Periradicular Disease (PRD):

Development of a Novel Explant Culture Model

to Investigate

the Cytokine Network

by

Colin Alexander Murray

B.D.S., F.D.S., R.C.S. (Edin), F.D.S.(Rest. Dent.) R.C.S. (Edin)

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Infection and Immunity Section,

University of Glasgow Dental School and Hospital,

University of Glasgow

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 6

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This thesis is the original work of the author.

Colin Alexander Murray

PREFACE

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(2004) Evidence for Interleukin 18 (IL-18) as a novel mediator in chronic periradicular disease. *Journal of Dental Research* **82,** C-506.

B Scientific meetings

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ABBREVIATIONS

°C	Degrees Centigrade
ACJ	Amelocemental junction
Ag	Antigen
APC	Antigen presenting cell(s)
ATCC	American Type Culture Collection
bp	Base pairs
BSA	Bovine serum albumin
CD	Cluster of differentiation antigen(s)
cDNA	Complimentary deoxyribonucleic acid
CFU	Colony forming units
CIA	Collagen induced arthritis
CO_2	Carbon dioxide
dH_20	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	2'deoxynucleotide 5' triphosphates
DMEM	Dulbecco's Modified Eagle's Medium
E.coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ENA-78	Epithelial-neutrophil activating protein
FCS	Foetal calf serum
FCγR	Fc gamma receptors on phagocytes
FFPE	Formalin fixed paraffin embedded
F. nucleatum	*Fusobacterium nucleatum*
g	Gram(s)
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H&E	Haematoxylin and eosin
H_2O_2	Hydrogen Peroxide
HRP	Horse radish peroxidase
HSP	Heat shock proteins
Ig	Immunoglobulin

ICAM-1	Intercellular adhesion molecule-1
IL-	Interleukin
IFN-γ	Interferon gamma
iNOS	Inducible nitric oxide synthesase
IRAK	Interleukin-1 (IL-1) receptor-associated kinase
JAK	Janus kinase
kDa	Kilodalton
kg	Kilogram(s)
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
M-CSF	Macrophage colony-stimulating factor
MCP-1	Monocyte chemoattractant protein-1
MGSA	Melanoma growth stimulator activity
MHC	Major histocompatability complex
MIP	Macrophage inhibitory protein
ml	Millilitre(s)
MMP	Matrix metalloproteinase(s)
mRNA	Message ribonucleic acid
N/A	Not applicable
NCTC	The National collection of type cultures
NFATc1	Nuclear factor of activated T cell transcription factor-c1
NFκB	Nuclear factor κB
ng/ml	Nanograms per millilitre
NK	Natural killer cell
No.	Number
OD	Optical density
OPG	Osteoprotegerin
pg/ml	Picograms per millilitre
PBMC	Peripheral blood mononuclear cell(s)
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PHA	Phytohemagglutinin
PCR	Polymerase chain reaction
P. intermedia	*Prevotella intermedia*

P. micros	*Peptostreptococcus micros*
PMN	Polymorphonuclear leukocyte(s)
PRD	Periradicular disease
PRS	Periradicular surgery
RA	Rheumatoid arthritis
RCT	Root canal treatment
rh	Recombinant human
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SAC	*Staphylococcus aureus* Cowan strain
SDS	Sodium dodecyl sulphate
SEB	*Staphylococcal* enterotoxin B
SEM	Standard error mean
SLF	Synovial-like fibroblasts
RANKL	Receptor activator of nuclear factor kappa B Ligand
RT-PCR	Reverse transcription polymerase chain reaction
Th1	$CD4^+$ effector T Helper 1 subset
Th2	$CD4^+$ effector T Helper 2 subset
TCR	T cell receptor
TGF-	Transforming growth factor
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF-α	Tumour necrosis factor- alpha
TRAF-6	TNF receptor-associated factor-6
VCAM	Vascular cell adhesion molecule
μl	Microlitre(s)
μg	Microgram(s)

SUMMARY

Periradicular disease (PRD), a localised chronic pathologic inflammatory reaction in response to continuous microbial stimuli from necrotic, infected dental root canals, represents a substantial health care burden. The efficacy of available therapies is sub-optimal and identification of new therapeutic targets is essential. Elucidation of functional interactions between PRD cell populations and tissue matrix and between PRD lesion and cells within the surrounding dentoalveolar bone matrix is prerequisite to this. ***I hypothesise that the cytokine milieu is central in orchestrating these interactions.*** I generated a novel human explant tissue culture system to investigate the pathogenesis of PRD. I aimed: (1) to investigate the expression of multiple cytokines, but particularly IL-18, within the human lesion and to elucidate their likely biological contribution towards PRD; (2) to investigate the presence of and functional interactions between inflammatory mediators within human PRD that influence bone homeostasis; and (3) to phenotype the contribution of the PRD fibroblast. Four hundred and fifty patients were recruited after obtaining informed consent. PRD tissue was obtained for investigations, of which 310 specimens were examined in a novel explant culture system. Endogenous cytokine release was readily detected *in vitro* confirming significant inflammatory activity within chronic PRD and facilitating a detailed analysis for the first time of the complex interactions between cytokine activities in PRD. Key findings are summarised as follows:

Responses to microbial ligands: TLR-1, -2, -4 and -6 mRNA expression was readily detectable within human PRD tissue biopsies. LPS (a TLR4 ligand) significantly increased expression of IL-1β and IFN-γ and moderately increased levels of TNF-α and IL-10 whereas SEB (acting primarily via TLR2) substantially increased IL-17A, TNF-α and IFN-γ levels.

Role of IL-18 in regulating cytokine responses: Factors regulating IFN-γ expression are of considerable importance as they may offer targets to modify $CD4^+$ effector Th_1 responses in the clinical setting. IL-18 significantly increased IFN-γ, IL-17A, IL-12, IL-1β and TNF-α expression. IL-18 in combination with IL-12 synergistically induced local expression of IFN-γ. Neutralisation experiments provided strong proof of concept that within PRD, IL-18 has an important effector role in host responses to pathogenic microorganisms. The precise contribution of $CD4^+$ effector Th_1/Th_2 responses to PRD lesion development is controversial. My data strongly support the hypothesis that PRD is Th_1 driven. Moreover,

although endogenous IL-18 may be required for the abolition of pathogens, in Th_1 dominated responses such as PRD, it likely leads to tissue destruction.

Additional cytokine activities in PRD: I extended my studies to include a panel of cytokines implicated in various chronic inflammatory diseases. IL-15 substantially increased IL-17A and IFN-γ and moderately decreased IL-10 expression. IL-17A also increased the expression of IL-8, TNF-α and IFN-γ within PRD explants. These data suggest upstream regulatory roles for IL-15 and IL-17 in PRD. Moreover, the recent description of Th_{17} cell lineages suggests that PRD may be driven not only via Th_1 but also by Th_{17} effector cells. In contrast, few anti-inflammatory cytokines were detected aside from IL-10 which although spontaneously expressed and occasionally modified by other cytokines clearly was not sufficient to regulate local responses.

A role for fibroblast activation in PRD: There is increasing interest in the contribution of tissue matrix cells in local inflammatory responses. Microbial products, live endodontic pathogens and cytokines (e.g. IL-17A, IL-18, TNF-α or IFN-γ) significantly increased IL-6 and/ or IL-8 within PRD fibroblast cultures.

Effects on bone modulatory cytokines: IFN-γ up-regulated RANKL expression and decreased OPG secretion within matched PRD explant cultures. IFN-γ could indirectly contribute towards the resorption of adjacent periradicular bone. Furthermore, IL-17A and TNF-α substantially increased RANKL and decreased OPG secretion within PRD explant cultures. Concomitant IFN-γ, IL-17A and TNF-α secretion, which were intrinsically expressed within unstimulated PRD cultures, likely promotes osteoclastogenesis leading to periradicular bone destruction.

In summary, I have developed a novel explant culture system that has defined the role of a variety of cytokines to pathogenesis for the first time in PRD. Clinical studies should now extrapolate inflammatory processes observed within *ex vivo* human PRD tissue to specific clinical signs symptoms and progression. These will in turn contribute to development of biomarkers for PRD progression and ultimately novel therapeutic opportunities.

CHAPTER 1

PERIRADICULAR DISEASE (PRD)

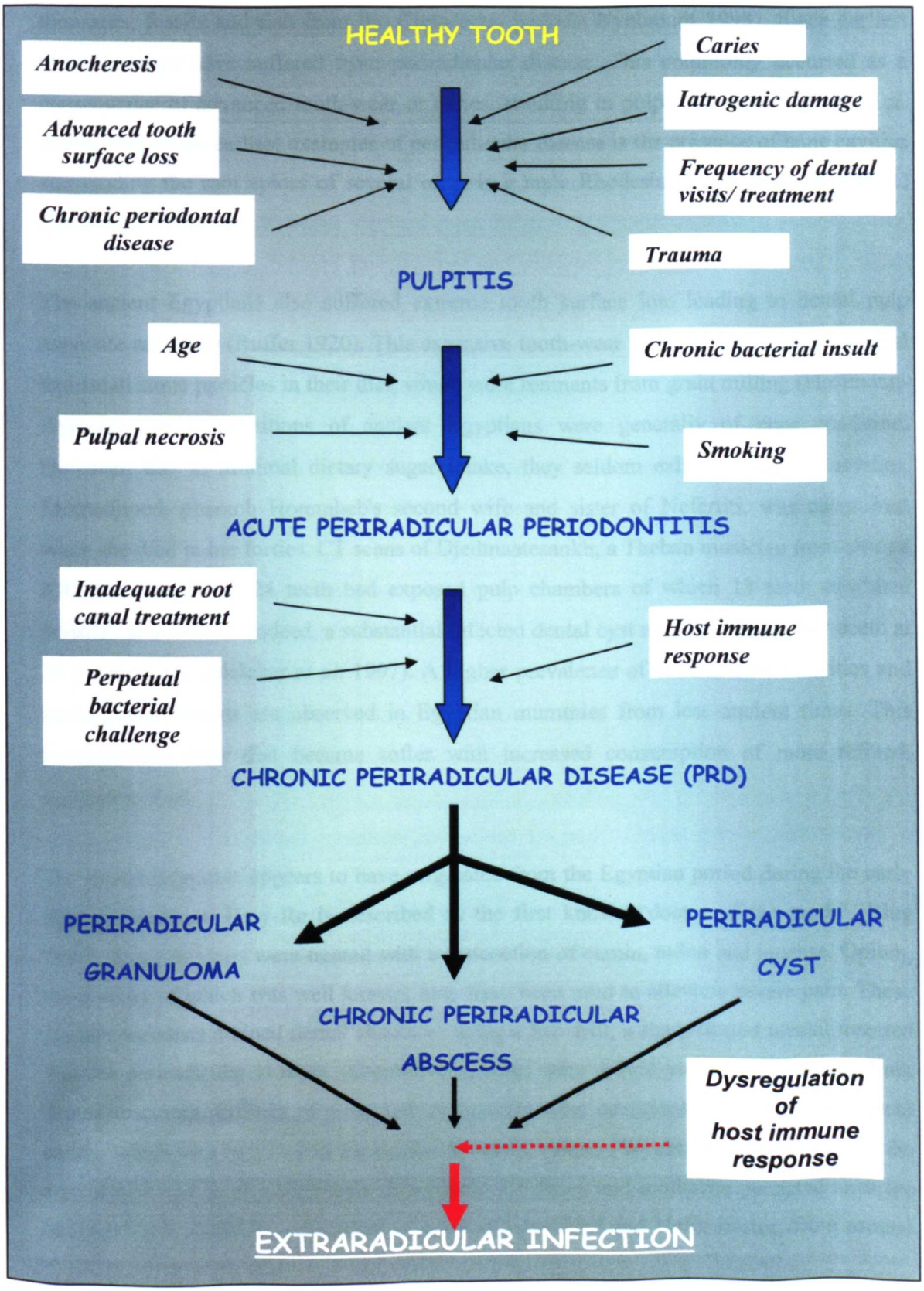
1 PERIRADICULAR DISEASE (PRD)

1.1 Introduction

Bacterial infiltration of the dental root canal system as a consequence of dental caries, iatrogenic trauma, advanced periodontal disease or traumatic tooth fracture evokes an inflammatory and immune response within the dental pulp. Persisting pathogenic challenge to the pulp culminates in dental pulp necrosis. The outcome of this inflammatory and immune response is apical periodontitis, whereby dento-alveolar bone destruction occurs at the dental root apex (Márton and Kiss 2000). Although generally localised, dysregulation of this chronic inflammatory process may lead to systemic dissemination of pathogens, resulting in extensive morbidity and potential mortality (Hollin *et al.* 1967, Currie and Ho 1993, Pappa and Jones 2005).

A number of pathogenic bacteria are implicated in eliciting this inflammatory reaction. These microorganisms are principally anaerobic and initially Gram-negative (Sundqvist 1994). Microorganisms as the aetiological agents of periradicular disease (PRD) have been established by studies demonstrating that in microbial free conditions, PRD does not develop (Kakehashi *et al.* 1965, Möller *et al.* 1981). These endodontic pathogens are responsible for instigating non-specific and antigenic-specific host immune responses. Initially, an inflammatory immune response occurs within the dental pulp and subsequently, the periradicular tissues (Yanagisawa 1980). Perpetuation of this chronic inflammatory reaction leads to the development of periradicular disease comprising periradicular granulomas, abscesses and periradicular bone cysts (Figure 1.1). Periradicular lesions comprise the most common type of osteolytic lesion within the facial skeleton. Moreover, PRD constitutes a substantial oral health disorder affecting a significant proportion of the population. It is, therefore, surprising that the specific aetiological agents of induction and the contribution of cell populations and growth factors associated with the perpetuation and resolution of PRD are still poorly understood (Figdor 2002).

Figure 1.1 **Diagrammatic illustration summarising a proposed cascade of events ultimately leading to chronic periradicular disease.**



1.2 Historical perspective

Dental diseases, in the form of caries and periodontal disease, have been demonstrated in dinosaurs, fossils and fish from the Cretaceous period (Wynbrandt 1998). Since earliest times, humans have suffered from periradicular disease. This commonly occurred as a consequence of advanced tooth-wear or caries, resulting in pulpal necrosis (Chazel *et al.* 2005). One of the earliest examples of periradicular disease is the presence of bone cavities surrounding the root apices of several teeth in a male Rhodesian skull dated 50,000 BC (Brothwell 1972).

The ancient Egyptians also suffered extreme tooth surface loss leading to dental pulp exposure and PRD (Ruffer 1920). This excessive tooth-wear was probably caused by sand and small stone particles in their diet, which were remnants from grain milling (Hoffmann-Axthelm 1981). Dentitions of ancient Egyptians were generally of poor condition. However, due to minimal dietary sugar intake, they seldom exhibited carious cavities. Mutnodjmed, pharaoh Horemheb's second wife and sister of Nefertiti, was edentulous when she died in her forties. CT scans of Djedmaatesankh, a Theban musician from around 850 BC, reveal that 24 teeth had exposed pulp chambers of which 13 teeth exhibited periradicular lesions. Indeed, a substantial infected dental cyst may have led to her death at 35 years of age (Melcher *et al.* 1997). A higher prevalence of carious dental cavities and periradicular lesions are observed in Egyptian mummies from less ancient times. This suggests that their diet became softer with increased consumption of more refined, cariogenic food.

The dental physician appears to have originated from the Egyptian period during the early third millennium. Hesy-Re is described as the first known "doctor of the tooth" (Ring 1985). Swollen gums were treated with a concoction of cumin, onion and incense. Opium, the toxicity of which was well known, may have been used to alleviate severe pain. These dental specialists drained dental abscesses using a fire-drill, a sharp heated utensil inserted into the periradicular abscess. Alternatively, holes were drilled into the jawbone to drain dental abscesses. Fillings of resin and chrysocolla were occasionally used to treat dental caries, which was believed to be caused by tooth worms (Wynbrandt 1998). Indeed, the supposition that toothworms were responsible for decay and toothache persisted until the Middle-Ages. Artificial teeth were occasionally provided and Nefer-ir-etes, from around

2600 BC, is mentioned as a maker of teeth. Dental extractions, which might have saved the lives of many a patient, were rarely if ever practised.

Dentistry, as a medical specialty, is otherwise seldom mentioned until the Graeco-Roman Period. Hippocrates attached significant importance to diseases of the dental system and invented crude dental forceps for dental extractions (Wynbrandt 1998). Up to the 12th century, carious cavities, periodontal diseases and misaligned teeth were common. No attempt was made to prevent these from occurring, and few remedies were available. However, by the Middle-Ages, cavities were being filled with waxes and resins. These materials were later succeeded by gold leaf and/ or lead in the mid-15th century. Teeth with symptomatic periradicular disease were treated by extraction rather than attempting to eradicate the periradicular infection and retain the functional dentition.

With the discovery of bacteria within necrotic root canals (Miller 1894), root canal treatment and the discipline of endodontics started to flourish in the late 19th and early 20th Centuries. Attempts to endodontically treat necrotic teeth associated with periradicular disease finally began to emerge as a routine form of dental treatment. At the beginning of the 1900s, the adoption of the 'focal infection theory' had a dramatic effect upon the medical and dental professions (Murray and Saunders 2000, Appendix 1). In order to eradicate 'oral sources' of systemic diseases, teeth with periradicular disease were once again extracted in preference over treatment to retain them. Consequently, the evolution of root canal treatment and progression of endodontic studies were hindered. It was not until the second half of the 20th century that root canal treatment and periradicular surgery re-emerged as the established treatment modalities for periradicular disease as opposed to dental extractions (Murray and Saunders 2000).

1.3 The healthy dental apparatus

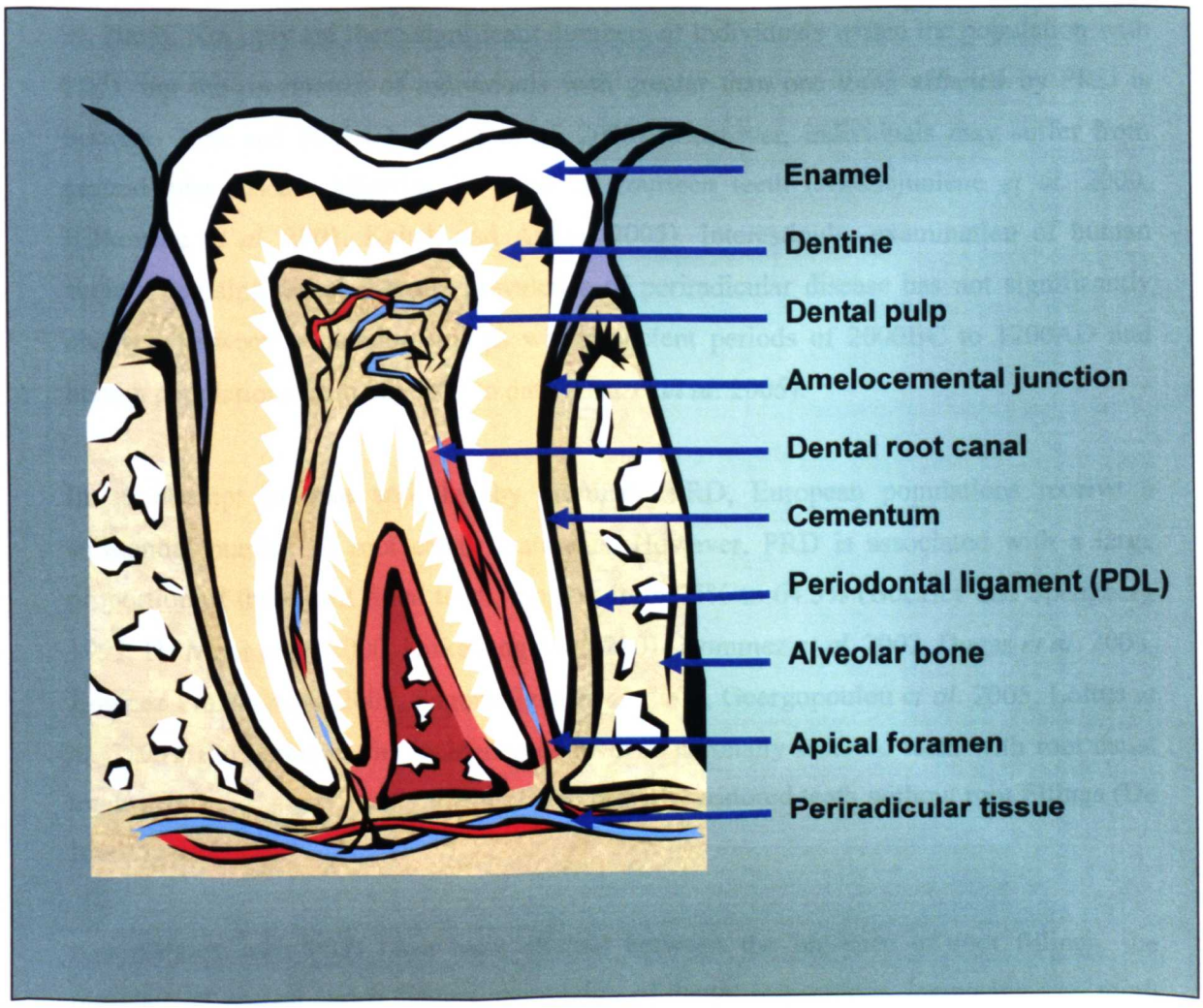
The healthy tooth contains an intact outer layer of enamel on the coronal occluding aspect. This overlies dentine, which encases the dental pulp residing within the dental pulp chamber and dental root canal (Figure 1.2). The pulp tissue is mesenchymal in origin with specialised odontoblast cells lining the periphery in close contact with the surrounding dentine (Garberoglio and Brannstrom 1976). The dental pulp consists of several tissue elements. These include myelinated and unmyelinated nerve fibres, vascular tissue,

connective tissue fibres, ground substance, interstitial fluid, and cellular components (Olgart and Bergenholtz 2003). Fibroblasts are the most numerous cells within the healthy pulp but macrophages, dendritic cells and T lymphocytes are also present (Jontell *et al.* 1998). B cells are extremely rare (Jontell *et al.* 1987) and mast cells are normally absent (Pulver *et al.* 1977).

The dental pulp communicates directly with the periodontal ligament (PDL) primarily through the apical foramen but also lateral canals and multiple foramina. The pulpal tissue within the dental root canal is encased by root dentine. This root dentine has an outer surface covering of cementum, which itself is covered by the periodontal ligament. The PDL consists of collagen fibres that are embedded into the cementum on the root surface and inserted into the adjacent alveolar bone, termed Sharpey's fibres. Thereby, the tooth is attached to the alveolar bone and retained within its socket. The predominant cell within the PDL is the fibroblast, this cell-type being responsible for the construction and maintenance of these collagen fibres (Mjör and Heyeraas 1998). In contrast, few macrophages and lymphocytes are seen within the healthy periodontal ligament.

Clinically, the healthy tooth is not tender to percussion and no tenderness is present when palpating the oral mucosa adjacent to the periradicular area. There is an absence of mucosal swelling and the patient is unaware of any symptoms. On radiographs, the lamina dura is found intact and the periodontal ligament space has a normal and consistent width around the entirety of the root. Furthermore, the width of the periodontal ligament space should be of similar dimensions to those of the adjacent teeth.

Figure 1.2 **Diagrammatic illustration of the anatomy of the healthy dental apparatus.**



1.4 Prevalence of PRD

Chronic apical periodontitis often develops without any subjective clinical symptoms from the patient. Therefore, intra-oral radiography plays an important role in establishing the presence of the PRD lesion. Several radiographic investigations within different population groups have been undertaken to elucidate the prevalence of PRD. These studies ascertain that PRD occurs within 33-80% of the adult population (Saunders *et al.* 1997, Aleksejuniene *et al.* 2000, De Moor *et al.* 2000, Kirkevang *et al.* 2001, Jiménez-Pinzón *et al.* 2003, Segura-Egea *et al.* 2004, Loftus *et al.* 2005, Kabak and Abbott 2005, Tsuneishi *et al.* 2005). Not only are there significant numbers of individuals within the population with PRD, but the prevalence of individuals with greater than one tooth affected by PRD is between 35% and 80% (De Moor *et al.* 2000). Moreover, individuals may suffer from periradicular disease affecting as many as fourteen teeth (Aleksejuniene *et al.* 2000, Kirkevang *et al.* 2001, Kabak and Abbott 2005). Interestingly, examination of human skeletal remains reveal that the prevalence of periradicular disease has not significantly changed between population groups within ancient periods of 2000BC to 1200AD and human populations from 1500AD to date (Chazel *et al.* 2005).

In an attempt to treat and thereby eliminate PRD, European populations receive a substantial number of root canal treatments. However, PRD is associated with a large proportion of these root filled teeth, ranging from 25% to 64.5% (Buckley and Spångberg 1995, De Moor *et al.* 2000, Kirkevang *et al.* 2001, Hommez *et al.* 2002, Dugas *et al.* 2003, Jiménez-Pinzón *et al.* 2003, Segura-Egea *et al.* 2004, Georgopoulou *et al.* 2005, Loftus *et al.* 2005). Furthermore, the incidence of PRD in coronally restored teeth with root canal treatment is also significantly greater than coronally restored teeth without root fillings (De Moor *et al.* 2000).

Associations with PRD have been elicited between the presence of root fillings, the presence of several caries lesions, the quality of dental restorations, increasing age, tooth type, higher frequency of dental visits, type II diabetes and smoking (Aleksejuniene *et al.* 2000, Kirkevang and Wenzel 2003, Jiménez-Pinzón *et al.* 2003, Kirkevang *et al.* 2004, Georgopoulou *et al.* 2005, Segura-Egea *et al.* 2005, Kabak and Abbott 2005). Nevertheless, others have not corroborated the latter finding (Bergström *et al.* 2004, Marending *et al.* 2005) or a relationship with age (Dammaschke *et al.* 2003, Frisk and

Hakeberg 2005). It can be concluded from these studies that PRD is a highly prevalent disease. Taken together, these recent investigations suggest that despite current advances in improving the oral health care of populations, the incidence of PRD within many geographic populations may actually be increasing rather than diminishing. In addition, current clinical endodontic procedures undertaken to eliminate the disease can be considered as sub-optimal.

1.5 Aetiology of PRD

1.5.1 Microflora within the infected dental root canal

It has long been established that bacteria propagate within root canals of teeth with necrotic pulps (Miller 1894, Macdonald *et al.* 1957, Winkler 1959, Shovelton and Sideway 1960). The involvement of bacterial infection in the initiation of PRD has been verified using animal models (Kakehashi *et al.* 1965, Möller *et al.* 1981). Bacterial induction of PRD has been more recently investigated in mice lacking Toll-like receptor (TLR)-4 expression. TLR-4 is a key receptor involved in the recognition and promotion of cellular responses to Gram-negative microorganisms (see Chapter 2.4). On pulp exposure to endodontic pathogens, TLR-4 deficient mice display reduced expression of essential inflammatory cytokine mediators. Furthermore, TLR-4 knockout mice suffer less infection-induced periradicular bone resorption than wild type controls (Hou *et al.* 2000a). However, the activation of LPS/TLR-4-independent mechanisms through endogenous ligands, for example heat shock protein 60 (Hsp60) (Ohashi *et al.* 2000), necrotic cellular products (Li *et al.* 2001), cytokines (Seibl *et al.* 2003) and extracellular degradation products may also contribute to lesion development.

The microflora of the infected root canal has been studied extensively (Sundqvist 1994). Following dental pulp necrosis, the microenvironment of the root canal system encourages the colonisation and proliferation of microbes. These microorganisms form complex polymicrobial populations (Fabricius *et al.* 1981, Sundqvist 1992b, Siqueira *et al.* 2004, Gomes *et al.* 2004). The negative and positive interactions between the multitudes of bacterial species combined with environmental selective pressures leads to the establishment of distinctive microbial populations within the infected canal (Grenier and Mayrand 1986, Sundqvist 1992a, Chávez de Paz *et al.* 2004). To date, over 300 different

microbial species have been identified from infected root canals (Sundqvist 1992a, Siqueira 2002). These microorganisms essentially conjugate in combinations of 4 to 7 species within the root canal (Molander *et al.* 1998, Siqueira *et al.* 2000). Theoretically, any one of these species has the potential to be an endodontic pathogen. However, a restricted set of 15 to 30 bacterial species/ phylotypes is most frequently identified from infected canals (Siqueira 2002, summarised in Table 1.2). Microbes within this set can predominantly be classed into six phyla that comprise *Spirochaetes*, *Fusobacteria*, *Actinobacteria*, *Firmicutes*, *Proteobacteria* and *Bacteroides* (Siqueira and Rôças 2005).

The microbial population within the infected dental root canal appears to vary between differing clinical situations (Siqueira 2002). In necrotic untreated cases, there is a preponderance of Gram-negative anaerobic bacteria within the infected root canal (Machado de Oliveira *et al.* 2000, Gomes *et al.* 2004). In contrast, the microbial flora of teeth with failed root canal treatment (RCT) and persisting root canal infection comprise bacteria with a mixed and predominantly Gram-positive anaerobic profile (Siren *et al.* 1997, Sundqvist *et al.* 1998, Pinheiro *et al.* 2003, Gomes *et al.* 2004). In these non-healing cases, *Enterococcus faecalis* emerges as one of the most prominent bacterial isolates (Sundqvist *et al.* 1998, Molander *et al.* 1998, Hancock *et al.* 2001, Siqueira and Róças 2004). However, the precise role of bacteria leading to the failure of root canal treatment or specific clinical symptoms is ambiguous. Others have either failed to detect *E. faecalis* in non-healing endodontic cases or have identified *E. faecalis* within only a few individuals (Rolph *et al.* 2001, Fouad *et al.* 2005). Differences in the isolation of *E. faecalis* and other suspected pathogens from root canals by various groups may be the consequence of differences in the geographic populations studied (Siqueira *et al.* 2005a), differences in the identification techniques utilised or differing sample sizes.

Certain bacterial species and bacterial associations within the root canal appear to result in differing clinical symptoms and conditions (Yoshida *et al.* 1987, Gomes *et al.* 1996, Foschi *et al.* 2005). Teeth lacking clinical symptoms appear to have lower bacterial growth from root canal isolates whilst anaerobic bacteria from the formerly termed *Bacteroides* species are associated with acute symptoms (Yoshida *et al.* 1987, Jacinto *et al.* 2003). Furthermore, *Fusobacterium nucleatum* has been associated with the development of the most severe forms of endodontic symptoms during root canal treatment (Chávez de Paz 2002). The microorganisms implicated in PRD produce a variety of virulence factors that

may contribute to their pathogenicity. These factors include proteolytic enzymes, cytotoxins, haemolysins, Gram-negative bacterial endotoxins and Gram-positive bacterial exotoxins (Socransky and Haffajee 1991).

Of all the bacterial-derived moieties, lipopolysaccharide (LPS) derived from Gram-negative bacteria plays a fundamental role in pulpal disease (Khabbaz *et al.* 2001) and the subsequent development of PRD (Nelson-Filho *et al.* 2002). Indeed, LPS contributes towards periradicular bone destruction (Pitts *et al.* 1982, Mattison *et al.* 1987) and LPS concentrations within the dental root canal correlate with the presence of periradicular lesions (Schein and Schilder 1975, Dahlen and Bergenholtz 1980, Horiba *et al.* 1991). Furthermore, LPS is detectable within the PRD lesion (Schonfeld *et al.* 1982) and its levels are increased within the periradicular tissue after infection of the pulp tissue (Yamasaki *et al.* 1992). Moreover, the administration of polymyxin B (PMB) into the rat PRD model has been shown to significantly reduce the amount of lesion-associated bone resorption by approximately 80% (Hong *et al.* 2004).

Nevertheless, others have demonstrated that C3H/HeJ mice, which are hyporesponsive to LPS, do not exhibit any differences in the size of established PRD lesions compared to wild type controls (Fouad and Acosta 2001). Furthermore, the addition of polymyxin B to extracts of rat (Wang & Stashenko 1993a) or human (Wang & Stashenko 1993b) periradicular lesions has no appreciable effect on the bone resorbing capacity of PRD explant tissues. Taken together, these data suggest that although LPS is implicated in PRD lesion development, cell wall components derived from Gram-positive bacteria and/ or other virulence factors, including fimbriae and lipoprotein, may also contribute towards the development of PRD. Indeed, the effects of LPS from endodontic pathogens may not be due to direct actions on bone-derived cells. More importantly, LPS may indirectly stimulate bone resorption by inducing cytokine expression within inflammatory cells (Wang & Stashenko 1991, Stashenko & Wang 1992). In response to the insult from these infecting pathogens and their pathogen associated molecular patterns (PAMPS), host defensive reactions within the dental pulp and periradicular tissue are activated to prevent further infection. Thereby, these virulence factors induce the expression of locally produced inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1 that results in the activation and recruitment of osteoclasts. This subsequently leads to destruction of the surrounding periradicular bone (Dewhirst *et al.* 1985). Although these

immune and inflammatory events are essential for host defence against bacterial challenge, an excessive and prolonged response is damaging to the periodontal ligament (PDL) and local periradicular tissue.

1.5.2 Microflora of the PRD lesion

There is controversy as to whether the periradicular lesion *per se* is sterile or whether microorganisms invade and inhabit the inflamed tissue (Nair 1997). Early studies provided evidence that PRD lesions contained significant numbers of bacteria (Stewart 1947, Hedman 1951, Winkler *et al.* 1972). Nonetheless, others were not able to corroborate these findings (Block *et al.* 1976, Langeland *et al.* 1977). Recent investigations have reinforced the conjecture that PRD tissue contains bacteria (Sunde *et al.* 2000, Hren *et al.* 2000, Chan *et al.* 2004). Moreover, when using conventional culture techniques, these bacteria are established as being viable (Tronstad *et al.* 1987, Iwu *et al.* 1990, Wayman *et al.* 1992, Abou-Rass and Bogen 1998). Indeed, it has been demonstrated that as many as 90% of PRD lesions contain cultivable bacteria (Hren *et al.* 2000). Nevertheless, microbes are frequently found in only very small numbers within the lesion (Siqueira and Lopes 2001). Furthermore, only a small percentage of PRD tissue has been shown to contain bacteria within the body of the lesion (Nair 1997). It therefore remains ambiguous as to whether the majority of these microorganisms are contaminants arising during surgical removal of the lesion. The possible contribution of these extraradicular microbes towards the perpetuation of PRD remains to be elucidated.

To date, the majority of microbiologic studies related to endodontic research have focussed on bacteria as the principal aetiological agent of PRD (summary of key endodontic pathogens in Table 1.1 and Table 1.2). Interestingly, a number of other candidate pathogens isolated from both infected root canals and periradicular lesions have recently been implicated in PRD. These microorganisms include viruses (Slots *et al.* 2003, Sabeti *et al.* 2003, Sabeti and Slots 2004, Slots *et al.* 2004), *Treponema* spirochetes (Siqueira and Róças 2003, Baumgartner *et al.* 2003, Lee *et al.* 2005, Róças and Siqueira 2005) and fungi (Siqueira and Sen 2004). The contribution of these microorganisms towards the initiation and perpetuation of PRD requires clarification. Furthermore, the interrelationship of these recently identified pathogens with currently defined endodontic bacterial pathogens in lesion development has not been established.

Table 1.1 **Endodontic pathogens associated with PRD.** Most commonly identified bacterial genera recovered from root canals with periradicular disease. Those marked * are genera frequently isolated from root canals of symptomatic teeth.

Anaerobic bacteria			
Gram -ve cocci	**Gram +ve cocci**	**Gram -ve rods**	**Gram +ve rods**
Veillonella	*Peptostreptococcus**	*Bacteroides** *Campylobacter* *Fusobacterium** *Porphyromonas** *Prevotella** *Selenomonas*	*Actinomyces* *Bifidobacterium* *Clostridium* *Eubacterium** *Lactobacillus* *Propionibacterium*
Facultative and anaerobic bacteria			
Neisseria	*Enterococcus* *Gemella* *Micrococcus* *Staphylococcus* *Streptococcus*	*Actinobacillus* *Capnocytophaga* *Eikenella* *Enterobacter* *Escherichia* *Haemophilus* *Klebsiella* *Proteus* *Pseudomonas*	*Actinomyces* *Bacillus* *Corynebacterium* *Lactobacillus* *Propionibacterium*

Table 1.2 **Occurrence and significance of proposed aetiopathogenic microorganisms of PRD.** (Adapted from Dahlen & Haapasalo, in Essential Endodontology, 1998)

Microorganism	Frequency of isolation	Level of virulence	Degree of resistance to treatment	Clinical situation of isolation
Staphylococci *S. aureus* *S. epidermidis*	+	+++	+++	Primary root canal infection
Streptococci (polysaccharide producing) *S. mutans* *S. salivarius* *S. sanguis*	+	+	++	Primary root canal infection
Streptococci (others) *S. anginosus (S.milleri)* *S. mitis* *S. intermedius*	+++	++	++	Primary root canal infection
Enterococci	++	+	+++	Primary root canal treatment
Peptostreptococci	++	++	+	Primary root canal infection/ acute infection
Gram-negative cocci *Neisseria* *Veillonella*	+	+	-	Primary root canal infection
Sporeformers *Bacillus*	+	?	?	Primary root canal infection
Gram-positive anaerobic rods *Corynebacterium* *Lactobacillus* *Propionibacterium*	++	+	++	Primary root canal infection
Other Gram-positive anaerobic rods *Actinomyces* *Eubacterium*	+++	++	++	Primary root canal infection/ Failed root canal treatment
Enteric rods	+	+	+++	Primary root canal infection
Gliding or corroding bacteria *Selenomonas spp.* *Campylobacter spp.* *Eikenella corrodens*	+	++	?	Primary root canal infection/ Acute infection
Porphyromonas *P.gingivalis* *P.endodontalis*	++	+++	+	Primary root canal infection/ Acute infection
Prevotella *P. intermedia* *P. buccae* *P. dentalis*	++	+++	+	Primary root canal infection/ Acute infection
Fusobacterium *F. nucleatum*	++	++	+	Primary root canal infection/ Acute infection
Spirochetes	+	?	-	Primary root canal infection/ Acute infection
Fungi *Candida*	+	?	?	?

1.6 Cellular composition of the PRD lesion

1.6.1 PRD: Stromal cells

Histological and immunohistochemical studies of chronic human PRD reveal that cells of stromal origin account for 50% of the total cellular population (Morse *et al.* 1975, Stern *et al.* 1981). The stromal cellular component of PRD essentially comprises vascular endothelium, epithelial cells and fibroblasts (Yu and Stashenko 1987). Fibroblasts constitute the most abundant host stromal cell type within the PRD lesion. These cell types perform an essential function in maintaining and remodelling the extracellular matrix (ECM) by synthesising connective tissue components (Everts *et al.* 1996, Lekic *et al.* 1997). Furthermore, fibroblast cells can be considered immune active cells that respond to various inflammatory cytokines and growth factors (Haase *et al.* 1998). Alterations in the balance between ECM synthesis and degradation to these inflammatory stimuli may result in pathological damage to the tissue matrix (Havemose-Poulsen and Holmstrup 1997). Therefore, PRD fibroblasts potentially play a central role in the development of periradicular disease. Fibroblasts within the PRD lesion may also contribute to the perpetuation of PRD through the production of matrix metalloproteinases (MMPs), which lead to matrix degradation (Shin *et al.* 2002, Chang *et al.* 2002, Wahlgren *et al.* 2002, Leonardi *et al.* 2005, refer to chapter 2.8).

1.6.2 PRD: Cells of innate immunity

The inflammatory cellular infiltrate within the PRD lesion is composed of neutrophils, macrophages, mast cells, lymphocytes, natural killer cells, plasma cells and eosinophils (Morse *et al.* 1975, Stern *et al.* 1981). Large numbers of neutrophils are present within the inflammatory lesion (Kontiainen *et al.* 1986). These neutrophils frequently form a dense wall at the interface of the root canal and PRD lesion (Nair 1987). Within periradicular granulomas, neutrophils are generally located within the granulomatous zone, although in lower abundance than neutrophils associated in exudative areas of lesions (Piattelli *et al.* 1990, Márton and Kiss 1993). Their primary function is the phagocytosis and killing of invading bacteria, thereby preventing the dissemination of microbes throughout the lesion (Walton and Ardjmand 1992).

Macrophages are a dominant inflammatory cell type within the PRD lesion (Stern *et al.* 1981, Kopp and Schwarting 1989, Kawashima *et al.* 1996). These cell types are recognised as central players in the natural resistance to infection (Mogensen and Virelizier 1987). The cytokine interferon-γ (IFN-γ) is a key mediator in converting macrophages from resting to activated states (Gajewski and Fitch 1988). On exposure to bacterial antigens, or their components such as LPS, macrophages produce cytokines including IL-12 and IL-18 that activate natural killer (NK), NKT and T cells (Ogasawara *et al.* 1998, Okamura *et al.* 1995b, 1998). Within the murine/ rat PRD pulp exposure model, it has been established that macrophage-associated antigen-presenting cells constitute a large proportion of immune cells within the lesion (Akamine *et al.* 1994, Okiji *et al.* 1994, Suzuki *et al.* 1999). Indeed, one of the earliest events in the initiation of the PRD lesion is a substantial influx of macrophages into the periradicular region (Kawashima *et al.* 1996). Furthermore, macrophages situated at the root apical foramen contain intracellular bacteria (Goldstein and McKinney 1981, Walton and Ardjmand 1992). These findings suggests that macrophages have a fundamental responsibility in impeding the dissemination of microbes into the PRD lesion, both at the onset of disease and consequently during chronic progression of the lesion.

Mast cells are present within both periradicular granulomas and cysts, accounting for approximately 2% of the human PRD lesion cell population (Mathiesen 1973, Kontiaianen *et al.* 1986). The contribution of mast cells within innate immunity as effector cells is well established (Féger *et al.* 2002). Within human PRD tissue, mast cells are situated in close proximity to T cells and localised to zones of inflammatory cell accumulation. Mast cells are located within central areas of periradicular granulomas and within sub-epithelial areas of periradicular cysts (Rodini *et al.* 2004, Ledesma-Montes *et al.* 2004). Additionally, mast cells within the periphery of periradicular cysts appear to be degranulated (Teronen *et al.* 1996). It has therefore been postulated that the production and secretion of histamine by mast cells may contribute to periradicular cyst expansion (Smith *et al.* 1989). In addition to their involvement within innate immunity, mast cells exhibit an effector role in the adaptive immune response via Fcγ receptor binding of IgG-coated bacteria (Chapter 2.2.3).

IHC investigations on human PRD tissue sections have determined natural killer (NK) cells to be present in the majority of inflamed PRD lesions though absent in scar tissue (Kettering and Torabinejad 1993). Conversely, others utilising IHC have found NK cells to

be detectable in only small numbers and in only a few PRD lesions (Suzuki *et al.* 2001, Liapatas *et al.* 2003). Clearly, the occurrence and specific roles of the NK cell within the PRD lesion remain to be properly evaluated.

1.6.3 PRD: Cells of adaptive immunity

Cells necessary for an effective adaptive immune response are present within the PRD lesion (Liapatas *et al.* 2003). T cells, in addition to macrophages, comprise the majority of the inflammatory cellular population both within human and animal PRD lesions (Bergenholtz *et al.* 1983, Torabinejad and Kettering 1985, Yu and Stashenko 1987, Stashenko and Yu 1989). During active stages of lesion development within the murine/ rat model, $CD4^+$ T effector cells are demonstrated to outnumber $CD8^+$ T cells. This ratio subsequently reverses into chronic stages of the disease (Stashenko and Yu 1989, Kawashima *et al.* 1996).

The predominance of T effector cells ($CD4^+$) over $CD8^+$ T cells has also been observed within the human PRD lesion (Kopp and Schwarting 1989, Matsuo *et al.* 1992, Walker *et al.* 2000). Nevertheless, others have not corroborated this finding within human PRD tissue (Torabinejad. and Kettering 1985, Kontiainen *et al.* 1986, Márton and Kiss 1993, Liapatas *et al.* 2003). It is suggested that differences in the observed $CD4^+/CD8^+$ ratios may be due to the analysis of tissue samples derived from differing clinical situations (Alavi *et al.* 1998), between differing lesion types (Suzuki *et al.* 2001), differing sizes of lesions (Matsuo *et al.* 1992) and differing stages in disease progression (Gao *et al.* 1988). Although a recent study has identified the presence of γδ T cells within the human PRD lesion (McCutcheon *et al.* 2004), current advances in the phenotyping and subsetting of T cells (see Chapter 2.2.6) are not reflected in the literature related to PRD. It is apparent that the precise role of T effector cells and T regulator cells within the human PRD lesion remains to be established.

B cells are prominent within the PRD lesion, producing IgG, IgA and to a lesser extent, IgE and minimal quantities of IgM (Pulver *et al.* 1978, Stern *et al.* 1981, Baumgartner and Falkler 1991a). Furthermore, these locally produced antibodies are reactive with endodontic pathogens (Baumgartner and Falkler 1991c, Kettering *et al.* 1991). However, the PRD lesion contains no germinal centre and B cells within the lesion are non-

proliferative. Furthermore, there appears to be no correlation between periradicular lesion expansion and the number of antibody-producing cells (Yu and Stashenko 1987). These data support the concept that PRD develops as a non-specific multibacterial infection (Takahashi *et al.* 1996). Moreover, data from the rat experimental model suggest that plasma cells may actually contribute towards tissue repair rather than lesion expansion (Akamine *et al.* 1994).

1.7 A postulated series of cellular events in PRD lesion development

1.7.1 Introduction

Periradicular disease is the result of a local inflammatory response to microbial challenge, mediated by an inflammatory cell infiltrate. This cellular reaction comprises components of both the innate and acquired immune systems. From investigations in the rodent pulp exposure model, it has been demonstrated that the influx of 'innate' immune cells into the periradicular region occurs within 3 days after pulp exposure (Akamine *et al.* 1994, Kawashima *et al.* 1996). Of clinical significance, this inflammatory infiltrate occurs within the periradicular tissue prior to necrosis of the pulp tissue (Suzuki *et al.* 1999). In response to the infiltration of endodontic pathogens, a myriad of inflammatory mediators are released by infiltrating immune cells (Yanagisawa 1980). The majority of data defining the immunopathogenesis of PRD has been derived from animal experiments. Critically, only a limited number of studies have investigated cellular components within the human PRD lesion. Furthermore, the majority of studies analysing human PRD have only been observational in nature, detailing histopathologic findings or determining the presence or absence of cell populations by using simple histopathology or immunohistochemistry (IHC) techniques.

1.7.2 Initiation of the PRD lesion

The influx of large numbers of polymorphoneutrophil leukocytes (PMNs) and monocytes acts as the first line of host defence to pathogens infiltrating the root canal (Akamine *et al.* 1994, Kawashima *et al.* 1996). This inflammatory infiltrate and consequent bone destruction occurs prior to total pulp necrosis (Yamasaki *et al.* 1994). On activation, these inflammatory cells spontaneously express genes for IL-1α, IL-1-β, IL-6 and TNF-α *in vivo*

and these are potentially involved in the recruitment and maturation of osteoclasts (Miller *et al.* 1996, Takeichi *et al.* 1996, Euler *et al.* 1998). LPS derived from suspected endodontic pathogens are capable of stimulating neutrophils *in vitro* to release significant amounts of MIP-1α and MIP-1β (Ko and Lim 2002). The synthesis of TNF-α by mast cells (Walsh *et al.* 1995) and stimulation of these cells by Substance P (released as a result of noxious stimuli within the lesion) may be involved in neurogenic inflammation. This neurogenic inflammation may further contribute towards the migration of leukocytes into the surrounding tissues (Kabashima *et al.* 2002). In addition to the release of cytokines, the local release of leukotrienes provides supplementary chemoattractant signals for neutrophil recruitment to the PRD inflammatory site (Torabinejad *et al.* 1991).

In the murine pulp exposure model, PRD lesions do not develop if methotrexate-induced neutropenia occurs prior to pulp exposure with bacterial pathogens. Conversely, methotrexate-induced neutropenia after the initiation of PRD has no effect on lesion development (Yamasaki *et al.* 1994). A reduction in the number of circulating leukocytes also has no significant effect upon PRD lesion development in rats with cyclophosphamide-induced neutropenia (Waterman *et al.* 1998). However, studies in P-/E-selectin knockout mice, which have elevated circulating phagocytic leukocytes but impaired PMN and monocyte infiltration into sites of inflammation (Kawashima *et al.* 1999), and a previous experiment in rats with cyclophosphamide-induced neutropenia (Kawashima *et al.* 1993) have produced contradictory findings. Following pulp exposure, increased periradicular bone destruction occurred within both these animal models. These two studies therefore suggest that neutrophils have a protective role in the initiation of PRD. Moreover, administration of PGG glucan in the rat pulp exposure model results in significantly less infection-stimulated periradicular bone resorption than control animals (Stashenko *et al.* 1995). PGG glucan stimulates the production of neutrophils and up-regulates their bactericidal and phagocytic activity, thus implying that neutrophils are important in limiting the onset of PRD. Taken collectively, it is evident from currently available studies that the precise role of the neutrophil in the initiation and development of the PRD lesion remains to be defined.

In addition to neutrophils, macrophages are rapidly localised to the apical tissues at the onset of periradicular inflammation (Akamine *et al.* 1994, Kawashima *et al.* 1996). During the acute stage of lesion development, factors such as macrophage-derived prostaglandins

(PG) are implicated in lesion progression. Fibroblasts may subsequently become a more important source of PGs in the chronic lesion (Miyauchi *et al.* 1996). Within expanding rat PRD lesions, class II molecule-expressing macrophages outnumber dendritic cell-like cells (Kaneko *et al.* 2001b). Macrophages, identified as ED1+/ OX6+, are localised to the outer region of the lesion where it is postulated they trigger lesion expansion through T cell activation (Suzuki *et al.* 1999). Dendritic cell-like cells subsequently become more prevalent in the chronic phase (Kaneko *et al.* 2001b). However, these studies have not used specific markers to clearly differentiate between dendritic cell subsets and macrophage populations. Instead, they have relied upon phenotypic characteristics such as the presence or lack of lysosomes and phagosomes to distinguish between these cell types (Kaneko *et al.* 2001b).

Macrophages contribute to initial lesion expansion by the release of inflammatory mediators, including nitric oxide (Suzuki *et al.* 1999, Lin *et al.* 2003) and the cytokines IL-1α and TNF-α (Tani-Ishii *et al.* 1995a, Wang *et al.* 1997). Macrophages express significant quantities of mRNA for MMP-1, COX-2 and IL-6 at an early stage in lesion development. Furthermore, these inflammatory mediators are also expressed during the chronic phase (Lin *et al.* 2002). Within murine/ rat experiments, a COX-2 inhibitor reduces mRNA expression of MMP-1 and IL-6, thereby reducing periradicular bone destruction. Taken together, these results imply that macrophages have an important role in the release of proinflammatory mediators that may contribute towards early lesion development. Whilst the macrophage has been analysed in the murine/ rat model, its involvement within human PRD lesion development and its cytokine repertoire is largely unknown. Nevertheless, one group has demonstrated a correlation between $CD11^+$ monocyte/ macrophage cell numbers and tenderness to tooth percussion within human PRD (Matsuo *et al.* 1992).

1.7.3 Transition from acute to chronic PRD

Following the initial inflammatory response, a network of T cell and macrophage-derived cytokines are implicated in regulating progress of the lesion from acute to chronic PRD (Márton and Kiss 1993, Sol *et al.* 1998). On stabilisation of the lesion, dendritic-like cells become more prevalent than macrophages and play a prominent role in the local immune defence against perpetual antigenic challenge (Kaneko *et al.* 2001a).

Cells of innate immunity clearly have a critical responsibility in early PRD lesion development. However, studies in RAG-2 SCID mice demonstrate that the limitation of infection to the confines of the root canal system and the subsequent prevention of sepsis also require the active involvement of the acquired immune response (Teles *et al.* 1997). Indeed, administration of immunosuppressive agents to the murine/ rat model results in increased bone loss and increased dissemination of infection (Yamasaki *et al.* 1994, Kawashima *et al.* 1996). However, the precise contribution of the acquired immune response in the development of PRD is ambiguous. Investigations of PRD experimental lesion development in athymic BALB/c *nu/nu* rats have produced conflicting results. In one study, *nu/nu* rats developed similar periradicular bone destruction to that seen within immunologically intact rats (Wallstrom *et al.* 1993). In contrast, others ascertained that *nu/nu* mice developed significantly less periradicular bone destruction than wild type controls (Tani-Ishii *et al.* 1995b). In early stages of infection, PRD lesions in BALB/c *nu/nu* mice are of an equivalent size as wild type controls. However, over longer time periods the PRD lesions are generally smaller and more circumscribed than those in controls (Tani-Ishii *et al.* 1995b). Furthermore, pulp exposure experiments within *scid* mice result in similar PRD lesions to their BALB/cj controls (Fouad 1997). More recently, it has been demonstrated that cyclosporin-A-induced immunosuppression, which inhibits the activation and proliferation of T cells, results in smaller PRD lesions. Moreover, the withdrawal of cyclosporin-A administration within these rat experiments leads to significant increases in the size of lesions (Kawahara *et al.* 2004). Taken together, these results suggest that during the acute immune response, the lack of T cells *per se* may not predispose animals to the dissemination of root canal infections. Furthermore, the involvement of T cell-independent specific immune responses may help to protect against systemic spread of infections from the root canal. These murine/ rat studies provide important evidence that T cells are critically involved in the perpetuation of the chronic PRD lesion. However, there is a paucity of data defining T cell-specific immune responses within the development of human periradicular disease.

The importance of the humoral response in PRD development has been demonstrated in monkeys immunised against pivotal infective endodontic microorganisms. These immunised monkeys develop smaller PRD lesions than their controls (Dahlen *et al.* 1982). PRD lesion development has also been compared between RAG-2 SCID, Igh-6 (B cell deficient), Tcrd Tcrd (T cell deficient) and Hc^0 (complement C5 deficient) mice. From this

study, Hou *et al.* (2000b) provide evidence that B cells and concomitant antibody-mediated mechanisms (possibly bacterial opsonisation) are of importance in the localisation of anaerobic root canal infections, thereby preventing their systemic dissemination

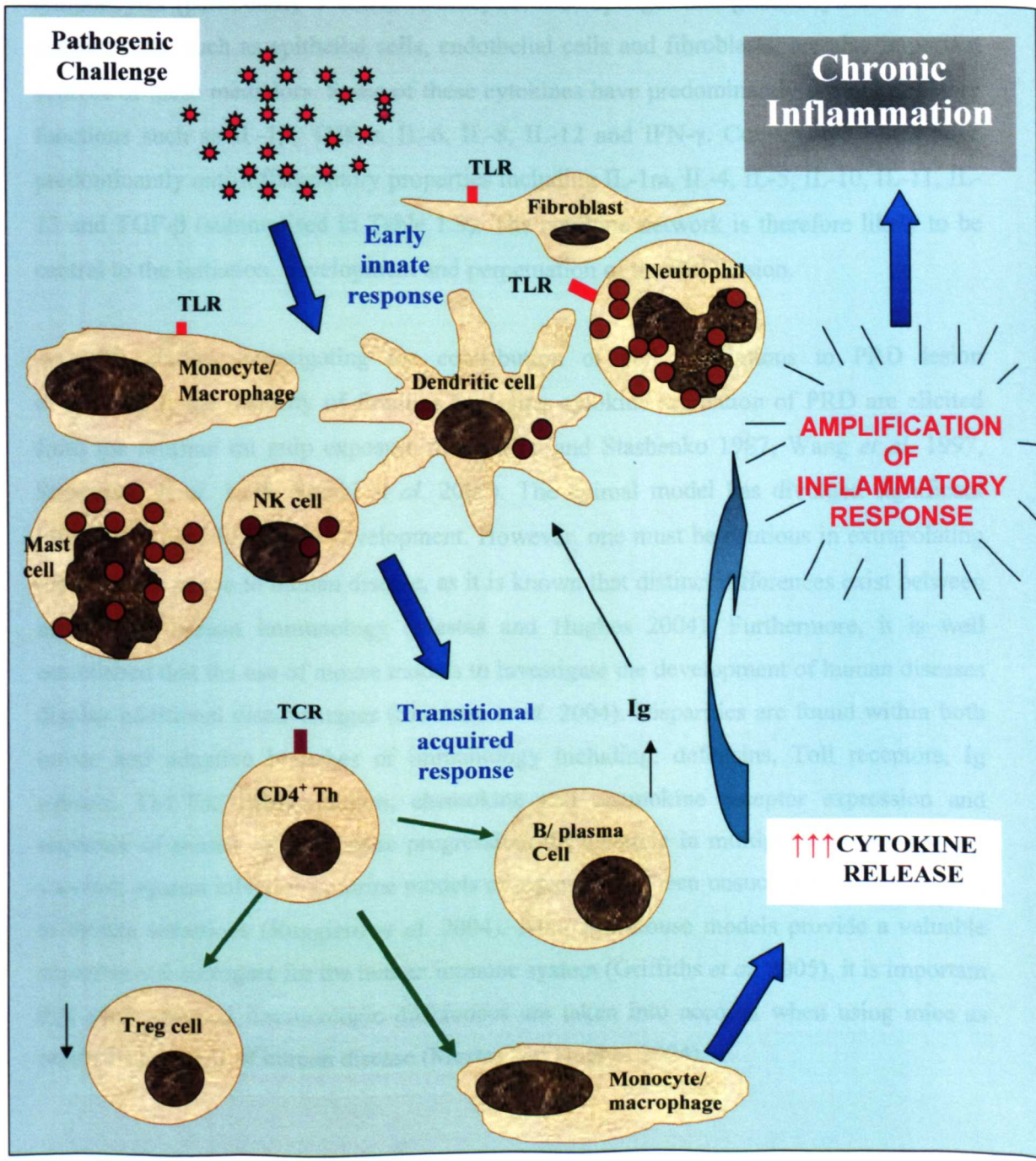
1.7.4 The chronic PRD lesion

Following the transitional phase from acute to chronic inflammation, the periradicular granuloma represents a reparative process of chronic localised inflammation comprised of granulomatous tissue (Gao *et al.* 1988). Periradicular cysts also develop due to perpetual low-grade chronic inflammation. These cysts, however, are lined by epithelium thought to be derived from epithelial rests of Malassez within or adjacent to the granulomatous tissue (Neville *et al.* 1995). Despite the differences in the chronic development of these two PRD lesions, previous histopathology studies and recent immunohistochemistry experiments ascertain that there are no significant differences in the constituents of the inflammatory infiltrate (Stern *et al.* 1981, Torabinejad & Kettering 1985, Gao *et al.* 1988, Matsuo *et al.* 1992, Liapatas *et al.* 2003).

Histopathologic examination of chronic PRD tissue continues to be the only available method to determine the precise nature of the chronic lesion. This allows the lesion to be classified as: 1) a periradicular cyst, 2) an abscess or 3) a periradicular granuloma (Linenberg *et al.* 1964). The true prevalence of each type of lesion is questionable. Periradicular cysts are reported to account for 6% (Block *et al.* 1976) to 55% (Priebe *et al.* 1954) and periradicular granulomas from 45% (Lalonde and Luebke 1968) to 95% (Block *et al.* 1976) of all PRD lesions. Differences in the sampling procedures, the population groups being studied and the use of differing histological methods (Nair *et al.* 1996) and diagnostic criteria may account for the large variability in these findings.

In summary, the murine/ rat pulp exposure model has elucidated substantial information on the initiation and development of the PRD lesion. This model has been extremely important in helping to characterise cellular events occurring within the acute lesion and its subsequent progression to chronic PRD. However, it is evident from the literature that information on the role of inflammatory cells in human PRD lesion development is limited and the dynamics of their interactions remain poorly defined. For a summary of the postulated cellular events in the development of the PRD lesion, see Figure 1.3.

Figure 1.3 A postulated series of cellular events involved in the initiation and development of the PRD lesion. Pathogenic micro-organisms stimulate resident cells of innate immune system and stromal cells through ligation of pattern recognition receptors. This induces rapid and spontaneous release of inflammatory mediators, including cytokines. The subsequent activation of cells of acquired immunity results in the further release of inflammatory mediators that act in numerous feedback loops upon recruited and local inflammatory cells. Continued amplification of this reaction, by the release of proinflammatory cytokines, results in the development of the chronic PRD lesion.



1.8 Cytokine regulation in PRD

1.8.1 PRD cytokines: Introduction

Cytokines are a diverse group of secreted proteins that act as communication molecules between virtually all host cells (refer to Chapter 2.5). They perform a fundamental function in orchestrating and modulating the host immune and inflammatory responses to infectious agents. Immune cells are the primary source of these inflammatory mediators including lymphocytes (particularly T cells), monocytes, macrophages and granulocytes. However, stromal cells, such as epithelial cells, endothelial cells and fibroblasts, are also important sources of these mediators. Some of these cytokines have predominantly proinflammatory functions such as IL-1β, TNF-α, IL-6, IL-8, IL-12 and IFN-γ. Conversely, others have predominantly anti-inflammatory properties including IL-1ra, IL-4, IL-5, IL-10, IL-11, IL-13 and TGF-β (summarised in Table 1.3). The cytokine network is therefore likely to be central to the initiation, development and perpetuation of the PRD lesion.

As with studies investigating the contribution of cell populations to PRD lesion development, the majority of findings analysing cytokine regulation of PRD are elicited from the murine/ rat pulp exposure model (Yu and Stashenko 1987, Wang *et al.* 1997, Stashenko *et al.* 1998, Sasaki *et al.* 2000). The animal model has divulged significant information on PRD lesion development. However, one must be cautious in extrapolating these results *per se* to human disease, as it is known that distinct differences exist between mouse and human immunology (Mestas and Hughes 2004). Furthermore, it is well established that the use of mouse models to investigate the development of human diseases display additional disadvantages (Shiohara *et al.* 2004). Disparities are found within both innate and adaptive branches of immunology including: defensins, Toll receptors, Ig subsets, Th1/Th2 differentiation, chemokine and chemokine receptor expression and sequence of events within disease progression, for example in multiple sclerosis. Indeed, vaccines against infectious murine models of disease have been unsuccessful when applied to human infections (Ruggiero *et al.* 2004). Although mouse models provide a valuable experimental surrogate for the human immune system (Griffiths *et al.* 2005), it is important that such essential immunologic differences are taken into account when using mice as preclinical models of human disease (Mestas and Hughes 2004).

Within studies investigating the pathogenesis of PRD, this is significantly demonstrated by the fundamental difference in the principal bone resorptive cytokines. In the murine model, IL-1α is the predominant cytokine responsible for bone resorption. In contrast, the principal cytokine promoting bone resorption in humans is IL-1β. Whilst the animal model will continue to offer great insight into lesion development, it is of paramount importance that further investigations are additionally undertaken on human PRD tissue to elucidate the contribution of the cytokine network in the development and perpetuation of the chronic PRD lesion.

Table 1.3 **Overview of cellular and cytokine involvement within innate and adaptive immune responses.**

Cells of innate immune responses	**Cells of acquired immunity**	**Innate cytokines with dominant proinflammatory effects linking acquired immunity**	**Innate cytokines with dominant suppressive effects linking acquired immunity**	**Cytokines characteristic of acquired immunity**
Dendritic cells APC cells Eosinophils Macrophages Neutrophils NK cells NKT cells γδ T cells (epithelial cells)	B cells $CD4^+$ cells $CD8^+$ cells	Type 1 IFN GM-CSF TNF-α (IFN-γ) IL-1 IL-12 IL-15 IL-17A IL-18	IL-10 IL-13 TGF-β	Th1 Responses IL-2 and IFN-γ Th2 Responses IL-4 and IL-5

1.8.2 PRD cytokines: Animal models

In murine experiments, bacterial invasion or LPS stimulation within the dental root canal induces up-regulated expression of the proinflammatory cytokines IL-1α, IL-1β and TNF-α within the pulp tissue by 3 h (Kawashima *et al.* 2005). Subsequently, resident macrophages and fibroblasts are activated within the tissue surrounding the root apex. This promotes the infiltration of IL-1α secreting macrophages and monocytes into the lesion (Kawashima *et al.* 1996, Hong *et al.* 2004). On experimental induction of the PRD lesion, macrophages expressing IL-1α and IL-1β are located within the vicinity of extensive bone resorption sites (Hamachi *et al.* 1995, Matsumoto *et al.* 1998). Indeed, IL-1α and TNF-α appear to play a critical role in the initiation and development of PRD. Expression of these cytokines is up-regulated within PRD tissue cells at an early stage of rodent pulp infection and bone resorption (Tani-Ishii *et al.* 1995a, Wang *et al.* 1997, Kawashima and Stashenko 1999).

However, neutralising studies in the rat indicate that TNF-α may not contribute significantly to periradicular bone resorption (Wang and Stashenko 1993a). The majority of inflammatory bone destruction appears to be directly related to the presence and effects of IL-1α (Wang and Stashenko 1993a, Sasaki *et al.* 2000). In P-/E-selectin knockout mice, which exhibit increased bone destruction, tissue levels of IL-2, IL-4, IL-10, TNF-α and IFN-γ are all increased but only IL-1α is significantly up-regulated compared with controls (Kawashima *et al.* 1999). Furthermore, bone resorptive activity in PRD extracts from the murine model is substantially inhibited with neutralising antibodies to IL-1 (Wang and Stashenko 1993a). Additionally, the *in vivo* administration of IL-1 receptor antagonist (IL-1ra) to mice significantly inhibits expansion of the PRD lesion (Stashenko *et al.* 1994). This IL-1-induced bone resorption is partly dependent on PGE_2 synthesis and synergistic effects between IL-1 and PGE_2 (Dewhirst *et al.* 1990).

The contribution of IL-1α, IL-1β and TNF-α in the development of the PRD lesion has been studied extensively within animal PRD models (Tani-Ishii *et al.* 1995a, Wang *et al.* 1997, Kawashima and Stashenko 1999, Bletsa *et al.* 2004). Macrophages and neutrophils are the principal sources of IL-1α and TNF-α. Their expression within the PRD lesion contributes towards the synthesis of downstream inflammatory mediators such as IL-6 and IL-8. These proinflammatory molecules also act on fibroblasts, thereby stimulating the

production of matrix metalloproteinases. The subsequent release of MMP1 and MMP3 may result in the degradation of extracellular matrix components as evidenced in other inflammatory bone diseases, for example rheumatoid arthritis (RA) (Okada *et al.* 1987). Moreover, Il-1β and TNF-α promote bone resorption by increasing the maturation and the bone resorbing activity of osteoclasts (Pfeilschifter *et al.* 1989).

Substantial evidence supports a destructive role for IL-1 and TNF-α in PRD lesion development. Nevertheless, it has been demonstrated that IL-1 receptor (IL-1R) or TNF receptor (TNFR) knockout mice suffer more severe periradicular bone destruction than wild type controls (Chen *et al.* 1999). Furthermore, IL-1R and TNFR double knockout mice suffer from far greater tissue damage than single knockout mice. Increased bacterial penetration into the periradicular tissues is also observed within double knockout mice. This suggests that IL-1 and TNF-α likely have important protective functions in host responses to mixed anaerobic infections. Recent rat PRD experiments have defined a role for LPS in inducing not only the early expression of IL-1α and TNF-α, but additionally iNOS, MMP-1 and TGF-β1 from macrophages (Hong *et al.* 2004). Interestingly, IL-6-/- mice develop periradicular lesions at an earlier stage than wild type controls and these lesions progress to be much larger (Huang *et al.* 2001). This indicates that IL-6 may actually have a protective role in lesion development.

1.8.3 PRD cytokines: The human lesion

Within human tissues, mRNA and/ or protein expression for several proinflammatory mediators have been identified. IL-1β has been detected within explant cultures of periradicular cysts (Meghji *et al.* 1989). In a subsequent study, the same group detected IL-1α and IL-1β protein expression within periradicular cyst explant supernatants and mRNA expression within all periradicular cyst tissue lesions examined (Meghji *et al.* 1996). An IHC investigation of periradicular cysts also demonstrated IL-1β and IL-1α expression in all examined lesions (n=12) and these cytokines were primarily localised to epithelial and endothelial cells (Bando *et al.* 1993). Honma *et al.* (1998) analysed periradicular cysts by *in situ* hybridisation and ascertained that mRNA expression for IL-1β, TNF-α, IL-6 and IL-8 was present within all lesions analysed. Expression of these inflammatory mediators was localised to fibroblasts, macrophages, T cells and plasma cells. Barkhordar *et al.* (1992)

also found significant levels of IL-1β within PRD lesions but could not establish a correlation with the degree of periradicular bone loss. High levels of IL-1β expression have been found within the root canal (Takeichi *et al.* 1996). Moreover, the concentration of IL-1β within the root canal exudate decreases during root canal treatment procedures (Matsuo *et al.* 1994). Interestingly, a trend towards raised IL-1β levels has been shown in symptomatic teeth (Lim *et al.* 1994).

As with IL-1β, TNF-α has been detected within root canal exudates from teeth associated with PRD lesions (Safavi and Rossomando 1991). Surprisingly, an investigation of periradicular cysts using ELISA methodology detected IL-8 and TNF-α protein expression within only 2 of 12 lesions examined (Bando *et al.* 1993). Danin *et al.* (2000) also observed limited TNF-α protein expression, with TNF-α being detected within only 2 of 25 PRD lesions by ELISA. From IHC experiments, others have found few cells expressing IL-1β and TNF-α within the periradicular granuloma lesion. This suggests that limited bone resorption may be occurring during chronic inflammatory stages of the disease (Artese *et al.* 1991).

Expression of IL-6 protein within the PRD lesion has also been measured. High levels of spontaneously secreted IL-6 and PGE_2 have been observed in periradicular cyst explant supernatants (Formigli *et al.* 1995, Meghji *et al.* 1996). Paralleling this finding, high concentrations of IL-6 are also detectable within root canal exudates of infected teeth (Takeichi *et al.* 1996). Furthermore, IL-6 is expressed at high levels within the PRD lesion compared to a lack of expression within healthy pulp controls (Barkhordar *et al.* 1999). The expression of significant quantities of IL-6 and GM-CSF protein within human PRD has been further confirmed in recent studies (Gervásio *et al.* 2002). Moreover, a trend towards increased IL-6 in addition to GM-CSF expression has been associated with symptomatic lesions (Radics *et al.* 2003). From IHC experiments, IL-6 expression is predominantly localised to epithelial and vascular endothelial cells (Bando *et al.* 1993). IL-8 has also been detected within the periradicular exudate within infected root canals. Furthermore, its concentrations correlate significantly with the presence of clinical symptoms (Shimauchi *et al.* 2001).

Using ELISA, TGF-β_1 protein has been detected within the majority of PRD lesions removed during periradicular surgery. Furthermore, a significant correlation has been

shown to exist between the size of the lesion and the expression of TGF-β_1 per milligram of tissue (Danin *et al.* 2000). From IHC experiments on human PRD tissues, TGF-β_1 expression has been identified within lymphocytes, fibroblasts and monocytes (Tyler *et al.* 1999). In comparison, TGF-β_1 expression is localised to macrophages in active phases of lesion development within the murine pulp exposure model. However, during chronic stages, TGF-β_1 expression is primarily located to osteoblasts (Lin *et al.* 2000).

Taken together, these data suggest that cytokines are likely to be central in the orchestration of events that lead to chronic PRD. A considerable problem in establishing the clinical significance of this cytokine network within the PRD lesion is the paucity of functional studies analysing human PRD tissue. The few studies that have investigated the regulatory function of cytokines within PRD have been performed using the rat/ murine pulp exposure model. The limited number of investigations analysing cytokine expression within human PRD have essentially been observational in nature. Cytokines act in a hierarchical structure, being released in a controlled, organised manner through "upstream" regulators that induce the expression of "downstream" cytokine mediators. Within any disease process it is necessary to identify dysregulated expression of key upstream inflammatory mediators. Through the identification and careful targeting of regulatory proinflammatory mediators or their signalling pathways, attenuation of a destructive immune response may become possible. Therefore, this thesis aims to start to address these issues by attempting to define more clearly the nature of the cytokine network within the human PRD lesion.

1.9 The role of T cell derived cytokines in PRD

1.9.1 Animal studies

Within the murine/ rat model, T cells infiltrate the PRD lesion between 7 and 14 days after pulp exposure (Kawashima *et al.*1996). $CD4^+$ T effector cells are an essential component of the immune response to infection and can be classified according to their cytokine profile as either T helper 1 (Th1) or T helper 2 (Th2) cells (refer to Chapter 2.2.6.3). Proinflammatory cytokines, representative of Th1 cells, and anti-inflammatory Th2 type cytokines are induced during the course of pulpal infection. Both sets of cytokines modulate the expression and activity of the principal bone resorptive cytokine IL-1 (Kawashima and Stashenko 1999). Of interest, extracts of the endodontic pathogen *E. faecalis* has immunosuppressive effects on the release of IL-2 and IL-4 from peripheral blood T cells. This suggests that endodontic pathogenic microorganisms may be capable of simultaneously modulating Th1 and Th2 type responses within the PRD lesion (Son *et al.* 2004).

Functional investigations within murine experiments implicate a Th1-driven process in the development of periradicular disease (Kawashima and Stashenko 1999). During the immediate four weeks following pulpal infection, Th1 type cytokines IL-12 and IFN-γ, in addition to the bone resorptive cytokines TNF-α and IL-1, are up-regulated in a linear fashion within the periradicular tissues (Kawashima and Stashenko 1999). This pattern of cytokine expression correlates with that of a predominantly Th1-type response. In contrast, Th2-type cytokines exhibit increased expression up until two weeks after infection and thereafter their levels decline. Furthermore, it has been established in the murine PRD model that *in vivo* elimination of the Th2 cytokines IL-10 and IL-6 markedly increases IL-1α production and this is correlated with increased bone resorption (Sasaki *et al.* 2000, Balto *et al.* 2001). Indeed, infection-stimulated PRD within IL-10 knockout mice results in lesions that are five times the size of those found in wild type controls. Moreover, locally produced IL-1α is increased 10-fold over controls (Sasaki *et al.* 2000). In contrast with IL-10, the Th2 cytokine IL-4 appears to have no observable effect upon PRD lesion size within the murine model. This demonstrates the heterogeneity amongst the Th2 cytokine group (Sasaki *et al.* 2000).

1.9.2 Human studies

Studies using human PRD pathological specimens establish that T cells comprise a significant component of the inflammatory cell infiltrate (Kontainen *et al.* 1986, Stashenko and Yu 1989). However, the precise nature of the T lymphocyte response within human PRD remains equivocal with conflicting published data. The majority of studies analysing the role of Th1 and Th2 type responses within human PRD have utilised immunohistochemistry (IHC). A Th1 dominated response has been proposed from a series of consecutive studies performed by one group of investigators. Using IHC, this group could not detect cellular expression of IL-4 protein within the human periradicular lesion. However, they established that many cells expressed IFN-γ protein (Kabashima *et al.* 1998). In contrast, the same group found that IL-4 protein was detectable in PRD regeneration tissues whereas there was no evidence of IFN-γ expression within these regeneration lesions (Kabashima *et al.* 2001a). A subsequent IHC study analysed particular subsets of chemokines and their receptors within the PRD lesion. From this, they determined a predominance of CCR5 and CXCR3 receptor expression compared with CCR3 expression (Kabashima *et al.* 2001b). Recently, the same group ascertained that IFN-γ and CXCR3 cellular expression was detectable within all inflammatory PRD lesions examined by IHC. In contrast, IL-4 expression was not observed and cells expressing CCR3 were only present within one lesion (Kabashima *et al.* 2004). Taken together, these findings support the hypothesis that the PRD lesion is balanced towards a Th1-type response. Furthermore, these results suggest that IL-4 and IFN-γ have a significant impact in determining the outcome of PRD towards repair or tissue destruction respectively.

However, others have determined that a Th2 process is central to the perpetuation of the PRD lesion. Hren *et al.* (1999) demonstrated that PRD lesions containing a higher number of *Streptococcus sp.* than anaerobic bacteria display a significant reduction in $CD8^+$ T cells. These results equate with previous findings wherein a relatively low index of IgG versus IgA is observed in antistreptococcal responses (Kettering *et al.* 1991). Hren *et al.* (1999) therefore suggest this might be representative of Th2-type immune responses in lesions that are predominantly infected with *Streptococci sp.*. Furthermore, using IHC on human PRD biopsy tissue, others have detected higher levels of IL-6 and IL-10 expression in comparison to IFN-γ (Walker *et al.* 2000). Using IHC on fifteen tissue specimens, De Sá *et al.* (2003) established predominance for IL-4 expression over lymphotoxin-α

expression, leading them to suggest that there was a preponderance of Th2 type cytokines within the PRD lesion. However any real interpretation of data from this study is difficult as such a small sample was analysed. Furthermore, the expression of only three cytokines was studied, which does not give a fully representative picture of Th1/ Th2 type cytokine responses within the lesion. More recently, an IHC investigation of human PRD lesions has analysed the presence of Th1 type (CCR1, CCR5 and CXCR3) compared with the expression of Th2 type (CCR2 and CCR3) chemokines and chemokine receptors (Silva *et al.* 2005). Interestingly, results from this study suggest that there may not be a predominance of either a Th1 or Th2 type response within the PRD lesion. Indeed, both responses appear to be occurring concomitantly.

Taken together, it is difficult to draw any real conclusions from these investigations. The majority of studies have investigated limited numbers of lesions and have analysed only a very small number of Th1/ Th2 type markers. The experimental techniques used have mostly been restricted to analysis of fixed biopsy tissue by immunohistochemistry. Moreover, the validity of some of the markers used to investigate and thereby robustly characterise the Th1/Th2 type response is questionable. However, it is of interest that the $CD4^+$ effector Th profile may be representative of the dominant pathogenic species present within the lesion. A Th1 type response may occur when the lesion is colonised predominantly by anaerobic microbes. Conversely, PRD infections that are dominated by aerobic bacteria, such as *Streptococci sp.*, may result in a Th2 driven pathway (Hren *et al.* 1999, Hren *et al.* 2000). In conclusion, it is abundantly clear that further investigations are necessary to define the role of Th1/ Th2 responses within human PRD and thereby determine the clinical significance of this response to lesion development and treatment success.

1.10 Treatment of periradicular disease

1.10.1 Root Canal Treatment

The objectives of root canal treatment are the biomechanical instrumentation of the root canal to reduce the reservoir of bacteria, disinfection of the canal to eliminate remaining bacteria followed by obturation of the root canal in combination with a coronal seal to prevent reinfection (Byström *et al.* 1987, Sjögren *et al.* 1997, Trope and Bergenholtz 2002). Cleaning of the canal, through irrigation and shaping does not completely eliminate bacteria colonising the root canal (Pitt Ford 1982, Byström *et al.* 1985a, Byström *et al.* 1985b, Matsuo *et al.* 2003). Therefore, the additional use of disinfectants is essential in attempting to eradicate bacteria from within the infected canal (Haapasalo and Ørstavik 1987, Ørstavik and Haapasalo 1990). Theoretically, the removal of noxious stimuli from the root canal should allow normal reparative processes to occur within the apical tissues, thereby leading to resolution of the PRD lesion.

However, even after careful preparation of the root canal, viable bacteria can be found in dentinal tubules within the deeper layers of dentine *in vitro* (Peters *et al.* 2001). Subsequent to *in vivo* clinical instrumentation and disinfection, viable bacteria are also detected (Chávez de Paz *et al.* 2003). A recent primate study has reaffirmed that after instrumentation and irrigation of the root canal with disinfectants, certain strains of bacteria may remain viable. Furthermore, the presence of these bacteria leads to persistence of the PRD lesion (Möller *et al.* 2004). To further reduce the bacterial burden, it is therefore conventional clinical practice to utilise an intracanal antimicrobial medicament between treatment visits. Currently, calcium hydroxide ($Ca(OH)_2$) is the intra-canal medicament of choice. Nevertheless, several studies have shown its relative inefficiency as an antimicrobial agent in root canal treatment (Stevens and Grossman 1983, Haapasalo and Ørstavik 1987, Reit and Dahlen 1988). There have been significant advances in the instruments and instrumentation techniques used to clean and shape root canals over the past decade. However, the intracanal medicament has a greater influence on successful healing of periradicular lesions than the type of instrumentation technique employed (De Rossi *et al.* 2005).

Unquestionably, successful root canal treatment is dependent upon the technical ability of the clinician to perform the procedure. The provision of an adequate root filling to the correct apical length, combined with comprehensive compaction of the root filling material are known to be significant factors in the success of this treatment (Buckley and Spangberg 1995, Kirkevang *et al.* 2000, Kojima *et al.* 2004, Orstavik *et al.* 2004, Segura-Egea *et al.* 2004). However, it is extremely difficult to instrument and disperse the antimicrobial agents to all aspects of the root canal system (Siqueira & Lopes 1999). Therefore, it is possible that even following careful instrumentation and obturation of the root canal, microorganisms may continue to colonise the canal (Nair *et al.* 1990). Furthermore, bacteria located within the periradicular tissue, not accessible to disinfectants or instrumentation, may also lead to treatment failure (Bystrom *et al.* 1987).

Figure 1.4 **Radiograph of a PRD lesion at diagnosis.**

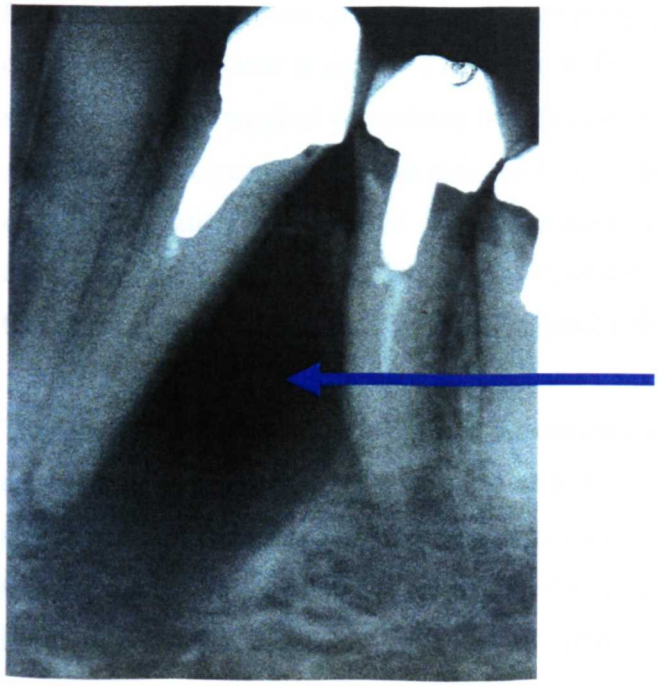
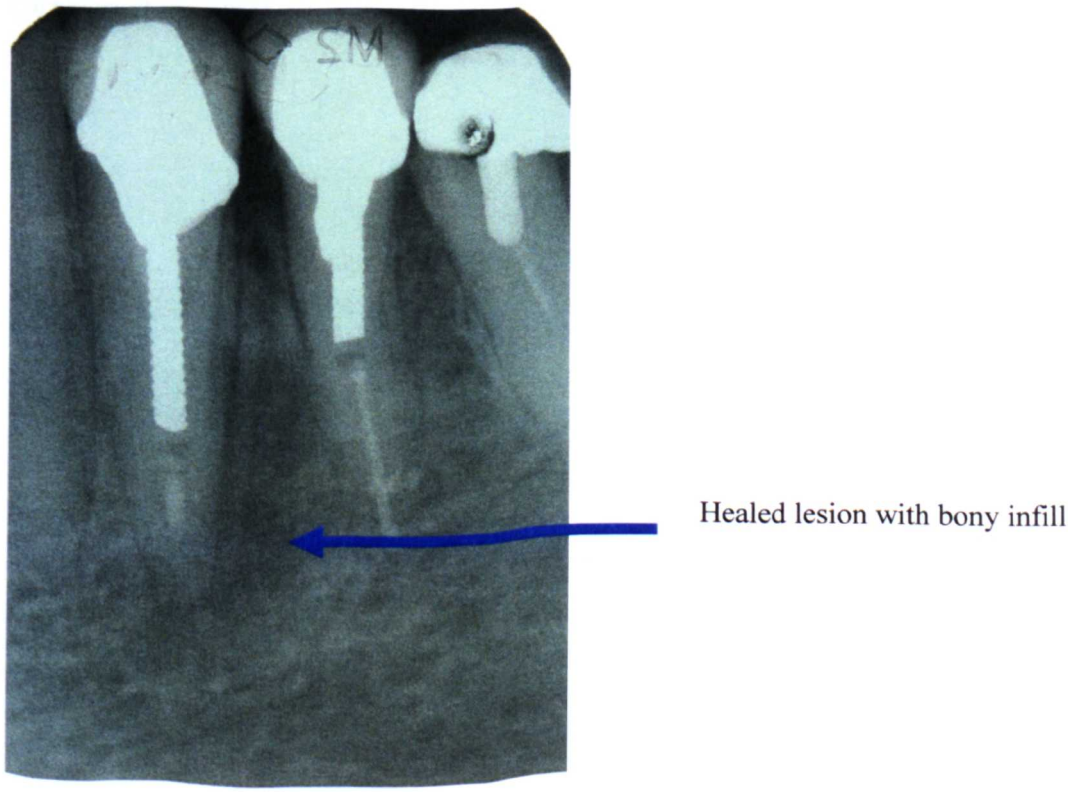


Figure 1.5 **Radiograph of healed lesion following root canal treatment.**



1.10.2 Periradicular surgery (PRS)

In teeth with failing root canal treatment and persisting periradicular lesions, non-surgical orthograde retreatment is generally regarded as the treatment of choice as this has a higher success rate than surgical procedures (Allen *et al.* 1989). Nevertheless, in certain clinical situations, re-root canal treatment may not be possible. Teeth restored with large post core crowns may suffer root fracture on post removal and some teeth may have inaccessible canals. Periradicular surgery (PRS) may therefore be the preferred treatment option in these cases (Royal College of Surgeons England 2001).

The surgical procedure aims to remove the necrotic and infected dental root apex, curettage and remove the periradicular lesion and seal off the apical aspect of the root canal. The apical seal of the root canal with a retrograde root filling has been shown to be a significant factor towards the successful outcome of PRS (Harty *et al.* 1970, Friedman 1991). There have been several significant advances in endodontic surgical procedures over the past decade. These include the use of improved root end filling materials such as mineral trioxide aggregate (Torabinejad *et al.* 1995, Bates *et al.* 1996), the use of magnification (Carr 1992) and the use of ultrasonics in root end preparation (Wuchenich *et al.* 1994, Taschieri *et al.* 2005).

Figure 1.6 **Radiograph of a PRD lesion prior to periradicular surgery (PRS).**

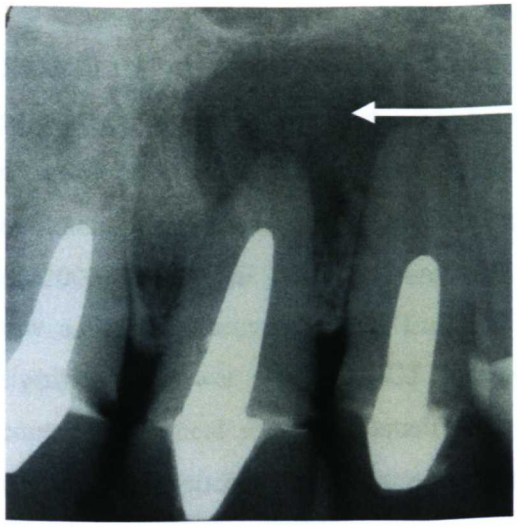


Figure 1.7 **Clinical photograph of periradicular surgery.**

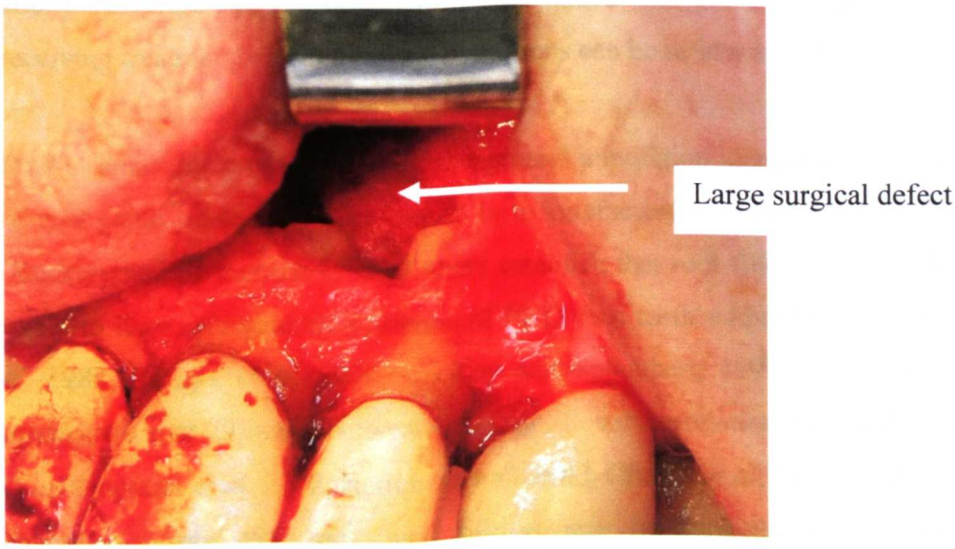
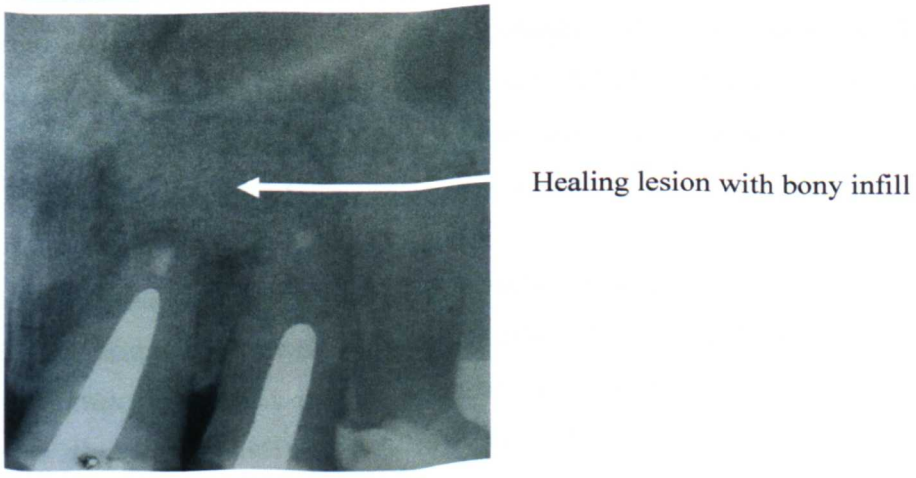


Figure 1.8 **Radiograph of the healing lesion after PRS.**



1.11 Success rates in treatment of PRD

Bacteria and other microorganisms play a central role in the initiation, progression and persistence of PRD. Endodontic treatment, therefore, aims to eliminate bacteria from the infected root canal and prevent reinfection by the clinical procedure of root canal treatment. Many studies report high success rates for RCT of greater than 90% (Lazarski *et al.* 2001, Alley *et al.* 2004). Conversely, other studies report the success of RCT to be as low as 45% (Meeuwissen and Eschen 1983). Epidemiologic studies consistently report significantly lower rates of RCT success than endodontic treatment undertaken within controlled clinical settings (Siqueira *et al.* 2005b). Indeed, the majority of investigations reporting high success rates in the treatment of PRD are limited to experiments undertaken on selected cases treated by endodontic specialists or supervised trainees (Boucher *et al.* 2002). Nevertheless, Cheung (2002) ascertained that success rates of RCT in patients receiving treatment within training institutions can be as low as 50%.

Studies analysing cross sections of the general population identify that the technical quality of root canal treatment is performed to a satisfactory standard in only 30-42% of cases (Buckley and Spångberg 1995). Others establish that root filled teeth are 5-12 times more likely to have radiographic evidence of PRD than teeth without root fillings (Petersson *et al.* 1986, Odesjo, *et al.* 1990, Buckley and Spångberg 1995). Furthermore, teeth with inadequate root fillings demonstrate a significant correlation with the presence of PRD (Lupi-Pegurier *et al.* 2002, Hommez *et al.* 2002, Chugal *et al.* 2003). Part of the reason for treatment failure with root canal fillings may be attributed to the lack of effective intracanal medicaments that eliminate a broad spectrum of bacteria during cleansing of the root canal. A recent meta-analysis of published root canal treatment literature calculated the success rate for root canal treatment to be 82.8% for vital teeth and 78.9% for non-vital teeth. This provides a cumulative success rate of 82% for teeth without a PRD lesion and 71.5% for teeth with a lesion (Kojima *et al.* 2004).

A significant problem in endodontic research is the lack of high quality controlled clinical studies that provide sound evidence on which clinicians can inform patients as to treatment outcomes. No studies investigating the outcome of re-root canal treatments have been published that provide scientific level of evidence (LOE) one. Only three LOE 2 and two LOE 3 studies have been published and the remainder of studies are in the low evidence

groups (Paik *et al.* 2004). Nevertheless, associations have been made between failed root canal treatment and missing adjacent teeth, greater plaque accumulation, the degree of marginal bone support, a history of trauma to the treated tooth, the lack of postoperative coronal restoration and older patients (Caplan and Weintraub 1997, Kirkevang *et al.* 2004, Orstavik *et al.* 2004). Most importantly, current research clearly identifies that the most significant factor influencing the success of root canal treatment is the presence and magnitude of a PRD lesion prior to commencing treatment (Friedman 1998, Chugal *et al.* 2001, Chugal *et al.* 2003, Dammaschke *et al.* 2003, Kojima *et al.* 2004, Orstavik *et al.* 2004, Wang *et al.* 2004a, Marending *et al.* 2005, Negishi *et al.* 2005).

In addition to localised tissue destruction, it is suggested that chronic persistence of periradicular disease may have an effect upon the host's systemic inflammatory mediators (Márton and Kiss 1992). Indeed, a reduction in peripheral blood levels of acute-phase proteins compared to levels in controls has been demonstrated after completion of endodontic treatment (Márton *et al.* 1988). However, within the endodontic literature it is only gradually being acknowledged and accepted that the host's immune response is an important factor in the outcome of endodontic treatment, this having previously been largely ignored (Marending *et al.* 2005).

The success of periradicular surgery has been reported to vary between 25% and 99% (Gutmann and Harrison 1994). A recent systematic review of periradicular surgery determined that the weighted average of success was 64%, whilst the outcome from 25.7% of cases remained uncertain and 15.7% of surgical cases were unsuccessful (Peterson and Gutmann 2001). Periradicular resurgery has been demonstrated to have a significantly greater failure rate than a first surgical procedure (Gagliani *et al.* 2005). Repeat surgery is reported to be associated with a weighted average healing rate of 35.7% and a failure of 38%. Furthermore, the success rate of non-surgical retreatment of failed periradicular resurgery cases is lower than non-surgical retreatment of failed endodontically managed cases (Çalişkan 2005). Clinical success or failure appears to be associated with the operator and the types of materials used within the surgical procedure (Peterson and Gutmann 2001, Wang *et al.* 2004b). It remains to be established if the use of modern materials, magnification, improved surgical techniques and better postgraduate training will result in improved surgical success rates over the longer-term.

Recently, a Swedish group established that within a low caries risk population group, which may be expected to undergo less root canal treatment over time than the general population, the number of endodontic procedures carried out between 1997 and 2003 had actually increased annually by 20% (Bjørndal and Reit 2004). However, the current success rates of RCT procedures are unsatisfactory. The population trend is towards an ageing population whom are retaining more teeth and will potentially require more root canal treatment procedures to retain their teeth. Taking these considerations together, the future cost burden to healthcare services to provide effective treatment of PRD will be substantial.

In summary, these studies clearly demonstrate that the current treatment strategy of root canal treatment, undertaken to prevent and resolve PRD, is at the very least sub-optimal. Furthermore, most of the data defining the immune component of PRD and the role of the host response in the initiation and progression of the disease has been derived from animal models. The distinct lack of functional investigations upon the human PRD lesion is a significant problem in being able to accurately understand and thereby treat this prevalent disease. There is unquestionable evidence that the presence of a PRD lesion prior to the start of treatment is the single most significant factor influencing the successful outcome of RCT. It is therefore surprising that there is such a lack in knowledge of the immunopathogenesis of the human PRD lesion.

Improved understanding as to the development of the PRD lesion may significantly help towards developing better treatment strategies that may increase treatment outcome. Thereby, the current morbidity to the patient may be significantly reduced. Furthermore, the substantial cost to the health service in financing re-treatment procedures for re-root canal treatment, periradicular surgery, re-surgery, dental extraction and/ or prosthetic rehabilitation may be significantly reduced. This thesis therefore aims to improve the current knowledge of the immunopathologic processes that contribute towards the development of the human PRD lesion.

CHAPTER 2

INFLAMMATORY CELLS AND CYTOKINES CONTRIBUTING TO INFLAMMATORY BONE DESTRUCTION

2 INFLAMMATORY CELLS AND CYTOKINES CONTRIBUTING TO INFLAMMATORY BONE DESTRUCTION

2.1 Introduction to immunity

The ability to combat pathogenic microbes that potentially would destroy the host is termed 'immunity'. Host immunity encompasses two functionally different but interrelated immune systems comprising non-specific, 'innate immunity' and the specific, adaptive or 'acquired system'. These two systems have evolved to provide protection from the invasion of pathogenic bacteria, fungi, and protozoa and altered self, as in viral infection. Innate and acquired immunity comprise cellular and humoral components interacting in a complex manner with the collective aim of eradicating pathogenic agents from the host (Janeway 1992). Innate immunity combines internal and external mechanisms to afford an instantaneous first line of defence against pathogenic challenge (Beutler 2004). The innate immune response is relatively efficient at preventing systemic infection and limiting pathogenic burden whilst sparing damage to host tissue (self tolerance). The rapid control over invading pathogens is achieved by the immediate activation of phagocytic antigen presenting cells (APCs). The concomitant release of down-regulatory mediators, for example the cytokine IL-10, tempers the potential for host tissue destruction arising from an exaggerated rapid inflammatory response. However, complete control of infection by the host is normally only achieved with the simultaneous induction of the adaptive immune response. Due to the requirement of T and B cell population expansion, an effective acquired immune response is only instigated a week after induction of innate immunity.

Activation of the inflammatory/ innate response is not directly pathogen specific, but is dependent upon ligand binding to germline encoded receptors (pattern-recognition receptors, PRRs). These PRRs, which include Toll-like receptors (TLRs), recognise pathogen associated microbial patterns (PAMPS) that are conserved between, and shared by classes of microbial pathogens not found in higher eukaryotes (refer to chapter 2.4). These receptors allow the recognition, uptake and phagocytosis of microbes by antigen-presenting cells. However, the innate system not only uses PRRs to recognise pathogens, but also utilises the complement system, specialised receptors on NK cells and certain intracellular receptors (Hoebe *et al.* 2004).

Innate and subsequent adaptive immune responses are not isolated sequential host events to infection but through cellular contacts and the secretion of soluble mediators, they interact with and impact upon one another. In particular, the cytokine milieu established during innate inflammatory responses to pathogens initiates the migration of antigen-specific T cells to lymph nodes where they meet antigen-presenting cells (APCs). The commencement of acquired immunity is dependant upon the innate system providing effective antigen presentation through class I and class II MHC antigens. These are predominantly located on macrophage and dendritic cells. In contrast to MHC-II binding of internalised exogenous antigenic molecules, MHC-I principally binds endogenous peptides from the cytosol (Harding *et al.* 2003). The concomitant engagement of T cell receptors with co-stimulatory molecules, including CD80, CD86 and CD40, is necessary to initiate an efficient acquired immune response (Borriello *et al.* 1997). In addition to the recognition of peptides, T cells also recognise microbial-derived and self-derived lipids bound to CD1 molecules (Harding *et al.* 2003).

Cells of innate immunity comprise mononuclear phagocytes and polymorphonuclear phagocytes. Pathogens are usually taken up by APCs and transported to secondary lymphoid organs. Antigen recognition within acquired immunity occurs through T cell antigen receptors (TCRs) and immunoglobulin (IgG) B cell receptors. A significant repertoire of these antigen receptors is present within the host, being expressed clonally on the surfaces of lymphocytes (van den Berg *et al.* 2004). The innate immune system developed long before acquired immunity and therefore defects in cells of innate immunity produce a more profound immunodeficiency state than lymphoid aplasia (Beutler 2004). Of critical importance in the crosstalk between cells of the innate and acquired immune responses is the release of soluble inflammatory mediators, including cytokines.

2.2 Contribution of immune cells to the cytokine network

2.2.1 Neutrophils

Polymorphonuclear leukocytes (PMN) are the first line of defence against microorganisms and act as critical effector cells within both innate and adaptive immune responses. Neutrophils are the most abundant leukocyte population, accounting for about 60% of all circulating leukocytes. In the average adult human, approximately 10 million PMN are

produced and released into the bloodstream every minute. However, due to their short lifespan before undergoing apoptotic cell death, their circulatory numbers remain constant. Neutrophil homeostasis is tightly regulated by dendritic cells and macrophages. Phagocytosis of apoptotic PMN leads to suppressed phagocyte secretion of IL-23, which in turn down-regulates IL-17 expression and thereby granulocyte-colony stimulating factor (G-CSF) production from γδ and αβ neutrophil-regulatory T cells (Stark *et al.* 2005).

The essential role of neutrophils as professional phagocytes has been extensively investigated. This has rather overshadowed research into their ability to orchestrate the immune response by the generation of their own inflammatory mediators including cytokines and chemokines (Kunkel *et al.* 1995, McDonald 2004). As individual cells, neutrophils produce lower cytokine quantities than macrophages or dendritic cells. However, in consideration of their vast numbers at inflammatory sites in comparison with other immune cells, they are an important source of cytokines. Through the release of these inflammatory mediators, PMN greatly influence the outcome of infectious diseases (Cassatella 1995). Neutrophils express TLR-4 and CD14 (Sabroe *et al.* 2002) and, in response to pathogenic challenge and concomitant cytokine stimulation, produce TNF-α, IL-1β, IL-8, IL-12 and IFN-γ (Cassatella *et al.* 1995b, Cassatella 1999, Witko-Sarsat *et al.* 2000, Ellis and Beaman 2002, Ethuin *et al.* 2004). Indeed, several agents have been identified that induce neutrophil expression of these proinflammatory cytokines. These include the cytokines IFN-γ, TNF-α, IL-1β, IL-4, IL-10, IL-13 and TGF-β, bacterial PAMPS, fungi, viruses, protozoa and matrix proteins (Meda *et al.* 1994). Through the release of these cytokines, neutrophils are essentially capable of linking innate and adaptive immunity.

In addition to the production of Th1 type cytokines described above, neutrophils also secrete the Th2 type cytokines IL-10 and IL-4. Furthermore, they have been implicated in driving Th2 type responses (Romani *et al.* 1997, Brandt *et al.* 2000). In a similar manner to dendritic cell sub-populations, it is suggested that PMNs may be divided into subsets capable of promoting either Th1 or Th2 driven host responses (Denkers *et al.* 2003). PMNs not only regulate T cell functions, but LPS-stimulated neutrophils are also responsible for inducing the production of IL-12 and TNF-α from dendritic cells (Bennouna and Denkers 2005). Taken together, it is evident that neutrophils perform a central role in controlling the

immediate innate immune response. Moreover, through their release of cytokines and chemokines, they subsequently influence the development of the adaptive response.

2.2.2 Antigen presenting cells: macrophages and dendritic cells

Macrophages and immature dendritic cells (DCs) are phagocytic antigen presenting cells that not only internalise and degrade bacteria but also present bacterial peptides on MHC-I and MHC-II molecules for T cell recognition. These phagocytic antigen-presenting cells are essential components within the interface between innate and adaptive immunity. They originate from bone marrow and circulating precursors and are ideally located within the peripheral tissues to swiftly respond to harmful pathogenic challenge. Dendritic cells can be distinguished in at least two subsets: myeloid derived DCs and dendritic cells derived from non-myeloid precursors, of which plasmacytoid DC (pDC) and Langerhans cells predominate (Banchereau *et al.* 2003).

Whilst DCs are the most important APCs for T cell priming, macrophages are important in presenting microbial antigens to effector T cells. Thereby, macrophages are essential in eliciting appropriate cytokine responses (Harding *et al.* 2003). Macrophages also interact with T lymphocytes by the release of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). Both ROI and RNI suppress lymphocyte function and this is further enhanced by the effect of low concentrations of IL-10 upon macrophages. Conversely, higher concentrations of IL-10 profoundly reduce macrophage derived ROI secretion. Furthermore, IL-10-induced suppression of TNF-α expression may also be capable of down-regulating the release of RNI (Bogdan *et al.* 1991). Importantly, the microbicidal activity of macrophages to pathogens is inhibited by IL-10 in a dose-dependant manner by a reduction in nitrogen oxide metabolites (Gazzinelli *et al.* 1992).

Through their virulence factors, pathogens induce DC maturation that consequently influences antigen presentation and T cell stimulation (Pierre *et al.* 1997, Rescigno *et al.* 1998). Pathogen recognition by dendritic cells occurs through cellular expression of Toll-like receptors (TLRs) and these are responsible for modulating co-stimulatory molecule expression. Gram-negative bacteria and LPS, particularly in the presence of IFN-γ, also increase macrophage expression of MHC-I, MHC-II, CD40 and CD86 (Yrlid *et al.* 2000, Svensson *et al.* 2001). However, long-term stimulation of macrophages with PAMPS leads

to down-regulated expression of MHC-II, thereby reducing their antigen processing capabilities (Chu *et al.* 1999). Dendritic cells are the most potent type of APC and are central to initiating both innate and acquired immunity (Banchereau and Steinman 1998). On encountering pathogens within peripheral tissues, DCs increase their antigen presentation properties, express co-stimulatory molecules and migrate to lymphoid organs to establish an immune response. Dendritic cells not only produce a core response to any pathogen but also exhibit stimulus-specific maturation and activation (Huang *et al.* 2001).

Dendritic cells and macrophages have complimentary roles in anti-bacterial immunity. Dendritic cells appear to be critical for the priming of naïve $CD8^+$ T cells. Macrophages however, are important in presenting bacterial antigens to effector T cells at infected tissue sites, producing and inducing cytokine expression and preventing bacterial replication (Harding *et al.* 2003). The first phase of the innate response to pathogenic challenge involves the rapid expression of monocyte-derived TNF-α, which in turn induces the release of pro-inflammatory cytokines such as IL-1 and IL-6. The second phase response involves the production of IL-10, which is detectable within 8 h of exposure to LPS. Furthermore, cytokines released by cells of innate immunity, including macrophages, are responsible for regulating the differentiation and activity of DCs. Indeed, macrophages are a significant source of type 1 cytokines and on encountering mycobacterial pathogens, release significant quantities of IL-12, TNF-α and IFN-γ (Wang *et al.* 1999). The local release of inflammatory cytokines, particularly IL-12 and IL-18, can further induce macrophage-derived cytokine secretion, including IFN-γ (Schindler *et al.* 2001).

2.2.3 Mast Cells

Mast cells are multifunctional, long-lived tissue dwelling cells distributed throughout connective and mucosal tissues. They originate from myeloid precursors and circulate as $CD34^+$ committed progenitor cells. Their differentiation into mature mast cells occurs only after tissue entry (Kirshenbaum *et al.* 1991). Human mast cells consist of two subsets that show functional, biochemical and morphological heterogeneity. Mast cells found in mucosal tissues contain only tryptase as a protease. In contrast, those located in connective tissue sites contain both tryptase and chymase.

Within tissue sites, mast cells interface with the external environment at epithelial surfaces and around blood vessels (Church and Levi-Scaffer 1997, Féger *et al* 2002). The preferential location of mast cells at the portals of entry to many infectious agents ensures their early contact with invading pathogens. On activation, mast cells spontaneously extrude granule-associated substances, such as histamine. Furthermore, they express lipid-derived mediators including leukotrienes minutes after exposure to pathogens (Brody and Metcalfe 1998). Within hours of activation, mast cells synthesise an array of cytokines including GM-CSF, IFN-γ, TNF-α, IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13 and IL-18, and several chemokines (Kobayashi *et al.* 2000). Mast cells are important players within several chronic inflammatory disease processes including rheumatoid arthritis (RA), Crohn's disease, interstitial cystitis and scleroderma (Krishnaswamy *et al.* 2001).

Mast cells are a major source of TNF-α and IL-4, which are decisive in initiating appropriate host immune and inflammatory responses to pathogenic bacteria (Li and Krlis 1999). Indeed, mast cell-derived TNF-α plays a crucial function in innate immunity by enhancing the recruitment of neutrophils to sites of pathogenic bacteria (Maurer *et al.* 1998). Bacterial recognition and killing is further aided by receptors for complement and Fcγ receptors that bind IgG coated bacteria to the mast cell membrane (Talkington and Nickell 2001). Of importance, mast cell-derived leukotriene B_4 recruits memory $CD4^+$ and effector $CD8^+$ T cells to sites of inflammation. Subsequently, mast cells are capable of processing and presenting bacterial antigens to these locally recruited T cells (Ott *et al.* 2003, Nigrovic and Lee 2005). By secreting biologically active products in response to pathogenic components and other stimuli generated during innate immune responses, mast cells modulate DC, T cell and B cell functions. This occurs in such a way that the ensuing acquired immune response may be enhanced and modified. Therefore, mast cells are not mere effector cells within innate immune responses but are critical immune cells linking and influencing both innate and acquired immunity (Galli *et al.* 2005).

2.2.4 Natural Killer (NK) Cells

Natural killer (NK) cells are an important component of innate immunity in the resistance to intracellular pathogens and tumours. Through their release of chemokines and cytokines, NK cells mediate inflammatory responses to induce haematopoiesis and regulate the developing acquired immune response (Moretta *et al.* 2005). NK cells comprise 10-15% of

peripheral blood lymphocytes and are defined by a lack of expression of CD3 and their expression of CD56. NK cells carry out their effector functions by balancing endogenous MHC-1 class-specific inhibitory derived responses with multiple activating receptor responses (Moretta 2005).

In response to pathogenic challenge, one of the principle immunomodulatory properties of NK cells is their ability to secrete IFN-γ. They are also capable of secreting TNF, GM-CSF, CCL3, CCL4 and CCL5, thereby contributing towards the recruitment of other inflammatory cells to the site of inflammation (Ferlazzo 2005). Various stimuli enhance NK cell activity that is accompanied by increased expression of Fas ligand (FasL), CD11a, CD11b, CD28 and CD44 and decreased levels of CD62L. NK cells do not have specific antigen receptors and lack the capacity to specifically recognise foreign antigens. As a consequence, the events that lead to NK cell activation are very different from those of other lymphocytes. For example, cytokines (IL-2, IL-12, IL-15, IL-18 and IL-21) and other ligands (Ly49, CD28, ICAM) can directly activate NK cell effector functions without the requirement for antigen-specific recognition. These act through their extensive range of cell surface receptors, including members of the C-type lectin or Ig superfamily, natural cytotoxic receptors (NCRs), cytokine receptors and a range of costimulatory molecules (Moretta 2005).

2.2.5 B lymphocytes

Similar to APCs, B cells recognise PAMPS such as LPS (Poltorak *et al.* 1998, Hoshino *et al.* 1999a). Indeed, PAMPs are capable of directly stimulating B cells and at high concentrations lead to polyclonal B cell activation (Möller 1975). Furthermore, CpG-containing DNAs and LPS modulate B cell effector function, promote B cell proliferation, induce plasmacytoid differentiation and class switching as well as regulating autoimmunity and Th1-related inflammation (Peng 2005). Many PAMP-related effects upon B cells occur through TLR ligation and signalling. Indeed, B cells express significant mRNA encoding TLR-1, and TLR-6-10 (Peng 2005). In contrast to T cells, B cells are efficient at recognising the geometry of pathogens. Pathogenic microorganisms are therefore able to directly crosslink B cell receptors (BCRs) thus activating B cells without the requirement of Th- cell-dependant isotype switching and memory B cell generation (Ruedl *et al.* 2000). Subsequently, BCR or CD40 ligation results in significant up-regulation of TLR

expression within the B cell. Of importance, B cells are 100 to 1000 times more potent than other antigen presenting cells, such as dendritic cells and macrophages, at inducing T cell activation to a specific antigen (Lanzavecchia 1990).

B cells are now recognised as being fundamental in the effector mechanism of tissue damage within chronic inflammatory bone diseases such as RA (Kotzin 2005). They release the key proinflammatory cytokine regulators TNF-α, IL-1, lymphotoxin, IL-6 and IL-10 (Duddy *et al.* 2004). The release of the latter two cytokines further stimulates B cell function via a feedback loop and in doing so, perpetuates chronic inflammation (Kotzin 2005). B cells also have central responsibilities within the inflammatory cascade through their effects on dendritic cells, their influence on germinal centre formation and the effective presentation of antigen to $CD4^+$ T cells, thereby mediating $CD4^+$ T cell activation. Interestingly, T cell infiltration into inflammatory sites and the ability of tissues to produce IL-1 and IFN-γ are inhibited in B cell-depleted mice (Kotzin 2005). This suggests that, at least in part, Th1 type inflammatory responses are governed by the presence of B cells.

2.2.6 T lymphocytes and adaptive responses

2.2.6.1 Introduction

Antigen recognition is the first stage in the activation and clonal expansion of antigen-specific T cells. Peptide antigens are recognised via the T cell receptor (TCR), which is situated alongside CD4 and CD8 coreceptor molecules. In addition, T cell activation requires costimulatory signalling through molecules such as CD28 and CD40 ligand. $CD8^+$ cytotoxic lymphocytes mediate resistance to infectious agents and tumours. Classically, they recognise antigens that are localised in the cytoplasm of target cells. These are subsequently processed and presented as peptide complexes with MHC-I molecules. $CD4^+$ T cells critically determine the outcome of any given infection. After leaving the thymus, naïve $CD4^+$ T cells are predominantly located in the lymph nodes and have the potential to differentiate into two functionally distinct T helper effector cell subsets, Th1 or Th2. Several factors influence the differentiation process toward Th1 or Th2 lineage including the type of APC, the concentration of antigen, the ligation of select costimulatory molecules and the local cytokine milieu (Szabo *et al.* 2003).

2.2.6.2 T Regulatory Cells

In an attempt to diversify and refine its ability to regulate adaptive immune responses, the immune system has evolved numerous mechanisms. Of these, naturally arising $CD4^+$ T regulatory (Treg) cells afford one of the critical mechanisms for the maintenance and the modulation of self-tolerance. They are responsible for suppressing the activation and expansion of self-reactive lymphocytes, which are implicated in autoimmune diseases. The majority of Treg populations have been defined on their phenotype and their ability to produce suppressor cytokines including IL-4, IL-10 and TGF-β. Treg cells frequently arise after antigen exposure and include regulatory Th2 cells, regulatory Th1 cells, IL-10 producing Tr1 cells, TGF-β-secreting Th3 cells, $CD8^+$ T cells, NK cells and γδ T cells (Piccirillo and Shevach 2004). However, the precise phenotype and exact mode of function of these cells remains poorly defined.

Regulatory $CD4^+$ T cells were originally recognised by their expression of the IL-2Rα chain (CD25). These $CD4^+CD25^+$ regulatory T cells constitute 5-15% of the peripheral $CD4^+$ T cell population and may be classified as naturally occurring (nTreg) or induced (iTreg) (Mittrücker and Kaufmann 2004, Piccirillo and Shevach 2004). In response to a number of different polyclonal stimuli, $CD4^+CD25^+$ regulatory T cells suppress both the proliferation and cytokine production of $CD4^+CD25^-$ responder (T-resp) T cells. This occurs in a cell contact-dependent manner, independent of IL-10 (Beacher-Allan *et al.* 2005). $CD4^+CD25^+$ regulatory T cells are also adept at modulating the responses of $CD8^+$ T cells, NK cells and the antigen specific responses of CD4 T cells to specific antigens (Beacher-Allan *et al.* 2005). These data suggest $CD4^+CD25^+$ cells may significantly contribute towards the regulation of immune responses. Interestingly, $CD4^+CD25^+$ regulatory T cells isolated from RA synovial joints exhibit increased suppressive effects compared with peripheral $CD4^+CD25^+$ T cells. Furthermore, activated synovial fluid T cells have a reduced susceptibility to suppression by these $CD4^+CD25^+$ regulatory T cells. This implies that in chronic inflammatory disease processes, such as RA, the degree of immune regulation is influenced by the balance between activated regulatory $CD4^+CD25^+$ T cells and local inflammatory cells (van Amelsfort *et al.* 2004). However, the potential contribution of T regulatory cells towards PRD pathologic processes has not been investigated.

2.2.6.3 $CD4^+$ effector Th1/ Th2 paradigm

During adaptive immune responses, lymphocytes differentiate to produce an appropriate immune response tailored specifically to the invading pathogens. Importantly, the early cytokine environment at the initial stage of T cell priming influences the type of immune response elicited against different pathogens. $CD4^+$ effector T lymphocytes can be divided into subclasses based upon their cytokine configuration and thereby, their potential functional activity (Mosmann *et al.* 1986). The avidity of the T-cell receptor (TCR) to the antigen/MHC complex, the nature of costimulatory molecules and the local cytokine milieu all influence the fate of the naïve $CD4^+$ Th0 precursor cell (Table 2.1). As such, effector Th1 development is induced by the presence of IL-12. Therefore, mice deficient in IL-12 or signal transducer and activator of transcription (Stat)4 exhibit impaired Th1 responses. Likewise, mice with targeted disruptions of IL-4 or its downstream signalling molecule Stat6 are unable to mount appropriate Th2 responses (Murphy *et al.* 2000). Despite intensive research, the mechanisms regulating the initiation and down-regulation of Th1/Th2 responses are still incompletely understood (Szabo *et al.* 2003).

The Th1 response is a critical element of host defence, responding to IL-12 derived from macrophages and dendritic cells in the presence of pathogens that require internalisation. Characteristic features of Th1 type responses include the secretion of IFN-γ, TNF-α, IL-2, IL-12 and TNF-β (LT) and isotype switching to immunoglobulin G2a (IgG2a). This essentially leads to the development of a dominant cell-mediated response. Thereby, macrophages, NK cells and cytotoxic T cells are activated, resulting in a prolonged inflammatory reaction (Mosmann and Coffman 1989). In addition to specific secreted components, Th1 cells preferentially express the IL-12Rβ2 chain, IL-18R, P-selectin glycoprotein ligand-1 and the chemokine receptors CXCR3 and CXCR5 (Ho and Glimcher 2002).

By binding to its IL-12Rβ2 receptor unit and signalling through Stat4, IL-12 is a key mediator directing the development of Th1 type responses. Furthermore, optimal Th1 development requires sustained IL-12 signalling (Athie-Morales *et al.* 2004). Nevertheless, provided IL-23 is present, IL-12 is not prerequisite for the generation of Th1 responses (Cooper *et al.* 2002). IFN-γ is critically involved in orchestrating protective functions

necessary for the elimination of most pathogenic microbes and is central to Th1 development. IFN-γ promotes phagocytosis and up-regulates microbial killing by inducing the availability of NO, hydrogen peroxide and superoxide in phagocytic cells. When combined with LPS, IFN-γ up-regulates IL-1 and TNF-α expression in macrophages (Collart *et al.* 1986, Sieling *et al.* 1994). IFN-γ is principally produced by macrophage/monocytes, which upon stimulation with IL-18 in combination with IL-12 secrete high levels of IFN-γ protein (Munder *et al.* 1998). IFN-γ signals through Stat1, thereby activating the T-box transcription factor T-bet, which is the principal inducer of IFN-γ and subsequent Th1 differentiation (Bettelli and Kuhcroo 2005, Kitamura *et al.* 2005). Indeed, the effects of T-bet are so potent that the expression of this transcription factor in previously differentiated Th2 cells reverses their phenotype and results in IFN-γ expression. Of importance, there appears to be a strong genetic influence that controls T-bet expression and therefore Th1 polarisation (Höhler *et al.* 2005). Th1 development is also suggested to result from IFN-γ-maintained expression of IL-12Rβ2, thus allowing cellular responsiveness to IL-12. Subsequent signalling by IL-12 activates the Stat4 pathway, thereby inducing expression of IL-18R and stabilising IFN-γ production. Consequently, the expression of IL-18R enables cellular responsiveness to IL-18. This ultimately leads to a Th1 cell capable of antigen driven IFN-γ production and antigen-independent cytokine production of IFN-γ by ligation of its IL-12 and IL-18 receptors (Berenson *et al.* 2004).

In contrast, the Th2 pathway is typically generated in the presence of large extracellular parasites e.g. helminths. Th2 type responses are largely characterised by the development of the humoral pathway and the production of Th2 type cytokines that promote B cell growth. The release of Th2 cytokines, including IL-4, IL-5, IL-10 and IL-13, govern what is considered to be a protective humoral type response, typified by the production of IgG1, IgA and IgE. Effector cells that use these antibody isotypes, including eosinophils via IL-5 and mast cells via IL-4, are thereby stimulated. IL-4 induces Stat6 activation, which promotes the expression of GATA-3. This is an essential transcription factor for both IL-4 and the development of Th2 cells (Bettelli and Kuchroo 2005). In addition to specific patterns of cytokine expression, Th2 cells preferentially express the chemokine receptors CCR3, CCR4 and CCR8 and inducible costimulatory molecule (ICOS) (Ho and Glimcher

2002). In contrast to Th1 cytokines, Th2 type cytokines down-regulate the production of IL-1 and TNF-α by macrophages treated with LPS (Zissel *et al.* 1996).

The fine balance of effects between Th1 and Th2 type subsets generally determine whether there is a successful outcome to an infectious disease or a perpetual chronic inflammatory reaction (Abbas *et al.* 1996). Although extensively investigated, many discrepancies are evident in attempting to define the exact nature of the Th1/Th2 response and the specific role of cytokine mediators. For example, the role of IL-1 is controversial with different groups, using differing *in vivo* murine models, suggesting that IL-1 establishes a type-2 response (Huber *et al.* 1998, Helmby and Grencis 2004). Conversely, others implicate IL-1 in driving Th1 type responses (Shibuya *et al.* 1998). IL-10, a general inhibitor of proliferative and cytokine responses in T cells, is produced by mononuclear phagocytes (Fiorentino *et al.* 1991, de Waal Malefyt *et al.* 1991, Hsu *et al.* 1992) and by both Th1 and Th2 lymphocytes (Del Prete *et al.* 1993). Furthermore, the roles of IL-18 and more recently IL-17 have been debated as to their preferential involvement in driving either Th1 or Th2 mediated responses. Indeed, it has recently been suggested that a third subset of $CD4^+$ T cells exist, termed Th_{17}, which are induced by the presence of IL-23 (Bettelli and Kuhcroo 2005).

A further confounding problem in defining Th1 or Th2 responses in disease processes is the identification of reliable markers that specifically identify $CD4^+$ T effector Th1 or Th2 cells. The elucidation of such stable markers would enable studies of diseased tissues to accurately determine a predilection towards a Th1 or Th2 pathway that results in chronic pathogenicity. Recently, the identification of another IL-1R family member, the T1/ST2 cell receptor, has been proposed as a suitable marker. Whilst IL-18 and IL-18R augment Th1 responses, T1/ST2 appears to be selectively expressed on Th2 cells (Mitcham *et al.* 1996, Xu *et al.* 1998a, Carter *et al.* 2001). Furthermore, T1/ST2 expression strongly correlates with IL-4 production, thus implicating T1/ST2 as a stable marker for Th2 cells *in vivo* (Löhning *et al.* 1998). However, others have demonstrated that ST2 expression is induced in activated human Th2 cells, but it is absent in resting cells implying that ST2 may not be a stable marker (Lécart *et al.* 2002). More recently, Dardalhon *et al.* (2005) have identified that the costimulatory molecule CD226 is expressed on the surface of Th1 cells and this regulates their expansion and effector functions.

In conclusion, it is evident from studies to date that the precise nature of Th1/ Th2 type responses and the role of cytokine regulation in the development of appropriate $CD4^+$ effector T cell responses still remain to be defined. Moreover, the endodontic literature is comparatively devoid of investigations accurately detailing the functional role of the $CD4^+$ effector T cell response within the human PRD lesion. Furthermore, the involvement of regulatory T cells within the initiation and perpetuation of the PRD lesion has not been reported.

Table 2.1 **Characterisation of $CD4^+$ Th subsets.**

Characteristic	Th1 cells	Th2 cells
Cytokine production	IFN-γ, IL-2, TNF, lymphotoxin α	IL-4, IL-5, IL-13, (IL-10 in mice)
Transcription factors	Stat1, Stat4, T-bet, NF-κB	Stat6, GATA3, NFAT
Inducing stimuli	IL-12, IFN-γ, IL-18, IL-27, PRR signalling	IL-4
Chemokine receptor expression	CXCR3, CCR5, CCR1	CCR3, CCR4, CCR8
Antibody isotypes	IgG1, IgG3, IgG2	IgG4, IgE
Effector response	Cell-mediated immunity Macrophage activation Antibody-mediated cellular cytotoxicity	Eosinophil activation Allergy

2.3 Innate immunity and PAMPs

Lipopolysaccharides, whose biological effects were first reported over 100 years ago (Pfeiffer 1892), are an essential component of the outer membrane of Gram-negative bacteria. They are heat stable, non-proteinaceous molecules composed of a predominantly lipophilic region, lipid A, and a covalently linked hydrophilic poly- or oligosaccharide portion (Holst *et al.* 1996). The lipid A domain represents a highly specific and conserved pathogen associated molecular pattern (PAMP) for infection by Gram-negative bacteria. Indeed, Lipid A is the bioactive component of LPS and is responsible for its toxicity (Dixon and Darveau 2005). In humans, the primary target cells of LPS virulence are the professional phagocytes of innate immunity i.e. peripheral monocytes, tissue macrophages, dendritic cells and neutrophils. These cells constitutively express the membrane bound form of the CD14 glycoprotein and Toll-like receptor (TLR)-4 (Haziot *et al.* 1988, Muzio *et al.* 2000). LPS is an important virulence determinant that exhibits several important properties including immunogenicity, induction of pro-inflammatory cytokines, and protection against phagocytosis and complement killing (Devine 2003).

LPS is a potent stimulator of cytokines from inflammatory cells. One of its most important properties is the induction of steady-state levels of IFN-γ mRNA and cell-associated immunoreactive IFN-γ (Fultz *et al.* 1993). IFN-γ further enhances the sensitivity, magnitude and maintenance of inflammatory monocyte responses to LPS *in vitro* (Hayes *et al.* 1995). Moreover, IFN-γ induces increased TLR4 mRNA expression in human monocytes and neutrophils *in vitro* (Muzio *et al.* 2000). In addition to IFN-γ, LPS activation of mononuclear cells leads to the secretion of TNF-α, IL-1β, IL-6, IL-8, IL-12, IL-15, IL-18 and M-CSF. LPS may significantly contribute towards infection associated tissue damage by inducing TNF-α production, thereby stimulating expression of pro-apoptotic genes and promoting the cytoplasmic apoptotic pathway (Alikhani *et al.* 2004). LPS derived from invading endodontic pathogens, which induce resident monocytes and neutrophils to secrete IL-1, IL-6, IL-8 and TNF-α, may therefore promote the degradation of hard tissue surrounding the dental root apex. However, in addition to stimulating the release of these proinflammatory mediators by neutrophils, LPS prepares these cells to rapidly respond to IL-10. Thereby, the extent of the inflammatory response, which may lead to adverse destructive pathways, is carefully regulated (Cassatella *et al.* 2005).

Gram-positive endodontic microorganisms such as *Streptococci* and *Staphylococci* are also capable of causing substantial tissue damage. Although not possessing LPS, these microbes possess a variety of virulent exotoxins including lipopeptides, peptidoglycans, lipoteichoic acid, double stranded (ds)RNA and unmethylated DNA fragments such as CpG-containing motifs. Although their biological properties are weaker than endotoxin, these virulence factors, in addition to Gram-positive pore forming toxins and superantigens, contribute to the overall toxic potential of bacterial challenge of the host (Beutler and Rietschel 2003). Bacterial and viral derived superantigens bind to the outer surface of both MHC-II molecules and the V_β region of the T cell receptor. Each superantigen can bind a variety of the different V_β gene products. Thereby, superantigens are capable of potently stimulating 2-20% of all T cells (Torres and Johnson 1998, Yamazaki and Nakajima 2004). Cell contact with mononuclear phagocytes and a combination of cytokines are usually required for the induction of IFN-γ (Billiau 1996). However, the *Staphylococcal* enterotoxin B (SEB) superantigen is capable of independently inducing IFN-γ production in human NK cells (D'Orazio *et al.* 1995). Indeed, SEB augments antigen-specific T cell responses by up-regulating IL-12 production in macrophages. The increased expression of IL-12 is then responsible for augmenting antigen-induced proliferation of Th1 type cells (Bright *et al.* 1999).

There are common elements in cellular responses to both Gram-negative and Gram-positive pathogens including the release of chemokines and cytokines, for example IL-6 and TNF-α. However, the pattern of gene expression induced by these two distinct PAMPs in leukocyte cell cultures *ex vivo* does not correlate well with *in vivo* protein expression of cytokine mediators in patients suffering with Gram-negative or Gram-positive sepsis (Feezor *et al.* 2003). Studies within humans have identified high levels of LPS within the PRD lesion and dental root canal exudates. However, within human PRD, the precise role of PAMPS upon immune cell activation and specifics of the subsequent induction of cytokine expression is largely unknown.

2.4 Toll-like receptors (TLRs)

Cellular recognition of invading pathogens by APCs is accomplished by pattern recognition receptors (PRR) binding invariant pathogen associated molecular patterns (PAMPS) (Bachmann and Kopf 2002). Members of the Toll-like family of receptors

(TLRs) perform a central role in initiating appropriate host innate responses to invading pathogenic microorganisms. To date, the expression of 11 human TLR paralogues have been found within humans. The cytoplasmic portion of TLRs is similar to that of the IL-1 receptor family and is termed the Toll/IL-1 receptor (TIR) domain. However the extracellular units of both types of receptors are structurally unrelated with TLRs bearing leucine-rich repeats (Takeda and Akira 2005).

Specific microbial virulence factors and invariant microbial structures can be distinguished by the differing Toll receptors. Lipoproteins, viral derived double stranded RNA (dsRNA), LPS, bacterial flagella and bacterial DNA containing unmethylated CpG motifs are recognised by TLR2, TLR3, TLR4, TLR5 and TLR9 respectively. In addition, TLR2 appears to recognise LPS derived from pathogens such as *Porphyromonas gingivalis*, which has a differing number of acetyl chains within the lipid A component from that of Gram-negative LPS (Takeda and Akira 2005). TLR recognition of pathogens induces TLR dimerisation, thereby activating their signalling pathways. As such, TLR2 is capable of forming heterophilic dimers with TLR1 or TLR6 but generally homodimers are formed. Furthermore, gene expression changes induced by pathogens upon first contact are primarily driven by TLRs (O'Neill 2004). However, PAMPS are not the only ligand activators of TLR-induced signalling. Interestingly, TLRs also recognise and are activated by endogenous host proteins such as extra cellular matrix proteins (ECM), heparin sulphate, β-defensins and heat shock proteins (Miyake 2004). Nonetheless, the crucial role of TLRs in the host defence against infection is emphasised by pathogens that are able to manipulate or inhibit TLRs, such as the Vaccina virus that inhibits TLR signalling (O'Neill 2004).

Individual TLR signalling pathways are divergent. Therefore, the activation of each TLR leads to slightly different patterns of gene expression profiles. Nevertheless, MyD88 is common to all TLR signalling pathways and culminates in the activation of NF-κB. Signalling of TLRs through MyD88 is essential for the induction of inflammatory cytokines such as TNF-α, IL-6, IL-12 and IL-18. However, signalling through MyD88-independent pathways, as can occur with TLR4 ligation, may also lead to late NF-κB activation. Excessive production of these inflammatory mediators would result in serious systemic disorders and mortality. TLR-mediated responses are therefore carefully modulated. This can occur through reduced expression of TLR4 and MD-2 that results in

LPS tolerance, reduced expression of IRAK-1, induction of the inhibitor IRAK-M, expression of SOCS1 or ubiquitination-mediated degradation of TLRs (Takeda and Akira 2005). In addition to microorganisms, TLR expression is regulated by many cytokines and chemokines such as colony stimulating factor (CSF-1), macrophage migration inhibitory factor (MIF), IL-2, IL-15, IL-1β, IFN-γ and TNF-α (Takeda *et al.* 2003) and inflammatory mediators such as histamine (Talreja *et al.* 2004).

Each TLR exhibits distinct tissue activity and their absolute levels within differing cell types is highly dynamic (Hopkins and Sriskandan 2005). Thereby, bronchial and gastrointestinal epithelial cells express distinct TLR patterns compared to the blood-brain barrier and endothelium. For example, TLR3 has a central role in protecting the penetration of the blood-brain barrier by West Nile virus whereas TLR2 and TLR4 on lung endothelial cells are sensitive to specific bacterial ligands and direct the migration of rolling neutrophils into inflamed tissues (Hopkins and Sriskandan 2005). In addition to the ligation of TLRs, co-expression of other molecules is often required for cellular responsiveness. This is classically demonstrated by the critical requirement for association of TLR-4 with CD14 and the MD-2 molecule for successful cell signalling to LPS (Miyake 2004).

Although TLR recognition is central to innate immunity, it is now recognised that these receptors are important in linking innate and acquired immune responses. TLR expression appears to be an essential component in the successful recognition and phagocytosis of bacteria, leading to expression of inflammatory cytokine and co-stimulatory molecule genes. In addition to phagocytosis-mediated antigen presentation, TLR-mediated cytokine gene expression instructs the development of antigen specific acquired immunity (Takeda and Akira 2005). Indeed, TLR family members represent critical PRRs, whose signals lead to the generation of effector responses including Th1 and CTL responses. Following TLR ligation, cytokines secreted from DCs, in combination with T cell CD28 engagement, are fundamental in allowing $CD4^+$ effector cell activation by overcoming the suppressive effects of T regulatory cells. The secretion of IL-6 and other cytokines makes $CD4^+$ cells refractory to suppression whilst not altering the activity of T regulatory cells (Pasare and Medzhitov 2004).

2.5 Cytokines: regulators of immune responses

2.5.1 Introduction

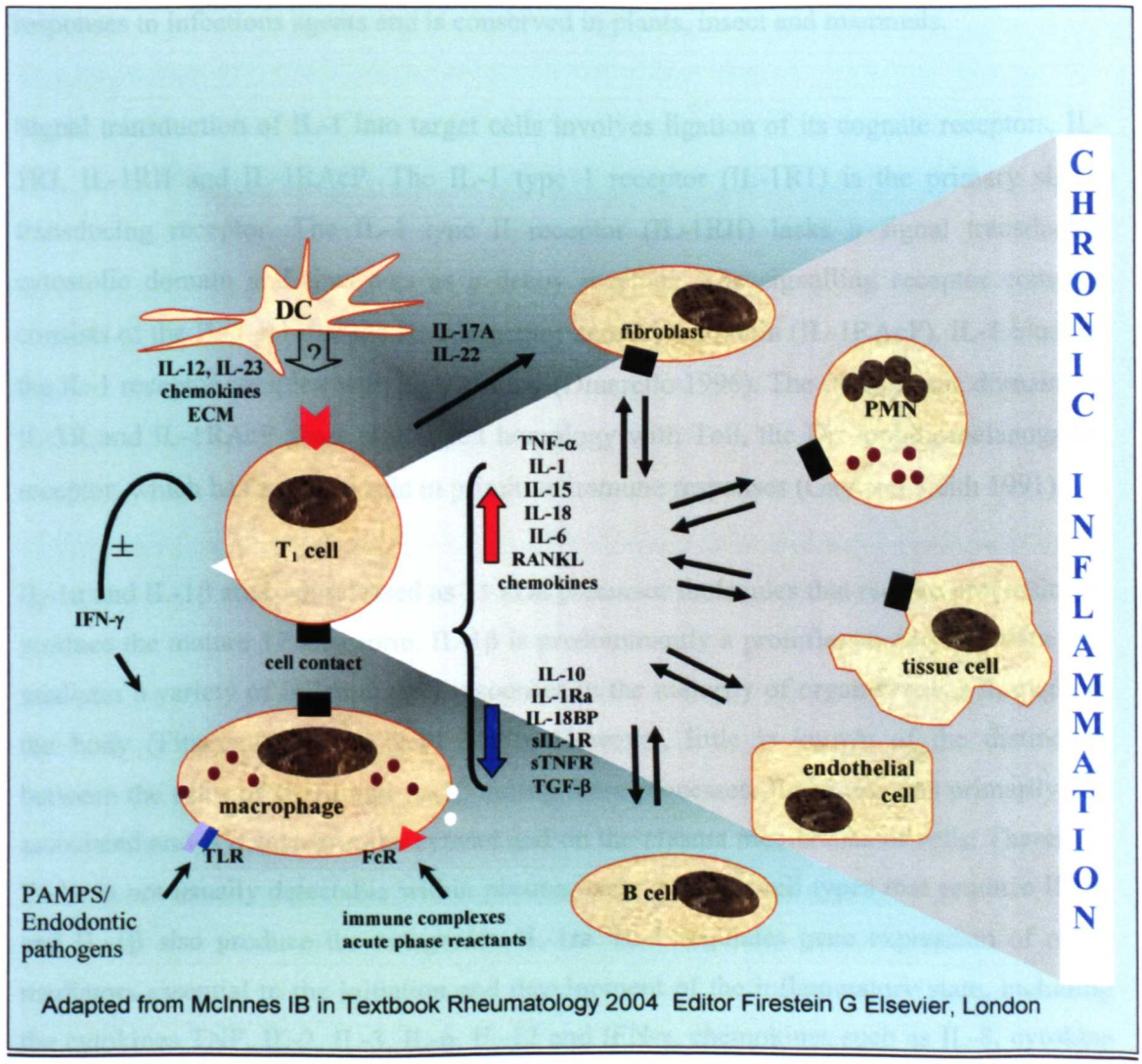
Cytokines are small, secreted proteins that govern host responses against pathogenic challenge. They regulate communication between APCs, lymphocytes and other cells involved within inflammatory and immune responses (Belardelli and Ferrantini 2002). Indeed, these inflammatory mediators orchestrate the initiation, progression and termination of all immunological processes. In addition to instructing host responses within inflammation and immunity, cytokines control cellular differentiation, maturation and activation and thereby, tissue repair and fibrosis. They frequently act simultaneously and may mutually modulate actions upon their cellular targets through synergistic enhancement/suppression or neutralisation effects.

Cytokines, such as IL-2, are traditionally considered to be characteristic of the acquired immune response. However, 'innate' cytokines derived from cells of innate immunity, including TNF-α, GM-CSF, IL-12, IL-15 and IL-18, are also involved in the generation of adaptive immune responses (summarised in Figure 2.1). Although cytokine expression is essential for host protection to inflammatory stimuli, they are also responsible for the pathogenesis of several chronic inflammatory disorders including rheumatoid arthritis (RA), Crohn's disease, ulcerative colitis, psoriasis, and atherosclerosis. Thereby, cytokines are capable of driving chronic inflammatory processes that exhibit concomitant tissue destruction (Andreakos *et al.* 2004).

Cytokines can be broadly classed according to generally accepted functional effects of $CD4^+$ effector Th1 and Th2 cells. Type 1 cytokines, promoting Th1 immune responses include IL-2, IL-12, IL-15, IL-18, IFN-γ, IFN-α and IFN-β and these generally drive cell-mediated immunity. By contrast, IL-4, IL-5, IL-10 and IL-13 are principally involved in Th2 type immune responses that primarily promote humoral responses. However, it is becoming increasingly evident that depending on the local cytokine milieu, these cytokines are capable of evoking either type of immune response. In turn, they determine whether the inflammatory response is directed towards tolerance or immunity (Banchereau and Steinman 1998).

In normal physiological circumstances, inducing stimuli result in transient cytokine production. Consequently, cytokine gene expression is rapidly down-regulated and cytokine proteins are degraded. However, in chronic inflammatory disorders such as PRD, these cytokines are up-regulated for extended periods of time. Importantly, dysregulated cytokine expression does not occur in a random and haphazard fashion. Conversely, they are expressed in an ordered hierarchical cascade of events.

Figure 2.1 **Schematic illustration representing cellular components of PRD and their contribution towards the cytokine network within the host response to infection.** Macrophages activated by endodontic pathogens and/ or microbial-derived products or acute phase reactants release a variety of pro-inflammatory cytokines and chemokines. Excessive levels of these mediators are carefully regulated by the concomitant secretion of antagonist molecules. The inflammatory reaction is further propagated by T cell activation upon cell contact with macrophages, inducing further secretion of proinflammatory cytokines. Activation and release of cytokines by stromal and other inflammatory cells results in a complex inflammatory cytokine network. Dysregulation of this inflammatory response leads to chronic inflammation and associated pathology.



2.5.2 Interleukin (IL)-1β

The IL-1 cytokine family is classically composed of IL-1α, IL-1β and IL-1ra. These three family members are structurally related to each other and bind to IL-1 receptors (IL-1R) on cell membranes (Dinarello 1994, 1996). Gene sequences encoding six new members of the IL-1 family have recently been elucidated and named from IL-1F5 to IL-1F10 (Barton *et al.* 2000, Busfield *et al.* 2000, Smith *et al.* 2000, Debets *et al.* 2001). IL-1α shares 22% sequence similarity with mature IL-1β and 18% with IL-1ra. IL-1α and IL-1β are potent agonists and recognise the same type 1 IL-1 receptor (IL-1R). IL-1 receptor antagonist (IL-1ra) acts as an antagonist to this receptor, thereby blocking the biological response of IL-1α and IL-1β. The IL-1 signalling system is critical for appropriate innate immune responses to infectious agents and is conserved in plants, insect and mammals.

Signal transduction of IL-1 into target cells involves ligation of its cognate receptors, IL-1RI, IL-1RII and IL-1RAcP. The IL-1 type 1 receptor (IL-1R1) is the primary signal transducing receptor. The IL-1 type II receptor (IL-1RII) lacks a signal transducing cytostolic domain and thus acts as a decoy receptor. The signalling receptor complex consists of the IL-1-R1 and the IL-1 Receptor accessory protein (IL-1RAcP). IL-1 binds to the IL-1 receptor complex with high affinity (Dinarello 1996). The cytoplasmic domains of IL-1R and IL-1RAcP share significant homology with Toll, the Drosophila melanogaster receptor, which has a central role in primitive immune responses (Gay and Keith 1991).

IL-1α and IL-1β are both released as 31 kDa precursor molecules that require processing to produce the mature 17 kDa form. IL-1β is predominantly a proinflammatory cytokine that mediates a variety of inflammatory responses in the majority of organ systems throughout the body (Fitzgerald and O'Neill 2000). However, little is known of the distinction between the roles of IL-1α and IL-1β during these processes. IL-1α remains primarily cell associated and is detected in the cytosol and on the plasma membranes of cells. Therefore, IL-1α is not usually detectable within plasma. Nearly all the cell types that produce IL-1α and IL-1β also produce the antagonist, IL-1ra. IL-1 regulates gene expression of many mediators essential to the initiation and development of the inflammatory state, including the cytokines TNF, IL-2, IL-3, IL-6, IL-12 and IFN-γ, chemokines such as IL-8, cytokine receptors, growth factors, adhesion molecules, matrix metalloproteinases and clotting

factors (Dinarello 1996). Through these multiple effects, IL-1 is fundamental in maintaining the health of the organism.

Accumulating evidence suggests that IL-1β levels are increased at sites of active bone resorption in a variety of chronic inflammatory bone and joint diseases (Tatakis 1993, Taubman and Kawai 2001). It is also well established that viruses, bacteria, yeasts and soluble microbial products up-regulate IL-1β expression (Dinarello 1996). Furthermore, high levels of IL-1β have been detected within dental root canal exudates from teeth associated with periradicular lesions (Matsuo *et al.* 1994) and within the lesion itself (Barkhordar *et al.* 1992). However, factors governing IL-1β expression within the human PRD lesion are unknown.

2.5.3 Interleukin-4

IL-4 is a 15 kDa globular glycoprotein originally described as a B cell growth factor (Paul and Ohara 1987). It signals through type I and type II receptor complexes that share a common gamma chain (γc) with IL-7 and IL-9 (Reinecker and Podolsky 1995). In addition to the γc chain, the type I receptor contains an IL-4Rα subunit whereas the type II receptor contains the IL-13Rα. The secreted extracellular domain of IL-4Rα, termed sIL-4Rα, binds IL-4 and antagonises its activity. Ligation of the IL-4R by IL-4 leads to Jak1 and Jak3 activation and subsequent nuclear translocation of Stat6 to induce its target genes. In addition, the IL-4Rα chain also recruits insulin receptor unit substrate-2 that results in the activation of the Akt signalling pathway (Mowen and Glimcher 2004).

IL-4 is a pleiotropic cytokine that regulates diverse T and B cell responses including cell proliferation, survival and gene expression. Produced by mast cells, basophils, NKT cells, T cells and bone marrow stromal cells, IL-4 is the hallmark cytokine regulating the differentiation of naïve $CD4^+$ T cells into effector Th2 type cells. IL-4 is responsible for up-regulating MHC-II on antigen presenting B cells, promoting antibody isotype switching to IgE and IgG1 and serving as a mitogen for B cells (Nelms *et al.* 1999). Through its ability to inhibit macrophage expression of IL-1, TNF-α and IL-6, it is considered as a predominantly anti-inflammatory cytokine (Hart *et al.* 1991).

2.5.4 Interleukin-6

IL-6, leukaemia inhibitory factor (LIF), oncostatin (OSM), IL-11 and cardiotrophin-1 (CT-1) are classed as gp130-signalling cytokines. These cytokines have common features that include functional redundancy, structural similarity and a signal-transducing component (gp130) in their receptors (Hirano 1998). Human IL-6 is synthesised as a precursor protein of 212 amino acids (Kerr *et al.* 2001). The IL-6 receptor consists of two polypeptide chains. The α chain (CD126) is an 80 kDa transmembrane glycoprotein that binds IL-6 with low affinity and its cytoplasmic domain is not necessary for signal transduction. A soluble form of the IL-6Rα has also been described which acts in an agonistic rather than an antagonistic manner (Hirano 1998). IL-6 activates gp130 through this soluble form, even on mast cells that lack the IL-6Rα on their membranes (Kishimoto *et al.* 1995). The β chain is a 130 kDa transmembrane glycoprotein that interacts with the IL-6/IL-6Rα complex to form a trimer. The trimer as such is not capable of signalling and the D1 domain of the gp130 is required for forming the higher order activation complex. The sequential assembly of the IL-6 signalling complex results in the activation of the Jak kinases followed by the phosphorylation and nuclear translocation of Stat3 and to a lesser extent Stat6 (Diehl and Rincón 2002).

IL-6 is an important multifunctional cytokine centrally involved in immune responses, inflammation, haematopoiesis as well as the regulation of the endocrine and nervous systems (Kerr *et al.* 2001). Several cell types produce IL-6 including leukocytes, monocytes, macrophages, T and B-lymphocytes, polymorphonuclear cells and mast cells. Furthermore, many nonimmune cells secrete IL-6 including endothelial and epithelial cells, keratinocytes, fibroblasts, adipocytes, osteoblasts, bone marrow stromal cells, synoviocytes, chondrocytes and trophoblasts. IL-1, IL-4, TNF-α and IFN-γ are all capable of modulating cellular IL-6 expression. A wide variety of biological activities in various cell populations are elicited by IL-6 and it plays a major role in cellular activation. IL-6 acts as a B cell differentiation factor responsible for the final maturation of B cells into plasma cells, which results in increased IgM, IgG and IgA production. IL-6 also stimulates the proliferation of thymocytes and peripheral T cells and promotes the activation, proliferation and differentiation of NK cells. Of importance, IL-6 may have a positive, negative or neutral effect on cell proliferation depending on the target cell type, its state of differentiation and cross-talk with locally expressed cytokines (Xing *et al.* 1998, Heinrich

et al. 1998, Jones *et al.* 2001). Thereby, IL-6 may exert pro- or anti-inflammatory effects within differing inflammatory conditions.

2.5.5 Interleukin-8

Acute inflammation is characterised by plasma and leukocyte extravasation from the blood compartments towards the injured tissue. PMNs are the first leukocyte population to migrate to areas of inflammation, and CXC chemokines are critical in the recruitment of PMNs to these inflammatory sites. IL-8, classed as the chemokine CXCL8, is a proinflammatory CXC type chemokine that signals through the CXCR1 and CXCR2 receptors. The predominant form of IL-8 is an 8.4 kDa protein containing 72 amino acid residues.

IL-8 is a potent chemoattractant and activator of neutrophils and is of pivotal importance for the generation of acute inflammatory responses (Witko-Sarsat *et al.* 2000). Several cells express IL-8 including T lymphocytes, epithelial cells, keratinocytes, fibroblasts, endothelial cells and neutrophils. IL-8 expression can be induced by bacterial PAMPS, fungi and related products, mitogens such as PHA, cytokines such as IL-1β and aggregated immune complexes (DeForge *et al.* 1992). Indeed, IL-8 expression can be induced by TNF-α and/or IL-1 within cultures of dermal and gingival fibroblasts (Larsen *et al.* 1989, Mielke *et al.* 1990, Kristensen *et al.* 1991, Takashiba *et al.* 1992), keratinocytes (Larsen *et al.* 1989, Kristensen *et al.* 1991) or endothelial cells (Kristensen *et al.* 1991). Of importance, IL-8 is a key modulator in the release of PMNs from the bone marrow (Terashima *et al.* 1998).

IFN-γ inhibits the expression of IL-8 in human monocytes stimulated with IL-2 (Gusella *et al.* 1993), IL-10 or LPS (Schnyder-Candrian *et al.* 1995). IFN-γ also interferes with LPS and TNF-α-induced IL-8 production in human PMNs by down-regulating gene transcription within these cells (Cassatella *et al.* 1993, Cassatella *et al.* 1995a). Moreover, IL-8 release is impaired by IFN-γ in cultures of human synovial or dermal fibroblasts (Rathanaswami *et al.* 1993, Maruyama *et al.* 1995), arthritic osteoblasts (Lisigonoli *et al.* 2002) and endothelial cells (Borgmann *et al.* 2002). Reduced IL-8 expression has been associated with impaired TNF-α-induced NF-κB and activator protein 1 activity (Borgmann *et al.* 2002). IL-8 expression has been reported within human PRD tissue.

However, factors that govern the expression of IL-8 within the PRD lesion have not been identified.

2.5.6 Interleukin-10

IL-10, an 18.5 kDa non-N-glycosylated polypeptide, was first described as "cytokine synthesis-inhibitory factor" and represents one of the most important immunoregulatory cytokines (Fiorentino *et al.* 1989). IL-10 signalling occurs through its receptor, a member of the class II cytokine receptor family. This consists of two subunits, the ligand specific IL-10R1 subunit and accessory IL-10R2 chain. These subunits are constitutively expressed on a variety of cells (Moore *et al.* 2001). IL-10 signalling predominantly activates the Jak/Stat3 pathway in myeloid cells and Stat5 in non-myeloid cells. Within myeloid cells, Stat3 is required for many of the anti-inflammatory effects induced by IL-10. However, several other IL-10 signalling pathways have been suggested (Moore *et al.* 2001).

IL-10 is produced as part of the homeostatic response to infection and inflammation and plays a critical role in limiting the duration and intensity of immune and inflammatory reactions (Moore *et al.* 2001). IL-10 production is carefully regulated as excessive production leads to an inability to control infectious pathogens. Conversely, insufficient IL-10 leads to pathology secondary to tissue injury. This is classically demonstrated in IL-10 gene-deficient mice, which develop chronic inflammatory disorders as a consequence of unregulated inflammatory cytokine production (Lang *et al.* 2002). Both Th1 and Th2 type $CD4^+$ T cell lines are capable of IL-10 production and human $CD8^+$ T cells also produce this cytokine (Yssel *et al.* 1992). In addition to peripheral T cells, activated human monocytes, macrophages, B cells and keratinocytes are important cellular sources of IL-10 (Powrie *et al.* 1997). Kinetic studies reveal that following the activation of T cells, monocytes and macrophages, IL-10 production occurs at a relatively late stage. Furthermore, maximal IL-10 mRNA expression in $CD4^+$ T cells and purified peripheral blood T cells is obtained after 24 h and IL-10 protein synthesis peaks between 24 h and 48 h after cellular activation (Yssel *et al.* 1992).

IL-10 inhibits the synthesis and gene expression of several proinflammatory cytokines including TNF-α, IL-1, IL-6, IL-8, IL-12, IL-18 and GM-CSF within monocytes, T and B lymphocytes, neutrophils and mast cells (Del Prete *et al.* 1993, Chernoff *et al.* 1995).

Furthermore, IL-10 modulates inflammatory reactions, regulates B cell functions (Fiorentino *et al.* 1989) and is a potent simulator of NK cells (Gerard *et al.* 1996, Zheng *et al.* 1996, Ignatius *et al.* 2000). Indeed, IL-10 is a dominant immunosuppressive and anti-inflammatory cytokine that deactivates macrophages and dendritic cells and suppresses antigen presentation cells (Moore *et al.* 2001). The inhibitory effects of IL-10 on T cells and NK cells occur indirectly through its effects upon macrophages and monocytes (Ding and Shevach 1992). Indeed, its major function appears to be the suppression of macrophage released cytokines, oxygen radicals, nitric oxide and chemokines (Bogdan *et al.* 1991, Gazzinelli *et al.* 1992, Roilides *et al.* 1997) by inhibiting NF-κB signalling (Wang, *et al.* 1995, McInnes *et al.* 2001, Denys *et al.* 2002). Furthermore, the inhibitory effect of PGE_2 on TNF-α and IL-6 expression from LPS stimulated macrophages is IL-10 dependent (Strassmann *et al.* 1994).

The effects of IL-10 in combination with LPS upon human monocyte-derived DCs reveal the activation of distinct functional pathways when compared with the actions of IL-10 alone. These effects can be categorised as: the inhibition of inflammation and immunity and the regulation of tissue remodelling; the tuning of cytokine/growth factor receptors: the stimulation of B cell function and lymphoid tissue neogenesis (Perrier *et al.* 2004). Although IL-10 generally suppresses the production of DC-derived chemokines, it is also capable of stimulating CCL13, CCL16 and CCL18 expression (Perrier *et al.* 2004). Taken together, it is evident that IL-10 is more than a mere deactivator of macrophage and DC function.

IL-10 possesses an important position within chronic inflammatory diseases. It is detectable at relatively high levels within inflamed joints and the addition of IL-10 neutralising antibodies to dispersed RA membrane cultures increases IL-1β and TNF-α expression. In contrast, the addition of exogenous IL-10 suppresses IL-1β and TNF-α production and reduces IL-8 secretion after 5 days. Taken together, these data indicate a relative deficiency of anti-inflammatory cytokines in diseased, chronically inflamed joints (Katsikis *et al.* 1994). Stimulation of antigen presenting cells with LPS induces IL-10 release that is further increased by the presence of damaged cells (Byrne *et al.* 2002, Huang *et al.* 2001). Furthermore, the expression of TNF-α and IL-1β by LPS-stimulated APCs in the presence of damaged cells is reduced (Byrne *et al.* 2002).

However, several inconsistencies exist in defining a pro- or anti-inflammatory role for IL-10. Priming of primary human macrophages with the type I interferon IFN-α diminishes the ability of IL-10 to suppress TNF-α production. Indeed, this actually contributes towards a proinflammatory gain in IL-10 activity (Sharif *et al.* 2004). Intravenous administration of IL-10 to healthy volunteers has proinflammatory effects through the release of IFN-γ, IP-10 and granzyme levels (Lauw *et al.* 2000). However, these proinflammatory effects may be counteracted by the inhibition of TNF-α, IL-1β, IL-6 and IL-8 expression (Chernoff *et al.* 1995, Pajkrt *et al.* 1997) and the down-regulation of T cell function (Chernoff *et al.* 1995). Nevertheless, it appears that the overall effect of IL-10 in mice and humans is to promote local effector mechanisms such as NK cell activation and the enhancement of expression of cytotoxic molecules.

More recently, homologues of IL-10 have been described that also exhibit potential immune regulatory roles (Conti *et al.* 2003). Of these, IL-19 and IL-20 are preferentially expressed in monocytes, IL-22 (summarised in chapter 2.5.10) and IL-26 have been exclusively detected within T cells and IL-24 expression has been located within both monocytes and T cells (Wolk *et al.* 2002).

2.5.7 Interleukin-12

IL-12 is a critical cytokine that bridges the division of cellular and humoral branches of host immunity. Bioactive IL-12p70 is a major Th1 driving cytokine produced by DCs interacting with $CD4^+$ T effector cells. The IL-12p70 molecule is a 75 kDa heterodimer comprising two disulphide-linked p35 (light chain) and p40 (heavy chain) subunits encoded by separate genes (Gubler *et al.* 1991, Wolf *et al.* 1991). Most cell types constitutively express IL-12 p35. Conversely, p40 is inducible and restricted to a subset of haemopoietic cells namely B cells, macrophages, granulocytes and DCs (Trinchieri *et al.* 1998). Optimal production of the bioactive IL-12p70 heterodimer by DCs both *in vivo* and *in vitro* requires two signals. These are provided by initial microbial stimuli, which predominantly up-regulate the expression of CD40 on DCs, and a T cell derived stimulus produced by CD40 ligand expression from interacting activated T cells (Cella *et al.* 1996, Snijders *et al.* 1998).

The IL-12 receptor is composed of IL-12Rβ1 and IL-12Rβ2 and is expressed mainly on activated T- and NK cells (Presky *et al.* 1996). Ligand binding to the IL-12R activates the Janus kinase (JAK)-Stat pathway of signal transduction. The specific cellular effects of IL-12 are principally due to activation of Stat4 (Thierfelder *et al.* 1996). Up-regulation of the IL-12R chains is enhanced by IFN-γ, TNF, IL-12 itself and co-stimulation with CD28. The IL-12Rβ2 is confined to Th1 cells and its expression correlates with responsiveness to IL-12 (Rogge *et al.* 1997, Szabo *et al.* 1997). Furthermore, expression of this subunit is increased on resting T cells by IL-18 (Chang *et al.* 2000).

Several cytokines such as IFN-γ are able to act on a variety of cells, thereby increasing their ability to secrete IL-12 (Hayes *et al.* 1998). Surprisingly, the Th2 type cytokines IL-4 and IL-13 can also induce IL-12 secretion (D'Andrea *et al.* 1995, Ma *et al.* 1996). A number of inhibitory factors of IL-12p70 production by DC cells have been elucidated. Most of these observations have been made using cultures of the human monocyte-derived DC. Among these inhibitors, IL-10 is the most potent mediator acting on several cell types of human and murine dendritic cells (De Smedt *et al.* 1997, Buelens *et. al.* 1997, Allavena *et al.* 1998, Corinti *et al.* 2001). TGF-β and surprisingly TNF are also capable of down-regulating IL-12 expression (Du and Sriram 1998, Ma and Trinchieri 2001).

IL-12 enhances the proliferation and cytolytic activity of activated NK cells and T cells and potently stimulates their production of IFN-γ (Trinchieri 1994). Furthermore, IL-12-induced secretion of IFN-γ requires the presence of low levels of both TNF-α and IL-1β (D'Andrea *et al.* 1993, Hunter *et al.* 1995). Most importantly, IL-12 induces the development of Th1 cells *in vitro* (Manetti *et al.* 1993) and *in vivo* (Afonso *et al.* 1994). In addition, IL-12 is a potent cofactor in the stimulation of growth, IFN-γ synthesis and cell adhesion of differentiated Th1 cells (Germann *et al.* 1993). IL-12 also has effects on B cells and, in synergy with IL-18, inhibits Ig production through up-regulated production of IFN-γ from these cells (Lauwerys *et al.* 1998). In response to infection, IL-12 expression occurs rapidly in DCs and this is independent of IFN-γ or responses from T cells (Gazzinelli *et al.* 1994, Scharton-Kersten *et al.* 1996). More recently, IL-12 has been demonstrated to induce the synthesis and secretion of biologically active IFN-γ from human neutrophils (Ethuin *et al.* 2004). Thus, in host responses to pathogenic challenge, IL-12 is a key cytokine regulating responses between innate and acquired immunity (Gately *et al.* 1998).

2.5.8 Interleukin-15

IL-15 is a member of the four-α-helix bundle cytokine family with closest homology to IL-2 and IL-21 (Bamford *et al.* 1994, Burton *et al.* 1994, Parrish-Novak *et al.* 2000). IL-15 was originally described as a T cell growth and activation factor with a molecular weight of 14-15 kDa (Grabstein *et al.* 1994). IL-15 uses the heterotrimeric IL-15 receptor (IL-15R) complex comprising the γ_c-chain, IL-2Rβ-chain (CD122) and a unique IL-15Rα subunit (Giri *et al.* 1995). The γ_c and IL-2Rβ subunits are responsible for intracellular signal transduction and the α subunit mediates specific high-affinity binding of IL-15 (Grabstein *et al.* 1994). Ligation of the IL-15Rαβγ complex results in signalling through Jak1/3 and Stat3/5 pathways (Giri *et al.* 1995, Waldmann 2002).

The importance of cytokines using the IL-2/ 15Rβ-chain for signalling is demonstrated in IL-2/ 15Rβ-knockout mice that are deficient in NK cells, TCRγδ cells and natural killer T (NK-T) cells (Suzuki *et al.* 1997). Interestingly these cells, which are severely affected by the deficiency of IL-2Rβ or IL-15Rα, are implicated in innate immunity. Therefore IL-15, and possibly some yet unidentified cytokine(s) that also use IL-2/15Rβ and IL-15Rα, is an essential mediator in the development of cytotoxic cells responsible for innate immunity. Macrophages and monocytes express a complex of biologically active IL-15 and IL-15Rα on their surface that can be further up-regulated by IFN-γ (Dubois *et al.* 2002). On macrophage/ monocyte activation, this complex is internalised and IL-15 is recycled in the endosomes and re-expressed on the cell surface (Dubois *et al.* 2002). IL-15 signalling through IL-15R is crucial for the functional maturation of APCs (both macrophages and DCs) and can stimulate IL-12 production. This activity is impaired in IL-15 deficient mice (Ohteki *et al.* 2001).

IL-15 is expressed in several cell types including macrophages, fibroblasts, dendritic cells, osteoclasts and endothelial cells (Grabstein *et al.* 1994, Doherty *et al.* 1996, McInnes and Liew 1998, Thurkow *et al.* 1997, Oppenheimer-Marks *et al.* 1998, Kurowska *et al.* 2002). Many of the biological properties attributed to IL-2 are also exhibited by IL-15. Under physiological conditions, IL-15 promotes the differentiation and proliferation of NK cells, NKT cells and $CD8^+$ T cells (Lodolce *et al.* 1998, Kennedy *et al.* 2000, Fehninger and Caligiuri 2001). Furthermore, in pathological processes, IL-15 stimulates the activation, recruitment, proliferation, survival, and effector function of several immune cells (Carson

et al. 1994, Kanegane and Tosato 1996, Alleva *et al.* 1997, Fehninger and Caligiuri 2001). Although initially thought to have limited effects on naïve $CD8^+$ or $CD4^+$ T cells, IL-15 induces the proliferation and maintenance of both memory and naïve $CD8^+$ and $CD4^+$ T cells (Marks-Konczalik *et al.* 2000, Nishimura *et al.* 2000, Niedbala *et al.* 2002). IL-15 alone is not capable of driving Th1 or Th2 type conditions. However, priming with IL-12 or IL-4 enables IL-15 to promote the differentiation of $CD8^+$ and $CD4^+$ Type 1 (IFN-γ) or Type 2 (IL-5) T cells respectively (Niedbala *et al.* 2002).

IL-15 is overexpressed in patients with active RA and acts as a chemoattractant and proliferation-inducing factor for synovial T cells (McInnes *et al.* 1996). IL-15 also increases synovial macrophage TNF-α production via processes that require direct contact between T cells and macrophages (Wilkinson and Liew 1995, Mc Innes *et al.* 1997). Furthermore, constitutive expression of IL-15 within synovial fibroblasts is capable of stimulating freshly isolated T cells (Miranda-Carus *et al.* 2004). A positive feedback loop is thereby generated with IL-15 inducing and maintaining T cell synthesis of TNF-α, IFN-γ and IL-17 which may further activate synovial fibroblast production of IL-6, IL-8 and IL-15 (Miranda-Carús *et al.* 2004). However, IL-15 activities are not strictly limited to T cells and macrophages but also extend to synoviocytes and neutrophils (McDonald *et al.* 1998a). Indeed, IL-15 induces IL-17 production from T cells (Ziolkowska *et al.* 2000), which in turn induces inflammatory cytokine expression by synoviocytes (Fossiez *et al.* 1996) and macrophages (Jovanovic *et al.* 1998). Furthermore, IL-15 also induces the proliferation and survival of hyperproliferating primary human fibroblast-like-synoviocytes (Kurowska *et al.* 2002).

Recent evidence has emphasised the protective effects of IL-15 during bacterial and fungal infection (Nishimura *et al.* 1996, Musso *et al.* 1998, Hirose *et al.* 1999, Tran *et al.* 2003). PBMCs mount a strong IL-15 response to infection with *C. albicans*, *E. coli* and *S. aureus*, which remarkably enhances their cytocidal (NK) activities (Tran *et al.* 2003). Furthermore, this response occurs early, peaking at 12 h post infection or later at 36 h, depending on the implicated pathogen. Taken together, these data provide important evidence of the essential role that IL-15 plays in the host's innate immune response against pathogenic microorganisms.

2.5.9 Interleukin-17A

2.5.9.1 Introduction

Interleukin 17A is a proinflammatory, 17 kDa dimeric cytokine implicated in cartilage destruction and bone loss (Yao *et al.* 1995, Fossiez *et al.* 1996). Additional molecules with the characteristic IL-17A conserved cysteine residues, though heterogeneity of function, have also been discovered and termed IL-17B-F (Aggarwal and Gurney 2002). $CD4^+$ T cells are the principal source of IL-17A however, it can also be found in eosinophils (Molet *et al.* 2001), $CD8^+$ T cells and neutrophils (Ferretti *et al.* 2003). IL-17A exhibits similar biological effects to, though less potent than, IL-1β and TNF-α. In addition, IL-17A enhances the effects of these two cytokines. Importantly, IL-17A demonstrates potent chemoattractant properties for neutrophils (Laan *et al.* 1999, Umemura *et al.* 2004). Thereby, T cell derived IL-17A may enhance and concentrate the actions of non-specific phagocytic effector cells at inflammatory sites (Stamp *et al.* 2004).

IL-17A secretion appears to predominantly be limited to activated $CD4^+$ $CD45RO^+$ T cells. Expression of IL-17A is induced by stimulation of these cells or PBMC cultures with lipopeptides, PMA, PHA, concanavalin A, anti-CD3 and/ or anti-CD28 (Infante-Duarte *et al.* 2000, Lenarczyk *et al.* 2000, Kim *et al.* 2005). In addition, several cytokines are capable of inducing IL-17A expression within PBMC cultures including IL-6, IL-15 and MCP-1 (Ziolkowska *et al.* 2000, Ferretti *et al.* 2003, Happel *et al.* 2003, Kim *et al.* 2005). Of importance, IL-23 appears to be a key cytokine regulating the expression of IL-17A (Aggarwal *et al.* 2003, Murphy *et al.* 2003). Interestingly, other proinflammatory cytokines including IL-1β, TNF-α, IL-18 and TGF-β are not capable of inducing IL-17A expression within PBMC cultures (Kim *et al.* 2005). It is accepted that IL-17-secreting $CD4^+$ effector T cells are central participants in autoimmune disorders (Wynn *et al.* 2005). However, it has only more recently been elucidated that $CD4^+$ effector cells that secrete IL-17 are developmentally distinct from Th1 or Th2 $CD4^+$ effector cells (Harrington *et al.* 2005, Park *et al.* 2005). IL-17A expression occurs through signal transduction dependent upon PI3K/Akt and NF-κB pathways (Kim *et al.* 2005).

In contrast to the limited cellular expression of IL-17A, the 130 kDa transmembrane IL-17A receptor is ubiquitously expressed and does not belong to other cytokine receptor

families (Yao *et al.* 1997). Indeed, IL-17R has been found on fibroblast-like synoviocytes (Kehlen *et al.* 2002), fibroblasts (Maertzdorf *et al.* 2002), airway epithelial cells (Kao *et al.* 2004) and intestinal epithelial cells (Awane *et al.* 1999). Ligation of IL-17R generally results in activation of NF-κB (Awane *et al.* 1999, Hata *et al.* 2002, Kehlen *et al.* 2002, Kao *et al.* 2004, Sebkova *et al.* 2004). However, IL-17A signalling can also act through AP-1 (Granet and Miossec 2004, Sebkova *et al.* 2004), MAPK (Hata *et al.* 2002, Hsieh *et al.* 2002, Kehlen *et al.* 2002) and JAK cellular pathways (Kao *et al.* 2004). Interestingly, IL-17A in combination with IL-1 and/or TNF-α has a synergistic effect upon transcription and nuclear translocation of AP-1 members, Ebr-1 and NF-κB in osteoblast cells (Granet and Miossec 2004) and in rheumatoid synoviocytes (Granet *et al.* 2004).

2.5.9.2 Biological effects of IL-17A

To date, the principal stimulatory effects elucidated for IL-17A is its capability to up-regulate IL-6 and/or IL-8 expression within fibroblast populations (Fossiez *et al.* 1996), fibroblast-like synoviocytes (Kehlen *et al.* 2002), keratinocytes (Teunissen *et al.* 1998), airway epithelial cells (Jones and Chan 2002), renal epithelial cells (Hsieh *et al.* 2002) and gastric epithelial cells (Sebkova *et al.* 2004). Indeed, IL-17A-induced secretion of IL-6, IL-8 and MCP-1 occurs rapidly after stimulation of human colonic myofibroblasts cultures (Hata *et al.* 2002). In addition to its individual effects upon the expression of cytokines and chemokines, IL-17A exhibits synergistic properties with several proinflammatory mediators. IL-17A exerts a strong synergistic effect with TNF-α in up-regulating IL-6 and/or IL-8 production from fibroblasts (Andoh *et al.* 2002, Hata *et al.* 2002, Maertzdorf *et al.* 2002). Furthermore, IL-6 and IL-8 expression is enhanced by IL-17A in combination with IL-1β (Hata *et al.* 2002). IFN-γ also synergises with IL-17A to significantly increase the secretion of IL-8 and MCP-1 from intestinal epithelial cells (Andoh *et al.* 2001) and ICAM expression on bronchial epithelial cells (Kawaguchi *et al.* 2001). Not only does IL-17A enhance the actions of cytokines, but it also supplements bFGF-, HGF- and VEGF-induced growth of vascular endothelial cells (Takahashi *et al.* 2005a).

In addition to its effects upon stromal cell-derived IL-6 and IL-8, IL-17A also induces IL-1β and TNF-α secretion from human PBMC-derived macrophages (Jovanovic *et al.* 1998). Individually, IL-17A and IL-17F are not capable of inducing G-CSF or GM-CSF expression from lung microvascular endothelial cells cultures. However, IL-17A and IL-

17F have differential regulatory roles in the production of G-CSF or GM-CSF in endothelial cells concomitantly stimulated with IL-1β and/or TNF-α (Numasaki *et al.* 2004a,b,c). Furthermore, IL-17A synergistically enhances the production of IL-8 and G-CSF from TNF-α stimulated human bronchial epithelial cells (Jones and Chan 2002). These studies establish that IL-17A synergises with prototypical Th1 cytokines to increase the expression of several pro-inflammatory mediators. However, it has also been ascertained that IL-17A synergises with Th2 cytokines. For example, IL-17A in combination with IL-4 or IL-13 synergistically increases IL-8 production from bronchial epithelial cells (Kawaguchi *et al.* 2001).

Taken together, these studies establish that the major role of IL-17A, and its related family members, is the co-ordination of local tissue inflammation. This primarily takes effect through the induction of proinflammatory and, in particular, neutrophil-attracting chemokines and cytokines from its target cells. As such, IL-17A has been implicated in a variety of inflammatory disorders including malignancy, allergy, human lung disease, RA and inflammatory bowel disease (Lubberts *et al.* 2001, Kolls and Lindén 2004).

2.5.9.3 IL-17A in inflammatory bone diseases

Elevated levels of IL-17A are detected within synovial fluid from patients with RA. IHC studies on synovial tissues demonstrate IL-17A protein to be localised to T cell rich areas (Chabaud *et al.* 1999, Kotake *et al.* 1999). In experimental models, IL-17A stimulates osteoclast differentiation and induces bone resorption through the induction of RANKL on the surface of osteoblasts. Conversely, OPG inhibits IL-17A-induced osteoclast differentiation (Kotake *et al.* 2001). Within the collagen induced arthritis (CIA) model, IL-17A is significantly involved in both early and late stages of disease progression. This occurs through up-regulated IL-6, IL-1 and RANKL expression (Lubberts *et al.* 2003). Furthermore, neutralisation of IL-17A within this CIA model significantly reduces the severity of CIA, which is demonstrated by suppressed levels of joint damage (Lubberts *et al.* 2004). Similarly, neutralisation of IL-17 and/ or IL-17R in mice infected with *Borrelia burgdorferi* alleviates severe destructive arthritis through the concomitant increase in $CD4^+$ $CD25^+$ T cells (Burchill *et al.* 2003, Nardelli *et al.* 2004).

In IL-17A knockout mice, collagen-induced arthritis is significantly diminished, though not completely when compared with controls. This strongly implicates IL-17A as being central to the pathogenesis of CIA (Nakae *et al.* 2003a). Indeed, in IL-1ra deficient mice, inactivation of IL-17A completely attenuates the spontaneous development of destructive arthritis (Nakae *et al.* 2003b). Furthermore, in the antigen-induced arthritis murine model, neutralisation of IL-17A attenuates joint inflammation and bone erosion (Koenders *et al.* 2005a). Reduction in joint erosion correlates with decreased levels of RANKL and interleukin-1. The significant contribution of IL-17A towards inflammatory arthritis has been further established by investigations of streptococcal cell wall (SCW)-induced arthritis in IL-1 knockout mice. In this model, IL-17A increases the extent of cartilage damage and up-regulates MMP expression (Koenders *et al.* 2005b). SCW-induced arthritis is attenuated in IL-17R knockout mice, thereby preventing bone erosion (Lubberts *et al.* 2005a). Of interest, IL-1 mRNA levels are significantly decreased in this model suggesting that in progressive chronic joint inflammation, IL-17A signalling takes place upstream of IL-1 (Lubberts *et al.* 2005b). In the SCW model, IL-17R deficiency results in the reduced accumulation of neutrophils to inflamed joints. This emphasises the importance of IL-17A in neutrophil chemoattraction and indicates that PMNs contribute towards inflammatory bone destruction. It has been demonstrated that IL-17A induces MMP production from chondrocytes. Moreover, MMP expression is synergistically increased when IL-17A is added in combination with IL-1, IL-6, TNF-α and/or oncostatin M (Koshy *et al.* 2002, van Bezooijen *et al.* 2002). Indeed, the induction of SCW arthritis in IL-17R-deficient mice results in reduced synovial expression of IL-1, IL-6, MMP-3, MMP-9 and MMP-13, which corresponds with an absence of cartilage erosion (Koenders *et al.* 2005c). Nevertheless, IL-17A is also capable of promoting cartilage matrix degradation independently of MMPs (Cai *et al.* 2001). The significant contribution of IL-17A towards inflammatory joint damage is now clearly established. Of importance, IL-23 appears crucial in promoting the development of IL-17A producing cells (Murphy *et al.* 2003).

2.5.9.4 IL-17A in response to pathogenic challenge

The majority of studies investigating the role of IL-17A in the host response to bacterial challenge have been undertaken within murine models. LPS exposure in mouse airways results in the release of IL-17A from T cells and neutrophils. The up-regulation of IL-17A expression leads to the mobilisation of neutrophils to sites of bacterial challenge (Ferretti *et*

al. 2003). Subsequently, endogenously produced IL-17A induces the local release of neutrophil-mobilising cytokines including IL-6 and MIP-2. These mediators further contribute towards the recruitment of neutrophils to the inflammatory site (Miyamoto *et al.* 2003a). The importance of IL-17A-regulated early migration of PMNs to infected tissues has been demonstrated in IL-17R-/- mice. These mice develop a normal adaptive immune response to infection with *Toxoplasma gondii*. However, as a consequence of impaired IL-17 signalling, they suffer a defective neutrophil response at the outset of infection resulting in increased rates of mortality (Kelly *et al.* 2005). Furthermore, leukocyte function-associated antigen 1 (LFA-1) knockout mice are more resistant to systemic *Listeria monocytogenes* infection than controls. This is essentially due to their development of a neutrophilia, which corresponds with an increase in serum IL-17A levels (Miyamoto *et al.* 2003b).

The importance of IL-17A in the initial recruitment of neutrophils to locations of pathogenic challenge has also been established in murine models of *Klebsiella pneumoniae* infection. Mice lacking the IL-17R show greater bacterial burden and increased mortality that correlates with impaired neutrophil recruitment (Ye *et al.* 2001a). Indeed, administration of IL-17A in this model leads to enhanced chemokine production, neutrophil recruitment and survival (Ye *et al.* 2001b). More recently, it has been elucidated from this infection model that TLR-4 signalling is the principal mechanism behind endogenous IL-17A production. Furthermore, $CD8^+$ T cells are a significant source of IL-17A, this occurring through an IL-23-dependent mechanism (Happel *et al.* 2005). The central involvement of IL-23 in the induction of IL-17A expression has been further demonstrated within activated naïve $CD4^+$ and $CD8^+$ T cells (Eijnden *et al.* 2005). The requirement of IL-17A in successful host responses to invading pathogens has also been investigated within other infectious models. Endogenous IL-17A, derived from $CD4^+$ T cells, mediates the *in vivo* development of experimentally induced intra-abdominal abscess formation in mice challenged with *Bacteroides fragilis* (Chung *et al.* 2003). Moreover, pre-treatment of the host with a specific IL-17A neutralising antibody attenuates abscess formation.

Recent evidence from animal experiments intimate that IL-17A expression may be essential for host survival against cecal ligation and puncture (CLP) induced sepsis. Through the actions of IL-17A, bacterial infection of the peritoneal cavity is contained

(Rice *et al.* 2005). Furthermore, from an analysis of a panel of 21 cytokines that induce the expression of innate response mediators, IL-17A is the most potent stimulatory cytokine for human β-defensin 2 and macrophage inflammatory protein 3 (MIP-3) (Kao *et al.* 2004). Through the induction of these inflammatory mediators, IL-17A would appear to be an important mediator in the interplay between innate and acquired immunity. However, the precise role of IL-17A in host defence against microbial infection remains to be defined. Nevertheless, studies to date emphatically support a central immunoregulatory position for IL-17A within host mechanisms against pathogenic challenge. It is therefore surprising that IL-17A has not been described within the PRD lesion.

2.5.10 Interleukin-22

Originally termed IL-TIF, two groups concomitantly identified interleukin 22 (IL-22) (Dumoutier *et al.* 2000a, Xie *et al.* 2000). IL-22 is a member of the IL-10 Class II cytokine family that includes IL-19, IL-20, IL-24 IL-26, IL-28A/B and IL-29. Although structurally related to IL-10, it shares only 22% homology and its biological activities are dissimilar. The gene for human IL-22 is localised on chromosome 12q15 in close proximity to IL-26, another IL-10 related factor (Dumoutier *et al.* 2000b). Comprising a six alpha helical bundle, the IL-22 structure closely resembles that of IL-10. In comparison to IL-10, which functions as a dimer, IL-22 is predicted to function as a monomer (Gurney *et al.* 2004). IL-22 readily binds to the second component of the IL-10 receptor (termed CRF2-4, IL-10R2 or IL-10Rβ). In combination with the IL-10R2 chain, IL-22 weakly binds its specific IL-22 receptor (termed IL-22R/IL-22R1/CRF2-9) thereby forming a unique signalling complex (Kotenko *et al.* 2001a). There appears to be no interaction between IL-10 and IL-22 upon biological events. Both require independent ligation with their respective R1 chains to create a binding site with the IL-10 R2 subunit (Wolk *et al.* 2005). Interestingly, IL-22R1 appears to be predominantly expressed on epithelial cells or stromal cell fibroblasts derived from various tissues (Boniface *et al.* 2005, Nagalakshmi *et al.* 2004a, Whittington *et al.* 2004).

IL-22 is expressed by T cells stimulated with cytokines such as IL-9 or via TCR stimulation (Xie *et al.* 2000, Dumoutier *et al.* 2000a) and NK cells (Wolk *et al.* 2002). In contrast to the high levels of IL-22 expression within T cells, other immune and stromal cells exhibit low IL-22 expression (Nagalakshmi *et al.* 2004a). Expression of IL-22

appears to be confined to memory $CD4^+$ T cells and is markedly enhanced within Th1 polarised populations rather than Th2 cell types. IL-22 mRNA expression within these $CD4^+$ cells is up-regulated by IL-12, anti-CD3+anti-CD28, or anti-CD3+ICAM. In agreement for a role within Th1 type responses, IL-22 mRNA expression is further enhanced by the action of IL-18 (Gurney *et al.* 2004). Of importance, IL-22 expression is closely regulated by a soluble decoy receptor termed IL-22BP/IL-22RA1/CRF2-10 (Dumoutier *et al.* 2001, Kotenko *et al.* 2001b, Xu *et al.* 2001).

Several cell lines are responsive to IL-22 including a murine acinar cell line (266-6), human kidney cell line (TK-10), rat and human hepatic cell lines (H4IIE and HepG2), human colon adenocarcinoma (SW480) and colonic epithelial cell line (Colo205) (Xie *et al.* 2000, Dumoutier *et al.* 2000c, Aggarwal *et al.* 2001, Nagalakshmi *et al.* 2004b). Within these cells, the expression of IL-22R1 appears to be the limiting factor for a cellular response to IL-22. Ligation of the IL-22R primarily results in activation of Stat1 and Stat3 signalling pathways (Nagalakshmi *et al.* 2004b). IL-22 can generally be considered as a proinflammatory molecule. It induces large increases in acute phase-mediators such as PAP1/Reg2, osteopontin (Aggarwal *et al.* 2001), serum amyloid A, alpha-1antichymotrypson and haptoglobin (Dumoutier *et al.* 2000c) and β-defensins and MMP3 from keratinocytes (Boniface *et al.* 2005, Wolk *et al.* 2004). Recently, IL-22 has been identified within synovial fibroblasts and macrophages. Furthermore, it increases the growth of synovial fibroblasts and induces the release of MCP-1 (Ikeuchi *et al.* 2005). It is of interest that despite its defined proinflammatory roles (Andoh *et al.* 2005), IL-22 induces IL-10 expression within the colonic epithelial cell line Colo205 (Nagalakshmi *et al.* 2004b).

2.5.11 TNF -α

TNF-α is a type II, 26 kDa transmembrane glycoprotein containing a C terminus that is external to the cell and a cytoplasmic domain. TNF is released from the cellular membrane by a protease of the metalloproteinase/ disintegrin/ cysteine-rich family called TNF-alpha converting enzyme (TACE) (Gearing *et al.* 1994). This results in a soluble homotrimer of 17 kDa subunits, which was originally described as a product of activated macrophages and monocytes with tumouricidal activity (Beutler *et al.* 1985). TNF-α is a highly pleiotropic cytokine belonging to a superfamily that incorporates 19 members (Feng 2005).

These family members comprise membrane-anchored and soluble cytokines that are predominantly involved in T cell-mediated immunity. TNF-α has a wide array of target cells including monocytes, macrophages, lymphocytes, eosinophils and neutrophils and is capable of enhancing proliferation of certain normal cells. TNF-α has potent proinflammatory effects and is a central mediator of septic shock, cachexia, autoimmunity and inflammatory diseases. The pleiotropic biological properties of TNF-α include leukocyte recruitment and activation, macrophage and fibroblast cellular proliferation, increased prostaglandin and matrix degrading MMP activity and bone destruction (Romas *et al.* 2002). Although TNF-α is predominantly pro-inflammatory in nature, it is also capable of exerting anti-inflammatory effects for example inhibiting IL-12 and IL-23 in macrophages pretreated with LPS and IFN-γ (Zakharova and Ziegler 2005).

TNF-α is a cytokine produced by a wide spectrum of cells types but primarily by monocyte-macrophages (Sunderkotter *et al.* 1994). The effects of TNF-α are mediated through its two functionally distinct membrane receptors. These comprise the 55 kDa type 1 receptor, TNF-R1 (p55), and the 75 kDa type II receptor, TNF-RII (p75). Most cell types express both receptors, including macrophages, lymphocytes, neutrophils and fibroblasts (Aderka 1996). Through the proteolytic cleavage of the extracellular domain of these receptors, TNF-α activity is antagonised by the release of sTNF-R1 and sTNF-R2 (Engelmann *et al.* 1989, Engelmann *et al.* 1990). Ligation of the TNF receptors leads to the activation of Rel/NF-κB and MAP kinase signal transduction pathways (Campbell *et al.* 2003). Importantly, TNF-α signalling is closely related to bone resorption (Gravallese *et al.* 2001). Indeed, TNF-α is one of the most potent osteoclastogenic cytokines produced in inflammation and is central to the pathogenesis of destructive inflammatory bone disorders including RA (Romas *et al.* 2002). Furthermore, TNF-α modulates osteoclast formation and function through its effects upon the expression of RANKL (Feng 2005). TNF-α has been described within PRD tissues. However, factors that are capable of regulating TNF-α expression within the human PRD lesion remain to be elucidated.

2.5.12 IFN-γ

Interferon- γ (IFN-γ) is a pleiotropic cytokine belonging to the Type II interferon family. It is a potent mediator of both innate and adaptive immunity, contributing significantly to host defence against various microbes. The principal cellular sources of IFN-γ are

activated natural killer cells (NK cells), $CD4^+$ Th1 cells and $CD8^+$ T cells (Rady *et al.* 1995, Young and Hardy 1995, Munder *et al.* 1998). However, expression of IFN-γ mRNA is readily detected in other cell types including B cells, dendritic cells and macrophages (Pang *et al.* 1992, Fenton *et al.* 1997, Yoshimoto *et al.* 1997, Gessani and Belardelli 1998). IFN-γ binds to the IFN-γR1 and IFN-γR2 receptor chains, thereby activating Jak1, Jak2 and Stat1 and occasionally Stat3 and Stat5 pathways (Ramana *et al.* 2002, Pestka *et al.* 2004). Under certain conditions, IFN-γ may also enhance activation of NF-κB (Cheshire *et al.* 1997). The expression and secretion of IFN-γ by NK cells, T cells and macrophages can be induced by the pro-inflammatory cytokines IL-12, IL-18 and IL-2 (Hardy and Sawada 1989, Trinchieiri *et al.* 1995, Puddu *et al.* 1997). Indeed, IL-18 is a principal inducer of IFN-γ (Gracie *et al.* 2003). Conversely, expression of the IL-18 antagonist, IL-18 binding protein (IL-18BP), is induced by IFN-γ in a variety of cellular systems including PBMCs (Corbaz *et al.* 2002), monocytes (Veenstra *et al.* 2002), fibroblast synoviocytes (Möller *et al.* 2003) and immortalised and primary keratinocytes (Mühl *et al.* 2000, Paulukat *et al.* 2001). This regulatory pathway has been shown to operate both *in vitro* and *in vivo*. Although exceptions exist, it appears that IFN-γ is the prime regulator of IL-18BP gene induction. Thereby, IFN-γ-induced IL-18BP may serve to control IL-18 and subsequent IFN-γ bioactivity in a classical negative loop.

Among the many biological activities of IFN-γ, activation of monocytes/ macrophages and the promotion of T cell differentiation towards a Th-1 type immune response are of central importance (Nathan *et al.* 1984, Gajewski and Fitch 1988). IFN-γ is primarily pro-inflammatory in nature, up-regulating several inflammatory mediators such as IL-12 (Fantuzzi *et al.* 1996, Ma *et al.* 1996), IL-15 (Doherty *et al.* 1996), TNF-α (Collart *et al.* 1986, Hayes *et al.* 1995) and inducible nitric oxide synthase (iNOS) (Xie *et al.* 1993). On exposure to bacterial antigens or their components such as LPS, macrophages produce IL-12 and IL-18, thereby activating NK, NKT and T cells (Okamura *et al.* 1995a, Ogasawara *et al.* 1998, Okamura *et al.* 1998). These cells in turn produce IFN-γ that further activates local macrophages in a positive feedback loop. IFN-γ therefore plays a critical role in the first line of host defence by augmenting the Th1 type response (Qiu *et al.* 2001). In mice spleens challenged with LPS, the predominant IFN-γ producing cells are NK cells and NKT cells whereas macrophages, T cells and dendritic cells contribute less (Varma *et al.* 2002).

Nevertheless, not all the properties displayed by IFN-γ are proinflammatory in nature (Mühl and Pfeilschifter 2003). IL-1β production can be inhibited by IFN-γ due to a reduction of mRNA steady-state levels without affecting mRNA stability, thereby suggesting a transcriptional mechanism of regulation (Schindler *et al.* 1990). IFN-γ has also been shown to suppress spontaneous intracellular production of proinflammatory IL-1β in synovial fluid macrophages derived from RA patients (Ruschen *et al.* 1989). Indeed, IFN-γ promotes the secretion of IL-1Ra from PBMCs and THP-1 cells, thereby modulating the effects of IL-1β (Kline *et al.* 1995). Furthermore, IFN-γ also enhances the release of IL-1Ra from synoviocytes (Seitz *et al.* 1994), keratinocytes (Gueniche *et al.* 1994) and neutrophils (McDonald *et al.* 1998b). In addition to the modulation of IL-1Ra expression, IFN-γ may also disturb IL-1 bioactivity by inducing the release of type II IL-1 receptors (Groves *et al.* 1995). By inhibiting production of pro-inflammatory IL-1 and IL-8, up-regulating the production of cytokine antagonists such as IL-1Ra and IL-18BP and by inducing expression of members of the SOCS family, IFN-γ has the potential to modulate inflammatory responses in either pro- or anti-inflammatory directions.

Indeed, these anti-inflammatory properties of the principally pro-inflammatory cytokine IFN-γ may be essential to controlling the extent of inflammatory conditions, thereby avoiding concomitant tissue destruction. Although IFN-γ has been described within the PRD lesion, the dynamics of IFN-γ expression and factors regulating this are currently poorly defined.

2.6 Interleukin-18

2.6.1 Introduction

2.6.1.1 Structure of IL-18

The novel pleiotropic cytokine IL-18 was originally isolated through toxic shock generated by the injection of mouse livers with heat-killed *Propionibacterium acnes* and challenged with LPS (Okamura *et al.* 1995b). Subsequently, the full-length IL-18 cDNA clone sequence was identified as a precursor polypeptide of 192 amino acids, possessing an atypical leader sequence of 35 amino acids (Okamura *et al.* 1995b, Ushio *et al.* 1996). This was mapped in the human gene to chromosome 11q22.2-q22.3 (Nolan *et al.* 1998).

Human IL-18 is a cytoplasmic protein synthesised as a 24 kDa biologically inactive, proIL-18 precursor molecule. The IL-18 precursor is devoid of a signal peptide and, like its family member IL-1, requires cleavage by interleukin-1 converting enzyme (ICE/ caspase-1) to generate a mature active polypeptide (Gu *et al.* 1997, Ghayur *et al.* 1997, Fantuzzi *et al.* 1999). IL-18 serum levels appear to be critical to the outcome of innate immune responses to LPS and this is primarily regulated by caspase-1 (Joshi *et al.* 2002). However, caspase-1 independent processing pathways, including neutrophil proteinase-3 (Sugawara *et al.* 2001), are also capable of generating the biologically active 18.3 kDa IL-18 molecule (Fantuzzi *et al.* 1998, Tsutsui *et al.* 1999). To date, IL-18 does not possess sequence similarities to other proteins but its molecular structure (Bazan *et al.* 1996), receptor family, signal transduction pathways and processing enzyme (Gu *et al.* 1997) relate it to the IL-1 superfamily (Ushio *et al.* 1996). Human proIL-18 shares 65% homology with its mouse counterpart and contains five structural cysteines. Mature IL-18 contains four cysteines, of which Cys^{112} and Cys^{163} are highly conserved across species and are essential for IL-18 function (Pei *et al.* 2005).

2.6.1.2 Cellular sources of IL-18

IL-18 was originally defined as being preferentially produced by phagocytic immune cells associated with acute inflammatory immune responses (Okamura *et al.* 1995a, Ushio *et al.* 1996, Seki *et al.* 2001). However, IL-18 mRNA and pro-IL-18 protein are ubiquitously expressed in many cell types including Kupffer cells (Okamura *et al.* 1995b, Matsui *et al.* 1997), T- and B-cells (Klein *et al.* 1999), dendritic cells (Stoll *et al* 1998, Gardella *et al.* 1999, Mee *et al.* 2000), osteoblasts (Udagawa *et al.* 1997), keratinocytes (Stoll *et al.* 1997, Sampanthanarak *et al.* 2005), intestinal epithelial cells (Takeuchi *et al.* 1997, Pizarro *et al.* 1999), Langerhans cells (Matsui *et al.* 1997), corneal epithelial cells (Burbach *et al.* 2001), oral epithelial cells (Sugawara *et al.* 2001), airway epithelial cells (Cameron *et al.* 1999), glucocorticoid-secreting adrenal cortex cells (Conti *et al.* 1997), astrocytes and microglia (Conti *et al.* 1999), pancreatic islet cells (Frigerio *et al.* 2002), chondrocytes (Olee *et al.* 1999), squamous carcinoma cells (Martone *et al.* 2004) and adipocytes (Skurk *et al.* 2005). Under normal physiological conditions, IL-18mRNA is considerably stable. In contrast to proIL-1β, many of these cells store substantial quantities of proIL-18. Thereby, these cell populations may respond swiftly to inflammatory/ immune stimuli by enabling the rapid release of constitutively expressed IL-18. Through this, IL-18 likely plays a central role in

regulating early innate immune responses. In a similar fashion to IL-1β, cellular secretion of IL-18 operates via the purinergic receptor P2X-7 (Mehta *et al.* 2001).

Whilst most of the aforementioned cells produce both pro, 24 kDa, and biologically active 18.3 kDa IL-18, monocytes and macrophages produce a wide variety of IL-18 forms (Kikkawa *et al.* 2001). Some of these are inactive dimers whilst other IL-18 forms have weak IFN-γ-inducing activity such as IL-18 type 2. This variant has been found at high levels in the plasma of approximately 30 percent of normal subjects, bound to plasma IgM (Shida *et al.* 2001a). It is suggested that this IL-18 derivative may have some responsibility in the development of Th2 type responses involving IgE production in association with atopic lesions (Shida *et al.* 2001b).

2.6.1.3 Cellular activation by IL-18

IL-18 signalling through its receptor unit (refer to chapter 2.6.2) initiates the cascade of kinases MyD88, IRAK (Robinson *et al.* 1997, Adachi *et al.* 1998) and TRAF-6 (Kojima *et al.* 1998). Consequently, IL-18 induces the activation of transcription factors AP-1 (Barbulescu *et al.* 1998, Greene *et al.* 2000) and NF-κB (Matsumoto *et al.* 1997, Kojima *et al.* 1999, Takayama *et al.* 1999, Morel *et al.* 2001, Chandrasekar *et al.* 2003, Weinstock *et al.* 2003). Furthermore, the synergistic effects of IL-12 with IL-18 are probably due to Stat4-up-regulated AP-1-mediated IFN-γ promoter activation (Schindler *et al.* 2001, Nakahira *et al.* 2002). The importance of IRAK in IL-18 signalling and function has been demonstrated in IRAK deficient mice challenged by LPS or IL-18. These mice exhibit dramatically reduced IFN-γ production from Th1 and NK cells. Furthermore, IL-18-induced NK cell cytotoxicity is also severely impaired (Kanakaraj *et al.* 1999). Nevertheless, IL-18 is also capable of operating through IRAK independent pathways (Thomas *et al.* 1999, Wald *et al.* 2001). IL-18-induced IFN-γ expression is primarily mediated by the mitogen activated protein kinase p38 (MAPK p38) and p42/44 also known as ERK1/2 (Fukao *et al.* 2000). Moreover, IL-18 stimulation of synovial fibroblasts, which leads to up-regulated VCAM expression, activates three separate signal transduction pathways: the Ras/Raf-1/ERK/AP-1, P13-kinase/Akt, or IRAK/NF-κB pathways (Morel *et al.* 2002). In contrast, IL-18 signal transduction in epithelial cells is primarily through the MAPK p38 pathway rather than NF-κB (Lee *et al.* 2004a).

2.6.2 IL-18 Receptor

2.6.2.1 IL-18 receptor structure

IL-18 acts by binding to the heterodimeric IL-18 receptor complex comprising the IL-18Rα and IL-18Rβ chains (Torigoe *et al.* 1997, Debets *et al.* 2000). Although IL-18 does not bind to the IL-1 receptor type 1, the ligand binding IL-18Rα chain is a member of the IL-1 receptor family identified as IL-1R-related protein 1 (IL-1Rrp/IL-1R5) (Parnet *et al.* 1996, Torigoe *et al.* 1997, Thomassen *et al.* 1998, Hoshino *et al.* 1999b). IL-18Rα represents the extracellular ligand-binding site for IL-18. However, co-expression of IL-18Rβ is essential for the high affinity binding of IL-18 and consequential signal transduction (Hoshino *et al.* 1999, Debets *et al.* 2000, Wu *et al.* 2003, Cheung *et al.* 2005). IL-18Rβ is structurally related to and was originally identified as IL-1R accessory protein like molecule (IL-1RAcPL/IL-1R7) (Greenfeder *et al.* 1995, Born *et al.* 1998, Kim *et al.* 2001). Importantly, IL-18 binding to the extracellular IL-18Rα chain is essential for recruitment of the IL-18Rβ subunit. This functional complex of IL-18, IL-18Rα and IL-18Rβ has a mass of approximately 160 kDa (Azam *et al.* 2003).

Cellular expression of IL-18R is central to the development of an appropriate immune response. Furthermore, clinical studies demonstrate that dysregulated expression of the IL-18R may contribute towards disease processes (Kobashi *et al.* 2001, McQuaid *et al.* 2003, Itoi *et al.* 2004). Interestingly, both IL-18R components harbour extracellular Ig-folds and an intracellular domain homologous to the cytostolic part of the Drosophila Toll protein, which is characteristic of IL-1R and IL-1R-like molecules (Debets *et al.* 2000). Indeed, IL-18R signalling operates through similar downstream effector pathways to those resulting from TLR ligation. The binding of IL-18 to this IL-18R complex induces formation of an IL-1R-associated kinase (IRAK)/TNF receptor associated factor-6 (TRAF6) complex that predominantly results in NF-κB activation in Th1 cells (Parnet *et al.* 1996, Matsumoto *et al.* 1997, Torigoe *et al.* 1997, Thomassen *et al.* 1998).

2.6.2.2 Biological role of IL-18 receptor

IL-18R is widely expressed on several cell types, including T cells, B cells, NK cells, macrophages, neutrophils, endothelial cells, epithelial cells, smooth muscle cells and mast cells (Olee *et al.* 1999, Kunikata *et al.* 1998, Yoshimoto *et al.* 1998, Okamura *et al.* 1998, Lauwerys *et al.* 1999, Gerdes *et al.* 2002, Gutzmer *et al.* 2003, Dahl *et al.* 2004, Lorey *et al.* 2004). Moreover, 24 of 39 analysed cell lines demonstrate constitutive expression of IL-18R, which occurs independently of cellular maturation in a non-lineage-specific manner (Nakamura *et al.* 2000). IL-18Rα expression was originally thought to occur on Th1 cell surfaces after T cell activation but not Th2 cells, thus explaining the predominate biological Th1 type immune responses afforded by IL-18 (Tomura *et al.* 1998, Xu *et al.* 1998, Yoshimoto *et al.* 1998, Debets *et al.* 2000). However, through the use of more specific antibodies, IL-18Rα subunit expression but not that of IL-18Rβ has been detected on almost all $CD4^+$ and $CD8^+$ T cells. Furthermore, T cell activation appears not to be a prerequisite for inducing expression of the IL-18Rα subunit (Smeltz *et al.* 2001). Th1 cells have therefore been phenotypically characterised by preferential surface expression of both IL-12Rβ2 and IL-18Rα (Rogge *et al.* 1997, Szabo *et al.* 1997, Xu *et al.* 1998b). However, FACS analysis reveals a poor correlation between the Th1 differentiation status of $CD4^+$ T cells with levels of IL-18Rα and IL-12Rβ2 expression. Clearly, the status of Th1 differentiation can therefore not be defined by the expression of these receptors alone (Smeltz *et al.* 2002).

Myeloid and monocytic cell lines, which constitutively express IL-18R, secrete IL-18 that may contribute towards autocrine proliferation of these cells (Nakamura *et al.* 2000). Moreover, up-regulation of the IL-18 receptor complex on these cells *in vivo* increases their responsiveness to IL-18, which may increase TNF-α production from these cells (Puren *et al.* 1998). Inflammatory cytokines such as IL-1β, IFN-α and TNF-α enhance IL-18R expression on NK cells, T-lymphocytes, monocyte dendritic cells and chondrocytes (Dai *et al.* 2005). Increased expression of both receptor subunits leads to specific *in vitro* responses that may reflect patterns occurring within disease processes *in vivo* (Nakamura *et al.* 2000, Sareneva *et al.* 2000, Gutzmer *et al.* 2003). IL-1β and TNF-α commonly coexist at chronic inflammatory sites and are not required for physiological T-lymphocyte or NK cell activation. It is therefore interesting that IFN-γ alone is unable to promote IL-18R but,

when combined with IL-1β and TNF-α, IL-18R expression is induced. This may indicate important pathways that distinguish physiological processes from pathological responses. Furthermore, IL-18R ligation induces the expression of cytokines such as IL-1β, IL-6 and TNF-α, which are up-regulated in many inflammatory diseases of an infectious aetiology (Puren *et al.* 1998, Ross 1999).

IL-12, IL-15 and IL-21 up-regulate IL-18Rα expression on NK cells and T cells (Fehniger *et al.* 1999, Tominaga *et al.* 2000, Strengell *et al.* 2002). However, the ability of IL-12 to increase IL-18Rα expression is severely impaired in IFN-γ-/- mice. Furthermore, the addition of IFN-γ has no effect upon IL-18Rα expression but, when combined with IL-12, IL-18Rα expression is enhanced (Smeltz *et al.* 2001, Nakahira *et al.* 2001). Studies on T cells derived from 5CC7 TCR transgenic mice demonstrate that in the absence of IL-4, IL-12 alone is sufficient and IFN-γ is dispensable for IL-12Rβ2 and IL-18Rα expression and Th1 differentiation (Smeltz *et al.* 2002). However, in the presence of IL-4, IL-12-induced Th1 differentiation additionally requires IFN-γ. This effect occurs through IFN-γ limiting the suppressive effects of IL-4 on both IL-18Rα and IL-12Rβ2 receptor expression whilst simultaneously neutralising IL-4. In addition, IFN-γ enhances IL-18Rα expression to greater levels than those induced by IL-12 alone and almost completely restores IL-12Rβ2 expression (Smeltz *et al.* 2002). Furthermore, IL-12-induced expression of steady state mRNA for IL-18Rβ chain is not increased in Stat4 deficient cells. This implies that Stat4 is essential for IL-12 induction of the functional IL-18R complex (Yoshimoto *et al.* 2000).

In response to infections and foreign antigens, endogenous IL-18 activity influences the development of Th1 as well as Th2 type immune responses. T cells cultured with IL-18, but without T cell receptor stimulation, produce the Th2 cytokines IL-4 and IL-13 and evolve into Th2 T cells in a Stat6-dependent manner (Yoshimoto *et al.* 2000). Furthermore, basophils expressing IL-18Rα produce large amounts of IL-4 and IL-13 in response to stimulation with IL-3 and IL-18 (Yoshimoto *et al.* 1999). From experiments on IL-4-/- mice and Stat6-/- T cells, it has been ascertained that IL-4 plays a central role in down-regulating IL-18Rα expression. However, TCR stimulation is required for this effect (Smeltz *et al.* 2001). Subsequent to IL-18 stimulation of $CD4^+$ T cells, IFN-γ secretion correlates with the level of cellular IL-18Rα expression. Importantly, this is tightly regulated by IL-4, which decreases IL-18Rα expression and thereby reduces the production of IFN-γ (Tominaga *et al.* 2000, Smeltz *et al.* 2001).

2.6.3 IL-18 Binding Protein

2.6.3.1 Introduction to cytokine inhibitors

Binding proteins, including soluble receptors that constitute the ligand binding extracellular domain of the cell surface receptor, are key regulators of cytokine activities. Thereby, they are critical in regulating appropriate host responses to pathogens (Fernandez-Botran *et al.* 1996). These soluble receptors are derived from receptor mRNA splice variants or by proteolytic cleavage of cytokine cell surface receptors. However, the affinity for their ligand is generally lower than that of the corresponding cell surface receptor. Binding proteins may either agonise or antagonise the activity of the cytokine they bind. For example, soluble IL-6R binds IL-6 to form a complex that confers responsiveness to IL-6 onto a variety of cells that do not express transmembrane IL-6R but do express gp130 such as endothelial cells (Romano *et al.* 1997). Conversely, soluble TNF receptors act as antagonists to TNF activity (Engelmann *et al.* 1989, Engelmann *et al.* 1990).

However, the IL-18 binding protein (IL-18BP) is not the counterpart of the cell surface receptor IL-18Rα chain. Nevertheless, its affinity to IL-18 is comparable with the cell surface IL-18 receptor (Okamura *et al.* 1995). Several viruses produce molecules that are homologous to binding proteins. Through the release of such molecules, they are able to escape challenges from host immunity (Alcamí and Smith 1995). In addition to soluble receptors for IL-1β, interferons and certain chemokines, poxviruses secrete active viral proteins homologous to IL-18BP, thereby implicating its significance as an immunoregulator (Xiang and Moss 1999, Born *et al.* 2000, Smith *et al.* 2000).

2.6.3.2 Introduction to IL-18BP

In contrast to IL-1β, IL-18 is constitutively expressed in humans and rodents at both gene and protein levels (Puren *et al.* 1999). Therefore, IL-18 activity requires careful regulation. This close control takes place through the binding of IL-18 with the naturally occurring and constitutively expressed IL-18BP (Novick *et al.* 1999). IL-18BP, a 38 kDa circulating decoy receptor, possesses high-affinity binding to IL-18 (dissociation constant of 400 p*M*) and potent anti-inflammatory properties (Aizawa *et al.* 1999, Novick *et al.* 1999, Xiang

and Moss 1999, Kim *et al.* 2000, Faggioni *et al.* 2001, Yatsiv *et al.* 2002, Banda *et al.* 2003). Importantly, the high affinity of IL-18BP to its ligand and the low dissociation rate of this complex suggest that IL-18BP is not merely a carrier protein (Novick *et al.* 2001).

Located in chromosome 11q13 at the inverted position of the nuclear mitotic apparatus protein 1, the human IL-18BP gene encodes for four isoforms each derived from a single mRNA by splice variants (Novick *et al.* 1999, Kim *et al.* 2000). Approximately 60% of the mature human IL-18BP resembles the extracellular domain of Ig-like receptors, which includes a highly conserved pair of cystienes and tryptophan residue (Aizawa *et al.* 1999, Novick *et al.* 1999). The isoforms share homology in their carboxyl termini whereas at the N terminal, one-third to two-thirds of the amino acids are different. Only isoforms IL-18BPa and IL-18BPc retain the intact immunoglobulin and are therefore biologically functional. Indeed, IL-18BPa is the most abundant and most potent inhibitor of IL-18 (Novick *et al.* 1999, Kim *et al.* 2000). Although present within humans, IL-18BPb and IL-18BPd are unable to neutralise IL-18 biological activity (Kim *et al.* 2000). With a single immunoglobulin domain, IL-18BP resembles the extracellular segment of a cytokine receptor with immunoglobulin like structures. Nevertheless, it is a novel protein distinct from members of the IL-1 and IL-18 receptor family (Kim *et al.* 2002a).

2.6.3.3 Effectors of IL-18BP expression

IL-18BP is constitutively expressed in normal tissues at the mRNA level. However, the spleen and lungs and to a lesser extent the colon, small intestine and prostate express significant quantities of IL-18BP (Novick *et al.* 1999). Healthy individuals have readily detectable levels of IL-18BP at concentrations of 2.15 ng/ml within the serum (Novick *et al.* 2001). Thereby, IL-18BP may serve as a natural buffer to IL-18, which is also constitutively expressed within the human blood compartment (Puren *et al.* 1999). IFN-γ up-regulates IL-18BPa expression in several cell lines including keratinocytes, colon carcinoma/epithelial cells and renal mesangial cells. Furthermore, IL-12 promotes IFN-γ-up-regulated IL-18BP expression within monocytes (Veesntra *et al.* 2002). This suggests that by regulating IL-18 concentrations, IL-18BP may act as a negative feedback inhibitor of the Th1 immune response (Mühl *et al.* 2000, Paulukat *et al.* 2001).

IFN-α is also capable of inducing IL-18BPa in the sera of chronic hepatitis C patients. This occurs independently of IFN-γ and results in decreased circulatory levels of IL-18. These effects have been further confirmed in isolated macrophages (Kaser *et al.* 2002). IL-12 also regulates cellular expression of IL-18BP through decreasing its expression in resting PBMCs from healthy donors. Conversely, IL-12 increases IL-18BP expression in RA synovial tissue cells and activated PBMCs (Kawashima *et al.* 2004). Given the high affinity of IL-18BP for IL-18, IL-18BP may be an effective therapeutic neutralising inhibitor within immune and inflammatory diseases associated with up-regulated expression of IL-18.

2.6.3.4 Biological significance of IL-18BP

The major function of IL-18BP is to regulate the inflammatory activity of IL-18 by specifically neutralising the biological activity of the mature form of IL-18 (Novick *et al.* 1999, Kim *et al.* 2000, Novick *et al.* 2001). Indeed, IL-18BP effectively neutralises IFN-γ production from IL-18 stimulated KG-1 cells (Aizawa *et al.* 1999). Moreover, in a similar manner to IL-18 neutralising antibodies (Fantuzzi *et al.* 1998), IL-18BP prevents LPS-induced expression of IFN-γ (Novick *et al.* 1999). Furthermore, IL-18BPa transgenic mice, with concanavalin A induced hepatotoxicity are significantly protected by reduced IFN-γ expression after mitogenic challenge (Fantuzzi *et al.* 2003). IL-18BPa increases spontaneous and IL-1β-induced prostaglandin production by the inhibition of IFN-γ (Reznikov *et al.* 2000). However, in contrast to antibodies to IL-18, the IL-18BP does not exhibit species specificity. Human IL-18BP therefore neutralises both human and murine IL-18 (Novick *et al.* 1999). *In vitro*, IL-18BP can effectively diminish IL-18-induced NF-κB activation, thereby decreasing IFN-γ and IL-8 expression.

IL-18BP likely has a significant role in regulating appropriate host immune responses to pathogenic challenge. Administration of IL-18BPa to mice challenged with LPS diminishes circulating levels of IFN-γ. This demonstrates that IL-18BP functions as an inhibitor of the early Th1 response *in vivo* (Novick *et al.* 1999). Furthermore, IL-18BP reduces *Staphylococcus epidermidis*-induced IFN-γ expression in human whole blood cultures by up to 77% (Stuyt *et al.* 2003a). Up-regulated expression of IL-18BP may also be responsible for reducing IFN-γ in *ex vivo* whole blood cultures obtained from patients

with septic shock (Oberholzer *et al.* 2000). Taken together, these findings establish that IL-18BP is an important immunoregulator (Grobmyer *et al.* 2000, Novick *et al.* 2001).

Within the murine model, IL-18BP has been used *in vivo* to determine its effects upon several diseases in which IL-18 has been implicated as an important participant of the pathological process. Indeed, the administration of IL-18BP protects against CIA in mice (Plater-Zyberk *et al.* 2001), reduces levels of metastasis of murine melanoma cells (Carrascal *et al.* 2003) and improves myocardial dysfunction (Dinarello 2001). Furthermore, IL-18BP prevents endogenous IL-18-induced murine diabetes by reducing IFN-γ expression (Nicoletti *et al.* 2003). In the NOD mouse model, the development of cyclophosphamide-induced diabetes is significantly reduced by prophylactic administration of IL-18BP:FcIg (Zaccone *et al.* 2005). Interestingly, inhibition of *in vivo* IL-18 activity by IL-18BP in mice leads to the stimulation of tissue neovascularisation in response to ischaemic injury (Mallat *et al.* 2002). IL-18BP promotion of post ischaemic neovascularisation results in increased expression of VEGF, increased phosphorylation of Akt and increased numbers of culture differentiated BM-EPCs. Furthermore, the proangiogenic properties of IL-18BP are also associated with its known anti-inflammatory and antiatherosclerotic effects (Gracie *et al.* 1999, Mallat *et al.* 2001, Plater-Zyberk *et al.* 2001, Ten Hove *et al.* 2001). Findings from these studies suggest that IL-18BP and/ or other IL-18 inhibitors may be suitable candidates for the treatment of ischaemic disease.

Whereas a combination of the ligand binding and the non-ligand binding extracellular domains of IL-18R are required to neutralise IL-18 activity, IL-18BP effectively neutralises IL-18 at equimolar concentrations (Reznikov *et al.* 2002). It has therefore been suggested that isoform IL-18BPa, which posses the highest affinity for IL-18, may represent a suitable candidate with which to inhibit IL-18-induced pathology in human diseases (Dinarello 2000). *In vivo* experiments on mice receiving a lethal dose of LPS have shown that pre-administration of IL-18BP:Fc reduces LPS-induced IFN-γ expression, resulting in significant reductions in host mortality (Faggioni *et al.* 2001). Blocking IL-18 function *in vivo* with IL-18BP:Fc effectively attenuates DSS induced intestinal inflammation. This effect occurs through attenuating the expression of MMP-3, -7, -9, -10 and -13, thereby indicating a role for IL-18 in the initiation of IBD associated intestinal damage (Sivakumar *et al.* 2002).

In human diseases, correlations between serum levels of IL-18 and IL-18BP have been demonstrated. Furthermore, correlations between disease status and IL-18 and IL-18BP serum concentrations are also suggested (Kawashima *et al.* 2001, Ludwiczek *et al.* 2002). However, as with many cytokines and their inhibitors, others have demonstrated that the correlation between circulatory IL-18 and IL-18BP concentrations may not be representative of the disease status. Furthermore, the circulatory IL-18/ IL-18BP ratio may not reflect the host response to treatment of chronic diseases at localised sites (Bresnihan *et al.* 2002). Indeed, IL-18BP is significantly up-regulated in human Crohn's disease and this correlates with an increase in IL-18 expression. However, despite increased expression of IL-18BP, free biologically active IL-18 is readily detectable within the tissues (Corbaz *et al.* 2002). It is therefore apparent from studies to date that the complex biology between IL-18 and IL-18BP remains to be defined.

2.6.4 Functional properties of IL-18

IL-18 is predominantly proinflammatory in nature, sharing similar biological properties to IL-12 and is associated with numerous pathological processes. However, IL-18 and IL-12 are not structurally related. IL-18 promotes the expression of several key inflammatory mediators. These include inducing IFN-γ production (Okamura *et al.* 1995a, Ushio *et al.* 1996, Kohno *et al.* 1997, Kojima *et al.* 1999), enhancing IL-2 expression (Kohno *et al.* 1997) and up-regulating GM-CSF from human PBMC or enriched T cells (Micallef *et al.* 1996, Ushio *et al.* 1996). In addition to promoting inflammatory mediators, IL-18 also down-regulates the expression of IL-10 in Con A-stimulated PBMC but has no apparent effect upon IL-4 expression (Ushio *et al.* 1996). Conversely, within the IL-10-/- murine model, IL-10 fails to inhibit the production of IL-18 in response to inflammatory stimuli (Zediak and Hunter 2003).

The most potent biological function of IL-18 is its IFN-γ inducing activity from T cells, NK cells and NK T cells in combination with IL-12 (Micallef *et al.* 1996, Fehniger *et al.* 1999, Leite-de-Moraes *et al.* 1999, Otani *et al.* 1999, Walker and Rotondo 2004). Indeed, the synergy between IL-12 and IL-18 results in the rapid secretion of IFN-γ from NK cells. This effect appears to be dependent upon MAPK p38, which stabilises IFN-γ mRNA by the MAPK p38 signalling pathway (Mavropoulos *et al.* 2005). However, antagonists such

as PGE_2 carefully regulate the degree of IL-18-induced IFN-γ expression (Walker and Rotondo 2004). In addition to IL-12, IL-2, IL-10 and IL-21 also have the ability to augment IL-18-induced IFN-γ expression by NK cells (Son *et al.* 2001, Cai *et al.* 1999, Strengell *et al.* 2003). Although NK cells are an important source of IFN-γ, IL-18 also induces IFN-γ expression in macrophages and vascular cells (Tenger *et al.* 2005). IL-18 in combination with IL-12 not only enhances IFN-γ expression, but additionally induces the production of IL-3, IL-6 and TNF-α from NK cells (Lauwerys *et al.* 1999). Furthermore, IL-18 alone induces IL-12 and TNF-α expression within PBMC cultures (Yoshida *et al.* 2001). The production of TNF-α and IL-1β by monocytes through direct contact with lymphocytes is also augmented by IL-18 (Dai *et al.* 2004a).

Like IL-12, IL-18 enhances allospecific CTL activity *in vitro* and augments NK cell cytotoxic activity and proliferation (Okamura *et al.* 1995a,b, Ushio *et al.* 1996, Bazan *et al.* 1996, Lauwerys *et al.* 1999). These actions are mediated in a Fas ligand-dependent (Tsutsui *et al.* 1996) and perforin-dependent manner (Hyodo *et al.* 1999) that is independent of TNF-α (Dao *et al.* 1998). The complex regulatory circuit involved in apoptotic cell death is in part mediated by IL-18-induced IFN-γ production, thereby up-regulating expression of the receptor for Fas Ligand, Fas antigen (Watanabe-Fukunaga *et al.* 1992). Although IL-18 is not required for early NK cell development, in combination with IL-12 it plays an important role in the functional maturation of NK cells (Takeda *et al.* 1998).

In addition to its important effects upon NK cells, IL-18 acts on other immune cells and stromal cell populations participating in inflammatory responses. IFN-γ secretion, induced from activated B cells by IL-12 and IL-18, inhibits IgE production (Yoshimoto *et al.* 1997). Additionally, IL-18 is synergistically capable of up-regulating IFN-γ from epidermal cells (Sugaya *et al.* 1999). IL-18 and IL-12 share the capacity to induce IFN-γ secretion by activated Th1 cells. However, their induction pathways appear to be independent, since neutralising antibodies to IL-12 do not block IFN-γ production induced by IL-18 and vice versa. Furthermore, IL-18 is more potent than IL-12 in inducing IFN-γ expression from T-cells (Okamura *et al.* 1995, Micallef *et al.* 1996) and natural killer cells (Tsutsui *et al.* 1996, Okamura *et al.* 1995, Ushio *et al.* 1996, Gu *et al.* 1997). Moreover, even in the presence of saturated amounts of IL-12, IL-18 is able to up-regulate the

expression of IFN-γ. Many of these IL-18-mediated effects upon cytokine expression, including the induction of IFN-γ (Kojima *et al.* 1999, Tsuji-Takayama 1999, Strengell *et al.* 2003) and IL-2 gene expression (Matsumoto *et al.* 1997), are regulated via NF-κB activation.

IL-18 induces the proliferation of murine T cells, Th1 clones and human enriched T cells stimulated with mitogen, anti-CD3 or antigen (Kohno *et al.* 1997, Jacobsen *et al.* 1995). IL-18-mediated T-cell proliferation takes place through an IL-2 dependent pathway (Micallef *et al.* 1996). IL-18 induces the release of Th1 type cytokines by T cells and macrophages and also stimulates the production of mediators such as CXC-chemokine IL-8, MGSA (CXCL1) and ENA-78 in rheumatoid arthritis synovial fibroblasts. Macrophage-derived chemokines and nitric oxide is also enhanced by IL-18 (McInnes *et al.* 2001, Morel *et al.* 2001). IL-18 promotes the release of IL-8 and IL-1β from mononuclear cells by inducing TNF-α secretion from $CD3^+/CD4^+$ cells (Puren *et al.* 1998). Furthermore, IL-18-induced release of monocyte-derived TNF-α and IL-1β is dependant upon cell-cell contact with T cells (Dai *et al.* 2004a). Not only does IL-18 induce cytokine release from dendritic cells and macrophages but it is also involved in the maturation of myeloid dendritic cells. However, it is not responsible for the differentiation of monocytes into dendritic cells (Li *et al.* 2004).

Many of these described effects impart a key role for IL-18 in orchestrating acquired immune responses. Nevertheless, through several actions, IL-18 may be involved in early stages of the initial inflammatory immune response. Indeed, IL-18 activates human neutrophils and induces their expression and secretion of IL-1β and IL-6 (Jablonska and Jablonski 2001, Jablonska *et al.* 2002). Furthermore, the initial recruitment of cells of innate immunity to inflammatory sites is fundamentally influenced by IL-18 (Umemura *et al.* 2004, Taube *et al.* 2004). IL-18 promotes neutrophil accumulation *in vivo*, whereas IL-18 neutralisation suppresses the severity of footpad inflammation following carrageenan injection (Leung *et al.* 2001). IL-18 induces the production of MMPs from chondrocytes (Dai *et al.* 2005), NK cells, U937 monocytes (Abraham *et al.* 2003) and PBMC (Nold *et al.* 2003). Some of these effects occur in a TNF-α dependent manner and are down-regulated by IL-4 and/or IL-10. Consequently, these IL-18-induced proteases are responsible for the degradation of the surrounding extracellular matrix and basal lamina (Ishida *et al.* 2004). Thereby, T cells (Komai-Koma *et al.* 2003) and neutrophils are

recruited into the inflammatory site (Cannetti *et al.* 2003). Moreover, IL-18-induced expression of IL-8, further contributes to the influx of these inflammatory cells (Wang *et al.* 2001a). In addition to its effects upon cytokines, IL-18 up-regulates ICAM-1 expression in human monocytes (Yoshida *et al.* 2001, Stuyt *et al.* 2003b) and KG-1 cells (Kohka *et al.* 1998). However, these predominantly proinflammatory IL-18 properties are carefully counterbalanced by the local release of down-regulatory mediators such as histamine (Takahashi *et al.* 2001, Itoh *et al.* 2002) and heat shock proteins (Wang and Chang 2005).

These data clearly demonstrate that IL-18-mediated effects are predominantly proinflammatory in nature. Nevertheless, IL-18 also possesses anti-inflammatory properties. IL-18 induces Th2 type cytokine expression including IL-4, IL-5, IL-10 and IL-13 from murine naïve spleen cells (Xu *et al.* 2000) and IL-4 from basophils (Yoshimoto *et al.* 1999). Furthermore, in the absence of IL-12, IL-18 stimulates $CD4^+$ T cells and macrophages to secrete IL-5, GM-CSF, IL-6, and G-CSF. In turn, these mediators induce haematopoietic cell proliferation leading to neutrophilia and eosinophilia in mice (Ogura *et al.* 2001). Intraperitoneal administration of IL-18 into mice has also been shown to reduce circulating leukocyte numbers by inducing IFN-γ release from NK cells (Hosohara *et al.* 2002). Not only does IL-18 stimulate proinflammatory cytokine secretion from NK cells but, when combined with IL-2, it is a potent co-inducer of IL-13 from NK and T cells. This effect appears to be IFN-γ-dependent (Hoshino *et al.* 1999c).

IL-18 is an angiogenic mediator, inducing microvascular endothelial cell migration and endothelial cell tube formation in a matrigel matrix *in vitro* and *in vivo* (Park *et al.* 2001). Functional IL-18 and IL-18 receptor are expressed by human atheroma-associated endothelial cells, smooth muscle cells, and mononuclear phagocytes. In combination with its ability to induce IFN-γ expression in smooth muscle cells, this suggests that IL-18 may have a paracrine pro-inflammatory role in atherogenesis (Gerdes *et al.* 2002). Conversely, Mallet *et al.* (2002) have demonstrated that the neutralisation of IL-18 by IL-18BP leads to neovascularisation after ischemia-induced pathology. This therefore implies that endogenous IL-18 may not always be proangiogenic. Indeed, IL-18 has been demonstrated to suppress tumour growth by its inhibitory effects upon angiogenesis (Coughlin *et al.* 1998, Cao *et al.* 1999). Clearly, the precise relationship of IL-18 with angiogenesis requires further clarification.

2.6.5 IL-18 and the Th1/ Th2 paradigm

After its discovery, IL-18 was found to exert a powerful synergy with IL-12 upon the induction of IFN-γ (Micallef *et al.* 1996). Furthermore, the costimulatory effects of IL-18-induced IFN-γ expression were selectively targeted towards Th1 type cells but not Th2 cells (Kohno *et al.* 1997). However, Th1 cell development is not directly induced by IL-18. It is established that IL-12-induced Th1 polarisation is required prior to IL-18-induced expression of IFN-γ from differentiated Th1 cells (Robinson *et al.* 1997, Stoll *et al.* 1998). Therefore, the predominant effect of IL-18 on Th1 development was suggested to be its role in the up-regulation of IL-12Rβ2 expression. Up-regulation of the IL-12 receptor enhances the capability of IL-12-mediated signalling. This would be of critical importance if IL-12 levels within the microenvironment were below threshold levels required for successful Th1 development (Chang *et al.* 2000). Furthermore, IL-18 in combination with anti-CD3 induces IFN-γ, IL-13, GM-CSF and IL-8 expression from Th1 cells but not from Th2 cells (Hata *et al.* 2004).

In consideration of its potent proinflammatory properties within the Th1 pathway, IL-18 was surprisingly demonstrated to be capable of inducing T cells to differentiate into Th2 cells independently of IL-4 (Xu *et al.* 2000). Additionally, IL-18 in combination with IL-2 is a potent co-inducer in the production of the Th2 type cytokine IL-13 in both murine NK and T cells (Hoshino *et al.* 1999). Moreover, IL-18 can direct Th2 responses by inducing IL-4 expression from NKT cells, irrespective of the presence of IL-12. Subsequently, this NKT cell-derived IL-4 contributes towards the adaptive Th2 immune response by up-regulating expression of the early activation marker CD69 on B cells (Leite-de-Moraes *et al.* 2001). Furthermore, IL-18 has been demonstrated to increase IL-4 expression in allergen stimulated T cells (Tarkowski *et al.* 2002).

The first *in vivo* investigation demonstrating the capability of IL-18 to induce a Th2 phenotype determined that it was a contributory factor towards allergic asthma (Wild *et al.* 2000). The broader role of IL-18 on Th1/Th2 orientation has been further investigated in IL-18 transgenic mice. These mice have higher serum levels of IgE, IgG1, IL-4 and IFN-γ and their splenic T cells produce higher levels of IFN-γ, IL-4, IL-5 and IL-13 than wild type controls (Hoshino *et al.* 2001). Indeed, IL-18 can induce IL-4 and IL-13 expression independently of TCR engagement through CD40 ligand expression on $CD4^+$ effector cells

(Yoshimoto *et al.* 2000, Konishi, *et al.* 2002). Additionally, it has been established that IL-18 can directly regulate Th2 cytokine expression in response to gastrointestinal nematode infections (Helmby *et al.* 2001). Of importance, it has recently been elucidated within the murine model that the determining factor as to whether IL-18 mediates a Th1 or Th2 type response to intracellular pathogens is dependent upon the genetic background of the host (Wei *et al.* 2004).

2.6.6 IL-18 and host responses to pathogens

2.6.6.1 Introduction

IL-18 induces a myriad of potent immunomodulatory functions, many of which are central to the containment of microbial infection. IL-18 is a critical mediator in host defence mechanisms against intracellular bacteria, including *Listeria*, *Shigella*, *Salmonella* and *Mycobacterium tuberculosis* (Dybing *et al.* 1999, Sugawara *et al.* 1999, Biet *et al.* 2002). The expulsion of invading pathogens for host survival requires a robust Th1 response and IL-18 is fundamental to this (Kobayashi *et al.* 1998, Takeda *et al.* 1998, Sugawara *et al.* 1999). The single most important factor in generating an efficient response against pathogenic insult is the appropriate induction of IFN-γ expression (Mastroeni *et al.* 1999). Through its up-regulatory effects upon IFN-γ expression, IL-18 significantly contributes towards efficient eradication of these microorganisms from the host (Kawakami *et al.* 1997). Furthermore, IFN-γ expression is also indirectly up-regulated by IL-18 through the induction of TNF-α (Kawakami *et al.* 1999). Host antimicrobial activity is further enhanced by the synergistic expression of NK cell and $CD8^+$ derived IFN-γ, which is synergistically induced by IL-12 in combination with IL-18 (Zhang *et al.* 1997, Berg *et al.* 2002). The importance of IL-18 within host resistance to pathogenic insult is emphasised by several pathogens. Pathogens including viruses and enteric protozoa, are capable of neutralising or cleaving IL-18 to render it inactive. Through these mechanisms, microorganisms may subvert immunity thereby prolonging their survival within the host (Xiang and Moss 1999, Born *et al.* 2000, Smith *et al.* 2000, Que *et al.* 2003).

2.6.6.2 Infection of in vitro cell cultures

IL-18 mediates resistance against a wide variety of infectious pathogens. This primarily occurs through increased cellular expression of IFN-γ. This important property was initially recognised in experiments using whole blood cell cultures stimulated with the Gram-negative derived PAMP, LPS (Puren *et al.* 1998, Manigold *et al.* 2000). Following early studies which established that IL-18 expression was induced by LPS, numerous *in vitro* cell culture experiments have been performed. These have explored the capability of several live and killed microbial pathogens and their mitogenic components to induce IL-18 expression.

IL-18 mRNA expression was subsequently demonstrated to be directly increased in PBMC cultures stimulated with LPS, LTA or *Staphylococcus aureus* Cowan strain 1 (SAC) (Marshall *et al.* 1999). Indeed, SAC up-regulates protein production of IL-10, IL-12, IL-18 and IFN-γ within PBMC cultures and this occurs independently of CD14 (Böcker *et al.* 2001). Interestingly, lipoteichoic acid (LTA), a structural component of SAC, is not capable of inducing IL-18 expression, although it induces IL-10 in a dose dependent manner. The Gram-positive mitogen SAC is also capable of increasing IL-18 production within whole blood cultures (Oberholzer *et al.* 2001). The secretion of IL-18 from cells challenged with microorganisms or their associated microbial products is mediated through TLR interactions (Seki *et al.* 2001, Adachi *et al.* 2001). Therefore following stimulation with LPS, TLR-deficient macrophages do not secrete IL-18 (Seki *et al.* 2001). Importantly, IL-18 expression induced within LPS-stimulated PBMC or monocyte cultures can be inhibited by histamine or prostaglandin E_1 respectively. Following pathogenic infection, the potential for tissue damage resulting from increased IL-18 expression may therefore be countered by down-regulation of IL-18 expression arising from the local release of histamine or prostaglandins (Takahashi *et al.* 2004, Takahashi *et al.* 2005a).

Staphylococcus epidermidis infection of whole blood cultures induces increased IFN-γ production through endogenously expressed IL-18, IL-12 and TNF-α and independently of IL-1 (Stuyt *et al.* 2003a). The staphylococcal superantigen, SEA also induces IFN-γ expression within PBMC cultures. NK cells, but in particular NK-type T cells, are the main source of this SEA-induced IFN-γ and its expression appears to be independent of IL-18 (Ami *et al.* 2002). Non-pathogenic *lactobacillus* strains and a pathogenic *Streptococcus*

pyogenes strain induce mRNA and protein production within PBMC cultures for IL-1β, IL-6, TNF-α, IFN-γ and IL-18. Conversely, these microbes only weakly up-regulate IL-10 expression and have no appreciable effect upon IL-4 (Miettinen *et al.* 1998). Infection of PBMC cultures with live *Helicobacter pylori* dose dependently induces expression of IFN-γ, IL-10 and IL-18 (Stassi *et al.* 2002). *Helicobacter pylori* infection of gastric epithelial cells also induces increased IL-18 expression and this enhances increased IL-8 production within these cell cultures (Day *et al.* 2004). Interestingly, the pre-treatment of PBMC cultures with killed *H. pylori* prior to infection with the live microorganism significantly down-regulates IFN-γ and IL-18 expression (Stassi *et al.* 2002).

Bacteria are not the only microorganisms that have effects upon cellular expression of IL-18 as fungi (Netea *et al.* 2002) and viruses (Manigold *et al.* 2003) also modulate IL-18 production. Indeed, IFN-γ production in whole blood cultures infected by *Candida albicans* is regulated in an IL-18, IL-12 and IL-1β dependent manner (Netea *et al.* 2002). In addition to PBMC and whole blood cell cultures, infection of other cell types by microorganisms and their virulence factors elicit IL-18 expression. Indeed, *Candida albicans* and LPS stimulate IFN-γ production from oral epithelial cells by inducing the secretion of mature IL-18 (Rouabhia *et al.* 2002). Infection of dendritic cells by *Listeria monocytogenes* induces the expression of IL-18 (Kolb-Mäurer *et al.* 2003). Furthermore, *Chlamydia trachomatis* infection of epithelial cells and dendritic cells results in IL-18 and IL-12 production, which drives IFN-γ expression from NK cells thereby, resolving the infection (Lu *et al.* 2000a, Hook *et al.* 2005).An involvement for IL-18 in linking innate and acquired immune responses is further emphasised by human β-defensin-2-induced secretion of IL-18 by keratinocytes (Sampanthanarak *et al.* 2005).

In contrast, some pathogens have been demonstrated to decrease the expression of IL-18 in cell cultures. For example, mouse macrophages infected with *Salmonella* exhibit decreased IL-18 expression. This has been further confirmed *in vivo* with *Salmonella* inoculated mucosal sites, which also exhibit reduced IL-18 expression. This suggests that some intracellular microorganisms may be capable of limiting the protective host response through their actions upon IL-18 (Elhofy and Bost 1999). This is in agreement with other pathogens including viruses that neutralise IL-18 bioactivity, thereby circumnavigating host immunity and prolonging their survival within the host (Xiang and Moss 1999, Born *et al.* 2000, Smith *et al.* 2000, Que *et al.* 2003).

2.6.6.3 Animal models of infection

IFN-γ and IL-18 are diminished in the sera of ICE-deficient mice exposed to *P. acnes* and LPS. Furthermore, ICE-deficient mice and mice lacking IFN-γ or its receptor are resistant to septic shock induced by endotoxin. This suggests that IL-18 has a central responsibility in inducing this type of septic shock (Li *et al.* 1995). Supporting this view is the observation that liver damage arising by infection with *Propionibacterium acnes* and septic shock induced by LPS challenge are alleviated in IFN-γ-deficient mice (Tsuji *et al.* 1999) and prevented by neutralising IL-18 activity (Okamura *et al.* 1995b). Part of the destructive element of IL-18 in lethal *E. coli* and *Salmonella typhimurium* infection has been demonstrated by a significant reduction in neutrophil-mediated tissue damage after IL-18 neutralisation (Natea *et al.* 2000). Conversely, *P. acnes*-primed IL-18 knockout mice exposed to LPS are highly susceptible to septic shock, although they have reduced endotoxin-induced liver damage (Sakao *et al.* 1999).

Several investigations have analysed the effects upon the exogenous administration or neutralisation of IL-18 in mice inoculated with a variety of pathogens. IL-18, particularly in combination with IL-12, has a beneficial synergistic action in the protection of mice challenged by *Cryptococcus neoformans* infection. This appears to be regulated through the early production of IFN-γ by NK and γδ T cells concomitant with suppressed IL-4 expression (Qureshi *et al.* 1999). This IL-18/IL-12 cytokine combination has also been demonstrated to afford protection against *Brucella* infection in mice. In contrast, either cytokine alone has only a limited effect against this infection. The protective synergistic effects of IL-12 and IL-18 appear to be through inducing increased IFN-γ expression (Pasquali *et al.* 2002). Interestingly, *Brucella abortus* infection selectively limits IL-18 expression from mouse splenocytes without affecting endogenous IFN-γ production (Fernández-Lago *et al.* 2005). The combined effect of IL-18 and IL-12 upon IFN-γ expression is also crucial in protecting mice infected with *Trypanosoma cruzi*. Within this infection, increased host expression of IFN-γ is related to a potent and early Th1 type response that is subsequently modulated by IL-13 expression (Antúnez and Cardoni 2001).

Although IFN-γ up-regulation is critical to a successful outcome from pathogenic insult, host up-regulation of IL-18 is also important. Indeed, IL-18 has been shown to be more potent than either IFN-γ or IL-12 in protecting both primary and secondary host immune

responses to *Listeria monocytogenes* infection (Neighbors *et al.* 2001). This has been further demonstrated by the administration of exogenous GM-CSF, which leads to increased clearance of *Pneumocystis carinii* infection within neonatal mice (Qureshi *et al.* 2005). Neonatal mice infected with group B streptococci (GBS) have significantly elevated IL-18 plasma levels and neutralisation of IL-18 increases mortality and bacterial burden. Furthermore, the administration of exogenous IL-18 markedly improves their survival, thereby implying an important role for IL-18 in protecting the host against neonatal GBS infection (Cusumano *et al.* 2004). Conversely, neutralisation of endogenous IL-18 in adult CD1 mice inoculated with GBS results in decreased mortality, although their incidence of arthritis is decreased (Tissi *et al.* 2004). Furthermore, administration of exogenous IL-18 within these CD1 mouse experiments actually increases mortality rates.

The transgenic IL-18 knockout mouse has allowed the detailed investigation of the role IL-18 plays within a number of infectious diseases. IL-18-/- mice are susceptible to *Leishmania major* infection and in comparison to wild type controls, express significantly lower IFN-γ levels and greater quantities of IL-4 (Wei *et al.* 1999). Experiments from *Streptococcal pneumonia* infected IL-18 knockout mice suggest that IL-18, and not IL-12, is required for an efficient early antibacterial host reaction to this pathogen (Lauw *et al.* 2002). The requirement for IL-18 in the early response to pathogenic challenge is further reinforced within IL-18 knockout mice infected with *E. coli.* IL-18-/- mice exhibit increased numbers of neutrophils. However, these are less active thereby rendering *E. coli* infected mice to disseminating infection (Weijer *et al.* 2003). The importance of IL-18 has been further demonstrated within IL-18 knockout mice with *Yersinia* (Hein *et al.* 2001) or *Cryptococcus neoformans* (Kawakami *et al.* 2000) infections. Disseminated *Candida albicans* infections in IFN-γ-/- and IL-18-/- mice have established that in addition to increased IFN-γ expression, successful host resistance to this pathogen requires monocyte recruitment, which is dependent upon IL-18 (Stuyt *et al.* 2003c, Netea *et al.* 2003).

However, induced infections in IL-18 knockout mice also reveal that IL-18 may adversely influence the outcome to certain infections, including *Pseudomonas aeruginosa* (Schultz *et al.* 2003) and *Streptococcus pneumoniae* (Zwijnenburg *et al.* 2003) or be dispensable (Graefe *et al.* 2003). Furthermore, *Staphylococcus aureus* infections in these mice are less severe than controls but they develop significantly more severe septic arthritis than controls. This is accompanied by a reduction in the levels of antigen-induced splenic T-cell

proliferation, decreased IFN-γ and TNF-α synthesis, but increased IL-4 expression (Wei *et al.* 1999).

In addition to bacteria, IL-18 is also important against viral infection. IL-18 reduces morbidity within BALB/c mice infected with herpes simplex virus (Fujioka *et al.* 1999). Murine experiments also establish that IL-12 and IL-18 act in synergy to clear vaccinia virus infection from the host (Gherardi *et al.* 2003). However, similar to bacterial infections, experimental viral infections in the IL-18 knockout murine model have produced conflicting results. In contrast to previous studies, it has recently been demonstrated that endogenous IL-18 impairs viral clearance during influenza infection (Van Der Sluijs *et al.* 2005). IL-12Rβ1-deficient mice, which suffer from significantly reduced coxsackie virus-induced myocarditis, also have decreased levels of IL-1β and IL-18 (Fairweather *et al.* 2003). Furthermore, although IL-18 has a role in modulating respiratory syncytial virus-induced airway inflammation, this is distinct from being responsible for viral clearance (Wang *et al.* 2004). It is apparent from these studies that the role for IL-18 in host immunity to viral challenge remains to be clarified. Nonetheless, the expression of vIL-18BPs by distinct poxvirus genera, which cause local infection or general viral dissemination, emphasise the importance of IL-18 in response to viral infections (Smith *et al.* 2000).

It is evident that several discrepancies exist between studies investigating the effects of IL-18 within host responses to invading pathogens in both wild type and IL-18 knockout mouse models. Recent investigations have started to elucidate some of the possible mechanisms behind this. *Streptococcus pnuemoniae* infections within IL-18 knockout mice establish that the differing effects of IL-18 to host infection appear to be dependent upon the individual microorganism strain used, the infection type and the host genetic background (Paterson *et al.* 2005). Furthermore, the levels of inflammatory cytokines, including IL-18, prior to infection impact upon the varying effects within and mortality rates of the host (Culshaw *et al.* 2005). In addition, the route or site of administration of pathogenic challenge influences host outcome to infections within these murine models (Baldwin *et al.* 2003).

2.6.6.4 Human infectious diseases

In pathogenic challenge to the host, the Th1/ Th2 balance is central to defining disease outcome. However, current data on the contribution of IL-18 towards pathogenic clearance in many human infectious diseases is conflicting. Such contradictory findings for the role of IL-18 are classically demonstrated in human leprosy. In resistant tuberculoid leprosy (TL), protective IFN-γ production is associated with increased IL-18mRNA expression within lesions. In addition, monocytes and TL patients show increased IL-18 mRNA expression following *in vitro* challenge with bacterial antigen (García *et al.* 1999). Furthermore, *in vitro* challenge of T and NK cells of TL patients by exogenous IL-18 results in increased IFN-γ production compared to cells from patients with susceptible lepromatous leprosy (LL). Surprisingly, serum IL-18 levels in the LL cohort are found to be much higher than patients with TL (Yoshimoto *et al.* 1999). This raises the possibility that IL-18 may contribute towards the development of Th2 responses, characteristic of LL. Human patients with active *M. tuberculosis* infection have raised plasma IL-18 levels (Morosini *et al.* 2003) and *in vitro* stimulation of PBMC cultures leads to increased IL-18 (Yamada *et al.* 2000, Inomata *et al.* 2005). However, they exhibit a decreased ability to produce IL-18 and IFN-γ in response to antigen compared to healthy responsive controls (Vankayalapati *et al.* 2000, Morosini *et al.* 2003, Vankayalapati *et al.* 2003). This demonstrates that the precise contribution of IL-18 towards protective Th1 type responses in such disease processes requires clarification.

Septic patients have increased plasma IL-18 concentrations compared with severely injured patient controls. Moreover, this is particularly evident within patients suffering from Gram-positive sepsis (Oberholzer *et al.* 2001). IL-18 serum levels in patients with fatal necrotising fasciitis are also raised compared with those of survivors (Lungstras-Bufler *et al.* 2004). In severe myeloidosis, caused by the Gram-negative bacillus *Burkholderia*, patients have elevated IFN-γ levels. Increased endogenous IL-18 expression is a contributing factor towards this (Lauw *et al.* 1999). IL-18 serum levels are also increased within patients with HIV infection (Ahmed *et al.* 2002, Stylianou *et al.* 2003) and renal allograft dysfunction (Hu *et al.* 2003). Conversely, IL-18 serum levels are decreased in patients with hepatitis C virus hepatitis. Interestingly, plasma IL-18 levels within these patients are related to hepatic histological activity (Schvoerer *et al.* 2003). Nevertheless, *in vitro* studies have demonstrated that IL-18 plays an important role in the protective

response to a number of bacterial infections including *Salmonella yersinia*, *Chlamydia* and *Shigella* (Mastroeni, *et al.* 1999, Bohn *et al.* 1998, Lu *et al.* 2000, Sansonetti *et al.* 2000).

The data derived from these studies clearly demonstrate that the role of IL-18 in host responses to human infectious diseases is extremely complex. The exact contribution of IL-18 towards host protection and pathogenic clearance or inflammatory dysregulation and morbidity within many of these infectious diseases remains to be elucidated.

2.6.7 Contribution of IL-18 to general systemic diseases

IL-18 is reported as being involved in the initiation or development of a multitude of inflammatory processes. Indeed, serum IL-18 levels have been measured in human patients suffering from a variety of general inflammatory diseases. Increased serum levels of IL-18 have been recorded in patients with Graves' ophthalmology (Myśliwiec *et al.* 2003), Behçet's disease (Hamazaoui *et al.* 2002, Oztas *et al.* 2005), allergic asthma (Wong *et al.* 2001), fatal cardiovascular disease (Blankenberg *et el.* 2002), psychiatric disorders (Kokai *et al.* 2002), type I diabetes (Nicoletti *et al.* 2001), acute kidney allograft rejection (Striz *et al.* 2005), adult-onset Still's disease (Kawaguchi *et al.* 2001, Saiki *et al.* 2004), fulminant hepatic failure (Yumoto *et al.* 2002), systemic lupus erythematosus (Robak *et al.* 2002, Amerio *et al.* 2002, Wong *et al.* 2002, Wong *et al.* 2000, Park *et al.* 2004), ANA-positive subacute cutaneous lupus erythematosus (Maczynska *et al.* 2006), Crohn's disease (Furuya *et al.* 2002, Ludwiczek *et al.* 2005), coeliac disease (Merendino *et al.* 2003, Lettesjö *et al.* 2005), ulcerative colitis (Wiercinska-Drapalo *et al.* 2005), idiopathic pulmonary fibrosis (Kitasato *et al.* 2004), heart failure (Mallat *et al.* 2004), systemic sepsis and in patients with liver disease such as autoimmune hepatitis and primary biliary cirrhosis (Grobmyer *et al.* 2000, McGuinness *et al.* 2000, Yamano *et al.* 2000).

However, the mere fact that IL-18 expression is up-regulated within the peripheral blood compartment of these patients compared with their control cohort needs to be interpreted with some caution. In several chronic inflammatory diseases, IL-18 serum levels are found to correlate with disease severity, including primary biliary cirrhosis (Yamano *et al.* 2000) acute pancreatitis (Endo *et al.* 2001, Grobmyer *et al.* 2000, McGuinness *et al.* 2000), SLE (Wong *et al.* 2000, Amerio *et al.* 2002), coronary heart disease (Blankenberg *et al.* 2003), atopic dermatitis (Tanaka *et al.* 2001b, Hon *et al.* 2004) and pulmonary tuberculosis

(Akgun *et al.* 2005). Conversely, others suggest that IL-18 serum levels do not specifically correlate with disease severity such as in SLE (Robak *et al.* 2002) and ulcerative colitis (Furuya *et al.* 2002). Whether raised IL-18 serum concentration is a contributing factor to the disease process or a mere by-product of the chronic inflammatory reaction requires further clarification.

Nonetheless, increased IL-18 mRNA and protein expression is demonstrated within intestinal epithelial cells and lamina propria mononuclear cells in Crohn's disease (Pizarro *et al.* 1999) and in samples from involved mucosal areas (Monteleone *et al.* 1999). Furthermore, expression of mature IL-18 and both IL-18 receptors are up-regulated in coeliac disease (Salvati *et al.* 2002). IL-18 expression is also increased in autoimmune non-obese diabetic (NOD) mice (Rothe *et al.* 1997) and BDC2.5 TCR transgenic mice with diabetes (André-Schmutz *et al.* 1999). However, its role in the development of diabetes remains to be defined, with the administration of exogenous IL-18 being shown to alternatively inhibit (Rothe *et al.* 1999) and promote (Oikawa *et al.* 2003) diabetes development. IL-18 expression is increased in PBMC cultures from patients with multiple sclerosis (Huang *et al.* 2004a). Furthermore, IL-18 is mitigated as a major contributing factor in atopic diseases including allergic asthma (Wild *et al.* 2000), bronchial asthma, allergic rhinitis (Verhaeghe *et al.* 2002) and atopic dermatitis (El-Mezzein *et al.* 2001). Indeed, following gut ischaemia/ reperfusion, IL-18 levels are significantly elevated and contribute towards lung inflammation (Yang *et al.* 2005). These findings suggest that IL-18 may be a central mediator of inflammatory pathology within humans (Dayer 1999, McInnes *et al.* 2000). It has therefore been postulated that an IL-18 inhibitor may offer desirable therapeutic benefits (Dinarello 2000). However, in other disease processes, neutralisation of IL-18 may have adverse consequences. *In vivo* murine experiments have demonstrated that IL-18 exerts a protective effect against the development of chronic graft-versus-host disease (Okamoto *et al.* 2000). Furthermore, IL-18 inhibition accelerates mortality within acute graft-versus-host disease as a consequence of increased FAS antigen mediated donor T-cell apoptosis (Reddy *et al.* 2001).

IL-18 exhibits strong antitumoural activities, protecting experimental animals against repeated challenges with tumour cells through NK cell activation (Micallef *et al.* 1997). Decreased tumour growth and inhibition of metastasis occurs with the administration of IL-18, both alone (Akamatsu *et al.* 2002) and in combination with IL-2 (Arai *et al.* 2000,

Coughlin *et al.* 2000). Furthermore, IL-18 gene transfer into murine tumours exerts potent antitumour effects that appear to be mediated by local IL-18 expression (Osaki *et al.* 1999, Liu *et al.* 2002, Hikosaka *et al.* 2004, Zhang *et al.* 2004). Moreover, the simultaneous expression of IL-18 and IL-12 by engineered melanoma cells confers significant antitumour properties in mice (Kishida *et al.* 2001). Indeed, vaccination with IL-18 gene-modified, superantigen-coated tumour cells elicits potent antitumour immune responses (Wang *et al.* 2001b). However, in contrast to these antitumour effects, IL-18 together with other cytokines may be responsible for the development of hepatic metastases of melanoma *in vivo* through up-regulating the expression of vascular cell adhesion molecule-1 and melanoma cell adherence (Vidal-Vanaclocha *et al.* 2000). Of significance, the initial published results of phase 1 trials of IL-18 therapy in cancer patients are not encouraging (Golab and Stoklosa 2005). Further trials and investigations into the involvement of IL-18 in the initiation, progress and metastasis of malignant tumours are therefore required to provide evidence of safety and efficacy of proposed IL-18 therapy.

More recently, studies have analysed relationships between IL-18 polymorphisms and disease processes. These investigations have identified a possible link of IL-18 polymorphisms with the pathogenesis of atopy (Kruse *et al.* 2003), insulin dependant diabetes mellitus (Kretowski *et al.* 2002), atopic eczema (Novak *et al.* 2005) and the development of post-injury sepsis (Stassen *et al.* 2003). Conversely, others have determined no association between IL-18 polymorphisms with periodontal disease (Folwaczny *et al.* 2005), autoimmune thyroid disease (Ide *et al.* 2003) or Type 1 diabetes (Ide *et al.* 2004, Novota *et al.* 2004, Martin *et al.* 2005). Furthermore, studies are divided over the relationship between IL-18 polymorphisms and certain diseases processes. For example, IL-18 gene polymorphisms are suggested as a contributing factor in rheumatoid arthritis (Gracie *et al.* 2005) and asthma (Higa *et al.* 2003). Conversely, others have determined that IL-18-promoter polymorphisms are not associated with RA (Rueda *et al.* 2005) or confer susceptibility to sarcoidosis (Zhou *et al.* 2005) and that the genetic effect of IL-18 polymorphisms is not associated with asthma (Shin *et al.* 2005). It is evident that in order to obtain any true meaning in the relationship between gene polymorphisms and disease processes, larger population samples will have to be analysed.

2.6.8 Contribution of IL-18 to bone metabolism

Bone remodelling involves the carefully coordinated partnership between bone deposition and bone resorption. Osteoblasts are responsible for the synthesis and deposition of the extracellular bone matrix (Ducy *et al.* 2000). Conversely, osteoclasts are central to bone resorption (Teitelbaum 2000). Osteoclast and osteoblast functions are intimately related and closely balanced between one another. Bone is continuously being resorbed or destroyed by osteoclasts whilst concomitantly being replaced by osteoblasts. Systemic hormones and local mediators closely regulate the dynamic process of bone deposition and resorption (refer to chapter 2.7).

Osteoblastic stromal cells are known to express IL-18 mRNA (Atkins *et al.* 2000). In mouse osteoblast/ bone marrow-spleen cell co-cultures, osteoblastic stromal cell-derived IL-18 inhibits osteoclastogenesis through GM-CSF but not IFN-γ production (Udagawa *et al.* 1997). The inhibitory effect of IL-18-induced T cell-derived GM-CSF on osteoclast formation was further confirmed in cell co-cultures derived from GM-CSF-/- mice (Horwood *et al.* 1998). Subsequent studies have demonstrated that IL-18 mRNA is expressed in a mouse stromal cell line ST2, a mouse osteoblast cell line MC3T3-E1 and mouse calvarial osteoblasts. Furthermore, it is suggested that IL-18 inhibits osteoclastogenesis by up-regulating osteoprotegerin (OPG) *in vitro* (Makiishi-Shimobayashi *et al.* 2001).

When combined with IL-12, IL-18 synergistically inhibits *in vitro* osteoclast formation (Horwood *et al.* 2001) and osteoclast bone resorbing activity (Yamada *et al.* 2002). In addition to up-regulated IFN-γ expression, these effects take place through unknown molecules. Furthermore, the mitogenic action of IL-18 on primary osteoblasts occurs independently of both GM-CSF and IFN-γ. This datum suggests that IL-18 may act directly on osteoblasts through its receptors (Cornish *et al.* 2003). IL-18 transgenic mice have deformed cortical bone plates and a reduced trabecular lumbar bone turnover rate compared with wild-type controls. These biological effects occur as a result of increased IL-18 expression and thereby significantly increased IFN-γ and reduced IL-4 expression within bone marrow cells (Kawase *et al.* 2003). IL-18 appears to have little influence upon the activity of mature osteoclasts. Its central attribute within the bone compartment may therefore be to limit osteoclast formation (Cornish *et al.* 2003). However, the majority of

findings from these studies are limited to animal *in vitro* experiments. It therefore remains unclear as to whether IL-18 influences bone mass or bone remodelling *in vivo* within humans.

Osteoblasts are an important source of a number of key proinflammatory mediators. Exposure of osteoblasts to either Gram-positive *S. aureus* or the Gram-negative pathogen *Salmonella* elicits marked induction of mRNA levels encoding IL-1β and IL-18. Indeed, murine osteoblasts infected with *S. aureus* or *Salmonella* express IL-1β and IL-18 mRNA as early as 6 h post infection, which peaks at 12 h post infection (Marriott *et al.* 2002). However, elevated mRNA levels within these cells 24 h post-infection does not translate into intracellular accumulation or secretion of mature IL-1β or IL-18 protein, even in the presence of caspase-1 activity. This demonstrates that bacterial pathogenic stimuli are not sufficient to promote translation of IL-1β and IL-18 in these cells (Marriott *et al.* 2002). It has also been established that bacterial infection of osteoblasts induces the production of IL-6, IL-12 (Bost *et al.* 1999), MCP-1 (Bost *et al.* 2001) and GM-CSF (Bost *et al.* 2000). IL-18 is known to be an angiogenic inhibitory factor (Coughlin *et al.* 1998) and through down-regulating angiogenesis may lead to aberrant bone formation (Gerber and Ferrara 2000).

Despite the demonstration of direct anti-bone destructive effects *in vivo*, IL-18 is highly expressed within the synovial joints of human RA patients (Gracie *et al.* 1999, Yamamura *et al.* 2001, Tanaka *et al.* 2001). Moreover, up-regulated expression of IL-18 within RA joints appears to be related to and responsible for locally increased production of TNF-α and IL-1β (Joosten *et al.* 2003). It has also been established within the murine model that IL-18 promotes collagen-induced arthritis (Leung *et al.* 2000). Furthermore, this effect is abrogated in mice lacking IL-18 (Wei *et al.* 2001). A proinflammatory role for IL-18 in destructive processes within CIA has further been elucidated in BB rats. In this model, the neutralisation of endogenous IL-18 with anti-IL-18 antibodies attenuates CIA (Ye *et al.* 2004). Increased interleukin-18 levels are also found at diseased sites associated with increased periodontal destruction (Johnson and Serio 2005) and may be associated with incipient periodontal attachment loss in patients with juvenile idiopathic arthritis (Miranda *et al.* 2005).

The direct effects of IL-18 on osteoblasts and osteoclasts appear to be the suppression of bone resorption. In the murine model, however, IL-18 induces joint inflammation independently of IL-1, although cartilage destruction occurs through IL-18-induced expression of IL-1β (Joosten *et al.* 2004). In the murine streptococcal cell wall arthritis model, IL-18 also induces joint destruction that appears to be independent of IFN-γ (Joosten *et al.* 2000). Furthermore, IL-18 induces RANKL production by T cells in RA synovitis and conversely has no effect upon OPG expression (Dai *et al.* 2004b). Thereby, IL-18 is capable of indirectly stimulating osteoclast formation through the up-regulation of RANKL. Indeed, IL-18-induced osteoclast formation is as effective as IL-1β but less potent than TNF-α (Dai *et al.* 2004b).

Taken together, these data suggest that the direct actions of IL-18 on bone cells *in vitro* may be anti-destructive in nature. Nevertheless, the overall effect of IL-18 within the cytokine milieu may be to induce the secretion of downstream inflammatory mediators that result in and perpetuate bone destruction. Indeed, there is accumulating evidence that IL-18 may directly contribute towards inflammatory bone destruction. IL-18 expression has not been characterised within the PRD lesion. It is therefore of importance to investigate its expression and potential involvement within the inflammatory reaction occurring within PRD.

2.7 Regulation of inflammatory bone resorption

2.7.1 Introduction

Alveolar bone is a specialised dynamic connective tissue comprising a mineralized matrix that endows it with elasticity and strength. The synthesis of organic matrix by osteoblasts and bone resorption by osteoclasts enables bone remodelling to take place in accordance with surrounding mechanical influences. Osteoblasts differentiate from mesenchymal stem cells through a series of progenitor stages to form mature matrix-secreting osteoblasts that are progressively transformed into osteocytes. The two major functions of osteoblasts are to synthesise the components of bone matrix and to control the bone resorbing activities of osteoclasts. However, osteoblasts have a third and equally important function in inflammatory bone diseases, the ability to secrete cytokines (Bost *et al.* 1999) and chemokines (Bost *et al.* 1999, Gasper *et al.* 2002). Following bacterial exposure, cytokine

and chemokine production by osteoblasts likely serves to recruit macrophages, neutrophils and activated T lymphocytes to the infected periradicular tissue. Thereby, osteoblasts are capable of sustaining an inflammatory response by maintaining the activated status of recruited inflammatory cells.

Osteoclasts are large multinucleated cells that are infrequently seen in normal adult bone but present at bone resorption sites within Howship's lacunae (Martin *et al.* 1998). Osteoclasts are the main protagonists of bone destruction and are haematopoietic in origin. They are formed through the fusion of mononuclear osteoclast precursor cells of the monocyte-macrophage lineage (Baron *et al.* 1986, Roodman 1999). Osteoclasts regulate bone resorption by attaching to the surface and secreting protons into the extracellular compartment formed beneath their ruffled border. Monocytes, macrophages, lymphocytes and endothelial cells are also implicated in bone remodelling by direct contact with bone cells. Subsequent to the initiation of periradicular disease, dento-alveolar bone destruction is therefore controlled by the numerous cell protagonists within the lesion that interface with bone tissue, the immune system and vascular component of the PRD tissue.

Fundamental to the bone remodelling process are the interactions between osteoblasts and osteoclasts (Epker and Frost 1965). Physical factors such as mechanical stimulation and polypeptides, including cytokines and hormones, are responsible for controlling the equilibrium between osteoblast and osteoclast activities. Through direct cell contact, osteoblasts regulate both osteoclast differentiation and osteoclast bone resorption activities (Jimi *et al.* 1996, Udagawa *et al.* 1999). This finding led to the discovery of cytokines within the TNF family that are central to the control of bone remodelling (Simonet *et al.* 1997, Tsuda *et al.* 1997, Lacey *et al.* 1998, Yasuda *et al.* 1998a,b).

Among the array of mediators that can directly act upon osteoblast and osteoclast precursors, receptor activator of nuclear factor-κB ligand (RANKL) has been identified as the principal regulatory protein. Up-regulation of this cytokine by factors acting on bone lining cells or other stromal cells provides a novel system that modulates osteoclastogenesis. Osteoprotegerin (OPG) is a naturally occurring antagonist of RANKL and a gene product that is distinct from the RANKL receptor, RANK (Bucay *et al.* 1998, Emery *et al.* 1998). Several recent studies have elucidated that activated T cells can support osteoclast formation from mononuclear precursor cells. These data directly

implicate lymphocytes in the pathogenesis of enhanced bone loss that is associated with immune mediated inflammatory disorders such as RA (Horwood *et al.* 1999, Kotake *et al.* 2001, Weitzmann *et al.* 2001).

2.7.2 Osteoprotegerin (OPG)

Osteoprotegerin (OPG), originally termed osteoclastogenesis-inhibitory factor (OCIF), was discovered as a soluble protein capable of suppressing osteoclast survival. OPG is a secreted TNFR-related protein that acts as a decoy receptor for RANKL. Furthermore, it interferes with stromal cell-osteoclast interactions and contributes towards a marked osteoporosis phenotype (Simonet *et al.* 1997, Tsuda *et al.* 1997, Akatsu *et al.* 1998, Yasuda *et al.* 1998b). Thereby, it is functionally responsible for regulating bone density and bone mass in animals (Simonet *et al.* 1997, Yasuda *et al.* 1998b). OPG is expressed ubiquitously and abundantly by a wide variety of cell types including bone marrow stromal cells, dendritic cells, lymphoid cells, endothelial cells, fibroblasts and monocytes (Simonet *et al.* 1997, Tan *et al.* 1997, Tsuda *et al.* 1997, Akatsu *et al.* 1998, Kwon *et al.* 1998, Yasuda *et al.* 1998a).

Primarily, the *in vitro* biological effects of OPG oppose those of RANKL. These include the inhibition of differentiation, survival and fusion of osteoclastic precursor cells, suppression of osteoclastic activation and promotion of osteoclast apoptosis (Akatsu *et al.* 1998, Fuller *et al.* 1998, Hakeda *et al.* 1998, Lacey *et al.* 1998, Miyamoto *et al.* 1998, Yasuda *et al.* 1998b, Burgess *et al.* 1999, Jimi *et al.* 1999, Lacey *et al.* 2000, O'Brien *et al.* 2000, Udagawa *et al.* 2000). Interestingly, the stimulation of human RA-derived synovial fibroblast cultures with TNF-α promotes OPG mRNA and protein expression (Kubota *et al.* 2004).

2.7.3 Receptor activator of NF-κB Ligand (RANKL)

Subsequent to the discovery of OPG, RANKL was identified as the ligand for OPG (Lacey *et al.* 1998, Yasuda *et al.* 1998a) and was termed OPG Ligand, TRANCE and osteoclast differentiation factor. Structurally, RANKL is smaller than OPG and is expressed in three distinct forms. These include a cell bound peptide of 317 amino acids (Lacey *et al.* 1998, Yasuda *et al.* 1998a), a truncated ectodomain created from the cell bound form by the

enzymatic cleavage by TNF-α converting enzyme protease (Lum *et al.* 1999) and a primary secreted molecule (Kong *et al.* 1999a, Nagai *et al.* 2000). The cell bound form is the most common and is expressed by many cell types (Lacey *et al.* 1998, Yasuda *et al.* 1998a). In contrast, the primary secreted form is limited to activated T cells (Kong *et al.* 1999b) and a squamous cell carcinoma cell line (Nagai *et al.* 2000). Various skeletal and extraskeletal cell types are capable of expressing RANKL including stromal cells, osteoblasts, osteoclasts, mesenchymal periosteal cells, chondrocytes and endothelial cells (Wong *et al.* 1997, Anderson *et al.* 1997, Lacey *et al.* 1998, Yasuda *et al.* 1998a). On osteoblastic cells, RANKL naturally exists as a surface transmembrane protein. However, cleavage of this membrane protein by MMPs results in a secreted form (Lum *et al.* 1999).

In direct contrast to OPG, RANKL is a potent positive regulator for osteoclastogenesis with downstream signalling events mediated by its transmembrane signalling receptor RANK (Yasuda *et al.* 1998a, Boyle *et al.* 2003). A combination of RANKL and colony stimulating factor-1 (CSF-1) are necessary to induce gene expression that typifies the osteoclast lineage, resulting in the development of mature osteoclasts (Lacey *et al.* 1998). Indeed, RANKL in the presence of M-CSF is sufficient to promote osteoclast differentiation (Matsuzuki *et al.* 1998, Quinn *et al.* 1998) and stimulate osteoclast activation (Fuller *et al.* 1998, Burgess *et al.* 1999, Jimi *et al.* 1999) survival (Lacey *et al.* 2000) and adherence to bone surfaces (O'Brien *et al.* 2000).

RANKL signals through the integral type I transmembrane receptor protein RANK, which is preferentially located on osteoclasts and dendritic cells. On signalling through its receptor, RANKL induces a strong activation of the NF-κB transcription factor. NF-κB activity appears to be critical for osteoclast differentiation as mice deficient in NF-κB proteins p50 and p52 have deficient osteoclastogenesis (Iotsova *et al.* 1997). However, signalling through RANK can also occur through Erk, Akt, JNK and MAPK pathways (Phan *et al.* 2004, Feng *et al.* 2005).

2.7.4 The OPG/ RANKL/ RANK regulatory axis

RANKL and OPG expression is carefully regulated to balance bone density and resorption positively and negatively by controlling the activation state of RANK on osteoclasts (Simonet *et al.* 1997, Akatsu *et al.* 1998, Kong *et al.* 1999, Boyle *et al.* 2003). Both the

promoter region of RANKL (Gao *et al.* 1998) and that of OPG (Thirunavakkarasu *et al.* 2000) contain binding sites for the osteoblastic transcription factor cbfa-1 (Ducy *et al.* 2000). Cbfa-1 is essential for osteoblast differentiation and function and therefore, the generation of normal bone. Indeed, cbfa-1 deficient mice not only lack mature osteoblasts but also lack osteoclasts (Ducy *et al.* 2000). This suggests that cbfa-1 and RANKL are essential factors for the osteoblast and osteoclast lineages respectively. Furthermore, they may represent a molecular link for the osteoblastic regulation of osteoclast biology and bone resorption.

The regulation of RANKL and OPG gene expression, in addition to OPG secretion, by human and rodent osteoblastic lineage cells has been extensively studied. Many known osteotropic cytokines and hormones regulate either RANKL or OPG or the expression of both and in either comparable or opposite directions. Dysregulation of the delicate balance between RANKL and OPG expression is implicated in the pathogenesis of postmenopausal osteoporosis, rheumatoid arthritis, Paget's disease, periodontal disease, benign and malignant bone tumours, bone metastases, and hypercalcaemia of malignancy (Hofbauer and Heufelder 2001). Furthermore, administration of OPG in animal models has been demonstrated to prevent or mitigate these disorders (Hofbauer and Heufelder 2001). Although the dominant effects of RANKL/RANK/OPG are related to bone biology, this molecular triad is also associated with pathological events underlying cardiovascular diseases and malignancy (Theoleyre *et al.* 2004).

2.7.5 Cytokine contribution towards inflammatory bone destruction

A multitude of factors is involved in the maintenance of bone homeostasis (Rodan 1998). For decades, multiple cytokine systems have been proposed as central regulators of osteoclast function (Suda *et al.* 1997). Growth factors such as those of the TGF-β superfamily that includes bone morphogenic proteins (Pfeilschifter and Mundy 1987), hormones and cytokines have all been implicated in regulating the equilibrium between bone resorption and deposition (Jilka *et al.* 1998, Manolagas 1995). However, cytokines are not obligatory for osteoclastogenesis but are simply involved in modulating this process (Phan *et al.* 2004).

Bone erosion is inhibited by IL-4 interacting directly with osteoclasts and indirectly by inhibiting the production of proinflammatory cytokines (Mirosavljevic *et al.* 2003). Indeed, RANKL-induced bone resorption is dose dependently inhibited by the direct effects of IL-4 upon both osteoclast precursors and mature osteoclasts (Wei *et al.* 2002, Mangashetti *et al.* 2005). Osteoclastogenesis is also indirectly suppressed by IL-4 down-regulating the transcription factors NFATc1 and c-Fos (Kamel Mohamed *et al.* 2005). Furthermore, IL-4 suppresses the expression of RANKL mRNA and protein and increases the secretion of OPG by fibroblast-like synoviocytes (Lee *et al.* 2004b). Lee *et al.* (2004b) suggest that IL-4 inhibits osteoclast formation by suppressing RANKL expression rather than altering RANK expression whilst IL-10 has no appreciable effect upon RANKL. Conversely, others have demonstrated IL-10 to have an inhibitory effect upon osteoclastogenesis (Hong *et al.* 2000). Furthermore, IL-10 knockout mice display greater bone loss in pathogen-free conditions (Al-Rasheed *et al.* 2004) and to bacterial and antigenic challenge (Al-Rasheed *et al.* 2003, Finnegan *et al.* 2003, Gjertsson *et al.* 2003, Sasaki *et al.* 2004a). Through their inhibition of cellular proliferation, IL-4 and IL-13 are generally anti-inflammatory in nature. Nevertheless, they are also capable of up-regulating IL-6 expression from osteoblasts. IL-6 is a potent stimulator of osteoclast recruitment. Therefore, increased concentrations of IL-13 and IL-4 may inadvertently lead to bone resorption through inducing increased IL-6 expression (Frost *et al.* 2001).

The proinflammatory cytokines IL-1 and TNF-α are implicated in both the formation and functional activity of osteoclasts. TNF-α-induced osteoclastogenesis occurs as the result of direct stimulation of macrophages that are exposed to a stromal environment expressing permissive levels of RANKL (Lam *et al.* 2000, Kudo *et al.* 2002). IL-1 and TNF-α are also capable of regulating OPG and RANKL gene expression in osteoblastic cells (Hofbauer *et al.* 1999). Indeed, TNF-α enhances the expression of RANKL by osteoblasts and stromal cells (Hofbauer *et al.* 1999). Furthermore, TNF-α induces the expression of RANK on osteoclasts, leading to a higher activation status of these cells by locally expressed RANKL (Kobayashi *et al.* 2000). IL-1 mediates the osteoclastogenic effects of TNF-α by enhancing stromal cell expression of RANKL and directly stimulates the differentiation of osteoclast precursors (Wei *et al.* 2005). Furthermore, cytokines such as TNF-α are also capable of inducing osteoclast differentiation independently of the RANKL-RANK-TRAF6 axis (Kim *et al.* 2005). Through these effects, cytokines can thereby shift the balance of bone

remodelling towards bone resorption and are therefore implicated in the pathogenesis of several inflammatory bone diseases including rheumatoid arthritis (Chu *et al.* 1991) and osteomyelitis (Meghji *et al.* 1998). IL-6 promotes osteoclast formation through cellular interactions between osteoblastic cells and progenitor cells. This property is related to further stimulation of IL-1β release (Kurihara *et al.* 1990, Rozen *et al.* 2000, Ishmi *et al.* 1990). Although IL-6 has little direct effect in regulating the RANKL/RANK/OPG system, interactions between IL-6 and the PGE_2 signalling system result in increased osteoclastogenesis (Liu *et al.* 2005). IL-8 is also a potent stimulator of bone destruction and has a direct stimulatory effect upon osteoclastogenesis and bone destruction (Bendre *et al.* 2003).

The presence of M-CSF is essential for human osteoclast formation from circulating mononuclear precursors. This induces both the proliferation and differentiation stages of human monocyte-osteoclast formation (Fujikawa *et al.* 2001). IL-17 is also capable of stimulating osteoclast differentiation and thereby inducing bone resorption. Indeed, IL-17 enhances the effect of TNF-α-induced osteoclast resorption *in vitro* (van Bezooijen *et al.* 1999). The effects of IL-17-mediated bone destruction occur in a prostaglandin dependant manner. Furthermore, they appear to be mediated through the induction of RANKL on the surface of osteoblasts as OPG inhibits IL-17A-induced osteoclast differentiation (Kotake *et al.* 2001). The local release of IL-17A in chronic inflammatory bone disorders may result in up-regulated expression of RANKL from local stromal cells, thereby contributing towards osteoclastogenesis (Page and Miossec 2005). Moreover, IL-17-mediated increase in RANKL expression is potently enhanced by the concomitant addition of IL-1β or TNF-α.

IL-12 inhibits osteoclast formation in co-cultures of mouse adult spleen cells and osteoblastic cells treated with M-CSF and RANKL (Horwood *et al.* 2001) and additionally, in bone marrow cell cultures (Nagata *et al.* 2003). IL-12 potently induces the production of IFN-γ from T and NK cells (Yoshimoto *et al.* 1998). Interestingly, IFN-γ is capable of inhibiting osteoclast formation in cell co-culture systems by interfering with the RANKL-RANK signalling pathway (Takayanagi *et al.* 2000). Furthermore, IFN-γ prevents osteoclast formation by directing the differentiation of osteoclast progenitors towards an alternative cytocidal lineage to that of the osteoclast (Fox and Chambers 2000). After T

cell activation, IFN-γ may therefore have a key responsibility in protecting against the destruction of calcified tissue. Nevertheless, *in vivo* studies using the pulp exposure model in murine knockout mice have failed to support an individual role for IL-12, IL-18 or IFN-γ in either protecting against or contributing towards infection stimulated bone destruction (Sasaki *et al.* 2004b).

Although many of the aforementioned effects of cytokines upon bone remodelling are related to the modulation of RANKL expression, osteoclasts can additionally be formed and activated independently of RANKL and RANK. Indeed, this may occur through the proinflammatory cytokines TNF-α (Azuma *et al.* 2000, Kobayashi *et al.* 2000) and IL-1 (Jimi *et al.* 1999, Fox *et al.* 2000). TNF-α appears to activate the generation of mature osteoclasts, whereas IL-1 stimulates the resorbing activity of osteoclasts (Jimi *et al.* 1999, Kobayashi *et al.* 1999, Azuma *et al.* 2000, Fox *et al.* 2000). This provides evidence that these inflammatory cytokines are partially capable of circumventing the RANKL-RANK pathway, especially in inflammatory diseases. However, RANK-deficient mice are resistant against the hypercalcaemic and bone resorptive effects of IL-1 and TNF-α (Li *et al.* 2000). Furthermore, OPG can prevent bone loss in a model of adjuvant arthritis that is characterised by local overproduction of IL-1 and TNF-α (Kong *et al.* 1999). These observations suggest that the direct contribution of IL-1 and TNF-α to bone resorption is minimal. Therefore, it is likely that the majority of bone destructive properties related to TNF-α and IL-1 are mediated through their potent induction of RANKL (Brändström *et al.* 1998, Vidal *et al.* 1998, Hofbauer *et al.* 1999).

2.7.6 RANKL/ OPG within PRD

OPG mRNA expression was initially identified within human PDL, human pulp cells and human gingival fibroblasts (Sakata *et al.* 1999). Furthermore, expression of OPG mRNA is increased in PDL cultures stimulated with IL-1β and TNF-α but not by TGF-β or IL-6. Despite the production of RANKL by PDL cells, endogenously expressed OPG is capable of preventing osteoclastogenesis within bone marrow cell co-cultures. This may provide a mechanism by which root resorption is prevented (Hasegawa *et al.* 2002a). Conversely, the pattern of OPG and RANKL mRNA expression within human PDL cell cultures derived from deciduous teeth is capable of inducing osteoclastogenesis. This may be a significant factor contributing towards root resorption and exfoliation of the deciduous dentition

(Hasegawa *et al.* 2002b). Using IHC methodology, OPG protein has been detected within inflamed dental pulp tissue and its cellular expression is primarily localised to fibroblasts, endothelial cells and chronic inflammatory cells (Kuntz *et al.* 2001).

RANKL production is increased by $CD4^+$ T lymphocytes stimulated with the periodontal/ endodontic pathogen *Actinobacillus actinomytemcomitans*. Within the murine model of periodontitis, infection with this microorganism enhances alveolar osteoclastic bone resorption. Of importance, these effects are abolished by the administration of OPG (Teng *et al.* 2000). *A. actinomytemcomitans* has also been shown to increase RANKL mRNA expression in both PDL and gingival fibroblast cultures. However, this pathogen has no effect upon OPG mRNA expression within these cell types (Belibasakis *et al.* 2005b). Recently, RANKL mRNA expression has been demonstrated to be present within the human PRD lesion and absent in healthy periodontal ligament controls (Sabeti *et al.* 2004). From experiments using IHC within the rat PRD model, RANKL protein expression within the periradicular tissues increases after pulp exposure and reaches maximal expression at day 14 (Zhang and Peng 2005). However, the presence of RANKL protein and mediators that are capable of modulating RANKL and OPG expression within the human PRD lesion remain to be determined.

2.8 Fibroblasts

2.8.1 Fibroblast origins

Fibroblasts are relatively long-lived flat, oval nucleated, elongated spindle-shaped cells derived from mesenchyme (Postlethwaite *et al.* 2004). They are distinguished from other cells by their characteristic morphology, their ability to adhere to plastic culture vessels and the absence of markers defining other cell lineages. Originally, fibroblasts were thought to be a homogeneous cell population. However, investigations have determined that cellular proliferation, size, shape, collagen synthesis and responsiveness to inflammatory mediators vary between fibroblasts originating from different hosts and differing host sites (Castor *et al.* 1962, Elias *et al.* 1987, Raghu *et al.* 1988, Chen *et al.* 2005). Thereby, fibroblasts comprise a diverse group of distinct functionally differentiated cell types that can be categorised on their gene expression profiles (Fries *et al.* 1994a, Chang *et al.* 2002c). Furthermore, even within a specific disease process, fibroblasts form

a heterogeneous population and the differing characteristic phenotypes of these subpopulations are stable (Kasperkovitz *et al.* 2005). Indeed, these distinct phenotypes are maintained *in vitro* after prolonged cell culture (Brouty-Boyé *et al.* 2000, Marinova-Mutafchieva *et al.* 2000).

Developmentally, fibroblasts are classically derived from the primary mesenchyme. However, within the head and neck region fibroblasts are ectodermal in origin, being derived from neural crest tissue (Komuro 1990). Subsequent to trauma or inflammation, fibroblasts may also differentiate from local epithelial structures (Iwano *et al.* 2002). Furthermore, in renal fibrosis the tubular epithelium contributes towards more than a third of the fibroblast population by a process termed epithelial-mesenchymal transition (Kalluri and Neilson 2003). Fibroblasts are also generated from circulating fibrocytes that are primarily derived from $CD14^+$ PBMCs (Abe *et al.* 2001) and a population of $CD34^-$ mononuclear cells (Zvaifler *et al.* 2000).

2.8.2 Physiological roles of fibroblasts

The predominant function of fibroblasts is to maintain the structural integrity of connective tissue. They synthesise extracellular matrix (ECM) products that afford mechanical strength and tissue architecture to organs and tissues of the body (Buckley *et al.* 2001). Of importance, the ECM also serves as a scaffold for cell adhesion of extravasated leukocytes (Shimizu and Shaw 1991). Matrix deposition can be increased by fibroblast stimulation with TGF-β, IL-1 and mast cell tryptase. Conversely, fibroblasts stimulated with IL-4 and/or interferon exhibit decreased collagen synthesis (Konttinen *et al.* 2000). Fibroblasts are also a central cell line regulating wound healing, they have an essential supporting role in haematopoiesis and help define the bone marrow stromal niche (Dexter 1982). Furthermore, thymic function and the generation of secondary lymphoid tissue are in part controlled by chemokines and cytokines released by resident fibroblasts (Parsonage *et al.* 2005). In addition to these physiological regulatory functions, fibroblasts are capable of differentiating into a number of connective tissue cells including cartilage, bone, adipocytes and smooth muscle cells (Harris 1994). Importantly, inflammatory cytokines are critical factors that influence fibroblast differentiation (Yamasaki *et al.* 2004).

2.8.3 Contribution of fibroblasts to inflammation and immunity

In addition to providing the supporting framework for other cell types, there is substantial evidence that fibroblasts perform a central active role within inflammatory processes (Buckley *et al.* 2004, Chen *et al.* 2005). The secretion of IL-8 and other molecules such as IL-16 (Franz *et al.* 1998), MCP-1 and MIP-1α by fibroblasts aid in the attraction of macrophages and $CD4^+$ T cells to inflammatory sites (Ritchlin 2000). Thereby, fibroblasts are frequently the first cell line that comes into contact with extravasated leukocytes such as T cells and macrophages. Moreover, the selective accumulation, survival, differentiation and retention of leukocytes at sites of inflammation are mediated by the stromal address code that fibroblasts contribute towards defining (Parsonage *et al.* 2005). Through fibroblast and immunocyte expression of transmembrane IL-1R type 1 and cell surface associated IL-1, interactions of cell-surface bound ligand and membrane-receptor likely occurs between fibroblasts and inflammatory cell lines.

Infiltration of haematopoietic cells at damaged tissue sites is regulated by fibroblasts through CD40-CD40 ligand (L) interactions (Zhang *et al.* 1998). Fibroblasts also appear to exercise an important regulatory function in initiating immune responses by inhibiting the production of IL-12 by dendritic cells, thereby precluding the development of Th1 type responses (Berthier *et al.* 2003). Fibroblasts possess Toll-like receptors and upon stimulation with PAMPS express a variety of MMPs and proinflammatory cytokines (Kyburz *et al.* 2003, Zeisel *et al.* 2005). Synovial-like fibroblasts exposed to *Salmonella enterica* are capable of enhancing the maturation of osteoclast precursors into active multinucleated osteoclast-like cells (Zhang *et al.* 2004). Furthermore, fibroblast-like synoviocytes (FLS) not only augment bone resorption but may also directly contribute to bone resorption (Pap *et al.* 2003). Taken together, this clearly demonstrates that, upon encountering microbial products, fibroblasts are capable of eliciting an innate immune response. Following activation by these microbes and their associated PAMPS, fibroblasts act as central players in adjusting the equilibrium between repair and destruction of the surrounding tissue matrix.

Dermal fibroblasts were originally recognised as primary targets of proinflammatory cytokines such as IL-1 and TNF-α (Dayer *et al.* 1977, Butler *et al.* 1994). Upon stimulation with these cytokines, fibroblasts express chemokines such as IL-8 and MCP-1, matrix

metalloproteinases (MMPs) and prostaglandins (Dayer *et al.* 1986, Larsen *et al.* 1989). Differing fibroblast populations secrete distinct patterns of chemokines, cytokines and matrix proteins. The production of these inflammatory mediators epitomises their influential role as effector cells in inflammatory reactions. Moreover, secretion of such inflammatory products suggests that fibroblasts are not mere bystanders within inflammatory events (Buckley *et al.* 2004). Fibroblasts themselves express IL-1 and thus contribute to paracrine and autocrine activation of neighbouring cells (Spörri *et al.* 1996, Rezzonico *et al.* 1998). Up-regulation of fibroblast-derived cytokines, including IL-6 and chemokines such as CXCL12 and CXCL13 (Hjelström 2001), may contribute towards the perpetuation of chronic inflammatory diseases including RA (Buckley *et al.* 2000), Sjögren's syndrome (Amft *et al.* 2001) and liver cirrhosis (Grant *et al.* 2002).

Fibroblasts may be classified according to their expression of Thy-1, a 25 kDa glycoprotein (Phipps *et al.* 1989). Upon challenge with cytokines, the differential expression of Thy-1 leads to specific patterns of response from fibroblasts. Whereas Thy-1$^-$ fibroblast subsets stimulated with TNF-α produce IL-1α, fibroblasts expressing Thy-1 do not secrete IL-1α (Phipps *et al.* 1990). Furthermore, only the Thy-1$^-$ subset responds to IFN-γ stimulation, which results in up-regulated expression of class II MHC antigens (Phipps *et al.* 1989). However, both Thy-1$^-$ and Thy-1$^+$ subsets produce IL-6, which acts as an autocrine growth factor (Fries *et al.* 1994b). More recently, the response of fibroblasts to exogenous IFN-γ has been further scrutinised. These investigations establish that stimulation of peripheral tissue-derived fibroblasts with either Th1 (IFN-γ) or Th-2 (IL-4) type cytokines induces gene expression more akin to lymphoid tissue (Parsonage *et al.* 2003).

Fibroblast activity is influenced by cell contact with T cells. It is known that cytokine activated fibroblast-like synoviocytes (FLS) bind T cells through an ICAM-1 dependent pathway (Bombara *et al.* 1993, Shingu *et al.* 1994). Furthermore, direct contact between fibroblasts and mitogen-activated T cells leads to down-regulation in the synthesis of fibroblast-derived collagen types I and III (Rezzonico *et al.* 1998). Additionally, contact with blood-derived activated T cells or synovial CD4$^+$ and CD8$^+$ T cell clones up-regulates FLS production of MMPs and prostaglandin (Burger *et al.* 1998). This effect appears to be dependent upon membrane bound cytokines, including TNF-α and IL-1α. Cytokines

produced by IL-1β and TNF-α- activated fibroblasts, such as IL-7 and IL-15, may also provide positive feedback loops for T cell activation (McInnes *et al.* 2000).

Through the inappropriate production of stromal derived factor (SDF-1) and IFN-β, fibroblasts may contribute to the perpetuation of chronic inflammation (Buckley *et al.* 2001). In addition to propagating chronic inflammatory disorders, LPS-stimulated fibroblasts may directly influence inflammatory bone destruction by releasing RANKL and OPG (Wada *et al.* 2004). Indeed, murine synovial fibroblasts promote osteoclast formation from macrophages through RANKL expression (Wu *et al.* 2005). Fibroblast-like synoviocytes have been shown to express IL-18R chains, although the majority of cultured FLS express only one chain, thereby rendering them unresponsive to IL-18 (Möller *et al.* 2002, Kawashima and Miossec 2003). Importantly, FLS have been shown to secrete large amounts of IL-6 and IL-8 and limited TNF-α, IL-1, IL-15 and IL-18 (Georganas *et al.* 2000, Hirth *et al.* 2002). These data implicate fibroblasts as immune active cells that are potentially responsible for regulating inflammatory processes, from acute through to chronic stages (Buckley 2003).

2.8.4 PRD fibroblasts

Periodontal ligament (PDL) fibroblasts are defined by their tissue localisation within the periodontal ligament surrounding the dental root. They are responsible for maintaining the rapid and extensive remodelling of the collagen attachment fibres that support the tooth in the alveolar bone socket (Bordin *et al.* 1984). Gingival fibroblasts, which coordinate the construction of the gingival soft tissue, are additionally situated within the region of the dental attachment to alveolar bone. These two fibroblast populations manifest distinct morphological characteristics and they may be further divided into heterogeneous sub-populations (Rose *et al.* 1987, Piche *et al.* 1989, McCulloch and Bordin 1991). Furthermore, as a result of their neural crest origin, PDL and gingival fibroblasts exhibit greater similarity in their gene profiling with osteoblasts than fibroblasts derived from dermal tissues (Lallier *et al.* 2005).

Periradicular disease lesions contain approximately equal numbers of inflammatory cells and fibroblastic cells (Stern *et al.* 1981). There are a substantial number of studies analysing the effects of inflammatory mediators upon PDL (Belibasakis *et al.* 2005b),

gingival (Imatani *et al.* 2001, Chu *et al.* 2004, Tardif *et al.* 2004, Belibasakis *et al.* 2005a, b) and pulp fibroblasts (Hosoya and Matsushima 1997, Chu *et al.* 2004, Coil *et al.* 2004, Huang *et al.* 2004, Chan *et al.* 2005, Tancharoen *et al.* 2005). In contrast, few studies have investigated the phenotype of fibroblasts derived from chronically inflamed PRD tissue lesions. Maeda *et al.* (2004) established that fibroblasts obtained from 3 human PRD lesions have the potential to differentiate into osteoblastic or cementoblastic cells. From this study, they suggest that PRD-derived fibroblast cells have the ability to contribute to osseous healing after root canal treatment. Conversely, through the production of cytokines and/or metalloproteinases that indirectly or directly promote matrix degradation, fibroblasts may actively contribute to the pathogenesis of PRD.

Only one study has analysed the cytokine profile of cultured fibroblasts from PRD lesions. Furthermore, these fibroblasts were isolated from only periradicular cysts. These cyst-derived fibroblasts secrete significantly greater quantities of IL-6 compared with fibroblasts derived from healthy pulp or gingival tissues (Kusumi *et al.* 2004). Interestingly, secreted levels of IL-1β, IL-8, TNF-α and IFN-γ from the PRD cyst-derived fibroblast cultures are not significantly different from pulp and gingival fibroblasts. Thereby, IL-6 may be a significant contributing factor towards PRD cystic expansion. Given the important role that fibroblasts perform in the regulating matrix structure both in health and in inflammation, the paucity of research defining the PRD fibroblast is somewhat surprising.

2.9 Aims of Study

Over two thirds of the Scottish adult population is affected by periradicular disease. In an attempt to treat this common ailment, root canal procedures and dental extractions cost the National Health Service within Scotland greater than £1.5m per annum. Furthermore, this figure does not incorporate the additional costs of providing the definitive coronal restoration required to seal the root canal access cavity, the subsequent provision of cuspal coverage coronal restorations, re-root canal treatments, re-surgery and/or the replacement of lost teeth with dental prosthesis (and their long-term replacement). Moreover, the indirect costs incurred by businesses resulting from lost working time as a consequence of patients undergoing such time consuming treatment procedures is substantial.

In contrast to many other chronic inflammatory diseases, PRD is a readily accessible, intact inflammatory tissue lesion in which immunoregulatory events may be explored. Given the high prevalence within and the substantial cost and morbidly to populations worldwide, it is remarkable that significant deficiencies exist in the understanding of processes integral to the immunopathogenesis of the human PRD lesion. This is particularly evident in the lack of knowledge as to the expression of novel inflammatory cytokines and their involvement in the initiation, development and perpetuation of the disease process. It is also surprising that functional investigations, which analyse the dynamics of immune and inflammatory mediators, have not been undertaken upon the human PRD lesion. This is astonishing when one considers the quantity of diseased tissue available from routine dental extractions and periradicular surgery that could be analysed. It is now established that fibroblasts have a central active role within chronic inflammatory disease processes. Furthermore, it has been recognised since early studies that fibroblasts constitute a significant proportion of the cellular population within the PRD lesion. However, PRD-derived fibroblasts have not been isolated, analysed and phenotyped and their potential involvement in PRD progression remains to be elucidated.

As scientific understanding of pathologic processes that lead to chronic PRD is inadequate, I hypothesised that PRD explant tissue cultures would permit detailed investigation of inflammatory events within human PRD. The specific aims of this thesis were:

1. To develop a novel human PRD explant culture model in which to investigate the functional role of likely inflammatory mediators involved in the initiation and perpetuation of the human PRD lesion.

2. Upon establishing a PRD explant tissue system, to investigate the expression of IL-18 within the human lesion and to elucidate its likely biological contribution towards inflammatory responses occurring within PRD.

3. To investigate the presence of and functional interactions between inflammatory mediators within the human PRD lesion that influence bone homeostasis.

4. To undertake investigations that would begin to phenotype the PRD fibroblast.

CHAPTER 3

GENERAL

MATERIALS AND METHODS

3 GENERAL MATERIALS AND METHODS

3.1 General Materials

3.1.1 General reagents

DMEM was supplemented with 100 U/ml penicillin, 100 μg streptomycin, 2 mM L-glutamine, 1.25 mg/ml amphoterecin and 10% foetal calf serum (FCS) obtained from Sigma (Poole, UK). A murine monoclonal antibody (mAb) raised against recombinant human IL-18 (clone 1.51 E3E1), gifted by A Jackson (Imperial Cancer Research Fund, Leeds, UK), was used for immunohistochemistry experiments. Vectastain® *ELITE* kit, 3,3'-diaminobenzidine (DAB) tetrahydrochloride, Vector® SG, V ector® NovaRED and secondary biotinylated antibodies were from Vector Laboratories Ltd (Peterborough, UK). IL-18Rα and β and IL-18BP RT-PCR primers (Table 1), TRIzol® Reagent and SuperScript™ II reverse transcriptase and Taq polymerase were from Invitrogen (Paisley, Scotland). PVDF membrane, ECL Plus™ and horseradish peroxidase (HRP) conjugated anti-mouse Ig secondary Ab for Western blotting were obtained from Amersham Biosciences (Buckinghamshire, UK).

3.1.2 Sample collection

80 dental periradicular tissue biopsies (60 periradicular granulomas and 20 radicular cysts) were immunohistologically examined. Specimens were obtained from patients (36 male and 44 female) undergoing periradicular surgery under local anaesthesia within the Restorative or Oral Surgery Departments, University of Glasgow or University of Dundee Dental Schools. Patients were in good health and not taking long-term medication for chronic conditions. The mean age of patients was 43 years (range 17-82 years). Local dental ethical committee approval was obtained for the project protocol. The diameter of PRD lesions was recorded from radiographs. The tissues were immediately fixed in 10% neutral buffered formalin at room temperature for processing for immunohistochemistry. A routine pathological diagnosis was concomitantly obtained. Control tissue for IHC experiments consisted of healthy periodontal ligament (PDL) tissue obtained from non-carious, unrestored third molar teeth extracted under local anaesthetic from systemically healthy patients. PDL tissues were carefully dissected off the mid-third of the root with a

sterile Swan-Morton® scalpel (Swann Morton, Sheffield, UK). Care was taken to avoid contamination from attached periodontal tissue or apical tissue. PDL tissue was immediately fixed in 10% neutral buffered formalin at room temperature for processing for immunohistochemistry. Further control experiments were also carried out on healthy pulp of sectioned teeth extracted for orthodontic purposes.

For RT-PCR experiments, PRD tissues were obtained from 90 patients, 41 male and 49 female, undergoing periradicular surgery under local anaesthesia within the Restorative and Oral Surgery Departments, University of Glasgow and University of Dundee Dental Schools. The mean age of patients was 45 years (range 18-84 years). Tissue was collected in RNA*Later*® (Ambion) or TRIZOL® Reagent (Invitrogen Life Technologies, Scotland) and stored at minus 20°C until further analysis. Control tissue for RT-PCR consisted of healthy pulpal tissue obtained from non-carious, unrestored third molar teeth extracted under local anaesthetic from systemically healthy patients. Crowns and the coronal third of the roots were cleaned with PBS and any adherent soft tissue removed. The teeth were sectioned horizontally below the amelocemental junction with a sterile high-speed diamond bur and sterile water coolant. The pulpal tissue was removed aseptically with a sterile barbed endodontic broach and immediately stored in RNA*Later* at -20^0C until analysis.

PRD tissues obtained from 25 patients (14 male/ 11 female) undergoing routine dental extractions within the Oral Surgery Department, University of Glasgow Dental School were obtained for Western blot experiments. The mean age of patients was 35 years (range 19-71 years). Periradicular tissues were carefully dissected off the dental root apex with a sterile Swan-Morton® scalpel (Swann Morton, Sheffield, UK), avoiding contamination with adjacent periodontal ligament cells or attached periodontal tissue. PRD tissues were snap-frozen in liquid nitrogen and stored in liquid nitrogen until further analysis. Control tissue for Western blotting comprised 10 healthy pulp tissues obtained from non-carious, unrestored third molar teeth extracted under local anaesthetic from systemically healthy patients. Crowns and coronal third of the roots were cleaned and adherent soft tissue removed. Teeth were washed with PBS and horizontally sectioned below the ACJ with a sterile high-speed bur and sterile water coolant. The pulpal tissue was removed aseptically with sterile barbed broaches, snap-frozen in liquid nitrogen and stored in liquid nitrogen until analysis. A summary of the patient study population is given in Table 3.1.

Table 3.1 **Characterisation of study population.** Specific numbers of patients with periradicular disease and healthy donors (dental pulp controls) for individual laboratory procedures. Age (years) is shown as the mean and range.

	IHC	*RT-PCR*	*Tissue Culture*	*Western Blot*	*TaqMan PCR*	*Healthy Pulp Control*
Number of specimens	80	90	310	25	23	20
Age (years)	17-82	18-84	9-76	19-71	18-55	16-43
mean	43	45	36	35	31	24
Male/Female	36/44	41/49	181/129	14/11	13/10	8/12

3.2 General Methods

3.2.1 PCR

3.2.1.1 RNA extraction

Samples stored at –20°C were thawed at RT, homogenised using a Polytron PT 2100 electric homogeniser (Kinematica, Switzerland) and 0.2 ml of chloroform (Prolabo, France) added per 1 ml of TRIZOL® Reagent to each sample. Samples were shaken vigorously for 15 seconds and left at RT for 3 minutes. Samples were freeze/ thawed three times and subsequently centrifuged at 1200 g at 4°C for 15 minutes. The upper aqueous layer was transferred to a sterile polypropylene tube and an equal volume of isopropyl alcohol (BDH Laboratory supplies, England) added to precipitate the RNA. Following 10 min incubation at RT, the sample was centrifuged at 1200 g to pellet the RNA. Subsequent to removal of the supernatant and washing in 75% ethanol (Hayman, Essex, UK), the pellet was air dried for 5 minutes and then resuspended in 22 μl of sterile water and incubated at 55°C for 10 minutes. RNA concentration was calculated by measuring the OD using a Bioquest spectrophotometer (Cecil Instruments, Cambridge, England). The purity of the sample was assessed from the ratio of the OD at 260 nm to the OD at 280 nm.

3.2.1.2 PCR amplification

5 μg of the total RNA obtained from samples was used for reverse transcription. Prior to reverse transcription, samples were treated with RNA free DNAase® (Ambion, Huntingdon, UK) for 15 minutes at 37°C then denatured at 70°C for 10 min. The RNA was reverse-transcribed into cDNA using the SuperScript™ II reverse transcriptase system according to the manufacturer's recommendations. RT reaction products (1 μl) were mixed with either IL-18Rα, IL-18Rβ, IL-18 or IL-18BP (a, c or b and d) sense or anti-sense primer pair (1 μl) each in a 10 μl total reaction mix (025 mM $MgCl_2$, 2 μM dNTPs, Taq polymerase and 1 μl 10x buffer). 10 μl of the reaction products were run on 2% w/v agarose gels with a 100 bp DNA Ladder (Promega, Southampton, UK). The PCR reaction was performed on the Genius PCR machine (Techne). Mock RT reaction products, obtained in the absence of reverse transcription or H_2O served as templates for negative control studies. The housekeeping gene, β-actin served as an mRNA integrity control,

whereas cDNA derived from LPS/ IFN-γ stimulated THP-1 cells and/or collagenase digested rheumatoid arthritis synovial tissue served as positive controls. Details of primer sequences are provided in Table 3.2.

3.2.1.3 Agarose gel electrophoresis

A 2% agarose gel (Bioline) containing 0.001% ethidium bromide (Sigma Chemicals, Dorset, UK) was set in a gel tank (Life Technologies, UK) containing a 20-toothed comb. Once set, the gel was overlaid with 1x TBE Buffer. Along with samples, a 1 kB DNA Ladder (Advanced Biotechnologies, Surrey, UK) was loaded onto the gel to allow sizing and appropriate quantification of the products within the sample. 10 μl of sample with 5 μl of orange G solution containing 50% glycerol and 50% TBE were added to individual wells. Electrophoresis of samples was undertaken at 100 volts for 60 minutes to allow migration of the cDNA within the gel from cathode to anode. The gel block was removed at the end of the run and visualised using UVI-photo software programme.

3.2.1.4 Quantitative PCR

24 μl of reaction mixture (12.5 μl Master mix, 1 μl probe, 0.75 μl of forward and reverse primers and 9 μl H_20) was added into each well in a 96 well plate (Thermofast ABgene, Epsom, UK). Taqman® probes (Table 3.3) were obtained from Biosource, UK and Taqman® master mix from Eurogentech, Southampton, UK. Hypoxanthine Ribosyl Transferase (HPRT) or GAPDH served as control genes (12.5 μl master mix, 1.25 μl probe and 10.25 μl H_20). Thereafter 1 μl of cDNA, standards or water as a negative control was added into triplicate wells. Following thermocycling, cDNA levels were quantified using an ABI prism 7700 sequence detector (Applied Biosystems, Foster City, CA). Products were expressed as a percentage of HPRT or GAPDH. Details of probe sequences are in Table 3.3. For analysis of TLR-1,- 2, -4 and -6 expression, 10 μg of each total RNA was subjected to first strand cDNA synthesis with a High-Capacity cDNA Archive kit (Applied Biosystems, UK). Real-time PCR on 1/30 of the cDNA pool was performed in duplicate on an ABI 7500 system (Applied Biosystems). Universal PCR Master Mix, TaqMan® probes, sense and anti-sense primers were obtained as Assays-on-Demand™ kits (Applied Biosystems, UK) and used according to the manufacturer's protocol.

Table 3.2 **Primer sequences for RT- PCR.**

Target	Sequence 5'-3'	*Product Size (bp)*
β-actin		
Sense	5'GTGGGGCGCCCCAGGCACCA3'	548
Antisense	5'CTCCTTAATGTCAC GCACG ATTTC3'	
IL-18BPa		
Sense	5'TATATCTAGAGCCACCATGAGACACAACTGGACACCA3'	779
Antisense	5'ATATCTAGATTAATGATGATGATGATGATGA-CCCTGCTGCTGTGGACTGC3'	
IL-18BPb+d		
Sense	5'TATATCTAGAGCCACCATGAGACACAACTGGACACCA3'	568
Antisense	5'ATATCTAGATTAATGATGATGATGATGATGCA-GGCTGCTCTGGCAGAGC3'	
IL-18BPc		
Sense	5'TATATCTAGAGCCACCATGAGACACAACTGGACACCA3'	805
Antisense	5'ATATCTAGATTAATGATGATGATGATGATGAGGT-TGTGCTGCTGCTGGCC3'	
IL-18		
Sense	5'ATCAGGATCCTTTGGCAAGCTTGAATCTAAATTTATC3'	487
Antisense	5'ATAGGTCGACTTCGTTTTGAACAGTGAACATTATAG3'	
IL-18Rα		
Sense	5'ACT TGT CAT TAG GTT GGC GG	305
Antisense	5'ACT CAG TCA CCC ACT GGT CC	
IL-18Rβ		
Sense	CCG CAT CAC ATA AGC AAG AC	606
Antisense	ACC ACT CCT CTC TTT TCT TTC A	

Table 3.3 **TaqMan primer sequences and probes used for quantitative PCR analysis of IL-18 expression.**

Description	*Sequence 5'-3'*
IL-18 Forward Primer	CGCCTCTATTTGAAGATATGACTGATT
Il-18 Reverse Primer	CCTCTAGGCTGGCTATCTTTATACATACT
IL-18 Probe	TGACTGTAGAGATAATGCACCCCGGACC

3.2.2 Immunohistochemistry

3.2.2.1 Tissue processing

Formalin fixed tissues were embedded in paraffin wax and 5 μm serial sections were collected on silane coated glass or Superfrost Plus slides (BDH Laboratory Supplies, UK). Prior to immunohistochemistry, two sections in each sample were used for morphological examination that was based upon haematoxylin and eosin staining. Dried sections (60°C for 35 minutes) were dewaxed in xylene and rehydrated in graded alcohol solutions to PBS. Endogenous peroxidase was inhibited with 0.3% H_2O_2 (Sigma) in methanol for 30 min. Antigens for IL-18, CD3, CD68, IL-17, CD57, CD25 and Ki-67 were unmasked by heating in 1L 0.01 M pre-hated citrate buffer, pH 6.0, under pressure within a microwave (650 W) for 8 minutes. Antigen retrieval for OPG was undertaken by heating 1L preheated EDTA, pH 8.0, under pressure within a microwave for 8 minutes. Antigen retrieval for mast cell tryptase was performed using 0.1% trypsin solution (Sigma) in Tris buffer (pH 7.6) containing 0.1% calcium chloride. Slides were incubated within this solution for 15 minutes at 37°C. After washing with PBS, a wax ring was drawn around each specimen and slides were incubated with blocking solution at RT for 1 h using 20% serum of the species in which the secondary antibody was raised in PBS. The blocking solution was tapped off and sections were then incubated overnight at 4°C with primary Ab in a humidified chamber. Subsequent to further washing in PBS at RT, slides were incubated with secondary Ab (Vector, Peterborough, UK). Primary antibodies used for IHC are detailed in Table 3.4.

3.2.2.2 Visualisation of products

Following a 30-minute incubation with secondary antibodies at RT, a standard avidin biotin protocol was followed (Vectastain® *ELITE*, Vector Laboratories). The product was viewed with DAB or Vector® NovaRED (Vector Laboratories) and sections counterstained with Harris's haematoxylin (BDH Ltd, Merck Ltd, Lutterworth, Leicester, UK). For double staining, sections were first stained with anti-IL-18 Ab and developed with DAB. After quenching of residual HRP activity, sections were incubated with anti-CD68 (Dako, Cambridgeshire, UK) or anti-CD3 (Novocastra Laboratories, Newcastle upon Tyne, UK)

and stained with Vector® SG or Vector® NovaRED (Vector Laboratories, England). Finally, sections were dehydrated in ethanol, cleared in xylene (BDH Laboratories, England) then mounted in DPX mountant (BDH Ltd- Merck Ltd). Control tissues comprised human tonsil sections, periodontal ligament cells and sections of asymptomatic teeth. Specificity of staining for IL-18 and IL-17 was confirmed by the addition of the corresponding recombinant proteins to establish blocking of antibody activity prior to immunostaining. Negative controls included the omission of one or both primary antibodies or the addition of isotype-matched control antibodies of irrelevant specificity.

Table 3.4 **Primary antibodies used for IHC experiments.**

Antibody	Manufacturer	Source	Concentration/ Dilution
Anti-CD3	Novacastra NCL-CD3-PS1	Mouse monoclonal	1:100
Anti-CD20	DakoCytomation Clone L26	Mouse monoclonal	1:200
Anti-CD25	Novocastra	Mouse monoclonal	1:100
Anti-CD57	Serotec MCA1305	Mouse monoclonal	10μg/ml
Anti-CD68	DakoCytomation Clone PG-M1	Mouse monoclonal	1:100
Anti-IL-17	R&D BAF 317	Goat Polyclonal	0.5μg/ml
Anti-IL-18	Clone E3E1, Andrew Jackson	Mouse monoclonal	0.4μg/ml
Anti-Mast Cell Tryptase	Novocastra NCL-MCTRYP-428	Mouse	1:200
Anti-Human neutrophil defensin	Novocastra NCL-DEFENSIN	Mouse	1:500
Anti-Ki-67	DakoCytomation Clone M1B-1	Mouse	1:100
Anti-OPG	R&D AF805	Mouse	15μg/ml

3.2.3 Western Blotting

3.2.3.1 Protein preparation

Western blot analysis was performed, following a standard protocol. Briefly, snap frozen specimens were ground under liquid nitrogen. Pulverised tissues were denatured in RIPA Buffer containing 50 mM Tris-HCL pH 8.0, 150 mM NaCl, 1mM EDTA, 1% NP40, 0.05% SDS, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml Aprotinin, 1 μg/ml Pepstatin and 1 μg/ml Leupeptin. Samples were stored on ice for 15 minutes then subjected to three rapid freeze-thaw cycles. Soluble protein was harvested after centrifugation at 12,000 g for 15 minutes, the supernatant collected and stored at –20°C until analysis.

3.2.3.2 Gel electrophoresis

10 μl of protein sample was heated with 2 μl of sample buffer at 95°C for five minutes and then spun down for 2 minutes. Subsequently, protein lysates were loaded into a 12% SDS-PAGE electrophoresis gel. Protein samples were fractitionated on 12% SDS-polyacrylamide gels at 200V for 45 minutes in a BioRad mini-PROTEAN III electrophoresis chamber.

3.2.3.3 Transfer of protein

Following electrophoresis, Hybond-P PVDF membrane (Amersham Life Science) was prewetted with methanol. The Western blot sandwich was created with the gel, PVDF membrane and Whatman 3MM filter paper and wetted with transfer buffer. Proteins were transferred to PVDF membranes by means of a semi dry transfer apparatus (BioRad Laboratories, Hemel Hempstead, UK) at 15V for 25 minutes. Following protein transfer, non-specific protein binding was blocked for 1 h at RT with 5% fat free milk in TBS/ 0.05% Tween20, pH 7.6 and membranes probed overnight at 4°C with relevant primary antibodies (summary of primary antibodies in Table 3.5).

3.2.3.4 Visualisation of proteins

Following three 5 minute washes with TBST, bound antibody was detected by incubation with the relevant secondary antibody for 60 min at RT on a shaker. Membranes were washed and developed by enhanced chemiluminescence, using ECL Plus™ (Amersham Biosciences, UK) for 5 minutes then exposed to X-ray films (KODAK scientific imaging film, X-OMAT AR) in a dark room for 2 minutes and 10 minutes. Molecular weights of products were compared with Precision Plus molecular weight markers (Bio-Rad, UK) and recombinant human cytokine controls.

Table 3.5 **Primary antibodies used in Western blotting experiments.**

Antibody	Manufacturer	Source	Concentration
Anti-IL-17	R&D BAF 317	Goat Polyclonal	0.5 μg/ml
Anti-IL-18	R&D MAB 318	Mouse monoclonal	1 μg/ml
Anti-IL-18Rα	R&D MAB 840	Mouse monoclonal	0.5 μg/ml
Anti-IL-18BPa	R&D AF 119	Goat monoclonal	0.2 μg/ml
Anti-RANKL	R&D MAB626	Mouse monoclonal	1 μg/ml
Anti-OPG	R&D AF805	Goat	0.1 μg/ml
Anti-NFκB p65	Delta Biolabs DB033	Rabbit	0.2 μg/ml

Table 3.6 **Secondary antibodies and controls used for IHC and Western blotting.**

Secondary Antibody	Manufacturer	Source	Working Dilution
Biotinylated anti-Goat	Vector Laboratories BA-5000	Rabbit	1:200
Biotinylated anti-Mouse	Vector Laboratories BA-2000	Horse	1:200
Biotinylated anti-Rabbit	Vector Laboratories BA-1000	Goat	1:200
Biotinylated anti-Rat	Vector Laboratories BA-4000	Horse	1:200
IgG1	DakoCytomation X0931	Mouse monoclonal	1:6
Anti-Mouse HRP	Amersham Biosciences	Sheep	1:5000
Anti-Goat HRP	Amersham Biosciences	Donkey	1:10000
Anti-Rabbit HRP	Amersham Biosciences	Donkey	1:5000

3.2.4 PRD tissue explant culture

3.2.4.1 Patient recruitment for explant culture

Specimens were obtained from consecutive patients undergoing periradicular surgery with local anaesthesia by the author within the Department of Restorative Dentistry or routine dental extractions within the Oral Surgery Department, University of Glasgow Dental School and Hospital. Patients were in good health and not taking long-term medication for chronic conditions. A total of 310 specimens were collected for tissue explant culture. Tissue biopsies were collected from 181 male patients (age range 9 to 76, mean 35) and 129 female patients (age range 11 to 70, mean 39). Specimens were collected in DMEM on ice and used in explant culture experiments within three hours. Periradicular tissues obtained upon dental extraction were carefully dissected off the dental root apex with a sterile Swan-Morton® scalpel (Swann Morton, Sheffield, UK). Care was taken to avoid contamination from adjacent periodontal ligament cells or attached periodontal tissue (Figures 3.1 and 3.2). PRD tissues were stored in DMEM on ice until explant culture experiments.

Figure 3.1 **Figure of periradicular lesion attached to root apex of extracted tooth.**

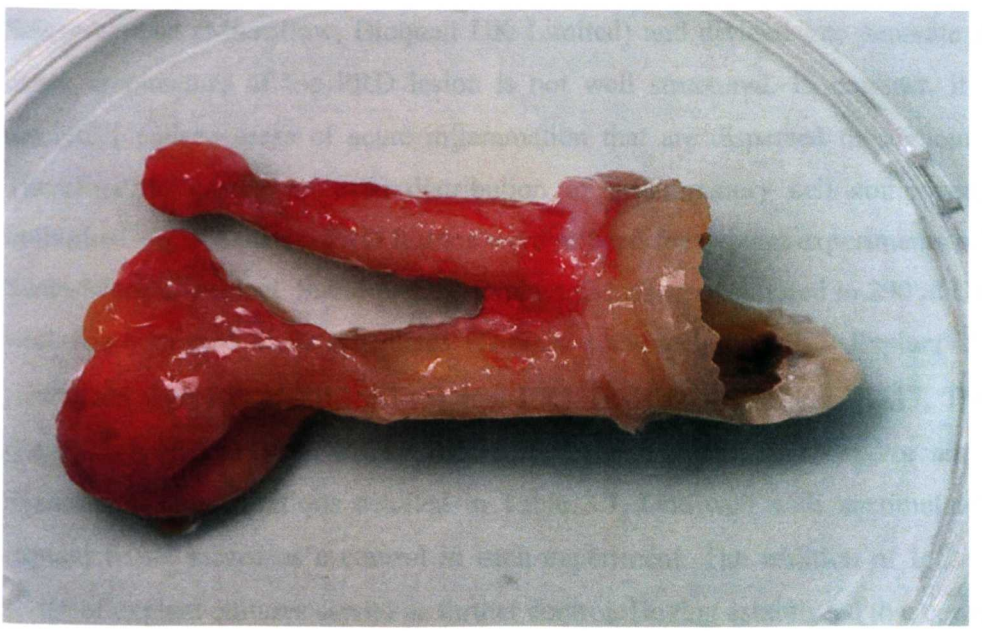
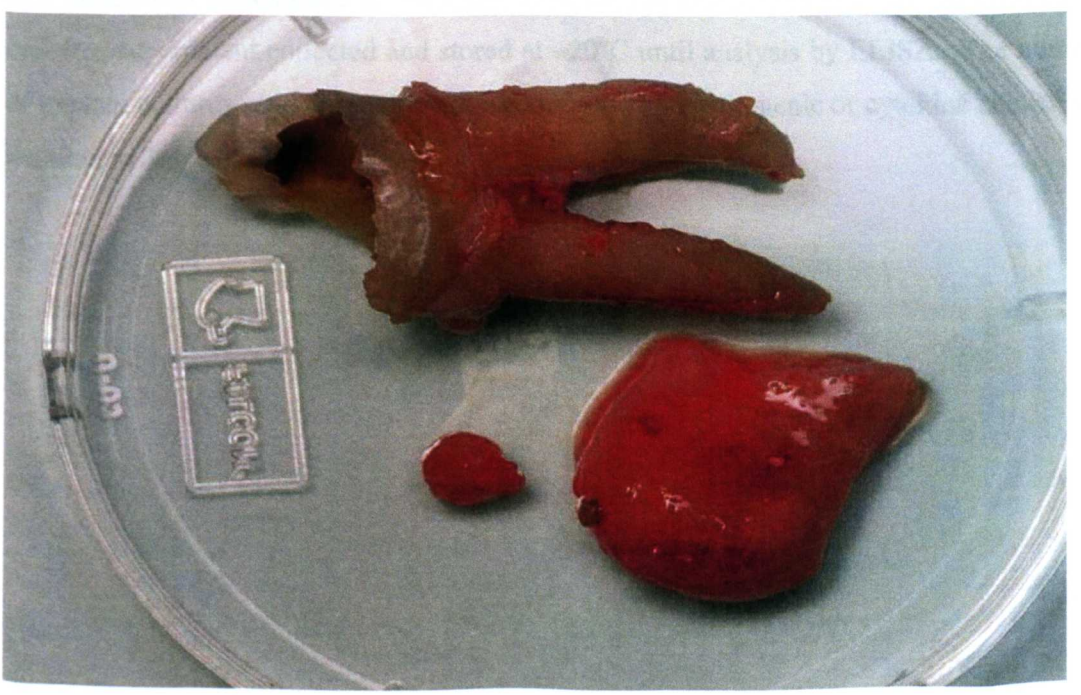


Figure 3.2 **Figure of dissected PRD lesion detached from root apex.**



3.2.4.2 Preparation of explant specimens

Freshly collected PRD tissue was weighed, minced with a sterile scalpel in a Class II Safety cabinet (Microflow, Bioquell UK Limited) and divided into separate pieces. The tissue architecture of the PRD lesion is not well structured. In contrast, it consists of discrete localised areas of acute inflammation that are dispersed throughout the lesion. Therefore, to ensure an even distribution of inflammatory cell components between individual explant plates, PRD lesions were minced for explant experiments with a sterile Swan-Morton® scalpel. Subsequently, explant tissues were cultured in 290 μl DMEM in 24 well flat-bottomed Cellstar® plates (Greiner Labortechnik, UK). Explant tissues were stimulated with LPS, SEB, IgG (Sigma, UK), IL-10, IL-12, IL-15, IL-17, TNF-α, IFN-γ (PeproTech EC), TGFβ_1, MCSF, IL-18, IL-22 and/or anti-IL-18 and/or antiST2 (R&D Systems) at concentrations detailed in Table 3.7. One well with unstimulated, matched explant tissue served as a control in each experiment. The addition of IgG to an initial series of explant cultures served as further control. Having established that control IgG had no impact upon explant tissue cytokine expression, the addition of IgG was not used in subsequent experiments due to the small volume of tissue available for explant culture. In addition to unstimulated and IgG controls, immunostaining was also performed on 5 tissues to assess the cellular composition after culture. After 18 to 72 h incubation at 37°C in a humidified atmosphere of 5% CO_2/ 95% air, the explant culture was centrifuged, the cell-free supernatant collected and stored at –20°C until analysis by ELISA. The numbers of explant cultures used for each cytokine analysed after mitogenic or cytokine challenge is detailed in Table 3.8 and Table 3.9.

Table 3.7 **Mitogens, recombinant human proteins and antibodies used within PRD explant, PBMC and fibroblast cultures.**

MOIETY	SOURCE	CONCENTRATION	ORIGIN	kDa
LPS	Sigma L4391	1 μg/ml	E-Coli	-
SEB	Sigma SO812	10 ng/ml	-	-
SAC	Sigma	1 mg/ml	*Staphylococcus aureus* Cowan strain	-
PHA	Sigma	5 μg/ml	-	-
IL-10	PeproTech EC 200-10	20 ng/ml	-	18.6
IL-12	R&D 219-IL	10 ng/ml	-	75
IL-15	PeproTech EC 200-15	50 ng/ml	-	12.9
IL-17A	PeproTech EC 200-17	50 ng/ml	-	15.5
Il-18	R&D	100 ng/ml	-	18
IL-22	R&D 782-IL	10 ng/ml	-	16.5
IFN-γ	R&D MAB285	5 ng/ml	-	17
TNF-α	PeproTech EC 200-10	0.1 ng/ml	-	17.5
MCSF	R&D 216-MC	1.5 ng/ml	-	18.5
TGF-β1	R&D 240-B	2 ng/ml	-	25
Anti IL-18	R&D MAB 318	1 μg/ml	mouse	-
Anti IL-12Rβ1	R&D MAB839	1 μg/ml	mouse	-
OPG	PeproTech EC 450-14	100 ng/ml	-	19.9
RANKL	PeproTech EC 310-01	100 ng/ml	-	20.0
IgG	Sigma 5381	1 mg/ml	mouse	-
Anti ST2	R&D MAB523	1 μg/ml	mouse	-

Table 3.8 **Details of 18 h PRD tissue explant cultures challenged with moieties and relevant cytokine measured.**

MOIETY ADDED TO EXPLANT	ANALYTE MEASURED										
	IL-1β	**IL-6**	**IL-8**	**IL-10**	**IL-12**	**IL-17A**	**IL-18**	**TNF-α**	**IFN-γ**	**RANKL**	**OPG**
LPS	12	20	18	18	2	13	18	46	24	12	9
LPS+ ANTI IL-18	7	15	13	7	6	5	6	16	10	5	-
SEB	**4**	**12**	**14**	**14**	**-**	**9**	**15**	**12**	**12**	**8**	**7**
SEB+ ANTI IL-18	**4**	**8**	**9**	**5**	**-**	**2**	**6**	**8**	**8**	**-**	**-**
SAC	4	-	-	-	-	-	-	23	5	-	-
PHA	-	-	-	-	-	-	-	20	-	-	-
Anti-ST2	-	12	-	12	-	12	-	12	12	-	-
LPS+ Anti-ST2	-	9	-	9	-	9	-	9	9	-	-
IgG Control	10	10	10	10	10	10	10	10	10	10	10

Table 3.9 **Numbers of PRD explant cultures challenged with cytokines and corresponding cytokine measured.**

STIMULANT	ANALYTE MEASURED										
	IL-1β	IL-6	IL-8	IL-10	IL-12	IL-17A	IL-18	TNF-α	IFN-γ	RANKL	OPG
IL-10	-	23	25	N/A	-	5	23	20	17	9	7
IL-10+ IL-18	-	10	9	N/A	-	-	N/A	8	6	-	-
IL-12	**15**	**9**	**24**	**16**	**N/A**	**2**	**11**	**22**	**20**	**8**	7
IL-12+ IL-18	**15**	**3**	**20**	**9**	**N/A**	**2**	**N/A**	**11**	**16**	-	-
IL-15	-	19	27	16	4	9	22	20	21	6	4
IL-15+ IL-18	-	8	9	6	-	-	N/A	7	8	-	-
IL-17A	**8**	**39**	**20**	**34**	**3**	N/A	**41**	**38**	**22**	**13**	7
IL-17A+ IL-18	-	**10**	**11**	**5**	-	N/A	N/A	**4**	**10**	**2**	-
IL-18	55	89	77	63	29	22	N/A	126	80	23	10
IL-22	-	**9**	**4**	**5**	-	**7**	**2**	12	6	3	3
TNF-α	-	27	28	20	-	8	23	N/A	15	12	16
IFN-γ	-	**20**	**20**	**16**	-	**5**	**16**	**16**	N/A	**14**	**17**
MCSF	-	5	7	11	-	1	19	12	3	17	20
TGF-β1	-	**5**	**3**	**5**	-	**1**	**11**	**8**	**4**	**11**	**11**
OPG	-	9	10	8	-	10	21	12	7	4	N/A
RANKL	-	**9**	**10**	**8**	-	**10**	**22**	**12**	**7**	N/A	**3**

3.2.4.3 Peripheral blood cell separation

In addition to unstimulated internal control cultures in fibroblast and tissue explant culture experiments, peripheral blood monocytes (PBMC) were collected from 20 healthy volunteers (8 male, 12 female) as a further control. Wash media was prepared from RPMI containing amphotericin, streptomycin, L-glutamine and penicillin (all Sigma). 25 ml of peripheral blood was collected by venepuncture from the anticubital fossa of volunteers in sodium heparinised Vacutainer® tubes (Becton Dickson, UK). Blood, diluted with an equal volume of RPMI, was carefully layered onto 10 ml Histopaque®-1077 (Sigma, Poole, Dorset, UK) and centrifuged at 350 g for 25 minutes to allow separation of blood into its component layers. Following centrifugation, the interface containing peripheral blood mononuclear cells (PBMC) was carefully removed using a sterile Pasteur pipette and placed in a separate universal container. This PBMC suspension was further washed three times with fresh RPMI to obtain a pure pellet of PBMC and cells were suspended in RPMI/ 10% (v/v) heat-inactivated FCS.

Cell numbers were calculated from a 1 in 10 dilution of the cell suspension in cell counting fluid by placing 10 μl of this fluid on a haemocytometer (Weber, England) under a coverslip. Using a light microscope, the number of live cells was counted over 16 squares and averaged over four quadrants. The number was multiplied by the dilution factor of 10 and then multiplied by the volume of the suspension to give the total number of cells. The suspension was then adjusted to 1x 10^6 viable cells/ml and cells were cultured in flat-bottomed 24 well Cellstar® plates (Greiner Bio-One GmbH, Germany) with RPMI and 10% FCS. Cells were stimulated with cytokines and mitogens (for concentrations see Table 3.7) for 18 h at 37°C in a humidified incubator with 5%CO_2/95%O_2. After 24 h, cell-free supernatants were harvested and stored at –70°C until analysis by ELISA.

3.2.4.4 Whole blood cultures

As a further control to culture experiments, whole blood was collected from 20 healthy human volunteers (8 male, 12 female) by venepuncture from the anticubital fossa in heparinised Vacutainer® tubes. Whole blood was diluted by one third with RPMI, containing amphotericin, streptomycin, L-glutamine and penicillin (all Sigma) and without FCS.

Whole blood cell samples were cultured in flat-bottomed 24 well Cellstar® plates (Greiner Bio-One GmbH, Germany) in RPMI. Cultures were stimulated with equivalent cytokine concentrations as PMBC culture controls (for concentrations see Table 3.7) for 18 h at 37°C in a humidified incubator in 5%CO_2/95%O_2. After 18 h, whole blood supernatants were spun down at 12, 000g for 10 minutes, the cell-free supernatant harvested and stored at –70°C until analysis by ELISA.

3.2.5 Fibroblast cultures

3.2.5.1 Cell culture

PRD tissue was carefully dissected from the root apex with a sterile Swan-Morton® scalpel as previously described for tissue explant cultures to avoid contamination with PDL fibroblasts. PRD tissue was subsequently minced and placed in DMEM supplemented with 10% FCS, penicillin/ streptomycin/ amphotericin in T25 Cellstar® flasks (Greiner Bio-One GmbH, Germany) and placed in a humidified 5% CO_2 incubator at 37°C. Fibroblasts were allowed to outgrow from the PRD tissue fragments over a three-week period and the culture medium changed every 3-4 days. At sub-confluence, fibroblasts were washed twice with PBS then detached with 0.025% trypsin and 0.05% EDTA (Sigma, Poole, UK) at 37°C for 5 min and seeded into a T75 Cellstar® flask (Greiner Bio-One GmbH, Germany). Once PRD fibroblasts had reached 80% confluence, this procedure was repeated and all fibroblasts cultures were then used for experiments at passage 3-4. Prior to experiments, confluent fibroblast cultures were grown in reduced serum for 24 hours and experiments were performed in serum free media. For all cell culture experiments, cell counts and cell viability were assessed by trypan blue dye exclusions. Cell numbers were calculated from a 1 in 10 dilution of the cell suspension in Trypan blue (Sigma) cell counting fluid and placing 10 μl of this on a haemocytometer as previously described.

3.2.5.2 Analysis of cytokines from resting PRD fibroblasts

Culture supernatant was collected at a variety of time points from unstimulated fibroblast cultures and spun down to remove non-adherent cellular debris. Supernatant was immediately frozen and stored at -20°C until analysis. Supernatants were analysed for cytokine levels by ELISA. For Western Blots, protein lysates were obtained from the cell pellet as described in 3.2.3.1.

3.2.5.3 Mitogenic stimulation of PRD fibroblast cultures

Confluent fibroblast cultures in T75 flasks were stimulated by mitogens or cytokines at concentrations described in Table 3.7. Following mitogenic stimulation, culture supernatant was collected at a variety of time points and spun down to remove non-adherent cellular debris. Supernatant was immediately frozen and stored at -20°C until analysis. Supernatants were analysed for cytokine levels by ELISA.

3.2.5.4 Microbial infection of PRD fibroblast cultures

The bacterial strains tested came from culture collections of *Fusobacterium nucleatum* (ATCC 25586), the gram negative rod *Prevotella intermedia* (ATCC 25611) and *Peptostreptococcus micros* (NCTC 11808). Bacterial strains were maintained in brain-heart infusion agar plates, prereduced anaerobically, sterilised and supplemented with hemin (5 mg/l) for anaerobes. Plates were incubated in anaerobic conditions (80% nitrogen, 10% carbon dioxide and 10% hydrogen) in a Don Whitley MK 3 anearobic chamber at 37 °C (Don Whitley Scientific Services, Shipley, UK). After five days growth, bacteria were removed from the agar plates with sterile cotton tipped applicator stick and placed in PBS. Bacterial suspensions were obtained by centrifugation at 3000 g for 15 minutes, washing three times in PBS and finally resuspended in PBS. The density of the live *F. nucleatum*, *P. intermedia* and *P. micros* prepared inoculum was measured spectrophotometrically (Dynex Technologies MRXII) and subsequently adjusted to a turbidity of 2 McFarland standard (6 x 10^8 CFU/ml). Live cells were washed a further twice in PBS solution and then added at this concentration to fibroblast cultures in T-75 flasks. Fibroblasts were used at passage 3-4 in serum free media without the addition of antibiotics or antifungals. After culture at the specified time intervals, the supernatant was collected, centrifuged and stored at –70°C until analysis by ELISA.

3.2.6 Enzyme Linked Immonosorbent Assays (ELISA)

Explant culture and fibroblast culture supernatants were analysed by ELISA for IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-γ (Cytosets, Biosource International), IL-18 (Bender MedSystems Ltd.), IL-17A, RANKL (PeproTech EC) and OPG (R&D, Duoset). 96 well Immulon-4 HBX microtiter plates (Dynex, Basingstoke, UK) were incubated

overnight with 100 μl capture Ab at 3°C. Following four washes with PBS/ 0.05%Tween-20, non-specific protein sites within wells were blocked with 300 μl PBS/ 0.5%BSA or as otherwise directed by the manufacturer overnight at 3°C. After four further washes with PBS/ 0.05%Tween-20, 50 μl of standards or supernatant samples were incubated with relevant biotinylated, capture Ab or as directed by the manufacturer at RT on a shaker for 2 h. Streptavidin-HRP was added after thorough washing with PBST and incubated for 45 min at RT. Colour was developed with tetramethyl benzidine (TMB) and the reaction stopped with 0.2M H_2SO_4. Absorbency was determined at 450 nm (reference filter 600 nm) on a microplate plate reader (Dynex Technologies MRXII). Cytokine concentrations (pg/ml) were calculated with reference to a standard curve, established by serial 1:2 dilutions of standard concentration as detailed in Table 3.10.

3.2.7 Statistical analysis

Supernatant cytokine concentrations were compared using Student's *t*-test for each stimulated culture against corresponding paired non-stimulated control (significant results of $p \leq 0.05$ are marked *, results of $p \leq 0.01$ are indicated **). In addition to comparison of paired specimens, Mann-Whitney U test was used to compare stimulated culture supernatant cytokine concentrations with whole population controls for each cytokine assayed (significant results are marked †). Analysis was undertaken using Minitab Software (Minitab Inc, USA). *P* values of <0.05 were considered significant, otherwise not significant (NS). Results are represented as means ± SEM. Additive cytokine effects are desribed in the context of the combination of cytokines inducing the sum of their individual effects. Synergistic effects are described when the combined effect(s) is significantly greater than the sum effect of individual cytokines.

Table 3.10 **Details of ELISA used in culture experiments.**

Analyte Measured	Source	Detection Range	Sensitivity
IL-1β	Biosource, CytoSets	31.25-2000 pg/ml	>2 pg/ml
IL-4	Biosource, CytoSets	31.25-2000 pg/ml	>5 pg/ml
IL-6	Biosource, CytoSets	31.25-2000 pg/ml	>2 pg/ml
IL-8	Biosource, CytoSets	31.25-2000 pg/ml	>7pg/ml
IL-10	Biosource, CytoSets	31.25-2000 pg/ml	>10 pg/ml
IL-12p40	Biosource, CytoSets	31.25-2000 pg/ml	>2 pg/ml
IL-17A	PeproTech EC	31.25-2000 pg/ml	>10 pg/ml
IL-18	Bender MedSystems	78.1-5000 pg/ml	>55pg/ml
IFN-γ	Biosource, CytoSets	31.25-2000 pg/ml	>4 pg/ml
TNF-α	Biosource, CytoSets	31.25-2000 pg/ml	>3 pg/ml
RANKL	PeproTech EC	31.25-2000 pg/ml	>10pg/ml
OPG	R&D Systems, Duoset	78.1-5000 pg/ml	>70pg/ml

3.3 General solutions and chemicals

Phosphate Buffered Saline 2.5l, 10X, pH, 7.5

NaCl	200g
$Na_2HPO_42H_20$	35.5g
KCL	5g
KH_2PO_4	5g
dH_20	to 2.5L

Trypsin solution 0.1%, 1l, pH 7.35

Trypsin	1g
Glucose	1g
1% phenol red solution	1.5ml
dH_20	to 1L

Ethylenediamine tertraacetate, EDTA (Versene), pH 7.35

EDTA tetrasodium salt	100mg
PBS	500ml

Electrophoresis Transfer buffer, pH 8.0

Methanol	800ml
Tris Base 20mM	12.1g
Glycine	57.6g
dH_20	to 4L

Tris Buffered Saline/ Tween, pH 8.0

Tris HCL 10mM pH 8.0	40 ml of 1M
NaCl 150mM	120 ml of 5M
Tween 20, 0.05%	2ml
dH_20	to 4L

Electrophoresis Buffer, 5X

Tris Base	15.1g
Glycine	72g
SDS	5g
dH_20	to 1L

Sample Buffer

dH_20	4ml
TrisCl 0.5M pH6.0	1ml
Glycerol	0.8ml
10% SDS	1.6ml
2 Mertcaptoethanol	0.4ml
Bromophenol Blue 0.1%	0.2ml

RIPA Buffer stock solution, pH 7.4

Tris base	790g
dH_20	75ml
NaCl	900g
10% NP-40	10ml
10% Na-deoxycholate	2.5ml
EDTA 100mM	1ml

RIPA Buffer working solution

RIPA Buffer stock solution	1ml
DTT 1mM	1µl
PMSF 200mM	5µl
Aprotinin 10µg/ml	1µl
Pepstatin 1µg/ml	1µl
Leupeptin 1µg/ml	1µl

CHAPTER 4

A NOVEL *EX-VIVO* HUMAN PRD EXPLANT CULTURE MODEL TO INVESTIGATE CYTOKINE REGULATION *IN-VITRO*

4 A NOVEL *EX-VIVO* HUMAN PRD EXPLANT CULTURE MODEL TO INVESTIGATE CYTOKINE REGULATION *IN VITRO*

4.1 Introduction

The host immune response to microbial challenge of the dental pulp and the subsequent infiltration of pathogens and their associated PAMPS into the periradicular tissue is central to the pathogenesis of human periradicular disease (Márton and Kiss 2000). Infiltration of bacteria into the dental root canal leads to the stimulation of macrophages and fibroblasts resident within the surrounding periradicular bone. Activation of these local cell populations leads to the concomitant recruitment of macrophages and monocytes into the periradicular site (Kawashima *et al.* 1996). Considerable data support the hypothesis that cytokines orchestrate the complex processes underlying this prevalent human inflammatory disease. Several studies have identified the expression of significant quantities of cytokines and chemokines within the PRD lesion, including TNF-α, IL-1-β, IL-6 and IL-8 (Meghji *et al.* 1989, Artese *et al.* 1991, Safavi and Rossomando 1991, Barkhordar *et al.* 1992, Formigli *et al.* 1995, Meghji *et al.* 1996, Takeichi *et al.* 1996, Honma *et al.* 1998).

To date, the rat/ murine pulp exposure model has imparted the majority of findings on the involvement of the cytokine network within PRD. These animal experiments clearly demonstrate the relevance of the host immune response in the initiation and progression of PRD (Stashenko *et al.* 1998). In contrast, few studies have investigated the role of the host immune response and its relationship to root canal treatment success within humans. This is surprising given that the patient's non-specific immune response may represent the most significant predictor for root canal treatment outcome (Marending *et al.* 2005). Indeed, a variety of patient-dependent factors influence the successful outcome of root canal treatment. These include patient gender, pulp diagnosis, periapical diagnosis, the size and the presence of an existing periradicular lesion and/ or a sinus tract (Chugal *et al.* 2001). Of all these factors, the presence and size of the PRD lesion is the strongest predictor of treatment outcome (Chugal *et al.* 2001). It therefore seems remarkable that only a small number of published studies have analysed functional immunological components of the human PRD lesion *in vitro, ex vivo*.

Human explant tissue cultures provide useful models in which to study human tissue *in vitro, ex vivo*. This methodology enables functional experiments to be undertaken on tissue

that preserves its inflammatory constituents. Explant cultures have been used extensively within immunologic research to investigate the effects of mitogenic and cytokine stimulation upon the release of inflammatory mediators (Monteleone *et al.* 1999, Dionne *et al.* 2003, Zhao *et al.* 2004 and Maerten *et al.* 2004). By using such experimental techniques, the inflammatory and immune responses of diseased tissue to specific aetiological pathogens and inflammatory agents can be elucidated *in vitro*, *ex vivo*. In contrast to cell culture experiments, explant cultures allow the complex interaction between the many cellular components of the disease process to be studied. Development of human explant culture models thereby reduces the absolute reliance upon animal experimentation.

The concentrations of a limited number of secreted inflammatory mediators have been analysed from human explant PRD tissue (Danin *et al.* 2003). The capacity for unstimulated PRD explant tissues to produce immunoglobulins has previously been examined (Baumgartner and Falkler 1991a, Baumgartner and Falkler 1991b). Furthermore, the specificity of these secreted antibodies to specific endodontic pathogenic bacteria has been investigated (Baumgartner and Falkler 1991c, Kettering *et al.* 1991). However, no studies have investigated the functional effects of stimuli upon PRD explant tissue cultures and the capacity of inflammatory mediators to modulate the expression of endogenous mediators.

Within PRD tissues, endotoxin concentrations are reported to correlate with inflammatory levels (Schonfield *et al.* 1982). Although LPS levels are significantly raised within the PRD lesion, it is suggested that LPS has minimal direct bone resorbing capacity. Instead, LPS appears to exert its bone destructive effects through the induction of proinflammatory cytokines (Wang & Stashenko 1993). It is therefore surprising that the effects of Gram-negative endotoxins or Gram-positive exotoxins upon inflammatory mediator expression within human PRD have not been explored. An appropriate combination of cytokines and immunocompetent cells is essential for the host to mount a successful immune response. Therefore, the identification of novel inflammatory mediators within the PRD lesion and the nature of their involvement in the inflammatory reaction is of considerable importance. I therefore investigated if PRD tissue would serve as a useful human tissue explant model with which to explore the cytokine network. Furthermore, I investigated the effects of PAMPS upon cytokine responses within PRD tissue ex-vivo, which likely reflects processes involved in perpetuation of the PRD lesion *in vivo*.

4.2 Endogenous cytokine expression within unstimulated human PRD tissue explant cultures

In order to gain a better understanding of cellular-derived events occurring within chronic PRD, I first established a novel human PRD tissue explant culture system. Fresh PRD tissue was obtained from patients sequentially undergoing periradicular surgery or routine dental extractions (described in section 3.2.4.1). Harvested tissue and/ or its culture supernatant were analysed either at the time of collection or at one of several time points during tissue culture as indicated (Figure 4.1). PRD explant tissue was initially assessed to determine its potential as an *in vitro, ex-vivo* human tissue explant model in which to investigate the cytokine network within chronic inflammatory disease. I therefore cultured human PRD tissue explants for 18 and 72 h to analyse the spontaneous expression of a panel of cytokines that are central to inflammatory responses.

Cytokine concentrations within culture supernatants from unstimulated human PRD explant tissue were measured by ELISA (described in chapter 3.2.6). After 18 h culture, 'resting' PRD explant tissues consistently expressed high levels of proinflammatory cytokines. Conversely, concentrations of prototypical anti-inflammatory cytokines such as IL-4 were low or not detectable (Figure 4.2A). Furthermore, substantial proinflammatory cytokine release from resting PRD explant tissue was observed within supernatants after 72 h culture (Figure 4.2B). The inherent production of inflammatory cytokines without any stimulation suggested that the PRD tissues were highly immunologically active. This was further confirmed by analysing NF-κB expression, which was readily detectable within the lesions using Western blots (Figure 4.2C)

Importantly, IL-4 was barely detectable within unstimulated explant culture supernatants at either 18 h or 72 h (n=30). Indeed, IL-4 protein expression was not inducible by either mitogenic or cytokine stimulation of explant tissue in subsequent experiments. The expression of IL-4 was therefore not included in any further analysis of PRD tissues.

Figure 4.1 Development of a novel PRD explant culture system. Diagrammatic representation of the source of human PRD tissue and the laboratory experiments performed on the collected tissue and/ or culture supernatant. Tissue culture supernatant was harvested after 18 h or 72 h of culture for explant experiments.

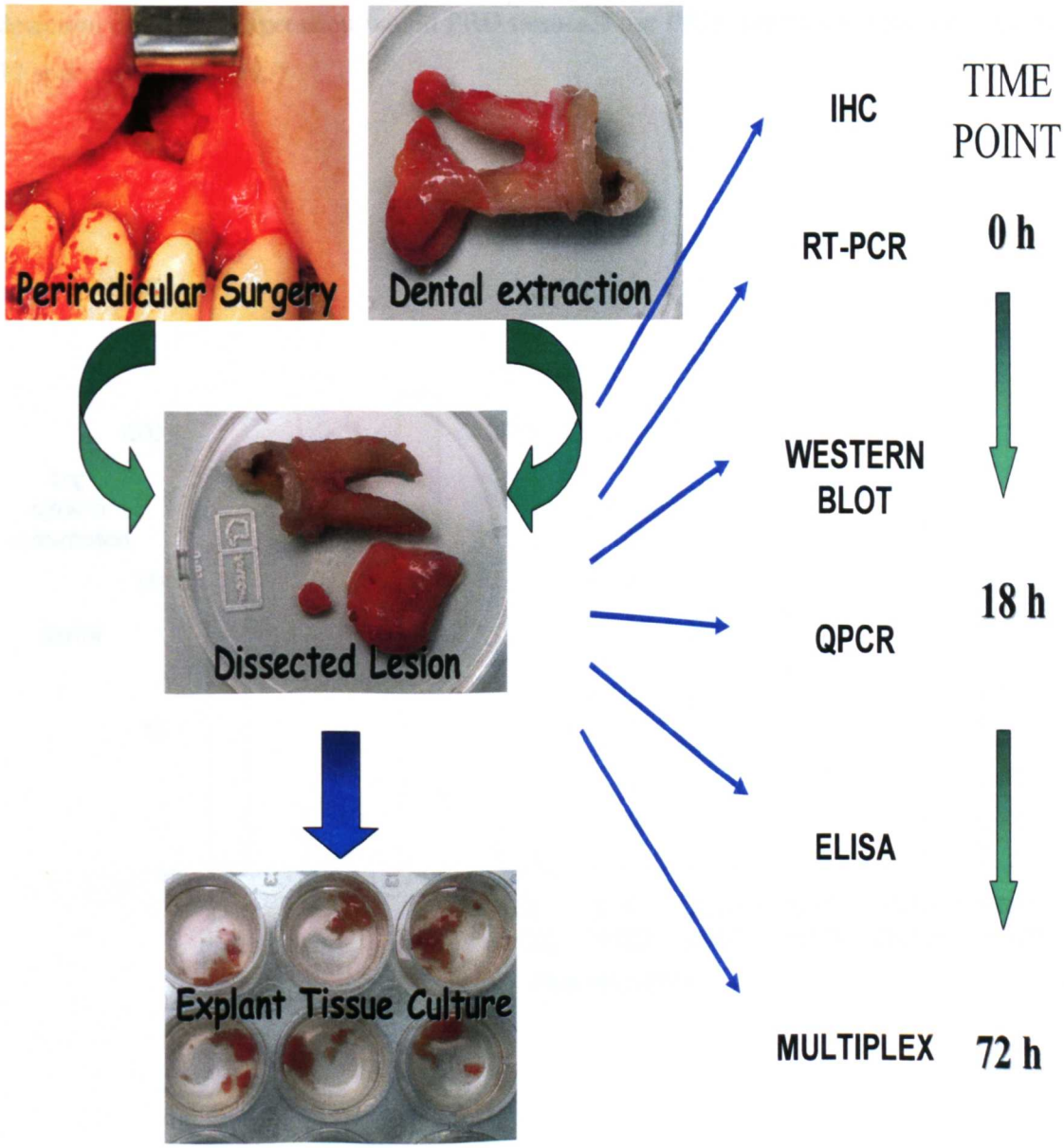
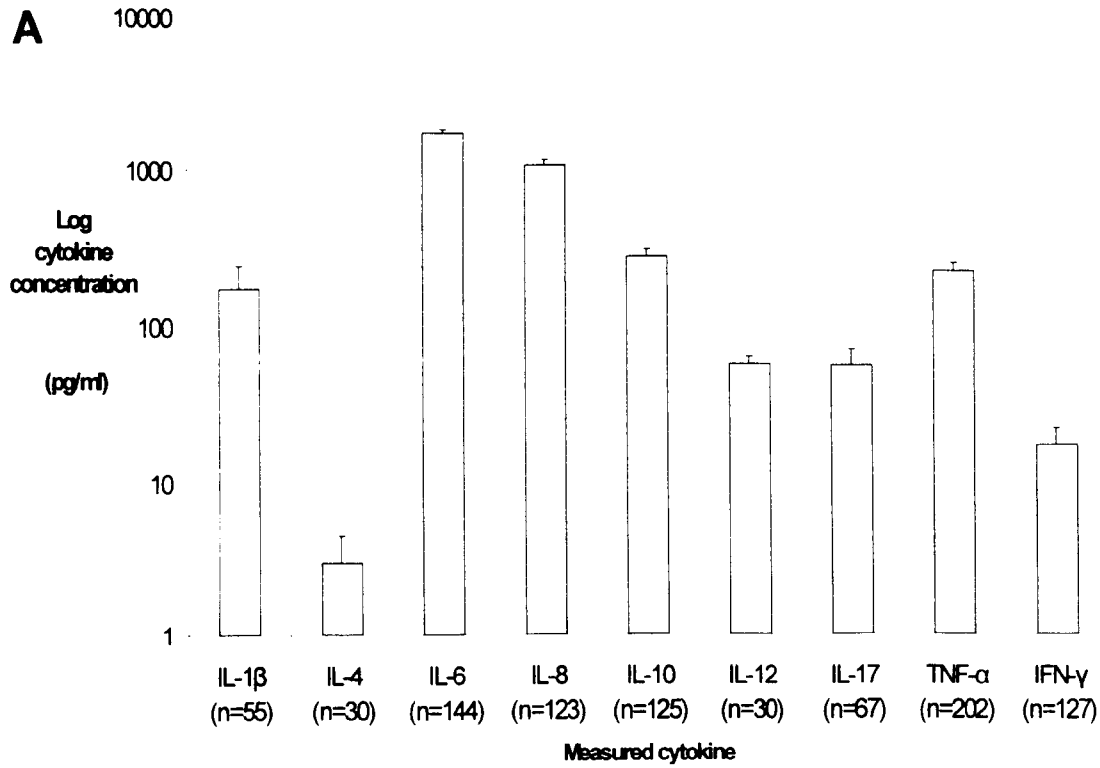
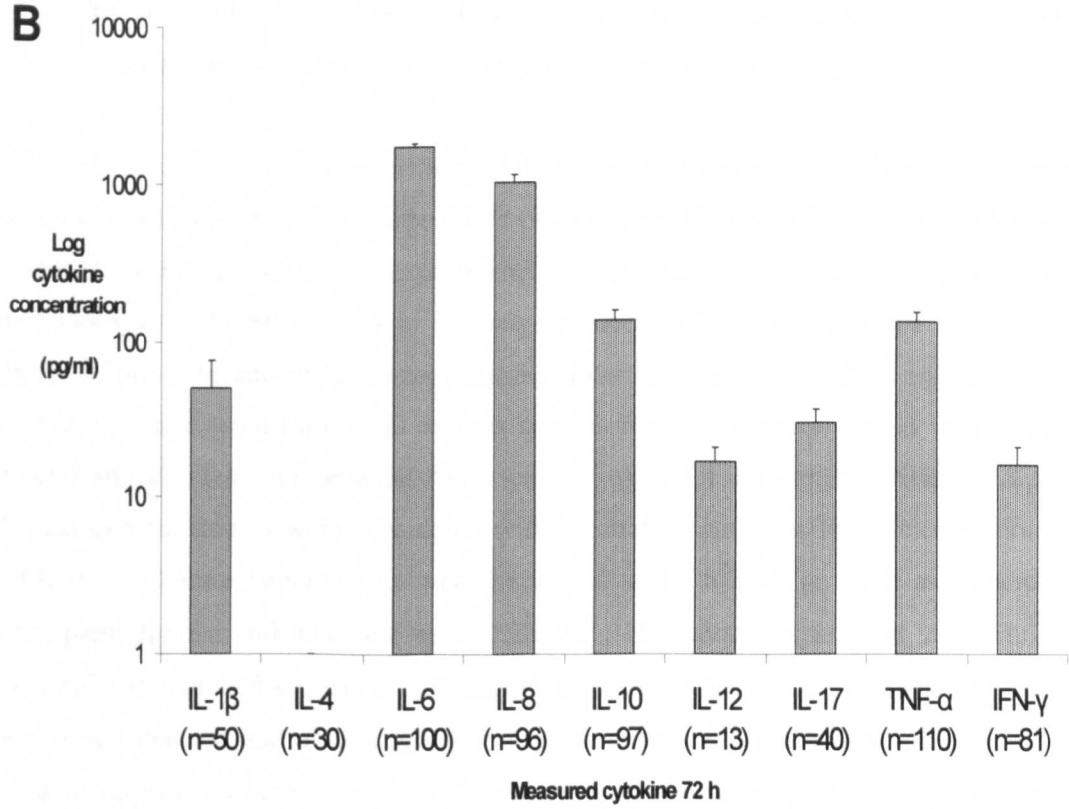
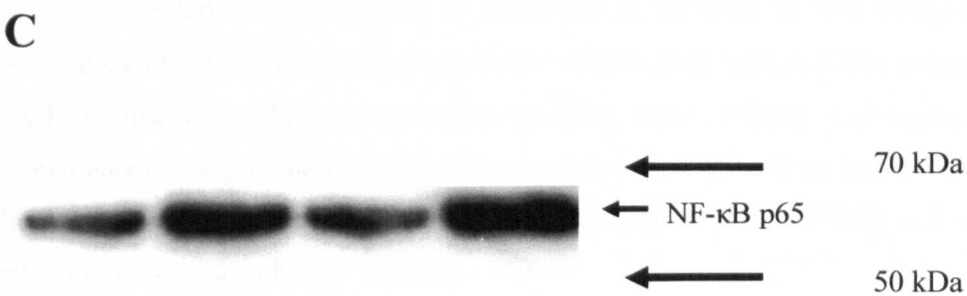


Figure 4.2 Development of a novel human PRD explant culture system. 'Resting' levels of spontaneously released cytokines within PRD tissue explant culture supernatants were assessed. Unstimulated PRD explant tissue culture supernatant was harvested (A) at 18 h and (B) at 72 h. The expression of a panel of cytokines was analysed by ELISA. Bars represent the mean concentration for each cytokine and the standard error of the mean. The number of unstimulated PRD tissues from individual donors used in experiments for the measurement of each individual cytokine is given in parenthesis. (C) Western blot for detection of NF-κB expression within PRD tissues. Four PRD specimens representative of 12 specimens analysed.




B
10000
1000
100
10
1
Log cytokine concentration (pg/ml)
IL-1β (n=50)
IL-4 (n=30)
IL-6 (n=100)
IL-8 (n=96)
IL-10 (n=97)
IL-12 (n=13)
IL-17 (n=40)
TNF-α (n=110)
IFN-γ (n=81)
Measured cytokine 72 h


C
70 kDa
NF-κB p65
50 kDa

4.3 *In vitro* effects of Gram-negative mitogens upon endogenous cytokine expression within human PRD tissue explant cultures

Using ELISA, the previous experiments established that unstimulated *ex-vivo* PRD tissue spontaneously expressed detectable levels of cytokines for up to 72 h culture. I therefore undertook a further series of experiments to determine if this novel explant culture methodology could be utilised to investigate functional tissue responses *in vitro*. The effects of pro- and anti-inflammatory stimuli upon endogenous cytokine expression were therefore studied within the PRD explant system. Each PRD tissue biopsy was weighed, minced and divided into separate tissue culture wells for culture (described in 3.2.4.2). Human explant tissues were stimulated with a panel of mitogens (for concentrations see Table 3.7). Culture supernatants were harvested at 18 h and/ or 72 h as indicated in subsequent figures and analysed by ELISA. In each explant experiment, one well with unstimulated matched explant tissue served as control. An initial series of experiments established that the addition of IgG, serving as control, had no modulating effect on the cytokine expression being analysed. Due to the small volume of tissue available for each individual explant culture experiment, IgG was not used in subsequent experiments.

To reflect the likely bacterial aetiology of PRD, explant tissues were stimulated with Gram-positive or Gram-negative derived moieties (for concentrations see table 3.7). Initially, I undertook experiments to determine if bacterial derived mitogens had any modulatory effects upon cytokine expression within 18 h explant tissue cultures. As LPS has been observed at high levels within the PRD lesion, I firstly investigated its effects upon a panel of key cytokine inflammatory mediators at 18 h tissue culture. In addition to the unstimulated explant tissue controls, the addition of LPS to PBMC and whole blood cell cultures served as further controls.

At 18 h culture, the addition of LPS to human PRD tissue explant cultures resulted in substantially increased levels of the proinflammatory cytokines IL-1β ($p=0.046$) and IFN-γ ($p=0.032$) (Figure 4.3A and B). Furthermore, LPS promoted a moderate increase in the expression of TNF-α ($p=0.02$) and IL-10 ($p=0.046$) that were at levels of statistical significance. The addition of LPS to PRD explants had no observable effect upon the expression of IL-6, IL-8 or IL-17A at 18 h culture compared with paired unstimulated control tissues. The order from greatest to least increase for LPS-induced cytokine

expression was IL-1β, IFN-γ, IL-10, TNF-α, and a minimal, non-significant increase in IL-17A.

Having determined that functional responses of a significant nature were detectable within PRD tissue explant cultures at 18 h, I next undertook a series of experiments to determine if these effects were sustained over longer-term culture. Explant tissue cultures were therefore maintained for 72 h with LPS and the supernatant harvested and subsequently analysed by ELISA for the quantitative expression of endogenous secreted cytokines. After 72 h culture, the expression of IFN-γ ($p=0.004$) was substantially increased by the addition of LPS to PRD tissue explants (Figure 4.5A and B). LPS also induced a moderate increase in TNF-α ($p=0.013$) expression, which was at levels of significance. LPS-induced expression of IL-1β ($p=0.017$) at 72 h culture was minor, although at a level of statistical significance. The increase in IL-1β expression was markedly lower than that observed at 18 h culture. The addition of LPS to 72 h cultures produced only a minor increase in IL-10 ($p=0.01$) expression, although this was at a level of statistical significance. In contrast, 72 h tissue explant culture with LPS resulted in a modest reduction of IL-8 ($p=0.0176$) expression, that reached levels of statistical significance. Compared with corresponding matched unstimulated control tissues LPS had no observable effect upon IL-6 or IL-17A expression at 72 h culture.

It can clearly be seen from Figure 4.5C that LPS substantially up-regulated IFN-γ expression. Furthermore the effect of LPS-induced IFN-γ expression occurred in a steady manner throughout the period of culture to 72 h. LPS also induced moderate levels of TNF-α throughout the culture period to 72 h, although this was not as marked as the effect upon IFN-γ. Interestingly, LPS induced a substantial increase in the expression of IL-1β at 18 h, which was as great as that of IFN-γ. Nevertheless, increased IL-1β levels were not sustained over longer-term culture. This may be the result of endogenous enzymatic cleavage of IL-1β over longer culture conditions. In 18 h PBMC control cultures, LPS induced significant increases in the proinflammatory cytokines IL-1β, IL-6, IL-8, IL-12 and TNF-α and the anti-inflammatory cytokine IL-10 (Figure 4.4A and B). The pattern of increased cytokine expression in PBMC controls and absence of an effect upon IFN-γ at 18 h is in accordance with others (Iwadou *et al.* 2002).

The cellular composition of the PRD lesion was examined by IHC experiments to identify potential cellular sources of these inflammatory cytokines (Figure 4.9).

Figure 4.3 ***In vitro*** **effects of 18 h Gram-negative-derived mitogenic stimulation upon cytokine expression within PRD tissue explants.** PRD explant tissues were stimulated with LPS for 18 h and supernatant collected and analysed by ELISA for a panel of cytokines. (A) The mean concentration of each cytokine after LPS stimulation of explants compared with their corresponding unstimulated controls. (B) The percentage change in culture supernatant cytokine concentration between LPS stimulated and unstimulated paired control tissues. The number of PRD tissues used in experiments for the measurement of each individual cytokine is given in parenthesis. Bars represent the mean concentration for each cytokine and the standard error of the mean.

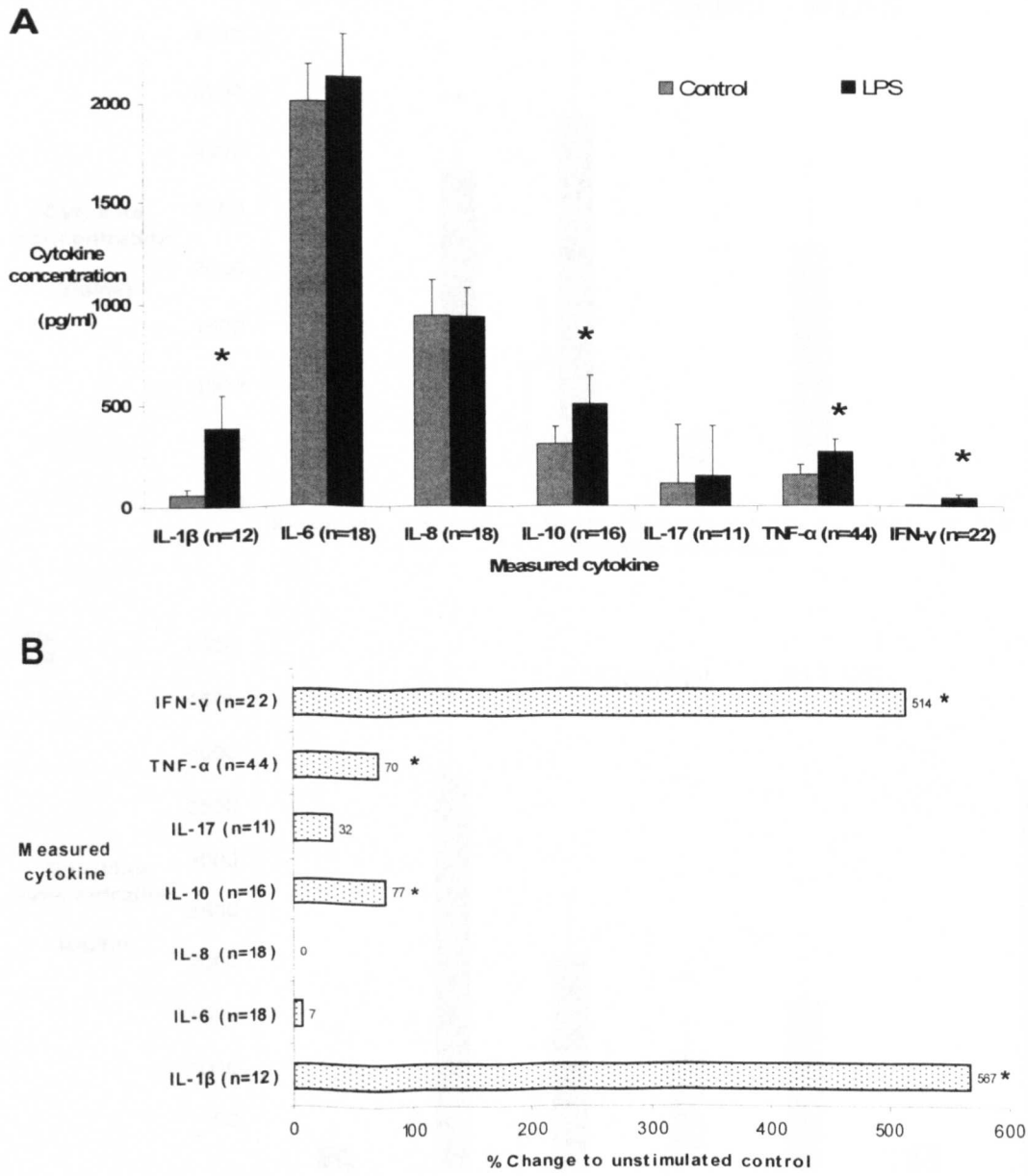
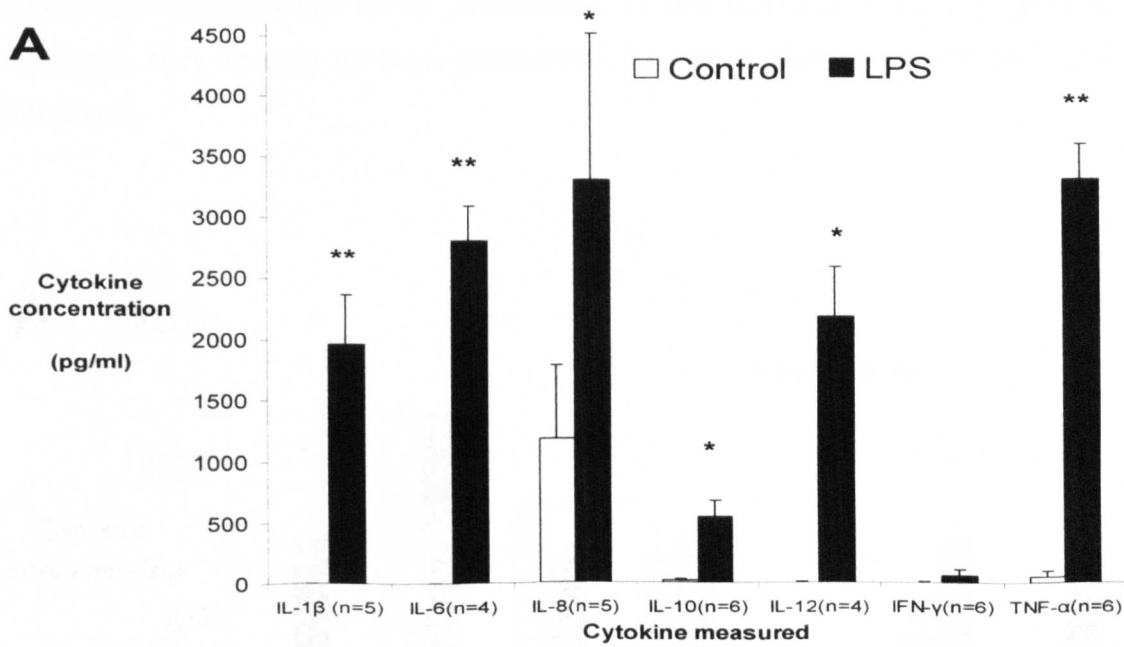


Figure 4.4 ***In vitro*** **effects of 18 h LPS stimulation upon cytokine expression within PBMC and whole blood cell cultures**. (A) The mean concentration of cytokines after LPS stimulation compared with unstimulated control in 18 h PBMC cultures. (B) The mean concentration of cytokines after LPS stimulation compared with unstimulated control in 18 h whole blood cell cultures. The total number of separate patient derived cultures stimulated with LPS is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.



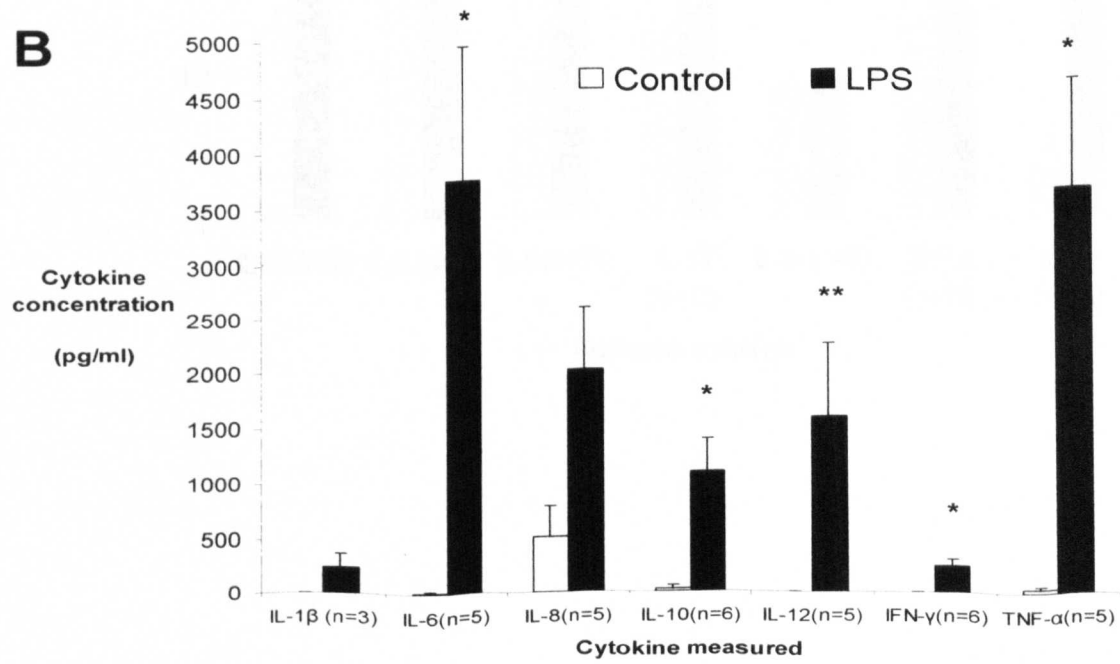
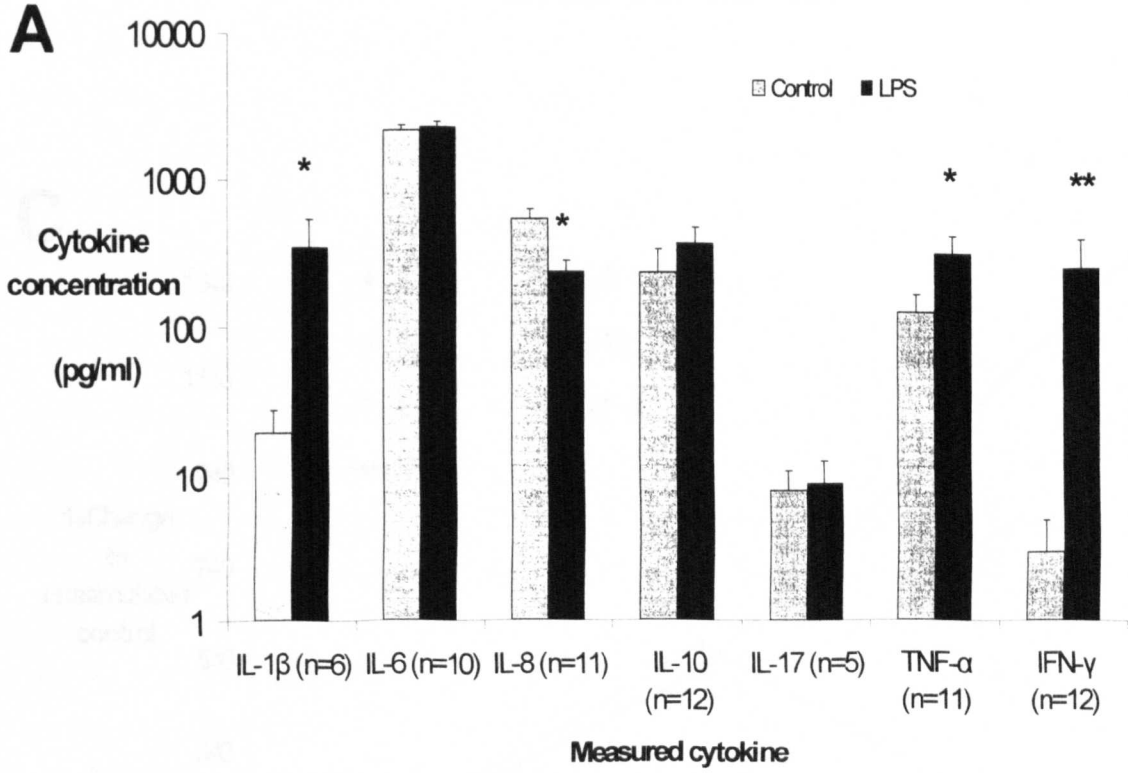
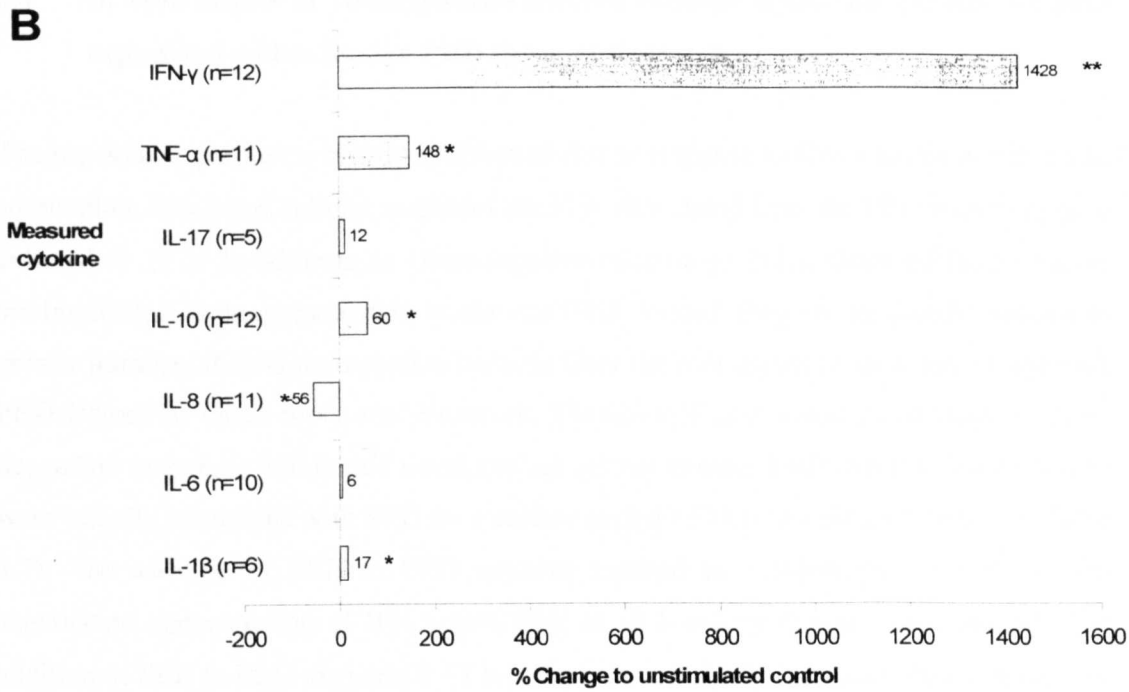
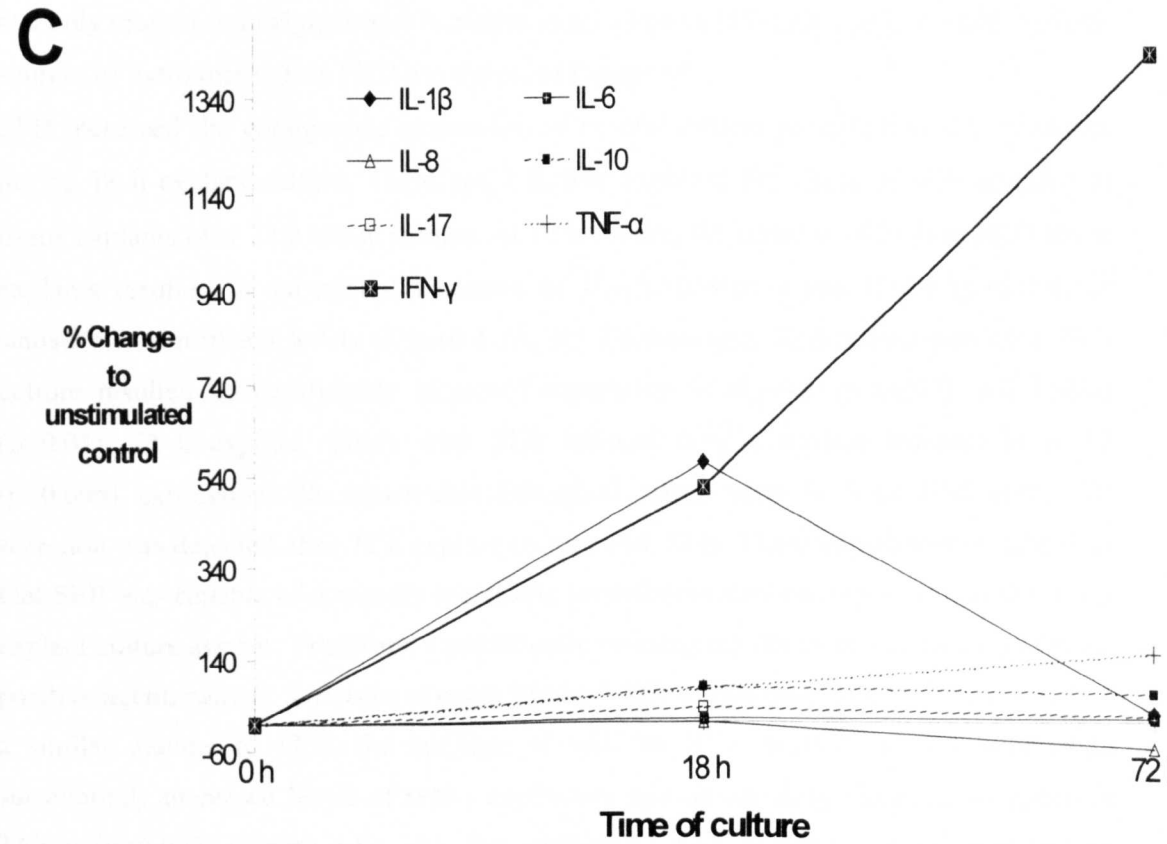


Figure 4.5 Effects of 72 h Gram-negative mitogenic stimulation upon PRD tissue explant cytokine expression. PRD explant tissues were stimulated with LPS for 72 h and supernatant collected and analysed by ELISA for a panel of cytokines. (A) The mean concentration of each cytokine after LPS stimulation of explants compared with their matched unstimulated controls. (B) The percentage change in culture supernatant cytokine concentration between LPS stimulated and unstimulated paired control tissues. (C) Time-line chart representing percentage changes of each analysed cytokine compared with unstimulated control over full period of culture with LPS stimulation. The number of PRD tissues used in experiments for the measurement of each individual cytokine is given in parenthesis. Bars represent the mean concentration for each cytokine and the standard error of the mean.




B
IFN-γ (n=12)
1428
**
TNF-α (n=11)
148 *
Measured cytokine
IL-17 (n=5)
12
IL-10 (n=12)
60 *
IL-8 (n=11)
*56
IL-6 (n=10)
6
IL-1β (n=6)
17 *
-200
0
200
400
600
800
1000
1200
1400
1600
% Change to unstimulated control


C
1340
1140
940
740
540
340
140
-60
IL-1β
IL-6
IL-8
IL-10
IL-17
TNF-α
IFN-γ
%Change to unstimulated control
0 h
18 h
72 h
Time of culture

4.4 *In vitro* effects of Gram-positive-derived moieties upon endogenous cytokine expression within human PRD tissue explant cultures

The previous experiments clearly established that in response to Gram-negative mitogenic stimulation, functional cellular responses could be elucidated from the PRD tissue explants cultured to 72 h. In addition to Gram-negative micro-organisms, Gram-positive bacteria are implicated in the perpetuation of chronic PRD. Indeed, they are frequently isolated in greater numbers than Gram-negative bacteria from the root canals of teeth associated with PRD lesions or failed root canal treatment. Therefore, I next investigated Gram-positive dependent responses within this novel explant culture system. PRD explant tissue cultures were initially stimulated with SEB for a culture period of 18 h (for concentration see Table 3.7). The addition of SEB to PRD explants resulted in a substantial increase in the supernatant concentration of IFN-γ ($p=0.001$) at 18 h culture (Figure 4.6A and B). The addition of SEB to PRD explants at 18 h culture also markedly increased the expression of IL-17A ($p=0.023$) and TNF-α ($p=0.01$). These results presumably reflect a predominant T cell-mediated response as SEB is a superantigen acting via TCR $V_\beta 8$ MHC class-II cross linking. There was no observed effect of SEB upon IL-6 or IL-8 expression at 18 h culture and only modest (non-significant) increases in IL-1β ($p=0.117$) and IL-10. Possible cellular sources of cytokines within PRD are shown in Figure 4.9.

SEB increased the endogenous expression of several critical proinflammatory cytokines during 18 h explant culture. Therefore, I further explored the effect of SEB addition to tissue explants over 72 h tissue culture. At 72 h culture, the addition of SEB to PRD tissue explants resulted in substantial increases of IL-1β ($p=0.011$) and IFN-γ ($p=0.004$) at statistically significant levels (Figure 4.7A, B). Furthermore, SEB stimulation over 72 h culture resulted in significantly increased expression of IL-17A ($p=0.027$) and TNF-α ($p=0.01$). 72 h explant culture with SEB induced only a modest increase in IL-10 ($p=0.096$) expression. No observable biological effect upon IL-6 or IL-8 ($p=0.138$) secretion was detected after 72 h explant culture with SEB. These experiments established that SEB was capable of markedly increasing proinflammatory mediators within the novel explant culture system. Therefore, I additionally investigated the effects of an intact Gram-positive agent, namely *S. aureus* (Cowan Strain, SAC) within this tissue culture model. In a similar manner to SEB, the addition of SAC to 18 h explant cultures resulted in substantially increased levels of IFN-γ expression and significantly increased secretion of TNF-α ($p=0.005$) (Figure 4.8A, B). The addition of SAC to the tissue explants had no observable effect upon IL-6 secretion.

Figure 4.6 ***In vitro*** **effects of the addition of Gram-positive-derived moieties upon cytokine expression within 18 h PRD tissue explant cultures**. PRD explant tissues were stimulated with SEB for 18 h and supernatant collected and analysed by ELISA for a panel of cytokines. (A) The mean concentration of each cytokine after SEB stimulation of explants compared with their corresponding unstimulated controls. (B) The percentage change in culture supernatant cytokine concentration between SEB stimulated and unstimulated paired control tissues. The number of PRD tissues used in experiments for the measurement of each individual cytokine is given in parenthesis. Bars represent the mean concentration for each cytokine and the standard error of the mean.

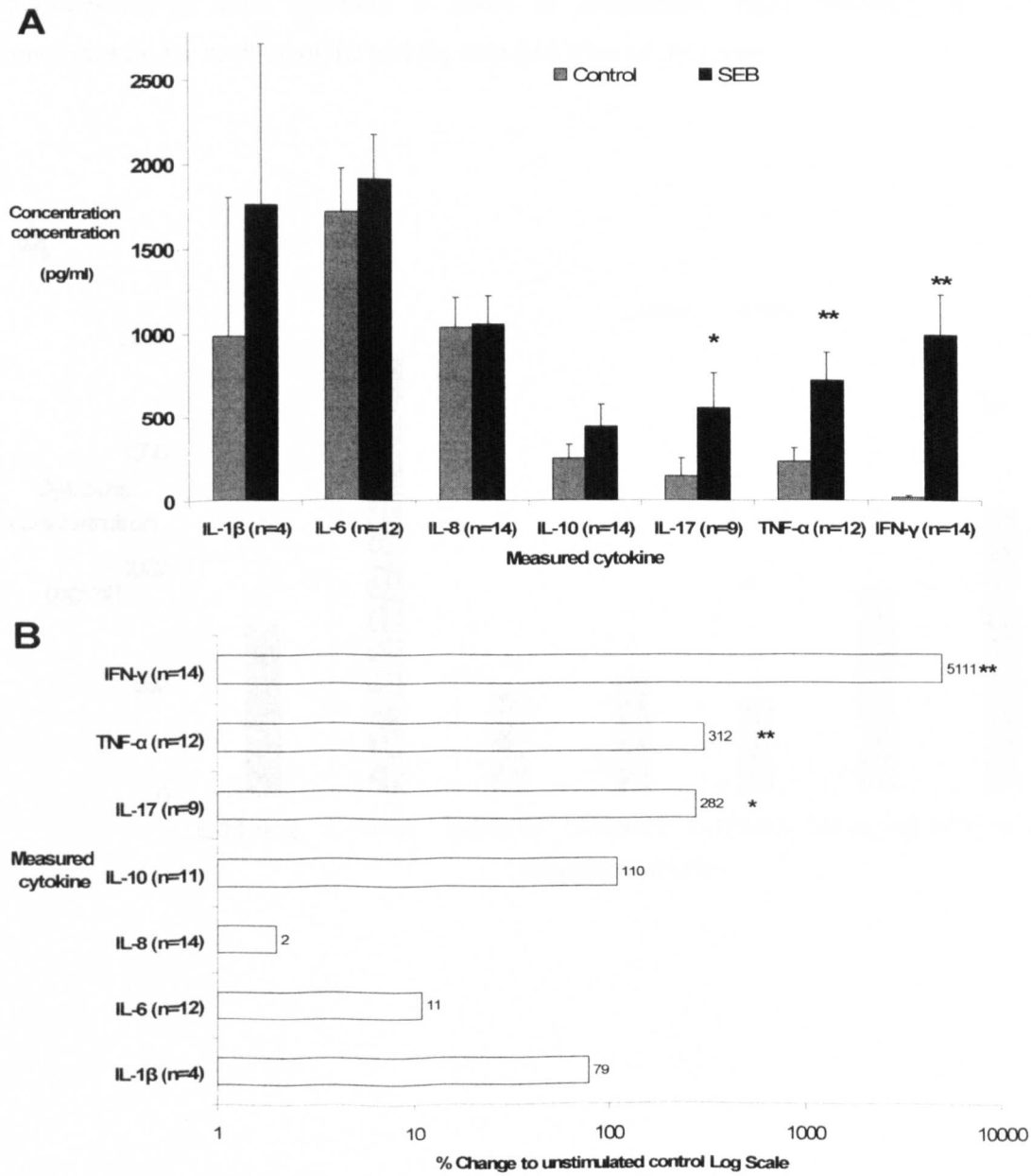
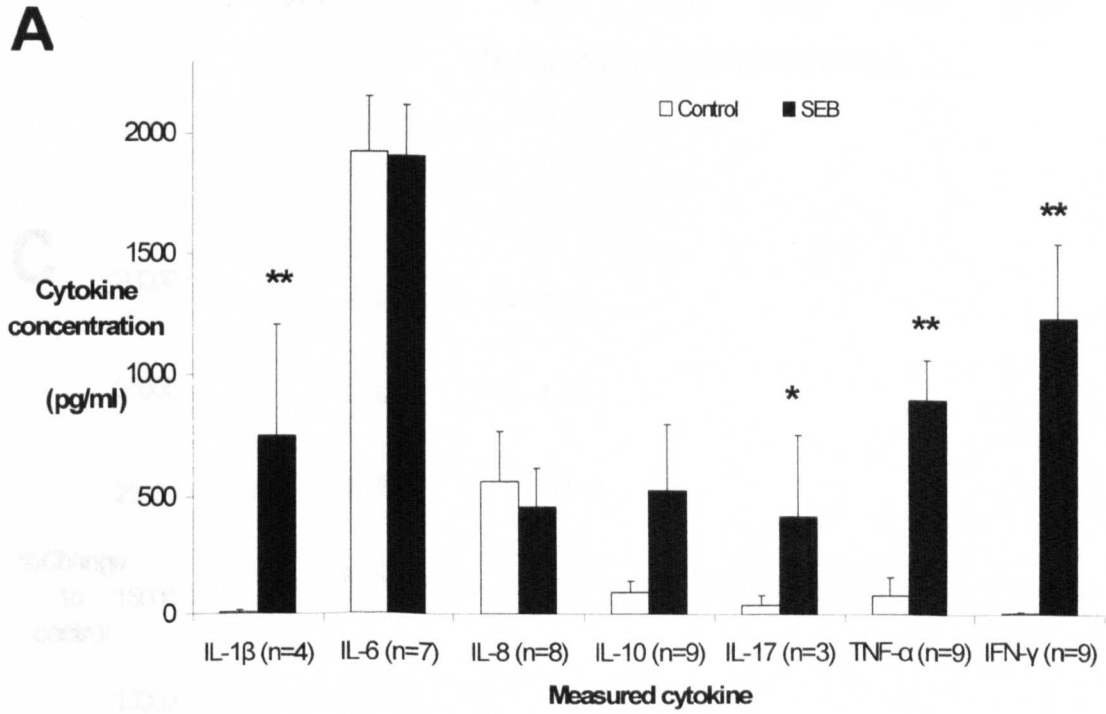
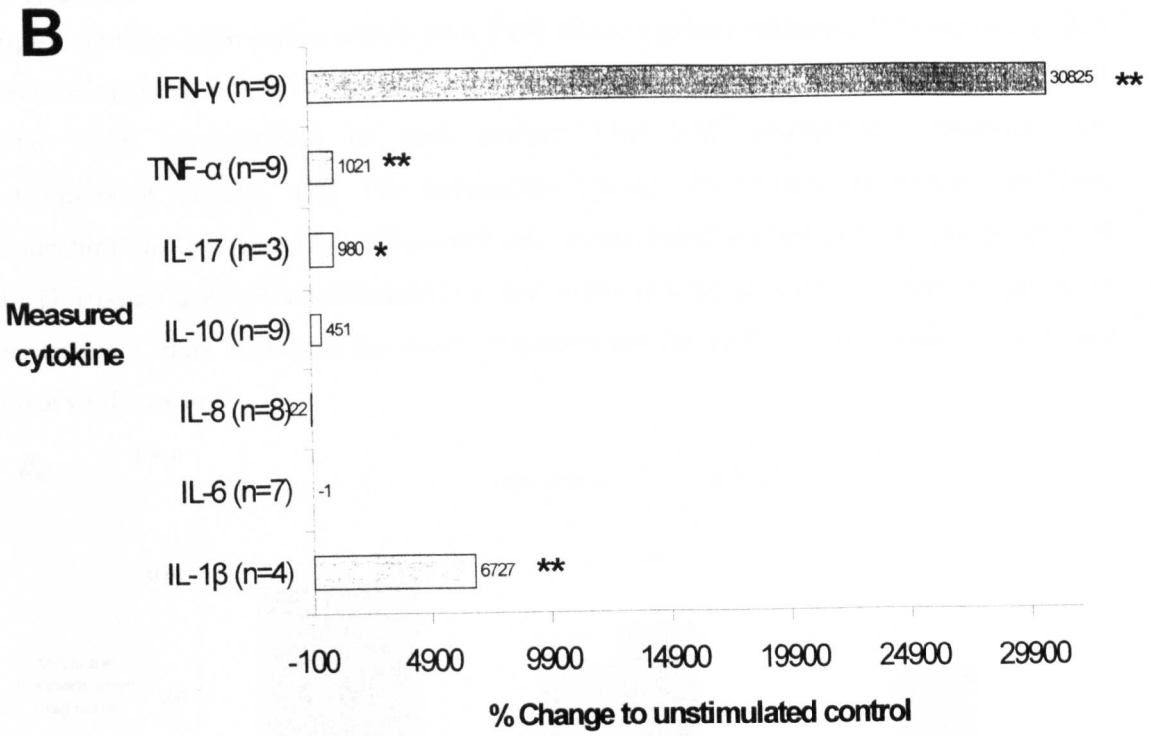


Figure 4.7 ***In vitro*** **effects of the addition of Gram-positive-derived moieties upon cytokine expression within 72 h PRD tissue explant cultures.** PRD explant tissues were stimulated with SEB for 72 h and supernatant collected and analysed by ELISA for a panel of cytokines. (A) The mean concentration of each cytokine after SEB stimulation of explants compared with their corresponding unstimulated controls. (B) The percentage change in culture supernatant cytokine concentration between SEB stimulated and unstimulated paired control tissues. (C) Time-line chart representing percentage changes for each analysed cytokine compared with unstimulated control over full period of culture with SEB stimulation. The number of PRD tissues used in experiments for the measurement of each cytokine is given in parenthesis. Bars represent the mean concentration for each cytokine and the standard error of the mean.




B
IFN-γ (n=9)
TNF-α (n=9)
IL-17 (n=3)
IL-10 (n=9)
IL-8 (n=8)
IL-6 (n=7)
IL-1β (n=4)
30825
**
1021
**
980
*
451
22
-1
6727
**
Measured cytokine
-100
4900
9900
14900
19900
24900
29900
% Change to unstimulated control

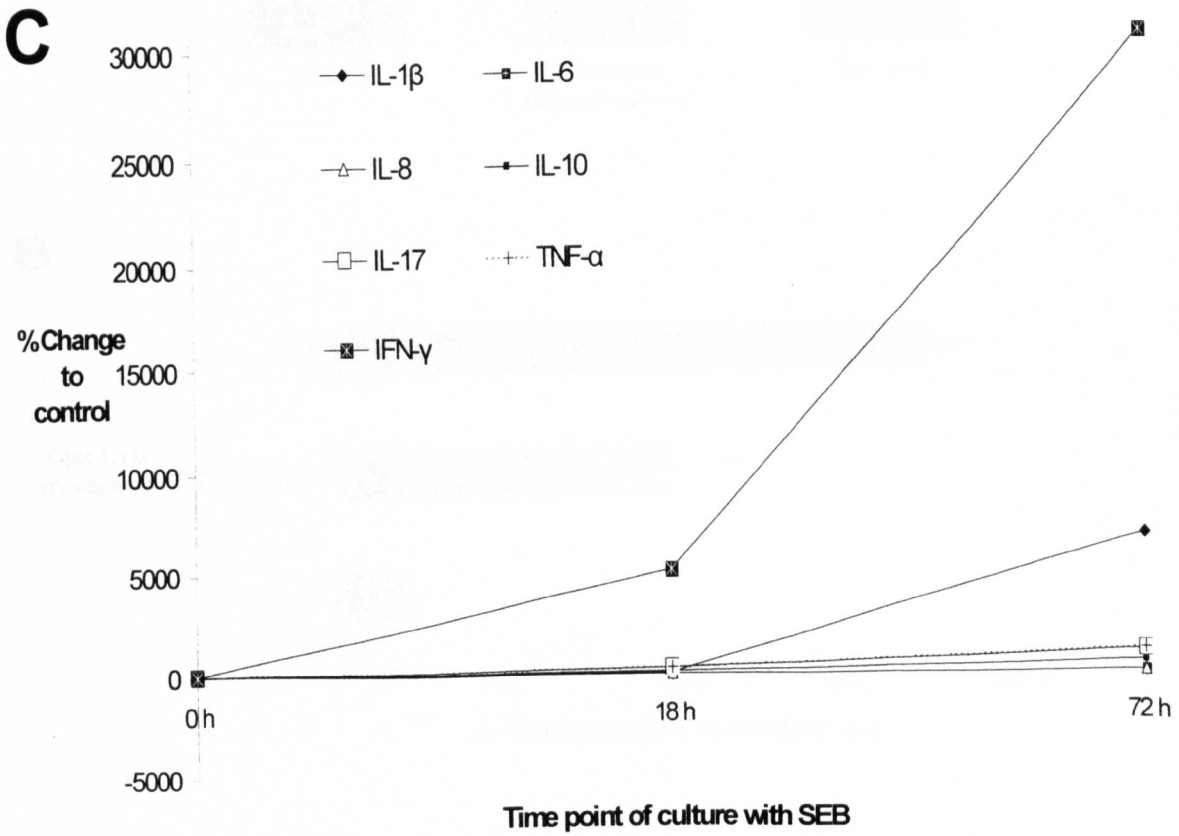

C
30000
25000
20000
15000
10000
5000
0
-5000
IL-1β
IL-6
IL-8
IL-10
IL-17
TNF-α
IFN-γ
%Change to control
0h
18h
72h
Time point of culture with SEB

Figure 4.8 ***In vitro*** **effects of the addition of the Gram-positive-derived mitogen SAC upon cytokine expression within 18 h PRD tissue explant cultures**. PRD explant tissues were stimulated with SAC for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of each analyte after SAC stimulation compared with unstimulated control. (B) The percentage change in culture supernatant cytokine concentration between SAC stimulated and unstimulated control tissues. The number of PRD tissues used in experiments for the measurement of each cytokine is given in parenthesis. Bars represent the mean concentration for each cytokine with the standard error of the mean.

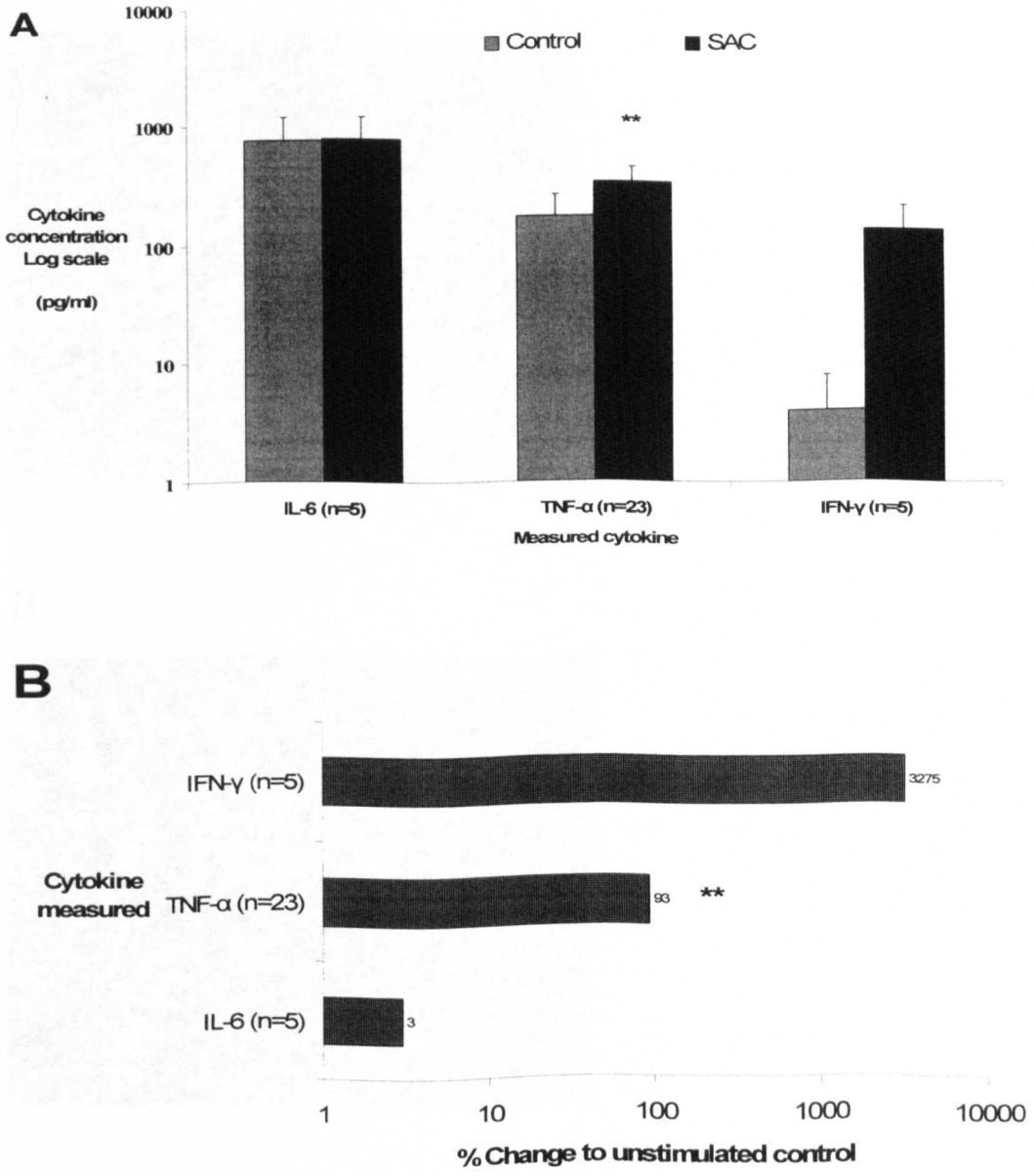
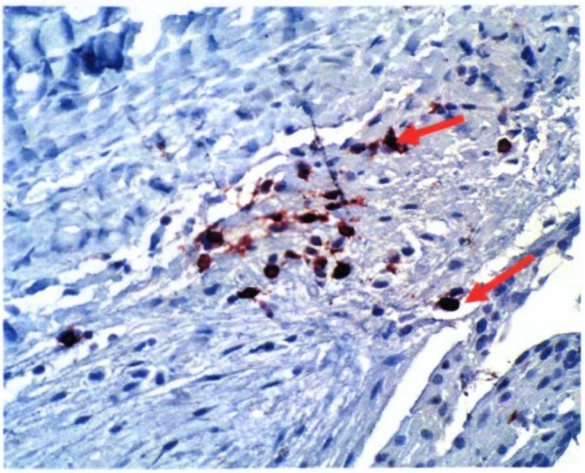


Figure 4.9 Potential cellular sources of secreted cytokines within PRD. Representative pictures of immunohistochemistry experiments undertaken upon 25 PRD biopsy specimens, tissues counterstained with H&E. (A) $CD20^+$ B cells. (B) Neutrophils identified by anti-human neutrophil defensins. (C) Mast cells identified by anti-mast cell tryptase. (D) NK T cells identified by anti-CD57. (E) Cellular staining positive for CD25. (F) $CD68^+$ macrophages. (G) PRD fibroblasts. (H) $CD3^+$ T cells, no counterstain. Arrows identify examples of positively stained cells.

A



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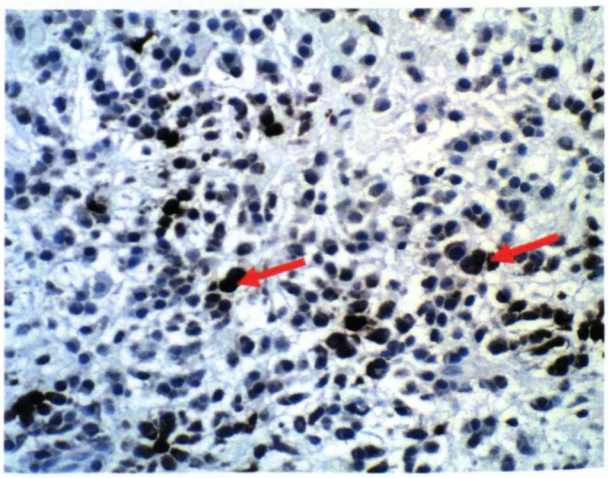

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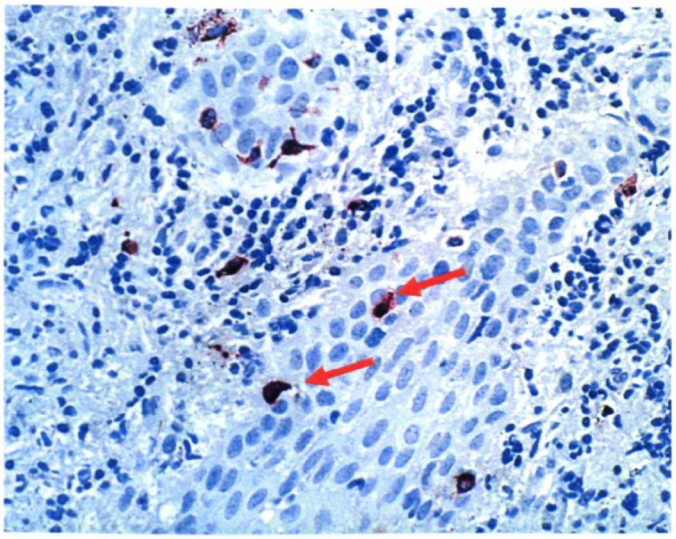

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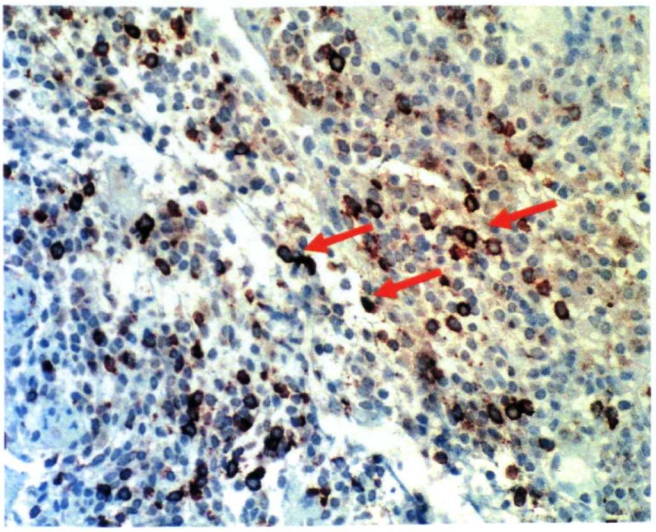

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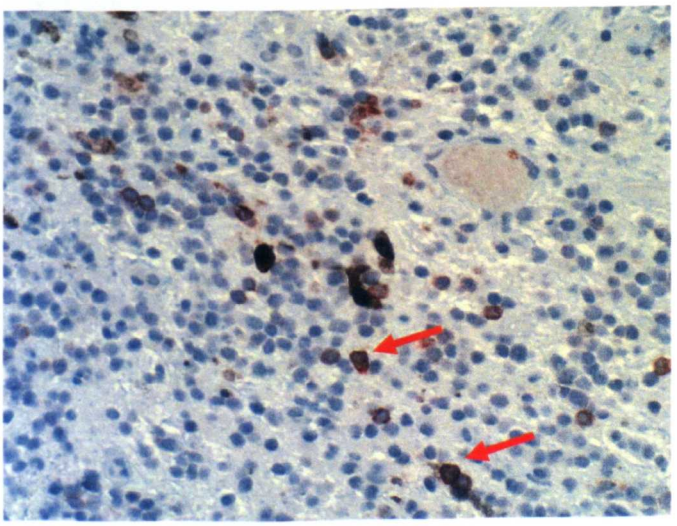

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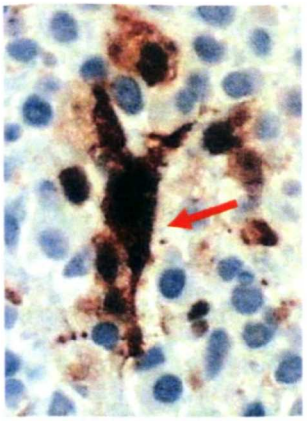

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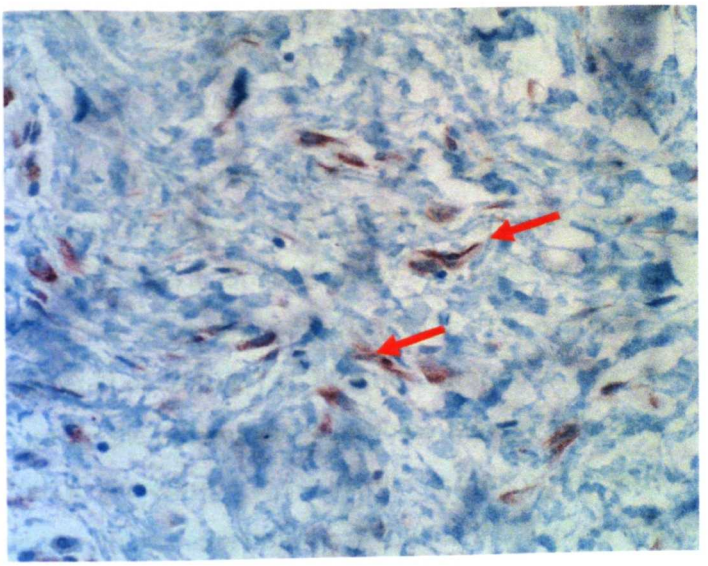

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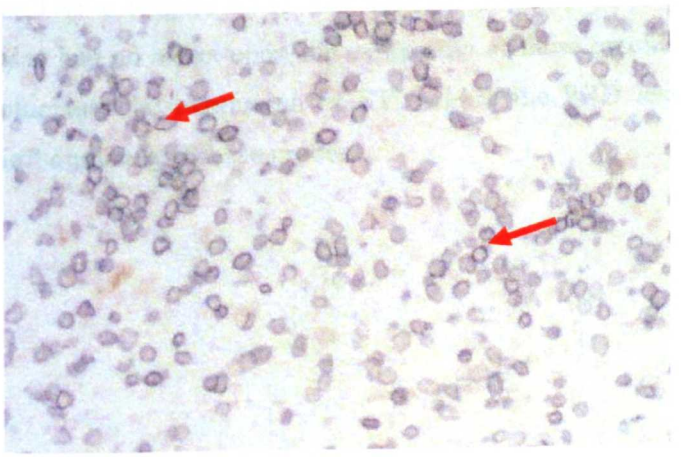


4.5 Regulation of cellular responses to pathogenic moieties

Having observed that the PRD tissue explants were responsive to Gram-negative and Gram-positive moieties, I next investigated if gene expression for Toll-like receptors was present within the tissue explants. Explant cultures were stimulated with either SEB or LPS for 18 h and tissue collected and analysed by real-time PCR for mRNA expression of TLR-1, -2, -4 and –6. Expression for all four TLR mRNA was detectable within both unstimulated control tissue and mitogen stimulated explants (Figure 4.10). However, the use of only two PRD tissue specimens does not allow for any detailed analysis. Nevertheless, these data establish that TLR expression is present within PRD and that the tissue is responsive to mitogenic stimuli. However, further studies are necessary to elucidate through which TLR pathways endodontic-derived PAMPs exert their inflammatory effects within the PRD lesion.

LPS activates macrophages via TLR, inducing the production of proinflammatory cytokines including IL-1β and TNF-α, thereby activating fibroblasts and other cell types to produce T1/ST2. Subsequently, ST2 binds to macrophages and selectively represses the expression of several proinflammatory cytokines including IL-6, IL-12, IFN-γ and TNF-α (Sweet *et al.* 2001). T1/ST2 may therefore provide an effective endogenous mechanism to regulate the inflammatory response through its interaction with macrophages (Sweet *et al.* 2001). Therefore, I examined the effects of the addition of an ST2 neutralising antibody upon cytokine expression within the PRD explant cultures. However, neutralisation of ST2 in the PRD tissue cultures had no effect upon the expression of IL-6, IL-10, IL-17A, IFN-γ or TNF-α (Figure 4.11). The addition of anti-ST2 concomitant with LPS to PRD explants also had no effect upon the expression of these cytokines. Further studies are necessary to elucidate the expression of ST2 within the PRD lesion and define its relevance to the inflammatory disease process.

Figure 4.10 ***In vitro*** **effects of mitogenic stimulation upon mRNA TLR expression within 18 h PRD tissue explant cultures**. PRD tissue explants were stimulated with LPS or SEB for 18 h. Stimulated and unstimulated matched control tissues were subsequently analysed by real-time PCR for TLR mRNA expression. Bars represent relative mRNA expression between all analysed samples after normalisation to GAPDH. Experiments are representative of two PRD explant culture experiments.

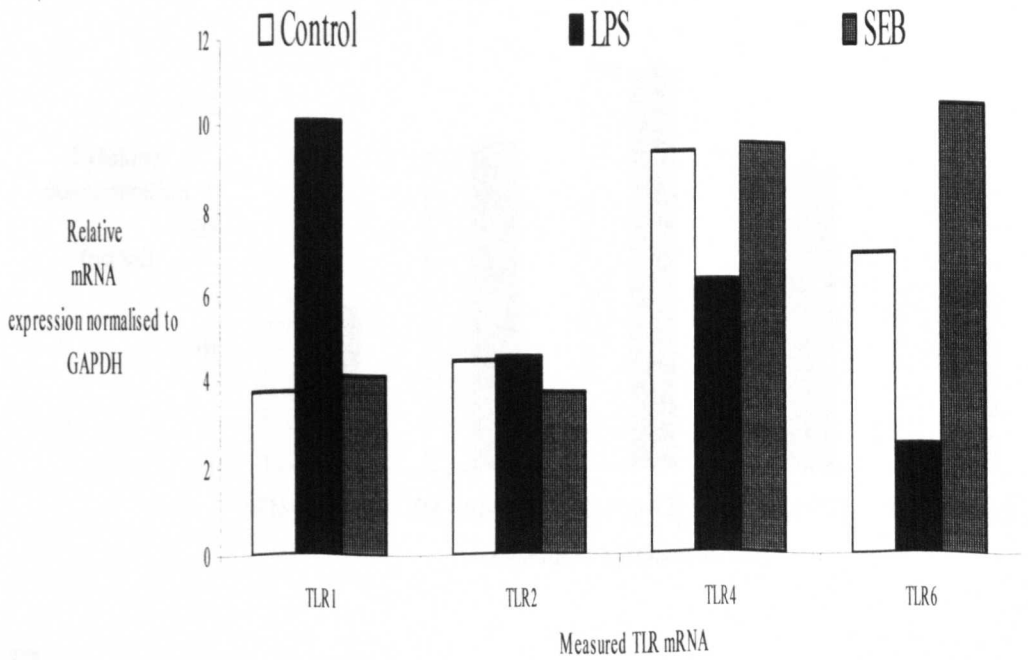
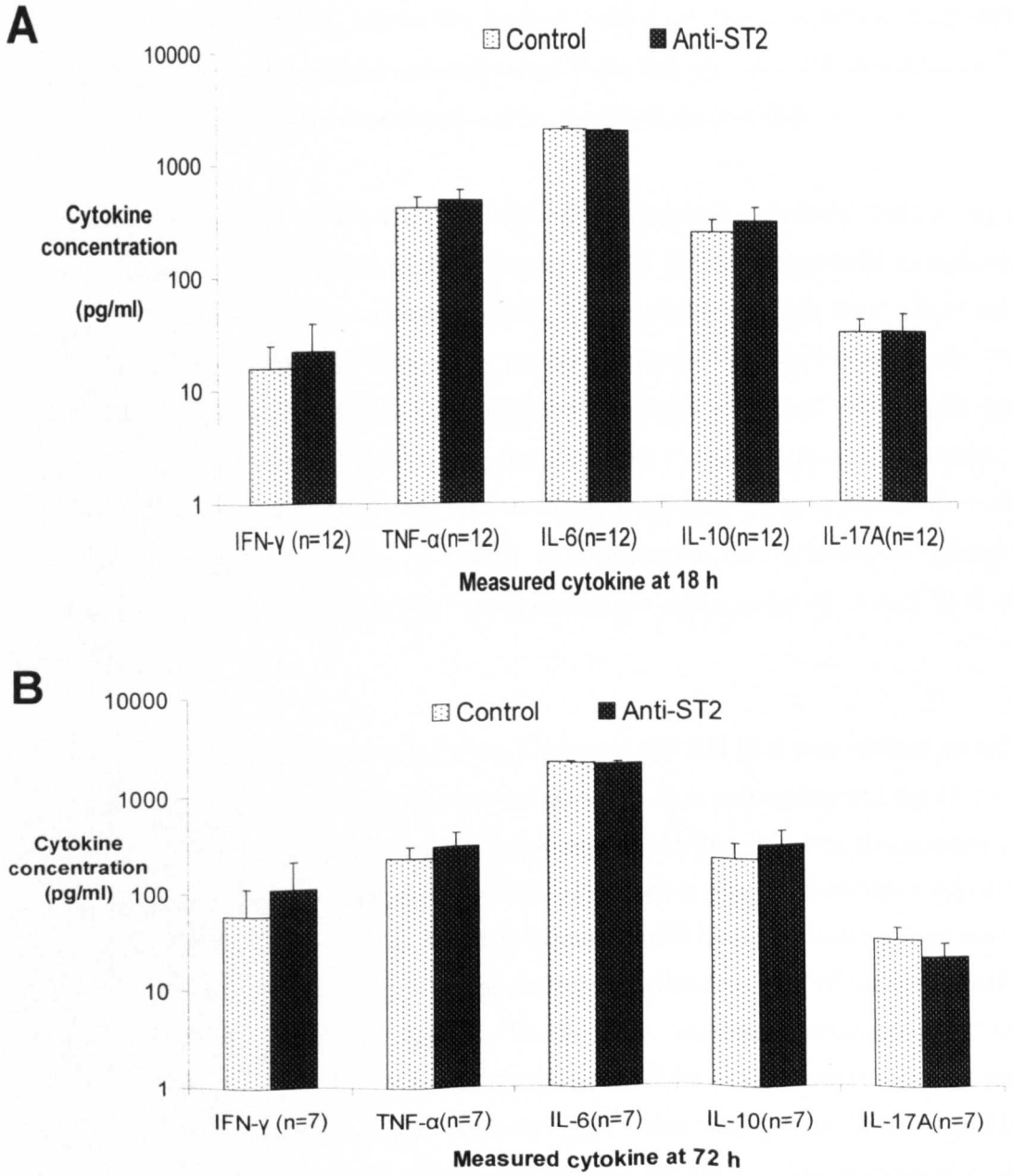


Figure 4.11 ***In vitro*** **effects of the addition of neutralising antibody to ST2 upon cytokine expression within PRD tissue explant cultures.** Anti-ST2 antibody was added to PRD explant tissues and supernatant collected and analysed by ELISA. (A) The mean concentration of each cytokine after neutralisation of ST2 compared with matched control for 18 h culture. (B) The mean concentration of each cytokine after neutralisation of ST2 compared with matched control for 72 h culture. The number of different patient-derived PRD tissues used in experiments for the measurement of each cytokine is given in parenthesis. Bars represent the mean concentration for each cytokine with the standard error of the mean.



4.6 Discussion

PRD tissues for explant culture were obtained sequentially from 310 patients undergoing periradicular surgery by the author or routine dental extractions. Previous studies demonstrate that of lesions derived from extracted teeth, 35-63% are periradicular abscesses, 5-50% are granulomas and 15-24% are periradicular cysts (Nair et al 1996, Vier and Figueiredo 2002). These lesions develop from the same infective origin and progress by similar processes. Furthermore, no significant differences have been found between the types or proportions of immunocompetent cells contributing towards these differing lesion types (Stern *et al.* 1981, Torabinejad and Kettering 1985, Gao *et al.* 1988, Matsuo *et al.* 1992, Liapatas *et al.* 2003). Given the limited volume of tissue available after dental extraction and the similar cellular composition of these lesions, the whole tissue lesion was used for each explant culture experiment without a pathological diagnosis.

Tissue explant cultures retain all their cellular components including their structural elements. Thereby, investigation of these integrated cell models can provide experimental advantages over isolated cell-culture systems. Tissue explant models have been widely used to investigate cytokine pathways in a variety of human diseases (Lordan *et al.* 2001). Surprisingly, no published studies have analysed functional responses within PRD tissue explant cultures. Recent evidence suggests that the cytokine network is central in orchestrating the cellular events that lead to the development of the periradicular lesion. Therefore, in initial experiments I sought to characterise the spontaneous release of endogenous cytokines by human PRD explant tissues cultured for 18 h and 72 h after surgical retrieval.

Significant release of monokines including TNF-α, IL-1β and IL-6 was evident within 18 h cultures. Although these cytokines were detectable at 72 h, culture beyond the 18 h point did not yield significant further accumulation suggesting that this was the optimal time point at which to observe functional activities. The high levels of constitutive expression of IL-6 and other cytokines within the PRD explant model likely indicates a high intrinsic activation status of the cells. This was also reflected by the detection of substantial NF-κB protein expression within the lesions. Furthermore, continued production of these inflammatory mediators to 72 h suggests the retention of the *in vivo* features present within the PRD lesion before removal from the bony crypt. Substantial release of IL-12 and IFN-γ, but little or no IL-4 or IL-5, strongly suggest that the PRD lesions examined in these

experiments were predominantly of a Th1 phenotype. This is in contrast to the observations from immunohistochemical experiments by others whom have reported a predominating Th2 phenotype (Walker *et al.* 2000, De Sá *et al.* 2003).

On encountering bacteria and their associated PAMPS from infected root canals, local host cells release inflammatory mediators to attract immune cells to the periradicular tissues. This establishes a localised inflammatory reaction within the tissue surrounding the dental root apex. Unless root canal treatment is undertaken to eradicate these pathogens or in cases where root canal treatment has failed, continual bacterial challenge from the root canal perpetuates this inflammatory process. This subsequently leads to the development and propagation of the chronic PRD lesion, resulting in periradicular granulomas or cysts.

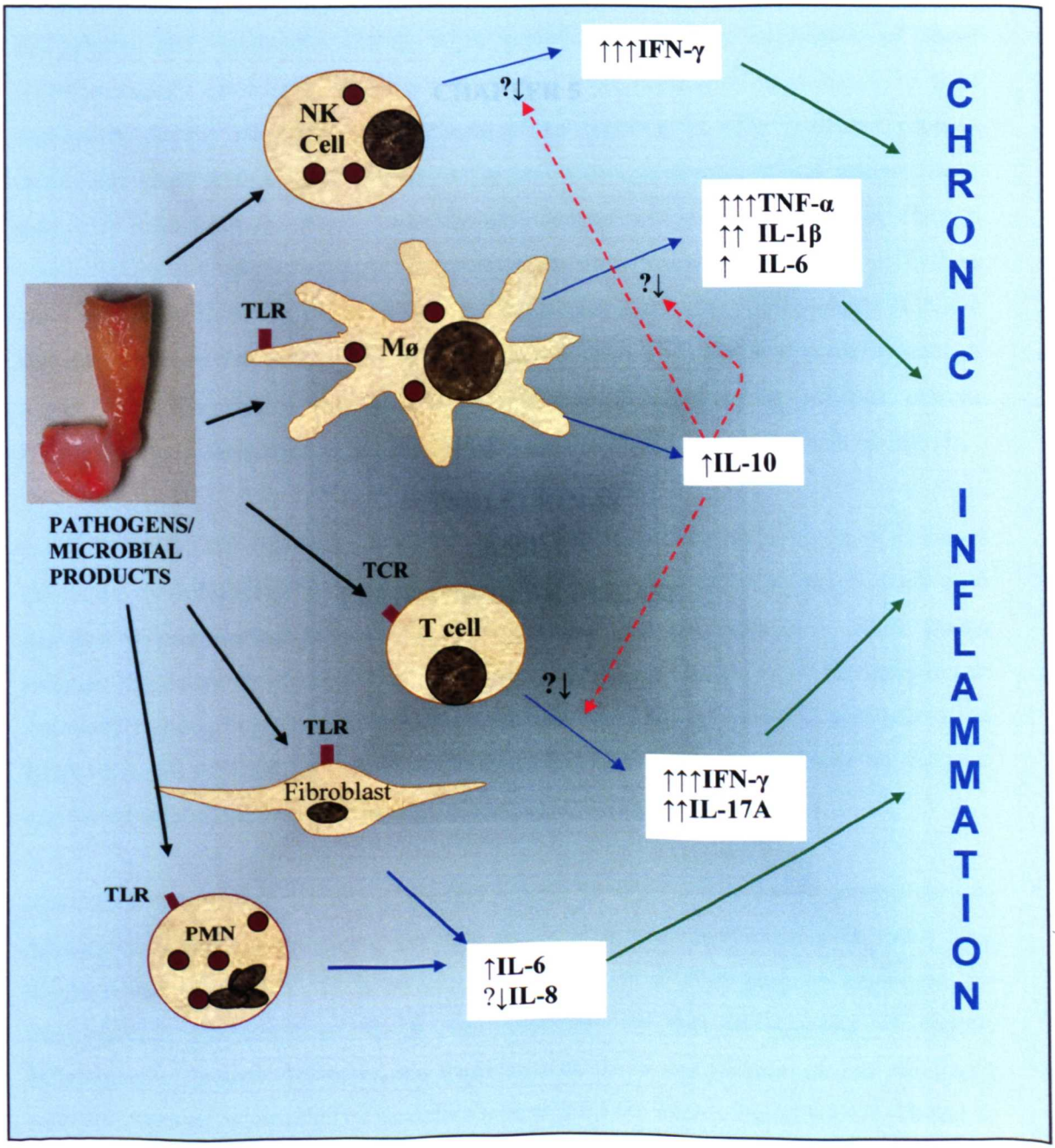
Substantial levels of LPS have been detected within the root canals of teeth associated with PRD lesions (Schein and Schilder 1975, Dahlen and Bergenholtz 1980, Horiba *et al.* 1991). Furthermore, high concentrations of LPS have been observed within the PRD tissue itself (Schonfeld *et al.* 1982). Nevertheless, it has been suggested that LPS alone may not be directly responsible for lesion development. More importantly, it has been purported that the induction of inflammatory cytokines by LPS may be more significant in the initiation and perpetuation of PRD than direct effects of LPS (Wang and Stashenko 1993a, Wang and Stashenko 1993b). Moreover, in cases of persisting PRD arising after failed RCT, the root canal flora is primarily composed of Gram-positive microorganisms (Siren *et al.* 1997, Sundqvist *et al.* 1998, Pinheiro *et al.* 2003, Gomes *et al.* 2004). The release of exotoxins by these Gram-positive pathogens may therefore be as, if not more influential than bacterial-derived LPS in contributing towards lesion development and chronicity. It is therefore of central importance to establish the contribution of PAMPS in eliciting endogenous cytokine responses, which may directly contribute towards the inflammatory reaction occurring within the PRD lesion. I therefore investigated the functional effects of Gram-positive and Gram-negative moieties upon endogenous cytokine expression within the PRD lesion by developing the novel human PRD tissue explant culture system.

The Gram-negative derived mitogen, LPS promoted a strong inflammatory response within PRD tissue explant cultures. Addition of LPS to explant cultures resulted in significant increases in proinflammatory cytokines IL-1β, TNF-α and IFN-γ and had no effect upon IL-6, IL-8 and IL-17A. This powerful proinflammatory reaction to LPS challenge is likely regulated by concomitant IL-10 release by cellular components of the PRD lesion.

Interestingly, LPS exerted this potent proinflammatory effect upon cytokine expression over long-term cultures at 72 h. The T cell superantigen SEB, a component of some Gram-positive bacteria, also induced a strong proinflammatory effect that resulted in increased secretion of the inflammatory mediators IL-1β, IL-17A, TNF-α and IFN-γ. Moreover, the addition of SEB to explant cultures substantially increased secreted levels of TNF-α and IFN-γ at both 18 h and in long-term culture to 72 h. Rapid production of these cytokines likely propagates the release of downstream inflammatory mediators including chemokines and matrix metalloproteinases. Thereby, further contact with bacterial products from the dental root canal results in chronic inflammation within the PRD lesion and concomitant destruction of the local dento-alveolar bone matrix.

It is most probable that the observed effects of these PAMPs upon endogenous cytokine expression within the PRD explants were regulated via TLR signalling pathways. Indeed, the experiments established that Toll-like receptors were expressed within the lesion. However, further work is required to study a larger number of tissues in order to elucidate the relevance of their expression within PRD and thereby, their contribution to destructive inflammatory pathways. Surprisingly, IL-4 was neither constitutively expressed within PRD tissue explants nor was it inducible by explant stimulation with microbial-derived moieties. In contrast, the stimulation of explants with microbial moieties resulted in substantially increased quantities of IFN-γ. These data further support the hypothesis that the human PRD lesion is dominated by a Th1 phenotype. A summary of the data from these experiments is presented in Figure 4.12.

Figure 4.12 Diagrammatic illustration summarising cytokine and potential cellular events occurring within the chronic inflammatory PRD lesion in response to PAMPs. NK- Natural Killer cell; Mø- macrophage/ dendritic cell; PMN- neutrophil. Microbial challenge of cells within the periradicular lesion and surrounding periradicular tissue induces a marked pro-inflammatory response with release of cytokines that exhibit potent inflammatory properties. Their continued release results in chronic inflammation. The concomitant secretion of IL-10 likely counters and down-regulates the extent of this inflammatory cascade, preventing exaggerated tissue destruction (broken red arrow).



CHAPTER 5

INTERLEUKIN-18
AND
PERIRADICULAR DISEASE

5 INTERLEUKIN-18 AND PERIRADICULAR DISEASE

5.1 Introduction

Recent evidence from animal studies implies a prominent role of pathways of innate and acquired immunity in the pathogenesis of PRD. Cytokines have garnered considerable interest as potential mediators of these inflammatory pathways. Several studies have identified the expression of IL-1, IL-6, IL-8 and TNF-α at the initiation of PRD and subsequently during chronic stages of the disease (Tani-Ishii *et al.*1995, Wang *et al.* 1997, Kawashima and Stashenko 1999). It is established that the expression of these proinflammatory cytokines and the presence of LPS are capable of inducing IL-18 gene expression thereby directing the synthesis of an inactive 24 kDa precursor product (Nakanishi *et al.* 2001). IL-18 augments the production of several critical inflammatory mediators implicated in chronic inflammatory diseases and inflammatory bone disease. These include the up-regulation of the proinflammatory cytokines IL-1β, IL-6 or TNF-α, the chemokines IL-8, MCP-1 and MIP-1a, the intracellular adhesion molecule ICAM-1 and inflammatory mediators GM-CSF, iNOS and iCox-2. Through these proinflammatory actions, IL-18 mediated processes are implicated in contributing towards chronic inflammatory diseases such as RA (reviewed in Gracie *et al.* 1999, McInnes *et al.* 2000).

IL-18 is capable of regulating cytokine production during the early phase of bacterial infections by influencing IL-12 production. Thereby, IL-18 provides a protective effect to the host in contributing towards the eradication of microbial pathogens within innate immune responses. Recent evidence further implicates a role for IL-18 within early innate immunity through its properties of neutrophil recruitment and activation. Nevertheless, the range of IL-18 activities so far reported suggest that this novel cytokine plays an essential regulatory role within cell-mediated immunity against foreign pathogens.

Elevated levels of both IL-18 mRNA and protein have been detected in several chronic inflammatory diseases embracing infection and bone destruction (Gracie *et al.* 1999). This suggests that although IL-18 is an essential component of host immune responses, the inappropriate production of IL-18 may contribute to the pathogenesis of chronic inflammatory diseases. However, the expression of IL-18 and its receptor has previously not been reported within PRD. I therefore investigated the expression of both IL-18 and its corresponding signalling receptor within the PRD lesion. Furthermore, using the PRD

explant culture model that I had established from the previous set of experiments, I investigated the functional effects of rhIL-18 addition to human PRD tissue cultures upon endogenous cytokine expression. Thereby, I intended to elucidate the mechanisms by which IL-18 may regulate inflammatory events occurring within the human PRD lesion.

5.2 IL-18 is expressed within inflamed human periradicular tissues

Given the importance of IL-18 in the host response within infectious diseases, I undertook initial experiments to determine if IL-18 mRNA expression was detectable within the PRD lesion. Using RT-PCR, IL-18 mRNA was detected in 70 of 90 PRD tissues (Figure 5.1A). However, in non-inflamed pulp tissue samples IL-18 mRNA was not expressed (n=11 tissues). In addition to β-actin, THP cells stimulated with LPS and IFN-γ and/or RA synovium served as positive controls. Negative controls comprised H_2O without the addition of cDNA. Having established that IL-18 mRNA was expressed within inflamed PRD lesions, I also determined its relative expression by using quantitative-PCR within a set of 23 PRD lesions (Figure 5.1B)

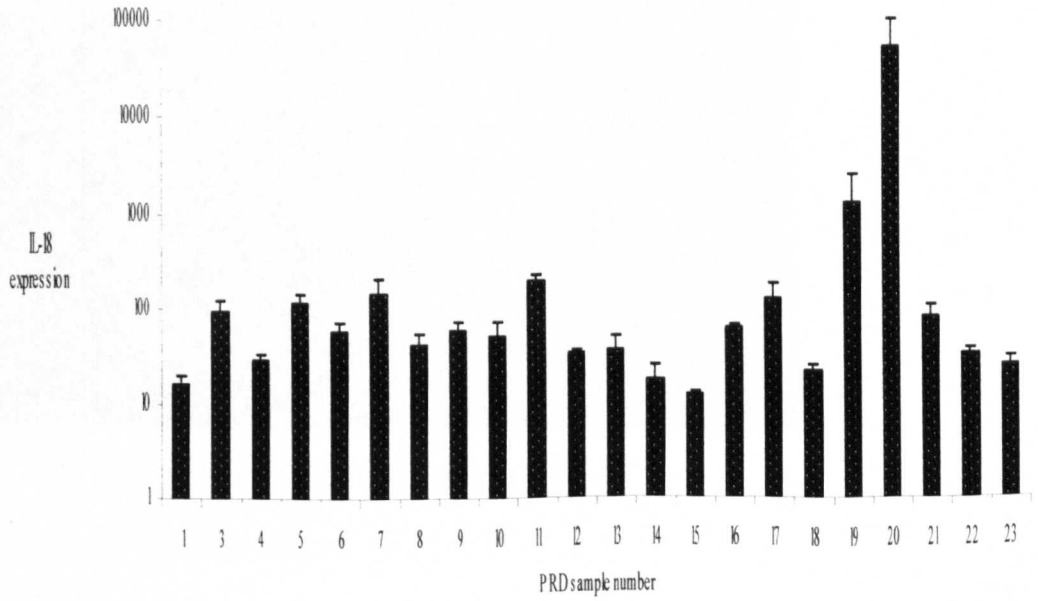
Using immunohistochemistry, I next sought to determine the cellular expression of IL-18. Widespread IL-18 expression was observed throughout the PRD lesion, within leukocyte aggregates and in diffuse interstitial tissue areas (Figure 5.1C). Double IHC staining revealed that the majority of IL-18 expressing cells were $CD68^+$, indicative of macrophage origin. In contrast, $CD3^+$ T cells were consistently IL-18 negative (Figure 5.1D-F). Tonsil tissue was additionally stained as a positive control. Periodontal ligament, a further control, exhibited no IL-18 expression commensurate with the absence of inflammatory infiltrate. Staining was neutralised by the addition of rhIL-18 to the anti-IL-18 antibody prior to section incubation. The specificity of the antibody to detect IL-18 was additionally confirmed by a lack of neutralisation when the IL-18 antibody was preincubated with the structurally related rhIL-1β protein.

Since currently available anti-IL-18 antibodies cannot distinguish mature and pro forms of IL-18 using IHC, I performed Western blot analysis to confirm the presence of mature IL-18. The majority of PRD samples (n=14) exhibited IL-18 present in both pro (23 kDa) and mature (18 kDa) forms, whereas in non-inflamed pulp tissues (n=12), only pro-IL-18 was detected (Figure 5.1G). This suggested that IL-18 was present in healthy mucosal tissue but cleaved to the mature biologically form only in the context of inflammation.

Figure 5.1 Identification of IL-18 expression within periradicular disease. (A) IL-18 mRNA detected within inflamed PRD tissues by RT-PCR. (B) Relative expression of IL-18 within PRD tissues to GAPDH by Q-PCR in 23 PRD lesions. (C) IHC single stain for IL-18 within PRD, arrows represent IL-18 positive cells. (D) Double stain IHC for IL-18 within PRD; blue arrow-$CD3^+$ cells, orange arrow- IL-18 positive cells. (E) Double stain IHC for IL-18 within PRD; blue arrow-$CD68^+$ cells, orange arrow- IL-18 positive cells (F) Western Blot of PRD tissues for IL-18, establishing mature IL-18 to be present within inflamed PRD tissues.

A

B



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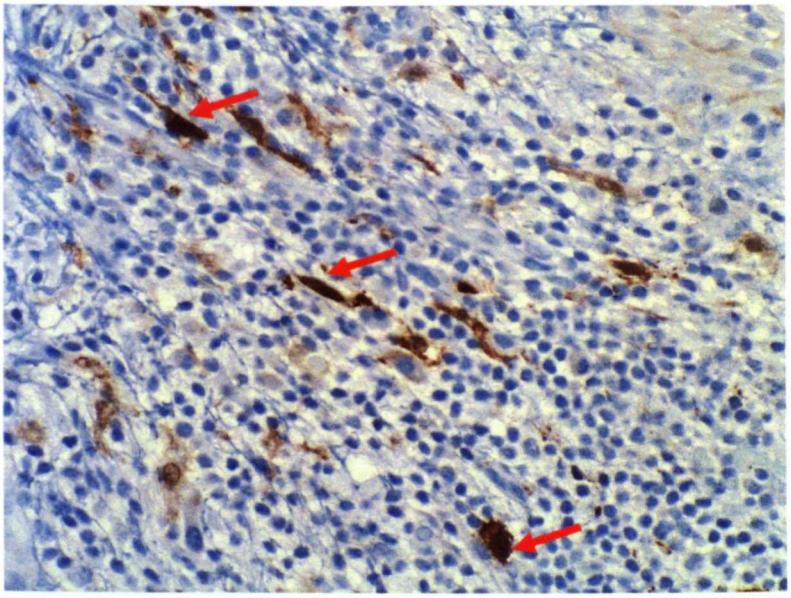

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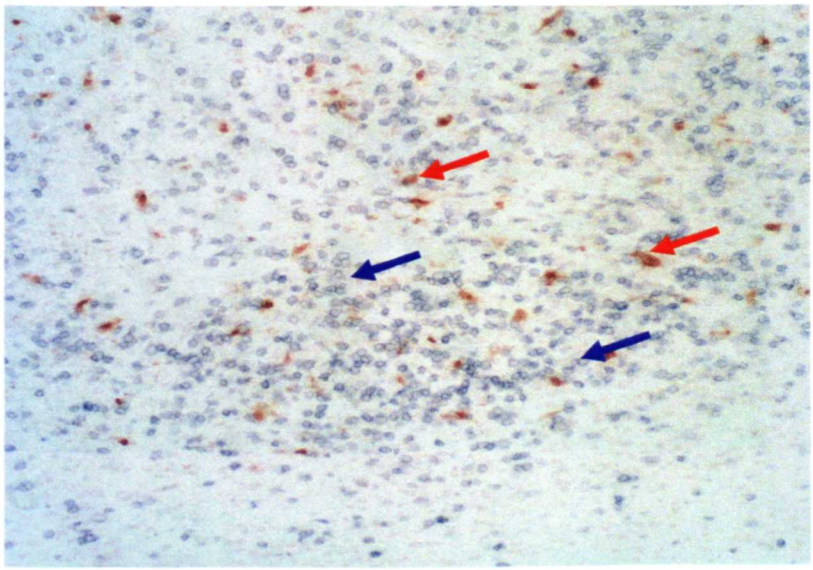

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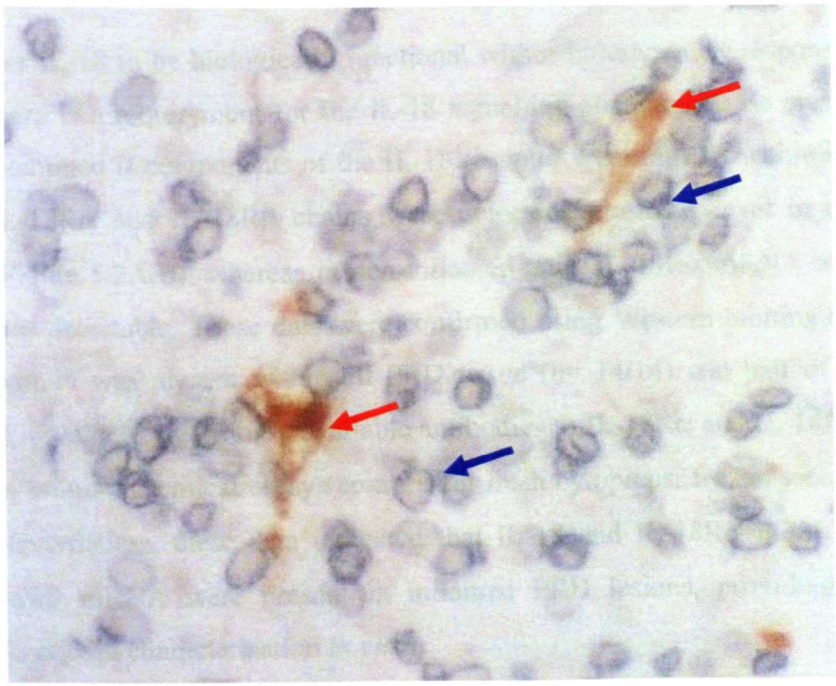

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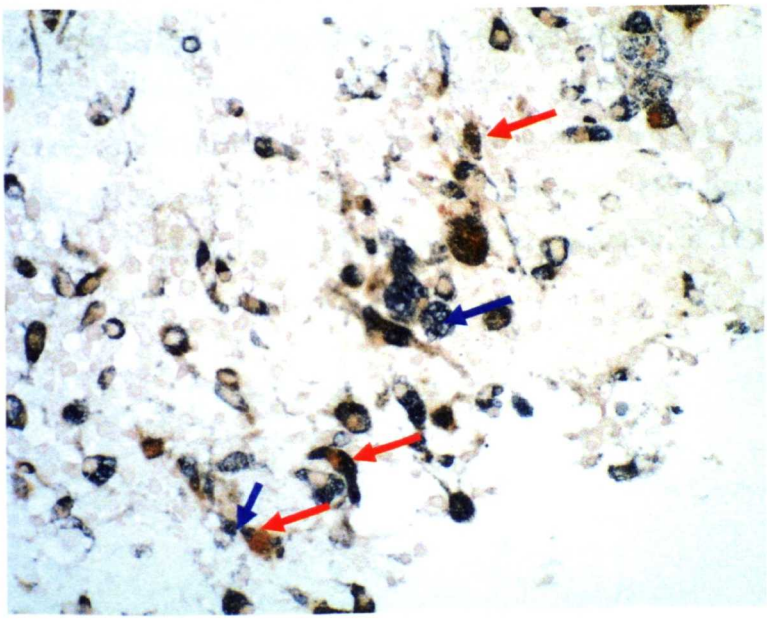

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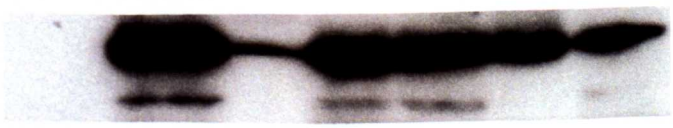


24 kDa Pro IL-18

18 kDa Mature IL-18

5.3 IL-18 receptors and IL-18BP are expressed in inflamed human PRD tissues

For IL-18 to be biologically functional within inflammatory responses in the PRD lesion, there is a requirement for the IL-18 signalling apparatus to be present. Therefore, I next examined if components of the IL-18 receptor were present within inflamed PRD tissues. IL-18Rα and IL-18Rβ chains were detected at mRNA level in inflamed PRD tissues (Figure 5.2A,B), whereas in non-inflamed pulp, IL-18Rα mRNA but no IL-18Rβ mRNA was detectable. These data were confirmed using Western blotting that revealed IL-18Rα protein was present within all PRD tissue (n= 14/14) and half of pulp tissue (n = 3/6) (Figure 5.2C). Currently available antibodies to IL-18Rα and IL-18Rβ were not functional in immunochemical assays comprising fresh cytopreps, frozen sections or FFPE sections. Nevertheless, these data indicated that IL-18 and IL-18Rα mRNA and protein and IL-18Rβ mRNA were present in inflamed PRD lesions, providing rationale for further functional characterisation *in vitro*.

IL-18 expression is tightly regulated by the concomitant production of IL-18BP. I therefore also investigated the expression of IL-18BP within the PRD lesion (Figure 5.3A,B). mRNA expression for IL-18BPa subunit was detectable within only 5/11 PRD tissue lesions and IL-18BPc mRNA was observed in 3/11 lesions. Gene expression for IL-18BPb and d subunits was not detectable within any of the PRD tissues examined. Using Western blotting, IL-18BPa was present within the majority of PRD protein lysates (n=18/20, Figure 5.3C)

Figure 5.2 Identification of IL-18 receptor within periradicular disease tissues. (A) IL-18Rα mRNA detected by RT-PCR within inflamed PRD tissues. Results are representative of three independent experiments with similar results. Stimulated PBMC were used as positive controls for cDNA quantification. (B) IL-18Rβ mRNA expression detected within inflamed PRD tissues. (C) IL-18Rα protein expression detected within PRD tissue protein lysates.

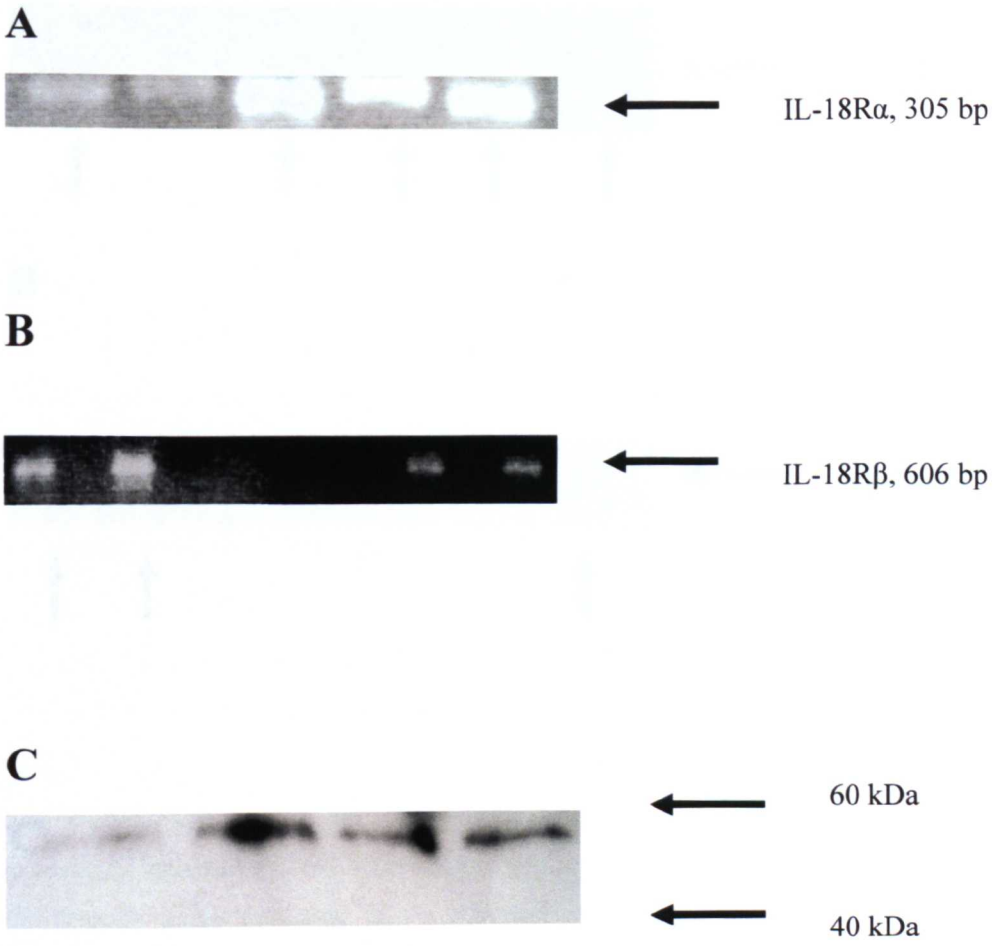
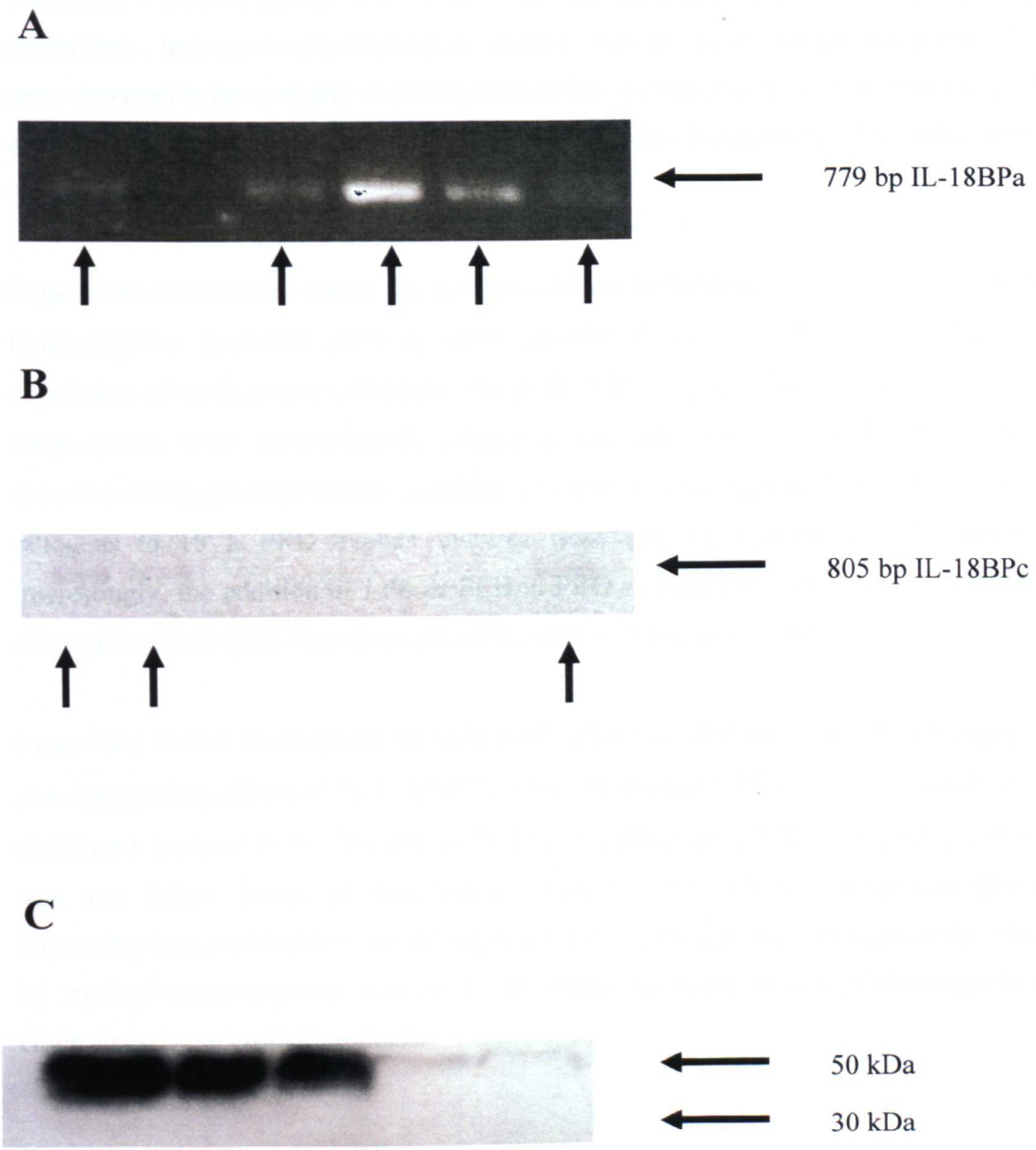


Figure 5.3 Identification of IL-18BP within PRD. (A) PCR detection of IL-BPa gene expression within PRD tissues, positive specimens identified by arrow. (B) mRNA for IL-18BPc chain detectable within only 3/11 PRD specimens by RT-PCR (C) Western blot detection of IL-18BPa within 18/20 PRD protein specimens.



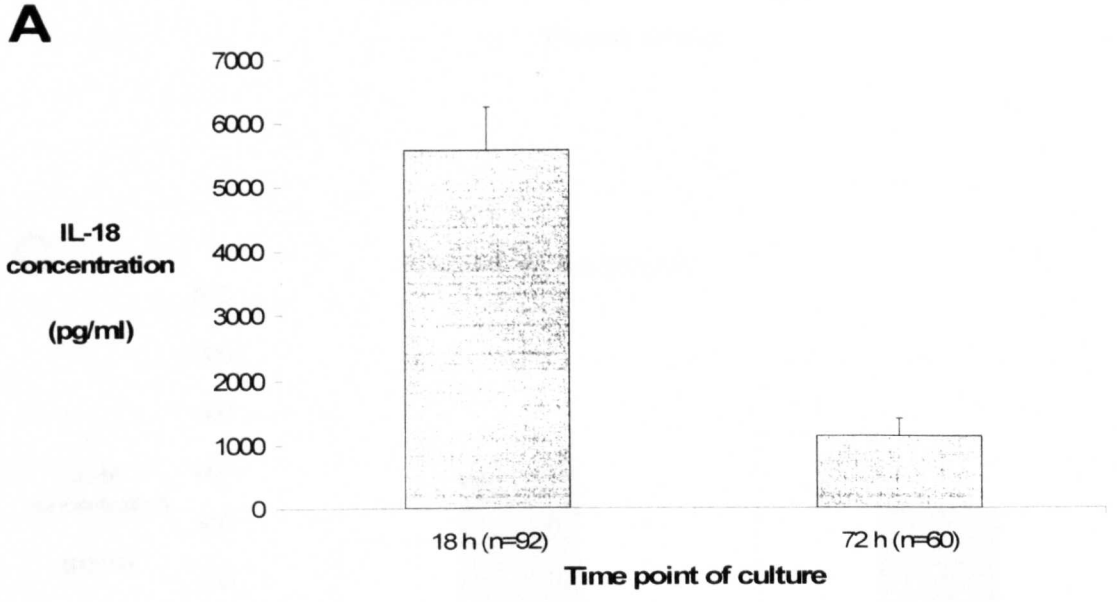
5.4 *In vitro* effects of Gram-negative and Gram-positive mitogens upon IL-18 expression within human PRD explant cultures

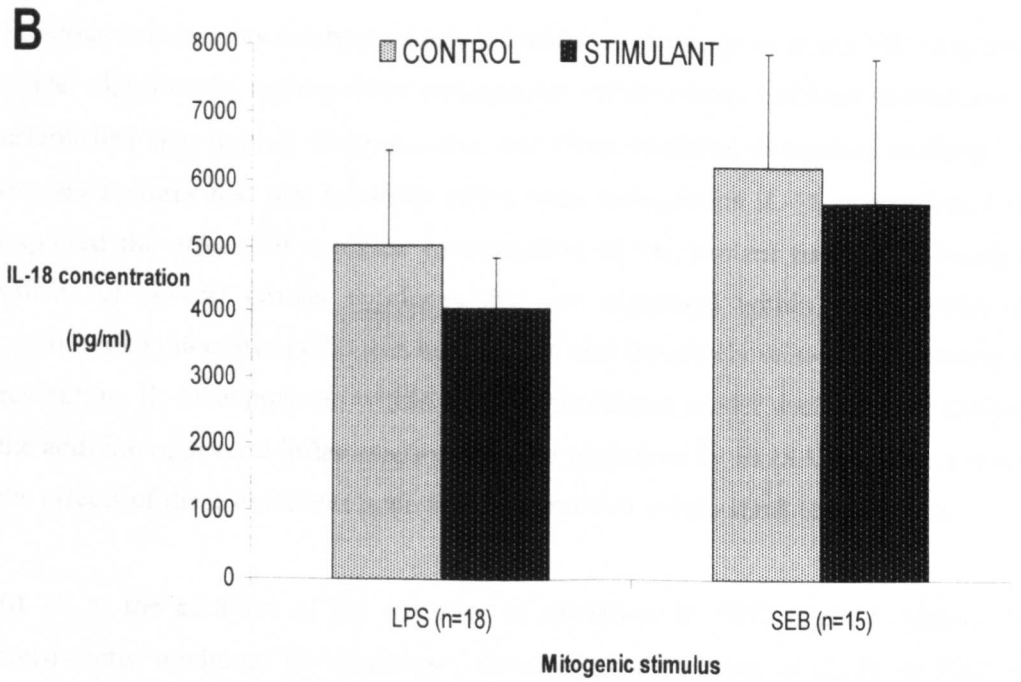
The previous series of experiments ascertained that IL-18 was present within inflamed PRD tissue lesions and that its receptor subunits were also expressed within these tissues. Therefore, I next examined if secreted IL-18 was detectable within the explant culture model that I had previously established. Indeed, high levels of spontaneous IL-18 release were observed in the majority of PRD tissue explant culture supernatants examined at 18 h. Furthermore, moderate levels of secreted IL-18 were also detectable at 72 h explant culture (Figure 5.4A).

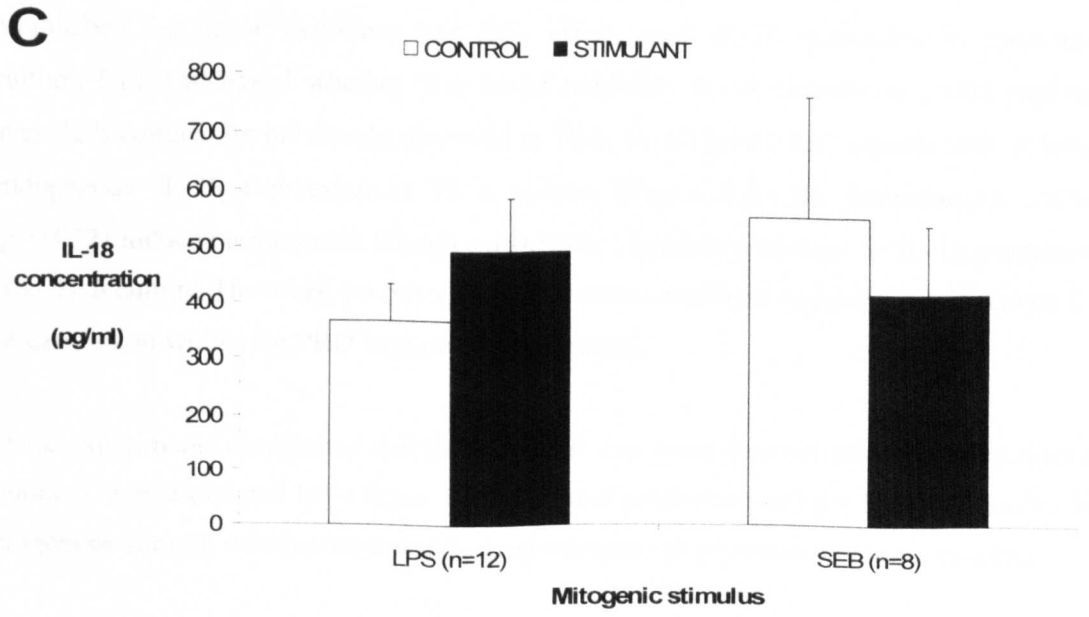
Experiments undertaken within the previous chapter demonstrated that Gram-positive and Gram-negative microbial products were capable of inducing marked changes in the expression of endogenous cytokines within the PRD explant culture system. Moreover, these effects were predominantly related to the induction of significantly increased secretion of key proinflammatory cytokines. I therefore investigated if the addition of these mitogens to 18 h PRD explant cultures were able to modulate IL-18 expression. Interestingly, the addition of LPS or SEB to PRD explant tissue cultures at 18 h had no observable effect upon the expression of secreted IL-18 (Figure 5.4B).

I therefore further investigated the effects of mitogenic challenge upon IL-18 expression over longer-term culture to 72 h. After 72 h explant culture, LPS induced a minimal but not significant increase in IL-18 expression. SEB slightly reduced IL-18 expression but this was also below levels of significance (Figure 5.4C). These experiments provided interesting data establishing that the expression of IL-18, although spontaneously released by 'resting' tissue explants, was not further inducible by the effects of Gram-positive or Gram-negative microbial products.

Figure 5.4 ***In vitro*** **effects of mitogenic stimulation upon IL-18 expression within PRD explant cultures**. 'Resting' levels of spontaneously released IL-18 within PRD tissue explant culture supernatants were initially assessed. (A) Unstimulated PRD explant tissue culture supernatant was harvested at 18 h and at 72 h and IL-18 concentrations determined by ELISA. PRD explant tissues were subsequently stimulated with LPS or SEB and supernatant collected and analysed by ELISA. (B) The mean concentration of IL-18 supernatant after 18 h mitogen stimulation compared with unstimulated control. (C) The mean concentration of IL-18 supernatant after 72 h mitogen stimulation compared with unstimulated matched control. The total number of PRD explant tissues analysed in each experiment is given in parenthesis. Bars represent the mean IL-18 concentration with the standard error of the mean.




B
CONTROL
STIMULANT
8000
7000
6000
5000
4000
3000
2000
1000
0
IL-18 concentration
(pg/ml)
LPS (n=18)
SEB (n=15)
Mitogenic stimulus


C
CONTROL
STIMULANT
800
700
600
500
400
300
200
100
0
IL-18
concentration
(pg/ml)
LPS (n=12)
SEB (n=8)
Mitogenic stimulus

5.5 *In vitro* effects of cytokine addition to human PRD explant cultures upon endogenous IL-18 expression

Previous experiments established that the addition of mitogens to the PRD explant culture model significantly up-regulated endogenous inflammatory cytokine expression. Having established that neither Gram-positive nor Gram-negative mitogenic challenge of PRD explant cultures had any inducible effect upon endogenous IL-18 expression, I therefore explored the effects of cytokine manipulation of the explant tissues. I investigated the effects of several critical cytokines that are expressed within inflammatory immune responses to infectious pathogens and that are also intimately related to inflammatory bone resorption. IL-18 expression within this explant culture model was therefore analysed after the addition of several inflammatory cytokine mediators by ELISA. Initially, I investigated the effects of these cytokines upon IL-18 expression within short-term culture.

At 18 h, the addition of the majority of cytokines to PRD explant cultures failed to consistently modulate IL-18 release. However, the addition of IL-10 to PRD explants induced a significant reduction in IL-18 expression ($p<0.010$). The addition of IFN-γ ($p=0.143$) and TNF-α ($p=0.058$) resulted in minor reductions of IL-18 expression that were both below levels of statistical and biological significance (Figure 5.5A,B). Having established that these cytokines had little effect upon IL-18 expression in short-term culture, I next analysed whether they could modulate IL-18 expression within explants over 72 h culture. As previously observed at 18 h, IL-10 ($p=0.0101$) significantly reduced endogenous IL-18 expression at 72 h culture (Figure 5.6A,B). Interestingly, MCSF ($p=0.032$) induced a moderate though statistically significant increase in IL-18 expression after 72 h culture. However, no other cytokines were capable of regulating endogenous IL-18 expression within the PRD explant culture system.

These experiments established that IL-18, which was spontaneously released in substantial amounts in unstimulated PRD tissue, is at maximal production and not further inducible by exogenous stimuli, whether bacterial-derived mitogens or pro-inflammatory cytokines.

Figure 5.5 ***In vitro*** **effects of 18 h cytokine stimulation upon IL-18 expression within PRD explant cultures**. PRD explant tissues were stimulated with a panel of cytokines for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of IL-18 after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IL-18 concentration between stimulated and unstimulated matched control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-18 concentration with the standard error of the mean.

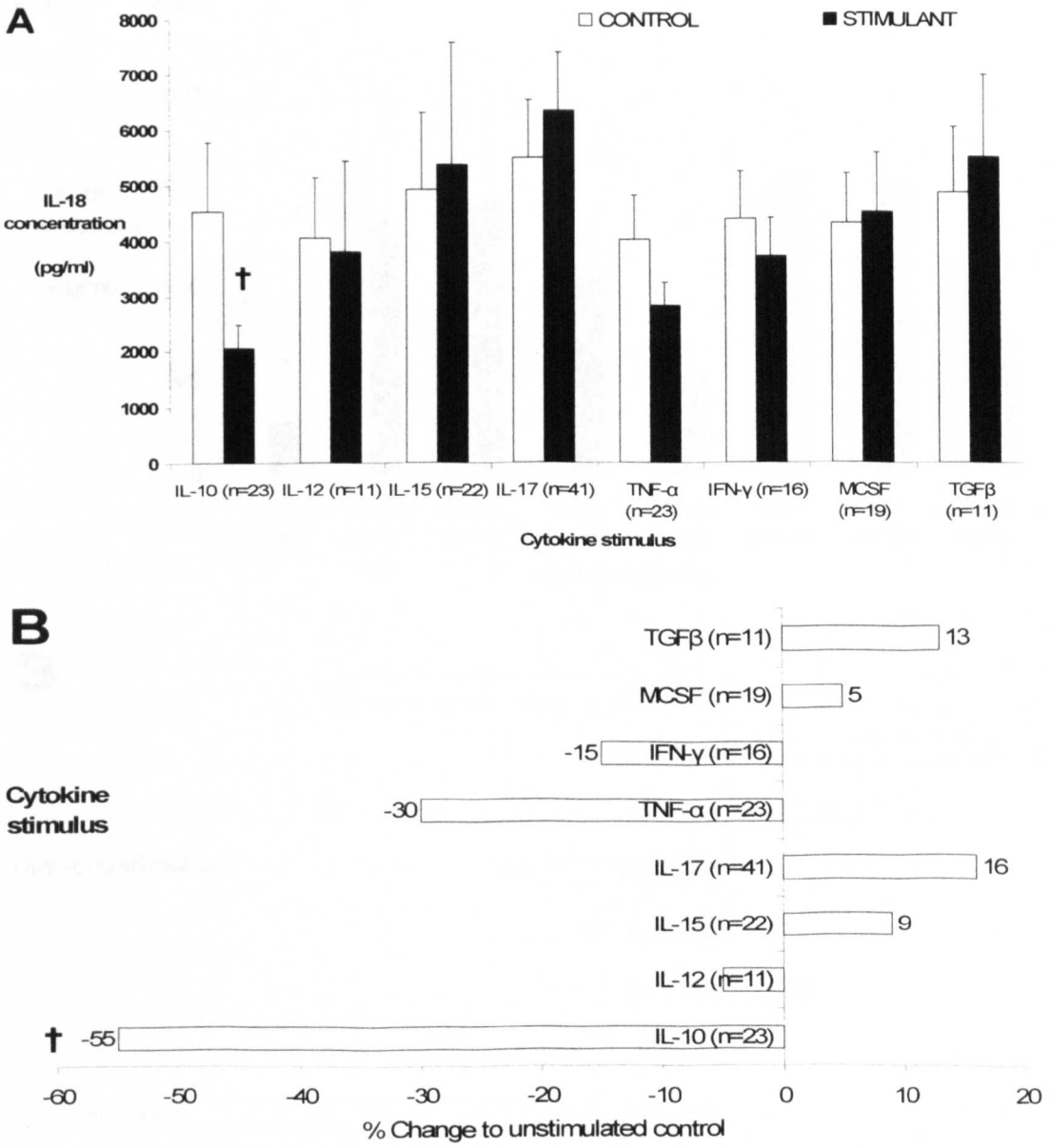
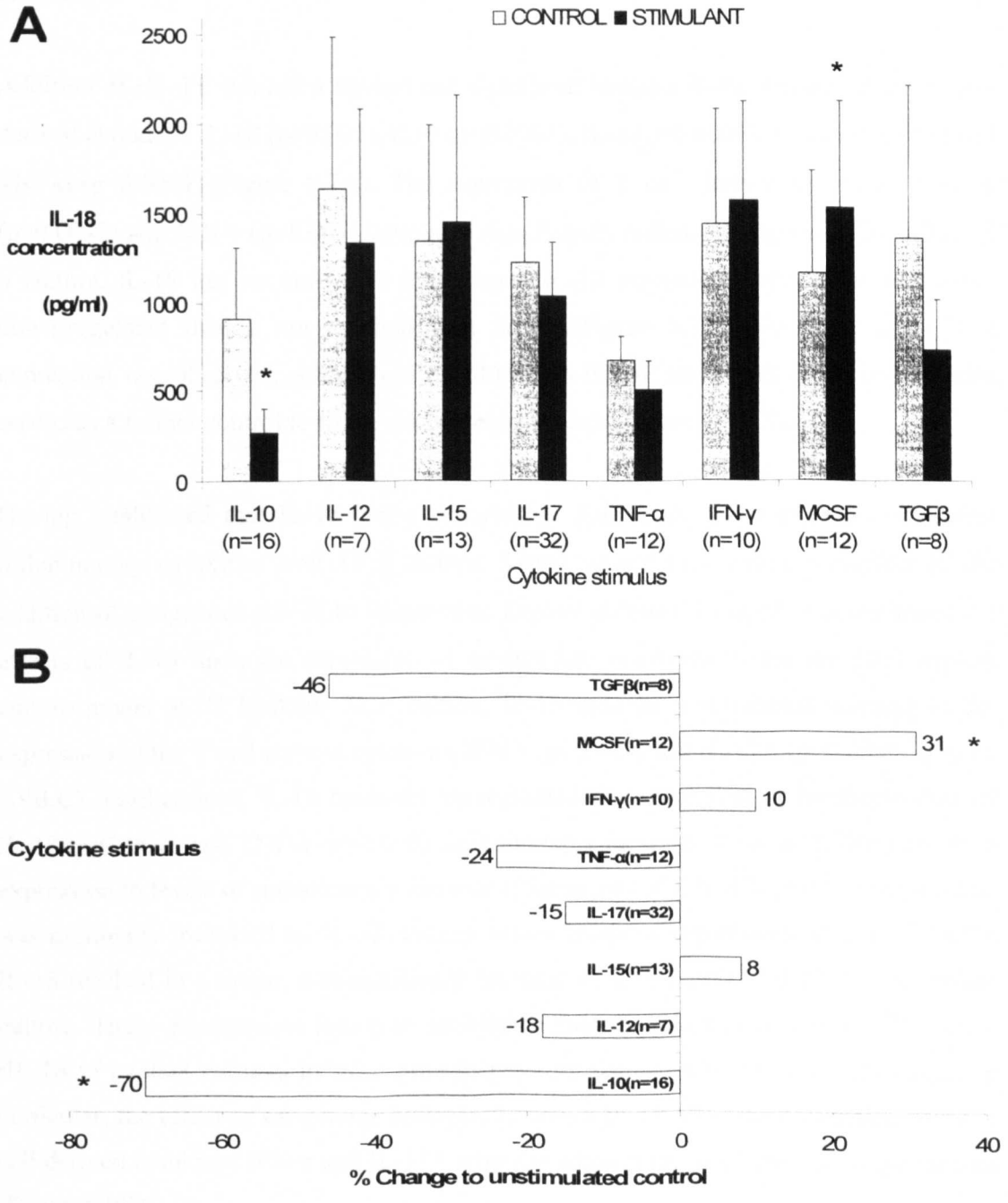


Figure 5.6 ***In vitro*** **effects of 72 h cytokine stimulation upon IL-18 expression within PRD explant cultures**. PRD explant tissues were stimulated with a panel of cytokines for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of IL-18 after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IL-18 concentration between stimulated and unstimulated matched control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-18 concentration with the standard error of the mean.



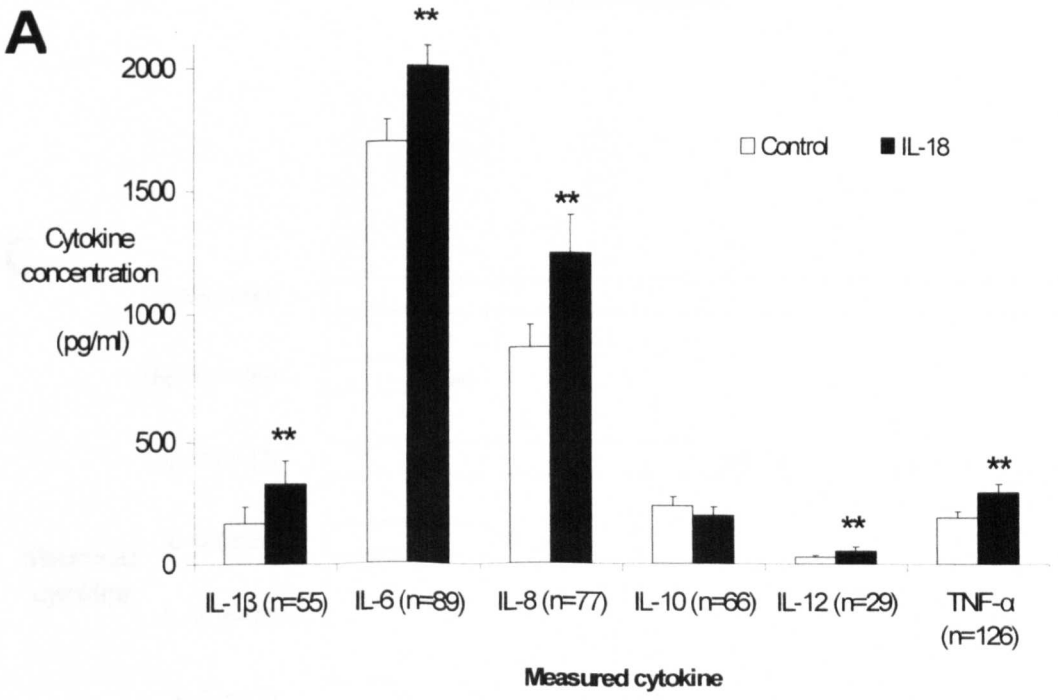
5.6 *In vitro* effects of exogenous IL-18 addition to PRD explant cultures

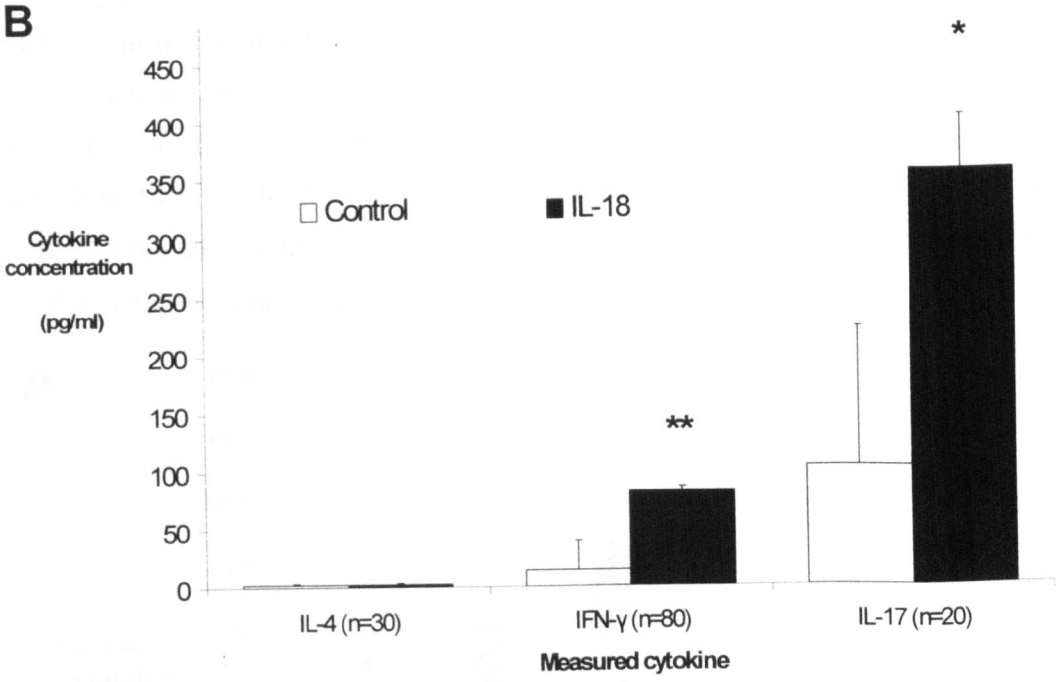
From the Western blot experiments, IL-18 was detectable in its 18 kDa biologically active form within PRD lesions. It was therefore of interest to explore the potential contribution of IL-18 to inflammatory pathways within the PRD lesion. I therefore investigated the effect of adding additional bioactive rIL-18 to the 18 h PRD explant culture. IL-18 was used at concentrations defined as optimal through previous experiments within our laboratory and subsequently by others (Gracie *et al.* 1999, Monteleone *et al.* 1999, Dai *et al.* 2004)

Addition of rIL-18 induced a modest but significant increase in the release of monocyte-derived cytokines IL-1β ($p=0.001$), IL-6 ($p=0.0001$), IL-8 ($p=0.0001$), IL-12 ($p=0.001$) and TNF-α ($p=0.0001$) (Figure 5.7A). The expression of T cell derived cytokines IL-17A ($p=0.0042$) and IFN-γ ($p=0.007$) were also significantly enhanced (Figure 5.7B). After 18 h culture, IL-18 had no consistent effect upon IL-10 expression, which was minimally down-regulated though not at significant levels (Figure 5.7C). No effect upon IL-4 expression was observed. Addition of IL-18 to 18 h PBMC and whole blood cell cultures served as a further control to tissue explant experiments (Figure 5.8A,B).

Having established that IL-18 had a stimulatory effect upon the expression of several inflammatory cytokines over 18 h culture, I next sought to examine the effect of the addition of exogenous rIL-18 to longer-term explant cultures. I therefore investigated the effects of IL-18 upon the expression of several key cytokines within the PRD explant culture model at 72 h. After 72 h culture, IL-18 induced a substantial increase in the expression of the T cell derived cytokines IFN-γ ($p=0.009$) and IL-17A ($p=0.0008$) (Figure 5.9B,C). Furthermore, IL-18 modestly up-regulated the expression of monocyte derived IL-1β ($p=0.064$), and TNF-α ($p=0.009$) and minimally increased IL-6 ($p=0.0001$) and IL-8 expression to levels of statistical significance (Figure 5.9A,C). IL-12 ($p=0.076$) expression was minimally increased by IL-18, though below levels of significance (Figure 5.9A,C). IL-18 resulted in a minor, non-significant decrease of IL-10 ($p=0.177$) after 72 h explant culture. These experiments therefore established that the addition of biologically active rIL-18 to explant cultures induced proinflammatory pathways within the PRD lesion. In particular, the effect of exogenous biologically active IL-18 upon the expression of the T cell derived cytokines IFN-γ and IL-17A over the whole term of culture was quite marked (Figure 5.9D).

Figure 5.7 ***In vitro*** **effects of IL-18 manipulation in 18 h PRD explant cultures.** PRD explant tissues were stimulated with rhIL-18 for 18 h and culture supernatant collected and analysed by ELISA. (A) The mean concentration of a panel of monokines after explant tissue stimulation with rhIL-18 compared with unstimulated control. (B) The mean concentration of a panel of T cell-derived cytokines after explant tissue stimulation with IL-18 compared with unstimulated control. (C) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated matched control tissues. The total number of PRD explant tissues stimulated with IL-18 for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.




B
*
450
400
350
Control
IL-18
Cytokine concentration
(pg/ml)
300
250
200
150
**
100
50
0
IL-4 (n=30)
IFN-γ (n=80)
IL-17 (n=20)
Measured cytokine

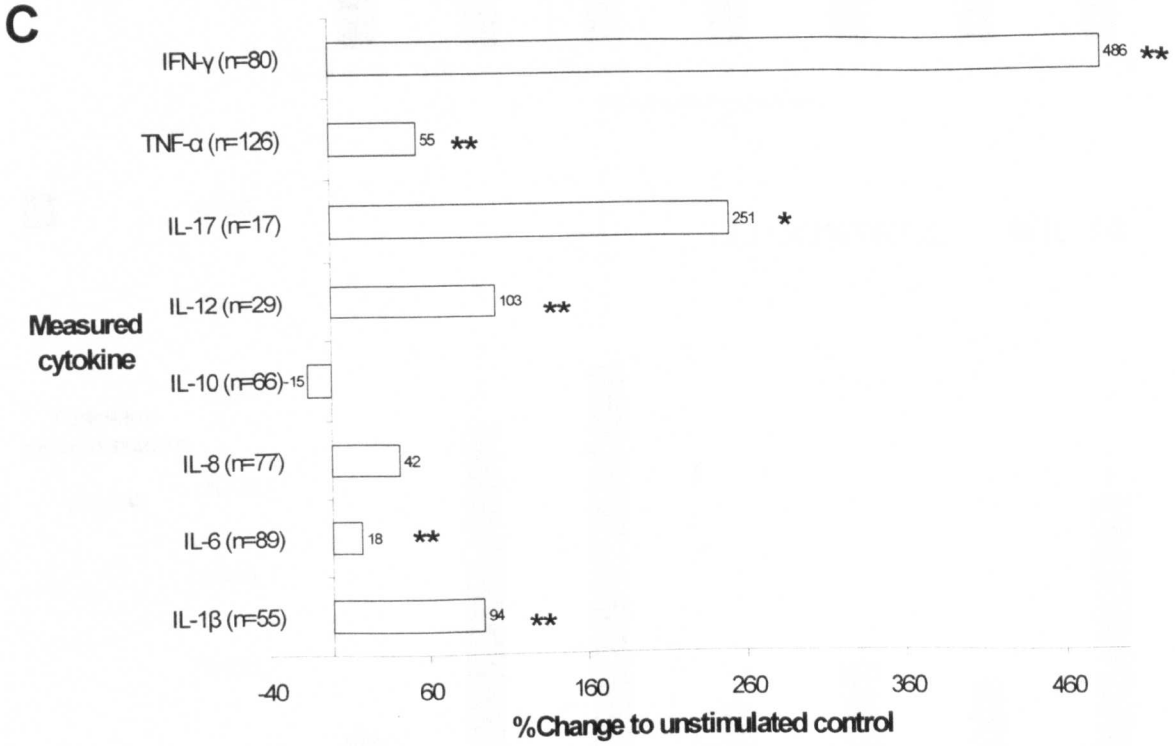
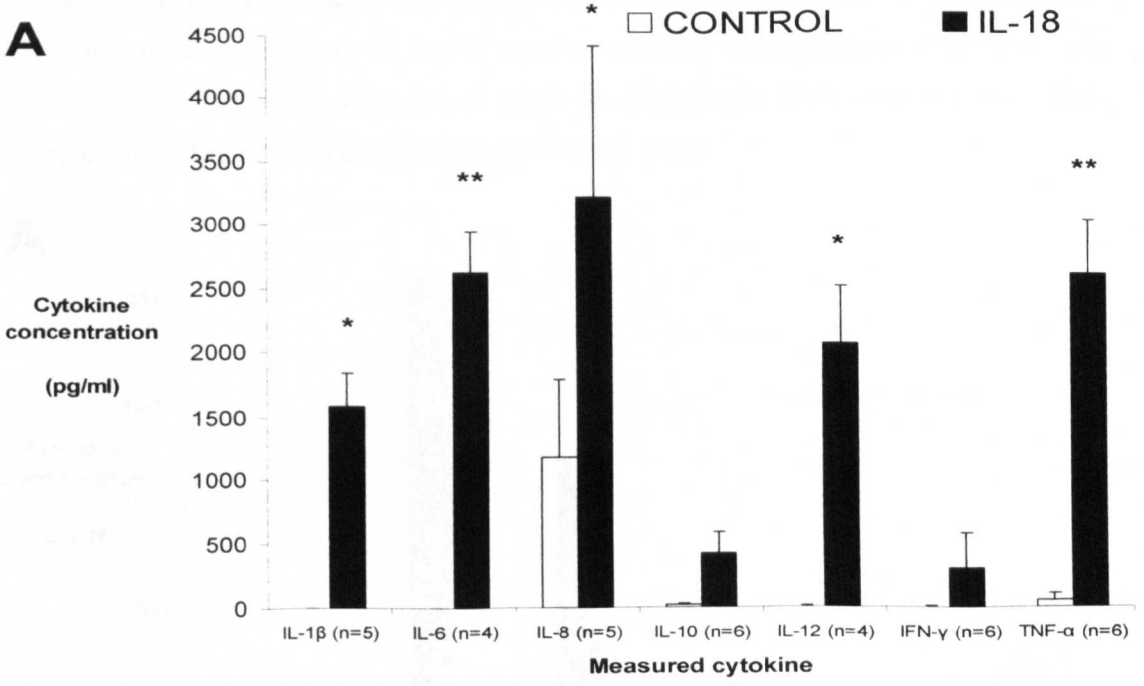

C
IFN-γ (n=80)
486
**
TNF-α (n=126)
55
**
IL-17 (n=17)
251
*
IL-12 (n=29)
103
**
Measured cytokine
IL-10 (n=66)
-15
IL-8 (n=77)
42
IL-6 (n=89)
18
**
IL-1β (n=55)
94
**
-40
60
160
260
360
460
%Change to unstimulated control

Figure 5.8 ***In vitro*** **effects of 18 h IL-18 stimulation upon cytokine expression within PBMC and whole blood cell cultures.** (A) The mean concentration of cytokines after IL-18 stimulation compared with unstimulated control in 18 h PBMC cultures. (B) The mean concentration of cytokine after IL-18 stimulation compared with unstimulated matched control in 18 h whole blood cell cultures. The total number of separate patient derived cultures stimulated with each cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.



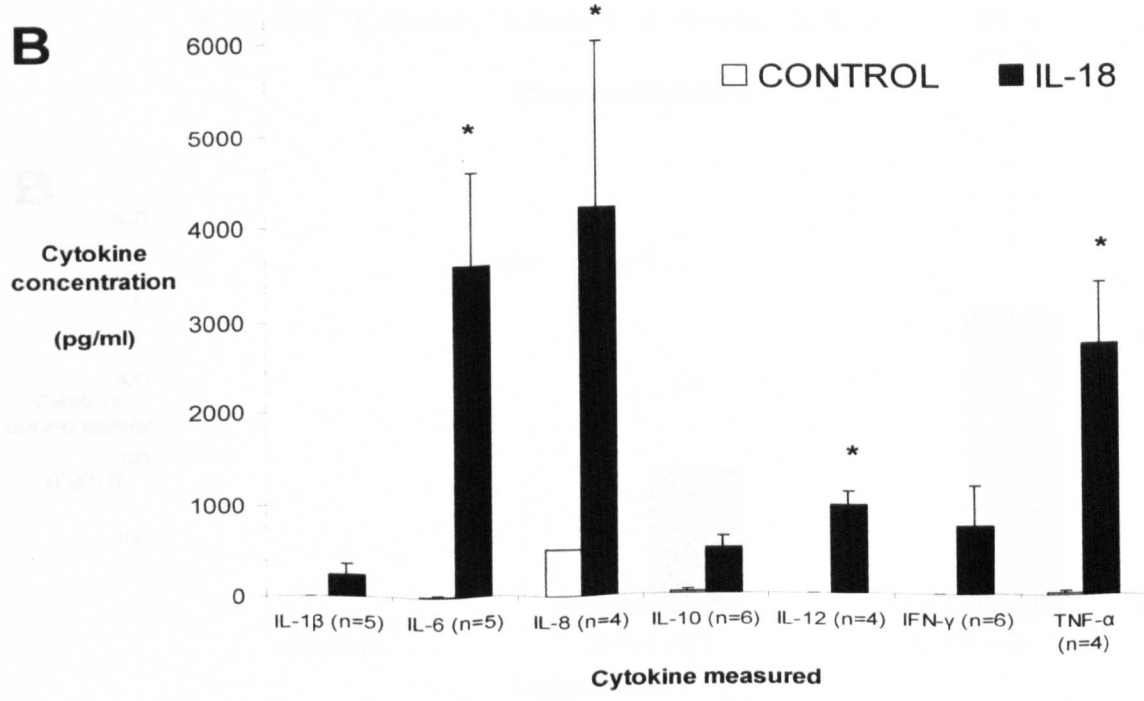
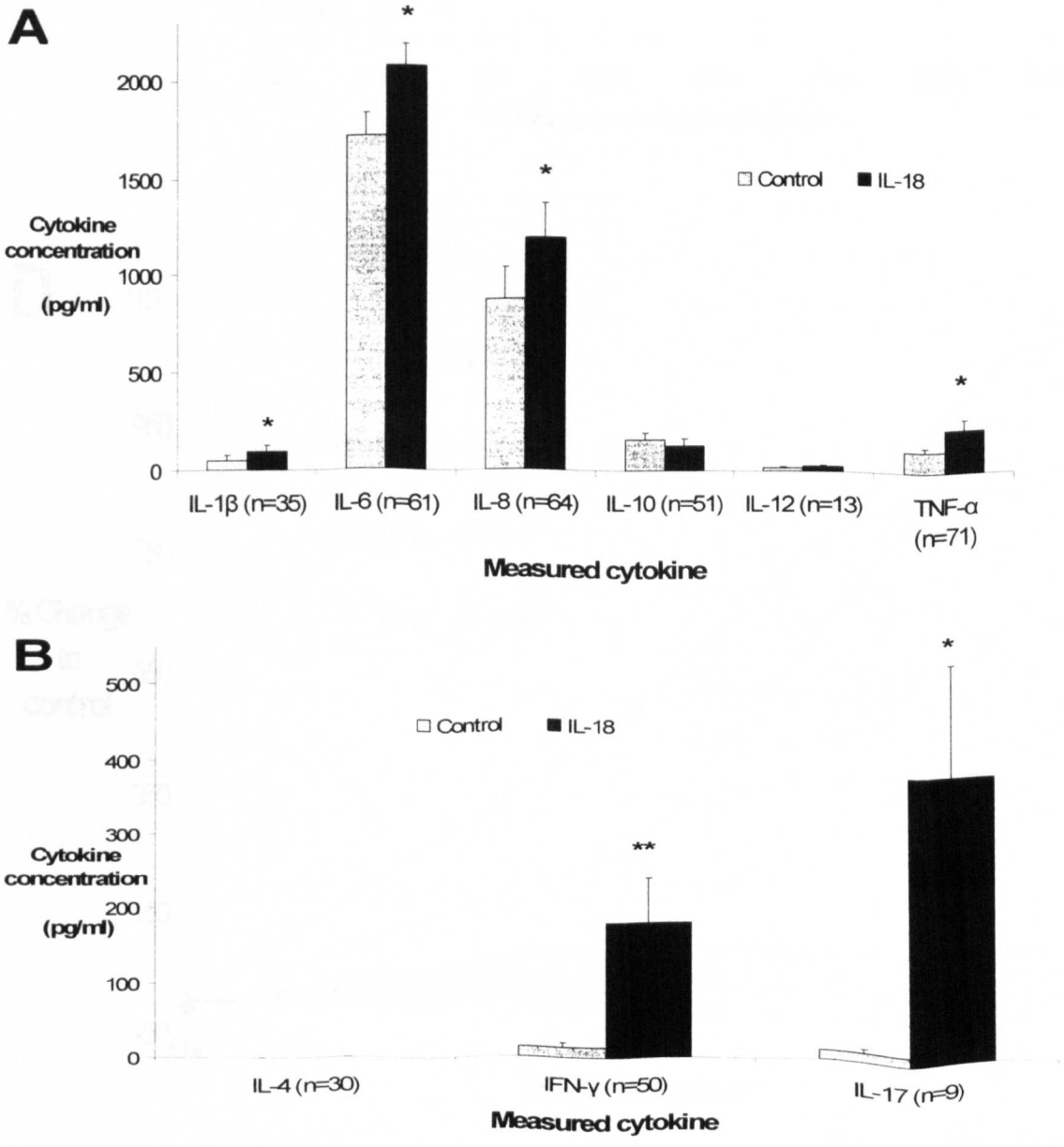
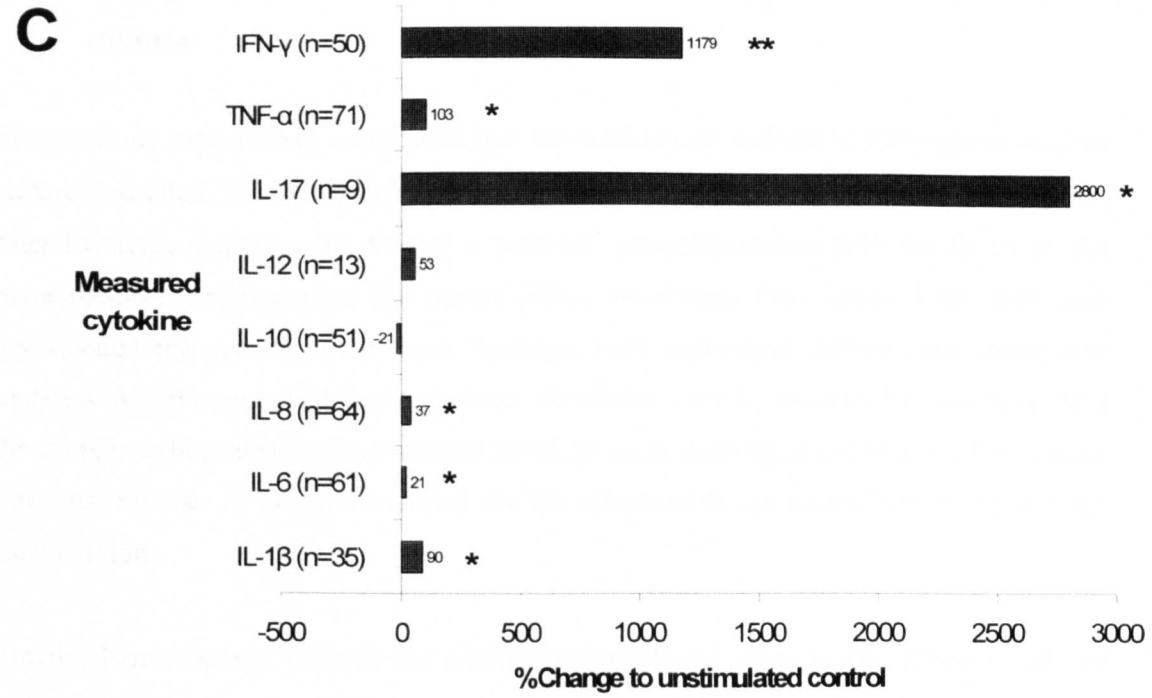
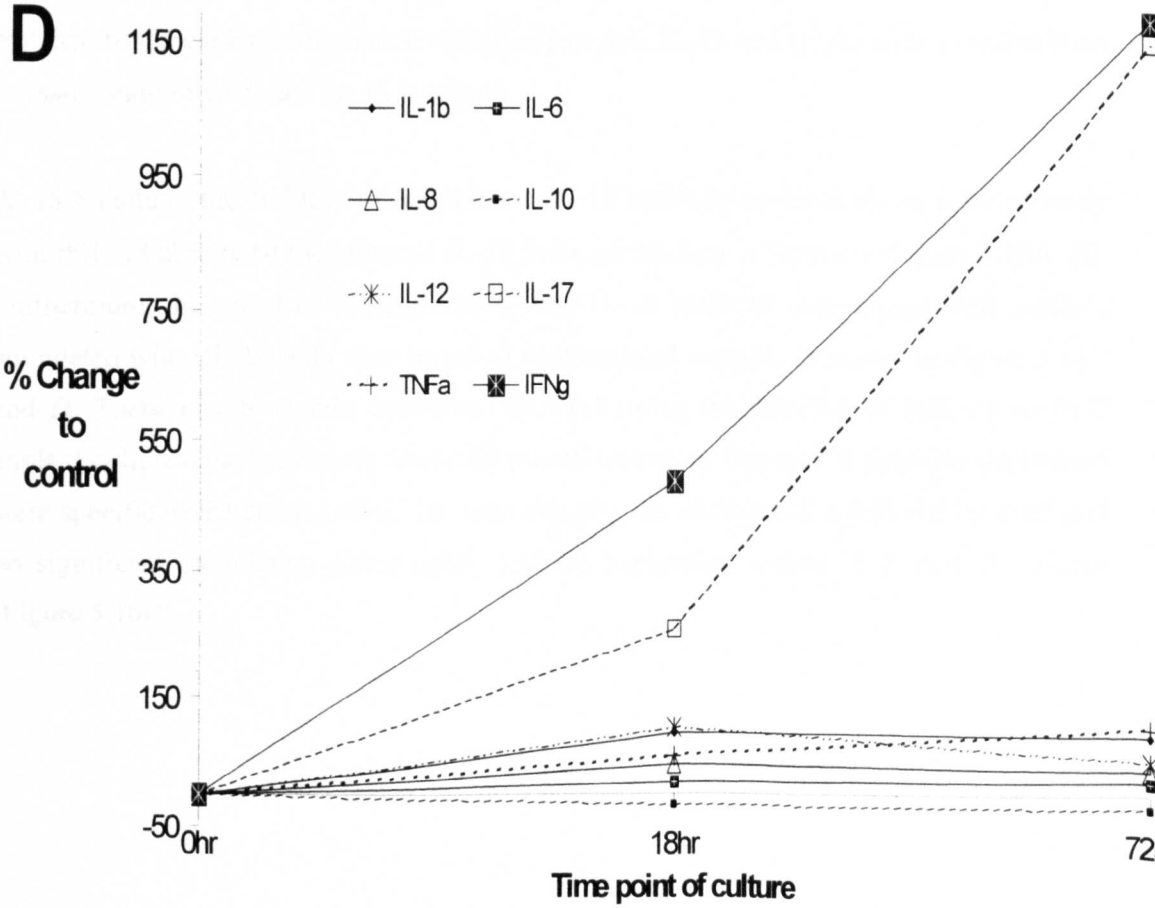


Figure 5.9 ***In vitro*** **effects of IL-18 manipulation in 72 h PRD explant cultures.** PRD explant tissues were stimulated with IL-18 for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of a panel of monokines after explant tissue stimulation with IL-18 compared with unstimulated control. (B) The mean concentration of a panel of T cell-derived cytokines after explant tissue stimulation with IL-18 compared with unstimulated control. (C) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated matched control tissues. (D) Percentage change of cytokine expression within PRD explants after IL-18 stimulation over whole time of culture to 72 h. The numbers of PRD explant tissues stimulated with IL-18 for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.




C
IFN-γ (n=50)
TNF-α (n=71)
IL-17 (n=9)
IL-12 (n=13)
IL-10 (n=51)
IL-8 (n=64)
IL-6 (n=61)
IL-1β (n=35)
1179
103
2800
53
-21
37
21
90
**
*
*
*
*
*
Measured cytokine
-500
0
500
1000
1500
2000
2500
3000
%Change to unstimulated control


D
1150
950
750
550
350
150
-50
IL-1b
IL-6
IL-8
IL-10
IL-12
IL-17
TNFa
IFNg
% Change to control
0hr
18hr
72h
Time point of culture

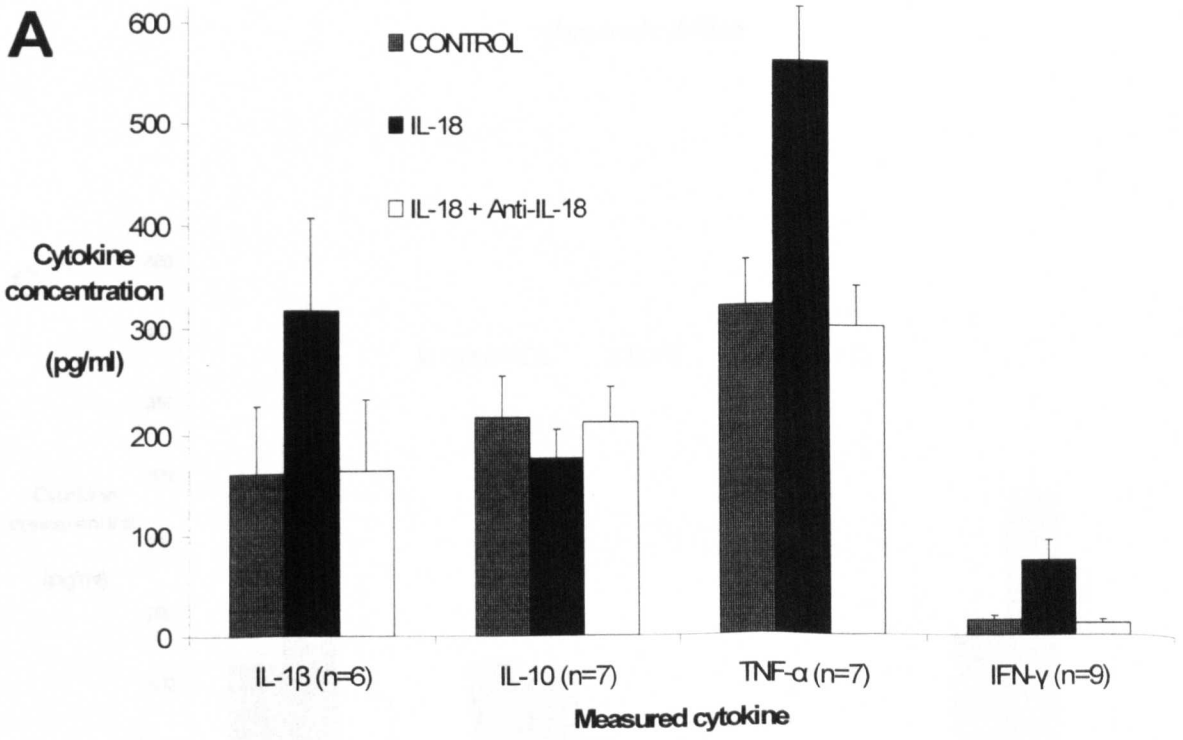
5.7 The specificity of *in vitro* effects of IL-18 manipulation in PRD explant cultures

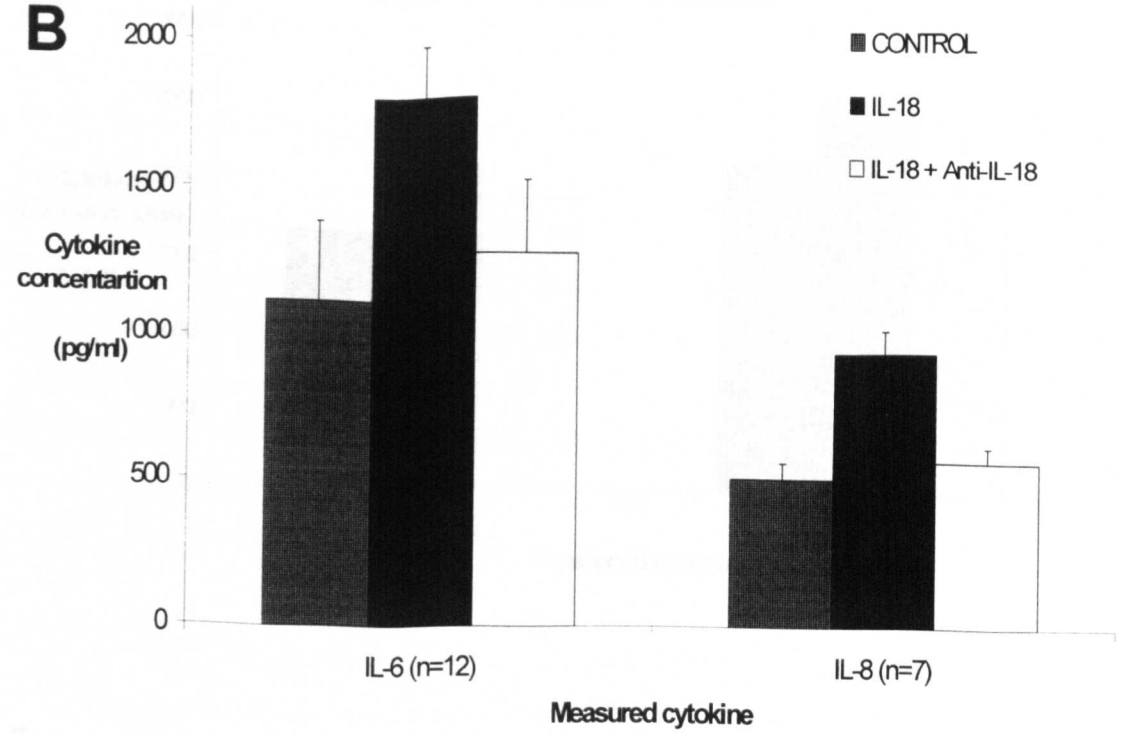
The previous experiments established that the addition of rhIL-18 to PRD tissue explant cultures resulted in up-regulated pro-inflammatory endogenous cytokine expression. Thereby, these experiments defined a potential proinflammatory role for IL-18 in the perpetuation of inflammation that occurs within the chronic PRD lesion. I therefore next investigated the specificity of these findings. Each individual culture experiment was undertaken within matched tissue explants stimulated with IL-18 or IL-18+anti-IL-18 and the corresponding unstimulated control from the same individual donor for 18 h culture. The total number of patients analysed for the effects of IL-18 neutralisation is given for each cytokine.

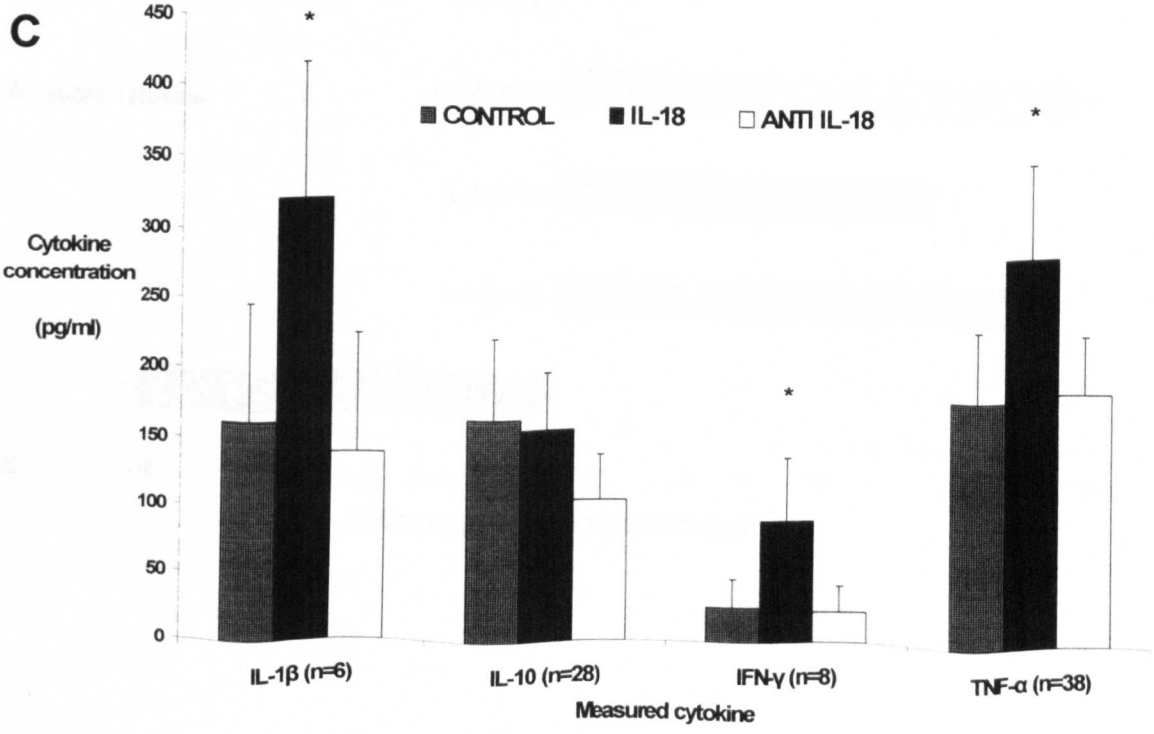
Finally, I investigated whether the addition of anti-IL-18 alone to the cultures had any inducible or suppressive effects upon the expression of the panel of cytokines previously analysed at 18 h explant culture and compared this with IL-18 addition to matched explants. Each individual culture experiment analysing these effects was undertaken within matched tissue explants stimulated with IL-18 or anti-IL-18 and unstimulated control from the same individual donor for 18 h culture.

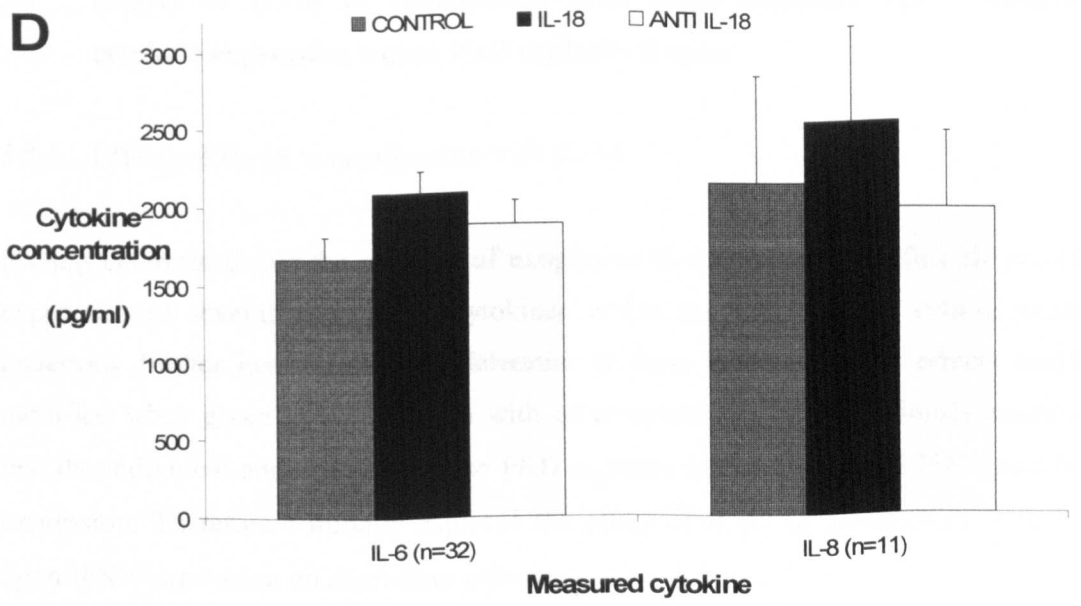
At 18 h culture, the addition of neutralising IL-18 antibody to the explants concomitantly with rhIL-18 abrogated the effect of IL-18 induced cytokine expression (Figure 5.10A, B). Furthermore, the effect of the addition of anti-IL-18 alone in comparison with explants stimulated with rIL-18 with their matched unstimulated controls is shown in Figure 5.10C and D. These results clearly confirmed that following the addition of rhIL-18 to PRD explant cultures, the previously observed proinflammatory changes in cytokine expression were specific to the actions of IL-18. Interestingly, the addition of anti-IL-18 by itself had no significant modulating effect upon cytokine expression within 18 h explant cultures (Figure 5.10C).

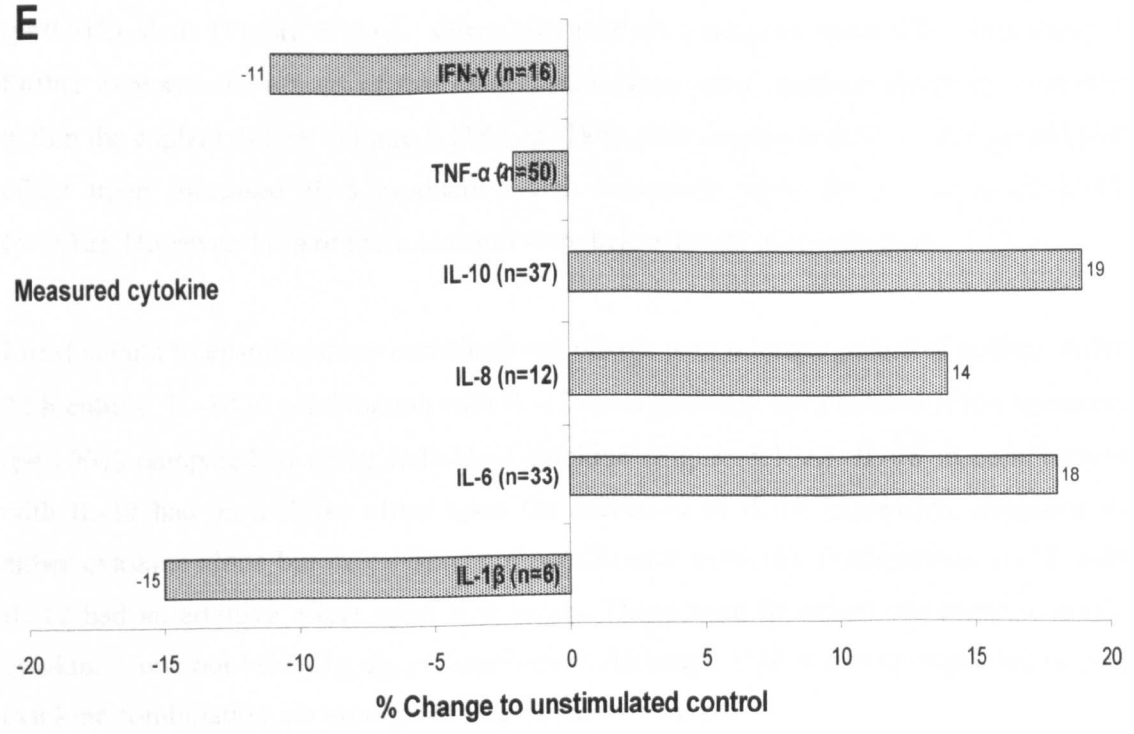
Figure 5.10 Specificity of IL-18 effects within PRD explant cultures. PRD explant tissues were stimulated with IL-18 and neutralising anti-IL-18 for 18 h and supernatant collected and analysed by ELISA. (A, B) The mean concentration of a panel of cytokines after explant tissue stimulation with IL-18 or anti-IL-18+IL-18 compared with unstimulated control from matched donors (C,D) The effects of anti-IL-18 alone upon cytokine expression after 18 h culture in comparison to rhIL-18. (E) Total effects of the addition of anti-IL-18 neutralising antibody to PRD explants upon endogenous cytokine expression. The numbers of PRD explant tissues stimulated with IL-18 and/ or anti-IL-18 for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.




B
2000
1500
1000
500
0
Cytokine concentartion (pg/ml)
CONTROL
IL-18
IL-18 + Anti-IL-18
IL-6 (n=12)
IL-8 (n=7)
Measured cytokine


C
450
400
350
300
250
200
150
100
50
0
Cytokine concentration (pg/ml)
CONTROL
IL-18
ANTI IL-18
*
*
*
IL-1β (n=6)
IL-10 (n=28)
IFN-γ (n=8)
TNF-α (n=38)
Measured cytokine


D
CONTROL
IL-18
ANTI IL-18
3000
2500
2000
1500
1000
500
0
Cytokine concentration (pg/ml)
IL-6 (n=32)
IL-8 (n=11)
Measured cytokine


E
-11
IFN-γ (n=16)
TNF-α (n=50)
IL-10 (n=37)
19
IL-8 (n=12)
14
IL-6 (n=33)
18
-15
IL-1β (n=6)
Measured cytokine
-20
-15
-10
-5
0
5
10
15
20
% Change to unstimulated control

5.8 Effects of IL-18 in combination with other cytokines upon endogenous cytokine expression within PRD explant cultures

5.8.1 Effects of IL-18 in combination with IL-12

Having determined that the addition of exogenous IL-18 induced significantly increased expression of several endogenous cytokines within the PRD explant culture model, I undertook further investigations to determine if these IL-18 mediated effects could be modified when given in combination with other cytokines. I had previously established that the individual addition of IL-18 to PRD explants induced increased IFN-γ and IL-12 expression. Therefore I initially explored the effect of IL-12 in combination with IL-18 upon IFN-γ expression on short-term cultures.

At 18 h culture, the synthesis of IFN-γ was synergistically enhanced by the combined addition of IL-12 and IL-18 compared to the administration of IL-18 ($p=0.001$) or IL-12 ($p=0.012$) alone (Figure 5.11A). Given this marked synergism upon IFN-γ expression, I further explored the effects of this combination upon other immunoregulatory cytokines within the explant system (Figure 5.11A). IL-18 in combination with IL-12 had an additive effect upon increased IL-8 production and conversely upon the reduction of IL-10 ($p=0.12$). However, both of these changes were below levels of significance.

I next sought to examine these combinational effects over a longer period of culture. After 72 h culture, IL-18 in combination with IL-12 synergistically up-regulated IFN-γ secretion ($p=0.001$) compared to either individual cytokine (Figure 5.12A). IL-18 in combination with IL-12 had an additive effect upon the reduction of IL-10 expression compared to either cytokine alone but below levels of significance ($p=0.16$). Furthermore, IL-18 with IL-12 had an additive effect upon IL-8 levels. These were increased compared to either cytokine alone but below levels of significance. Although TNF-α was up-regulated by this cytokine combination, no synergistic effects were observed.

Figure 5.11 ***In vitro*** **effects of IL-18 in combination with IL-12 upon cytokine expression within 18 h PRD explant cultures.** PRD explant tissues were stimulated with IL-18 and IL-12 for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of a panel of cytokines after explant tissue stimulation with IL-18 and/ or IL-12 compared with unstimulated control. (B) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated control tissues. The numbers of PRD explant tissues stimulated with IL-18 for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.

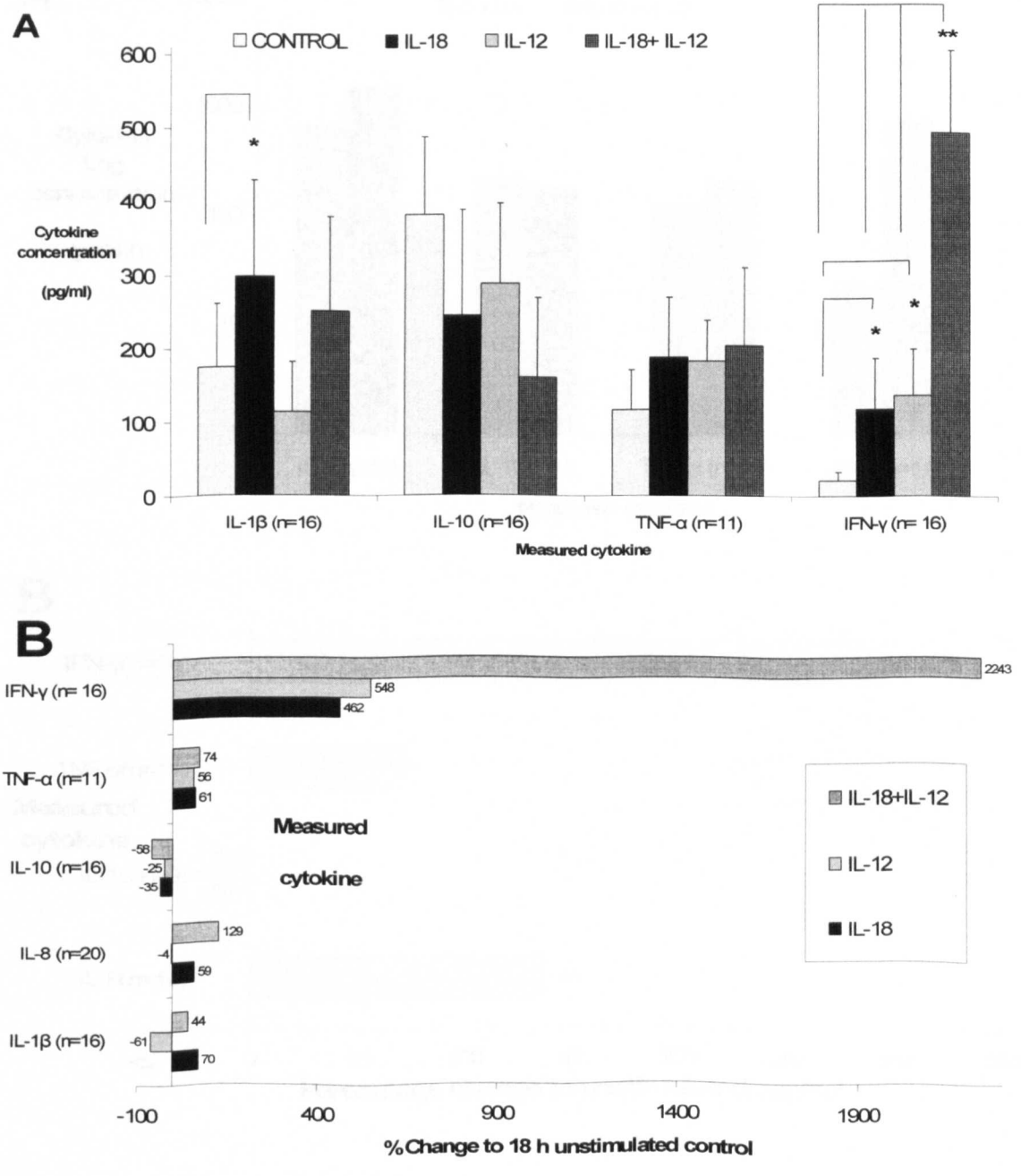
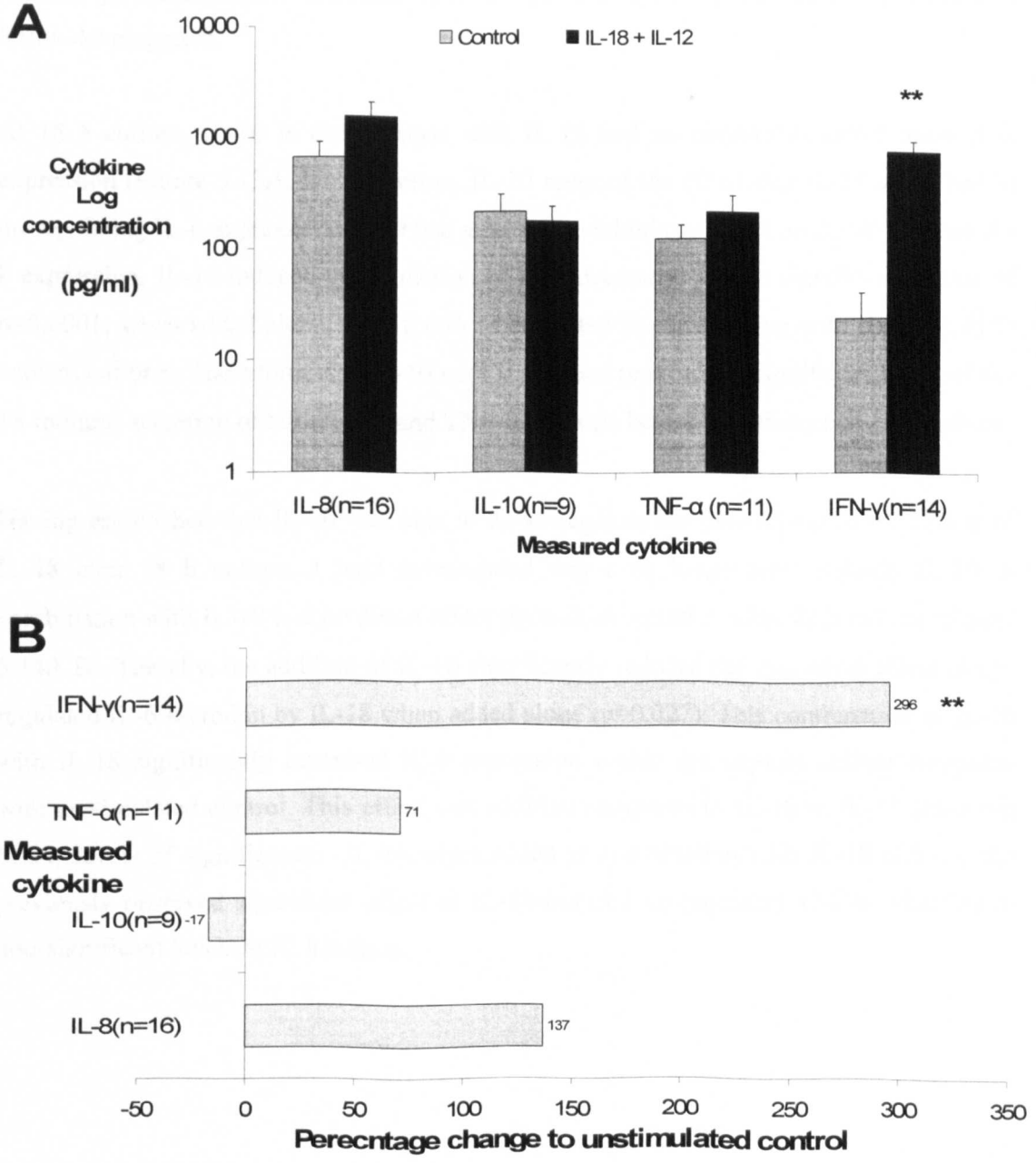


Figure 5.12 ***In vitro*** **effects of IL-18 in combination with IL-12 upon cytokine expression within 72 h PRD explant cultures.** PRD explant tissues were stimulated with IL-18 and IL-12 for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of cytokines after explant tissue stimulation with IL-18 and IL-12 compared with unstimulated control. (B) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated control tissues. The numbers of PRD explant tissues stimulated with IL-18 for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.



5.8.2 Effects of IL-18 in combination with IL-10

IL-10 is generally classed as a cytokine with predominantly anti-inflammatory properties. The previous investigations had established that exogenous IL-18 was capable of driving a proinflammatory cytokine response within the explant culture system and that this was synergistically enhanced by the concomitant addition of IL-12. I therefore next investigated the addition of IL-10 in combination with IL-18 to PRD explant cultures to determine whether IL-10 was capable of down-regulating the previously observed IL-18-induced proinflammatory response upon several inflammatory cytokines implicated in lesion development.

At 18 h culture, IL-10 in combination with IL-18 had no observable effect upon IL-6 expression (Figure 5.13A, B). Therefore, IL-10 reduced the effect that IL-18 alone had in up-regulating IL-6 expression. IL-10 had a moderate inhibitory effect on IL-18-induced IL-8 expression. IL-18-induced up-regulation of IL-8 decreased form a significance value of $p=0.0001$, when added alone, to $p=0.066$ when added in combination with IL-10 to PRD explant cultures. The addition of IL-10 with IL-18 also reduced the significant effect of IL-18-induced secretion of both IFN-γ and TNF-α to levels below significance at 18 h culture.

Having established that IL-10 was able to down-regulate the pro-inflammatory effects of IL-18 over 18 h culture, I next investigated this over longer-term culture. IL-10 in combination with IL-18 had no direct effect upon IL-6 secretion after 72 h culture (Figure 5.14A,B). Thereby, the addition of IL-10 significantly reduced the individual effect of up-regulated IL-6 secretion by IL-18 when added alone ($p=0.027$). This combination of IL-10 with IL-18 significantly increased IL-8 expression within the explant culture compared with unstimulated control. This effect was additive compared to IL-10 or IL-18 alone but below levels of significance. IL-10 when added in combination with IL-18 reduced the previously observed significant effect of IL-18-induced up-regulated TNF-α secretion to non-significant levels at 72 h culture.

Figure 5.13 ***In vitro*** **effects of IL-18 in combination with IL-10 upon cytokine expression within 18 h PRD explant culture.** PRD explant tissues were stimulated with IL-18 and IL-10 for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of a panel of cytokines after explant tissue stimulation with IL-18 and IL-10 compared with unstimulated control. (B) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated control tissues. The numbers of PRD explant tissues stimulated with IL-18/ IL-12 for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.

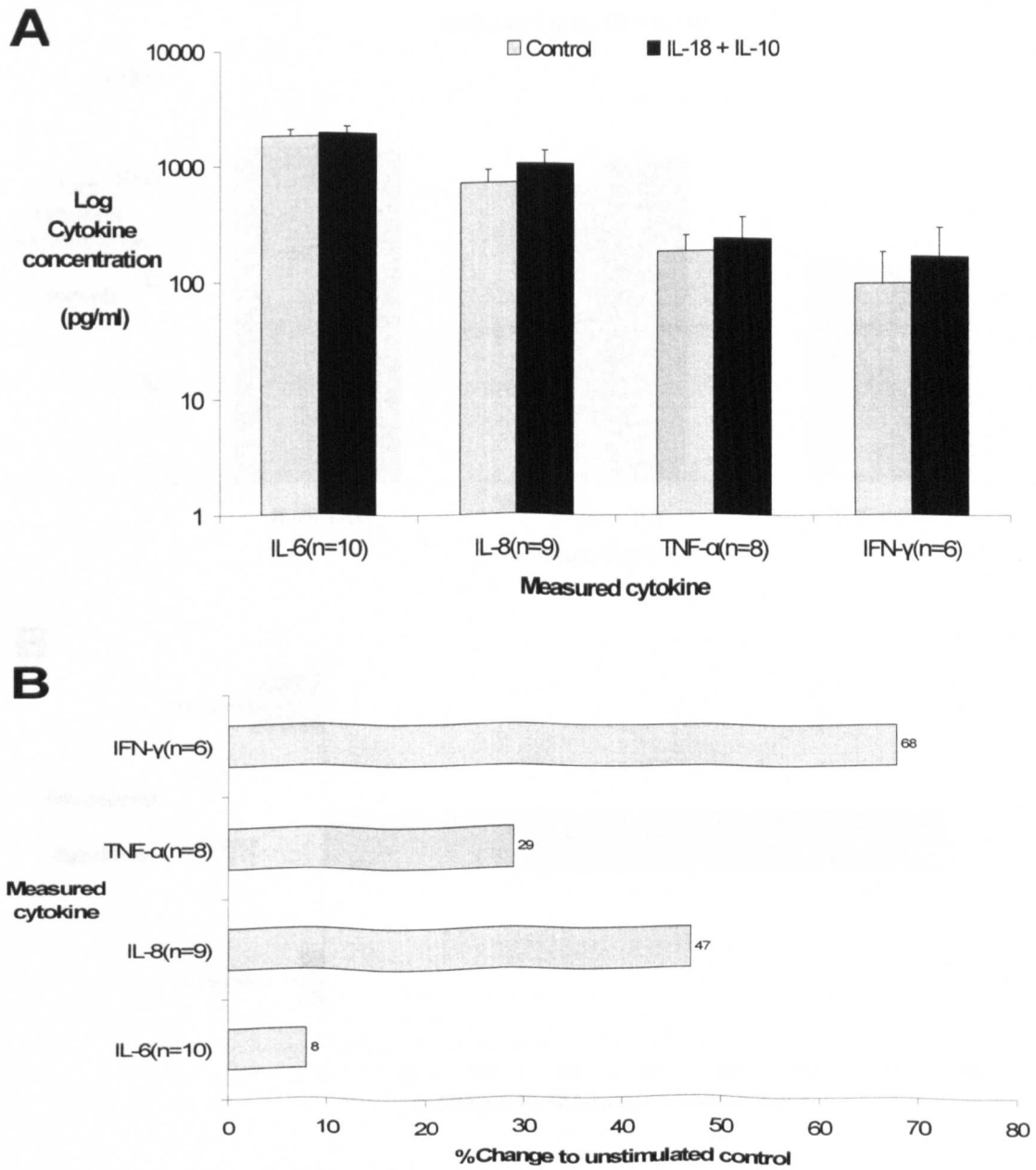
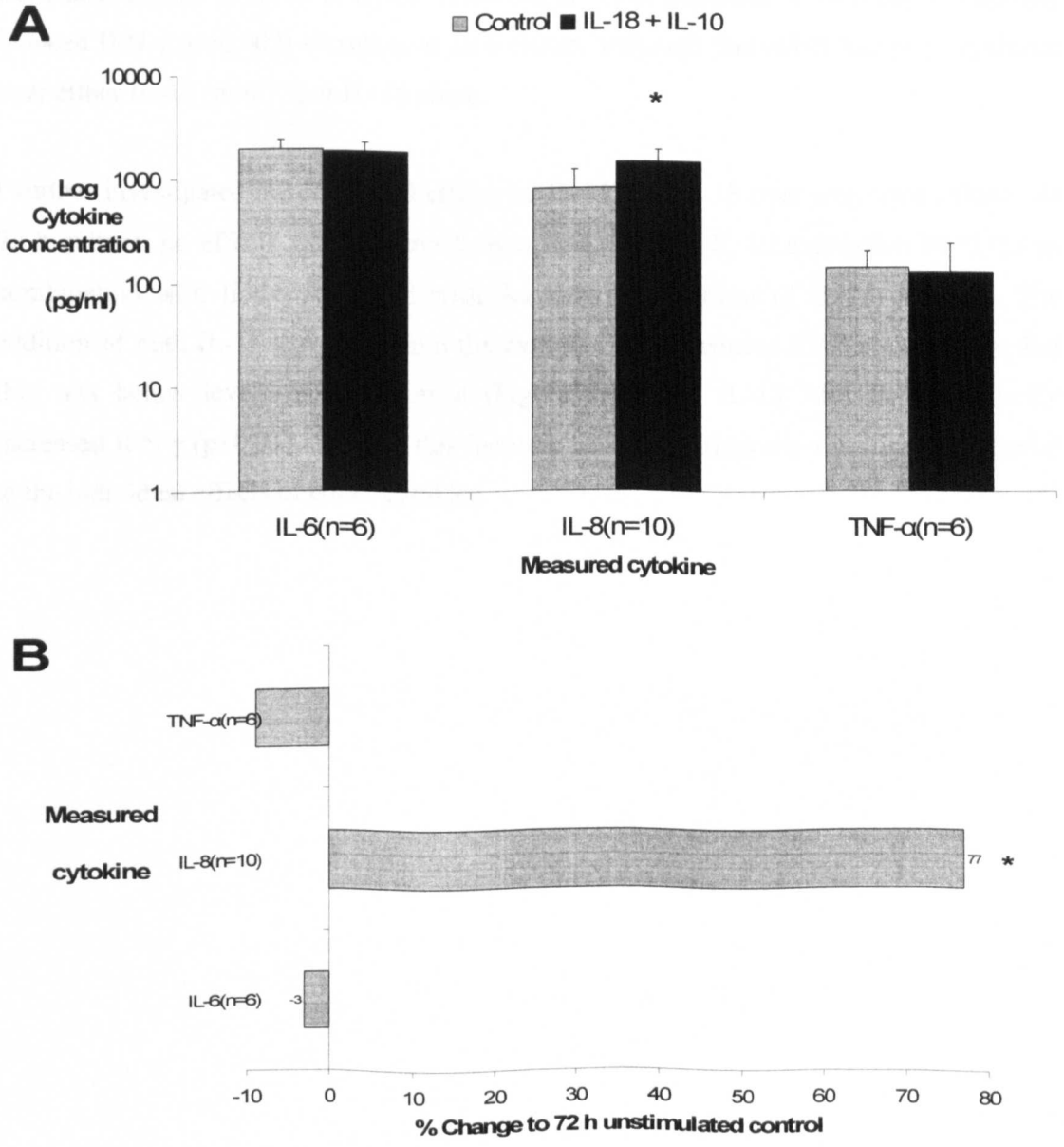


Figure 5.14 ***In vitro*** **effects of IL-18 in combination with IL-10 upon cytokine expression within 72 h PRD explant culture.** PRD explant tissues were stimulated with IL-18 and IL-10 for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of a panel of cytokines after explant tissue stimulation with IL-18 and IL-10 compared with unstimulated control. (B) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated control tissues. The numbers of PRD explant tissues stimulated with IL-18/ IL-12 for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.



5.8.3 Effects of IL-18 in combination with IL-15

IL-15 is a cytokine with many proinflammatory properties. I therefore investigated whether IL-15 in combination with IL-18 exhibited any synergistic effects upon the expression of cytokine inflammatory mediators within the PRD explant system. After 18 h culture, IL-15 in combination with IL-18 had no direct effects upon IL-8 secretion (Figure 5.15A,B). Interestingly, the addition of IL-15 resulted in a significant reduction of IL-8 secretion when compared with IL-18 alone ($p=0.05$). No effects were observed upon IL-6, IL-8, IL-10 or TNF-α expression by the combination of IL-15 and IL-18 compared with the individual actions of IL-15 or IL-18. However, IL-15 in combination with IL-18 markedly induced IFN-γ ($p=0.002$) secretion at 18 h culture although this effect was not significant over either IL-18 ($p=0.15$) or IL-15 alone.

I further investigated the combined effects of IL-15 and IL-18 over long-term culture. At 72 h culture, no effects were observed upon IL-6, IL-8 or IL-10 expression by IL-15 in combination with IL-18 compared with the individual actions of IL-15 or IL-18. The addition of both IL-15 and IL-18 into the explant culture reduced TNF-α expression, but this was below levels of significance (Figure 5.16A,B). IL-18 with IL-15 markedly increased IFN-γ ($p=0.01$) although this increase was not statistically significant compared to the individual effects of either cytokine.

Figure 5.15 ***In vitro*** **effects of IL-18 in combination with IL-15 upon cytokine expression within 18 h PRD explant culture.** PRD explant tissues were stimulated with IL-18 and IL-15 for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of a panel of cytokines after explant tissue stimulation with IL-18 and IL-15 compared with unstimulated control. (B) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated control tissues. The numbers of PRD explant tissues stimulated with IL-18/ IL-15 for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.

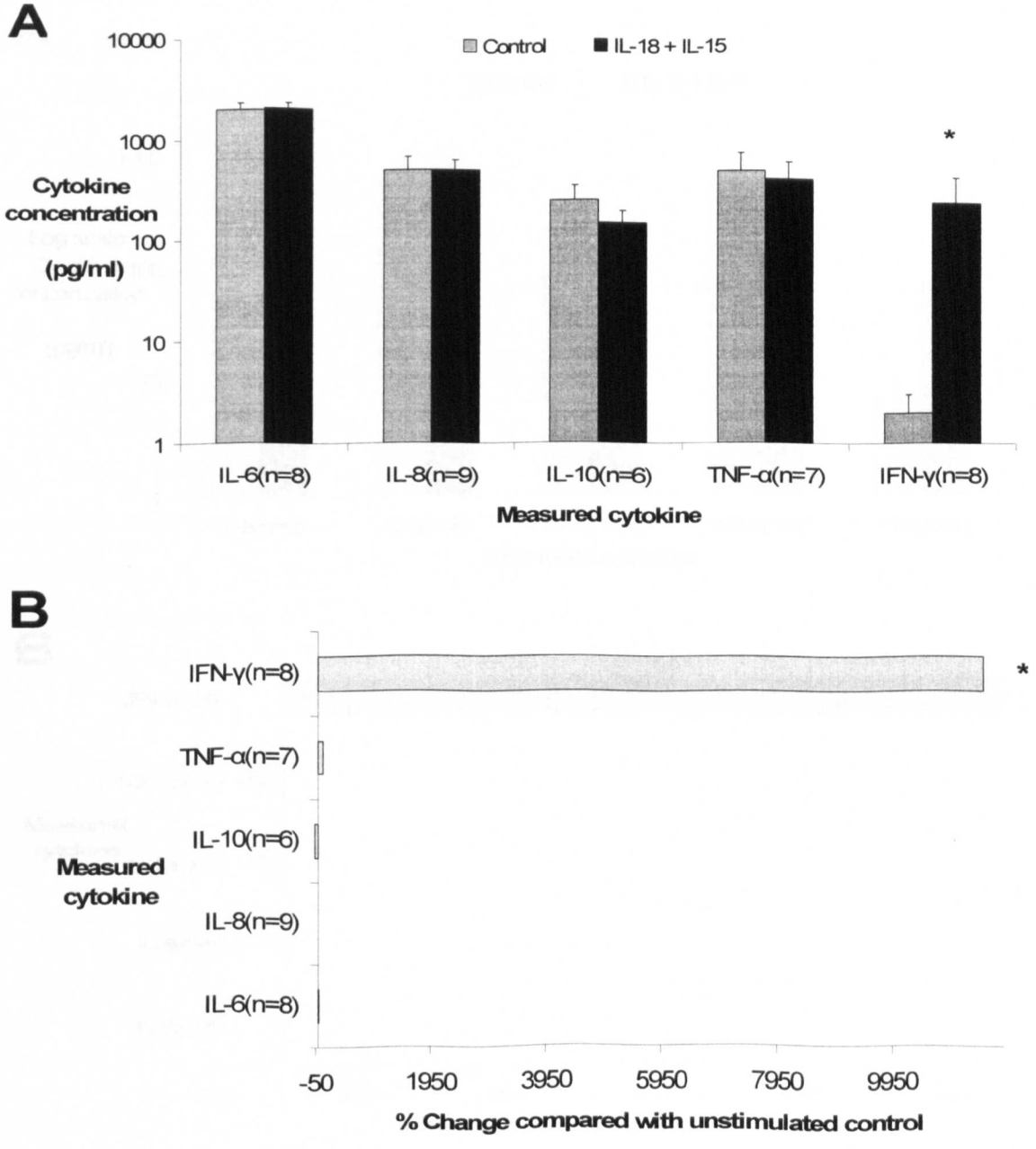
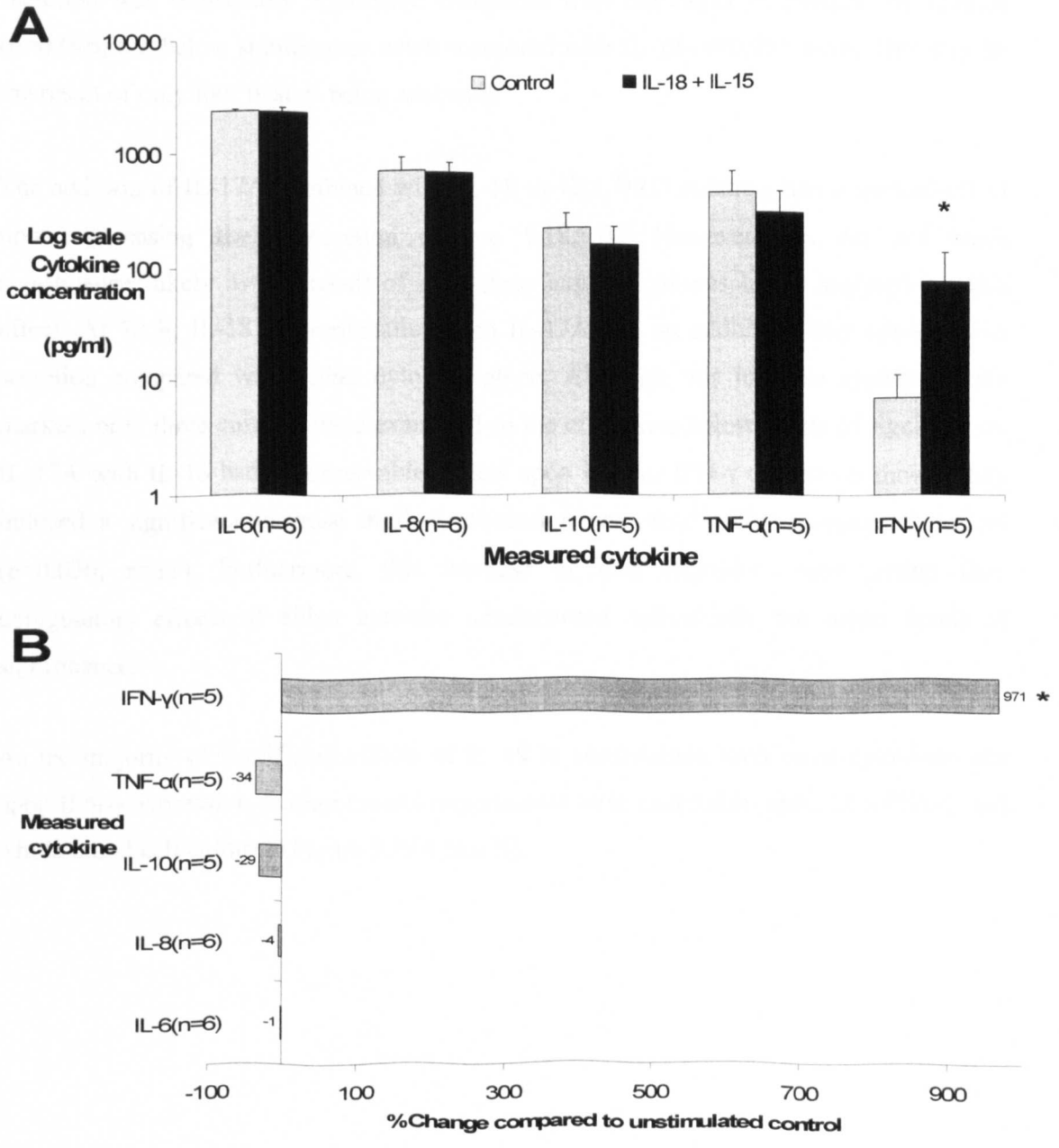


Figure 5.16 ***In vitro*** **effects of IL-18 in combination with IL-15 upon cytokine expression within 72 h PRD explant culture.** PRD explant tissues were stimulated with IL-18 and IL-15 for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of a panel of cytokines after explant tissue stimulation with IL-18 and IL-15 compared with unstimulated control. (B) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated control tissues. The numbers of PRD explant tissues stimulated with IL-18/ IL-15 for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.



5.8.4 Effects of IL-18 in combination with IL-17A

The concomitant addition of IL-17A and IL-18 to explant cultures resulted in a significant increase in IL-8 expression compared with unstimulated control ($p=0.030$). However, this was only marginally greater than IL-18-induced up-regulation of IL-8 alone (Figure 5.17A,B). Although IL-18 in combination with IL-17A increased IFN-γ expression, this combination had no additional effect upon IL-6, IL-10 or IFN-γ explant concentrations compared with the administration of either individual cytokine. IL-17A in combination with IL-18 markedly increased TNF-α secretion at 18 h ($p=0.003$). This increased TNF-α secretion was statistically significant compared with individual stimulation by IL-17A ($p=0.046$) but below significance when compared with IL-18 ($p=0.09$) alone. This may be the result of only four tissues being analysed.

The addition of IL-17A combined with IL-18 to 72 h PRD cultures, had a marked effect upon decreasing IL-10 secretion (Figure 5.18A,B). However this did not reach significance, likely as the result of only three explant cultures being analysed for this effect. At 72 h, IL-18 in combination with IL-17A had an additive effect upon TNF-α secretion compared with either cytokine alone. Although this increase appeared quite marked, only three cultures were examined so the effect was below levels of significance. IL-17A with IL-18 had no observable effects upon IL-6 or IFN-γ expression though they induced a significant increase in IL-8 expression compared with unstimulated control ($p=0.030$, $n=11$). Furthermore, this increase in IL-8 expression was greater than upregulatory effects of either cytokine administered individually but below levels of significance.

As the majority of significant effects of IL-18 in combination with other cytokines was upon IFN-γ expression, further control experiments were undertaken upon 18 h PBMC and whole blood cell cultures (Figure 5.19A and B).

Figure 5.17 ***In vitro*** **effects of IL-18 in combination with IL-17A upon cytokine expression within 18 h PRD explant culture.** PRD explant tissues were stimulated with IL-18 and IL-17A for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of a panel of cytokines after explant tissue stimulation with IL-18 and IL-17A compared with unstimulated control. (B) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated control tissues. The numbers of PRD explant tissues stimulated with IL-18/ IL-17A for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.

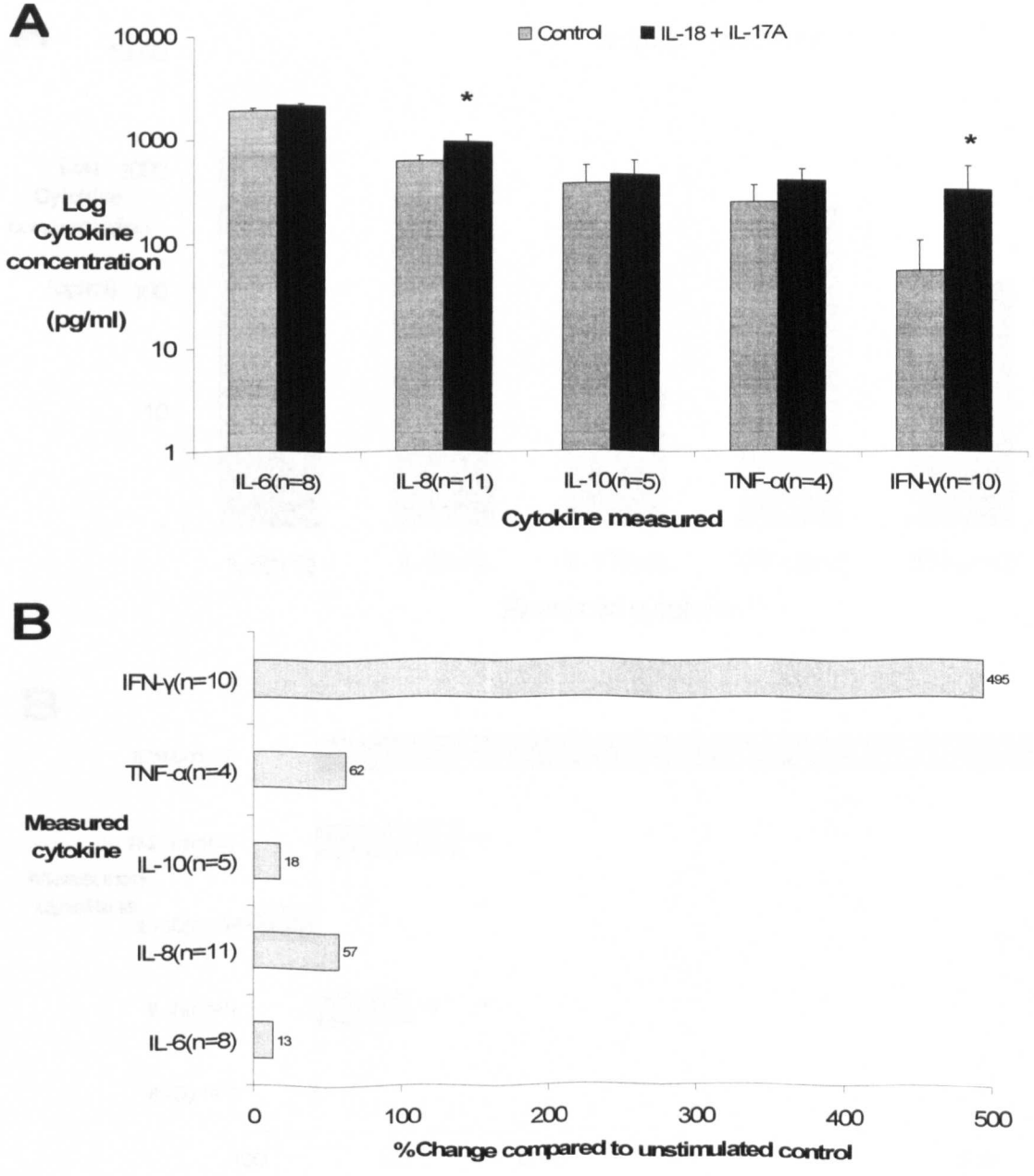


Figure 5.18 ***In vitro*** **effects of IL-18 in combination with IL-17A upon cytokine expression within 72 h PRD explant culture.** PRD explant tissues were stimulated with IL-18 and IL-17A for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of a panel of cytokines after explant tissue stimulation with IL-18 and IL-17A compared with unstimulated control. (B) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated control tissues. The numbers of PRD explant tissues stimulated with IL-18/ IL-17A for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.

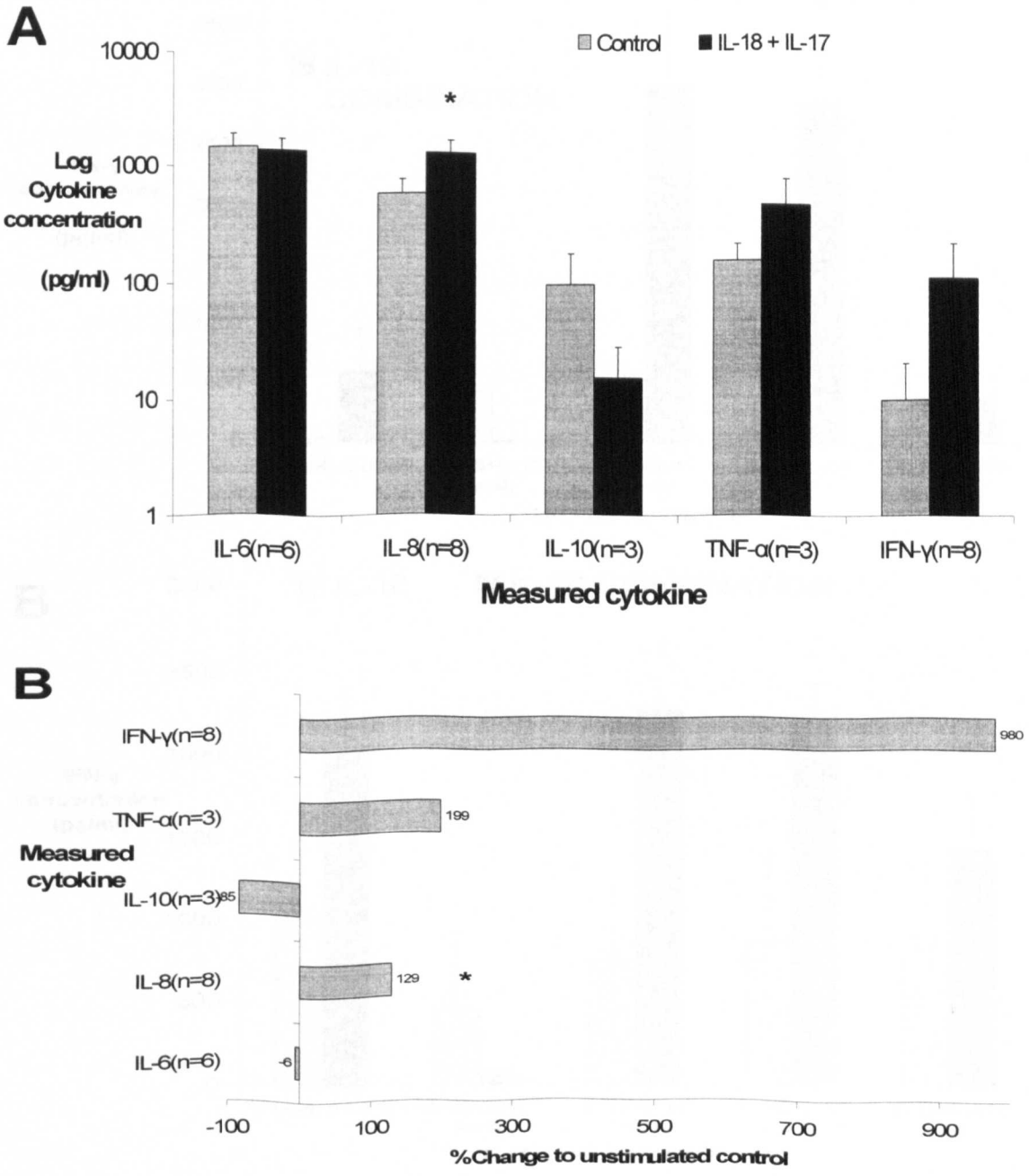
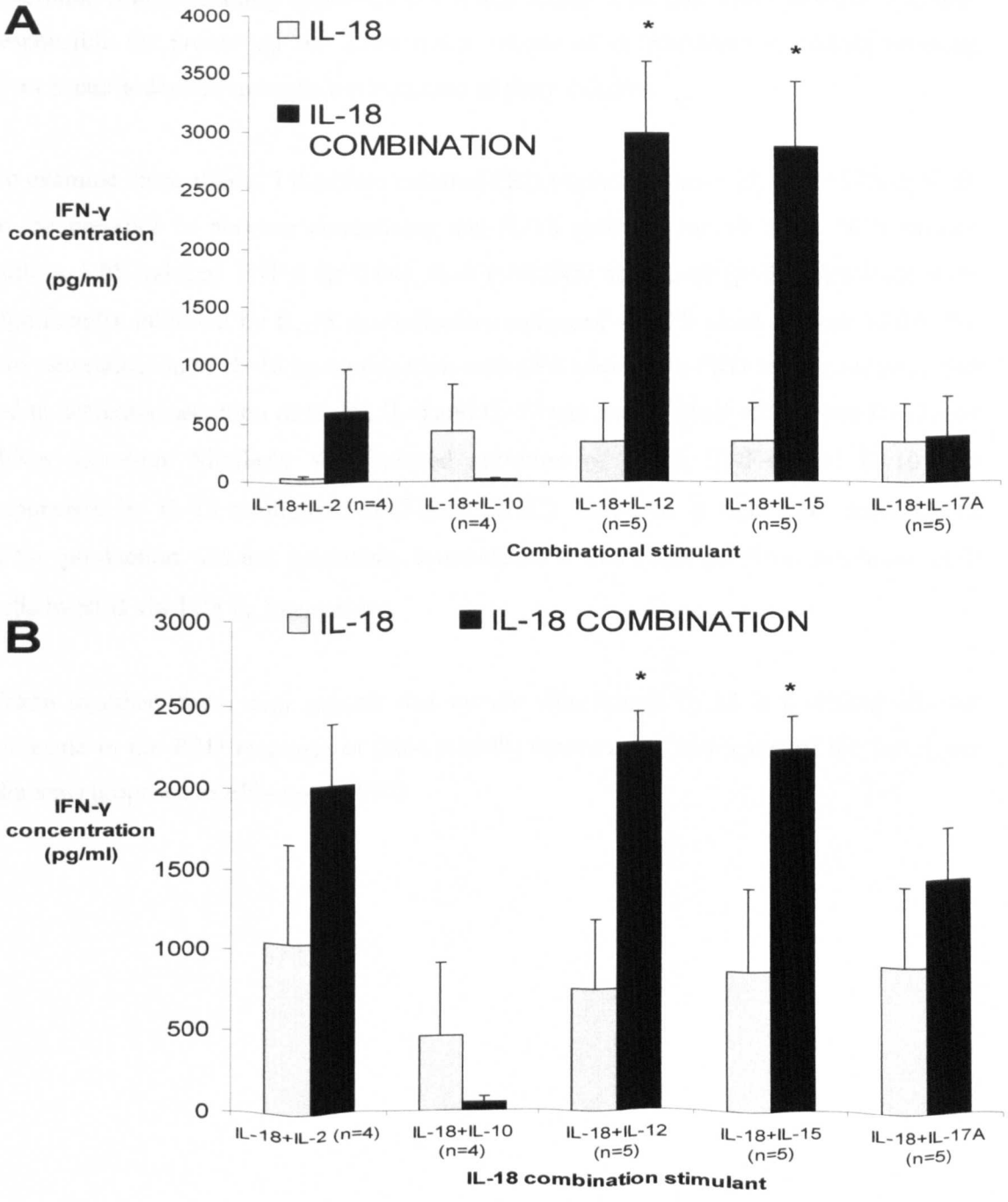


Figure 5.19 ***In vitro*** **effects of 18 h IL-18 stimulation in combination with other cytokines upon IFN-γ expression within PBMC and whole blood cell cultures.** (A) The mean concentration of IFN-γ after IL-18 stimulation compared with IL-18 in combination with other cytokines in 18 h PBMC cultures. (B) The mean concentration of IFN-γ after IL-18 stimulation compared with IL-18 in combination with other cytokines in 18 h whole blood cell cultures. The total number of separate patient derived cultures stimulated with each cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.



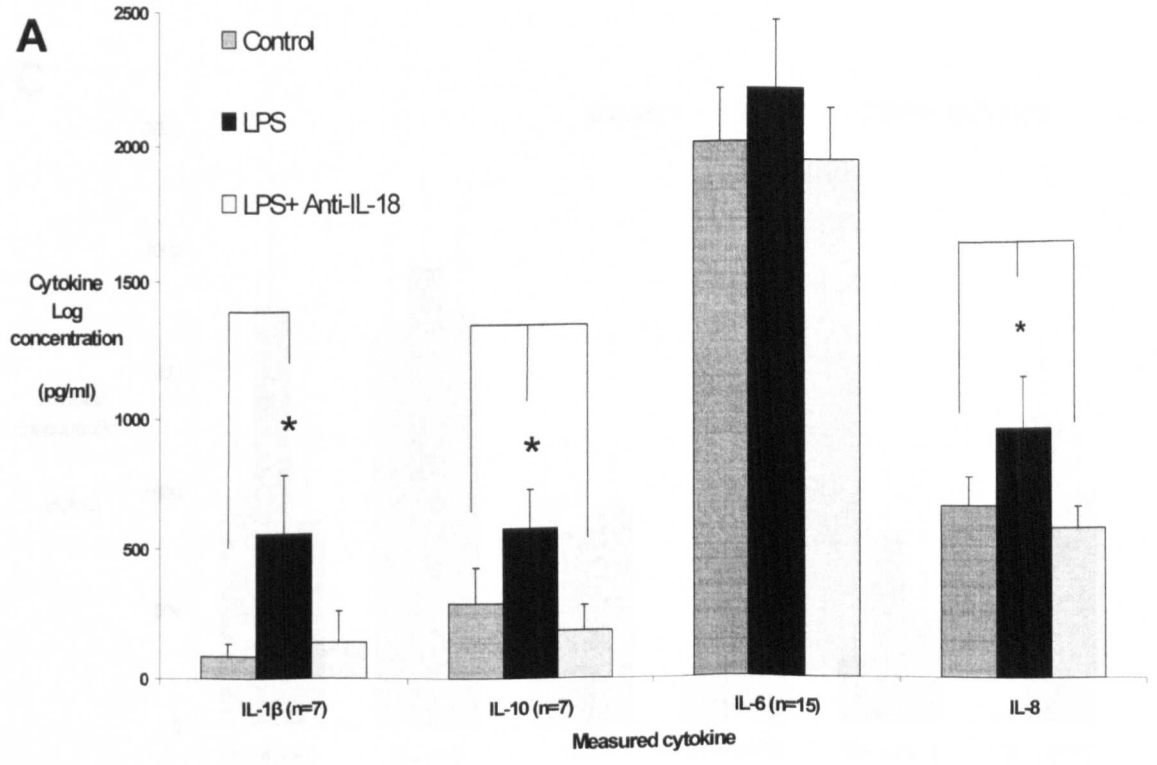
5.9 A role for IL-18 in bacterial product induced cytokine production in PRD

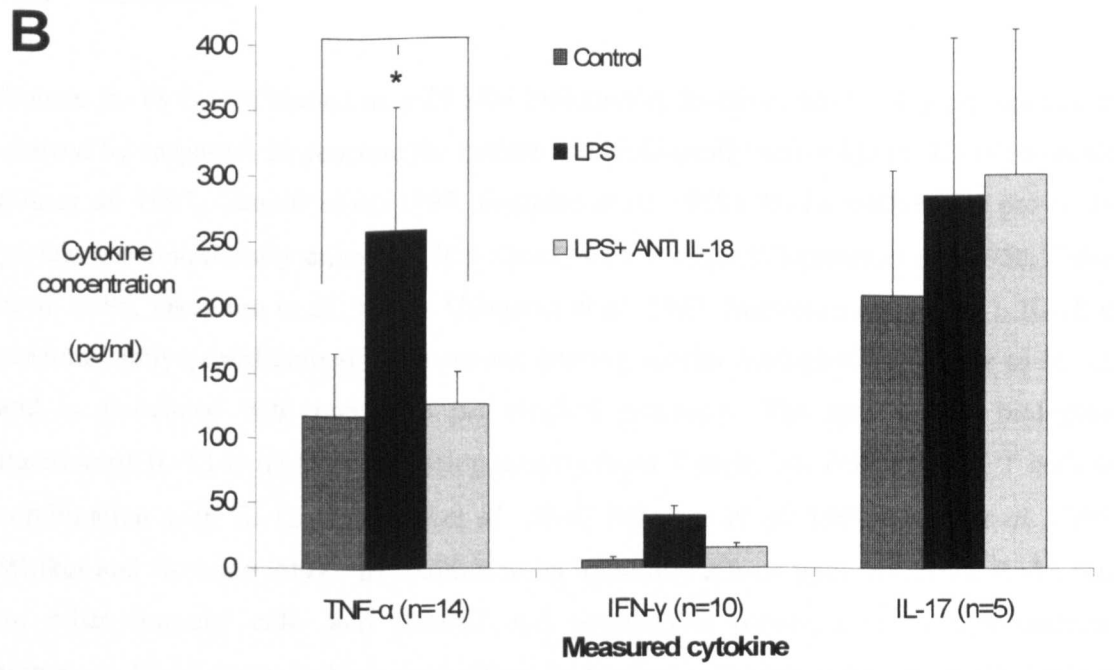
The foregoing data addressed the experimental role of exogenous IL-18 within the novel PRD explant culture system. IL-18 was clearly able to induce proinflammatory cytokine responses within these explant cultures. Of importance, IL-18 substantially increased the expression of IFN-γ. The previous chapter established that this experimental system could be utilised to investigate the effects of Gram-positive and Gram-negative mitogenic stimuli upon the induction of the inflammatory cytokine cascade. It was therefore of interest to determine if endogenously expressed IL-18 was acting as an upstream cytokine regulator, responsible for promoting the downstream release of inflammatory cytokines resulting from bacteria-derived mitogenic stimulation of these cultures.

To examine these effects, I therefore cultured PRD explant tissues with either LPS or SEB, in the presence or absence neutralising anti-IL-18 antibody for 18 h. At 18 h explant culture, LPS-induced TNF-α ($p=0.04$), IL-8 ($p=0.045$) and IL-10 ($p=0.045$) release were significantly inhibited by IL-18 neutralisation compared to LPS alone (Figure 5.20A, B). The neutralisation of IL-18 in combination with LPS addition to PRD explant cultures also led to decreased secretion of IL-1β, IL-6 and IL-12 and had minimal effect upon IL-17A or IFN-γ secretion. Similarly SEB-induced secretion of IL-1β, TNF-α and IL-10 was suppressed by IL-18 neutralisation (Figure 5.20C). However, a significant reduction in IFN-γ production was not detectable, commensurate with expected direct activation of T cells by SEB via $TCRV_{\beta}$ interactions.

Taken together, these data suggest that mature (functional) IL-18 is a critical effector molecule in the PRD response to these stimuli, representing surrogates of the infectious elements proposed as effectors in PRD.

Figure 5.20 ***In vitro*** **effects of IL-18 neutralisation upon mitogen-induced expression of cytokines within PRD explant cultures.** PRD explant tissues were stimulated with anti-IL-18 and LPS for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of IL-1, IL-6, IL-8 and IL-10 after explant tissue stimulation with anti-IL-18+LPS compared with matched LPS-stimulated or unstimulated control tissues. (B) The mean concentration of TNF-α and IFN-γ after explant tissue stimulation with anti-IL-18+LPS compared with matched LPS-stimulated or unstimulated control tissues. (C) The mean concentration of a panel of cytokines after explant tissue stimulation with anti-IL-18+SEB compared with matched SEB-stimulated or unstimulated control tissues. The numbers of PRD explant tissues stimulated with LPS±anti-IL-18 for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.

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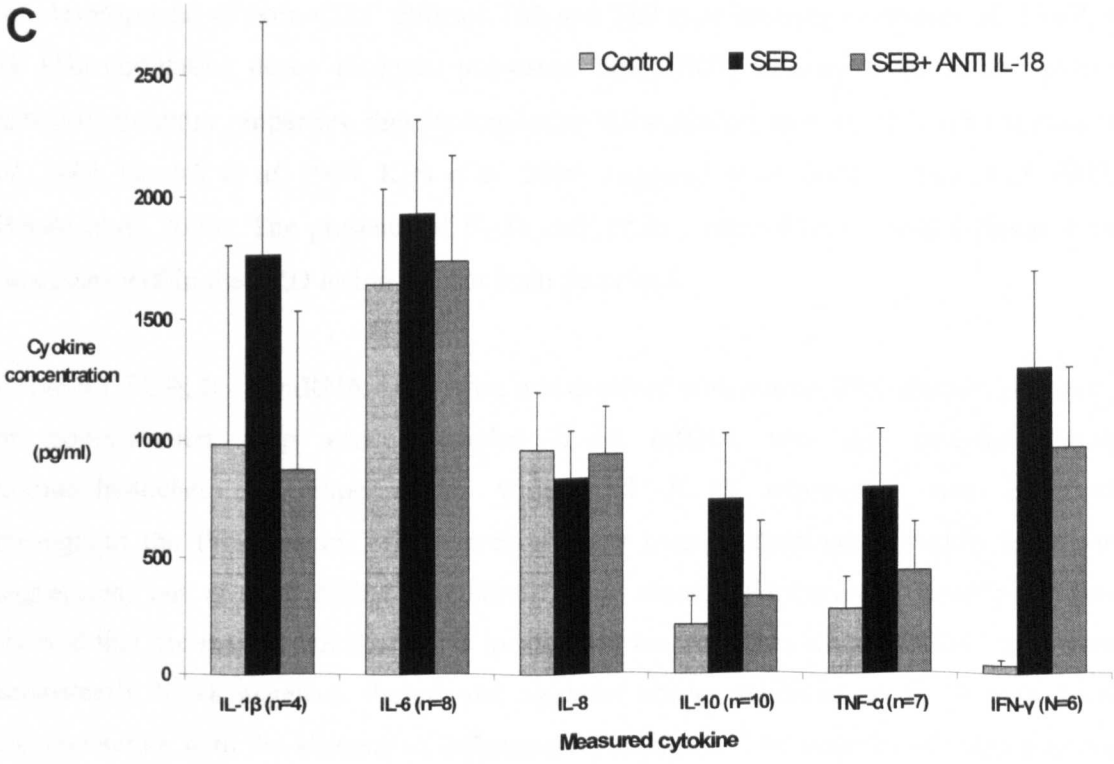
Measured cytokine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IL-8
IL-10 (n=10)
TNF-α (n=7)
IFN-γ (N=6)
Measured cytokine

5.10 Discussion

Human IL-18 is synthesised as a 24 kDa biologically inactive, proIL-18 precursor that is cleaved by caspase-1 to generate the mature and biologically active 18kDa IL-18 molecule (Gu *et al.* 1997, Ghayur *et al.* 1997, Fantuzzi *et al.* 1999). IL-18 mRNA and pro-IL-18 protein are ubiquitously expressed in a variety of cell types (Okamura *et al.* 1995a, Ushio *et al.* 1996, Okamura *et al.* 1995b, Udagawa *et al.* 1997, Sugawara *et al.* 2001). IL-18 is predominantly proinflammatory in nature, sharing similar biological properties to IL-12, and is associated with numerous pathological processes. The most potent biological function of IL-18 is its IFN-γ inducing activity from T cells, NK cells and NK T cells in combination with IL-12 (Micallef *et al.* 1996, Fehniger *et al.* 1999, Otani *et al.* 1999, Walker and Rotondo 2004). In addition to its important effects upon NK cells, IL-18 acts on other immune cells and stromal cell populations participating in inflammatory responses. IL-18 exerts its biological effects by binding to the heterodimeric IL-18 receptor complex comprising the IL-18Rα and IL-18Rβ chains (Torigoe *et al.* 1997, Debets *et al.* 2000). In response to infections and foreign antigens, endogenous IL-18 activity influences the development of both $CD4^+$ effector Th1 and Th2 type immune responses. IL-18BP, a 38 kDa circulating decoy receptor, possesses high-affinity binding to IL-18 and potent anti-inflammatory properties, thereby regulating inflammatory activity of IL-18 (Aizawa *et al.* 1999, Novick *et al.* 1999, Kim *et al.* 2000, Faggioni *et al.* 2001, Yatsiv *et al.* 2002, Banda *et al.* 2003). The presence of IL-18 and/ or its contribution towards inflammatory processes within the PRD lesion has not been described.

Using RT-PCR, IL-18 mRNA expression was detected with human PRD tissues. However, in non-inflamed pulp tissue samples IL-18 mRNA was not detected. From immunohistochemistry experiments, widespread IL-18 expression was observed throughout the PRD lesion. This expression was seen predominantly within leukocyte aggregates, but also in diffuse interstitial tissue areas. Furthermore, double staining showed that the majority of IL-18 was localised to macrophages whereas $CD3^+$ cells were consistently IL-18 negative. Periodontal ligament tissue, exhibited no IL-18 expression commensurate with the absence of inflammatory infiltrate. The majority of PRD samples exhibited IL-18 as being present in both pro (23 kDa) and mature (18 kDa) forms, whereas in non-inflamed pulp tissues, only pro-IL-18 was detected. This suggested that IL-18 was present in resting mucosal tissue but cleaved to mature form only in the context of inflammation.

Expression of IL-18R regulates IL-18 bioactivity and IL-18 responsiveness requires the presence of both IL-18R chains. It has been demonstrated within SFL cultures that only a small percentage of these cells express both IL-18R subunits. Thereby SFL exhibit a lack of responsiveness to stimulation by exogenous IL-18 (Möller *et al.* 2002). Experiments were therefore undertaken to identify expression of both IL-18 receptor subunits within the PRD lesion. IL-18Rα and IL-18Rβ chains were detected at mRNA level in inflamed PRD tissues whereas in non-inflamed pulp, IL-18Rα mRNA but no IL-18Rβ was present. These data were further confirmed by Western blotting, which identified IL-18Rα protein as being present within all PRD tissue specimens and half of pulp tissue samples. Current available antibodies to IL-18Rβ were not functional in immunochemical assays. Nevertheless, these data indicated that IL-18 and IL-18Rα mRNA and protein and IL-18Rβ mRNA were present in inflamed PRD lesions, providing rationale that IL-18 may have functional effects within the novel *in vitro* explant culture system previously established.

High levels of spontaneous IL-18 release were observed in the majority of tissue explants examined. Addition of a range of cytokines or mitogens failed to consistently enhance IL-18 release suggesting prior maximal induction *in vivo*. Addition of IL-10 however, resulted in a 55% reduction in IL-18 release ($p<0.05$). Since the majority of this detected IL-18 was likely to represent pro form, further investigations were undertaken to analyse the effect of adding exogenous mature IL-18 to determine if further tissue responses could be detected. rIL-18 induced significant increases in the release of IL-1β, IL-6, IL-12, TNF-α and IFN-γ within PRD tissue explant cultures. No effect on IL-4 expression was observed. The coincident addition of neutralising anti-human IL-18 antibody abrogated this effect of IL-18, confirming specificity. Since IL-18 conventionally activates T cells in synergy with IL-12, I also investigated as to whether synergistic effects on IFN-γ release could be detected. Whereas IL-18 or IL-12 addition alone induced IFN-γ release in PRD explants, this synthesis was markedly enhanced by addition of IL-12 and IL-18 together. These data suggest that IL-18 is capable of regulating downstream effector cytokines within the PRD lesion.

Microbial agents are clearly identified effector stimuli in the initiation of PRD. Although these agents appeared to have no direct effect upon modulating IL-18 expression, the

results from mitogenic stimulation of the explant cultures with neutralising anti-IL-18 antibody clearly suggest that mature (functional) IL-18 was a critical effector molecule in the up-regulation of endogenous proinflammatory cytokines within the PRD explant culture system. This further suggests that IL-18 likely plays a critical role in the regulation of downstream inflammatory mediators within PRD responses to infectious elements.

IL-12, IL-18 and IFN-γ are reported to have opposing regulatory functions upon inflammatory bone destruction. They are responsible for the development of inflammatory responses within the PRD lesion whilst at the same time having inhibitory effects on osteoclasts. *In vivo* experiments have demonstrated that inflammatory bone resorption, induced by the infection of dental root canals in IL-18-/-, IL-12-/- and IFN-γ-/- mice leads to only a slight reduction in PRD lesion development compared with wild type controls (Sasaki *et al.* 2004b). Furthermore, the infusion of rIL-12 into these knockout mice also exerts no significant effect upon lesion development. Together, these data suggest that, at least individually, there is considerable functional redundancy of these cytokines in infection stimulated-bone resorption within the murine pulp exposure model (Sasaki *et al.* 2004b). The absence of a significant effect on the development of inflammatory PRD within IL-18-/-, IL-12-/- and IFN-γ-/- mice may therefore reflect the balance between a reduction in proinflammatory signals against a reduction in osteoclast inhibition. However, it may be postulated that a deficiency in a combination of these cytokines may result in a significant alteration in PRD lesion development.

Increased IL-18 expression has been observed within tissues derived from patients with chronic inflammatory diseases for example Crohn's disease and RA thereby suggesting a possible role in disease pathogenesis (Monteleone *et al.* 1999, Gracie *et al.* 1999). However, early studies did not take into account the role of IL-18BP, which exhibits an exceptionally high affinity for IL-18. Indeed, IL-18BP was later confirmed to be present within CD lesions (Kim *et al.* 2000). Likewise, mRNA expression for IL-18BPa and c subunits was detected within the PRD lesion. Although the appropriate IL-18BP neutralising isoforms were detectable in PRD tissues, substantial expression of free IL-18 was observed in PRD explant culture supernatants. This highlights the complexity of the regulation of IL-18 activity. The significance of the gene expression of IL-18BP and its role in the inhibition of the proinflammatory effects of IL-18 within the PRD lesion therefore requires further investigation. Principal biological effects of IL-18 within PRD inflammatory responses are summarised in Figure 5.21.

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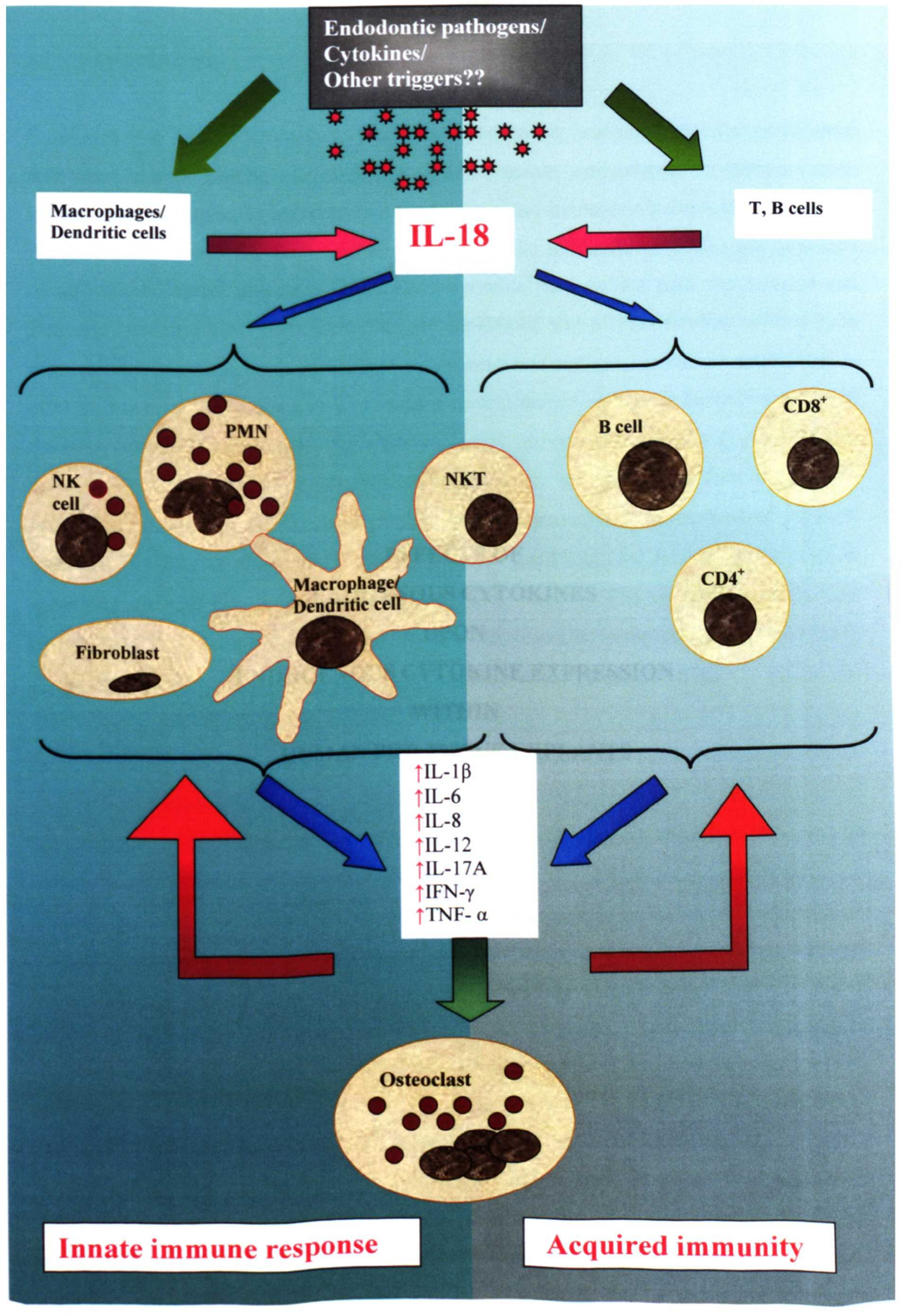
Figure 5.21 Schematic summary of IL-18 orchestrated events within PRD.

Substantial IL-18 expression is present within the PRD lesion and is likely essential to effective host responses against endodontic pathogens. However, precise initiators of IL-18 secretion remain to be defined. IL-18 activates cells of both innate and acquired immunity, inducing the release of several proinflammatory cytokines including IL-1β, IL-6, IL-8, IL-12, IL- 17A, TNF-α and IFN-γ. These cytokines perpetuate the inflammatory reaction by acting in positive regulatory loops to induce further release of inflammatory mediators. IL-1β, IL-6, IL-8, IL-17A, and TNF-α activate osteoclasts, thereby promoting tissue destructive pathways within the surrounding dentoalveolar bone matrix. These adverse destructive effects are likely moderated by the down-regulatory actions of IL-12 and IFN-γ, which prevent osteoclastogenesis. In addition to clearance of endodontic pathogens within PRD, IL-18 is therefore central to modulation of proinflammatory pathways leading to tissue damage and anti-inflammatory pathways resulting in tissue repair.

Figure 5.21 Schematic summary of IL-18 orchestrated events within PRD.



CHAPTER 6

EFFECTS OF EXOGENOUS CYTOKINES UPON ENDOGENOUS CYTOKINE EXPRESSION WITHIN HUMAN PRD TISSUE EXPLANTS

6 EFFECTS OF EXOGENOUS CYTOKINES UPON ENDOGENOUS CYTOKINE EXPRESSION WITHIN HUMAN PRD TISSUE EXPLANTS

6.1 Introduction

Cytokines are key inflammatory mediators involved in orchestrating successful host responses against pathogenic challenge. They regulate communication between both immune and stromal cells involved in inflammatory and immune responses (Belardelli and Ferrantini 2002). Indeed, these mediators modulate the initiation, evolution and cessation of all immunological processes. They form complex partnerships with one another and frequently act simultaneously. Cytokines are capable of mutually modulating actions upon their cellular targets through synergistic enhancement or suppression and by neutralisation effects. It is established that cytokines play a central mediating role in the pathogenesis of several chronic inflammatory disorders including rheumatoid arthritis (RA), Crohn's disease, ulcerative colitis and atherosclerosis. They are responsible for directing chronic inflammation and are thereby accountable for the concomitant destruction of adjacent tissue (Andreakos *et al.* 2004). Depending on the local cytokine milieu, many of these cytokines are capable of promoting either $CD4^+$ effector Th1 or Th2 type immune responses. The pathway of this response in turn determines whether the host immune status is directed towards tolerance or immunity (Banchereau and Steinman 1998).

In normal physiological circumstances, inducing stimuli lead to the transient cellular expression of cytokines. Following this, their gene expression is promptly down-regulated and they are swiftly degraded. However, in chronic inflammatory disorders, for example PRD, these cytokines are up-regulated and expressed for extended periods of time. Dysregulated expression of these cytokines does not occur in a random fashion. Indeed, they are generally expressed in an ordered hierarchical cascade of events. Several inflammatory cytokines have been detected within the human PRD lesion. The majority of studies on human PRD material have identified the presence of cytokines within biopsy tissue specimens by utilising *in-situ* hybridisation or immunohistochemistry. In contrast, only a few studies have examined the concentrations of cytokines released from unstimulated periradicular cysts and these investigations have analysed only a limited number of cytokines. To date, no published studies have attempted to characterise inflammatory events within the human PRD lesion by undertaking functional investigations upon explant PRD tissue cultures of human origin.

Experiments within the previous two chapters utilising the novel PRD explant culture model elucidated that several cytokines are spontaneously released by human PRD tissue explants. I also established that human PRD explants were responsive to bacterial-derived mitogens. Furthermore, these moieties were capable of modulating endogenous cytokine expression within PRD tissue cultures and these effects were quantifiable. Subsequently, I was able to explore the biology of the novel inflammatory cytokine, interleukin-18, within this culture system and established the importance of IL-18 expression in mitogen-driven inflammatory responses. In addition, I was able to add this proinflammatory mediator to the PRD tissue cultures and characterise its effects upon endogenous cytokine expression.

The chronically inflamed PRD lesion is surrounded by dento-alveolar bone that is constantly remodelling. In addition to physiological homeostasis, this remodelling is influenced through direct interactions with the adjacent cellular environment of the PRD tissue. The cytokine milieu established within the lesion is primarily a result of stimulation from pathogens and their microbial components present within the root canal and cellular components of the lesion. These cytokines significantly contribute towards protecting the host from disseminating spread of infectious endodontic pathogens. However, this cytokine network also plays a central role in the perpetuation of chronic PRD. Additionally, the release of inflammatory cytokine mediators from cellular constituents of the PRD lesion likely impact upon neighbouring matrix tissue cells including osteoblasts and osteoclasts. Thereby, the cytokine network within the PRD lesion likely influences the overall pattern of surrounding dento-alveolar bone remodelling towards either destruction or repair.

In this chapter, I therefore further investigated the cytokine milieu within the PRD lesion by utilising the novel PRD explant culture system that I had previously developed. I analysed the effects of exogenous inflammatory cytokines, which are implicated in contributing towards perpetuation of the lesion and the concomitant destruction of adjacent alveolar bone, upon endogenous cytokine expression.

6.2 *In vitro* effects of inflammatory mediators upon IL-6 expression within PRD tissue explant cultures

I had earlier identified that unstimulated PRD explant tissues spontaneously secreted high levels of IL-18. By using the novel experimental PRD explant culture model, I had also been able to establish that endogenous expression of critical inflammatory cytokines could be regulated *in vitro* by the addition of exogenous IL-18 to tissue cultures. The previous experiments also demonstrated that unstimulated PRD explant tissue expressed high levels of spontaneously secreted IL-6. These data suggest that within the PRD lesion, IL-6 might be an important contributor to the cytokine network. It was therefore of importance to identify potential upstream regulators of IL-6 expression. Therefore, I first investigated the effects of the addition of a number of exogenous cytokines to the PRD explant culture model upon IL-6 expression within short-term cultures.

After 18 h culture, the expression of IL-6 protein within PRD tissue explant supernatants was moderately increased by the addition of the proinflammatory cytokine IL-12 ($p=0.028$) and minimally increased by IL-17A ($p=0.020$) and IFN-γ ($p=0.015$) (Figure 6.1A, B). The addition of MCSF ($p=0.006$) to PRD implant cultures also induced a statistically significant response, resulting in moderately decreased IL-6 supernatant levels. The addition of IL-10 ($p=0.07$), IL-15, IL-22, TNF-α or TGF-β had no observable effect upon IL-6 secretion at 18 h culture. In addition to the matched, unstimulated explant tissue controls, cytokine stimulation of PBMC and whole blood cell cultures served as further controls (Figure 6.2A, B).

I next investigated the effects of these inflammatory mediators over longer-term explant culture. After 72 h, none of the added cytokines had any effect upon IL-6 cytokine expression within the PRD explant cultures (Figure 6.3A, B).

Figure 6.1 ***In vitro*** **effects of 18 h cytokine stimulation upon IL-6 expression within PRD explant cultures.** PRD explant tissues were stimulated with a panel of key cytokines for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of IL-6 after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IL-6 concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-6 concentration with the standard error of the mean.

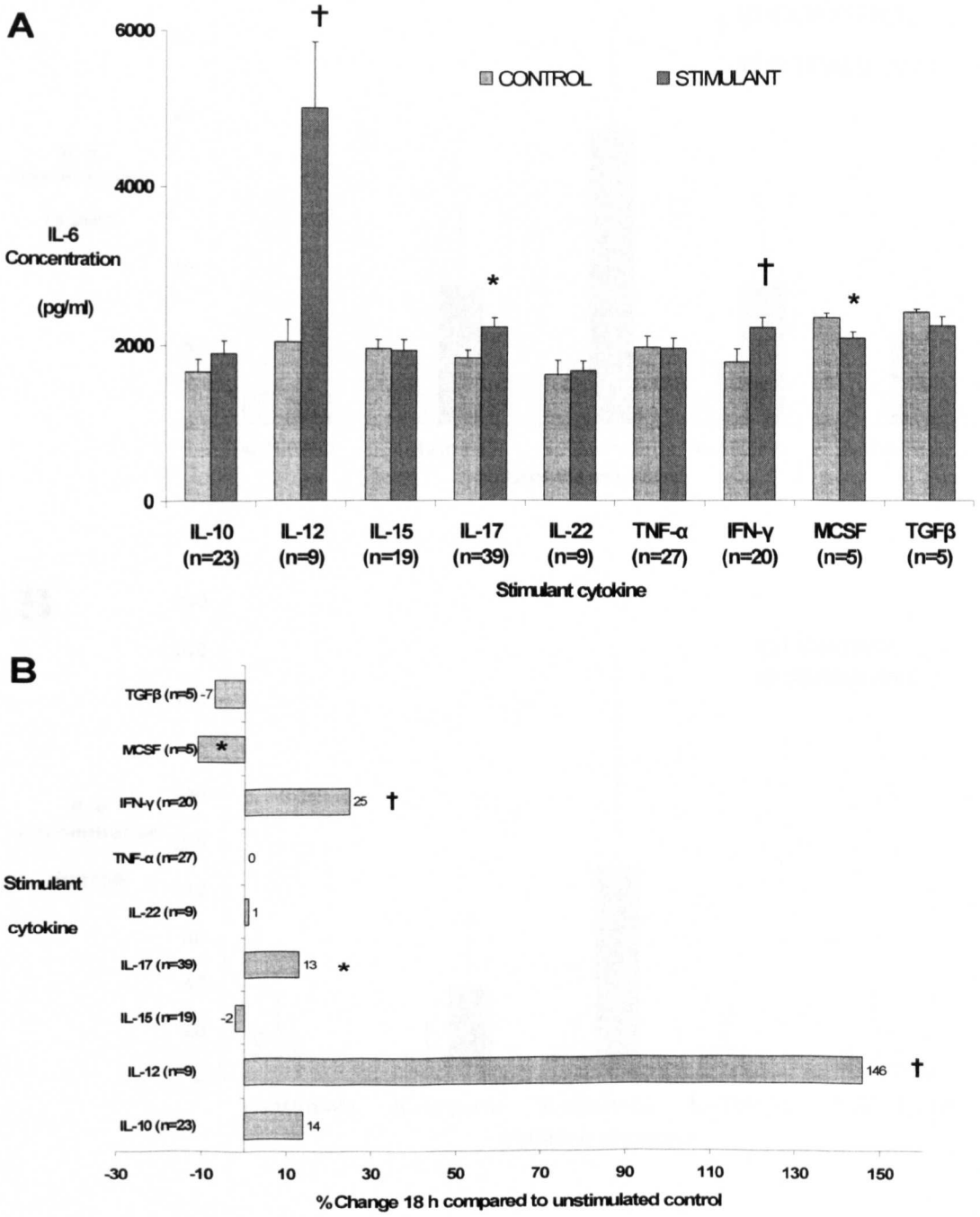
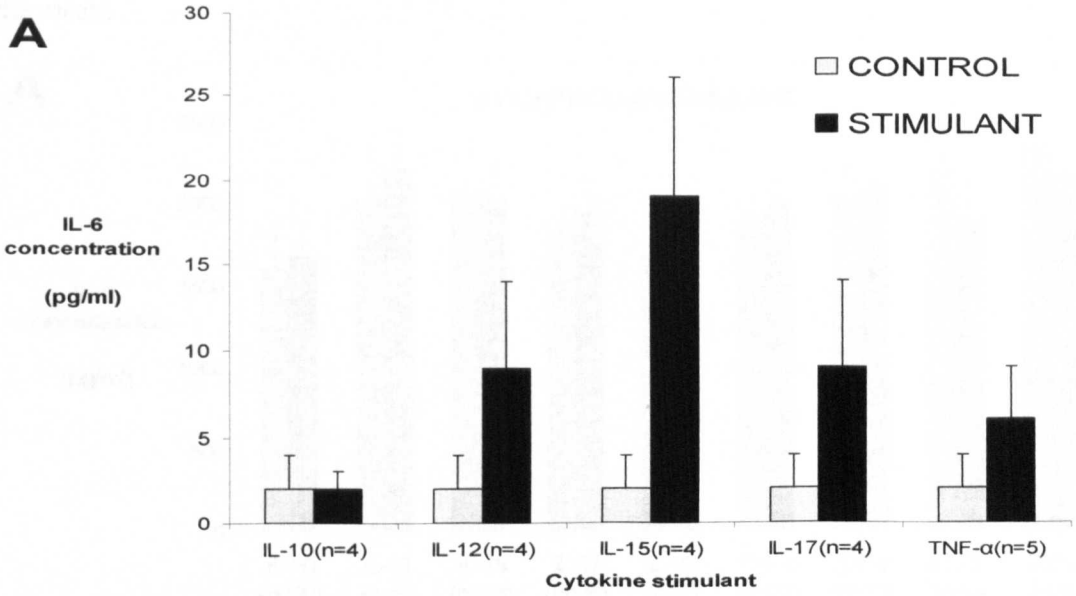


Figure 6.2 ***In vitro*** **effects of 18 h cytokine stimulation upon IL-6 expression within PBMC and whole blood cell cultures**. (A) The mean concentration of IL-6 after cytokine stimulation compared with unstimulated control in 18 h PBMC cultures. (B) The mean concentration of IL-6 after cytokine stimulation compared with unstimulated control in 18 h whole blood cell cultures. The total number of separate patient derived cultures stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-6 concentration with the standard error of the mean.



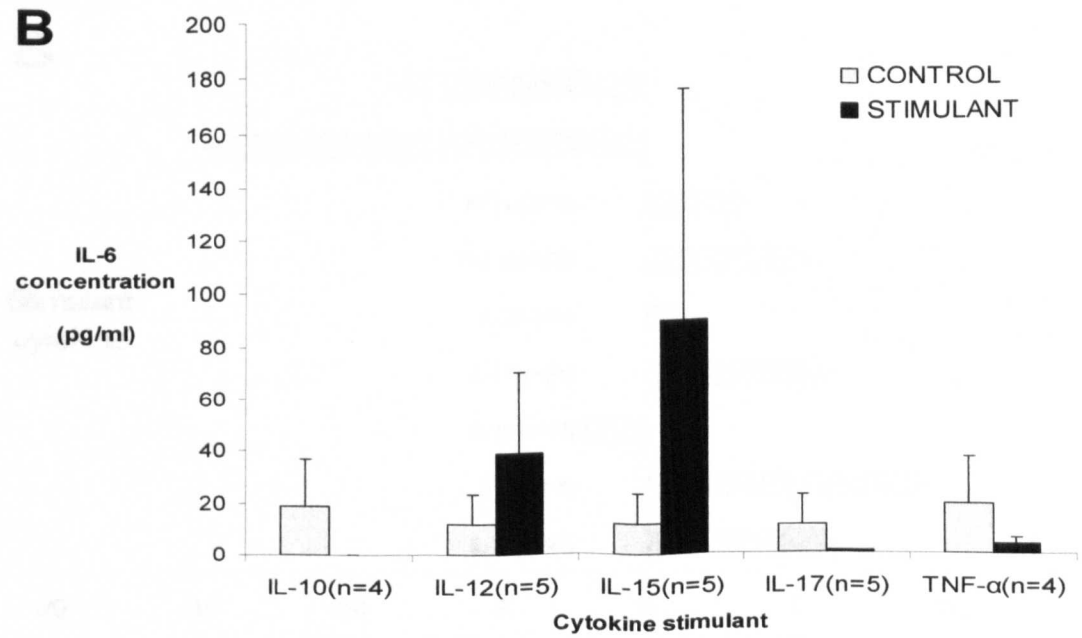
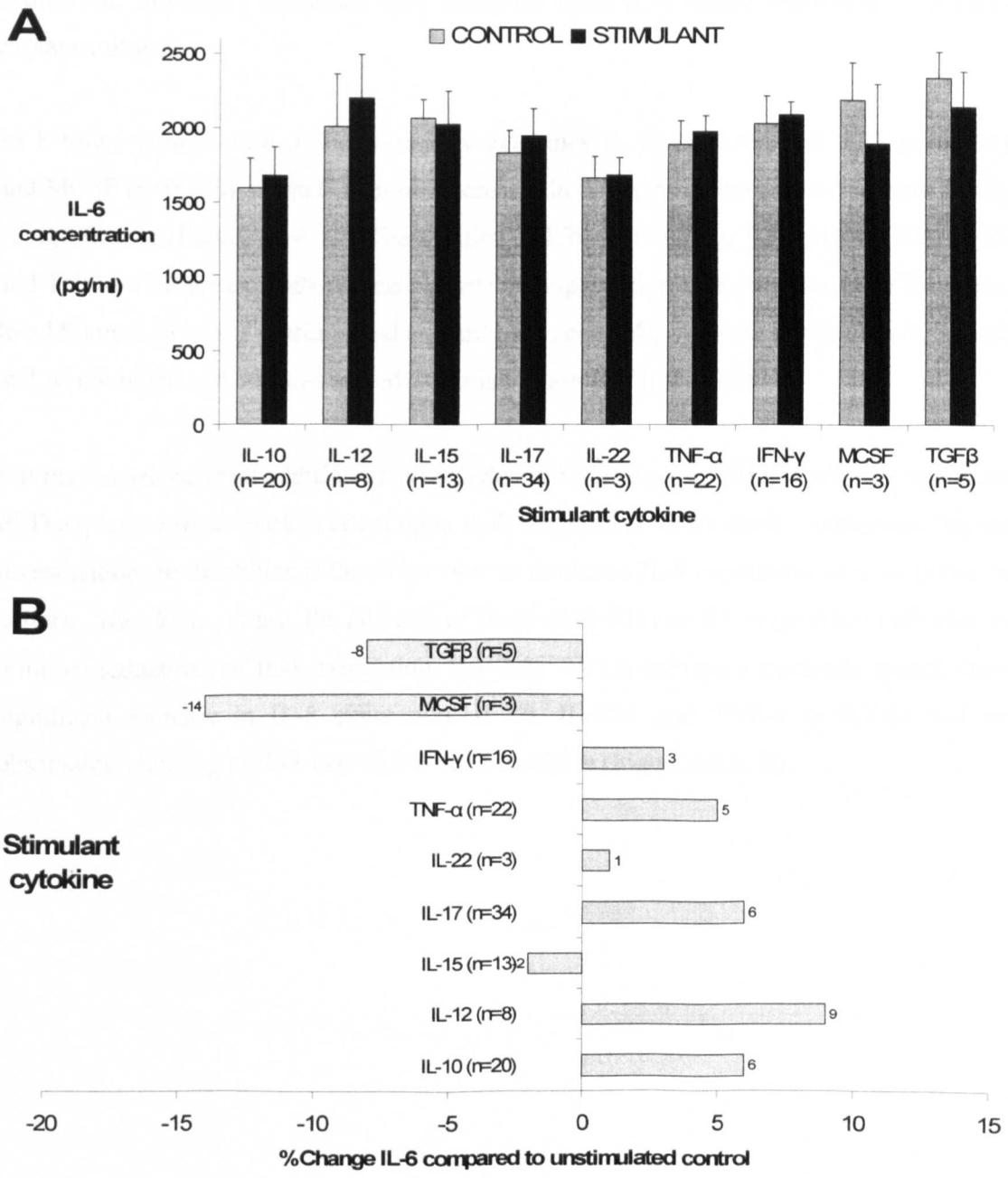


Figure 6.3 ***In vitro*** **effects of 72 h mitogenic stimulation upon IL-6 expression within PRD explant cultures.** PRD explant tissues were stimulated with a panel of key cytokines for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of IL-6 after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IL-6 concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-6 concentration with the standard error of the mean.



6.3 *In vitro* effects of inflammatory mediators upon IL-8 expression within PRD tissue explant cultures

The previous experiments established that within the PRD explant system, IL-6 expression was inducible by key inflammatory cytokine mediators at 18 h culture. Having previously ascertained that unstimulated PRD explants also produced significant quantities of IL-8, elucidation of upstream regulators of IL-8 production is therefore of importance. Therefore, I next investigated whether the same panel of cytokines could modulate IL-8 expression. Initially, I examined their influence upon IL-8 within short-term 18 h PRD explant cultures.

18 h tissue culture with proinflammatory cytokines IL-12 ($p=0.081$), IL-17A ($p=0.161$) and MCSF ($p=0.176$) resulted in minor increases in IL-8 expression that were below levels of significance (Figure 6.4A, B). The addition of IFN-γ ($p=0.372$), TGF-β ($p=0.147$), IL-15 and TNF-α to tissue explants had no observable impact upon IL-8 secretion at 18 h culture. In addition to matched unstimulated explant tissue controls, cytokine stimulation of PBMC and whole blood cell cultures served as further controls (Figure 6.5A, B).

Having determined that addition of the selected inflammatory cytokine mediators to human PRD explants had no marked effect upon IL-8 secretion at 18 h culture, I undertook further investigations to determine if they were able to modulate IL-8 expression over longer-term culture. After 72 h culture, the addition of IL-15 ($p=0.031$) or IFN-γ ($p=0.031$) resulted in minimal reductions of IL-8 expression. IL-12 ($p=0.053$) induced a moderate though non-significant increase in IL-8 expression. IL-10, IL-17A and TNF-α ($p=0.192$) had no observable effect upon IL-8 expression at 72 h culture (Figure 6.6A, B).

Figure 6.4 ***In vitro*** **effects of 18 h cytokine stimulation upon IL-8 expression within PRD explant cultures**. PRD explant tissues were stimulated with a panel of key cytokines for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of IL-8 after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IL-8 concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-8 concentration with the standard error of the mean.

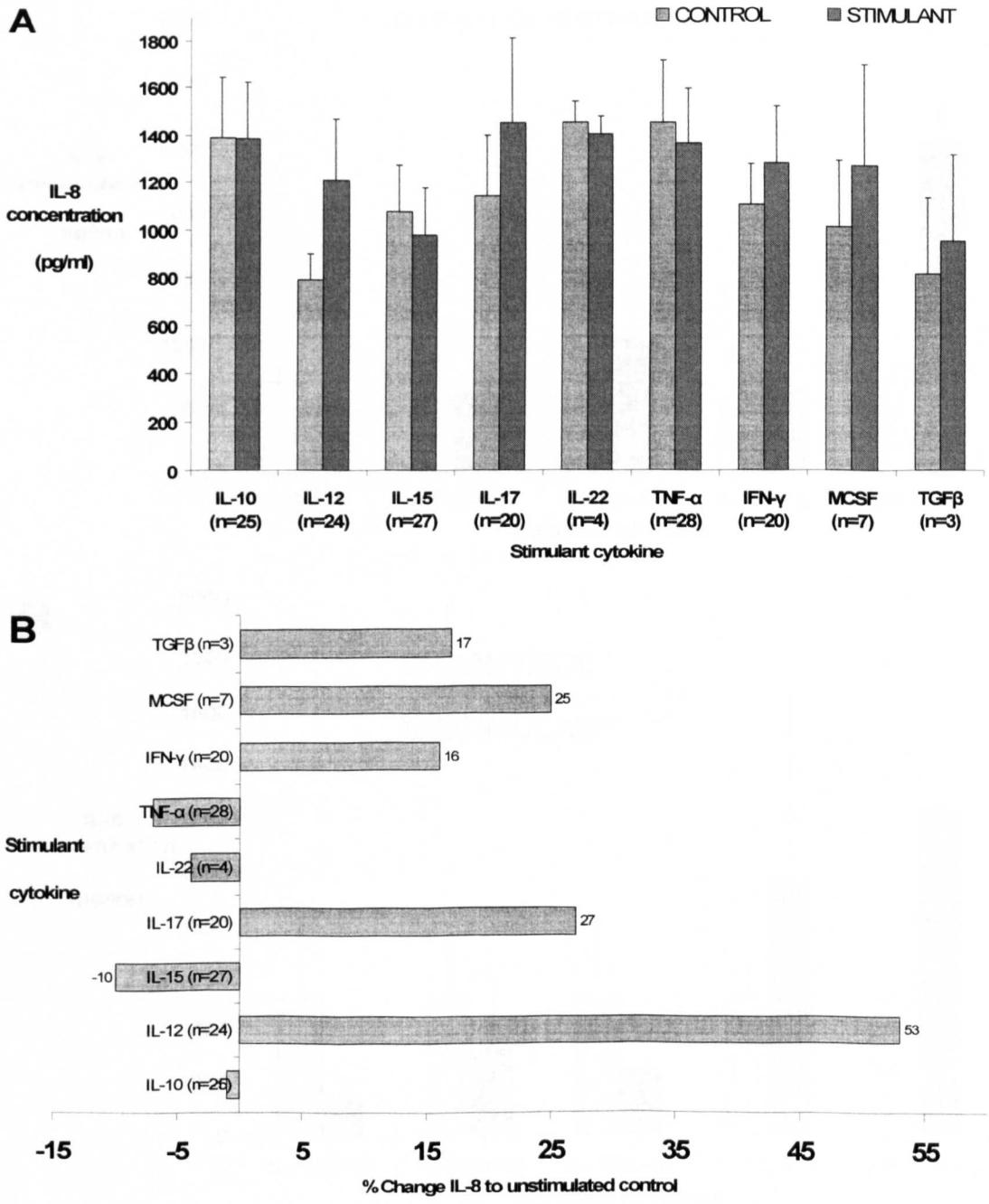


Figure 6.5 ***In vitro*** **effects of 18 h cytokine stimulation upon IL-8 expression within PBMC and whole blood cell cultures.** (A) The mean concentration of IL-8 after cytokine stimulation compared with unstimulated control in 18 h PBMC cultures. (B) The mean concentration of IL-8 after cytokine stimulation compared with unstimulated control in 18 h whole blood cell cultures. The total number of separate patient derived cultures stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-8 concentration with the standard error of the mean.

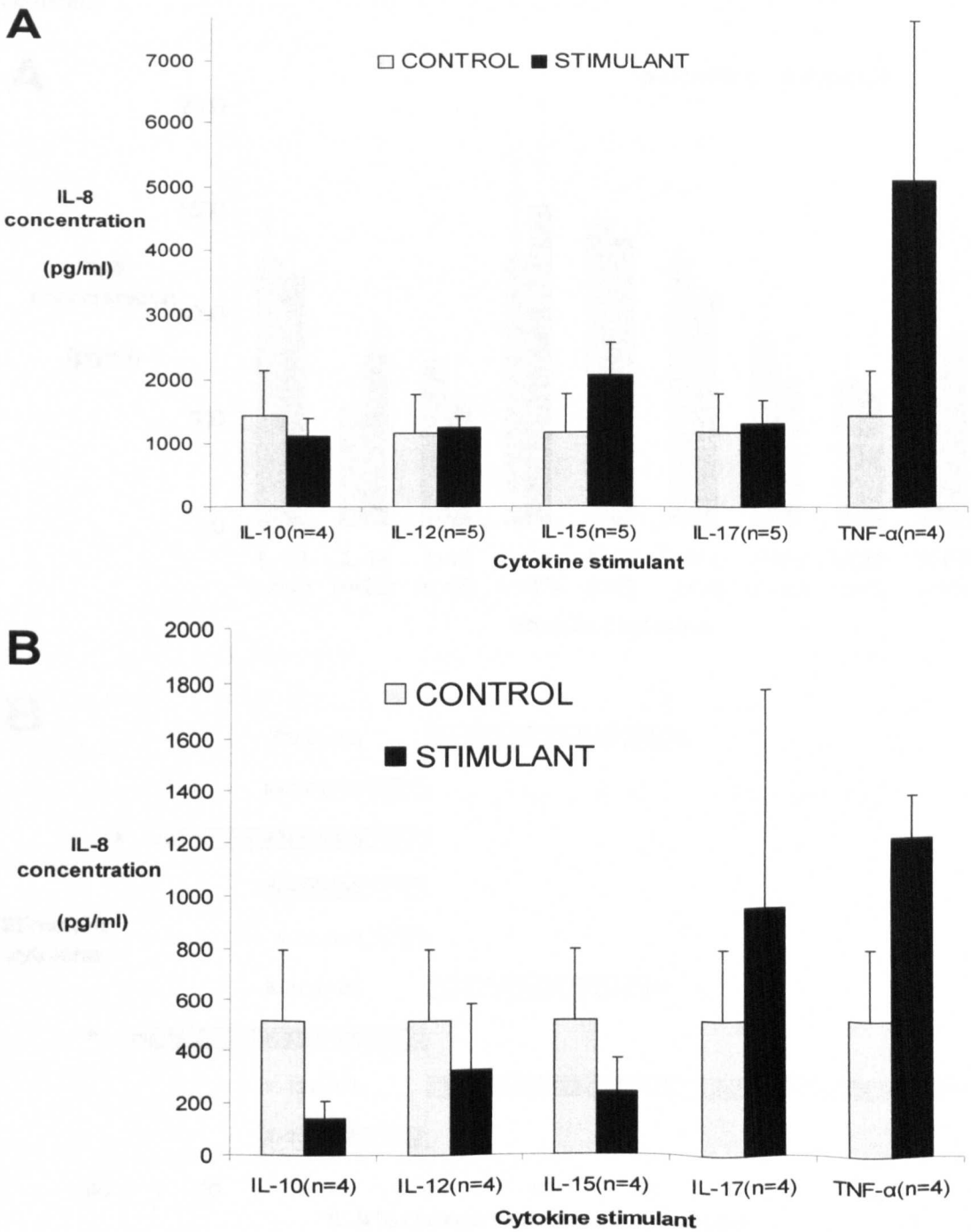
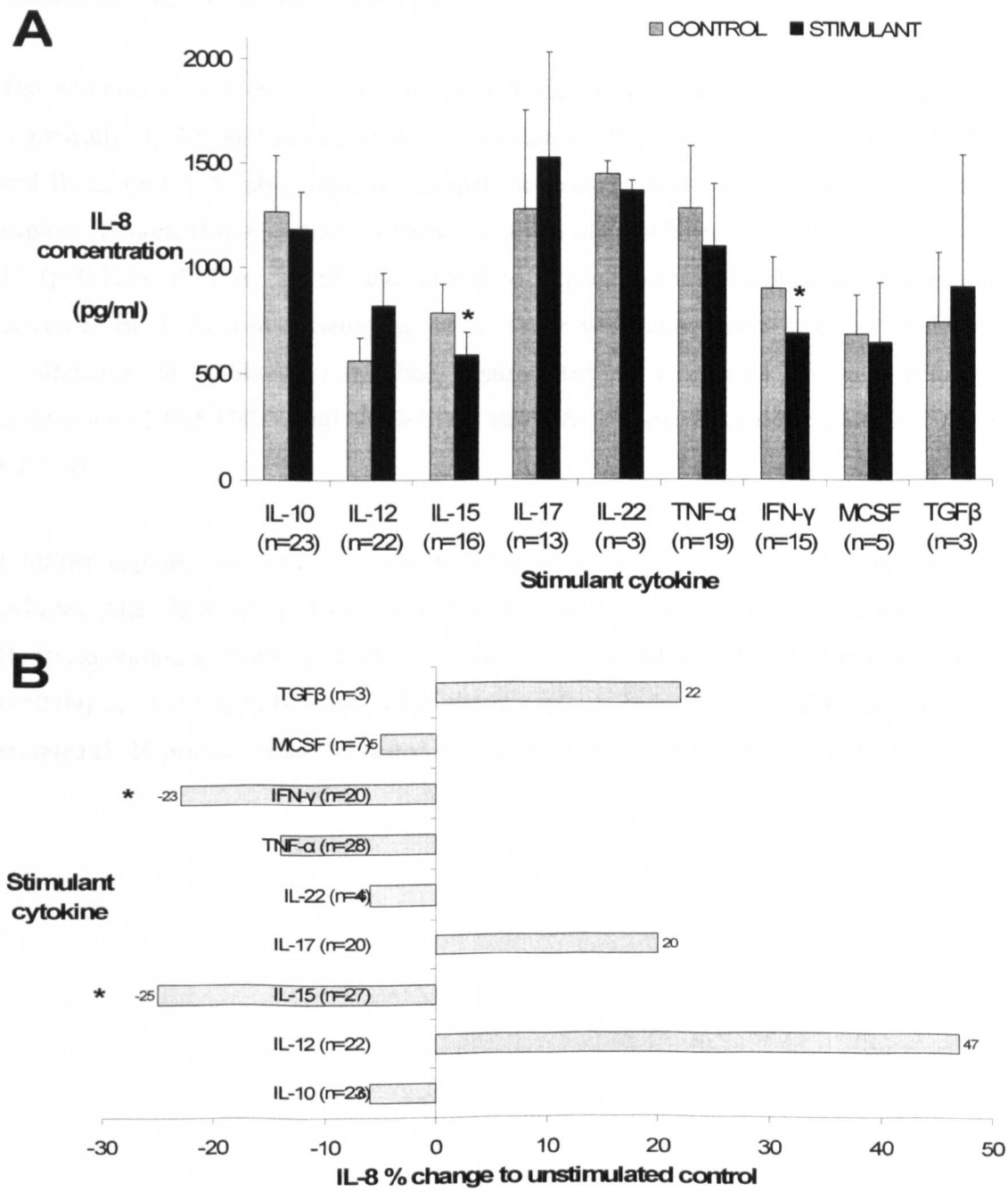


Figure 6.6 ***In vitro*** **effects of 72 h mitogenic stimulation upon IL-8 expression within PRD explant cultures.** PRD explant tissues were stimulated with a panel of key cytokines for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of IL-8 after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IL-8 concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-8 concentration with the standard error of the mean.



6.4 *In vitro* effects of inflammatory mediators upon IL-10 expression within PRD tissue explant cultures

The previous experiment demonstrated that exogenous cytokine addition to PRD explant tissue cultures had minimal modulatory effects upon the expression of endogenous IL-8. I therefore undertook further investigations to determine if the same panel of exogenous cytokines had any effect upon the secretion of the predominantly antiinflammatory cytokine, IL-10. Initially, I explored the modulation of IL-10 expression by these mediators in short-term PRD explant culture.

The addition of TNF-α ($p=0.015$) to PRD tissue explants resulted in a moderate and statistically significant increase in IL-10 secretion at 18 h culture (Figure 6.7A,B). IFN-γ and IL-22 ($p=0.158$) also induced minimal increases of IL-10 expression in 18 h PRD explant cultures. However, these were below levels of significance. Addition of IL-12, IL-15 ($p=0.121$), IL-17A, MCSF and TGF-β to explant cultures resulted in only minor decreases in IL-10 concentrations at 18 h. These were also below levels of statistical significance. In addition to matched unstimulated explant tissue controls, cytokine stimulation of 18 h PBMC and whole blood cell cultures served as further controls (Figure 6.8A,B).

I further explored the effects of the addition of these cytokines to long-term explant cultures. After 72 h explant tissue culture, TNF-α ($p=0.05$) induced a moderate increase of IL-10 expression at levels of statistical significance. The addition of IL-15 ($p=0.08$), IFN-γ ($p=0.09$) or IL-12 ($p=0.0143$) to PRD tissue explants induced only minor increases of secreted IL-10 protein within harvested supernatants at 72 h culture (Figure 6.9A,B).

Figure 6.7 ***In vitro*** **effects of 18 h cytokine stimulation upon IL-10 expression within PRD explant cultures**. PRD explant tissues were stimulated with a panel of key cytokines for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of IL-10 after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IL-10 concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-10 concentration with the standard error of the mean.

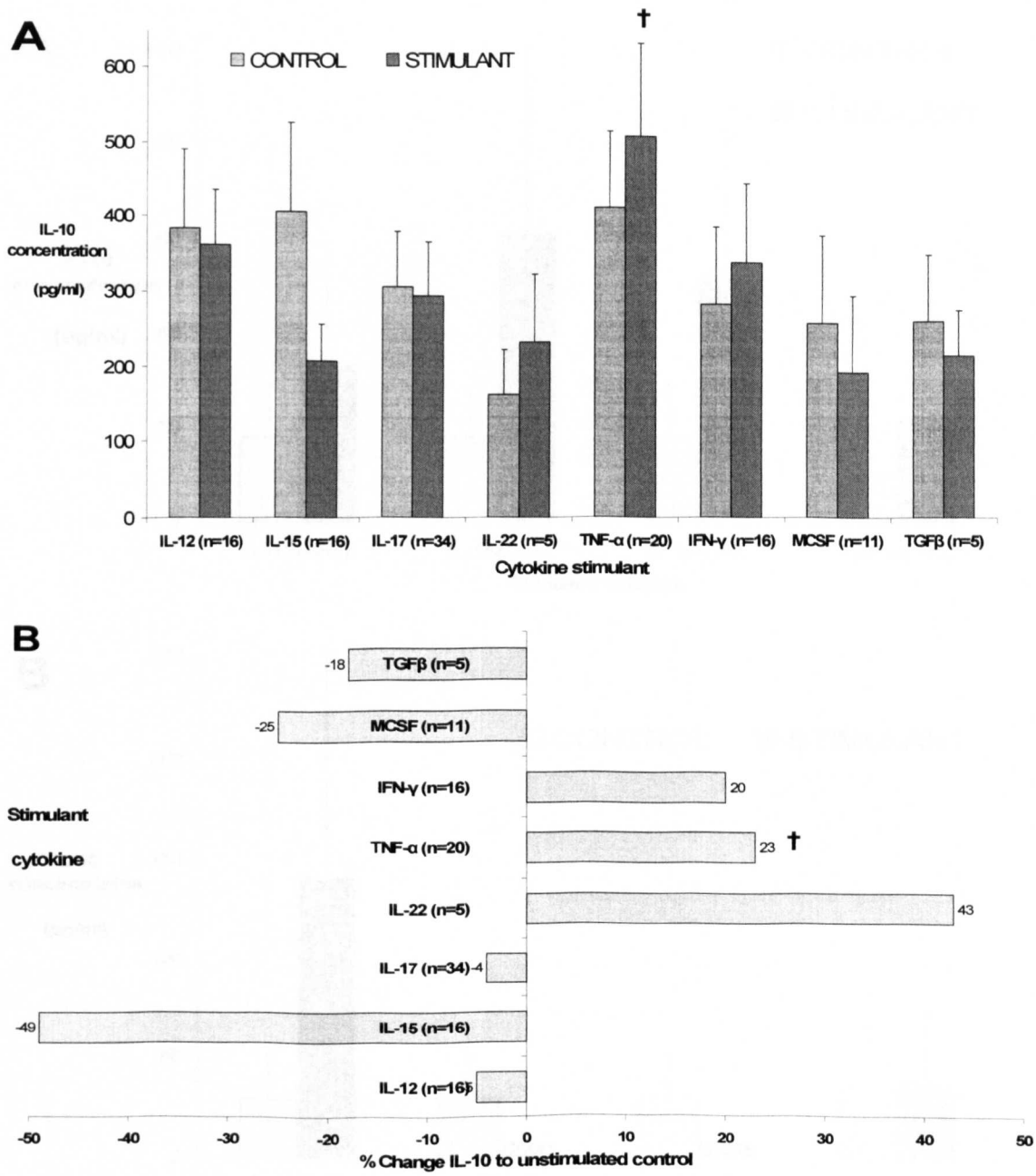
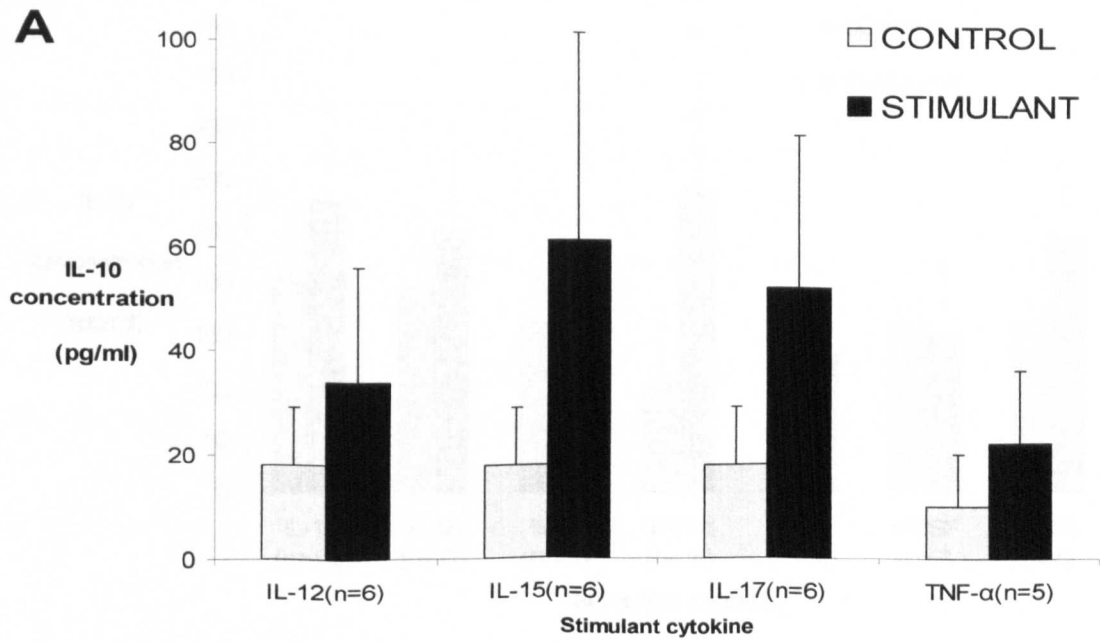


Figure 6.8 ***In vitro*** **effects of 18 h cytokine stimulation upon IL-10 expression within PBMC and whole blood cell cultures.** (A) The mean concentration of IL-10 after cytokine stimulation compared with unstimulated control in 18 h PBMC cultures. (B) The mean concentration of IL-10 after cytokine stimulation compared with unstimulated control in 18 h whole blood cell cultures. The total number of separate patient derived cultures stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-10 concentration with the standard error of the mean.



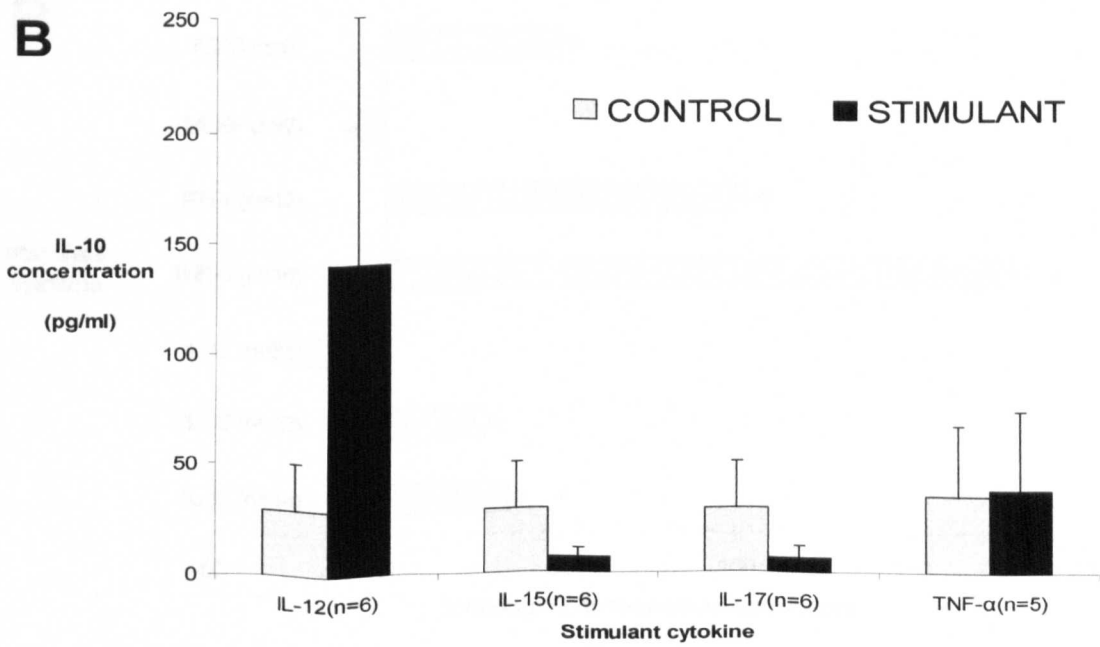
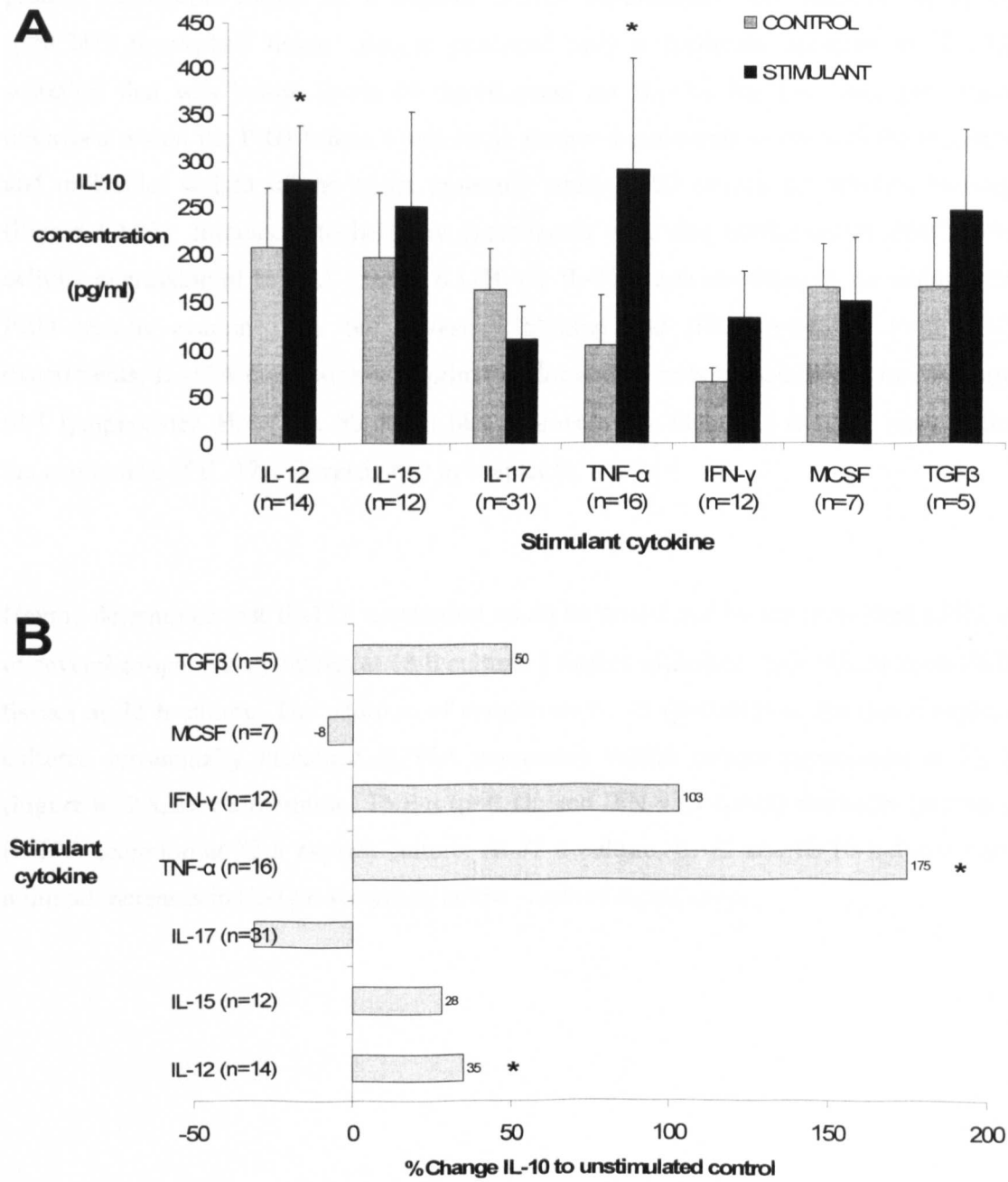


Figure 6.9 ***In vitro*** **effects of 72 h mitogenic stimulation upon IL-10 expression within PRD explant cultures**. PRD explant tissues were stimulated with a panel of key cytokines for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of IL-10 after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IL-10 concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-10 concentration with the standard error of the mean.



6.5 *In vitro* effects of inflammatory mediators upon IL-17A expression within PRD tissue explant cultures

I next explored the effects of the same panel of cytokines upon the expression of the recently discovered and novel cytokine, IL-17A over short-term PRD tissue explant culture. The addition of IL-15 ($p=0.043$) to PRD tissue explants markedly increased IL-17A expression at 18 h culture (Figure 6.10A, B). Stimulation with exogenous IL-22 ($p=0.004$), IFN-γ ($p=0.058$) or TNF-α ($p=0.014$) resulted in minor increases of IL-17A protein expression within 18 h explant culture supernatants. The addition of IL-10 ($p=0.241$) to explant tissue cultures produced only a moderate decrease in IL-17A secretion that was below levels of significance. As IL-17A has not been previously described within the PRD lesion, I undertook further experiments to confirm the presence and molecular weight of the target molecule within PRD lesions by Western blotting (Figure 6.11A). Immunohistochemistry experiments were also performed to identify the cellular expression of IL-17A (Figure 6.11B, C). IL-17A was identified in the majority of PRD lesions examined by both Western blotting and IHC ($n=24/25$). From IHC experiments, IL-17A expression was primarily located to cells exhibiting the morphology of T lymphocytes. However, fibroblast-like cells were also identified as being positive for the expression of IL-17A (investigated in chapter 8).

Having determined that IL-17A expression could be modulated by the individual addition of several exogenous cytokines at 18 h culture, I further examined their effects upon PRD tissues at 72 h culture. The addition of exogenous IL-15 ($p=0.049$) to the tissue explant cultures substantially increased IL-17A expression within culture supernatant at 72 h (Figure 6.12A,B). Furthermore, TNF-α ($p=0.11$) and IFN-γ ($p=0.048$) markedly increased IL-17A secretion at 72 h explant culture. At 72 h culture, IL-22 and IL-10 induced only minimal increases in IL-17A secretion, below levels of significance.

Figure 6.10 ***In vitro*** **effects of 18 h cytokine stimulation upon IL-17A expression within PRD explant cultures**. PRD explant tissues were stimulated with a panel of key cytokines for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of IL-17A after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IL-17A concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-17A concentration with the standard error of the mean.

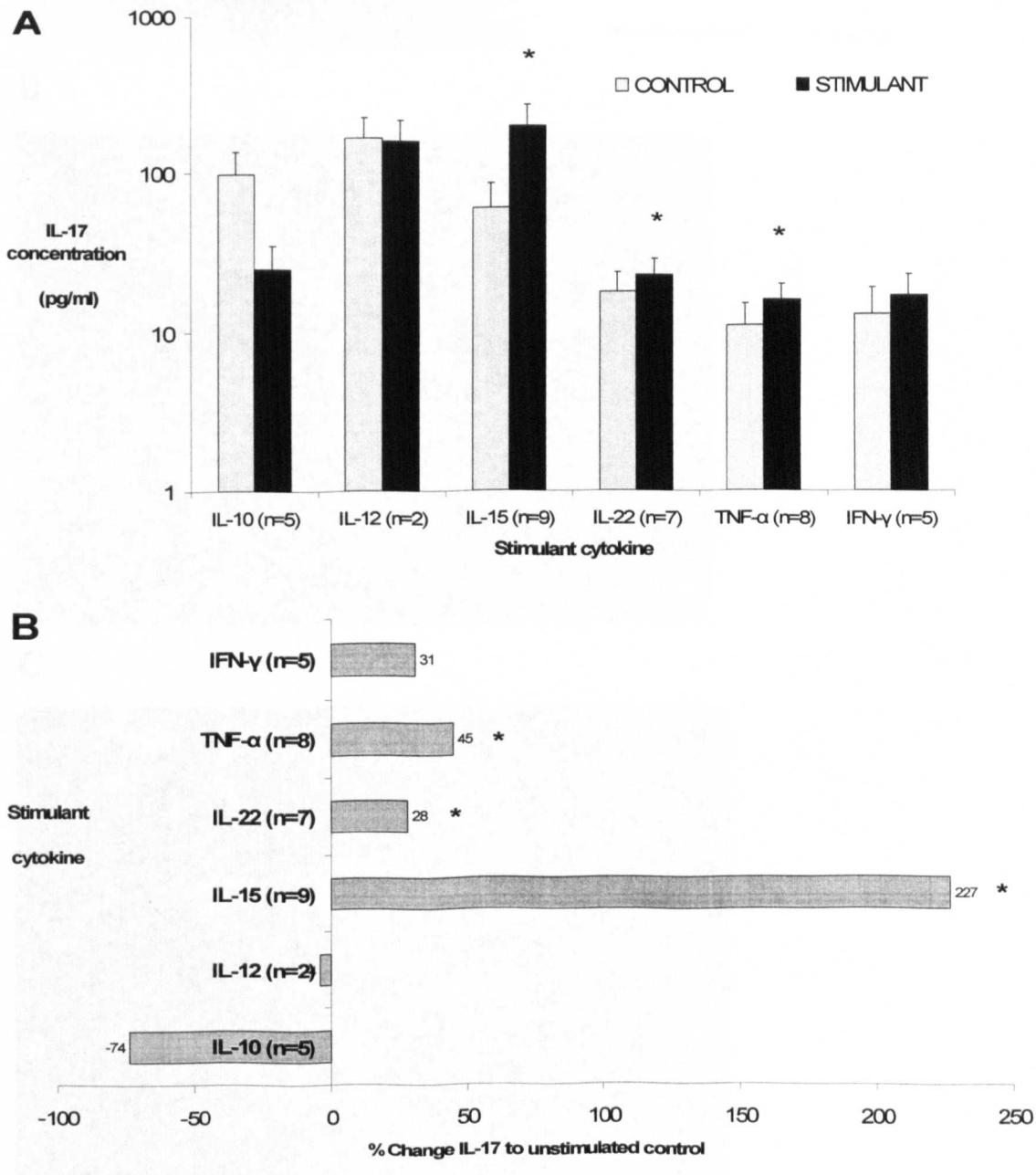
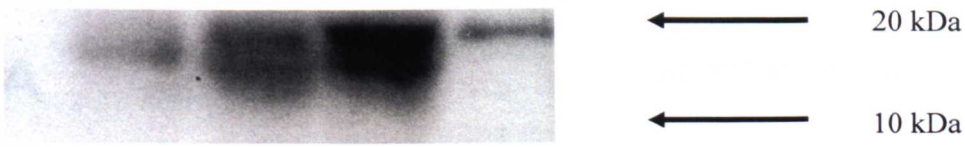


Figure 6.11 Confirmation of IL-17A expression in PRD tissue. (A) Western blot of 4 PRD tissue protein lysates confirming the molecular weight of IL-17A. (B) Immunohistochemical detection of IL-17A within PRD tissue biopsies, low power magnification. (C) High power resolution of IL-17A within PRD tissues, orange arrows represent IL-17A positive cells (T lymphocytes), blue arrows represent IL-18 positive cells (macrophages) in close proximity.

A



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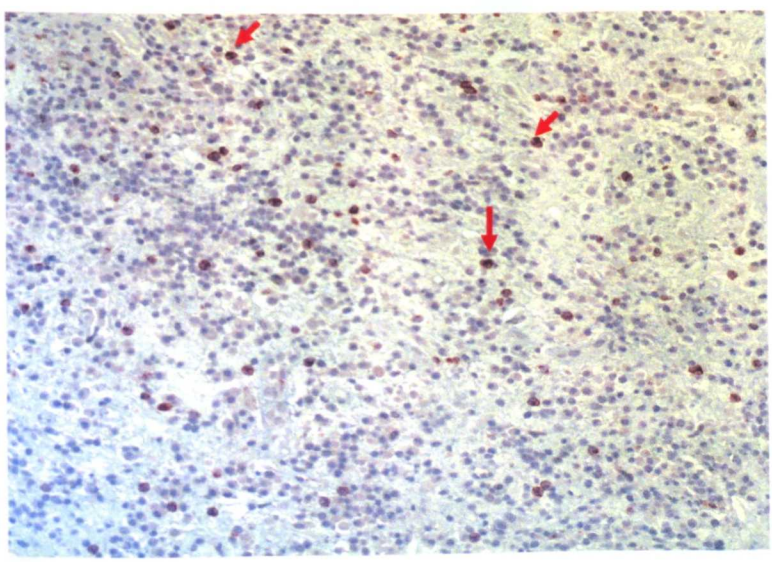

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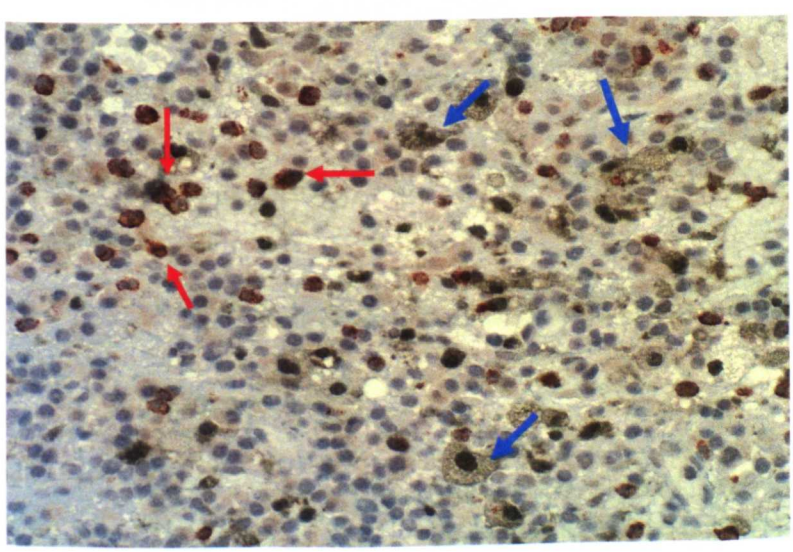
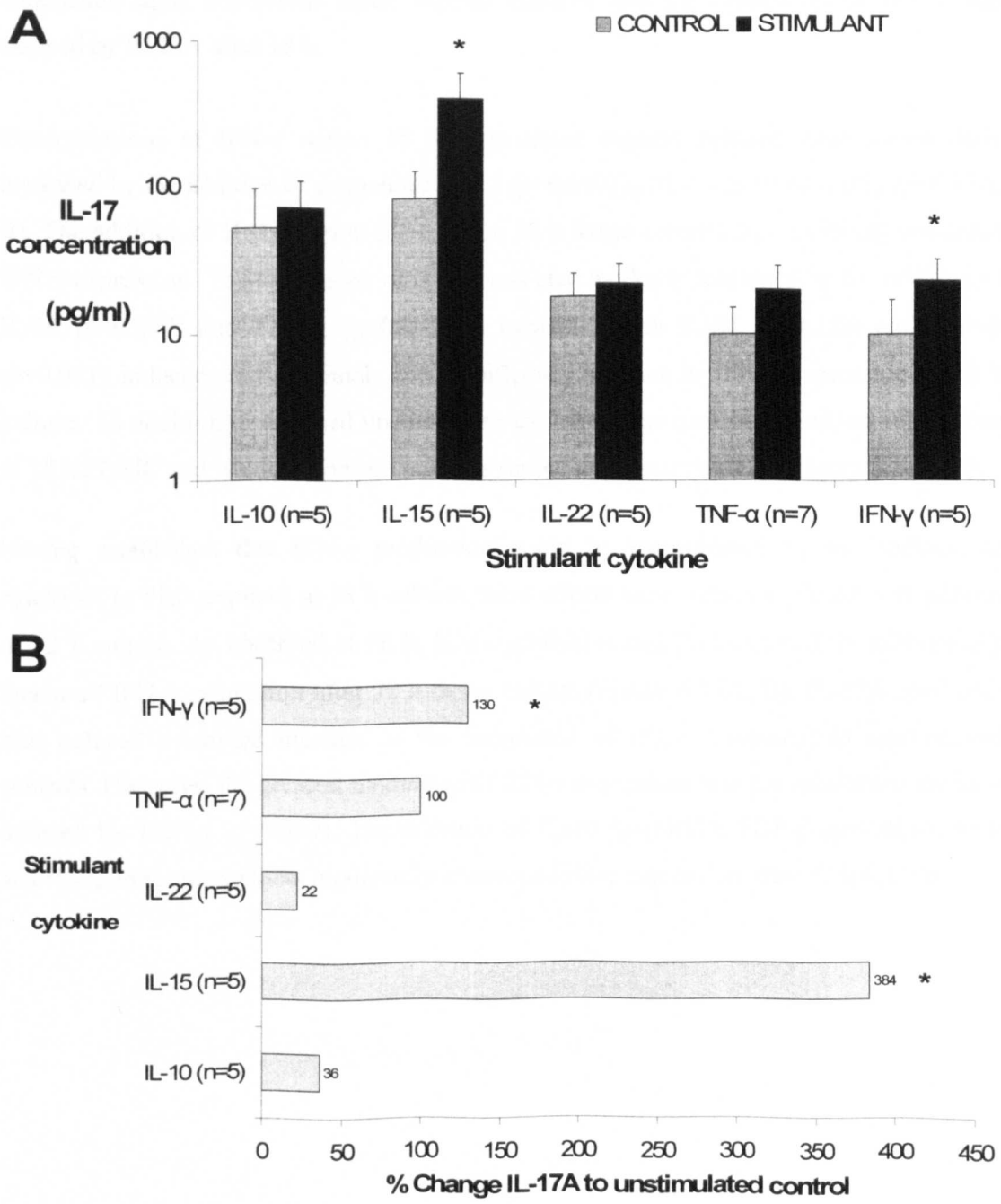


Figure 6.12 ***In vitro*** **effects of 72 h mitogenic stimulation upon IL-17A expression within PRD explant cultures**. PRD explant tissues were stimulated with a panel of key cytokines for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of IL-17A after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IL-17A concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-17A concentration with the standard error of the mean.



6.6 *In vitro* effects of inflammatory mediators upon IFN-γ expression within PRD tissue explant cultures

From the previous experiments, I ascertained that within the novel PRD explant culture system, proinflammatory cytokine expression could be manipulated and further induced by the addition of exogenous cytokines. I therefore investigated the effects of these inflammatory mediators upon the expression of a cytokine that is central to inflammatory immune responses of infectious aetiology, namely IFN-γ. Experiments were initially undertaken upon short-term tissue explant cultures and the expression of IFN-γ was assayed by ELISA after 18 h.

Concentrations of IFN-γ within 18 h PRD tissue explant cultures were substantially increased by the addition of exogenous IL-12 ($p=0.040$) or IL-15 ($p=0.003$) (Figure 6.13A, B). The addition of IL-17A ($p=0.05$) to these 18 h tissue cultures also markedly increased IFN-γ expression. The expression of IFN-γ was also modestly increased by the addition of IL-10 ($p=0.047$) and TNF-α ($p=0.085$) to explants. Both IL-22 ($p=0.270$) and TGF-β ($p=0.061$) induced only minimal, non-significant increases in IFN-γ expression at 18 h culture. In addition to matched unstimulated explant tissue controls, cytokine stimulation of 18 h PBMC and whole blood cell cultures served as further controls (Figure 6.14A, B).

Having established that IFN-γ production could be manipulated by the addition of cytokines to PRD explants at 18 h culture, these effects were further explored over periods of 72 h culture. As observed at 18 h, IL-12 ($p=0.024$) and IL-15 ($p=0.022$) substantially increased IFN-γ expression after 72 h tissue culture (Figure 6.15A, B). IL-17A ($p=0.040$) also induced a marked increase in the expression of IFN-γ compared to unstimulated controls. However, the greatest modulator of IFN-γ expression was the substantial increase induced by TNF-α ($p=0.037$). The addition of IL-10 ($p=0.057$), TGF-β ($p=0.0006$, $n=4$) and IL-22 to tissue explants moderately increased IFN-γ expression after 72 h culture.

Figure 6.13 ***In vitro*** **effects of 18 h cytokine stimulation upon IFN-γ expression within PRD explant cultures**. PRD explant tissues were stimulated with a panel of key cytokines for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of IFN-γ after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IFN-γ concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean IFN-γ concentration with the standard error of the mean.

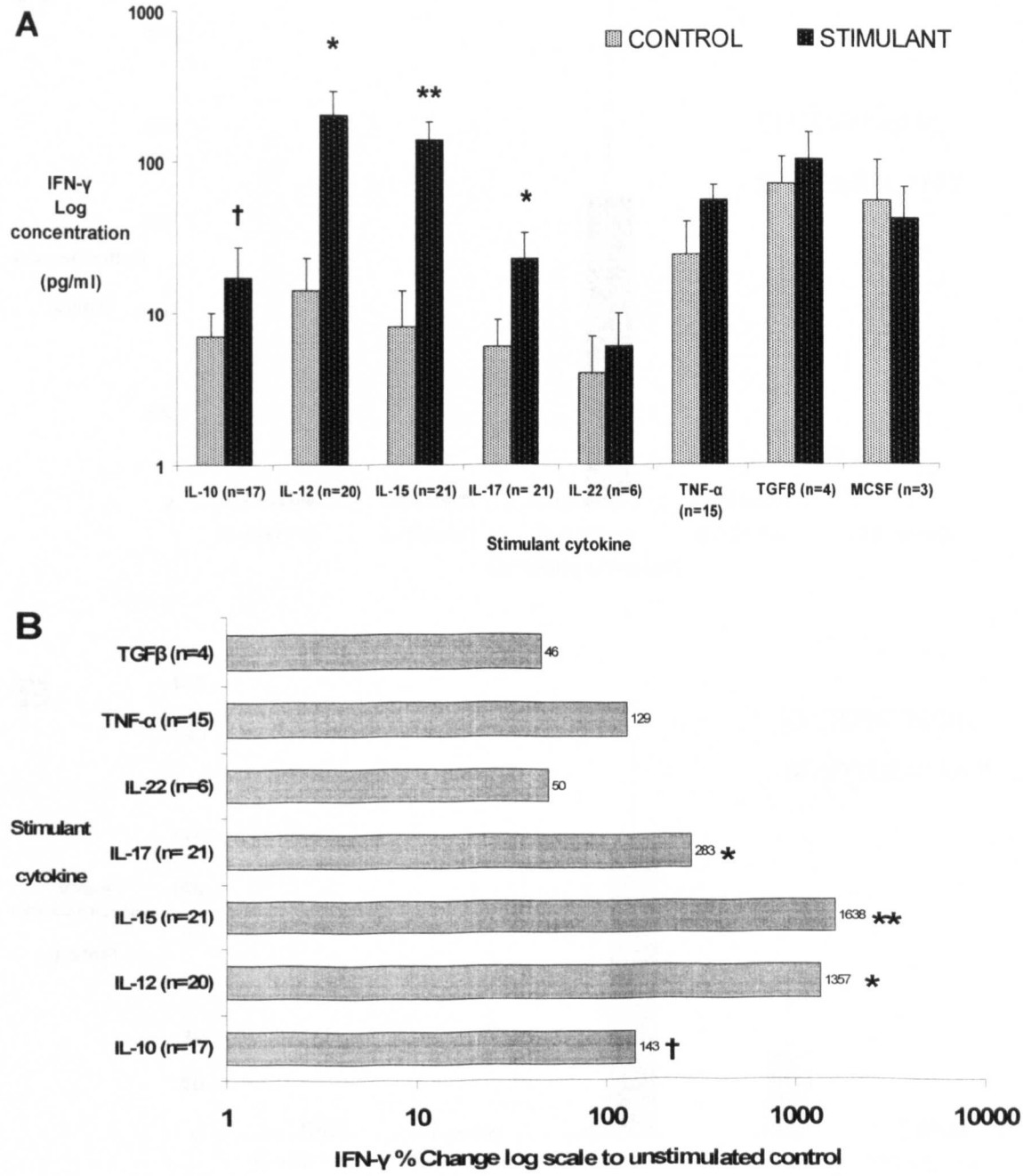
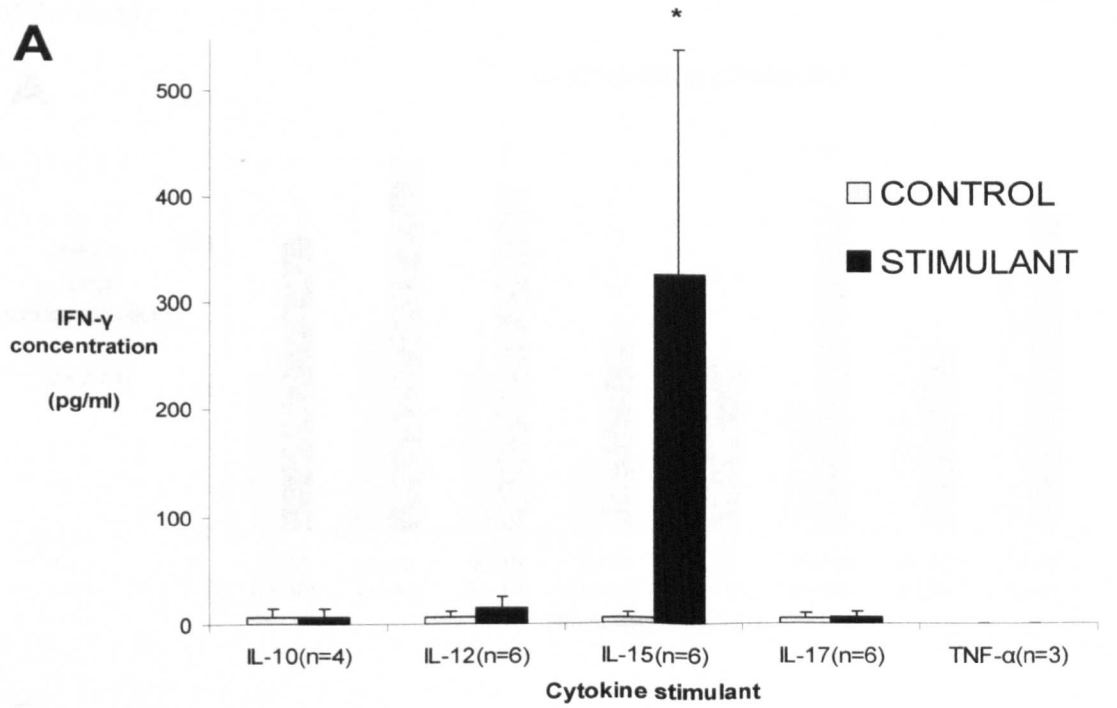


Figure 6.14 ***In vitro*** **effects of 18 h cytokine stimulation upon IFN-γ expression within PBMC and whole blood cell cultures.** (A) The mean concentration of IFN-γ after cytokine stimulation compared with unstimulated control in 18 h PBMC cultures. (B) The mean concentration of IFN-γ after cytokine stimulation compared with unstimulated control in 18 h whole blood cell cultures. The total number of separate patient derived cultures stimulated with each cytokine is given in parenthesis. Bars represent the mean IFN-γ concentration with the standard error of the mean.



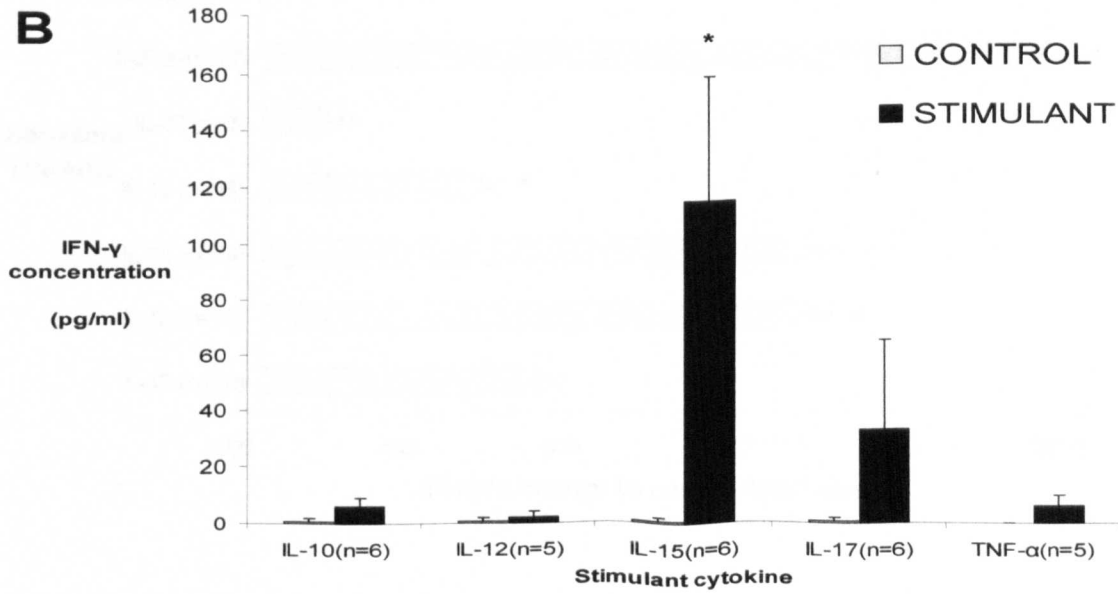
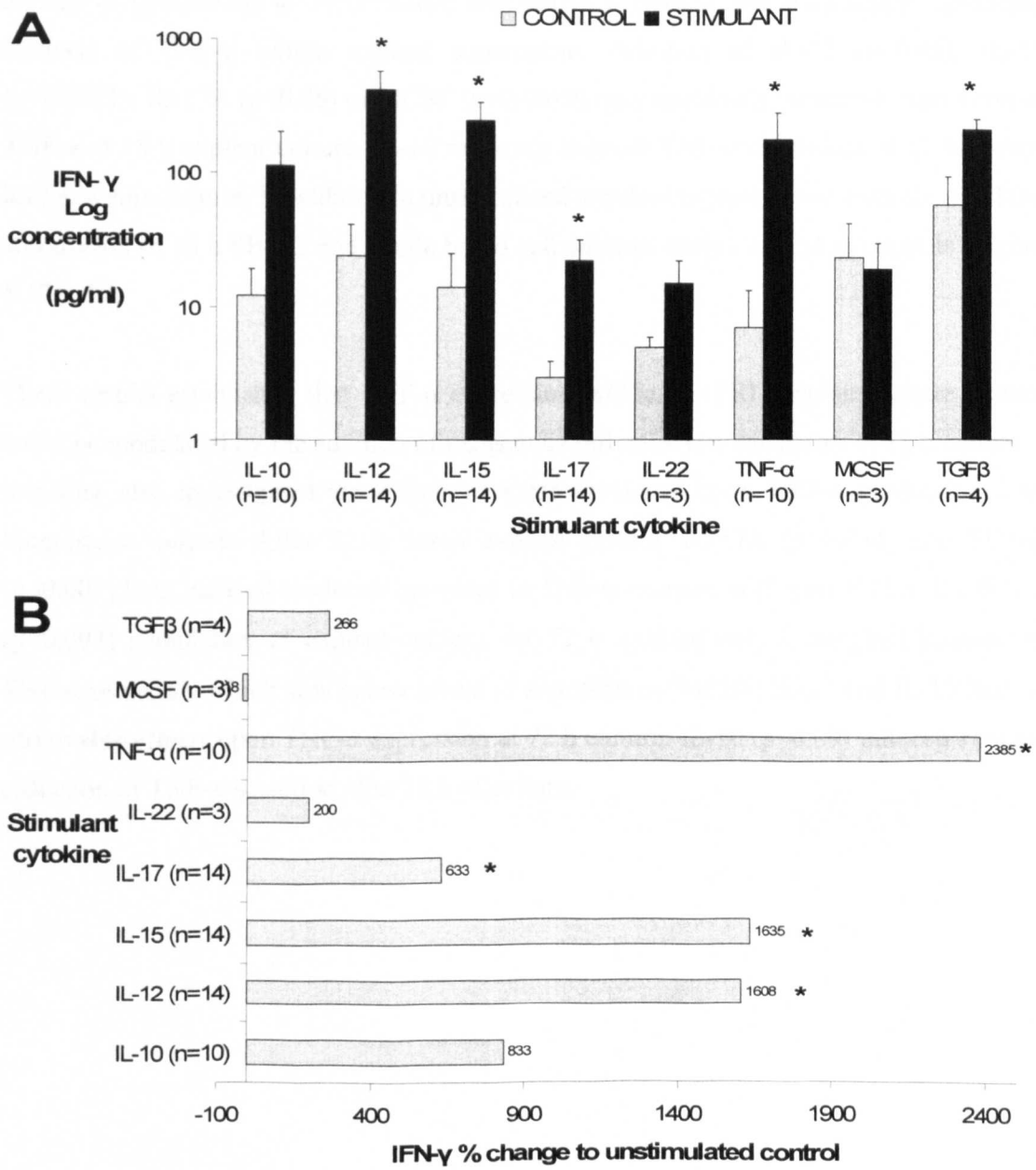


Figure 6.15 ***In vitro*** **effects of 72 h cytokine stimulation upon IFN-γ expression within PRD explant cultures**. PRD explant tissues were stimulated with a panel of key cytokines for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of IFN-γ after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IFN-γ concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean IFN-γ concentration with the standard error of the mean.



6.7 *In vitro* effects of inflammatory mediators upon TNF-α expression within PRD tissue explant cultures

Finally, I investigated the effects of cytokine addition to PRD tissue explants upon endogenous expression of the proinflammatory cytokine TNF-α over short-term, 18 h culture. 18 h stimulation of PRD tissue explants with IFN-γ ($p=0.027$) resulted in a marked increase in TNF-α expression within culture supernatants (Figure 6.16A, B). The addition of TGF-β ($p=0.0026$) to PRD tissues resulted in a modest and statistically significant increase of TNF-α within explant supernatant. Addition of IL-12 ($p=0.08$), IL-15 ($p=0.0287$), IL-17A ($p=0.06$) or MCSF ($p=0.0019$) only minimally increased expression of TNF-α at 18 h explant culture. IL-10 modestly reduced TNF-α expression at 18 h, below levels of significance. In addition to unstimulated matched explant tissue controls, cytokine stimulation of 18 h PBMC and whole blood cell cultures further served as controls (Figure 6.17A, B).

These results established that TNF-α expression within the PRD explant culture system could be modulated by the addition of exogenous inflammatory mediators at 18 h culture. I therefore also investigated the effects of these cytokines upon TNF-α production over longer-term culture. After 72 h tissue explant culture, IL-17A ($p=0.044$) and TGF-β ($p=0.007$) both induced moderate increases in TNF-α expression (Figure 6.18A, B). IFN-γ ($p=0.097$) stimulation of explant cultures for 72 h induced only a marginal increase in TNF-α secretion, which was below levels of significance. MCSF, IL-22 and IL-15 had no observable effects upon TNF-α expression at 72 h culture. IL-10 ($p=0.09$) induced a minor reduction of TNF-α secretion after 72 h of culture.

Figure 6.16 ***In vitro*** **effects of 18 h cytokine stimulation upon TNF-α expression within PRD explant cultures**. PRD explant tissues were stimulated with a panel of key cytokines for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of TNF-α after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant TNF-α concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean TNF-α concentration with the standard error of the mean.

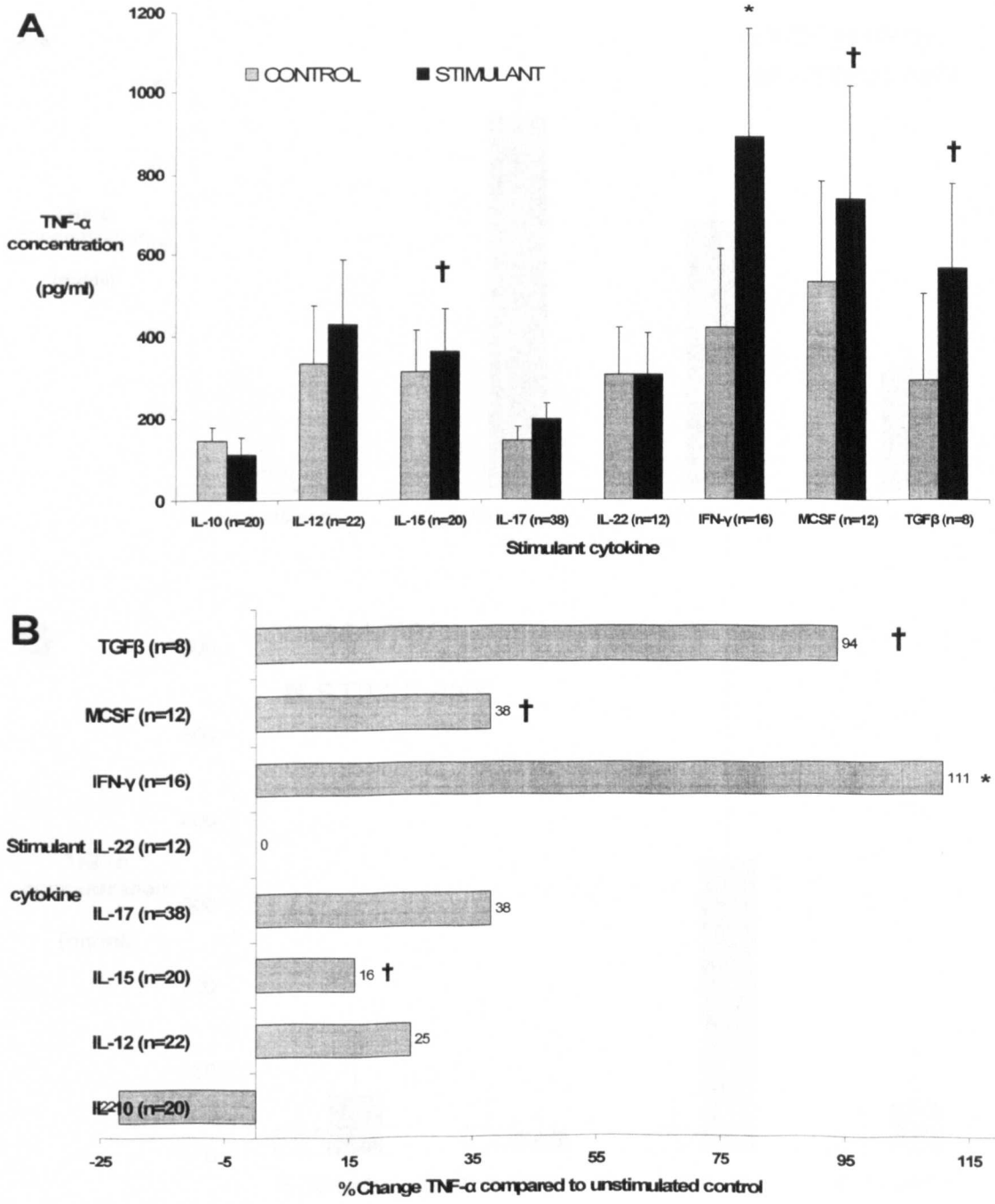


Figure 6.17 ***In vitro*** **effects of 18 h cytokine stimulation upon TNF-α expression within PBMC and whole blood cell cultures.** (A) The mean concentration of TNF-α after cytokine stimulation compared with unstimulated control in 18 h PBMC cultures. (B) The mean concentration of TNF-α after cytokine stimulation compared with unstimulated control in 18 h whole blood cell cultures. The total number of separate patient derived cultures stimulated with each cytokine is given in parenthesis. Bars represent the mean TNF-α concentration with the standard error of the mean.

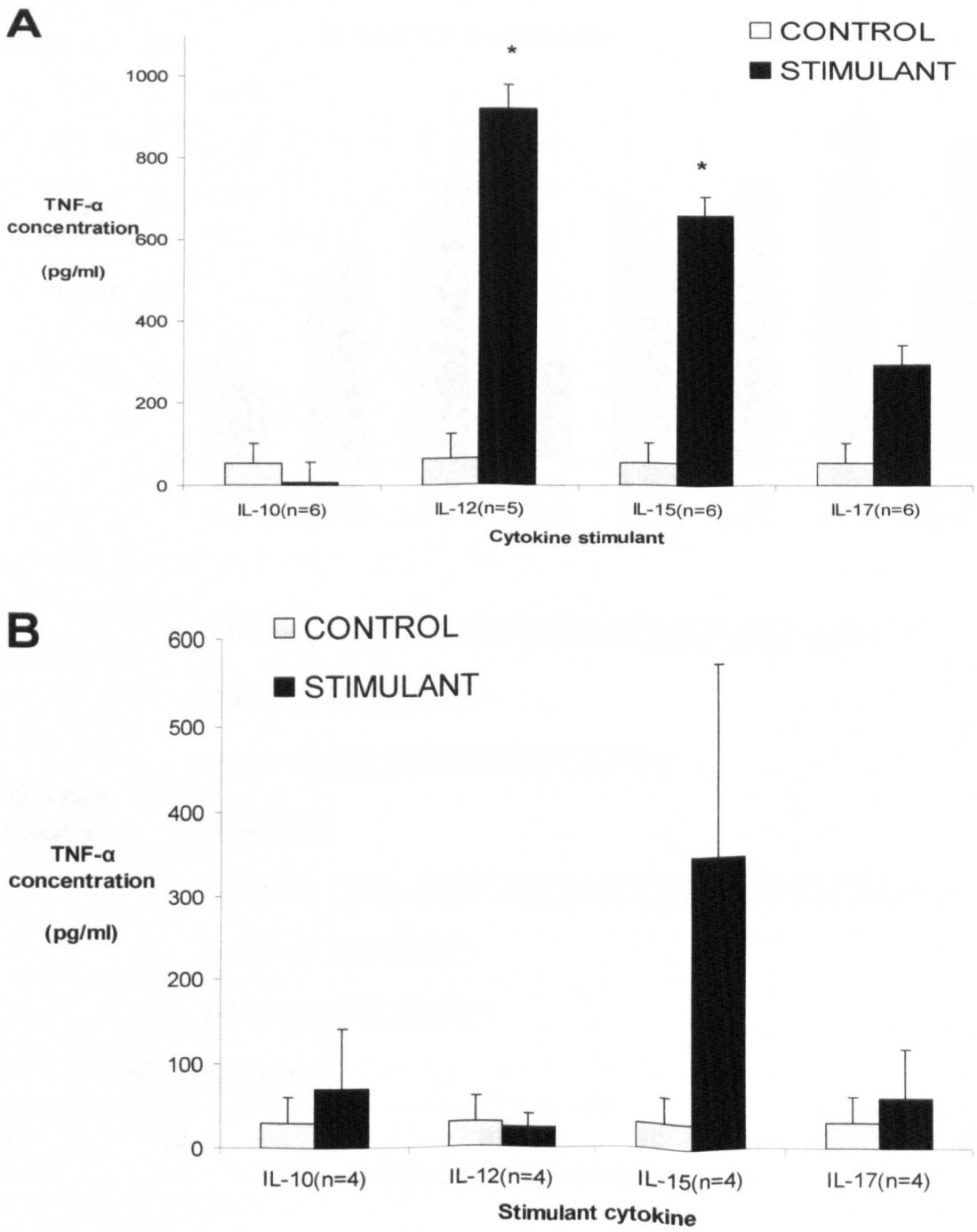
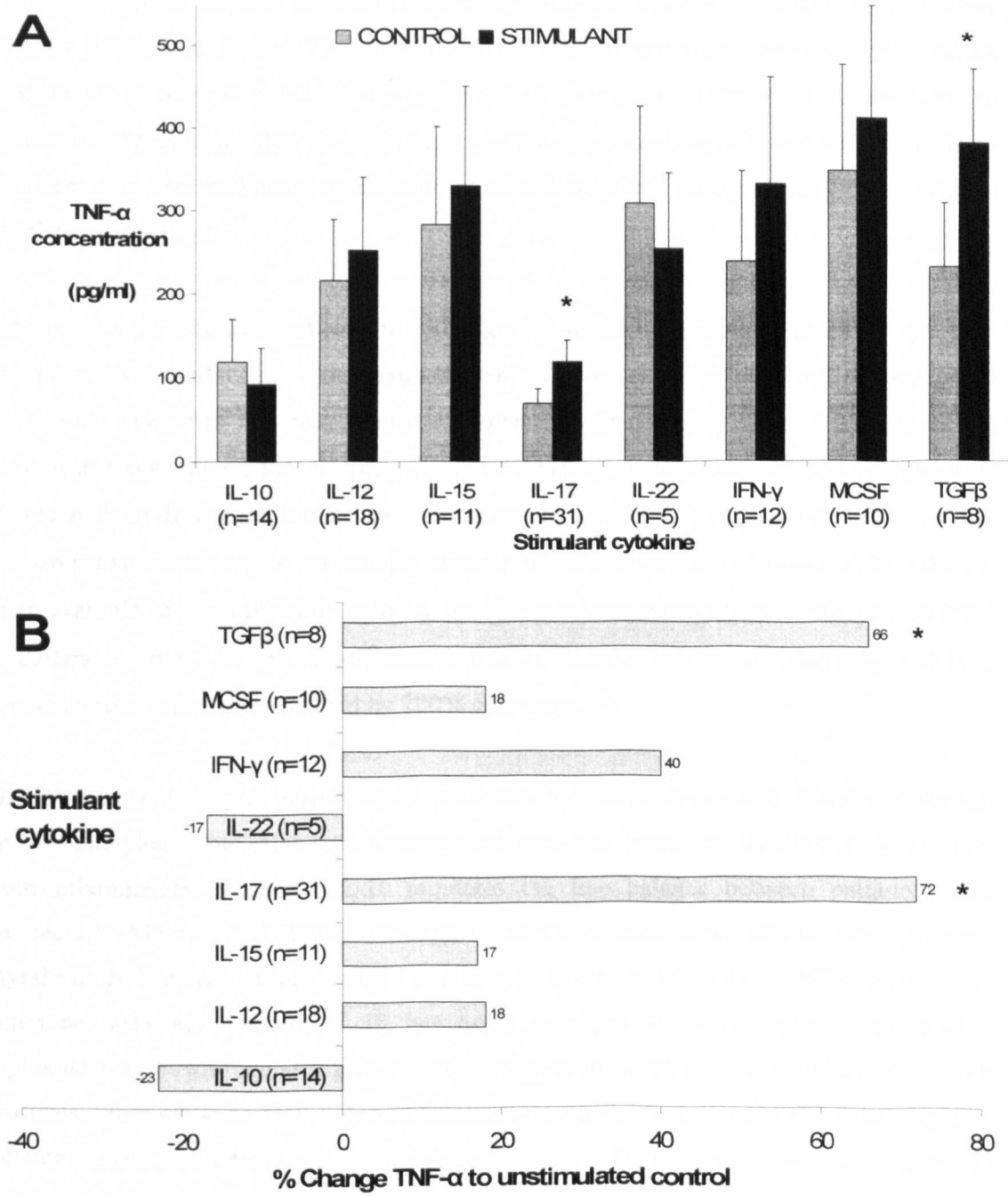


Figure 6.18 ***In vitro*** **effects of 72 h cytokine stimulation upon TNF-α expression within PRD explant cultures**. PRD explant tissues were stimulated with a panel of key cytokines for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of TNF-α after cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant TNF-α concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated with each cytokine is given in parenthesis. Bars represent the mean TNF-α concentration with the standard error of the mean.



6.8 Discussion

The cytokine network is central to the perpetuation of inflammatory responses and thereby chronic persistence of pathological lesions. Recent evidence supports the opinion that the cytokine network has a central role in initiation and development of the periradicular lesion. In previous experiments, I established that 18 h and 72 h after surgical retrieval, PRD tissue explants spontaneously released plenteous quantities of cytokines. The substantial constitutive release of several key inflammatory cytokines within unstimulated cultures suggests they are important contributors towards prolonging chronic inflammatory events within the lesion. The identification of novel upstream cytokines that induce expression of these secreted inflammatory cytokines is of key importance. I ascertained in previous experiments that endogenous cytokine expression within PRD explant tissue cultures could be modulated by addition of bacterial-derived mitogens or cytokine stimuli, in the form of IL-18.

In this chapter, I further utilised the PRD explant model to investigate potential upstream inflammatory mediators. I determined their effects upon secretion of endogenously expressed cytokines that may contribute towards inflammatory events within the PRD lesion and potentially, the destruction of surrounding alveolar bone. The key important findings from these experiments are summarised in Table 6.1. The table takes into account findings that are seen to be statistically significant but may not be of biological importance. For example, a 15% increase induced by one cytokine stimulant at levels of statistical significance may be of little biological significance when compared to cytokine concentrations that have changed by 200% or greater.

IL-10 is a type 2 cytokine primarily produced by macrophages and T cells. Although possessing pleiotropic effects on immuno-inflammatory reactions, IL-10 is predominantly anti-inflammatory. Thereby, IL-10 regulates the fine balance between pathology and protection (Moore *et al.* 2001). Through its ability to inhibit proinflammatory cytokine synthesis and reduce surface antigen expression, IL-10 down-regulates inflammatory and immune responses. Indeed, IL-10 has been described as a macrophage deactivating cytokine that down-regulates proinflammatory responses such as the release of oxygen radicals, nitric oxide or TNF-α. It was therefore of interest to find that PRD tissue explants spontaneously expressed moderate quantities of IL-10. In agreement with its acknowledged anti-inflammatory role, IL-10 decreased expression of proinflammatory

mediators IL-17A and TNF-α within the PRD explant culture model. In contrast, IL-10 had no effect upon IL-6 or IL-8 and slightly increased expression of IFN-γ in long-term culture. Reductions in IL-17A and TNF-α expression within PRD cultures may reflect a negative feedback loop, whereby IL-10 limits the inflammatory response to prevent pathologic damage.

IL-4, another Th2 type cytokine, inhibits bone erosion by interacting directly with bone osteoclasts and indirectly by inhibiting the production of proinflammatory cytokines. Bone resorption induced by RANKL is dose dependently inhibited by direct effects of IL-4 upon both osteoclast precursors and mature osteoclasts (Wei *et al.* 2002, Mangashetti *et al.* 2005). Interestingly, IL-4 was not detectable within resting PRD tissue cultures and was not inducible by other cytokines. This suggests predominance of Th1 type responses within the PRD lesion. It may be postulated that the absence of protective, antiinflammatory effects afforded by IL-4 may contribute towards lesion expansion. However, in contrast to IL-10, PRD lesion size within the murine model is not affected by IL-4 expression, indicating the heterogeneity amongst Th2 type cytokines (Sasaki et al 2000). Bone protective responses may therefore be significantly driven by IL-10-mediated pathways within the human PRD tissue lesion.

IL-12 is an essential proinflammatory cytokine predominantly produced by phagocytic and dendritic cells. In contrast to IL-4 and IL-10, it stimulates innate and $CD4^+$ effector Th1 type immune responses and is essential for resistance against infection. I had previously ascertained that IL-12 was spontaneously secreted by unstimulated PRD explant cultures. The addition of IL-12 to PRD explant cultures resulted in a powerful pro-inflammatory response, resulting in up-regulated expression of IL-6, IL-8 and IFN-γ. Interestingly, after long term culture IL-12 induced a moderate increase in IL-10 secretion. Similar to TNF-α and IFN-γ, the late increase induced by IL-12 upon IL-10 secretion may form part of a negative feedback loop to prevent exaggerated destructive proinflammatory responses.

Addition of IL-17A to PRD tissue cultures induced a strong proinflammatory response with significantly increased levels of TNF-α and IFN-γ and increased IL-6 and IL-8 expression. To my knowledge, IL-17A has not previously been reported to induce increased expression of IFN-γ. Previous studies have established a synergistic effect between IL-17A and IFN-γ upon increasing IL-6 and IL-8 expression (Andoh *et al.* 2001). The release of IL-17A within the PRD lesion may therefore form a regulatory loop

whereby IFN-γ expression is increased and concomitant production of IL-17A with IFN-γ results in further increased expression of IL-6 and IL-8. The proinflammatory cytokines IL-15, TNF-α and IFN-γ further increased IL-17A expression. IL-17A is capable of stimulating osteoclast differentiation and inducing bone resorption through induction of RANK on the surface of osteoblasts (Kotake *et al.* 2001). IL-17A expression within the PRD lesion may therefore be a major contributing factor towards associated periradicular bone resorption. The potentially damaging effects of these proinflammatory cytokines upon promoting IL-17A-induced matrix destruction may in part be tempered by endogenous release of IL-10, which modestly decreased IL-17A expression. In contrast, others have demonstrated that IL-10 has no effect upon IL-17 expression (Chabaud *et al.* 1999).

NK cells are important innate producers of IFN-γ at early stages of host infection (Daniels *et al.* 2001, Pien and Biron 2000). Recent data suggest that through cytokine and chemokine expression, NK cells contribute toward the development of other adaptive responses. It is well established that the ability of professional APCs to mediate many of their coactivating functions is by the production of soluble mediators such as IL-12. The ability of NK and NKT cells to rapidly secrete IFN-γ, a key molecule in the development of adaptive immune responses, and chemokines (MIP-1a) that can recruit selective subsets of leukocytes, suggest that another role of NK cells is to modulate the delicate balance of immune development in response to viral or microbial challenge. Thereby, innate NK cell IFN-γ production will provide early immune regulation that can alter the outcome and quality of the adaptive immune response (Ortaldo and Young 2003). It is therefore of great importance that addition of IL-12, IL-15 and TNF-α to PRD explants induced substantially increased levels of secreted IFN-γ. Furthermore, addition of IL-17A to explants contributed towards increased IFN-γ expression. Cellular sources of inducible IFN-γ secretion within PRD tissues remain to be identified, but NK cells are likely to play their part. The release of IFN-γ following the activation of cells with cytokines or bacterial derived mitogens likely leads to further increased expression of proinflammatory cytokines including IL-6, IL-17A and TNF-α, thereby perpetuating the inflammatory reaction.

TGF-β_1 is a multifunctional growth factor released by inflammatory cells in response to bacterial products and tissue injury (Wahl 1994) and osteoblasts, fibroblasts and osteoclasts (Bonewald 1999). In addition to its anti-inflammatory properties, TGF-β_1 induces proinflammatory effects including recruitment and activation of neutrophils,

monocytes and T cells (Brandes *et al.* 1991). Furthermore, TGF-β_1 triggers production of bone resorptive cytokines such as IL-1, TNF-α, and IL-6 (Brandes *et al.* 1991, Wahl 1992) and directly induces mononuclear phagocyte osteoblast precursors to differentiate into active osteoclast cells in the presence of M-CSF and absence of RANKL (Itonaga *et al.* 2004). Despite finding that TGF-β_1 can induce osteoclastogenesis without an absolute requirement for RANKL it plays a pivotal role in inducing both RANKL gene expression and protein formation in a dose-dependant manner (Zhang *et al.* 2003). The role of TGF-β_1 within inflammatory bone destruction is rather unclear. Within animal models, TGF-β_1 down-regulates proinflammatory cytokine production and exerts protective effects in collagen induced arthritis (Kuruvilla *et al.* 1991) and suppression of TGF-β_1 by NK cells promotes antibody-induced joint inflammation (Kim *et al.* 2005). Conversely, TGF-β_1 promotes inflammation by enhancing neutrophil infiltration and induces angiogenesis within joint tissues. Addition of TGF-β_1 to PRD explant tissues had limited effects upon modulating endogenous cytokine expression. However, it significantly increased TNF-α expression at both 18 h and 72 h explant culture and interestingly induced IL-10 in long-term culture. The late increase in IL-10 expression may serve to counter-regulate potentially destructive proinflammatory effects from increased TNF-α expression induced by TGF-β_1.

Table 6.1 Effect of endogenous cytokine stimulation upon endogenous cytokine expression within PRD explant cultures. Table represents the major biological effects of a panel of cytokines added to PRD explant cultures. Time point of culture is given in number of hours at time of analysis (blue represents 18 h, red represents 72 h). Measured cytokine within explant supernatant by ELISA is displayed in top row. Recombinant human cytokine added to explant cultures are indicated in left column. ↑- increased expression of analysed cytokine. ↓- decreased expression of analysed cytokine. Number of arrows corresponds with the quantity of change in measured cytokine concentration. ?- represents a query as to the meaningful biological significance of induced change in cytokine expression in the context of specific quantity of change and its relationship with changes induced by other cytokine stimulants. A dash represents no observable change in cytokine concentration compared with control.

Cytokine measured	IL-6		IL-8		IL-10		IL-17A		TNF-α		IFN-γ	
Time ⟶ / ↓Stimulant	18 h	72 h	18 h	72 h	18 h	72 h	18 h	72 h	18 h	72 h	18 h	72 h
IL-10	-	-	-	-	N/A	N/A	↓	-	↓	↓	-	↑?
IL-12	↑↑	-	↑	↑?	-	↑?	-	-	-	-	↑↑↑	↑↑↑
IL-15	-	-	↓?	-	↓?	-	↑↑	↑↑	↑?	-	↑↑↑	↑↑↑
IL-17A	↑?	-	-	-	-	↓?	N/A	N/A	↑?	↑	↑↑	↑↑
IL-22	-	-	-	-	↑?	-	↑?	-	-	-	-	-
TNF-α	-	-	-	-	↑?	↑	↑	↑	N/A	N/A	↑	↑↑↑
IFN-γ	↑	-	-	↓?	-	↑	↑	↑↑	↑↑	↑	N/A	N/A
M-CSF	↓?	-	-	-	-	-	-	-	↑?	-	-	-
TGF-β	-	-	-	-	-	↑	-	-	↑	↑	-	-

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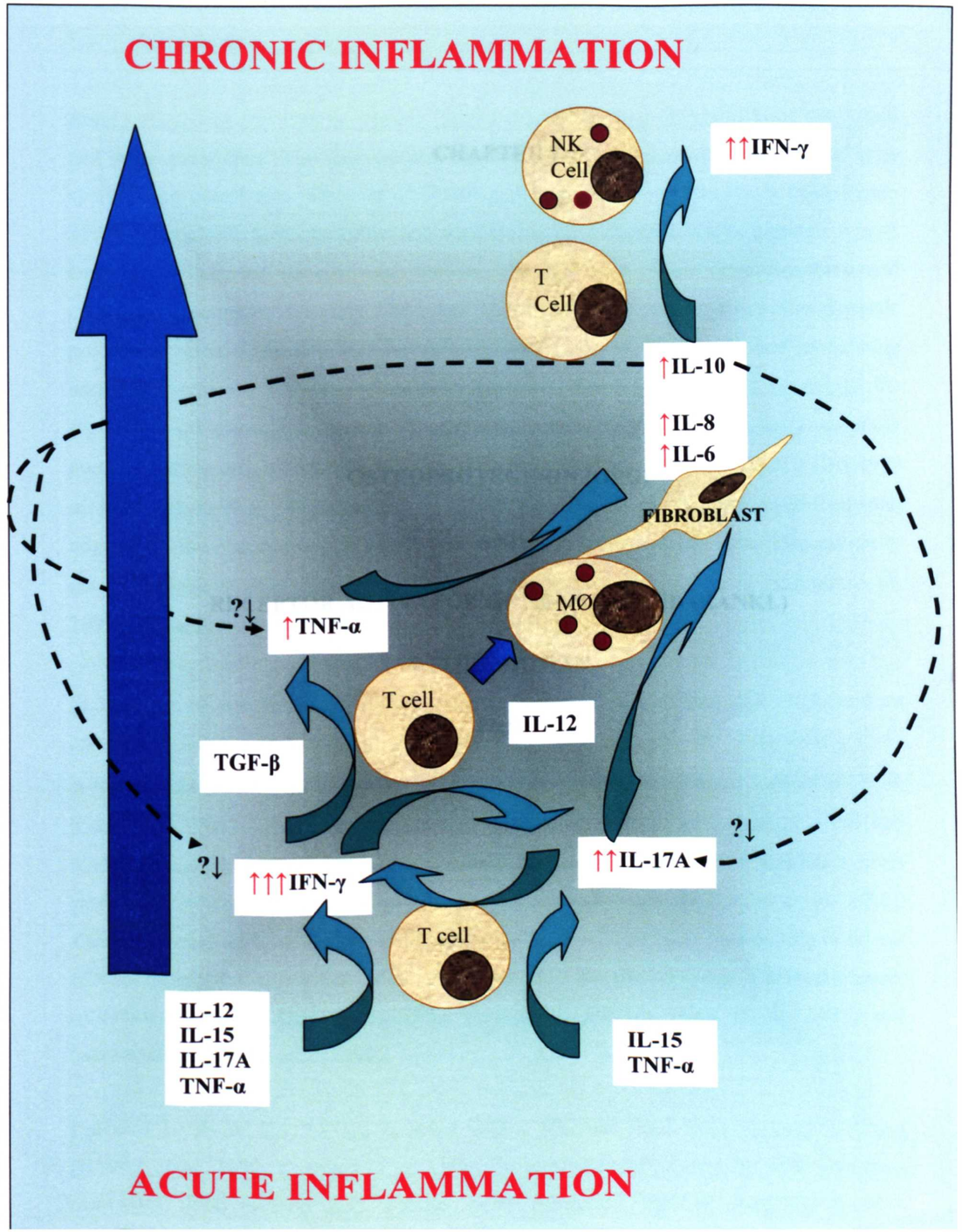
Figure 6.19 **Schematic representation of the key cytokine inducers within the PRD cytokine network involved in regulating inflammatory bone resorption.**

Spontaneous expression of proinflammatory cytokines IL-12, IL-15, IL-17A and TNF-α is readily detectable within PRD. These moieties likely induce T and NK cell activation and thereby contribute towards IFN-γ secretion. IL-15 and TNF-α promote IL-17A expression that likely feedbacks in a regulatory loop to induce increased expression of IFN-γ, which in turn perpetuates the inflammatory response by promoting further increases in secreted IL-17A. However, continued secretion of IL-12, IFN-γ and TNF-α stimulates macrophage release of IL-10 which operates in a negative feedback loop to decrease IL-17A and TNF-α expression. Thereby adverse destructive inflammatory response may be down-regulated. In addition to the contribution of immune cells to inflammatory events, IFN-γ, Il-12 and IL-17A promote fibroblast secretion of IL-6 and/or IL-8 which likely contributes towards matrix destruction.

Figure 6.19 **Schematic representation of the key cytokine inducers within the PRD cytokine network involved in regulating inflammatory bone resorption.**



CHAPTER 7

OSTEOPROTEGERIN (OPG)

AND

RECEPTOR ACTIVATOR OF NF-κB LIGAND (RANKL)

WITHIN HUMAN

PRD

7 OSTEOPROTEGERIN (OPG) AND RECEPTOR ACTIVATOR OF NF-κB LIGAND (RANKL) WITHIN HUMAN PRD

7.1 Introduction

Bone remodelling involves the closely regulated and opposing effects of bone deposition and bone resorption. The key cellular modulators of this dynamic process are bone synthesising osteoblasts (Ducy *et al.* 2000) and bone resorbing osteoclasts (Teitelbaum 2000). Osteoclastic bone resorption and osteoblastic bone deposition are intimately related and closely balanced between one another. Through their effects upon osteoblast and osteoclast precursors, systemic hormones and local mediators moderate the dynamic process of bone deposition and resorption. Among the multitude of bone modulating molecules, receptor activator of nuclear factor-κB ligand (RANKL) is central to the regulation of osteoclastogenesis. Conversely, osteoprotegerin (OPG) is a naturally occurring antagonist of RANKL with an inhibitory effect upon bone resorption (Bucay *et al.* 1998, Emery *et al.* 1998). In order to balance bone density and resorption positively and negatively, the expression of RANKL and OPG is carefully coordinated. This primarily occurs through modulating the activation state of RANK on osteoclasts (Simonet *et al.* 1997, Akatsu *et al.* 1998, Kong *et al.* 1999, Boyle *et al.* 2003).

In addition to the RANKL/RANK/OPG regulatory axis, cytokines are implicated as important contributors in bone remodelling processes (Manolagas 1995, Jilka 1998). TNF-α induces osteoclastogenesis through direct stimulation of macrophages (Lam *et al.* 2000, Kudo *et al.* 2002). Furthermore, IL-1 and TNF-α are capable of regulating OPG and RANKL gene expression in osteoblastic cells (Hofbauer *et al.* 1999). IL-8 also has a direct stimulatory effect upon osteoclastogenesis and bone destruction (Bendre *et al.* 2003). Thereby, cytokines have the potential to alter the balance from bone remodelling in favour of bone resorption. Cytokines are therefore central to the pathogenesis of several chronic inflammatory bone diseases including rheumatoid arthritis (Chu *et al.* 1991) and osteomyelitis (Meghji *et al.* 1998).

Elevated levels of IL-17A are detected within synovial fluid from patients with RA (Kotake *et al.* 1999, Chabaud *et al.* 1999). In experimental models, IL-17A stimulates osteoclast differentiation and induces bone resorption through inducing RANKL expression on the surface of osteoblasts. Conversely, OPG inhibits IL-17A-induced

osteoclast differentiation (Kotake *et al.* 2001). Within the collagen induced arthritis (CIA) model, IL-17A up-regulates RANKL expression thereby contributing towards disease progression (Lubberts *et al.* 2003). Neutralisation of IL-17A within this model significantly reduces the severity of CIA, resulting in markedly suppressed joint damage (Lubberts *et al.* 2004). Local release of IL-17A in chronic inflammatory bone disorders results in up-regulated expression of RANKL from local stromal cells, thereby contributing towards osteoclastogenesis (Page and Miossec 2005). Moreover, IL-17-mediated increases in RANKL expression is potently enhanced by IL-1β or TNF-α.

IL-17A, IL-1β and TNF-α exhibit predominantly bone resorptive properties. Conversely, IL-12 inhibits osteoclast formation in co-cultures of mouse adult spleen and osteoblastic cells treated with M-CSF and RANKL (Horwood *et al.* 2001). IL-12 also inhibits osteoclast formation in bone marrow cell cultures (Nagata *et al.* 2003). Of importance, IL-12 induces IFN-γ production from T and NK cells (Yoshimoto *et al.* 1998). Subsequently, IFN-γ potently inhibits osteoclast formation by interfering with the RANKL-RANK signalling pathway (Takayanagi *et al.* 2000b). IFN-γ also prevents osteoclast formation by directing osteoclast progenitor differentiation towards an alternative cytocidal lineage to the osteoclast (Fox and Chambers 2000). It can be understood from these studies that the balance between RANKL and OPG expression is influenced by the overall cytokine milieu. Therefore, a dominant expression of cytokines that increase RANKL expression and directly promote bone resorption such as IL-17A, IL-1β and TNF-α will likely drive inflammatory processes towards localised tissue destruction.

There is limited information on expression of RANKL and OPG within the PRD lesion. Furthermore, no data is currently available defining modulators of RANKL and OPG expression within PRD. I therefore undertook investigations to determine if RANKL or OPG were present within the human PRD lesion. Furthermore, I studied the effects of mitogen or cytokine addition to PRD explant cultures upon RANKL and OPG expression.

7.2 Detection of OPG and RANKL within human PRD tissue

From my previous experiments it was apparent that many cytokines recognised as mediators of inflammatory bone resorption were detectable within the novel PRD explant tissue culture model. Furthermore, I established that expression of inflammatory cytokines could be induced or suppressed by addition of either bacterial-derived microbial products or inflammatory cytokines to the novel explant culture system. I therefore next investigated if the principal mediators responsible for directing bone homeostasis, OPG and RANKL, were detectable within the explant culture model. I initially investigated if secreted RANKL and OPG could be detected by ELISA as previously described within unstimulated 18 h and 72 h PRD explant culture supernatant.

RANKL and OPG were spontaneously released by unstimulated PRD explant tissues at 18 h and 72 h culture (Figure 7.1). Using immunohistochemistry staining, OPG was expressed by a variety of cell types including lymphocytes and stromal cells. Available antibodies for detection of RANKL were not functional on the FFPE sections. OPG and RANKL expression was confirmed by Western blotting (Figure 7.2).

Figure 7.1 Spontaneous release of OPG and RANKL within human PRD explant tissue cultures. 'Resting' levels of spontaneously released OPG and RANKL within PRD tissue explant culture supernatants were assessed. Unstimulated PRD explant tissue culture supernatant was harvested at 18 h (A) and at 72 h (B). RANKL and OPG expression were analysed by ELISA. Bars represent the mean concentration for each mediator with the standard error of the mean. The number of unstimulated PRD tissues used in experiments for the measurement of each individual cytokine is given in parenthesis.

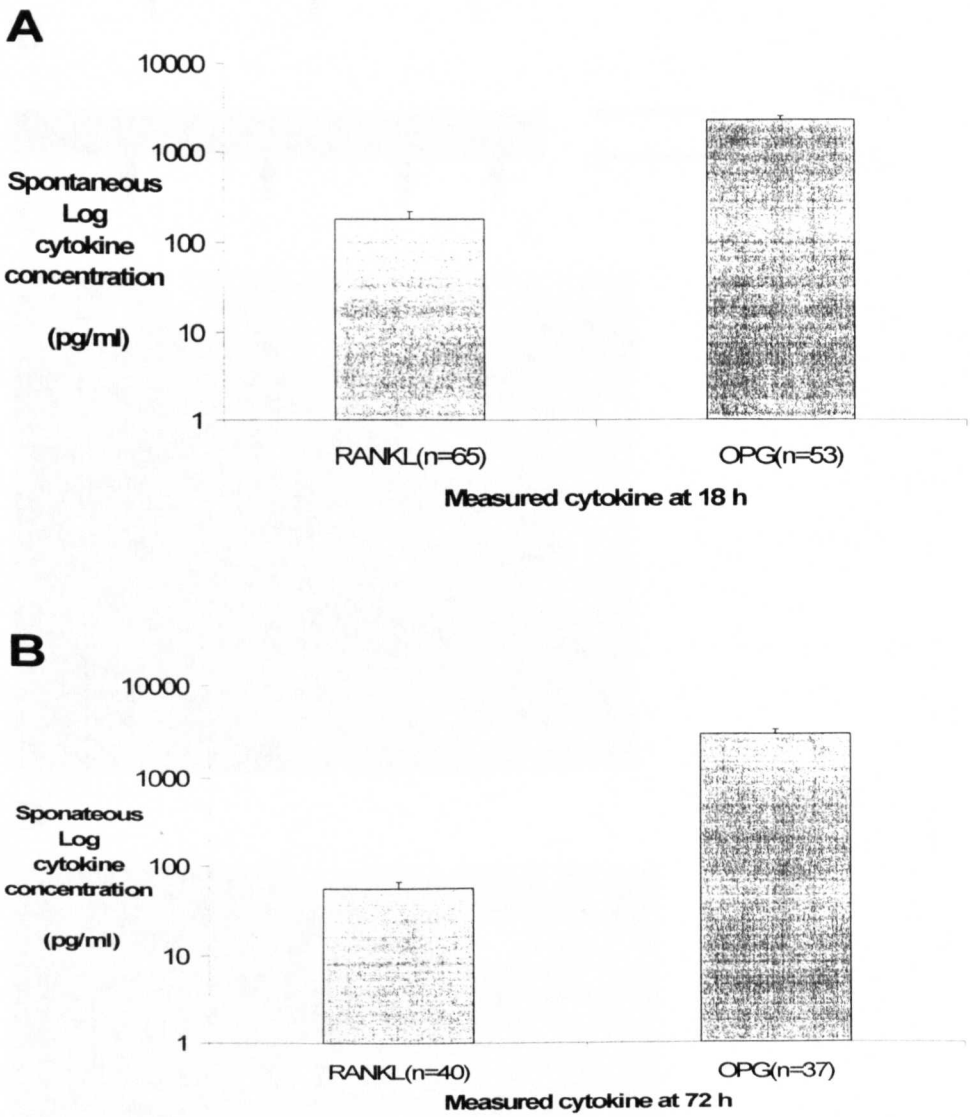
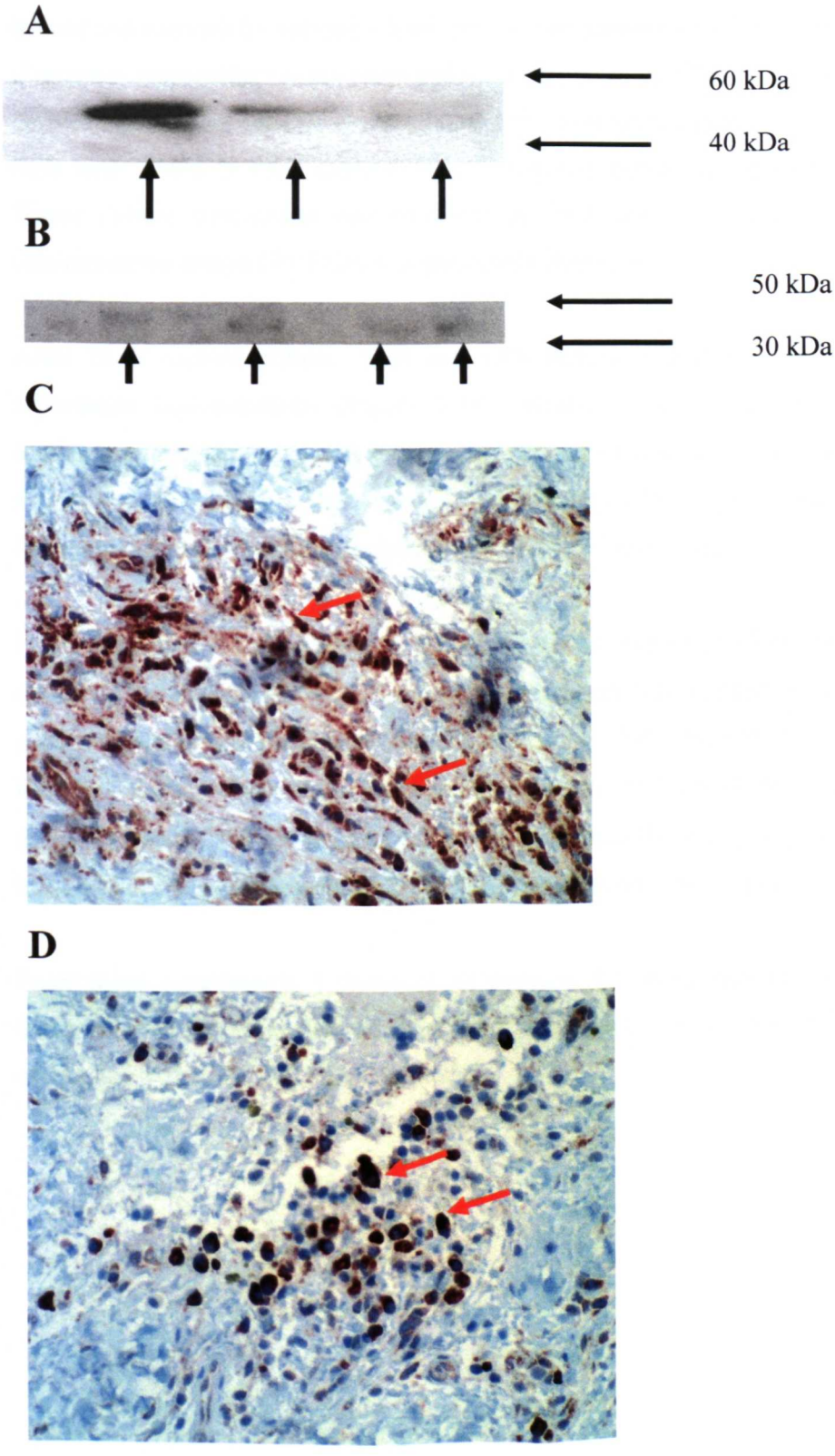


Figure 7.2 **Detection of OPG and RANKL within human PRD tissue.** (A) Western blot of three PRD specimens for OPG protein. Representative of 15 analysed PRD specimens. (B) Low levels of RANKL detectable in 8/15 PRD specimens by Western blotting. OPG expression located primarily to (C) fibroblasts and (D) inflammatory cells.



7.3 *In vitro* effects of mitogenic addition to PRD explants upon endogenous OPG and RANKL expression

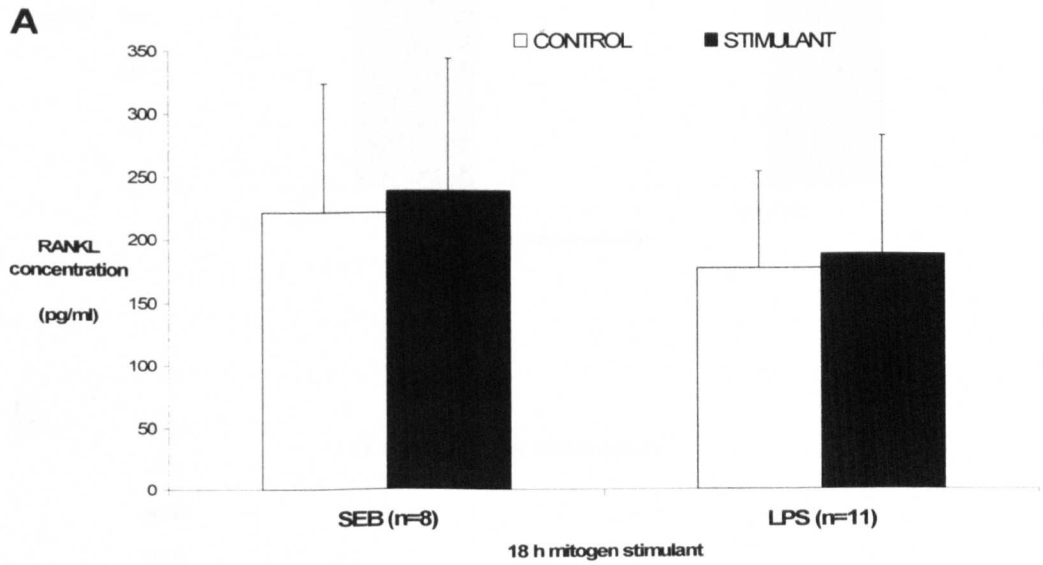
The previous experiments established that OPG and RANKL were expressed within PRD lesions and secreted by explant tissues into culture supernatants. I next sought to determine if moieties responsible for initiation and perpetuation of the PRD lesion, namely microbial-derived agents were capable of modulating OPG or RANKL expression. Therefore, LPS or SEB were added to PRD explant tissue cultures at previously described concentrations. Tissue culture supernatant was harvested at 18 h and 72 h, and OPG and RANKL concentrations assayed by ELISA as previously described.

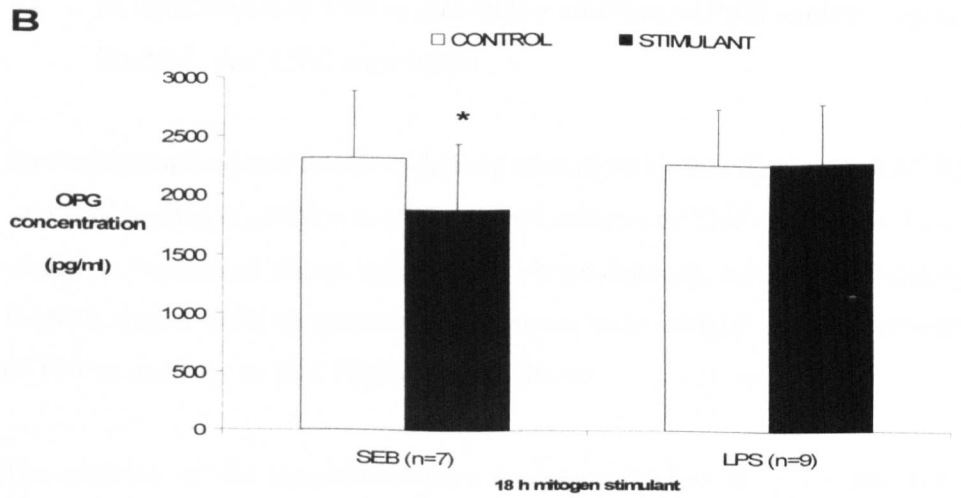
After 18 h explant culture, SEB and LPS had no significant effect upon RANKL supernatant concentrations (Figure 7.3A). However, SEB negatively regulated OPG secretion, inducing a moderate decrease in OPG expression that was at levels of statistical significance ($p=0.047$) at 18 h culture (Figure 7.3B). LPS had no observable effect upon OPG explant supernatant concentration at 18 h PRD tissue culture.

I next investigated the addition of these moieties to long-term PRD explant cultures and examined RANKL and OPG expression. SEB induced only a minimal increase in RANKL concentration after 72 h culture and the addition of LPS resulted in a slight decrease in RANKL, neither observation being of significance. At 72 h culture, SEB only slightly down-regulated OPG expression, not at levels of significance ($p=0.27$). As at 18 h, LPS had no effect upon OPG secretion at 72 h culture (Figure 7.3C and D).

From these experiments, I therefore ascertained that these specific Gram-negative and Gram-positive moieties had no significant biological impact upon OPG and RANKL expression within the PRD explant model.

Figure 7.3 ***In vitro*** **effects of 18 h and 72 h mitogenic stimulation of PRD explants on RANKL and OPG supernatant levels.** PRD explant tissues were stimulated with LPS or SEB for 18 h and 72 h. Supernatants were collected and analysed by ELISA for RANKL and OPG. (A) The mean concentration of RANKL after mitogenic stimulation of explants compared with their corresponding unstimulated controls at 18 h. (B) The mean concentration of supernatant OPG after mitogenic stimulation of explants compared with their corresponding unstimulated controls at 18 h. (C) The mean concentration of RANKL after mitogenic stimulation of explants compared with their corresponding unstimulated controls at 72 h. (D) The mean concentration of OPG after mitogenic stimulation of explants compared with their corresponding unstimulated controls at 72 h. The number of PRD tissues used in experiments for the measurement of each individual cytokine is given in parenthesis. Bars represent the mean concentration for each cytokine and the standard error of the mean.

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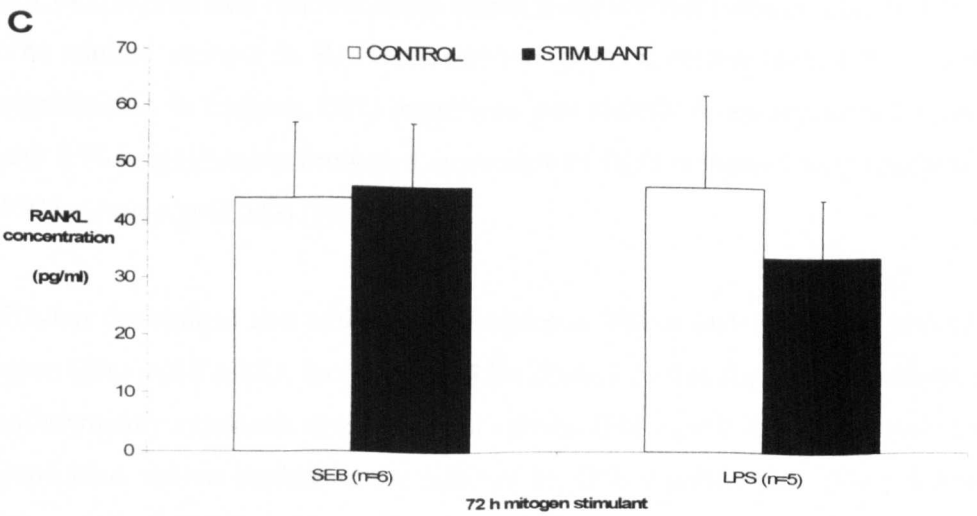
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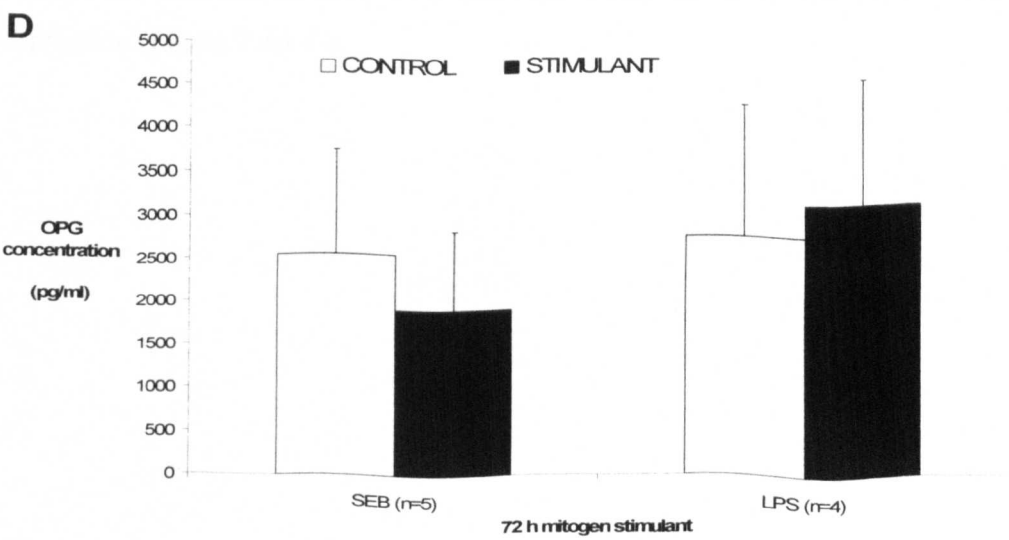
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OPG concentration (pg/ml)
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72 h mitogen stimulant

7.4 *In vitro* effects of TNF-α and IFN-γ addition to PRD explants upon endogenous RANKL and OPG expression

Having determined that bacterial-derived stimuli had little effect upon RANKL and OPG, I next investigated if addition to PRD explant cultures of TNF-α or IFN-γ, two key cytokine mediators implicated within inflammatory bone diseases, had any modulatory effect upon RANKL and/or OPG expression. Experiments were initially undertaken with the addition of TNF-α or IFN-γ to 18 h PRD explant cultures.

The addition of the proinflammatory mediators TNF-α or IFN-γ resulted in increased RANKL supernatant concentrations within tissue explant cultures after 18 h (Figure 7.4A). The marked increase in RANKL secretion ($p=0.05$) arising from IFN-γ addition was of significance. In contrast, OPG expression was slightly down-regulated by TNF-α at 18 h and IFN-γ significantly decreased expression of OPG compared with unstimulated control PRD cultures ($p=0.046$) (Figure 7.4B).

Having determined that addition of exogenous TNF-α and IFN-γ had observable effects upon OPG and RANKL secretion at 18 h culture, I further explored the effects of these two inflammatory cytokines over long-term culture. IFN-γ ($p=0.05$) significantly reduced OPG expression within explant tissue cultures at 72 h. Furthermore, IFN-γ induced a 156% increase in RANKL concentration compared with control, although this was just below levels of statistical significance. At 72 h culture, TNF-α minimally down-regulated culture supernatant concentrations of OPG although it had no observable effect upon RANKL expression (Figure 7.5A-C).

Figure 7.4 ***In vitro*** **effects of TNF-α and IFN-γ manipulation upon RANKL and OPG in 18 h PRD explant cultures.** PRD explant tissues were stimulated with TNF-α or IFN-γ for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of RANKL after 18 h explant tissue stimulation with TNF-α or IFN-γ compared with unstimulated control. (B) The mean concentration of OPG after 18 h explant tissue stimulation with TNF-α or IFN-γ compared with unstimulated control. The total number of PRD explant tissues stimulated with TNF-α or IFN-γ and analysed for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.

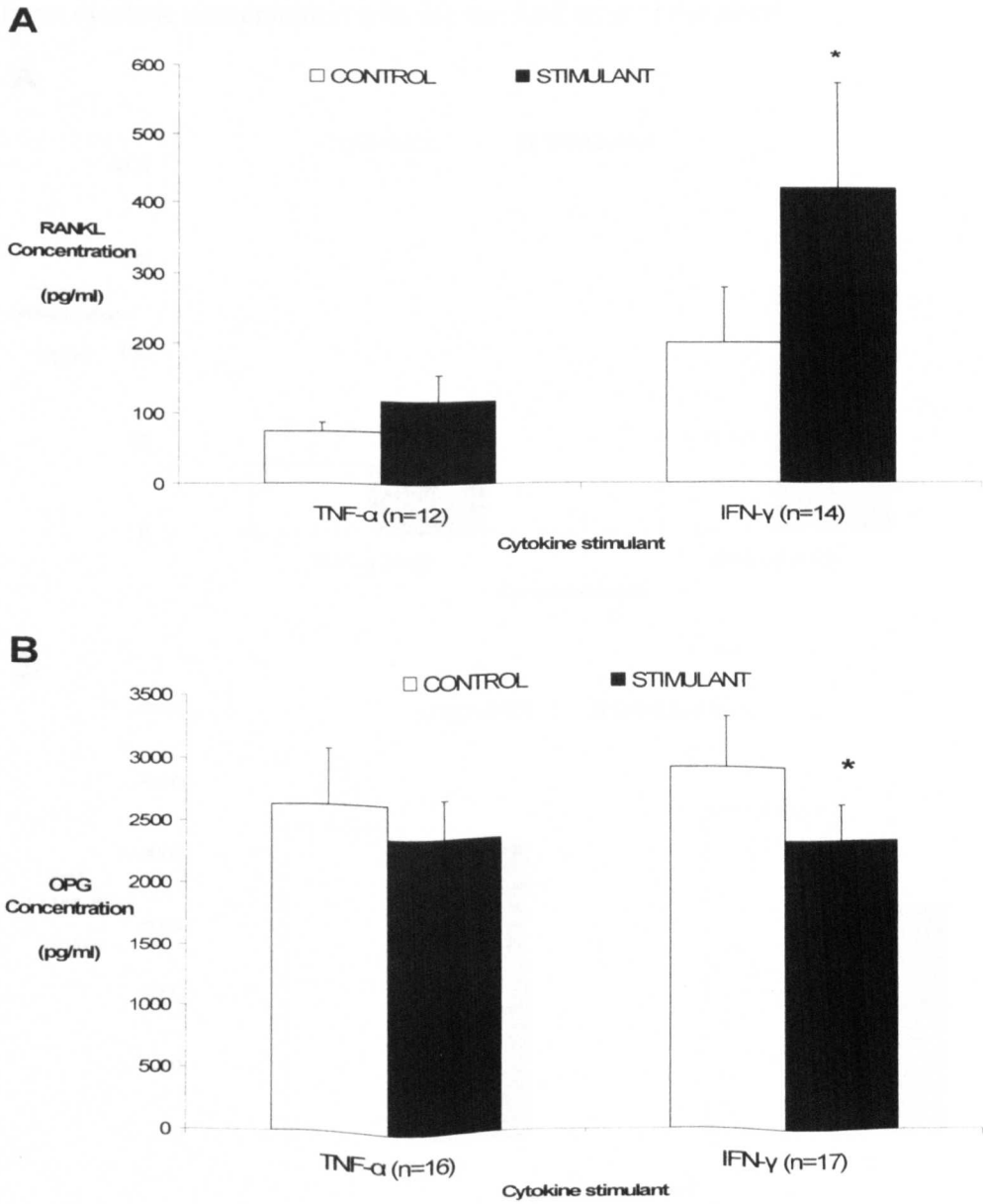
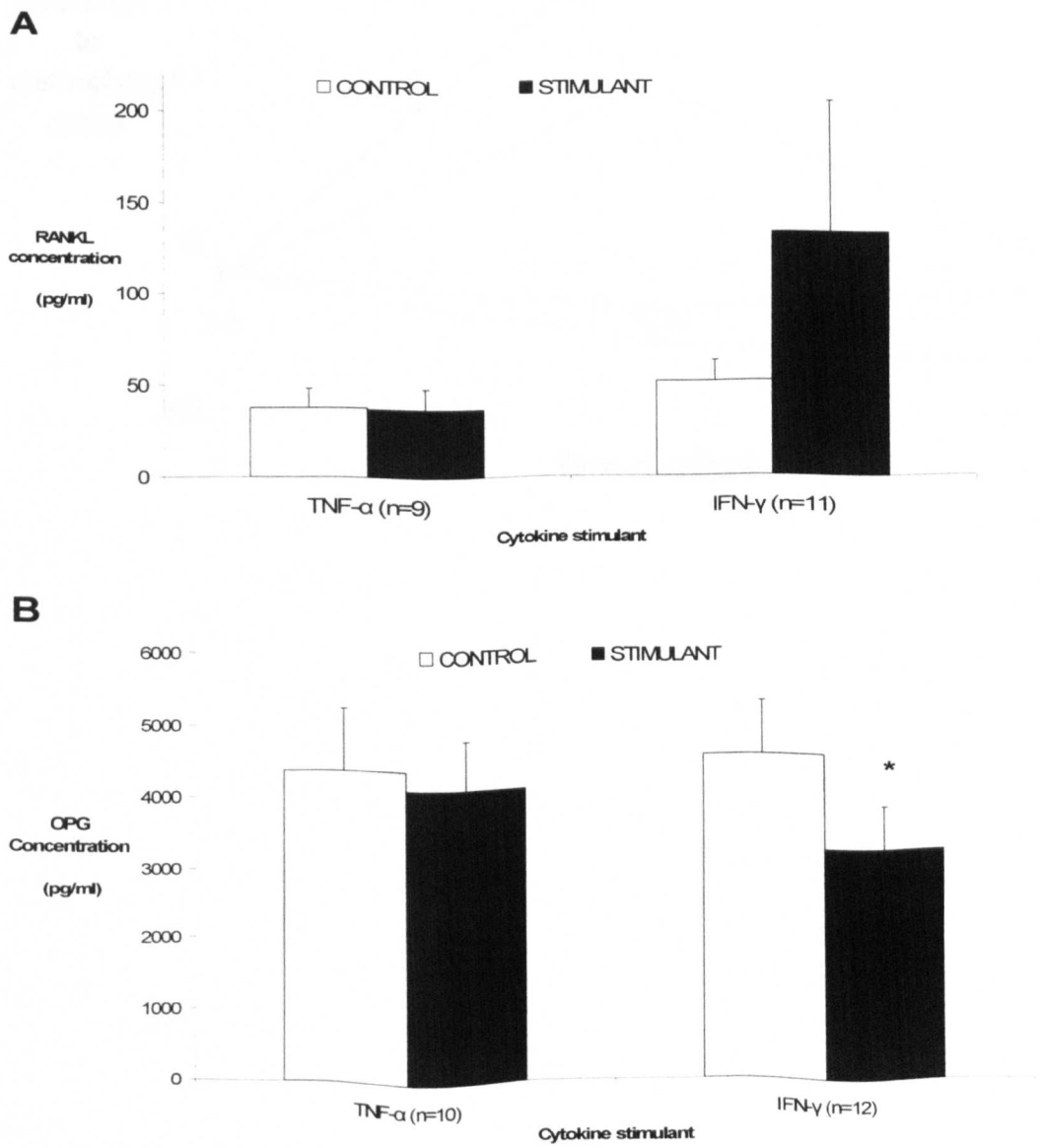
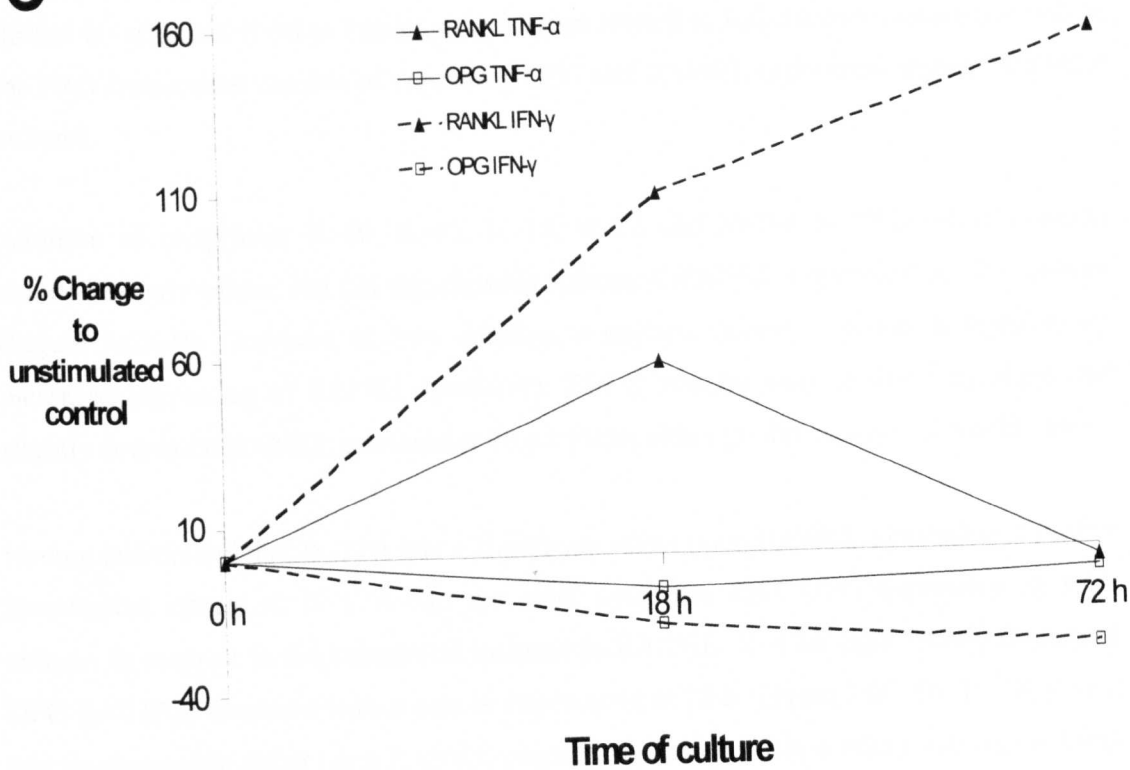


Figure 7.5 ***In vitro*** **effects of TNF-α and IFN-γ manipulation upon RANKL and OPG in 72 h PRD explant cultures.** PRD explant tissues were stimulated with TNF-α or IFN-γ for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of RANKL after explant tissue stimulation with TNF-α or IFN-γ compared with unstimulated control. (B) The mean concentration of OPG after 72 h explant tissue stimulation with TNF-α or IFN-γ compared with unstimulated control. (C) Time-line chart representing the percentage change in culture supernatant cytokine concentrations between stimulated and unstimulated control tissues. The total number of PRD explant tissue cultures stimulated with TNF-α or IFN-γ for either RANKL or OPG is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.



C


160
110
60
10
-40
% Change to unstimulated control
RANKL TNF-α
OPG TNF-α
RANKL IFN-γ
OPG IFN-γ
0 h
18 h
72 h
Time of culture

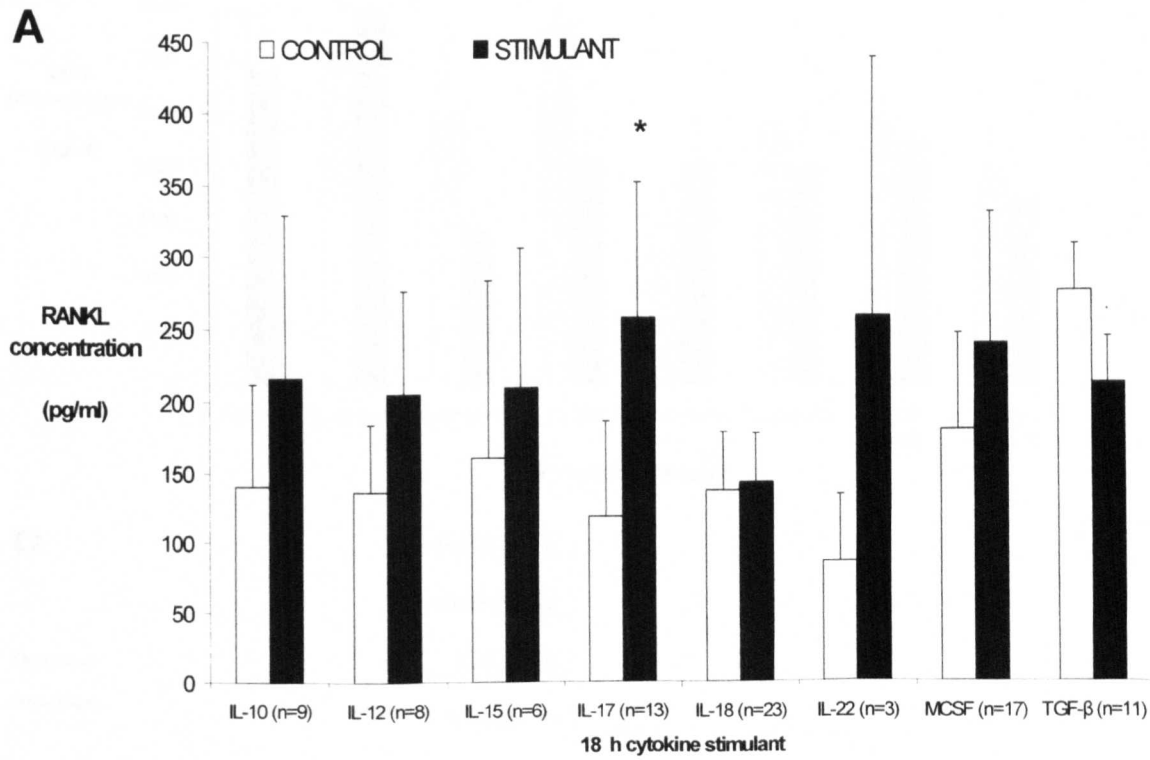
7.5 *In vitro* effects of cytokine addition to 18 h PRD explant cultures upon endogenous RANKL and OPG expression

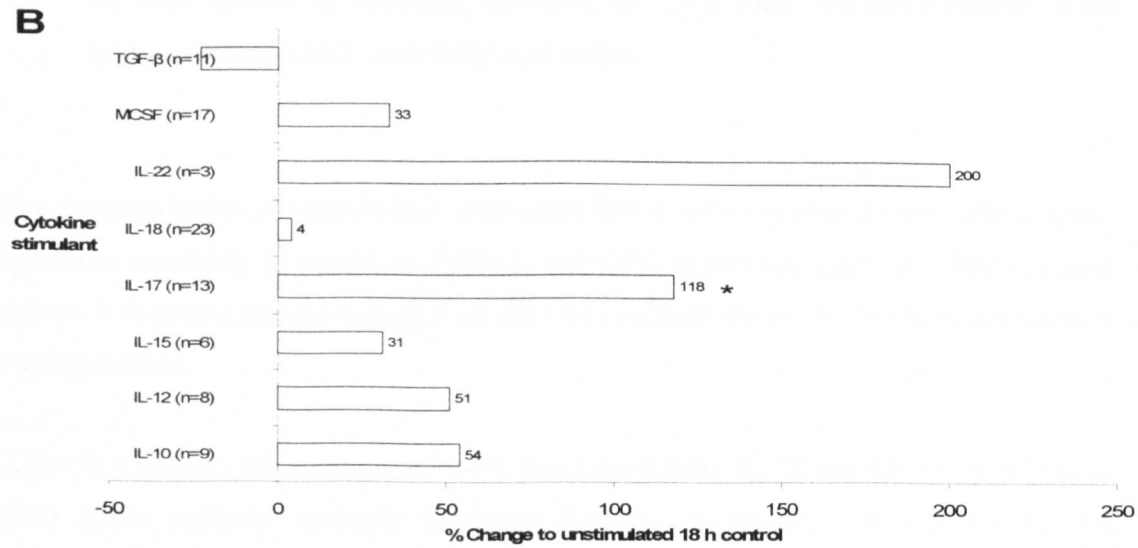
The previous experiments established that OPG and RANKL expression within the PRD culture model were modulated by addition of TNF-α or IFN-γ to explant tissues. I therefore further investigated if other important cytokines related to inflammatory processes within the PRD lesion were capable of regulating OPG and RANKL expression within 18 h PRD cultures.

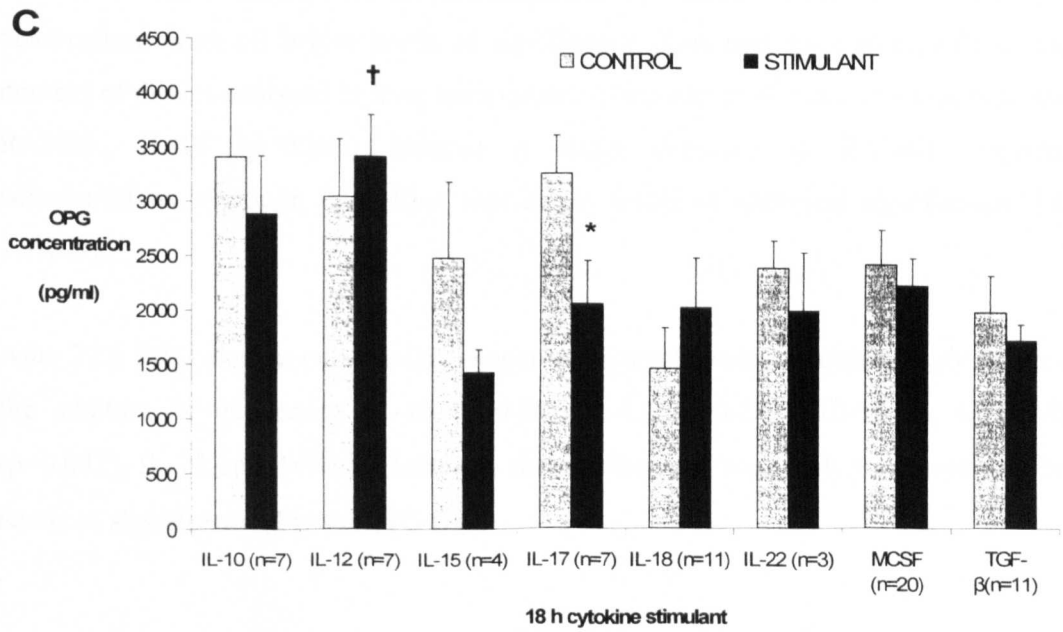
Addition of exogenous IL-10, IL-12, IL-15, IL-22 and MCSF to PRD tissue explants resulted in only minor, but not significantly increased RANKL expression at 18 h culture (Figure 7.6A-B). However, IL-17A addition to explant cultures resulted in significantly increased expression of RANKL ($p=0.019$). TGF-β was the only analysed cytokine that slightly decreased RANKL secretion at 18 h culture, although this was not of significance.

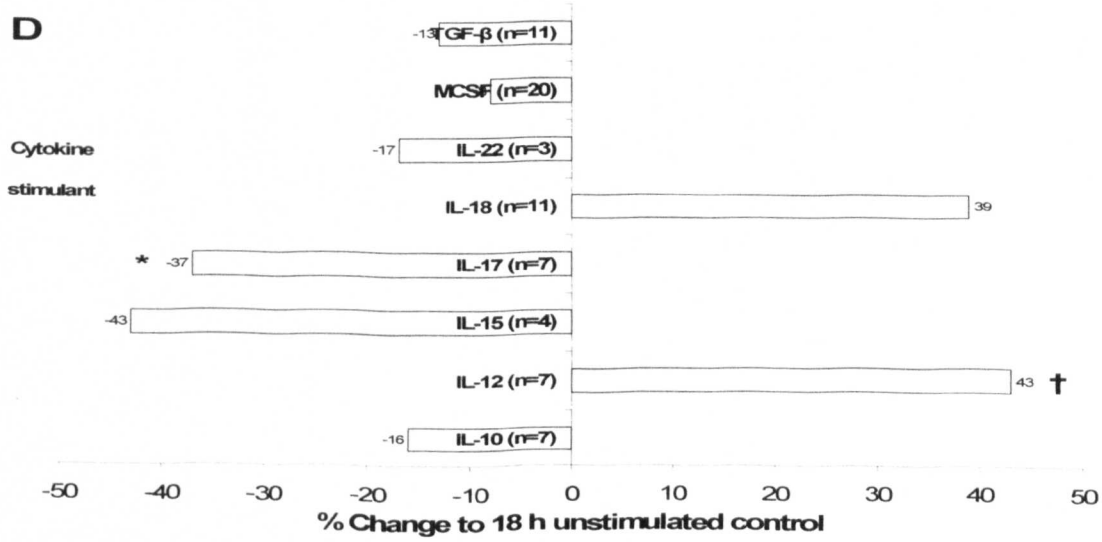
Having determined that IL-17A had a significant effect upon RANKL expression, I further investigated effects of IL-17A and the other cytokines upon OPG expression at 18 h culture. In contrast to the substantial increase in RANKL, IL-17A significantly decreased OPG ($p=0.032$) secretion within culture supernatant at 18 h (Figure 7.6C-D). IL-18, which had no observable effect upon RANKL production, induced only a minor increase in OPG ($p=0.135$) expression, below levels of significance. Interestingly, the addition of IL-12 to the explants moderately increased OPG ($p=0.032$) expression after 18 h culture at levels of statistical significance.

Figure 7.6 ***In vitro*** **effects of cytokine manipulation upon RANKL and OPG in 18 h PRD explant cultures.** PRD explant tissues were stimulated with cytokines for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of RANKL after 18 h explant tissue stimulation with cytokines compared with unstimulated control. (B) The percentage change in culture supernatant RANKL concentration between stimulated and unstimulated paired control tissues. (C) The mean concentration of OPG after 18 h explant tissue stimulation with cytokines compared with unstimulated control. (D) The percentage change in culture supernatant OPG concentration between stimulated and unstimulated paired control tissues. The total number of PRD explant tissues stimulated by each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.




B
TGF-β (n=11)
MCSF (n=17)
IL-22 (n=3)
IL-18 (n=23)
IL-17 (n=13)
IL-15 (n=6)
IL-12 (n=8)
IL-10 (n=9)
33
200
4
118
*
31
51
54
Cytokine stimulant
-50
0
50
100
150
200
250
% Change to unstimulated 18 h control


C
CONTROL
STIMULANT
†
*
OPG concentration (pg/ml)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
IL-10 (n=7)
IL-12 (n=7)
IL-15 (n=4)
IL-17 (n=7)
IL-18 (n=11)
IL-22 (n=3)
MCSF (n=20)
TGF-β(n=11)
18 h cytokine stimulant


D
-13
TGF-β (n=11)
MCSF (n=20)
-17
IL-22 (n=3)
IL-18 (n=11)
39
*
-37
IL-17 (n=7)
-43
IL-15 (n=4)
IL-12 (n=7)
43
†
-16
IL-10 (n=7)
Cytokine stimulant
-50
-40
-30
-20
-10
0
10
20
30
40
50
% Change to 18 h unstimulated control

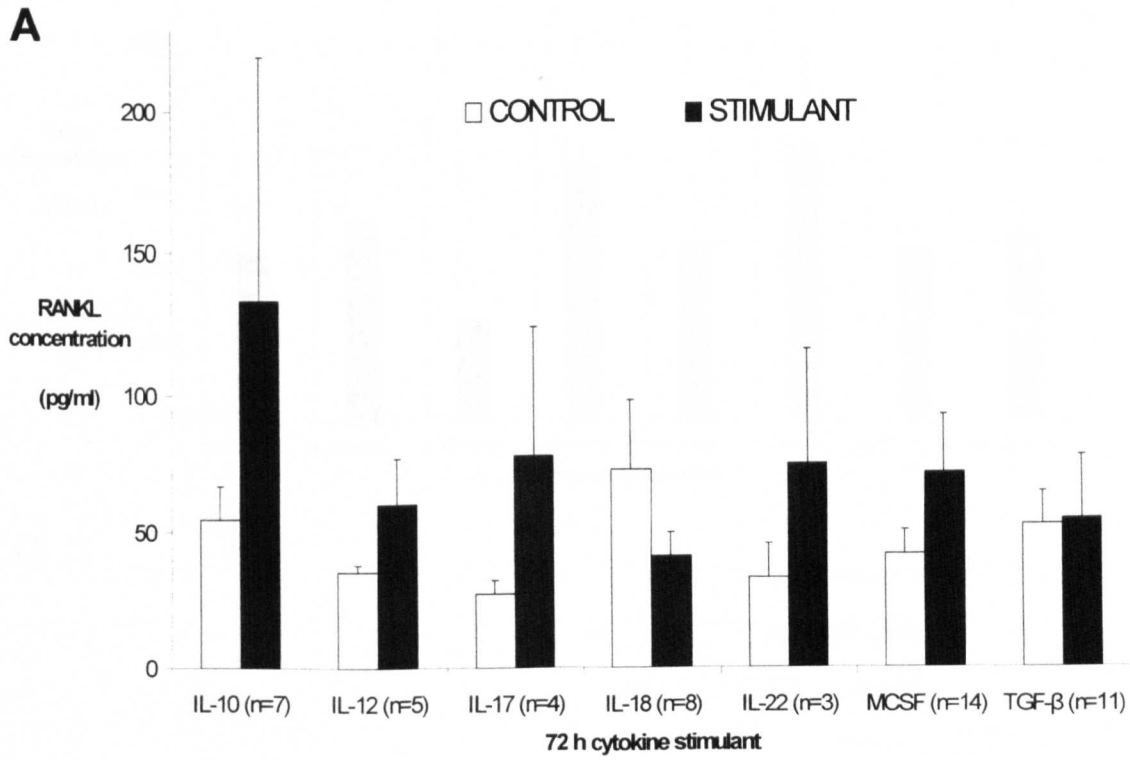
7.6 *In vitro* effects of cytokine addition to 72 h PRD explant cultures upon endogenous RANKL and OPG expression

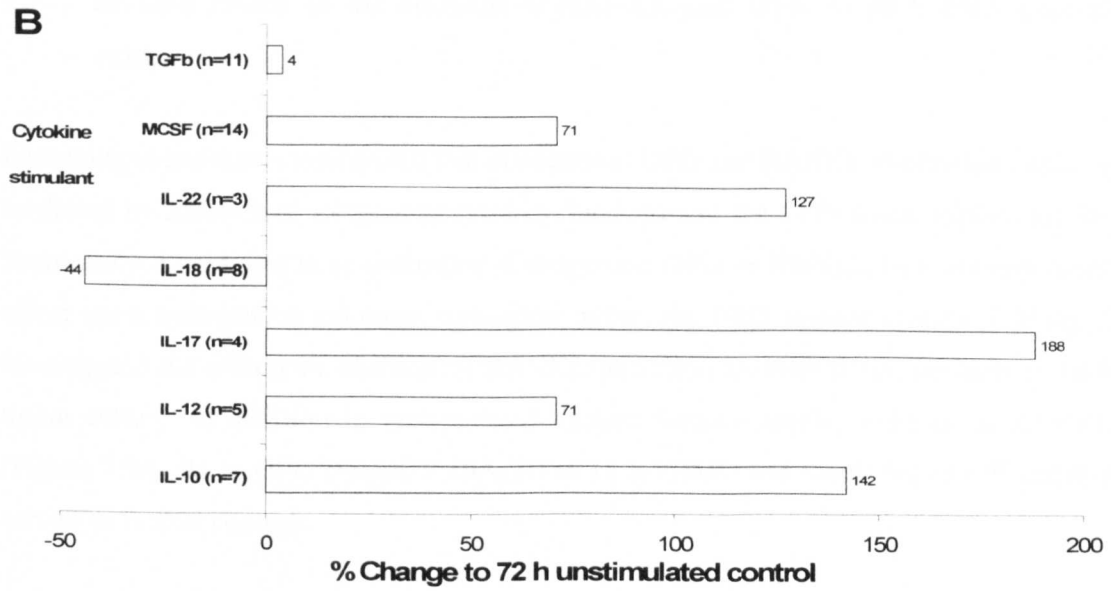
The previous series of experiments ascertained that a select number of key inflammatory cytokines were able to modulate RANKL and OPG expression after 18 h PRD explant culture. I therefore next investigated whether these effects were exhibited over longer-term explant culture.

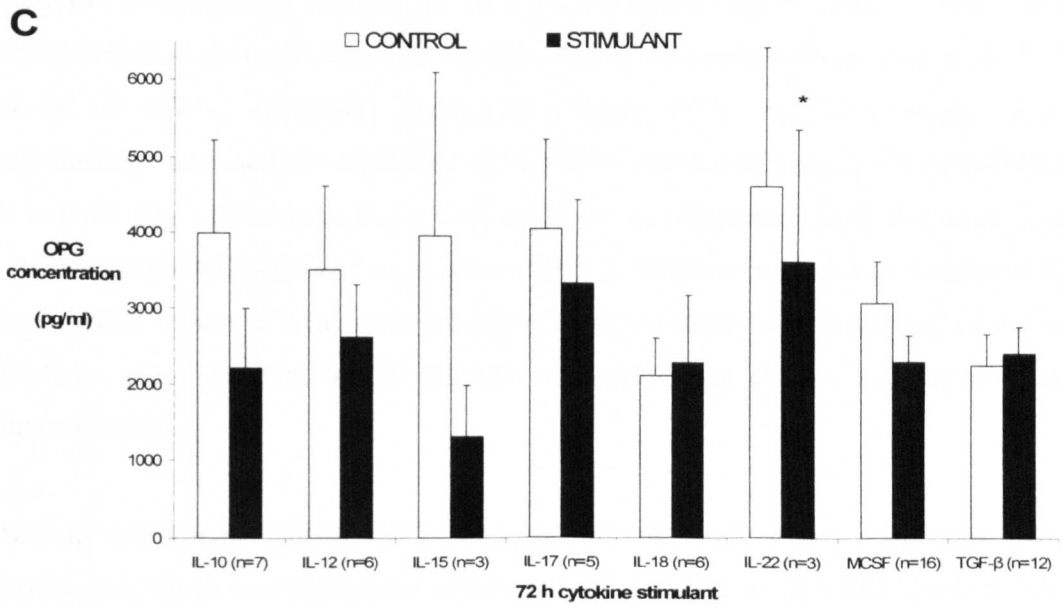
After 72 h culture, the addition of IL-10, IL-12 ($p=0.194$), IL-22 and MCSF ($p=0.186$) to PRD tissue explants modestly increased RANKL expression. Furthermore, IL-17A induced a 188% increase in the concentration of secreted RANKL. However, these observations were all below levels of significance. This may be a result of the smaller number of tissues analysed in long-term cultures. In contrast to these observed increases in RANKL, IL-18 ($p=0.206$) induced a slight decrease in RANKL supernatant concentrations, although this effect was below levels of statistical significance (Figure 7.7A-B).

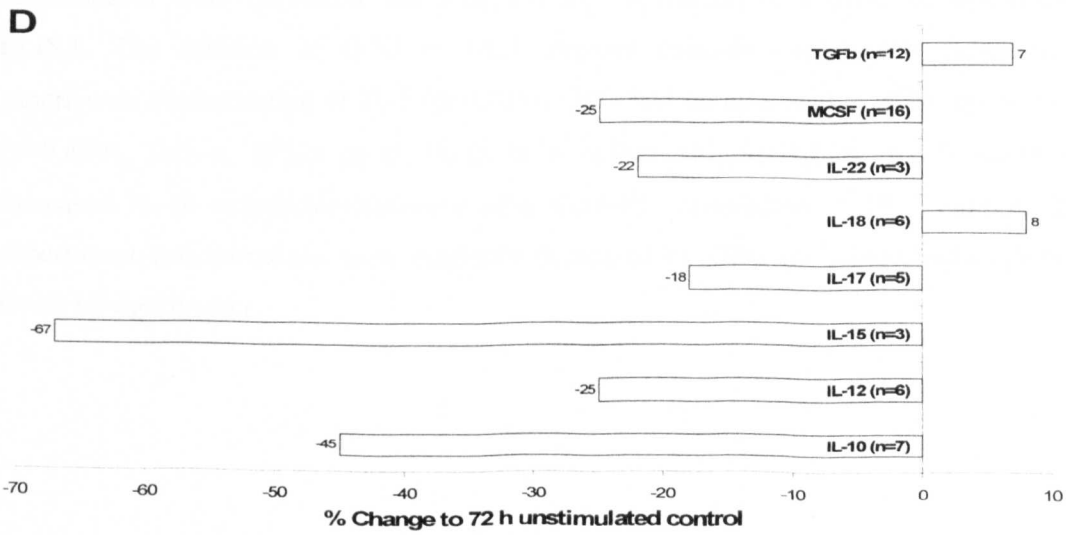
After 72 h PRD explant culture, OPG supernatant levels were correspondingly reduced by the addition of exogenous IL-10 ($p=0.08$), IL-12 ($p=0.230$), IL-15, IL-17A, MCSF ($p=0.082$), IL-22 and TGF-β. However, these reductions were only moderate and below levels of significance (Figure 7.7C-D).

Figure 7.7 ***In vitro*** **effects of cytokine manipulation upon RANKL and OPG in 72 h PRD explant cultures.** PRD explant tissues were stimulated with cytokines for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of RANKL after 72 h explant tissue stimulation with cytokines compared with unstimulated control. (B) The percentage change in culture supernatant RANKL concentration between stimulated and unstimulated control tissues. (C) The mean concentration of OPG after explant tissue stimulation with cytokines compared with unstimulated control. (D) The percentage change in culture supernatant OPG concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated by each individual cytokine and measured for RANKL and OPG is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.




B
TGFb (n=11)
4
Cytokine
MCSF (n=14)
71
stimulant
IL-22 (n=3)
127
-44
IL-18 (n=8)
IL-17 (n=4)
188
IL-12 (n=5)
71
IL-10 (n=7)
142
-50
0
50
100
150
200
% Change to 72 h unstimulated control


C
CONTROL
STIMULANT
6000
5000
4000
3000
2000
1000
0
OPG
concentration
(pg/ml)
*
IL-10 (n=7)
IL-12 (n=6)
IL-15 (n=3)
IL-17 (n=5)
IL-18 (n=6)
IL-22 (n=3)
MCSF (n=16)
TGF-β (n=12)
72 h cytokine stimulant


D
TGFb (n=12)
7
-25
MCSF (n=16)
-22
IL-22 (n=3)
IL-18 (n=6)
8
-18
IL-17 (n=5)
-67
IL-15 (n=3)
-25
IL-12 (n=6)
-45
IL-10 (n=7)
-70
-60
-50
-40
-30
-20
-10
0
10
% Change to 72 h unstimulated control

7.7 *In vitro* effects of the addition of RANKL and OPG to 18 h PRD explant cultures

Foregoing experiments established that endogenous OPG and RANKL expression could be mediated by addition of exogenous cytokine mediators to the PRD tissue explant model. Conversely, I next sought to determine if exogenous OPG or RANKL had any regulatory effect upon endogenous cytokine expression within the PRD explant system. Initially, I investigated the effects of addition of RANKL and OPG to PRD tissue explants at 18 h tissue culture. In addition to unstimulated explant tissue controls, addition of RANKL (Figure 7.9A, B) or OPG (Figure 7.10A, B) to 18 h PBMC and whole blood cell cultures served as further controls.

Addition of exogenous RANKL to 18 h PRD explant cultures induced only a modest increase in IL-8 ($p=0.08$) secretion. RANKL had no observable effects upon IL-6, IL-17A, IL-18 or TNF-α ($p=0.138$) expression (Figure 7.8A, B). Interestingly, RANKL significantly increased the expression of both IFN-γ ($p=0.0089$) and IL-10 ($p=0.046$) at 18 h culture. The effect upon IL-10 was moderate in comparison with the large increase observed in RANKL-induced expression of IFN-γ. Whether increased expression of IFN-γ by RANKL is part of a negative regulatory loop to reduce bone resorption or a positive feedback loop, inducing further RANKL expression (see chapter 7.4) requires further investigation.

Having established that RANKL was capable of modulating endogenous cytokine expression, I next investigated the effects of OPG addition to 18 h PRD explant cultures. Supernatants were harvested and analysed for expression of a panel of cytokines by ELISA. The addition of OPG to 18 h explant cultures significantly increased the supernatant concentration of IL-8 ($p=0.021$). OPG had no appreciable effect upon IL-17A ($p=0.489$), TNF-α, IFN-γ or IL-18 at 18 h culture (Figure 7.11A, B). In contrast to increased IL-10 expression observed after RANKL stimulation at 18 h culture, IL-10 supernatant concentrations were modestly decreased by OPG ($p=0.262$), although below levels of significance.

Figure 7.8 ***In vitro*** **effects of RANKL manipulation upon cytokine expression in 18 h PRD explant cultures.** PRD explant tissues were stimulated with RANKL for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of cytokines after explant tissue stimulation with RANKL compared with unstimulated control. (B) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated by RANKL and analysed for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.

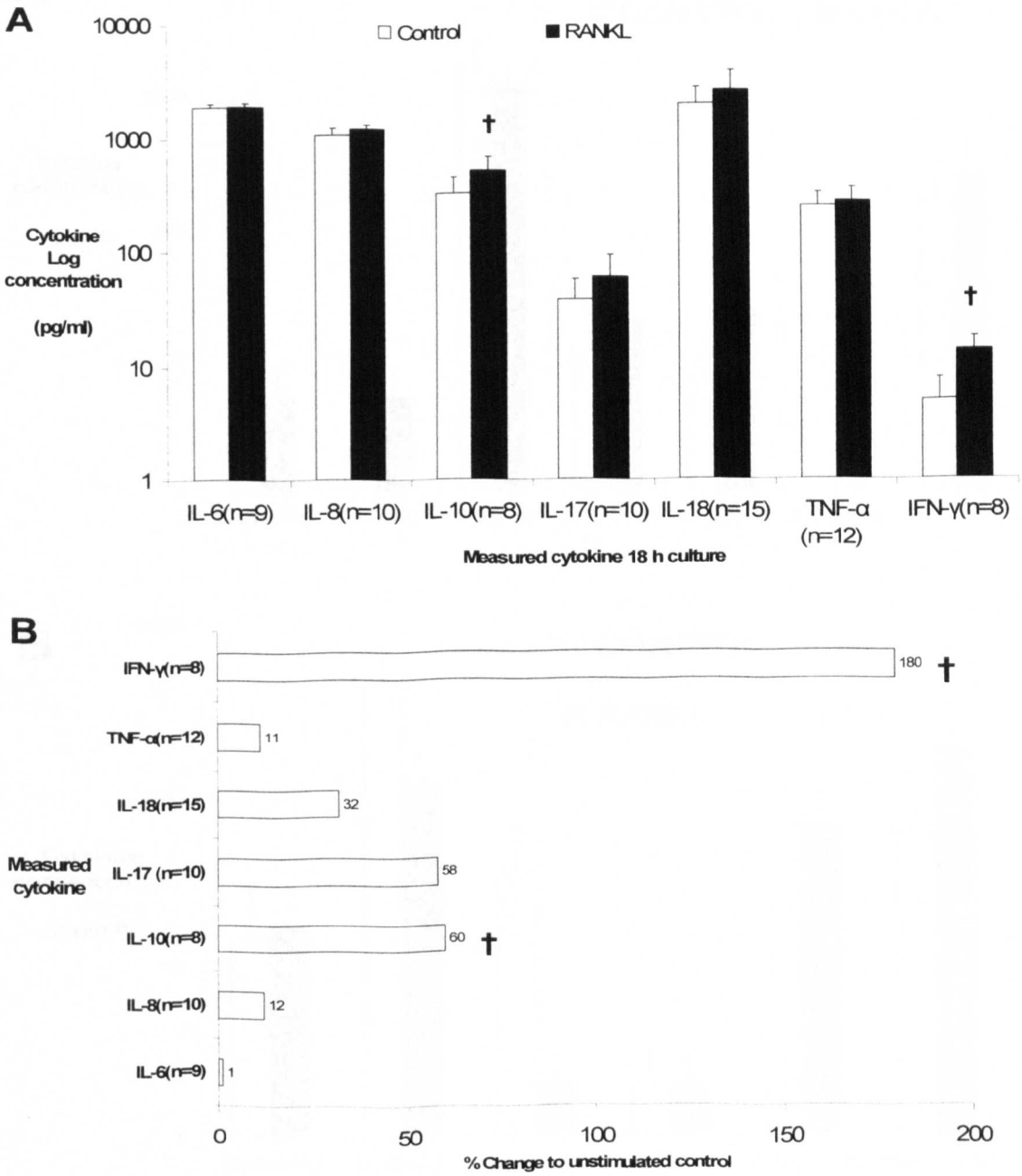


Figure 7.9 ***In vitro*** **effects of 18 h RANKL stimulation upon cytokine expression within PBMC and whole blood cell cultures.** (A) The mean concentration of cytokines after RANKL stimulation compared with unstimulated control in 18 h PBMC cultures. (B) The mean concentration of cytokines after RANKL stimulation compared with unstimulated control in 18 h whole blood cell cultures. The total number of separate patient derived cultures stimulated with each cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.

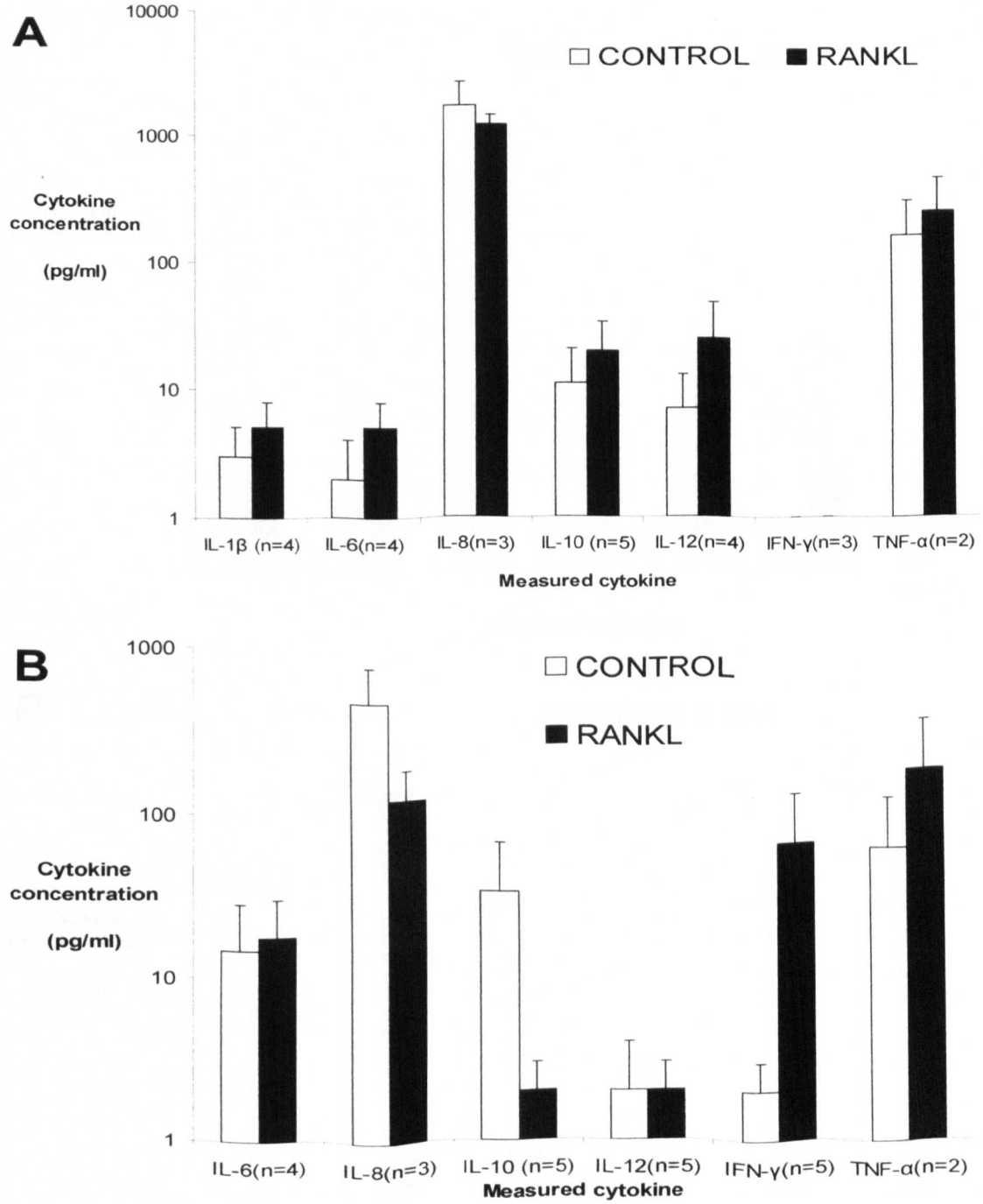
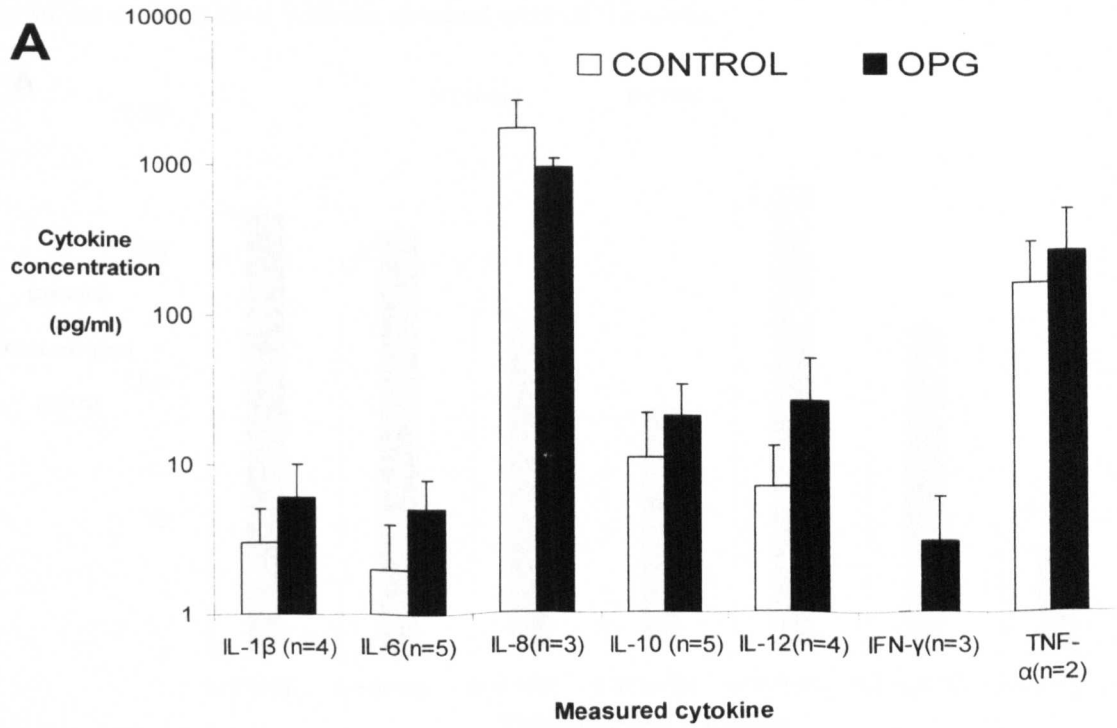


Figure 7.10 ***In vitro*** **effects of 18 h OPG stimulation upon cytokine expression within PBMC and whole blood cell cultures**. (A) The mean concentration of cytokines after OPG stimulation compared with unstimulated control in 18 h PBMC cultures. (B) The mean concentration of cytokines after OPG stimulation compared with unstimulated control in 18 h whole blood cell cultures. The total number of separate patient derived cultures stimulated with each cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.



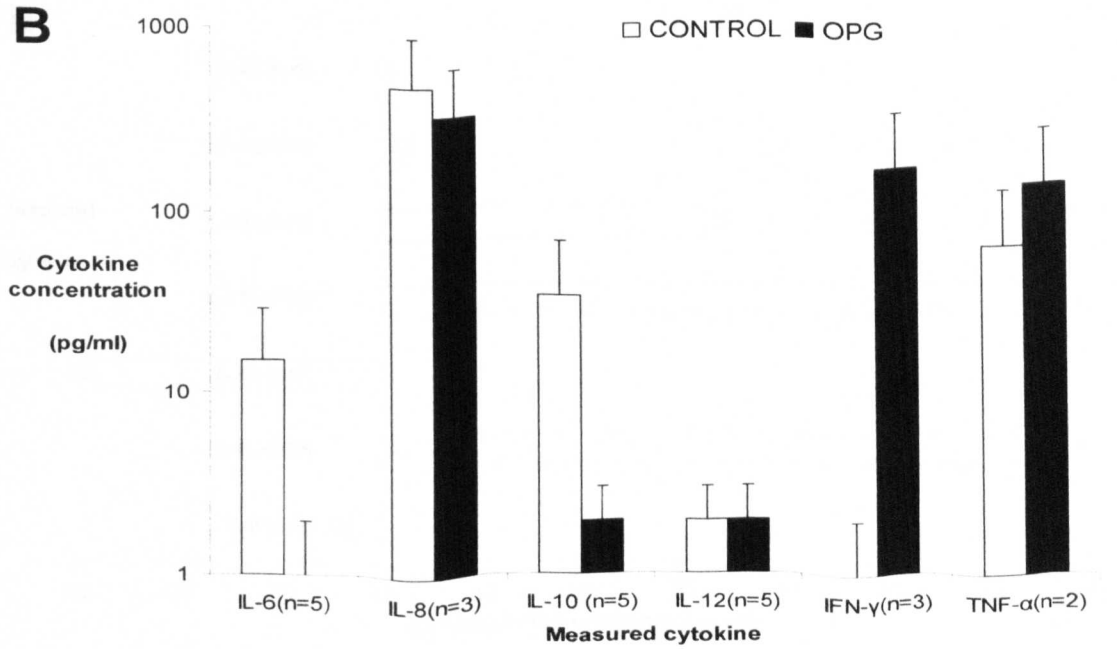
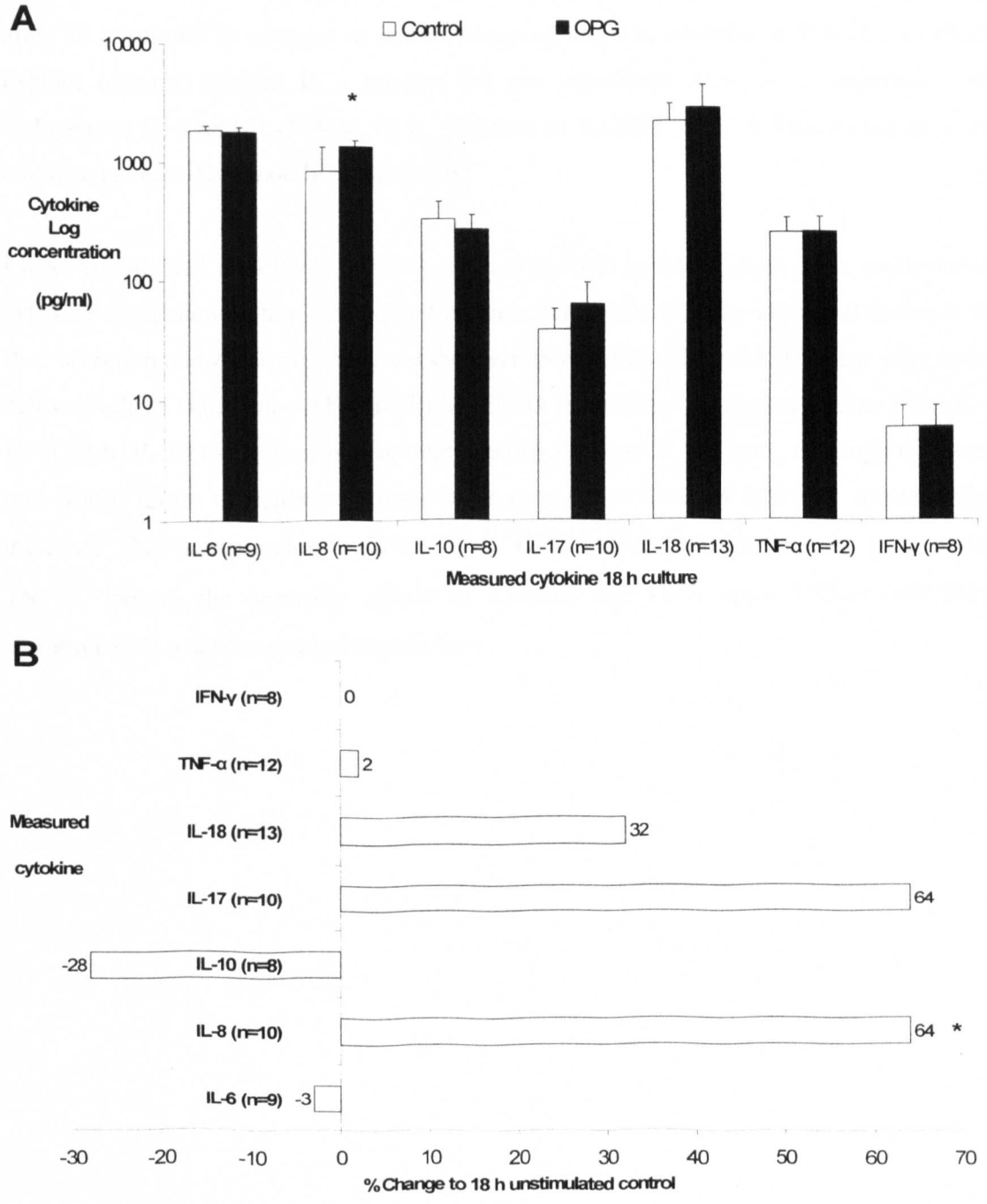


Figure 7.11 ***In vitro*** **effects of OPG manipulation upon cytokine expression within 18 h PRD explant cultures.** PRD explant tissues were stimulated with OPG for 18 h and supernatant collected and analysed by ELISA. (A) The mean concentration of cytokines after explant tissue stimulation with OPG compared with unstimulated control. (B) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated by OPG and analysed for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.



7.8 *In vitro* effects of the addition of RANKL and OPG to 72 h PRD explant cultures

The previous experiments established that RANKL and OPG were capable of modulating endogenous cytokine expression at 18 h PRD tissue culture. Therefore, I further investigated the effects of the addition of exogenous RANKL to the explant model over a longer period of culture to 72 h. At 72 h explant culture, RANKL induced only a minor increase in secreted IL-10 ($p=0.135$) and IL-18 ($p=0.14$), below levels of significance (Figure 7.12A, B). However, RANKL significantly increased TNF-α expression ($p=0.04$) after 72 h culture. In contrast to results observed at 18 h, addition of RANKL to PRD explant cultures resulted in a modest but not significant decrease in expression of endogenous IL-8 and IL-17A at 72 h. Addition of RANKL to 72 h PRD tissue explant cultures had no effect upon IL-6 expression.

I next investigated the effects of OPG addition to PRD explant tissues upon endogenous cytokine expression within 72 h explant cultures. OPG induced only a minimal decrease in IL-8 secretion and modestly increased the expression of IL-18 ($p=0.15$). These were both below levels of significance (Figure 7.13A, B). In contrast to the observed effect upon IL-10 at 18 h, IL-10 expression was up-regulated by OPG at 72 h culture, although this was just below levels of statistical significance ($p=0.083$). Whereas RANKL substantially increased TNF-α expression at 72 h culture, OPG induced a large decrease in secreted TNF-α. Indeed, the opposing effects of RANKL and OPG upon TNF-α ($p=0.084$) secretion at 72 h almost reached significance.

Figure 7.12 ***In vitro*** **effects of RANKL manipulation upon cytokine expression within 72 h PRD explant cultures.** PRD explant tissues were stimulated with RANKL for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of cytokines after explant tissue stimulation with RANKL compared with unstimulated control. (B) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated by RANKL and analysed for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.

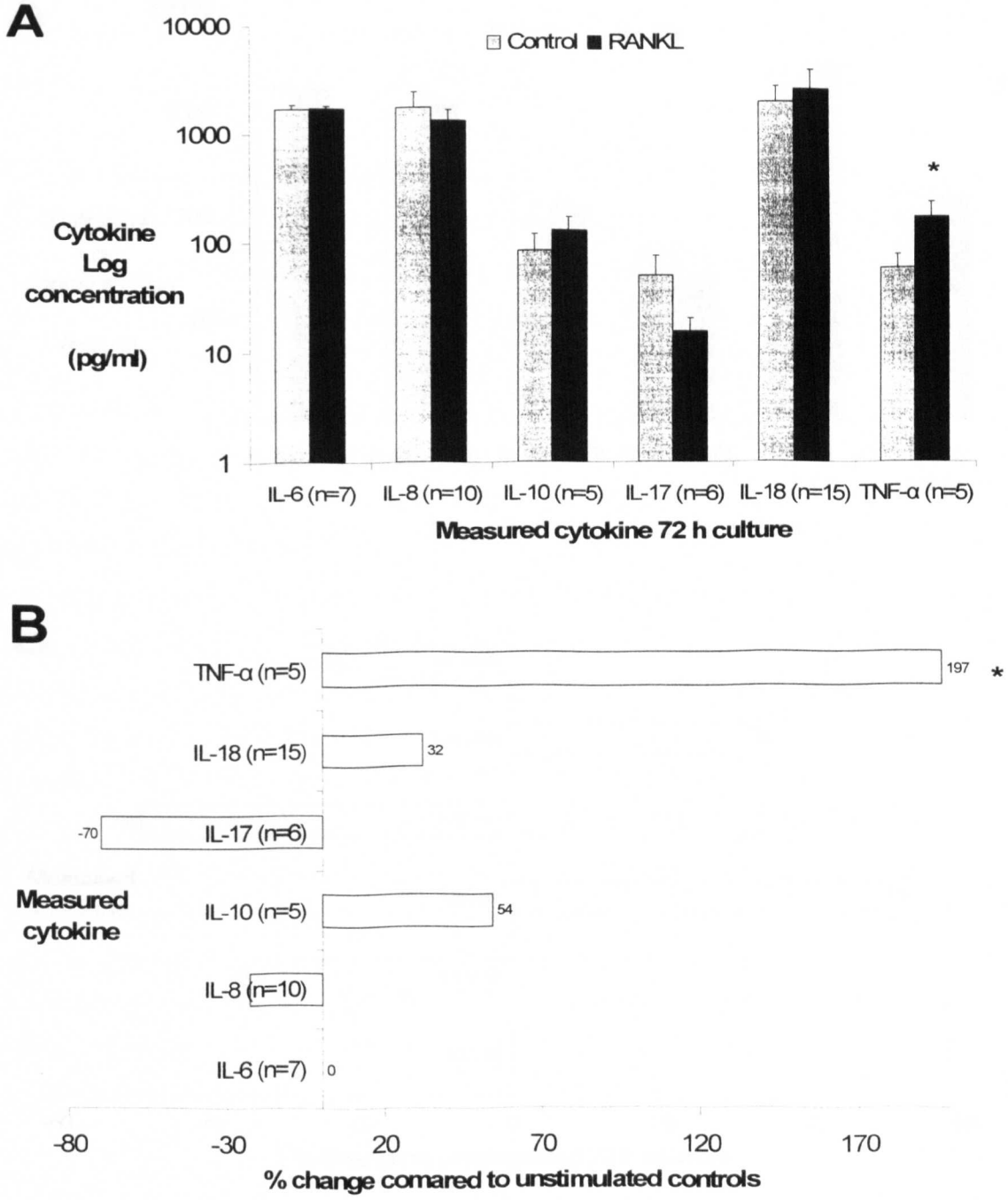
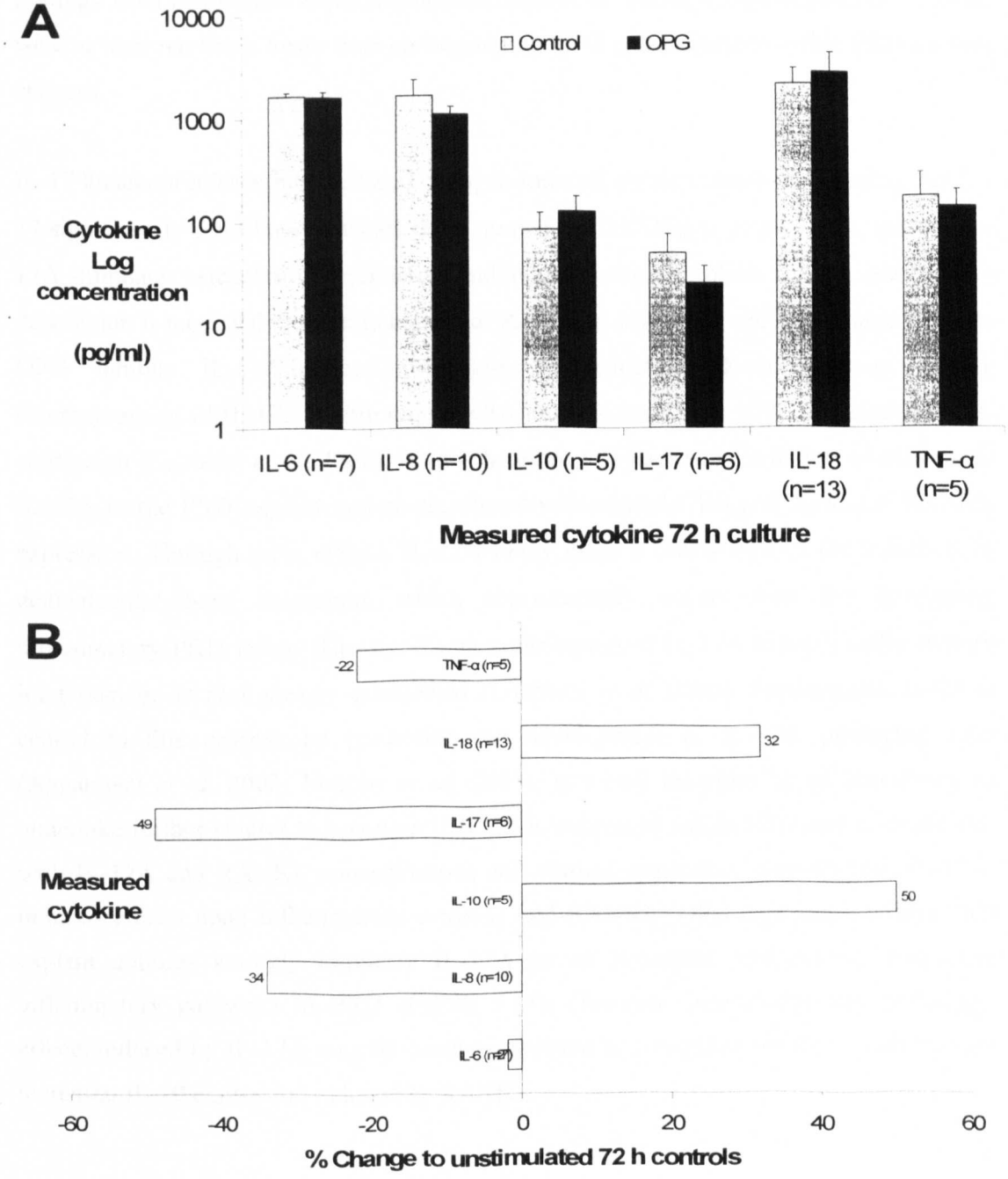


Figure 7.13 ***In vitro*** **effects of OPG manipulation upon cytokine expression within 72 h PRD explant cultures.** PRD explant tissues were stimulated with OPG for 72 h and supernatant collected and analysed by ELISA. (A) The mean concentration of cytokines after explant tissue stimulation with OPG compared with unstimulated control. (B) The percentage change in culture supernatant cytokine concentration between stimulated and unstimulated control tissues. The total number of PRD explant tissues stimulated by OPG and analysed for each individual cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.



7.9 Discussion

Physiological bone remodelling is regulated by osteoblastic bone deposition and bone resorption by osteoclasts (Ducy *et al.* 2000, Teitelbaum 2000). This equilibrium is maintained by expression of a multitude of growth factors, cytokines and hormones. Nevertheless, bone homeostasis is primarily governed through expression of and complex interactions between RANKL, RANK and OPG. I established that RANKL and OPG were spontaneously expressed within 'resting' PRD explant tissue cultures. In agreement with findings from cell culture experiments (Mandelin *et al.* 2005), concentrations of RANKL were at least ten times lower than corresponding OPG concentrations within PRD explant cultures.

IL-17 knockout mice exhibit reduced collagen-induced arthritis thereby suggesting that IL-17 significantly contributes towards development of CIA (Nakae *et al.* 2003). Indeed, IL-17A stimulates osteoclast differentiation and induces bone resorption. IL-17A related bone destruction is mediated through induction of RANK on osteoblast cell membranes whereas OPG inhibits IL-17A-induced osteoclast differentiation (Kotake *et al.* 2001). Overexpression of IL-17A within murine CIA joints is associated with increased RANKL expression (Lubberts *et al.* 2002). It was therefore of interest that addition of exogenous IL-17A to the PRD explant system significantly decreased OPG and increased RANKL expression. Through these effects, IL-17A likely plays a central role in the induction of dentoalveolar bone resorption, which concomitantly occurs with the developing inflammatory PRD lesion. The significant contribution of IL-17A towards inflammatory joint damage is now clearly established (Lubberts *et al.* 2005). Furthermore, IL-23 is central to this process by promoting the development of IL-17A producing cells (Aggarawal *et al.* 2003, Murphy *et al.* 2003). It would therefore be of importance to undertake further studies to ascertain if IL-23 is expressed within PRD and correlate this with IL-17A and RANKL concentrations and clinical symptoms. Nevertheless, IL-17A-induced effects upon inflammatory cytokine and RANKL/ OPG expression within PRD explant cultures strongly implicate IL-17A as an important mediator of destructive inflammatory pathways in PRD (Figure 7.14). However, proinflammatory pathologic effects induced by IL-17A may be counter-regulated in a negative feedback loop through increased IL-10 expression induced by RANKL.

In vitro studies establish that by up-regulating OPG expression, IL-18 inhibits osteoclastogenesis (Makiishi-Shimobayashi *et al.* 2001). Conversely, Dai *et al.* (2004b) recently demonstrated that IL-18 induces RANKL production from T cells in RA synovitis. Moreover, IL-18 has no effect upon OPG expression and therefore, through up-regulating RANKL expression, IL-18 indirectly stimulates osteoclast formation. Indeed, IL-18-induced osteoclast formation is as effective as IL-1β but less potent than TNF-α (Dai *et al.* 2004b). Although IL-18 was readily detectable within PRD tissues, the addition of IL-18 to explant cultures had little effect upon OPG and RANKL expression. It is therefore unlikely that IL-18 contributes towards destruction of surrounding dentoalveolar bone through the RANKL/OPG axis. Nevertheless, the direct effects of IL-18 upon local osteoblast and osteoclast cell populations within dentoalveolar tissues remain to be elucidated.

IL-4 inhibits bone erosion by interacting directly with osteoclasts and indirectly by inhibiting production of proinflammatory cytokines. RANKL-induced bone resorption is dose dependently inhibited by direct effects of IL-4 upon mature osteoclasts (Mangashetti *et al.* 2005) and osteoclastogenesis is indirectly suppressed by IL-4 down-regulating the transcription factors NFATc1 and c-Fos (Kamel Mohamed *et al.* 2005). Furthermore, IL-4 suppresses RANKL mRNA and protein expression and promotes OPG secretion by fibroblast-like synoviocytes (Lee *et al.* 2004). It was therefore of importance that secreted IL-4 was not detectable within PRD explant cultures. Neither was IL-4 expression inducible by addition of cytokines or bacterial moieties to the PRD explant tissue system (chapter 4.2). Deficient IL-4 expression within the PRD lesion likely impacts upon homeostasis of the surrounding bone environment, whereby RANKL expression may be ineffectively down-regulated and the ability to induce OPG expression may be impaired.

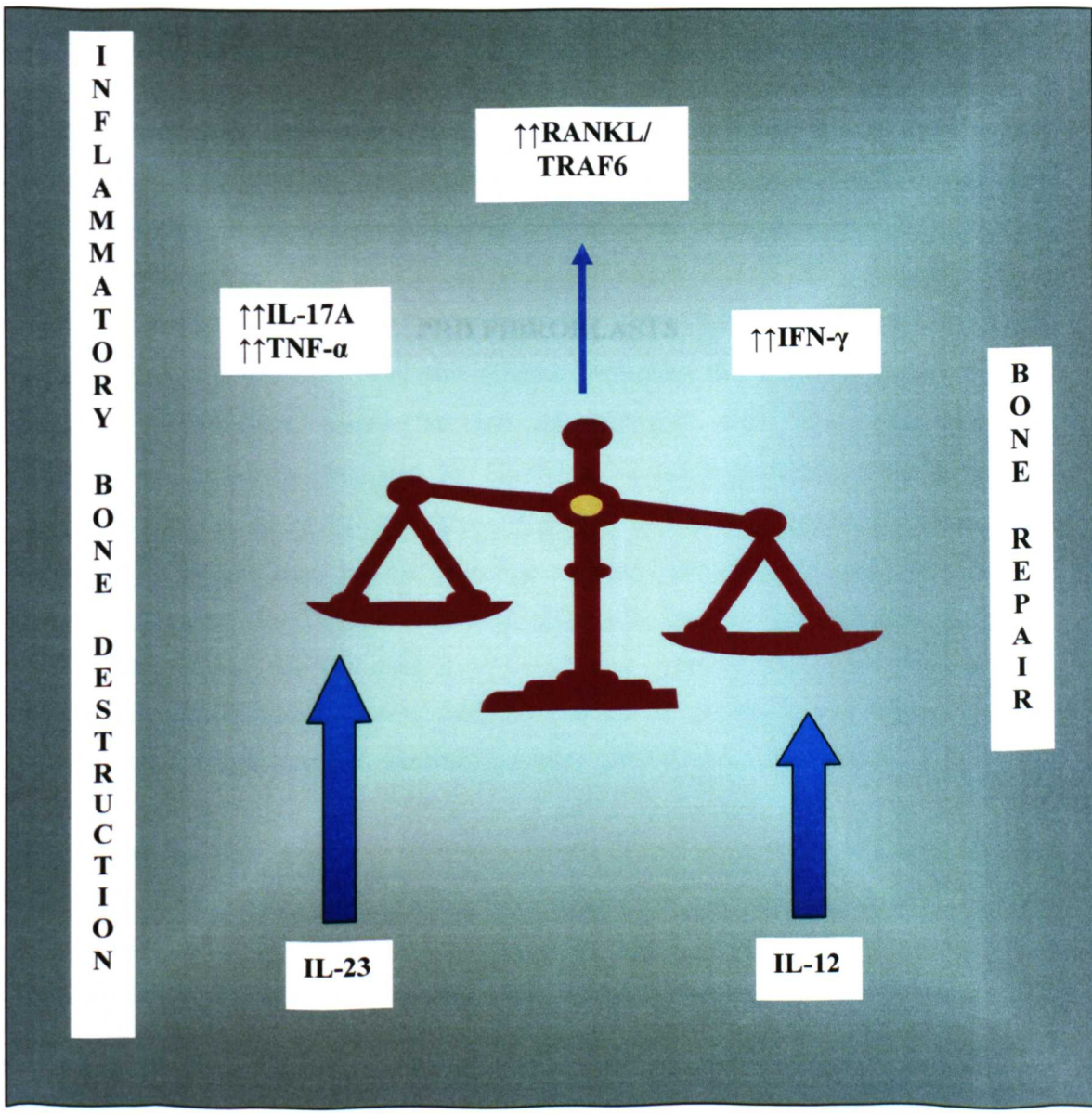
Osteoclastogenesis occurs by TNF-α stimulation of macrophages exposed to a stromal environment expressing permissive levels of RANKL (Lam *et al.* 2000, Kudo *et al.* 2002). IL-1 and TNF-α also regulate OPG and RANKL gene expression in osteoblastic cells (Hofbauer *et al.* 1999). Interestingly, stimulation of synovial fibroblasts from RA patients with TNF-α promotes OPG mRNA and protein expression (Kubota *et al.* 2004). Within the PRD lesion, TNF-α reduced OPG expression and induced a slight increase of RANKL although these effects were minimal. These data signify that TNF-α has limited effects upon RANKL and OPG expression by cellular constituents of the PRD lesion. The

contribution of TNF-α towards dentoalveolar bone resorption is unlikely mediated by its influence upon RANKL/OPG expression from cellular components of the tissue lesion.

Through interfering with the RANKL-RANK signalling pathway, IFN-γ inhibits osteoclast formation (Takayanagi *et al.* 2000b). IFN-γ also prevents osteoclast formation by directing differentiation of osteoclast progenitors towards an alternative cytocidal lineage to the osteoclast (Fox and Chambers 2000). After T cell activation, IFN-γ thereby has a key responsibility in protecting against calcified tissue destruction. It was therefore surprising that IFN-γ addition to explant cultures significantly increased RANKL and significantly decreased OPG expression. Furthermore, addition of OPG or RANKL to explant cultures resulted in substantially increased IFN-γ expression. Contrary to its well established bone protective properties, it may be postulated that through effects upon the RANKL/ OPG ratio, IFN-γ has the potential to indirectly contribute to localised periradicular bone resorption.

Fibroblasts are an important source of RANKL that contributes towards bone erosion in rheumatoid arthritis and loosening of hip prosthesis (Takayanagi *et al.* 2000a, Mandelin *et al.* 2005). TNF-α, IL-1β, IL-6 and IL-11 have no apparent effect upon RANKL expression at mRNA or protein levels in fibroblasts derived from the joint/ tissue interface. In contrast, TNF-α stimulation of tissue interface fibroblasts leads to increased expression of OPG (Mandelin *et al.* 2005). As there is no data describing RANKL and OPG expression within PRD fibroblasts, I next undertook investigations to determine expression of these mediators within PRD fibroblast cultures. These experiments are described in chapter 8.

Figure 7.14 Schematic illustration of effects of inflammatory mediators upon RANKL expression within the PRD lesion. Bone resorption is carefully balanced between RANKL expression, which is inducible by IL-17A and bone protective effects exhibited by IL-12 and IFN-γ. Increased expression of RANKL, induced by IL-17A, propagates increased TNF-α expression, which likely contributes towards pathologic bone destruction. IL-17A expression, induced by IL-23, leads to increased secretion of a cascade of proinflammatory cytokines whilst concomitantly decreasing OPG expression. IL-12 and IFN-γ are considered to be bone protective. However, through inducing increased RANKL expression from cellular components of the PRD lesion, IFN-γ may also contribute towards tissue destructive pathways. Potentially destructive effects of IFN-γ may be counter-regulated by IL-12, inducing increased OPG expression.



CHAPTER 8

PRD FIBROBLASTS

8 PRD FIBROBLASTS

8.1 Introduction

Fibroblasts constitute a significant cellular component of the tissue microenvironment disseminated throughout the body. Through the synthesis and release of connective tissue constituents, fibroblasts are central to remodelling of the extracellular matrix (Everts *et al.* 1996, Lekic *et al.* 1997). Furthermore, fibroblasts are capable of responding to a variety of growth factors, which include cytokines (Haase *et al.* 1998). In response to these inflammatory mediators, fibroblasts carefully regulate the balance between ECM synthesis or tissue degradation (Havemose-Poulsen and Holmstrup 1997). Chronic dysregulation of this tightly controlled equilibrium leads to pathologic tissue damage such as occurs within periradicular disease.

A multitude of investigations have been undertaken upon fibroblasts derived from chronic inflammatory lesions that are associated with concurrent bone degradation. It is established that fibroblasts have the capacity to instruct reparative and destructive processes occurring within chronic inflammatory periodontal diseases (Takashiba *et al.* 2003, Hosokawa *et al.* 2005) and rheumatoid arthritis (Pap *et al.* 2005). Fibroblasts derived from chronically inflamed tissues are known to release effector molecules that act on a variety of cells including lymphocytes, monocytes and mesenchymal cells. The wide array of inflammatory mediators produced by fibroblasts includes cytokines, chemokines and matrix-metalloproteinases (MMPs). Through production and release of these inflammatory molecules, it is now accepted that fibroblasts directly contribute towards the perpetual matrix destruction that occurs concomitantly with chronic inflammatory reactions. Furthermore, it has recently been established that synovial fibroblasts derived from rheumatoid arthritis joints express mediators central to regulating the delicate balance between bone resorption and deposition, including OPG (Kubota *et al.* 2004).

Evidence of the role played by fibroblasts within several chronic inflammatory diseases reinforces the concept that during development of pathologic diseases, resident mesenchymal cells may act as effector cells. Infiltrating T lymphocytes and monocytes are implicated in the initiation and perpetuation of periradicular disease. Through secretion of chemoattractant mediators, fibroblasts likely perform an important function in recruitment of leukocytes at an early stage in PRD lesion development. Moreover, cytokine-stimulated

fibroblasts derived from inflamed tissues modulate the inflammatory response by inducing cytokine expression from recruited immune cells, including IL-17A from T cells (Numasaki *et al.* 2004). Nevertheless, the role of fibroblasts in PRD lesion initiation and/or progression has received scant attention. Indeed, there is little published research phenotyping the PRD fibroblast.

The effects of suspected endodontic pathogens upon whole blood cultures obtained from PRD patients (Matsushita *et al.* 1999), periodontal ligament cells (Chang *et al.* 2002b,c) and pulp-derived fibroblasts (Hosoya and Matsushima 1997, Chang *et al.* 2002b, Yang *et al.* 2003a,b,c) have been investigated. Although these studies have analysed oral tissue-derived fibroblasts, they are unlikely to be representative of PRD-derived fibroblast responses as it is recognised that there is significant heterogeneity between and within fibroblast populations (Reichenbertg *et al.* 2005). Surprisingly, only one reported study has analysed effects of cytokines or pathogens upon fibroblasts originating from PRD tissue. However, fibroblasts analysed were only derived from periradicular bone cysts (Kusumi *et al.* 2004). There is clearly a lack of information about spontaneous or inducible release of cytokines by fibroblasts originating from periradicular disease tissues. I therefore investigated the potential for PRD-derived fibroblasts to contribute towards the initiation and perpetuation of the PRD lesion by analysing their expression of key inflammatory mediators.

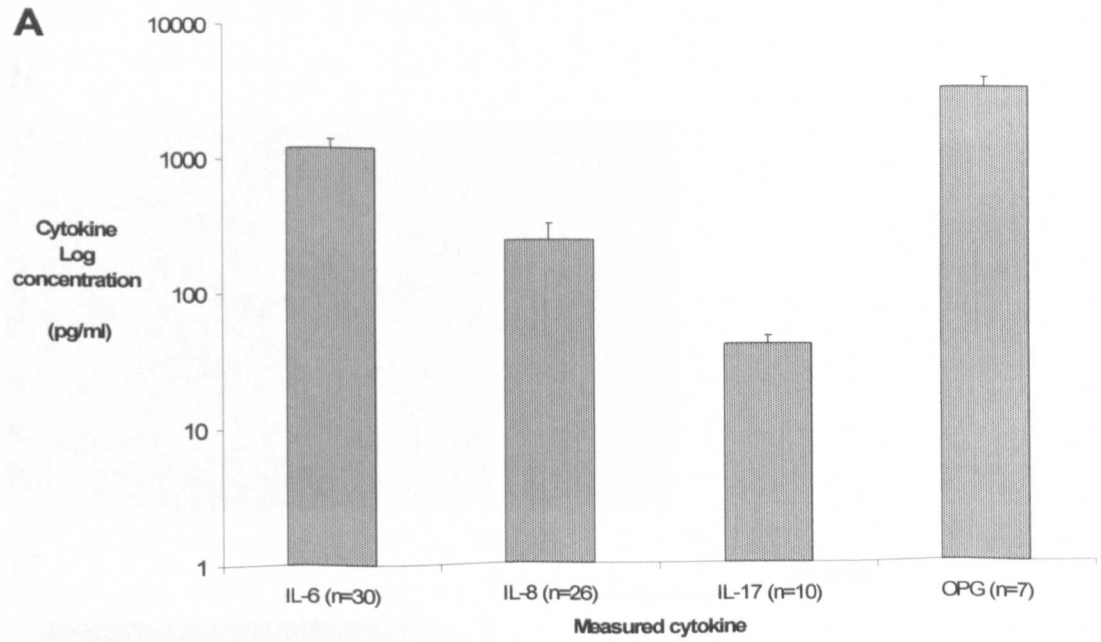
In the following series of fibroblast cell culture experiments, *Prevotella intermedia*, *Peptostreptococcus micros* and *Fusobacterium nucleatum* were used as live infective agents. *Prevotella intermedia* are Gram-negative, short round-ended anaerobic rods, *Peptostreptococcus micros* are Gram-positive cocci and *Fusobacterium nucleatum* are Gram-negative rods. These microorganisms were selected as they are implicated in endodontic infections. Moreover, they are frequently isolated from necrotic root canals of teeth presenting with clinical symptoms and are correlated with the development of extraradicular infections (Peters *et al.* 2002, summarised in Table 1.1). Indeed, *Fusobacterium nucleatum* is associated with development of the most severe forms of endodontic symptoms arising from root canal treatment (Chávaz de Paz 2002). This may occur due to the ability of *F. nucleatum* to bind MMP-9, thereby increasing the tissue invasive potential of this microorganism (Gendron *et al.* 2004).

8.2 Spontaneous cytokine production by unstimulated PRD fibroblast cultures

PRD fibroblasts have never been phenotypically analysed or formally characterised. No data is currently available defining their cytokine repertoire or ability for cytokine processing and release. Therefore, I initially undertook investigations to determine if unstimulated, "resting" PRD fibroblast cultures secreted detectable levels of cytokines. Fibroblast culture supernatants were analysed for a panel of cytokines. These cytokines were selected on the basis that they are reported to be produced by fibroblast populations derived from other chronic inflammatory diseased tissues. Levels of other cytokines, such as those of predominantly T cell or macrophage origin, were additionally measured as control. Therefore, supernatants from unstimulated PRD fibroblast cultures were assessed for levels of spontaneously secreted IL-1β, IL-6, IL-8, IL-10, IL-12, IL-17A, IL-18, TNF-α, IFN-γ, OPG and RANKL by ELISA.

IL-1β, IL-10, IL-12, IL-18, TNF-α, IFN-γ and RANKL were not detected within the culture supernatant of unstimulated PRD fibroblasts. However, substantial levels of OPG and IL-6 and moderate levels of IL-8 were detectable within culture supernatants of resting PRD fibroblasts (Figure 8.1A). Production of such quantities of inflammatory cytokines indicates that unstimulated PRD fibroblasts were in a high state of cellular activation. Furthermore, NF-κB p65 was readily detectable in cell lysates by Western blotting (Figure 8.1B). Surprisingly, low levels of IL-17A were also detected from fibroblast cultures, the significance of which is unknown. As the detection of secreted IL-17A and OPG were novel findings, these data were extended to confirm the molecular weight of these molecules by Western blotting (Figure 8.2A, C). Confirmation of cellular localisation of OPG and IL-17A to fibroblasts was also investigated by immunohistochemistry (Figure 8.2B, D).

Figure 8.1 Spontaneous cytokine release by PRD fibroblast cultures. (A) 'Resting' levels of spontaneously released cytokines within PRD fibroblast culture supernatants were assessed. Supernatant from unstimulated PRD explant fibroblast cultures was harvested at 18 h. Culture supernatants were analysed by ELISA for the expression of a panel of secreted cytokines. Bars represent the mean concentration for each cytokine and the standard error of the mean. The number of different patient-derived unstimulated fibroblast populations used in experiments for the measurement of each individual cytokine is given in parenthesis. (B) High activation status of resting PRD fibroblasts with substantial NF-κBp65 expression by Western blot of fibroblast cellular protein lysates. Three blots representative of 10 differing PRD fibroblast populations.



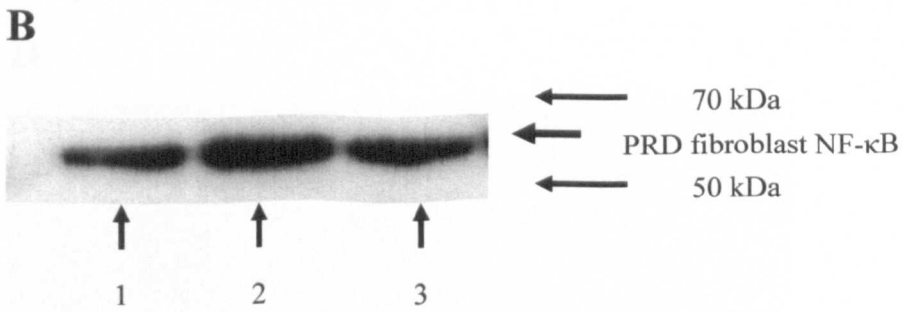
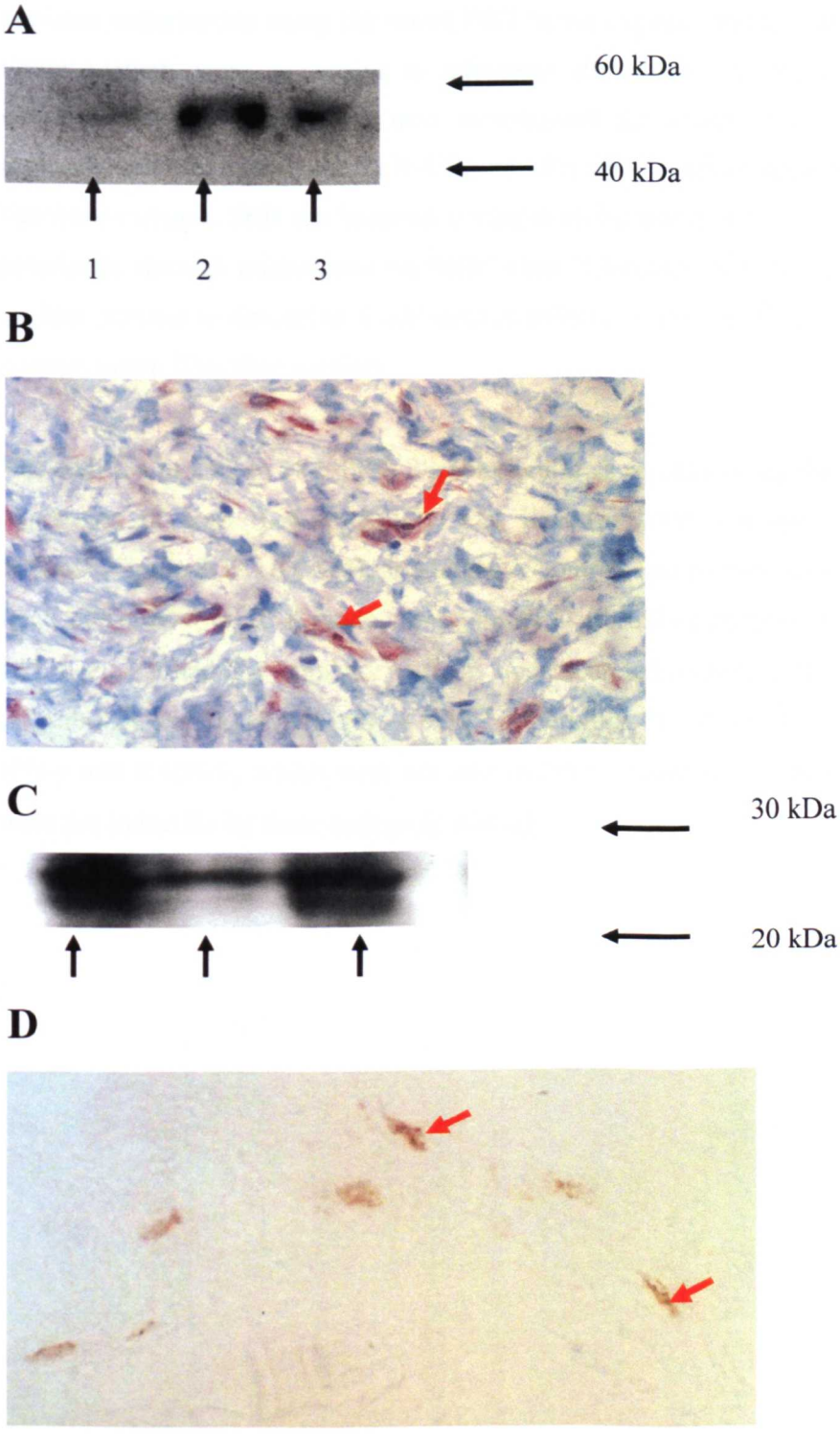


Figure 8.2 Confirmation of OPG and IL-17A expression by PRD fibroblasts. (A) Western blot confirming the molecular weight of OPG within PRD fibroblasts. (B) IHC identifying OPG localisation to PRD fibroblasts, represented by arrows. (C) Western blot of PRD fibroblasts for IL-17A. (D) IHC detection of IL-17A localised to fibroblasts, indicated by arrows (no counterstain). IHC experiments are representative of staining from 20 human PRD tissues, Western blots are representative of 15 PRD tissues.

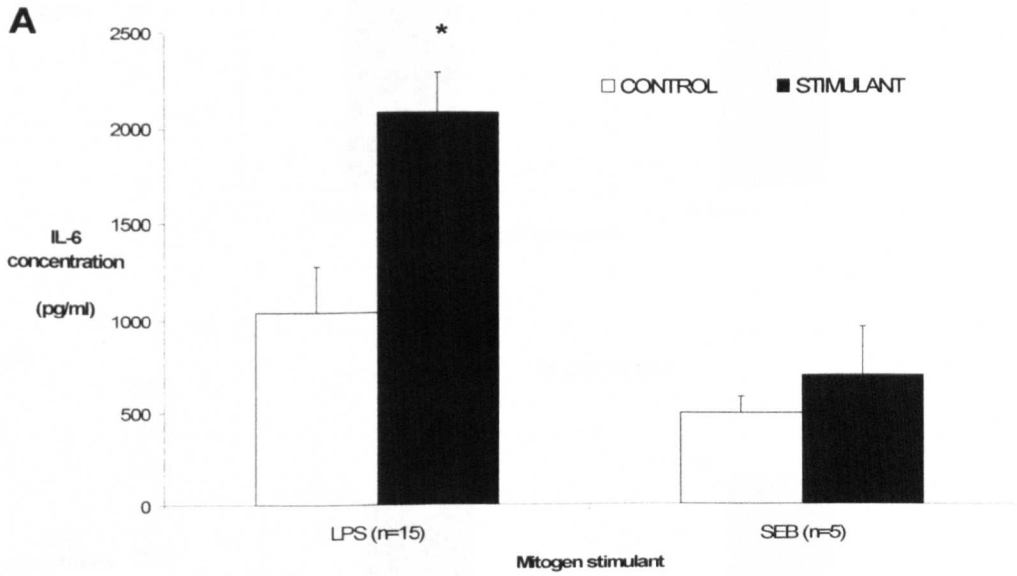


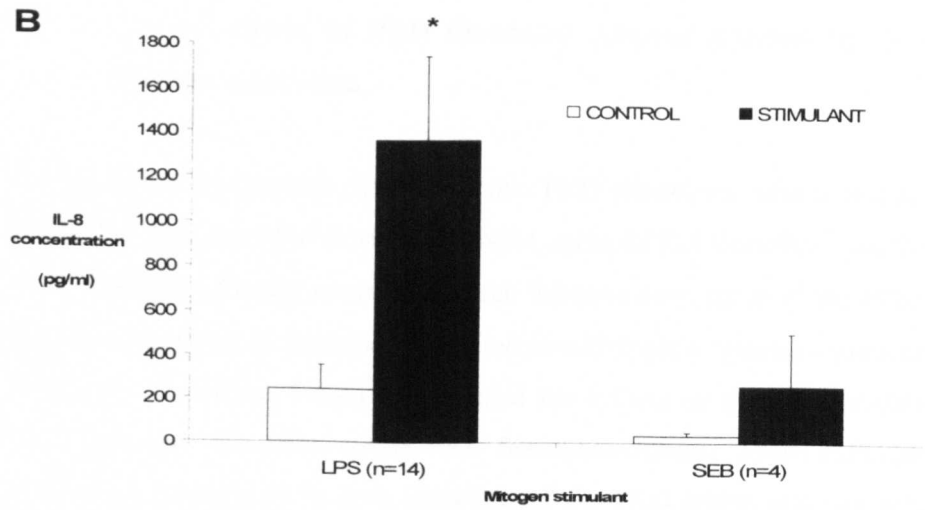
8.3 *In vitro* effects of mitogenic stimulation upon PRD fibroblast cultures

Having established that PRD fibroblasts were capable of spontaneously expressing inflammatory cytokines, I hypothesised that fibroblasts may contribute towards chronic inflammatory events within the PRD lesion. Perpetual challenge from the dental root canal by microorganisms and their by-products leads to chronic inflammatory PRD. From previous experiments using the novel PRD tissue explant model, I ascertained that PRD tissue explants were responsive to mitogenic stimulation. As the candidate stimuli of chronic PRD are microbes, I next investigated the effect of a pathogen associated molecular pattern, namely the TLR-4 ligand LPS, upon cytokine responses in PRD-derived fibroblast cultures. SEB is a bacterial virulence factor that promotes immunological effects principally through interactions via MHC class II binding. Having been used in the PRD explant cultures to determine T cell derived effects, it was therefore utilised as a negative control within fibroblast cultures.

The addition of LPS to 18 h PRD fibroblast cultures resulted in significant increases in the expression of both IL-6 ($p=0.0001$) and IL-8 ($p=0.008$) compared with unstimulated controls (Figure 8.3A, B). SEB, as a negative control, had no biologically significant effect upon either IL-6 or IL-8 production from PRD fibroblast cultures. Interestingly, neither LPS nor SEB had any regulatory effects upon secreted protein expression of either OPG (Figure 8.3C) or IL-17A (Figure 8.3D). Furthermore, IL-1β, IL-10, IL-12, IL-18, TNF-α, IFN-γ and RANKL, which were not spontaneously secreted by PRD fibroblast cultures, were not inducible by these mitogenic stimuli.

Figure 8.3 Effects of bacterial derived moieties upon cytokine expression within 18 h PRD fibroblast cultures. PRD fibroblasts were stimulated with LPS or SEB for 18 h and supernatant collected and analysed by ELISA for a panel of cytokines. (A) The mean concentration of IL-6 after 18 h stimulation of fibroblasts compared with their corresponding unstimulated controls. (B) The mean concentration of IL-8 after 18 h stimulation of fibroblasts compared with their corresponding unstimulated controls. (C) The mean concentration of IL-17A after 18 h stimulation of fibroblasts compared with their corresponding unstimulated controls. (D) The mean concentration of OPG after 18 h stimulation of fibroblasts compared with their corresponding unstimulated controls. The number of different patient-derived PRD fibroblast populations used in experiments for the measurement of each individual cytokine is given in parenthesis. Bars represent the mean concentration for each cytokine and the standard error of the mean.

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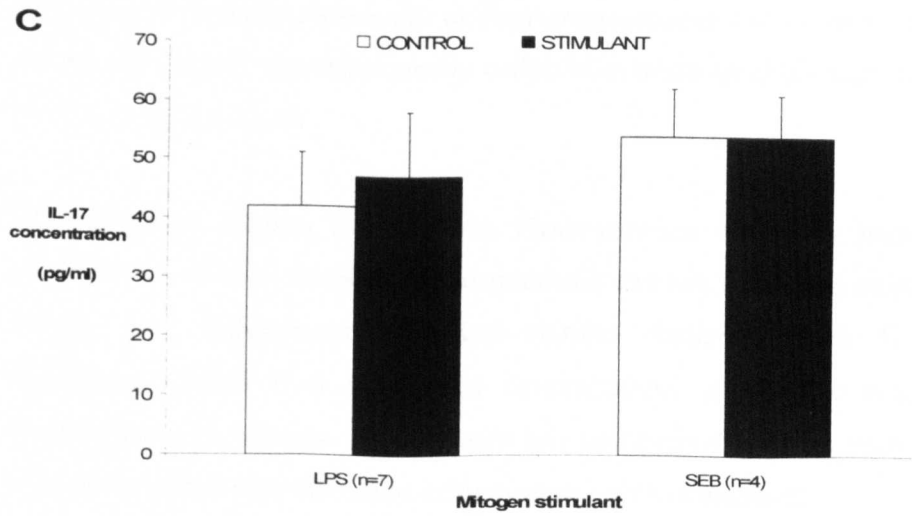
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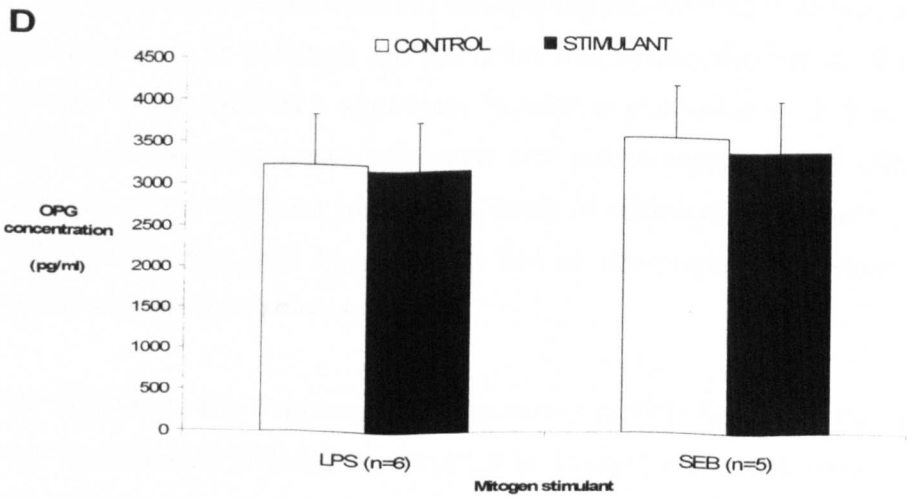
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SEB (n=5)
Mitogen stimulant

8.4 *In vitro* effects of PRD fibroblast cultures infected by live bacteria upon cytokine expression

The previous experiments determined that PRD fibroblasts were responsive to stimulation from the Gram-negative derived microbial agent LPS. I therefore hypothesised that PRD-derived fibroblasts may contribute to the inflammatory status of the PRD lesion by being directly responsive to microbes suspected as aetiological agents of induction in endodontic infections. Therefore, I next investigated the effects of three key endodontic pathogens upon cytokine expression within PRD fibroblast cultures. These pathogens were selected as they are implicated in both initiation of the PRD lesion and exacerbation of clinical symptoms (see chapter 1). PRD fibroblasts were infected with live *Fusobacterium nucleatum*, *Prevotella intermedia* or *Peptostreptococcus micros* up to 48 h culture. Cell culture supernatant was subsequently collected at times specified and analysed by ELISA for a panel of cytokines.

PRD fibroblast cultures infected with *Fusobacterium nucleatum* secreted significantly greater levels of IL-6 compared to unstimulated controls at all time points to 48 h culture (Figure 8.4). Furthermore, fibroblast cultures challenged with *F. nucleatum* had significantly raised IL-8 supernatant concentrations at all time points compared to unstimulated cell cultures. *F. nucleatum* had no observable effect upon IL-17A or OPG expression within PRD fibroblast cultures at any given time point.

Prevotella intermedia infection of fibroblast cultures resulted in significantly increased IL-6 expression at 18 h culture and this effect was further observed at 48 h (Figure 8.5). *P. intermedia* also induced a significant increase in expression of IL-8 at 4 h culture. IL-8 levels were increased at each subsequent time point compared to non-infected control to 48 h culture. However, these were below levels of statistical significance. Infection of PRD fibroblast cultures with *P. intermedia* had no observable effect upon IL-17A or OPG secretion at any time points to 48 h.

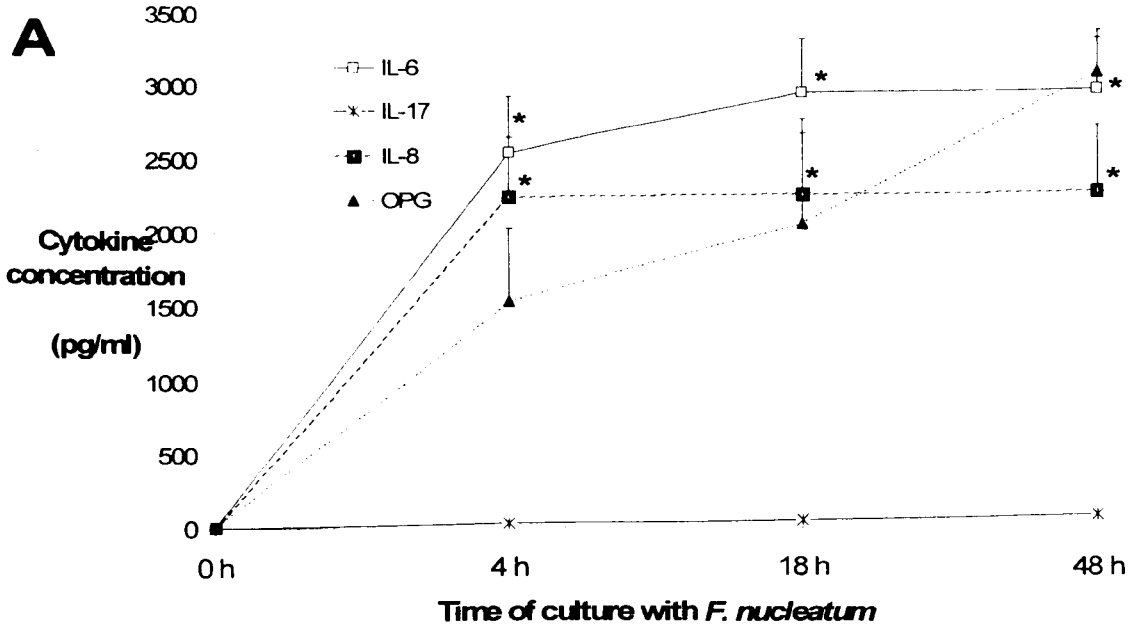
The addition of live *Peptostreptococcus micros* to PRD fibroblasts significantly increased IL-6 expression at 18 h culture (Figure 8.6). In comparison with controls, IL-6 expression was increased in fibroblast cultures infected with *P. micros* at all other time points. However, these raised concentrations were below levels of significance. *P. micros* also induced a significant increase in IL-8 expression at 4 h culture. In comparison to control

cultures, IL-8 levels were moderately increased at all time points to 48 h, but below levels of significance. These data suggest that the predominant effect of *P. micros* upon PRD fibroblasts was the early up-regulation of IL-6 and IL-8 expression. IL-17A and OPG supernatant concentrations were only minimally reduced by *P. micros* at all time points to 48 h but below levels of significance. Of interest, the increase in IL-6 and IL-8 concentrations induced by *P. micros* were not as great as those resulting from infection with the Gram-negative pathogens *P. intermedia* or *F. nucleatum*.

I had previously established that within resting PRD fibroblast culture supernatants, TNF-α was not detectable by ELISA. Surprisingly, *F. nucleatum* or *P. intermedia* infection of fibroblast cultures induced detectable levels of secreted TNF-α within culture supernatants (Figure 8.7). Although these were modest levels, infection of PRD fibroblast cultures with *F. nucleatum* resulted in significant levels of TNF-α production.

Individual addition of the three endodontic pathogens to PRD fibroblast cultures did not induce secretion of detectable levels of IL-1β, IL-4, IL-10, IL-12, IL-18, IFN-γ or RANKL into culture supernatant at any time points. Further experiments would be necessary to elucidate if a combination of pathogens has any effects upon these inflammatory mediators.

Figure 8.4 Effects of *Fusobacterium nucleatum* infection of PRD fibroblast cultures upon cytokine expression. Fibroblast cultures were stimulated with *F. nucleatum* for a variety of time points as indicated and supernatant collected and analysed by ELISA for IL-6, IL-8, OPG and IL-17A. (A) The mean concentration of each cytokine after infection of fibroblast cultures with *F. nucleatum* (B) The percentage change in culture supernatant cytokine concentration between *F. nucleatum* stimulated and unstimulated paired control fibroblast cultures. Vertical lines represent the standard error of the mean. Figures are representative of three separate experiments.



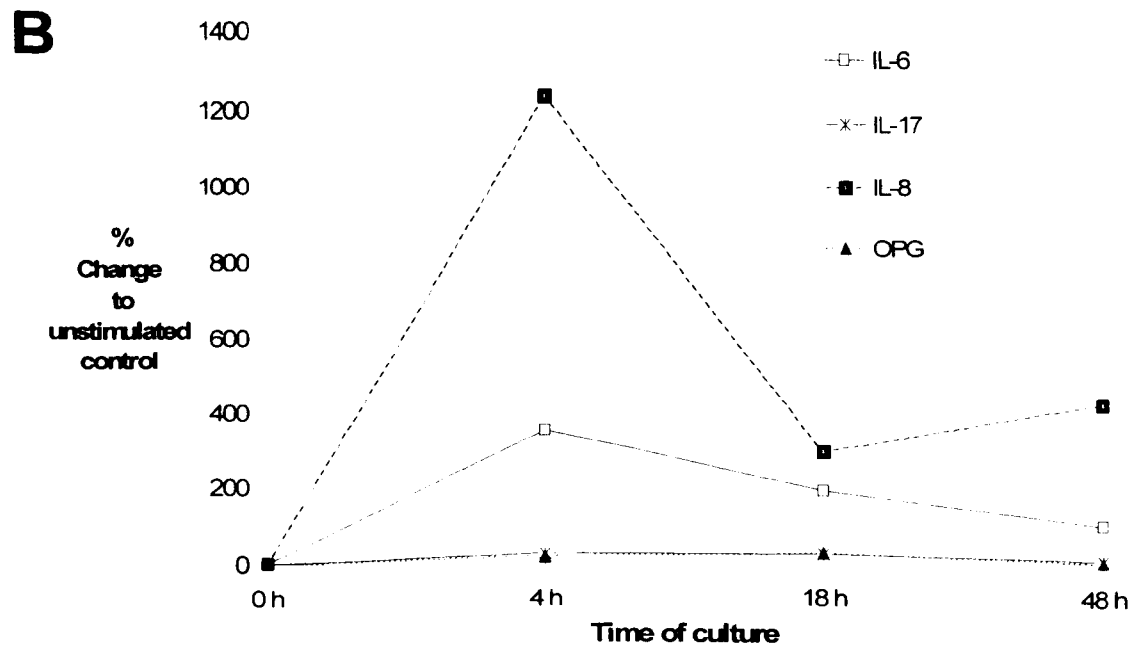


Figure 8.5 Effects of *Prevotella intermedia* infection of PRD fibroblast cultures upon cytokine expression. Fibroblast cultures were stimulated with *P. intermedia* for a variety of time points as indicated and supernatant collected and analysed by ELISA for IL-6, IL-8, OPG and IL-17A. (A) The mean concentration of each cytokine after infection of fibroblast cultures with *P. intermedia* (B) The percentage change in culture supernatant cytokine concentration between *P. intermedia* stimulated and unstimulated paired control fibroblast cultures. Vertical lines represent the standard error of the mean. Figures are representative of three separate experiments.

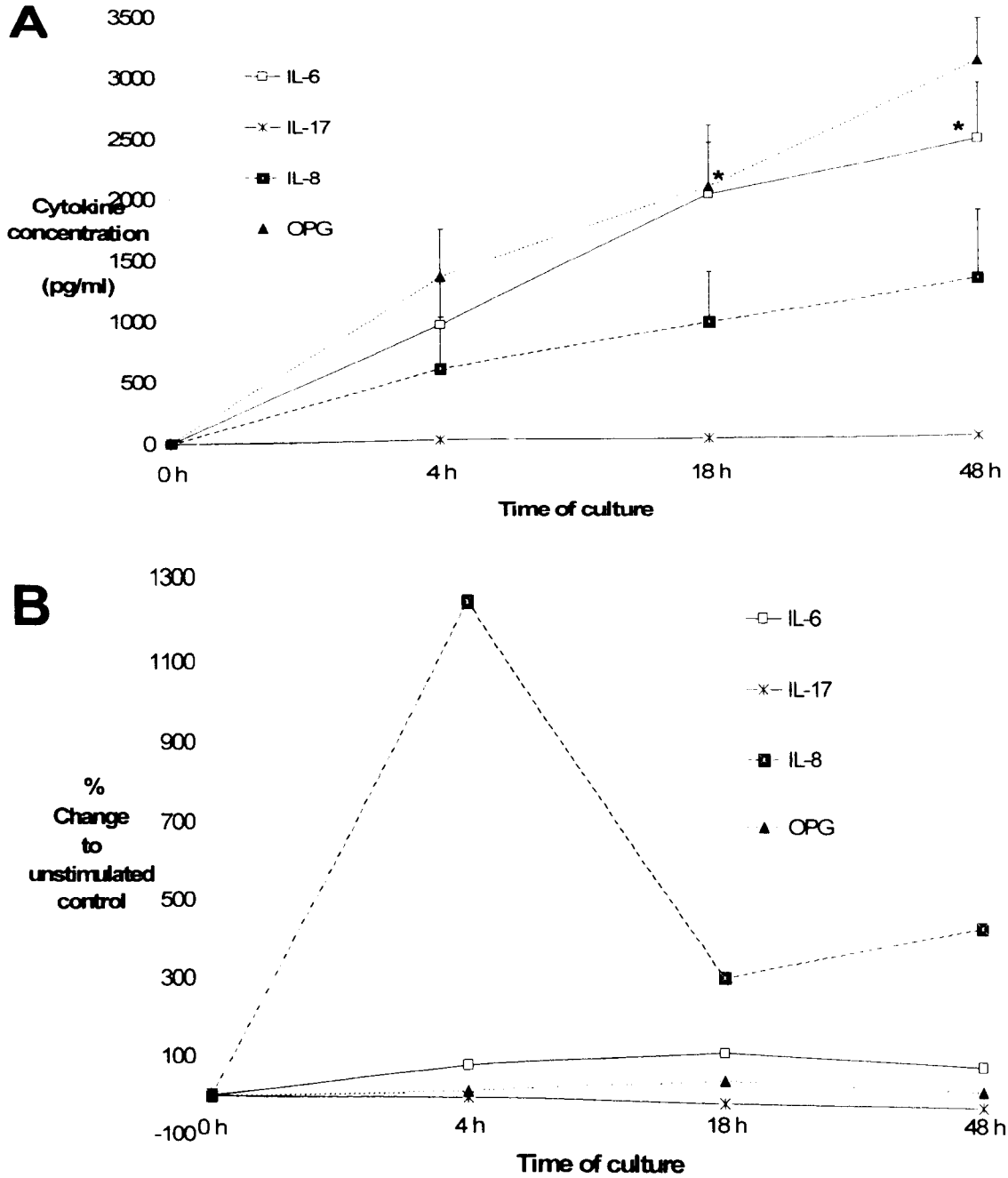


Figure 8.6 Effects of *Peptostreptococcus micros* infection of PRD fibroblast cultures upon cytokine expression. Fibroblast cultures were stimulated with *P. micros* for a variety of time points as indicated and supernatant collected and analysed by ELISA for IL-6, IL-8, OPG and IL-17A. (A) The mean concentration of each cytokine after infection of fibroblast cultures with *P. micros*. (B) The percentage change in culture supernatant cytokine concentration between *P. micros* stimulated and unstimulated paired control fibroblast cultures. Vertical lines represent the standard error of the mean. Figures are representative of three separate experiments.

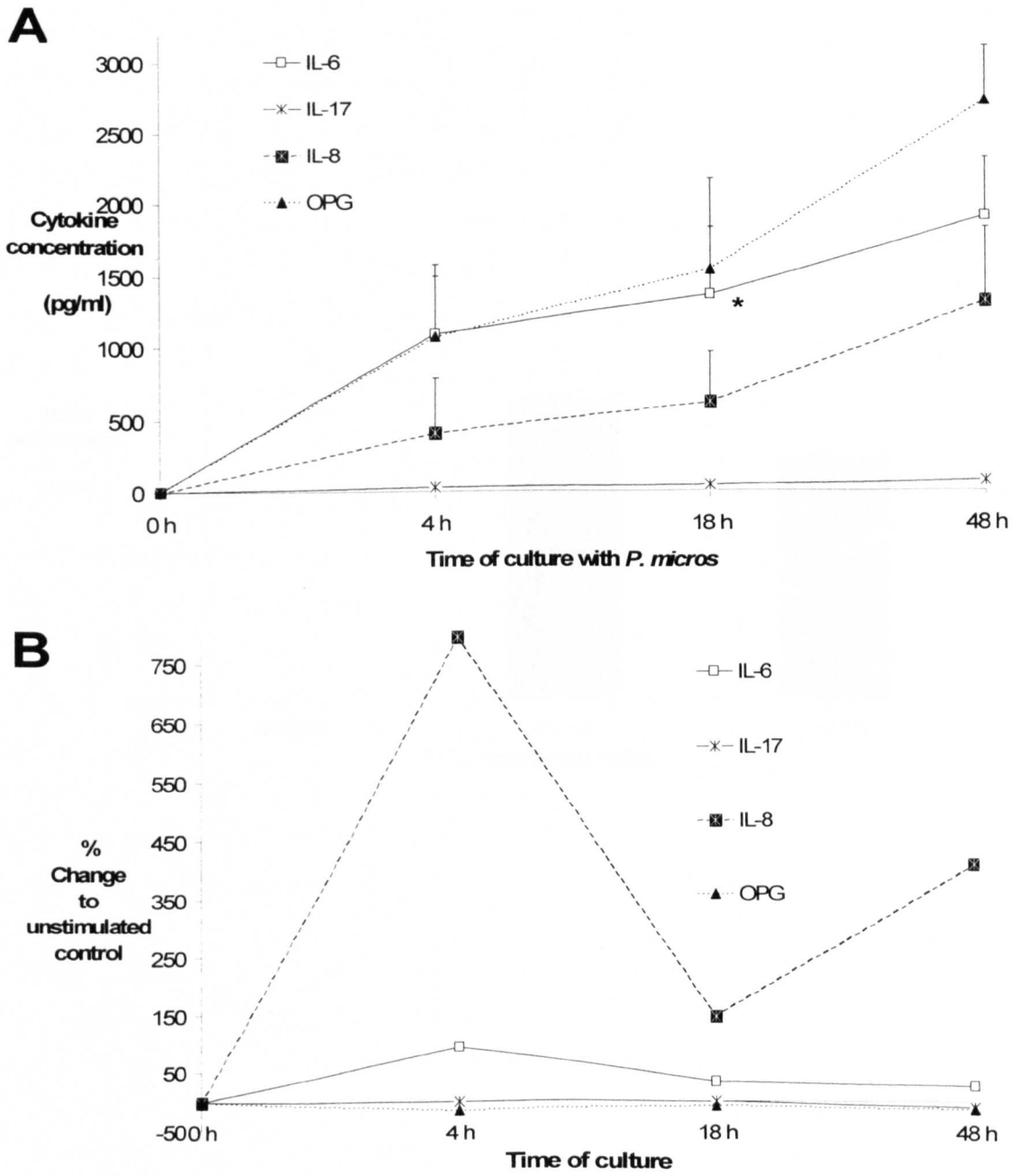
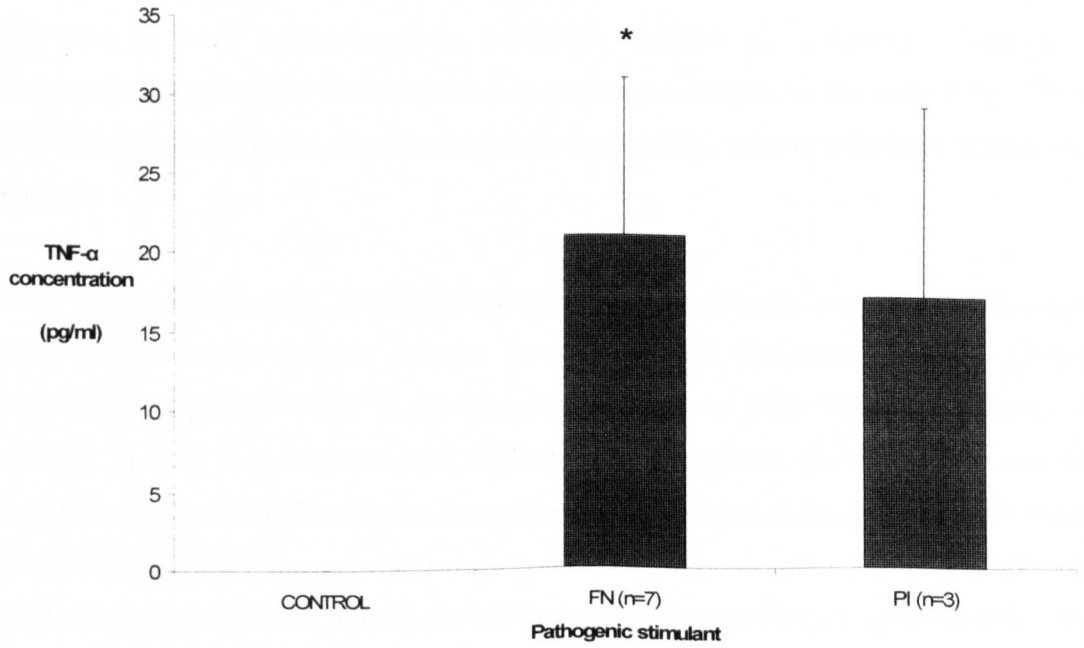


Figure 8.7 Effects of bacterial infection of PRD fibroblast cultures upon TNF-α expression. Fibroblast cultures were stimulated with live endodontic pathogens for 18 h and supernatant collected and analysed by ELISA for TNF-α. The mean concentration of TNF-α after stimulation of fibroblast cultures with endodontic pathogens compared with unstimulated controls. The number of PRD fibroblasts derived from separate patients used in experiments for the measurement of TNF-α is given in parenthesis. Bars represent the mean concentration for TNF-α and the standard error of the mean. FN- *F. nucleatum*, PI- *P. intermedia.*



8.5 IL-18 biology within PRD fibroblast cultures

From the previous series of experiments, I ascertained that cytokine expression within PRD fibroblast cultures could be manipulated by the addition of either live micro-organisms or microbial-derived agents. Furthermore, investigations undertaken in chapter 5 established that cytokine secretion within the novel PRD tissue explant system could be regulated by the addition of rhIL-18 to cultures. It was therefore of interest to elucidate if IL-18-mediated effects within PRD explant cultures could be attributable, at least in part, to stimulation of resident fibroblasts within the PRD lesion. Initially I examined PRD-derived fibroblasts for cellular expression of IL-18, IL-18R and IL-18BP. Subsequently, I explored the effects of IL-18 addition to PRD-derived fibroblast cultures upon endogenous cytokine expression. Recent literature clearly defines fibroblasts as comprising a heterogeneous cell population. This is applicable to fibroblasts from differing tissue sites and even to those originating from individual diseased tissue lesions. Therefore, to investigate the effects of IL-18 upon a representative sample of the potentially diverse PRD fibroblast population, fibroblasts derived from a large number of patient lesions were studied.

From Western blotting, IL-18 protein was not detectable in either pro (24 kDa) or mature form within protein cellular extracts of PRD-derived fibroblasts. Indeed, previous experiments ascertained that IL-18 was not secreted by PRD fibroblast cultures. In contrast, IL-18R was expressed by PRD-derived fibroblasts. IL-18R expression was detectable within 13/16 fibroblast cultures derived from PRD lesions obtained from sixteen patients (Figure 8.8A) and was increased upon LPS addition to cultures (Figure 8.8B). This datum suggested that PRD fibroblasts may be capable of responding to addition of IL-18 to cultures. Interestingly, IL-18BPa was detectable within cellular extracts of 3 of 4 PRD fibroblast populations (Figure 8.8C). The contribution of PRD fibroblast-derived IL-18BPa in regulating IL-18 concentrations within the PRD lesion requires further investigation.

Having established that the IL-18R was present within the fibroblast cultures, I next investigated if PRD fibroblasts were responsive to exogenous IL-18. The addition of IL-18 to 18 h PRD fibroblast cultures resulted in significantly increased supernatant levels of IL-6 ($p=0.008$) and IL-8 ($p=0.002$) compared with unstimulated control cultures. Conversely, IL-18 had little effect upon IL-17A or OPG expression (Figure 8.9A). The responsiveness of PRD fibroblasts to IL-18 contrasts with studies upon fibroblast populations derived from

other chronic inflammatory diseased tissues. Several of these investigations have determined fibroblast cultures to be unresponsive to IL-18. Therefore, I investigated the specificity of IL-18-mediated effects upon IL-6 and IL-8 expression. A neutralising antibody to IL-18 was added concomitantly with rhIL-18 to 18 h PRD fibroblast cultures. Indeed, neutralisation of IL-18 with anti-IL-18 abrogated previously observed fibroblast responses, confirming specificity of IL-18-induced cytokine expression (Figure 8.9B)

The addition of IL-18 in combination with other cytokines to the PRD explant culture system synergistically enhanced the expression of several proinflammatory mediators. Therefore, I also explored if the concomitant addition of IL-18 and other cytokines to PRD fibroblast cultures exerted synergistic effects upon endogenous cytokine expression. Cell culture supernatants were harvested at 18 h and cytokine concentrations measured by ELISA.

IL-18 in combination with TNF-α ($p=0.04$) and IL-17A ($p=0.02$) had additive effects upon IL-6 secretion from 18 h PRD fibroblast cultures compared to stimulation with IL-18 alone (Figure 8.10A). No additive or synergistic effects upon IL-6 expression were observed when IL-18 was added to fibroblast cultures with IL-12 or IL-15. IL-18 in combination with IL-12 ($p=0.02$), IL-15 ($p=0.013$), IL-17A ($p=0.039$) and TNF-α ($p=0.04$) had a significant additive effect upon IL-8 expression within 18 h PRD fibroblast cultures compared with IL-18 stimulation alone (Figure 8.10B).

Figure 8.8 Presence of IL-18R and IL-18BP within PRD fibroblast cultures. Western blot to determine IL-18R expression by PRD fibroblasts. (A) Bands of 55 kDa were present in 10/14 cultures, protein extracts from two representative fibroblast populations are shown. (B) LPS addition to PRD fibroblast cultures increased IL-18R expression. Figure is representative of four repeated experiments upon four differing PRD fibroblast populations. (C) IL-18BPa detected within PRD fibroblast cellular protein extracts, representative of two repeated experiments upon four separate PRD fibroblast populations.

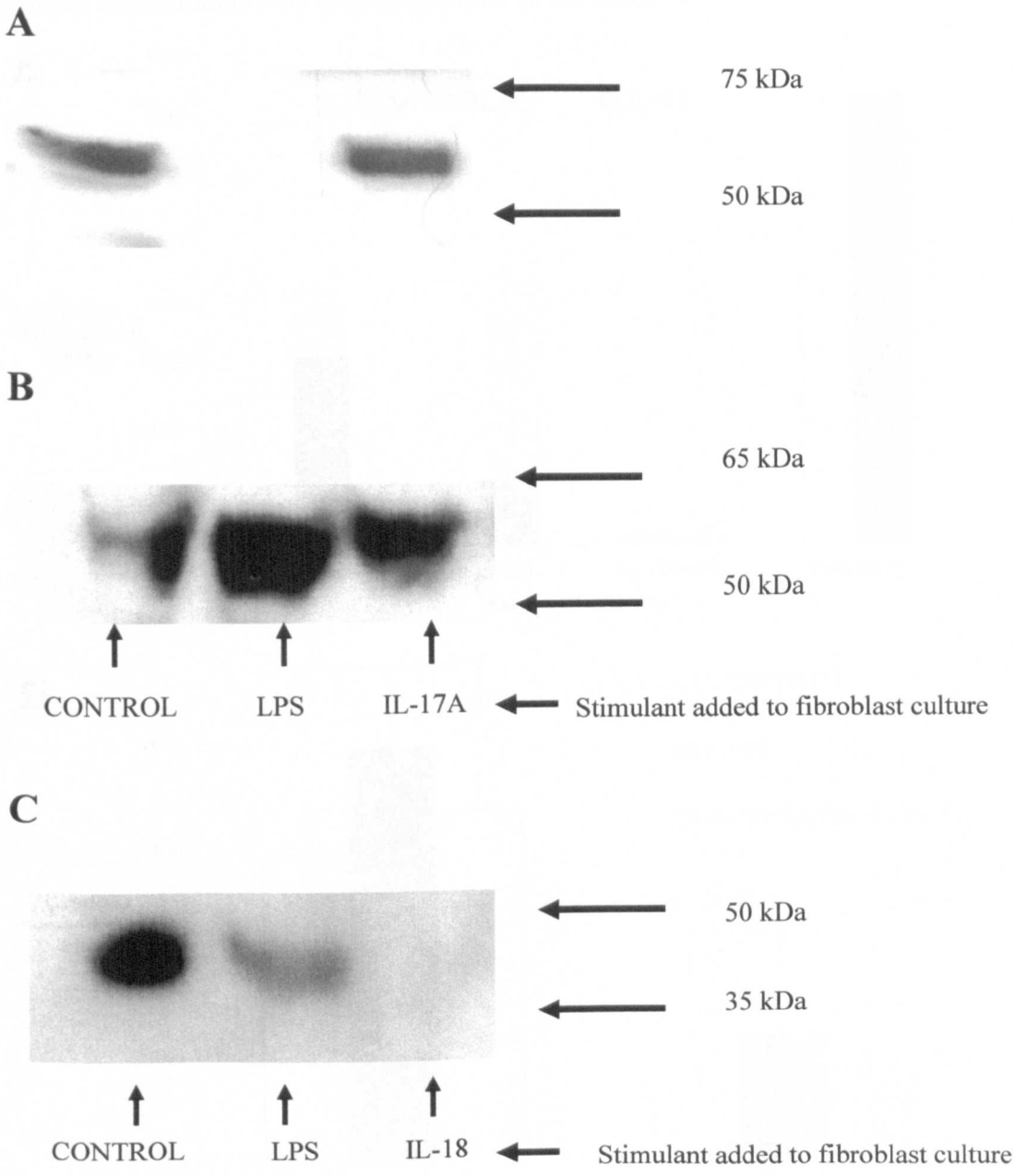


Figure 8.9 ***In vitro*** **effects of IL-18 addition to PRD fibroblast cultures upon endogenous cytokine expression.** PRD fibroblast cultures were stimulated with neutralising anti-IL-18 and/or rhIL-18 for 18 h and supernatant collected and analysed for cytokine expression by ELISA. (A) The mean concentration of fibroblast derived cytokines after 18 h IL-18 stimulation compared with unstimulated control on a large number of PRD fibroblasts. (B) Specificity of IL-18-mediated effects upon IL-6 and IL-8 expression within 18 h fibroblast cultures. The total number of fibroblast cultures derived from separate PRD lesions stimulated with each cytokine is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.

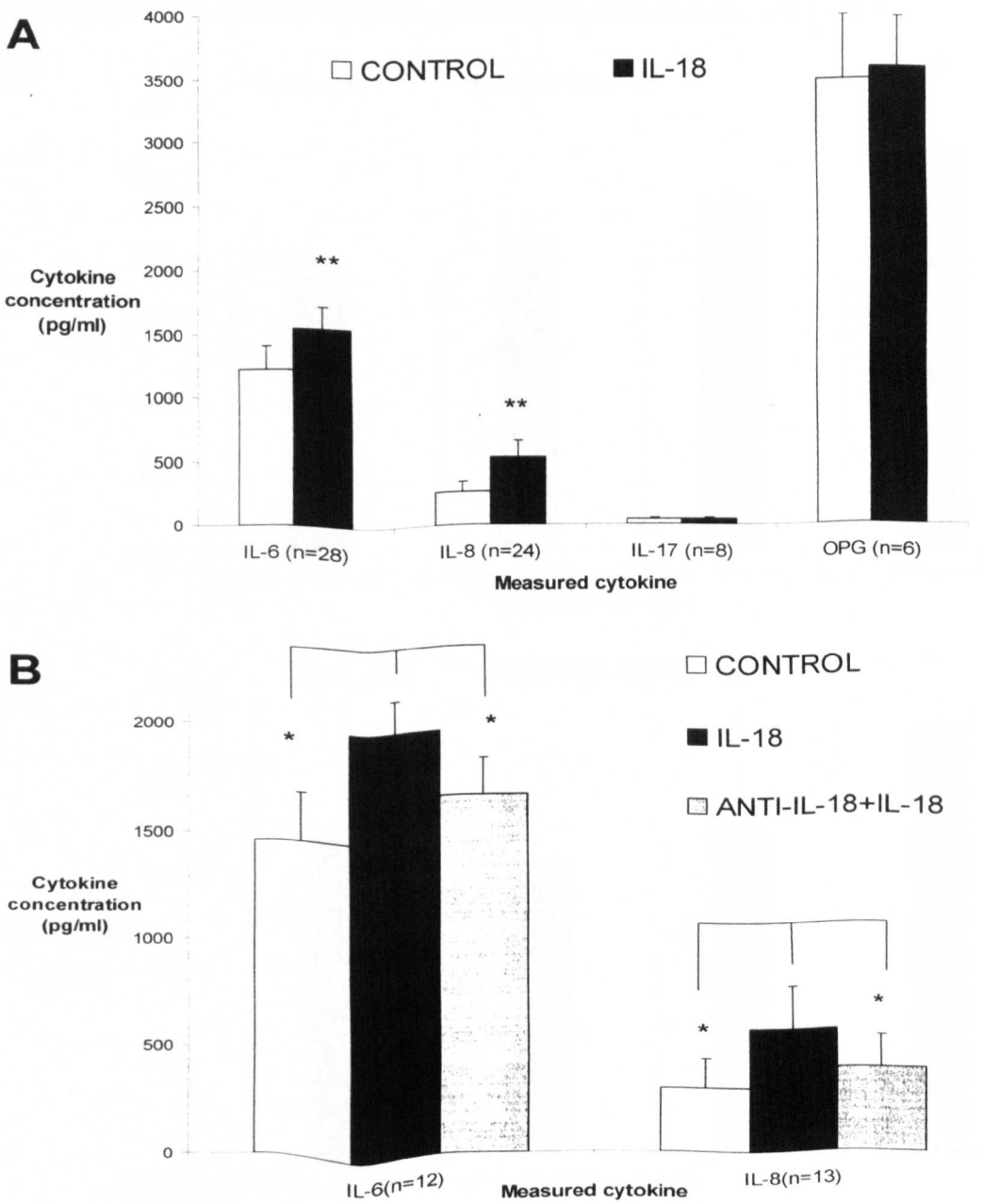
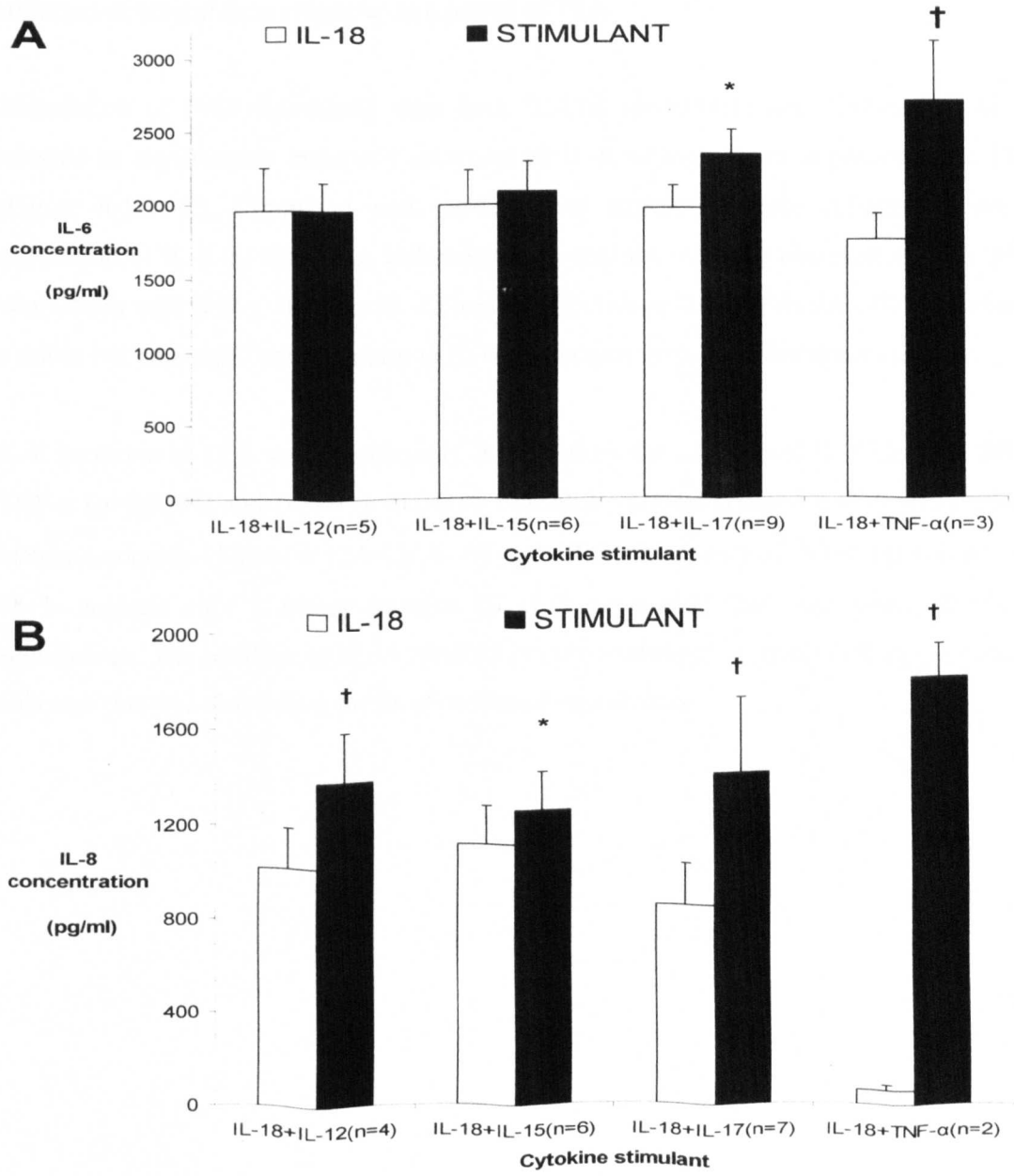


Figure 8.10 ***In vitro*** **effects of IL-18 in combination with other cytokines upon endogenous cytokine expression within PRD fibroblast cultures.** PRD fibroblast cultures were stimulated with IL-18 and another cytokine and supernatant collected and analysed for endogenous cytokine expression by ELISA. (A) The mean concentration of IL-6 after 18 h stimulation of PRD fibroblast cultures by IL-18 concomitantly with other cytokines in comparison with IL-18 alone. (B) The mean concentration of IL-8 after 18 h stimulation of fibroblast cultures by IL-18 concomitantly with other cytokines compared with matched cultures with IL-18 alone. The total number of different patient PRD fibroblast cultures stimulated with each cytokine combination is given in parenthesis. Bars represent the mean cytokine concentration with the standard error of the mean.



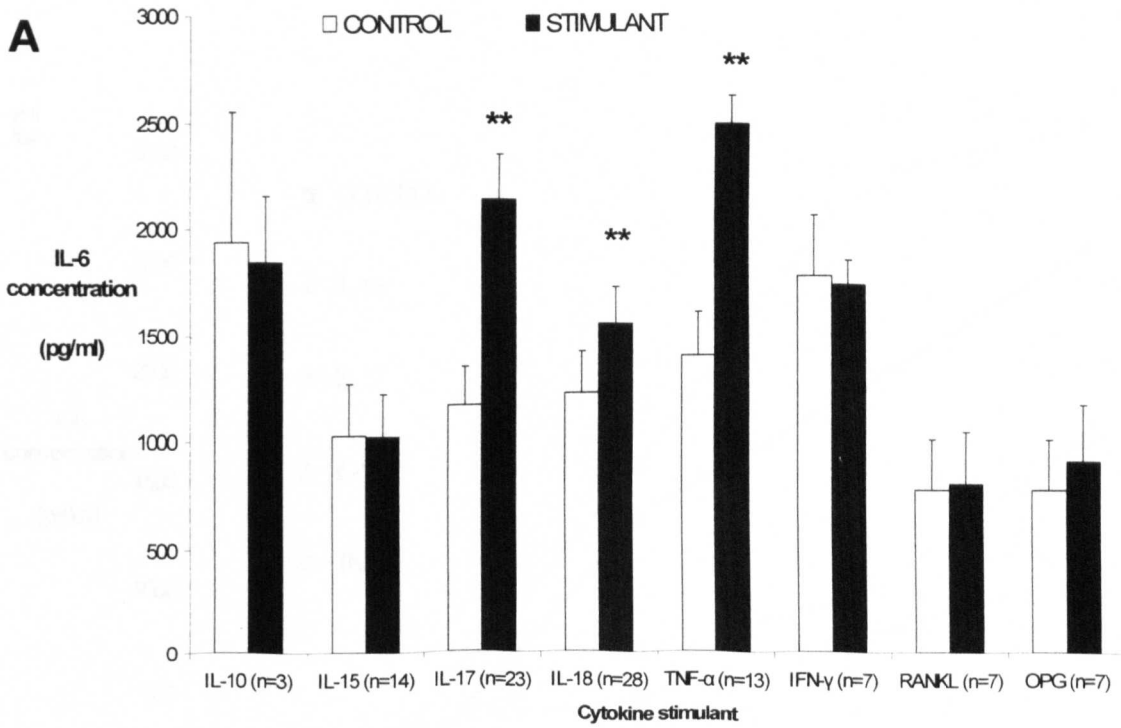
8.6 *In vitro* effects of exogenous cytokines upon PRD fibroblast IL-6 and IL-8 expression

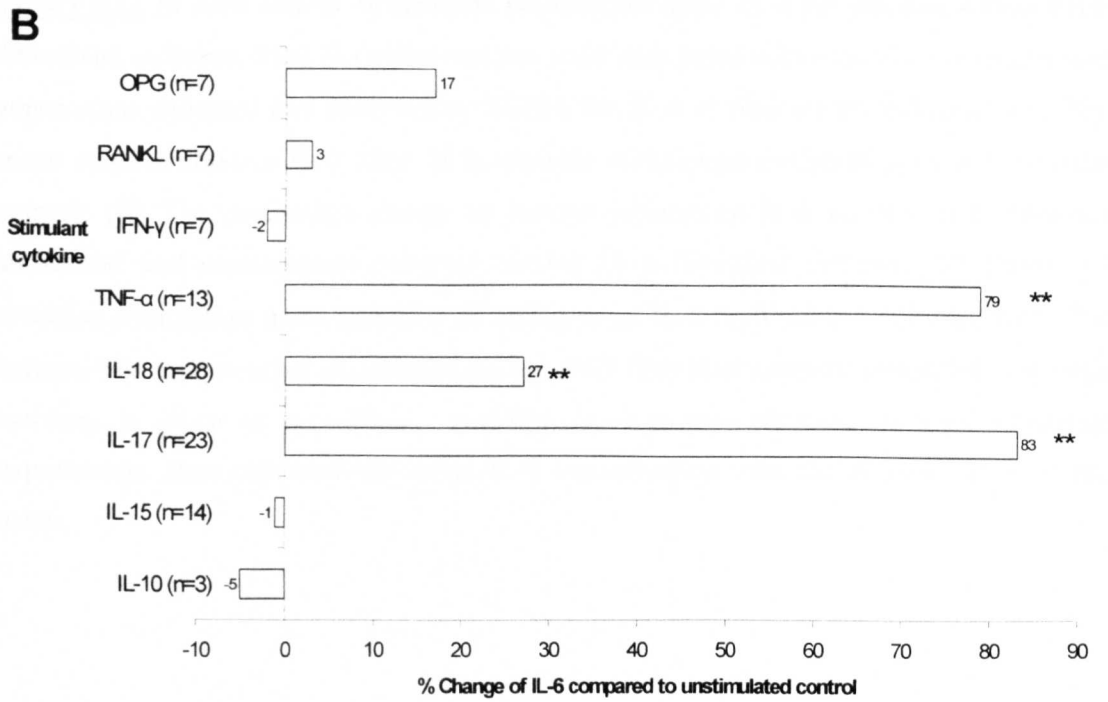
The previous experiments established that through detectable changes in expression of secreted cytokines, PRD fibroblast cultures were responsive to infection by endodontic pathogens and the addition of exogenous IL-18. Cytokine regulatory loops likely influence the chronicity of disease processes. Having established that IL-18 could modulate cytokine expression within PRD fibroblast cultures, I further investigated if endogenous cytokine responses within PRD fibroblast biology could be modulated by the addition of other exogenous cytokines. Cytokines were added to fibroblast cell cultures and supernatant collected at several time points up to a period of 72 h.

Stimulation of PRD fibroblasts with both IL-17A ($p=0.0001$) and TNF-α ($p=0.0001$) resulted in significantly enhanced secretion of IL-6 within culture supernatants at 18 h (Figure 8.11A-C). Compared with unstimulated matched control cultures, increased expression of IL-6 by these two inflammatory cytokines was also observed at 72 h. 18 h stimulation with IFN-γ, IL-10 or IL-15 had no effect upon IL-6 expression. OPG promoted a minor but non-significant increase in IL-6 production from PRD fibroblast cultures.

IL-8 secretion at 18 h was significantly increased by the addition of IL-17A ($p=0.0080$), TNF-α ($p=0.0001$) and IFN-γ ($p=0.04$) to fibroblast cultures compared with unstimulated matched controls (Figure 8.12A-C). IL-10 and OPG stimulation of fibroblast cultures for 18 h induced only a minor increase in IL-8 expression that was below levels of significance. The addition of IL-15 resulted in only a minimal decrease in IL-8 expression, although this was also below levels of statistical significance.

Figure 8.11 ***In vitro*** **effects of cytokine stimulation upon IL-6 production within PRD fibroblast cultures.** PRD fibroblast cultures were stimulated with a panel of cytokines and supernatant collected and analysed by ELISA for IL-6 at time points indicated. (A) The mean concentration of IL-6 after 18 h cytokine stimulation compared with unstimulated control. (B) The percentage change in IL-6 culture supernatant concentration between stimulated and unstimulated matched control 18 h fibroblast cultures. (C) Effects of cytokine stimulation upon secretion of endogenous IL-6 in fibroblast cultures over 72 h culture. The total number of different patient PRD fibroblast cultures stimulated with each cytokine is given in parenthesis. Timeline chart is representative of three fibroblast experiments. Bars represent the mean IL-6 concentration with the standard error of the mean.




B
OPG (n=7)
RANKL (n=7)
IFN-γ (n=7)
TNF-α (n=13)
IL-18 (n=28)
IL-17 (n=23)
IL-15 (n=14)
IL-10 (n=3)
Stimulant cytokine
17
3
-2
79
27
83
-1
-5
**
**
-10
0
10
20
30
40
50
60
70
80
90
% Change of IL-6 compared to unstimulated control

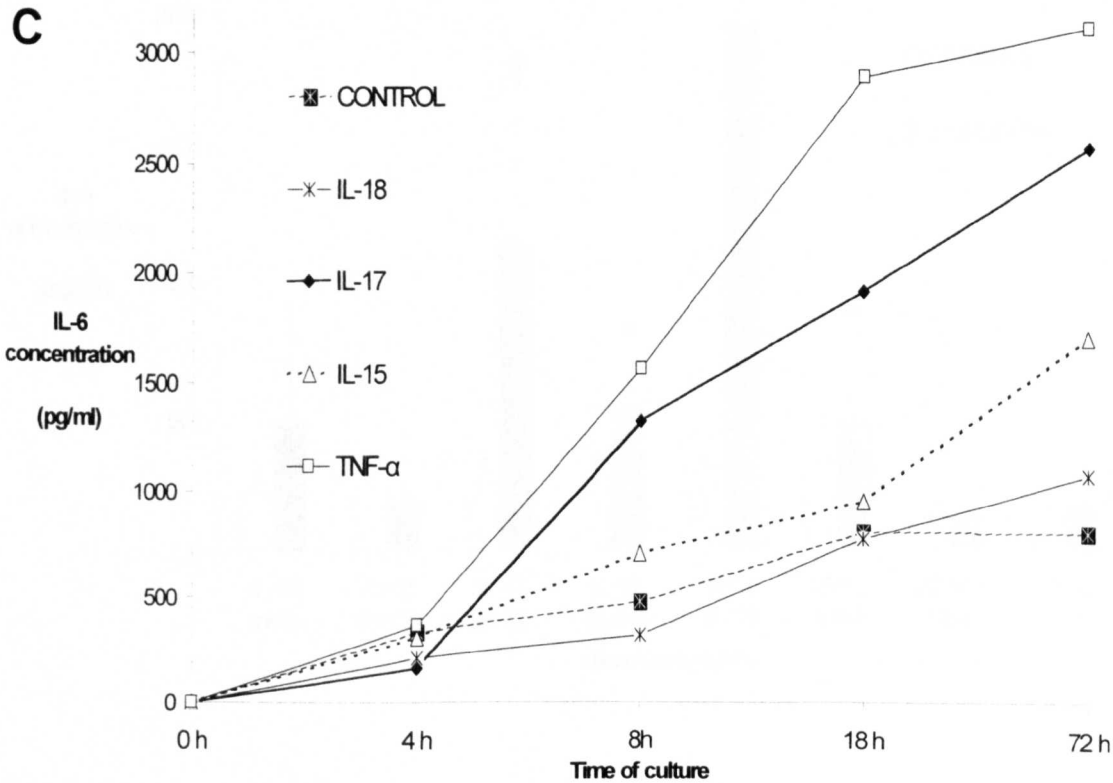
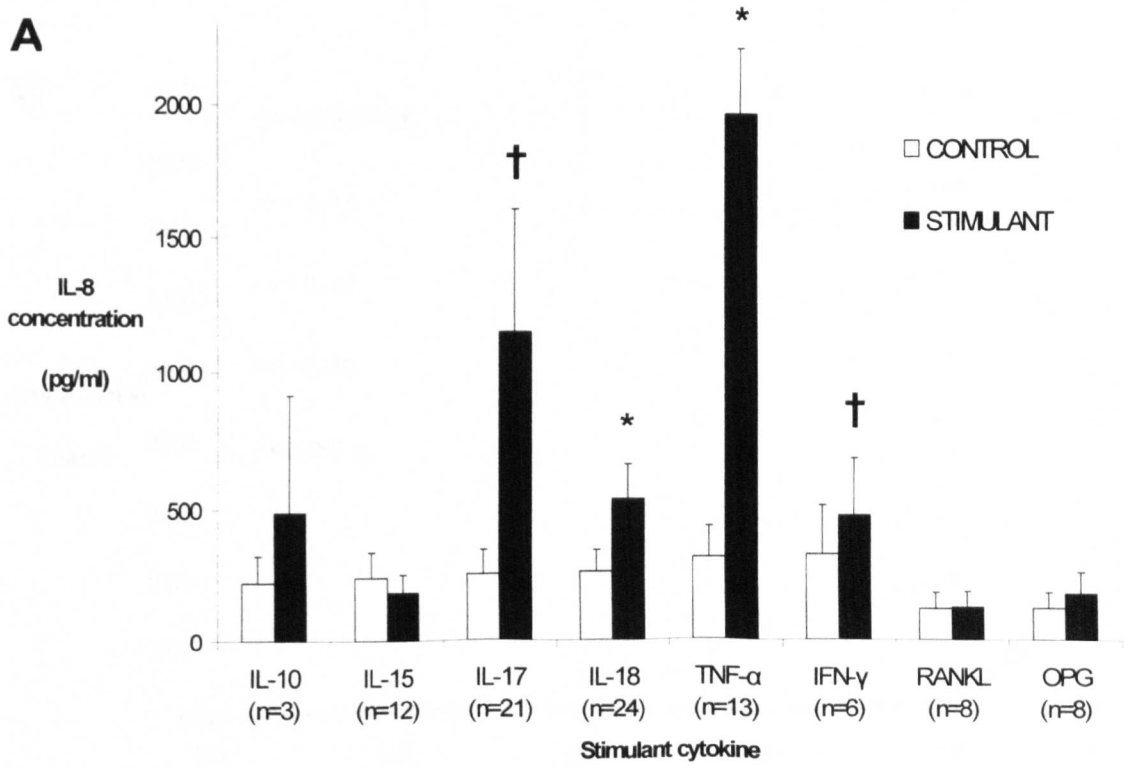
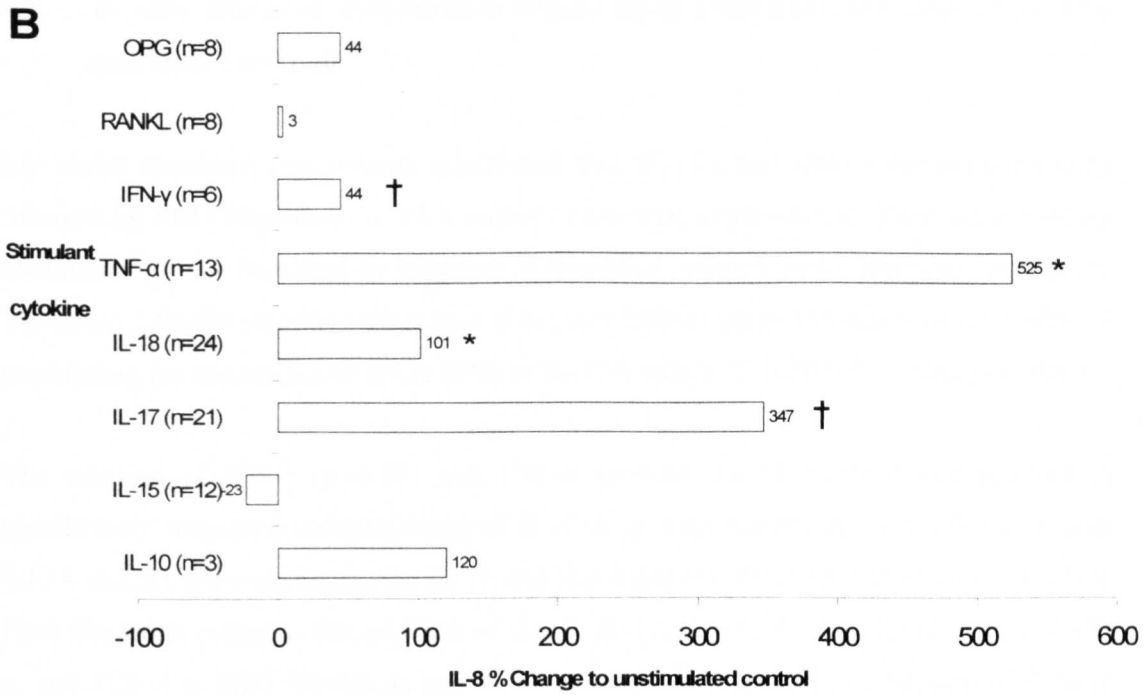
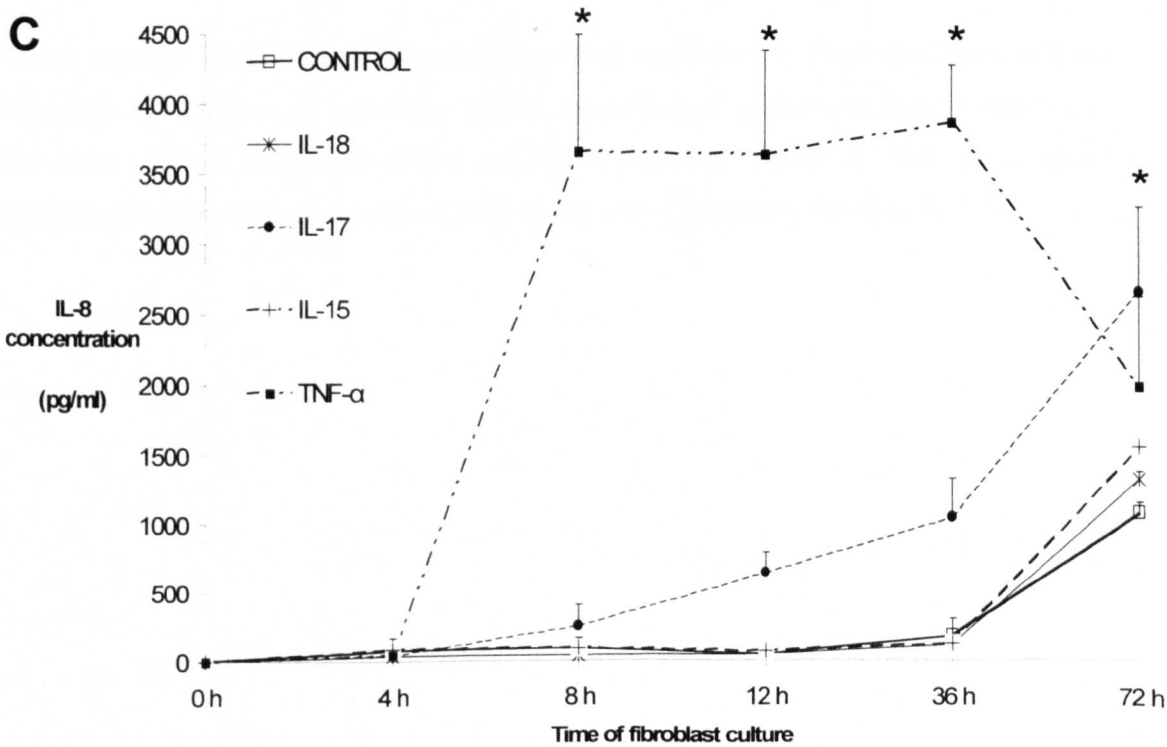

C
3000
2500
2000
1500
1000
500
0
IL-6 concentration (pg/ml)
CONTROL
IL-18
IL-17
IL-15
TNF-α
0 h
4 h
8h
18 h
72 h
Time of culture

Figure 8.12 ***In vitro*** **effects of cytokine stimulation upon IL-8 production within PRD fibroblast cultures.** PRD fibroblast cultures were stimulated with a panel of cytokines and supernatant collected and analysed by ELISA for IL-8 at time points indicated. (A) The mean concentration of IL-8 after 18 h cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IL-8 concentration between stimulated and unstimulated matched control 18 h fibroblast cultures. (C) Effects of cytokine stimulation upon secretion of endogenous IL-8 in fibroblast cultures over 72 h culture. The total number of different patient PRD fibroblast cultures stimulated with each cytokine is given in parenthesis. Timeline chart is representative of three fibroblast experiments. Bars represent the mean IL-8 concentration with the standard error of the mean.




B
OPG (n=8)
44
RANKL (n=8)
3
IFN-γ (n=6)
44
†
Stimulant
TNF-α (n=13)
525
*
cytokine
IL-18 (n=24)
101
*
IL-17 (n=21)
347
†
IL-15 (n=12)
23
IL-10 (n=3)
120
-100
0
100
200
300
400
500
600
IL-8 % Change to unstimulated control


C
4500
4000
3500
3000
2500
2000
1500
1000
500
0
CONTROL
IL-18
IL-17
IL-15
TNF-α
IL-8
concentration
(pg/ml)
*
*
*
*
0 h
4 h
8 h
12 h
36 h
72 h
Time of fibroblast culture

8.7 *In vitro* effects of exogenous cytokines upon PRD fibroblast-derived IL-17A and OPG secretion

My initial fibroblast experiments established that IL-17A and OPG were spontaneously released by PRD fibroblasts at 18 h culture. However, expression of these inflammatory mediators was not regulated by infection of fibroblast cultures with endodontic pathogens. Therefore, I finally sought to determine if any key inflammatory cytokines were capable of modulating the expression of either OPG or IL-17A within 18 h PRD fibroblast cultures.

The addition of IFN-γ ($p=0.01$) and TNF-α ($p=0.04$) to PRD fibroblasts resulted in significantly increased concentrations of IL-17A in supernatants at 18 h culture (Figure 8.13A and B). Conversely, IL-12, IL-15 and IL-18 had no effect upon IL-17A secretion in PRD fibroblast cultures. The addition of IL-10, IL-12, IL-15, IL-17A, IL-18, IFN-γ, TNF-α, and TGF-β to PRD fibroblasts had no observable effect upon the expression of OPG at 18 h culture (Figure 8.14A and B).

Taken together, these data demonstrate that OPG expression by PRD fibroblasts was not inducible by addition of microbial agents or individual proinflammatory cytokines to fibroblast cultures. In contrast, IFN-γ and TNF-α were both capable of inducing increased expression within PRD fibroblast cultures of the proinflammatory cytokine IL-17A.

Figure 8.13 ***In vitro*** **effects of cytokine stimulation upon IL-17A production within PRD fibroblast cultures.** PRD fibroblast cultures were stimulated with a panel of cytokines and supernatant collected and analysed by ELISA for IL-17A at 18 h. (A) The mean concentration of IL-17 after 18 h cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant IL-17A concentration between stimulated and unstimulated matched control 18 h fibroblast cultures. The total number of different patient PRD fibroblast cultures stimulated with each cytokine is given in parenthesis. Bars represent the mean IL-17A concentration with the standard error of the mean.

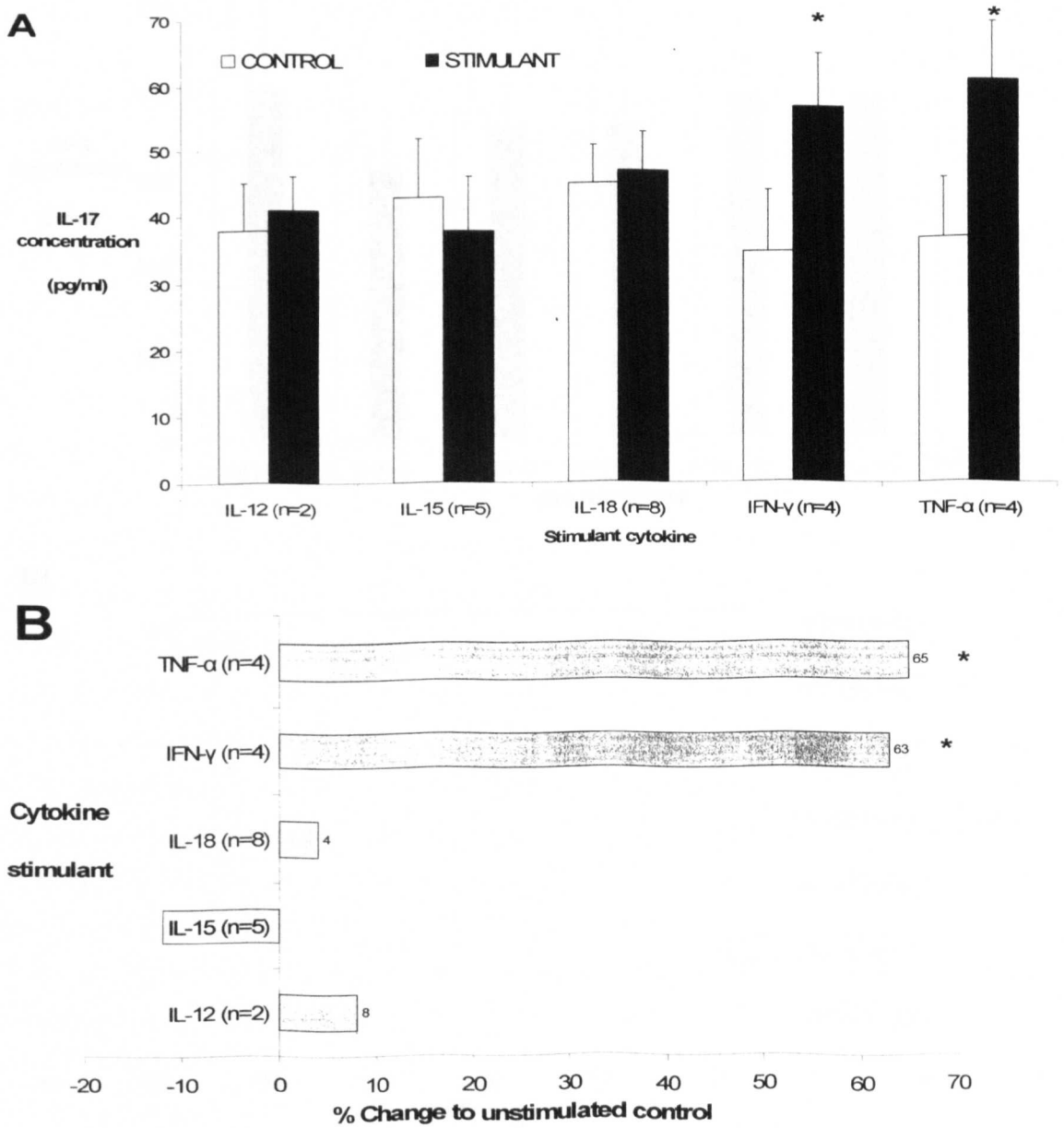
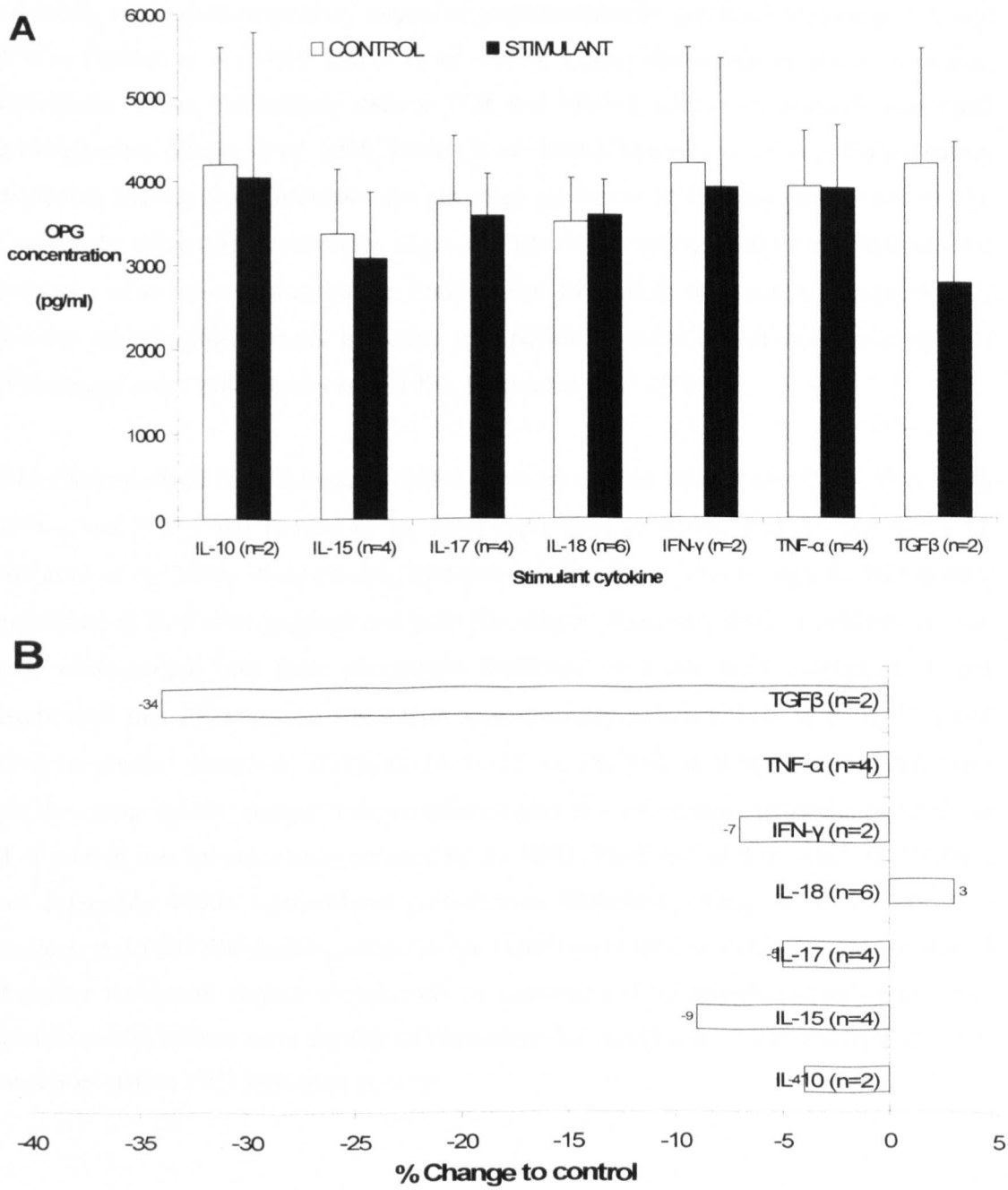


Figure 8.14 ***In vitro*** **effects of cytokine stimulation upon OPG production within PRD fibroblast cultures.** PRD fibroblast cultures were stimulated with a panel of cytokines and supernatant collected and analysed by ELISA for OPG at 18 h. (A) The mean concentration of OPG after 18 h cytokine stimulation compared with unstimulated control. (B) The percentage change in culture supernatant OPG concentration between stimulated and unstimulated matched control 18 h fibroblast cultures. The total number of different patient PRD fibroblast cultures stimulated with each cytokine is given in parenthesis. Bars represent the mean OPG concentration with the standard error of the mean.



8.8 Discussion

8.8.1 Introduction

IL-1 and/ or TNF-α expression at sites of infection by monocytes and macrophages followed by activation of resident tissue cells, including epithelial cells and fibroblasts, triggers an inflammatory cytokine cascade. In turn, this network amplifies or suppresses inflammation by inducing further release of cytokines and growth factors. Fibroblasts were originally recognised as primary targets of proinflammatory cytokines including IL-1 and TNF-α (Butler *et al.* 1994, Dayer *et al.* 1977). Upon stimulation by these cytokines, fibroblasts secrete chemokines such as IL-8 and MCP-1, matrix metalloproteinases and prostaglandins (Dayer *et al.* 1986, Larsen *et al.* 1989). The release of such inflammatory mediators implies that fibroblasts are not mere producers of the structural tissue matrix. Conversely, these data establish the concept of fibroblasts acting as effector cells in disease processes of an inflammatory nature. Furthermore, fibroblasts are reported to express IL-1, thereby contributing towards autocrine and paracrine activation of neighbouring cells (Fuhlbrigge *et al.* 1988, Sporri *et al.* 1996, Rezzonico *et al.* 1998).

Fibroblasts derived from human periradicular cysts express mRNA for IL-1β, IL-6, IL-8, TNF-α and IFN-γ and limited or no gene expression of IL-10, TGF-β1 and GM-CSF (Kusumi *et al.* 2004). Furthermore, cyst-derived fibroblasts secrete significantly greater quantities of IL-6 than gingival and pulp fibroblasts. However, PRD fibroblasts are not well characterised and their phenotypic attributes have not been elucidated. I first established that PRD-derived fibroblasts spontaneously secreted IL-6, IL-8, IL-17A and OPG in culture. However, IL-1β, IL-10, IL-12, IL-18, TNF-α, IFN-γ and RANKL were not detectable within "resting" culture supernatants. It is of interest that moderate levels of IL-8 protein was spontaneously secreted by the PRD fibroblasts as IL-8 gene expression is not detectable within unstimulated pulp-derived fibroblasts (Yang *et al.* 2003a). This suggests that PRD fibroblasts cultured in my experiments were in a highly activated state. I therefore undertook further experiments to determine if microbial-derived agents, live microbes or cytokines were capable of regulating the expression of secreted inflammatory mediators within PRD fibroblast cultures.

8.8.2 Effects of microbes and microbial products upon PRD fibroblasts

Live periodontal pathogens (Steffen *et al.* 2000) or their microbial products (Steffen *et al.* 2000, Imatani *et al.* 2001) modulate IL-8 expression within gingival fibroblast cultures. Furthermore, infection of human pulp fibroblast cultures with the endodontic pathogens *Porphyromonas gingivalis*, *P. intermedia* or *Porphyromonas endodontalis*, increases IL-8 mRNA expression (Yang *et al.* 2003a). IL-8 gene expression within *P. endodontalis* infected pulp fibroblasts gradually increases after 2, 6 and 24 h culture. In contrast, subsequent to *P. intermedia* infection, pulp fibroblast gene expression for IL-8 reaches maximal levels at 2 h and declines to baseline levels between 6 and 24 h. The reason for differences in achieving maximal IL-8 gene expression by these differing microbial pathogens are likely related to differences in lipid A and polysaccharide structures (Yang *et al.* 2003a).

Despite these observations, no published studies report the effects of endodontic pathogens or microbial components upon secreted IL-8 expression within human PRD fibroblast cultures. Similar to studies investigating fibroblast populations originating from other oral sources, PRD fibroblast cultures were responsive to *E. coli*-derived LPS and live microbial pathogens. LPS induced a significant increase in concentrations of secreted IL-8 at 18 h PRD fibroblast culture. Paralleling gene expression observed within pulp fibroblast cultures (Yang *et al.* 2003a), PRD fibroblasts cultured with *P. intermedia* exhibited maximal IL-8 protein expression at an early stage of infection. This early inducible effect upon IL-8 secretion was also observed in fibroblast cultures infected with *F. nucleatum* and *P. micros*. However, only *F. nucleatum* induced significantly increased levels of secreted IL-8 to 48 h fibroblast culture.

In addition to IL-8, LPS derived from *P. gingivalis*, *Actinobacillus actinomytemcomitans* and *E. coli* increase IL-6 secretion from fibroblasts derived from healthy gingival tissue (Imatani *et al.* 2001). Furthermore, IL-1α, TNF-α and *Bacteroides spp.* up-regulate IL-6 mRNA expression within human pulp fibroblast cultures (Yang *et al.* 2003b). Infection of gingival fibroblast cultures with *A. actinomytemcomitans* increases IL-6, IL-1β and TNF-α mRNA although only secreted IL-6 protein is detectable within culture supernatants (Belibasakis *et al.* 2005a). The addition of *P. intermedia*-derived LPS to human dental pulp fibroblasts increases IL-6 mRNA expression by 1 h and this is sustained to 8 h culture. Interestingly, this effect is inhibited by antibodies to CD14 and reduced by 50% by the

addition of IFN-γ, IL-10 and/or IL-4 (Tokuda *et al.* 2001). Endodontic pathogens derived from infected root canal and cyst exudates also increase IL-6 expression within periodontal ligament fibroblast cultures (Ogura *et al.* 1994). In consideration of these data, it is surprising that no studies have evaluated the effects of microbial agents or endodontic pathogens upon secreted IL-6 within PRD-derived fibroblast cultures.

I established that *E. coli*-derived LPS and live endodontic pathogens were capable of modulating IL-6 protein expression within cultures of PRD fibroblasts. At 18 h culture, *E. coli*-derived LPS induced a significant increase in secreted IL-6 protein within PRD fibroblast supernatants. Furthermore, PRD fibroblasts infected with *F. nucleatum* significantly increased their secretion of IL-6 at all time points to 48 h culture. *P. intermedia* induced maximal IL-6 protein expression at 18 h culture and significantly increased IL-6 concentrations were further detected after 48 h infection. The maximal effect upon increased IL-6 expression at 18 h was also observed in PRD fibroblast cultures infected with *P. micros.*

In whole blood culture experiments, Gram-negative endodontic pathogens induce TNF-α more strongly than Gram-positive bacteria (Matsushita *et al.* 1998). Furthermore, Gram-positive bacterial cell fragments stimulate less cytokine release from human PBMC cultures than LPS (Safavi and Nichols 2000). *Yersinia enterocolitica* infection of synovial fibroblast cultures transiently induces TNF-α mRNA expression at 4 h and 24 h although this does not translate into secreted TNF-α protein (Meyer-Bahlburg *et al.* 2004). The addition of *E. coli*-derived LPS to PRD fibroblast cultures did not induce TNF-α expression. Interestingly, infection of PRD fibroblasts by both Gram-negative endodontic pathogens, *P. intermedia* and *F. nucleatum*, resulted in low but detectable levels of secreted TNF-α. In contrast, *P. micros*, a Gram-positive microbe, did not induce detectable levels of TNF-α from the PRD fibroblast cultures. Addition of *F. nucleatum*-derived LPS to human pulp cell cultures increases production of IL-1β in a dose dependent manner that is reduced by IL-1ra (Lu *et al.* 2002). However, I was not able to detect secreted IL-1 protein within resting PRD fibroblast cultures and neither was it inducible by microbial agents.

In contrast to *Streptococcus mutans*, the endodontic pathogen *P. endodontalis* behaves as a weak stimulator of inflammatory cytokines, and has little effect upon the production of IFN-γ and TNF-α from mononuclear cells. However, it is also less able to induce IL-10.

Through its limited stimulatory effects upon host cytokine expression, it is postulated that *P. endodontalis* may escape the immune response mounted by leukocytes and thereby contribute towards bone destruction occurring at PRD sites (Jiang and Schilder 2002). Infection of fibroblast cultures with *Bacteroides spp.* results in increased mRNA expression and/ or secretion of MMPs (Chang *et al.* 2002a,b), and tissue plasminogen activator (Yang *et al.* 2003b). Furthermore, the periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis* induce MMP-2 secretion from human PDLF, which is counter-regulated by TGF-β (Chang *et al.* 2002a). These experiments define an important role for fibroblasts in promoting tissue destructive pathways. Further investigations are therefore required to elucidate if PRD-derived fibroblasts spontaneously express MMPs and thereafter, examine the effects of endodontic pathogens upon MMP expression by PRD fibroblasts.

Infection of PRD fibroblast cultures with endodontic pathogens had no effect upon expression of secreted OPG, which is also the case with pathogenic challenged SFL cultures (Zhang *et al.* 2004). Conversely, the addition of *E. coli*-derived LPS to PDL fibroblasts stimulates OPG expression through inducing TNF-α and IL-1β (Wada *et al.* 2004). Interestingly, bacterial infection of fibroblasts co-cultured with osteoclast precursors up-regulates RANKL expression and they become competent in promoting the maturation of osteoclast precursors into multinucleated TRAP positive bone-resorbing multinucleated cells (Zhang *et al.* 2004). Furthermore, *A. actinomytemcomitans* induces RANKL gene expression in human gingival fibroblast cultures through a pathway independent of IL-1, IL-6, TNF-α or PGE_2 (Belibasakis *et al.* 2005a). *E. coli*-derived LPS also stimulates RANKL expression in PDL fibroblasts through the induction of TNF-α and IL-1β (Wada *et al.* 2004). Although I did not analyse RANKL mRNA expression, secreted RANKL was not detected within the supernatant of PRD fibroblast cultures either at rest or after the addition of microbial agents or infection with live microbes. Furthermore, RANKL protein was not detected within fibroblast cellular lysates by Western blotting. In addition to increasing OPG secretion by FLS, IL-4 suppresses RANKL mRNA and protein expression (Lee *et al.* 2004b). The lack of detectable IL-4 within PRD explants combined with the inability of endodontic pathogens to increase OPG expression from PRD fibroblasts are likely contributing factors promoting inflammatory bone destruction during development of PRD.

8.8.3 Effects of cytokines on PRD fibroblasts

Synovial fibroblasts express IL-18 protein and IL-18 mRNA (Möller *et al.* 2001) and release IL-18 in culture (Schuler and Aicher 2004). In contrast, others have been unable to detect IL-1β, IL-15 and IL-18 protein within FLS cultures, although high levels of IL-6 are secreted (Hirth *et al.* 2002). Gingival and dermal fibroblasts express IL-1β mRNA and ICE, however LPS does not induce IL-18 secretion and induces only low levels of IL-1β (Tardif *et al.* 2004). I was unable to detect secreted IL-18 or IL-1β within PRD fibroblast cultures. In addition, Western blotting revealed a lack of constitutive IL-18 protein expression and IL-18 was not induced by infection of PRD fibroblast cultures with endodontic pathogens.

IL-18 bioactivity is regulated by the expression of IL-18R in various cell types. Fibroblast-like synoviocytes (FLS) constitutively express IL-18Rα mRNA but do not express IL-18Rβ mRNA at rest or upon stimulation with IL-1 or IL-12. Although IL-1β and to a lesser extent IL-12 induce increased IL-6 production from FLS cultures, IL-18 exerts no effects upon IL-6 expression (Kawashima and Miossec 2003, Kawashima *et al.* 2003). Therefore it is postulated that in RA, the inflammatory effects of IL-18 likely occur via T cells and macrophages with no direct action upon FLS (Kawashima and Miossec 2003). In contrast, Morel *et al.* (2002) demonstrated that IL-18 modulates inflammatory responses in RA-derived FLS by up-regulating VCAM expression. I therefore hypothesised that IL-18 might also participate in PRD fibroblast activation. Initially, I established that PRD fibroblasts expressed IL-18R and thereafter, investigated the biological effects of exogenous IL-18 upon long-term PRD fibroblast cultures. From these experiments, I ascertained that cultured PRD fibroblasts were responsive to IL-18. Expression of IL-6 and IL-8 was significantly up-regulated by IL-18 addition to cultures. Of importance, addition of IL-17A or TNF-α with IL-18 additively increased both IL-6 and IL-8 secretion by PRD fibroblasts. I had previously detected substantial levels of secreted IL-17A, IL-18 and TNF-α within the PRD tissue explant culture system. The concomitant release of these cytokines within the PRD lesion may significantly contribute towards PRD fibroblast activation, thereby promoting proinflammatory destructive pathways by these cell types.

IL-17A induces IL-6 and IL-8 expression in synovial fibroblasts (Fossiez *et al.* 1996, Kehlen *et al.* 2002) and increases IL-6 secretion from periodontal fibroblasts in a dose dependent manner (Takahashi *et al.* 2005b). I therefore postulated that IL-17A might

contribute towards PRD fibroblast activation. Importantly, IL-17A increased expression of both IL-6 and IL-8 in PRD fibroblast cultures. IL-17A stimulates greater production of IL-6 and IL-8 than either IFN-γ or IL-15 from SLF cultures (Hwang *et al.* 2004). In agreement with these data, PRD fibroblasts secreted substantially greater quantities of IL-6 and IL-8 after IL-17A addition to cultures than IL-15. IL-17A possesses additive effects with IL-1β upon the secretion of IL-6 and IL-8 from fibroblast-like synoviocytes (Kehlen *et al.* 2003). Further experiments are required to determine if IL-17A exhibits additive or synergistic properties when combined with other inflammatory mediators upon endogenous cytokine expression by PRD fibroblasts.

In addition to the effects of IL-17A upon PRD fibroblasts, it was surprising to find that IL-17A was detectable within these cultures. PRD fibroblast-derived IL-17A was significantly increased by the addition of the inflammatory cytokines TNF-α and IFN-γ to cultures. It is therefore possible that through IL-17A secretion, PRD fibroblasts contribute towards T-cell activation and lesion expansion. IL-17A and TNF-α alone have little effect upon SLF-derived OPG. However, when these mediators are combined with IL-1, OPG production is greatly increased, likely reflecting a negative feedback loop to control bone destruction (Granet *et al.* 2004). I also ascertained that the individual addition of cytokines to PRD fibroblast cultures did not modulate expression of secreted OPG. Whether a combination of cytokines is able to influence OPG expression by PRD fibroblasts requires investigation.

Within pulp cell cultures, IL-1α and TNF-α stimulate IL-6mRNA expression (Yang *et al.* 2003b). The addition of IL-1α to gingival fibroblast cultures decreases TIMP expression whereas TGF-β leads to increased TIMP expression (Yang *et al.* 2002). Furthermore, IL-1β induces greater increases in IL-6 production by PDL fibroblasts than gingival fibroblasts (Shimizu *et al.* 1992). However, no studies have evaluated effects of these inflammatory mediators upon PRD-derived fibroblasts. I established that addition of TNF-α to PRD fibroblast cultures significantly increased concentrations of secreted IL-6, IL-8 and IL-17A. In addition, IFN-γ promoted increased expression of IL-8 and IL-17A within PRD fibroblast cultures.

Certain fibroblast populations secrete IL-10 when challenged with IL-1β or TNF-α and the release of low quantities of TNF-α has been observed within fibroblast populations such as SFLs (Hirth *et al.* 2002). However, Hirth *et al.* (2002) suggest that IL-10 expression detected in FLS experiments may be from macrophage contamination in early passage

cultures. Similarly, TNF-α is predominantly produced by macrophages and its reported production by fibroblast cultures may be a result of culture contamination with other cell types. Nevertheless, secreted IL-10 was not evident within the PRD fibroblast cultures and it was not inducible by infection with live endodontic pathogens or microbial derived moieties. The absence of IL-10 and other monokines suggests that PRD fibroblast cultures were not contaminated with other cell types. However, stimulation of PRD fibroblasts with endodontic pathogens elicited TNF-α production, albeit at low concentrations. Although IL-10R mRNA is not constitutively expressed by human pulp fibroblasts, stimulation with *P. intermedia*-derived LPS induces IL-10R mRNA expression and is inhibited by antibodies to CD14 (Tokuda *et al.* 2003). It is likely that PRD-derived fibroblasts express IL-10R as addition of IL-10 to fibroblast cultures induced a moderate increase in IL-8 expression.

8.8.4 Conclusions

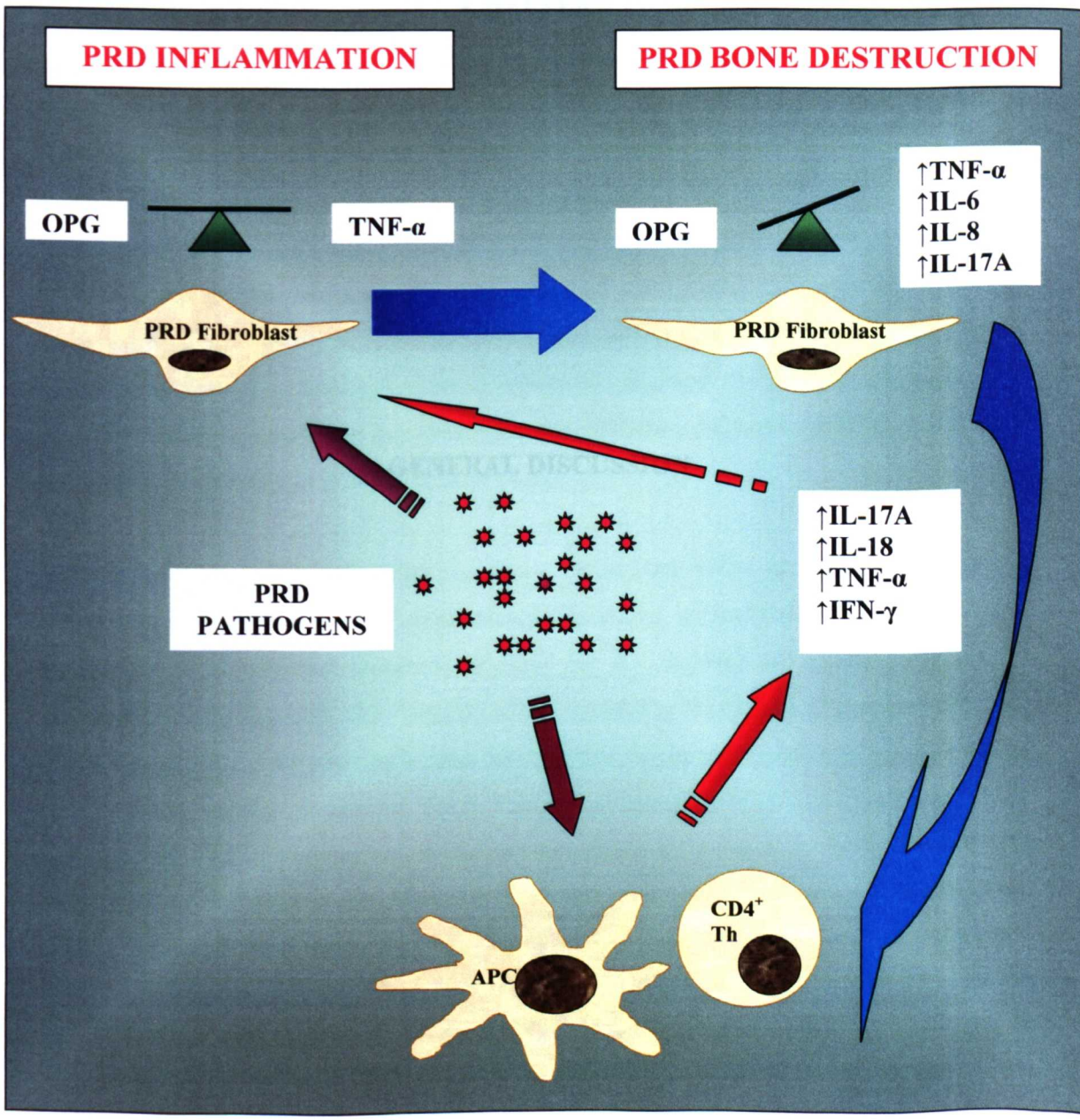
Fibroblasts were traditionally recognised as target cells, orchestrating tissue repair in response to mediators released by monocytes and lymphocytes such as IL-1β or TNF-α. However, recent evidence establishes that through the release of a variety of growth factors, including GMCSF and CSF, fibroblasts are capable of regulating the development and activation of haematopoietic cells and their precursors. Indeed, my experiments clearly demonstrated that PRD fibroblasts were responsive to a wide panel of inflammatory cytokines. Furthermore, upon stimulation with these inflammatory mediators, PRD fibroblasts released effector molecules that are capable of acting on a variety of cells, including monocytes and macrophages.

Fibroblasts have never been directly implicated in the pathogenesis of PRD. Upon stimulation with TNF-α, IL-17A, IL-18, or IFN-γ, expression of inflammatory mediators such as IL-6 and IL-8 were up-regulated by PRD fibroblasts. These findings suggest that excessive quantities of endogenous TNF-α, IL-17A, IL-18, or IFN-γ, which were readily detectable within PRD explant cultures, may induce further secretion of inflammatory mediators by PRD fibroblasts. On the basis of these results, the tissue destructive properties of PRD fibroblasts may be supported by their production of high quantities of IL-6 and IL-8. Furthermore, secretion of such pro-inflammatory mediators likely supports continued recruitment of inflammatory cells to the periradicular tissue. Thereby, PRD fibroblasts may contribute towards the perpetual chronic inflammatory reaction, ultimately

leading to destruction of the periradicular tissue matrix. Undoubtedly, numerous cell types contribute to the pathogenesis of PRD. However, data from my experiments implicate PRD fibroblasts as central participants within this process.

Bacterial product ligation of TLRs allow SLFs to participate in innate immunity, resulting in up-regulated ICAM-1, MMP-1, MMP-3, MMP-13 and inflammatory cytokine expression (Kyburz *et al.* 2003). I demonstrated that PRD fibroblasts were responsive to all three endodontic pathogens used in culture experiments. This is likely to be mediated, at least in part, through TLR expression. Further investigations are necessary to define TLR expression within this cell population and elucidate downstream inflammatory mediators activated upon TLR ligation and signalling. Through infection-induced secretion of substantial quantities of IL-6 and a limited amount of TNF-α, PRD fibroblasts likely trigger acute phase responses. IL-8 potently attracts neutrophils to inflammatory sites and IL-8 secretion by PRD fibroblasts was induced by endodontic pathogens. Indeed, within the PRD lesion, fibroblasts are a major source of this chemokine. Marked release of IL-8 may therefore promote the recruitment of PMNs to the inflamed periradicular area. Importantly, these data imply that PRD fibroblasts are capable of directly contributing towards the innate immune response. The contribution of fibroblasts towards inflammatory processes within PRD is summarised in Figure 8.15.

Figure 8.15 Diagrammatic summary of PRD fibroblast contribution towards inflammatory processes within chronic inflammatory PRD. Endodontic pathogens and endogenous cytokines activate PRD fibroblasts to release proinflammatory cytokines that recruit and activate inflammatory cells and osteoclasts. Deficiency of an inducible increase in OPG secretion by PRD fibroblasts tips their contribution within the chronic inflammatory reaction towards a predominantly pro-inflammatory destructive effect. PRD fibroblasts release several inflammatory mediators that perpetuate inflammation by positive feedback regulatory loops, thereby promoting bone destruction.



CHAPTER 9

GENERAL DISCUSSION

9 GENERAL DISCUSSION

9.1 Introduction

Periradicular disease (PRD) develops as a localised chronic pathological inflammatory immune reaction in response to continuous microbial stimuli from necrotic, infected dental root canals. Human periradicular disease is characterised by alveolar bone resorption at the dental root apex culminating in patient morbidity and occasionally mortality. Host susceptibility to PRD is determined by complex interactions between bacteria, host factors and environmental components. In order to successfully attenuate the immune response and eradicate the disease, understanding the complex pathologic processes that contribute towards PRD is of importance. Epidemiologic studies undertaken within differing geographical populations establish that PRD is remarkably prevalent (Saunders *et al.* 1997, Loftus *et al.* 2005, Kabak and Abbott 2005). These employed different criteria in the types of population groups recruited, differing types of radiographs analysed, differences in radiographic and/ or clinical examinations and differing protocols for assessing the presence of PRD. Nevertheless, there is agreement amongst the plethora of studies that PRD represents a substantial healthcare burden, afflicting between 40-70% of the adult population (Kirkevang *et al.* 2001, chapter 1.4).

The majority of patients attending for dental extractions suffer acute dental symptoms, which are primarily associated with acutely inflamed periradicular lesions (Vier and Figueiredo 2002). Apical abscesses account for the majority of periradicular lesions attached to extracted teeth (63.7%) and of the remaining lesions, 24.5% are periradicular cysts. Importantly, periradicular lesions derived from symptomatic extracted teeth, whether cystic or not, display comparable signs of acute inflammation (Vier and Figueiredo 2002). Furthermore, IHC investigations reveal no significant differences in composition of the inflammatory infiltrate in periradicular cysts and periradicular granulomas (Stern *et al.* 1981, Torabinejad and Kettering 1985, Gao *et al.* 1988, Matsuo *et al.* 1992, Liapatas *et al.* 2003). Given these data and small tissue volumes available from each lesion, PRD lesions used in tissue explant culture experiments from extracted teeth were not subjected to pathological examination to differentiate between periradicular cysts or granulomas.

PRD poses a primary health care issue of significance in its own right. However, it also provides an ideal model system in which to explore cytokine interactions in chronic human

inflammatory responses. I therefore developed a novel explant culture system whereby PRD tissues were harvested during surgical procedures or after dental extractions and immediately cultured. Cytokines released at sites of infection have the capacity to modify normal bone remodelling processes. Thereby, cellular secretion of such inflammatory mediators contributes towards physiological bone remodelling or pathologic bone destruction. It was therefore of relevance that spontaneous release of endogenous cytokines was detected within PRD explant cultures implying that tissues remained immunologically active for 72 hours, with expression of endogenous factors necessary to sustain such activity.

Although expression of prototypical proinflammatory cytokines has been described within PRD tissue, this is the first study to investigate factors that modulate their expression within human lesions. Furthermore, the endodontic literature is bereft as to the contribution of novel pleiotropic cytokines in development of inflammatory PRD. I therefore explored IL-18 biology within the human PRD lesion and further investigated the effects of several other novel cytokines upon endogenous inflammatory mediator expression. Laboratory-based research using human diseased tissue is frequently difficult due to the many inherent patient variables, for example smoking status, age, clinical symptoms and genetic background. A further confounding factor is clinical accessibility in obtaining appropriate chronically inflamed human tissues in which to study inflammatory events. Furthermore, there is a significant problem in interpreting data from investigations analysing the immunopathogenesis of human PRD as relatively low numbers of lesions have been examined (summarised in Table 9.1). However, experiments within this thesis were undertaken upon a **substantial number** of PRD lesions. The large quantity of lesions analysed strongly support that my experiments are representative of the overall disease state.

Table 9.1 **Summary of studies investigating cytokine expression within human chronic inflammatory PRD tissues.**

Author	Specimen	No	Mediator studied	Method	Control
Meghji *et al.* 1989	PRD cysts	5	IL-1	Explant culture/ cell culture	rhIL-1
Artese *et al.* 1991	Granulomas	10	IL-1β, TNF-α, CD-3, CD-19, anti Leu M5	IHC- frozen	Nil
Barkhordar *et al.* 1992	PRD	8	IL-1β	ELISA-tissue homogenates	Healthy pulp
Bando *et al.* 1993	PRD cysts	12	ICAM, ELAM, IL-1α, IL-1β, TNF-α, IL-8	IHC- frozen	Healthy gingival, buccal mucosa
Lim *et al.* 1994	Granulomas	22	IL-1β	ELISA-tissue homogenates	Healthy pulp, Inflamed gingiva
Formigli *et al.* 1995	PRD Cysts	35	TRAP, IL-6, PGE_2	ELISA, RAI	Peripheral serum
Meghji *et al.* 1996	PRD Cysts	16	IL-1α, IL-1β, TNF-α, IL-6, LPS	RT-PCR, ELISA	Medium only
Honma *et al.* 1998	PRD Cysts Gingival fibroblasts	10	IL-1α, IL-1β, TNF-α, IL-6, IL-8, CD3, CD68, L26, vimentin	*in situ* hybridisation	Nil
Hren *et al.* 1998	PRD Granulomas	17	ICAM, IL-2R	FACS	Peripheral blood
Kabashima *et al.* 1998, 2004	PRD Granulomas	8/ 12	IL-1α, IL-1β, IL-4, IL-6, IFN-γ, iNOS, CXCR3	IHC- frozen	PDL
Barkhordar *et al.* 1999	PRD	6	IL-6	ELISA-tissue homogenates	Inflamed pulp, Healthy pulp
Tyler *et al.* 1999	Granulomas PRD Cysts	17	TGF-β_1, TGF-α	IHC- FFPE *in situ* hybridisation	Isotype matched Ab
Danin *et al.* 2000	Granulomas PRD Cysts	25	TNF-α, TGF-β_1	ELISA-tissue homogenates	Internal
Walker *et al.* 2001	Granulomas PRD Cysts	12/ 12	IL-2, IL-4, IL-6, IL-10, IFN-γ, CD20, CD68, CD45RO	IHC- FFPE	Tonsil
Gervásio *et al.* 2002	PRD Cysts	24	IL-3, IL-6, GM-CSF	ELISA-tissue homogenates	Healthy pulp
Radics *et al.* 2003	Granulomas PRD Cysts	42	IL-6, GM-CSF	ELISA-tissue homogenates	Healthy pulp
De Sá *et al.* 2003	Granulomas	15	IL-4, IL-6, LT-α	IHC- FFPE	Non-immune serum

9.2 Effects of microbial moieties upon PRD explants

To identify stimuli responsible for inducing cytokine production, I initially evaluated the capacity for microbial components to promote cytokine expression by developing a novel *in vitro ex vivo* PRD tissue explant model. LPS addition to PRD explants resulted in significantly increased expression of IL-1β and IFN-γ and moderately increased levels of TNF-α and IL-10. Furthermore, SEB substantially increased culture levels of IL-17A, TNF-α and IFN-γ. Of importance, macrophage and T cell-derived pro-inflammatory cytokines IL-1α, IL-1β, TNF-α and IFN-γ are recognised as mediating tissue destruction at the initiation and subsequent progression of PRD (Tani-Ishii *et al.* 1995, Kawashima and Stashenko 1999). Of these, IL-1 is significantly up-regulated at the onset of pulpal and periapical inflammation (Tani-Ishii *et al.* 1995). Within the rodent pulp exposure model, IL-1α is central to pathogenesis of the periradicular lesion and IL-1β is a key bone resorptive cytokine in the human PRD lesion. Spontaneous secretion of these cytokines in unstimulated PRD tissue explants and their significantly increased expression induced by LPS and/ or SEB reinforces the important contribution of bacteria and their microbial products to destructive pathways in lesion development.

LPS-induced production of proinflammatory cytokines is likely mediated through ligation of TLRs. TLR-1, -2, -4 and -6 mRNA expression was readily detectable within human PRD tissue biopsies (chapter 4.5). Pre-incubation of IFN-γ-primed monocytes with LPS nullifies the effect of LPS-induced IL-12 production (Wittmann *et al.* 1999). Interestingly, LPS-stimulated PRD explant cultures expressed significantly increased levels of IL-12. It may be speculated that only low levels of LPS were present within analysed lesions, thereby implicating a predominantly Gram-positive microbial flora. Indeed, the microbial flora of teeth with failed root canal treatment (RCT) or persisting root canal infection consists primarily of anaerobic bacteria with a Gram-positive profile (Pinheiro *et al.* 2003, Gomes *et al.* 2004). Cells other than monocytes could contribute towards the LPS-induced secretion of IL-12. LPS-activated macrophages in a co-culture system suppress fibroblast growth, which is reversed by the addition of hydrocortisone. This may provide a mechanism whereby the removal of pathogens during RCT allows reparative processes to take place with normal proliferation of fibroblasts (Metzger *et al.* 1997).

9.3 A potential role for IL-18 in PRD

IL-18 is produced by activated blood monocytes and tissue macrophages such as Kupffer cells. An important function of IL-18 is the regulation of growth, differentiation and modulation of functionally distinct subsets of T helper cells required for Th1 cell mediated immune responses (Nakanishi *et al.* 2001). IL-18 induces production of IFN-γ, IL-2 and GM-CSF and stimulates IL-2R α-chain expression, thereby supporting $CD4^+$ effector Th1 cell proliferation. The soluble circulating antagonist, IL-18BP is responsible for regulating IL-18 activity (Aizawa *et al.* 1999). Although there is no significant homology between IL-18BP and IL-18R components, IL-18BP has the immunoglobulin domain homologous to the third IgG domain of the decoy receptor of IL-1, type II IL-1R. As it lacks a transmembrane domain, IL-18BP exists only as a soluble circulating protein and thus belongs to a novel family of soluble receptor-like proteins including OPG and cytokine like factor 1 (Novick *et al.* 1999). IL-18BP neutralises IL-18 effector functions (Novick *et al.* 1999) establishing that the IL-18 system operates through elaboration of its receptors and binding protein.

Although IL-18 expression has been widely reported across a range of conditions, little direct functional analysis has been possible on primary inflamed human tissues. This is principally due to difficulties in obtaining the appropriate effector lesion. Demonstration that cytokine blockade is important in therapeutic development is best exemplified by TNF-α neutralisation in rheumatoid arthritis synovial tissues *in vitro* in which a hierarchical role for TNF-α in IL-6 and IL-1β production was proposed. This was a critical step, leading to the development of infliximab and successful clinical product development. The PRD lesion represents an intriguing inflammatory tissue in this respect. Unstimulated PRD explant tissues secreted substantial quantities of IL-18 and both mRNA and mature protein IL-18 expression were observed within PRD tissues. Within the novel human PRD explant model, IL-18 significantly increased IFN-γ and IL-17A expression and moderately increased IL-12, IL-1β and TNF-α. Through induction of these potent proinflammatory mediators, IL-18 likely contributes towards the plentiful expression of IL-1β and IFN-γ observed within human PRD tissues (Barkhordar *et al.* 1992, Kabashima *et al.* 1998). Furthermore, IL-18 is likely responsible for, at least in part, the substantial expression of TNF-α and IL-1β present within root canal exudates of teeth associated with PRD lesions (Safavi and Rossomando 1991, Takeichi *et al.* 1996).

The innate cellular immune system is responsible for rapidly initiating cytokine and cellular responses essential for early microbicidal host defences. IFN-γ and TNF-α expression are central to the development of host inflammatory responses and pre-requisite to effective host immunity to invading microbial pathogens and clearance of microbial pathogens. It is therefore of importance that IL-18 induces the expression of both these cytokines from a variety of cell types (Nakanishi *et al.* 2001). Critically, addition of exogenous IL-18 to *ex-vivo* PRD explant cultures significantly increased expression of IFN-γ and TNF-α. Furthermore, IL-18 in combination with IL-12 synergistically induced IFN-γ expression within PRD tissue cultures, which may occur independently of TCR engagement (Nakanishi *et al.* 2001). Of much significance, IL-18 neutralisation experiments established that IL-18 has an important effector role when bacterial-derived stimuli were added to chronic PRD explant lesions. Although endogenous IL-18 is required for the abolition of many pathogens, in Th1 dominated responses, it may lead to tissue destruction.

Increased expression of TNF-α induces MMP-2 and MMP-9 activity from a variety of human cells, thereby promoting localised matrix destruction (Ueda and Matsushima 2001). The contribution of IL-18-up-regulated expression of TNF-α in destructive inflammatory processes is classically demonstrated in RA. Through inducing increased TNF-α expression, it is likely that IL-18 indirectly contributes to inflammatory-mediated destruction of the surrounding periradicular tissue matrix during lesion development. Within the human PRD lesion, it is conceivable that IL-18 has a role in host protection to microbes from the infected root canal by enhancing IFN-γ expression. Through the induction of IFN-γ, specific T-cell and B cell-mediated responses may be promoted against pathogenic endodontic bacteria. However, such pathways could equally be associated with pathological destruction. In addition to TNF-α, these destructive features include increased expression of IL-1β and neutrophil activation (Wyman *et al.* 2002). IL-1 is a potent bone-resorbing factor that inhibits mediators responsible for promoting bone formation and may contribute towards radicular cyst expansion (Tanebe *et al.* 2004). Up-regulated expression of IL-1β and IL-17A, demonstrated by IL-18 addition to the PRD explant model, may therefore promote destructive pathways in dento-alveolar bone within the vicinity of the dental root apex. From immunostaining experiments, the majority of the direct effects of IL-18 within the PRD lesion would seem to occur via macrophages. The data depicting IL-18 expression in freshly harvested tissues, in combination with results from *ex vivo* culture

experiments provide strong proof of concept that IL-18 is implicated in the host response within PRD.

It has recently been demonstrated that IL-18 and IL-12 are capable of up-regulating TLR-2 and TLR-4 expression in monocytes through their capacity to increase IFN-γ secretion from T cells (Radstake *et al.* 2004). Interestingly, IL-18 substantially increased TLR-1 mRNA expression within PRD explant tissue cultures. Whether this is a direct effect of IL-18 upon TLR-1 expression or the actions of IL-18-induced downstream mediators requires further investigation. My experiments implicate IL-18 as a major contributor towards inflammatory processes within the PRD lesion. Nevertheless, mRNA and protein expression for IL-18BP was readily detectable within PRD tissues (chapter 5.3). The relevance of this expression in the regulation of endogenous IL-18 within PRD requires further exploration.

9.4 Unravelling the cytokine network within PRD

The pro-inflammatory and antiapoptotic cytokine, IL-15 has a multifactorial role in chronic inflammatory disease processes such as RA. Synovial macrophage expression of TNF-α is stimulated by T cells in an IL-15-dependent manner (McInnes *et al.* 1997) and IL-15 induces IL-17 production (Ziolkowska *et al.* 2000, Ferretti *et al.* 2003). Therefore, IL-15 occupies a central and upstream position within inflammatory cascades contributing to chronic inflammatory diseases. Individually, IL-15 is unable to stimulate cytokine production and therefore does not drive antigen-specific T cells to exhaustion. However, in the presence of IL-12, IL-15 induces IFN-γ expression from $CD4^+$ and $CD8^+$ cells (Liew 2003). Conversely, in IL-4 mediated Th2 conditions, IL-15 induces the production of IL-5 from these T cell subsets (Niedbala *et al.* 2002). The individual addition of IL-15 to PRD tissue explant tissues significantly increased expression of T cell derived cytokines IL-17A and IFN-γ. Increased expression of these two cytokines may be the result of IL-15 interacting with endogenously expressed IL-12, which was readily detectable within PRD tissue explants. In addition to increased IL-17A and IFN-γ secretion, IL-15 moderately decreased IL-10 expression. These data implicate IL-15 as a potent proinflammatory mediator contributing towards destructive inflammatory processes within human PRD.

IL-10 is central to orchestrating the balance between pathology and host protection. Indeed, the phenotype of IL-10-/- mice divulges this as the most essential of its many

functions. A critical responsibility of IL-10 in infectious disease is moderating pathological events arising from host inflammatory responses by suppressing production of proinflammatory type 1 cytokines, including IFN-γ, IL-12 and TNF-α and prohibiting T cell functions by inhibiting accessory CD28/B7.1 receptor interaction (Schandené *et al.* 1994). Furthermore, IL-10 and IFN-γ are known to antagonise one another (De Waal Malefyt *et al.* 1991, Fiorentino *et al.* 1991). As a result, significant attention has focussed upon the anti-inflammatory properties of IL-10. Nevertheless, depending on the dose and route of administration, IL-10 also induces pro-inflammatory effects and can be produced simultaneously with IFN-γ as IL-10 is capable of stimulating IFN-γ secretion (Carson *et al.* 1995, Shibata *et al.* 1998). As expected, LPS stimulation of PRD explant tissue cultures significantly increased IL-10 production (Huang *et al.* 2001, Corinti *et al.* 2001, Byrne and Reen 2002). Within human monocyte cultures, gene expression of IL-10 is related to increased expression of IL-1β, IL-7, IL-10, IL-1Rap, IL-4R, TNFR, IL-17R, IL-12R and IL-1RA genes and decreased expression of IL-15, TNF-α, IL-18, IL-24, IL-2Rγ, IL-1Rα (Jung *et al.* 2004). Addition of IL-10 to PRD explants down-regulated IL-17A and TNF-α protein expression. Microbial-induced expression of IL-10 likely moderates the developing inflammatory response within PRD, although inducible IL-10 expression may alone be insufficient to abrogate destructive inflammatory pathways.

It is logical that IFN-γ potentiates inflammatory events when endodontic pathogens that activate TLRs are present. Conversely, in response to endogenous cytokines TLRs may also limit inflammation. Thereby, the excessive activity of cytokines such as IL-1, which can lead to extensive tissue destruction, may be carefully regulated. Furthermore, IFN-γ down-regulates destructive effects of IL-1 by inhibiting IL-1R through a Stat 1 dependent manner (Hu *et al.* 2005). Interestingly, IFN-γ was significantly inducible within *ex vivo* PRD tissues by the addition of IL-18 and IL-12. As both IL-18 and IL-12 were spontaneously secreted within PRD explant cultures, their concomitant expression likely contributes towards enhanced levels of IFN-γ expression within the lesion. Thereby, increased levels of IFN-γ may propagate inflammatory processes within the developing lesion. Conversely, through its effects upon osteoblasts and osteoclasts, increased IFN-γ expression may reduce destruction of the surrounding dento-alveolar bone. Whether the predominating effects of IFN-γ are proinflammatory or anti-inflammatory within PRD remains to be clarified.

By inducing cytokine and chemokine secretion, IL-17A is a key component in T cell-triggered inflammation. IL-17A is central to chronic inflammatory processes including rheumatoid arthritis (Kotake *et al.* 1999), inflammatory bowel disease (Fujino *et al.* 2003), psoriasis (Teunissen *et al.* 1998), atopic dermatitis (Toda *et al.* 2003) and periodontal disease (Takahashi *et al.* 2005b). In chronic periodontitis, IL-17A is significantly increased in gingival crevicular fluid (GCF) samples and gingival cells (Vernal *et al.* 2005). In contrast, *in situ* expression of IL-17A mRNA is higher in gingivitis tissues as opposed to tissue associated with destructive periodontitis lesions. However, the frequency of IL-17A positive samples is higher in periodontitis patients (Oda *et al.* 2003). Using Western blotting, Takahashi *et al.* (2005) identified IL-17A protein in 10 of 17 periodontal explant samples and using ELISA in only five of these tissues. Nevertheless, IL-17A concentrations correlate with the amount of localised periodontal destruction, thereby implicating a role for IL-17A in disease progression (Johnson *et al.* 2004).

IL-17A, a predominantly pro-inflammatory cytokine, was consistently detected within unstimulated human PRD explant cultures. It was therefore of importance to elucidate factors contributing towards endogenous IL-17A expression within the PRD lesion. Initially, I examined the effects of microbial moieties upon IL-17A secretion within PRD explant cultures. Although PBMC cultures from gingivitis and periodontitis patients stimulated by *Porphyromonas gingivalis* outer membrane protein (OMP) express comparable levels of IL-17A mRNA and protein (Oda *et al.* 2003), *E. coli*-derived LPS had little modulatory effect upon IL-17A expression within the PRD explant model. Nevertheless, IL-17A may be involved in early stages of PRD inflammatory responses to invading pathogens, as it was up-regulated by addition of other bacterial-derived moieties, for example SEB (chapter 4.4).

High levels of IL-6 are expressed in RA and correlate with disease activity. IL-6 stimulates the secretion of immunoglobulin by plasmacytes, activates and promotes proliferation of T lymphocytes, increases neutrophils and platelets, induces acute phase proteins including CRP, fibrinogen, haptaglobin and serum amyloid-A, regulates the proliferation and differentiation of osteoclasts and induces bone resorption (Matsuno *et al.* 1998). Although IL-6 and TNF-α are regulated by distinct mechanisms, these two cytokines have overlapping and synergistic actions (Matsuno *et al.* 2002). Of importance, IL-17A consistently increased IL-6 expression within PRD tissue explants (chapter 6.2), as has been observed in cultures of cartilage, synovium and bone tissues (Chabaud *et al.* 1998,

Moseley *et al.* 2003). IL-17A-induced secretion of IL-6 may therefore contribute towards the recruitment of neutrophils to inflamed periradicular sites and high levels of expression of both IL-17A and IL-6 likely contributes towards degradation of the surrounding periradicular tissues. Within my experiments, IL-17A was added at a concentration of 50 ng/ml as previously described in explant culture experiments (LeGrande *et al.* 2001).

IL-8 is present within periradicular and inflamed pulp tissue, although barely expressed within healthy pulps (Shimauchi *et al.* 2001, Huang *et al.* 1999). Both IL-4 and IL-10 suppress neutrophil-derived IL-8, conversely IFN-γ increases its expression (Kunkel *et al.* 1995). The substantial levels of spontaneously released IL-8 within unstimulated PRD explants likely propagate continued infiltration of immune cells into developing inflammatory PRD lesions. IL-17A induces IL-6 and IL-8 expression in several cell lines, including epithelial cells, vascular endothelial cells and fibroblasts (Kawaguchi *et al.* 2001, Laan *et al.* 2001, Jones and Chan 2002) and moderately increased IL-8 secretion within PRD explant cultures. Endogenous IL-8 expression was also increased by addition of exogenous IL-18 to fibroblast and tissue explant cultures. Up-regulated expression of IL-8, by the effects of IL-17A and IL-18 upon PRD fibroblasts and other cell populations, likely enhances the recruitment of neutrophils and activated T cells (Laan *et al.* 1999, Laan *et al.* 2001), thereby exacerbating localised periradicular tissue pathology.

Several of the biological processes activated by IL-17A are similar to, though less potent than IL-1β. Destructive pathways induced by IL-1β and TNF-α within joint compartments are synergistically or additively augmented by IL-17A (Moseley *et al.* 2003). In support of predominantly pro-inflammatory properties, IL-17A increased TNF-α and IFN-γ expression within PRD explants (Figure 6.18 and Figure 6.13). IL-17A-medaited inflammatory pathways may subsequently be enhanced through positive feedback loops whereby TNF-α and IFN-γ further increase IL-17A secretion (Figure 6.10 and Figure 6.12). IL-15 substantially increased levels of secreted IL-17A within PRD explants. Thereby, IL-15 likely amplifies pro-inflammatory responses within PRD from acute through to chronic stages. Within PRD explants, IL-10 moderately reduced IL-17A expression. Perpetuation of an exaggerated inflammatory reaction, arising from continuous IL-17A secretion within the PRD lesion, may therefore be tempered by concomitant IL-10 expression. However, in rheumatoid synovial explant cultures IL-17A production is inhibited by IL-4 and IL-13 but not IL-10 (Chabaud *et al.* 1999). Therefore, the absence of or low IL-4 expression within PRD tissues may further direct inflammatory pathways

towards a predominating proinflammatory profile. There is evidence that IL-17 acts upstream of several inflammatory mediators. For example, IL-17A promotes the release of human β-defensin 2, which subsequently induces IL-18 secretion (Niyonsaba *et al.* 2005). The many observed effects of IL-17A upon PRD explant cultures strongly implicate IL-17A as an upstream mediator of inflammatory processes within PRD. However, the position of IL-17A in the hierarchical structure of the inflammatory network within PRD remains to be established.

TNF-α occupies a dominant position in cytokine hierarchy and regulates the synthesis of several cytokines. In addition to its effects upon cytokines, TNF-α potently up-regulates E-selectin, ICAM-1 and VCAM-1 expression, thereby enhancing leucocyte recruitment (Mueller 2002). Furthermore, TNF-α is a powerful inducer of MMP-1, MMP-3 and MMP-13 (Nishikawa *et al.* 2002). Spontaneous TNF-α expression was readily detected within PRD explant cultures and substantially increased by exogenous IFN-γ and modestly increased by IL-15, IL-17A and IL-12 (chapter 6.7). These cytokines were spontaneously expressed within PRD cultures and likely contribute towards intrinsic and inducible TNF-α expression. In turn, increased TNF-α secretion likely perpetuates inflammatory processes by up-regulating IL-17A and IFN-γ expression in a positive feedback loop and contributing towards destruction of periradicular tissues by inducing MMP production.

9.5 Biology of Th1 vs Th2 within PRD

The precise contribution of $CD4^+$ effector Th1/Th2 responses to PRD lesion development is controversial. T cells constitute a significant component of the inflammatory cell infiltrate within PRD (Kontainen *et al.* 1986, Stashenko and Yu 1989). A predominance of Th2 type responses have been reported within human PRD tissues (Walker *et al.* 2000, De Sá *et al.* 2003). The low quantities or frequent absence of detectable T cell-derived cytokines such as IL-4 within unstimulated tissue explant culture supernatants is therefore surprising. These data question the capacity of Th2 cells to contribute efficiently towards inflammatory processes within chronic PRD. Generation of substantial quantities of the archetypal Th1 cytokine IFN-γ within stimulated PRD explant cultures, the presence of high levels of TNF-α and lack of ability to induce IL-4 strongly support the hypothesis that inflammatory reactions within PRD are Th1 driven (Kabashima *et al.* 1998, Kabashima *et al.* 2004). In addition to patterns of cytokine expression, identification of cell surface markers including IL-18R and CCR5 provide further evidence of predominating Th1

immune responses (Xu *et al.* 1998, Loetscher *et al.* 1998, Uguccioni *et al.* 1998) and I consistently detected IL-18R protein expression within PRD tissues. Furthermore, CCR5 predominates over CCR3 expression within PRD biopsy tissues (Kabashima *et al.* 2001b). Collectively, these data reinforce the conjecture that inflammatory processes within PRD are promoted by a prevailing $CD4^+$ effector Th1 response. Interestingly, in a background of IL-12, IL-15 induces a Th1 type response and in the presence of IL-4, which was barely detectable within PRD explants, IL-15 induces a Th2 dominated response (Niedbala *et al.* 2002). Addition of IL-15 to PRD explants significantly increased IFN-γ production (chapter 6.6). Inducible IFN-γ production allied with endogenous IL-12 expression and absence of IL-4 support the supposition that PRD predominantly comprises a $CD4^+$ effector Th1 type response.

In vivo elimination of the Th2 cytokine IL-10 in addition to IL-6 results in substantially increased IL-1α expression within murine PRD tissues, which correlates with increased periradicular bone resorption (Balto *et al.* 2001, Sasaki *et al.* 2000). Indeed, IL-10 knockout mice develop infection-stimulated PRD lesions up to five times the size of those developing within wild type controls and locally produced IL-1α is increased 10-fold (Sasaki *et al.* 2000). In contrast to IL-10 within the murine model, the Th2 cytokine IL-4 has no influence upon PRD lesion size, indicating the heterogeneity amongst Th2 type cytokines (Sasaki *et al.* 2000). Surprisingly, deficiency of IL-12, IL-18 or IFN-γ within the murine model appears to have no effect upon development of infection-induced PRD. In consideration of these data, Sasaki *et al.* (2000) suggest that targeting of Th2-derived cytokines may be a therapeutic option to prevent inflammatory bone loss. Whether pharmacologic use of Th2 type cytokines, for example IL-10, would be of any therapeutic value in the treatment of human PRD lesions remains to be investigated.

9.6 Cellular contribution towards destructive pathways within PRD

Several cellular pathways could contribute to tissue damage arising from PRD. The stromal cell component of PRD essentially comprises vascular endothelium, epithelial cells and fibroblasts and accounts for 50% of the total cell population (Morse *et al.* 1975, Stern *et al.* 1981, Yu and Stashenko 1987). The inflammatory cellular infiltrate of the PRD lesion consists of neutrophils, macrophages, mast cells, lymphocytes, natural killer cells, plasma cells and eosinophils (Morse *et al.* 1975, Kontiainen *et al.* 1986). Release of IL-1β or TNF-α by monocytes/ macrophages and subsequent activation of resident tissue cells,

including fibroblasts, endothelial cells and stromal cells, provokes a cascade that amplifies or suppresses inflammation by inducing further release of cytokines and/or growth factors.

Macrophage and fibroblast derived cytokines were abundantly detected within unstimulated and stimulated PRD explants, including IL-1, TNF-α, IL-6, IL-18 and the chemokine IL-8. In addition to activating inflammatory mediator expression within these cells, several cytokines exhibit chemoattractant properties. IL-1β has marked chemoattractant effects upon $CD4^+$ T effector cells (Hunninghake *et al.* 1987), IL-1α upon human keratinocytes (Gyulai *et al.* 1994), IL-18 upon T lymphocytes (Yoshimura *et al.* 2001) and IL-18 upon neutrophils and dendritic cells (Leung *et al.* 2001, Gutzmer *et al.* 2003). Furthermore, RANKL likely contributes towards increased numbers and persistence of antigen presenting dendritic cells and subsequent proliferation of T cells (Josien *et al.* 2000) within PRD. However, regulation of cellular migration and proliferation by cytokines is complex. The effects are dependent upon cytokine concentrations within the local cytokine milieu and may be exerted through indirect actions upon cells. Endogenous release of IL-1, TNF-α, IL-6, IL-8, IL-17A and IL-18 therefore likely co-ordinates migration and activation of inflammatory cells into the PRD lesion. Despite significant T cell infiltrate within the lesions, it was somewhat surprising that concentrations of T cell derived cytokines, for example IL-4, were low in unstimulated PRD explant cultures. In contrast, IL-17A was detected in modest quantities within unstimulated lesions.

Fibroblasts have never been directly implicated in pathogenesis of the PRD lesion. However, PRD-derived fibroblasts secreted a variety of cytokines that exert pleiotropic effects on monocytes/ macrophages. Through release of such inflammatory mediators, fibroblasts significantly contribute towards inflammatory pathways within PRD and potentially matrix degradation of the surrounding tissues. Importantly, microbial products, live endodontic pathogens or cytokines (for example IL-17A, IL-18, TNF-α or IFN-γ) significantly increased secreted IL-6 and/ or IL-8 within PRD fibroblast cultures (chapter 8). Interestingly, IL-17A and IL-18 also induced these inflammatory cytokines within PRD explant cultures. IL-17A and IL-18 likely exaggerate inflammatory pathways within PRD by inducing and maintaining macrophage and T cell synthesis of TNF-α, IFN-γ and IL-17A. These in turn increase IL-6 and IL-8 secretion from fibroblasts, which enhance the recruitment of PMNs and other immune cells into the inflamed periradicular site. Therefore, through the release of such inflammatory mediators, PRD fibroblasts contribute towards perpetuation of inflammatory responses within PRD and thereby lesion chronicity.

IL-17A is predominantly produced by T cells. It was therefore surprising to detect IL-17A within PRD fibroblast cultures and within fibroblast cell lysates and its expression was significantly increased by addition of TNF-α or IFN-γ to cultures. In addition to inflammatory effects induced by IL-6 and IL-8 expression, PRD fibroblasts likely contribute towards T-cell activation and lesion expansion by their production of IL-17A. TNF-α and IL-17A exert little individual effect upon expression of SLF-derived OPG and cytokines added individually to PRD fibroblast cultures did not modulate OPG expression. However, SFL-derived OPG production is greatly increased when TNF-α or IL-17A are combined with IL-1, likely reflecting a negative feedback loop to control bone destruction (Granet *et al.* 2004). The effect of cytokine combinations upon OPG expression by PRD fibroblasts therefore requires further investigation. Lack of IL-4 protective effects within PRD combined with an inability of endodontic pathogens to increase OPG expression from PRD fibroblasts likely contribute towards inflammatory bone destruction concomitantly occurring with the inflammatory PRD lesion. On the basis of these results, tissue destructive properties of PRD fibroblasts are supported by production of significant quantities of IL-6 and IL-8. Moreover, through the expression of inflammatory mediators (and possibly MMPs) PRD fibroblasts may contribute directly and/ or indirectly to dento-alveolar bone destruction. MMP expression within PRD fibroblasts and mediators of their induction remain to be elucidated. Although numerous cell types contribute to the pathogenesis of PRD, data from my experiments implicate PRD fibroblasts as central participants in these processes.

Experiments on NK cells establish that IL-12 and IL-15 induce significantly more IL-10, MIP-1alpha, MIP-1beta and TNF-α both at protein and transcript levels than IL-18 combined with IL-12. Indeed, IL-12 and IL-18 induce very little IL-10 from NK cells and optimal release of GM-CSF is obtained by NK cell stimulation with IL-15 and IL-18 (Fehniger *et al.* 1999). The substantial levels of IFN-γ induced by the addition of IL-18 and IL-12 to the PRD explant cultures suggests that NK cells may significantly contribute towards inflammatory events within the PRD lesion. Furthermore, NKT cells were consistently detected within PRD sections by IHC (Figure 4.9) and may represent a key cellular source of IL-18-mediated IFN-γ expression. Further studies are necessary to evaluate the contribution of NK and NKT cells to inflammatory events within PRD.

$CD68^+$ macrophage-like cells in human PRD lesions are situated in close proximity to blood vessels, near the epithelium of cysts and in central regions that may be related to

bacterial ingress (Rodini *et al.* 2001). My IHC experiments agreed with these observations and established that they are an important source of IL-18 which was abundantly expressed within inflamed PRD tissues. Thereby, macrophages are important contributors to proinflammatory events within PRD. CD25 molecules are strongly up-regulated on activated T cells and can therefore be considered valuable markers of T cell activation. Immunohistochemical staining revealed $CD25^+$ cells within inflamed PRD lesions. Interestingly, this T cell subset was situated in close contact with IL-18 expressing macrophages. During chronic infections, suppressive cytokines including IL-10 and TGFβ are produced and moderate immune responses to prevent an exaggerated inflammatory reaction and harmful pathology. It is likely that some of these inflammatory suppressors are produced by $CD4^+CD25^+$ Treg cells (Mittrücker and Kaufmann 2004). Indeed, mice lacking $CD4^+CD25^+$ cells show enhanced resistance to *Candida albicans* infection however, this is associated with severe pathology (Mittrücker and Kaufmann 2004). Whilst a proportion of $CD25^+$ cells observed within the PRD lesion may be Treg cells, there is currently no data defining Treg cell expression within human PRD. Undoubtedly, the use of FACS analysis to accurately define the cellular composition of PRD would be of substantial benefit in establishing the contribution of specific cell subsets to tissue destructive pathways within PRD.

9.7 Mediators of inflammatory dentoalveolar bone destruction

Healthy adult dentoalveolar bone undergoes continuous dynamic remodelling. The equilibrium between bone deposition and bone resorption is closely regulated by the opposing actions of osteoblasts and osteoclasts upon the bone matrix. Osteoclast activity and bone metabolism is primarily modulated through the relative expression of the key bone mediators, RANKL and OPG. RANKL-induced bone resorption is inhibited by secretion of its decoy receptor OPG (Akatsu *et al.* 1998, Fuller *et al.* 1998, Lacey *et al.* 1998, Yasuda *et al.* 1998b, Lacey *et al.* 2000, O'Brien *et al.* 2000). Data from previous described experiments established the importance of microbial pathogens and microbial derived products in regulation of inflammatory pathways within PRD (chapter 4.3 and 4.4). Although *E. coli*-derived LPS had no effect upon OPG or RANKL protein expression, other microbial-derived moieties likely influence dentoalveolar bone destruction as SEB reduced the expression of OPG within PRD tissue explant cultures (chapter 7.3). In addition to the RANKL/ OPG regulatory axis, cytokines significantly contribute towards the coordination of bone metabolism. In the rodent pulp exposure

model, administration of IL-1 receptor agonist (IL-1ra) leads to reduced periradicular lesion expansion (Stashenko *et al.* 1994), thereby implicating IL-1α as a key modulator of periradicular bone destruction (Wang and Stashenko 1993). Of importance, substantial levels of IL-1β and TNF-α expression were present within unstimulated PRD explant cultures. Through actions upon stromal-osteoblastic cells, IL-1β and TNF-α increase local levels of RANKL expression. However, potentially destructive pathways arising from concomitant release of IL-1β and TNF-α are moderated by a simultaneous increase in OPG production from stromal-osteoblasts (Hofbauer *et al.* 1999).

T cells are present in the bone microenvironment and their impact upon skeletal turnover is only now being unravelled. Depending upon local factors, T cells produce mediators of bone resorption such as RANKL or participate in inhibitory pathways of osteoclast formation such as that used by IL-18 (Horwood *et al.* 1998, Horwood *et al.* 1999, Kong *et al.* 1999). Increased RANKL expression contributes towards bone destruction by ameliorating Fas-mediated apoptosis of osteoclasts thereby increasing their life span (Wu *et al.* 2005). IL-18 is central to T cell development and activation and inducing the secretion of IFN-γ from $CD4^+$ effector Th1 cells, NK cells and NKT cells. Although IFN-γ is required for an effective immune response to pathogens, it potently inhibits osteoclast formation (Fox and Chambers 2000), suppresses osteoclastogenesis by interfering with the RANKL signalling pathway (Takayanagi *et al.* 2000) and directs progenitor cell differentiation towards cell lineages other than that of osteoclasts (Quinn and Gillespie 2005). In view of its described bone protective properties, it was therefore surprising that IFN-γ significantly up-regulated RANKL expression and decreased OPG secretion within matched PRD explant tissue cultures. Indeed, the opposing effect of IFN-γ upon OPG and RANKL expression was at levels of significance. IFN-γ induces cytokine expression from macrophages and other inflammatory cells that initiate bone destructive pathways thereby emphasising complexity of the modulatory role for IFN-γ in osteoclastic responses. Through its effects upon cellular constituents within the PRD lesion, IFN-γ may therefore indirectly contribute towards promoting resorption of adjacent periradicular bone.

IL-1, TNF-α, IL-15 and IL-17A enhance osteoclast formation by stimulating production of RANKL and M-CSF from bone lining cells. However, IL-18, IL-1β and TNF-α have no effect upon RANKL expression within resting T cells (Dai *et al.* 2004). In contrast, RANKL expression is increased and sustained for 2 days in PHA-stimulated T cells and synovial T cells stimulated with these pro-inflammatory cytokines. Within PDL cultures,

OPG gene expression is increased by TNF-α and IL-1β (Sakata *et al.* 1999). PRD fibroblasts spontaneously expressed substantial levels of OPG. Through secretion of high quantities of OPG, PRD fibroblasts may provide protection against pathologic bone destruction. Surprisingly, TNF-α and other cytokines had no observable effects upon OPG expression within PRD fibroblast cultures. The inability of cytokines or bacterial pathogens and their cell wall components to modulate PRD fibroblast-derived OPG may result in an inefficiency to counter-regulate increased RANKL expression induced by IL-17A, TNF-α and IFN-γ expression from other cellular components of the PRD lesion. Further work is therefore required to determine if a combination of microorganisms or cytokines have modulatory effects upon PRD fibroblast-derived OPG expression.

IL-17A markedly increases RANKL within CIA mice joints, thereby inducing joint erosion (Lubberts *et al.* 2003) and dose-dependently enhances TNF-α-induced osteoclastic bone resorption *in vitro* (van Bezooijen *et al.* 1999). Following addition of TNF-α to Saos-2 human osteoblastic cell cultures, the RANKL/OPG ratio increases after 2 h and gradually decreases thereafter (Kim *et al.* 2002). A similar effect for TNF-α upon the RANKL/OPG ratio was observed within PRD explant tissues. Indeed, IL-17A and TNF-α substantially increased RANKL expression within PRD explant cultures. Of importance, IL-17A and TNF-α also decreased OPG secretion within matched explant tissue cultures. This suggests that concomitant expression of IL-17A and TNF-α, both intrinsically expressed by unstimulated PRD cultures, promotes a powerful pro-osteoclastogenic reaction that likely contributes towards periradicular bone destruction. A positive regulatory loop is likely created, whereby increased IFN-γ and TNF-α expression induced by up-regulated IL-17A promotes further RANKL expression thereby sustaining pathologic damage to adjacent tissues.

IL-6 promotes osteoclast formation through cellular interactions between osteoblastic cells and progenitor cells. This is related to IL-6-mediated release of IL-1β and minimal direct effect upon the RANKL/RANK/OPG system (Ishimi *et al.* 1990, Kurihara *et al.* 1990, Rozen *et al.* 2000). Furthermore, interactions between IL-6 and the PGE_2 signalling system lead to increased osteoclastogenesis (Liu *et al.* 2005). IL-8 is also a potent stimulator of bone resorption and has a direct stimulatory effect upon osteoclastogenesis and bone destruction (Bendre *et al.* 2003). Therefore, the high quantities of spontaneous and inducible IL-6 and IL-8 expressed within PRD tissues likely contributes towards periradicular bone destruction. Increased IL-6 and IL-8 expression by PRD fibroblasts

stimulated by endogenously expressed IL-17A, IL-18 and/ or TNF-α may contribute significantly towards local matrix degradation.

Taken together, results from the PRD explant culture model and PRD fibroblast cultures suggest that substantial expression of potent inflammatory cytokines, including IL-6, IL-8, IL-17A, IL-18 and TNF-α significantly promotes a strong proinflammatory lesion phenotype. Through direct interactions upon cells within the bone matrix regulating bone remodelling and cells capable of secreting RANKL within the PRD lesion, these cytokines are likely contributors towards destructive pathways within PRD. IL-4 is a potent inhibitor of RANKL and osteoclastogenesis (Wei *et al.* 2002, Mirosavljevic *et al.* 2003, Mangashetti *et al.* 2005). The absence in expression of essential down-regulatory mediators, including IL-4, within PRD tissues combined with concomitant production of pro-inflammatory cytokines IL-17A, IL-1β and TNF-α further reinforces the destructive inflammatory nature of PRD.

9.8 Clinical implications and future work

Previously, bacteriologic culture tests were used as clinical indicators for the end point of root canal treatment. However, this is an unreliable procedure and is now infrequently undertaken other than for research purposes (Trope and Grossman 1985). To determine the inflammatory status of PRD lesions, it has been suggested that the identification and measurement of appropriate biological markers may be of clinical relevance. Therefore, a small number of studies have investigated the potential for sampling of the root canal fluid in teeth associated with PRD. Concentrations of host inflammatory mediators within the root canal have then been quantified and correlated with clinical findings.

Root canal concentrations of PGE_2 are directly associated with clinical symptoms and negatively correlate with increasing lesion dimensions (Takayama *et al.* 1996). TNF-α (Safavi and Rossomando 1991), immunoglobulins (Kuo *et al.* 1998), nitrite (Takeichi *et al.* 1998, Shimauchi *et al.* 2001), IL-1β (Matsuo *et al.* 1994, Shimauchi *et al.* 1996, Takeichi *et al.* 1996, Kuo *et al.* 1998), IL-6 (Takeichi *et al.* 1998) and IL-8 (Shimauchi *et al.* 2001) have all been detected within root canals of teeth associated with PRD. IL-1ra levels in root canal exudates correspond with levels of IL-1β expression and their ratio appears to correlate with clinical symptoms (Shimauchi *et al.* 1998). Root canal concentrations of IL-8 correlate with symptomatic teeth associated with PRD lesions (Shimauchi *et al.* 2001). In

addition to associations between cytokine concentrations and clinical parameters, significant reductions in root canal levels of MMP-8 occur during root canal treatment procedures (Wahlgren *et al.* 2002). In contrast, root canal concentrations of IL-1β or TNF-α do not correlate with clinical symptoms (Ataoğlu *et al.* 2002) and others have failed to detect TNF-α within periradicular exudates recovered from necrotic infected canals (Takeichi *et al.* 1998).

However, the literature relating to root canal sampling must be interpreted with caution. The majority of these studies have analysed only small numbers of canals and reproducibility of findings have not been investigated by follow-up studies. Nevertheless, root canal sampling may allow development of non- or minimally-invasive techniques within the clinical environment during routine clinical procedures. The quantification of inflammatory mediators may elicit information for the clinician of lesion status and thereby, aid in defining treatment prognosis. However, the current lack of understanding of inflammatory dynamics and mechanisms of repair and destruction within human PRD is a major obstacle in developing such host-based biological markers. My experiments demonstrated that human PRD tissues inherently produce substantial quantities of a number of proinflammatory cytokines. The majority of observed functional effects were derived from positive addition of inflammatory mediators to *ex vivo* explant tissues. Future work within this *ex vivo* model requires investigations to additionally investigate the effects of inhibitors and neutralising antibodies upon inflammatory mediator expression. Indeed, experiments are planned to examine the effect of TNF-α inhibitors upon endogenous cytokine expression within the PRD explant model.

Clinical studies are now necessary to attempt to correlate observed inflammatory processes within the *ex vivo* human PRD tissue explant model with the presence or absence of specific clinical signs and/ or symptoms. By developing such studies, an improved understanding of how inflammatory events within PRD relate to clinical complications may significantly contribute towards the development of reliable biological markers that indicate inflammatory disease status. Thereby, root canal exudates could be effectively sampled and clinicians informed as to whether canals can be successfully obturated and treatment therefore completed. Importantly, this may aid the clinician in advising the patient as to the predicted outcome of the treatment procedure (Spangberg 2000).

In addition to sampling of root canal exudates, identification of novel inflammatory mediators and their contribution to proinflammatory pathways may allow the development of therapeutic devices that could be placed within the root canal or inserted directly into the PRD lesion. Thereby, destructive inflammatory responses may be impaired and tissue repair promoted. Cytokines are centrally involved within pathologic processes sustaining inflammatory PRD. The therapeutic blockade of upstream proinflammatory cytokines within the lesion may therefore be advantageous. This approach has been successfully employed in several chronic inflammatory diseases including biologic agents that target TNF-α in RA and Crohn's disease (Taylor 2003). More recently, a clinical trial has also established the efficacy of targeting IL-15 in RA patients (Baslund *et al.* 2005). Although data from the PRD explant experiments would suggest an upstream proinflammatory role for IL-18, inhibition of IL-18 may prove too expensive for therapeutic value. The use of small molecule approaches including P2X7 receptor inhibition, caspase I inhibition or IL-1R cascade signalling inhibitors may provide more suitable therapeutic benefits. The effects of these molecules upon inflammatory processes within PRD require investigation within the PRD explant model system.

9.9 Conclusion

The successful identification of therapeutic targets that attenuate destructive immunoinflammatory pathways within PRD is greatly sought after. To achieve this, a functional understanding of complex interactions between differing cell populations, between these cells and tissue matrix and between cells of the PRD lesion and cells within the matrix of the surrounding dentoalveolar bone is prerequisite. The cytokine milieu is central in orchestrating these interactions yet to date is poorly defined within human PRD, the majority of data being derived from observational studies. Results of experiments undertaken within this thesis support the working hypothesis that the development of a novel PRD explant culture model would enable detailed investigations of the cytokine network within PRD. Indeed, the spontaneous release of endogenous cytokines was readily detected within explant cultures, confirming significant inflammatory activity within chronic PRD. Importantly, the PRD model facilitated analysis of functional cellular/ tissue responses to exogenous stimuli and established the contribution of bacterial moieties to proinflammatory destructive pathways.

Taken together, results from my experiments strongly implicate IL-18 as a key modulating moiety driving inflammatory processes within human PRD. Indeed, addition of IL-18 to PRD tissue explants up-regulated expression of proinflammatory cytokines including IL-1β, IL-6, TNF-α, IFN-γ and IL-17A and moderately reduced IL-10 secretion. Of importance, experiments utilising the novel PRD explant model provide strong proof of concept that IL-18 regulates host responses to pathogenic microorganisms within PRD. Cell culture experiments defined periradicular fibroblasts as a target of IL-18-mediated processes within PRD inflammatory responses. Through increased production of IL-6 and IL-8, PRD fibroblasts likely contribute towards destructive inflammatory events arising from up-regulated expression of IL-18 within the lesion. Indeed, the effects of exogenous IL-18 upon endogenous cytokine expression clearly suggest that IL-18 promotes proinflammatory pathways within the lesion. Nevertheless, IL-18-mediated effects may not necessarily be detrimental to the surrounding periradicular tissues. Through induction of GM-CSF, IL-18 inhibits osteoclast maturation (Udagawa *et al.* 1997, Horwood *et al.* 1998) and induces PG expression (Makiishi-Shimobayashi *et al.* 2001). Therefore, IL-18 secreted by cellular components of PRD tissues may have the capacity to impede local resorption of dentoalveolar bone. The precise contribution of IL-18 towards destructive or compensatory pathways within the periradicular tissues requires further exploration.

Having established that the PRD lesion serves as a useful *in vitro ex vivo* experimental model, future work is necessary to establish associations between underlying immunologic observations within PRD lesions to clinical parameters. Thereby, identification of novel proinflammatory molecules and pathways that significantly contribute towards destructive inflammatory events may be elucidated. Potentially, this may facilitate the development of appropriate pharmacologic agents that attenuate destructive inflammatory responses leading to lesion chronicity and the identification of inflammatory mediators that may provide diagnostic information from root canal sampling.

Conclusions from experiments undertaken within this thesis may be summarised against the original aims of the study as follows:

1. Human PRD provided an effective *in vitro ex vivo* novel explant culture model in which to investigate functional dynamics of likely inflammatory mediators contributing to the initiation and perpetuation of PRD.

2. Substantial IL-18 expression was observed within PRD tissue and IL-18 biology significantly contributes towards proinflammatory pathways within the PRD lesion.

3. Inflammatory mediators directly related to bone regulatory pathways, including cytokines, RANKL and OPG were readily detectable within the human PRD lesion. Furthermore, several factors were identified that modulate expression of these inflammatory moieties.

4. PRD fibroblasts were successfully cultured *in vitro*. Through the release of inflammatory mediators including IL-6, IL-8, IL-17A, TNF-α and OPG, PRD fibroblasts are important regulators of inflammatory processes within human PRD.

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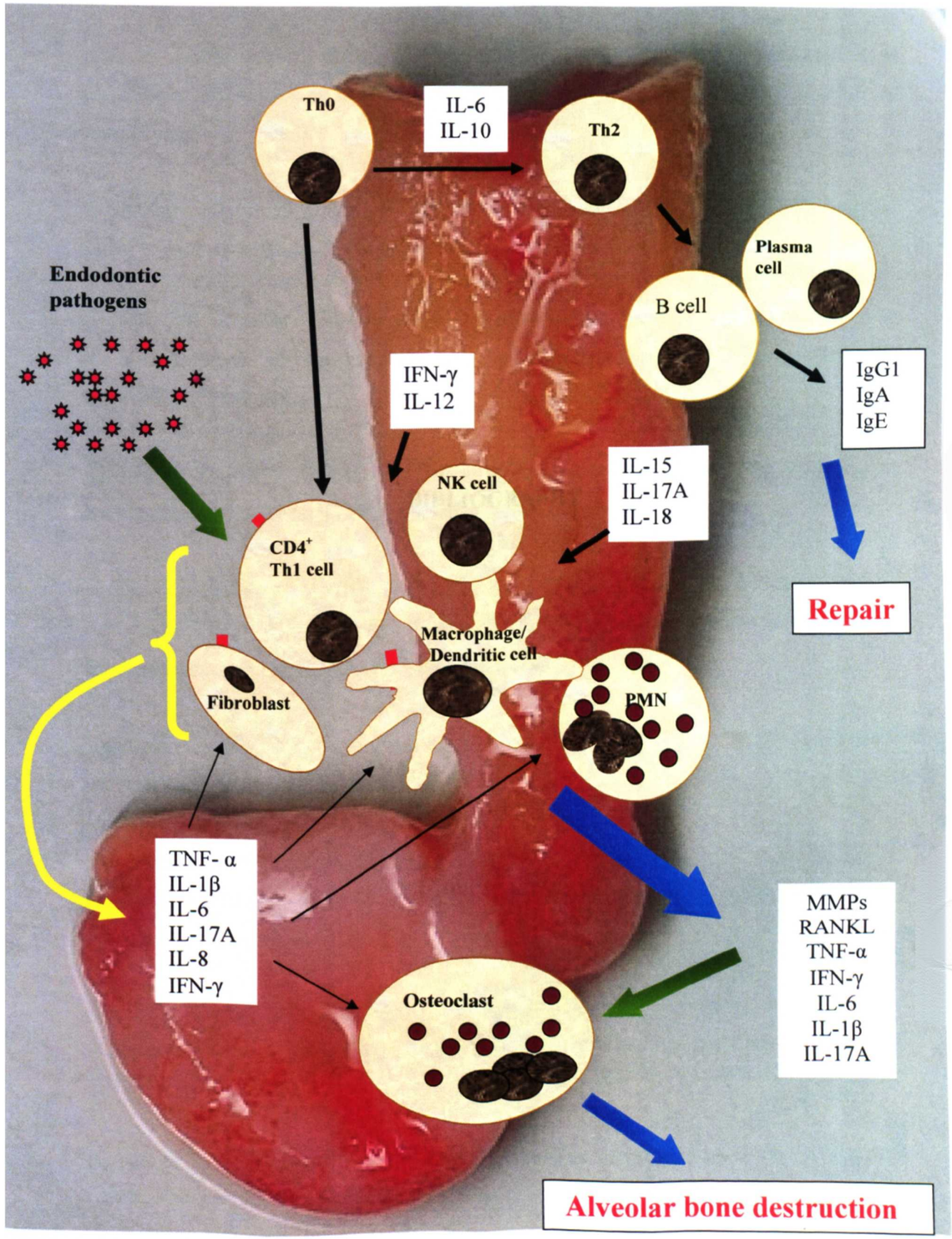
Figure 9.1 **Schematic illustration summarising inflammatory processes within chronic inflammatory PRD.**

PAMPs originating from contents of infected dental root canals enter the periradicular tissues and bind to PRRs, including Toll-like receptors-1, -2, -4 and -6, activating cells of innate immunity and resident stromal cells. Upon activation, inflammatory mediators including cytokines are released leading to an inflammatory cascade which is enhanced by cell-cell contact. Following activation of innate immunity, acquired immune responses are instigated. Secreted cytokines act in autocrine or paracrine manners and in positive or negative regulatory feedback loops. Substantial expression of proinflammatory cytokines within the lesion, including IL-1β, IL-6, IL-8, IL-17A, IL-12, IL-18, TNF-α and IFN-γ, promotes a powerful inflammatory reaction that results in further cell activation. Continued release of these proinflammatory mediators perpetuates inflammatory events within the PRD lesion. High levels of spontaneous and inducible IFN-γ, TNF-α and IL-12 expression implicate a predominating $CD4^+$ effector Th1 pathway. Deficient antiinflammatory Th2 type cytokine expression within PRD, for example the absence of IL-4, likely contributes towards dysregulated inflammatory pathways which promote tissue destruction. Release of MMPs, RANKL, TNF-α, IL-1β, IL-6, IL-8 and IL-17A by PRD fibroblasts and other cellular constituents of the PRD lesion results in the activation of cells within the bone matrix, including osteoclasts, and bone destruction. IFN-γ and IL-18 are essential in successful host responses to endodontic pathogens and may be protective against local matrix degradation by preventing osteoclastogenesis. Conversely, through inducin g an increase in the RANKL/ OPG ratio and promoting proinflammatory cytokine expression (including TNF-α, IL-1β, IL-6, IL-8 and IL-17A) IFN-γ and IL-18 respectively contribute towards destructive pathways in surrounding dento-alveolar bone.

Figure 9.1 **Schematic illustration summarising inflammatory processes within chronic inflammatory PRD.**



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APPENDIX 1

Root canal treatment and general health: a review of the literature

C. A. Murray & W. P. Saunders

University of Glasgow Dental School, Glasgow, UK

Abstract

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Review The focal infection theory was prominent in the medical literature during the early 1900s and curtailed the progress of endodontics. This theory proposed that microorganisms, or their toxins, arising from a focus of circumscribed infection within a tissue could disseminate systemically, resulting in the initiation or exacerbation of systemic illness or the damage of a distant tissue site. For example, during the focal infection era rheumatoid arthritis (RA) was identified as having a close relationship with dental health. The theory was eventually discredited because there was only anecdotal evidence to support its claims and few scientifically controlled studies.

There has been a renewed interest in the influence that foci of infection within the oral tissues may have on general health. Some current research suggests a possible relationship between dental health and cardiovascular disease and published case reports have cited dental sources as causes for several systemic illnesses. Improved laboratory procedures employing sophisticated molecular biological techniques and enhanced culturing techniques have allowed researchers to confirm that bacteria recovered from the peripheral blood during root canal treatment originated in the root canal. It has been suggested that the bacteraemia, or the associated bacterial endotoxins, subsequent to root canal treatment, may cause potential systemic complications. Further research is required, however, using current sampling and laboratory methods from scientifically controlled population groups to determine if a significant relationship between general health and periradicular infection exists.

Keywords: arthritis, bacteraemia, focal infection, health, root canal treatment.

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Introduction

Bacteria were first demonstrated scientifically in the diseased dental pulp by Miller (1894). The medical profession took notable interest in the importance of the infected dental pulp and oral tissues when William Hunter (1900) theorized that microorganisms present in the oral cavity could disseminate throughout the body, resulting in systemic disease. Following further studies (Davis 1912, Billings 1914, Rosenow 1914), a number of research articles appeared in the literature investigating the effect that diseased tissue sites, including oral tissues, had upon systemic illness. Researchers presented the idea that the dissemination of microorganisms, or their associated toxins, throughout the body from a focus of circumscribed infection at a particular tissue site could initiate or exacerbate systemic disease or damage a distant tissue (Daland 1916, Gilmer 1916, Hunter 1921, Thompson 1925). This concept became known as the Focal Infection Theory.

Correspondence: Professor W. P. Saunders, Department of Adult Dental Care, University of Glasgow Dental School, 378 Sauchiehall Street, Glasgow G2 3JZ, Scotland, UK (e-mail: w.saunders@dental.gla.ac.uk).

The focal infection theory

During the period 1910 to the 1940s, the medical and dental literature contained several references

concerning focal infections of dental origin (Mayo 1914, Rosenow 1917, Mayo 1922, Bedell 1925, Peck 1925, Price 1926, Rosenow 1927). Billings (1912) and his coworkers' efforts were instrumental in gaining the medical profession's broad acceptance that a localized, low grade chronic infection could produce disease elsewhere in the body. Billings declared 'when the focal infection, wheresoever it may be located, *seems* to be related to the systemic disease, radical measures should be instituted to remove it'. Mayo (1914) concluded that 'root abscesses and pus pockets connecting with them are often the source of acute and chronic rheumatism'. Rosenow (1917) reinforced the concept of a focus of infection from which microorganisms could enter the bloodstream causing systemic illness, proclaiming 'these results call for a public health propaganda so that the people may be fully informed of the dangers to their continued health that arise from infected teeth'.

Concomitant with the focal infection theory there arose the hypothesis of elective localization (Rosenow 1919). This theory suggested that bacteria located within a certain focus of infection could cause systemic infections by localizing within a specific distant target organ or tissue. Rosenow insisted that enclosed lesions that could only drain into the circulation, such as a necrotic pulp, were the most dangerous foci of infection.

Goadby (1911) demonstrated that cultivated microorganisms from oral infections were capable of inducing rheumatoid arthritis (RA) in the knee joints of rabbits. Other published articles claimed a clearly defined link between infective rheumatoid arthritis and foci of infection, with oral sepsis cited as a main contributing origin (Billings 1913, Hartzell & Henrici 1916). Research revealed that the primary infective agent in rheumatoid sufferers was apparently the *Streptococcus* organism which was prevalent in the oral tissues (Shandalow 1928, Cecil *et al.* 1929).

The subject of focal infection was debated and recommendations proposed that were based on limited scientific research, few controlled studies and anecdotal evidence. These early papers promoted the ritual of wholesale dental extractions and tonsillectomies as the complete cure for many systemic ailments. The theory of focal infection was taught vigorously, citing infected teeth or their surrounding structures as being responsible for a wide range of diseases (Holman 1928). Rhoads & Dick (1932) regarded all pulpless teeth as probable foci of infection and determined that the extraction of teeth in several individuals improved their various medical conditions. Rhein *et al.* (1926) suggested that the extraction of healthy teeth was justifiable in the prevention of focal infection. In America, the reaction against root canal treatment was so strong that the teaching of clinical endodontic techniques was stopped altogether in most institutions in favour of dental extractions (Grossman 1971). Consequently the progress in root canal treatment, still very much in its infancy, was severely curtailed for over 40 years.

Few scientists had published any doubts concerning the focal infection theory during its early development (Howe 1919, Miller 1926). However, by the 1930s researchers began to question the evidence supporting the focal infection theory (Valentine & Van Meter 1930, Bernhardt & Hench 1931), despite review articles upholding its claims (Holman 1928). Cecil & Archer (1927) recorded that from 200 rheumatoid cases, 62% of those who had undergone dental extractions were either cured or had improved. They concluded that success in treatment was dependent upon a relentless search for foci of infection and advocated prompt removal of such foci early in the course of the disease. The tonsils and teeth were determined as the most common foci of infection. Ten years later Cecil & Angevine (1938) questioned the validity of these results and denounced the link between RA and oral disease because, from the original study, there was only a small percentage of RA patients whose condition could have resulted from a focal infection. Their second study demonstrated that chronic focal infection played a comparatively small role in patients with RA and thus disputed the role of focal infection in systemic disease. Despite the indication from earlier papers of a relationship between *Streptococcus* sp. and rheumatoid arthritis, it proved difficult to reproduce these results with further research (Nye & Waxelbaum 1930, Dawson *et al.* 1932).

Reimann & Havens (1940) suggested that Hunter's views expressed 30 years previously had been misinterpreted. They stated that Hunter had laid the blame of systemic disease on 'ill fitting dentures and crowns and not on apical abscesses about pulpless teeth'. In reviewing the literature and from experiments they had performed, Reimann & Havens concluded that 'the removal of local infections in the hope of influencing remote or general symptoms and disease must still be regarded as an experimental procedure not devoid of hazard.' Whilst others (Slocumb *et al.* 1941, Ensign 1945) continued to support the concept of focal infection, particularly in relation to specific clinical entities including heart disease, chronic infectious

arthritis and glomerulonephritis, they accepted that a more logical and scientific approach to focal infection should be adopted. In a critical appraisal reviewing the relationship between focal infection and rheumatoid disease, Freyberg (1946) postulated that removal of foci of infection should only be part of a broad spectrum of treatment and that in chronic cases removal of these foci would be of little benefit. A focus of infection was deemed important in RA patients though it was proposed that it should only be corrected as a prophylactic measure at an early stage in the disease process rather than as a therapeutic intervention.

Researchers who continued to declare their allegiance to the focal infection theory (Shuster 1941) acknowledged that not every focus of infection was responsible for a focal infection and that anaemia, emotional strain, exposure, malnutrition, endocrine imbalance, fatigue, senility, trauma, age and infectious disease all had a role to play in lowering the patient's resistance to disease (Arnett & Ennis 1933). Those sceptical of the focal infection theory (Easlick 1951, Mitchell & Helman 1953) claimed that most of the research up until 1950 exhibited no foci of infection that could be located in the afflicted individual and that there was often no dramatic improvement in the patient's medical condition after the removal of the focus. Mitchell & Helman (1953) also noted that many persons in perfect health had as many septic foci as patients suffering from diseases such as arthritis. The early focal infection studies were crude, with no control cohorts and unreliable bacteriological culture techniques had been used which affected the findings. These criticisms of the focal infection theory led to its gradual demise, although case reports of focal infection from an oral source were occasionally published during the 1950s (Knapp 1952).

Subsequently, the emphasis in research on the focal infection theory graduated toward investigating potential systemic effects as a consequence of bacteraemia following oral and medical procedures. Studies within dentistry started to analyse closely the only apparent identifiable form of a focal infection and elective localization from dental origin, that of infective endocarditis (Jones & Newsom 1931). The link between focal infection and an oral origin for infective endocarditis arose after acute episodes of infection, whereas no chronic infection from a dental origin could clearly be identified as initiating or aggravating systemic illness.

The rise of the focal infection theory during the early decades of the 20th century owed as much to social and economic factors as to scientific validity (Dussault & Sheiham 1982). In Britain, the increasing popularity of Hunter's focal infection theory offered a convincing argument for the lowly regarded dental profession to gain better public credibility and to help support a law amendment for the compulsory registration of dental practitioners. Support of the focal infection theory helped to achieve this aim when in 1921 a Dental Act was passed acknowledging the professional status of dentists in Britain.

Concurrent with fulfilling the professional objectives of dentists in Britain, there was also a favourable political and social environment to promote the focal infection theory at the beginning of the century. There were great concerns over the poor physical condition of entrants into the British Armed Forces and the appalling health of large sections of the community. These concerns led to the creation of a Ministry of Health in 1919. The focal infection theory emerged at a time where there was great political interest in the physical health of the population in Britain, thus helping to fuel its popularity. Criticism of the theory was stronger in the United States, where dentistry was already well established as an autonomous profession, yet it was only after the passing of the Dental Act in 1921 that British dentists began to question Hunter's views. By the 1940s there was sufficient scientific data available to discredit the focal infection theory, and although the Dental Board of the United Kingdom maintained its support for the theory, it had served the profession's objectives and lost its popularity.

In recent years, however, the issue of focal infection has once again resurfaced with the publication of scientific review papers (Hughes 1994, Newman 1996). A popular book (Meinig 1986) has questioned whether the dental profession has chosen to ignore a very significant and much researched concept in relation to the population's general health. This has particularly called into question the role of root canal treatment and its importance in the long-term effect on an individual's health (International Academy of Oral Medicine and Toxicology 1998, HTTP://www.rheumatic.org/teeth.htm, HTTP://www.netset.com/~docws/page13.html, HTTP://www.zip.com.au/~rgammal/root_therapies.htm). However, the possible link between general health and root canal treatment has been strongly disputed by dental governing bodies (British Dental Association 1996).

General bacteraemia, infective endocarditis and prosthetic joints

The earliest published studies analysing the species of microorganisms in a bacteraemia, originating from a focus of infection, used rudimentary laboratory equipment and techniques that could only cultivate a limited number of microorganisms (Richards 1932). However, these studies were useful in confirming that bacteraemia was possible from foci of infections within a variety of tissue sites, including the oral cavity. Whilst bacteraemias related to oral procedures were only beginning to be explored, the medical profession had already recorded bacteraemias following various medical procedures (Lake 1919, Barrington & Wright 1930, Elliot 1939a, Rhoads *et al.* 1955). Several papers followed, investigating different aspects of the postoperative bacteraemia consequent to dental extractions (Burket & Burn 1937, Faillo 1942, McEntegart & Porterfield 1949, Schirger *et al.* 1960). Murray & Moosnick (1941) proved that a bacteraemia could even occur without dental operator intervention by the chewing of paraffin wax in diseased mouths. Although it was apparent that a bacteraemia occurred after dental extractions, it was suggested that apical infections were 'effectively walled off by the associated inflammatory reaction' and thus did not contribute toward a bacteraemia (Elliot 1939b). Burket & Burn (1937) claimed that a reduced bacteraemia occurred following the administration of local anaesthetic, compared with extractions performed under general anaesthesia, although this claim was contradicted by Robinson *et al.* (1950).

The focal infection theory motivated many researchers to investigate the role of dental extraction and its relationship to endocarditis and other cardiac diseases including valvular defects, congenital defects and rheumatic heart disease (Abrahamson 1931, O'Kell & Elliot 1935, Feldman & Trace 1938, Hopkins 1939, Palmer & Kempf 1939, Geiger 1942). These studies paved the way for research into the relationship between bacteraemia and endodontic procedures (Ross & Rogers 1943, Beechen *et al.* 1956, Bender *et al.* 1960). Kennedy *et al.* (1957) showed that bacteraemia could occur in monkeys after the introduction of *Streptococcus haemolyticus* into the root canal. Other oral manipulations were also demonstrated to produce a bacteraemia, including chewing (Cobe 1954), periodontal scaling (Bandt *et al.* 1964), tooth brushing (Rise *et al.* 1969, Sconyers *et al.* 1973), oral prophylaxis (DeLeo *et al.* 1974), dental flossing (Ramadan *et al.* 1975) and the removal of osteosynthesis plates (Otten *et al.* 1987). Not only was the bacteraemia following dental extractions implicated with bacterial endocarditis but noninvasive procedures including scaling and root canal therapy were noted as possible causes of potentially fatal bacteraemia (Eisenbud 1962).

However, these studies had their limitations, especially in relation to the sensitivity of blood culture techniques. Thus large variations in the frequency of bacteraemia following dental extractions were recorded, ranging from 34 to 100%, depending on the different techniques employed at the time of extraction (Lazansky *et al.* 1949, Coffin & Thompson 1956) and the laboratory methods sanctioned by the authors (Hockett *et al.* 1977). It was also evident that the sole use of aerobic culturing techniques was an inadequate environment for the growth of all microorganisms originating within the oral cavity and that in order to detect a broader range of microorganisms, better anaerobic techniques would have to be employed (Rogosa *et al.* 1960, Bender *et al.* 1961, Khairat 1966, Goldberg 1968).

The improved laboratory skills and materials enabled a more accurate analysis of the quantitative and qualitative aspects of bacteraemias during the 1950s. Refinements of anaerobic culturing techniques combined with the increasing availability of antibiotics allowed researchers to determine the effect of antibacterial prophylaxis on bacterial endocarditis consequent to bacteraemia from an oral origin (Bender & Pressman 1945, Peterson 1951, Cates & Christie 1951, Merrill *et al.* 1951, Bender *et al.* 1958, Bender *et al.* 1963, Baltch *et al.* 1982, Oakley 1987). As a consequence of studies analysing antimicrobial agents in the prevention of endocarditis (Everett & Hirschmann 1977, Von Reyn *et al.* 1981, Oakley & Somerville 1981) guidelines were developed for the prophylactic use of antimicrobial agents for medically 'high risk' patients during various dental procedures (Guntheroth 1984, Kaye 1986, Pallasch 1989). However, there were no clear published guidelines relating specifically to nonsurgical root canal treatment (Bender & Montgomery 1986), although articles in the literature have discussed this issue with varying opinions (McGowan 1982, Bender *et al.* 1984, Lavelle 1996).

In addition to antibiotic prophylaxis of bacterial endocarditis, other methods of reducing bacteraemias from an oral origin were sought. The use of a presurgery mouthwash was demonstrated to cause a

significant reduction in bacteraemia from oral sources (Rise *et al.* 1969, Scopp & Orvieto 1971). Wank *et al.* (1976) were unable to show a reduction in bacteraemia with improved oral hygiene. However, Sconyers *et al.* (1973), Madsen (1974) and Silver *et al.* (1977) confirmed that greater bacteraemia occurred with higher levels of gingival inflammation although plaque levels did not appear to be directly correlated. Therefore, 'high risk' dental patients, including cardiac patients and elderly patients with native valve infective endocarditis, require meticulous oral hygiene regimes (Diener *et al.* 1964, Silver *et al.* 1977, Friedlander & Marshall 1994). Despite these recommendations, cases of infective endocarditis and subsequent deaths as a result of dental treatment or oral neglect are still reported in the literature (Younessi *et al.* 1998).

The greatest peak in detectable bacteraemia after extractions has been noted to occur within 30 s of the procedure (Roberts *et al.* 1992). Whilst this bacteraemia may cause no harm in the healthy host, bacteraemia in the susceptible host such as immunocompromised patients, the elderly, RA patients and those with damaged heart valves, may potentially cause serious systemic problems. Okabe *et al.* (1995) discussed the factors affecting bacteraemia after dental extractions and indicated a close relationship between increasing age, presence of gingival inflammation, the number of teeth extracted and the length of time taken for extractions, with bacteraemia.

Another concern of bacteraemia from an oral origin has been the role of oral bacteria in the infection of prosthetic joints, in particular late infection of total hip replacements (THR). A number of retrospective anecdotal case reports have speculated that microorganisms derived from an oral origin were responsible for late infection of prosthetic joints (Rubin *et al.* 1976, Jacobsen & Murray 1980, Mulligan 1980, Lindqvist & Slatis 1985, Strazzeri & Anzel 1986, Sullivan *et al.* 1990, Waldman *et al.* 1997). However, none of these studies have been able to confirm a positive origin from the oral cavity by the use of ribotyping. This has raised much debate as to whether individuals with prosthetic joints, undergoing dental treatment, should receive antibiotic prophylaxis (Little 1983, Tsevat *et al.* 1989). LaPorte *et al.* (1999), in reviewing almost 3000 THR patients, concluded that dental procedures were implicated in 6% of late infections in their patient sample. However, there are no controlled prospective studies that have determined if root canal treatment procedures increase the probability of infection in these patients.

Root canal flora

After Miller (1894) showed the presence of microorganisms within the root canal, multiple studies characterized the different species of microorganisms within the root canal (Henrici & Hartzell 1919, Haden 1925, Thoma 1928, Winkler & van Amerongen 1959, Sciaky & Sultzenu 1961, Engstrom 1964). An experiment by Kakehashi *et al.* (1965) revealed the significance of microorganisms in the root canal. They demonstrated that exposed pulps in gnotobiotic rats did not develop periradicular lesions. However, pulp exposures in ordinary rats led to pulpal necrosis that was determined to be caused by oral microorganisms invading the pulp tissue.

Not only were microorganisms capable of infecting root canals from the oral environment, but Robinson & Boling (1941) demonstrated that systemic bacteria were able to enter inflamed dental pulps, this process being termed anachoresis. MacDonald *et al.* (1957) identified the presence of bacteria in the canals of nonvital, intact, traumatized teeth and suggested that bacteria were capable of entering the root canal system of these nonvital teeth through the individual's general circulation or via the lymphatics and blood vessels from the periodontium.

Consequently, root canal treatment procedures were developed to attempt to eradicate these microorganisms biomechanically (Grossman 1945, Ingle & Zeldow 1958, Cvek *et al.* 1976, Bystrom & Sundqvist 1981) or with intracanal antimicrobial agents (Auerbach 1953, Matsumiya & Kitamura 1960, Shovelton & Sidaway 1960, Melville & Slack 1961, Shih *et al.* 1970). Several researchers indicated that better anaerobic culture techniques were needed to cultivate all the microorganisms present within the root canal (Brown & Rudolph 1957, Engstrom & Frostell 1961, Crawford & Shankle 1961, Melville & Birch 1967). Subsequently, specific canal medicaments could be used to eliminate these microorganisms prior to obturation of the root canal.

The benefit of determining the microflora of the root canal was questioned after studies revealed no difference in success rates in teeth that showed a positive culture at preobturation with those that were negative (Seltzer *et al.* 1963, Bender *et al.* 1964, Eggink 1982). Morse (1971) thought that there were so many inherent shortcomings with the sampling and culturing techniques that culturing of the root canal contents was of little value for clinical purposes. This was especially true for teeth with no radiographic

periradicular radiolucencies where it was considered by Stobberingh & Eggink (1982) that the possibility of a positive microbiological result was so low that root canal sampling was of no benefit. Sims (1973) suggested that sterility was unattainable in the root canal and that disinfection of the root canal need only be surgically safe. However, Pitt Ford (1982) demonstrated that a high incidence of persisting periradicular inflammation occurred from canals that had not had all the microorganisms removed by the combination of both mechanical instrumentation and intracanal medicaments. Bystrom *et al.* (1987) proved that a careful aseptic technique was capable of eliminating bacteria from root canals with consequent healing of periradicular lesions.

Developments in microbial laboratory techniques continued during the early 1970s, with a greater research emphasis being placed on the culturing of anaerobic organisms that may inhabit the root canal. Enhanced methods to grow these groups of bacteria were developed (Aranki & Freter 1972, Fulghum *et al.* 1973, Crawford *et al.* 1974, Matusow 1979). Even the process of sampling the root canal contents was improved by using an anaerobic sampling technique (Berg & Nord 1973), although the use of media supplemented with blood, rather than exposure of the microorganisms to oxygen during sampling, was deemed to be more important (Carlsson *et al.* 1977). The use of rubber dam provided an aseptic technique under which to perform the root canal treatment and microbiological sampling.

Several studies were undertaken that evaluated the quality and location of microorganisms within the root canals of teeth in relation to varying dental signs and symptoms (Taklan 1974, Wittgow & Sabiston 1975, Rowe & Binnie 1977, Griffee *et al.* 1980, Yoshida *et al.* 1987, Hashioka *et al.* 1992, Gomes *et al.* 1996). It became possible for the first time to culture and identify the presence of anaerobic microorganisms to the species level (Kantz & Henry 1974). Many articles established that the use of certain culture media and an anaerobic environment were more effective in producing successful cultures from root canal samples (Zielke *et al.* 1976, Keudell *et al.* 1976, Goodman 1977, Zielke *et al.* 1979, Griffee *et al.* 1981). A predominance of anaerobic microorganisms was shown to be present (Bergenholtz 1974). Consequently, it is now known that the ecology of the root canal flora is very diverse, containing complex bacterial interrelationships (Sundqvist *et al.* 1979, Baumgartner & Falkler 1991, Sundqvist 1992a, 1992b, Baumgartner & Watkins 1994, Sundqvist 1994).

Endodontic bacteraemia

With the understanding that a bacteraemia could occur during root canal procedures (Ross & Rogers 1943) further research was conducted to measure the bacteraemia after root canal treatment (Bender *et al.* 1960, Klotz *et al.* 1965). These studies illustrated that it was far more probable that a bacteraemia would occur if root canal instrumentation was performed beyond the apex of the root than when maintained within the confines of the root canal system. The postoperative bacteraemia following endodontic procedures was shown to last no longer than 10 min (Bender *et al.* 1960), the microorganisms within the circulation being cleared by phagocytes initially and then by the reticuloendothelial system.

It was not until 1976 that a study measuring bacteraemia subsequent to surgical and nonsurgical root canal treatment, applying an aseptic technique and improved anaerobic culture mediums, was published (Baumgartner *et al.* 1976). The authors were able to confirm from their 30 patient sample that a bacteraemia was not produced if root canal instrumentation remained within the root canal. In a follow-up study, Baumgartner *et al.* (1977) demonstrated that nonsurgical root canal treatment resulted in a much lower bacteraemia incidence (3.3%: as a result of over-instrumentation), than surgical flap reflection (83.3%), periradicular curettage (33.3%) or tooth extraction (100%). No further studies that analysed root canal treatment-induced bacteraemia were conducted or published for nearly 20 years.

By the 1980s improved transport media (Carlsson & Sundqvist 1980) enhanced consistently superior results of bacterial growth from the culturing of root canals. The use of good aseptic techniques and careful culturing methods in both anaerobic and aerobic environments were sufficient to determine that a relatively large and varied population of bacteria, in terms of species and bacterial concentration, were present in infected root canals (Zavistoski *et al.* 1980). The root canal hosts more than 100 different bacterial species (Tronstad 1992).

Whilst culturing oral anaerobic bacterial samples was an efficient method of identifying bacteria from root canals, it was confirmed that the use of DNA probes was a far more reliable and precise method for the identification of microorganisms recovered from the root system (Loesche *et al.* 1992). VMGA III has been proved to be an effective transport medium for oral microorganisms allowing a high and consistently reliable

recovery rate of anaerobic organisms, probably as a result of its low redox potential (Dahlen *et al.* 1993).

Recent studies analysing root canal treatment fostered bacteraemia have used these improved anaerobic techniques and more sophisticated diagnostic tools. Debelian *et al.* (1992) published further evidence that a bacteraemia occurred if root canal instrumentation was carried beyond the apex, and the most likely bacteria involved were anaerobic types. Subsequent studies by the same authors demonstrated that the root canals contained a diverse group of microorganisms with 132 strains being isolated and the number of species contained within an individual canal varying from one to 11. *Fusobacterium nucleatum*, the most common isolate within the root canal was located from 53% of root canals studied. They also showed that a bacteraemia could occur even if instrumentation was maintained within the root canal and that the most common organisms present within the associated bacteraemia were anaerobes (Debelian *et al.* 1995). Using SDS-PAGE electrophoresis they established that the organisms in an endodontic-related bacteraemia were identical to those arising from within the root canal (Debelian *et al.* 1996). These results were repeated with ribotyping using a DNA hybridization method (Debelian *et al.* 1997) and then using both phenotype and genetic methods (Debelian *et al.* 1998). Bacteraemias occurred in 31 to 54% of the root canal treatments, however, this study was based on a small sample group and intentional instrumentation through the apex resulted in the higher recordings of bacteraemia. Nonetheless, this study showed far greater bacteraemias resulting from root canal treatment procedures than figures produced in the past, because of more sensitive culturing and identification techniques applied.

Oral disease and general health

Over the past 25 years there has been an increased interest in the significance of oral disease and its relationship with systemic disorders. Many researchers have studied the significance of systemic disease on oral health though there has been limited scientific work undertaken to study the effect of oral disease on general health. There has been an increase in the number of case reports published in the medical literature citing dental infection as an associated factor in several systemic illnesses including: uveitis (Sela & Sharav 1975, Murphy *et al.* 1979), intracranial abscesses (Hollin *et al.* 1967, Henig *et al.* 1978, Ingham *et al.* 1978, Churton & Greer 1980, Aldous *et al.* 1987, Marks *et al.* 1988, Saal *et al.* 1988), childhood hemiplegia (Hamlyn 1978), cerebral infarction (Syrjanen *et al.* 1989), bacteriospermia and subfertility (Bieniek & Riedel 1993), necrotizing fasciitis (Gallia & Johnson 1981, Steel 1987, Stoykewych *et al.* 1992), mediastinitis (Hendler & Quinn 1978, Zachariades *et al.* 1988, Musgrove & Malden 1989), fatal endocarditis (Kralovic *et al.* 1995) and toxic shock syndrome (Egbert *et al.* 1987, Navazesh *et al.* 1994). Septicaemia has been reported to occur as a possible complication of root canal treatment (Lee 1984).

The probability of a systemic disorder occurring from oral foci of infection has been suggested to be particularly prevalent in susceptible individuals with a lowered immune response. These individuals include cirrhotic patients (Borowsky *et al.* 1979), patients taking regular steroids (Latronica & Shukes 1973), the elderly (Navazesh & Mulligan 1995) and RA patients (Ainscow & Denham 1984, Stoll *et al.* 1996). Such articles have aroused a renewed interest in the focal infection theory. However, despite the availability of more accurate culturing and microbiological identification techniques and improved scientific methodology, most of these recent case reports provide only anecdotal evidence of a possible oral source for the bacteria. The bacterial species recovered from the infected tissue in these patients have been assumed to be derived from the oral cavity but they have not had their origin reliably confirmed by ribotyping methods. Consequently, there is only speculation that a dental focus may have been the origin of the microorganisms and the possible cause of the systemic illness.

There is continued interest in attempting to determine if oral disease and associated oral microorganisms are aetiological factors in systemic disorders. Advanced research techniques over the past 10 years have taken into account the many deficiencies in older, traditional studies and thus not only has the primary factor in a disease process been analysed, but also the interrelationship of other risk factors (Stamler 1979). This has resulted in the dental profession scrutinizing more closely the correlation between dental disease and systemic disorders. In particular, the association between periodontal or dental disease and cardiovascular disease has been investigated (Genco *et al.* 1997, Loesche 1997, Beck & Offenbacher 1998). A relationship has been suggested between dental health and acute myocardial infarction (Mattila *et al.* 1989, Mattila 1993), vascular disease and oral

infections (Syrjanen 1990), coronary heart disease and dental health (DeStefano *et al.* 1993, Mattila *et al.* 1993, Mattila *et al.* 1995, Joshipura *et al.* 1996), missing teeth and ischaemic heart disease (Paunio *et al.* 1993), periodontal disease and cardiovascular disease (Beck *et al.* 1996, Beck *et al.* 1998) and acute cerebrovascular ischaemia and dental disease (Grau *et al.* 1997, Loesche *et al.* 1998). Seymour & Steele (1998) have suggested that, even taking into account the multitude of risk factors associated with coronary heart disease (The Pooling Project Research Group 1978), there is strong evidence that dental health is an important factor in the development of cardiovascular disease. Other researchers have suggested that a relationship may exist between periodontal disease and spontaneous abortions and premature births (Boyd *et al.* 1994) and periodontal disease and preterm low birthweight (Offenbacher *et al.* 1996).

In addition, pyrexia or fever of unknown origin (PUO) has been described as a possible symptom of oral infection in the medical literature for many years (Alt & Barker 1930). This is defined as episodes of fever of 38.3 degrees centigrade or more over a three-week period with an unclear cause upon examination after 1 week of hospitalization (Petersdorf & Beeson 1961). This definition has changed little over the years. Oral sepsis has been implicated in several case reports (Berry & Silver 1976, Levinson & Barondess 1979, Hedstrom *et al.* 1980, Siminoski 1993), although the evidence of an oral cause has been anecdotal. However, some authors have indicated an oral cause for PUO to be as high as 14% (Naschitz & Yeshurun 1985). This has led to debate about the potential role of oral disease in these cases (Huebner & Groat 1986, Samra *et al.* 1986, Gordon *et al.* 1988). The involvement of pyrogens within the root canal (Shinoda *et al.* 1987) or from periodontal disease (Katz *et al.* 1992) have been postulated as the most probable initiating causes in certain cases of PUO. These published case reports have once again raised the possibility of a link between various oral disease processes and general systemic illness (Van Velzen *et al.* 1984, Meurman 1997).

Oral disease, endodontics and rheumatoid arthritis

Rheumatoid disease, a chronic systemic illness, is a common autoimmune disease of unknown aetiology that affects approximately 1–3% of the population (Freese 1985). Despite the many indications of a possible relationship between RA and dental disease proclaimed during the focal infection theory era, few articles have been published analysing this relationship using current research techniques. Cecil & Angevine (1938) provided evidence to discredit the focal infection theory and demonstrated that there was no apparent link between oral disease and RA. Nordh (1965) showed no correlation between chronic oral inflammatory conditions and the presence of rheumatoid arthritis serum factors, whilst other researchers concluded that infections of the ear, nose or throat played no role in the aetiology of rheumatoid arthritis (Davidson *et al.* 1949). These findings may have led to a reduction in the quantity and quality of research investigating any further potential links between RA and oral disease. Despite the demise of the focal infection theory, there are still occasional publications linking dental foci of infection with RA (Morer 1977, Shimizu *et al.* 1977).

Scientific interest in the link between RA and oral disease was again raised when it was demonstrated that children suffering from rheumatoid arthritis and rheumatic fever had significantly more carious teeth than control cohorts, suggesting a possible correlation between the incidence of caries and rheumatoid arthritis (Drecka-Kuzan 1971). This had also been recorded by Matlashenko (1968) who showed that 44.7% of a group of RA patients had complicated forms of caries.

Malmstrom (1975), Malmstrom & Calonius (1975), Malmstrom & Jokinen (1975) and Malmstrom & Natvig (1975) analysed the significance of periradicular lesions within RA sufferers. The link between rheumatoid patients and the occurrence of periradicular lesions was found to be tenuous but some important findings were made. No relationship was shown between the histological features or number of periradicular lesions present and the incidence of rheumatoid arthritis (Malmstrom & Calonius 1975). A weak link was detected between the presence of rheumatoid factor in periradicular lesions and the rheumatoid patient group. Rheumatoid factor occurred in 6% of the RA group (Malmstrom & Jokinen 1975), although no rheumatoid factor could be found in the gingival tissues. The most convincing association observed was the immunological and histological reaction occurring in a periradicular lesion which was similar to the inflammatory reaction occurring in rheumatoid arthritis (Malmstrom & Natvig 1975). The authors also observed a positive correlation for the presence of IgG rheumatoid factor in the periapical tissues of rheumatoid sufferers. Despite the findings

from this series of articles, there have been no other papers published investigating the relationship of periaradicular periodontitis and rheumatoid arthritis.

Articles describing general oral disease patterns in RA patients, with conflicting results, do however, exist. Several authors have noted an increased number of missing teeth in rheumatoid patients compared to an unaffected population sample (Malmstrom & Calonius 1975, Tolo & Jorkjend 1990, Kasser *et al.* 1997). Blair & Chalmers (1976) had also recorded a greater number of missing teeth in RA patients and suggested the significantly earlier age of becoming totally edentulous in the RA sample could have been a result of extractions performed under the influence of the focal infection theory. These findings were in contrast to those of other research groups, who found no significant difference in tooth loss for rheumatoid arthritis patients compared with an age and sex matched systemically healthy population (Yavuzyilmaz *et al.* 1992, Arneberg *et al.* 1992). An epidemiological study of 10 523 individuals in the United States also reported no correlation between self-reported arthritis and tooth loss (Eklund & Burt 1994).

Several studies have investigated the periodontal condition of rheumatoid patients against population controls. Helminen-Pakkala (1972) observed that male RA patients had similar periodontal status to their controls, yet female RA patients had improved periodontal health compared to their controls. Sjostrom *et al.* (1989) obtained similar results observing that RA patients had better periodontal condition than the control population group. Yavuzyilmaz *et al.* (1992) also showed that RA patients were not a risk group for advanced periodontal disease. However, Kasser *et al.* (1997) obtained contrasting results and concluded that patients with longstanding active RA had a substantially increased frequency of periodontal disease compared with controls.

There have also been articles analysing the impact of RA on the well-being of affected patients and its potentially crippling nature in the orofacial region (Trimble *et al.* 1983, Larheim *et al.* 1983, Chalmers & Blair 1992, Kallenberg *et al.* 1997). In a review article by Donaldson (1995) it was noted that temporomandibular joint (TMJ) involvement in RA patients varied from between 50 and 55%. Some research is now being directed at investigating the role that oral microorganisms may have in initiating septic arthritis (Jackson *et al.* 1999), however, further research is required to ascertain whether oral microorganisms play an aetiological role in RA.

Current research

Considerable research has attempted to analyse the relationship of periodontal disease to general systemic disease (Van Dyke *et al.* 1986). In contrast, the role of periradicular disease and root canal therapy in relation to systemic health has received less consideration. The emphasis in current research is toward investigating the relationship between periodontal disease and coronary heart disease, although the association between periodontal disease and RA is also being studied (Gargiulo *et al.* 1982, Yusof *et al.* 1995, The & Ebersole 1996). There is evidence that antigen injected into the gingivae of mice is capable of inducing inflammation in the knee joint (Lens & Beersten 1988). It has been postulated that antigenic stimulation from inflamed gingival tissue in humans may potentially have the same effect. In two classic studies, administration of antigen through the root canal of primates and rabbits caused both a local and systemic immunological response (Barnes & Langeland 1966, Okada *et al.* 1967). Further animal studies have also shown that antigenic stimulation within the root canal elicits a strong immune response, stimulating the production of circulating antibodies (Steffen *et al.* 1976, Dahlen *et al.* 1982). It may therefore be possible for chronic latent periradicular lesions to stimulate arthritic inflammation in a similar manner to that displayed from the gingival tissues.

The development of advanced laboratory techniques to identify microorganisms has resulted in a greater interest in oral microbiology (Loesche *et al.* 1992). In particular, the development of the polymerase chain reaction (PCR) (Birtles 1993) has fostered the identification of many previously unculturable and unidentifiable oral microorganisms that may play a role in systemic disease (Riggio *et al.* 1996). Using these highly sensitive microbiological procedures, Debelian *et al.* (1997, 1998) have confirmed that microorganisms present in bacteraemia resulting from root canal procedures originated from the root canal. These modern laboratory methods may allow researchers to identify the origin of microorganisms, claimed by authors to be from a dental source, that have been implicated in the aetiology of septic arthritis and other systemic disease (Patrick & Lewis 1992, Marshall *et al.* 1993, Payne & Wolfe 1994). They will also help to determine if chronic dental infection has any influence upon chronic RA.

Despite all the research suggesting a link between RA and periradicular disease, no contemporary

sampling and analysing techniques have been used to study the species of microorganisms present either in the root canal or in the bacteraemia resulting from root canal therapy in systemically ill RA patients compared with a healthy population sample. Some authors have stated that there is no relationship between chronic periradicular disease and rheumatoid arthritis (Sugita 1994) although controlled scientific evidence to support this claim is lacking. Newman (1996), in reviewing the theory of focal infection, has suggested a possible link between systemic disease and RA with oral disease.

Conclusion

Further research is required to determine whether patients who are systemically ill with RA or PUO and THR patients have a greater incidence of periradicular disease and whether root canal treatment and any subsequent bacteraemia may have adverse effects on these population groups compared to matched healthy cohorts. There have been no publications to suggest that root canal treatment has any adverse systemic effects since Rogers (1976) and Ehrmann (1977) dismissed any relationship between endodontics and focal infection in review articles. With an enhanced awareness amongst the population toward their general health and an increasing concern that disturbance of the periradicular tissues may potentially cause systemic upset, it is necessary to ascertain whether root canal therapy is indeed exacerbating or causing ill health. There is currently an international body of dental practitioners who refuse to perform root canal treatment, reciting the research performed 70–80 years ago to justify this stance. However, there is neither recent scientific evidence nor studies to support this view. Further scientific research is required to establish the relationship between pulpally induced or treatment-fostered periradicular disease and systemic health.

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