Interleukin-1α Production in Periodontal Disease: 
The Interaction of Bacteria and Gene Polymorphisms

Nagihan Bostancı

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Center for Adult Oral Health
Barts and The London School of Medicine and Dentistry
Queen Mary, University of London
London

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DEDICATED TO MY BELOVED FAMILY
(SEVGİLİ ANNEM, BABAM VE KARDEŞLER İME)
# CONTENTS

ABSTRACT ............................................................................................................................................ 7  
LIST OF FIGURES ................................................................................................................................ 9  
LIST OF TABLES ................................................................................................................................ 11  
ABBREVIATIONS ............................................................................................................................... 14  
CHAPTER 1 ......................................................................................................................................... 17  
  1.1 PERIODONTAL DISEASE .................................................................................................... 18  
  1.1.1 Chronic periodontitis ................................................................................................................ 20  
  1.1.2 Aggressive periodontitis ........................................................................................................... 20  
  1.2 AETIOLOGY OF PERIODONTAL DISEASE ...................................................................... 22  
  1.2.1 Bacterial risk factors ................................................................................................................ 22  
  1.2.2 Smoking ................................................................................................................................... 27  
  1.2.3 Psychological factors ............................................................................................................... 29  
  1.2.4 Systemic risk factors ................................................................................................................ 29  
  1.2.5 Genetic factors ......................................................................................................................... 30  
  1.3 PATHOGENESIS OF PERIODONTITIS ............................................................................... 31  
  1.4 HOST RESPONSES TO PERIODONTAL MICROORGANISMS ........................................ 33  
  1.4.1 Polymorphonuclear leukocytes (PMNs) .................................................................................. 35  
  1.4.2 Monocytes/macrophages .......................................................................................................... 35  
  1.5 MODIFIERS OF HOST RESPONSE ...................................................................................... 36  
  1.5.1 Systemic modifiers ................................................................................................................... 38  
  1.5.2 Smoking ................................................................................................................................... 38  
  1.5.3 Genetic modifiers ..................................................................................................................... 39  
  1.6 CANDIDATE GENE APPROACH and PERIODONTAL DISEASE .................................... 45  
  1.6.1 Candidate Genes in Periodontal Disease .................................................................................. 46  
  1.7 IL-1α: A CANDIDATE GENE IN PERIODONTAL DISEASE ............................................ 47  
  1.7.1 Expression and synthesis of IL-1α ........................................................................................... 49  
  1.7.2 Maturation and secretion IL-1α ............................................................................................... 50  
  1.7.3 Biological and pathological properties of IL-1α ...................................................................... 50  
  1.8 IL-1α versus IL-1β ................................................................................................................... 52  
  1.9 IL-1α in periodontal disease ..................................................................................................... 54  
  1.9.1 The role of IL-1α in bone resorption ........................................................................................ 54  
  1.10 IL-1A gene polymorphisms and periodontal disease ............................................................... 55  
  1.10.1 Interleukin-1A gene polymorphisms in aggressive periodontitis ............................................. 56  
  1.10.2 IL-1A gene polymorphisms in chronic periodontitis ............................................................ 58  
  1.11 The functional importance of IL-1A gene polymorphisms ..................................................... 58  
  1.12 AIMS OF THE STUDY ........................................................................................................... 59  
CHAPTER 2 ......................................................................................................................................... 61  
  2.1 INTRODUCTION ..................................................................................................................... 62  
  2.1 MATERIAL & METHODS ....................................................................................................... 62
ABSTRACT

There is good evidence that interleukin-1 alpha (IL-1α) plays a key role in both the host response to bacteria and the tissue destruction associated with periodontal disease. This thesis investigated the interaction of bacterial, genetic and pharmacological factors in the regulation of IL-1α in periodontal disease. To investigate the stimulation of IL-1α by periodontal pathogens, human monocytes were challenged with a wide range of periodontal species. Most of the species tested stimulated high levels of IL-1α. Induction by P. gingivalis was notably weak. Co-stimulation with P. gingivalis antagonised the ability of other bacterial species to induce IL-1α, and this antagonism was due to the specific nature of its LPS. The association of periodontal disease with host genetic variation was investigated by screening the IL-1A promoter region by direct sequencing in patients with aggressive periodontitis. Three single-nucleotide polymorphisms (SNPs) were identified including rs3783521 C>T, rs1800794 C>T, rs1800587 C>T. However, the prevalence of polymorphic alleles between patients and healthy subjects was similar. To test if any of these polymorphisms effect transcription of IL-1A, the polymorphisms of interest were introduced into an IL-1α luciferase reporter construct by site-directed mutagenesis. Only one of these polymorphisms, rs1800587 (-899), resulted in a significant change in transcription using transient transfection assays. The T allele of this SNP reduced both the basal activity and the response to LPS or periodontopathogens. Therefore, the rs1800587 SNP might play a role in the regulation of IL-1A gene transcription induced by bacteria. The central role played by IL-1α in the pathogenesis of periodontal disease suggests that immunomodulation may offer a therapeutic option. Sub-antimicrobial concentrations of doxycycline inhibited IL-1α production in response to bacterial stimulation by human monocytes. This effect appears to be at translational level rather than transcription. It does not appear to involve the regulation of NF-κB activity, or the suppression of IL-1α transcription, or doxycycline chelation properties. In conclusion, IL-1α is a pivotal cytokine involved in periodontal disease, and its regulation is a complex event governed by bacterial factors, host genetics and pharmacological agents.
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LIST OF FIGURES

Chapter 1

Figure 1.1 Diagrammatic representation of the microbial complexes in dental plaque
Figure 1.2 Schematic representation of the model of oral bacterial colonization
Figure 1.3 Schematic representation of the pathogenesis of periodontal diseases
Figure 1.4 Schematic representation of spectrum of periodontal diseases
Figure 1.5 Host response characteristics as a function of bacterial challenge level
Figure 1.6 Schematic representation of the host–environment interaction in the course of infection
Figure 1.7 Schematic representation of the classic relationship of phenotype, environment, and genotype
Figure 1.8 Schematic representation of SNP comprising a C→T substitution
Figure 1.9 Schematic representation of SNP functional assays
Figure 1.10 Schematic representation of the inflammatory cascade triggered by IL-1α

Chapter 2

Figure 2.1 Schematic representation of the Limulus Amebocyte Lysate (LAL) assay
Figure 2.2 IL-1α production by monocytic cell lines stimulated with *E. coli* LPS
Figure 2.3 The effects of LPS concentration and serum levels on IL-1α production by human monocytes
Figure 2.4 The effect of cell density of Monomac-6 cells on IL-1α production
Figure 2.5 The effect of stimulation time on IL-1α production by Monomac-6 cells
Figure 2.6 Total protein concentrations in the culture supernatants
Figure 2.7 Dose-response study of the effect of periodontal pathogen(s) supernatants on IL-1α production by Monomac-6 cells
Figure 2.8 The effect of heat treatment on periodontopathogen induced IL-1α production by Monomac-6 cells
Figure 2.9 The effect of LPS depletion from periodontopathogen(s) supernatants on IL-1α production

Chapter 3

Figure 3.1 Schematic representation of the RgpA/B and Kgp proteases of *P. gingivalis*
Figure 3.2 Schematic representation of the suggested role of gingipains in the pathogenesis of periodontitis
Figure 3.3 Flow cytometric analysis of isolated PBMCs
Figure 3.4  Production of IL-1α by monocytes in response to bacterial stimuli
Figure 3.5  Effects of bacterial supernatants on cell viability
Figure 3.6  Effects of different P. gingivalis strains on the antagonistic activity
Figure 3.7  The effect of LPS depletion and heat treatment on periodontopathogen induced IL-1α production by Monomac-6 cells
Figure 3.8  Growth assay of P. gingivalis and effect of culture length on total protein levels
Figure 3.9  Effect of culture length on stimulation of IL-1α
Figure 3.10  The levels of total IL-1α in Monomac-6 cells treated with P. gingivalis supernatant or its LPS
Figure 3.11  The role of P. gingivalis LPS in C. rectus mediated IL-1α production by Monomac-6 cells
Figure 3.12  The role of priming of Monomac-6 cells with P. gingivalis LPS in C. rectus mediated IL-1α production

Chapter 4

Figure 4.1  A schematic representation of Polymerase Chain Reaction (PCR)
Figure 4.2  Schematic diagram of the IL-1A gene.
Figure 4.3  A schematic representation of automated DNA sequencing
Figure 4.4  Example of DNA sequence output
Figure 4.5  The traces as displayed by the GAP4 contig editor
Figure 4.6  A diagram of the TaqMan SNP Genotyping Assay
Figure 4.7  Ethidium-bromide stained agarose gel showing results of PCR amplification (the promoter)
Figure 4.8  Ethidium-bromide stained agarose gel showing results of PCR amplification (the intron and exon)
Figure 4.9  Electropherogram showing different genotypes (Patient1)
Figure 4.10  Electropherogram showing different genotypes (Patient 2)
Figure 4.11  LD plot showing linkage disequilibrium
Figure 4.12  Real-time PCR cycle threshold plotted against DNA concentration

Chapter 5

Figure 5.1  Schematic representation of TOPO Cloning
Figure 5.2  Schematic representation of generation of IL-1 A gene reporter construct
Figure 5.3  Schematic representation of reporter system
Figure 5.4  RT-PCR of a 2.1 kb promoter fragment of IL-1A gene
Figure 5.5  The genomic fragment cleavage by restriction enzymes in TOPO
Figure 5.6  Analysis of recombinants obtained after cloning of IL-1A promoter
Figure 5.7  DNA sequence output showing mutated alleles
Figure 5.8  Representative image of THP-1 cells transfected with control pEGFP-N3
Figure 5.9  Allele-specific transcriptional activity in THP-1 cells
Figure 5.10  Allele-specific transcriptional activity in the presence of bacteria in THP-1 cells
Figure 5.11  Representative image of human HEK 293T transfected with pEGFP-N3
Figure 5.12  Allele-specific transcriptional activity in HEK 293T cells
Figure 5.13  Representative image of HeLa cells transfected with pEGFP-N3
Figure 5.14  Allele-specific transcriptional activity in HeLa cells
Figure 5.15  Representative image of human skin fibroblasts transfected with pEGFP-N3
Figure 5.16  Allele-specific transcriptional activity in human skin fibroblasts
Figure 5.17  TRANSFAC analysis for transcriptional binding sites for SNP I
Figure 5.18  TRANSFAC analysis for transcriptional binding sites for SNP II
Figure 5.19  TRANSFAC analysis for transcriptional binding sites for SNP III

Chapter 6

Figure 6.1  A schematic representation of non-antimicrobial activity of tetracyclines
Figure 6.2  The levels of IL-1α, IL-1β, IL-6 and IL-8 in human monocytes
Figure 6.3  The levels of IL-1α, IL-1β, TNF-α, IL-6 and IL-8 in Monomac-6 cells
Figure 6.4  Effects of doxycycline on cell viability
Figure 6.5  The effect of pre-incubation with doxycycline prior to challenge
Figure 6.6  Effect of doxycycline on the IL-1A gene promoter activity
Figure 6.7  Effect of Doxycycline on the NF-κB promoter activity
Figure 6.8  The ion-chelation properties of doxycycline
Figure 6.9  Cytotoxicity of ions or ion chelators in Monomac-6 cells
Figure 6.10  Kinetics of pro-inflammatory cytokine release in the presence of doxycycline
Figure 6.11  Kinetics of anti-inflammatory cytokine release in the presence of doxycycline
LIST OF TABLES

Chapter 1
Table 1.1 Abbreviated version of the 1999 Classification of Periodontal Diseases
Table 1.2 Specific features of chronic periodontitis
Table 1.3 Specific features of aggressive periodontitis
Table 1.4 Hypothetical way of classifying periodontal diseases
Table 1.5 Reports of literature search summarizing findings on the association between periodontitis and polymorphisms
Table 1.6 Increased expression of various genes by IL-1α
Table 1.7 Reported IL-1A gene polymorphisms in aggressive periodontitis

Chapter 2
Table 2.1 Bacterial strains and growth conditions

Chapter 3
Table 3.1 P. gingivalis strains and serotypes
Table 3.2 Overall changes in total IL-1α levels in Monomac-6 and human monocytes
Table 3.3 Arg-X and Lys-X protease activities in isogenic mutants of P. gingivalis

Chapter 4
Table 4.1 Pre-screening methods for SNP discovery
Table 4.2 Examples of web-based resources available for assessment of SNPs
Table 4.3 SNPs in the IL-1A gene promoter
Table 4.4 Patient Selection Criteria
Table 4.5 The PCR primer sequences.
Table 4.6 Components of the PCR mix.
Table 4.7 The primers used for direct sequencing of the IL1 A gene
Table 4.8 TaqMan genotyping probe and primer sequences.
Table 4.9 The thermal cycling conditions for PCR
Table 4.10 Genotype and allele frequencies of the identified IL-1A SNPs
Table 4.11 Measures of linkage disequilibrium (LD)
Table 4.12 Frequency of IL-1A promoter region haplotypes
Table 4.13 Genotype and allele frequencies for the 3 SNPs examined in patients with generalized aggressive periodontitis
Table 4.14 Allele frequencies for the 3 SNPs in different populations
Table 4.15  Allele frequencies for the 3 SNPs according to smoking

Chapter 5

Table 5.1  The 5'→ 3' sequences of the sense and antisense primer pairs
Table 5.2  Primers for the pGL3 basic vector sequence
Table 5.3  The primers for Site-Directed Mutagenesis
Table 5.4  Mutant Strand Synthesis Reaction
Table 5.5  RT-PCR cycling parameters for the QuickChange Site-Directed Mutagenesis
Table 5.6  Allele-specific transcriptional activity in the presence or absence of bacteria in THP-1 cells
ABBREVIATIONS

3' 3 prime
5' 5 prime
A Adenine
bp Base pair
BSA Bovine serum albumin
C Cytosine
COX-2 Cyclooxygenase-2
CuSO₄ Copper sulphate
D Dalton
DMEM Dulbecco's modified eagle's medium
DNA Deoxyribonucleic acid
DNase Deoxyribonuclease
dNTP Deoxynucleoside triphosphate
EDTA Ethylenediaminetetra-acetic acid disodium salt
ELISA Enzyme Linked Immunosorbent Assay
ER Endoplasmic reticulum
FCS Fetal calf serum
FeCl₃ Iron (III) chloride
Fc gammaR Fc gamma receptors
G Guanine
GAP Generalized aggressive periodontitis
GCF Gingival crevicular fluid
GM-CSF Granulocyte macrophage colony-stimulating factor
H₂O Water
H₂O₂ Hydrogen peroxide
HTLV-1 T-lymphotropic virus type 1 tax
ICAM-1 Intracellular adhesion molecule-1
ICE Interleukin-1 converting enzyme
IFN-γ Interferon-gamma
IgG₂ Immunoglobulin G₂
IL-1 Interleukin-1
IL-1α Interleukin-1 alpha
IL-1Ra Interleukin-1 receptor antagonist
IL-1RI Interleukin-1 type I receptor
IL-1RACp Interleukin-1 receptor accessory protein
IL-6  Interleukin-6
IL-8  Interleukin-8
iNOS  Inducible nitric oxide synthase
kbp  Kilobase pair
LAL  Limulus Amebocyte Lysate
LAP  Localized aggressive periodontitis
LDH  Lactate-dehydrogenase
LPS  Lipopolysaccharide
M-CSF  Macrophage colony stimulating factor
mg/ml  Milligrams per millilitre
μl  microlitre
MMPs  Matrix metalloproteinase
mRNA  Messenger RNA
MTT  3-[4, 5-dimethylthiazol-2-yl]-2, 5- diphenyl tetrazolium bromide
NaN₃  Sodium Azide
NF-κB  Nuclear Factor-{kappa} B
NO  Nitric oxide
OPG  Osteoprotegerin
PBMC  Human peripheral blood mononuclear cells
PBS  Phosphate buffered Saline
PGE₂  Prostaglandin E₂
PLA₂  Phospholipases A₂
PMA  Phorbol-12-myristate-13 Acid
PMNs  Polymorphonuclear leukocytes
PCR  Polymerase Chain Reaction
PDL  Periodontal ligament
RANKL  Receptor activator of nuclear factor κ B ligand
RNA  Ribonucleic acid
RT-PCR  Reverse Transcription-polymerase chain reaction
SNPs  Single nucleotide polymorphisms
T  Thymine
Taq  Thermus aquaticus
TAE  Tris Acetate EDTA Buffer
TEMED  N,N,N',N'-tetramethylethylenediamine
TLRs  Toll-like family of receptors
TNF-α  Tumour necrosis factor-alpha
TTM  Tetrathiomolybdate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Tween 20</td>
<td>Polyoxethylene (20) sorbitan monolaurate</td>
</tr>
<tr>
<td>U</td>
<td>Uracil</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular adhesion molecule-1</td>
</tr>
<tr>
<td>VNTRs</td>
<td>Variable number tandem repeats</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>A. actinomyetemcomitans (A.a)</td>
<td>Aggregatibacter actinomyetemcomitans</td>
</tr>
<tr>
<td>C. rectus (C.r)</td>
<td>Campylobacter rectus</td>
</tr>
<tr>
<td>E.coli (E.c)</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>F. nucleatum (F.n)</td>
<td>Fusobacterium nucleatum</td>
</tr>
<tr>
<td>P. gingivalis (P.g)</td>
<td>Porphyromonas gingivalis</td>
</tr>
<tr>
<td>P. intermedia (P.i)</td>
<td>Prevotella intermedia</td>
</tr>
<tr>
<td>P. nigrescens (P.n)</td>
<td>Prevotella nigrescens</td>
</tr>
<tr>
<td>S. sanguis (S.s)</td>
<td>Streptococcus sanguis</td>
</tr>
<tr>
<td>S. mutans (S.m)</td>
<td>Streptococcus mutans</td>
</tr>
<tr>
<td>T.denticola (T.d)</td>
<td>Treponema denticola</td>
</tr>
<tr>
<td>V. atypica (V.a)</td>
<td>Veillonella atypica</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction
"If it were not for the great variability among individuals medicine might as well be a science and not an art" Sir William Osler, 1892

1.1 PERIODONTAL DISEASE

Periodontal disease has been defined as any condition affecting the periodontium or tooth supporting tissues including the periodontal ligament, the gingival connective tissue and the alveolar bone. More than 40 different conditions have been classified as periodontal diseases at the International Workshop for the Classification of Periodontal Disease and Conditions in 1999 (Armitage, 1999) (Table 1.1). Several classification systems for periodontal diseases have been adopted over the years based on the understanding of the nature of these diseases. Advances in research methodologies have led to changes in disease paradigms. For the period from approximately 1870 to 1920, the diseases were classified on the basis of their clinical features. The concepts of classical pathology were used from 1920 to 1970 based on results from histopathological studies. Current classification systems for periodontal diseases are mainly based on the Infection/Host Response paradigm since the 1970s (Armitage, 2002).

Gingivitis and periodontitis are the most common forms of periodontal disease. The primary aetiology of both gingivitis and periodontitis is the presence of oral bacteria that accumulate as biofilm communities on tooth surfaces (Haffajee and Socransky, 1994) and the majority of periodontal tissue destruction is caused by an inappropriate host response to those microorganisms and their products (Page and Kornman, 1997; Schenkein, 2006).

Gingivitis is characterised by the presence of gingival inflammation, without loss of connective tissue attachment and can reverse to a healthy state after removal of the dental biofilm (Loe, 1965; Teles et al., 2006). Periodontitis results from a persistent bacterial infection, which causes chronic inflammation resulting in loss of gingival connective tissue and alveolar bone. Studies show that periodontitis is preceded by gingivitis and the duration of onset and the intensity of the inflammatory process vary considerably from person to person, as well as between teeth and tooth sites within the same person.
Chapter 1: Introduction

CURRENT CLASSIFICATION OF PERIODONTAL DISEASES AND CONDITIONS

I. Gingival diseases
   A. Dental plaque-induced gingival diseases
   B. Non-plaque-induced gingival diseases

II. Chronic periodontitis
   - mild: 1-2mm CAL
   - moderate: 3-4mm CAL
   - severe: >5mm CAL
   A. Localized
   B. Generalized

III. Aggressive periodontitis
   - mild: 1-2mm CAL
   - moderate: 3-4mm CAL
   - severe: >5mm CAL
   A. Localized
   B. Generalized

IV. Periodontitis as a manifestation of systemic diseases
   A. Periodontitis associated with hematological disorders
   B. Periodontitis associated with genetic disorders
   C. Not otherwise specified (NOS)

V. Necrotizing Periodontal Diseases
   A. Necrotizing ulcerative gingivitis (NUG)
   B. Necrotizing ulcerative periodontitis (NUP)

VI. Abscesses of periodontium
   A. Gingival abscess
   B. Periodontal abscess
   C. Pericoronal abscess

VII. Periodontitis associated with endodontic lesions
    A. Combined periodontic-endodontic lesions

VIII. Developmental or acquired deformities and conditions
    A. Tooth-related factors that modify or predispose to plaque-induced gingival diseases/periodontitis conditions
    B. Mucogingival deformities and conditions
    C. Occlusal trauma

Table 1.1 Abbreviated version of the 1999 Classification of Periodontal Diseases. A new classification which was agreed upon at the International Workshop for a Classification of Periodontal Diseases and Conditions in 1999 on October 30–November 2, 1999. Over 40 different gingival diseases and seven major categories of destructive periodontal diseases were listed. Adapted from Armitage (1999).
1.1.1 Chronic periodontitis

Two main forms of destructive periodontal disease are currently recognized, chronic and aggressive periodontitis. Although prevalence figures vary with race and geographic region, chronic periodontitis is rather prevalent affecting about 35% of the population (Albandar et al., 1999; Gjermo et al., 2002; Sheiham and Netuveli, 2002). Chronic periodontitis is designated as localized or generalized depending on whether more or less than 30% of sites are affected (Goodson et al., 1982). In earlier literature this clinical form was referred to as adult periodontitis since it was believed that only adults developed the disease (Page and Schroeder, 1976). However, epidemiologic data clearly show that the disease can also be found in children and adolescents (Albandar and Tinoco, 2002). The main clinical features and characteristics of chronic periodontitis are listed in Table 1.2 (Armitage, 1999).

| Most prevalent in adults, but can occur in children and adolescents |
| Level of destruction is consistent with the presence of local factors |
| Subgingival calculus is a frequent finding (~40-60%) |
| Can be associated with local predisposing factors (e.g., tooth-related or iatrogenic factors) |
| May be modified by and/or associated with systemic diseases |
| Can be increased by diabetes mellitus, cigarette smoking and emotional stress |
| Associated with a variable microbial pattern (i.e. no single bacterial species) |
| Slow to moderate progression rate, but may have periods of rapid progression |

Table 1.2 Main clinical features and characteristics of chronic periodontitis (1999 Classification).

1.1.2 Aggressive periodontitis

Aggressive periodontitis encompasses distinct types of periodontitis that affect people who appear otherwise healthy. Patients with aggressive periodontitis (formerly termed juvenile periodontitis, early onset periodontitis or rapidly progressive periodontitis) are characterized by a rapid and severe periodontal destruction in mainly younger individuals (Albandar and Tinoco, 2002; Caton and Lowenguth, 1993; Page et al., 1983). Epidemiological surveys have shown that the prevalence of aggressive periodontitis varies widely among races, regions and countries. The estimated prevalence of aggressive periodontitis ranges from 0.1% to 15% among Caucasians, Hispanics and African-Americans (Albandar et al., 1997a; Loe and Brown, 1991).
Aggressive periodontitis is subdivided into localized (LAP) or generalized (GAP) (Armitage, 2004; Ranney, 1993). In the case of generalized aggressive periodontitis, subjects display bone loss in at least three permanent teeth other than first molars and incisors. The localized form usually occurs in adolescence with characteristically high levels of antibodies against putative pathogens in serum, whereas GAP is usually diagnosed in people less than 35 years of age with low levels of antibodies against putative pathogens. However, GAP is not necessarily confined to individuals younger than 35 years of age. Generally accepted features of aggressive periodontitis are shown in Table 1.3 (Armitage, 1999). Currently, it cannot be concluded whether the localized and generalized forms of aggressive periodontitis are two different diseases, with distinct aetiological factors and pathogenesis, or whether they are two stages of the same disease.

<table>
<thead>
<tr>
<th>Primary features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Besides periodontitis, subjects are systemically healthy</td>
</tr>
<tr>
<td>Rapidly progressing periodontal destruction</td>
</tr>
<tr>
<td>Often show familial aggregation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary features (often but not always)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No clear relationship between the amount of dental plaque and level of tissue destruction</td>
</tr>
<tr>
<td>High prevalence of <em>Aggregatibacter actinomycetemcomitans</em> (formerly named <em>Actinobacillus actinomycetemcomitans</em>) and <em>Porphyromonas gingivalis</em></td>
</tr>
<tr>
<td>Hyper-responsive macrophage-phenotype with increased production of inflammatory mediators</td>
</tr>
<tr>
<td>Progression of attachment loss and bone loss may be self-arresting</td>
</tr>
</tbody>
</table>

Table 1.3 Features of aggressive periodontitis that are common to both the localized and generalized forms of the disease (1999 Classification).

In summary, there is still uncertainty regarding the aetiological factors that might be involved and much is yet to be learned in order to explain why some individuals develop severe periodontal disease while others are relatively resistant to disease progression beyond marginal gingivitis. It is very likely that both chronic and aggressive periodontitis are a constellation of diseases rather than a single entity. The clinical manifestations of these diseases are altered by important environmental and host-modifying conditions including oral hygiene, smoking, emotional stress, and diabetes. With increased understanding of periodontal infections and increasing information about the genetic factors controlling host responses to these infections, it is may be possible to sub classify the forms of periodontitis into discrete microorganism/host genetic polymorphism groups.
This thesis will focus on understanding the host response to bacteria and the genetic factors controlling host responses to these bacterial infections.

| Group A - Set #1 of microorganisms + Set #1 of genetic polymorphisms |
| Group B - Set #2 of microorganisms + Set #2 of genetic polymorphisms |
| Group C - Set #3 of microorganisms + Set #3 of genetic polymorphisms |

Table 1.4 Hypothetical way of classifying periodontal diseases. Adapted from Armitage (2004).

1.2 AETIOLOGY OF PERIODONTAL DISEASE

Aetiology is defined as the study of causation of disease and includes both the direct cause and factors associated with cause. Two components can be defined within this; risk factors and risk determinants (or susceptibility factors). The term risk factor refers to an aspect of personal behaviour, an environmental exposure, or an inherited characteristic, which on the basis of epidemiological evidence is known to be associated with the increased rate of a subsequently occurring disease (Last, 1982). Risk factors are associated with a disease but do not necessarily cause the disease. Some risk factors may be modified to reduce one's risk of initiation or progression of disease, such as smoking cessation or improved oral hygiene while non-modifiable risk factors are usually intrinsic to the individual and therefore not easily changed such as genetic factors. A risk factor that cannot be modified is often referred to as a risk determinant (Albandar, 2002).

Periodontal disease is considered to have multiple aetiological factors (Nunn, 2003). Microbial dental plaque biofilms are the principal aetiologic factor of periodontitis, whereas several other local and systemic factors related to the host have important modifying roles in its pathogenesis.

1.2.1 Bacterial risk factors

The aetiology of various forms of periodontal disease has long been recognized to be related to bacterial accumulations and plaque composition. Microorganisms in dental biofilms are aetiological factors essential for the initiation of the inflammatory process locally at the infection site, and in the absence of these, the inflammatory response does not take place (Haffajee and Socransky, 2006; Moore and Moore, 1994). The evidence for the infectious nature of periodontal disease comes from several sources, including:
1. Clinical and microbiological data linking accumulated dental biofilms to the initiation, progression and recurrence of disease in periodontitis.
2. Clinical studies which demonstrate that elimination of dental biofilms can be correlated with clinical improvement.
3. Genetic and experimental data linking virulence factors possessed by different dental biofilm bacteria to pathogenic events known to occur during periodontitis.

The role of bacteria in periodontal diseases was initially established in experimental gingivitis models (Löe et al., 1965) including studies on beagle dogs (Theilade et al., 1966). Experimental gingivitis studies provided the first evidence that accumulation of dental plaque biofilms on clean tooth surfaces results in the development of an inflammatory process around gingival and periodontal tissues and that the inflammation may resolve subsequent to removal of the microbial biofilm (Silness and Löe, 1964). Since this advance, an enormous research effort has been devoted to the study of periodontal-disease-associated microflora spanning from classic bacterial culture methods to modern approaches at the molecular, whole genomic and proteomic levels (Gmur and Thumheer, 2002; Griffen et al., 1998; Klein and Goncalves, 2003; Kumar et al., 2003; Lyons et al., 2000; Mayanagi et al., 2004). Early studies of the bacterial composition of subgingival plaque samples used cultural techniques (Nyvad and Kilian, 1987; Socransky et al., 1977). These have yielded considerable insights into the colonizing micro-organisms that can be identified by means of cultivation. In the following years, poly or monoclonal antibodies were used to enumerate species directly in plaque samples (Wilson et al., 1993). More recently use of the checkerboard DNA–DNA hybridization techniques have provided not only information on these species, but also enabled the detection of unusual micro-organisms that are present in low numbers or those that are difficult to enumerate by culture (Ramberg et al., 2003; Ximenez-Fyvie et al., 2000b).

Certain bacterial species have been associated with periodontitis. Specific genetic variants of A. actinomycetemcomitans have been shown to significantly correlate with aggressive forms of periodontal diseases and to be prevalent in aggressive periodontitis patients from certain geographic areas, particularly among subjects of African descent (DiRienzo et al., 1994; Fine et al., 2006; Haubek et al., 2001; Kilian et al., 2006). There is also evidence showing that P. gingivalis is strongly associated with chronic
periodontitis (Holt and Ebersole, 2005; Slots and Ting, 1999). This bacterium is consistently detected in high levels in cross-sectional studies in persons with severe attachment loss and deep probing depths (Albandar et al., 1997b; Grossi et al., 1994). However, evaluation of these pathogens as risk factors for identification of attachment loss over time has resulted in conflicting evidence. Some studies indicated that none of these pathogens were useful in predicting periodontal disease progression (Listgarten et al., 1991; MacFarlane et al., 1988).

Most recent evidence suggests that periodontal diseases have a multi-bacterial aetiology and therefore it is important to gain insight into the bacterial composition of dental plaque rather than the specificity of certain bacteria (Socransky and Haffajee, 2002). In the consensus report from the World Workshop in 1996, *P. gingivalis*, *A. actinomycetemcomitans* and *Tannerella forsythensis* were described as being the most likely candidate organisms that could be referred to as risk indicators in periodontitis (Zambon, 1996). Once it became clear that no single pathogen was a causative agent for periodontal disease, studies shifted towards investigating how different organisms act in concert. Five sets of bacteria, or complexes that were consistently (>60%) found together in periodontitis were identified in 1998 (Figure 1.1) (Socransky et al., 1998). The most pathogenic complex designated as “the red complex”, comprises of *P. gingivalis*, *T. forsythensis*, and *Treponema denticola*. The red complex bacteria exist in high numbers in lesions of chronic periodontitis (Umeda et al., 1996; Ximenez-Fyvie et al., 2006) and exhibit a strong relationship with inflammatory or other indicators of periodontitis such as pocket depth and bleeding on probing (Socransky et al., 1998). The other complex is designated as “the orange complex” including *Fusobacterium nucleatum*, *Prevotella intermedia*, *Prevotella nigrescens*, *Peptostreptococcus micros*, *Campylobacter* spp., *Eubacterium nodatum*, *Streptococcus constellatus* and is considered less pathogenic. The red complex is closely associated with the orange complex and bacteria belonging to “the orange complex” have also been related to clinical parameters such as pocket depth (Socransky et al., 1998) but to a lesser degree than bacteria from the “red complex”. There is also “the green complex”, including *Capnocytophaga* spp., *Campylobacter concisus*, *A. actinomycetemcomitans* (serotype a) and *Eikenella corrodens*. Moreover, *Streptococcus* spp including *Streptococcus mitis*, *Streptococcus sanguis* and *Streptococcus oralis* form “the yellow complex”. Also “the purple complex” is made up by *Actinomyces odontolyticus*, and *Veillonella parvula*. *Actinomyces naeslundii*,
Selenomonas noxia and *A. actinomycetemcomitans* (serotype b) are not clustered with other species. Species of the yellow, green and purple complexes together with *A. naeslundii* are considered to be host compatible.

**Figure 1.1** Diagrammatic representation of the microbial complexes in dental plaque. Socransky et al., (1998) carried out a study to define species associations that occur in subgingival biofilms *in vivo*. Over 13,000 subgingival biofilm samples from 185 subjects with different states of periodontal health and disease were tested. Associations between all pairs of species were measured by various similarity coefficients and the resulting similarity matrices subjected to cluster analysis. These analyses supported the hypothesis that there were distinct complexes of microorganisms in subgingival biofilms. Adapted from Socransky and Haffajee (2005).

A large number of bacteria exist as a part of the normal ecology of the oral cavity, and these consist of diverse types of microorganisms that differ greatly in their virulence potential (Moore and Moore, 1994; Socransky and Haffajee, 2005). However, various bacterial species may have different effects on the pathogenesis of these diseases, and therefore may possess variable risk for periodontitis (Wolff et al., 1994; Zambon, 1996). The formation of a complex of periodontopathogenic bacteria is likely the result of the interactions between pathogenic and commensal organisms (Socransky and Haffajee, 2005). Interactions among different bacterial cells have been proposed to drive the maturation of the dental biofilm. These interactions can occur at several levels including physical contact, metabolic exchange, and signal mediated communications (Kolenbrander et al., 2006). The sequential nature of colonizing species is showed in Figure 1.2 (Courtesy of Dr. P.E. Kolenbrander). However, not all the bacteria present
might necessarily be part of transition from health to disease. Some longitudinal studies suggest that *T. forsythensis* and *C. rectus* are the major species that characterized sites converting from health to disease (Tanner et al., 1998). It still remains unsolved as to how these shifts correlate with the appearance of gingivitis and in the susceptible host with the development of periodontitis.

**Figure 1.2** The model of oral bacterial colonization. This figure shows recognition of salivary pellicle receptors by early colonizing bacteria, and coaggregations between early colonizers, fusobacteria and late colonizers of the tooth surface. Adapted from Kolenbrander et al. (2006).

The characteristics of microbiological progression from periodontal health to gingivitis and eventually to periodontal disease are vast and complicated (Albandar et al., 1999; Haffajee et al., 2006; Socransky and Haffajee, 1994). The transition from health to disease in the periodontal tissues is accompanied by a change of the plaque microflora from predominantly gram-positive aerobic cocci to predominantly gram-negative rods (Listgarten, 1976). Total bacterial numbers also increase during microbial transition from health to disease. Approximately 10^2–10^3 bacteria are found in healthy subgingival plaque (Tanner et al., 1996), increasing to 10^4–10^6 organisms during gingivitis and escalating as high as 10^5–10^8 organisms during periodontitis (Darveau et al., 1997; Socransky et al., 1963). However, it must be acknowledged that in some forms of periodontal disease the patients exhibit minimal levels of subgingival plaque (Brown et al., 1996).
Initial biofilm formation of up to 2 days of undisturbed plaque accumulation is predominated by streptococci (~63-86% of total bacterial number isolated), in particular *S. mitis* and *S. oralis* along with some *Actinomyces, Veillonella* (Nyvad and Kilian, 1987; Palmer et al., 2001; Socransky et al., 1994). As plaque matures, the bacterial population shifts to predominantly rods and filamentous bacteria (Listgarten, 1976; Tinanoff et al., 1976). After 4 days of plaque accumulation, there is an increase in the numbers of *F. nucleatum* which facilitates biofilm development due to its coaggregation properties (Kroes et al., 1999). Subgingival plaque is probably formed by the spread of supragingival plaque down into the gingival sulcus (Theilade and Theilade, 1985). Few studies have examined the composition of subgingival and supragingival plaque samples in periodontal health and various states of periodontal diseases (Haffajee and Socransky, 1994; Moore and Moore, 1994; Ximenez-Fyvie et al., 1999). The most commonly detected species in both health and disease are *A. naeslundii* and *F. nucleatum*. There are also other gram-negative periodontopathogenic bacteria such as *P. gingivalis, T. denticola* in the both supragingival and subgingival plaque of healthy subjects and subjects with periodontitis. However, subjects with periodontitis seem to have higher proportions of these organisms in their supragingival plaque (Ximenez-Fyvie et al., 2000a).

It is likely that subject-specific factors such as genetic make-up prevent straightforward categorization of a microorganism as being absolutely causative of periodontal disease (Paster et al., 2006). There are studies that report inter-individual variation in dental plaque (Palmer et al., 2003; Palmer et al., 2001). It remains unclear whether the individual differences in flora composition are a result of different stages of disease progression, or are related to host, or environmental influences. It is possible that variation between individuals in the microflora of the oral cavity could be attributed to differences in host factors that modulate colonization of an individual by a specific set of species. Although periodontitis has been viewed for years as primarily the outcome of bacterial infection, it has become apparent that pathogens are necessary but not sufficient for disease activity to occur (Albandar, 2005; Page et al., 1997).

1.2.2 Smoking

Cigarette smoking has long been recognized as a risk factor for periodontal disease (Preber et al., 1980). The positive association between smoking and periodontal disease
has been reported in many epidemiological, clinical and in vitro studies (Albandar et al., 2000). Cross-sectional and longitudinal data provide strong support for the statement that the risk of developing periodontal disease as measured by clinical attachment loss and alveolar bone loss increases with increased smoking between two and seven times (Johnson and Hill, 2004; Tomar and Asma, 2000). In a report derived from data of the United States Third National Health and Nutrition Examination Survey, it was calculated that 41.9% of periodontitis cases (6.4 million cases) in the adult population were attributable to current cigarette smoking and 10.9% (1.7 million cases) to former smoking (Tomar and Asma, 2000). A study of twins indicated that the degree of alveolar bone loss and the number of teeth lost were greater in the twins with a high smoking exposure when compared to their twin partners with a low smoking exposure (Bergstrom and Floderus-Myrhed, 1983).

The study of genetic polymorphisms and smoking indicates that the interaction between polymorphisms and smoking may play a role in susceptibility to periodontal disease (McGuire and Nunn, 1999; Meisel et al., 2002). In a study of 323 maintenance patients, bleeding on probing was associated with a genotype of IL-1 in those who had never smoked but the effect was not significant in smokers, where the authors suggest that the effect is overshadowed by the effect of smoking (Lang et al., 2000).

Several studies have demonstrated that the severity of periodontitis appears to be related to the duration of tobacco use and amount of tobacco intake (Grossi et al., 1995). Periodontal treatment tends to result in a less successful outcome in smokers than in non-smokers (Hughes et al., 2006; Scabbia et al., 2001; Trombelli and Scabbia, 1997). A number of studies have established that smoking is associated with reduced gingival inflammation and bleeding (Bergström and Bostrom, 2001; Muller et al., 2001). Reduced bleeding might reflect an underlying disruption of the immune response and that this may account for the increased loss of clinical attachment and alveolar bone (Barbour et al., 1997; Palmer et al., 2005). Modulation of the host response by smoking will be discussed in the following sections.
1.2.3 Psychological factors

A hypothesis of an increased risk for destructive periodontal diseases due to psychological disorders has long been promoted. Several studies have suggested that psychological stress may be associated with more acute forms of disease particularly necrotizing periodontal diseases (Horning and Cohen, 1995). Studies have demonstrated that individuals under psychological stress are more likely to develop clinical attachment loss and loss of alveolar bone (Hugoson et al., 2002; Pistorius et al., 2002; Wimmer et al., 2002). It is well established that psychological stress can down-regulate the cellular immune response (Rozlog et al., 1999). A recent study suggested that host response to \textit{P. gingivalis} infection may be compromised in psychologically stressed individuals (Hourihane et al., 2003). Another study assessed the effect of stress on the host immune system and showed that stimulation with \textit{P. gingivalis} LPS significantly enhanced secretion of nitric oxide by macrophages, with the stressed animals showing significantly higher levels of nitric oxide than controls. Despite existing evidence from case control and cross sectional studies, no longitudinal studies have been published that confirm psychological stress as a risk factor for periodontal disease. Perhaps the relationship is due to the fact that individuals under stress are less likely to perform regular good oral hygiene and prophylaxis (Croucher et al., 1997).

1.2.4 Systemic risk factors

Periodontitis as a manifestation of systemic disease has been classified as separate periodontal disease category (Armitage, 1999) (Table 1.1). In fact, in most of these systemic diseases, there is either decreased host resistance to infections or perturbations in gingival connective tissue that increases its susceptibility to inflammation-induced destruction. It should be noted that diabetes mellitus is not on this list. Diabetes mellitus can be a significant modifier of all forms of periodontitis but there are insufficient data to conclude that there is a specific diabetes mellitus-associated form of periodontitis.

Diabetes mellitus is one of the strongest systemic risk factors for periodontitis (Kinane and Chestnutt, 1997) and it is considered to be a modifier of existing periodontitis rather than a disease that causes periodontitis (Löe, 1993). Diabetes mellitus has been shown to be positively associated with clinical attachment loss (Genco, 1996). The severity of periodontitis in the diabetic patient appears to be related to the control of the diabetes
Chapter 1: Introduction

(Emrich et al., 1991; Tervonen and Oliver, 1993). In a longitudinal study of non-insulin dependent diabetics, severe periodontal disease at baseline was found to be a significant risk factor for poor glycemic control (Taylor et al., 1996). Similarly, poor glycemic control in non-insulin dependent diabetics has been shown to be associated with significantly greater alveolar bone loss compared to well-controlled non-insulin-dependent diabetics (Taylor, 2001). In addition, it has also been suggested that effective periodontal therapy can have a positive effect on the control of diabetes (Thorstensson, 1995).

Associations between periodontal disease and other systemic diseases such as osteoporosis, rheumatoid arthritis have also been reported, although it is not clear what role these relationships play (Mercado et al., 2001; Wactawski-Wende, 2001). With the discovery of emerging risk factors, inflammatory responses, and genetic regulators of these responses, it is quite possible that these associations actually reflect common underlying risk factors rather than any sort of causal relationship. The possible role of systemic diseases and systemic exposures in initiating or modifying the progress of periodontal disease is clearly a complex issue.

1.2.5 Genetic factors

A risk factor that cannot be modified is often referred to as a determinant (Genco, 1996). Risk determinants or susceptibility factors are demographic variables including age, gender and genetic factors.

There is clear evidence that genetic factors affect the age of onset, severity, and risk of developing periodontitis (Hart and Kornman, 1997; Hassell and Harris, 1995). While microbial and other environmental factors initiate and modulate periodontal disease, individuals are known to respond differently to common environmental challenges, and this differential response is influenced by the individual's genetic profile (Schenkein, 2002). Evidence for a genetic contribution to periodontitis comes from several sources including familial aggregation of periodontal diseases (Boughman et al., 1988; Marazita et al., 1996), linkage studies (Hart et al., 2000b; Li et al., 2004b) and twin studies (Michalowicz et al., 1991; Michalowicz et al., 2000). Linkage studies of aggressive periodontitis have supported an autosomal dominant transmission (Hart et al., 2000b).
Studies comparing disease in monozygotic and dizygotic twins have suggested that about 50% of the population variance in attachment loss could be accounted for by genetic factors (Michalowicz et al., 2000). Family studies of patients with aggressive periodontitis verify that there is a genetic contribution to this form of disease (Boughman et al., 1988). As well as clinical and epidemiological studies, animal models using susceptible and resistant mice have also provided strong evidence for a genetic predisposition to periodontal disease (Baker et al., 2000; Gemmell et al., 2003). However, studies of the prevalence of different bacterial species in human twins (Michalowicz et al., 1999) or in susceptible versus resistant mouse strains suggest that host genetics alone can not explain this variance in the incidence of P. gingivalis or P. intermedia (Baker, 2005). Thus, it seems that genetic factors have more influence on the host response to infection than on bacterial colonization itself (Baker et al., 2000).

1.3 PATHOGENESIS OF PERIODONTITIS

Periodontitis, a series of infections of the periodontal tissues arises from a complex biofilm of commensal and periodontopathic bacteria and their products, triggering an aberrant human inflammatory response. The bacteria colonizing the hard and soft tissues of the oral cavity are known to profoundly influence oral health and disease (Henderson and Wilson, 1998). Over 700 species have been identified in the human oral cavity (Kazor et al., 2003; Paster et al., 2001). These microorganisms produce several bioactive end products including endotoxins (lipopolysaccharides), exotoxins (e.g. leukotoxin, cytolethal distending toxin) and proteases (e.g. gingipains) (Curtis et al., 2001; Nishihara and Koseki, 2004; Potempa et al., 2000).

These bacterial factors are more likely to challenge and disturb immune responses, and some of them may be chemotactic for polymorphonuclear leukocytes, activate the plasma proteinase cascade systems, or stimulate inflammatory and resident tissue cells to produce cytokines, including IL-1α, IL-1β, TNF-α, platelet activating factor and prostanoids (e.g. prostaglandins, leukotrienes). Reactive oxygen species have also been implicated in the pathogenesis of periodontitis. The overproduction of reactive oxygen species may be a key component of hyper-inflammation associated with periodontitis (Chapple and Matthews, 2007). The pathogenesis of periodontitis is therefore largely attributed to localized inflammation which results from interactions between host and
microbial factors (Ishikawa, 2007). Understanding how the precise nature of host interactions with bacterial factors may clarify what determines the shift from health to periodontal disease. It is noteworthy that the consensus view of the host's involvement in the development of periodontal diseases comes over a hundred years later than when it was first mooted by Miller in 1890, in The Infection/Host Response Paradigm (Armitage, 2002).

Although bacterially induced host responses may contribute to the progression of periodontal inflammation and tissue destruction, the intensity of the inflammatory process varies considerably from person to person (Schenkein, 2006). Resistant individuals successfully control the microbial challenge and the immuno-inflammatory pathways activated by bacteria, preventing periodontal destruction. Classic longitudinal studies have demonstrated the variation that exists in host susceptibility to a bacterial challenge. The vast majority of individuals (~80%) exhibit moderate attachment loss, whereas 7-10% develop severe generalized periodontitis, exhibiting rapid attachment loss (Loe et al., 1986). It is now considered that these variations may be related to complicated genetic, behavioural and environmental predisposing risk factors, including oral hygiene, smoking, emotional stress and diabetes (Brown and Loe, 1993; Kornman et al., 2000; Page, 1991; Schenkein, 2006) (Figure 1.3).

A significant portion of the host predisposition to disease may relate to innate and acquired host defences, subsequent to inflammatory activation. Activation of immune responses occurs via a complex network of inflammatory mediators originating in both myeloid and non-myeloid cells. The variations in these host immune responses may in part be related to specific genetic polymorphisms (Kornman, 2001; Kornman and Duff, 2001).
1.4 HOST RESPONSES TO PERIODONTAL MICROORGANISMS

The host immune and inflammatory response to the microbial challenge is a critical determinant of susceptibility to develop the destructive disease, under the influence of multiple behavioral, environmental, and genetic factors (Cohen, 2002; Jenner and Young, 2005; Schumann, 2004). There is little doubt that patient susceptibility to periodontal disease plays a major role in determining the ultimate disease outcome (Meng et al., 2007).Susceptibility to periodontal disease involves the interplay between bacteria, the host and environmental factors. It has been suggested that periodontitis is a continuous spectrum with defined clusters of individuals presenting with a varying host susceptibility component (Chapple, 2002). The host employs a variety of defence strategies to control and protect itself against bacterial invasion. The oral epithelium represents the first barrier against the microbial biofilm. In addition to providing a physical barrier against invading pathogens, epithelial cells play an important role in innate host immune defences by producing a consortium of antibacterial peptides such as beta-defensins (Dale et al., 2001; Hosokawa et al., 2006; Ouhara et al., 2006). Interactions between bacteria and epithelial cells lead to the activation of several complex signalling cascades, which ultimately regulate the immune responses.
The way in which the host responds to the bacteria is determined by the nature and control of both the innate and adaptive immune responses (Berglundh and Donati, 2005). Innate immunity is responsible for initiation of the inflammatory process, acting as the first line of host defence and is critical for the successful eradication of an infection (Alexander and Rietschel, 2001; Bullard et al., 1996). It is also linked to the adaptive immune response, which helps clear the infection and builds specific immunity with a memory component. Therefore it is difficult to separate their relative roles in the pathology of periodontitis (Dixon et al., 2004). The innate processes of the host immune response primarily depend on the activity of macrophages and polymorphonuclear leukocytes (PMNs) (Mathur and Michalowicz, 1997). Their shared role in the periodontium is the defence against the invasion of pathogens and the maintenance of local homeostasis and tissue integrity. These cells can respond to the bacteria by signalling thorough the Toll-like receptors (TLRs) which can directly initiate the innate immune response, which, in turn, may control the emergence and/or balance of the adaptive immune responses (Schroder et al., 2005).
1.4.1 Polymorphonuclear leukocytes (PMNs)

Polymorphonuclear (PMNs) leukocytes are an important line of defence against dental biofilms accumulated at the gingival margin. Bacterially activated neutrophils also produce chemotactic and vasoactive mediators that perpetuate the host inflammatory response. PMNs can be found within the gingival sulcus and migrate through the junctional epithelium in all stages of periodontal disease (Delima and Van Dyke, 2003; Tonetti et al., 1998). Their specific functions include adherence to host substrata, directed migration along chemical gradients, recognition and phagocytosis of microorganisms, followed by bactericidal activity. The importance of PMNs in protective responses against bacterial invasion is underlined by the prevalence and severity of periodontitis in patients with syndromes characterised by dysfunctional PMNs, such as leukocyte adhesion deficiency (Hart et al., 1994). Data suggests that the neutrophils of patients with localized aggressive periodontitis have abnormal functions (Lavine et al., 1979; Van Dyke, 1985) and it appears that a hyperresponsiveness of the neutrophil results in enhanced tissue destruction in periodontal disease (Kantarci et al., 2003). When activated, PMNs release lysosomal proteolytic enzymes such as Cathepsin G and matrix metalloproteinase-8 (MMP-8) and secrete arachidonic acid metabolites both of which can lead to periodontal tissue damage (Claesson et al., 2002; Pippin et al., 1995; Taichman et al., 1977). In addition, PMNs are capable of directly secreting pro-inflammatory mediators, as well as inducing the production of these cytokines from surrounding cells and tissues (Galbraith et al., 1997). Thus, they are able to directly contribute to the inflammatory response and attachment loss observed in periodontitis (Kantarci and Van Dyke, 2002).

1.4.2 Monocytes/macrophages

Monocytes arise from precursors in the bone marrow, which enter the peripheral circulation. At this point they are still considered immature cells, but upon entering the tissues, they differentiate into macrophages. Like PMNs, these cells are highly motile, but they are long-lived cells and are not terminally differentiated like neutrophils, meaning that they can undergo further proliferation once they exit the circulation and can form large multinucleated giant cells, or osteoclasts. Like PMNs they can respond to chemotactic gradients and share similar enzymatic systems to combat foreign antigens,
but because of their longer life spans macrophages usually predominate in chronic inflammation.

Monocytes and macrophages both function as phagocytes, engulfing and destroying pathogens as well removing damaged tissues. Monocytes can enter the tissues and function as antigen-presenting cells, where they engulf foreign materials, digest them and display these antigens on their cell surface. Bacterial antigens such as lipopolysaccharide, can interact with monocytes/macrophage receptors including CD14 and TLRs to stimulate production of inflammatory cytokines and other mediators (Dixon et al., 2004; Ren et al., 2005). TLR-based recognition of pathogens enables the host to mount and maintain effective defence against pathogens. Monocytes as well as monocyte-derived dendritic cells are identified as the major TLR-expressing cells in human blood (Yang et al., 1998). Ten members of the TLR family (TLR1 to TLR10) have been described (Du et al., 2000a; Takeda et al., 2003). Three of them, namely TLR1, TLR2, TLR4 are highly expressed in monocytes (Ozinsky et al., 2000). Human monocytes also express low levels of TLR3, TLR7, and TLR9 (Hornung et al., 2002).

Monocytes both initiate and enhance the immune response by the secretion of a number of proinflammatory cytokines such as IL-1α and TNF-α and a number of chemokines which influence the recruitment of additional monocytes, PMNs and lymphocytes into the gingival tissues (Amano, 2003; Taubman et al., 2005). The interaction of lipopolysaccharide with macrophages also stimulates production of prostanoids, in particular prostaglandin E2 (Blix and Helgeland, 1998; Shapira et al., 1994). Macrophages also act as antigen-presenting cells in the initial stages of the immune response and play a vital role in the effector stages as microbicidal cells (Ishikawa et al., 1997). The presence of different subpopulations of macrophages in various forms of periodontal tissues may indicate participation of these cells in the local immune response in periodontal disease (Schlegel Gomez et al., 1995).

1.5 MODIFIERS OF HOST RESPONSE

While periodontopathic bacteria and the inflammation they provoke are essential for disease progression, environmental risk factors such as tobacco smoking, psychosocial stress and systemic diseases such as diabetes modify the host response and may be major determinants of the enormous variation in susceptibility (Page et al., 1997). It is likely
then that the amount of inflammatory mediators produced by an individual's monocytes, whether locally in the GCF or from isolated peripheral blood monocytes in response to periodontal microorganisms, is a characteristic of that individual's host response in periodontitis. Individuals with a normal response would have a sufficient level of inflammation which will have beneficial effects to fight the infection, and will not be sufficient to trigger periodontal tissue breakdown. Hyperresponsive individuals would produce much higher levels of inflammatory mediators than normal individuals at the same given challenge, and would exhibit disease expression at a lower microbial load threshold. This has been reported in studies of periodontitis in diabetic patients (Salvi et al., 1997; Takeda et al., 2006). When stimulated with lipopolysaccharide, monocytes isolated from diabetic patients produced higher levels of PGE₂, IL-1 and TNF-α than those of nondiabetic patients with similar periodontal status (Salvi et al., 1998). There are also hyporesponsive individuals producing lower inflammatory mediator levels than normal individuals for a similar challenge (Molvig et al., 1988). Several factors might influence an individual's monocyte responsiveness, including genetic factors and environmental factors such as stress, smoking and systemic diseases (Medvedev et al., 2006; Rallabhandi et al., 2006). Inter-individual differences in monocytic synthesis of cytokines have been reported in relation to different genetic backgrounds (Baker et al., 2000). These genetic factors are intrinsic to the individual and do not vary with time. These behavioral and environmental factors may change over time and affect the individual's response differently at different time-points. This will be further discussed in the following sections.

![Figure 1.5](image_url) **Figure 1.5** Host response characteristics as a function of bacterial challenge level. This curve represents the host response levels to lipopolysaccharide (LPS). There are three types of response including normal, hypo and hyper. Adapted from Champagne et al. (2003).
1.5.1 Systemic modifiers

Systemic diseases can modify all forms of periodontitis through their effects on the normal immune and inflammatory defences. As discussed previously, polymorphonuclear leukocyte deficiencies and a number of genetic disorders such as Down's syndrome and chronic granulomatous disease confer increased susceptibility to periodontitis (Cohen et al., 1985; Gabre, 2000; Orner, 1976). Many of these cases are rare.

Diabetes mellitus is a major systemic risk factor for periodontitis. There is a higher incidence and greater severity of periodontal disease in adults with either both type I and type II diabetics (Emrich et al., 1991; Tervonen and Oliver, 1993). A review found considerable evidence to suggest that diabetes and periodontitis are directly related (Kinane and Chestnutt, 1997). Other studies have also shown that the level of diabetic control is an important factor in this relationship and may modify the response to dental plaque (Aldridge et al., 1995; Guzman et al., 2003). It seems that a bi-directional relationship between periodontal disease and glycemic control exists (Nishimura et al., 2003). Poorly controlled diabetic patients respond less successfully to periodontal therapy relative to well-controlled and non-diabetic patients (Westfelt et al., 1996).

1.5.2 Smoking

Tobacco smoking is among the most important risk factors in the aetiology and progression of periodontal diseases (Bergstrom, 1989; Bergstrom, 2003; Haffajee and Socransky, 2001a). Several mechanisms behind this relationship have been proposed including physiologic effects of smoking on vasoconstriction, re-vascularization of wounds, as well as host response alteration by smoking (Albandar et al., 2000; Barbour et al., 1997). Tobacco smoking increases the risk of periodontal destruction by stimulating inflammatory responses and impairing protective or reparative host responses (Ryder, 2007; Sorensen et al., 2004). Smoking is associated with reduced gingival bleeding (Bergström and Bostrom, 2001; Persson et al., 2001) and reduced bleeding may reflect an underlying disruption of the immune response.

Inflammatory components in the GCF and serum have been studied in relation to smoking. Levels of inflammatory substances in smokers vary from levels in non-smokers
but the significance of this is not clear. Studies have shown altered gingival crevicular fluid inflammatory cytokine profiles such as IL-1, IL-8 (Ryder et al., 1998a; Ryder et al., 1998b) and altered proteolytic regulation in smokers (Chang et al., 2003). However, smoking was not found to alter GCF levels of IL-1β and IL-1ra (Bostrom et al., 2000). Compared to non-smokers, smokers also have lower serum immunoglobulin and antibody levels against bacteria such as *P. gingivalis* (Quinn et al., 1996; Tangada et al., 1997). Smoking has also been shown to influence polymorphonuclear leukocyte function including chemotaxis and phagocytosis (van der Vaart et al., 2004). In another study smokers had a lower level of IgG2, which may impair neutrophil function and in this way aggravate periodontal disease (Fredriksson et al., 1999).

The relationship between some bacterial strains associated with periodontal disease and smoking has been studied (Haffajee and Socransky, 2001b; Preber et al., 1992; van Winkelhoff et al., 2001). The studies suggest that smoking could influence the host control of bacteria. In a study where bacteria were quantified, it was suggested that smoking favours a habitat for the establishment of bacterial species such as *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* in shallow pockets (Eggert et al., 2001). In summary, the results of these studies do not suggest a single mechanism for the effects of smoking, but it is likely that smoking modifies the host immune response in a variety of ways and therefore influences disease expression (Wittebole et al., 2007).

1.5.3 Genetic modifiers

Periodontal disease is associated with a variety of bacterial species; however, neither the type nor the proportion of bacteria fully explains the population variance in this disease (Johnson et al., 1988). Some individuals might be very susceptible and will develop aggressive forms of periodontal disease at a relatively young age, while others might be resistant and will never develop periodontal disease. If not everyone is equally prone to disease, this raises the question as to why some people are more susceptible than others. Genes play a role in the predisposition to and progression of periodontal diseases (Hassell and Harris, 1995; Sofaer, 1990). There is evidence that host genetics may affect the susceptibility to severity of periodontal diseases (Hodge and Michalowicz, 2001). The presence of microorganisms is required, but not sufficient, for the development of an infectious disease, such as periodontitis. A large body of published data suggests that in
humans, susceptibility to infectious diseases is influenced by the genetic make-up of the host (Casanova and Abel, 2004; Segal and Hill, 2003). Clinical phenotypes in the course of an infectious disease result from the complex interaction between environmental (microbial and non-microbial) and host (genetic and non-genetic) factors (Figure 1.5). Genetic epidemiological studies, studies of concordance rates between monozygotic and dizygotic twins and complex segregation studies have implicated human genetics as a central factor in susceptibility to infectious disease (Abel and Dessein, 1998; Kwiatkowski, 2000).

![Figure 1.6](https://example.com/figure6.png)

**Figure 1.6** A schematic showing the stages of the host-environment interaction in the course of infection. The complex process that goes from exposure to an infectious agent to the development of a clinical infectious disease is under host and environmental control. Host factors might be genetic (for example, mutation in a gene involved in immunity to infection) or non-genetic. Environmental factors might be microbial (for example, virulence factors) or related to the mode of exposure (for example, smoking), and might have an impact at each stage of the interaction. Adapted from Casanova and Abel (2004).

To understand the potential clinical relevance of genetic variability on periodontitis it is necessary to understand how genes contribute to genetic diseases (Figure 1.6). Diseases can be divided into two categories based on genetics; Mendelian diseases and multifactorial diseases (also called complex traits). With Mendelian genetics, the interaction between genes and environment that creates the phenotype is dominated by the genetic component (i.e. cystic fibrosis, Papillon Lefèvre syndrome, amelogenesis imperfecta) (Hewitt et al., 2004; Rommens et al., 1989). Diseases like atherosclerosis and
periodontitis are referred to as complex diseases because multiple genes contribute to the phenotype either individually or through interactions with each other or the environment (Hemminki et al., 2006; Rosenberg et al., 2002). In contrast to Mendelian diseases, multifactorial diseases do not follow a simple pattern of familial distribution or transmission. In the case of multifactorial diseases, what is inherited is a higher risk of developing the disease rather than the certainty of disease (Belmont and Leal, 2005).

It is increasingly evident that genetic variance plays an important role in the determination of individual susceptibility to multifactorial diseases (Pritchard, 2001). There are several genetic alterations such as mutations and polymorphisms. Mutations are rare (less than 0.1%) and can have deleterious effects on protein functions, therefore mutations are the primary cause of a clinical phenotype or a disease. DNA sequence polymorphisms are usually defined as variation present at greater than 1% frequency in the population and that by itself is not sufficient to cause a disease (Nielsen, 2004; Wang et al., 1998). Nevertheless, DNA sequence polymorphisms may contribute to susceptibility to a disease or variation in functional properties of a protein. The most common are single nucleotide polymorphisms (SNPs) which occur on average every 1000 nucleotides across the genome (Collins and Morton, 1998; Hinds et al., 2005; Sachidanandam et al., 2001). A single nucleotide polymorphism defines two alleles for which there could be three genotypes amongst individuals in a given population (Figure 1.8). A genotype is a description of the two alleles at a particular locus. Each individual will have one or maximum two forms of DNA. The latter is possible if the parents differed from each other at these alleles and the child inherited a different form from each parent.

There are an estimated 7–10 million SNPs in the 3 billion–nucleotide human genome (Wheeler et al., 2005). SNPs are often inherited together as a consequence of their
physical proximity on a single chromosome and travel as blocks from generation to generation, which is known as linkage (Stephens et al., 2001). Linkage disequilibrium refers to the non-random association of alleles at different loci. Groups of co-inherited SNPs on the same chromosome are called haplotypes and identification of the state of one polymorphism may predict the state of the others (Judson and Stephens, 2001).

Figure 1.8 Example of SNP comprising a C→T substitution. Data generated from PCR product sequenced with ABI BigDye Terminator sequencing kit (Applied Biosystems). Electropherogram shows the genomic DNA from individual homozygous for C at the site of the SNP (A) and individual homozygous for T (B). The base substitution is denoted by a black arrow.

There are also other types of DNA polymorphisms such as minisatellite variable number tandem repeats (VNTRs), microsatellite polymorphisms and insertions or deletions (Reich et al., 2001). VNTRs are not evenly dispersed in the genome and the majority of microsatellite polymorphisms occur at di-nucleotide repeat sequences with very low frequency (1 every 30000 base pairs). The role of VNTRs will not be discussed further due to their low frequency.

Although the majority of DNA polymorphisms are probably functionally neutral, a proportion of them can exert allele-specific effects on the regulation of gene expression or function of the coded protein, thus causing between-individual differences in various biological traits and in susceptibility to disease (Hart et al., 2000a; Toomes et al., 1999).
The precise location of a polymorphism within the gene can be important (Chakravarti, 1999). SNPs are classified according to their position in genes into coding and noncoding polymorphisms. Noncoding SNPs may occur in the promoter region of the gene, within introns, and 5' and 3' untranslated regions (Cargill et al., 1999; Sonenberg, 1994). Noncoding SNPs do not alter the base pair sequence in the part of the DNA that codes for the protein but may alter the rate of transcription, the processing or the stability of the mRNA. Polymorphisms occurring in promoter regions of genes may potentially affect the process of transcription whereby RNA polymerase, the enzyme responsible for transcribing the DNA into mRNA, is recruited to the gene and the mRNA synthesized (Hoogendoorn et al., 2003). Polymorphisms occurring in promoter regions may also modulate or eliminate transcription factor binding, generate binding sites, or result in binding by a different transcription factor (Morley et al., 2004). Introns and the regions around the exons that do not code for proteins are also important, because they contain sequences that may dictate other attributes of how protein production is regulated (Roy and Penny, 2006). Coding SNPs are located in the exons of genes and classified into synonymous and nonsynonymous (Fallin et al., 2001; Tabor et al., 2002). Synonymous SNPs change the codon (set of three nucleotides in these exons code for the amino acids that are used to build proteins) into another that codes for the same amino acid. Therefore, there is no change in the amino acid. Nonsynonymous SNPs change the amino acid with functional consequences and may affect protein structure (Wray et al., 2003). Such coding polymorphisms have classically been implicated in monogenic Mendelian disorders (Sidransky, 2006).

The reason for the current enormous interest in SNPs is the hope that they could be used as markers to identify genes that predispose individuals to common, multifactorial disorders (Loos et al., 2005). The human genes that contribute to these phenotypes can be sought following a hypothesis-driven or genome-wide approach. The hypothesis-driven approach selects candidate genes based on data obtained in animal models, on human data obtained in vitro, or on previous knowledge regarding disease pathogenesis (Fortier et al., 2005; Pharoah et al., 2004). Genome-wide association studies using large numbers of bi-allelic SNPs have been proposed as a potentially powerful method for identifying genes involved in complex diseases (Abel et al., 2006; Nelson et al., 2004). Other approaches have been applied such as linkage analyses using markers of genome variation in large families with multiple affected individuals (Terwilliger and Weiss,
Chapter 1: Introduction

Different experimental designs can be used to conduct genetic association studies (Cardon and Bell, 2001). The most widely used are case-control and cohort studies. For complex disease susceptibility genes, direct association approaches that compare the distribution of genetic marker frequencies in individuals affected by a disease (case) and in those not affected by the disease (controls) are expected to have greater power than traditional linkage studies (Rees, 2002; Risch, 2000). A higher frequency of the SNP in cases than in controls is considered evidence that the allele/genotype is associated with increased risk. However, both linkage analysis and association studies typically leave open the question of whether a disease-associated genetic variant is functionally important (Reich and Lander, 2001).

There are a variety of experimental methodologies to investigate the functional significance of SNPs including DNA footprinting and gel-shift assays, allele-specific analysis of mRNA, assays of secreted protein and reporter gene assays (Figure 1.9) (Rebbeck et al., 2004). The most commonly used assay to investigate the functional effects of SNPs on gene expression is reporter gene assay (Alam and Cook, 1990). This assay involves constructing a plasmid in which the putative regulatory region spanning the position of the SNP(s) from the gene of interest is linked to the coding sequence for a reporter gene whose expression can be measured (Figure 1.9B). The reporter gene construct is then transfected into a cell and expression assayed and then constructs with the two allelic forms of the SNP are compared.

A) Secreted protein assay  
B) Reporter gene assay

Figure 1.9 The approaches for investigating the functional significance of SNPs A) Secreted protein assay: An approach is to compare levels of protein produced from the gene of interest in individuals of differing genotype, for example, homozygous AA or BB or heterozygous B. B) Reporter gene assay: The DNA sequence of interest, for example a promoter region spanning an SNP, is placed upstream of a reporter gene whose expression can be measured. The reporter gene construct is then inserted (transfected) into a cell and expression assayed. Adapted from Knight (2005).
1.6 CANDIDATE GENE APPROACH and PERIODONTAL DISEASE

Since SNPs are the most common source of human genetic variation, (Suh and Vijg, 2005), associating SNPs with human disease phenotypes has potential for direct clinical application by providing genetic markers for diagnostic and prognostic purposes and, possibly, novel therapeutic targets. Thus there has been enormous effort expended so far to identify gene polymorphisms associated with risk for periodontal diseases (Kinane et al., 2005).

Large scale screening for SNPs is still very complex (Cantor and Nelson, 2005). Alternative approaches include selecting candidate genes coding for particular features of a disease (Goldstein et al., 2003). This is in contrast to genome-wide scans and other approaches that rely on linkage disequilibrium between loci to identify genotype-disease associations, for which knowledge of function is not required. A good candidate gene should have an expression pattern consistent with the disease phenotype and genes implicated in a disease may be expressed to a different degree in patients and controls. There are three types of candidate genes; functional candidate genes; positional candidate genes and expressional candidate genes (Hunter et al., 2005). Functional candidate genes are identified from an existing knowledge of the phenotype and the potential function of the gene involved after clinical or physiological studies of affected individuals (Tabor et al., 2002). Positional candidate genes are based on the involvement of the gene to a marked chromosomal location after genetic linkage analyses (Cantor and Nelson, 2005). Expressional candidate genes are determined through differences in gene expression using microarrays.

The most promising approach for statistically powerful candidate gene association studies involves; i) the selection of candidate genes on the basis of different types of information, including available linkage results and results obtained with mouse models of the phenotype ii) determination of the haplotype structures for these genes in control populations and testing for association between phenotype and haplotype status iii) assessing function of selected haplotypes using experimental systems, including in vitro systems and in vivo animal models (Collins et al., 1997; Goldstein and Weale, 2001; Johnson et al., 2001; Lohmueller et al., 2003; Rebbeck et al., 2004).
There has been a great interest in exploring SNPs in those genes involved in the control of the inflammatory response. To date many studies have focused on cytokine genes due to their significant role in orchestrating the immune response (Bidwell et al., 1999). Interindividual differences in cytokine profiles appear to be due, at least in part, to allelic polymorphism within regulatory regions of cytokine genes. Many studies have examined the relationship between cytokine gene polymorphisms, cytokine gene expression in vitro, and the susceptibility to and clinical severity of diseases.

1.6.1 Candidate Genes in Periodontal Disease

Genetic polymorphisms in a candidate gene approach have been explored as risk factors for periodontitis. There is a genetic basis to many aspects of the periodontal host response (Schenkein, 2006). Mutations of genes have been identified in Mendelian inherited syndromes that include significant periodontitis as a future clearly indicating that single gene defects can predispose to periodontitis (Hart et al., 2000b). This has facilitated studies to evaluate the association of an array of genetic polymorphisms with periodontitis.

Pro-inflammatory and regulatory cytokines have been selected as possible candidate genes as a result of their direct involvement in the pathogenesis of periodontal disease (Gemmell et al., 1997; Landi et al., 1997; Oido-Mori et al., 2001; Taylor et al., 2004). Inflammatory cytokines including TNF-α, IL-1α, IL-1 β, IL-6, and IL-8 are present in the diseased periodontal tissues, and their uncontrolled production seems to play a role in tissue destruction (Bascones et al., 2005; Okada and Murakami, 1998). This approach is based on the hypothesis that increased or unregulated production of inflammatory cytokines in response to periodontopathogens occurs in subjects carrying functional polymorphisms in these genes. There are reports that some polymorphisms in the genes encoding IL-1, TNF, Fc gamma receptors (Fc gammaR), IL-10 and the vitamin D receptor, may be associated with periodontitis in certain ethnic groups. Table 1.5 summarizes the results of a PubMed search on published gene polymorphisms and periodontitis (Takashiba and Naruishi, 2006).

Polymorphisms in the IL-1 system have been the main focus of attention because of an important regulatory role of IL-1 in inflammation and host response in periodontal
disease (Taylor et al., 2004). Since TNF-α plays an important role in both the immune and inflammatory responses, several studies have investigated the role of genetic variants such as microsatellite polymorphisms and SNPs in the TNF-α gene in relation to periodontal disease (Craandijk et al., 2002; Endo et al., 2001; Folwaczny et al., 2004; Kinane et al., 1999). They have failed to find any significant associations between TNF-α gene polymorphisms and periodontitis. However, other studies found a correlation between the TNF-α -308 genotype and levels of TNF-α production by neutrophils in periodontal disease (Galbraith et al., 1998). IL-6 is expressed by a wide variety of cells in the periodontal lesion and in common with IL-1 and TNF-α, enhances bone resorption. It has been suggested that polymorphisms in the IL-6 gene may be one of the protective factors associated with lower susceptibility to chronic periodontitis (Holla et al., 2004; Komatsu et al., 2005). Several other candidate genes have also been examined for association with periodontal disease. These studies have not supported a role for the tested polymorphisms in IL-10 (Hennig et al., 2000; Yamazaki et al., 2001) or IL-4 (Michel et al., 2001; Pontes et al., 2004; Scarel-Caminaga et al., 2003).

<table>
<thead>
<tr>
<th>Category (n)</th>
<th>Gene (n)</th>
<th>Correlation (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Cytokine (66) and its receptor</td>
<td>IL-1 (36)</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>TNF (14)</td>
<td>6</td>
</tr>
<tr>
<td>HLA allele (20)</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Immuno- receptor (21)</td>
<td>FcR (14)</td>
<td>13</td>
</tr>
<tr>
<td>Protease (6)</td>
<td>MMP (3)</td>
<td>2</td>
</tr>
<tr>
<td>Enzymes and hormones</td>
<td>Cathepsin C (6)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Vitamin D - R (6)</td>
<td>5</td>
</tr>
<tr>
<td>Others (16)</td>
<td></td>
<td>9</td>
</tr>
</tbody>
</table>

Table 1.5 Reports of literature search summarizing findings on the association between periodontitis and polymorphisms in genes affecting host response and metabolism. Adapted from Takashiba and Naruishi (2006).

1.7 IL-1α: A CANDIDATE GENE IN PERIODONTAL DISEASE

The observation that severe periodontitis affects only 7–15% of individuals means that not all individuals are at equal risk for this disease. Therefore, it is important to establish who is at risk and what characteristics can be used to identify those individuals at the greatest risk. The association between the risk factor and the disease should be consistent
with current understanding of the disease process, and the factor should remain associated with the disease after controlling for other known risk factors and background variables. Such a factor could be used to screen individuals successfully and classify them accurately into high- and low-risk groups, and if this factor is targeted by specific interventions in high-risk individuals the probability of disease occurrence in these individuals should be lowered. Although it is likely that the IL-1 genes are likely to be among many other such genes that contribute to risk for periodontitis, the biology of IL-1 and the association of high innate levels of IL-1 with severe disease provide compelling evidence that this molecule is important in understanding periodontal destruction. This is discussed in the following sections.

IL-1α is a multifactorial proinflammatory cytokine that plays an important role in inflammation and host responses to infection. The IL-1α gene, a member of the large IL-1 gene family is located in the region 2q12-q21 of human chromosome 2, with 7 exons and 6 introns (Furutani et al., 1986; Lafage et al., 1989; March et al., 1985). The original identification of IL-1 as an endogenous pyrogen, lymphocyte-activating factor, hemopoietin-1, and osteoclast-activating factor, serves to demonstrate its pleiotropic activity (Allan et al., 2005; Dinarello, 2002; Tanaka et al., 2005).

The IL-1 gene family of cytokines includes IL-1α and IL-1β and IL-18 and six new members named IL-1F5 to IL-1F10 (Nicklin et al., 2002). The IL-1 family also has another member, IL-1 receptor antagonist (IL-1Ra), a natural competitive inhibitor to IL-1 activity. Comparison of human IL-1α, IL-1β and IL-1RA amino acid sequences yields < 25% identity (Dinerallo, 1996). IL-1 family members act by binding to specific receptors. IL-1α and IL-1β bind to IL-1 type I receptor (IL-1RI) and this results in recruitment of the IL-1 receptor accessory protein (IL-1RAcP) which is a required signalling component (Cullinan et al., 1998). A third member of the family, the IL-1 receptor antagonist (IL-1ra), also binds to the type I IL-1 receptor but does not interact with IL-1RAcP. Therefore, IL-1ra functions as an antagonist preventing the action of the agonist IL-1s by blocking the receptor (Hannum et al., 1990). Additional regulation is provided by the type II, or decoy, IL-1 receptor, which binds and sequesters the agonist IL-1s without inducing any signaling response of its own (Colotta et al., 1993).
1.7.1 Expression and synthesis of IL-1α

The IL-1A gene does not contain a classical transcription initiation motif or TATA box (Furutani et al., 1986). The main elements such as NFκB and NF-IL6 regulating IL-1α expression are located upstream of transcription site (Figure 1.10) (Furutani, 1994; McDowell et al., 2005; Stylianou and Saklatvala, 1998). Several studies have shown the functional significance of the proximal promoter region in IL-1α gene regulation (Alheim et al., 1996; Bailly et al., 1996a; McDowell et al., 2005; Zaldivar et al., 2002). Transactivation of the IL-1α promoter in T cells by the T-lymphotropic virus type 1 tax (HTLV-1) protein suggests that NF-κB may be involved in the transcriptional regulation of IL-1α (Mori and Prager, 1996). The NFκB sites are also likely to be involved in the induction of IL-1α by other factors such as glucocorticoids (Awad et al., 2002; Miyazaki et al., 2000). Recently, an NF-IL-1α binding site has been shown to be important for aberrant constitutive expression of IL-1α in fibroblasts from patients with systemic sclerosis (Kawaguchi et al., 2003). There is also evidence that both NFκB and AP-1 may be induced by IL-1α itself, suggesting a possible positive feedback loop (Niu et al., 2004; Werman et al., 2004; Zaldivar et al., 2002).

![Fig. 1.10. A schematic representation of the structural organisation of human IL1A gene including the proximal promoter, exons 1-2 and intron 1. The major transcription factor sites for NFκB (black), NF-IL6 (hatched), are shown with numbers below each coloured box indicating the boundary of each region and the distance in base pairs from the transcriptional initiation site (shown as an arrow). Adapted from Stylianou and Saklatvala (1998)](image)

The transcription of IL-1α is stimulated during inflammatory and infectious processes by the immune complexes, certain coagulation and complement cascade proteins, viral or bacterial products, in particular LPS (Calvano et al., 2005; Hazuda et al., 1988). It is also induced or enhanced by certain cytokines such as macrophage colony stimulating factor (M-CSF) (Hanamura et al., 1997) and interferon-gamma (IFN-γ) (Kobayashi et al., 1995). Other cytokines such as TNF-α stimulate the production of IL-1α by keratinocytes (Kutsch et al., 1993) and gingival fibroblasts (Kobayashi et al., 1999). IL-1α also
Chapter 1: Introduction

stimulates its own production by monocytes (Manson et al., 1989). In addition, stimulants of non-microbial origins such as phorbol esters, retinoic acid and stress can also stimulate transcription and synthesis of IL-1α (Jarrous and Kaempfer, 1994; Mandinov et al., 2003; Murphy et al., 2003).

After stimulation in vitro by LPS, mRNA for IL-1α appears in the monocyte cells after 15 minutes, accumulates over 4 hours and peaks at 6 hours then rapidly declines. The half-life of the IL-1α mRNAs may vary depending on cell type but is generally in the range of 6-8 hours (Godambe et al., 1993; Perregaux and Gabel, 1998).

1.7.2 Maturation and secretion IL-1α

Upon cell activation, IL-1α is translated as 31-kDa precursor molecule (pro-IL-1α). Pro-IL-1α remains in the cytosol after its translation and does not accumulate in organelles. Pro-IL-1α is as fully active as the mature form, remains intracellular for the main part and acts at this level (Hu et al., 2003; Mosley et al., 1987; Werman et al., 2004). IL-1α is undetectable in the serum of patients with inflammatory diseases, with the exception of severe pathological states in which cell death likely allows release of the cytokine (Watanabe and Kobayashi, 1996). Under some circumstances, pro-IL-1α is cleaved to yield a 17-kDa mature cytokine species that retains biologic activity; a calpain-like protease has been implicated in this cleavage (Carruth et al., 1991; Kobayashi et al., 1990). Studies have demonstrated the existence of a transmembrane IL-1α form of 23 kDa (Bailly et al., 1990b; Brody and Durum, 1989; Kurt-Jones et al., 1985). This IL-1α form is bioactive and was demonstrated at the surface of monocytes and B lymphocytes. IL-1α differs from most other cytokines by lacking a signal sequence, thus not trafficking through the endoplasmic reticulum (ER)-Golgi pathway; the precise mechanisms of IL-1α secretion are thus largely unknown (Andrei et al., 1999; Minnich-Carruth et al., 1989).

1.7.3 Biological and pathological properties of IL-1α

Interleukin-1α is one of the most potent proinflammatory cytokines involved both in physiological immune responses and in the development of various immuno-pathological disorders. IL-1α is a multifunctional cytokine that plays a major role in acute and chronic inflammation. The importance of IL-1α in the initiation and maintenance of adequate responses to bacterial invasion has been clearly established and reported in some detail
Data obtained from studies using IL-1R1-deficient mice suggest that IL-1α is an important factor in response to tissue damage and infection, but is not required for normal development and homeostasis (Labow et al., 1997).

The biological effects of IL-1α are multiple and directed at many cell types and organs, but its principal role is considered the initiation of the defence reaction. IL-1α is made by a variety of cells, in particular monocytes, macrophages and epithelial cells, keratinocytes, gingival and dermal fibroblasts, neutrophils and endothelial cells (Bailly et al., 1990a). Major differences are observed in the amount of secreted IL-1α from these different cell types (Kobayashi et al., 1999; Kong et al., 2006; Lonnemann et al., 1989; Murphy et al., 2003; Roubenoff et al., 1998; Sugano et al., 2004).

IL-1α regulates major innate immune processes by modulating the production of potent inflammatory molecules, such as nitric oxide (NO), type II phospholipase (PL) A₂, cyclooxygenase (COX)-2, prostaglandins, leukotrienes, platelet-activating factor and other cytokines (Clausen et al., 1996) (Figure 1.10). The metabolites of arachidonic acid, prostaglandins and leukotrienes in turn are also endogenous regulators of IL-1α (Endres et al., 1989). IL-1α can also up-regulate adhesion molecules (ICAM-1) on endothelial cells (Scholz et al., 1996). IL-1α is likely to play a pivotal role in the pathogenesis and maintenance of chronic diseases by augmenting the transcription of other pro-inflammatory genes such as IL-8 and IL-6 (Werman et al., 2004).

IL-1α is also a major inductive factor in osteoclastic bone resorption and can simultaneously inhibit osteoblastic activity (Suda et al., 2004; Tanabe et al., 2005; Wei et al., 2005; Yu and Ferrier, 1993). Connective tissue remodelling occurs in growth and development and is regulated by the interplay of cell-cell and cell-matrix interactions involving the production of enzymes, activators and inhibitors and cytokines and growth factors (Reynolds and Meikle, 1997). Proteinases such as the metalloproteinases (MMPs) are key enzymes in tissue degradation and IL-1α can directly activate MMPs thereby contributing to tissue destruction (West-Mays et al., 1995).
As listed in Table 1.6, IL-1α induces the expression of genes associated with inflammation at the transcriptional level. It is clear that IL-1α has been associated with a wide range of vital functions including immune cell recruitment, cell proliferation, angiogenesis and invasiveness of different tumour cells, tissue destruction, bone resorption, blood pressure and central nervous cell functions (Dinarello, 2000; Dinarello, 2002; Voronov et al., 2003). Moreover, clinical studies show that IL-1α is a significant modifier of inflammation in vivo, as exemplified by patient responses to IL-1 inhibitors (Choy and Panayi, 2001; Dayer et al., 2005). With all these capabilities, IL-1α appears to act as key cytokine in many chronic diseases ranging from rheumatoid arthritis (Buchan et al., 1988; Niki et al., 2001), Alzheimer’s disease (Hayes et al., 2004), juvenile osteoarthritis, systemic sclerosis (Du et al., 2000b) to periodontitis (Dayan et al., 2004).

1.8 IL-1α versus IL-1β

IL-1α and IL-1β display amino acid and nucleotide homologies of 26% in humans (Dower et al., 1986). IL-1α and IL-1β bind to the same receptors, and it generally has been assumed that the two forms of the cytokine elicit similar responses (Dinarello, 1996; Patarca and Fletcher, 1997). However, there is evidence that distinguishes specific roles for IL-1α and IL-1β causing inflammatory disease and that IL-1α is an essential mediator of Yersinia enterocolitica-induced intestinal inflammation that cannot be
compensated for by the endogenous levels of IL-1β (Dube et al., 2001). Endogenously produced IL-1α and IL-1β differ dramatically in the subcellular compartments in which they are active. Unlike pro IL-1α, pro IL-1β only has marginal biological activity and needs to be cleaved by the IL-1-converting enzyme (ICE) or caspase-1 to yield the active IL-1β peptide (Cerretti et al., 1992). IL-1α is mainly active as an intracellular precursor (31 kDa) or as a membrane-associated form (23 kDa), but is only marginally active as a secreted 17 kDa molecule. IL-1α is only rarely secreted by living cells, except for activated macrophages, and in contrast to IL-1β, IL-1α is not commonly detected in blood or in body fluids, except during severe disease, in which case the cytokine may be released from dying cells. Despite the fact that in vitro IL-1α apparently shares the vast majority of effects of IL-1β, its physiological role remains unclear (Dinarello, 1996).

Although there is a significant amount of data supporting the role of IL-1 in the initiation and progression of periodontal disease, most studies have focused on the role of IL-1β in periodontal disease (Delaleu and Bickel, 2004; Figueredo et al., 1999; Graves and Cochran, 2003; Hou et al., 1995; Jandinski et al., 1991). Little is known about the role of IL-1α in inducing an inflammatory response to periodontopathogens. Its relative pathogenic contribution during the progression of periodontitis remains to be fully elucidated.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>IL-1α, IL-1β, IL-1Ra, TNF-α, IL-12, IL-3, IL-6, IL-12, GM-CSF, IL-8 and other chemokines</th>
</tr>
</thead>
</table>
| Cytokine receptors | IL-2 (P55)  
| | IL-2, IL-3, GM-CSF |
| Proinflammatory mediators | Cyclooxygenase, type-2  
| | Cytosolic and secretory phospholipase A₂  
| | Endothelin-1 |
| | Gamma glutamyl transferase |
| Growth factors | PDGF A chain; fibroblast growth factor; keratinocyte growth factor  
| | Hepatocyte growth factor  
| | Nerve growth factor  
| | Melanoma growth stimulatory activity  
| | Insulin-like growth factor |
| Tissue remodelling | Stromelysin, gelatinases, elastase, collagenases, tissue inhibitor of metalloproteinases 1 |
| Adhesion molecules | ICAM-1, ELAM, VCAM-1, lymphocyte L-selectin |

Table 1.6 Increased Expression of Various Genes by IL-1α. IL-1α induces the expression of several genes associated with inflammation. Adapted from Dinarello (1996).
1.9 IL-1α in periodontal disease

A number of lines of evidence support a pivotal role for IL-1α in mediating the host response to periodontal pathogenic bacteria and the associated tissue damage. Firstly, levels of IL-1α are undetectable in the gingival crevicular fluid (GCF) of healthy teeth but are highly elevated in severe periodontal disease (Ishihara et al., 1997; Mathur et al., 1996; Rasmussen et al., 2000). Secondly, successful treatment results in the reduction of IL-1α concentration to pre-disease levels (Holmlund et al., 2004). Thirdly, in a primate model of acute periodontal disease the use of IL-1 receptor antagonists to block IL-1 activity is able to significantly reduce inflammation and tissue damage (Delima et al., 2002; Graves et al., 2000; Graves and Cochran, 2003). Finally, the ectopic expression of IL-1α in the oral epithelia of transgenic mice shows that expression of IL-1α is sufficient to induce all the cardinal features of periodontal disease even in the absence of bacteria (Dayan et al., 2004).

1.9.1 The role of IL-1α in bone resorption

IL-1α is produced in response to periodontal pathogens mainly by monocytes in the periodontal tissues but is also produced by other cells including gingival keratinocytes, gingival fibroblasts, human polymorphonuclear leukocytes (Henderson et al., 2002; Johnson and Organ, 1997; Kobayashi et al., 1999; Matsuki et al., 1993; Stoufi et al., 1987; Takeichi et al., 1994; Zhou et al., 2005).

Alveolar bone resorption is a hallmark of periodontitis. IL-1α is one of the most potent bone-resorbing factors associated with inflammation. A number of studies have found IL-1α can simultaneously inhibit osteoblast and promote osteoclast activity (Nakamura and Jimi, 2006; Tanabe et al., 2004; Wang and Stashenko, 1993). Further studies demonstrate that increased IL-1α synthesis by bone marrow cells may play an important role in the differentiation and survival of osteoclasts induced by Aggregatibacter (Actinobacillus) actinomycescomitans LPS (Ueda et al., 1998). In vivo periodontal disease models show that regulation of receptor activator of nuclear factor kappaB ligand (RANKL) and osteoprotegerin (OPG) expression is important for the control of alveolar bone destruction (Teng et al., 2000). Recent studies have shown that IL-1α induces RANKL expression by stromal cells (Wei et al., 2005) and decreases OPG production by osteoblasts (Tanabe et al., 2005) and thus stimulates bone resorption. Moreover, the
degradation of collagen is a key step in bone resorption and MMPs have long been implicated in collagen degradation (Song et al., 2006). IL-1α increases the expression of several MMPs including MMP-1, MMP-3 and MMP-7 (Wisithphrom et al., 2006). Collectively, the literature on IL-1α suggests that it is involved in the regulation of bone resorption generally and that includes alveolar bone resorption.

1.10 IL-1A gene polymorphisms and periodontal disease

It has been reported that genetic polymorphisms at the IL-1 gene locus including IL-1α may identify individuals who are susceptible to periodontitis, although there is disagreement about which variant of the IL-1 gene confers susceptibility. The IL-1A gene demonstrates interindividual differences in protein production rates in response to stimulation and these high and low producer phenotypes have a molecular basis (Endres et al., 1989). A number of polymorphisms have been reported in the IL-1A gene (Bailly et al., 1996b; Bensen et al., 2003; Dominici et al., 2002). Although many single-nucleotide polymorphisms (SNPs) have been identified in the coding and regulatory regions of the IL-1A gene (http://www.ncbi.nlm.nih.gov/entrez), most clinical studies have used only one or two of the SNPs that are reported to exist in this region (Kaijzel et al., 2002; McDowell et al., 1995). The IL-1A gene variations have been associated with earlier onset or more severe disease expression of Alzheimer disease (Dominici et al., 2002), cardiovascular disease (Um et al., 2003), osteoporosis (Duncan et al., 1999), polycystic ovary syndrome (Kolbus et al., 2006), primary open angle glaucoma (Wang et al., 2006) and gingival overgrowth (Bostanci et al., 2006).

The possible role of IL-1A gene polymorphisms in the periodontal diseases was first suggested in 1997 (Kornman et al., 1997a). In that study, the IL-1 composite genotype formed by the carriage of allele 2 at both IL-1A -889 and IL-1B +3953 was found to be associated with an increased risk for severe chronic periodontitis with an odds ratio of 6.8 (95% confidence interval: 1.01–45.95). Since then, several studies have evaluated the association of IL-1 genotypes with the severity of periodontal disease (Kornman et al., 1997a; McDevitt et al., 2000), increased bleeding on probing (Lang et al., 2000) and tooth loss (McGuire and Nunn, 1999). The composite genotype has also been reported to be correlated to the bacterial composition of plaque from periodontitis patients (Agerbaek et al., 2006; Socransky et al., 2000). However, contradictory reports showed that the
simultaneous presence of allele 2 at the IL1A-889 and IL1B +3953 loci is not positively associated with severity of chronic periodontitis (Meisel et al., 2002; Papapanou et al., 2001) or tooth loss (Cattabriga et al., 2001). The IL-1 composite genotype is also limited to specific racial groups. Whilst this genotype is apparently predictive of periodontal disease severity in people of European population, it is so infrequent among people of Chinese subjects that correlation with disease severity cannot be made (Armitage et al., 2000).

1.10.1 Interleukin-1A gene polymorphisms in aggressive periodontitis

IL-1A gene polymorphisms, including IL-1A-889 and IL-1A+4845 have been studied by different groups in various ethnic populations (see Table 1.7). Since there is strong linkage disequilibrium between IL-1A-889 SNP and IL-1A+4845 SNP in exon 5 (G to C transition) some of the studies have chosen to test the latter (Gore et al., 1998; McGuire and Nunn, 1999).

Regarding the relationship between IL-1A genotype and aggressive periodontitis susceptibility, conflicting results have been presented based on different ethnic populations, including Caucasian-American, African-American, European-Caucasian and Asian populations (Anusaksathien et al., 2003; Brett et al., 2005; Gonzales et al., 2003; Hodge et al., 2001; Moreira et al., 2007; Rogers et al., 2002; Walker et al., 2000). Studies on early-onset periodontitis found that allele 1 (C) of IL-1A-889 and IL-1B+3953 was transmitted more frequently with this clinical phenotype. In addition, both polymorphisms were in strong disequilibrium with each other in Caucasians but not in African Americans (Diehl et al., 1999). When the association of these polymorphisms with aggressive periodontitis was investigated in a Chinese population, the frequency of allele 2 at the IL-1A+4845 has been correlated with an increased risk in male patients (Li et al., 2004a).

In contrast, studies on generalized early onset periodontitis in Scottish Caucasian patients failed to find any association between aggressive periodontitis and genotype with respect to the IL-1A-899 genotype (Hodge et al., 2001). An investigation of this genotype in Brazilian and Japanese populations did not reveal any association with aggressive periodontitis (Tai et al., 2002). The contradictory results found in several studies may
also be explained by differences in the diagnostic criteria used to define populations, the low number of subjects studied, and the characteristics of the control population (Table 1.7). Since aggressive periodontitis is a complex, oligogenic disorder, IL-1A genetic variations may have an important but not exclusive influence on disease risk.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Genotype reported</th>
<th>Subjects</th>
<th>Study design</th>
<th>Principal findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diehl et al., 1999</td>
<td>IL-1A-889</td>
<td>28 African-American and seven Caucasian American</td>
<td>Linkage analysis</td>
<td>Allele 1 of IL-1A-889 was transmitted more frequently with the EOP phenotype</td>
</tr>
<tr>
<td>Hodge et al., 2001</td>
<td>IL-1A-889</td>
<td>56 GEOP patients and 56 controls with Caucasian heritage</td>
<td>Cross-sectional</td>
<td>No assoc.</td>
</tr>
<tr>
<td>Rogers et al., 2002</td>
<td>IL-1A-889</td>
<td>119 Caucasian EOP patients and 60 healthy controls in Perth Australia</td>
<td>Cross-sectional</td>
<td>No assoc.</td>
</tr>
<tr>
<td>Walker et al., 2000</td>
<td>IL-1A+4845</td>
<td>37 African-Americans with LJP</td>
<td>Cross-sectional</td>
<td>No assoc.</td>
</tr>
<tr>
<td>Trevilatto et al., 2002</td>
<td>IL-1A-889</td>
<td>Brazilian family, 14 individuals) with AgP</td>
<td>Family study</td>
<td>No assoc.</td>
</tr>
<tr>
<td>Gonzales et al., 2003</td>
<td>IL-1A+4845</td>
<td>28 North European AgP patients and 33 controls, 16 Central American AgP patients and 14 controls</td>
<td>Cross-sectional</td>
<td>No assoc.</td>
</tr>
<tr>
<td>Quappe et al., 2004</td>
<td>IL-1A-889</td>
<td>36 AgP patients, 75 healthy controls and 75 subjects of unknown periodontal status (reference population) in Chile</td>
<td>Cross-sectional</td>
<td>No assoc.</td>
</tr>
<tr>
<td>Tai et al., 2002</td>
<td>IL-1A+4845</td>
<td>47 GEOP patients and 97 healthy controls in Japan</td>
<td>Cross-sectional</td>
<td>No assoc.</td>
</tr>
<tr>
<td>Liu et al., 2004</td>
<td>IL-1A+4845</td>
<td>122 GAgP and 96 healthy controls in Chiese</td>
<td>Case-control</td>
<td>Association in male</td>
</tr>
<tr>
<td>Anusaksathien et al., 2003</td>
<td>IL-1A-889</td>
<td>123 Thai subjects, including CP, AgP and healthy controls</td>
<td>Cross-sectional</td>
<td>No assoc.</td>
</tr>
<tr>
<td>Moreira et al., 2006</td>
<td>IL-1A-889</td>
<td>55 GAgP and 41 healthy controls</td>
<td>Case-control</td>
<td>No assoc.</td>
</tr>
</tbody>
</table>

Table 1.7 Reported IL-1A gene polymorphisms in aggressive periodontitis. GAP, (generalized) aggressive periodontitis; G-EOP, (generalized) early onset periodontitis; No assoc., not related to disease susceptibility.
1.10.2 IL-1A gene polymorphisms in chronic periodontitis

In contrast to aggressive periodontitis, several reports on chronic periodontitis indicate that the IL-1A genotype is associated with this form of periodontal disease (Laine et al., 2001; McDevitt et al., 2000; Papapanou et al., 2001; Shirodaria et al., 2000). However, other studies failed to find an association between IL-1A genotype and chronic periodontitis (Armitage et al., 2000; Lopez et al., 2005). IL-1A gene polymorphisms have been investigated with respect to chronic periodontitis, smoking, ethnic group, oral microbial pathogens present in the plaque biofilm and other periodontal clinical parameters related to inflammatory status or to the progression of the disease (Cattabriga et al., 2001; Cullinan et al., 2001; Kornman et al., 1997a; Laine et al., 2001; Moreira et al., 2007). It has been suggested that the IL-1A allele 2 (T) genotype is correlated with the levels of specific bacterial complexes including *T. forsythensis*, *P. gingivalis*, *T. denticola* and suggests that there may be a correlation between a tendency of genotype-positive people to harbour these bacteria in their plaque (Socransky et al., 2000). A 5-year longitudinal study of a group of subjects of almost exclusively European heritage showed an interaction between the IL-1A periodontitis-associated genotype and age, smoking and presence of *P. gingivalis* (Cullinan et al., 2001). Studies including Chinese, Greek, Japanese and Thai populations did not find a relationship between IL-1 genotypes and chronic periodontitis disease susceptibility or severity (Anusaksathien et al., 2003; Armitage et al., 2000; Sakellari et al., 2006). Overall, the results appear contradictory and it is difficult to draw any firm conclusion concerning the association of IL-1A gene polymorphisms with chronic periodontitis. Besides ethnic differences, it appears that confounding factors, such as smoking, gender or age may collectively complicate the study outcomes on IL-1A polymorphisms in chronic periodontitis.

Polymorphisms in the IL-1A gene linked with periodontitis appear to be dependent upon ethnic background, and also may indicate that different genetic polymorphisms are associated with different forms of periodontitis, namely chronic periodontitis or aggressive periodontitis (Hodge et al., 2001; Parkhill et al., 2000).

1.11 The functional importance of IL-1A gene polymorphisms

The suggestion that IL-1A gene polymorphisms influence the pathogenesis of periodontal disease needs to be supported by evidence from studies at the molecular and
cellular level. However, the number of studies describing the functional importance of IL-1A polymorphisms is relatively few. There is evidence supporting the hypothesis that various combinations of alleles on the IL-1A gene differ in the way they produce IL-1α cytokine in vivo. A study reported that significantly elevated levels of IL-1α in plasma were detected with the IL-1A-899 (TT) homozygous as compared to the IL-1A CC or CT (Hulkkonen et al., 2000). Another study reported that carriage of the IL-1A-889 allele 2 (T) resulted in increased IL-10 plasma levels in healthy individuals (Kilpinen et al., 2002).

There is evidence that the presence of allele 2 (T) at position IL-1A-899 was associated with a four-fold increase of GCF IL-1α protein levels in non-smoker chronic periodontitis patients (Shirodaria et al., 2000). Furthermore, periodontitis patients carrying the IL-1A-889 T allele had significantly increased serum levels IL-6 and C-reactive protein (D’Aiuto et al., 2004). However, it is not clear whether these promoter polymorphisms are causally related to the regulation of IL-1α transcription. A single in vitro study has tested the effect of the IL-1A-889 polymorphism on transcriptional activity in reporter gene assays (Dominici et al., 2002). This study reported that allele 2 (T) at position IL-1A-899 was associated with a 1.8-fold increase at basal transcription rate compared with a construct containing the allele 1 (C) in PCIJ cells (human pancreatic cell line). The same TT genotype was also associated with five fold higher levels of secreted IL-1α compared with the CC genotype when PMBC cells were stimulated with LPS for 3 hours. Interestingly, those differences disappeared after 6 hours of cell stimulation. The basis for such observations will be further discussed in Chapter 5.

1.12 AIMS OF THE STUDY

As mentioned in this review, the available evidence suggests that IL-1α is an important mediator of periodontal disease. Furthermore it appears that genetic polymorphisms in the IL-1A gene may play an important role in influencing susceptibility to disease by regulating transcription and thereby increasing IL-1α production. Therefore the hypothesis to be tested here is that polymorphisms in the IL-1A gene promoter regulate IL-1A transcriptional activity induced by a range of periodontopathogens. This in turn provides a mechanism to directly influence pathogenesis of periodontal disease by
increasing the IL-1α associated bystander damage. The studies proposed here aim to identify polymorphisms in the promoter region of the IL-1A gene, to investigate their effect on transcriptional activity, and to investigate their interaction with a range of periodontopathogens.

The specific objectives of the studies include:

1. To investigate how periodontopathogens may regulate IL-1α production through multiple pathways
2. To identify the common IL-1A gene promoter polymorphisms present in patients with aggressive periodontitis
3. To test the detected polymorphisms to ascertain whether there was a significant difference between patients with aggressive periodontitis and unrelated healthy individuals in terms of allele frequencies or genotype frequencies
4. To test functional impact of identified polymorphisms on transcriptional activity using *in vitro* reporter gene assays and *in silico* analysis
5. To investigate if different periodontopathogens interact with specific IL-1α polymorphisms
6. To identify the mechanism by which doxycycline modulates inflammation including IL-1α production including:
   - The regulation of IL-1α production in monocytes by doxycycline
   - The effect of metal ions on IL-1α production and if doxycycline regulates cytokine response via the chelation of metal ions
   - To test the specificity of the response to doxycycline for IL-1α and other cytokines
CHAPTER 2

IL-1α Stimulation by Periodontal Bacteria
2.1 INTRODUCTION

As mentioned previously, the initiation and progression of periodontal disease depends on the presence of bacterial plaque. Tissue destruction is an indirect result of the presence of bacteria and their ability to trigger a host inflammatory response. The local host response to periodontal bacteria includes the recruitment of polymorphonuclear neutrophils and monocytes/macrophages (Dixon et al., 2004; Kornman et al., 1997b). Monocytes are a major part of the inflammatory infiltrate and can be found within gingival connective tissues of patients with periodontitis (Zappa et al., 1991). These cells play a critical role in regulation of the local inflammatory host response, in part through their ability to secrete pro-inflammatory cytokines including IL-1α in response to gram-negative bacteria and their products (Matsuki et al., 1991; Stoufi et al., 1987).

The stimulation of IL-1α production by bacterial products has been studied by several groups. However, there has not been a systematic comparison of the effects of a full range of periodontal pathogenic bacterial species. Previous studies using primary monocytes or cell lines have investigated only either a single species, a number of strains of a single species or compared relatively small number of different species (Henderson et al., 2002; Sugano et al., 2004). Therefore, the aims of these pilot studies were to investigate how different bacteria may induce IL-1α production by monocytes and categorize bacteria in classes of response. In preliminary studies the aim was to compare a range of culture conditions for IL-1α production by monocytes. Different bacterial species were compared to identify qualitatively different classes of bacterial stimulants. Characterization of bacterial factors within the supernatants was carried out including heat treatment, LPS depletion. These results served for the basis of experimental work described in later chapters.

2.1 MATERIAL & METHODS

2.1.1 Cell Culture

Monomac-6, THP-1 and U-937 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Mascheroder, Braunschweig, Germany). MonoMac-6 cells were cultured in RPMI-Glutamax (Gibco BRL Life Technologies, UK) supplemented with 10% fetal bovine serum, 1% non-essential amino acids (Gibco BRL), 1% sodium pyruvate (Gibco BRL) and 9 μg/ml bovine insulin (Sigma-Aldrich). THP-1
Chapter 2: IL-1α Stimulation by Periodontal Bacteria

and U-937 cells were cultured in RPMI-Glutamax (Gibco BRL Life Technologies, UK) supplemented with 10% fetal bovine serum. All cell lines were cultured as a suspension in humidified conditions containing 5% CO₂ at 37°C. They were sub-cultured at 2x10⁶/ml every 2-3 days. This was followed by centrifugation of suspension at 1000 rpm for 5 min. The cells were then seeded in T75cm² flasks as required.

Cells were cultured at the density of 1 x 10⁶ cells per ml in 96-well flat-bottom plates and challenged with bacterial supernatants. The bacterial supernatants were added to the monocytes (200μl/well) at dilutions of 1: 250, 1: 100, 1: 50 and were incubated at 37°C in a 5% CO₂ humidified atmosphere. Control cultures were incubated in the absence of bacterial supernatant. At the end of each study all plates were simultaneously thawed and exposed to three freeze-thaw cycles to lyse the cells. The cell free culture supernatants were collected by centrifuging at 1000 rpm for 5 minutes and stored at -80°C until the cytokine assays were performed. The IL-1α concentrations were measured in the supernatant (secreted compartment) and in the lysate (cell-associated compartment). All experiments were carried out in triplicate.

2.1.2 Bacterial strains and growth conditions

Bacterial supernatants were from cultures of P. gingivalis W50 and W83, F. nucleatum ATCC 25586, C. rectus ATCC 33238, A. actinomycetemcomitans Y4, P. intermedia NCTC 9336, V. atypica NCTC 11830, and P. nigrescens Mu14. Supernatants from a culture of S. sanguis NCTC 7863 were used as a negative control and LPS from the non-oral bacterium Escherichia coli 026:B6 (Sigma-Aldrich) was used as a positive control. Bacterial cultures were grown in the appropriate growth medium either aerobically in a 5% CO₂ incubator or in an anaerobic chamber containing an atmosphere of in 80% N₂, 10% H₂, and 10% CO₂ (Table 2.1). Cultures were grown to the same density (approximately 5x10⁷ colony forming units/ml) and harvested by centrifugation at 10000 x g for 15 min at 4°C. The culture supernatants were collected, filter-sterilized and stored at -80°C until used.
<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Growth Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. actinomycetemcomitans</em> (A.a) Y4</td>
<td>Tryptic soy broth with 0.6% yeast extract</td>
</tr>
<tr>
<td><em>S. sanguis</em> (S.s) -NCTC 7863</td>
<td>Tryptic soy broth containing 5% defibrinated sheep blood</td>
</tr>
<tr>
<td><em>S. mutans</em> (S.m) -NCTC 10449</td>
<td>Schaedler broth (Difco)</td>
</tr>
<tr>
<td><em>F. nucleatum</em> (F.n) -ATCC 25586</td>
<td>Tryptic soy broth (Difco) supplemented with 5 g of hemin</td>
</tr>
<tr>
<td><em>P. gingivalis</em> (P.g) -W50 and W83</td>
<td>YPD broth with 1% yeast, 2% peptone and 2% dextrose</td>
</tr>
<tr>
<td><em>C. rectus</em> (C.r)-FDC285</td>
<td>Veillonella agar plates (Difco)</td>
</tr>
<tr>
<td><em>V. atypica</em> (V.a) -NCTC 11830</td>
<td>Trypticase-yeast extract-gelatin-volatile Fatty acids broth with 5% rabbit serum</td>
</tr>
<tr>
<td><em>P. intermedia</em> (P.i)-NCTC 9336</td>
<td>Trypticase-yeast extract-gelatin-volatile Fatty acids broth with 5% rabbit serum</td>
</tr>
<tr>
<td><em>P. nigrescens</em> (P.n) - Mu14</td>
<td>Trypticase-yeast extract-gelatin-volatile Fatty acids broth with 5% rabbit serum</td>
</tr>
<tr>
<td><em>T. denticola</em> (T.d)-ATCC 35404</td>
<td>Trypticase-yeast extract-gelatin-volatile Fatty acids broth with 5% rabbit serum</td>
</tr>
</tbody>
</table>

Table 2.1 Bacterial strains and growth conditions. Bacterial cultures were grown in the appropriate growth medium either aerobically in a 5% CO₂ incubator or in an anaerobic chamber containing an atmosphere of in 80% N₂, 10% H₂, and 10% CO₂.

2.1.3 Total protein assay of bacterial culture supernatants

Protein quantitation was performed by the Bradford method using Bio-Rad colorimetric protein assay (Bradford, 1976). To quantify total protein, bacterial culture supernatants were defrosted and 50 µl was taken for measurement of protein concentration by Bio-Rad colorimetric protein assay. Bradford Assay is an accurate method commonly used to determine the total protein concentration of a sample. According to the principle of this technique the binding of the acidic dye solution Coomassie Brilliant Blue G-250 with the proteins in the supernatants results in a qualitative colorimetric reaction that can be read in a microplate reader at 595 nm, and compared against a standard curve of bovine albumin of known concentrations.

2.1.4 Endotoxin removal by Polymyxin B

To assess the relative role of LPS of periodontal pathogens on IL-1α production by Monomac-6, bacterial supernatants were passed through polymyxin B coated columns to remove endotoxin components according to the manufacturer's protocol (Detoxigel, Pierce Biochemicals). Detoxi-Gel AffinityPak Columns were regenerated by washing with 5ml of 1% sodium deoxycholate (Sigma-Aldrich, UK) followed by 5ml of pyrogen-free water to remove detergent. 500µl of bacterial supernatants were applied to the
column, and 500μl of pyrogen-free water were added. Bottom and top caps were replaced after the sample had entered the gel bed and incubated for 2 hours at room temperature. Samples were eluted with 1ml pyrogen-free water and stored at −70°C until use in experiments. Columns were regenerated as before and stored in 25% ethanol at 2-8°C.

2.1.5 Endotoxin Testing

To confirm that polymyxin B coated columns had removed LPS from supernatants, the endotoxin content of elutriated supernatants was measured by the Limulus Amebocyte Lysate (LAL) (Cambrex Biotech). The Limulus Amebocyte Lysate (LAL) test is a qualitative test for Gram-negative bacterial endotoxin. The principle is as below.

![Figure 2.1](image.png)

**Figure 2.1** The Limulus Amebocyte Lysate (LAL) assay. Gram-negative bacterial endotoxin catalyzes the activation of a proenzyme in the Limulus Amebocyte Lysate (Young et al., 1972). The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme (coagulase) hydrolyses specific bonds within a clotting protein (coagulogen) also present in Limulus Amebocyte Lysate. Once hydrolyzed, the resultant coagulin self-associates and forms a gelatinous clot.

The use of LAL for the detection of endotoxin was performed according to the manufacturer’s instructions. Briefly, samples were tested to check the pH was between 7.0 and 7.5. Sterile Water for Injection (USP) was used to reconstitute E.coli Control Standard Endotoxin. The vial was vortexed for 15 minutes. The standard endotoxin was
diluted to give concentrations ranging from 1 EU/ml to 0.01 EU/ml. The lysate was reconstituted by addition of 0.25 ml of the negative control (USP), positive sample control, control standard endotoxin dilution or sample to be tested to the lyophilized lysate in the test vial. The vials were incubated one hour in a 37°C water bath. Care was taken to avoid any microbial endotoxin contamination.

At the end of the incubation period, each vial was carefully removed and inverted 180°. The sample vials were compared to the control vials. A positive test was characterized by the formation of a solid gel, which remains intact after inversion. This was observed in the positive control vials and in the positive sample control vials. The lysate sensitivity was 0.01EU/ml. All test sample vials failed to form a solid clot after inversion and were therefore scored as endotoxin negative.

2.1.6 Statistical analysis

Student's t-test and a one-way analysis of variance were used and data were considered significant at P<0.05.

2.2 RESULTS

2.2.1 Induction of IL-1α production in Monomac-6, THP-1 and U-937 cells

A variety of established cell lines exist that exhibit monocyte-like characteristics. The ability of the three cell lines including human Monomac-6, THP-1 and U-937 to synthesize and secrete IL-1α in response to E. coli LPS was examined at 6 hours.

The cell lines were cultured with a range of Escherichia coli (E. coli) LPS doses between 10 ng/ml and 100 ng/ml for 6 hours. Detectable levels of IL-1α were measured from all cell lines. In all cases unstimulated cells produced very low levels of IL-1α. Monomac-6 and THP-1 cell lines produced IL-1α in a dose dependent manner as can be seen in Figure 2.2. However, Monomac-6 cells produced four times more IL-1α than THP-1 cells. U-937 cells did not respond to LPS, which is consistent with previous studies (Hass et al., 1991).
Figure 2.2 IL-1α production by monocytic cell lines stimulated with *E. coli* LPS. Monomac-6, THP-1 and U-937 were exposed to *E. coli* LPS (10 and 100 ng/ml) for 6 hours. The cell-associated and secreted amounts of IL-1α were quantified by ELISA. The data are the mean ± standard deviations (S.D.) of triplicate assays.

### 2.2.2 Monomac-6 cells as a model for IL-1α production

Monomac-6 cells were stimulated with *E. coli* LPS in the presence or absence of fetal bovine serum. *E. coli* LPS induced a dose dependent increase in IL-1α production (Figure 2.3). The addition of fetal bovine serum significantly increased the production of IL-1α in a dose dependent manner. Un-stimulated cells produced very low levels of IL-1α compared to stimulated cells. Cell-associated levels in unstimulated cells were higher than secreted levels (p<0.05). Although a considerable proportion of IL-1α protein is cell-associated, determination of both cell associated and secreted compartments is necessary in order to assess overall cytokine synthesis to approximate the *in vivo* conditions. Therefore further experiments tested total IL-1α levels.
Chapter 2: IL-1α Stimulation by Periodontal Bacteria

Figure 2.3 The effects of LPS concentration and fetal bovine serum levels on IL-1α production by Monomac-6 cells. Monomac-6 cells were exposed to 1, 10 and 100 ng/ml of *E. coli* LPS for 6 hours. The cell-associated and secreted amounts of IL-1α were quantified by ELISA. The data are the mean ± standard deviations (S.D.) of triplicate assays.

2.2.3 Determination of the optimal number of Monomac-6 cells

To investigate the source of variation between experiments, the effect of cell number on IL-1α production was analysed. Three different concentrations of cells were tested; 1x10^5, 2x10^5 and 1x10^6 cells per ml. In these experiments *E.coli* LPS (10ng/ml) was used as a stimulant. The highest concentration of cells produced approximately 7 times more IL-1α than others (Figure 2.4).

Hence, a concentration of 1x10^6 cells per ml was chosen for subsequent experiments. These data suggest that variation in cell plating could be a key factor in variation between different experiments.
2.2.4 Time-dependent production of IL-1α by Monomac-6 cells

Previous studies reported that the half-life of the IL-1α mRNAs may vary depending on cell type being in the range of 6-8 hours (Godambe et al., 1993; Perregaux and Gabel, 1998). To determine which time was likely to give the clearest results in this system, levels of IL-1α production were compared at 1, 2, 4 and 6 hours. Monomac-6 cells were stimulated with *E. coli* LPS (100ng/ml) for the indicated time periods. The results indicated that production of IL-1α increases over 2 hours and peaks at 6 hours (Figure 2.5). Therefore all subsequent experiments were studied at 6 hours.
2.2.5 IL-1α production by Monomac-6 cells in response to periodontopathogens

This study aimed to examine the production of IL-1α by Monomac-6 cells exposed to different concentrations of supernatants from oral bacteria. The effect of nine different types of bacterial supernatant on the production of IL-1α was tested. The bacterial supernatants from A. actinomycetemcomitans (A.a), F. nucleatum (F.n), C. rectus (C.r), P. intermedia (P.i), V. atypica (V.a), T. denticola (T.d), P. gingivalis (P.gW50 and W83), P. nigrescens (P.n) and S. sanguis (S.s) were tested. The supernatants were added to culture medium at dilution ratio of 1: 250, 1: 100 and 1: 50.

Total protein levels differed between different bacterial cultures but levels appeared to be significantly higher for P. intermedia (Figure 2.6). As expected, there was a significant difference between inoculated bacterial media and bacterial culture supernatants (p<0.05).
Chapter 2: IL-1α Stimulation by Periodontal Bacteria

Figure 2.6 Total protein concentrations in the culture supernatants expressed as μg/ml. Samples were defrosted and 50 μl was taken for measurement of protein concentration by Bio-Rad colorimetric protein assay. Quantitative colorimetric reaction was read in a microplate reader at 595 nm, and compared against a standard curve of bovine albumin of known concentrations.

To assess the importance of concentration of supernatant components on cytokine production a wide range of doses were tested. The results indicated that despite varying the concentrations added (1: 250, 1: 100 and 1: 50), levels of cytokine production increased by at most 4-39% at highest dosage (Figure 2.7). Minimal IL-1α was measured in cultures with inoculated media (IOM). All bacterial supernatants significantly increased IL-1α production. Amongst the different species tested A. actinomycetemcomitans (A.a), F. nucleatum (F.n), C. rectus (C.r), P. intermedia (P.i) and V. atypica (V.a) were high inducers of IL-1α production consistent with a previous report (Zubery et al., 1998). In contrast, T. denticola (T.d), P. gingivalis (P.g W50 and W83), P. nigrescens (P.n) and S. sanguis (S.s) consistently induced lower levels. There was no significant differences between the two strains of P. gingivalis (p=0.67).
Chapter 2: IL-1α Stimulation by Periodontal Bacteria

Figure 2.7 Dose-response study of the effect of periodontal pathogen(s) supernatants on IL-1α production by Monomac-6 cells. Monomac-6 cells were plated at a density of 1x10⁵ cells/ml in 96-well dishes. Cells were exposed to a range of periodontopathogens. Bacterial supernatants were added to cell culture media at various dilutions (1:250, 1:100 and 1:50). After 6 h incubation the cells were lysed by subjecting those to three cycles of freezing and thawing to assess total IL-1α. The supernatants were harvested and assayed for IL-1α by ELISA. The data are the mean ± standard deviations (S.D.) of triplicate assays.

2.2.6 Effect of heat treatment of bacterial supernatants

In previous studies it has been shown that different periodontal bacterial species or different fractions of the same bacteria have different heat vulnerability profiles (Choi et al., 2001). To further reveal differences between bacterial supernatants on IL-1α production, the supernatants were subjected to heat treatment. *E. coli* LPS (10ng/ml) was used as a positive control. The supernatants were heated to boiling for 30 minutes. They were added to cell culture media at 1:100 dilutions and compared to unheated supernatants.

Fig. 2.8 shows the impact of heat treatment on the supernatant's capacity to stimulate cytokine production. As expected, *E. coli* LPS was resistant to heat treatment. Our data show an important interspecies heat vulnerability profile from different bacterial supernatants. Heat-treatment of culture supernatants of *A. actinomycetemcomitans*, *P. intermedia*, and *T. denticola* resulted in significant decline in their capacity to induce cytokine production (Figure 2.8). In contrast, IL-1α-inducing capacity of *C. rectus*, *P.*
*gingivalis* and *V. atypica* was significantly increased (*p*<0.05). In addition, *F. nucleatum* cytokine-inducing capacity was not significantly affected by heat-treatment.

![Graph showing the effect of heat treatment on periodontopathogen induced IL-1α production by Monomac-6 cells. The bacterial supernatants were heated to boiling for 30 minutes. The cells were exposed to unheated or heated bacterial supernatants for 6 hours and the cells were lysed by subjecting those to three cycles of freezing and thawing to assess total IL-1α by ELISA. The data are the mean ± standard deviations (S.D.) of triplicate assays. *P*<0.05]

**Figure 2.8** The effect of heat treatment on periodontopathogen induced IL-1α production by Monomac-6 cells. The bacterial supernatants were heated to boiling for 30 minutes. The cells were exposed to unheated or heated bacterial supernatants for 6 hours and the cells were lysed by subjecting those to three cycles of freezing and thawing to assess total IL-1α by ELISA. The data are the mean ± standard deviations (S.D.) of triplicate assays. *P*<0.05

### 2.2.7 The effect of LPS depletion

To assess the relative role of LPS of periodontal pathogens on IL-1α production by Monomac-6, bacterial supernatants were passed through polymyxin B coated columns to remove endotoxin components. LPS-depleted supernatants were then assessed for their ability to stimulate IL-1α production. LPS-depleted supernatants were added to cell culture media at 1:100 dilutions and compared to un-treated supernatants. As expected, *E. coli* LPS completely lost its activity by polymyxin B treatment. In cultures stimulated by *C. rectus, P. intermedia, V. atypica* and *P. gingivalis* approximately 99%, 99.3%, 99.7% and 86% of the IL-1α production was abrogated by polymyxin B, respectively. Treatment with polymyxin B inhibited the IL-1α-inducing capacity of *A. actinomycetemcomitans* and *F. nucleatum* by 52% and 71% respectively (Figure 2.9).
Figure 2.9 The effect of LPS depletion from periodontopathogen(s) supernatants on IL-1α production. The supernatants were passed through polymyxin B coated columns and the cells were exposed to untreated or LPS-depleted bacterial supernatants for 6 hours. After 6 h incubation the cells were lysed by subjecting those to three cycles of freezing and thawing to assess total IL-1α. The supernatants were harvested and assayed for IL-1α by ELISA. The data are the mean ± standard deviations (S.D.) of triplicate assays. *P<0.05.

These studies suggest that bacteria can induce IL-1α production by a range of mechanisms. At least three different classes of bacterial stimulants can be identified:

1. Those partly affected by depletion of LPS and heat-labile, *F. nucleatum* and *A. actinomycetemcomitans*
2. Those requiring LPS and heat-labile; *P. intermedia, P. nigrecens*
3. Those requiring LPS and enhanced by heat treatment; *C. rectus, V. atypica, P. gingivalis*

### 2.3 DISCUSSION

This study demonstrated that the more mature monocytic cell line, Monomac-6 (Ziegler-Heitbrock et al., 1994) is more susceptible to bacterial stimulation than THP-1 or U-937 cell lines (Schwende et al., 1996). These results extend the finding of others showing that Monomac-6 does not need to be pre-cultured with PMA to show significant IL-1α secretion in response to LPS or periodontal pathogens (Hass et al., 1991; Pradines-Figueres and Raetz, 1992). These results imply that Monomac-6 is a more efficient and time saving model system to study monocyte activation in periodontal disease. The
present results also confirmed previous studies that the amount of IL-1α production is highly dependent upon monocyte culture conditions and under the experimental conditions assayed, maximum IL-1α production by Monomac-6 is obtained once these cells are stimulated in the presence of 10% FCS for 6 hours at density of 1x10^6/ml (Danis et al., 1990). Un-stimulated cells did not produce detectable levels of secreted IL-1α but did produce low but significant levels of cell-associated IL-1α. These results are similar to previously published reports and confirm that under normal conditions, 94-95% of IL-1α remains cell-associated (Matsushima et al., 1986; Watanabe and Kobayashi, 1994).

Although a considerable proportion of IL-1α protein is cell-associated, determination of both cell associated and secreted compartments is necessary in order to assess overall cytokine synthesis to approximate the in vivo conditions.

This study also demonstrated that a broad range of oral bacteria stimulate the expression of IL-1α in Monomac-6 cells as previously reported (Henderson et al., 2002; Reddi et al., 1995; Sugano et al., 2004). Previous studies have investigated only either a single species, a number of strains of a single species or compared relatively small number of different species. In this thesis bacterial culture supernatants were used as a stimulant. One problem with this type of strategy is that culture supernatants from each bacterial species will contain a mixture of bacterial endotoxin, bacterial waste products and bacterial enzymes. The exact content is likely to change not only between species but also between batches of bacterial cells. This would reflect slight variation of culture medium and incubation period of bacteria. Indeed, total protein levels differed between different bacterial cultures but levels appeared to be significantly higher for only P. intermedia. However, the results indicated that despite P. intermedia having the highest protein concentration, levels of IL-1α production were lower than others which had lower protein.

The results here also indicated that a range of periodontal pathogens have the capacity to induce IL-1α production in Monomac-6 cells; nevertheless the nature of the responsible component for this effect may vary among them. This capacity may be attributed to either LPS or to proteinaceous component depending upon the specific species. In support of this data, Monomac-6 cells have been shown to produce cytokines in response to an extract of A. actinomycetemcomitans surface associated proteins. This cytokine inducing activity was heat sensitive suggesting that the active components were
proteinaceous (Reddi et al., 1996; Reddi et al., 1995). Different bacterial virulence factors may act through a range of distinct signalling mechanisms to stimulate the production and release of cytokines (Darveau et al., 2002; Saklatvala, 1995). Toll-like receptors 2 and 4 have recently been identified as possible signal transducers for various bacterial ligands (Bainbridge and Darveau, 2001; Yoshimura et al., 2002). To further clarify the mechanisms involved the ability of different bacterial virulence factors to interact with different TLRs to stimulate IL-1α production could be investigated further.

In summary, this study demonstrates that a broad range of oral bacteria stimulate the expression of IL-1α in Monomac-6 cells and the nature of the responsible component for this effect may vary among them.
CHAPTER 3

Antagonistic Effects of Porphyromonas gingivalis
3.1 INTRODUCTION

As mentioned previously more than 700 species can colonize the oral cavity (Aas et al., 2005) and approximately 500 species may be detected in subgingival plaque (Paster et al., 2001). Evidence suggests that the most pathogenic complex is “the red complex” which includes P. gingivalis, T. forsythensis, and Treponema denticola (Lakshmyya et al., 2007; Socransky et al., 1998). Although there are strong indications that certain species are the likely aetiologic agents of periodontal diseases, there are almost limitless possibilities for bacterial interactions in the ecology of the oral cavity microflora, including synergism, commensalism, and antagonism. The mixed nature of the bacterial infection in periodontal disease will have a direct influence on the nature and extent of the host response to the bacterial challenge. Cytokines are central to this response and appropriate production of cytokines results in development of protective immunity. However inappropriate production of cytokines can lead to tissue destruction and disease progression. P. gingivalis has been reported to be a major component of the pathogenic microbiota in various periodontal diseases (Holt et al., 1999). Despite a profound inflammatory response, the host immune system is usually not able to eliminate P. gingivalis infection. P. gingivalis might have the potential to suppress the normal immune response to a wide range of periodontal pathogens. This chapter will focus on P. gingivalis and its properties of modulating cytokines in ways that evade the innate host immune response, and permit its colonization and propagation in the oral cavity.

3.1.1 Porphyromonas gingivalis

P. gingivalis is a gram-negative, black-pigmented anaerobe and is characterised as a bona fide periodontal pathogen within “the red complex” (Holt and Ebersole, 2005). Several approaches for identifying P. gingivalis strains have been reported, including serotyping and whole genomic digest (Genco and Loos, 1991; Parent et al., 1986). More recently, 22 unique but closely related P. gingivalis types have been identified using heteroduplex analysis (Leys et al., 1999). The effects of different strains of P. gingivalis have been compared in several animal studies, indicating a range of host responses (Evans et al., 1992; Genco et al., 1991; Laine and van Winkelhoff, 1998). In most animal studies, P. gingivalis strains W50 and W83, which are indistinguishable from one another, proved to be particularly virulent and strongly associated with human
Chapter 3: Antagonistic Effects of *P. gingivalis*

periodontitis (Griffen et al., 1999). Other strains, such as 381, 49417, A7A1-28, are also considered as virulent strains (Ebersole et al., 1995; Lin et al., 2005).

*Porphyromonas gingivalis* expresses several virulence factors including capsular polysaccharide (K-antigen), fimbriae, the cysteine proteases (the Arg- and Lys-gingipains) and lipopolysaccharide (LPS). Studies have been performed to determine the distribution of the specific serotypes of *P. gingivalis* strains. *P. gingivalis* has been classified into serotypic groups based on antisera binding to the capsule (K-antigen, K1 to K6) (Aduse-Opoku et al., 2006; Laine et al., 1996), fimbriae (filamentous component, types I to V) (Nakagawa et al., 2000) or outer membrane antigens (A, B, C) of *P. gingivalis* (Ebersole and Steffen, 1995). *P. gingivalis* strain 381 is reported as K antigen negative (K-neg) or capsule deficient (van Winkelhoff et al., 1993). Some *P. gingivalis* strains and their serotypes are shown in Table 3.1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Capsular serotype</th>
<th>Fimbrial serotype</th>
<th>Outer membrane serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>W50</td>
<td>K1</td>
<td>V</td>
<td>C</td>
</tr>
<tr>
<td>W83</td>
<td>K1</td>
<td>V</td>
<td>C</td>
</tr>
<tr>
<td>381</td>
<td>K-neg</td>
<td>I</td>
<td>A</td>
</tr>
<tr>
<td>A7A128 (ATCC53977)</td>
<td>K3</td>
<td>II</td>
<td>B</td>
</tr>
<tr>
<td>FAY19m-1</td>
<td>–</td>
<td>III</td>
<td>–</td>
</tr>
<tr>
<td>ATCC 49417</td>
<td>K4</td>
<td>II</td>
<td>–</td>
</tr>
<tr>
<td>A7A128 (ATCC53977)</td>
<td>K3</td>
<td>II</td>
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<tr>
<td>ATCC 49417</td>
<td>K4</td>
<td>II</td>
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</tr>
<tr>
<td>HG1690</td>
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<tr>
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<td>–</td>
</tr>
<tr>
<td>HG184</td>
<td>K2</td>
<td>–</td>
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</tr>
</tbody>
</table>

Table 3.1 *P. gingivalis* strains and serotypes

3.2 Major virulence factors of *P. gingivalis*

Bacterial pathogens are highly evolved and have developed strategies to enable them to survive and grow within their specific hosts. These specialized survival strategies are dependent upon the co-ordinated expression of appropriate genes which facilitate the processes of colonization, acquisition of growth nutrients, evasion of host defences, and, in some cases, invasion of host tissues prior to transmission to a new host environment (Hornef et al., 2002). These gene products can be broadly referred to as the virulence determinants of the pathogen. In the following sections, some of the major virulence
factors of *P. gingivalis* that may have particular relevance to the survival of the organism in an inflammatory site and the potential consequences of these factors to the aetiology of periodontal disease will be reviewed.

### 3.2.1 Effects of *P. gingivalis* LPS on host innate and immune mechanisms

Gram-negative bacteria are protected from their environment by the outer membrane, which contains the unique glycolipid, LPS. LPS consists of three regions: a phosphorylated glucosamine disaccharide substituted with fatty acids known as lipid A that possesses the majority of the activity inducing an inflammatory response, a highly variable O-polysaccharide, and a conserved core oligosaccharide (Seydel et al., 2000). The LPS of *P. gingivalis* differs from that of other Gram-negative bacteria in that the protein structure of *P. gingivalis* LPS lacks heptose and 2-keto-3-deoxyoctonate, (Koga et al., 1985) and *P. gingivalis* LPS has weak endotoxic activity, although it is significantly mitogenic (Mayrand and Holt, 1988; Ogawa et al., 1994).

*P. gingivalis* LPS is a significant antigen in periodontitis patients and may account for a significant proportion of the immune response directed towards this organism (Farida et al., 1986). *P. gingivalis* LPS has several properties which are associated with the bacterium's role in the pathogenesis of periodontitis. Recent studies indicate that *P. gingivalis* LPS can activate mononuclear phagocytes to induce proliferation, cytokine production, and CD14 expression. This provides evidence that this bacterial component can act as a critical regulatory factor in the chronic inflammatory response associated with periodontitis (Roberts et al., 1997).

*P. gingivalis* LPS induces a variety of cytokines from a number of different cells. Human gingival fibroblasts secrete the pro-inflammatory cytokines IL-1β, IL-6 and IL-8 in response to incubation with *P. gingivalis* lipopolysaccharide. In addition, *P. gingivalis* lipopolysaccharide also induces monocytes-macrophages to secrete TNF-α, IL-1, nitric oxide, prostaglandin E2 at higher concentrations compared to the equivalent dose of *E. coli* LPS (Bramanti et al., 1989; Shapira et al., 2000; Zhou and Amar, 2006). Neutrophils exposed to *P. gingivalis* lipopolysaccharide show increased expression of the transcription factors NF-κB and AP-1 and the pro-inflammatory cytokine IL-8 (Sugita et al., 1998). Models of murine periodontitis have been used to show that *P. gingivalis*
lipopolysaccharide induces bone resorption via the stimulation of local production of IL-1α and IL-1β (Lino and Hopps, 1984; Nishida et al., 2001). *P. gingivalis* LPS can interact with CD14 on osteoclasts to induce the expression of IL-1β and IL-6, which in turn initiates bone resorption (Miyata et al., 1997).

Lipopolysaccharide of *P. gingivalis* evokes a highly unusual host cell response. Unlike *E. coli* LPS which interacts with Toll-like receptor 4 (TLR-4), *P. gingivalis* LPS interacts mainly with Toll-like receptor 2 (TLR-2) (Wang and Ohura, 2002). Macrophages have recently been shown to be stimulated by *P. gingivalis* LPS via TLR-2 to secrete pro-inflammatory cytokines (Hirschfeld et al., 2001). In addition, *P. gingivalis* LPS appears to be able to antagonise TLR-4 and competes with other bacterial proteins for TLR-4 (Hajishengallis et al., 2002; Wang et al., 2001; Yoshimura et al., 2002). Interactions between TLR-2 and TLR-4 agonists may be important in the regulation of inflammatory reactions in periodontitis (Bainbridge and Darveau, 2001). Although the precise effect of antagonistic LPS on the pathogenesis of periodontitis remains to be elucidated, this antagonistic activity would allow the microorganisms to escape from the innate immune system. It has been suggested that although LPS is generally considered a bacterial component that alerts the host to infection, LPS from *P. gingivalis* may selectively modify the host response as a means to facilitate its pathogenic effects (Darveau et al., 2002).

### 3.2.2 Extracellular proteolytic enzymes and their role in periodontitis

*P. gingivalis* produces a variety of proteinases with the ability to degrade host proteins and modulate the immune response (Curtis et al., 2001). While the precise roles of the cysteine proteases of *P. gingivalis* in these processes are still under investigation, there is a plethora of host target proteins with critical functions in host defence and tissue integrity that are susceptible to proteolytic inactivation by these enzymes. This has led to the suggestion that cysteine proteinases may be important virulence factors in the periodontal diseases (Lamont and Jenkinson, 1998). Although, gingipains are the major cysteine proteinases synthesized by *P. gingivalis*, there are other proteases of *P. gingivalis* such as a protease-hemagglutinin, a dipeptidyl peptidase and periodontain. At least one of these proteinases, periodontain can inactivate the human serpin α1 proteinase
inhibitor, which is the primary endogenous regulator of human neutrophil elastase (Nelson et al., 1999).

The Arg-gingipains (RgpA/B) and Lys-gingipain (Kgp) cysteine proteases are believed to be the main endopeptidases produced by the bacterium (Kesavalu et al., 1997b). Current data suggests that the Arg-gingipains and Lys-gingipains represent the majority of surface exposed protein on *P. gingivalis* W50 (Veith et al., 2002). Three genes encode the major extracellular Arg-X and Lys-X specific cysteine proteinases of *P. gingivalis*. The extracellular and cell-associated protease activity with specificity for arginine peptide bonds is derived from two genes, rgpA and rgpB (for Arg-gingipain A and B). The gene encoding gingipain R associated with hemagglutinin/adhesion domains is referred to as rgpA and the gene that encodes gingipain R without this C-terminal hemagglutinin/adhesion extension is referred to as rgpB. The extracellular and cell-associated cysteine protease activity with specificity for lysine peptide bonds is derived from a single gene, Kgp (for Lys-gingipain) (Figure 3.1).

The construction and phenotypic analysis of isogenic protease mutants of *P. gingivalis* have confirmed putative functions for these enzymes. In *P. gingivalis* rgpA isogenic mutants, protease activity is reduced to about 50% of the wild type. Otherwise, the mutants are phenotypically similar to the wild type (Fletcher et al., 1995; Rangarajan et al., 1997; Tokuda et al., 1998). Similarly, an rgpB mutant of *P. gingivalis* W50 retains only 50% of total Arg-X activity (Rangarajan et al., 1997b). The double mutant lacking both rgpA and rgpB abolishes all Arg-X activity (Nakayama et al., 1995; Aduse-Opoku et al., 2000). Inactivation of the kgp of *P. gingivalis* leads to total loss of lysine specific protease activity confirming that this gene is the sole source of this activity in *P. gingivalis* (Okamoto et al., 1998; Barkocy-Gallagher et al., 1999; Lewis et al., 1999; Shi et al., 1999; Aduse-Opoku et al., 2000).
Clinical studies support the role of gingipains in periodontitis (Figure 3.2). Several studies have demonstrated that periodontal infection by *P. gingivalis* leads to the development of a significantly elevated antibody response to the gingipains. In a survey of patients with chronic periodontitis, 92% showed a serum IgG response to the RgpA-Kgp proteinase–adhesin complexes (O'Brien-Simpson et al., 2000). Patients with generalized aggressive periodontitis also possess elevated levels of both arginine and lysine-gingipain-specific IgG in their sera, suggesting that gingipain-specific antibodies may be important for the control of *P. gingivalis* infections (Gibson et al., 2005).

*In vivo* studies in a mouse model using specifically engineered protease mutants have reinforced the view that the cysteine enzymes play a key role in the infection process. (Fletcher et al., 1995; Kesavalu et al., 1997a; Tokuda et al., 1998). More recently, the contribution of rgpA, rgpB and kgp to the virulence of *P. gingivalis* W50 has been shown in the murine lesion model using isogenic mutants lacking each proteinase (O'Brien-Simpson et al., 2001). These data are also supported by studies which have examined the protective effect of immunization by means of purified preparations of cysteine enzymes. A recent study reported that mice immunized with the RgpA proteinase and its associated adhesins protected against *P. gingivalis*-induced periodontal bone loss whereas the RgpB proteinase did not result in a protective immune response (Gibson and Genco, 2001). However, these studies suggest protease gene inactivation can elicit pleiotropic effects in *P. gingivalis*, and hence the decrease in virulence observed in the above studies may not solely be a function of decreased proteolysis.
P. gingivalis gingipains also activate a broad range of host proteins and are considered key virulence factors in the onset and development of chronic periodontitis and host defence evasion (Imamura, 2003). Gingipains stimulate expression of matrix metalloproteinases (MMPs) in fibroblasts and activate secreted latent MMPs that can destroy periodontal tissues (Potempa et al., 2000). P. gingivalis proteinases degrade several plasma host proteinase inhibitors including α-antitrypsin, α2-macroglobulin, antichymotrypsin, antithrombin III and antiplasmin (Grenier, 1996).

The RgpA/B proteinases can directly degrade CD14 on the surface of monocytes thereby inhibiting lipopolysaccharide activation (Sugawara et al., 2000). Gingipains can also degrade components of the complement system and inactivate other receptors including TLR-2 and TLR-4 (Jagels et al., 1996; Kishimoto et al., 2006). Thus gingipains can perturb the host-defence systems and thereby facilitate sustained colonization by P. gingivalis. The cysteine proteases of P. gingivalis can degrade TNF-α, IL-6, and IL-8 and probably other cytokines (Baba et al., 2002; Banbula et al., 1999; Calkins et al., 1998; Kobayashi-Sakamoto et al., 2003; Mezyk-Kopec et al., 2005; Mikolajczyk-Pawlinska et al., 1998; Oleksy et al., 2002). Thus the proteases can directly disrupt the normal function of the cytokine network.

While these studies suggest that gingipains could have a catastrophic effect on the cytokine network in the periodontal tissues through degradative effects, it is also clear that these products of P. gingivalis can stimulate the production of cytokines. The arginine-specific proteases from P. gingivalis activate protease-activated receptors on human oral epithelial cells and induce IL-6 secretion (Lourbakos et al., 2001). In addition, P. gingivalis infection induces gene expression of IL-6 and MMP-13 in stromal cells/osteoblasts, and gingipains may be involved in inducing several other proinflammatory factors (Ohno et al., 2006). Indeed, while arginine-specific protease can functionally inactivate IL-1, an internal peptide derived from RgpA can stimulate IL-6 synthesis by human fibroblasts and mononuclear cells (Sharp et al., 1998). The conflict between up-regulation of synthesis and proteolytic degradation of cytokines complicates the interpretation of the true biological significance of proteolysis in this system.
3.2.3 Capsule

Bacterial capsules are accepted as important virulence determinants and the interaction between capsule and the host’s immune system in invasive infections may decide the outcome of infection (Moxon and Kroll, 1990). The capsule of *P. gingivalis* is a major surface antigen and it consists of a polysaccharide heteropolymer that surrounds the outer membrane. Capsular polysaccharides of gram-negative bacteria play an important role in maintaining the structural integrity of the cell in hostile environments (Aduse-Opoku et al., 2006). Based on the antigenicity of the polysaccharide K antigens of the capsule, 6 different capsular serotypes have been reported (Laine et al., 1996). *P. gingivalis* strain 381 has no capsular-polysaccharide layer and lacks K antigen and does not induce a capsular-specific antibody response (Fan et al., 2001). The presence of capsule has also been correlated with virulence of *P. gingivalis* using in vivo model systems. Encapsulated strains of *P. gingivalis* such as W50 are also more resistant to phagocytosis by polymorphonuclear cells than strain 381 (Sundqvist et al., 1991).

3.2.4 Fimbriae

Fimbriae play an important role in the adherence of *P. gingivalis* to oral surfaces and may also trigger host responses (Amano et al., 2004). *P. gingivalis* fimbriae have been shown to stimulate the release of IL-1α, IL-1β, TNF-α, IL-6 from macrophages and
fibroblasts and TNF-α, IL-6 and IL-8 from polymorphonuclear cells (Hamada et al., 2002; Hanazawa et al., 1992). Furthermore, \textit{P. gingivalis} fimbriae have been shown to stimulate bone resorption in a murine embryonic calvarial bone resorption assay by activating osteoclasts (Kawata et al., 1994). Five \textit{P. gingivalis} fimbrial types (types I–V) have been described based on their antigenicity (Nakagawa et al., 2000). Recent clinical investigations have demonstrated the close relationship between the organisms with type II fimA and the development of periodontitis (Kato et al., 2006).

3.2.5 GroEL heat shock protein

GroEL, a homologue of the Heat shock protein 60 family, is a major HSP in various bacterial infections and is widely recognized as an important molecule in periodontitis (Maeda et al., 2000; Nair et al., 1999). GroEL of \textit{P. gingivalis} can stimulate the expression of pro-inflammatory cytokines in macrophages (Kesavalu et al., 2002; Ueki et al., 2002). The \textit{P. gingivalis} heat shock protein GroEL is highly antigenic and antibodies to \textit{P. gingivalis} GroEL are detectable in gingival tissue extracts (Tabeta et al., 2000).

3.3 SUMMARY AND AIMS

There is good evidence that \textit{P. gingivalis} is one of the key bacterial species involved periodontal diseases in humans (Holt et al., 1999). The substantial rise in the numbers and proportions of \textit{P. gingivalis} in subgingival plaque samples from diseased sites in the periodontium suggests that this organism is well-adapted to and benefits from the hostile conditions which prevail subgingivally during the inflammatory disease (Socransky et al., 1999). Despite a profound inflammatory response, the host immune system is usually not able to eliminate \textit{P. gingivalis} infection.

The production of pro-inflammatory cytokines including IL-1, by host cells upon sensing bacterial products is crucial to the innate and adaptive immune responses to infection. These cytokines enhance the bactericidal capacity of phagocytes, recruit additional innate cell populations to sites of infection, and direct the ensuing specific immune response to the invading microbes. A number of lines of evidence support a pivotal role of IL-1α in mediating the host response to periodontal pathogenic bacteria (Dayan et al., 2004; Wang and Stashenko, 1993). So far the studies have demonstrated that most periodontopathogens induced a significant level of IL-1α release from both Monomac-6
and primary monocytes, while *P. gingivalis* induced IL-1α to only a minimal level. It is puzzling that *P. gingivalis* is strongly associated with disease but does not induce IL-1α production. The stimulation of IL-1α at the site infection by other periodontopathogens could be antagonised by *P. gingivalis*.

As reviewed in detail above, *P. gingivalis* seems to have the ability to perturb the innate immune response rendering the host susceptible to disease via its virulence factors such as LPS and gingipains. *P. gingivalis* LPS exhibits unusual characteristics and can interact with TLR-2 and is able to antagonize the effects of other bacteria. *P. gingivalis* also produces proteases that may directly degrade CD14 and lead to further reduction in response to LPS from other bacteria. Therefore, it was hypothesized that *P. gingivalis* may directly alter IL-1α activities via either inactivation or degradation, and its major virulence factors may play an important role in the deregulation of IL-1α at the site of inflammation.

The aims of the studies described in this chapter are:

- To compare the ability of supernatants from a number of the major periodontal pathogens including *A. actinomycetemcomitans*, *P. gingivalis*, *F. nucleatum*, *C. rectus*, *P. intermedia*, *V. atypica* and *P. nigrescens* for their ability to stimulate IL-1α production in both the MonoMac-6 cell line and primary monocytes.
- To investigate if *P. gingivalis* culture supernatants can antagonize IL-1α production stimulated by other pathogens, when cells were exposed to combinations of supernatants.
- To investigate the underlying mechanisms of antagonism of cytokine production by *P. gingivalis*.

### 3.4 MATERIALS & METHODS

#### 3.4.1 Bacterial strains and growth conditions

Bacterial supernatants were obtained from cultures of *P. gingivalis W50* and *W83, 381, F. nucleatum* ATCC 25586, *C. rectus* ATCC 33238, *A. actinomycetemcomitans* Y4, *P. intermedia* NCTC 9336, *V. atypica* NCTC 11830, and *P. nigrescens* Mu14. Supernatants from a culture of *S. sanguis* NCTC 7863 were used as a negative control and LPS from
Chapter 3: Antagonistic Effects of *P. gingivalis*

the non-oral bacterium *Escherichia coli* 026: B6 (Sigma-Aldrich) was used as a positive control. *P. gingivalis* W50 protease mutant strain (*kgp*, Lys-gingipain) and the protease (Arg-gingipain)-double defective mutants (*rgpA, rgpB*) were kindly supplied by Prof. M.A. Curtis, Molecular Pathogenesis Group, Queen Mary, University of London (Aduse-Opoku et al., 2000). To investigate the role of *P. gingivalis* gingipain cysteine proteinases on cytokine production, *P. gingivalis* supernatant was pre-treated with 1mM proteinase inhibitor Na-p-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) (Sigma-Aldrich) for 1h at 4°C. Bacterial cultures were grown in the appropriate growth medium (see Chapter 2, Table 2.1) either aerobically in a 5% CO₂ incubator or in an anaerobic chamber containing an atmosphere of in 80% N₂, 10% H₂, and 10% CO₂. Cultures were grown to the same density (approximately 5x10⁷ colony forming units / ml) and harvested by centrifugation at 10000 x g for 15 min at 4°C. The culture supernatants were collected, filter-sterilized and stored at -80°C until used. For some studies, culture supernatants were heat-inactivated at 100°C for 20 min. The supernatants of bacteria were directly diluted in culture medium to final dilutions of 1:250 and 1:50.

3.4.2 Stimulation of human primary monocytes and Monomac-6

Peripheral blood was obtained from three healthy individuals by venous puncture and collected in EDTA vacutainers (Becton Dickinson). All participants signed a written consent form. Protocols for the study were approved by the East London and City Health Authority London Research Ethics Committee. Human peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences) according to the manufacturer's instructions. The cells were washed twice with PBS containing EDTA (2mM) and bovine serum albumin (2%), pH 7.3. Cell viability was determined by Trypan blue exclusion. PBMC count was performed with a haernocytometer and processed for magnetic purification. The Monocyte Isolation Kit II (Miltenyi Biotec, Germany) was used. This technique relies upon an indirect magnetic labelling system allowing the isolation of unstimulated monocytes from human peripheral blood mononuclear cells (PBMC) (Hafsi et al., 2004). The purity of isolated monocytes was evaluated by flow cytometry on a FACScan flow cytometer (Becton Dickonson) using a CD14 FITC-conjugated antibody (Figure 3.3). Purified monocytes were re-suspended in RPMI-Glutamax (Gibco BRL Life Technologies, UK) supplemented with 10% heat-inactivated fetal bovine serum (Bio-
Whittaker, Maryland). Monocytes were then seeded in 96-well microtitre plates at a
density of 1x10^6 cells per ml and stimulated with bacterial culture supernatants at 37°C
for 6 hours in a 5% CO₂ atmosphere.

The myelomonocytic cell line Monomac-6 was obtained from the German Collection of
Microorganisms and Cell Cultures (Mascheroder, Braunschweig, Germany). MonoMac-6
cells were cultured in RPMI-Glutamax (Gibco BRL Life Technologies, UK)
supplemented with 10% fetal bovine serum, 1% non-essential amino acids (Gibco BRL),
1% sodium pyruvate (Gibco BRL) and 9 µg/ml bovine insulin (Sigma-Aldrich).
MonoMac-6 cells were cultured at a density of 1 x 10^6 cells per ml in 96-well flat-bottom
plates and challenged with bacterial supernatants. The bacterial supernatants were added
to the monocytes (200µl/well) at dilutions of 1:250 (0.8µl) and 1:50 (4µl) and were
incubated at 37°C in a 5% CO₂ humidified atmosphere. In preliminary experiments, a 6
hour incubation period was found to be suitable for detection of IL-1α production. To
assay total IL-1α (cell associated and secreted) cells were lysed by three freeze-thaw
cycles. The cell free culture supernatants were collected by centrifuging at 1000 rpm for
5 minutes and stored at -80°C until the cytokine assays were performed. Control cultures
were incubated in the absence of bacterial supernatant. All experiments were carried out
in triplicate.

Figure 3.3. Flow cytometric analysis of isolated PBMCs. PBMCs were isolated using a Monocyte
Isolation Kit II. Non-monocytes were indirectly magnetically labeled using a cocktail of biotin-conjugated
antibodies against CD3, CD16, CD19, CD56 (specific for T cells, NK cells, B cells, NK and CD3+ T cells,
respectively) and Anti-Biotin MicroBeads. The dot plots show nucleated live cells gated according to LDS
and propidium iodide staining. Enriched cells were stained with antibiotin-PE and CD14-FITC. A) Gated
on CD45+ events only CD3, CD19 and CD56-PE. B) Gated on CD45+ events only CD14-PE.
3.4.3 Cell viability assays

Cell viability was determined by measurement of lactate dehydrogenase (LDH) release using the CytoTox96 non-radioactive cytotoxicity assay (Promega, USA). In brief, Monomac-6 cells or primary human monocytes were exposed to bacterial culture supernatants and then incubated for 6 hours at 37°C. A sample of (50 µl/well) supernatant was carefully removed and transferred into an optically clear 96-well plate. Reaction solution was added to each well and incubated for 30 min in darkness. The enzyme reaction was then stopped by the addition of 1M HCl. The absorbance at 490nm was measured using an ELISA reader (Model 680, Bio-Rad Laboratories). The activity of the enzyme released from damaged cells into the supernatant was measured, and the activity was expressed as a percentage of the total LDH activity released from cells lysed by exposure to 0.1% Triton X-100 for 45 min. Values shown represent the mean ± standard deviation of three wells.

3.4.4 Protease Assays

P. gingivalis wild type (W50) and protease gene knockouts (E8 and K1A) were grown in BHI broth with haemin and menadione under identical conditions to achieve maximal Arg-X and Lys-X active proteases. The enzyme assays for Arg-X and Lys-X protease activities using the synthetic substrates N-α-benzoyl-DL-arginine-p-nitroanilide (DL-BRpNA) and N-α-acetyllysine-p-nitroanilide (Ac-LyspNA) respectively, were performed according to the published protocols (Rangarajan et al., 1997).

3.4.5 Determination of cytokine production

Cytokine production from the different experiments was determined in the cell free culture supernatant by commercially available specific ELISA (R&D Systems, Minneapolis, MN, USA) according to standard procedures. The absorbance at 450 nm was read using a microplate reader (Model 680, Bio-Rad Laboratories) with a wavelength correction set at 570nm. A standard curve was generated using a four parameter logistic (4-PL) curve-fit (Microsoft Office Excel) for each set of samples assayed. The cytokine concentrations of the samples were calculated using the standard curve.
3.4.6 Determination of cytokine degradation

To control for any effects of degradation of IL-1α by *P. gingivalis* supernatants, recombinant human (rh) IL-1α (50 ng/ml) was added to cultures. The cytokine concentration in the supernatants was determined by ELISA and compared to the basal levels of a control incubated for 6 hours in the absence of bacterial supernatant.

3.4.7 Preparation of LPS from *P. gingivalis*

*P. gingivalis* was a gift from Dr. Minnie Rangarajan (Molecular pathogenesis Group, ICMS). It was prepared as follows. *P. gingivalis* W50 was grown anaerobically in brain-heart infusion supplemented with haemin (5 μg/ml) for 24 hours. The cells were harvested, washed with water and freeze-dried. *P. gingivalis* LPS was prepared by the cold MgCl2-ethanol procedure as described previously (Darveau and Hancock, 1983). LPS was lyophilized to determine the yield and stock solutions were prepared in pyrogen-free distilled water (1 mg/ml) and vortexed prior to use. No protein was detected in LPS preparations as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining for protein using the enhanced colloidal gold procedure.

3.4.8 Determination of endotoxin levels in the bacterial supernatants

Bacterial endotoxin was assessed using The Chromogenic Limulus Amebocyte Lysate (LAL) Test (QCL1000, Cambrex Bio Science, and Walkersville). Purified *P. gingivalis* LPS and bacterial supernatants were dissolved in pyrogen-free water and serial dilutions were made. The absorbance of the sample was determined spectrophotometrically at 405 nm and the concentration of endotoxin was calculated from a standard curve. In the case of polymyxin B treatment, endotoxin was removed from bacterial supernatants using a polymyxin B column (Detoxigel, Pierce Biochemicals) according to the manufacturer’s instructions (see in Chapter 2). As assessed by LAL assay (using the purified LPS as a standard), the concentration of LPS in *P. gingivalis* and *C. rectus* supernatant was 35.7 and 38 μg/ml, respectively. In addition, pretreatment of bacterial supernatants with polymyxin B resulted in a dramatic reduction in LPS amount of both supernatants (0.55 and 0.39 μg/ml, respectively).
3.5 Statistical analysis

Student's t-test and a one-way analysis of variance were used and data were considered significant at P<0.05.

3.6 RESULTS

3.6.1 Stimulation of IL-1α production by culture supernatants from periodontal pathogens

When the monocytic cell line, Monomac-6 was exposed to bacterial supernatants of cultured periodontal pathogens a significant up-regulation of the total level of IL-1α was seen after 6 hours (Figure 3.4A). Unstimulated Monomac-6 cells or those exposed to noninoculated culture media showed very low levels of IL-1α production. The most pronounced inducers were *A. actinomycetemcomitans*, *F. nucleatum*, *C. rectus* and *V. atypica*, whereas *P. gingivalis* was consistently the least potent treatment. Some putative pathogenic species including *P. intermedia* and *P. nigrescens* produced only a modest induction of IL-1α. When primary CD14 positive monocytes were stimulated with bacterial supernatants in a similar manner, IL-1α production was significantly up-regulated after 6 hours (Figure 3.4B). The pattern of IL-1α production was somewhat different to Monomac-6 cells with responses to *A. actinomycetemcomitans* and *F. nucleatum* being relatively lower and responses to *P. intermedia* and *P. nigrescens* being significantly greater. However, the response to *P. gingivalis* was still lowest.

A)
Figure 3.4 Production of IL-1α by human monocytes in response to bacterial stimuli. Monomac-6 cells (A) and freshly isolated human monocytes (B) were plated at a density of 1x10⁶ cells/ml in 96-well dishes. Cells were exposed to culture supernatants from periodontopathogens. Bacterial supernatants were added to cell culture media at 1:250 dilution. After 6 h incubation the cells were lysed and cell-free supernatants were harvested to assess total IL-1α (secreted and cell-associated) by ELISA. The data are the mean±standard error of mean (SEM) n=3.

Monomac-6 cells and isolated human monocytes showed almost no toxic effects in the presence of bacterial supernatants except A. actinomycetemcomitans (Figures 3.5A and 3.5B, respectively). Whilst A. actinomycetemcomitans induced some toxicity it was still able to significantly elevate levels of IL-1α. In the present study, most supernatants were diluted no less than 1:250 and for P. gingivalis in combination with other supernatants it was diluted 1:50 in medium.

The toxicity testing demonstrated that the inability of P. gingivalis to elicit a significant IL-1α response was not related to its toxicity to the Monomac-6 cells or isolated human monocytes (Figure 3.5A and B). Interestingly the levels of LDH release in cells treated with both C. rectus and P. gingivalis were not increased compared to cells treated with either bacterium alone. It has been previously shown that P. gingivalis can partially neutralize the leukotoxicity of A. actinomycetemcomitans (Johansson et al., 2000) and it is possible a similar mechanism is involved in this situation. Although the amount of leukotoxin in the A. actinomycetemcomitans supernatants used was not defined, 1:250
dilution induced similar cytotoxicity to <5ng/ml of purified leukotoxin shown in a previous study (Kelk et al., 2003).

3.6.2 Antagonism of IL-1α production by *P. gingivalis*

To investigate if *P. gingivalis* could affect the IL-1α stimulating activity of other periodontal species a 5-fold excess of *P. gingivalis* supernatant was mixed with other bacterial supernatants before addition to monocytes. Several species showed an altered capacity to induce IL-1α in the presence of *P. gingivalis* supernatant (Table 3.2A). A small (21±4%) but significant (P<0.05) synergistic effect was seen in combination with *A. actinomycetemcomitans* whilst all the other bacteria showed a reduction in IL-1α production. The *C. rectus* induction of IL-1α production appeared to be the most sensitive to the presence of *P. gingivalis* showing a 72±7% reduction in total IL-1α levels.

One possible explanation for the antagonistic effects and the low IL-1α production by *P. gingivalis* supernatant is that the proteolytic activity of the supernatant acts to degrade any IL-1α produced. To evaluate the direct degradation of IL-1α by *P. gingivalis* supernatant, recombinant human IL-1α (50 ng/ml) was incubated with culture supernatant (at 1:250 and 1:50) at 37°C in PBS for 6 hours. Subsequent ELISA analysis was able to detect 96% of the added IL-1α protein. This confirms that the proteolytic degradation of IL-1α was not a significant factor in this experimental system and did not account for the low cytokine production seen in the presence of *P. gingivalis* supernatant. Similar effects of co-stimulation with *P. gingivalis* and other pathogens were also seen in primary monocytes (Table 3.2B). In all cases the addition of *P. gingivalis* significantly decreased total IL-1α production of between 69 and 84%. Interestingly the stimulating effect of *A. actinomycetemcomitans* in combination with *P. gingivalis* seen in Monomac-6 cells was not seen and instead in primary monocytes IL-1α production was reduced by 84%.
Figure 3.5 Effects of bacterial supernatants on cell viability. Monomac-6 (A) and freshly isolated human monocytes (B) were exposed to culture supernatants from periodontopathogens for 6 hours. Cytotoxicity was determined by the release of the cytosol enzyme lactate dehydrogenase (LDH). The activity was expressed as a percentage of the total LDH activity released from cells lysed by exposure to 0.1% Triton X-100 for 45 min. The data are the mean±standard deviations (S.D.) n=3. *P<0.05
### A) Bacteria

<table>
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<tr>
<th>Bacteria</th>
<th>Singles</th>
<th>Co-stimulation</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.11±7.56</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>P. gingivalis</em> (<em>P.g</em>)</td>
<td>35.89 ± 4.23</td>
<td>45.15 ± 18.05</td>
<td>26.84 ± 12.4↑</td>
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<td><em>A. actinomycetemcomitans</em> (<em>A.a</em>)</td>
<td>1874.89 ± 89.45</td>
<td>2272.88 ± 47.90</td>
<td>21.22 ± 16.5↑</td>
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<td><em>F. nucleatum</em> (<em>F.n</em>)</td>
<td>3423.25 ± 344.99</td>
<td>2468.14 ± 405</td>
<td>27.90 ± 16.0↓</td>
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<td><em>C. rectus</em> (<em>C.r</em>)</td>
<td>1754.78 ± 138.16</td>
<td>500.27 ± 74.48</td>
<td>71.49 ± 7.43↓</td>
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<td><em>V. atypica</em> (<em>V.a</em>)</td>
<td>3103.12 ± 641.74</td>
<td>2423.10 ± 364</td>
<td>21.91 ± 9.89↓</td>
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<tr>
<td><em>P. intermedia</em> (<em>P.I</em>)</td>
<td>284.45 ± 84.21</td>
<td>159.57 ± 38.42</td>
<td>43.90 ± 9.26↓</td>
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<tr>
<td><em>P. nigrescens</em> (<em>P.n</em>)</td>
<td>121.24 ± 15.74</td>
<td>64.40 ± 32.80</td>
<td>46.33 ± 1.44↓</td>
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<td><em>E. coli</em> LPS (<em>E.c</em>)</td>
<td>1109.86 ± 89.87</td>
<td>464.90 ± 39.83</td>
<td>64.80 ± 2.59↓</td>
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### B) Bacteria

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<th>Co-stimulation</th>
<th>% change</th>
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<td>Control</td>
<td>117.72±0.87</td>
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<td>-</td>
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<tr>
<td><em>P. gingivalis</em> (<em>P.g</em>)</td>
<td>208.54±27.79</td>
<td>255.28±34.57</td>
<td>22.41 ± 6.2↑</td>
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<td><em>A. actinomycetemcomitans</em> (<em>A.a</em>)</td>
<td>559.22 ± 88.06</td>
<td>88.78 ± 37.51</td>
<td>83.86 ± 2.49↓</td>
</tr>
<tr>
<td><em>F. nucleatum</em> (<em>F.n</em>)</td>
<td>417.77 ± 113.8</td>
<td>123.17 ± 11.81</td>
<td>69.19 ± 7.30↓</td>
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<tr>
<td><em>C. rectus</em> (<em>C.r</em>)</td>
<td>594.66 ± 90.11</td>
<td>144.49 ± 64.17</td>
<td>75.20 ± 7.43↓</td>
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<td><em>V. atypica</em> (<em>V.a</em>)</td>
<td>1242.16 ± 268.84</td>
<td>206.81 ± 25.52</td>
<td>82.82 ± 4.14↓</td>
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<tr>
<td><em>P. intermedia</em> (<em>P.I</em>)</td>
<td>1209.46 ± 16.34</td>
<td>239.52 ± 10.86</td>
<td>80.19 ± 2.60↓</td>
</tr>
<tr>
<td><em>P. nigrescens</em> (<em>P.n</em>)</td>
<td>1369.58 ± 140.96</td>
<td>346.22 ± 37.83</td>
<td>74.56 ± 2.40↓</td>
</tr>
</tbody>
</table>

**Table 3.2** Overall changes in total IL-1α levels in Monomac-6 (A) and human monocytes (B) treated with a range of bacterial supernatants in the presence of *P. gingivalis* supernatant Monomac-6 or freshly isolated monocytes were plated at a density of 1 x 10⁶ cells/ml in 96-well dishes (100μl/well). Cells were treated with a range of periodontopathogens or LPS from *E. coli* (10 ng/ml) with (co-stimulation) or without (single) supernatant from *P. gingivalis* for 6 hours. After 6 h incubation the cells were lysed and cell-free supernatants were harvested to assess total IL-1α (secreted and cell-associated) by ELISA. Changes induced by co-stimulation represent mean changes in three separate experiments with mean±standard error. Figures for co-stimulation represent mean changes in three separate experiments with mean±standard error. Figures for co-stimulation represent mean changes in three separate experiments with mean±standard error. Figures for co-stimulation represent mean changes in three separate experiments with mean±standard error. Figures for co-stimulation represent mean changes in three separate experiments with mean±standard error. Portions were adjusted for the stimulating effect of *P. gingivalis* alone. ↑% increase, ↓% decrease,* P<0.05.
3.6.3 The effect of different *P. gingivalis* strains on the antagonistic activity

Before testing the ability of mutants to antagonise the effects of *C. rectus*-mediated IL-1α production it was necessary to confirm that the other pathogenic strains produced similar effects. The antagonistic activity of *P. gingivalis* was not restricted to the W50 strain. A comparison of the antagonistic ability of this strain with W83 and 381 (nonencapsulated) showed no significant differences when used in combination with *C. rectus* (Figure 3.6). Subsequent experiments used strain W50 which is the parental strain for the mutants that were tested in later experiments.

![Figure 3.6](image)

**Figure 3.6** Effects of different *P. gingivalis* strains on the antagonistic activity. Monomac-6 cells were plated at a density of 1 × 10⁶ cells/ml in 96-well dishes. Cells were exposed to *C. rectus* with or without supernatant from *P. gingivalis* (W50, W83 or 381). After 6 h incubation the cells were lysed by subjecting those to three cycles of freezing and thawing to assess total IL-1α. The supernatants were harvested and assayed for IL-1α by ELISA. The data are the mean ± standard deviations (S.D.) of triplicate assays. *P<0.05

3.6.4 Mechanism of immune suppression

Since the most dramatic antagonistic interaction in Monomac-6 cells was between *C. rectus* and *P. gingivalis* this combination of bacterial supernatants was used to investigate further the mechanism of antagonistic activity. Removal of LPS from *P. gingivalis* and *C. rectus* supernatants by polymyxin B almost completely eliminated the ability to stimulate IL-1α production by 94 and 99% respectively. Heat treatment of supernatants
resulted in an increase in the stimulating ability of the supernatants by 44 and 48% respectively and suggests that the active components are not heat-sensitive (Figure 3.7).

![Figure 3.7](image_url)

**Figure 3.7** The effect of LPS depletion and heat treatment on periodontopathogen induced IL-1α production by Monomac-6 cells. The bacterial supernatants were passed through polymyxin B columns or heated to boiling for 30 minutes. The cells were exposed to untreated or LPS-depleted or heated bacterial supernatants for 6 hours. After 6 h incubation the cells were lysed and cell-free supernatants were harvested to assess total IL-1α (secreted and cell-associated) by ELISA. The data are the mean±standard deviations (S.D.) n=3.

**Role of gingipains of P. gingivalis in inhibition of IL-1α stimulation by C. rectus**

The LPS and protease content of *P. gingivalis* cultures are known to vary significantly with time in culture (Rangarajan et al., 1997). Thus cultures grown between 1 and 6 days were compared for their ability to induce IL-1α from Monomac-6 cells and antagonize *C. rectus* activity. Samples or cells were withdrawn throughout the course of logarithmic growth. These investigations were performed by examining growth in appropriate medium as detailed in Materials & Methods. Growth of *P. gingivalis* W50 was significantly increased over four days followed by reduced cell proliferation (Figure 3.8 A). The levels of protein in culture supernatants increased steadily throughout the course of growth (Figure 3.8 B). However, culture supernatants did not show any significant change in the ability to induce IL-1α over the culture period (Figure 3.9 A) but showed a significant progressive increase ($R^2=0.95$) in their ability to antagonize *C. rectus* activity (Figure 3.9 B).
Figure 3.8 Growth assay of *P. gingivalis* W50 (A) and effect of culture length on total protein levels (B)

Cultures were grown as described in Materials and Methods. A) Bacterial growth at the indicated time points was evaluated via measurement of the optical density at 540 nm with a UV-visible spectrophotometer. B) Supernatants withdrawn at the indicated time points (1-6 day-old). Total protein concentrations were measured by Bio-Rad colorimetric protein assay. Quantative colorimetric reaction was read in a microplate reader at 595 nm, and compared against a standard curve of bovine albumin of known concentrations. The data are the mean±standard deviations (S.D.) n=3
Figure 3.9 Effect of culture length on stimulation of IL-1α (A) or inhibition of IL-1α production stimulated by C. rectus in Monomac-6 cells (B). Cultures were grown as described in Materials and Methods. Supernatants withdrawn at the indicated time points (1-6 day-old). Monomac-6 cells were plated at a density of $1 \times 10^6$ cells/ml in 96-well dishes. Cells were exposed to P. gingivalis with and without C. rectus for 6 hours. After incubation the cells were lysed and cell-free supernatants were harvested to assess total IL-1α (secreted and cell-associated) by ELISA. The data are the mean±standard deviations (S.D.) n=3.

Using specific protease mutants lacking the Lys-gingipain kgp (K1A) or Arg-gingipains rgpA rgpB expression (E8) it was possible to test their role in the antagonism of C. rectus activity. Enzyme assays for Arg-X and Lys-X protease activities using N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and N-α-acetyllysine-p-nitroanilide (AcKpNA), respectively, were performed to confirm the phenotypes of these mutants as described in
Chapter 3: Antagonistic Effects of *P. gingivalis*

Materials & Methods. Compared to *P. gingivalis* W50, *P. gingivalis* E8 mutant (rgpA rgpB) had Arg-X protease activity below the detection limit of the assay without any significant effect on the Lys-X protease activity in culture supernatants. In contrast, Arg-X activity in culture supernatants of K1A (kgp) only reached a maximum of ≈ 40% of levels in W50.

A complementary approach used the proteinase inhibitor TLCK or heating to block protease activity in the wild-type *P. gingivalis* supernatants. Treatment with 1mM TLCK completely abolished both Arg-X and Lys-X activity (Table 3.3). Both K1A and E8 *P. gingivalis* protease-mutant strains remained capable of antagonizing *C. rectus* mediated IL-1α production by respectively 68.4±4.9% and 49.6±2.8% (wild type inhibition was 68.9±4.7%). In addition, heat-treatment or chemical blockage of cysteine protease activity of *P. gingivalis* did not remove the inhibitory activity. Together this data suggests that protease activity does not play a significant role in the antagonistic activity of *P. gingivalis*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Enzyme activities U/ml/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Arg-X</td>
</tr>
<tr>
<td>W50</td>
<td>wild-type</td>
<td>5.36±0.14</td>
</tr>
<tr>
<td>K1A</td>
<td>kgp::erm</td>
<td>1.90±0.27*</td>
</tr>
<tr>
<td>E8</td>
<td>rgpA::tetQ</td>
<td>0.30±0.07*</td>
</tr>
<tr>
<td>TLCK-treated</td>
<td>wild-type</td>
<td>0.48±0.11*</td>
</tr>
</tbody>
</table>

*P<0.05 compared to wild-type W50

Table 3.3. Arg-X and Lys-X protease activities in isogenic mutants of *P. gingivalis*. Culture supernatants of *P. gingivalis* W50 and K1A and E8 were assayed for Arg-X and Lys-X activity using the substrates DL-BAPNA and Nα-acetyl-L-lysine-p-nitroanilide (Ac-LyspNA) respectively. Arg-X and Lys-X activities were normalized with respect total protein in the culture supernatants and activities are expressed as units ml⁻¹ per mg protein. The figures shown are the mean±standard deviations (S.D.) of triplicate assays and statistical differences were calculated by Student’s *t* test.

Role of *P. gingivalis* LPS in inhibition of IL-1α stimulation by *C. rectus*

LPS is another important component of the bacterial supernatant. The ability of purified *P. gingivalis* LPS to stimulate IL-1α production by Monomac-6 cells was tested. *P. gingivalis* LPS caused a significant concentration-dependent stimulation of IL-1α.
production (Figure 3.10). Purified LPS was a relatively poor inducer of IL-1α from Monomac-6 cells. *P. gingivalis* supernatant was able to induce slightly higher IL-1α than 100 ng/ml of its purified LPS. In addition, measurement of *P. gingivalis* culture supernatants by the LAL assay showed that the 1:250 dilution used to stimulate cells contained approximately 142 ng/ml.

![Graph](image)

**Figure 3.10** The levels of total IL-1α in Monomac-6 cells treated with *P. gingivalis* supernatant or its LPS. Monomac-6 cells were plated at a density of 1 x 10^6 cells/ml in 96-well dishes. Cells were exposed to *P. gingivalis* supernatant or LPS from *P. gingivalis* (10, 100, 1000 ng/ml). After 6 h incubation the cells were lysed and cell-free supernatants were harvested to assess total IL-1α (secreted and cell-associated) by ELISA. The data are the mean±standard errors (SEM) of three independent experiments. *p<0.05

Since purified LPS has only a limited ability to induce IL-1α it was possible to test its antagonistic activity when mixed with *C. rectus* supernatant. Whilst 10 ng/ml purified LPS had no significant effect the addition of 100 or 1000 ng/ml was able to significantly antagonize stimulation of IL-1α by *C. rectus* (Figure 3.11). The level of IL-1α inhibition seen with 100-1000 ng/ml LPS was not significantly different from *P. gingivalis* culture supernatant (1:50) containing 714 ng/ml LPS (p=0.16 and p=0.07, respectively). In addition, polymyxin B treatment of *P. gingivalis* supernatant reduced LPS concentrations from 714 ng/ml to 11.2 ng/ml at the (1:50) dilution used, and subsequently inhibited the *C. rectus*-stimulated IL-1α production by only 10% (Figure 3.11). Collectively these findings provide evidence that the LPS in *P. gingivalis* culture supernatants appears to be
essential and present in sufficient quantities to account for the observed inhibition of C. rectus-induced IL-1α production by Monomac-6 cells.

Figure 3.11  The role of P. gingivalis LPS in C. rectus mediated IL-1α production by Monomac-6 cells. Monomac-6 cells were plated at a density of 1×10⁶ cells/ml in 96-well dishes. Cells were exposed to C. rectus with purified P. gingivalis LPS (10, 100, 1000 ng/ml) or P. gingivalis supernatant. After 6 h incubation the cells were lysed and cell-free supernatants were harvested and assayed for total IL-1α (secreted and cell-associated) by ELISA. The data are the mean±standard errors (SEM) of three independent experiments. *p<0.05

To learn more about the possible mechanisms by which P. gingivalis LPS acts to antagonize C. rectus activity, Monomac-6 cells were pre-treated with 1 μg/ml purified P. gingivalis LPS. Priming of Monomac-6 cells with P. gingivalis LPS largely abolished responsiveness to C. rectus (Figure 3.12). There was no obvious effect when pre-treated cells were exposed to a combination of C. rectus and P. gingivalis compared to C. rectus alone. The result of LPS pre-treatment suggests that Monomac-6 cells were rendered either directly or indirectly unresponsive to C. rectus.
Chapter 3: Antagonistic Effects of *P. gingivalis*

**Figure 3.12** The role of priming of Monomac-6 cells with *P. gingivalis* LPS in *C. rectus* mediated IL-1α production. Monomac-6 cells were pretreated for 1 h with *P. gingivalis* LPS (1 μg/ml) or medium alone. Monomac-6 cells were plated at a density of 1x10⁶ cells/ml in 96-well dishes and subsequently challenged with *C. rectus* or *P. gingivalis* or both supernatant for 6 h. After 6 h incubation the cells were lysed and cell-free supernatants were harvested to assess total IL-1α (secreted and cell-associated) by ELISA. The data are the mean±standard deviations (S.D.) of triplicate assays. **p<0.05

3.7 DISCUSSION

This study demonstrated that a broad range of oral bacteria stimulate the expression of IL-1α in Monomac-6 cells and primary monocytes as previously reported (Henderson et al., 2002; Reddi et al., 1995; Sugano et al., 2004). The weakest stimulators of IL-1α were the non-pathogenic *S. sanguis* and surprisingly *P. gingivalis*. Most previous studies considered the stimulation of cytokine production for each bacterial species in isolation. Therefore, IL-1α production by monocytic cells was compared when treated with single bacterial supernatants to those treated with mixtures. A number of previous studies suggested that *P. gingivalis* might evade the normal responses through its ability to suppress the normal innate immune mechanisms (Darveau et al., 1998). In the present study *P. gingivalis* supernatants inhibited the capacity of most studied pathogens to stimulate IL-1α production by monocytes. The most pronounced inhibition was seen in combination with *C. rectus*, where the overall level of IL-1α production was reduced by approximately 72% in the presence of *P. gingivalis*. In the case of *A. actinomycetemcomitans*, co-stimulation of the cells with *P. gingivalis* caused a synergistic effect in IL-1α production by Monomac-6 cells, but a reduction in primary...
human monocytes. The basis for this difference is not clear but could reflect increased sensitivity to *P. gingivalis* stimulation by primary monocytes compared to Monomac-6 cells. There is no reason to believe that all oral bacterial species that are weak stimulators of cytokine production act to suppress immune responses. In preliminary experiments with *Treponema denticola*, we have shown that culture supernatants elicit only a very small level of IL-1α production on their own but are highly synergistic when combined with other periodontal pathogenic species (Javier Fernandez, M Clinic Dent Thesis, 2006).

*P. gingivalis* expresses a number of potential virulence factors. In this study the culture supernatant contains a mixture of bacterial endotoxin, bacterial metabolic products and enzymes. The most frequently studied components are the LPS and the Arg- and Lys- gingipain cysteine proteinases which are found in large quantities in culture supernatant (Curtis et al., 2001; Shapira et al., 1998). In previous studies it has been shown that proteolytic cleavage by *P. gingivalis* can degrade and inactivate cytokines including IL-6, IL-8 and TNF-α (Banbula et al., 1999; Calkins et al., 1998; Mezyk-Kopec et al., 2005; Oleksy et al., 2002) and the CD14 receptors (Duncan et al., 2004; Sugawara et al., 2000). However, the possibility that the antagonistic actions of *P. gingivalis* reported here were the result of proteolytic breakdown of the IL-1α is not supported by our data. When cultures containing *P. gingivalis* supernatant were supplemented with IL-1α protein at known concentrations almost all could be detected by ELISA after 6 hours. The reasons that we did not observe proteolytic degradation in our system could reflect the presence of serum proteins, including protease inhibitors and the relatively short (6 hour) period of incubation. Moreover, the *P. gingivalis* protease-mutant strains still retained their capacity to antagonize *C. rectus*-mediated IL-1α production. In addition, heat-treatment or chemical inhibition of protease activity in the wild-type *P. gingivalis* strain supernatant did not abolish its inhibitory capacity, but were in fact slightly enhanced. This may reflect the normal complexing of the gingipains with LPS in the supernatant which acts to mask LPS activity. The act of heating unmasks the LPS and results in increased inhibitory activity (Takii et al., 2005). Overall the data suggests that gingipains do not play a significant role in the antagonism of activity of *C. rectus* by *P. gingivalis* as measured by changes in IL-1α production.
Chapter 3: Antagonistic Effects of *P. gingivalis*

To investigate the involvement of LPS in the observed inhibition of IL-1α production the activity of purified *P. gingivalis* LPS was tested. This purified LPS could not stimulate high levels of IL-1α production by monocytes which is in agreement with previous findings (Roberts et al., 1997). In contrast, depletion of LPS from *C. rectus* culture supernatants using polymyxin B columns largely abolished its ability to stimulate IL-1α production. Adding purified *P. gingivalis* LPS was sufficient to suppress IL-1α stimulation in response to the *C. rectus*. Interestingly LPS concentrations in *P. gingivalis* supernatants are similar to the level of purified LPS needed to inhibit IL-1α production. In addition, *P. gingivalis* supernatant depleted of LPS using polymyxin B was unable to block *C. rectus* induced IL-1α production.

One possible explanation for the observed antagonistic effect is that although *P. gingivalis* LPS is a weak stimulus for proinflammatory cytokines it is a potent inducer of IL-1R antagonist (IL-IRA) (Ogawa et al., 1994). Indeed, it has been shown that high levels of IL-1RA may down-regulate IL-1α production in response to LPS in monocytes (Granowitz et al., 1992). Alternatively, the antagonistic effect could be attributed to competition of the two species for common signalling receptors, such as CD14 and Toll-like receptors (TLRs) (Darveau et al., 2004; Hajishengallis et al., 2002; Yoshimura et al., 2002; Zhou et al., 2005). Although it has not been conclusively shown that *P. gingivalis* and *C. rectus* possess any components that initiate common signalling pathways, the present work supports a putative LPS cross-talk between these two species. We have shown that priming of Monomac-6 cells with *P. gingivalis* LPS results in a significant decrease in the capacity for *C. rectus* to stimulate IL-1α production either alone, or in combination with *P. gingivalis*. Previous studies have demonstrated that *P. gingivalis* LPS can antagonize the LPS of *E. coli* as well as other species (Yoshimura et al., 2002). There is evidence that binding of *P. gingivalis* LPS to both TLR2 and TLR4 is involved in this inhibition (Coats et al., 2003; Darveau et al., 2002; Nassar et al., 2002). It is also important to draw a distinction between the phenomenon of endotoxin tolerance which is normally thought to require a prolonged stimulation of immune cells and this study which involves a more acute short term assay (Muthukuru et al., 2005). It is possible that the same mechanisms are involved, but equally studies suggest that the observed antagonism could reflect direct signalling by *P. gingivalis* LPS rather than a generalized suppression of immune responsiveness (Coats et al., 2003; Darveau et al., 2002).
In summary, this work demonstrates an antagonism between \textit{P. gingivalis} and \textit{C. rectus} in terms of IL-1\(\alpha\) production that appears to rely on the integration of positive and negative signals through cell signalling rather than any direct interaction between components in the supernatants. This study suggests that \textit{P. gingivalis} appears to play a role in the short term inhibition of innate immune responses. Although \textit{P. gingivalis} LPS exhibits a weak cytokine stimulating capacity, it may cause an early inactivation of immune cell responses to other bacteria. In this situation the other periodontal bacterial species may be more likely to persist therefore favouring the establishment of chronic inflammatory lesion typical of periodontitis.
CHAPTER 4

Aggressive Periodontitis and IL-1A SNPs
4.1 INTRODUCTION

As described in Chapter 1, the association of IL-1 genotypes with periodontal disease has been widely studied. In 1997, Kornman and co-workers found an association between carriage of two alleles in the IL-1A-889 and IL-1B+3953 genes, and an increased severity of chronic periodontitis (Kornman et al., 1997a). This has resulted in the production of a commercially available periodontal susceptibility test (PST, Interleukin Genetics). The composite IL-1 genotype has been extensively investigated with respect to both chronic and aggressive periodontitis, smoking, ethnic group, oral microbial pathogens present in the plaque biofilm and other periodontal clinical parameters related to inflammatory status or to the progression of the disease (Shapira et al., 2005). However, there is conflicting data in the literature regarding the specific role of IL-1 polymorphisms in periodontal disease (Kinane et al., 2005). It has been suggested that these polymorphisms are a useful marker in specific populations (Armitage et al., 2000; Walker et al., 2000) but unlikely to be relevant in aggressive periodontitis (Hodge et al., 2001).

The contradictory results found in several studies may be explained by differences in the diagnostic criteria used to define populations, the low number of subjects studied, difficulties in selecting a control group (due to the continuous variability of chronic periodontitis and the different age of onset of disease between individuals) (Greenstein and Hart, 2002). In contrast, identifying the role played by genetics in generalized aggressive periodontitis patients may be easier since it has a widely accepted definition in terms of phenotype and age of onset compared to chronic periodontitis (Hodge and Michalowicz, 2001).

A number of techniques are available for rapid SNP genotyping. A key requirement of a SNP genotyping method is that it distinguishes unequivocally between the allelic variants present in homozygous and heterozygous forms. The choice of technology depends on whether a few SNPs are to be screened in many individuals or many different SNPs are to be examined in a few individuals. There are several methods to discriminate alleles including by differential hybridization, primer extension, ligation and cleavage of an allele-specific probe (Twyman and Primrose, 2003; Wang et al., 1998). The most reliable system for re-sequencing genes in multiple individuals is still nucleotide sequencing itself (Chan, 2005; Sanger et al., 1977). Pre-screening methods have been widely used for
the discovery of SNPs or mutations in human disease populations and are especially useful to identify SNPs that are rare (Table 4.1). The available high-throughput methods make genotyping of a large number of samples fast and inexpensive. The TaqMan assay (Applied Biosystems) is one of high-throughput methods. However, TaqMan analysis requires basic information regarding the existence and abundance of different polymorphisms. The information available in public data bases is currently incomplete to allow all potential polymorphisms to be identified using this technique in patients with periodontitis.

<table>
<thead>
<tr>
<th>Method</th>
<th>Accuracy</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequencing</td>
<td>High</td>
<td>(Livingston et al., 2004)</td>
</tr>
<tr>
<td>Single-strand conformation polymorphism (SSCP)</td>
<td>Low</td>
<td>(Wang et al., 2003)</td>
</tr>
<tr>
<td>Cleavage fragment length polymorphism (CFLP)</td>
<td>Medium</td>
<td>(Casadei et al., 2001)</td>
</tr>
<tr>
<td>Denaturing high-performance liquid chromatography (DHPLC)</td>
<td>Medium</td>
<td>(Glatt et al., 2001)</td>
</tr>
</tbody>
</table>

Table 4.1 Pre-screening methods for SNP discovery. Listed pre-screening methods for SNP discovery, broadly categorized according to their principles of SNP detection, and comparing their accuracy under one set of conditions, excluding human error. Sequencing was Adapted as the gold standard. Assuming one set of conditions, no human error, and a 100% accuracy of sequencing as the arbitrary standard; high = 100%, medium = 90–100%, low = 60–90% Adapted from (Suh and Vijg, 2005).

There are several web-based resources available for assessment of single nucleotide polymorphisms (Table 4.2). The NCBIEntrez SNP database (http://www.ncbi.nlm.nih.gov/entrez) currently lists 15 polymorphisms in 5' upstream region of the IL-1A gene of which five are located in the putative promoter (2.1 kb of the genomic sequence upstream of the transcriptional start site). Many of the reported polymorphisms are based on studies of relatively small numbers of individuals (often only 46) and in all cases periodontal status is unknown. Surveys of the abundance of polymorphisms generally aim to identify those polymorphisms with a frequency above about 4%. However, since severe periodontal disease has an incidence of about 8% in the general population, it is possible that any given polymorphism abundant in this sub-population could be missed or classed as rare from a small survey of random individuals.
Therefore, the aims of this chapter were:

1. To identify the common IL-1A gene promoter polymorphisms present in patients with aggressive periodontitis

2. To test the detected polymorphisms to ascertain if there was a significant difference between patients with aggressive periodontitis and unrelated healthy individuals in terms of allele frequencies or genotype frequencies.

In this thesis two strategies were used for detecting all polymorphisms including direct sequencing using BigDye v2 and TaqMan analysis. The first strategy was to directly sequence the 2.1 kb genomic sequence upstream of the transcriptional start site as well as first exon and intron by surveying the frequency and occurrence of all variation within this region including SNPs, insertion/deletions and VNTRs (variable number of tandem repeats) within a Caucasian population of diseased individuals. Secondly, after identifying the existence and abundance of different polymorphisms in the promoter region of the IL-1A gene, TaqMan analysis was performed to provide information regarding the abundance of polymorphisms in the IL-1A gene in a range of ethnic groups including both diseased and control individuals.
SNPs can be numbered relative to their locations to the ATG start site. Reported studies have used either rs ID numbers in the database or previously reported positions (Table 4.3). There are several transcriptional start sites in the human IL-1A promoter but Ets data confirms that the most common start site is what has been considered here (see in Appendix IV). To avoid unnecessary confusion, throughout this thesis these polymorphisms are referred to by their rs SNP ID numbers in the NCBIEntrez SNP database, instead of the base locations reported in the original publications. Furthermore, for the sake of clarity these SNPs have been referred to I-V in the thesis text.

Table 4.3 SNPs in the IL-1A gene promoter. Position column indicates base positions with respect to the translation initiation site. They are allocated to the positions -990, -1303, -1287 and -2037 upstream of the translational initiation codon. SNP ID column refers to their rs SNP ID numbers in the NCBIEntrez SNP database. Rare allele frequencies were taken from NCBIEntrez SNP database. No data = there is no data available in database. Furthermore, for the sake of clarity these SNPs have been referred to I-V in the thesis text.

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Position</th>
<th>Sequence</th>
<th>Allele</th>
<th>Rare Allele frequency (%)</th>
<th>ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1800587</td>
<td>(-990)</td>
<td>C/T</td>
<td>33% 39% 12%</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>or</td>
<td>(-899)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs3783521</td>
<td>(-1607)</td>
<td>C/T</td>
<td>27% 10% 67%</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>rs1800794</td>
<td>(-1303)</td>
<td>C/T</td>
<td>33% 2% 12%</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>rs3783571</td>
<td>(-1287)</td>
<td>A/G</td>
<td>Non no data 2%</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>rs3783570</td>
<td>(-2037)</td>
<td>C/T</td>
<td>Non non 2%</td>
<td>V</td>
<td></td>
</tr>
</tbody>
</table>

4.2 MATERIAL & METHODS

4.2.1 Patient Cohort

The study was approved by the Local Research Ethics Committee (P/00/021 East London and The City Health Authority) and by the Local Research Ethics Committees in the Faculty of Medicine, Queens University, Belfast. Informed consent was obtained from all patients involved in the study.
The patients for this study were a cohort of 150 subjects with generalized aggressive periodontitis (GAP). One hundred and ten subjects with GAP were collected during an MRC-funded clinical study of risk and prognostic factors at the Royal London Hospital. Subjects were recruited from among new patients attending the Periodontal Consultant Assessment clinic and criteria for their inclusion are indicated in Table 4.4. All participants were interviewed with respect to systemic health history and current medication, and smoking habits were recorded according to a simplified protocol: 0 = subjects who had never smoked; 1 = former smokers 2 = current smokers. This group was ethnically diverse with an approximate breakdown of 74% white Caucasian, 11% Asian and 15% Afro-Caribbean. 117 healthy controls were recruited from both staff within Barts and the London School of Medicine and Dentistry and within Institute of Cell and Molecular Science. The age and gender distribution was reasonably well balanced, with cases being on average 33.7 years old (16-42, SD 5.3) and controls 33.7 years old (18-53, SD 7.6). Forty subjects with GAP (24 females and 16 males with the mean age of 30 ± 5 years ranging from 16 to 37 years) were identified from referrals to the Periodontal Department, School of Dentistry, Queen’s University, Belfast (collaboration with Prof Gerry Linden).

| Age 16-40 years |
| No systemic conditions |
| 6 teeth CAL > 6mm |
| No medication in the last 6 months |
| No periodontal treatment in the last 6 months |

Table 4.4 Patient Selection Criteria. Subjects were recruited from among new patients attending the Periodontal Consultant Assessment clinic and criteria for their inclusion are indicated.

### 4.2.2 Sample collection and preparation

A 5 ml venous blood sample was taken from each patient using a standard vacutainer containing sodium citrate and stored at -20°C prior to extraction of DNA.

### 4.2.3 DNA extraction

Genomic DNA was extracted from 2 ml of blood using a salt precipitation technique as described previously (Miller et al., 1988) and outlined in the Appendix III. DNA yields
were quantified using Nanodrop, ND-1000 (NanoDrop Technologies, USA) and the concentration was expressed as ng/µl (Appendix III, Table 1-2). The purity of the extracts was determined from the ratio of the absorbancy values at 260 nm and at 280 nm, to assess for protein contamination. The samples were stored at 4°C until further analysis.

4.2.4 Direct DNA sequencing and allele genotyping

Polymerase chain reaction (PCR)

The strategy to identify polymorphisms was based on PCR amplification of putative promoter, the first exon and first intron which directly sequenced using BigDye v2. The sequence for the human IL-1A gene was obtained using the Ensemble genome browser. The accession number is AF536338 (Appendix IV). PCR primers were designed with assistance of the online PCR primer design programme, Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi) and all primers were checked on the BLAST online sequence search engine to ensure they had low similarity with other known human cDNA sequences (http://www.ncbi.nlm.nih.gov/BLAST). All primers were synthesized by Invitrogen, UK (Table 4.5).

PCR is a commonly used method for amplifying defined target DNA sequences of interest. Two oligonucleotide primers (15–25 nucleotides) which are specific for the target sequence are used. After the primers are added to denatured template DNA, they bind specifically to complementary DNA sequences at the target site. In the presence of a suitably heat-stable DNA polymerase and DNA precursors (the four deoxynucleoside triphosphates, dATP, dCTP, dGTP and dTTP), they initiate the synthesis of new DNA strands which are complementary to the individual DNA strands of the target DNA segment (Figure 4.1). The PCR is a chain reaction because newly synthesized DNA strands will act as templates for further DNA synthesis in subsequent cycles. After about 25 cycles of DNA synthesis, the products of the PCR will include, in addition to the starting DNA, about 10^5 copies of the specific target sequence, an amount which is easily visualized as a discrete band of a specific size when submitted to agarose gel electrophoresis.
Chapter 4: Aggressive Periodontitis and IL-1A Polymorphisms

Figure 4.1 A schematic representation of Polymerase Chain Reaction (PCR). PCR is an *in vitro* method for amplifying DNA sequences using defined oligonucleotide primers. Oligonucleotide primers A and B are complementary to DNA sequences located on opposite DNA strands and flanking the region to be amplified. Adapted from Human Molecular Genetics 2 (Strachan, 1999).

Genomic polymerase chain reaction (PCR) was carried out on a Thermal Cycler (Hybaid) in a 10x PCR buffer containing 10 mM Tris-HCl (pH 8.3), 200 ng of genomic DNA, 50 ng/μl mix of forward and reverse primer, 10mM dNTPs (Sigma), 50mM MgCl₂ and 4U Bio-X-Act Polymerase (Table 4.6). Each primer set was optimised for MgCl₂ and temperature using four MgCl₂ concentrations (1, 1.5, 2, and 2.5 mM) and four temperatures (50°C, 55°C, 60°C, 65°C). Optimized annealing temperature was 60°C and the MgCl₂ concentration for primers was 2.5 mM. 10 μl of the PCR reaction was loaded onto a 1% agarose gel using loading buffer and run for 40 min at 100V. 1kb DNA Ladder (New England Biolabs, Hitchin, UK) was used as marker to estimate size of product. The samples were then stored at 4°C until further analysis.
Chapter 4: Aggressive Periodontitis and IL-1A Polymorphisms

<table>
<thead>
<tr>
<th>Primer</th>
<th>5'-Primer (forward)</th>
<th>3'-Primer (reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter</td>
<td>TACGTTCCACTGTCCTTCCTCC</td>
<td>ACCGCCAATGAAATGACTCC</td>
</tr>
<tr>
<td>First exon</td>
<td>TCAATAACTCTGGAATATCT</td>
<td>CAAAATGAGAGAATCATT</td>
</tr>
<tr>
<td>First intron</td>
<td>AATTACAGTCAGATTCAGAA</td>
<td>TGTAACAGTTCTTCAGGTCTT</td>
</tr>
</tbody>
</table>

Table 4.5 The PCR primer sequences. The primers were designed using Primer3 web programme and synthesized by Invitrogen, UK. The 5'→3' sequences of the sense (S) and the 3'→5' antisense (AS) primer pairs are shown.

<table>
<thead>
<tr>
<th>Stock concentration</th>
<th>Concentration required</th>
<th>Volume used</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X OptiBuffer</td>
<td>1X</td>
<td>5 µl</td>
</tr>
<tr>
<td>50 mM MgCl Solution</td>
<td>1-2.5 mM</td>
<td>1-2.5 µl</td>
</tr>
<tr>
<td>100 mM dNTPs</td>
<td>2 mM</td>
<td>1 µl</td>
</tr>
<tr>
<td>Genomic DNA (25 ng/µl)</td>
<td>50 ng</td>
<td>2 µl</td>
</tr>
<tr>
<td>Primer mix 100 µM</td>
<td>2 µM</td>
<td>2 µl</td>
</tr>
<tr>
<td>BIO-X-ACT Long (4 u/µl)</td>
<td>4 u</td>
<td>1 µl</td>
</tr>
<tr>
<td>Water (ddH₂O)</td>
<td>x</td>
<td>37.5-39 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Table 4.6 Components of the PCR mix. Polymerase chain reaction (PCR) was carried out on a Thermal Cycler (Hybaid, UK) in a 10X PCR buffer containing 10 mM Tris-HCl (pH 8.3), 200 ng of genomic DNA, 50 ng/µl mix of forward and reverse primer, 10mM dNTPs (Sigma), 50mM MgCl₂ and 4U Bio-X-Act long polymerase.

Sequencing of PCR products

Seven primer pairs were used to sequence directly the 2.1 kb genomic sequence upstream of the transcriptional start site as well as the first exon and intron (Table 4.7). The strategy is outlined in Figure 4.2.

A)
Chapter 4: Aggressive Periodontitis and IL-1A Polymorphisms

Figure 4.2 A) Schematic diagram of the IL-1A gene. Boxed white regions depict the DNA sequences corresponding to the 2.1 kb genomic sequence upstream of the transcriptional start site as well as first exon and intron. The numbers are distance in base pairs from the transcriptional initiation site (black arrow). B) Schematic diagram of the sequencing primers. Arrows indicate the position of all 5 currently reported polymorphisms in the 2.1 kb genomic sequence upstream of the transcriptional start site (5'–500bp).

Table 4.7 The primers used for direct sequencing of the IL-1A gene to detect polymorphisms. The primers were designed using Primer3 programme and synthesized by Invitrogen, UK. The 5’ to 3’ sequences of the sense (S) and the 3’ to 5’ antisense (AS) primer pairs are shown.

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’-Primer (forward)</th>
<th>3’-Primer (reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AGAAGAGGCTGACTGTTGTTCA</td>
<td>ATACATTTTCAGGTGAGGC</td>
</tr>
<tr>
<td>2</td>
<td>AAATATCTTCAGGCTTTCAAT</td>
<td>TGGTGCTATAATAGGGTTAAT</td>
</tr>
<tr>
<td>3</td>
<td>TTACATCCAAGTGTTGTTTATT</td>
<td>TTATATAATCTTGTTGAGCCAGC</td>
</tr>
<tr>
<td>4</td>
<td>TCATTGCTAAGTAGCTGGTG</td>
<td>TAAGGCATAATAACGTTCAGA</td>
</tr>
<tr>
<td>5</td>
<td>ATTCAGAAGAGAAGAGGATC</td>
<td>ATGGAAGAGATGGCCATG</td>
</tr>
<tr>
<td>6</td>
<td>TCAATAACTTCTGGAATATCT</td>
<td>CAAAATGAGAGAATTCATT</td>
</tr>
<tr>
<td>7</td>
<td>TCAATAACTTCTGGAATATCT</td>
<td>CAAAATGAGAGAATTCATT</td>
</tr>
</tbody>
</table>

PCR products were prepared for sequencing using the ABI BigDye Terminator sequencing kit (Applied Biosystems, Warrington, Cheshire, UK) in accordance with the manufacturer’s instructions. Firstly, PCR primers and dNTPs were removed before sequencing. 3.5 µL of PCR product was incubated with 1.5 µl of ExoSaplT (Amersham, UK) for 37 min at 37°C followed by 20 min at 80°C to inactivate the enzymes (Appendix V-14). This method employs shrimp alkaline phosphatase (SAP) and exonuclease I (ExoI) to degrade nucleotides and single stranded DNA (primers) remaining after PCR (Werle et al., 1994). PCR products are sequenced by controlled termination of their replication and the assay is based around the Sanger method of DNA sequencing (Sanger et al., 1977). With either the forward or the reverse primer used for the PCR in the
sequencing reaction, AmpliTaq DNA polymerase catalyses complementary DNA strand synthesis using dNTPs in the reaction mix. Four separate fluorescent dyes are labelled onto a base-specific dNTPs. The order of the nucleotides is determined using argon ion laser, which scans the fragments on the gel measuring their emission spectrum and determining the order which dNTPs where incorporated (Figure 4.3A). This produces an emission plot along the gel, called electropherogram, which reveals the sequence of the DNA (Figure 4.3B). The information is recorded and data is stored in a computer database.

A)

B)

Figure 4.3 A) Principles of automated DNA sequencing. Four separate fluorescent dyes are used as labels for the base-specific reactions (the label can be incorporated by being attached to a base-specific dNTP). During the electrophoresis run, a laser beam is focused at a specific constant position on the gel. As the individual DNA fragments migrate past this position, the laser causes the dyes to fluoresce. Maximum fluorescence occurs at different wavelengths for the four dyes, and the information is recorded electronically and the interpreted sequence is stored in a computer database. Adapted from Human Molecular Genetics 2 (Strachan, 1999). B) Example of DNA sequence output (an electropherogram). This figure shows a typical output of sequence data from an ABI 3700 DNA analyzer automated DNA sequencer as a succession of dye-specific (and therefore base-specific) intensity profiles.
Each 10-μl sequencing reaction contained 50 ng of purified PCR product, 1.5 pmol of sequencing primer, 1 μl of BigDye Terminator mix, 1.5 μl of 5x sequencing dilution buffer (400 mM Tris, pH 9.0, 10 mM MgCl₂), and water to volume. Cycling conditions were 94°C for 1 min and 25 cycles of 94°C for 30 s, 50°C for 30 s, and 60°C for 4 min, finishing with a single 72°C extension step for 5 min. Sequencing products were ethanol precipitated, air-dried, resuspended in 25 μl ddH₂O, and analyzed on an ABI 3700 DNA analyzer (See detailed method in Appendix V.14).

Sequence analysis

The sequence analysis was carried out using the GAP4 analysis software by Staden (Bonfield and Staden, 2002). The forward and reverse sequences for each patient were aligned for comparison with a reference sequence imported from Ensembl. Because no sequence reading is 100% reliable, the search for mutations is not quite as simple as comparing a suspected polymorphic sequence against a wild type. Differences between two sequences can be due to mutations or to base calling errors. Thus the problem is recognising the real mutations within a background of base calling errors. The program TRACE_DIFF was used which normalizes and subtracts the pairs of reference and patient traces to produce a new set of traces which represent their differences. The difference traces are effectively flat except where a difference occurs and this provides a much simpler visual method of searching for mutations (Figure 4.4). While sequence alignment was performed and polymorphic ranking scores were identified using software, genotypes were read by eye.
4.2.5 TaqMan analysis

SNPs for TaqMan analysis were selected on the basis of having relatively high minor allele frequencies (>10%). In addition to direct sequencing results, the 3 SNPs were also checked on public databases including the National Center for Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/SNP) and the SNP Consortium database (http://snp.cshl.org). TaqMan analysis were performed to provide information regarding the abundance of polymorphisms in the IL-1A gene in a range of ethnic groups including both disease and control individuals. Further analyses were done if there was a significant difference between patients and unrelated healthy individuals, in terms of allele frequencies or genotype frequencies. Genotyping was carried out by Pre-made TaqMan Genotyping assays (Applied Biosystems, Warrington, UK). TaqMan assay IDs and the context sequence (the nucleotide sequence surrounding the SNP site provided in the (+) genome strand orientation relative to NCBI reference genome is shown in Table 4.8. The TaqMan SNP Genotyping Assay exploits the 5'-exonuclease activity of AmpliTaq Gold DNA polymerase to cleave a doubly labeled probe hybridized to the SNP-containing sequence of DNA (Livak et al., 1995). Cleavage separates a 5'-fluorophore from a 3'-quencher leading to detectable fluorescent signal (Kutyavin et al., 2000). The use of two probes, one specific to each allele of the SNP and labeled with two fluorophores, allows detection of both alleles in a single tube. Fluorogenic probes with a minor groove binder (MGB) produce enhanced allelic discrimination, because the MGB stabilizes the double-stranded probe template structure. This provides enhanced
mismatch discrimination between these shorter probes, resulting in improved allele specificity (Figure 4.5).

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Assay ID</th>
<th>Forward primer</th>
<th>Reporter dyes</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3783521 (C/T)</td>
<td>C-1839900-10</td>
<td>ACTGGCTACATTCTCTATTACCTCTT[A/G]</td>
<td>Allele 1 = VIC, Allele 2 = FAM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTCCTCTTAGCAACACACAAATG</td>
<td></td>
</tr>
<tr>
<td>rs1800794 (C/T)</td>
<td>C-9546482-30</td>
<td>CAGGAACAGAGGAATACCTTTATCCAA</td>
<td>Allele 1 = VIC, Allele 2 = FAM</td>
</tr>
<tr>
<td>rs1800587 (C/T)</td>
<td>C-9546481-20</td>
<td>ACTGGCTACATTCTCTACCTT[G/A]</td>
<td>Allele 1 = VIC, Allele 2 = FAM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTCCTCTTAGCAACACACAAATG</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.8. TaqMan genotyping probe and primer sequences. TaqMan assay IDs and the context sequence (the nucleotide sequence surrounding the SNP site provided in the (+) genome strand orientation relative to NCBI reference genome) are shown.

Figure 4.5 A diagram of the TaqMan SNP Genotyping Assay. (A) Probe binding and primer extension in a TaqMan SNP Genotyping Assay. (B) Allelic discrimination is achieved by the selective annealing of matching probe and template sequences, which generates an allele-specific (fluorescent dye-specific) signal. Briefly, during PCR, each TaqMan MGB probe anneals specifically to its complementary sequence between the forward and reverse primer sites. AmpliTaq Gold DNA polymerase cleaves only probes that are hybridized to the target and cleavage separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter. Adapted from Applied Biosystems, UK.
Sample Preparation

The amount of genomic DNA in samples was quantified before using TaqMan SNP Genotyping Assays as recommended by Applied Biosystems (Appendix III). DNA samples were diluted with DNase-free water (Sigma, UK) to deliver a final DNA concentration in the range of 1 to 20 ng per well. 2µl of diluted genomic DNA was delivered to the bottom surface of a MicroAmp Optical 384-Well Reaction Plate by pipeting and the DNA samples were dried down completely by evaporation at room temperature. The plate was covered with a lint-free tissue while drying. 10 samples were randomly selected and included as replicates for each genotype tested.

PCR amplification

AmpliTaq Gold DNA polymerase from the TaqMan Universal PCR Master Mix amplifies target DNA using sequence-specific primers and TaqMan MGB probes from the SNP Genotyping Assay. The reaction mix was prepared for each assay. 5 µl of the reaction mix was added to the prepared DNA reaction plate. The plate was sealed with the cover (MicroAmp Optical Adhesive Film). Applied Biosystems 7900HT Fast Real-Time PCR System was used for PCR amplification. The thermal cycling conditions for PCR are shown in Table 4.9.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitaq Enzyme activation</td>
<td>95°C</td>
<td>10 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>92°C</td>
<td>15 sec</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60°C</td>
<td>1 min</td>
</tr>
</tbody>
</table>

Table 4.9 The thermal cycling conditions for PCR. Applied Biosystems 7900HT Fast Real-Time PCR System was used for PCR amplification.

Allelic Discrimination Plate Read and Analysis

After PCR amplification, an endpoint plate read was performed using an Applied Biosystems Real-Time PCR System. The Sequence Detection System (SDS) Software uses the fluorescence measurements made during the plate read to plot fluorescence values based on the signals from each well. Genotype calls for individual samples were made by plotting the normalized intensity of the reporter dyes in each sample well on a
cluster plot. A clustering algorithm in the data analysis software assigns individual sample data to a particular genotype cluster (Figure 4.6).

![Cluster plot](image)

Figure 4.6 Cluster plot of DNA samples generated from TaqMan assay for rs3783521 SNP. DNA samples were genotyped simultaneously on 384-well plate. Genotype calls for individual samples were made by plotting the normalized intensity of the reporter dyes in each sample well on a cluster plot. A clustering algorithm in the data analysis software assigns individual sample data to a particular genotype cluster. Data points Blue= Allele 1/1 homozygous, Green= Allele 1/2 heterozygous, Red= Allele 2/2 homozygous. Circled area shows failed samples.

### 4.3 Statistical analysis

Two-group comparisons for demographic values were assessed with Mann–Whitney U-tests and \( P<0.05 \) was considered to be statistically significant. The association of allele and genotype frequencies in cases and controls was analysed by the chi-square test. The genotype frequencies in the control population were in Hardy-Weinberg equilibrium. The Hardy-Weinberg principle dictates that the genotype frequencies in the population are determined solely by the allele frequencies in the population (Balding, 2006). The computation is straightforward: if \( p \) and \( q \) are the allele frequencies in the population, where \( p+q=1 \), then the genotype frequencies will be \( p^2 \) (for homozygous of type one), \( 2pq \) (for heterozygous), \( q^2 \) (for homozygotes of the other type). In initial analysis,
genotype and allele frequencies between cases and controls were compared without categorizing subjects by ethnicity or smoking. In a separate analysis, genotype and allele frequencies were compared between smoker and non-smoker cases and controls. Furthermore, genotype and allele frequencies were compared between Caucasian cases and controls. Since Afro-Caribbeans and Asians were only in small numbers, no further statistical analysis was done on these groups.

The population genetics program Haploview was utilized to estimate haplotype frequencies based on the EM algorithm (Barrett et al., 2005). Pair-wise linkage disequilibrium (LD) between SNPs was evaluated using two statistical measures, \(D'\) and \(r^2\) which are respectively useful for modelling recombination rates and association power. Haplotype frequencies of three SNPs were derived from genotype data using the Phase and Harlequin software programmes (Stephens and Donnelly, 2003).

4.4 RESULTS

4.4.1 Identification of genetic polymorphisms in the IL-1A gene

DNA samples were amplified by PCR using primers corresponding to the promoter and the region including intron and exon. The PCR product generated a 2,100bp or 500bp fragment which was run on ethidium-bromide stained agarose gel and viewed under ultraviolet (UV) illumination as illustrated in Figure 4.7 and Figure 4.8, respectively. One sample (lane 4) failed to amplify on this run and was repeated on the next run.

![Figure 4.7](image)

**Figure 4.7** Ethidium-bromide stained agarose gel showing results of PCR amplification of DNA samples. A 2,100bp fragment was generated by amplification with DNA polymerase. The DNA was analyzed on 1% agarose gel containing ethidium bromide. Lanes: lane 1, New England Biolabs’s 1kb DNA Ladder; lanes 2-8, amplified genomic DNA for 7 patients.

124
Figure 4.8 Ethidium-bromide stained agarose gel showing results of PCR amplification of region including both Exon 1 and Intron 1. A 500 bp fragment was generated by amplification with DNA polymerase (arrow indicates expected size of PCR product). The DNA was analyzed on 1% agarose gel containing ethidium bromide. Lanes: lane 9 indicates 100 bp Gene ruler DNA Ladder (Fermentas, UK), lanes 1-8, amplified genomic DNA for seven patients.

Screening of the first intron and the first exon of the IL1A gene in 15 patients revealed no genetic variation related to aggressive periodontitis. Three polymorphic PCR fragments SNP I C>T (rs1800587), SNP II C>T (rs3783521) and SNP III C>T (rs1800794) were detected in the IL-1A putative promoter region of Caucasian patients with GAP. Three SNPs already had a database SNP ID number (http://www.ncbi.nlm.nih.gov/entrez).

The base substitutions in all PCR fragments were confirmed by direct sequencing by both the forward and reverse strand. Figures 4.9-4.10 show DNA sequence outputs (electropherograms) for two patients. The single blue peaks show the sample does not contain the SNP; they are in the homozygous forms CC. The double peaks (red and blue) show the sample contains the SNP C>T in heterozygous form.

The genotype data and allele frequencies for all SNPs are shown in Table 4.10. Minor allele (Allele 2 or T) frequencies for SNP I, SNP II, SNP III were 36.25%, 32.5% and 33.75%, respectively. None of the patients carried previously reported SNP IV C>T (rs3783571) and SNP V G>A (rs3783570). All patients had C or G at these positions, respectively.
Chapter 4: Aggressive Periodontitis and IL-1A Polymorphisms

Figure 4.9 Electropherograms showing different genotypes (Patient 1). Electropherograms were produced by fluorescence-based sequencing using an ABI 3700 showing the genomic DNA from an individual. These electropherograms show dye-specific (base-specific) intensity profiles. The single blue peaks in the left (A) and middle traces (B) show the sample does not contain the SNP; they are in the homozygous forms CC. The double peaks in the right trace with N letter (C) show the sample contains the SNP C>T in heterozygous form. The change was also confirmed by sequencing the reverse strand.

Figure 4.10 Electropherogram showing different genotypes (Patient 2). Electropherograms were produced by fluorescence-based sequencing using an ABI 3700 showing the genomic DNA from an individual. These electropherograms show dye-specific (base-specific) intensity profiles. The double peak (arrow) in the left trace (A) and middle trace with letter N (B) shows the sample contains the SNP C>T in heterozygous form. Single blue peak in the right trace (C) shows the sample does not contain the SNP; it is in the homozygous form CC. The change was also confirmed by sequencing the reverse strand.

Table 4.10 Genotype and allele frequencies of the identified IL-1A SNPs in Caucasian patients with GAP. The base substitutions in all PCR fragments were confirmed by direct sequencing.
4.4.2 The LD analysis and haplotype profiles of the IL-1A gene promoter

Using the haploview program, it was possible to examine measures of LD (Figure 4.11). Pair-wise LD between SNPs was evaluated using two statistical measures, \((D')\) and \(r^2\), which respectively help modelling recombination rates and association power (Table 4.11). Using an \(r^2\) value of over 0.3 as a threshold to represent useful LD for association studies (Pritchard and Przeworski, 2001), only SNP I (rs1800587) & SNP III (rs1800794) gave \(r^2 = 0.74\). The similar LD linkage of the IL-1A gene closely resembled those seen in the Caucasian data from the International HapMap project (http://www.hapmap.org/cgi-perl/gbrowse).

<table>
<thead>
<tr>
<th>Measures of LD</th>
<th>Haploview Output</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3783521</td>
<td>0.74</td>
</tr>
<tr>
<td>rs1800794</td>
<td>1.76</td>
</tr>
<tr>
<td>rs3783521</td>
<td>0.765</td>
</tr>
<tr>
<td>rs1800587</td>
<td>2.28</td>
</tr>
<tr>
<td>rs1800794</td>
<td>0.877</td>
</tr>
<tr>
<td>rs1800587</td>
<td>12.29</td>
</tr>
</tbody>
</table>

Table 4.11 Measures of linkage disequilibrium (LD) for three studied SNPs in the IL-1A gene promoter region. Pairwise LD in the IL-1A gene was evaluated by \(D'\)and \(r^2\) in GAP.

Figure 4.11 LD plot showing linkage disequilibrium between studied SNPs in the IL-1A gene promoter. The haploview program was used. The SNPs were numerated as follows rs3783521 = 1 rs1800587 = 2 rs1800794 = 3

Haplotype frequencies of three SNPs were derived from genotype data using the Phase and Harlequin software programmes (Stephens and Donnelly, 2003). A summary of haplotype probabilities is presented in Table 4.12. Most common haplotypes were \((C\ T\ T)\), \((C\ C\ C)\) and \((T\ C\ C)\) occurring at \(> 25\%\) frequency in the studied population.
Chapter 4: Aggressive Periodontitis and IL-1A Polymorphisms

<table>
<thead>
<tr>
<th>haplotype</th>
<th>Harlequin Output frequency</th>
<th>Phase Output frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTT</td>
<td>0.29804</td>
<td>0.280811</td>
</tr>
<tr>
<td>CCC</td>
<td>0.29743</td>
<td>0.343748</td>
</tr>
<tr>
<td>TCC</td>
<td>0.28653</td>
<td>0.277871</td>
</tr>
<tr>
<td>CCT</td>
<td>0.01852</td>
<td>0.030921</td>
</tr>
<tr>
<td>TTT</td>
<td>0.01473</td>
<td>0.029542</td>
</tr>
<tr>
<td>CTC</td>
<td>0.01314</td>
<td>0.016679</td>
</tr>
<tr>
<td>TTC</td>
<td>0.01308</td>
<td>0.013137</td>
</tr>
</tbody>
</table>

Table 4.12 Frequency of IL-1A promoter region haplotypes. Haplotypes were estimated from three SNPs using both Harlequin and Phase programmes. Seven different haplotypes occurred in the studied population. Of the 7 possible haplotypes, three represented greater than 25% of haplotypes in the studied population.

4.4.3 TaqMan Analysis

Since DNA concentration is a critical variable in PCR reactions, this was optimized using patient samples (1-2), as well as control DNA (human genomic DNA, Promega, UK). DNA amount used was diluted to 0.625, 1.25, 2.5, 5, 10, 20 ng/μl. 2 μl of DNA was added to each well and dried overnight and then a 5μl reaction ran using primers for SNP III (rs1800794) and 60 cycles to make detection as sensitive as possible.

Results are shown in Figure 4.12. Graphs represent the cycle threshold plotted against DNA concentration for each sample. Amplification of sample1 appeared to be inhibited at 20 ng/μl, reaching the cycle threshold of 60. Sample 2 gave amplification for all concentrations used. As the concentration of DNA independently was also assayed by nano-drop methodology it is possible to be confident of the amount of the concentration used. The most likely explanation for differences is the presence of different levels of salt in the samples. Since all of the samples tested had a CT value around 18-20 at 1ng per well, further analysis was carried out using DNA at 1ng per well.
Figure 4.12 Real-time PCR cycle threshold plotted against DNA concentration (ng/μl). (A) Control human DNA, Promega, UK, (B-C) Patient DNA samples. DNA amount used was diluted to 0.625, 1.25, 2.5, 5 ng/μl. 2 μl of DNA was added in each well and dried overnight and then ran a 5 μl reaction using primers for SNP III (rs1800794) and 60 cycles to make detection as sensitive as possible.
4.4.5 Study Groups and Genotype Distributions

For each assay, 10 samples were randomly selected and included as replicates for each genotype tested. All replicates gave the same genotype. Relatively few samples between 3 and 8 failed to react for each SNP (96.1–98.5% completion rate).

Patient demographics

The study included a total of 267 subjects, 150 cases and 117 controls. The GAP group consisted of 88 females and 61 males between the ages of 16 and 42 years (mean 33.77±5.36 of years). The healthy group consisted of 65 females and 48 males ranging in age from 18 to 53 years with a mean age of 33.76 ± 7.6 years. No significant differences were detected between cases and controls groups regarding the mean age (P>0.05). The frequency of female and males were similar between cases (F:M, 55.6%:41%) and controls (F:M, 58.7%:40.7%). GAP group consisted of 73% of Caucasian, 15% of Afro-Caribbean and 12% of Asian. Control group included 85% of Caucasian, 12% of Asian and 3% of Afro-Caribbean. Smoking status was divided in to three categories (none: smoker: previous smoker). The frequency of smokers was higher in cases (43% P<0.05) than in controls (16%). The frequency of previous smokers in cases and controls was 6%, 3%, respectively.

4.4.6 Genotype Distributions and Allele Frequencies

Only three SNPs in the 2.1 kb promoter of the IL-1A gene were polymorphic in the Western European population (Northern Ireland). In this section, these three SNPs including SNP I, II and III were analysed in a mixed population including Caucasians, Asians and Afro-Caribbeans. Further analyses were done if there was a significant difference between patients and unrelated healthy individuals, in terms of allele frequencies or genotype frequencies.

The frequencies of the genotypes and alleles in GAP patients and controls are presented in Tables 4.13. Allele 2 (T) frequencies for SNP I, SNP II, SNP III were 34.2%, 26.7%, 34.2% in cases, 29.8%, 29.6%, and 30.6% in controls, respectively. No statistically significant differences were found between patients and controls for any of the genotype or allele frequencies investigated (SNPI, SNPII, SNP III).
(Chi-squared (for genotypes) = 0.22, 0.23, 0.37, respectively. Chi-squared (for allele frequencies) = 0.64, 0.56, 0.36, respectively).

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Genotypes</th>
<th>Genotypes frequencies / Allele frequencies (2N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1800587,SNP1</td>
<td>CT</td>
<td>Genotype N (%)</td>
</tr>
<tr>
<td></td>
<td>Allele 1 (C)</td>
<td>44 (38.5) / 160 (70.2)</td>
</tr>
<tr>
<td></td>
<td>Allele 2 (T)</td>
<td>12 (10.5) / 68 (29.8)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>58 (50.8) / 65 (43.92)</td>
</tr>
<tr>
<td>rs3783521,SNP2</td>
<td>CT</td>
<td>Genotype N (%)</td>
</tr>
<tr>
<td></td>
<td>Allele 1 (C)</td>
<td>50 (43.4) / 162 (70.4)</td>
</tr>
<tr>
<td></td>
<td>Allele 2 (T)</td>
<td>9 (7.8) / 68 (29.6)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>56 (48.6) / 76 (51.7)</td>
</tr>
<tr>
<td>rs1800794,SNP3</td>
<td>CT</td>
<td>Genotype N (%)</td>
</tr>
<tr>
<td></td>
<td>Allele 1 (C)</td>
<td>53 (45.6) / 161 (69.3)</td>
</tr>
<tr>
<td></td>
<td>Allele 2 (T)</td>
<td>9 (7.75) / 71 (30.6)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>54 (46.65) / 75 (50.3)</td>
</tr>
</tbody>
</table>

Chi-squared analysis = 0.22 / 0.64 (Genotype / allele frequency) (aggressive vs. control)

Table 4.13 Genotype and allele frequencies for the 3 SNPs examined in patients with generalized aggressive periodontitis (GAP, n=150) and healthy controls (n=117). There were no significant differences (P > 0.05) in allele frequencies between the unstratified cases and the controls for any of the 3 SNPs. For significance at the 0.05 level, chi-square should be greater than or equal to 3.84.

4.4.7 Genotype Distributions and Allele Frequencies and ethnic background

The three SNPs were examined in three different populations, namely Caucasians, Afro-Caribbeans and Asians. Allele frequency distribution differed among the different populations (Table 4.14). Only two of controls were Afro-Caribbeans and carried allele 1 (C) for all SNPs studied. SNPs II and III may be a population specific SNP since the SNP II and III T allele accounts for 4% of Afro-Caribbeans allele frequencies in the diseased group. SNP frequencies were not significantly different between control and cases for all populations. Minor allele frequencies for Caucasians were 30%, 28%, 31% in controls and 28%, 31%, and 30% in cases, respectively (Table 4.14). There was no significant difference in the prevalence of the carriage of rare alleles (allele 2) in the three studied SNPs observed between a group of generalized aggressive periodontitis patients (Caucasians) and a control group (Table 4.14).
### Table 4.14 Allele frequencies for the 3 SNPs in different populations. There were no significant differences ($P > 0.05$) in allele frequencies between the stratified cases (GAP) and the controls (according to ethnic background) for any of the 3 SNPs. For significance at the 0.05 level, chi-square should be greater than or equal to 3.84.

#### 4.4.8 Genotype Distributions and Allele Frequencies and smoking status

Table 4.15 shows the genotype frequencies in smokers and non-smokers. The frequency of smokers was 65% in cases, but only 19% controls were smokers. No statistically significant differences were found between smoker GAP and non-smoker GAP for any of the genotype (CC versus CT/TT) investigated (SNP I, SNP II, and SNP III). Chi-squared for genotypes $=1.02$, 0.39, 0.23, respectively. No statistically significant differences were found between smoker controls and non-smoker controls for any of the genotype (CC versus CT/TT) investigated (SNP I, SNP II, and SNP III). Chi-squared for genotypes $=3.1$, 3.7, 1.2, respectively.
Table 4.15 Genotype and allele frequencies for the 3 SNPs according to smoking status in patients with generalized aggressive periodontitis (GAP, n=150) and healthy controls (n=117). There were no significant differences (P >0.05) in allele frequencies between the stratified cases and the controls (according to smoking status) for any of the 3 SNPs. For significance at the 0.05 level, chi-square should be greater than or equal to 3.84.

4.5 DISCUSSION

This study screened the upstream region of the IL-1A gene including first intron, first exon and promoter region (2.1 kb of the genomic sequence upstream of the transcriptional start site). Direct sequencing of exon 1 and intron 1 of the IL1A gene from patients with aggressive periodontitis revealed no genetic variation. Previous studies of IL-1A transcription suggested that inclusion of the first intron sequence in reporter constructs did not affect transcriptional activity (Huang et al., 1999). Therefore, further investigations were focused on the promoter region upstream of the IL-1A gene. Three previously described polymorphisms were observed in Caucasians with generalized aggressive periodontitis: a) SNP I C->T substitution, b) SNP II C->T substitution and c) SNP III C->T substitution (http://www.ncbi.nlm.nih.gov/entrez). No additional unreported polymorphisms were detected. No individuals were identified carrying the previously reported SNP IV C->T or SNP V G->A polymorphisms (http://www.ncbi.nlm.nih.gov/entrez). All patients had C or G at these positions, respectively. Interestingly, the SNP database showed a very low frequency of these polymorphisms, with 2.7±1.3% only being carried in African/Sub-Saharan Africans. It is
likely that these polymorphisms are restricted to certain ethnic groups. Therefore, no further studies were conducted in relation to the SNP IV and SNP V polymorphisms.

In the present study, 40 West European subjects with generalized aggressive periodontitis were screened for three polymorphisms and 55% of them carried allele 2 (T) either as a heterozygote or homozygous for SNP II. This was a similar frequency to that in the public database as shown in Table 4.3. The frequency of heterozygotes varied between different ethnic populations the lowest being in Sub-Saharan Africans (1%). A high proportion of the patients (65%) carried allele 2 (T), either as heterozygotes or homozygotes for SNP III. This frequency was higher than in the public database where an average frequency of 24 ± 25% for heterozygotes is reported. The frequency of heterozygotes in the database varies between different ethnic populations, the highest in European (48-57%) and absent within Sub-Saharan Africans. However, in all cases periodontal status was unknown. There is no public data available for either the SNP II or SNP III polymorphisms relating to periodontal disease. To our knowledge, this is first identification of the SNP II or SNP III polymorphisms in patients with generalized aggressive periodontitis.

Of the three identified SNPs, only SNP I (referred to -889) has been previously associated with periodontal diseases (Shirodaria et al., 2000) as a part of genetic susceptibility test for periodontal disease (Kornman et al., 1997a). A high proportion of Caucasian patients (65%) carried allele 2 (T), either heterozygote or homozygous status for the SNP I. However, according to published reports and the SNP database, carriage rates of SNP I alleles differ across populations. The frequency of carriage of allele 2 (T) varies with the highest being in Caucasians (35%) and lowest in Chinese and Japanese (6%, 9%, respectively).

The association of the SNP II and the SNP III polymorphisms with periodontal disease has not been studied previously. No studies have addressed the relationship of these SNPs to the risk of developing aggressive periodontitis. These polymorphisms has been reported in the SNP database (http://www.ncbi.nlm.nih.gov/entrez). However, in all cases periodontal status was unknown. In the present study, the SNP II genotype frequency (number of individuals with either CT or TT genotypes) for the case and the
control population was 48.2%, 51.2%, respectively. This was a similar frequency to a public database, where SNP genotyping data is available from European, African American, Asian and Sub-Saharan African populations with an average heterozygote frequency of 44±15% (http://www.ncbi.nlm.nih.gov/entrez). However, there was no statistical difference regardless of smoking or ethnic background. In the previous chapter, when only Western European subjects with generalized aggressive periodontitis were screened, this heterozygote frequency was 55%. The SNP III allele 2 carriage rate (number of individuals with either CT or TT genotypes) for the case and the control population was 49.7%, 53.4%, respectively. These frequencies are higher than those reported in the public database where allele frequency is available from European, African American, Asian, and Sub-Saharan African populations with average frequency of 24 ± 25% at heterozygote status. However, there was no significant association between SNP III genotypes and aggressive periodontitis.

The SNP I genotyping frequency (number of individuals with either CT or TT genotypes) for the control population was 49%. This was somewhat different than other reports where carriage rates vary considerably; 57.1% in European Caucasians (Hodge et al., 2001), 41.5% in Brazilians (Moreira et al., 2007), of 45% in Caucasians (Rogers et al., 2002), 32.5% in Italians (Scapoli et al., 2005). This may be due to variation between different populations. Indeed, when the data was stratified based on ethnic background, allele 2 was absent in Afro-Caribbeans while the frequency of the same allele in the control Caucasians was 31% and only 25% in Asians.

The results presented here are in accordance with other reported studies that have observed no significant differences in the frequencies of the SNP I (-889) alleles or genotypes between a population of individuals with aggressive periodontitis and controls in Caucasians, African-Americans, Thai ethnic group, Central Americans, Brazilians (Anusaksathien et al., 2003; Brett et al., 2005; Gonzales et al., 2003; Hodge et al., 2001; Moreira et al., 2007; Rogers et al., 2002; Walker et al., 2000). Since there is strong linkage disequilibrium between IL-1A -889 SNP and IL-1A+4845 SNP in exon 5 (G to C transition), some of the studies have chosen to test the latter (Gore et al., 1998; McGuire and Nunn, 1999). Tai et al., (2002) examined the IL-1A+4845 SNP in a Japanese population with generalized aggressive periodontitis and found no significant difference
between patients and controls, although there was a significant association with the IL-1RN intron 2 variable numbers of tandem repeat genotype (Tai et al., 2002). However, there was no similar association with IL-1RN intron 2 variable numbers of tandem repeat genotype in a UK population (Parkhill et al., 2000). In addition, Liu et al., (2004) investigated the IL-1A+4845 SNP in a Chinese population with generalized aggressive periodontitis and found significant differences between patients and controls (Li et al., 2004a). The contradictory results found in several studies may also be explained by differences in the diagnostic criteria used to define populations, the low number of subjects studied, and the characteristic of the controls such as age, unknown periodontal status.

Several studies have used control subjects of unknown periodontal status (Brett et al., 2005; Walker et al., 2000) and therefore likely to fail to find associations. The use of subjects of unknown periodontal status as controls involves the possibility of chance deviations, especially if there is no data available of the prevalence of studied polymorphisms. To avoid these problems, the control subjects included in the present study were periodontally healthy, ethnically mixed population with mean age of 33.7. In the study presented here the control subjects were matched for age. Control subjects not only belonged to dental hospital staff often used as control populations, since it is also possible that the high level of oral hygiene of the control population used in the previous studies may have masked any genetic predisposition to periodontitis. A relatively small sample size is one of the major reasons for the lack of reproducibility of case–control association studies and is a key limiting factor (Kinane et al., 2005). Current evidence from complex diseases (with large heritable components) indicates that the predicted odds ratios for many genes may be 1.5 or less (Cardon and Bell, 2001) and to identify such associations, as a general rule at least 100 cases and controls should be genetically characterized. In the present study, 150 cases and 117 controls were included and the sample size was adequate to be reasonably confident of concluding that there was no association with the IL-1A promoter polymorphisms and generalized aggressive periodontitis.

It has been widely recognized that the two most important environmental risk factors for periodontal disease are smoking and the presence of specific bacteria (Barbour et al.,
When smoking status was included in this thesis analysis as a covariate there was still no significant difference between the patient and control groups. Unfortunately, the control subjects were not well matched for smoking status. The low numbers of smokers (65% versus 19%) in the control group complicates interpretation of the data. In particular, smoking may affect both disease prevalence and severity. Therefore, due to the uneven distribution of smokers in present case and control populations, it may be reasonably assumed that part of the difference in the clinical condition between generalized aggressive periodontitis patients and periodontally healthy controls might be attributed to the detrimental impact of smoking on periodontal tissues. It should also be considered that a gene-environmental interaction between smoking and the IL-1 polymorphisms has been recently reported (Meisel et al., 2004; Meisel et al., 2002). Contrary to previous reports, in agreement with the data presented here smoking status was not found to influence the results (Hodge et al., 2001). Further studies are, therefore, needed to determine whether and to what extent differences in IL-1A polymorphisms may affect the onset and severity of the periodontal lesions in generalized aggressive patients with different smoking exposure.

Given the complex biology of IL-1 regulation and the extensive polymorphisms in the IL-1 gene cluster, it is very likely that if IL-1 genes influence disease, then a combination of specific alleles will be important rather than individual alleles. In the present study, between tested SNPs, only SNP III & SNP I gave strong LD with $r^2 = 0.74$. A correlation between physical distance and the degree of LD in the IL-1 cluster has been described (El-Omar et al., 2000). Since these two SNPs are ~300bp away from each other, it is likely that they will be in a strong LD. In addition, the possibility of other polymorphisms that linked to the studied three polymorphisms may not be excluded. There is linkage disequilibrium across the IL-1 cluster (Cox et al., 1998; Danis et al., 1995; Diehl et al., 1999; Scapoli et al., 2005), and it is likely that combinations of specific alleles are more important rather than individual alleles (Chen et al., 2006). Therefore further studies will be required to make dense SNP typing within the IL-1 gene cluster to fully determine an extended informative haplotype structure and its possible associations with aggressive periodontitis.
In contrast to aggressive periodontitis, several reports on chronic periodontitis indicate that the IL-1A genotype is significantly associated with this form of periodontal disease (Laine et al., 2001; McDevitt et al., 2000; Papapanou et al., 2001; Shirodaria et al., 2000). However, other studies have failed to find an association between the IL-1A genotype and chronic periodontitis (Armitage et al., 2000; Lopez et al., 2005). In an interesting study, Socransky and co-workers have reported that IL-1 (allele 2) genotype appears to be associated with the levels of specific bacterial complexes with an increase in those containing periodontal pathogens (Socransky et al., 2000). Another study reported an interaction between IL-1 genotype, *P. gingivalis*, smoking and age and stated that IL-1 genotype is a contributory but not essential risk factor for periodontal disease progression (Cullinan et al., 2001). Previously published reports, as well as the data presented here suggest that the nature of the underlying genetic susceptibilities may differ between aggressive and chronic periodontal disease.

The main findings of the present study can be summarized as follows: a) no significant difference in the prevalence of the carriage of either allele 1 or allele 2 in the three studied SNPs was observed between an ethnically mixed group of generalized aggressive periodontitis patients and a control group of periodontally healthy subjects; (b) No statistical difference was observed when comparing SNPs between the control and aggressive periodontitis groups, when only a Caucasian population was included in the analysis (c) When smoking status was included in the analysis as a covariate, the distribution of genotypes for three SNPs in smokers and non-smokers showed no statistical significance either in controls or in cases. d) Using the haploview program, it was possible to examine measures of linkage disequilibrium (LD) and haplotype frequencies for those three SNPs. The analysis showed that SNP III & SNP I had the strongest linkage disequilibrium ($r^2 =0.74$), whereas SNP II & SNP III has the weakest LD. Nevertheless, these results in this chapter do not exclude a role for the identified SNPs in periodontal disease. Periodontal disease is a complex disease in which many interacting gene variants will act synergistically with confounding environmental factors such as smoking or bacterial flora could interact with the genotype. Therefore further studies will be required to make wide SNP typing within the IL-1 gene cluster to fully determine an extended informative haplotype structure and its possible associations with aggressive periodontitis.

138
CHAPTER 5

Functional Analysis of IL-1A SNPs
5.1 INTRODUCTION

Whilst there is evidence for transcriptional regulation of IL-1α gene expression (Alheim et al., 1996; Bailly et al., 1996a; Kawaguchi et al., 2003; Mori and Prager, 1996), polymorphisms in the promoter region of the IL-1A gene may be important for IL-1α gene expression and protein production. There are four reports describing the functional importance of IL-1A gene polymorphisms. There is evidence that the C/T single base variation in the IL1A promoter (SNP I or previously called -889) was associated with fourfold increase of GCF IL-1α protein levels in chronic periodontitis patients (Shirodaria et al., 2000). Furthermore, periodontitis patients carrying the IL-1A-889 T allele has been associated with significantly increased serum interleukin-6 or C-reactive protein levels (D'Aiuto et al., 2004). There is also evidence that the TT genotype of the -889 polymorphism significantly increased plasma levels of IL-1β (Hulkkonen et al., 2000). However, it is not clear whether these promoter polymorphisms are of functional relevance in the regulation of IL-1α transcription. A single in vitro study has tested the effect of SNP I (-889) C->T substitution on transcriptional activity (Dominici et al., 2002) and reported that the SNP IT allele was associated with a 1.8-fold increase at basal transcription rate compared with a construct containing the allele C in PC1J cells.

There are a number of possible genetic associations between polymorphisms in the IL1A gene and periodontal disease; however there is as yet no consensus on their functional importance. Therefore, the objective of the present study was to investigate if different bacteria interact with specific IL-1A promoter polymorphisms. Reporter gene assays were used to assess the relative importance of polymorphic variation in the proximal promoter region on IL-1A gene expression. Since more than 55% of patients carried studied SNPs, it is possible that presence of those polymorphisms may lead to altered IL-1α expression levels and so contribute to aberrant or inappropriate immune function. Therefore, we investigated the effects of these alleles in a reporter assay performed in LPS or periodontopathogen stimulated cell lines. Further examination of the SNPs was performed using the web-based transcription factor binding site identification program TFSEARCH. Analysis for transcriptional binding sites revealed that these SNPs were located in putative regulatory element sequences. Therefore they were chosen for replication in reporter constructs.
5.2 MATERIAL & METHODS

5.2.1 Cloning strategy of the IL-1A promoter construct

The 5'-flanking region of the human IL-1A gene was amplified by using a standard PCR protocol. The obtained PCR fragment was initially cloned into the plasmid vector pCR2.1-TOPO (TOPO Cloning Kit; Invitrogen). The amplified DNA fragments were cloned in a KpnI- and BglII-digested pGL3 basic plasmid vector (Promega) using Quick stick DNA ligase (Bioline, UK) to generate pGL3-IL-1A reporter constructs. The polymorphisms of interest were introduced into pGL3-IL-1A reporter construct by oligonucleotide-directed PCR mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene, UK). Nucleotide sequences of the cloned DNA fragments were confirmed in each case by sequencing.

5.2.2 Construction of IL-1 A Promoter/Luciferase Reporter Gene Plasmids

The IL-1A promoter sequence was amplified by PCR using Bio-X-Act Polymerase (Bioline, London, UK). Commercially available genomic DNA (Human genomic DNA, Promega) with the homozygous genotype (C/C) was used as the template. To facilitate directional cloning of the PCR fragment into the pGL3-basic plasmid, a KpnI site was introduced to the 3' end of the forward primer, and a BglII site to the 5' end of the reverse primer (Table 5.1).

<table>
<thead>
<tr>
<th>5'-Primer (forward)</th>
<th>3'-Primer (reverse)</th>
<th>Annealing</th>
</tr>
</thead>
<tbody>
<tr>
<td>TACGTTCACGTCCCTTCC</td>
<td>ACCGCAATGAAATGACTCC</td>
<td>60 °C</td>
</tr>
<tr>
<td>GAAGATCTTTCTTAAGCTGAGTCAGTCTTC</td>
<td>GGGGTACCCCCAAAAACATGCAAGGACCC</td>
<td>58°C</td>
</tr>
</tbody>
</table>

Table 5.1 The 5'→ 3' sequences of the sense (S) and antisense (AS) primer pairs are listed. Sites for KpnI and BglII in the primers used for IL-1A cloning are underlined.

5.2.3 Cloning into TOPO vector

The PCR fragment was initially cloned into the plasmid vector pCR2.1-TOPO (TOPO Cloning Kit; Invitrogen) to make sub-cloning easier. After 5 minutes at room temperature, the ligation reaction was placed on ice prior to transforming competent cells. Detailed information about cloning protocol is provided in the Appendix V-1.
Chapter 5: Functional Analysis of IL-1A SNPs

The pCR TOPO vectors are ideal for general subcloning. These vectors are ready for direct ligation of unpurified PCR products. The key to TOPO Cloning is the enzyme DNA topoisomerase I, which functions as both a restriction enzyme and a ligase. Its biological role is to cleave and rejoin DNA during replication (Figure 5.1).

![Diagram](image)

**Figure 5.1** TOPO TA Cloning of PCR product (Invitrogen, UK)

### 5.2.4 Cloning into pGL3 Vector System

The promoterless pGL3-basic vector (Promega, Southampton, UK) containing a luciferase reporter gene was used for promoter constructs. The pGL3-Basic Vector lacks eukaryotic promoter and enhancer sequences, allowing maximum flexibility in cloning putative regulatory sequences. The pGL3 Vectors contain a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells (Gould and Subramani, 1988) (Figure 5.2).

The amplified fragments in TOPO were cleaved with *KpnI* and *BglII* (Appendix V-9). The products were run on 1% agarose (w/v) gel in 1X TAE, with 1 μg/ml ethidium bromide, in 1X TAE running buffer. Generuler1kb DNA Ladder (Fermentas, UK) was also run on the gel to determine the size of bands. Under a UV illuminator bands were cut out of the gel and transferred into sterile microfuge tubes and DNA bands were purified using a MinElute Gel Extraction Kit (Qiagene, Crawley, UK) according to manufacturer’s intructions (Appendix IV-4) and ligated into the multiple cloning site of pGL3 basic vector which was cut with the same two enzymes. Ligation was performed using Quick stick DNA ligase (3U/μl, Bioline, UK) following a standard protocol (Appendix V-5). Following ligation, the vector was transformed into JM109 (Promega)
competent cells. Plasmid DNA was purified from bacterial cells using Qiagen chemistry and procedures (Qiagen, UK) as detailed in Appendix V-8.

The plasmid DNA was sequenced using Big Dye Terminator chemistry (Applied Biosystems) in both directions using primers specific for the pGL3-basic luciferase expression vector (RV primer 3 and GL primer 2, Promega Corp, UK) (Table 5.2) to confirm that the haplotypes present in genomic DNA were faithfully represented. The chromatographs were recorded at a wavelength of 260nm. The sequencing results were compared with known human genomic DNA sequences using BLAST online search engine (http://www.ncbi.nlm.nih.gov/BLAST) to confirm amplification and sequencing of the correct region (Appendix V-16).

Figure 5.2 Schematic representation of generation of IL-1 A reporter construct. The PCR fragment was initially cloned into the plasmid vector pCR2.1-TOPO and the amplified fragments were cleaved with Kpn I and Bgl II ligated into the multiple cloning site of pGL3 basic vector which was cut with the same two enzymes. pGL3-Basic vector circle map adapted from Promega, UK.
5.2.5 Site-Directed Mutagenesis

The polymorphisms of interest were introduced into IL1A construct plasmid by oligonucleotide-directed PCR mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene, UK). This method is based on the capability of DNA polymerases (Pfu polymerase) to be used for the synthesis of entire plasmids, using a pair of completely complementary primers. The second key element of the procedure is the use of the restriction enzyme DpnI. This enzyme only cuts DNA that is methylated or hemi-methylated (G\textsuperscript{m6}ATC) by the dam methylase of E. coli. Thus, non-mutated parent DNA or mutants-wild-type heteroduplexes are inactivated by DpnI (Appendix V. 11).

Mutagenic primers were designed using web-based Primer Design Program available online at http://bioinformatics.org/primere. Primers should be between 25 and 45 bases in length, the desired mutation should be in the middle of the primer with ~10 –15 bases of correct sequence on both sides. The bases in red are the point mutation to be introduced (Table 5.3).

<table>
<thead>
<tr>
<th>5'-3' Primer (Forward)</th>
<th>Mutation and position</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTTGCCTAAAGAGGAA\textbf{TCAAGGTAAGCAGAAATG}</td>
<td>SNP II (RS3783521) C&gt;T</td>
</tr>
<tr>
<td>CAACTCACACAAGCT\textbf{GTTTTCCTCCCAGATCC}</td>
<td>SNP III (RS1800794) C&gt;T</td>
</tr>
<tr>
<td>GTAACCAGGCAACA\textbf{TATTGAAGGCTCATATG}</td>
<td>SNP I (RS1800587)9 C&gt;T</td>
</tr>
</tbody>
</table>

Table 5.3 The primers for Site-Directed Mutagenesis. *The red bold letters indicate the introduced mutation. Numbers in column two refer to mutation and ID number.

Briefly, two primers were prepared and simultaneously annealed and were extended using parental vector as template with Pfu polymerase using PCR as outlined in Table 5.4 and 5.5. 1 μl of DpnI-treated DNA was transformed into DH5α cells by a standard
transformation procedure (Appendix V.11.3). The bacteria (1/10) were plated onto LB plates with ampicillin. Plasmid DNA was isolated by miniprep and once mutagenesis was confirmed by sequencing, a large scale preparation was made. Mutated plasmid DNA was prepared with an Endo-free Maxiprep plasmid DNA isolation kit (Qiagen, UK).

<table>
<thead>
<tr>
<th>PCR Mix component</th>
<th>Volume per 50 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA, 200 ng/µL</td>
<td>1 µL</td>
</tr>
<tr>
<td>Polymerase buffer, 10x</td>
<td>5 µL</td>
</tr>
<tr>
<td>dNTPs, 12.5 mM</td>
<td>1 µL</td>
</tr>
<tr>
<td>Mutant sense primer (1.25 ng)</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Mutant antisense primer (1.25 ng)</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>37 µL</td>
</tr>
<tr>
<td>Tfu polymerase, 2.5 U/µl</td>
<td>1 µL</td>
</tr>
</tbody>
</table>

Table 5.4 Mutant Strand Synthesis Reaction

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>1</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Step 2</td>
<td>12</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55°C</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68°C</td>
<td>7 minutes</td>
</tr>
</tbody>
</table>

Table 5.5 RT-PCR cycling parameters for the QuickChange Site-Directed Mutagenesis

5.2.6 Transient Transfections and Reporter Gene Assays

The process of introducing nucleic acids into eukaryotic cells by nonviral methods is defined as “transfection”. Using various chemical, lipid or physical methods i.e. electroporation, this gene transfer technology is a powerful tool for studying gene function in the context of a cell. Chemicals like calcium phosphate (Loytner et al. 1982) and DEAE-dextran (Vaheri and Pagano, 1965; McCutchan and Pagano, 1968) or cationic lipid-based reagents (Felgner et al. 1987) coat the DNA, neutralizing or even creating an overall positive charge to the molecule. Physical methods like microinjection or
electroporation simply punch through the membrane and introduce the DNA directly into the cytoplasm.

Firefly luciferase is widely used as a reporter gene for studying gene regulation. It is a very sensitive genetic reporter due to the lack of any endogenous luciferase activity in mammalian cells or tissues and the functional enzyme is created immediately upon translation (de Wet et al., 1987). Firefly luciferase is a 62,000 dalton protein, catalyzes ATP-dependent D-luciferin oxidation by oxygen into oxyluciferin with emission of light centered on 560 nm.

![Diagram of reporter gene system](image)

Figure 5.3 Schematic representation of reporter systems (adapted from Promega, UK).

### 5.2.7 β-Galactosidase Assay

The pSV-β-Galactosidase Control Vector is a positive control vector for monitoring transfection efficiencies of mammalian cells (Hall et al., 1983). The SV40 early promoter and enhancer drive transcription of the lacZ gene, which encodes the β-Galactosidase enzyme. The pSV-β-Galactosidase Control Vector can be co-transfected with DNA of interest and it provides cell extracts that can be assayed for both luciferase and β-galactosidase activities. In this manner, we used the pSV-β-Galactosidase Vector (Promega, UK) as an internal control for transient expression assays.
5.2.8 Transient transfection into Monomac-6 cells

Transient transfection of cultured cell lines with reporter plasmids is commonly used to determine the transcriptional activity of promoter sequences. Gene transfection is known to be cell type-dependent. However, gene transfection in monocytes has proven difficult. A number of non-viral gene transfer methods have been used to improve monocyte transfection, including calcium-phosphate precipitation (Thompson et al., 1999), DEAE-dextran (Mack et al., 1998) and electroporation (Klan and Steinhilber, 2003) and nucleofection (Martinet et al., 2003). Although the reasons underlying the resistance of monocytic cells to DNA transfection is not known precisely, it has been proposed that much of the exogenous DNA enters the cell via endocytosis, resulting in degradation of the DNA by abundant lysosomal nucleases.

In this study, a range of gene transfection systems for Monomac-6 cells including a variety of liposomal agents (Fugene-Roche, Lipofectamine 2000, Invitrogen, UK), diethylaminoethyl (DEAE)-dextran (CellPhect Transfection Kit, Amersham Biosciences, UK) and nucleofection (Amaxa, UK) were used. Transfection efficiency of these systems was optimized and compared several methods for DNA-mediated cell transfection to determine which would be optimally applicable to Monomac-6 cells.

pEGFP-N3 vector (BD Biosciences Clontech, Oxford, UK) was used to express EGFP, the red-shifted, humanized Aequorea protein in the cell line as a transfection marker (Chalfie et al., 1994). pEGFP-N3 expresses green fluorescent protein under the control of a CMV promoter. The cell populations were photographed using a fluorescent microscope.

Monomac-6 cells were refractory to transfection by liposomal agents or DEAE-dextran. Despite many attempts transfection rates were below 3% and the construct was not able to drive detectable levels of luciferase. The Nucleofector technology, a new electroporation-based gene transfer technique yielded higher transfection efficiency (~20-30%). However, this method was associated with higher cell death (70-80%).

Monomac-6 cells were difficult to transfect. Thus, to substantiate this point, various cell lines from different origins including THP-1 monocytes, human primary fibroblasts,
HeLa and embryonic kidney cell line 293T were transfected with the IL-1 A-luciferase reporter plasmid or pEGFPN3 vector.

5.2.9 Transient transfection into THP-1 cells

A variety of established cell lines exist that exhibit monocyte-like characteristics including THP-1. Preliminary experiments (see in Chapter 2) showed that the more mature monocyteic cell line, Monomac-6 (Ziegler-Heitbrock et al., 1994) is more susceptible to bacterial stimulation than untreated THP-1 or U-937 cell lines (Schwende et al., 1996). However, the Monomac-6 cell line was difficult to transfect. Therefore, THP-1 cells were tested and transfected according to the published method (Warny et al., 2000).

Human monocytic THP-1 cells (American Type Culture Collection) were grown in RPMI-Glutamax supplemented with 10% FCS, in a humid atmosphere containing 5% CO₂. THP-1 cells were transiently transfected using the DEAE-dextran procedure. Briefly, 2 x 10⁷ THP-1 cells were suspended in 1 ml prewarmed Tris-buffered saline and incubated for 10 minutes at 37°C with 80 µg DEAE-dextran (Pharmacia). For transfection, THP-1 cells were transfected with 5 µg DNA of the luciferase IL-1A reporter plasmid and co-transfected with 2 µg of pSV-beta-galactosidase vector as internal control. Transfection was stopped by adding 25 ml Tris-buffered saline. After washing, cells were cultured for 48 hours before stimulation. After stimulation, for 6 hours with a range of periodontopathogens or LPS, THP-1 cells (2 x 10⁶ cells per stimulus) were washed in PBS. Cell lysis and luciferase assay were performed using the Luciferase Assay System from Promega Corp. following the instructions of the manufacturer (Appendix V.12). Luciferase activity was normalized for pSV-beta-galactosidase activity measured with β-galactosidase enzyme assay as detailed in Appendix V.13. All transfection experiments were performed in triplicate.

5.2.10 Transient Transfection into Human fibroblasts, HeLa and HEK 293T

Human primary skin fibroblasts were kindly provided by Dr. Baki Akgul, Cancer Research UK, Institute of Cell and Molecular Science, Queen Mary, University of London. Fibroblasts derived from the normal skin of the healthy controls and the human derived epithelial-like cell line HeLa (ATCC, CCL-2) were chosen as much of the
previous analysis of the IL1A gene promoter had been performed in these cells (McDowell et al., 2005). In addition, the human embryonic kidney cell line HEK 293T was included for comparison since it is known to be easy to transfect. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, UK) plus 10% fetal calf serum (FCS, Sigma, UK).

Plasmids were transfected into cultured cells by using the FuGene 6 transfection reagent (Roche) per the manufacturer's instructions. Before transfection, 2 x 10^5 cells in 2 ml of medium were plated in a six-well plate overnight. On the day of the experiment all transfections were performed on 6 well plates with sub-confluent (60%) cells by the non-liposomal formulation method. Cells were transfected with 1.5 µg of reporter plasmid and 0.5 µg pSV-beta-galactosidase internal control vector using FuGENE 6 reagent (Roche Molecular Biochemicals, Germany) at a 3:1 ratio in a polystyrene tube, incubated at room temperature for 15 min and 100µl of complex were added to cells. Control transfections were performed using the equivalent amount of pGL3 empty vector (Promega). Cells were harvested at 48 hours post-transfection. Cells to be assayed for luciferase activity were lysed in reporter lysis buffer and cellular debris was removed by centrifugation. Cell lysates were assayed using the Luciferase Reporter Assay System (Promega) on a luminometer. Luciferase activity was normalized to β-galactosidase to standardize transfection efficiency.

5.3 Statistical analysis

The nonparametric Kruskal-Wallis test and Dunnett's multiple comparisons test were used to compare luciferase levels under the various experimental conditions, using GraphPad software. A P value less than 0.05 was considered statistically significant.

5.4 RESULTS

5.4.1 Cloning of pGL3-IL-1A reporter construct

A fragment of the promoter region of the human IL-1A gene was amplified by PCR using human genomic DNA as template and the forward primer with Kpn I site and the reverse primer with Bgl II site (Figure 5.4A). The PCR fragment was initially cloned into the plasmid vector pCR2.1-TOPO (TOPO Cloning Kit; Invitrogen). Figure 5.4B shows an agarose gel picture for DNA of 15 colonies containing the IL-1A gene promoter.
Chapter 5: Functional Analysis of IL-1A SNPs

fragment. Five positive colonies (lanes 1-3-4-5-6) were selected for preparation of Minipreps and Maxipreps (Appendix V.8). They were subjected to endonuclease digestion with Kpn I and Bgl II. Following digestion, the DNA product was run on an agarose gel and the DNA band was excised as indicated by the red arrow (Figure 5.5A). Following digestion of the pGL3 basic luciferase with Kpn I and Bgl II and purification, these fragments were inserted into the pGL3 basic luciferase reporter as previously described in Materials and Methods (Figure 5.5B). To analyse recombinants obtained after cloning of the IL-1A gene promoter fragment into the pGL3 basic vector, the plasmid DNA was then digested with Kpn I and Bgl II to release the insert from the vector (Figure 5.6).

A) RT-PCR of IL-1A gene promoter B) Screening after TOPO cloning

![Image of RT-PCR and screening results]

**Figure 5.4** A) RT-PCR of a 2.1 kb promoter fragment of IL-1A gene. B) PCR products were subcloned into the TOPO cloning vector as detailed in Material & Methods. Invitrogen's 1kb DNA ladder was used to estimate size of PCR products.

A) TOPO Cloning B) pGL3 vector digestion analysis

![Image of TOPO cloning and pGL3 vector digestion analysis results]

**Figure 5.5** The genomic fragment cleavage by restriction enzymes A) The 2.1 kb genomic fragment was released by Kpn I and Bgl II digestion and separated from the vector backbone (2.7 kb). Since TOPO vector has also Bgl II cut site, 1.3 kb fragment (the lowest band) was released from the vector backbone. GeneRuler 1kb DNA Ladder (Fermentas, UK) B) Kpn I and Bgl II digestion of analysis of pGL3 vector (4.8kb). The DNA was analyzed on 1% agarose gel containing ethidium bromide. Lanes: Lane 1, Invitrogen's 1kb DNA Ladder; Lanes 3-5 are digested recombinant plasmid DNA.
Figure 5.6 Analysis of recombinants obtained after cloning of IL-1A promoter fragment into the pGL3 Basic Vector. A 2.1kb fragment was generated by amplification with DNA polymerase. 2 μl was then ligated into the pGL3 Basic Vector. The ligation reaction was transformed into JM109 Competent Cells. Plasmid DNA was isolated using the QIA Spin Miniprep Kit DNA (Qiagen, UK). To analyse the generated construct, the plasmid DNA was then digested with Kpn I and Bgl II to release the insert from the vector backbone (4.8 kb). The DNA was analyzed on 1% agarose gel containing ethidium bromide. Lanes: lane 1, Invitrogen’s 1kb DNA Ladder; lanes 2-9, digested recombinant plasmid DNA.

5.4.2 Identification of the mutated constructs

The polymorphisms of interest were introduced into the IL1A gene reporter construct plasmid by oligonucleotide-directed PCR mutagenesis as detailed in Materials & Methods. Direct sequencing of the mutated constructs showed that the bases at the SNP I, SNP II and SNP III were all T (Figure 5.7A). The other sequences were exactly the same as the IL-1A gene sequence registered in Genebank (AF536338) indicating successful construction (Figure 5.7B).

Figure 5.7 DNA sequence output showing mutated alleles. A) Sequence of the mutated Allele 2 (T) IL-1A constructs B) Sequence of the Allele 1 (C) IL-1A construct
5.4.3 Allele-Specific Transcriptional Activity in THP-1 cells

Since transfection efficiency in Monomac-6 cells was less than 3% and the construct was not able to drive luciferase activity, further studies were performed in THP-1 monocytes. Figure 5.8 shows images of THP-1 cells transfected with control pEGFPN3 vector which is CMV-driven green fluorescent protein. The enhanced green fluorescent protein (EGFP) was monitored by fluorescence microscope (blue light, 20 X objectives). Transfection efficiency was around 50%.

![Image of THP-1 cells transfected with control pEGFPN3 vector.](image)

**Figure 5.8** Representative image of THP-1 cells transfected with control pEGFP-N3 vector. pEGFP-N3 vector was used to express EGFP, the red-shifted, humanized Aequorea protein in the cell line as a transfection marker. The cell populations were photographed using a fluorescent microscope (X20 objective, blue light).

*Basal IL-1A promoter activity (non-induced)*

The rates of transcription of the wild-type construct where all SNPs were set to the more common allele (allele 1, C) were compared with those containing allele2 (T). There were statistically significant differences in mean basal values of luciferase activity among the four reporter constructs tested. The non-parametric test showed $F$-value of 4.4, $P < 0.05$, for overall variation among the basal levels of IL-1A CC and IL-1A TT construct luciferase activity. ANOVA with Dunnett's multiple comparisons test was then used to compare the results of each of the variant constructs with that of the CC. Only the SNP I (-889) showed statistically significant differences ($P < 0.05$) compared to IL-1A CC. Conversion of SNP II and SNP III from allele 1 (C) nucleotide to allele 2 (T) had no significant effect on transcription at the basal level ($p=0.7516$, $p=0.1155$, respectively) (Figure 5.9). Conversion of SNP I from allele 1 (C) nucleotide to allele 2 (T) resulted in a significant decrease ($34.01 \pm 15.23\%$ $p=0.0045$) in basal transcriptional activity.
Chapter 5: Functional Analysis of IL-1A SNPs

Figure 5.9  Allele-specific transcriptional activity in THP-1 cells. Luciferase activities of constructs harbouring 2.1 kb fragments with either the Allele 1(C) or the Allele 2 (T) site (three independent experiments in duplicate) were compared in transfection studies using THP-1 cells. Luciferase levels are expressed as a percentage of control (Allele 1(C)) after standardization for \(\beta\)-galactosidase activity. Bars indicate mean values with standard error of mean. The statistical significance of differences was determined by the non-parametric Kruskal-Wallis test and Dunnett’s multiple comparisons. A \(P\) value less than 0.05 was considered statistically significant.

**Bacteria-induced IL-1A promoter activity**

Since only SNP I allele 2 (T) had a significant effect on basal transcriptional activity, further investigations were done to test if bacterial stimulation could influence transcription of the construct carrying T allele of this SNP. All bacterial stimulants significantly increased (~42%) transcriptional activity compared to the unstimulated one \( (P<0.05)\). Nevertheless, there were no significant differences between *E.coli* LPS and tested periodontopathogens including *A. actinomycetemcomitans*, *C. rectus*, *P. gingivalis* and *P. intermedia*. SNP I allele 2 (T) showed significant decrease in transcriptional activity when compared with allele 1 (C) with or without bacterial stimulant (Figure 5.10). Table 5.6 summarizes mean data (% decrease) from three independent experiments.
Chapter 5: Functional Analysis of IL-1A SNPs

Figure 5.10 Allele-specific transcriptional activity in the presence of bacteria in THP-1 cells. *A. actinomyctematum (A.a), C. rectus (C.r), P. gingivalis (P.g) and P. intermedia (P.i) and E.coli LPS (E.C) were used as stimulant. Luciferase activities of the constructs harbouring 2.1 kb fragments with either the Allele 1(C) (IL-1WT) or the Allele 2 (T) (SNP I or rs1800587-889) site (one out of three independent experiments which was performed in triplicates Mean ± SD) were compared in transfection studies using THP-1 cells. Firefly luciferase activity was normalized to co-transfected β-gal activity. **P<0.05

Table 5.6 Allele-specific transcriptional activity in the presence or absence of bacteria in THP-1 cells. *A. actinomyctematum (A.a), C. rectus (C.r), P. gingivalis (P.g) and P. intermedia (P.i) and E.coli LPS (E.C) were used as stimulant. Reduction in luciferase activity of the IL-1A gene reporter construct carrying SNP I with either the allele 1(C) or the allele 2 (T) site was calculated from three independent experiments in triplicates. *P < 0.05.

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>NS</th>
<th>E.coli LPS</th>
<th>A.a</th>
<th>C.r</th>
<th>P.g</th>
<th>P.i</th>
</tr>
</thead>
<tbody>
<tr>
<td>% decrease in the activity</td>
<td>41.5±15.0*</td>
<td>60.4±21.3*</td>
<td>46.8±16.6*</td>
<td>41.09±14.77*</td>
<td>45.43±28.6*</td>
<td>51.02±12.1*</td>
</tr>
</tbody>
</table>

5.4.4 Allele-Specific Transcriptional Activity in Different Cell Lines

Transcriptional activity of the IL-1A promoter constructs was compared in different cell lines of different origin to investigate potential cell type-specific control of gene expression. The transient transfection studies were conducted in embryonic kidney cell line 293T, human primary skin fibroblasts and HeLa cell line to explore whether the studied polymorphisms influence the basal rate of transcription of the IL-1A gene.
The human embryonic kidney cell line HEK 293T

Transfection efficiency was ~ 80% in the human embryonic kidney cell line HEK 293T when transfected with control pEGFPN3 vector (Figure 5.11).

![Image](image_url)

**Figure 5.11** Representative image of human HEK 293T transfected with pEGFP-N3 vector. pEGFP-N3 vector was used to express EGFP, the red-shifted, humanized Aequorea protein in the cell line as a transfection marker. The cell populations were photographed using a fluorescent microscope (X40 objective, blue light).

The rates of transcription of the wild-type construct where all SNPs were set to the more common allele (allele 1, C) were compared with those containing allele 2 (T). As shown in Figure 5.12, conversion of SNP II and SNP III from allele 1 (C) nucleotide to allele 2 (T) had no significant effect on transcription at the basal level (P>0.05). Conversion of SNP I from allele 1 (C) nucleotide to allele 2 (T) resulted in a significant decrease of basal transcriptional activity (a 14% decrease, p=0.03). The pilot data indicate that the C>T substitution at the SNP I of the IL-1A gene promoter decreases the basal transcriptional rate of IL-1A gene in the human embryonic kidney cell line HEK 293T (Figure 5.12).
Figure 5.12 Allele-specific transcriptional activity in HEK 293T cells. Luciferase activities of constructs harbouring 2.1 kb fragments with either the Allele 1(C) or the Allele 2 (T) site (one experiment which was performed in triplicates) were compared in transfection studies using in the human embryonic kidney cell line HEK 293T. Firefly luciferase activity was normalized to co-transfected [beta]-galactosidase activity. Bars indicate mean values with standard deviations. The statistical significance of differences was determined by the non-parametric Kruskal-Wallis test and Dunnett’s multiple comparisons. A P value less than 0.05 was considered statistically significant. *p<0.05

HeLa Cell Line

The transient transfection studies were also conducted in the HeLa cells. The transfection efficiency was more than 70% when transfected with control pEGFPN3 vector (Figure 5.13).

Figure 5.13 Representative image of HeLa cells transfected with pEGFP-N3 vector. pEGFP-N3 vector was used to express EGFP, the red-shifted, humanized Aequorea protein in the cell line as a transfection marker. The cell populations were photographed using a fluorescent microscope (X40 objective, blue light).
Any of the studied SNPs did not show significant transcription differences at the basal level by allele ($P>0.05$) in HeLa cells (Figure 5.14). The pilot data suggest that the C>T substitution at the position of all identified SNPs in the IL-1A gene promoter did not change the basal rate of transcription of IL-1A gene in the HeLa cells.

![Figure 5.14](image) Allele-specific transcriptional activity in HeLa cells. Luciferase activities of constructs harbouring 2.1 kb fragments with either the Allele 1(C) or the Allele 2 (T) site (one experiment which was performed in triplicates) were compared in transfection studies using in HeLa cells. Firefly luciferase activity was normalized to co-transfected [beta]-galactosidase activity. Bars indicate mean values with standard deviations. The statistical significance of differences was determined by the non-parametric Kruskal-Wallis test and Dunnett's multiple comparisons. A P value less than 0.05 was considered statistically significant.

**Human skin fibroblasts**

The studies were also carried out in human primary skin fibroblasts. Transfection efficiency was ~ 40% when transfected with control pEGFPN3 vector (Figure 5.15).

Conversion of SNP II and SNP III from allele 1 (C) nucleotide to allele 2 (T) had no significant effect on transcription at the basal level ($P>0.05$) (Figure 5.16). Conversion of SNP I from allele 1 (C) nucleotide to allele 2 (T) resulted in a significant decrease of basal transcriptional activity (42% decrease, $p<0.05$) (Figure 5.16). The pilot data indicate that the C>T substitution for SNP I of the IL-1A gene promoter decreased the basal rate of transcription of IL-1A gene in human primary skin fibroblasts.
Figure 5.15 Representative image of human primary skin fibroblasts transfected with pEGFP-N3 vector. pEGFP-N3 vector was used to express EGFP, the red-shifted, humanized Aequorea protein in the cell line as a transfection marker. The cell populations were photographed using a fluorescent microscope (X20 objective, blue light).

Figure 5.16 Allele-specific transcriptional activity in human skin fibroblasts. Pilot results are presented from one experiment which was performed in triplicates (Mean ± SD). Firefly luciferase activity was normalized to co-transfected β-gal activity to control for transfection efficiency. The statistical significance of differences was determined by the non-parametric Kruskal-Wallis test and Dunnett's multiple comparisons. A P value less than 0.05 was considered statistically significant.

5.5 TRANSFAC analysis for transcriptional binding sites for identified SNPs

In the absence of experimental or epidemiologic evidence, the potential functional consequences of a SNP can be predicted using various bioinformatics tools to query large online databases and algorithms, namely "in silico" referring to modeling research conducted with computers only (Zhu et al., 2004).
Putative transcription factor binding sites were identified by the programme the TFSEARCH. The effect of different SNP alleles in the IL-1A promoter may alter affinity of the DNA for trans-acting factors. Therefore using the TFSEARCH site which uses a transcription factor binding site profile database in the TRANSFAC databases (http://www.cbrc.jp/research), it is possible to make an assessment in silico of possible changes to promoter binding sites (Wingender et al., 2001). For each SNP within an aligned region, two allelic versions of a 20-bp alignment slice centred the SNP were searched for putative transcriptional binding sites using the TRANSFAC databases. Transcriptional binding sites with a relative matrix score exceeding 70% were selected.

5.5.1 TRANSFAC analysis for transcriptional binding sites for SNP I

Analysis of the database indicated that the presence of T creates a consensus binding site for the transcription factors Nkx-2, GATA-1-2-3, Pbx-1, CDP CR, CdxA. The C allele abolishes most of the consensus binding sites with the exception of CDP CR, CdxA. In addition, the presence of T in position -899 abolishes the consensus binding site for v-Myb and c-Rel (Figure 5.17).

![Figure 5.17 TRANSFAC analysis for transcriptional binding sites for SNP I. Two allelic versions of a 20-bp alignment slice centred around the SNP were searched for putative transcriptional binding sites using the TRANSFAC databases. Transcriptional binding sites with a relative matrix score exceeding 70% were selected.](image_url)
5.5.2 TRANSFAC analysis for transcriptional binding sites for SNP II

Analysis of the database indicated that the presence of T creates a consensus binding site for the transcription factors Pbx-1, GATA-1-2, Nkx-2, Tst-1, CDP CR, HSF2. The C allele abolishes most of the consensus binding sites (Figure 5.18).

![Figure 5.18 TRANSFAC analysis for transcriptional binding sites for SNP II. Two allelic versions of a 20-bp alignment slice centered around the SNP II were searched for putative transcriptional binding sites using the TRANSFAC databases. Transcriptional binding sites with a relative matrix score exceeding 70% were selected.]

5.5.3 TRANSFAC analysis for transcriptional binding sites for SNP III

Analysis of the database indicated that the presence of T creates a consensus binding site for the transcription factors HNF-3b, c-Ets, Tst-1, v-Myb, E2F. In addition, the presence of T abolishes the consensus binding site for GATA-2 (Figure 5.19).
Figure 5.19 TRANSFAC analysis for transcriptional binding sites for SNP III. Two allelic versions of a 20-bp alignment slice centered around the SNP III were searched for putative transcriptional binding sites using the TRANSFAC databases. Transcriptional binding sites with a relative matrix score exceeding 70% were selected.

5.6 DISCUSSION

The objective of the present study was to test the hypothesis that the IL-1A gene contains physiologically relevant polymorphisms that influence IL-1A gene regulation. There is evidence for transcriptional regulation of IL-1A gene expression (Alheim et al., 1996; Bailly et al., 1996a; Kawaguchi et al., 2003; Mori and Prager, 1996). In order to investigate whether the IL-1A promoter SNPs were associated with functional regulation of IL-1A expression, firefly luciferase reporter constructs were made and studied in vitro.

Studies described in this thesis show that Monomac-6 cells are difficult to transfect. This is consistent with previous reports (Martinet et al., 2003). To confirm that this construct was not intrinsically problematic, various cell lines including THP-1 monocytes, human skin fibroblasts, HeLa cells and 293T embryonic kidney cell line were transfected with the IL-1A-luciferase reporter plasmids. High efficiency (>60%) transfection was possible in all these cell lines. The cell lines were chosen to represent a variety of human tissues.
types and represent a balance between choosing cell lines with different tissue origins and robustness of the assay conditions. The activity of promoters varied widely between cell lines. This in part is likely to represent quantitative differences in the transfection efficiencies of different cell lines. There were also qualitative differences in the responses of the cell lines to individual promoter haplotypes. Although one can question the specific choice of cell lines used, this observation supports the importance of using multiple cell lines in this analysis.

Using the other cell lines it was possible to compare the reporter constructs carrying different polymorphisms. Firstly, only SNP I allele 2 (previously reported as -889) showed functional activity compared with allele 1 (see Figure 5.10). These transfection studies provided evidence that the SNP I allele 2 (T) is associated with a decreased basal transcription rate compared with constructs harbouring the allele 1 (C). Secondly, TRANSFAC databases analysis indicated that the presence of T in position -899 abolishes the consensus binding site for v-Myb and c-Rel transcription factors. The previous studies show that c-Rel proteins act as transcription factors and share extensive homology with the p50 and p65 subunits of the NF-κB complex (Ghosh et al., 1990; Hansen et al., 1994). NF-κB regulates a wide variety of inflammatory and immunoregulatory genes, and together with other factors has been reported that it is a positive trans-acting factor in controlling IL-2 gene transcription (Tan et al., 1992). Transactivation of the IL-1α promoter in T cells by the HTLV-1 Tax protein has been described suggesting that NF-κB may be involved in the transcriptional regulation of IL-1α (Mori and Prager, 1996). The NF-kappa B/rel family of transcription factors has also been shown to regulate IL-1β promoter activity (Hiscott et al., 1993). NF-kappaB/Rel transcription factors have also been implicated in the differentiation of monocytes to macrophages or dendritic cells (Baltathakis et al., 2001). Differences in the binding affinity of c-Rel transcription factors may be the basis for the observed differences in transcriptional activity of the two alleles. However, the effects of SNPs in the binding site are unlikely to be simple. While some binding sequence alterations made by SNPs may totally interrupt gene expression, others may only influence the level of expression. Considering that gene transcription is a complex process involving many transcription factors, a single position change may not influence all of them. Therefore, the fact that more SNPs are located in the transcription factor binding sites may demonstrate a differential requirement for gene expression under a given condition.
Since only SNPI allele 2 (T) had a significant effect on basal transcriptional activity, further investigations were done to test if bacterial stimulation could influence transcription of the construct carrying T allele of this SNP. Exposing transfectants to bacterial stimuli elevated basal luciferase activity, nevertheless, there were no significant differences between E.coli LPS and tested periodontopathogens including A. actinomycetemcomitans, C. rectus, P. gingivalis and P. intermedia. The presence of SNP rs1800587 allele 2 (T) decreased significantly luciferase expression driven by the IL-1A promoter in both un-stimulated and stimulated THP-1 cells suggesting a role for IL-1A rs1800587 polymorphism in the complex regulation of IL-1A gene transcription. The relationship between IL-1A genotype and specific bacteria is unknown. While it is conceivable that cytokine polymorphisms may result in a non-protective immune response, thus allowing specific pathogens to persist, the results of the present study demonstrate that the presence of the IL-1A polymorphic allele is associated with a decreased stimulated transcription rate. Socransky and co-workers have reported that IL-1 (allele 2) genotype influences the levels of specific bacterial complexes with an increase in those containing periodontal pathogens (Socransky et al., 2000). Another study has also shown an interaction between IL-1 genotype, P. gingivalis, smoking and age (Cullinan et al., 2001). In these studies, the IL-1 genotypes were defined by either presence of either IL-1A (-899) or IL-1B (+3953) and no genotyping data was given for each gene, therefore it is difficult to compare these results with IL-1A SNP I (-889) alone.

As mentioned in Chapter 1, a few studies have suggested a functional effect of the polymorphism SNP I (-899). Notably, carriage of allele 2 (T) in the (-889) locus resulted in an almost three fold increase of interleukin-1α protein levels in a small group of chronic periodontitis patients (Shirodaria et al., 2000). Interestingly, differences in IL-1α in patients according to IL-1A genotype were higher in non-smokers (four fold) than in heavy smokers (2.8 fold), emphasizing the possible role of confounding factors in cytokine regulation. Furthermore, periodontitis patients carrying the SNP I T allele had significantly higher serum levels of interleukin-6 or C-reactive protein concentrations than subjects carrying C allele (D’Aiuto et al., 2004). There is also evidence that the TT genotype of the SNP I polymorphism significantly increased plasma levels of IL-1β (Hulkkonen et al., 2000).
A single *in vitro* study has tested the effect of SNP I (rs1800587) C->T substitution on transcriptional activity in reporter gene assays (Dominici et al., 2002). In this study, the SNP I (T) allele was associated with a 1.8-fold increase at basal transcription rate compared with a construct containing the allele C in PCIJ cells (human pancreatic cell line). The same TT genotype was also associated with significantly higher (five fold) levels of secreted IL-1α in compared with the CC genotype when PMBC cells were stimulated with LPS for 3 hours. Interestingly, no differences were observed after 6 hours of cell stimulation between the two genotypes. However, the effect on IL-1α mRNA level was small, showing only a 5% difference between the two genotypes in nonstimulated cells. It was concluded that the substantial difference in the protein level might be due to differences in the instability of the mRNA as a consequence of the polymorphism irrespective of the promoter activity.

These results are in contrast to the findings of this thesis in that the SNP I (T) allele is associated with a significant decrease in the basal or stimulated rate of IL-1α transcription *in vitro* in THP-1 cells. The differences between the previous study and the present findings may reflect the different cell lines employed. PCIJ is a transformed cell line whilst monocytic cells are more likely to be involved in the inflammatory processes. Cell-type specific differences may be the results of differential expression of essential transcription factors or underlying differences in transcriptional control. Since different cell types use different receptors, signalling pathways and transcription factors, it is possible that the same SNPs may show tissue-specific differences in function (Knight, 2005). Indeed, the pilot data in the present study suggest that the SNP I C>T substitution has no effect on the basal rate of transcription of the IL-1A gene in HeLa cells. In addition, it is also important to consider the other possible reasons for the differential expression including the effect of construct size. The previous study (Dominici et al., 2002) cloned a 1.4 kb promoter region of the IL-1A gene compared to 2.1 kb used in this thesis. Expression levels may differ between long and short constructs (McDowell et al., 2005). The present results suggest that the regulation of IL1A gene transcription may be cell type-specific, which may be physiologically relevant when considering different disease processes localised to different tissues or organs.

The functional significance of the SNP II C->T and the SNP III C->T are unknown. The physiological significance of those polymorphisms has not been directly investigated. A
recent study investigating the relationship of polymorphisms in the IL-1α gene to the risk of osteoporosis found no association between fracture risk or bone turnover and carriage of the alleles of the SNP III polymorphism (Knudsen et al., 2007). There have been no published studies of the effect of these polymorphisms on transcriptional activity using reporter gene assays. TRANSFAC data suggests that the polymorphisms could alter transcription factor binding sites. However, in the present study, no effect of the polymorphic sites was seen in assays of transcription of the IL-1A promoter when tested in vitro in THP-1, human skin fibroblasts or HeLa cells. The effects of polymorphisms in the binding site are unlikely to be simple. Considering that gene transcription is a complex process involving many transcription factors, a single position change may have little effect. Use of the TRANSFAC database is limited by considering only a subset of transcription factors and analysis suggest the role possible candidate factors play to explain observed functional differences. It seems better to test whether polymorphisms are functional or non-functional on the basis of standardised reporter gene assays. To understand further differential transcriptional activity of individual alleles in the reporter assays, an electrophoresis mobility shift assay using specific antibodies for these transcription factors would be a logical method. Overall, the data presented in the present study indicates that the SNP I polymorphism affects the transcriptional levels of IL-1A gene at least in the reporter system.

It should be emphasized that whereas the data presented in this thesis are compatible with the above interpretation, not all components of this hypothesis have been tested experimentally. Firstly, I have not tested the effects of the identified polymorphisms on IL-1α mRNA or protein levels in response to bacterial stimulants in human monocytes derived from subjects genotyped for the identified polymorphisms. This could answer functional effects of the identified polymorphisms at the translational level. Periodontal disease is considered as a complex disease in which many interacting gene variants act synergistically with environmental factors, as noted earlier, confounding factors such as smoking, a pathogenic flora contribute could exert an influence in addition to the genotype. Secondly, it is likely that polymorphisms would interact with environmental factors in order to confer susceptibility to disease. It can not be formally excluded that other polymorphisms, linked to the SNP I polymorphism might influence the expression of IL-1α. There is linkage disequilibrium across the IL-1 cluster (Cox et al., 1998; Danis et al., 1995; Diehl et al., 1999; Scapoli et al., 2005), and it is likely that a combination of
specific alleles is more important rather than individual alleles (Chen et al., 2006). Further studies are needed to understand haplotype structure between identified polymorphisms and also test the influence of haplotype context on the molecular function of a polymorphism in a gene promoter.
CHAPTER 6

Modulation of IL-1α in Periodontal Diseases by Doxycycline
6.1 INTRODUCTION

IL-1α plays a critical role in stimulating the innate host response and this capacity prepares the host to defend itself against bacteria (Glaccum et al., 1997). However, the resulting inflammation, while effective in protecting the host from being overrun by bacteria, can cause collateral damage. If IL-1α has a pivotal role in the progression of periodontitis then pharmacological inhibition of IL-1α activity may be of therapeutic benefit to patients with periodontitis. There are several potential targeting strategies which could be employed for reducing activity at the stage of transcription, during translation or synthesis. Conceptually, this can be accomplished with use of

I) natural inhibitors such as IL-1 receptor antagonists, soluble receptors to IL-1 or monoclonal antibodies (Carteron, 2000).

II) drugs that can inhibit the synthesis and/or release of IL-1α or increase the synthesis of endogenous inhibitors of IL-1α

IL-1α is regulated in vivo by soluble IL-1 receptor antagonist (IL-1Ra), a 22kD glycosylated protein (Dower et al., 1992; Harrison et al., 1990). However, IL-1Ra is not the only endogenous inhibitor of IL-1α. The IL-1 family receptors also include IL-1sR-I and IL-1sRII generated by proteolytic cleavage of their membrane-anchored counterparts (Colotta et al., 1994; McMahan et al., 1991). Soluble cytokine receptors have been used in several inflammatory diseases to antagonize IL-1 responses (Opal and DePalo, 2000). Treatment of periodontal disease in a model of Macaca fascicularis with soluble receptors including IL-1 receptor type I plus soluble TNF-α receptor was shown to inhibit progression of inflammation in the alveolar bone and limit the destructive effects of periodontal pathogens as well as osteoclast activity (Assuma et al., 1998; Delima et al., 2001; Graves et al., 1998). Compared with control animals, intrapapillary injection of soluble receptor antagonist of IL-1 and TNF-α reduced 80% of the inflammatory cell numbers and alveolar bone loss by 50% as measured by computer-assisted densitometric image analysis (Assuma et al., 1998). Since these studies used a combination of IL-1 and TNF inhibitors, the precise role of IL-1 was not defined. Moreover, a recent study showed that treatment of periodontal disease using human soluble IL-1 receptor type I as a specific inhibitor (sIL-1RI) alone is also highly effective in reducing progression of inflammation (by 50-70%), loss of attachment (by 30-70%) and alveolar bone (by 60-168
65%) in a *Macaca fascicularis* primate model (Delima et al., 2002). Those studies not only further support the pivotal role of IL-1 in periodontal disease progression but also suggest that reduction in IL-1 activity by specific inhibition could reduce disease progression. However, the modulation of IL-1α by soluble receptors is still limited to *in vitro* or preclinical animal periodontitis models and it remains to be tested in a clinical environment.

A second possible approach is the pharmaceutical inhibition of IL-1α as adjunctive or alternative therapy for periodontal disease. Several anti-inflammatory drugs and antibiotics have been investigated as adjuncts to mechanical non-surgical periodontal therapy. The only systemic host modulatory agent currently approved for adjunctive use with nonsurgical periodontal procedures, however, is Periostat (20mg doxycycline hyclate) (Caton, 1999). Doxycycline, a member of the tetracycline family, is a broad-spectrum antibiotic effective against a wide range of gram-positive and gram-negative organisms. Doxycycline has been studied extensively in several diseases including osteoarthritis, rheumatoid arthritis, adult periodontitis that are characterized by high levels of pro-inflammatory mediators and protease activities (Brown et al., 2004; Lamparter et al., 2002; Lee et al., 2004; Preshaw et al., 2004b). In each of these disorders, the beneficial effects of doxycycline are not due to their antimicrobial effects but rather their efficacy in inhibiting pro-inflammatory and protease activities.

### 6.2 Cytokine modulation by Tetracyclines

Since cytokines are involved in the destruction of both periodontal tissue and alveolar bone and they can stimulate increased production of MMPs (MacNaul et al., 1990), another non-antimicrobial mechanism by which the Tetracyclines may inhibit tissue breakdown could be through inhibition of cytokines. There is evidence to support inhibition of extracellular matrix breakdown by Tetracyclines through inhibition of cytokines (D'Agostino et al., 2001; Kirkwood et al., 1999; Krakauer and Buckley, 2003; Milano et al., 1997; Sandler et al., 2005; Shapira et al., 1997; Shapira et al., 1996). In addition, processing of cytokines such as TNF-α can be mediated by matrix metalloproteinase enzymes (Gearing et al., 1994). A recent study reported that antagonists of IL-1 or TNF-α alone or in combination down regulated MMP-1, MMP-3
and MMP-13 expression in cultures of cartilage from osteoarthritic patients (Kobayashi et al., 2005).

In addition to in vitro and animal studies, there are also studies which have monitored GCF levels of proteolytic enzymes or cytokines in clinical trials of non-surgical periodontal therapy with adjunctive subantimicrobial dose doxycycline (SDD) (Choi et al., 2004; Emingil et al., 2004; Golub et al., 2001; Gurkan et al., 2005). The findings of these studies suggest that a combination of SDD with non-surgical therapy improves clinical parameters of periodontal disease and decreases GCF inflammatory mediator levels.

6.3 IL-1α Modulation by Tetracyclines

6.3.1 In vitro and animal evidence of modulation of IL-1α by Tetracyclines

Tetracyclines, especially doxycycline are able to exert a range of biological activities including differential inhibition of the activity of members of the matrix metalloproteinase (MMP) family, inhibition of MMP synthesis, inhibition of cytokine synthesis, inhibition of activated B cell function, inhibition of nitrous oxide synthesis by lipopolysaccharide activated macrophage. However, data on modulation of IL-1α production by doxycycline is limited.

**Figure 6.1** A schematic representation of non-antimicrobial activity of tetracyclines and their chemically modified counterparts

It is generally considered that metal chelation is key to their non-bactericidal properties (Golub et al., 1987; Golub et al., 1995; Greenwald et al., 1987). However, some evidence
has suggested that inhibition of proteases by doxycycline is an indirect result of inhibiting the secretion of IL-1 (Rawdanowicz et al., 1994; Shlopov et al., 2001; Solomon et al., 2000). A recent study investigated the effects of doxycycline in an experimental murine dry eye model and showed that doxycycline decreased mRNA levels of IL-1α as well as IL-1β and TNF-α in the corneal epithelium (De Paiva et al., 2006).

6.4 Doxycycline as direct catalytic inhibitor of MMPs

Doxycycline has been shown to inhibit MMP activity and alter pro-collagenase and collagenase conformation by binding to their Ca\(^{2+}\) (Golub et al., 1995; Smith et al., 1996). This was the first mechanism proposed for the ability of tetracyclines to inhibit activity of metalloproteinases. This mechanism is supported by elimination of the ability of tetracyclines to inhibit collagenase activity \textit{in vitro} when excess Ca\(^{2+}\) or excess Zn\(^{2+}\) is present (Golub et al., 1983; Yu et al., 1991).

Although tetracyclines could directly inhibit protease activity by binding diivalent cations, there is reason to believe that other mechanisms may also be involved. The interaction kinetics of MMP-8 with doxycycline suggest that doxycycline acts as a non-competitive inhibitor of collagenase \textit{in vitro} (Sorsa et al., 1994). In addition, the chemically modified tetracycline (CMT-5) which lacks a Ca\(^{2+}\) or Zn\(^{2+}\) binding site can inhibit the oxidative activation of pro-MMP (Sorsa et al., 1998). Tetracyclines can also inhibit activation of MMPs by scavenging reactive oxygen intermediates such as HOCl generated by peripheral blood neutrophils (Gabler and Creamer, 1991). This property of tetracyclines appears not to be dependent on their metal-ion binding properties. Moreover, inhibition of the proinflammatory secretory enzyme phospholipase A2 (sPLA2) by Tetracyclines cannot be blocked by addition of excess calcium (Pruzanski et al., 1992; Pruzanski et al., 1998). A recent study showed that cysteine proteinase inhibition by doxycycline was not affected by the presence of calcium (Imamura et al., 2001). These findings suggest that the inhibitory activity of tetracyclines cannot be completely accounted for by the ability to bind metal-like ions. Their exact mechanisms of action independent of chelation are still unclear.
The use of tetracyclines and their analogues in the treatment of periodontal diseases has been reviewed in several articles (Golub et al., 1998; Preshaw et al., 2004a; Rifkin et al., 1993; Vernillo et al., 1994). Tetracyclines, mainly doxycycline, tend to be highly concentrated in the gingival crevicular fluid at levels 5-10 times greater than those found in serum (Pascale et al., 1986). In addition, the gradual release of doxycycline from teeth in active form also may contribute to increased exposure, and maintain effectiveness during the post-treatment period (Baker et al., 1983). Doxycycline has a long half-life, which makes convenient twice-a-day dosing possible. Subantimicrobial doses of doxycycline have no antimicrobial activity and therefore do not produce any microbial resistance after prolonged use (Kornman and Karl, 1982; Thomas et al., 1998).

The concentrations required to achieve the direct inhibition of MMP activity by doxycycline are higher than clinically achievable levels (Gilbertson-Beadling et al., 1995). This may imply that subantimicrobial doses of doxycycline may decrease MMP activities due to indirect mechanisms such as cytokine inhibition. Little work has been carried out on the effect of doxycycline on monocytes which are involved in cell mediated immune responses, including IL-1α production. Therefore, the aims of this study were to identify the mechanism by which doxycycline modulates cytokine production, including IL-1α. More specifically these studies aimed to test;

- the regulation of IL-1α production in monocytes by doxycycline
- the effect of metal ions on IL-1α production and if doxycycline regulates cytokine response via the chelation of metal ions
- the specificity of the response to doxycycline in IL-1α and other cytokines

6.5 MATERIALS & METHODS

6.5.1 Bacterial stimuli and materials

Bacterial supernatant was obtained from cultures of A. actinomycetemcomitans Y4. Cultures were grown in appropriate growth medium (see Table 2.1) in an anaerobic chamber containing an atmosphere of 80% N₂, 10% H₂, and 10% CO₂. Cultures were grown to approximately 5x10⁷ colony forming units/ml and harvested by centrifugation at 10000g for 15 min at 4°C. The culture supernatants were collected, filter-sterilized and stored at -80°C until used. The supernatant of bacterium was directly diluted in culture
Chapter 6: Modulation of IL-1α

medium to final dilutions of 1:250, a concentration that has been shown to induce cytokine production in the absence of cell toxicity (Bostanci et al., 2006). LPS from the non-oral bacterium Escherichia coli 026: B6 (Sigma-Aldrich, UK) was used as a positive control. Doxycycline was dissolved in distilled water (stock of 50 mg/ml) and filter-sterilized and stored at -80°C. Doxycycline, when used, was added simultaneously with bacterial stimuli. Other chemicals including ammonium tetrathiomolybdate and copper II sulphate were also purchased from Sigma-Aldrich, UK.

6.5.2 Cell culture

Peripheral blood was obtained from healthy individuals by venous puncture and collected in EDTA vacutainers (Becton Dickinson). All participants signed a written consent form. Protocols for the study were approved by the East London and City Health Authority London Research Ethics Committee. Human peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences, UK) according to the manufacturer’s instructions. The cells were washed twice with PBS containing EDTA (2mM) and bovine serum albumin (2%), pH 7.3. Cell viability was determined by Trypan blue exclusion. PBMC count was performed with a haemocytometer and processed for magnetic labelling.

The Monocyte Isolation Kit II (Miltenyibiotec, Germany) was used to isolate unstimulated monocytes from human peripheral blood mononuclear cells (PBMC) using an indirect magnetic labelling system. The purity of isolated monocytes was evaluated by flow cytometry on a FACScan flow cytometer (Becton Dickonson) using a CD14 FITC-conjugated antibody. Purified monocytes were re-suspended in RPMI-Glutamax (Gibco BRL Life Technologies, UK) supplemented with 10% heat-inactivated fetal bovine serum (Bio-Whittaker, Maryland). The myelomonocytic cell lines, MonoMac-6 and THP-1 were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). MonoMac-6 cells were cultured in RPMI-Glutamax (Gibco BRL Life Technologies, UK) supplemented with 10% fetal bovine serum, 1% non-essential amino acids (Gibco BRL), 1% sodium pyruvate (Gibco BRL) and 9μg/ml bovine insulin (Sigma-Aldrich, UK). THP-1 cells were cultured in RPMI-Glutamax (Gibco BRL Life Technologies, UK) supplemented with 10% fetal bovine serum. The assays were performed with cells at a density of 1x10⁶ cells per ml.

173
6.5.3 ELISAs for cytokine production

The cells were seeded in 96-well microtitre plates at density of 1x10^6 cells/ml and incubated in the presence of either 10 ng of LPS or bacterial supernatant from A. actinomycetemcomitans Y4 (A.a) with or without doxycycline at various concentrations (2.5 μg/ml to 50 μg/ml) for 6 hours at 37°C in a 5% CO₂ atmosphere. In some experiments, cells were preincubated with doxycycline for 1 hour and before exposure to bacterial challenge. To assay cytokine production, the cell free culture supernatants were collected by centrifuging at 1000 rpm for 5 minutes and stored at -80°C until the cytokine assays were performed. The concentrations of TNF-α, IL-1α, IL-1β and IL-6, IL-8 were determined by an enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols. The absorbance at 450 nm was read using a microplate reader (Model 680, Bio-Rad Laboratories) with a wavelength correction set at 570nm. A standard curve was generated using a four parameter logistic (4-PL) curve-fit (Microsoft Office Excel) for each set of samples assayed. The values of the samples were assigned according to the standard curve.

For further analysis, levels of a number of cytokines were assessed in the culture supernatants using multiplex immunoassays. The Proteoplex 16 well Human Cytokine Array Kit, Merck Biosciences, UK) was used for the assessment of TNF-α, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, IFN-γ and GM-CSF. The minimum detectable concentration in this assay was 5 pg/ml for each analyte.

6.5.4 Transfection and luciferase reporter gene assays

Since Monomac-6 cells were resistant to transfection, THP-1 cells were used. 2 x 10^7 THP-1 cells were suspended in 1 ml prewarmed Tris-buffered saline and incubated for 10 minutes at 37°C with 80 μg DEAE-dextran (Pharmacia). For transfection, THP-1 cells were transfected with 5 μg DNA of the luciferase IL-1A reporter plasmid or 1.5 μg of a luciferase reporter plasmid containing five sequential NF-κB binding sites upstream of a minimum promoter element, NF-κB-Luc (PathDetect NF-κB Cis-Reporting System, Stratagene, UK). 2 μg of pSV-beta-galactosidase vector (Promega, UK) was transfected as an internal control. Transfection was stopped by adding 25 ml Tris-buffered saline. After washing, cells were cultured for 24 hours before stimulation. THP-1 cells (2 x 10^6 cells per stimulus) were stimulated with culture supernatant of A. actinomycetemcomitans.
or E.coli LPS for 6 hours. After stimulation, luciferase assay was performed using the Luciferase Assay System (Promega, UK) following the instructions of the manufacturer (Appendix V.12). Luciferase activity was normalized for pSV-beta-galactosidase activity measured with β-galactosidase enzyme assay as detailed in Appendix V.13. All transfection experiments were performed in triplicate.

6.5.5 Cell viability assays

LDH assay

Cell viability was determined by measurement of lactate dehydrogenase (LDH) release using the CytoTox96 non-radioactive cytotoxicity assay (Promega, UK). In brief, Monomac-6 cells or primary human monocytes were exposed to bacterial supernatants and then incubated for 6 hours at 37°C. 50 µl/well supernatant was carefully removed and transferred into an optically clear 96-well plate. Reaction solution was added to each well and incubated for 30 min in darkness. The enzyme reaction was then stopped by the addition of 1N HCl. The absorbance at 490nm was measured using an ELISA reader (Model 680, Bio-Rad Laboratories). The activity of the enzyme released from damaged cells into the supernatant was measured, and the activity was expressed as a percentage of the total LDH activity released from cells lysed by exposure to 0.1% Triton X-100 for 45 min. Values shown represent the mean ± standard deviation of three wells.

MTT Assay

Cytotoxicity of copper sulfate (CuSO₄), tetrathiomolybdate (TTM), Iron (III) chloride (FeCl₃) or Ferrichrome in Monomac-6 cells was determined by the use of the MTT assay (Mosmann, 1983). The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenase activity. The key component is 3-[4, 5-dimethylthiazol-2-yl]-2, 5- diphenyl tetrazolium bromide. Solutions of MTT solubilized in tissue culture media or balanced salt solutions, without phenol red, are yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured.
In brief, Monomac-6 cells (1 x10⁶) were plated into each well of a 96-well plate in the presence or absence of CuSO₄ (250 µM), TTM (400nM), FeCl₃ (25µM) and Ferrichrome (25 µg/ml) incubated at 37°C for 6 hours. After 6 hour incubation, the medium was then removed and 100 µl of MTT reagent (0.5 mg/ml) was added to all wells. After a 3 hours additional incubation, 100 µl of acidified isopropanol was added to all wells and incubated 1 hour at 37°C; then, the absorbance of solution was determined with ELISA plate reader (Model 680, Bio-Rad Laboratories) using a test wavelength of 570 nm. The net absorbance taken from the wells with cells cultured with control medium was taken as the 100% viability value. The percentage of viability of the treated cells was calculated from the net absorbance of the sample divided by net absorbance of the control times 100.

6.6 Statistical analysis

The results were statistically analysed using Student’s t-test with P values <0.05 considered significant.

6.7 RESULTS

6.7.1 The effect of doxycycline on cytokine production by human monocytes

Several pro-inflammatory cytokines are considered to participate in the inflammatory process associated with tissue destruction in periodontal disease. The effect of doxycycline on the production of several key pro-inflammatory cytokines using freshly isolated human monocytes was investigated. To determine the effects of doxycycline on the production of proinflammatory cytokines, either E. coli LPS or culture supernatant from A. actinomyetemcomitans was added to the cells together with or without doxycycline (from 5 µg/ml to 25 µg/ml). Exposure of freshly isolated human monocytes to 10 ng/ml E. coli LPS or culture supernatant from A. actinomyetemcomitans for 6 h induced a marked increase in the levels of IL-1α, IL-1β, IL-6 and IL-8 production (Figure 6.2 A, B, C, D). Doxycycline at concentrations of 5-25 µg/ml caused a dose-dependent inhibition of IL-1α, IL-1β, IL-6 and IL-8 by 21-70%, 31-74%, 18-84% and 24-85%, respectively.
Chapter 6: Modulation of IL-1α

A) IL-1α

![Graph showing IL-1α levels with varying Dox concentrations.](image)

B) IL-1β

![Graph showing IL-1β levels with varying Dox concentrations.](image)

C) IL-6

![Graph showing IL-6 levels with varying Dox concentrations.](image)
Figure 6.2 The levels of IL-1α, IL-1β, IL-6 and IL-8 in human monocytes regulated on activation by A. actinomyctemcomitans or E.coli LPS in the presence or absence of doxycycline (Figures A-D). Freshly isolated CD14+ monocytes were plated at a density of 1 x 10^6 cells/ml in 96-well dishes. Cells were exposed to culture supernatants from A.a or E.c LPS (10 ng/ml) in the presence or absence of doxycycline (5 to 25 μg/ml). After 6 h incubation, cell-free supernatants were collected to assess cytokine release by ELISA. The data are means ± SD (n=3) representative of one of three separate experiments. *Doxycycline treated cells are statistically different as compared with untreated controls. *P < 0.05

6.7.2 The effect of doxycycline on cytokine production by Monomac-6 cells

To eliminate problems of variation associated with use of the primary cells Monomac-6 cells were used for most of the following experiments. The effect of doxycycline on the production of several key pro-inflammatory cytokines using Monomac-6 cells was investigated. Previous studies have shown that these cells provide a good model of cytokine production in response to bacterial stimulation (Bostanci et al., 2006).

As shown in Figure 6.3 Monomac-6 cells produced variable levels of IL-1α (A), TNF-α (B), IL-1β (C), IL-6 (D), IL-8 (E). The levels of these cytokines significantly increased in response to either E. coli LPS or culture supernatant from A. actinomyctemcomitans as compared with untreated controls. Doxycycline (10-25 μg/ml) significantly inhibited the stimulated or un-treated control levels of IL-1α, TNF-α, IL-1β, IL-6 and IL-8 by 39 to 63%, 55 to 73%, 79 to 86%, 97 to 98%, and 87 to 98%, respectively.
A) IL-1α

![Graph showing modulation of IL-1α with different concentrations of Dox.]

B) IL-1β

![Bar graph showing modulation of IL-1β with different concentrations of Dox.]

C) TNF-α

![Bar graph showing modulation of TNF-α with different concentrations of Dox.]

179
D) IL-6

![Graph showing IL-6 levels with and without doxycycline]

E) IL-8

![Graph showing IL-8 levels with and without doxycycline]

Figure 6.3 The levels of IL-1α, IL-1β, TNF-α, IL-6 and IL-8 in Monomac-6 cells in response to culture supernatant from *A. actinomycetemcomitans* in the presence or absence of doxycycline (Figures A-E). Monomac-6 cells were exposed to culture supernatants from *A.a* (1:250) in the presence or absence of doxycycline (10-25 μg/ml). After 6 hours incubation, cell-free supernatants were collected to assess cytokine release by ELISA. The data are the mean±standard deviations (S.D.) n=3. *Doxycycline treated cells are statistically different as compared with untreated controls.* P < 0.05

The toxicity testing demonstrated that the ability of doxycycline to inhibit a significant cytokine response was not related to its toxicity to the Monomac-6 cells or isolated human monocytes. It should be noted that even the highest concentration of doxycycline used did not appear to be toxic as determined by levels of LDH release (Figures 6.4A-B).
Chapter 6: Modulation of IL-1α

A) Monomac-6

B) Human isolated monocytes

Figure 6.4 Effects of doxycycline on cell viability. Monomac-6 (A) and freshly isolated human monocytes (B) were incubated with the indicated concentrations of doxycycline for 6 hours. Cytotoxicity was determined by the release of the cytosol enzyme lactate dehydrogenase (LDH). The activity was expressed as a percentage of the total LDH activity released from cells lysed by exposure to 0.1% Triton X-100 for 45 min. The data are the mean±standard deviations (S.D.) n=3.

Monomac-6 cells were also pre-incubated with doxycycline for 1 hour prior to challenge with either 10 ng/ml *E. coli* LPS, or culture supernatant from *A. actinomycetemcomitans* for 6 hours. Pre-incubation effectively suppressed IL-1α production induced by these two bacterial stimuli by 57% to 65% at 5 μg/ml to 50 μg/ml (Figure 6.5), indicating that doxycycline suppresses pathways through which *E.coli* LPS or other bacterial signals are transmitted into the cell and thus suppresses pro-inflammatory cytokine production by monocytes and Monomac-6 cells. Since doxycycline is removed before exposure to bacterial stimuli it must induce a lasting change in the cell rather than act by interfering in the interaction of the bacterial components and the cell.
Figure 6.5 The effect of pre-incubation with doxycycline prior to challenge. Monomac-6 cells were plated at a density of $1 \times 10^6$ cells/ml in 96-well dishes. Cells were exposed to culture supernatants from A.a (1:250). Doxycycline (5 to 25 µg/ml) was added 1 h prior to activation. After 6 h incubation, cell-free supernatants were collected to assess cytokine release by ELISA. The data are the mean ± standard deviations (S.D.) n=3. *Doxycycline treated cells are statistically different as compared with untreated controls. *P < 0.05

6.7.3 Doxycycline does not affect expression of IL-1α promoter in THP-1 cells

If doxycycline suppresses IL-1α production one obvious mechanism is via the inhibition of transcription. To investigate the effect of doxycycline on transcriptional regulation of IL-1α gene, a luciferase reporter construct pGL3-IL-1α was transfected into THP-1 monocyctic cells. After transfection, cells were allowed to recover for 24 hours then treated with culture supernatant from A. actinomycetemcomitans or E. coli LPS in the presence or absence of doxycycline for 6 hours. Background activity of the construct was low but significantly increased over 60% in the presence of bacterial stimulants (Figure 6.6). In the presence of doxycycline there was no evidence of reduced transcriptional activity.
Figure 6.6 Effect of doxycycline on the IL-1A gene promoter activity. THP-1 cells were transfected with 5 µg DNA of the luciferase IL-1A reporter plasmid and 2 µg of pSV-beta-galactosidase vector as internal control. After 24 hours incubation, cells were exposed to A.a supernatant with or without doxycycline for 6 hours. Luciferase levels are expressed after standardization for [beta]-galactosidase activity. Bars show the mean and SD of triplicate assays.

6.7.4 Effect of Doxycycline on E. coli LPS induced NF-κB activation

LPS-induced production of several cytokines, including IL-1α and TNF-α, is dependent on the activation and translocation into the nucleus of NF-κB. Since doxycycline inhibits secretion of multiple cytokines, the potential inhibition of NF-κB activation by doxycycline was examined. To study the effects of doxycycline on the transcription factor NF-κB, a luciferase reporter plasmid containing five sequential NF-κB binding sites upstream of a minimum promoter element, NF-κB-Luc (PathDetect NF-κB Cis-Reporting System, Stratagene) was used. NF-κB-Luc was transiently transfected into Monomac-6 cells and after 24 hours, cells were treated with doxycycline and LPS for 6 hours. After a further 6 hours, activity of the NF-κB promoter construct was measured. Addition of LPS induced a 2-fold increase compared to control cells (Figure 6.7).
Figure 6.7. Effect of doxycycline on the NF-κB promoter construct activity upon transient transfections. THP-1 monocytic cells were transfected with 1.5 μg of reporter plasmid and 0.5 μg pSV-beta-galactosidase internal control vector using Dextran. Cells were exposed to E. coli LPS with or without doxycycline for 6 h. Luciferase activity was measured in the extracts using a luciferase assay system that was normalized for β-galactosidase activity. Bars show the mean and SD of triplicate assays. *p<0.05

However the presence of doxycycline failed to reduce transcriptional activation of the NF-κB reported in LPS-stimulated cells. These results suggest that doxycycline did not interfere with the LPS induced activation of NF-κB and that the inhibitory effect of doxycycline on the production of cytokines might act elsewhere in the pathway from signal to cytokine.

6.7.5 The ion-chelation properties of doxycycline

Since doxycycline, like other tetracyclines can act as a chelating agent, the inhibitory effect of doxycycline on cytokine production in Monomac-6 cells could result from changes in levels of specific ions. In addition, secretion of IL-1α is believed to involve a copper containing complex (Mandinova et al., 2003). An attractive hypothesis is that reduced IL-1α production in the presence of doxycycline could result from depletion of copper levels. Therefore, the ability of Cu²⁺ to regulate cytokine secretion at secreted and cell- associated level was assessed. 250 μM copper increased secretion of IL-1α by 88% (Figure 6.8). There was a slight increase in the cell associated level (12%). Under the same conditions, TTM (400nM), a specific copper chelator, inhibited secretion of IL-1α by 31%. In addition, the effect of iron was tested under same conditions. Fe^{3+} had a slight
effect on IL-1α by reducing both secreted and cell associated levels. In contrast, Ferrichrome, a specific iron chelator had no effect on the total levels of IL-1α. The inhibiting effect of doxycycline on IL-1α was not abolished when excess amounts of either copper were added (Figure 6.8A) or iron (Figure 6.8B). The MTT assay testing demonstrated that the ability of ions or their chelators to regulate IL-1α production was not related to their toxicity to the Monomac-6 cells (Figure 6.9).

A)

![Graph A](image)

B)

![Graph B](image)

**Figure 6.8** The ion-chelation properties of doxycycline. Monomac-6 cells were plated at a density of 1 x 10⁶ cells/ml in 96-well dishes. Cells were exposed to culture supernatants from A.a (1:250) with or without doxycycline (10 µg/ml), CuSO₄ (250 µM), TTM (400nM), FeCl₃ (25µM), Ferrichrome (25 µg/ml). After 6 h incubation, cell-free supernatants were collected to assess cytokine production by ELISA. The data are the mean ± standard deviations (S.D.) n=3.
Chapter 6: Modulation of IL-1α

6.7.6 Kinetics of cytokine release in the presence of doxycycline

A cytokine array analysis was further employed to demonstrate the kinetics of cytokine inhibition in Monomac-6 cells by doxycycline. Using multiplex cytokine analysis doxycycline activity was tested on the production of several key pro- or anti-inflammatory cytokines. The Monomac-6 cells produced various levels of cytokines in response to culture supernatant from *A. actinomycetemcomitans*, including IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12, GM-CSF and TNF-α. The cells produced low- (<5 pg/ml) to undetectable levels of IL-2, IL-4, IL-7 and IFN-γ.

A reduction in pro-inflammatory cytokine levels was demonstrated over 4 h with the administration of 10 μg/ml doxycycline to the cells. The cytokine inhibition was observed already at 2 h in IL-1α and IL-1β, IL-6, IL-8, whereas a change in TNF-α levels was observed at 4 h. TNF-α, IL-1α, IL-1β, IL-6 and IL-8 production were inhibited by 23 to 27%, 55 to 67%, 40 to 43%, 46 to 97%, and 87 to 98%, respectively (Figure 6.10 A-E). In contrast, the production of IL-10 and IL-12 increased by 46% to % 100 in the presence of doxycycline (Figure 6.10 F-G).
Chapter 6: Modulation of IL-1α

A) TNF-α

B) IL-1α

C) IL-1β
Chapter 6: Modulation of IL-1α

D) IL-6

![Graph showing IL-6 levels over time with Aa and Aa+Dox groups.]

E) IL-8

![Graph showing IL-8 levels over time with Aa and Aa+Dox groups.]

F) IL-10

![Graph showing IL-10 levels over time with Aa and Aa+Dox groups.]
6.8 DISCUSSION

Doxycycline has been studied at sub-antimicrobial concentrations in human and animal diseases that are characterized by high levels of pro-inflammatory mediators and protease activities, the diseases such as rheumatoid arthritis and periodontitis (Brown et al., 2004; Madan et al., 2007; O'Dell et al., 2006; Preshaw et al., 2004a; Salvi and Lang, 2005).

The present study demonstrated that doxycycline inhibits the production of several cytokines by human monocytes and the Monomac-6 monocytic cell line, when
stimulated by \textit{E. coli} LPS and culture supernatant from \textit{A. actinomycetemcomitans.} Inhibition of bacteria-induced cytokine secretion by doxycycline occurred in a dose-dependent manner within a concentration of 5-25 \(\mu\text{g/ml}\). In subsequent studies a concentration of 10 \(\mu\text{g/ml}\) doxycycline was used as it did not affect cell viability. Doxycycline concentrations of 2 \(\mu\text{g/ml}\) to 10 \(\mu\text{g/ml}\) have been reported in GCF, gingival tissue or plasma after oral administration of this drug (Lavda et al., 2004; Sakellari et al., 2000). Thus, the concentrations used in the present study are approximately in the range of those found in patients after the administration of a subantimicrobial dose. The kinetics of cytokine production in Monomac-6 cells showed that doxycycline is more effective in modulating pro-inflammatory cytokine production rather than anti-inflammatory ones. Doxycycline displayed an early (at 2 h) inhibitory action towards IL-1\(\alpha\) and IL-1\(\beta\), IL-6, IL-8, whereas a change in TNF-\(\alpha\) level was observed at 4 h. Under the culture conditions of the study, cells produced low- (<5 pg/ml) to undetectable levels of IL-3, IL-4, IL-7 and IFN-\(\gamma\) during 4 h of incubation. These findings are in agreement with previous reports showing that doxycycline inhibits the release of TNF-\(\alpha\), IL-1\(\beta\), IL-6 by human monocytes (Krakauer and Buckley, 2003; Shapira et al., 1997). It therefore appears that doxycycline has an inhibitory effect on the production of a number of cytokines as a putative mechanism of action not specifically restricted to IL-1\(\alpha\).

Most of the studies that have addressed gene regulation by doxycycline have found that its effects occur via a post-transcriptional or translational mechanism (Kirkwood et al., 1999; Shapira et al., 1996; Uitto et al., 1994). Therefore, in the present study using reporter assays as an \textit{in vitro} model, the hypothesis that doxycycline suppresses IL-1\(\alpha\) production via the inhibition of transcription was tested. Based on the regulation of the IL-1A gene promoter activity in monocytes, the results suggest that doxycycline decreases IL-1\(\alpha\) protein levels due to the inhibition of protein translation rather than transcription at the promoter level. This result is supported by a previous study demonstrating that doxycycline inhibits LPS-induced TNF-\(\alpha\) and IL-1 secretion by monocytes but not accumulation of cytokine mRNA (Shapira et al., 1996).

The transcription factor NF-\(\kappa\)B is an important regulator of genes encoding proinflammatory cytokines, including IL-1, IL-6, IL-8, and TNF-alpha (Karin and Delhase, 2000; May and Ghosh, 1998). It is of interest that the results of the present
study revealed that although treatment with LPS induced activation of NF-κB, treatment with doxycycline did not inhibit this activation. In the present study, analysis of the NF-κB transcription pathway suggested that it may not play a significant role in attenuating production of cytokines after exposure to doxycycline. This concept is supported by a recent study demonstrating that doxycycline-modulated inhibition of NO production in murine LA4 cells is independent of the NF-κB pathway (Hoyt et al., 2006).

Doxycycline, like other tetracyclines is known to bind divalent cations such as calcium, copper, and zinc (Golub et al., 1983; Yu et al., 1991). Recent studies have indicated that cytokine release may depend upon metal ions (Bar-Or et al., 2003; Lewis et al., 2003; Mandinova et al., 2003). Indeed, a copper-dependent release of IL-1α from the U937 human monocytic cell line has been documented (Brewer, 2003; Mandinova et al., 2003). Earlier studies have also shown a linear relationship between the concentration of iron in GCF (26 to 170 μM) and severity of periodontal disease (Wang et al., 1990). Analysis of the effect of copper in the present study suggested that intracellular transport of this ion is involved in the regulation of IL-1α release. This effect was also verified by the use of TTM, a specific copper chelator (Miller and Engel, 1960) and since TTM reduced both intra-cellular and secreted IL-1α levels by Monomac-6 cells. The effects of TTM on TNF-α production have also been recently demonstrated (Brewer et al., 2004). A potential hypothesis could be that reduced IL-1α production in the presence of doxycycline could result from the depletion of copper levels. However, in the present study, when excess amounts of copper were added to the culture media, there was no change in the inhibitory effect of doxycycline on bacterial stimulation. In addition, the presence of excess biological concentrations of iron did not change the activity of doxycycline. Whilst a strong iron chelating activity for doxycycline has been demonstrated, this was only at a concentration 250 μg/ml, which is higher than the clinically achievable levels at sub-antimicrobial doses (Grenier et al., 2000). Taken together, these findings suggest that the inhibitory activity of tetracyclines can not be fully accounted by their ability to bind metal-like ions, and therefore it seems unlikely that these actions are mainly dependent on ion chelation.

Previous studies have shown that the addition of excess Ca^{2+} (mM concentrations) or excess Zn^{2+} (μM concentrations) can eliminate the inhibition of collagenase activity by
tetracyclines (Golub et al., 1983; Yu et al., 1991). However, the concentrations of doxycycline (250 μg/ml) required to achieve the direct inhibition of MMPs activity are very high (Gilbertson-Beadling et al., 1995). The chemically modified tetracycline 5 (CMT-5) which lacks Ca\(^{2+}\) and Zn\(^{2+}\) binding sites is also able to inhibit the oxidative activation of pro-MMP (Sorsa et al., 1998). These results are also supported by other studies showing that proinflammatory secretory enzyme phospholipase A2 (sPLA2) inhibition by doxycycline can not be eliminated by the addition of excess calcium up to 50 mM (Pruzanski et al., 1992; Pruzanski et al., 1998) and cysteine proteinase inhibition by doxycycline is not affected in the presence of calcium (Imamura et al., 2001). Taken together, these results suggest that subantimicrobial doses of doxycycline may decrease MMP activities via indirect mechanisms including cytokine inhibition.

In summary, the results of this study indicate that doxycycline is an inhibitor of secretion of cytokines by human monocytes stimulated with *E.coli* LPS or *A. actinomycetemcomitans* supernatant. This inhibitory effect does not appear to involve regulation of NF-κB activity or the suppression of transcription of IL-1α. The inhibitory effects are also independent of the ability of doxycycline to chelate metal ions. The data presented here suggest that low doses of doxycycline below the threshold for antimicrobial actions may be useful for treatment of periodontal diseases due to anti-inflammatory activity.
CHAPTER 7

General Discussion
Chapter 7: General Discussion

7.1 GENERAL DISCUSSION

Periodontal diseases are multifactorial infectious diseases that manifest clinically by destruction of the supporting tissues of the teeth. The host immune and inflammatory response to periodontopathogenic bacteria is a critical determinant of susceptibility to develop the destructive periodontal disease, under the influence of multiple behavioral, environmental, and genetic factors (Cohen, 2002; Jenner and Young, 2005; Schumann, 2004).

The observation that severe periodontitis affects only 7–15% of individuals means that not all individuals are at equal risk of developing the disease. Genetic factors are important determinants of periodontitis susceptibility and progression (Kinane et al., 2005). SNPs are the most common source of human genetic variation (Suh and Vijg, 2005), their association with human disease phenotypes has the potential to be used as diagnostic and prognostic markers and potentially lead to the development of novel therapeutic targets. Since large scale screening for SNPs is still very complex (Cantor and Nelson, 2005), alternative approaches which can be used for identification of disease associated SNPs include selection of candidate genes coding for particular features of a disease (Goldstein et al., 2003).

This thesis included a candidate gene approach using the IL-1A gene. IL-1α was selected on the basis of available information from both human and transgenic animal studies for the potential function of the IL-1A gene involved in the pathogenesis of periodontal disease (see Chapter 1.8). This approach was based on the hypothesis that increased or unregulated production of IL-1α in response to periodontopathogens may occur in subjects carrying functional polymorphisms of this gene. Firstly, studies were conducted to investigate how different periodontopathogens may regulate IL-1α production by monocytes. This was followed by screening of exon, intron and promoter regions of IL-1A for polymorphisms and then an examination of the identified polymorphisms to determine if there was a significant difference between patients and unrelated healthy individuals, in terms of allele frequencies or genotype frequencies. Moreover, the functional impact of identified polymorphisms on transcriptional activity and their interactions with periodontopathogens was assessed using in vitro reporter gene assays and in silico analysis.
The stimulation of IL-1α production by bacterial products has been previously studied. However, there has not been a systematic comparison of a full range of periodontal pathogenic bacterial species (Henderson et al., 2002; Sugano et al., 2004). Studies in Chapter 2 aimed to determine the optimal culture conditions for bacterial-stimulated production of IL-1α in monocytes and included a systematic comparison of a full range of periodontal pathogenic bacterial species. The amount of IL-1α production was highly dependent upon monocyte culture conditions and under the experimental conditions assayed, maximum IL-1α production by Monomac-6 was obtained once these cells were stimulated in the presence of 10% FCS for 6 hours at density of 1x10^6/ml (Pradines-Figueres and Raetz, 1992; Schwende et al., 1996; Ziegler-Heitbrock et al., 1994). The results indicated that a range of periodontal pathogens have the capacity to induce IL-1α production in Monomac-6 cells; nevertheless the nature of the component responsible for this effect may vary between them. This activity was due to either LPS or to the proteinaceous components of the bacteria depending upon the specific species tested.

The studies in Chapter 2 demonstrated that most periodontopathogens induced a significant level of IL-1α release from Monomac-6, while *P. gingivalis* induced IL-1α to only a minimal level. It was surprising that *P. gingivalis* is strongly associated with periodontal disease but did not induce IL-1α production. In addition, the stimulation of IL-1α at the site of infection by other periodontopathogens could be antagonised by *P. gingivalis*. Therefore, the studies in Chapter 3 compared IL-1α production by monocytic cells when treated with single bacterial supernatants to those treated with combination of *P. gingivalis*. *P. gingivalis* supernatants inhibited the capacity of most pathogens studied to stimulate IL-1α production by monocytes. The most pronounced inhibition was seen in combination with *C. rectus*, where the overall level of IL-1α production was reduced by approximately 72% in the presence of *P. gingivalis*. Further studies investigated the mechanism of antagonistic activity and showed that *P. gingivalis* LPS appeared to be the crucial component for this antagonistic activity. This study suggests that *P. gingivalis* appears to play a role in the short term inhibition of innate immune responses. Although *P. gingivalis* LPS exhibited a weak cytokine stimulating capacity, it may cause an early inactivation of immune cell responses to other bacteria. In this situation other periodontal bacterial species may be more likely to persist, therefore favouring the establishment of a chronic inflammatory lesion typical of periodontitis.
Genetic polymorphisms within the IL-1 gene cluster, including IL-1α, may identify individuals who are susceptible to periodontitis, although there is disagreement about which variant of the IL-1 gene confers susceptibility. The NCBI Entrez SNP database (http://www.ncbi.nlm.nih.gov/entrez) currently lists 15 polymorphisms in 5' upstream region of the gene of which five are located in the putative promoter (2.1 kb of the genomic sequence upstream of the transcriptional start site) (Table 4.1). Many of the reported polymorphisms are based on studies of relatively small numbers of individuals (often only 46) and in all cases periodontal status is unknown. Surveys of the abundance of polymorphisms generally aim to identify those polymorphisms with a frequency above about 4%. However, since severe periodontitis has an incidence of about 8% in the general population, it is possible that any given polymorphism abundant in this subpopulation could be missed or classed as rare from a small survey of random individuals. Therefore, in Chapter 4 two strategies were used to identify the common IL-1A gene promoter polymorphisms including direct sequencing and TaqMan analysis. The first strategy used was to sequence directly the 2.1 kb genomic sequence upstream of the transcriptional start site as well as the first exon and intron by surveying the frequency and occurrence of all variation within this region including SNPs, insertion/deletions and VNTRs within a Caucasian population of subjects with generalized aggressive periodontitis. Three previously described polymorphisms in the public database were observed in patients with generalized aggressive periodontitis, designated here as SNP I, SNP II and SNP III. No additional novel polymorphisms were detected. SNPs for further analysis were selected on the basis of having relatively high minor allele frequencies (>10%).

After identifying the existence and abundance of different polymorphisms in the promoter region of the IL-1A gene, TaqMan analysis was performed to provide information regarding the abundance of polymorphisms in the IL-1A gene in a range of ethnic groups including both disease and control individuals. Further analyses were done if there was a significant difference between patients and unrelated healthy individuals, in terms of allele frequencies or genotype frequencies. Of the three identified SNPs, only SNP I (referred to as -889) has been previously associated with periodontitis (Kornman et al., 1997a; Shirodaria et al., 2000). The association of the SNP II and the SNP III polymorphisms with periodontal disease has not been studied previously. The results presented in Chapter 4 showed no significant differences in the prevalence of the carriage
of either allele of the three studied SNPs between an ethnically mixed group of generalized aggressive periodontitis patients and a group of periodontally healthy subjects. In addition, no statistical difference was observed when comparing SNPs between the control and aggressive periodontitis groups, when only the Caucasian population was included in the analysis. When smoking status was included in the analysis as a covariate, the distribution of genotypes for three SNPs in smokers and non-smokers showed no statistical significance between controls and cases. Within the limitations of the study, results suggested that there was no association with the IL-1A gene promoter polymorphisms and generalized aggressive periodontitis. The results are in agreement with other studies that have reported no significant differences in the frequencies of the SNP I (referred to as -889) alleles in populations of individuals with aggressive periodontitis and healthy subjects (Anusaksathien et al., 2003; Brett et al., 2005; Gonzales et al., 2003; Hodge et al., 2001; Moreira et al., 2007; Rogers et al., 2002; Walker et al., 2000).

Nevertheless, these results in Chapter 4 do not exclude a role for the identified SNPs in periodontal disease. Periodontal disease is a complex disease in which many interacting gene variants will act synergistically with confounding environmental factors such as smoking or bacterial flora could interact with the genotype. In addition, an important feature of the complex disease is that most susceptible individuals are at elevated risk due to the combined effects of several susceptibility alleles (Bertram et al., 2007). If the complex disorder is influenced by multiple rare variants in a gene, each of minor or modest effect, each risk-increasing variant may be too rare to achieve a level of significance in association studies. Given the complex biology of IL-1 regulation and the extensive polymorphisms in the IL-1 gene cluster, it is very likely that if IL-1 genes influence disease, then a combination of specific alleles will be important rather than individual alleles. There is linkage disequilibrium across the IL-1 cluster (Chen et al., 2006; Cox et al., 1998; Danis et al., 1995; Diehl et al., 1999; Scapoli et al., 2005). Therefore further studies will be required to make wide SNP typing within the IL-1 gene cluster to fully determine an extended informative haplotype structure and its possible associations with aggressive periodontitis. Moreover, further studies are needed to determine whether and to what extent differences in IL-1A polymorphisms may affect the onset and severity of the periodontal lesions in generalized aggressive patients with different environmental exposures.
There are a number of possible genetic associations between polymorphisms in the IL-1A gene and periodontal disease (Shirodaria et al., 2000), but as yet there is no consensus on their functional importance. Whilst there is evidence for transcriptional regulation of IL-1α gene expression (Alheim et al., 1996; Bailly et al., 1996a; Kawaguchi et al., 2003; Mori and Prager, 1996), polymorphisms in the promoter region of the IL-1A gene may be important for IL-1α gene expression and protein production. Studies in Chapter 5 aimed to investigate the functional impact of the polymorphisms identified on transcriptional activity and their interactions with periodontopathogens using in vitro reporter gene assays and in silico analysis.

In silico investigation for transcriptional binding sites using TFSEARCH revealed that these SNPs are located in putative regulatory element sequences. However, the effects of polymorphisms in the binding site are unlikely to be simple and use of the TRANSFAC database is limited by considering only a subset of transcription factors. Therefore it was decided to test whether these polymorphisms have functional significance on the basis of standardised reporter gene assays.

Reporter gene assay studies in THP-1 monocytes showed that the SNP I allele 2 (T) resulted in decreased basal and stimulated levels of IL-1A transcription compared with constructs containing the allele 1 (C). This is a novel finding since a single previous in vitro study testing the effect of SNP I (rs1800587) C->T substitution on transcriptional activity in reporter gene assays (Dominici et al., 2002) showed that the SNP I T allele was associated with a 1.8-fold increase at basal transcription rate compared with a construct containing the allele C in PCIJ cells (human pancreatic cell line). The differences between the previous study and the present findings may reflect the different cell lines employed. PCIJ is a transformed cell line whilst monocytic cells are more likely to be involved in the inflammatory processes. Cell-type specific differences may be the results of differential expression of essential transcription factors or underlying differences in transcriptional control. Since different cell types use different receptors, signalling pathways and transcription factors, it is possible that the same SNPs may show tissue-specific differences in function (Knight, 2005). Indeed, the pilot data in the present study suggest that the SNP I C>T substitution has no effect on the basal rate of transcription of the IL-1A gene in HeLa cells. In addition, it is also important to consider the other possible reasons for the differential expression including the effect of construct
size. The previous study (Dominici et al., 2002) cloned a 1.4 kb promoter region of the IL-1A gene compared to 2.1 kb used in this thesis. Expression levels may differ between long and short constructs (McDowell et al., 2005). The present results suggest that regulation of IL1A gene transcription may be cell type-specific, which may be of physiological relevance when considering different disease processes localised to different tissues or organs.

The functional significance of the SNP II C->T and the SNP III C->T are unknown. The physiological significance of those polymorphisms has not been directly investigated. TRANSFAC data suggests that the polymorphisms could alter transcription factor binding sites. However, in this thesis, no effect of the polymorphic sites was seen in assays of transcription of the IL-1A promoter when tested in vitro in THP-1, human skin fibroblasts or HeLa cells. However, these results do not completely exclude the role of SNP II and SNP III since there is a strong linkage between SNP III & SNP I (-899). In addition, the possibility of other polymorphisms that are linked to the studied three polymorphisms may not be excluded. Using the three SNPs, the distribution of IL-1A promoter haplotypes in Caucasians with aggressive periodontitis were also estimated in the present study. However, the influence of haplotype context on the transcriptional activity of the IL-1A gene has not been investigated. Further experiments should define the major haplotype combinations that might influence the regulation of IL-1A promoter activity in response to periodontopathogens.

The central role played by IL-1α in the pathogenesis of periodontal disease suggests that immunomodulation may offer a suitable therapeutic target for investigation. In Chapter 6, the present studies have confirmed the previous findings by showing that doxycycline inhibits the production of several cytokines, including IL-1α, by human monocytes. This inhibitory effect did not appear to involve regulation of NF-kB activity or the suppression of transcription of IL-1α. The inhibitory effects are also independent of the ability of doxycycline to chelate metal ions. The data presented suggest that low doses of doxycycline below the threshold for antimicrobial actions may be useful for treatment of periodontal diseases due to anti-inflammatory activity.
7.2 FUTURE STUDIES

It is evident from data obtained from this thesis that bacteria can induce IL-1α production by a range of mechanisms. Different bacterial virulence factors may act through a range of distinct signalling mechanisms to stimulate the production and release of cytokines (Darveau et al., 2002; Saklatvala, 1995). Toll-like receptors (TLRs) 2 and 4 have recently been identified as possible signal transducers for various bacterial ligands (Bainbridge and Darveau, 2001; Yoshimura et al., 2002). To further clarify the mechanisms involved, the ability of different bacterial virulence factors to interact with different TLRs to stimulate IL-1α production could be investigated further.

In addition, this thesis also demonstrates an antagonism between P. gingivalis and C. rectus in terms of IL-1α production that appears to rely on the integration of positive and negative signals through cell signalling rather than any direct interaction between components in the supernatants. One possible explanation for the observed antagonistic effect is that although P. gingivalis LPS is a weak stimulus for proinflammatory cytokines it is a potent inducer of IL-1Receptor antagonist (IL-IRA) (Ogawa et al., 1994). Regulation of IL-1RA in the present system could be studied. Alternatively, the antagonistic effect could be attributed to competition of the two species for common signalling receptors, such as CD14 and TLRs (Darveau et al., 2004; Hajishengallis et al., 2002; Yoshimura et al., 2002; Zhou et al., 2005). It would be interesting to investigate if P. gingivalis and C. rectus possess any components such as LPS that initiate common signalling pathways involved in this antagonism.

Recent periodontitis infection models suggest that a consortium of bacteria rather than a single species is associated with more severe periodontal tissue destruction (Lakshmyya et al., 2007). In vitro studies using mixed bacteria in a macrophage-epithelial cell coculture model have reported that complexes of bacteria may induce higher levels of proinflammatory mediators (Bodet et al., 2006). Therefore, further studies would be useful to investigate cytokine regulation in in vivo infection models using current technologies such as microarrays. Preliminary experiments using T. denticola as a stimulant in Monomac-6 cells showed that culture supernatants elicit only a very small level of IL-1α production on their own, but are highly synergistic when combined with other periodontal pathogenic species (Javier Fernandez, M ClinDent Dissertation, 2006, 200
QMUL, and unpublished data). Since *T. denticola* is strongly implicated as part of the pathogenic consortium in the aetiology of periodontitis, further studies would be useful to investigate its putative role in IL-1α regulation (Lakshmyya et al., 2007).

The failure to find any association with IL-1A genotype and risk of aggressive periodontitis is similar to findings from some other studies but in contrast to other studies showing associations between IL-1 genotype and risk of aggressive periodontitis (Hodge et al., 2001). The relationship between genotype and periodontal disease remains unclear, partly due to the absence of carefully controlled large studies. A more definitive approach to these studies requires additional large population studies (e.g. of the order of 2000-3000 racially defined subjects and matched controls) with careful disease phenotyping using standard criteria.

Data from this study also indicates that the SNP I (-899) C→T polymorphism affects the transcriptional levels of IL-1A gene, at least in the reporter system, and it might play a role in regulating IL-1A gene transcription induced by bacteria. It should be emphasized that whereas the data presented in this thesis are compatible with the above interpretation, not all components of this hypothesis have been tested experimentally. Firstly, the effects of the identified polymorphisms on the IL-1α mRNA or protein levels in response to bacterial stimulants in human monocytes derived from subjects genotyped for the identified polymorphisms have not been tested. This could answer functional effects of the identified polymorphisms at the translational level. Secondly, since periodontal disease is considered as a complex disease, confounding factors such as smoking and specific microflora could exert an influence on the outcome by their interactions with genotype. It is likely that polymorphisms would interact with environmental factors in order to confer susceptibility to disease. Further analysis is required to consider these factors. Furthermore, the possibility that other polymorphisms, linked to the SNP I polymorphism, may influence the expression of IL-1α, cannot be formally excluded. There is linkage disequilibrium across the IL-1 cluster (Cox et al., 1998; Danis et al., 1995; Diehl et al., 1999; Scapoli et al., 2005), and it is likely that a combination of specific alleles is more important than individual alleles (Chen et al., 2006). Further studies are needed to understand haplotype structure between identified polymorphisms and also test the influence of haplotype context on the molecular function.
of a polymorphism in a gene promoter. Finally, to further understand differential transcriptional activity of individual alleles in the reporter assays, electrophoresis mobility shift assay using specific antibodies for these transcription factors would be a logical method to use in the future studies.

The results from this thesis have suggested that doxycycline inhibits the production of several cytokines including IL-1α by human monocytes. This inhibitory effect does not appear to involve regulation of NF-κB activity or the suppression of transcription of IL-1α. The inhibitory effects are also independent of the ability of doxycycline to chelate metal ions. MAPK signalling pathways play an important role in the translational regulation of pro-inflammatory cytokine synthesis (Clark et al., 2003; Fleenor et al., 2003). Since data from this thesis suggest that regulation is post-transcriptional, rather than transcriptional, to determine a mechanism for the cytokine inhibition, MAPK signalling pathways such as p38 MAPK protein could be examined.

The data presented here suggest that low doses of doxycycline below the threshold for antimicrobial actions may be useful for treatment of periodontal diseases due to anti-inflammatory activity. Although subantimicrobial dose doxycycline has been tested clinically as an adjunct for the management of periodontitis, further clinical studies to investigate further the role of doxycycline treatment or other IL-1 attenuating therapies in the management of periodontitis would be valuable. The possibility of targeting such therapies on the basis of levels of IL-1 production or IL-1 genotyping may merit investigation in the future.

### 7.3 CONCLUSIONS

In conclusion, IL-1α is a pivotal cytokine involved in periodontal disease and its regulation is a complex event governed by bacterial factors, host genetics and pharmacological agents. Clear differences in responses to different bacteria and specific combinations of bacteria may have particular implications for the understanding of disease aetiology and progression.

It is often difficult to assess SNP function on the basis of nucleotide sequence alone. This is particularly true when SNPs do not alter an amino acid or do not disrupt protein
function or structure (Rebbeck et al., 2004). The present results demonstrate that SNP I (-889) has a novel functional role in IL-1α regulation, even if it may not prove to be a risk determinant for aggressive periodontitis. An important feature of a complex disease like periodontitis is that most susceptible individuals are at elevated risk due to the combined effects of several susceptibility alleles and their interaction with confounding factors, such as smoking and the presence of a pathogenic flora. From the viewpoint of experimental design, further association studies are still required, with larger number of cases and controls in homogeneous populations, including analysis of all possible confounding factors. New methods are required to consider simultaneously interactions between genes and environmental factors. These approaches should be incorporated into SNP based associations in periodontal diseases. Such genetic information from those approaches would have a potential as diagnostic and prognostic biomarkers for periodontal disease susceptibility and possibly, for development of novel therapeutic targets aimed at preventing the development of periodontal disease.
APPENDIX I Standard buffers and reagents

**Luria Broth (LB)**

- 10 g bacto-tryptone
- 5 g bacto-yeast extract
- 10 g NaCl

Made up to 1L with dH₂O and pH adjusted to 7.2 and autoclaved.

**LB Agar**

- 15 g Bacto agar in 1L LB

**1.0% Agarose**

- 1 g of agarose in 100 ml of 1x TAE buffer. Heated in microwave to melt.

**Ethidium Bromide (10% EtBr)**

- 100 mg of EtBr in 1ml dH₂O. Stored in the dark.

**10x Tris Acetate EDTA (TAE)**

- 48.46 g (0.4 M) Tris base
- 3.72 g (0.01 M) EDTA-Na₂-salt
- 12.01 g (0.2 M) Glacial Acetic Acid

Made up to 1L with dH₂O. Used at 1X by diluting 1:10 in dH₂O and pH adjusted to 8.3.

**Phosphate buffered saline (PBS)**

- 5.84 g Sodium Chloride
- 11.5 g Di-sodium hydrogen orthophosphate
- 2.96 g Sodium dihydrogen orthophosphate

Made up to 1L with dH₂O and pH adjusted to 7.5
APPENDIX II ELISA ASSAY

Solutions required

PBS Phosphate Buffered Saline

Wash buffer- 0.005 % Tween 20 in PBS.

Block buffer- 1% Bovine Serum Albumin (BSA, >98% electrophoresis, Sigma), 5% Sucrose in PBS with 0.05% NaN₃

Reagent diluent- 1% BSA in PBS, 0.2μm filtered.

Substrate Solution- tetramethyl benzidine dihydrochloride (TMB, Sigma-Aldrich) substrate dissolved 0.1 M C₆H₄O₇, 0.1 M Na₂H, 0.00075 % H₂O₂

Stop Solution- 2N H₂SO₄

Reagent preparation

1. Dissolve 10 PBS tablets into 1.0 L of deionised water to prepare wash buffer and add 500μl of Tween 20

2. Prepare all solutions required (see above).

3. Reconstitute the IL-1α standard with 1.0 ml of deionised water. This reconstitution produces a stock solution of 50 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

4. Pipette 500 uL of reagent diluent into 8 tubes and prepare a dilution series. The 500 pg/mL serves as the high standard and reagent diluent serves as the zero standard.

Protocol

1. Add 100 μl anti-human IL-1α (2mg/ml) antibody in to each well for overnight at room temperature 100 μL Assay Diluent to each well.

2. Aspirate and wash 4 times

3. Add 300 μL block buffer to each well. Incubate for 1 hr on the shaker.

4. Aspirate and wash 4 times

5. Add 100 μL Standard or samples to each well. Incubate for 2 hrs at room temperate on a horizontal orbital shaker set at 500 rpm.

6. Aspirate and wash 4 times.

7. Add 100 μL biotinylated detection antibody to each well. Incubate for 2 hrs on the shaker.

8. Aspirate and wash 4 times

9. Add 100 μL Streptavidin-HRP to each well. Incubate for 20 min at room temperature protected from light.
10. Aspirate and wash 4 times
11. Add 100 μL substrate solution to each well. Incubate for 20 min at room temperature protected from light.
12. Add 50 μL stop solution to each well. Read at 450 nm within 30 min with a correction at 570 nm.

Figure 1 A standard curve was generated using a four parameter logistic (4-PL) curve-fit (Microsoft Office Excel) for each set of samples assayed. The values of the samples were assigned in relation to the standard curve.
APPENDIX III DNA extraction

Reagents

Solution 1
0.32 M Sucrose (filter sterilised)
10 mM Tris-Cl, pH 7.6 (sterile)
5 mM MgCl2 (sterile)
0.02 % Sodium Azide (sterile)
1 % Triton X-100
Sterile distilled deionised water to 500 ml

Solution 2
50 mM Tris-Cl, pH 8
20 mM EDTA, pH 8
2 % SDS
Sterile distilled water to 500 ml

Solution 3
Add NaCl to 300ml of sterile distilled deionised water keep adding salt until salt comes out of solution.

DNA Extraction method using salt precipitation

1. Place an aliquot of solution 1 of adequate size and some distilled water in the fridge to cool before use.
2. For blood samples of 2ml add distilled water to make up to 12.5 ml
3. Centrifuge at 2500/3000 revs/min for 15min at 15°
4. Remove supernatant by pouring gently to leave around 1ml (leave pellet plus pink layer).
5. Add distilled water up to a volume of 4ml and ensure pellet is dissolve (pipette up and down if needed to break up pellet) add solution1 up to a volume of 8 ml and then add distilled water up to a final volume of 12 ml.
6. Mix thoroughly to disperse pellet.
7. The above stages result in cell lysis
8. Incubate on ice for 2min (volume of 12ml)
9. Centrifuge at 2500/ 3000 rev/min at 4°C for 15min
10. Remove supernatant by pouring gently and saving pellet
11. Vortex to get pellet of tube.
Appendices

12. This pellet should appear light pink in colour. If a dark red pellet is present, repeat previous steps.

13. Resuspend the pellet in 2.5 ml of **solution 2** and vortex vigorously (ensure pellet broken up)

14. Add proteinase K to supernatant to yield a final concentration of 100mcg/ml. Incubate at 60° with shaking for 1 hr at 37°C or overnight.

15. Incubate on ice for 10min

16. add 1ml of **solution 3**

17. Mix by invert several times

18. Centrifuge at 2000/ 2500 revs/min for 15 min at 20°C

19. Transfer supernatant using a large bore pipette to a sterile tube which is correctly labelled. Precipitate DNA by adding two volumes of 100% ethanol (cool before use) to the supernatant

20. Invert the tube gently until the DNA precipitates a 'hairball'

21. This can be stored at −20 °C overnight if necessary

22. Spin at 2000 rev/min for 15 min

23. Remove ethanol

24. Resuspend the DNA in 500ul of TE, gently invert the tube.

25. DNA samples should be stored at 4°C.

![Figure Typical Spectrophotometer plot for DNA quantification](image-url)

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APPENDIX IV IL-1 A DNA sequence

Accession number: AF536338

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4021 GGTTGGGTGATATTCTAACCCAAGTTAGCTGTTTCTCAACCAAGTTCTCTTTGAAAAATT
4081 CAACAACCACCTTTGGGGAATTATTTACAACAGAGGAGTGAGGATGGGACCAGGATAGGT RS3783570 (-2037)
4141 ATTGCCTATGTTGGTGGAACCAGGGTTTTTTTCCTGGATTACCAAAGAGATGGTATGCAT
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5221 GCGAAGAAGACTGAC EXON I
5281 'AAAGGTATTOTCCTCACATCTCTGGCTATTAAAGTATTlý--ý-: --, -
5341 --. ýý-. -TT-CTCTTTGGCTGTTTTCTCTCACATTGCCTTCTCTAAAGCTACAGCCTCTCCT
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6721 CTGAAJGAACTGTTACAGGTAAGGAATAAGATTTATCTCTTGTGATTTAATGAGGGTTTCA
6781 EXON 2

Figure 2. The DNA sequence of the human IL-1A putative promoter. Yellow highlights indicate positions of single-nucleotide polymorphisms. Numbers in brackets indicate base positions with respect to the translation initiation site blue highlights indicate the translated region (exon 1). Red underlined sequences are both forward and reverse primers DNA sequence.
APPENDIX V MOLECULAR BIOLOGY PROTOCOLS

V.1 TOPO Cloning Reaction

Set-up

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Final Volume 6μl

V.2 Performing the TOPO Cloning Reaction

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).
2. The reaction was placed on ice and proceeded to transforming competent cells.

V.3 Chemical Transformation Protocol

1. 2 μl of the TOPO cloning reaction from the TOPO Cloning Reaction was added into a vial of One Shot Chemically Competent E. coli and mixed gently.
2. The cells were incubated on ice for 30 minutes.
3. The tubes were then placed in a 42°C water bath for 30 seconds exactly in order to heat shock cells then placed back on ice for 2 minutes. Then 250 μl of SOC broth was added to each tube which was placed in an Inova 4000 shaking incubator (New Brunswick Scientific) at 225 rpm for ~60 minutes at 37°C.
4. 10-50 μl of medium was then added to each pre-warmed plate which was evenly spread. The plates were then incubated upside down for 16 hours at 37°C and colonies viewed the next day.

IV.4 Isolation of DNA from agarose

MinElute Gel Extraction Kit, Quiagene was used to isolate PCR products from agarose gel.

1. After running the PCR product on an agarose gel, the DNA band was excised from the gel using a clean scalpel blade and placed into a 1.5ml centrifuge tube. The volume of the gel was then estimated by weight (1g = 1ml).
2. 3 volumes of binding buffer (Buffer QG) was added to the excised gel at a 1 volume equalling the volume of the gel. (100mg~100ml). The mixture was agitated gently and incubated in a water bath at 50°C for 10 minutes. The microfuge tube was vortexed every 2-3 minutes to help dissolve the gel.

3. After gel slice has dissolved completely, 1 gel volume of isopropanol was added and the mixture placed a MinElute column in a provided collection tube to bind DNA.

4. The DNA-matrix was pelleted by centrifugation at 13000 rpm in a benchtop minicentrifuge for 1 minute. Supernatant was discarded and the DNA/matrix washed in 500 μl binding buffer and centrifuged at 13,000 rpm for 1 minute.

5. The supernatant was discarded and the DNA-matrix washed twice further in 750μl wash buffer PE and centrifuged at 13,000rpm for 1 minute. The supernatant was removed

6. MinElute column centrifuged at 13,000rpm for additional 1 minute to remove residual ethanol from buffer PE.

7. MinElute column placed into a clean 1.5 ml microcentrifuge tube and 10 μl elution buffer (Buffer EB) was added and was incubated at RT for 1 minute and, centrifuged at 13,000rpm for 1 minute. The supernatant was removed again and placed into a fresh 0.5ml microfuge tube which was stored at -20°C until required.

8. The recovery of DNA was estimated by running on a 1% agarose gel and the concentration estimated by UV spectrophotometer (A260) where 50 μg/ml = 1 absorbance unit (Nanodrop, UK).

V.5 Ligation

| 4x QS ligase buffer | 5 μl |
| DNA to be ligated | 14 μl (100ng) |
| Quick -Stick ligase (3U/μl) | 1 μl |
| Total volume | 20 μl |

The above reaction mixture was incubated at RT for 5 minutes and stored at -20°C.

V.6 Pouring Luria-Bertani Broth (LB Broth)/Agar plate

1. The LB/Agar was melted using a microwave and left to cool to about 45°C.

2. When cool to touch, 800 μl of ampicillin (50 mg/ml) was added to the 400 ml of LB/Agar, 20 ml of which was poured per plate (9 cm diameter Sterilin). The top of the agar was then flamed to sterilize and remove air bubbles.

3. The agar plates were dried upside down in a 37°C incubator for 1 hour, then wrapped in cling film and stored at 4°C until required.
V. 7 Transformation of JM 109 Cells

1. 2 μl of ligation mixture was added into sterile Eppendorf (on ice) tubes followed by 50 μl of JM 109 competent cells (Promega).

2. The ligation-cell mixture was incubated on ice for 20 minutes. The tubes were then placed in a 42°C water bath for 47 seconds exactly to heat shock cells then placed back on ice for 2 minutes. Then 700 μl of 2% LB broth was added to each tube which was placed in an Inova 4000 shaking incubator (New Brunswick Scientific) at 225 rpm for ~60 minutes at 37°C.

3. 50 μl of medium was then added per plate which was evenly spread. The plates were then incubated upside down for 16 h at 37°C and colonies viewed the next day.

V. 8 Purification of Plasmid DNA from Bacterial cells

V. 8.1 Mini-Prep Bacterial Cell Culture

To each falcon tube containing 3 ml of sterile 2% LB broth, 6 μl of ampicillin (50 mg/ml) was added. Sterile pipette tips were then dabbed onto selected colonies and transferred into the LB broth. The falcon tubes were placed into a shaking incubator at 225 rpm, at 37°C for 16 hours.

QIA Spin Miniprep Kit DNA (Qiagen) was used.

1. The 3ml bacterial culture described above (mini-prep) was centrifuged at 4000rpm, 4°C for 15 minutes. The supernatant was then discarded and the falcon tubes blotted upside down on paper towel to remove excess media.

2. The pellet was re-suspended in 250 μl of cell re-suspension solution then the re-suspended cells were transferred to a 1.5 ml tube. The cells were then lysed by adding 250 μl of lysis solution and inverting the tubes four times.

3. 350 μl of neutralization solution was then added and the tube inverted four times. The lysate was then centrifuged at 13,000 rpm in a bench top microcentrifuge for 5 min.

4. The supernatant from above step was applied to QIA-prep Spin Column by decanting and centrifuged at 13,000 rpm in a bench top micro centrifuge for 1 minute.

5. The QIA-prep Spin Column was washed by adding 0.5 ml Buffer PB and centrifuged at 13,000 rpm in a bench top micro centrifuge for 1 minute.

6. The column was washed again by adding 0, 75 ml Buffer PE and centrifuged at 13,000 rpm for 30-60 s. The mini-column was then centrifuged at 13,000rpm in a bench top microcentrifuge for 2 minutes to dry the resin. The column was removed and transferred to a 1.5 ml micro- centrifuge tube.
7. 50 µl of dH2O was applied to the mini-column which was left for 1 minute. The DNA was then eluted by centrifuging the mini-column -microfuge tube assembly for 1 minute at 13,000 rpm. The purified plasmid DNA was stored at -20°C until required.

V.8.2 Maxi-prep Bacterial culture

Selected bacterial colonies were cultured in 6 ml of sterile 2% LB broth with 12 µl of ampicillin (50mg/ml) for 12 hours. The 6 ml culture was then poured into 500 ml of 2% LB broth with ampicillin (100µg/ml) and left overnight in a shaking incubator at 37°C, 225 rpm for no longer than 16 hours.

1. The 500 ml bacterial culture described above was divided into two 250 ml Beckman centrifuge tubes and centrifuged at 4,000 rpm for 15 minutes.
2. The bacterial pellet was then re-suspended in 10ml of buffer P1 containing RNAse A. Each bacterial suspension was transferred into a 40 ml centrifuge tube (Beckman). 10 ml of buffer P2 (lysis buffer) was then added to the bacterial suspension which was then mixed by inverting the centrifuge tube four times. The bacterial suspension was incubated at room temperature for 5 minutes.
3. 10 ml of chilled buffer P3 (precipitation buffer) was then added to the suspension which was mixed by inverting the centrifuge tube four times. The bacterial suspension was then incubated on ice for 20 minutes and centrifuged at 12,000 rpm for 30 minutes at 4°C.
4. A QIAGEN-tip 500 column was equilibrated by applying 10 ml of buffer QBT, (equilibration buffer) then allowing the column to empty by gravity flow. Both supernatants from the original maxi prep were then added to one QIAGEN-tip which was allowed to enter the resin by gravity flow.
5. The QIAGEN-tip was then washed twice with buffer QC and the DNA eluted with 15ml buffer QF. Elute was transferred to a 40ml tube and the DNA precipitated with 0.7 volume of iso-propanol at room temperature.
6. The precipitate was immediately centrifuged at 9,500 rpm at 4°C for 30 minutes. The supernatant was then discarded and the DNA washed twice with 5ml 70% ethanol.
7. The DNA was air dried for 5 minutes and re-suspended in 1ml distilled H2O. The purified plasmid DNA was stored at -20°C until required.
V.9. Restriction Digestion

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>x μl (2 μg)</td>
</tr>
<tr>
<td>Enzyme specific buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>0.5 μl (2-10U)</td>
</tr>
<tr>
<td>dH₂O</td>
<td>y μl</td>
</tr>
</tbody>
</table>

Total volume 20 μl

The above reaction was incubated at 37°C for 3 hours. Restriction products were viewed by agarose gel electrophoresis.

V.10. DNA precipitation

1. Sodium acetate was added to the DNA solution, in a 1.5 ml microfuge tube, at a ratio of 1 part DNA to 10 parts sodium acetate (pH 3.0) followed by a 2.5x volume of 100% alcohol.
2. The solution was left for 60 minutes at 4°C and centrifuged for 10 minutes at 13,000 rpm. The supernatant was then discarded and the pellet washed with 75% alcohol.
3. The solution was then re-centrifuged at 13,000 rpm for 5 minutes and the supernatant discarded leaving the pellet to dry.
4. The pellet was redissolved in a suitable volume of water or TE buffer, pH 8.0

V.11 Site-Directed Mutagenesis

V.11.1 Mutant Strand Synthesis Reaction

1. Prepare the sample reactions as indicated in Material and Methods Table 5.3.
2. Cycle each reaction using the cycling parameters outlined in Material and Methods Table 5.4
3. Following PCR, place the reaction was cooled on ice for 2 minutes and then subjected to restriction enzyme digestion.

V.11.2 DpnI digestion

1. Add 1 μl (10 U) of the Dpn I restriction enzyme directly to the PCR reaction
2. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 2 hours to allow complete cleavage of digest the nonmutated supercoiled DNA.
V.11.3 Transformation

1. Gently thaw *E. coli* cells on ice and aliquot 50 μl of cells to a prechilled 14-ml polypropylene round-bottom tube (BD Falcon).
2. Transform competent *E. coli* with 1 ml of *DpnI*-treated DNA.
3. Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 minutes.
4. Heat pulse the transformation reactions for 45 seconds at 42°C and then place the reactions on ice for 2 minutes.
5. Add 0.5 ml of LB broth preheated to 42°C and incubate the transformation reactions at 37°C for 1 hour with shaking at 225–250 rpm.
6. Plate between 10 and 50 μl of each transformation reaction on ampicillin agar plates (10 mg/ml).
7. Incubate the transformation plates at 37°C for 16 hours.

The desired mutations were verified by sequencing the constructs as detailed in sequencing section (V.14).

V.12 Luciferase Reporter Assay

V.12.1 Protocol for Preparing Cell Lysates

1. Lysis buffer was diluted from 5X stock and equilibrated to room temperature.
2. The growth medium was removed from cells and then cells were rinsed with PBS. 200 μl of 1X lysis buffer was added to cover the cells and incubated at RT for 30 minutes.
3. Attached cells were removed from the dish using a scraper and cells were transferred to a microcentrifuge tube.
4. Tubes were placed on ice for 10 minutes and the microcentrifuge tubes were vortexed 10–15 seconds, and then centrifuged at 12,000 x g for 15 seconds at RT.
5. The supernatants were transferred new tubes and stored at –70°C until used.

V.12.12 Luciferase Assay Protocol

1. The samples were taken out and adjusted to room temperature before experiments. 20μl of cell lysate were added to each well and mixed with 100μl of Luciferase Assay Reagent.
2. The luciferase activity was measured in relative light units with a luminometer. The values determined were subsequently standardized to the amount of input protein and are indicated as relative light units per microgram of protein.
V.13 β-Galactosidase Assay

-Z- buffer:
10mM KCl
60mM Na₂HPO₄
40mM NaH₂PO₄
1mM MgSO₄
50mM β-mercaptoethanol
Adjust pH to 8 with NaOH at 25°C.
ONPG (4mg/ml in Z buffer)
1 M Na₂CO₃

1. 50 μl of cell extract was added to 250 μl of Z-buffer. 50 μl of ONPG (ortho-nitrophenyl-beta-D-galactopyranoside) was added to mixture and vortexed briefly.
2. Samples were incubated at 37°C for 30 minutes or until a faint yellow color has developed and the reaction was stopped by adding 250 μl of 1M sodium carbonate and mixed by vortexing briefly.
3. Optically clear 96-well plates were used to read in a plate reader. 100 μl of mixture was added to each well and absorbance of samples was read at 405nm in a plate reader.

V.14 Direct sequencing

1. Exosap 1.5 μl in each well
   a. PCR product 3.5 μl
   b. Total volume = 5 μl
2. Cover
3. Spin
4. PCR machine - GC, Exosap (programme), 10 μl (min vol), heated lid, approx 37 min
5. Make up Big dye master mix;
   a. 1 μl Bigdye
   b. 1 μl water
   c. 3 μl better buffer
6. 5 μl of this mix per sample
7. Add 1 μl of primer (forward or reverse)
8. Cover and spin
9. PCR machine - GC, BigDye programme, 10 μl, appox 2.46 hrs.
10. Ethanol/ammonium acetate solution - 60 μl per well
    a. Invert several times to mix and centrifuge at 3100 rcf, 4°C 30 min
11. Prepare spreadsheet for 3700
12. Add Cold 70% ethanol – 100 μl per well and spin for 2 min at 3100 rcf at 4°C
13. Repeat step 12
14. Remove ethanol spin for 1 min at 250 rcf at 4°C
15. Leave to dry 10 min
16. Resuspend in 10 μl water
17. Into fresh microplate add 8 μl hydide formamide
18. Transfer 10 μl of sample into microplate and mix by pipetting
19. Spin for 2 min 3100 rcf
20. Place in 3700 in holder and run for 4 hours

DNA sequences obtained were analysed for sequence identity with known human genomic DNA sequences using the BLAST online search engine (http://www.ncbi.nlm.nih.gov/BLAST). Below figure shows an example of BLAST sequence match obtained.

V.15 Example of an electropherogram obtained form sequencing PCR or plasmids

![Figure 3 Part of an electropherogram of DNA sequenced from a PCR product](image)

V.16 Example of BLAST search results obtained from plasmid sequences

Reference

RID: 1172009972-32520-153901653553.BLASTQ3

**Database:** All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, environmental samples or phase 0, 1 or 2 HTGS sequences) 5,001,276 sequences; 19,821,175,605 total letters

**Taxonomy report**

Distribution of 38 Blast Hits

Sequences producing significant alignments:

<table>
<thead>
<tr>
<th>Score E (bits)Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1423 0.0</td>
<td>Homo sapiens interleukin 1, alpha</td>
</tr>
<tr>
<td>1423 0.0</td>
<td>Homo sapiens BAC clone RP11-67L14</td>
</tr>
<tr>
<td>1409 0.0</td>
<td>TPA: TPA_exp: Homo sapiens</td>
</tr>
<tr>
<td>1409 0.0</td>
<td>Homo sapiens BAC clone RP11-285D1</td>
</tr>
<tr>
<td>165 3e-37</td>
<td>Human gene for interleukin 1 alpha</td>
</tr>
<tr>
<td>107 7e-20</td>
<td>Mus musculus interleukin-l alpha gene</td>
</tr>
</tbody>
</table>

Alignments

>gi|22385323|gb|AF536338.1| Homo sapiens interleukin 1, alpha (IL1A)
gene, complete cds
Length=18446

Score = 1423 bits (718), Expect = 0.0
Identities = 788/799 (98%), Gaps = 5/799 (0%)
Strand=Plus/Plus

AAACAATGCAAGGACCCACCTCTCTCTCTGAACCTCACCCACCCTAGTTTTCC

AAACAATGCAAGGACCCACCTCTCTCTCTGAACCTCACCCACCCTAGTTTTCC
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228


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243


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253


