................ 52

Macrophage dysfunction in oral disease .......................................................................................... 53

Macrophages in vitro: Generation M1 and M2 .............................................................................. 53

Results .................................................................................................................................................. 58

PMA as a macrophage differentiating factor: PMA induces THP-1 adherence, a marker of monocyte/macrophage differentiation .............................................................. 58

Vitamin D3 as a macrophage differentiating factor: Vitamin D3 treatment does not induce cell adherence, and induces a cytokine profile unlike M1 or M2 macrophages ............... 61

Polarisation of THP-1 cells into M1 and M2 like phenotypes using Th1 and Th2 cytokines 63

Treatment of THP-1 with PMA and IFNγ or IL-4 generates cell populations exhibiting a differential pro-inflammatory cytokine profile ..................................................................................... 65

Discussion .............................................................................................................................................. 72

Chapter 4 - Modulation of Macrophage responses by Porphyromonas gingivalis LPS. ......... 76

Introduction .............................................................................................................................................. 76

Porphyromonas gingivalis signalling pathways ................................................................................... 77

Results .................................................................................................................................................. 81

Effects of LPS on cytokine production during differentiation of non-polarised, PMA differentiated macrophages (Ms) ............................................................................................................. 81

Effects of LPS on cytokine production in M1 polarised macrophages .............................................. 85

Effects of LPS on cytokine production in M2 polarised macrophages .............................................. 89

Toll-like receptor activation by Porphyromonas gingivalis LPS ....................................................... 92

Surface expression of TLR2 and TLR4 in un-stimulated and P. gingivalis polarised macrophages .............................................................................................................................................. 95

Discussion .............................................................................................................................................. 97

Chapter 5 - Influence of oral squamous cell carcinoma culture supernatants on macrophage responses to Porphyromonas gingivalis ........................................................................ 105

Introduction .............................................................................................................................................. 105

Results .................................................................................................................................................. 108

H357 cytokine responses to P. gingivalis LPS ................................................................................. 108
List of figures

Figure 1-1: Schematic representation of APC driven T helper cell differentiation ................. 211
Figure 2-1: Diagram of tumour tissue samples used in the study ........................................... 39
Figure 2-2: Schematic representation of macrophage polarisation protocol ............................ 400
Figure 2-3: Schematic representation of H357/THP-1 transwell co-culture system ............... 422
Figure 3-1: A resting period of one day is sufficient for THP-1 cells to recover from PMA stimulation, but still exhibit a pro-inflammatory profile in response to PAMPs .................. 59
Figure 3-2: PMA or Vitamin D3 treated cells do not display M1 and M2 profiles ............... 622
Figure 3-3: TNFα expression in PMA, IL-4 or IL-13 treated THP-1 cells .............................. 644
Figure 3-4: Schematic representation of the differentiation protocol for THP-1 polarisation
................................................................................................................................. 68
Figure 3-5: IFNγ and IL-4 polarised macrophages display characteristic M1 and M2 cytokine profiles. .................................................................................................................................. 68
Figure 3-6: Expression levels of M2 markers IL-10 and TGF-β are higher IL-4 treated than IFNγ treated macrophages . ........................................................................................................................................ 69
Figure 3-7: TNFα, IL-1β and IL-6 production in response to P. gingivalis LPS, E. coli LPS and Pam3csk4 ........................................................................................................................................... 71
Figure 4-1: Schematic representation of MyD88 dependent and independent signalling pathways via TLR signalling ........................................................................................................ 78
Figure 4-2: Schematic representation of experimental protocol for polarising macrophages in the presence of LPS .............................................................................................................. 80
Figure 4-3: Exposure of PMA macrophages to P. gingivalis or E.coli LPS during polarisation ........................................................................................................................................ 83
Figure 4-4: Exposure of M1 macrophages to P. gingivalis or E.coli LPS during polarisation .... 87
Figure 4-5: Exposure of M2 macrophages to P. gingivalis LPS during polarisation:...... Error! Bookmark not defined. 0
Figure 4-6: TNFα production is dependent on TLR2 signalling .......... Error! Bookmark not defined. 3
Figure 4-7: IL-1β production is dependent on TLR2 signalling .......... Error! Bookmark not defined. 3
Figure 4-8: IL-6 production is dependent on TLR2 and TLR4 signalling...... Error! Bookmark not defined. 4
Figure 4-9: TLR2 and TLR4 expression in PMA, M1 and M2 macrophages ......................... 93
Figure 4-10: Percentage change in TLR2 and TLR4 expression in macrophages polarised in the presence of P. gingivalis LPS ........................................................................................................... 93
Figure 5-1: H357 dose response to P. gingivalis LPS .......................................................... 10907
Figure 5-2: H357 time course response to P. gingivalis LPS ............................................... 11008
Figure 5-3: TLR2 and TLR4 surface expression on H357 cells .......................................... 11109
Figure 5-4: IL-6 expression by macrophage co-cultured H357 cells................................. 11410
Figure 5-5: TNFα production in MØ, M1 and M2 macrophages polarised in the presence or absence of H357 conditioned media. ................................................................. 1153
Figure 5-6: IL-1β production in MØ, M1 and M2 macrophages polarised in the presence or absence of H357 conditioned media ......................................................................................... 1164
Figure 5-7: IL-6 production in MØ, M1 and M2 macrophages polarised in the presence or absence of H357 conditioned media .................................................................................. 1175
Figure 5-8: IL-6 production induced by conditioned media alone ..................................... 1196

7
**Figure 5-9:** IL-6 response to *P. gingivalis* LPS in when polarised with a series of dilutions of H357 conditioned media................................................................. 122

**Figure 5-10:** Response to *E. coli* LPS following polarisation in H357 conditioned media... 1240

**Figure 5-11:** Difference in cell number following culture with H357 conditioned media... 1261

**Figure 1-1:** TNFα production by MØ, M1 and M2 macrophages polarised in the presence of primary OSCC sample conditioned media......................................................... 122

**Figure 1-2:** Consitutive production of cytokines in primary OSCC samples versus control tissue (gingiva)................................................................. 125

**Figure 6-1:** Plot of TGF-β standard concentrations from chequerboard optimising technique .................................................................................................................. 1754

**Figure 6-2:** Plot of optimal concentrations for TGF-β capture and detect antibodies ...... 1765
List of tables

Table 2-1: List of reagents used in the study .................................................................. 35
Table 2-2: List of primer sequences and suppliers .......................................................... 37
Table 2-3 Concentrations of antibodies used in ELISA .................................................. 45
Table 3-1: Methods of M1 and M2 polarisation using THP-1 cells from previously published research ........................................................................................................... 55
Table 3-2: Summary of cell-type specific cytokine production in response to Pam3csk4 

Bookmark not defined.

Table 4-1: Summary of cytokine modulation in macrophages polarised with P. gingivalis or E. coli LPS .......................................................................................................................... 92
Table 4-2: Summary of toll like receptor utilisation by P. gingivalis LPS in M1 and M2 polarised macrophages .................................................................................................................. 95
Table 5-1: Viability count of cells polarised in the presence or absence of H357 conditioned media by Trypan blue exclusion .................................................................................................. 122
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This thesis is dedicated to Joanna Elizabeth Boyle (1983-2007), my beautiful best friend, who I miss every day with all my heart.

AUTHOR’S DECLARATION
At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

Work submitted for this research degree at the Plymouth University has not formed part of any other degree either at Plymouth University or at another establishment.

This study was financed with the aid of a studentship from the Natural Environment Research Council and carried out in collaboration with Plymouth Marine Laboratory.

A programme of advanced study was undertaken, which included a final year honours course in colloid chemistry, supervised information technology instruction and a postgraduate course on analysis and hazard assessment of marine pollution.

Relevant scientific seminars and conferences were regularly attended at which work was often presented; external institutions were visited for consultation purposes and several papers prepared for publication.

Publications:


Presentations and Conferences attended:


Word count of main body of thesis: 41,725

Signed

Date
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BD</td>
<td>Beckton Dickenson</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow derived macrophage</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca2+</td>
<td>Calcium</td>
</tr>
<tr>
<td>CCL -</td>
<td>C-C motif chemokine-</td>
</tr>
<tr>
<td>CCR-</td>
<td>C-C chemokine receptor type-</td>
</tr>
<tr>
<td>CD -</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagles medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecos phosphate buffered saline</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EcLPS</td>
<td><em>Escherichia coli</em> lipopolysaccharide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FASL</td>
<td>FAS ligand</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FC</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>Hu</td>
<td>Human</td>
</tr>
<tr>
<td>IFNβ</td>
<td>Interferon beta</td>
</tr>
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<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of nuclear factor kappa-B kinase</td>
</tr>
<tr>
<td>IKKα</td>
<td>Inhibitor of nuclear factor kappa-B kinase subunit alpha</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor-associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus Amebocyte Lysate</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M1</td>
<td>Classically activated macrophage</td>
</tr>
<tr>
<td>M2</td>
<td>Alternatively activated macrophage</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MCS-F</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MD-2</td>
<td>Myeloid differentiation factor-2</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>Mg2+</td>
<td>Magnesium</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Expanded Form</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>P. gingivalis</td>
<td>Porphyromonas gingivalis</td>
</tr>
<tr>
<td>PAM3CSK4</td>
<td>N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteiny-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PgLPS</td>
<td>Porphyromonas gingivalis lipopolysaccharide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate13-acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonucleocyte</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Real-time quantitative reverse transcriptase – polymerase chain reaction</td>
</tr>
<tr>
<td>R10</td>
<td>RPMI 1640 + 10% fetal bovine serum + 1% L-glutamine</td>
</tr>
<tr>
<td>RMPI 1640</td>
<td>Rosewell Park Memorial Institute 1640</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SHIP</td>
<td>Src homology 2-containing inositol-5'-phosphate</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
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<tr>
<td>TAB</td>
<td>TAK1 binding protein</td>
</tr>
<tr>
<td>TAK</td>
<td>TGF-β-activated kinase</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumour associated macrophage</td>
</tr>
<tr>
<td>Tc</td>
<td>Cytotoxic T cell</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TH</td>
<td>T-helper</td>
</tr>
<tr>
<td>TLR-</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF related adapter molecule</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>1alpha, 25-Dihydroxyvitamin D3</td>
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Overview of oral mucosal immunity

The primary function of the oral mucosal immune system is to selectively and discriminately tolerate oral microbiota and food antigens, whilst retaining the capacity to launch an immune response to pathogenic challenge. The mouth contains a plethora of site-specific bacteria (over 750 distinct species)\(^1\) and the teeth are the most densely populated tissues, with approximately 80% of the bacterial species inhabiting the dental plaque\(^2\). In comparison, the mucosal tissue is sparsely populated. Some oral bacteria are pathogenic, for example *Streptococcus mutans*, implicated in dental caries\(^3\), and *Porphyromonas gingivalis*, a periodontal pathogen\(^4\), however, most oral bacteria present are commensal; they benefit from inhabiting the oral environment, but do not adversely affect the host. The key role of the oral mucosal immune system is thus to discriminate between the pathogenic and commensal microbiota/food antigens.

The innate immune response in the oral mucosa

As part of the innate, mechanical barrier to pathogens, saliva has numerous protective and anti-microbial functions. It performs a physical 'sloughing' action on the epithelial cells, removing potentially infected cells from contact with the underlying immune cells. Saliva also contains secretory immunoglobulin A (sIgA) produced by activated B cells (plasma cells) distributed throughout the mucosa\(^5\), which can inhibit binding of bacteria to the mucosal surface, thus limiting colonisation\(^6\). In addition, antimicrobial peptides in the saliva play a role in mucosal immunity. Human Beta defensin 3 (HBD3) may have protective effects against *P. gingivalis* invasion by binding to antigens on the bacterial cell surface, such as hemaglutinin B (HaB), an extracellular adhesin, inhibiting the binding to the host cells\(^7\). Salivary lysozyme elicits its antibacterial effects by lysing peptidoglycan (a bacterial cell wall component), and activating bacterial autolytic enzymes\(^8\). To reinforce the mechanical barrier between foreign antigens and the host’s immune system, oral squamous epithelial cells
secrete mucins and a layer of anti-microbial peptides such as α- and β-defensins\(^9\). Thus; secretory factors contribute to innate immune defence against pathogens in the oral cavity.

The mechanism by which a pathogen initiates an immune response is dependant both on the cell it encounters, and the nature of the pathogen. Typically, professional antigen presenting cells (such as macrophages and dendritic cells) are the first point of contact between antigens and cells of the innate immune system. Highly conserved molecular patterns on a bacterial cell wall, such as lipopolysaccharide (LPS), can activate antigen presenting cells (APCs) and initiate an immune response. LPS binding protein (LBP) in the serum binds to the LPS and forms a complex with CD14 (a co-stimulatory molecule), MD2 and toll-like receptor (TLR) 4\(^1\) on the surface of the APC. Ligation of TLR 4 activates a downstream signalling cascade\(^1\), resulting in the activation of transcription factors and the expression of pro-inflammatory cytokines (such as TNFα, IFNγ, IL-1β, IL-18, IL-6), chemokines (e.g. IL-8, MCP-1) and the up-regulation of cell surface molecules (TLRs and B7 co-stimulatory molecules\(^1\)). The APCs internalise, process and present antigen to cells of the adaptive immune system to elicit a tailored, pathogen specific immune response.

As well as APCs, oral epithelial cells play an active role in innate immunity. Epithelial cells had conventionally been thought of as immunologically inert, however, recent studies have shown that these cells can acquire immune cell characteristics by up-regulating the expression of pattern recognition receptors (PRRs)\(^1\) in response to signals from the basolateral surface. A number of oral epithelial cell lines and primary gingival epithelial cells are unresponsive to a selection of bacterial pathogen associated molecular patterns (PAMPs); LPS - a TLR4 ligand, peptidoglycan (PGN) and lipotechoic acid (LTA) - TLR2 ligands, and muramyl dipeptide (MDP) – a nucleotide-binding oligomerization domain containing 2 (NOD)2 ligand. Following pre-treatment with IFNγ, the cells become responsive...
to PAMPs and up regulate interleukin (IL)-8 and granulocyte-macrophage-colony stimulating factor (GM-CSF) expression. This would suggest that epithelial cells are involved in the recruitment of neutrophils to the epithelial surface and differentiation of monocytes into pro-inflammatory macrophages, respectively. During inflammation, it is likely that there are high levels of IFNγ produced by T cells, rendering epithelial cells responsive to PAMPs. Thus, PAMPs provide the ‘danger’ signals needed to break tolerance and initiate an immune response, which oral epithelial cell immune responsiveness (activated by IFNγ) can perpetuate. In contribution to their active role in mucosal immunity, oral epithelial cells express the bacterial LPS receptor, TLR4. Although expression of its co-receptor, CD14, is low, oral epithelial cells can be rendered responsive to PAMPs via soluble, bio-active CD14 (sCD14) in the plasma and saliva. sCD14 can form a complex with MD2, LBP, LPS and TLR4, rendering the CD14 negative cells responsive to LPS and inducing cytokine expression. Interestingly, *P. gingivalis* has been shown to promote the shedding of membrane-bound CD14 from macrophages via the proteolytic enzymes, gingipains. This increase in soluble CD14 may render epithelial cells more responsive to microbiota that are normally tolerated by the mucosal immune system, but at the same time decrease macrophage intracellular signalling so that they cannot clear the *P. gingivalis* infection effectively, leading to epithelial damage but macrophage unresponsiveness.

In addition to oral epithelial cells, gingival fibroblasts (GFBs) can mediate inflammatory responses to pathogens such as *P. gingivalis*. GFBs express TLRs 1,2,3,4,5,-6 and 9 and can produce inflammatory cytokines such as IFNγ, IL-6, IL-1β, and IL-8, along with PGE2, ICAM-1 and HLA-DR. As such, GFBs may be involved in leukocyte recruitment, macrophage polarization, periodontal bone resorption, activation of epithelial cells, antigen presentation and induction of IL-2 production leading to T cell proliferation. Thus, GFBs and oral epithelial cells actively contribute to both innate and adaptive immune responses.
Communication between innate and adaptive immunity via antigen presentation is fundamental in the mucosal immune system. The oral cavity is sometimes considered simply an extension of the gastrointestinal tract, and whilst there are structural and functional similarities, the methods of antigen presentation differ in oral and gut mucosa. In the gut, mucosal microfold (M) cells internalise intra-lumenal antigens and present them to antigen presenting cells (dendritic cells (DCs) and macrophages (MΦ)) in the lamina propria. M cells are not present in the oral mucosa, thus intra-oral antigens are sampled by DCs extending spindly protrusions through the tight junctions of the epithelium. Rescigno et al. (2001) demonstrated this mechanism with a model of the gut epithelium, using the gut epithelial cell line CaCO2 co-cultured in transwells with DCs on the basolateral side.

When non-invasive bacteria were added apically to the epithelial monolayer (not basolaterally), DCs upregulated the expression of tight junction proteins occludin and claudin1, and formed new, tight-junction-like structures with the epithelial cells. By utilising this pathway of transepithelial sampling, the integrity of the epithelial barrier remained intact, whilst allowing the processing and presentation of antigen by DCs to induce an adaptive immune response.

The adaptive immune response in the oral mucosa

Following the initiation of an innate immune response, a pathogen-specific adaptive response is required to eliminate infection. Under physiological conditions, effector T helper (TH) cell subsets TH1, TH2, and the newly-described TH17 provide immune protection against a particular range of pathogens. TH1 cells defend the host against infection by intracellular bacteria and viruses (part of the cell-mediated immune response), and TH2 cells provide defence against extracellular bacteria and parasites (part of the humoral immune response). TH17 cells help eradicate fungi and both intra- and extra-cellular bacteria and have been implicated in mucosal immunity. Dysfunction of TH2 or TH1/TH17 leads to the development of allergy and autoimmunity, respectively. Under physiological conditions, regulatory T cells (Tregs) express high levels of the anti-inflammatory cytokines IL-10 and
TGF-β in order to suppress effector T cell function when an immune response is not needed\textsuperscript{30}.

Differentiation of T helper cell subsets is dependent on the cytokines/cell-cell stimuli they encounter\textsuperscript{27}. T\textsubscript{H}1 are differentiated from naive precursors (dependant on the transcription factor T-bet) in the presence of IL-12 expressed by APCs\textsuperscript{16}. T\textsubscript{H}1 express IL-2 needed for their proliferation, and IFNγ\textsuperscript{27} which activates pro-inflammatory macrophages\textsuperscript{31–33}, initiating a cell mediated immune response. T\textsubscript{H}2 are differentiated (dependant on GATA-3\textsuperscript{34}) in the presence of IL-4 expressed by APCs. They express high levels of IL-10, IL-4 and IL-13\textsuperscript{16}, which suppress the differentiation of T\textsubscript{H}1/ T\textsubscript{H}17, and promote a humoral immune response, and the differentiation of anti-inflammatory M2 macrophages\textsuperscript{31–33}.

Figure 1-1: Schematic representation of APC driven T helper cell differentiation
TH17 express high levels of IL-17, IL-21 and IL-22, which act on mucosal epithelial cells to produce inflammatory cytokines and chemokines (such as TNFα and IL-8) and antimicrobial peptides (such as β-defensin 2). IL-1β, IL-6, IL-21, IL-23, TNFα, and TGF-β (in a seemingly concentration-dependent manner) have all been implicated in TH17 differentiation, although the involvement of TGF-β has been hotly debated. Low concentrations of TGF-β in combination with pro-inflammatory cytokines such as IL-6 may lead to TH17 development, whereas high levels of TGF-β lead to Treg differentiation, which is amplified by the presence of retinoic acid. Nevertheless, TH17 differentiation is dependent on the transcription factors RORγt and RUNX1.

In cells stimulated with TGF-β alone (Treg-priming conditions), RUNX1 binds to Foxp3 to promote IL-10 expression and suppresses RUNX1/RORγt binding, thus silencing IL-17 expression. In cells stimulated with TGF-β + IL-6/IL-21 (TH17 conditions), RUNX1 binds to RORγt and suppresses RUNX1/Foxp3 binding, thus silencing IL-10 expression. Given the close, reciprocal regulation of anti-inflammatory Tregs and pro-inflammatory TH17 differentiation, it is possible that a genetic polymorphism in RUNX1 leads to the loss of Treg suppressive activity and promotion of a destructive TH17 response in the oral mucosa. Indeed, such a polymorphism has been identified in other chronic, inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, and psoriasis. Furthermore, IL-17 producing T cells have been associated with periodontal lesions and even oral cancer.

In oral cancer, invasion, migration and cervical metastasis of cancer cells is driven by MIP-3a, a T cell chemokine. Lymphocytes isolated from oral tumour infiltrates produce high levels of IL-1β, IL-6, TNFα and TGF-β compared with cells isolated from peripheral blood. CD4+CD25+ T cells isolated from peripheral blood mononuclear cells (PBMC) and tumour sites in oral cancer patients also express Treg markers, FoxP3, GITR, CD45RO, CD69, LAP, CTLA-4, CCR4, and IL-10. These cells exhibited a more suppressive effector function than the equivalent cells isolated from healthy patients. FoxP3 was also detected in T cells.
isolated from peripheral blood, suggesting that oral tumours suppress anti-tumoural immune responses both locally and systemically. CD4⁺FoxP3⁺ IL-17-producing T cells also infiltrate the tumour microenvironment. T_H₁7 cells were found in patients with early stage cancer, but switch to a prevalence of Tregs a later stages in disease.

Anti-tumoural responses are hypothesised to be mediated by a phenomenon called cancer immunoediting. Cancer immunoediting has 3 stages: elimination, equilibrium and escape. During the elimination phase, the immune system is alerted to malignant transformation via danger signals, and transformed cells are deleted. Tumour antigen presentation by APC to CD₈⁺ cytotoxic T cells (Tc) is required for tumour rejection and IFNγ is indispensable in this tumour cell killing. In the equilibrium phase, the immune system exerts more selection pressure on the transformed cells, which in turn develop further mutations, resulting in a continuum of eradication and emergence of newly transformed cells. Escape from this stage may be promoted by the pro-tumoural actions of tumour associated immune cells, described later in this chapter.

**Oral Mucosal Tolerance**

The oral mucosa is a complex immune site that discriminately responds to pathogenic microorganisms and disregards commensals and dietary antigens. This mechanism of discretionary non-responsiveness is described as ‘oral mucosal tolerance’. It is due to this immunological phenomenon that we do not elicit an immune response to food antigens or commensal microorganisms, but retain the capacity to orchestrate a deadly assault on pathogens when required. This is the fundamental role of the oral mucosal immune system. To prevent constant (and inappropriate) immune activation, the oral mucosal immune system has in place a variety of mechanisms to suppress activation and induce a state of immune ‘tolerance’ to antigenic stimuli.

Dendritic cells in peripheral tissues, such as dermal Langerhans cells (LC), are found in an immature state and constitutively endocytose antigens of microbial or self-origin. This is a
key factor in mucosal tolerance: endocytosis alone does not lead to DC activation. This mechanism of internalisation without activation leads to the presentation of antigen and apoptotic material to T cells without co-stimulation, thus inducing T cell non-responsiveness (anergy)\textsuperscript{58}. If these DCs remain un-stimulated, they do not up-regulate their expression of co-stimulatory molecules and become tissue resident LCs\textsuperscript{58}. However, it has been reported that LCs of the oral mucosa express high levels of Fc receptors (FcRs), co-stimulatory molecules and MHC I and II\textsuperscript{59}. The lack of CD83, a marker of mature DCs in oral LCs suggests that they are in an immature state\textsuperscript{59}, and these immature DCs would not be expected to express co-stimulatory molecules\textsuperscript{58}. Thus, LCs of the oral mucosa have the potential to be highly immunogenic, yet under physiological conditions, the oral mucosal immune system maintains a state of tolerance. It is likely then that oral LCs maintain tolerance via different mechanisms. For instance, ligation of FcεR1 by IgE on monocytes leads to the expression of IL-10, and up-regulates Indoleamine 2, 3-Dioxygenase (IDO) activity\textsuperscript{60,61}, leading to suppression of effector T cell activity. Is it possible that a similar mechanism is utilized in the oral LCs; in fact, isolated oral LCs have been shown to produce IL-10 in response to stimulation via TLR4, and induce tolerogenic Foxp3\textsuperscript{+}IL-10\textsuperscript{+} and TGF-β\textsuperscript{+} Tregs\textsuperscript{25,60} suggesting that they actively contribute to maintaining oral mucosal tolerance.

The presence of oral microbiota is also likely to play a role in mucosal immune homeostasis. Commensal microflora have been shown to maintain a constant presence of APCs at the epithelial surface\textsuperscript{14}. Repeated stimulation of APCs by bacterial PAMPs can also have a tolerising effect, for example, LPS from \textit{P. gingivalis} has been shown to down regulate the expression of TLRs 2 and 4 on macrophages in response to repeated stimulation, and the expression of pro-inflammatory cytokines is decreased in accordance\textsuperscript{62}. In addition, circulating, naturally occurring Tregs (nTregs) patrolling the tissues release anti-inflammatory cytokines to suppress unnecessary effector T cell activity\textsuperscript{63}. They express IL-10, which blocks T\textsubscript{H}1 and T\textsubscript{H}17 differentiation, and TGF-β, which induces the release of more IL-10
(which can also promote a $T_{H2}$ immune response), generating an anti-inflammatory environment and suppression of inappropriate pro-inflammatory effector responses; i.e. tolerance. This level of tolerance is maintained by a low dose of antigen, and small amounts of IL-2. High levels of pro-inflammatory cytokines expressed by DCs and macrophages, such as IL-6, can 'switch off' Tregs by blocking their activation, leading to an effector T cell response. Given that oral mucosal tolerance is maintained by a concurrent array of complex mechanisms, dysregulation in any one part may lead to a breakdown in tolerance and uncontrolled immune activation in response to oral microbiota.

**Aberrant innate cell function leads to pathology in the oral mucosa – key role of macrophages**

Macrophages prime the adaptive immune response according to the differentiation and/or activation signals they encounter. They have been shown to play a vital role in driving chronic inflammatory conditions such as rheumatoid arthritis (RA) and Crohn’s disease. They can be divided into distinct subsets according to their cytokine profile and phenotypic traits. Classical, pro-inflammatory macrophages (M1) are polarized by granulocyte macrophage-colony stimulating factor (GM-CSF) and activated by IFNγ produced by $T_{H1}$. Alternative, anti-inflammatory macrophages (M2), lead to the generation of a $T_{H2}$ response and are polarized by M-CSF. They can be further divided into subsets; M2a – activated by IL-4 and IL-13, M2b – activated by immune complexes + LPS and TLR ligation, and M2c - activated by IL-10. M2 type macrophages exhibit anti-inflammatory properties and express the regulatory cytokines, IL-10 and TGF-β which negatively regulate M1. M1 type macrophages express high levels of pro-inflammatory cytokines, TNFα, IL-1β, IL-18, IL-6, IL-12 and IL-23. Thus, polarisation of macrophages to an M1 phenotype favours a pro-inflammatory response and M2 differentiation favours an anti-inflammatory / regulatory response. Aberrant activation of M1 and M2 leads to development of inflammatory conditions (such as periodontitis) and immunosuppressive
conditions, such as oral cancer, respectively. Periodontitis is a chronic inflammatory disease affecting the supporting structures of the teeth (periodontium). Previous studies have shown that the oral pathogen *Porphyromonas gingivalis* plays a significant role in the development of periodontitis.

**Immune function in periodontitis**

*P. gingivalis* and oral epithelial cell interactions

*Porphyromonas gingivalis* is a gram-negative, anaerobic, rod-shaped and black pigmented bacterium. The vast array of potential virulence factors produced by the bacterium can initiate destruction of periodontal tissue. In the gingival sulcus, neutrophils form a barrier between the epithelium and the plaque biofilm which serves to protect the epithelium from invasion. However, *P. gingivalis* can circumvent killing by neutrophils and adhere to epithelial cells. Upon adherence, *P. gingivalis* can rapidly invade the epithelial cells by binding of its major fimbriae to β1 integrin receptors and remodelling the actin cytoskeleton via phosphorylation and activation of FAK (focal adhesion kinase) and paxillin. Invasion of epithelial cells allows *P. gingivalis* to evade recognition by the host immune system. Once inside the cells, *P. gingivalis* resides in the cytoplasm and accumulates around the nucleus. There, *P. gingivalis* can hijack host cytoskeletal machinery to facilitate the formation of intercellular tubules, allowing for safe passage to the neighbouring cells. Thus, epithelial cells are chronically exposed to *P. gingivalis*. Furthermore, *P. gingivalis* has been detected within epithelial cells at sites remote from its usual, sub-gingival habitat, such as the buccal epithelium.

Oral epithelial cells and *P. gingivalis* exhibit a dynamic relationship; upon invasion of epithelial cells, *P. gingivalis* induces major changes in its gene transcription and protein expression profiles, which in turn modulates transcriptional response of the epithelial cells. Thus, *P. gingivalis* and GEC adapt to facilitate long-term cohabitation. Epithelial cells
survive for up to 8 days after infection, allowing intracellular *P. gingivalis* to remain viable and spread between host cells, and evading recognition by immune cells.

*P. gingivalis* employs several mechanisms to avoid eradication by the immune system. It can inhibit chemically induced apoptosis in GECs by blocking activation of the effector caspase-3. The anti-apoptotic phenotype of *P. gingivalis* is conserved across strains and does not depend on the presence of fimbriae, as fimbriae-deficient strains were able to impede apoptosis. *P. gingivalis* infection promotes a survival of epithelial cells by inhibition of mitochondrial cell death pathways via phosphorylation of JAK1 and Stat3, and increased expression of Survivin and Stat3. However, in gingival fibroblasts, pro-apoptotic pathways are up-regulated by prompting translocation of apoptosis inducing factor to the nucleus indicating that *P. gingivalis* causes fibroblast apoptosis through a pathway independent of proteolytic activity and the classical apoptotic pathways involving caspase-3. *P. gingivalis* also suppresses the adaptive, anti-microbial T-cell responses by up-regulating IL-10 and PD-1, which in turn inhibits IFNγ production, and up-regulates the expression of the tolerance-inducing molecules B7-H1 and B7-DC receptors in gingival keratinocytes.

**Porphyromonas Gingivalis** LPS and Toll like receptor interactions

LPS is a virulent molecule located in the outer membrane of gram negative bacteria. Traditionally, LPS consists of inner and outer core regions, an external O-antigen (recognised by antibodies), and is anchored to the bacterial membrane by lipid A. The lipid A portion of LPS consists of a glucosamine disaccharide backbone with fatty acid residues attached. Lipid A is the bioactive component of LPS and confers its biological activity by binding TLR4 and myeloid differentiation factor 2 (MD-2) on host cells. The fatty acids, of which there are traditionally 6 (hexa-acetylated), such as in the archetypal enterobacteria *E.*
coli LPS, fit precisely into the binding pocket of MD-2. This initiates dimerization of the TLR4/MD-2 complex and an intracellular signalling cascade, leading to activation of transcription factors and production of inflammatory cytokines. Lipid A is a structurally conserved pathogen associated molecular pattern (PAMP), but its conformation can vary by the number of fatty acids, where the fatty acids are linked to the glucosamine backbone, and the number of phosphate groups. P. gingivalis lipid A has several unusual properties that distinguish it from *E. coli* Lipid A. First determined in 1993 by Ogawa et al, *P. gingivalis* lipid A has a tri-acylated, mono-phosphorylated chemical structure, that is, it consists of 2 glucosamine molecules (β-(1–6) linked) making up the disaccharide backbone, with 3 fatty acids (2 molecules of 3-hydroxy-15-methylhexadecanoic acid and 1 molecule of 3-hexadecanoyloxy-15-methylhexadecanoic acid) at the 2- and 2’-positions of the disaccharide backbone, respectively. Later, it was discovered that *P. gingivalis* also produces lipid A species of the tetra- and penta-acylated forms, with the extra fatty acids linked to the 3 and 3’ positions. Furthermore, *P. gingivalis* lipid A is distinct from *E. coli* lipid A in that it has no phosphate at the 4’-position (*E. coli* lipid A is phosphorylated at the 1 and 4’ positions), and it has fatty acids that possess more carbon atoms (16–17 atoms).

It is thought that these differences in lipid A conformation in *P. gingivalis* are responsible for its unusual biological activity. *P. gingivalis* LPS contains a heterogenous compilation of lipid A species, which are mostly tri- and tetra-acylated lipid A. This results in a less precise binding of the fatty acids to the binding pocket in MD-2, and a consequent lower level of bioactivity. As well as the number of fatty acid residues, the number of phosphate groups of lipid A can also affect biological activity. Curtis *et al* (2011) have shown that *P. gingivalis* grown under stress conditions (elevated temperature simulating oral inflammation) will change its lipid A conformation: *P. gingivalis* grown at 41°C produces mostly mono-phosphorylated, penta-acylated lipid A, whereas *P. gingivalis* grown at 37°C produced mostly non- and mono- phosphorylated, tetra-acylated lipid A. As the lipid A conformation
changed with temperature increase, the virulence of the lipid A increased, leading to more potent activation of TLR4 (measured by NFκB activation).

In addition to biological activity being affected by the number of fatty acid residues and phosphate groups in lipid A, it is also affected by the position of the phosphates. Like *P. gingivalis*, several bacterial species produce a lipid A that is penta-acyltated and non-phosphorylated, which does not activate TLR4. However, *P. gingivalis* also produces a mono-phosphorylated, penta-acylated form, and it is the unusual position of the phosphate (at the 4’ position) that confers its lower immunogenicity (Coats, 2011). This is exemplified by the fact that *P. gingivalis* LPS has consistently been demonstrated to exert lower endotoxin activity (lower cytokine production) in many studies, including this one.

The TLR signalling pathways utilised by *P. gingivalis* have been contested for a number of years. In a recent review, Kirkwood *et al.* suggest that *P. gingivalis* LPS preferentially utilizes toll-like receptor-2 and not toll-like receptor-4 as previously thought. However, other studies report that it is down to contaminants (namely lipoprotein) during the purification process which lead to the activation of TLR2. It is important to determine the utilisation of either TLR2 or TLR4 by *P. gingivalis*, as activation of either pathway may result in differential activation of the adaptive immune response in periodontal lesions. Studies have reported that *P. gingivalis* LPS binds TLR4 in gingival fibroblasts, and promotes IL-12p70, IFNγ, and production of other Th1-associated cytokines. In contrast, other studies indicated that in response to TLR2 activation these cytokines were produced in much smaller amounts, or not at all, suggesting that TLR2 stimulation, in the absence of simultaneous TLR4 stimulation, favours the development of a Th2-like immune response. It has now been generally accepted that Th1 cells are associated with (early) stable periodontal lesions, while Th2 cells are associated with progressive lesions, however, evidence is gathering for the involvement of Th17 cells in periodontitis. Indeed, *P. gingivalis* induces Th17 associated cytokines IL-1β, IL-6 and IL-23, but not Th1 related IL-12 from APCs in periodontitis. Furthermore, *P. gingivalis* gingipains preferentially degraded Th1 associated cytokines.
skewing the inflammatory microenvironment towards a Th17/tissue destructive type, associated with autoimmunity\textsuperscript{103}.

A further mechanism possessed by \textit{P. gingivalis} to induce autoimmunity is via the molecular chaperone GroEL. GroEL and human heat shock protein HSP 60 show marked structural similarity, and both are expressed in higher levels in periodontitis tissues than control tissue \textsuperscript{104}. \textit{P. gingivalis} GroEL binds to both TLR2 and TLR4 to induce NF-κB activity in monocytes \textsuperscript{105}, however, in a separate study it was reported that it was human HSP 60 not \textit{P. gingivalis} GroEL that activated monocytes up-regulated TNFα production to similar levels induced by \textit{P. gingivalis} LPS \textsuperscript{106}. Nevertheless, human HSP 60 is recognised by antibodies raised to \textit{P. gingivalis} GroEL \textsuperscript{106,107}, thus providing a potential mechanism of autoimmune tissue destruction induced by \textit{P. gingivalis} infection seen in periodontitis. Heat shock protein families and \textit{P. gingivalis} infection are implicated in other chronic inflammatory conditions, such as rheumatoid arthritis \textsuperscript{108} and atherosclerosis \textsuperscript{109,110}.

**Periodontal disease and its systemic implications**

There have been many studies into links with systemic inflammatory diseases and periodontitis. There is evidence to support the relationship between human periodontal disease and an increased risk for acute myocardial infarction\textsuperscript{111–113}. Furthermore, several bacteria associated with periodontal disease, including \textit{P. gingivalis}, have been detected in atherosclerotic plaque \textsuperscript{111,114,115}. Serum antibodies to \textit{P. gingivalis} have also been associated with coronary heart disease \textsuperscript{112}. It has been suggested that periodontal disease can lead to low-level bacteremia, an elevated leukocyte count, and systemic endotoxemias, which could affect endothelial integrity, metabolism of plasma lipoproteins, blood coagulation, and platelet function. Furthermore, it is well established that infection with \textit{P. gingivalis} induces local inflammation, which can lead to gingival ulceration and local vascular changes, which have the potential to increase the incidence and severity of transient bacteremias. Several studies have also demonstrated that patients with periodontal disease have elevated levels
of systemic inflammatory mediators. Extensive periodontal disease has been associated with increased levels of C-reactive protein (CRP)\textsuperscript{116}; moderately elevated CRP is a systemic marker of inflammation and a documented risk factor for cardiovascular disease\textsuperscript{117}.

It has long been established that infection with particular bacteria or viruses can lead to the malignant transformation of epithelial cells; the enterobacterium Helicobacter pylori, for example, is a recognised gastric carcinogen\textsuperscript{118}. Evidence is accumulating that the periodontal pathogen has a role in the development of oral cancer\textsuperscript{119–121}

**Immune function in oral cancer**

More than 90% of malignancies of the oral cavity are squamous cell carcinomas (OSCC), and as of 2010, holds sixth position in the ranking of cancer incidence worldwide\textsuperscript{122,123} Most prevalent in males\textsuperscript{124}, OSCC is a multifactorial disease and its initiation has been linked to oral leukoplakia\textsuperscript{125}, HPV infection\textsuperscript{126}, periodontal disease\textsuperscript{127}, oral bacterial species\textsuperscript{128}, alcohol and tobacco use\textsuperscript{129,130}. In the Western nations, the increase in incidence of OSCC among younger cohorts may be related to HPV transmission\textsuperscript{126}.

A key feature of OSCC is the infiltration of immune cells, including lymphocytes, neutrophils, eosinophils and monocytes\textsuperscript{131}. Once recruited to the tumour site by chemotactic factors such as MCP-1, expressed more highly in OSCC tissue than normal mucosa\textsuperscript{132}, monocytes differentiate into macrophages and can polarise into M1 or M2-like subsets with pro- and anti-tumoural properties. These tumour-associated macrophages (TAM) have been subject to intense investigation.

**Macrophages in oral cancer**

Characteristically, M1 macrophages amplify T\textsubscript{H}1 responses, providing a positive feedback loop in the anti-tumour response\textsuperscript{133} However, in the tumour microenvironment, these responses can be overridden and macrophages then facilitate tumour progression\textsuperscript{134}. Compared with macrophages within the surrounding tumour stroma, macrophages in direct
contact with cancer cells display impaired tumour antigen presentation due to low lysosome expression and defective phagosome-lysosomal apparatus. Highlighting the influential role of tumour cells over macrophage function. TAMs can be modulated by tumour cells to facilitate tumour growth and survival. They can induce immune suppression via production of anti-inflammatory cytokines such as IL-10 and TGF-β, production of angiogenic factors (VEGF) (reviewed in), and inhibition of tumour antigen presentation by down-regulating MHC molecules and up-regulating inhibitory co-stimulatory B7 molecules. IL-10 and TGF-β can further inhibit the anti-tumoural response by inducing Treg differentiation. Recently, pro-inflammatory cytokines (presumed to be anti-tumoural), have been shown to play a detrimental role in cancer. In response to pathogenic stimuli, macrophages produce inflammatory mediators such as reactive oxygen and nitrogen species, and cytokines (such as TNFα, IL-1β, IL-6 and IL-8), leading to oxidative and nitrative DNA damage, enhanced cell growth and proliferation, inhibition of apoptosis and production of angiogenic factors (reviewed in). TNFα and IL-6 are associated with poor prognosis in several carcinomas, and inhibitors of both are in clinical trials for the treatment of cancer.

TAMs display broad heterogeneity, differing both across and within disease sites. Monocytes stimulated with breast cancer supernatants increase expression of IL-10, IL-8, and the M2-associated chemokines CCL17 and CCL22 (T cell chemoattractants binding CCR4). However, upon culture with colon cancer cell supernatants, monocytes produced more of the M1-associated factors IL-12, TNFα, and ROS. In breast cancer tumours, at least two distinct TAM populations have been identified. These TAMs differentially express CD206 and MHC II, and display distant behavioural profiles; sessile, M2-like macrophages found in hypoxic regions and at the tumour borders, and migratory macrophages (less resembling the M2 phenotype) which are found perivascularly. Therefore, there is a very complex relationship between tumours and their associated macrophages, and TAM behaviour should be studied in a disease specific context, rather than inferred from other cancers.
In OSCC, macrophages expressing CD163 (an M2 marker) were found in elevated numbers, particularly in advanced lesions, and this significantly correlated with a poor outcome in patients with OSCC. Macrophages expressing high levels of M2-associated cytokines IL-10 and TGF-β have also been found in elevated numbers in OSCC microenvironments, and higher numbers of macrophages were present in metastatic versus non-metastatic OSCC. This led to a shorter survival time in patients with a higher percentage of TAMs, than patients with low TAM infiltration.

In general, tumour-associated macrophages (TAMs) acquire an M2-like phenotype, but in colorectal cancer and non-small cell lung carcinoma, the infiltrating macrophages display a more pro-inflammatory, M1-like phenotype, and some even develop into a mixed M1/M2 phenotype. However, in the tumour environment, macrophages exhibit extraordinary plasticity, adapting their phenotype and effector functions in response to local stimuli. It has been demonstrated both in vivo and in vitro that macrophage polarisation can be reversed by exposure to opposing polarising factors. This plasticity can be exploited to develop anti-cancer therapies. For example, the M1-polarising cytokine IFNγ reverses the anti-inflammatory/suppressive properties of TAMs. TAMs can also be switched to an anti-tumour phenotype by blocking NFκB signalling. Thus, localised IFNγ treatment or inhibition of NFκB signalling (specifically via activation of IKKβ) make suitable candidates for therapeutic development. A further mechanism proposed to modulate TAMs toward a more pro-inflammatory, anti-tumoural activation state, is bacterial LPS. LPS or analogues thereof have been used in human clinical trials as an anti-cancer therapy. The effects of LPS on OSCC cells has yet to be described.

Given the fundamental role macrophages play in driving both inflammatory (periodontitis) and immunosuppressive (cancer) conditions, investigating how their functions are affected by these disease states will provide insight into their pathogenic mechanisms. What is more,
these interactions have been implicated in diseases at sites distant from the oral mucosa, and so may have wider reaching implications in macrophage driven pathology in general.

Thus, the main objectives of the present study are as follows:

- To establish a robust, reproducible model of M1 and M2 macrophage polarisation using the THP-1 cell line, so that their interactions with pathogenic oral bacteria can be investigated.
- To determine what effect the presence of *P. gingivalis* LPS during the macrophage polarisation process has on cytokine expression in response to subsequent LPS stimulation.
- To determine the cytokine response of the OSCC cell line H357 to *P. gingivalis* LPS.
- To determine the effect of OSCC produced soluble factors on macrophage polarisation and subsequent responses to *P. gingivalis* LPS.
Chapter 2 – Materials and methods

Materials and reagents

The materials and reagents used in the study are listed in Table 2-1.

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<td>-</td>
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<td>IL-15 standard</td>
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<tr>
<td>Target gene</td>
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<tr>
<td>Human IL-10 Reverse</td>
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<tr>
<td>Human TGF-β1 Forward</td>
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<tr>
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<td>ATT GCC GAC AGG ATG CAG AA</td>
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</tr>
<tr>
<td>Human β actin Reverse</td>
<td>GCT GAT CCA CAT CTG CTG GAA</td>
<td>Eurofins MWG Operon, UK</td>
</tr>
</tbody>
</table>

Primers were either selected from published literature or designed using Applied Biosystems software.
General cell culture: Cell lines and growth conditions

THP-1
THP-1 (ATCC, TIB-202) are a monocytic cell line derived from a one-year old male with acute myeloid leukaemia. Cells were reactivated from liquid nitrogen at passage 9 and cultured in RPMI 1640 (Lonza, UK) supplemented with 10% foetal calf serum (LabTech, UK) and 1% L-glutamine (Lonza, UK) in a humidified incubator at 37°C with 5% CO₂. Cells were initially seeded at 4 x 10⁵ cells/ml and routinely sub-cultured using 1:4 split ratios and maintained for no more than 10 passages THP-1 cell culture media is referred to as R10 throughout this thesis.

H357
H357 cells were a kind gift from Professor Stephen Prime/Dr Simon Whawell, University of Sheffield. Cells were seeded in tissue culture treated flasks at 5 x 10³ cells/ml in 1:1 DMEM and Ham’s F12 (Lonza, UK), supplemented with 10% foetal calf serum and 1% L-glutamine. Cells were grown to 70-80% confluence then sub-cultured using Accutase™ cell detachment solution (eBioscience). H357 culture media is referred to as HM throughout this thesis. H357 were cultured in serum because it has been previously reported that serum must be present for epithelial cells to respond to LPS ¹⁵⁹

Primary tissue collection and culture

All samples of primary tissue were collected from Derriford Hospital (Plymouth, UK) and the Royal Devon and Exeter hospital (Exeter, UK) with full patient consent. The study was approved by the NRES Committee South West - Cornwall & Plymouth in September 2009, study number: 09/H0203/78.
Collection of control (non-tumour) mucosa:

Following routine wisdom tooth extraction, 0.5cm of the least inflamed mucosa from the distal incision was collected prior to placement of sutures. The excised tissue was placed in 7ml bijou tube containing culture medium, which was then tightly sealed with parafilm and kept on ice until processed.

Collection of oral squamous cell carcinoma tissue:

Following excision of tumour for biopsy, a section of the tumour was removed (see Figure 2-1) and placed in 7ml bijou tube containing culture medium, which was then tightly sealed with parafilm and kept on ice until processed.

Figure 2-1: Diagram of tumour tissue samples used in the study

Generation of M1- and M2- like THP-1 macrophages

Method adapted from Tjiu et al 160. 5 x 10^5 THP-1 cells were seeded in tissue culture plates and incubated with 5 ng/ml PMA for 6 hours. Next, all the culture media was removed, including any non-adhered cells and was replaced with fresh media containing either (a) 5 ng/ml PMA, (b) 20 ng/ml IFNγ + 5 ng/ml PMA (c) 20 ng/ml IL-4 + 5 ng/ml PMA or (d) 20 ng/ml IL-10 + 5 ng/ml PMA for a further 18 hours to generate PMA controls (MΦ_{PMA}), M1-like macrophages (MΦ_{IFNγ}) and M2-like macrophages (MΦ_{IL-4}), respectively.
Figure 2-2: Schematic representation of macrophage polarisation protocol

**Generation of H357 conditioned media**
To assess the effects of soluble factors produced by H357 on M1/M2 THP-1 polarisation, the usual polarisation protocol was followed (see Figure 2-2), but the cytokines (IFNγ/ IL24/ PMA) were added to H357 -conditioned media instead of R10. Conditioned media was generated by growing H357 to confluence in 75cm\(^2\) tissue culture flasks for 6 days. Culture media was removed and replaced with fresh media. After 24 hours incubation, the fresh media was collected and centrifuged (IEC Centra CL3R, International Equipment Company, UK) at 230 G for 5 minutes to remove any cells that had become loose in the medium. Conditioned media was transferred to a sterile 25ml universal tube and stored at -20ºC until used in experiments.

**LPS biological activity assay: Limulus Amebocyte Assay (LAL)**

The presence of LPS in conditioned medium used to stimulate the cells was assayed by a kinetic Limulus Amebocyte Lysate (LAL) assay (KQCL kit, Lonza Ltd, UK) according to the
manufacturer’s instruction. Briefly, a stock endotoxin standard (50 endotoxin units (EU)/ml) was prepared and vortex-mixed for 15 minutes. A 4 point standard dilution of 0.005, 0.05, 0.5 and 5 EU/ml was then prepared in pyrogen free glass tubes. The conditioned medium was diluted 1:10 and 1:20 and was heated at 75°C for 10 minutes. The assay was then set up by adding 50µl of each standard dilution and the test dilutions from the conditioned medium in duplicate wells on LAL reagent grade 96 wells plate (Lonza Ltd, UK). The plate was incubated for 10 minutes at 37°C inside the ELX808 Absorbance plate reader (Lonza Ltd, UK). In the meantime, the LAL reagent was reconstituted by adding 2.6mL of pyrogen free water and swirled gently to mix. The re-constituted LAL reagent (50µL) was then added to each well and the absorbance was read for 100 minutes and the level of the endotoxin in the condition medium was determined using WinKQCL™ 3.1.1 Endotoxin Detection and Analysis software (Lonza Ltd, UK).

**H357 and THP-1 co-culture experiments**

The experiments to measure the effects of H357-conditioned media on THP-1 polarisation raised the following questions:

1. *Does the effect of conditioned media on macrophage polarisation change if there is cross-talk between the macrophages and epithelial cells?*

2. *Does co-culture with macrophages affect the H357 response to P. gingivalis LPS?*

To test if cross-talk between the H357 and THP-1 affected macrophage polarisation, a series of co-culture experiments were set up to measure trans-well cross talk between THP-1 and H357. **Figure 2-3** a: co-culture. b: no co-culture control. H357s were harvested from culture flasks at approximately 70% confluence (or 6 days post subculture) and seeded into BD Falcon tissue culture inserts (VWR, UK) at 5 x 10^4 cells/cm^2 and grown to a confluent monolayer. THP-1 were seeded into BD Falcon companion plates and polarised in the usual manor, with (a) or without (b) **(Figure 2-3)** H357s in a tissue culture insert suspended above
the well. After the usual polarisation process, the H357s in tissue culture inserts were removed and transferred to a fresh companion plate containing 2ml HM. H357s were then stimulated with 1µg/ml *P. gingivalis* LPS or with media alone as a control. From the macrophages, the culture media was removed and replaced with fresh R10, with or without *P. gingivalis* LPS. Both cell lines were incubated with or without *P. gingivalis* LPS for 24 hours and the cell free supernatants were harvested and stored at -20°C until assay for cytokines by ELISA.

![Figure 2-3: Schematic representation of H357/THP-1 transwell co-culture system.](image)
BD Falcon tissue culture inserts containing 4.5 x 10³ H357 cells (upper well). The larger wells of the companion plate (lower well) contain 1x10⁶ THP-1 cells.

**Cell viability - Trypan blue exclusion test**

Viable cells with their membrane intact will not take up Trypan blue. To test for cell viability, a sample of cells (8µl) was mixed 1:1 with 0.4% Trypan blue (8µl) and transferred to a haemocytometer. All viable cells (refractive and pale) and all dead (un-refractive and blue) cells in 4, 16x16 squares were counted and percentage viability was calculated. Unless otherwise stated, all experiments were set up with >98% viability.

**THP-1 responses to *P. gingivalis* LPS**

To measure cytokine responses of polarised THP-1 cells to stimulation with various PAMPs (*P. gingivalis* LPS, *E.coli* LPS, heat-killed *P. gingivalis*, Pam3csk4) cells were polarised according to the afore mentioned protocol and stimulated with varying concentrations of PAMPs for 24 hours, unless otherwise stated within each chapter. Upon completion of the
incubation period, cell free supernatants were harvested and stored at -20°C until assay for cytokines by ELISA. Cells incubated in media alone served as negative controls.

**H357 responses to *P. gingivalis* LPS**

To assess the effects of *P. gingivalis* LPS stimulation on cytokines production in H357s, cells were cultured in the usual way and seeded at a density of 5 x 10^3 cells/cm^2 in 96 well tissue culture microplates (IWAKI). Dose-response curves were generated by incubating H357s with *P. gingivalis* LPS at 1000, 100 or 10 ng/ml. H357 with culture medium alone served as negative controls. After 24 hours, the supernatants were collected and stored at -20°C until assay for cytokines by ELISA.
Enzyme Linked Immunosorbent Assay (ELISA)

Concentrations of paired antibodies were optimised using the chequerboard technique (see appendix 1). Optimal concentrations were picked by ascertaining the lowest signal-to-noise ratio.

**ELISA reagents:**

- **ELISA wash buffer:** 500ml Distilled water
  - 2.5 Phosphate buffered saline tablets (Sigma, UK)
  - 250µl Tween 20

- **Blocking solution:** 2% BSA in PBS

- **Stop solution:** 1M sulfuric acid (H₂SO₄)

**Antibodies:**

**Human TNFα ELISA**

BD Pharmingen paired antibodies

Capture antibody: Murine anti-TNFα Mab1 IgG1

Detection antibody: Biotinylated murine anti-human TNFα Mab1

TNFα standard (NIBSC, UK) 5 ng = 25µl

**Human IL-6 ELISA**

BD Pharmingen paired antibodies

Capture antibody: Murine anti-IL-6 Mab1 IgG1

Detection antibody: Biotinylated murine anti-human IL-6 Mab1

IL-6 standard (NIBSC, UK) 5 ng = 25µl
Human IL-1β ELISA

BD Pharmingen paired antibodies

Capture antibody: Murine anti IL-1β Mab1 IgG1

Detection antibody: Biotinylated murine anti-human IL-1β Mab1

IL-1β standard (NIBSC, UK) 5 ng = 25µl

Table 2-3 Concentrations of antibodies used in ELISA

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<tr>
<th>Cytokine</th>
<th>Capture antibody (µg/ml)</th>
<th>Detection antibody (µg/ml)</th>
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</thead>
<tbody>
<tr>
<td>TNFα</td>
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<td>0.5</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>IL-6</td>
<td>4</td>
<td>0.5</td>
</tr>
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</table>

General ELISA protocol

50µl/well capture antibody in phosphate buffered saline (PBS) was added to a 96 well microtiter plate. Following overnight incubation at 4°C, unbound antibody was washed x3 in ELISA wash buffer and non-specific binding was blocked by incubation with 150µl/well BSA/PBS (2%) at room temperature for 4 hours. A standard curve for each cytokine from 5000-7pg/ml was prepared in R10 and used at 1 in 3 serial dilutions. Standards and test samples were added at 50µl per well and the plate was incubated overnight at 4°C. Samples were then removed by washing three times with ELISA wash buffer. The detection antibody was diluted in 2% BSA/PBS, added to the plate (50µl/well) and incubated at room temperature for 4 hours. Detection antibody was removed by washing three times with
ELISA wash buffer, then the enzyme streptavidin horseradish peroxidase was added (50µl/well) for 1 hour at room temperature. Colour reagent TMB microwell peroxidase was added (100µl/well) and colour was allowed to develop. The reaction was stopped using 50µl 1M sulfuric acid (H₂SO₄) and absorbance was measured at 450nm in a Versa Max microplate reader (Molecular Devices, UK)

Standard curve analysis was performed using SoftMax pro software and unknown concentrations of cytokines in the samples were calculated from the standard curve.

**Flow cytometry**

All cell preparation was done aseptically, and on ice. THP-1 or H357 cells were dissociated from the tissue culture plastic with Accutase cell dissociation solution at 37°C. Once lifted, medium containing serum was added to inhibit the enzymes. Cells were then transferred to sterile tubes and centrifuged at 230 G for 5 minutes. The supernatants were gently removed with a pipette and the pellets were re-suspended by gentle flicking of the tubes. 1ml of staining solution (1% BSA in sterile Ca²⁺ and Mg²⁺ free DPBS) was added and tubes were inverted 10 times to evenly distribute the cells. A 10µl sample was taken and mixed 1:1 with Trypan blue exclusion dye and cells were counted on a haemocytometer.

2x10⁶ cells were collected and centrifuged at 230 G for 5 min and re-suspended in 300µl staining solution, giving a concentration of 10⁶ cells/150µl. After incubation on ice for 30 minutes to block non-specific binding, 15µl containing 10⁵ cells was transferred to sterile 500µl Eppendorf tubes. To each tube, the appropriate amounts of antibody, isotype control or staining buffer were added. Tubes were vortexed at a moderate speed and briefly spun (~10 seconds) to make sure the cells and antibody were evenly mixed.

Samples were incubated on ice and in the dark for 30 minutes, following which 400µl staining buffer was added to wash any unbound antibody from the cells and centrifuged at 230 G for 5 minutes. Supernatants were removed and cells were re-suspended in 500µl PBS, filtered through a 35 µm cell strainer mesh into sterile polystyrene tubes (Beckton
Dickenson, UK) and processed in the BD FACSaria flow cytometer. Data was acquired using FACSDiva software (Becton Dickenson) and analysed using WinMDI software (Joseph Trotter, Scripps Research Institute, San Diego, USA.

Mean fluorescence intensity (MFI) was calculated using the following formula:

\[
\left( \frac{\overline{x}_{\text{test}} - \overline{x}_{\text{isotype control}}}{\overline{x}_{\text{isotype control}}} \right) \times 100
\]

<table>
<thead>
<tr>
<th>Table 4: List of antibodies used in flow cytometry</th>
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<tr>
<td>Anti-TLR4</td>
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<tr>
<td>Isotype control</td>
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</table>
Total RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction from THP-1 cell line

(Adapted from\textsuperscript{\textcopyright161})

Reagents:

- Guanidinium thiocyanate (4M)
- Sodium citrate
- N-laurosarcosine (sarkosyl) (10%)
- 2-mercaptoethanol
- Sodium acetate (anhydrous) (2M, pH4.0)
- Glacial acetic acid
- Water saturated phenol
- 1 –bromo-3-chloropropane
- Isopropanol (~100%)
- Ethanol (70%)
- RNase- and DNAse- free water

Denaturing solution:

Stock denaturing solution was prepared by dissolving 25g of guanidium thiocyanate in 29.3ml of water at 65°C, followed by 1.76ml of 0.75M sodium citrate, pH 7.0, and 2.64ml of 10% sarkosyl. Denaturing solution was activated before use by addition of 72µl 2-mercaptoethanol to 10ml of the stock solution.

2M sodium acetate, pH 4.0

16.42g of sodium acetate (anhydrous) was added to 35ml of water and 40ml glacial acetic acid. pH was adjusted to 4.0 with glacial acetic acid and the final volume was brought to 100ml with water.
Following experiments, culture supernatants were removed from THP-1 cells and 500µl of denaturing solution was added directly to the wells. Cells were rested in denaturing solution for 1 minute to allow complete lysis and the solution was pipetted up and down 10 times to ensure all the lysate was collected. The samples were allowed to sit for 5 minutes before being frozen at -20°C for later analysis.

To extract the RNA, 50µl of 2M sodium acetate, pH 4.0 was added to the cell lysates and mixed thoroughly by inversion. Then, 500µl water saturated phenol was added, mixed by inversion, followed by 100µl of 1-bromo-3-chloropropane, and shaken vigorously by hand to mix. Samples were cooled on ice for 15 minutes then centrifuged for 20 minutes, 15000g, at 4°C.

The upper aqueous phase containing mostly RNA was transferred to an RNAse- and DNAse free Eppendorf tube and an equal amount of isopropanol was added to precipitate the RNA. Samples were incubated at -20°C until needed for the next step.

Samples were centrifuged for 20 minutes, 15000g, at 4°C. The supernatant was discarded and the gel-like precipitate (RNA) was retained then dissolved in 300µl of denaturing solution. Next, 300µl of isopropanol was added and the samples were incubated at -20°C for at least 30 minutes, after which they were centrifuged for at 15000g for 10 minutes at 4°C. The supernatant was discarded and the pellet was re-suspended in 500µl of 75% ethanol and vortexed for 10 seconds to mix, and then incubated for 15 minutes at room temperature to dissolve any residual guanidinium thiocyanate. At this point, samples were stored at -20°C until RNA extraction could be completed.

Samples were centrifuged for at 15000g for 20 minutes at 4°C, and the supernatant was discarded. The pellet was air dried for around 15 minutes in a laminar flow hood at room temperature, and then the RNA was dissolved in 15µl of RNAse- and DNAse- free water and incubated for 15 minutes at 60°C to ensure complete solubilisation of the RNA. To remove any residual genomic DNA, samples were treated with the DNase 1 kit (Sigma, UK).
solublised RNA, 1.5µl of 10x reaction buffer and 1.5µl amplification grade DNase 1 (1 unit / µl) were added and samples were left to stand at room temperature for 15 minutes. To stop the reaction, 1.5µl stop solution was added to each tube. Samples were then incubated at 70°C for a further 10 minutes, then placed on ice ready for reverse transcription.

Reverse transcription
RNA quantity was determined using a NanoVue plus (GE Healthcare) spectrophotometer and purity was estimated using the A_{260}/A_{280} ratio.

0.5µg RNA was added to RNase and DNAse- free PCR tubes, and made up to a total volume of 9µl with water. A master mix of RT buffer (Applied Biosystems) and enzyme (50 U/µL MultiScribe™ MuLV, Applied Biosystems, UK) was prepared by adding 10µl buffer and 1µl enzyme per sample to a tube. RNA, water and master mix were added sequentially to PCR tubes. Tubes were briefly spun down to pool the ingredients and eliminate any air bubbles. The reverse transcription reaction was done by incubating the samples in a thermal cycler (Veriti, Applied Biosystems) at 37°C for 60 minutes and stopped by heating to 95°C for 5 minutes and held at 4°C. The cDNA generated was stored at -20°C until ready for qRT-PCR.

Quantitative real-time polymerase chain reaction (qRT-PCR)
For all PCR, Power SYBR green PCR master-mix (Applied Biosystems) was used.

cDNA generated from RNA samples was prepared for qRT-PCR. A master mix containing 10µl SYBR green, 1.25µl (12.5µmol) forward primer, 1.25µl (12.5µmol) reverse primer and 2.5µl water per sample was prepared for each assay. 1µl of cDNA was diluted in 4µl of water for each test. 15µl of master mix followed by 5µl diluted cDNA was added to the appropriate well in a 96 well PCR plate, in duplicate. qRT-PCR was run under the following cycling conditions on the Applied Biosystems StepOne Plus real-time PCR system:

- Hold at 95 °C for 10 minutes
• Denature at 95 °C for 15 seconds then anneal/extend at 60 °C for 60 seconds x 40 cycles.

Data analysis
Data was acquired and analysed on the Applied Biosystems StepOne software (Applied Biosciences, UK). Human β actin 1 was used as the internal reference gene (housekeeping gene), and non-polarised (PMA treated) macrophages were used as calibrator samples. Differences in gene expression levels were calculated relative to the calibrator sample using the ∆∆ Ct method.

Statistical analyses

For comparison between two independent treatment groups, tests for differences were analysed using Student's t-test (Figure 3-2, Error! Reference source not found., Figure 4-3 to 4-5, Figure 5-3 C and Figure 5-4 – 5-8) if the data were parametric, or Mann Whitney U test if data were non-parametric (Error! Reference source not found. C – EclPS). In experiments where more than two groups of treatments were being tested, a one-way analysis of variance (if the data were parametric) with the Holm-Sidak method for all pairwise comparison procedure to test for differences between groups (Figure 3-, Figure 4- to 4-12, Figure 5-4 and Figure 5-12), or the Kruskall-Wallis analysis of varience if the data was non-parametric (Figure 3-a – Pam3csk4) were used. Results were considered significant if the p value was ≤ 0.005.
Chapter 3 - Optimisation of an M1/M2 macrophage polarisation model using the cell line THP-1

Macrophages perform specific functions dependant on their location, origin, and factors encountered in the microenvironment. Macrophages differentiate from peripheral blood monocytes and (broadly speaking) undergo two types of activation; classical and alternative. In vivo, classically activated macrophages (M1) are polarized by granulocyte macrophage-colony stimulating factor (GM-CSF) and activated by IFNγ \(^{31,33}\). Alternatively activated macrophages (M2), are polarized by M-CSF \(^{31–33}\) and can be further divided into three subsets: M2a, M2b and M2c. M2a are activated by IL-4, IL-13 or both, M2b are activated by complement and TLR ligation, and M2c are activated by IL-10 \(^{32,68}\). M2 macrophages exhibit anti-inflammatory properties and express regulatory cytokines, IL-10, IL-1Ra and TGF-β. M1 type macrophages express high levels of pro-inflammatory cytokines, TNFα, IL-1β, IL-18, IL-6, IL-12 and IL-23 \(^{32}\).

**Macrophages in the oral mucosa**

The ratio of M1 to M2 macrophages in the oral mucosa may vary between health and disease. Under homeostatic conditions, the predominant macrophage population is likely to be regulatory; mucosal macrophages take on an M2 phenotype as a means of controlling aberrant inflammation to commensal bacteria and food antigens. During disease, however, the immune status of the oral mucosal tissues is prone to change. The cytokine milieu present in periodontal lesions is both Th1 and Th2 related, with more recent evidence suggesting that Th17 cytokines are also involved \(^{162,163}\).

Given the variety of M1 and M2 polarising cytokines present in lesions, macrophages may become polarised to an M1 or M2 phenotype, or both, dependant on disease state (i.e. health versus disease, acute versus chronic).
**Macrophage dysfunction in oral disease**

Disruption in macrophage number and function may play a more important role in driving disease than pathogenic causes alone. Whilst a known aetiological agent in periodontitis, the presence of pathogenic bacterium *Porphyromonas gingivalis* in the oral microflora does not presuppose disease. *Porphyromonas gingivalis* colonise epithelial cells in healthy and periodontally diseased patients, suggesting that a dysfunctional immune response plays a key role in disease progression. Investigations indicating that macrophages remain in an un-activated state in periodontal lesions credits this theory. Whilst rapid inflammation is seen in response to ceasing oral hygiene routines there is no change in macrophage number between healthy gingival tissues and periodontitis-associated tissues. In oral squamous cell carcinoma (OSCC), whilst the infiltrating macrophage count predicts poor prognosis, these macrophages develop a pro-tumoural, M2 like phenotype which also correlates with poor prognosis. These findings implicate macrophage dysfunction rather than number is integral to disease progression.

Despite the likelihood that M2 macrophages are present in both oral health and disease, their interactions with - and responses to - pathogenic oral bacteria, such as *P. gingivalis* are yet to be investigated.

**Macrophages *in vitro*: Generation M1 and M2**

Investigation of macrophage/oral pathogen interactions requires a robust *in vitro* model of M1 and M2 macrophages. *In vivo*, several factors have been shown to induce macrophage differentiation from monocytes with polarization into distinct phenotypes. Numerous methods have been developed using human peripheral blood monocytes, most using M-CSF to generate M2 and GM-CSF to generate M1. Other methods have used combinations of IL-1β, TNF-alpha or LPS to generate M1, and IL-4, IL-13, or both, to generate M2. Treatment with combinations of these factors up-regulates expression of distinctive M1 or M2 markers. Treatment with IFNγ leads to up-regulation of typical M1 receptors CD80 and
CD64 (FcγR1) \(^{172,173}\). IL-4 treatment up-regulates CD36 \(^{174}\) expression and activation of STAT6 \(^{175}\) and down-regulates CD14 \(^{172,173}\), iNOS \(^{174}\) and CD163 \(^{176}\), and inhibits phagocytic capacity \(^{177}\). Macrophages polarised with IL-10 up-regulate surface markers CD163 and CD16 \(^{172,173}\). Despite being the gold standard in human monocyte and macrophage research, primary cells are difficult to obtain and often yield low numbers. Immortalised cell lines can be used instead, although different researchers use different concentrations and combinations of factors to polarise monocytes into macrophages; there is no consensus on how to generate an effective M1/M2 model using cell lines. Among the variety in methodology there are similarities, particularly in the pro-monocytic cell line, THP-1 (see Table 3-1). Established by Tsuchiya et al. in 1980 \(^{178}\) from an acute myeloid leukaemia, THP-1 cells have been shown to better reflect the behaviour of primary cells than other monocytic cell lines \(^{179}\) and can be differentiated to monocytes, macrophages or dendritic cells \(^{179–181}\). Thus, the objective of this research was to establish a robust, reproducible model of M1 and M2 macrophage polarisation using THP-1, so that their interactions with pathogenic oral bacteria could be investigated.

As well as the human THP-1 cell line, many studies have used mouse primary cells or cell lines to generate M1 and M2 like macrophages, characterised by their expression of arginase (M2) and NOS (M1), and Ym and FIZZ-1. Whilst expression of iNOS and ARG-1 has been demonstrated in human cells \(^{182,183}\) their validity as M1/M2 markers in humans remains a bone of contention \(^{184,185}\).

In the present study, a series of experiments were designed to determine the method of generating M1 and M2 macrophages in vitro the displayed the most similar cytokine profile to primary M1 and M2 macrophages. Using the literature as a guide, the M1/M2 polarising properties of several known macrophage differentiation factors (PMA, vitamin D3, IL-13, IL-4 and IFNγ) were tested.
Table 3-1: Methods of M1 and M2 polarisation using THP-1 cells from previously published research

<table>
<thead>
<tr>
<th>Author</th>
<th>Differentiation protocol</th>
<th>Measured effects</th>
</tr>
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<tbody>
<tr>
<td>Caras (2011)\textsuperscript{151}</td>
<td>30 ng/ml PMA for 6 hours, then:</td>
<td>M1: Higher levels of TNF(\alpha), IL-6 and IL-1Ra M2: Lower levels of TNF(\alpha), comparable levels of IL-6, and higher levels of IL-1Ra (vs. PMA alone)</td>
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<tr>
<td></td>
<td>M1: PMA and 10 ng/ml LPS + 5 ng/ml IFN(\gamma) for 66 hours</td>
<td></td>
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<tr>
<td></td>
<td>M2: 25 ng/ml IL-4 + 25 ng/ml IL-13 for 66 hours</td>
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<tr>
<td></td>
<td>Cells were cultured for a further 24 hours in complete medium</td>
<td></td>
</tr>
<tr>
<td>Dabelic (2006)\textsuperscript{166}</td>
<td>10\textsuperscript{-7} M PMA for 48 hours.</td>
<td>Up regulation of Galectin-3 expression</td>
</tr>
<tr>
<td>Daigneault (2010)\textsuperscript{187}</td>
<td>200nM PMA for 3 days, followed by a rest period of 5 days</td>
<td>Generated macrophages closely resembling primary bone marrow derived macrophages (BMDM; assessed by morphology, adherence and CD14 expression),</td>
</tr>
<tr>
<td>Isa (2011)\textsuperscript{188}</td>
<td>Monocytes: 100 ng/ml PMA* for 72 hours M2 monocytes: IL-13 15 ng/ml and 1\textmu M rosiglitazone for 72 hours M2 M(\Phi): 100 ng/ml PMA for 6 hours, followed by addition of 15 ng/ml IL-13 and 1\textmu M rosiglitazone for 66 hours</td>
<td>M2 macrophages up-regulated CD206 and IL-1Ra expression. M2 Macrophages more sensitive to apoptosis induced by oxLDL than monocytes</td>
</tr>
<tr>
<td>Study</td>
<td>Treatment Details</td>
<td>Effects</td>
</tr>
<tr>
<td>-----------------</td>
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<td>-------------------------------------------------------------------------</td>
</tr>
<tr>
<td>MacKinnon (2008)</td>
<td>100 ng/ml PMA for 24 hours, 10 ng/ml IL-4 for 24 hours, 100 U/ml IFN-γ/100 ng/ml LPS for 24 hours</td>
<td>Up-regulation of Galectin-3 and CD98, Up-regulation of CD206 and galectin-3 expression, Inhibition of CD206 and galectin-3 expression</td>
</tr>
<tr>
<td>McLaren (2010)</td>
<td>160 nM PMA for 24 hours, 100 ng/ml</td>
<td>Monocyte to macrophage differentiation</td>
</tr>
<tr>
<td>Ribbens (2000)</td>
<td>4 ng/ml Vitamin D3 for 48 hours</td>
<td>Allows THP-1 responsiveness to stimulation</td>
</tr>
<tr>
<td>Smythies (2010)</td>
<td>4F, apoA-I, or vehicle for 7 days</td>
<td>Down-regulates: HLA-DR, CD86, CD11b, CD11c, CD14, TLR-4, CD49d+ CD32, LPS-induced mRNA of MCP-1, MIP-1, RANTES, IL-6, TNFα, monocyte adhesion to human endothelial cells, transendothelial migration and phagocytosis of dextran-FITC beads, Up-regulates IL-10</td>
</tr>
<tr>
<td>Spencer (2010)</td>
<td>M1: 20 ng/ml LPS + 20 ng/ml IFNγ overnight in serum free media, M2: 5 nM TPA in PBS for 5 min, then: 20 ng/ml IL-4 (M2a), 20 ng/ml IL-10 (M2c) overnight</td>
<td>M1: Higher levels of IL-1 and IL-12, lower levels of IL-10, M2: Lower levels of IL-1 and IL-12, higher levels of IL-10, Measured mRNA</td>
</tr>
<tr>
<td>Tjiu (2009)</td>
<td>320 nM PMA for 24 hours, 20 ng/ml</td>
<td>Up-regulation of CD206 and CD204 expression</td>
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<tr>
<td>Vey (1992)\textsuperscript{195}</td>
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<tr>
<td>Vitamin D3 (4 ng/ml) or IFNγ (500U/ml) for 48 hours.</td>
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<tr>
<td>TNFα, IL-1β and IL-6 production was evident in IFNγ stimulated cells, but not in Vitamin D3 stimulated cells. IL-1β co-cultured with activated and fixed T cells and Vitamin D3 treated THP-1 cells up-regulated their cytokine production; IFNγ treated THP-1 did not.</td>
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</table>
Results

PMA as a macrophage differentiating factor: PMA induces THP-1 adherence, a marker of monocyte – macrophage differentiation.

PMA is an established macrophage differentiation factor in THP-1 cells, but is also a potent macrophage activator. In a study by Daigneault et al (2010) THP-1 cells were treated with 200nM PMA for 3 days, followed by a rest period of 5 days to generate macrophages closely resembling primary bone marrow derived macrophages (BMDM; assessed by morphology, adherence and CD14 expression). It is yet to be established whether PMA treatment leads to the generation of macrophages displaying M1 or M2 profiles. To determine whether this protocol would lead to a differential expression of M1 and M2 markers in our model, and allow enough time for recovery from PMA activation, THP-1 cells were differentiated with 200nM PMA for 3 days and left for a series of resting periods of 0, 1, 2, 3, 4 and 5 days (Figure 3-1). Culture supernatant levels of TNFα, an M1 marker, in response to P. gingivalis LPS (A), heat killed P. gingivalis (B), E. coli strain K12 (C) and synthetic TLR2 ligand Pam3csk (D) were assayed by ELISA. Effective concentrations of stimulatory PAMPs were determined by dose/response experiments (data not shown) and was in accordance with current literature.

As resting time increased, TNFα production decreased in response to all stimuli. Background levels of TNFα were elevated at day 0, but had returned to negligible levels by day 1. A resting period of 1 day allows cells enough time to recover from PMA activation, but still exhibit a high level of TNFα production in response to stimulation with PAMPs (Figure 3-1). Following PMA treatment, THP-1 cells became strongly adherent to the culture plates. At a 200nM concentration of PMA, and at 1 day post treatment, cell adherence and high TNFα production indicate that cells have adopted an M1-like phenotype. However, as resting time increased, TNFα production decreased in response to stimulus, and reduced TNFα production is associated with M2 polarisation. Thus, PMA treatment generated an adherent macrophage population, but M1 or M2 phenotypes were not clear.
A. PgLPS (1µg/ml)

B. Heat killed Pg (10^7 cells/ml)
Figure 3-1: A resting period of one day is sufficient for THP-1 cells to recover from PMA stimulation, but still exhibit a pro-inflammatory profile in response to PAMPs. 25 ng/ml Data are expressed as the mean of triplicate samples in one experiment ± standard deviation.
**Vitamin D3 as a macrophage differentiating factor: Vitamin D3 treatment does not induce cell adherence, and induces a cytokine profile unlike M1 or M2 macrophages.**

Like PMA, vitamin D3 is a commonly used macrophage differentiation factor. Because of its purported anti-inflammatory properties, it was hypothesised that vitamin D3 mediated differentiation might generate an M2-like population of macrophages. To test this in the present study, THP-1 cells were treated with vitamin 10nM D3 for 4 days to differentiate them into macrophages. To keep differentiation periods the same for both PMA and vitamin D3 treatment (thus keeping the age and passage number of the cells equal), the concentration of PMA was reduced to 25nM and the differentiation period was extended to 3 days. Following differentiation, cells were washed 3 times in PBS and then stimulated with 1 µg/ml *P. gingivalis* LPS for 24 hours. Levels of phenotypic markers TNFα, IL-12, IL-6, IL-1β (M1), CCL18 (M2) were measured in the culture supernatant (Figure 3-2).

In response to *P. gingivalis* LPS, PMA treated macrophages produced higher levels of TNFα compared with vitamin D3 treated cells, however, the difference in TNFα production between cell types was minimal (Figure 3-2 A, p=0.02). Conversely, Vitamin D3 treated THP-1 cells expressed markedly higher levels of M1 associated cytokines IL-1β (Figure 3-2 B, p<0.001) and IL-6 (Figure 3-2 C, p<0.001) in response to *P. gingivalis* LPS compared to PMA treated cells. IL-12 (p70 subunit) and CCL18 were not detected in any of the culture supernatants (data not shown). These data suggest that the PMA/Vitamin D3 model of M1/M2 polarisation might not accurately reflect the behaviour of M1 and M2 macrophages *in vivo.*
Figure 3-2: PMA or Vitamin D3 treated cells do not display M1 and M2 profiles. THP-1 cells were treated with PMA or Vitamin D3, and then stimulated with *P. gingivalis* LPS for 24 hours. Cell free supernatants were assayed for TNFα, IL-1β and IL-6 by ELISA. Data represent means of 3 independent experiments performed in triplicate (n=3). Tests for statistically significant differences were performed using one way ANOVA.
Polarisation of THP-1 cells into M1 and M2 like phenotypes using Th1 and Th2 cytokines

The present data suggest that treatment of THP-1 cells with Vitamin D3 does not yield effective M2 like macrophages. Previous studies using the THP-1 cell line to generate M2 like macrophages have commonly used Th2 cytokines IL-4 and IL-13, either alone or in combination\(^\text{189,194}\). In order to establish whether further polarisation along M2 pathways using Th2 cytokines yields a more characteristic M2 cytokine profile, experiments were conducted to assess responses of IL-4/13 treated THP-1 cells to LPS stimulation, based on a method adapted from Tjiu et al (2009)\(^\text{160}\):

**To generate M2 (i):** 15 ng/ml 15 ng/ml 5 ng/ml IL-13 for 72 hours, followed by 100 ng/ml PMA for 72 hours, with 24 hours rest

**To generate M2 (ii):** 15 ng/ml 15 ng/ml 5 ng/ml IL-4 for 72 hours, followed by 100 ng/ml PMA for 72 hours, with 24 hours rest

**To generate M1 (i):** 100 ng/ml PMA for 72 hours, with 24 hour rest

THP-1 cells were incubated with PMA, IL-4 or 13 for 3 days, followed by PMA for 3 days to generate M1 and M2 (i) and M2 (ii), respectively. Following a 24 hour rest period, cells were stimulated with 1µg/ml *P. gingivalis* LPS or 1µg/ml *E. coli* LPS, or were incubated with media alone as a negative control.

No difference was observed in TNFα production in response to *P. gingivalis* or *E. coli* LPS between IL-4 and IL-13 polarised cells (Figure 3-3). PMA treated cells produced the highest levels of TNFα in response to both LPS, however, background levels of TNFα were also elevated in un-stimulated PMA treated cells (Figure 3-3). It is possible that greater up-regulation of TNFα was due to direct activation by PMA, rather than in response to PAMPs.
Figure 3-3: TNFα expression in PMA, IL-4 or IL-13 treated THP-1 cells. THP-1 cells were incubated with PMA for 3 days, or IL-4 or 13 for 3 days, followed by PMA for 3 days. Following a 24 hour rest period, cells were stimulated with 1ug/ml *P. gingivalis* LPS or 1ug/ml *E. coli* LPS, or were incubated with media alone as a negative control. Data are expressed as the mean of triplicate samples within one experiment ± standard deviation.
Treatment of THP-1 with PMA and IFNγ or IL-4 generates cell populations exhibiting a differential pro-inflammatory cytokine profile

According to Borthwick et al. 2011 (un-published data), and in conformity with other published material on THP-1 polarisation, addition of IFNγ to PMA treated THP-1 further polarises cells into an M1 like phenotype. To test this, experiments were conducted to determine if incubation with IFNγ generated a more robust M1 model. As there was a lack of discrepancy between IL-4 and IL-13 treated cells, IL-4 was used in subsequent experiments. Additionally, the order in which IL-4 and PMA were added was reversed, as previous studies have shown that IL-4 receptor expression is down-regulated during monocyte – macrophage differentiation.

THP-1 cells were incubated with 5 ng/ml PMA for 6 hours to initiate cellular adhesion and monocyte/macrophage differentiation. To generate a population of non-polarised macrophages, PMA was removed and refreshed with media containing 5 ng/ml PMA. Cells were cultured for a further 18 hours. To generate M1 and M2 like macrophages, after 6 hours initial PMA treatment, cells were incubated with 20 ng/ml IFNγ or 20 ng/ml IL-4, plus 5 ng/ml PMA, respectively. After a total of 24 hours, the polarisation process was complete and cells were ready for use in further experiments (see Figure 3-4).
M1 and M2 macrophages have been characterised according to their inflammatory cytokine profiles. M1 macrophages classically express high levels of TNFα, IL-12 and IL-23, whereas M2 typically produce anti-inflammatory cytokines IL-10, TGF-β and IL-1Ra in response to LPS/TLR interactions. To determine whether IFNγ macrophages and IL-4 macrophages took on differential cytokine profiles following polarisation, polarised cells were incubated for 24 hours with 1µg/ml of either *P. gingivalis* LPS (purported TLR2 ligand) or *E. coli* LPS (TLR4), see Error! Reference source not found. for schematic representation. Pro-inflammatory cytokines TNFα, IL-1β and IL-6 were measured by ELISA. Anti-inflammatory cytokines/chemokines CCL18, IL-1Ra and IL-10, and M1 associated cytokine IL-12p70 were also measured but were not detectable at protein level in the supernatant.

In response to *P. gingivalis* and *E. coli* LPS, the expression of M1 associated cytokines TNFα, IL-1β and IL-6 was higher in IFNγ treated macrophages than PMA and IL-4 treated
macrophages (Error! Reference source not found.5: A, B and C, \( p=0.01 \), \( p=0.014 \), \( p=0.006 \), respectively). As previously described\(^\text{197}\), up-regulation of TNFα, IL-1β and IL-6 was induced to a greater extent by \( E. \ coli \) LPS; stimulation of macrophages in all polarisation states by \( E. \ coli \) LPS resulted in higher inflammatory cytokine production than \( P. \ gingivalis \) LPS. Next, the expression level of M2 associated cytokines IL-10 and TGF-β were measured by qRT-PCR. Briefly, cells were polarised with IFNγ or IL-4, then stimulated with \( P. \ gingivalis \) LPS for 6 hours. Un-stimulated cells (no LPS) served as negative controls. Culture supernatant was removed and cells were lysed for RNA extraction (see methods chapter for details and primer sequences). Relative gene expression levels were calculated using the \( \Delta\Delta \text{Ct} \) method, normalised to housekeeping gene, β-actin and compared with unpolarised, MØ macrophages. THP-1 treated with IL-4 expressed higher levels of IL-10 and TGF-β mRNA than IFNγ treated cells in response to \( P. \ gingivalis \) LPS. Basal levels of both cytokines however were similar between M1 and M2 macrophages (Figure 3-6, A and B). At 24 hours stimulation, M2 still expressed higher levels of TGF-b, but IL-10 reached similar levels (Figure 3-6 C).

68
Figure 3-5: IFNγ and IL-4 differentiate macrophages with distinct M1 and M2 inflammatory cytokine profiles
Figure 3-6: Expression levels of M2 markers IL-10 and TGF-β are higher IL-4 treated than IFNγ treated macrophages. Gene expression levels of IL-10 (A) and TGF-β (B) were measured in M1 and M2 macrophages stimulated with *P. gingivalis* LPS for 6 hours. Unstimulated cells served as negative controls. IL-10 and TGF-β was also measured in unstimulated cells at 24 hours.

To determine whether lower levels of cytokine production in response to *P. gingivalis* LPS is a species-specific effect, or a general response to TLR2 activation, experiments were repeated using synthetic TLR2 ligand Pam3csk4 (Figure 3-7).

As observed in the previous experiment (*Error! Reference source not found.*), *E. coli* LPS and *P. gingivalis* LPS induced a stronger pro-inflammatory response in IFNγ macrophages than in IL-4 or PMA macrophages. Likewise, when stimulated with Pam3csk4, levels of IL-1β...
and IL-6 were higher in IFNγ macrophages than in IL-4 or PMA macrophages (Figure 3-7). There was no difference in TNFα or IL-1β production between PMA and IL-4 macrophages in response to Pam3csk4, but PMA macrophages produced higher levels of IL-6 compared with IL-4 macrophages, although this did not reach statistical significance. Interestingly, unlike LPS, there was no difference in TNFα production induced by pam3csk4 between any of the cell types (Figure 3-7, summarised in table 3-2). Furthermore, activation of TLR2 by Pam3csk4 induced far higher levels of inflammatory cytokines than *P. gingivalis* LPS, suggesting that it is not differential utilization of TLR2 that is responsible for discrepancy between *P. gingivalis* LPS and *E. coli* LPS.

**Table 3-2: Summary of cell-type specific cytokine production in response to Pam3csk4.** Crosses indicate levels of expression, +++ being high and + being low.

<table>
<thead>
<tr>
<th></th>
<th>IFNγ macrophages</th>
<th>IL-4 macrophages</th>
<th>PMA macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>IL-1β</td>
<td>+++</td>
<td>+</td>
<td>+</td>
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<tr>
<td>IL-6</td>
<td>+++</td>
<td>+</td>
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</table>
Figure 3d7: TNFα, IL-1β and IL-6 production in response to *P. gingivalis* LPS, *E. coli* LPS and Pam3csk4. Data are expressed as the mean ± standard deviation from three independent experiments. *NS = no significant difference.

Figure 3-7: TNFα, IL-1β and IL-6 production in response to *P. gingivalis* LPS, *E. coli* LPS and Pam3csk4. Data are expressed as the mean ± standard deviation from three independent experiments. *NS = no significant difference.
Discussion
There is no established protocol for differentiation/polarisation of THP-1 into M1 or M2 like macrophages. Previous work in our laboratory had used PMA and Vitamin D3 to generate M1 and M2 like THP-1 macrophages, respectively. PMA and Vitamin D3 are commonly used differentiation factors, but to date there are no published studies which have used Vitamin D3 to generate alternative macrophages. Although Daigneault et al.\(^{187}\) assessed ability of Vitamin D3 to generate a population of cells closely resembling primary monocyte derived macrophages, the group concluded that PMA was a more effective method of macrophage differentiation than Vitamin D3, and Vitamin D3 treated cells were excluded from further analysis. The study did not assess capacity of Vitamin D3 to differentiate THP-1 into an M2 like phenotype.

Vitamin D3 signals through the Vitamin D receptor in the nucleus and activates the PI3K pathway\(^{198,199}\). PI3K is associated with alternative macrophage polarisation\(^{189}\) and negatively regulates M1 markers, iNOS\(^ {200}\) and IL-12B\(^ {201}\). However, Vitamin D3 has also been shown to down-regulate M2 related cytokine IL-10 expression in THP-1 cells\(^ {202}\). Treatment of THP-1 with Vitamin D3 generated a population of cells which were highly proliferative and non-adherent. PMA treated THP-1, in contrast, generated a population of cells which were non-proliferative and adherent to cell culture plastic. Percentage viability was lower in vitamin D3 treated than PMA treated cells.

At all time points, TNFα production was higher in Vitamin D3 treated cells than PMA treated cells, at baseline and in response to \textit{P. gingivalis} LPS. A longer M2 differentiation period led to a reduction in TNFα, however, it remained higher than that of M1 cells, even after 7 days. This decrease could be a result of increased proliferation of vitamin D3 treated cells observed at the end of the experiment. PMA treated cell number did not increase over the experimental period. The higher level of cell death in vitamin D3 treated than PMA treated cells increased with differentiation time. It is possible that TNFα was released from intracellular stores upon cell death in the Vitamin D3 treated cells, or that it was up-regulated.
in response to apoptotic or necrotic cell debris. Neither IL-12 nor CCL18 were detected in supernatants from any cell types. Faulty antibodies/ELISA techniques were ruled out as standard curve worked well. Previous studies \(^{203}\) have shown that IL-12 is up-regulated in response to \(E. \text{coli}\) LPS, so it may be that \(P. \text{gingivalis}\) LPS (TLR2 ligand) does not induce IL-12 expression. However, none of the cell types replicated this.

The inflammatory cytokine profiles of PMA and vitamin D3 treated cells suggest that these treatments do not yield definitive M1 and M2 like macrophage populations. A 2012 study of the polarising capacity of vitamin D3 revealed that the compound did not induce expression of either M1 or M2 markers \(^{204}\). In the present study, vitamin D3 treatment resulted in expression of M1 characteristic cytokines, but was rejected from further use in polarisation protocols as the cells were non-adherent (adherence is a marker of macrophage differentiation) and there was higher level of cell death. Whilst PMA had undesirable activatory properties, these had returned to baseline levels after 24 hours. Thus, PMA was chosen for use in further polarisation experiments.

To establish whether IL-4, IL-13, or both could further polarise cells to an M2 phenotype, THP-1 cells were incubated with 20 ng/ml of IL-4 or IL-13 for 72 hours, followed by 100 ng/ml PMA for a further 72 hours. Cells were washed to remove PMA and allowed to rest for 24 hours before stimulation with a range of PAMPs (Figure 3-3). There was no difference in TNFα production in response to \(P. \text{gingivalis}\) or \(E. \text{coli}\) LPS between IL-4 and IL-13 polarised cells. PMA treated cells produced highest levels of TNFα in response to range of PAMPs however; background levels of TNFα were also elevated in un-stimulated PMA treated cells. It is possible that greater up-regulation of TNFα was due to direct activation by PMA, rather than in response to PAMPs.

It is widely accepted that IFNγ primes macrophages to take on pro-inflammatory M1 like characteristics \(^{170}\). Thus, we wanted to see if treatment with IFNγ would generate stronger M1 characteristics i.e. higher levels of TNFα, IL-1β and IL-6. In order to generate M1 like
macrophages from THP-1, cells were incubated with 25 ng/ml PMA for 6 hours, followed by PMA + 20 ng/ml IFNγ for a further 18 hours. The PMA concentration was lowered from 100 ng/ml to 25 ng/ml, as 100 ng/ml appeared to directly activate cells (Figure 3-3, PMA control) in accordance with previously published data. As there was no difference in TNFα production between IL-4 and IL-13 treated cells, IL-4 was used for all subsequent experiments to generate M2 like cells. Cells treated with 25 ng/ml PMA were included as a negative control for polarisation. In a previous study by Caras et al., PMA/IFNγ treatment period was increased from 24 hours to 72 hours because their cells became unstable and were dis-adhered by simply washing. However, in our model, THP-1 + PMA/cytokine were strongly adhered. A resting period following PMA treatment was not needed as baseline production of cytokines in un-stimulated cells was low.

TNFα, IL-6 and IL-1β production were highest in response to Pam3csk4, followed by E. coli LPS, with P. gingivalis LPS inducing lowest levels of cytokines in all cell types. In line with data published elsewhere, E. coli LPS consistently induced cytokine production in all cell types to a higher level than P. gingivalis LPS. This property of P. gingivalis LPS is thought to be a mechanism by which it evades host immune response.

IFNγ treated macrophages were consistently the highest responders to all stimuli, with IL-4 and PMA macrophages displaying similar cytokine profiles. Interesting results were obtained from response of different cell types to Pam3csk4; PMA, IFNγ and IL-4 macrophages displayed differential production of IL-6 and IL-1β in response to Pam3csk4, but this discrepancy between cell types was lost with respect to TNFα (Figure 3-7).

This lack of discrepancy in TNFα production could either be down to high specificity of Pam3csk4 for TLR2 masking any subtle differences between cell types, or that IFNγ and IL-4 macrophages display differential responses to TLR4 ligation, but not TLR2. Differential cytokine profiles between cell types in response to TLR4 ligand E. coli LPS are obvious. Although levels of cytokine production in response to P. gingivalis LPS were lower than that
of *E. coli* LPS, pattern of expression was similar. *Porphyromonas gingivalis* LPS is purported to be a TLR2 ligand, but this is still a contentious issue. Whether *P. gingivalis* LPS utilises TLR2 or TLR4 appears to depend on method of LPS purification and culture temperature, among other things. Investigation into whether lack of discrepancy in TNFα production between cell types in response to Pam3csk4 is TLR2 or stimulus specific is needed.

IFNγ and PMA treated macrophages display similar IL-6 up-regulation in response to Pam3csk4, whereas IL-4 macrophages produce very little. IL-6 production is similar between IFNγ and PMA macrophages (with very little IL-6 produced by IL-4 macrophages), whereas IL-1β production is similar in IL-4 and PMA macrophages (with very little IL-1β production in IFNγ macrophages). This could be indicative of differential utilization of TNFα, IL-1β and IL-6 pathways in response to TLR2 activation between cell types.

Monocytes/macrophages derived from THP-1 cells do not express IL-10 at the protein level in response to LPS. Barksby et al (2009) reported that IL-10 mRNA is up-regulated in THP-1 cells in response to *P. gingivalis* LPS. It is possible that in THP-1, IL-10 mRNA is synthesized de novo in response to stimuli, but not secreted in protein form. This would suggest that there is a difference in post translational processing of IL-10 in THP-1 and primary cells. Indeed, data from the present study reveal that IL-10 and TGF-β is expressed at the mRNA level, and that this expression is higher in IL-4 treated M2 macrophages than in IFNγ treated M1 macrophages.
Chapter 4 - Modulation of Macrophage responses by *Porphyromonas gingivalis* LPS.

Introduction

Chronic inflammation can arise when the immune system fails to clear initial infection by invading bacteria and becomes dysregulated. Such is the case in the oral inflammatory condition, periodontitis. *Porphyromonas gingivalis* is a well-known periodontal pathogen that invades epithelial cells and can spread throughout the epithelium to underlying tissues, where it evades recognition by immune cells. In fact, *P. gingivalis* had evolved several mechanisms to avoid immune recognition, thus promoting its survival and dissemination, and characteristics permissive to developing chronic inflammation. One such evasive strategy, once it has penetrated the epithelial barrier, involves *P. gingivalis* attaching to erythrocytes via complement receptor 1 and circulating through the bloodstream. The bacterium thereby translocates to sites distant from the oral mucosa where it plays a role in development of chronic inflammation outside of the oral cavity. Indeed, *P. gingivalis* has been isolated from atheromatous plaque, and has been shown to enhance uptake of oxidised low density lipoprotein (LDL) by macrophages and induce foam cell formation. With regard to epithelial cell invasion, *P. gingivalis* has been shown to preferentially colonise and invade oral squamous carcinoma cells over normal, healthy cells, and has been isolated from OSCC tumours. Like periodontitis, OSCC is associated with chronic inflammation and infiltration of macrophages. Thus, under a variety of pathological circumstances, *P. gingivalis* and its associated virulence factors - such as LPS - interact with macrophages at varying stages of activation/differentiation and modulate their behaviour. Infection of macrophages with other intracellular bacteria, such as *Listeria monocytogenes*, interrupts the interferon gamma signalling pathway so that macrophages cannot completely polarise to an M1 phenotype by interfering with STAT and its association with its transcriptional co activators. The effect of *P. gingivalis* LPS presence during macrophage polarisation, however, is yet to be characterised.
**Porphyromonas gingivalis** signalling pathways

*P. gingivalis* LPS is renowned for its unusual properties; it is well established that it has lower endotoxin activity than that of *E. coli* LPS, which is thought to be down to structural differences in the lipid A component and a differential use of TLR signalling pathways. TLR2 and TLR4 share a common signalling pathway (MyD88/MAPK) to regulate expression of inflammatory cytokines in response to LPS. In response to activation by their cognate ligands, TLR2 (dimerised with either TLR1 or 6) or TLR4 recruit the adapter molecule MyD88 via TRIF-related adapter molecule (TRAM) and TIR domain-containing adapter protein (TIRAP). MyD88 in turn activates IL-1 receptor-associated kinases (IRAKs), required for NFκB activation, via TNF receptor-associated factor 6 (TRAF6). TRAF6 activates the TGF-β -activated kinase 1/TAK1-binding protein 2/TAK1-binding protein 3 (TAK1/TAB2/TAB3) complex, which then activates the inhibitor of NFκB (IκB) kinase complex (IKKα, IKKβ and NEMO). The IκB complex phosphorylates IκB itself, leading to the release of NFκB from its inhibitor and its translocation to the nucleus. In the nucleus, NFκB induces the transcription of inflammatory cytokines via a raft of nuclear proteins; C/EBPδ, IκBζ, IκB-NS and Zc3h12a regulate IL-6 expression, AFT3 regulates IL-6 and IL-12p40 expression, and tristeraprolin (TTP) regulates TNFα expression.

TLR4 is unique in that it can also utilise MyD88 independent signalling pathways in response to ligation by LPS. Cells lacking MyD88 do not produce inflammatory cytokines, rather they up-regulate production of chemokine CXCL10 and type I interferons (IFNα and IFNβ). In this pathway, ligation of TLR4 leads to the recruitment of TRIF. TRIF activates IRF3 (and later NFκB) via recruitment of TRAF3 (and TAK1 activation). Activation of IRF3 via association with TRAF family member-associated NFκB activator (TANK), TANK binding kinase 1 (TBK1) and inducible IKK (IKKi) leads to downstream induction of type I interferons. In TLR4 signalling, TRAF3 is incorporated into the MyD88 complex where it is degraded by cIAP1 and cIAP2, inhibiting the MyD88 pathway (thus inflammatory cytokine
production), whilst leaving the type I interferon pathway active\(^{229}\) (see Figure 4-3 for summary).

![Figure 4-1: Schematic representation of MyD88 dependent and independent signalling pathways via TLR signalling](image)

Whether \textit{P. gingivalis} LPS signals through TLR2 or TLR4 remains a contentious issue. \textit{Porphyromonas gingivalis} undeniably utilises either TLR2 or TLR4 because TLR2/4 double knock-out cells from mouse bone marrow do not respond to \textit{P. gingivalis} LPS\(^{213}\). Many studies have shown \textit{P. gingivalis} LPS to signal via TLR2\(^{96,203,230–235}\), including one using C3H/HeJ mice (a mutant strain that are defective in TLR4), reporting that whilst these mice had a defective response to \textit{E. coli} LPS, they retained the capacity to respond to \textit{P. gingivalis} LPS\(^{236}\), indicating that \textit{P. gingivalis} LPS utilised TLR2. It has also been reported that \textit{P. gingivalis} LPS signals through a TLR1/2 heterodimer, and that CD14 and LBP must also be available\(^{234,237}\). Conversely, many other studies have reported that \textit{P. gingivalis} LPS signals through TLR4\(^{91,209,238–240}\). Several reasons for this discrepancy in TLR utilisation by \textit{P. gingivalis}...
*P. gingivalis* LPS have been put forward, including LPS purification methods, with some groups claiming that purified *P. gingivalis* LPS signals through TLR2, whereas live *P. gingivalis* signals through TLR2 or TLR4. A further study shows that *P. gingivalis* LPS signals through a combination of TLR2 and intracellular receptor, TLR7. Finally, Darveau *et al* (2004) suggest that *P. gingivalis* LPS signals through both TLR2 and TLR4. A consensus is yet to be reached. Thus, two objectives were generated for this study:

1) Determination of effects of *P. gingivalis* LPS during the macrophage polarisation process on cytokine expression in response to subsequent LPS stimulation

2) Confirmation of the TLR utilised in our model system.

To do this, macrophages were polarised according to the protocol established in chapter 3 in the presence or absence of LPS (*P gingivalis* or *E. coli*), at doses of 1 ng/ml or 1 µg/ml. Following polarisation, culture media was removed and cells were washed 3 times in PBS. Fresh media was added containing either 1 µg/ml *P. gingivalis* or *E. coli* LPS to activate the macrophages. Media alone (no LPS) served as a negative control (see Figure 4-11 for schematic representation). After 24 hours incubation, cell free supernatants were harvested and stored at -20°C until assay for TNFα, IL-1β and IL-6 by ELISA.
Figure 4-2: Schematic representation of experimental protocol for polarising macrophages in the presence of LPS
Results

Effects of LPS on cytokine production during differentiation of non-polarised, PMA differentiated macrophages (Mϕ)

Firstly, the polarisation of macrophages in the presence of *P. gingivalis* LPS was examined at two different doses (1 µg/ml and 1 ng/ml) to measure any effect on TNFα production in response to secondary stimulation, the dose of 1 µg/ml being concurrent with the literature. In macrophages differentiated with PMA + 1 µg/ml *P. gingivalis*, TNFα production was downregulated in response to stimulation with 1 µg/ml *P. gingivalis* LPS (Figure 4-3 a, p=0.007), however, when the macrophages were differentiated in the presence of *P. gingivalis* LPS at 1 ng/ml there was slight down-regulation in TNFα, but this was not significant (Figure 4-3 a, p=0.179). Then, we went on to examine whether polarisation with *P. gingivalis* LPS affected TNFα production in response to TLR4 ligand, *E. coli* LPS. In response to challenge by *E. coli* LPS, PMA + *P. gingivalis* LPS differentiated macrophages down-regulated TNFα production (Figure 4-3 d, p<0.001). As seen in response to *P. gingivalis* LPS stimulation, 1 µg/ml polarising LPS was needed for this down-regulation to be significant (1 ng/ml, Figure 4-3 d, p=0.363).

Next, we examined whether this down-regulation of TNFα production was observed when macrophages were polarised in the presence of PMA and *E. coli* LPS, either in response to challenge with TLR2 activating, *P. gingivalis* LPS (1µg/ml) or to challenge with the same stimulus (1µg/ml *E. coli* LPS). When macrophages were differentiated with PMA and 1 µg/ml or 1 ng/ml *E. coli* LPS and stimulated with 1 µg/ml *P. gingivalis*, TNFα production was down-regulated (p=0.06, p=0.05, Figure 4-3g). Likewise, when macrophages were differentiated with PMA and *E. coli* LPS at both 1 µg/ml or 1 ng/ml, TNFα production was down-regulated.
in response to 1 µg/ml *E. coli* LPS (p<0.001, Figure 4-3). 

Contrary to TNFα, when cells were differentiated in the presence of PMA + *P. gingivalis* or *E. coli* LPS at 1 µg/ml or 1 ng/ml, there was no difference in IL-1β or IL-6 production in response to either *E. coli* or *P. gingivalis* LPS: *Differentiation with PMA in the presence of LPS only affected TNFα production* (Figure 4-3b,c,e,f,h,l,k and l).
Figure 4-3: Exposure of PMA macrophages to *P. gingivalis* or *E.coli* LPS during polarisation: Effects on macrophage responses to LPS. PMA, M1 and M2 macrophages were polarised in the presence or absence of *P. gingivalis* or *E. coli* LPS. After 24 hours incubation, media was removed and cells were washed x3 in PBS. Macrophages were then challenged with 1 µg/ml *P. gingivalis* or *E. coli* LPS for 24 hours. Cells incubated for 24 hours in normal media (no LPS) served as negative controls. Supernatants were assayed for TNFα (a, d + g), IL-1β (b, e + h) and IL-6 (c, f +i) by ELISA. Data are expressed as the mean of three independent experiments performed in triplicate wells +/- standard deviation.
Effects of LPS on cytokine production in M1 polarised macrophages

After studying the effects of LPS on cytokine production on non-polarised, PMA differentiated Mø macrophages, we next examined the effects of LPS during the polarisation of macrophages into an M1 phenotype. When cells were polarised in the presence of IFNγ and 1 µg/ml P. gingivalis LPS, TNFα production was down-regulated in response to secondary challenge with P. gingivalis LPS (p=0.002, Figure 4-4 a), however, when cells were polarised with IFNγ and 1 ng/ml P. gingivalis LPS there was no significant change in TNFα production (p=0.532, Figure 4-4 a). When cells were polarised with IFNγ + P. gingivalis LPS followed by challenge with 1 µg/ml E. coli LPS, TNFα production was down-regulated (p=0.041, Figure 4-4 d). As reported with P. gingivalis LPS, secondary stimulation with E. coli LPS in cells polarised with IFNγ + 1 ng/ml P. gingivalis LPS had no effect on TNFα production (p=0.085, Figure 4-4 d).

When cells were polarised with IFNγ + 1 µg/ml E. coli LPS, TNFα production was down-regulated in response to secondary stimulation with 1 µg/ml P. gingivalis LPS (p=0.006, Figure 4-4 g) but when M1 polarisation occurred in the presence of 1 ng/ml E. coli LPS, there was no significant effect on TNFα production (p=0.110, Figure 4-4 g). The same effect was witnessed when M1 cells were polarised with IFNγ + E. coli LPS and challenged with 1 µg/ml E. coli LPS, with 1 µg/ml polarising E. coli LPS down-regulating TNFα production (p<0.001, Figure 4-4 j), but 1 ng/ml polarising E. coli LPS was not enough to elicit this response (p=0.431, Figure 4-4 j).

After measuring the effect of LPS polarisation in TNFα production, we went on to measure the effects on IL-1β production. When cells were polarised with IFNγ + 1 µg/ml P. gingivalis LPS then stimulated with 1 µg/ml P. gingivalis LPS, IL-1β production was down-regulated (p=0.026, Figure 4-4 b). Polarisation with 1 ng/ml P. gingivalis LPS did not result in IL-1β down-regulation in response to secondary stimulation with 1 µg/ml P. gingivalis LPS (p=0.118, Figure 4-4 b).
In response to challenge with *E. coli* LPS, M1 cells polarised in the presence of IFNγ and 1 µg/ml or 1 ng/ml *P. gingivalis* LPS down-regulated IL-1β production (*p*=0.04, *p*=0.027, Figure 4-4 e). Next, we examined the effects of *E. coli* LPS during M1 polarisation on cytokine response to *P. gingivalis* LPS or *E. coli* LPS. Cells polarised with IFNγ and 1 µg/ml and 1 ng/ml *E. coli* LPS down-regulated IL-1β production in response to *P. gingivalis* LPS (*p*=0.002, *p*=0.0034, Figure 4-4 h), whereas in response to secondary stimulation with *E. coli* LPS, IL-1β was only significantly down-regulated when the cells were polarised with 1 µg/ml *E. coli* LPS (*p*<0.001, Figure 4-4 k).

As seen in the PMA differentiated Mø macrophages, there was no change in IL-6 production in response to *P. gingivalis* or *E. coli* LPS when cells were polarised to an M1 phenotype in the presence of *P. gingivalis* or *E. coli* LPS (Figure 4-4 c, f, i and l).
IL21 (pg/ml)

0
400
800

IL26 (pg/ml)

0
200
400

p=0.027

TNFα (pg/ml)

0
2000
4000
6000
8000
10000

p=0.041

p=0.04

M1

P=0.026

Pre-treatment: IFNγ

- + - +

pgLPS

- + - +

Stimulus: pgLPS

Pre-treatment: IFNγ

- + - +

pgLPS

- + - +

Stimulus: pgLPS

Pre-treatment: IFNγ

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Stimulus: pgLPS

Pre-treatment: IFNγ

- + - +

pgLPS
Figure 4-4: Exposure of M1 macrophages to *P. gingivalis* or *E. coli* LPS during polarisation: Effects on macrophage responses to LPS. M1 macrophages were polarised in the presence or absence of *P. gingivalis* or *E. coli* LPS. After 24 hours incubation, media was removed and cells were washed x3 in PBS. Macrophages were then challenged with 1 µg/ml *P. gingivalis* or *E. coli* LPS for 24 hours. Cells incubated for 24 hours in normal media (no LPS) served as negative controls. Supernatants were assayed for TNFα (a, d + g), IL-1β (b, e + h) and IL-6 (c, f + i) by ELISA. Data are expressed as the mean of three independent experiments performed in triplicate wells +/- standard deviation.
Effects of LPS on cytokine production in M2 polarised macrophages

Finally, the effects of LPS during M2 polarisation on cytokine production were examined. When cells were polarised in the presence of IL-4 + 1 µg/ml *P. gingivalis* LPS, TNFα production was down-regulated in response to challenge with 1 µg/ml *P. gingivalis* LPS (p=0.004, Figure 4-5 a), whereas there was no significant effect on TNFα production when cells were polarised with 1 ng/ml *P. gingivalis* LPS (p=0.431, Figure 4-5 a). Likewise, TNFα production was down-regulated in response to challenge with 1 µg/ml *E. coli* LPS when polarised with 1 µg/ml *P. gingivalis* LPS but not at 1 ng/ml (p<0.001 and p=0.129, Figure 4-5 d).

Conversely to polarisation with *P. gingivalis* LPS, TNFα production was down-regulated in M2 macrophages when polarised in the presence of both 1 µg/ml and 1 ng/ml *E. coli* LPS in response to challenge with *P. gingivalis* LPS (p=0.024 and p=0.038, Figure 4-5 j). Furthermore, M2 macrophages down-regulated TNFα production in response to *E. coli* LPS when polarised with *E. coli* LPS at both 1 µg/ml and 1 ng/ml (p<0.001 and p=0.003, Figure 4-5 g).

The only change in IL-1β production in M2 macrophages was when cells were polarised in the presence of IL-4 + 1 µg/ml *P. gingivalis* LPS and challenged with *E. coli* LPS (p=0.049, Figure 4-5 e). Polarisation with *P. gingivalis* LPS followed by stimulation with *P. gingivalis* LPS had no effect on IL-1β production (p=0.063, Figure 4-5 b). Likewise, polarisation with *E. coli* LPS had no effect on IL-1β production in response to secondary stimulus with either *P. gingivalis* or *E. coli* LPS (Figure 4-5 k and h).

As with the other macrophage phenotypes, IL-6 production was not affected by polarisation with LPS (Figure 4-5 c, f, l and l).
M2

**Pre-treatment:** IL-4 | PgLPS
---|---
- | +
+ | -
+ | -
+ | -
+ | -

**Stimulus:** PgLPS
---
- | -
- | -
+ | -
+ | -
+ | -

**IL21 (pg/ml)**

<table>
<thead>
<tr>
<th>0</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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<td>-</td>
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<td>-</td>
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<td>+</td>
<td>+</td>
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</table>

**IL26 (pg/ml)**

<table>
<thead>
<tr>
<th>0</th>
<th>20</th>
<th>40</th>
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<tbody>
<tr>
<td>+</td>
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**p-values:**

- **p<0.001**
- **p=0.049**
Figure 4-5: Exposure of M2 macrophages to *P. gingivalis* or *E. coli* LPS during polarisation: Effects on macrophage responses to LPS. M2 macrophages were polarised in the presence or absence of *P. gingivalis* or *E. coli* LPS. After 24 hours incubation, media was removed and cells were washed x3 in PBS. Macrophages were then challenged with 1 µg/ml *P. gingivalis* or *E. coli* LPS for 24 hours. Cells incubated for 24 hours in normal media (no LPS) served as negative controls. Supernatants were assayed for TNFα (a, d + g), IL-1β (b, e + h) and IL-6 (c, f + i) by ELISA. Data are expressed as the mean of three independent experiments performed in triplicate wells +/- standard deviation.
Table 4-1: summary of cytokine modulation in macrophages polarised with *P. gingivalis* or *E. coli* LPS

<table>
<thead>
<tr>
<th>Polarised with:</th>
<th>Stimulated with:</th>
<th>1 µg/ml PgLPS</th>
<th>1 ng/ml PgLPS</th>
<th>1 µg/ml E. coli LPS</th>
<th>1 ng/ml E. coli LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. gingivalis</em> LPS (TLR2)</td>
<td>TNFα (PMA, M1 &amp; M2)</td>
<td>IL-1β (M1)</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td><em>E. coli</em> LPS (TLR4)</td>
<td>TNFα (PMA, M1 &amp; M2)</td>
<td>IL-1β (M1 &amp; M2)</td>
<td>TNFα (PMA)</td>
<td>IL-1β (M1)</td>
<td>IL-1β (M1)</td>
</tr>
</tbody>
</table>

**Toll-like receptor activation by Porphyromonas gingivalis** LPS

It is possible that differential cytokine responses to LPS following polarisation with *P. gingivalis* or *E. coli* LPS are due to differential utilisation of TLR signalling pathways. *E. coli* LPS is an established TLR4 ligand, but the TLR pathway utilised by *P. gingivalis* LPS is still contested. To confirm the role of TLR2 and TLR4 in *P. gingivalis* LPS signalling in our model, blocking antibodies to TLR2 and TLR4 were used in stimulation experiments.

Briefly, macrophages were incubated with blocking antibodies specific for putative *P. gingivalis* LPS receptor, TLR2, or established LPS receptor, TLR4. After 30 minutes incubation, any unbound antibody was removed by washing the cells 3 times in PBS. Cells were then stimulated with 1 µg/ml *P. gingivalis* LPS for 24 hours, and supernatants were collected and stored at -20°C until assay for TNFα, IL-1β and IL-6 by ELISA.
Figure 4-6: TNFα production is dependent on TLR2 signalling. Data represent the mean of three independent experiments, performed in triplicate, +/- standard deviation.

Figure 4-7: IL-1β production is dependent on TLR2 signalling. Data represent the mean of three independent experiments, performed in triplicate, +/- standard deviation.
Incubation with anti-TLR2 antibodies inhibited TNFα production in M1 (p<0.001) macrophages in response to *P. gingivalis* LPS, whereas blocking anti-TLR4 did not. (Figure 4-66).

Incubation with anti-TLR2 antibodies also inhibited IL-1β production, and whilst (p=0.006, Figure 4-77 ) there was evidence for inhibition of IL-1β production by anti-TLR4 antibodies this did not reach statistical significance (p=0.072, Figure 4-77). Incubation with anti-TLR2 and anti-TLR4 antibodies inhibited IL-6 production in M1 macrophages (p=0.001, p=0.001, Figure 4-88).

**Figure 4-8: IL-6 production is dependent on TLR2 and TLR4 signalling**. Data represent the mean of three independent experiments, performed in triplicate, +/- standard deviation.
Table 4-2: Summary of toll like receptor utilisation by *P. gingivalis* LPS in M1 and M2 polarised macrophages

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cell</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>M1</td>
<td>TLR2</td>
</tr>
<tr>
<td>IL-1β</td>
<td>M1</td>
<td>TLR2</td>
</tr>
<tr>
<td>IL-6</td>
<td>M1</td>
<td>TLR2 and TLR4</td>
</tr>
</tbody>
</table>

Surface expression of TLR2 and TLR4 in un-stimulated and *P. gingivalis* polarised macrophages

Polarising macrophages to an MØ, M1 or M2 phenotype in the presence of *P. gingivalis* LPS reduces their production of TNFα in response to secondary stimulation. IL-1β production was down-regulated in M1 only. Is this because *P. gingivalis* LPS reduced surface expression of LPS receptors TLR2 and TLR4?

To address this, THP-1 were polarised to PMA, M1 or M2 phenotypes according to the usual protocol (described in chapter 3), in the presence or absence of 1 µg/ml *P. gingivalis* LPS for 24 hours. TLR2 and TLR4 expression was assayed by flow cytometry.

In the non-polarised (PMA) macrophages, expression of both TLR2 and TLR4 was down-regulated when treated with *P. gingivalis* LPS during differentiation (Error! Reference source not found.10) the M1 polarised cells, TLR2 and TLR4 expression was also decreased (Error! Reference source not found.0). The presence of *P. gingivalis* LPS during M2 polarisation however led to an increase in TLR2 and TLR4 expression compared to polarisation without LPS (Error! Reference source not found.0Figure 4-8), though this was not statistically significant.

There was no significant difference in TLR2 or TLR4 expression between PMA, M1 and M2 macrophages (Figure 4-99).
Figure 4d60: Percentage change in TLR2 and TLR4 expression in macrophages polarised in the presence of *P. gingivalis* LPS. PMA, M1 and M2 macrophages were incubated with PE conjugated anti-TLR2 and anti-TLR4 antibodies and expression was analysed by flow cytometry. Data represent the mean of 5 independent experiments +/- standard deviation.

Figure 4d9: TLR2 and TLR4 expression in PMA, M1 and M2 macrophages. PMA, M1 and M2 macrophages were incubated with PE conjugated anti-TLR2 and anti-TLR4 antibodies and expression was analysed by flow cytometry. Data represent the mean of 5 independent experiments +/- standard deviation.
Discussion

When macrophages were polarised with 1 µg/ml *P. gingivalis* LPS, all cell types down-regulated TNFα production upon secondary stimulus with *P. gingivalis* LPS or *E. coli* LPS. Polarising with 1 ng/ml *P. gingivalis* LPS was not enough to elicit this effect in any of the cell types. The only cell type to down-regulate IL-1β production following *P. gingivalis* LPS polarisation was M1, but again polarising with 1 ng/ml *P. gingivalis* LPS was not enough to elicit the same effect. Both M1 and M2 down-regulated IL-1β in response to *E. coli* LPS when polarised with 1 µg/ml *P. gingivalis* LPS, although in the M1, 1 ng/ml was enough to replicate this response. There was no effect on IL-6 production in any of the cell types, under any of the polarisation conditions.

When polarised with *E. coli* LPS, all cell types down-regulated their TNFα response to *P. gingivalis* LPS challenge, and in the M2s, 1 ng/ml was enough to elicit the same response. As with *P. gingivalis* LPS polarising, the only cell type to down-regulate IL-1β in response to secondary *P. gingivalis* LPS challenge was M1, and this was only when polarised with 1 µg/ml *P. gingivalis* LPS. All cell types down-regulated TNFα production in response to *E. coli* LPS challenge, and in MØ and M2 cells, polarising with 1 ng/ml *E. coli* LPS was enough to elicit the same response, the M1 cells however needed the higher dose (1 µg/ml) for this down-regulation to happen. Only M1 down-regulated IL-1β production in response to *E. coli* LPS challenge. As seen with *P. gingivalis* LPS polarisation, there was no effect on IL-6 production in any of the cell types.

In summary, there was a down-regulation of M1 associated cytokines TNFα and IL-1β in response to a secondary LPS stimulus in macrophages polarised in the presence of LPS (summarised in Table 4-1). The down-regulation of cytokine production may be due to expression of negative regulators of TLR signalling. It has previously been demonstrated that *P. gingivalis* LPS induces regulatory factors in macrophages, leading to the formation of
regulatory NFκB p50 homodimers and up-regulation of IRAK-M expression in THP-1 macrophages. However, SOCS3 and 6 are reported to be up-regulated in response to P. gingivalis LPS in gingival epithelial cells. The effects of P. gingivalis LPS on expression of other negative regulators of TLR signalling, such as PPARγ, TOLLIP, SHIP are yet to be investigated. Given that IRAK-M and NFκB homodimers are preferentially induced by P. gingivalis LPS compared with E. coli LPS, and that the same levels of cytokine down-regulation are observed in both LPS species, it is not likely that up-regulation of negative regulators are the cause of cytokine down-regulation.

Differential transcriptional regulation of TNFα and IL-6/IL-1β production in response to TLR ligation may have some part to play in the differing levels of down-regulation seen in the present results. Nuclear proteins C/EBPδ, IκBζ, IκB-NS, AFT3 and Zc3h12a regulate IL-6 expression, whereas TNFα expression is regulated by TTP. It may be that these proteins are differentially regulated in endotoxin tolerance, although this remains to be investigated.

Alternatively, this down-regulation of cytokine production may be due to skewing of the macrophages towards an M2-like phenotype. Previous studies have reported that P. gingivalis up-regulates the M2 markers IRAK-M and IL-1Ra and that periodontal infection with P. gingivalis leads to the development of a population of myeloid derived suppressor cells (MDSCs) that have similar characteristics to M2 macrophages. This does not account for the down-regulation of cytokines in response to E. coli, however. The expression of M2 characteristic cytokines in the present study remains to be investigated, although a previous study has shown that IL-10 production is not up-regulated upon repeated exposure to LPS.

It has been argued that M2 polarisation is in fact a manifestation of endotoxin tolerance. A comprehensive study of gene expression on Pseudomonas aeruginosa LPS tolerised human macrophages by Pena et al (2001) revealed that tolerised macrophages exhibit similar gene
expression profiles as IL-10 polarised M2 macrophages, with an up-regulation of M2-associated chemokines CCL-22, CCL-24, scavenger receptors MARCO and CD23, CD163 growth factors VEGF and FGF-2, matrix metalloproteinase 7 and 9, wound healing factor versican and formyl peptide receptor ligand 1 (activation of formyl peptide receptor inhibits expression of M1 cytokine IL-12p70\(^{251}\)). They demonstrated a concomitant down-regulation of M1 associated inflammatory mediators TNFα, COX2 and tissue factor (TF), and chemokines CCL-3 and CCL-20\(^{250}\). Moreover, \(P. \text{gingivalis}\) LPS induces expression of tolerogenic molecules ILT-3 and B7-H1 on dendritic cells and human PBMCs, which is further increased upon a secondary stimulation with \(P. \text{gingivalis}\) LPS, suggesting the \(P. \text{gingivalis}\) plays a tolerogenic role\(^{252}\).

A study by Zaric et al (2011) reports that TNFα production is down-regulated in response to \(P. \text{gingivalis}\) and \(E. \text{coli}\) LPS tolerising, whilst IL-8 is unaffected in THP-1 cells. Physiologically, this leads to the same level of neutrophil recruitment but lower levels of neutrophil apoptosis induced by TNFα in \(P. \text{gingivalis}\) LPS tolerised cells\(^{253}\). The present study did not address IL-8, however a similar pattern was found in IL-6 production, which remained at the same levels as cells polarised without exposure to LPS, although this was the case for both \(E. \text{coli}\) and \(P. \text{gingivalis}\) LPS.

An earlier study by Martin et al (2001) reported that endotoxin tolerance induced by \(E. \text{coli}\) LPS in THP-1 led to down-regulation of TNFα, IL-1β and IL-6, whereas endotoxin tolerance induced by \(P. \text{gingivalis}\) LPS resulted in down-regulation of IL-1β production only\(^{234}\). These findings differ from the present study in that TNFα production was down-regulated by prior exposure to \(P. \text{gingivalis}\) LPS, but there was no change in IL-6 levels from naïve (LPS free) cells stimulated with LPS. However, both studies are in agreement that IL-1β was down-regulated by prior exposure to either LPS species. In contrast to the Martin et al (2001) study, and in common with results from the present study, Muthukuru et al (2005) reported that TNFα and IL-1β were down-regulated in response to repeated exposure of PBMCs to \(P. \text{gingivalis}\) LPS, but IL-6, IL-8 and IL-10 were down-regulated to a much lesser extent\(^{62}\). The
discrepancy in results may be born from the fact that the Martin et al (2001) study used THP-1 cells differentiated for 72 hours in 10 ng/ml PMA, washed 3 times and rested overnight, compared to the various polarising treatments used in the present study, i.e. macrophage polarisation may affect tolerogenic mechanisms induced by E. coli and P. gingivalis LPS.

Dobrovolskaia et al (2003) reported that both E. coli LPS and synthetic TLR2 ligand PAM3CSK4 induced homotolerance but not heterotolerance in murine macrophages. PAM3CSK4 pre-treatment up-regulated TNFα production in response to E. coli LPS. Porphyromonas gingivalis LPS also induced homotolerance, but like PAM3CSK, E. coli pre-treatment up-regulated TNFα production in response to secondary stimulus with P. gingivalis LPS. In contrast, results from the present study showed that E. coli LPS pre-treatment led to a down-regulation of TNFα production when subject to a secondary stimulation by P. gingivalis LPS. However, the studies are in agreement in that P. gingivalis pre-treatment led to a down-regulation of TNFα production when subject to a secondary stimulation by E. coli LPS.

The present study showed little difference in the down-regulation of cytokines in response to polarisation in the presence of either LPS species. Previous studies have shown that P. gingivalis LPS is a weaker inducer of endotoxin tolerance than E. coli LPS, and that this is down to differential use of TLRs and intracellular signalling pathways, with E. coli LPS reducing degradation of NFkb inhibitors IκKα and IκKβ and TLR4 surface expression upon secondary stimulation, whereas P. gingivalis LPS up-regulates TLR2 and CD14 expression and degrades inhibitory IκKβ, thereby rendering cells less responsive to tolerance. It may be that the different macrophage differentiation protocols between the two studies affect the response to secondary stimulation to LPS pre-treated cells.
LPS pre-treatment activates the MyD88 independent pathway (via TLR4 signalling) leading to production of IFNβ\(^{100}\), and IFNβ production leads to \textit{E. coli} induced endotoxin tolerance in THP-1 cells\(^{232,253}\). A previous study by Zaric \textit{et al} (2011) reported that \textit{P. gingivalis} signalling via TLR2 did not induce IFNβ production, thereby rendering the cells only partially tolerised (reduced TNFα production but IL-8 levels remained the same)\(^{253}\). Only TLR4, and not TLR2, can active the TRIF pathway, therefore inducing IFNβ mediated tolerance. However, there is still a down-regulation in cytokine production induced by \textit{P. gingivalis} LPS, a purported TLR2 ligand. Therefore, either \textit{P. gingivalis} LPS is signalling through TLR4, or another mechanism induces tolerance via TLR2 signalling. In order to ascertain \textit{P. gingivalis} LPS receptor utilisation in our model, MØ, M1 and M2 macrophages were pre-incubated with neutralising anti- TLR2 or TLR4 antibodies prior to stimulation with \textit{P. gingivalis} or \textit{E. coli} LPS.

Anti-TLR4 antibodies inhibited IL-6 production in M1 (p=0.001). IL-1β was also inhibited in M1, but this did not reach statistical significance. A study by Darveau \textit{et al} (2004) showed that \textit{P. gingivalis} LPS has the ability to signal through both TLR2 and TLR4\(^{213}\). Data from the present study also demonstrate that \textit{P. gingivalis} can utilise both TLRs under certain conditions; M1 production of IL-6 is dependent on TLR2 and TLR4.

Following confirmation of TLR utilisation by \textit{P. gingivalis} LPS, we sought to determine levels of TLR2 and TLR4 surface expression on M1 and M2 polarised macrophages, as this is yet to be described. M1 may express higher levels of TLR2 and 4 than MØ and M2 macrophages, but there was no statistically significant difference. This data could in part explain why M1 produce higher levels of inflammatory cytokines than M2. However, caution must be taken when interpreting these results. Expression levels of TLR2 and TLR4 were normalised to fluorescence produced by cells alone (auto-fluorescence) because the isotype control antibody was binding specifically to an unknown ligand on the M1 polarised macrophages. As this isotype control binding was only present in the M1 macrophages, and changed markedly between M1 and MØ/M2 macrophages, it was unsuitable to use as a
normalisation point\textsuperscript{255}. Therefore, further experiments to address expression levels of TLR2 and TLR4 are needed to confirm these findings.

After measuring the levels of TLR2 and TLR4 expression on the surface of MØ, M1 and M2 macrophages, we next sought to determine whether expression levels were modulated in response to polarisation in the presence of \textit{P. gingivalis} LPS. As shown in Error! Reference source not found.10, the presence of \textit{P. gingivalis} LPS during polarisation down-regulated TLR2 and TLR4 expression in MØ and M1 macrophages, but up-regulated TLR2 and 4 expression in M2 macrophages. Up-regulation of TLR2 on THP-1 cells in response to a single stimulation by \textit{P. gingivalis} LPS has been demonstrated in previous studies\textsuperscript{62,252,256}, but down-regulation of TLR2 and TLR4 upon the second stimulation has also been noted \textsuperscript{62}.

Contrary to MØ and M1 macrophages, there is an up-regulation of TLR2 and TLR4 surface expression in response to \textit{P. gingivalis} LPS polarisation in M2 macrophages, with a concomitant down-regulation of cytokine production. This further suggests that there is a different mechanism involved in this down-regulation of cytokines in response to repeated LPS exposure in M1 and M2 macrophages. The expression of anti-inflammatory cytokines remains to be investigated. It is possible that \textit{P. gingivalis} LPS might up-regulate TLRs in M2 macrophages in order to increase production of anti-inflammatory cytokines to aid resolution of inflammation. Indeed, data from the previous chapter shows that \textit{P. gingivalis} up-regulates IL-10 and TGF-β in M2 to a greater extent than M1. This provides an exciting avenue of investigation for future research.

When taken together with the down-regulation of cytokine production in response to polarisation with \textit{P. gingivalis} LPS, this suggests that the down-regulation of cytokines is due to a down-regulation of TLRs. However, previous studies have reported that endotoxin tolerance is not likely to be solely due to a down-regulation of TLR4/MD2 at the cell surface, as this is transient\textsuperscript{257}, and cells over expressing TLR4/MD2 are still readily tolerised\textsuperscript{258}.
Data from the present study demonstrates that M2 macrophages are sensitive to TNFα down-regulation at ng/ml concentrations of LPS, whereas M1 require µg/ml concentrations to elicit the same effect. This suggests that M2 macrophages may be more readily tolerised than M1, at least with respect to TNFα production, which given their anti-inflammatory role may prove a viable hypothesis. It has been previously reported that type 1 interferon priming before LPS (TLR4) stimulation in M2 macrophages leads to an increase in the use of the MyD88 independent (TRIF) pathway and autocrine production of IFNβ compared with M1. This preferential induction of IFNβ in M2 macrophages accounted for their signature low inflammatory cytokine production, and the lack of IFNβ in M1 led to an up-regulation of M1 cytokines. This provides a possible mechanism whereby M2 are more readily tolerised than M1.

This study demonstrates for the first time that M2 macrophages down-regulate cytokine production in response to repeated exposure to *P. gingivalis* and *E. coli* LPS, and that the cytokines down-regulated are different from M1. Thus, future studies should be designed to investigate the mechanisms of tolerance induction in M2 versus M1 macrophages.

To date, no studies have described the levels of TLR2 and TLR4 expression on M1 versus M2 macrophages. The present study tentatively suggests that M1 macrophages express higher levels of TLR2 and TLR4 than M2 macrophages, and further investigation is needed in order to confirm this finding.

The present data suggests for the first time that TLR signalling by *P. gingivalis* LPS may differ between M1 and M2 macrophages. Both anti-TLR2 and anti-TLR4 inhibited IL-6 production in M1 macrophages, but were unable to inhibit IL-6 production in M2, suggesting a possible difference in regulation of IL-6 production. Furthermore, IL-1β production was dependant on TLR2 signalling alone in M1 macrophages, whereas ligation of either TLR2 or TLR4 induced IL-1β in M2 macrophages. Taken together, the data presented in this study
suggest that TLR signalling pathways that lead to the production of inflammatory cytokines may be differentially regulated in M1 and M2 macrophages.
Chapter 5 - Influence of oral squamous cell carcinoma culture supernatants on macrophage responses to *Porphyromonas gingivalis*

**Introduction**

As of 2010, oral cancer is the 6th most common cancer across the globe, of which over 90% are squamous cell carcinoma (OSCC)\(^{123}\). OSCC is a multifactorial disease and has been linked to oral leukoplakia\(^{125}\), HPV infection\(^{126,260}\), periodontal disease\(^{127,261}\), oral bacterial species\(^{128}\), alcohol and tobacco use\(^{129,130}\). Despite intensive research into all aspects of OSCC, from epidemiology, through treatment and prevention, to the cellular and molecular basis of the disease, the global incidence and mortality of OSCC is increasing\(^{262}\). Thus, investigations into the mechanisms that drive oral cancer are required to provide novel therapeutic targets. To this end, the cell line H357, established by Prime et al (Sheffield, UK), has been used to investigate pathological mechanisms in OSCC\(^{263–265}\). To date, H357 have been shown to produce MMP -2 and -9\(^{265}\) αvβ1 integrin\(^{266}\), ICAM-1\(^{267}\), super oxide\(^{268}\) and TGF-β\(^{269}\), however the pro-inflammatory cytokine producing capability of these cells has not been investigated. Furthermore, the production of inflammatory cytokines by oral epithelial cells in general is not well established, particularly in response to the oral bacterium *P. gingivalis*. Several studies have shown that IL-1β, IL-6, IL-8, GM-CSF and TNFα are up-regulated in primary gingival epithelial cells and an oral epithelial cell line in response to viable and non-viable *P. gingivalis*\(^{270–272}\). Conversely, other studies have reported that *P. gingivalis* inhibits IL-8 and ICAM-1 in primary gingivalis epithelial cells\(^{273,274}\).

It has long been established that infection with particular bacteria or viruses can lead to the malignant transformation of epithelial cells; the enterobacterium *Helicobacter pylori*, for example, is a recognised gastric carcinogen\(^{118}\). Evidence is accumulating that the periodontal pathogen *P. gingivalis* has a role in the development of OSCC\(^{119–121}\), however, other groups have shown that *P. gingivalis* colonises control tissue rather than tumour...
Since OSCC cells and *P. gingivalis* may come into contact *in vivo*, the first part of this study was designed to assess the cytokine response of H357 to a major virulence factor of *P. gingivalis*: LPS.

Recently, OSCC research has focused on the interaction between tumour cells and non-tumour cells in their microenvironment, such as fibroblasts and infiltrating immune cells. Of the infiltrating immune cells, macrophages have been shown to play a pivotal role in promoting a pro-tumoural environment. Monocytes recruited to tumours by MCP-1, produced by the cancer cells, differentiate and polarise into functional effector macrophages depending on factors they encounter in their microenvironment. In OSCC, MCP-1 is significantly elevated in cancerous lesions compared to healthy mucosa. The accumulation of macrophages around the tumour site is associated with poor prognosis, which is even poorer if the macrophages develop into a suppressive, M2 phenotype.

M2 macrophages facilitate tumour growth and survival by immune suppression (via production of anti-inflammatory cytokines such as IL-10 and TGF-β), production of angiogenic factors (VEGF), and MMPs and cytokines to aid metastasis (reviewed in). Recently, pro-inflammatory cytokines have also been shown to play a detrimental role in cancer. In response to pathogenic stimuli, macrophages produce inflammatory mediators such as reactive oxygen and nitrogen species, and cytokines (such as TNFα, IL-1β, IL-6 and IL-8) leading to oxidative and nitrative DNA damage, enhanced cell growth and proliferation, inhibition of apoptosis and production of angiogenic factors (reviewed in). TNFα and IL-6 are associated with poor prognosis in several carcinomas, and inhibitors of both are in clinical trials for the treatment of cancer.

In the tumour environment, macrophages exhibit extraordinary plasticity, adapting their phenotype and effector functions in response to local stimuli. In general, tumour associated macrophages (TAMs) acquire an M2 like phenotype, but in colorectal cancer...
and non-small cell lung carcinoma, the infiltrating macrophages display a more pro-inflammatory, M1 like phenotype\textsuperscript{149,150}, and some even develop into a mixed M1/M2 phenotype\textsuperscript{151}. Macrophage polarisation however is not terminal; it has been demonstrated both \textit{in vivo} and \textit{in vitro} that macrophage polarisation can be reversed by exposure to opposing polarising factors\textsuperscript{152,153}. This plasticity can be exploited to develop anti-cancer therapies.\textsuperscript{152} One such mechanism proposed to overcome the immunosuppressive characteristics of TAMs and drive them to a more anti-tumoural activation state, is bacterial LPS. Indeed, LPS or analogues thereof, have been used in human clinical trials as an anti-cancer therapy\textsuperscript{155–158}. The effects of LPS on H357 cells is yet to be described.

OSCC and normal epithelial cells produce factors into the local microenvironment that affect macrophage behaviour and drive them to a particular activation state, which may be pro- or anti-tumoural. It is therefore necessary to investigate the effects of LPS on OSCC produced factors as they may influence macrophage behaviour in the microenvironment.

Evidently, TAM behaviour is far from clear; both pro- and anti-inflammatory mediators play a role in cancer, and macrophages spanning the spectrum of phenotypes have been described surrounding tumours. Thus, TAM polarisation is likely to be dictated by the tumour location and stage of progression. Because of this, it is vital that TAM behaviour is studied in the context of specific diseases, rather than making inferences from other cancer types.

Given the limited data on TAM behaviour in OSCC, and the lack of characterisation of cytokine responses in H357 cell, the present study had the following aims:

\begin{enumerate}
  \item To determine the cytokine response of H357 cells to \textit{P. gingivalis} LPS.
  \item To determine the effect of OSCC produced soluble factors on macrophage polarisation and subsequent responses to \textit{P. gingivalis} LPS.
\end{enumerate}
Results

H357 cytokine responses to *P. gingivalis* LPS

In this study, the response of H357 cells to *P. gingivalis* LPS was characterised in a time- or dose-dependent manner. Firstly, cytokine expression was measured in response to increasing doses of *P. gingivalis* LPS, at 0, 10, 100 and 1000µg/ml for 24 hours. H357 cells did not produce TNFα or IL-1β, either constitutively or in response to *P. gingivalis* LPS, but did produce high levels of IL-6 (Figure 5-1). Next, to see if time was a factor in cytokine production, H357 cells were incubated with 1 µg/ml *P. gingivalis* LPS for 6, 24 and 48 hours. Supernatants were harvested after stimulation and assayed for TNFα, IL-1β and IL-6 by ELISA. There was no difference in response over the time course (6, 24 or 48 hours) for TNFα or IL-1β or IL-6 (Figure 5-2).

![Figure 5-1: H357 dose response to *P. gingivalis* LPS.](image)

H357 cells were seeded at 5x10³ cells/cm² and grown to confluence. Culture media was removed and replaced with fresh media alone, or containing increasing doses of *P. gingivalis* LPS. After 24 hours incubation, supernatants were collected and stored at -20°C until assayed for cytokines by ELISA. Data represents mean ± standard deviation from two independent experiments performed in triplicate wells.
Figure 5-2: H357 cytokine response to *P. gingivalis* LPS time course. Culture media was removed from confluent H357 cells and replaced with fresh media containing 1 μg/ml *P. gingivalis* LPS for 6, 24 and 48 hours. Media without LPS served as a control. Data are representative of two independent experiments performed in triplicate wells ± standard deviation.
As neither time nor concentration of *P. gingivalis* affected cytokine release from H357 cells, the cells were tested for surface expression of the LPS receptors TLR2 and TLR4 by flow cytometry. Briefly, H357 cells were grown to confluence in 6 well plates and harvested using an enzymatic detachment solution (see methods and materials). Cells were washed in PBS and counted, and non-specific binding was blocked using 1% PBS/BSA for 30 minutes, on ice. Next, cells were washed and incubated with PE-conjugated anti TLR2 or TLR4 antibodies or an isotype matched control for a further 30 minutes in the dark, on ice (see methods and materials chapter 2: Flow cytometry p42).

**Surface expression of Toll-like receptors 2 and 4 on H357 cells**

![Flow cytometry histograms](attachment:image.png)

**Figure 5-3: TLR2 and TLR4 surface expression on H357 cells.** H357 cells were grown to confluence, harvested and assayed for TLR2 (a) and 4 (b) expression by flow cytometry. Histograms are representative of n=2 independent experiments. Mean fluorescence intensity (c) was normalised to isotype control and is representative of two independent experiments ± standard deviation. Student’s T-test was used to test for differences in MFI.
Both TLR2 and TLR4 were detected at low levels on the surface of H357 cells (Figure 5-3 a and b), suggesting whilst that they possess the receptor machinery needed to respond to LPS, the low levels may mean that any slight up-regulation in cytokine production induced by ligation of these receptors may go undetected. Compared with macrophages, levels of TLR expression a very low (see Figure 4-9). There is not a significant difference between levels of TLR2 and TLR4 expression (Figure 5-3 c, Figure 5-5, p=0.33). It has previously been reported that co culture with macrophages modulates the behaviour of cancer cells by increasing invasiveness via TNFα\(^{140}\). To see if co culture with macrophages could affect other properties of cancer cells, such as cytokine production, H357 cells were co cultured with PMA, M1 or M2 macrophages for 24 hours during exposure to \(P.\) gingivalis LPS in a trans-well co culture system (see methods and materials chapter 2: H357 and THP-1 co-culture experiments p38).

![Figure 5-4: IL-6 expression by macrophage co-cultured H357 cells.](image)

H357 cells were grown to confluence on trans-well inserts and exposed to 1 µg/ml \(P.\) gingivalis LPS in co culture for 24 hours. Supernatants were harvested and stored at -20°C until assay for cytokines by ELISA. Tests for statistically significant differences were performed using one way ANOVA.
Although perhaps a very slight increase in IL-6 production in response to *P. gingivalis* LPS, the only conditions where IL-6 increased significantly from control (no LPS) was during coculture with M1 (P=0.03), however, levels of IL-6 did not reach those of H357 cultured alone and challenged with LPS (P=0.533).

**Effects of H357 conditioned media on the macrophage cytokine responses to *P. gingivalis* LPS**

To measure the effects of factors produced by the OSCC cell line on the macrophage polarisation and subsequent response to *P. gingivalis* LPS, conditioned media from H357 cells was added to macrophage cultures during the polarisation process. Briefly, to generate conditioned media, H357 cells were grown to confluence in 25cm$^2$ flasks. At confluence, culture media was removed and replaced with fresh media. Cells were incubated for 24 hours and harvested into centrifuge tubes, where they were spun down to remove any cellular debris. The cell free supernatants were collected and stored at -20°C until needed for experiments. Conditioned media was then used in experiments to investigate whether factors produced by the H357 cells affected the response of macrophages to *P. gingivalis* LPS and the polarisation process. Macrophages were polarised in the presence or absence of H357 conditioned media and stimulated with 1 µg/ml *P. gingivalis* LPS for 24 hours. Cytokine production was measured by ELISA.

Compared to macrophages polarised in normal culture media (control), IL-1β (Figure 5-6) and IL-6 (Figure 5-7) production in response to *P. gingivalis* LPS was amplified in macrophages polarised in the presence of H357 conditioned media. This was observed in all polarisation conditions (PMA, M1 and M2: p<0.05). TNFα production, however, was not affected (Figure 5-5).

The addition of conditioned media alone was enough to up regulate IL-6 production from all macrophage phenotypes (p=0.001), however, this was around 10 fold lower than seen with
the addition of *P. gingivalis* LPS (Figure 5-8). The presence of H357 conditioned media alone did not effect TNFα or IL-1β production.

PMA, M1 and M2 macrophages retained their respective cytokine profiles, with M1 being the highest producers of TNFα, IL-1β and IL-6, therefore it is not likely that the conditioned media affected phenotypic polarisation *per se*, but the same amplification of cytokines production was seen across all phenotypes.
Figure 5-5: TNFα production in MØ, M1 and M2 macrophages polarised in the presence or absence of H357 conditioned media. Macrophages were polarised according to the usual protocol, in the presence or absence of H357 conditioned media for 24 hours. Cells were then stimulated with P. gingivalis LPS for a further 24 hours. TNFα production was measured by ELISA. Data represent mean of n=3 independent experiments ± standard deviation. Tests for significant differences between control media and conditioned media were conducted using Student's T test.
Figure 5-6: IL-1β production in MØ, M1 and M2 macrophages polarised in the presence or absence of H357 conditioned media. Macrophages were polarised according to the usual protocol, in the presence or absence of H357 conditioned media for 24 hours. Cells were then stimulated with *P. gingivalis* LPS for a further 24 hours. IL-1β production was measured by ELISA. Data represent mean of n=3 independent experiments ± standard deviation. Tests for significant differences between control media and conditioned media were conducted using Student’s T test.
Figure 5-7: IL-6 production in MØ, M1 and M2 macrophages polarised in the presence or absence of H357 conditioned media: IL-6 production in MØ, M1 and M2 macrophages polarised in the presence or absence of H357 conditioned media. Macrophages were polarised according to the usual protocol, in the presence or absence of H357 conditioned media for 24 hours. Cells were then stimulated with *P. gingivalis* LPS for a further 24 hours. IL-6 production was measured by ELISA. Data represent mean of n=3 independent experiments ± standard deviation. Tests for significant differences between control media and conditioned media were conducted using Student’s T test.
Figure 5-8: IL-6 production induced by conditioned media alone. Macrophages were polarised according to the usual protocol, in the presence or absence of H357 conditioned media for 24 hours then incubated with control media alone for a further 24 hours. Supernatants were harvested and assayed for IL-6 by ELISA. Data represent mean of n=3 independent experiments ± standard deviation. Tests for significant differences between control media and conditioned media were conducted using Student’s T test.
To see if the amplification of cytokine production in response to *P. gingivalis* LPS was dose dependant, THP-1 cells were polarised to their respective phenotypes in the presence of decreasing dilutions of conditioned media. Neat or diluted H357 conditioned media (1/5, 1/25 and 1/125) was added to the THP-1 cells during polarisation. Macrophages polarised in normal growth media (in the absence of conditioned media) served as controls. After completion of the polarisation process (24 hours), polarising media was removed, the cells were washed 3 times in PBS and replaced with fresh media (normal growth media) with or without 1 µg/ml *P. gingivalis* LPS and incubated for 24 hours. Cell free supernatants were harvested and assayed for IL-6 by ELISA.

Levels of IL-6 increased in line with increasing concentrations of conditioned media (Figure 5-9). This was universal across all macrophage cell types. As seen in the previous experiment (Figure 5-8), conditioned media alone was enough to induce IL-6 production from the macrophages without the need for stimulation by LPS.
Figure 5-9: IL-6 response to *P. gingivalis* LPS in when polarised with a series of dilutions of H357 conditioned media. Conditioned media at increasing concentrations (0, 1/125, 1/25, 1/5 and neat) were added to macrophages during the polarisation phase then challenged with *P. gingivalis* LPS. Unstimulated macrophages served as negative controls.
Epithelial cells have been shown to respond differentially to different bacterial species\textsuperscript{281–284}. To assess whether the amplification of cytokine production was an effect specific to \textit{P. gingivalis}, the experiment was repeated, this time including enterobacterium \textit{E. coli} LPS as a comparison. As previously found under normal polarisation conditions, stimulation of macrophages with \textit{E. coli} LPS resulted in higher cytokine production than stimulation with \textit{P. gingivalis} LPS. This pattern was repeated in macrophages polarised in H357 conditioned media. As seen with the \textit{P. gingivalis} LPS, IL-6 and IL-1β were both up-regulated beyond that of levels produced by normally polarised macrophages when they were polarised in the presence of H357 conditioned media (Figure 5-10). There was no amplification of TNFα production (data not shown). The amplification of cytokine production was not due to LPS in the conditioned media, as determined by LAL assay (data not shown).

At the end of the experiment, a Trypan blue exclusion assay was carried out to confirm cell viability following treatment with conditioned media / LPS. Briefly, following removal of supernatant, cells were washed three times in PBS and incubated with Accutase to detach them from the wells. Triplicate wells were pooled and counted by Trypan blue exclusion using a haemocytometer. Viability consistently remained over 97% throughout all the treatments (Table 5-1). Interestingly, incubation with conditioned media resulted in an increase in cell number compared to incubation with R10 (Figure 5-11) Moreover, the increase in cell number was higher in the M2 macrophages than the M1 macrophages. The addition of LPS resulted in a less marked increase in cell number, although it was still above that of R10 polarised cells, with \textit{E. coli} LPS reducing less than \textit{P. gingivalis} LPS.
Figure 5-10: Response to E. coli LPS following polarisation in H357 conditioned media. PMA, M1 and M2 macrophages were polarised in the presence or absence of H357 conditioned media and stimulated with P. gingivalis or E. coli LPS. Unstimulated macrophages served as controls.
Table 5-1: Viability count of cells polarised in the presence or absence of H357 conditioned media by Trypan blue exclusion

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Polarised with:</th>
<th>Stimulus</th>
<th>% Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>R10</td>
<td>Ctrl</td>
<td>98.46154</td>
</tr>
<tr>
<td>M1</td>
<td>R10</td>
<td>PgLPS</td>
<td>97.05882</td>
</tr>
<tr>
<td>M1</td>
<td>R10</td>
<td>EcLPS</td>
<td>100</td>
</tr>
<tr>
<td>M2</td>
<td>R10</td>
<td>Ctrl</td>
<td>100</td>
</tr>
<tr>
<td>M2</td>
<td>R10</td>
<td>PgLPS</td>
<td>98.85057</td>
</tr>
<tr>
<td>M2</td>
<td>R10</td>
<td>EcLPS</td>
<td>100</td>
</tr>
<tr>
<td>M1</td>
<td>CM</td>
<td>Ctrl</td>
<td>98.34711</td>
</tr>
<tr>
<td>M1</td>
<td>CM</td>
<td>PgLPS</td>
<td>100</td>
</tr>
<tr>
<td>M1</td>
<td>CM</td>
<td>EcLPS</td>
<td>97.05882</td>
</tr>
<tr>
<td>M2</td>
<td>CM</td>
<td>Ctrl</td>
<td>100</td>
</tr>
<tr>
<td>M2</td>
<td>CM</td>
<td>PgLPS</td>
<td>99.57082</td>
</tr>
<tr>
<td>M2</td>
<td>CM</td>
<td>EcLPS</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 5-11: Difference in cell number following culture with H357 conditioned media. THP-1 cells were polarised to M1 or M2 phenotypes in the presence or absence of H357 conditioned media for 24 hours. Cells were removed from triplicate wells, pooled, and counted using Trypan blue exclusion. Data are representative of n=1 experiments.
Effects of primary OSCC conditioned media on the macrophage cytokine responses to P. gingivalis LPS

It has been argued that cell lines do not accurately replicate the behaviour of primary cells\textsuperscript{285}. To confirm whether the amplification of cytokine production seen by H357 cell line conditioned media also occurs in response to primary tissues, the experiments were repeated using conditioned media from excised human OSCC tumours.

Freshly isolated OSCC specimens (soft palate/tonsil, posterior maxilla and floor of mouth) were washed 3 times in growth media and cultured in 1ml growth media for 48 hours. Supernatants were harvested and cell debris was removed by centrifugation. Condition media from culture supernatants was added to macrophages during the polarisation phase, as per previous experiments. After 24 hours incubation, conditioned media was removed, cells were washed 3 times with PBS and fresh media was added to the cells with or without P. gingivalis LPS. Supernatants were harvested after 24 hours and assayed for TNFα by ELISA.
Figure 5-12: TNFα production by MØ, M1 and M2 macrophages polarised in the presence of primary OSCC sample conditioned media. Macrophages were polarised in the presence or absence of a series of dilutions of OSCC conditioned media for 24 hours, then challenged with *P. gingivalis* LPS for a further 24 hours. Data represent the mean of n=3 independent experiments ± standard deviation.
In response to stimulation by *P. gingivalis* LPS, MØ and M2 macrophages polarised in the presence of OSCC conditioned media exhibited amplified TNFα production compared with macrophages polarised in normal growth media. However, this did not reach statistical significance. There was no change in TNFα production in the M1 macrophages polarised with OSCC conditioned media in response to *P. gingivalis* LPS compared with M1 macrophages polarised in normal growth medium (Figure 5-12).

Amplification of cytokine production in response to *P. gingivalis* LPS by macrophages polarised in the presence of conditioned media, and potentially primary OSSC specimens, would suggest that OSCC cells (primary or cell line) produce factors that affect macrophage cytokine production. Thus, primary OSSC sample supernatants were assayed for an array of cytokines by ELISA. Excised gingival epithelium was used as a non-cancerous control tissue. Freshly isolated tumour and control tissues were washed 3 times in media and cultured for 48 hours in 1ml fresh media. Supernatants were harvested and cellular debris was removed by centrifugation, and then stored at -80°C until assay for IL-10, TGF-β, TNFα, IL-15, IL-6 and IL-8 by ELISA.

Control tissues produced higher levels of all cytokines than the OSCC tissues, except IL-5, which was produced in higher quantities in the tumour tissue (p= 0.017). Neither control tissue nor tumour tissue produced any TNFα, but did produce IL-6, reflecting the behaviour of the H357 cells.
Figure 5-13: Consitutive production of cytokines in primary OSCC samples versus control tissue (gingiva). Samples of OSCC or gingiva were dissociated and cultured for 48 hours. Cytokine production was measured by ELISA. Data represent mean ± standard deviation from n=6 samples (6 OSCC vs 6 control samples).


Discussion

*P. gingivalis* has been associated with OSCC: it is present in higher numbers on transformed mucosa than healthy mucosa, it has been isolated from OSCC tumour cores and it invades squamous carcinoma cells 33% more than healthy tissues \(^1,119,219\). The cell line H357 has been used to investigate pathological mechanisms in OSCC \(^263–265\), but the response of these cells to *P. gingivalis* has not been investigated. Since OSCC cells and *P. gingivalis* are likely to come into contact *in vivo*, the first part of this study was designed to assess the cytokine response of H357 to a major virulence factor of *P. gingivalis*: LPS.

Following 24 hours stimulation with *P. gingivalis* LPS, there was no TNFα or IL-1β production in the H357 cells, either constitutively or in response to LPS. Previous studies using SCC cell lines have described a similar lack of TNFα or IL-1β production\(^286\). The same pattern has been reported using healthy excised gingival multilayers cultured *in vitro* \(^215\). In fact, Huang *et al* showed that IL-8 and ICAM-1 expression was down-regulated in response to *P. gingivalis* \(^274\). On the other hand, studies have shown that primary gingival epithelial cells do produce IL-8, MCP-1, TNFα and IL-1β in response to *P. gingivalis* LPS \(^271,287\).

Here, in contrast to TNFα and IL-1β, IL-6 was produced constitutively (between 1836-4430 pg/ml), in line with previous studies on squamous cell carcinoma cell lines\(^288\). Whilst potentially indicating a dose-response, this is not enough to conclude that the H357 cells are responsive to *P. gingivalis* LPS and requires further investigation. Furthermore, the 2006 Hagemann *et al* study showed no IL-6 production in response to *P. gingivalis* in another SCC cell line \(^286\).

It remains to be confirmed whether oral epithelial cells produce cytokines in response to bacterial challenge. There have been many different experimental set ups; primary epithelial cells or cell lines, viable or non-viable *P. gingivalis*, yet no consensus has been reached.
regard cytokine responses to *P. gingivalis* in epithelial cells. Data from the present study shows that H357 cells, at least, do not respond to *P. gingivalis* LPS.

In order for LPS to initiate pro-inflammatory cytokine production, it has to bind to its receptor on the surface of the cell, in a complex with CD14, LBP and MD2\(^{11}\). To see if the unresponsiveness to LPS seen in the present study was due to a lack of LPS receptors on the H357 cells, TLR2 and TLR4 expression was measured by flow cytometry. H357 cells were both TLR2 and TLR4 positive. There was no significant difference in expression between the TLRs. Concurrent with these results, human gingival epithelial cells have been shown to express TLR2 and TLR4\(^{289-291}\). The presence of LPS receptors TLR2 and TLR4 on the surface of H357 cells with a concurrent unresponsiveness to *P. gingivalis* LPS throws up a number of suggestions:

- Expression levels of TLR2 and TLR4 are low compared with macrophages (Figure 4-9). It may be that the levels of TLRs present on the surface of the H357 are not high enough to induce a cytokine response to LPS.
- Epithelial cells have been shown not express LPS co-receptor, CD14\(^{18,289}\). It may be that the cells are not responding to LPS because of lack of co-receptors.
- *P. gingivalis* up-regulates negative regulator of cytokine expression IRAK-M in epithelial cells\(^{292}\). There may be down-stream inhibition of cytokine production in response to *P. gingivalis* LPS TLR ligation.
- It has been reported that gingival epithelial cells up regulate TNFα, IL-1β and other cytokines in response to whole dead, *P. gingivalis*\(^{271,284}\). It may be that epithelial cells respond to other virulence factors of *P. gingivalis* beside LPS.
- Uehara *et al* (2002) demonstrated that for epithelial cells to become responsive to LPS, they first had to be primed by IFNγ\(^{15}\). It may be that epithelial cells need to interact with immune cells to become responsive to LPS.
To address this final point in our system, H357 and THP-1 cells were cultured together in a trans-well apparatus: H357 cells were grown on a culture insert and macrophages were grown in the well plate allowing cell-cell cross talk during the polarisation stage. Hagemann et al (2005) reported that co culture with macrophages modulated the behaviour of cancer cells and activated inflammatory JNK and NF-κB pathways via TNFα, which in turn lead to increased tumour invasiveness. In the present study, co culture with macrophages did not affect the H537 cytokine response to P. gingivalis LPS. The effects of co culture on H357 invasiveness was not tested, but presents an avenue for investigation in the future. The present study focused on the effects of H357 produced soluble factors on macrophage responses to P. gingivalis.

When THP-1 cells were polarised in the presence of H357 cell conditioned media, macrophage production of IL-1β and IL-6 was amplified in response to P. gingivalis LPS compared to THP-1 cells polarised in their normal media. This pattern of amplification was seen across the phenotypes (PMA, M1 and M2). In line with these results, a study by Caras et al, used conditioned media from colorectal cancer cell supernatants to polarise PMA treated THP-1, and found that they up-regulated TNFα, IL-6 and IL-1Ra production, generating a macrophage population with a mixed M1/M2 phenotype. When the experiment was repeated with an adenocarcinoma cell line, they found the same up regulation of TNFα, but not of IL-6 or IL-1Ra. Another study shows that in renal cell carcinoma, TAMs have a higher basal level of IL-6, IL-1β and TNFα but respond less vigorously to LPS challenge. The present study indicated the opposite; that conditioned media had no effect on TNFα production, and a greatly amplified IL-6. IL-1Ra however was not measured. Interestingly, the Caras study, and study by Stewart et al, which used breast cancer cell line supernatants that up-regulated TNFα, IL-6, RANTES and MCP-1 production, did not use LPS to stimulate the macrophages; the up regulation in cytokine production in the cells was due to factors in the conditioned media only. In the present study, it was only IL-6 that was up-regulated without the need for stimulation by LPS. This
suggests that different cancers manipulate local macrophages in ways specific to that tumour.

In the present study, cross talk between the THP-1 and H357 cells made no difference to the response of either cell type to \textit{P. gingivalis} LPS. In contrast, Hagemann \textit{et al} (2006) showed that effects on macrophage polarisation by epithelial cells differed when macrophages were exposed to SCC soluble factors via indirect co-culture or conditioned media\textsuperscript{286}. During co-culture, TNF\textgreek{a}, IL-1\textbeta and IL-10 (along with other cytokines) were increased. The unstimulated epithelial cells did not express TNF\textgreek{a}, IL-10, IL-12 and IL-1\textbeta, as concurrent with the present study.

To investigate whether the amplification of cytokine production was specific to \textit{P. gingivalis}, the experiments were repeated using \textit{E. coli} LPS. The same pattern of cytokine amplification was seen in response to \textit{E. coli} LPS as to \textit{P. gingivalis} LPS, in fact, the amplification was greater. \textit{E. coli} LPS promotes a more vigorous response than \textit{P. gingivalis} LPS\textsuperscript{197}, as described in previous chapters.

Pro-inflammatory cytokine expression by H357 cells had not, until now, been characterised. In this study, H357 cells did not produce TNF\textgreek{a} or IL-1\textbeta, but did produce constitutively high levels of IL-6. IL-6 can be either inflammatory or anti-inflammatory, depending on its mode of signalling (reviewed in \textsuperscript{295}) and the cell type it elicits its effects on. It can induce the production of soluble TNF receptor p55 and IL-1Ra\textsuperscript{296}, and inhibit TNF\textgreek{a} and IL-1\textbeta production in mononucleocytes\textsuperscript{297}. On the other hand, IL-6 has been shown to increase proliferation, cell growth and inhibit apoptosis in squamous cell carcinoma cell lines\textsuperscript{298,299}. Thus, inducing local macrophages to produce IL-6, as seen in these results, works in favour of the tumour.

Given the evidence supporting the infiltration of anti-inflammatory M2 macrophages in OSCC, and the immunosuppressive nature of tumours in general, it seems unusual for pro-inflammatory cytokines to be up-regulated in macrophages in the present study.
Nevertheless, inflammatory cytokines in the tumour microenvironment can play a role in the pathological process of cancer; TNFα has been shown to up regulate E selectin in endothelial cells, thus facilitating metastasis, and IL-1β acts in an autocrine manner to up-regulate TNFα production in SCC. IL-6 plays a pluripotent and complicated role in cancer, and has both inflammatory/anti tumoural and anti-inflammatory/pro-tumoural effects. It remains to be clarified whether anti-inflammatory cytokines are affected by H357 conditioned media. Whilst there were lower levels of inflammatory cytokines in the primary OSCC samples than the control samples, the conditioned media from both primary and H357 cell lines up-regulated inflammatory cytokine production in response to LPS. A previous study reported that levels of salivary IL-6 and IL-1β are detected in OSCC patients vs. control patients, but TNFα was not elevated. The cytokines amplified by OSCC conditioned media are the same cytokines found in elevated levels in OSCC patient saliva.

To confirm whether H357 cells behaved like OSCC in vivo, MØ and M2 macrophages were polarised in the presence of primary human OSCC conditioned media. In contrast to H357, conditioned media, OSCC conditioned media-polarised macrophages exhibited amplified TNFα production. However, this did not reach statistical significance. This may have been down to the inter-patient variability of the clinical samples, and the low number of experimental replicates (n=3). Furthermore, a difference in response to primary samples versus cell lines may arise from the fact that primary samples are not a homogenous population of cells, and are likely to contain a multitude of cell types. Therefore, the H357 cell line makes a viable model for studying cell specific interactions. As seen in the H357 conditioned media experiments, there was no change in TNFα production in the M1 macrophages polarised with OSCC conditioned media in response to P. gingivalis LPS compared with M1 macrophages polarised in normal growth medium (Figure 5).

Finally, we sought to determine if OSCC produced factors that may module macrophage behaviour. Primary samples of OSCC and excised gingival mucosa (from routine wisdom tooth extractions) were cultured for 48 hours and the supernatants were analysed for an
array of cytokines. Whilst OSCC tissues expressed most of the cytokines assayed for, almost all (bar IL-5) were expressed more highly in the control tissues. Neither control tissue nor tumour tissue produced any TNFα, but did produce IL-6, reflecting the behaviour of the H357 cells. In contrast to previous studies, IL-1β, IL-6 and TGF-β expression was lower than in control samples\textsuperscript{52,304}. This discrepancy may be down to small sample sizes and variation between laboratories and culture techniques, or the increased level of pro-inflammatory cytokines in the control tissue in the present study may be a result of inflammation in infected wisdom tooth.

Polarisation with conditioned media generated an unexpected result during viability tests. M2 macrophages cultured in conditioned media were increased in cell number compared to M2 cultured in R10. Furthermore, M2 increased in cell number to a greater extent than M1. If this were the case \textit{in vivo}, it may be that cancer cells promote the proliferation of M2 cells preferentially over M1 cells in an effort to aid tumour survival. Nevertheless, Trypan blue exclusion is not the optimal assay for cell enumeration; a more relevant protocol would be to use a proliferation assay kit, such as WST-1 to measure proliferation of each macrophage subset directly in the wells. It may be that not all the cells were harvested by the enzyme detachment and therefore cell count may not be accurate. Still, it is an interesting proposition given the association of high numbers of M2 macrophages commonly found in in tumour microenvironments. Further investigation to identify factors responsible for M2 proliferation is required. H357 cells produce high levels of TGF-β\textsuperscript{269}, which has been shown to induce proliferation in macrophages\textsuperscript{305}

Up-regulation of inflammatory cytokines promotes SCC metastasis. Results from the present study reveal that H357 cells up regulate pro-inflammatory cytokine production in surrounding macrophages, irrespective of their phenotype, most likely as a mechanism to promote
metastasis. The cytokines up-regulated by polarisation with conditioned media are those found to be elevated in OSCC sites.

In conclusion, this study shows for the first time that:

1) H357 do not produce TNFα or IL-1β constitutively
2) H357 produce high levels of IL-6 constitutively
3) H357 do not respond to *P. gingivalis* LPS
4) H357 and primary OSCC conditioned media amplifies macrophage cytokine production in response to *P. gingivalis* LPS.
Pathological interactions between oral bacteria and polarised macrophages have been implicated in a range of oral and systemic diseases. When monocytes are recruited to sites of inflammation, they differentiate into macrophages and polarise within a spectrum of activation states. Depending on the factors they encounter in their microenvironment, macrophages can be activated to an inflammatory, M1 phenotype or an anti-inflammatory, M2 phenotype. Macrophages infiltrate areas of inflammation, such as periodontal lesions and tumours, where aberrant M1 and M2 polarisation has been associated with disease development. Porphyromonas gingivalis, a pathogenic oral bacterium, has been identified both in periodontal lesions and oral squamous cell carcinomas. In OSCC tissues, macrophages have been shown to polarise to an M2 state. The polarisation state of macrophages in periodontitis is yet to be characterised, but it is likely that during the acute phase of inflammation, macrophages take on an M1 phenotype in order to eradicate invading pathogens. Later in disease progression, macrophages may switch to an M2 phenotype in an attempt to resolve inflammation. Indeed, M1 associated Th1 cells are associated with (early) stable periodontal lesions, while M2 associated Th2 cells are associated with progressive lesions. Thus, macrophages in a variety of differentiation and polarisation states are likely to come into contact with P. gingivalis. The present study set out to investigate these interactions with two core aims:

1) Effects of P. gingivalis LPS on M1 and M2 polarisation, and cytokine response to subsequent LPS challenge
2) Effects of OSCC cell conditioned media on M1 and M2 polarisation, and cytokine response to subsequent LPS challenge

The first step was to develop a model of M1 and M2 macrophage polarisation in vitro using THP-1 cells. PMA and vitamin D3 are commonly used to stimulate macrophage...
PMA activates the PKC signalling pathway and is a potent inducer of inflammation, whereas Vitamin D3 activates the M2 associated PI3K pathway and exhibits anti-inflammatory properties. It was thus hypothesised that PMA and Vitamin D3 could be used to generate M1 and M2 macrophages, respectively. However, when this method was tested, PMA and Vitamin D3 treated cells did not display typical M1 and M2 cytokine profiles in response to LPS stimulation. In response to LPS, Vitamin D3 treated cells produced markedly higher levels of IL-1β and IL-6 than PMA treated cells, and only marginally less TNFα. PMA treated cells became strongly adherent to the culture plates (a marker of macrophage differentiation), but Vitamin D3 treated cells remained mostly in suspension, indicative of incomplete differentiation. A previous study by Daigneault et al (2010) also assessed the ability of PMA and Vitamin D3 to generate macrophages. They reached the same conclusion; Vitamin D3 treatment leads to a poorly differentiated population of cells. Previous reports indicate that PMA treatment leads to development of macrophages with an M2-like phenotype. Our results, and others corroborate this: In the present study, PMA and IL-4 consistently displayed a similar cytokine profile. It may be that in the absence of M1 polarising factors, macrophages will default to an M2 phenotype to avoid excessive inflammatory responses.

In light of these findings, a polarisation method was adapted from a previous study by Tjiu et al (2009). The Th1 and Th2 cytokines IFNγ and IL-4, in addition to PMA, were used to further push the macrophages along their respective polarisation pathways. This method of polarisation resulted in the stable and reproducible production of signature M1 and M2 cytokine profiles in response to LPS; IFNγ treatment generated TNFα, IL-1β, IL-6 high/IL-10, TGF-β low expressing cells, whereas IL-4 treatment generated TNFα, IL-1β, IL-6 low/IL-10, TGF-β high expressing cells. Taken together, these data confirm that using IFNγ and IL-4 with PMA generates a population of cells better resembling M1 and M2 macrophages than
cells treated with PMA or Vitamin D3 alone, and that this is an effective method for generating M1 and M2 macrophages in vitro.

In addition to IFNγ, LPS is commonly used to polarise macrophages to an M1 phenotype. LPS is renowned for its stimulation of pro-inflammatory cytokine production in a range of cells. Before the present study, research into the effect of LPS on the ability of IFNγ or IL-4 to polarise macrophages had not been conducted. Furthermore, no studies had polarised macrophages using LPS from *P. gingivalis*. To address this, the newly established model of M1 and M2 polarisation described above was used to polarise THP-1 cells in combination with *P. gingivalis* LPS. *E. coli* LPS, used in previous in polarisation protocols, was included as a comparison. Non-polarised macrophages (PMA only) were used to assess the effects of LPS on macrophage polarisation in the absence of IFNγ and IL-4. The addition of LPS from *P. gingivalis* or *E. coli* during macrophage polarisation resulted in a down-regulation of pro-inflammatory cytokine production in response to a subsequent challenge with LPS, even in the presence of IFNγ. This raises an interesting point; using LPS along with IFNγ to generate M1 macrophages may be dampening the M1 pro-inflammatory cytokine response, rendering the macrophages less M1-like than when polarised with IFNγ alone. Moreover, LPS in combination with immune complexes or IL-1β polarises macrophages to an M2b phenotype. If immune complexes/IL-1β are present in macrophage environment, this may affect the direction of polarisation. Thus, data from the present study suggests that the addition of LPS during polarisation may not be the most effective method of generating M1 macrophages.

One explanation for the decreased cytokine production may be that TLR2 and TLR4 are down-regulated in response to LPS. In the present study, MØ and M1 macrophage TLR expression decreased following polarisation with *P. gingivalis*, in contrast to a previous study showing that TLR2 expression was up-regulated on THP-1 cells after 24 hours incubation with *P. gingivalis* LPS. Here however, TLR expression levels were increased in the M2 cells, with a concurrent down-regulation of cytokine production. This indicates that
there is another mechanism at play for the down-regulation of cytokines. Nevertheless, difference in levels of TLR modulated by *P. gingivalis* did not reach statistical significance, probably due to variance between samples. Therefore, further replications of the experiment are needed to confirm these findings.

*In vivo*, reduction of pro-inflammatory cytokine expression by macrophages in response to prolonged or repeated exposure to LPS may have either beneficial or detrimental effects in oral health and pathology. In the early stages of OSCC development for example, a pro-inflammatory environment might be benefit the host and promote an anti-tumour response. However, at later stages of disease, pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 may benefit tumour progression and facilitate metastasis. In normal mucosal tissues, down-regulation of cytokine production in response to repeated exposure to LPS is a mechanism employed by macrophages and dendritic cells to avoid over-zealous inflammation that would result in tissue damage – a phenomenon known as endotoxin tolerance. Several studies have assessed mechanisms of endotoxin tolerance in response to *P. gingivalis* LPS, however, each study reported different results regarding the ability of *P. gingivalis* to induce a tolerogenic state. This may be born from several differences in protocols; cell treatments, cell types and LPS structures differed between all the experiments.

Another explanation for the down-regulation of pro-inflammatory cytokines is that LPS is skewing the macrophages polarisation to an M2-like phenotype. It has in fact been argued that endotoxin tolerised cells exhibit an M2 profile. *Porphyromonas gingivalis* has been shown to activate the PI3K pathway associated with alternative macrophage polarisation and negative regulation of M1 markers, up-regulate expression of both tolerance and M2 associated molecules and negative regulator of TLR signalling, IRAK-M. These anti-inflammatory mechanisms exhibited by *P. gingivalis* LPS potentially explain the cytokine down-regulation seen in the present study. The effects of *P. gingivalis* LPS on expression of other negative regulators of TLR signalling, such as PPARγ, TOLLIP, SHIP are yet to be investigated and provide an interesting avenue for further investigation.
In the present study, a lower concentration of *E. coli* LPS was able to down regulate cytokine production in response to cross stimulation with different LPS, than *P. gingivalis* LPS. Whilst the present study did not directly address the question of endotoxin tolerance, the results would suggest that *E. coli* LPS more effectively down-regulates cytokine production than *P. gingivalis* LPS, in line with previously published data. Therefore, it is surprising that these M2-associated, regulatory mechanisms are up-regulated more strongly by *P. gingivalis* LPS than *E. coli* LPS, as *E. coli* down-regulates cytokine production to a greater extent than *P. gingivalis*. Furthermore, pro-inflammatory cytokine production was also down-regulated in M2 macrophages, albeit in a different way to M1. Taken together, these data suggest that down-regulation of cytokine production is likely due to endotoxin tolerance rather than M2 skewing.

Further investigation is needed to assess the levels of tolerance and M2 characteristic markers induced by polarisation with *P. gingivalis*, as the present study only measured effects on pro-inflammatory cytokine production. Whilst previous studies have been conducted into the mechanisms of endotoxin tolerance in macrophages, none so far have investigated the mechanisms of tolerance in M2, if indeed they can be tolerised at all. Thus, this is the first time that a potential tolerogenic mechanism has been described in M2 macrophages.

Whilst polarisation in the presence of LPS down-regulates cytokine production in response to secondary stimulus with LPS, polarisation with OSCC supernatants results in the exact opposite effect. Polarisation of macrophages in the presence of OSCC cell conditioned media led to the amplification of M1-associated cytokine production in response to LPS. This is surprising, given that OSCC TAMs have been shown to take on an M2-like profile. Furthermore, it has been demonstrated that TGF-β, produced in high levels in H357, up-regulates expression of negative regulator of TLR signalling, IRAK-M.
There is no consensus on whether *P. gingivalis* LPS signals through TLR2 or TLR4. *Porphyromonas gingivalis* LPS has been shown to signal via TLR2, a TLR1/2 heterodimer in concert with CD14 and LBP, TLR4, TLR2 and TLR7 or both TLR2 and TLR4. LPS purification methods have been suggested as a reason for this discrepancy, with contaminating proteins utilising other TLRs. Furthermore, bacterial viability is thought to account for some of these discrepancies, as purified *P. gingivalis* LPS signals through TLR2, but live *P. gingivalis* signals through both TLR2 and TLR4. In the present study, this was investigated using blocking antibodies to TLR2 and TLR4. For TNFα and IL-1β production in M1 polarised macrophages, *P. gingivalis* LPS required TLR2. For IL-26 production however, M1 macrophages could utilise TLR2 and TLR4. These data suggest that in the present model, *P. gingivalis* LPS predominantly uses TLR2 to induce inflammatory cytokine production, with the exception of IL-6, which can be induced by both TLR2 and 4. Whether this is down to the purification method or lipid A structure remains to be elucidated.

Primary OSSC samples and non-cancerous gingival mucosal samples (control) were screened for an array of cytokines: IL-10, TGF-β, TNFα, IL-15, IL-6 and IL-8. It must be born in mind that primary tissue samples contain a mixed population of cells, some of which may have produced the cytokines measured in the supernatants. Because of this, H357 provide a good model for investigating cell-specific interactions.

Whilst all of the cytokines were expressed in both tumour and control tissues, only IL-15 was expressed at higher levels in the tumour samples than the control samples; all the rest were expressed at higher levels in the control tissue. TNFα was not expressed by either sample, reflecting the behaviour of the H357 cells. TNFα has anti-tumour properties, including induction of apoptosis. It may be that tumour cells down-regulate TNFα production in order to promote survival. However, there was no production of TNFα in the control samples either, suggesting that epithelial cells in general do not produce TNFα.
The cytokines produced by OSCC samples may have differing effects on the local macrophages. IL-10 and TGF-β have been shown to polarise macrophages to an M2 phenotype. Tumour samples produced both of these M2 polarising factors, yet in response to conditioned media, macrophages up-regulated production of M1 associated cytokines. IL-10 and TGF-β were produced at higher levels in control samples than tumour samples. It may be that in the case of OSCC, a less immunosuppressive environment is beneficial to the tumour.

IL-15 is a T cell growth factor, similar to IL-2, that was discovered independently by two different groups. It stimulates B cell immunoglobulin synthesis, cellular proliferation and the generation of cytotoxic T cells, and NK cells. IL-15 has little effect on regulatory T cells however, suggesting an anti-tumour role for this cytokine. Macrophages express the IL-15 receptor, and in response to its ligation, they increase phagocytic activity and up-regulate IL-12, MCP-1 IL-6, IL-8 and TNFα expression, thus making IL-15 a potential candidate for causing the cytokine amplification seen in the present study.

Although typically thought of as a pro-inflammatory cytokine, IL-6 can exhibit anti-inflammatory, pro-tumoural effects, such as induction of soluble TNF receptor p55 and IL-1Ra production, and inhibition of TNFα and IL-1β production in mononucleocytes. IL-6 has also been shown to increase proliferation, cell growth and inhibition of apoptosis in squamous cell carcinoma cell lines, and has been implicated in tumour multi-drug resistance. Thus, the high constitutive levels of IL-6 production by OSCC cells with a concomitant up-regulation of IL-6 in macrophages may be an attempt by the tumour to promote an environment permissive to its survival. The role of IL-6 in the amplification of macrophage cytokine production provides and promising target for further investigations into the mechanisms of this phenomenon. A full screening of H357 supernatants is needed to identify other targets for further investigation.
One such potential target for investigation is IL-17. Recent studies have implicated IL-17 in OSCC; IL-17 and its receptors are present at higher levels in PMNs and PBMCs from OSCC patients than control, and Foxp3+ IL-17+ CD4+ T cells infiltrate OSCC tumours \(^{50,329}\). Furthermore, it has been shown to up-regulate TNFα and IL-1β in macrophages \(^{330,331}\), and IL-6 and IL-8 in gingival fibroblasts \(^{331,332}\) and rheumatoid synovial cells \(^{333}\). Macrophages express IL-17 receptor \(^{334}\), thus it can be hypothesised that if IL-17 is present in the conditioned media, it could be responsible for the cytokine amplification seen in the present study.

Manipulation of macrophage function in pathological scenarios is as potential target for therapy. Tumour produced factors influence the effector functions of macrophages in the microenvironment. As such, it serves that if tumour cytokine expression can be modulated, this in turn could modulate macrophage function. The cell line H357 has been used to investigate pathological mechanisms in OSCC \(^{263–265}\), but the response of these cells to \(P.\ gingivalis\) has not been investigated. Since OSCC cells and \(P.\ gingivalis\) are likely to come into contact \(in\ vivo\), a study was designed to assess the cytokine response of H357 to \(P.\ gingivalis\): LPS.

First, to assess whether H357 had the capacity to respond to LPS, the expression of LPS receptors TLR2 and TLR4 were measured on the cell surface by FC. H357 cells were positive for both TLR2 and TLR4, although there was no significant difference in expression between the TLRs. In line with the current findings, human gingival epithelial cells have also been shown to express TLR2 and 4 \(^{289–291}\), hence loss of TLR expression does not appear to be affected by cancerous transformation in the H357 cells. Secondly, after the expression of LPS receptors was confirmed, cytokine production in response to \(P.\ gingivalis\) LPS was addressed. In accordance with previously published data \(^{215,286}\), there was no TNFα or IL-1β production in the H357 cells, either constitutively or in response to LPS. IL-6 was produced constitutively, as reported in previous studies on squamous cell carcinoma cell lines \(^{286,288}\). TNFα, IL-1β and IL-6 production was not affected by the addition of LPS, despite the
expression of LPS receptors on the cell surface. However, it remains to be confirmed whether H357 cells express LPS co-receptor CD14, or the intracellular signalling pathways that lead to cytokine production.

In contrast to the current findings, other studies have shown that primary gingival epithelial cells do produce IL-8, MCP-1, TNFα and IL-1β in response to *P. gingivalis* LPS\(^{271,287}\). Therefore, it remains to be confirmed whether oral epithelial cells produce cytokines in response to bacterial challenge. Data from the present study shows that H357 cells, at least, do not respond to *P. gingivalis* LPS.

Hagemann *et al* (2006)\(^{286}\) showed that SCC cytokine expression was increased when macrophages were polarised by exposure to SCC soluble factors in a trans-well set up, but not in response to exposure via conditioned media\(^{286}\), indicating that macrophage/SCC cross talk was vital for cytokine up-regulation by SCC cells. In the present study however, trans-well co-culture had no effect on cytokine production in the H357 cells, suggesting that LPS unresponsiveness is a characteristic specific to these OSCC cells.

**Future directions and Closing remarks**

Given the pro-inflammatory and anti-inflammatory properties of LPS and tumour cell produced factors (respectively), it could be speculated that LPS polarising would up-regulate pro-inflammatory cytokine production, and tumour conditioned media polarising would down regulate pro-inflammatory cytokine production. Surprisingly, the opposite was true. It could be that in both cases, the immune system is working to overcome its respective challenges – down-regulation of cytokines in response to repeated LPS exposure to limit tissue damage and resolve inflammation, and up-regulation of inflammatory cytokines in response to tumour secreted factors in an attempt to drive the adaptive response to an anti tumoural one. In the first instance, it would be necessary to identify what in the OSCC conditioned media is promoting this effect by screening for a wide array of potential candidates. As IL-6 is produced in copious amounts, this would be a logical place to start. Given that the presence
of LPS or conditioned media during polarisation have opposing effects on macrophage responses to LPS, it would be of interest to examine whether OSCC conditioned media can override the tolerance-like down-regulation of cytokine production seen in response to LPS polarisation.

Data from the present study confirms that macrophages express TLR2 and TLR4 as reported in the literature. Previous research has suggested that factors produced by carcinomas can activate myeloid cells via TLR2, stimulating metastasis. Furthermore, Lewis lung cell carcinoma supernatants induce IL-6 and TNFα production from macrophages via TLR-2 and -6. Therefore, it could be hypothesised that factors produced by OSCC cells might activate macrophages via TLR pathways, resulting in the amplification of pro-inflammatory cytokines reported in this study. The effects of blocking TLR2 and TLR4 signalling in macrophages before the addition of OSCC conditioned media may prove an interesting avenue of investigation.

Based on the evidence from the present study, is not possible to determine whether the H357 cells are acting like normal epithelial cells or are more representative of oral squamous cell carcinoma cells. It has previously been reported that cancer cell lines grown as a monolayer do not truly replicate their behaviour in vivo and behave more like tumours if they are grown in 3-dimensional culture as tumour spheres. In fact, tumour cells grown in spheroids have been shown to modulate macrophage polarisation differentially from cells grown in a monolayer. These multi layered structures more accurately reproduce the tumour microenvironment, including development an anoxic core. Given that P. gingivalis has been isolated from OSCC cores, this provides an ideal model for the investigation of bacteria/tumour/macrophage interactions. Preliminary experiments in this lab show that H357 cells readily form spheroids (data not shown).

An unexpected result was revealed during viability tests after polarisation with conditioned media: M2 macrophages cultured in conditioned media had increased in cell number.
compared to M2 cultured in R10. Furthermore, M2 increased in cell number to a greater extent than M1. However, a more accurate method of cell enumeration needs to be executed before any conclusions are drawn from these data. Nevertheless, it is tempting to hypothesise that cancer cells promote the proliferation of M2 cells preferentially over M1 cells in an effort to aid tumour survival, especially given the association of high numbers of M2 macrophages commonly found in in tumour microenvironments. Moreover, H357 cells produce high levels of TGF-β, which has been shown to induce proliferation in macrophages. Thus, in light of this preliminary data, a more robust study into the effects of OSCC secreted factors on proliferation in M1 and M2 macrophages should be conducted.

_In vivo_, macrophages and OSCC cells will not only come into contact with _P. gingivalis_ LPS, but multitude of other virulence factors. Thus, a limitation of this study is that polarisation with and subsequent responses to _P. gingivalis_ were only carried out using its LPS. Indeed, it has been shown that membrane associate molecules, such as fimbriae, have different effects on macrophage function compared with LPS, and that both LPS and fimbriae in turn modulate macrophage function in a different way from the live bacterium. Therefore, future investigations should involve the use of live _P. gingivalis_ to address this issue.

In conclusion, the key findings from the present study report:

1) Development of a reproducible model of M1 and M2 polarisation using THP-1 cells
2) Exposure of macrophages to LPS during polarisation leads to a down-regulation of inflammatory cytokines in response to subsequent stimulation with LPS
3) M1 macrophages display higher levels of TLR2 and TLR4 on the cell surface than M2, however, this needs further investigation to clarify.
4) H357 cells express LPS receptors, TLR2 and TLR4
5) H357 cells do not produce TNFα or IL-1β, either constitutively or in response to LPS
6) H357 produce high levels of IL-6 constitutively; this is not affected by the addition of
LPS

7) H357 and primary OSCC conditioned media amplify macrophage cytokine production
in response to \textit{P. gingivalis} LPS.

The findings reported in the present study may have wider reaching implications; \textit{P. gingivalis} has been associated with the progression of non-orally related diseases, such
as atherosclerosis\textsuperscript{217,342–347} and rheumatoid arthritis\textsuperscript{348–350} (reviewed in \textsuperscript{351} and \textsuperscript{352}).
Therefore, the continued study of macrophage/ \textit{P. gingivalis} interactions may shed light
on pathogenic mechanisms not only in oral pathological conditions, but in a range of
diseases.
References


113. Akamatsu, Y. *et al.* Porphyromonas gingivalis induces myocarditis and/or myocardial infarction in mice and IL-17A is involved in pathogenesis of these diseases. *Archives of oral biology* **56**, 1290–8 (2011).


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Appendix 1

**TGFβ-1 ELISA Optimisation: antibody concentrations**

Antibodies were added to wells in combinations of concentrations as shown below:

- Coating antibody: 0.5 µg/ml Detection antibody: 0.5µg/ml
- Coating antibody: 0.5 µg/ml Detection antibody: 1µg/ml
- Coating antibody: 0.5 µg/ml Detection antibody: 2µg/ml
- Coating antibody: 1µg/ml Detection antibody: 0.5µg/ml
- Coating antibody: 1 µg/ml Detection antibody: 1µg/ml
- Coating antibody: 1 µg/ml Detection antibody: 2µg/ml
- Coating antibody: 2µg/ml Detection antibody: 0.5µg/ml
- Coating antibody: 2µg/ml Detection antibody: 1µg/ml
- Coating antibody: 2µg/ml Detection antibody: 2µg/ml
- Coating antibody: 4µg/ml Detection antibody: 0.5µg/ml
- Coating antibody: 4µg/ml Detection antibody: 1µg/ml
- Coating antibody: 4µg/ml Detection antibody: 2µg/ml
TGF-β1 ELISA Protocol

(LAB-January 2009)

BD Biosciences/Pharmingen paired antibodies
TGF-β1 coating antibody: (Purified Rat Anti-Mouse, Human, Pig) cat no: 555052 lot: 49897
TGF-β1 detection antibody: (Biotin Rat Anti-Mouse, Human, Pig) cat no: 555053 lot: 67324
NIBSC TGF-β1 standard 89/514: 160ng in 1.6ml R10 = 100 ng/ml, 50µl aliquots

1. Coat 96 well microtitre plate with 50µl per well coating antibody (stock 0.5mg/ml)@ 4µg/m in PBS. Leave O/N at 4°C.
2. Wash x3 with PBS/0.05%-Tween20 ELISA wash buffer.
3. Block with 150µl per well 2% BSA/PBS for 4 hours @ room temperature.
4. Wash x3 with PBS/0.05%-Tween20 ELISA wash buffer.
5. Prepare standard curve: Top concentration 5000pg/ml, then 1 in 3 dilutions (5000-7pg/ml).
6. Add standards and test samples 50µl per well. Leave O/N at 4°C.
7. Wash x3 with PBS/0.05%-Tween20 ELISA wash buffer.
8. Add 50µl per well detection antibody (stock 0.5mg/ml) @ 2µg/ml in PBS/1% BSA. Leave 4 hours @ RT.
9. Wash x3 with PBS/0.05%-Tween20 ELISA wash buffer.
10. Add 50µl per well Streptavidin-HRP (conjugate: R&D Systems, DY998 part 890803) @ 1/250 dilution in PBS/1% BSA. Leave at RT for 1 hour.
11. Wash x3 with PBS/0.05%-Tween20 ELISA wash buffer.
12. Add TMB substrate reagent (KPL, cat: 50-76-03) 50µl per well (A:B = 50:50).
13. Allow colour to develop @ RT. Stop colour reaction with 50µl per well 1.8M H₂SO₄.
14. Read absorbance @ 450nm
# Human TGF-β ELISA optimization plate layout

<table>
<thead>
<tr>
<th></th>
<th>Detection antibody</th>
<th>Capture antibody</th>
<th>Standard</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>5 ng/ml</td>
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<tr>
<td>B</td>
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<td>0.5µg/ml</td>
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</table>

1 in 3 dilutions

0µg/ml
TGFβ-1 ELISA Optimisation

Optical Density

(log10) TGFβ-1 Standard Concentration (µg/ml)

Figure 6-1 Plot of TGF-β standard concentrations from chequerboard optimising technique
Figure 6-2: Plot of optimal concentrations for TGF-β capture and detect antibodies
Publications