The role of B cells in periodontitis

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Author's declaration

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

Signed:

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Abstract

Introduction: Varying degrees of periodontal disease affect the majority of the population. Severe forms of periodontitis have a considerable impact on oral health and quality of life. Periodontitis results from imbalances in the oral microbiome and the host immune response. The mainstay of periodontal treatment – removal of dental plaque – is only partially successful. B cells infiltrate the gingiva of periodontitis patients, but their role in pathology has not been well characterised. The overarching aim of this research was to better characterise the role of B cells in periodontitis. Periodontitis shares similarities in risk factors and aspects of immunopathology with rheumatoid arthritis. Epidemiological evidence suggests patients with rheumatoid arthritis are more likely to have periodontitis, which cannot be completely explained by shared risk factors. This has led to the hypothesis that the two diseases are immunologically linked, and that periodontitis may precede, and cause, rheumatoid arthritis. A further objective of this research was to investigate whether the autoimmunity characteristic of rheumatoid arthritis emerges in periodontitis.

Results: B cell infiltrate in the gingiva of periodontitis patients was confirmed. Periodontitis patients were found to have elevated serum titers of anti-citrullinated peptide antibodies which were generally below the diagnostic threshold for rheumatoid arthritis, and were reduced following non-surgical periodontal treatment. In a murine model of periodontitis, subtle changes to B cell phenotype were observed in tissues regional to the oral cavity in mice with periodontitis, at an early stage of disease. Such changes included increased B cell expression of receptor activator of NfκB ligand in the gingiva, and increased proportions of GC B cells in the draining lymph nodes. Some of these trends were enhanced in mice with periodontitis exacerbated by interleukin-33 treatment. B cell-deficient mice were protected from the alveolar bone loss normally induced in the model of periodontitis.

Conclusion: B cells form a substantial proportion of the inflammatory infiltrate in the gingiva of periodontitis patients. Treatment of periodontitis can reduce titers of anti-citrullinated peptide antibodies in patients, potentially reducing their risk of developing rheumatoid arthritis. Evidence from B cell-deficient mice suggests that B cells contribute to pathological alveolar bone loss. Therefore, B cells may be worthy of targeting therapeutically in periodontitis.

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Abbreviations

AAP = American Academy of Periodontology
ABC = alveolar bone crest
ACR = American College of Rheumatology
ACPA = anti-citrullinated peptide antibody
ADCC = antibody-dependent cell-mediated cytotoxicity
AGEs = advanced glycation end-products
ANOVA = analysis of variance
AP = alkaline phosphatase
APC = antigen presenting cell
APRIL = a proliferation inducing ligand
ARRIVE = Animal Research: Reporting of In Vivo Experiments
AU = arbitrary units
BAFF = B cell activating factor
BALT = bronchus-associated lymphoid tissue
BCR = B cell receptor
BM = bone marrow
BMD = bone mineral density
BOP = bleeding on probing
Breg = regulatory B cell
CAIA = collagen antibody-induced arthritis
cAMP = cyclic adenosine monophosphate
CCP = cyclic citrullinated peptide
CD = cluster of differentiation molecule
CDC = Centre for Disease Control
cDNA = complementary DNA
CE = capillary electrophoresis
CEJ = cementoenamel junction
ChIP-Seq = chromatin immunoprecipitation sequencing
CIA = collagen-induced arthritis
CLR = C-type lectin receptors
CMC = carboxymethylcellulose sodium salt
COPD = chronic obstructive pulmonary disease
CRP = C-reactive protein

cRPMI = complete RPMI

Ct = cycle threshold

C3aR = C3a receptor

C5aR = C5a receptor

CCL = chemokine (C-C motif) ligand

CCR = chemokine (C-C motif) receptor

CXCL = chemokine (C-X-C motif) ligand

CXCR = chemokine (C-X-C motif) receptor

DAB = diaminobenzidine

DAPI = 4', 6-diamidino-2-phenylindole

DAS28 = disease activity score 28

DC = dendritic cell

DC-SIGN = DC-specific ICAM-3 grabbing non-integrin

Del-1 = developmental endothelial locus 1

dH_2O = distilled water

dLN = draining lymph node

DNA = deoxyribonucleic acid

DNase = deoxyribonuclease

dNTPs = deoxynucleotide triphosphates

dsDNA = double-stranded DNA

DTH = delayed hypersensitivity reaction

DTT = dithiothreitol

ECM = extracellular matrix

EAE = experimental autoimmune encephalitis

EDTA = ethylene diamine tetra-acetic acid

EFP = European Federation of Periodontology

ELANE = elastase, neutrophil expressed

ELISA = enzyme-linked immunosorbent assay

ERK = extracellular signal-regulated kinases

ESR = erythrocyte sedimentation rate

EU = ELISA units

EULAR = European League Against Rheumatism

FcR = Fc receptor

FCS = fetal calf serum

FITC = fluorescein isothiocyanate

fMet = N-formylmethionine

fMLP = N-formylmethionyl-leucyl-phenylalanine

FO = follicular

GC = germinal centre

GCF = gingival crevicular fluid

GEC = gingival epithelial cell

GF = germ free

GIMP = GNU Image Manipulation Program

GM = gingival margin

GPCR = G protein-coupled receptor

HBSS = Hank's balanced salt solution

HEL = hen egg lysozyme

HIV-1 = human immunodeficiency virus type 1

HRP = horse radish peroxidase

ICAM = intercellular adhesion molecule

IFN = interferon

IHC = immunohistochemistry

IL = interleukin

IL-1RAP = IL-1 receptor accessory protein

iNK = innate natural killer

iNOS = nitric oxide synthase

IRF1 = interferon regulatory factor 1

LAD = leukocyte adhesion deficiency

LC = Langerhan's cell

LDL = low-density lipoprotein

LFA-1 = lymphocyte function-associated antigen 1

LN = lymph node

LOA = loss of attachment

MACS® = magnetic activated cell sorting

MAPK = mitogen-activated protein kinase

MCP = monocyte chemoattractant protein

MHC = major histocompatibility complex

Micro-CT = micro-computed tomography

MIP = macrophage inflammatory protein

MMP = matrix metalloproteinase

MRI = magnetic resonance imaging

mRNA = messenger RNA

MZ = marginal zone

NAMPT = nicotinamide phosphoribosyltransferase

NGS = next generation sequencing

NK = natural killer

nfH_2O = nuclease-free H_2O

NfκB = nuclear factor κ-light-chain-enhancer of activated B cells

NIH = National Institute of Health

OD = optical density

OPG = osteoprotegerin

OVA = ovalbumin

PAD = peptidylarginine deiminase

PAMP = pathogen associated molecular pattern

PBS = phosphate buffered saline

PBST = PBS with tween

PCR = polymerase chain reaction

PD = periodontitis

PE = phycoerythrin

PFA = paraformaldehyde

PKA = protein kinase A

PKC = protein kinase C

PMA = phorbol 12-myristate 13-acetate

PMP = phenolphthalein monophosphate

PPD = probing pocket depths

PRR = pattern recognition receptor

RA = rheumatoid arthritis

RANK = receptor activator of NfκB

RANKL = receptor activator of NfκB ligand

RF = rheumatoid factor

RPMI = media developed by Moore et al., at *Roswell Park Memorial Institute*

RNA = ribonucleic acid

RNase = ribonuclease

RT = reverse transcriptase

SA = streptavidin

SD = standard deviation

SDS = sodium dodecyl sulfate

SEM = standard error of the mean

SFB = segmented filamentous bacteria

SHP-1 = Src homology region 2 domain-containing phosphatase 1

SOFAT = secreted osteoclastogenic factor of activated T cells

SPF = specific pathogen free

STAT1 = signal transducer and activator of transcription 1

TCR = T cell receptor

TD = T cell-dependent

Tfh = T follicular helper cell

Th = T helper cell

TI = T cell-independent

TMB = tetramethylbenzidine

TNF = tumour necrosis factor

TRAP = tartrate resistant acid phosphatase

Treg = regulatory T cell

T1DM = type 1 diabetes mellitus

UK = United Kingdom

US = United States

VEC = vascular endothelial cells

qRT-PCR = quantitative real time PCR

WT = wild-type

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Chapter 1: General introduction

1.1 Periodontitis

1.1.1 Clinical characterisation of periodontitis

PD is a chronic inflammatory disease which destroys the tissues and bone supporting the teeth, and can result in tooth loss. Diagnosis of PD is usually achieved through assessing gingival inflammation and destruction by testing for BOP and measuring LOA. LOA of the gingiva is evaluated by assessing the position of the GM relative to the CEJ in combination with measuring PPD (**Figure 1.1.1.1**). Radiographic evaluation of the level of the alveolar bone relative to the CEJ enables the degree of alveolar bone loss to be determined. Alveolar bone loss may be further calculated as the proportion of bone loss relative to tooth root length: < 30 %, 30-50 %, or > 50 % alveolar bone loss can be defined as mild, moderate, or severe respectively. In line with proposals from the EFP and AAP, these clinical parameters can be used as a guide for case definitions in periodontal research. At the 2005 EFP workshop, it was proposed that mild or incipient PD could be assigned to cases where there are two or more tooth sites (on different, non-adjacent teeth) with LOA of ≥ 3 mm, and severe PD considered where ≥ 30 % of teeth have LOA of ≥ 5 mm (Preshaw, 2009). In 2007 the CDC and AAP further distinguished between moderate and severe PD, stating that cases could be considered as moderate PD where there are two or more tooth sites with LOA of ≥ 4 mm, and severe PD where there are two or more tooth sites with LOA of ≥ 6 mm (Preshaw, 2009).

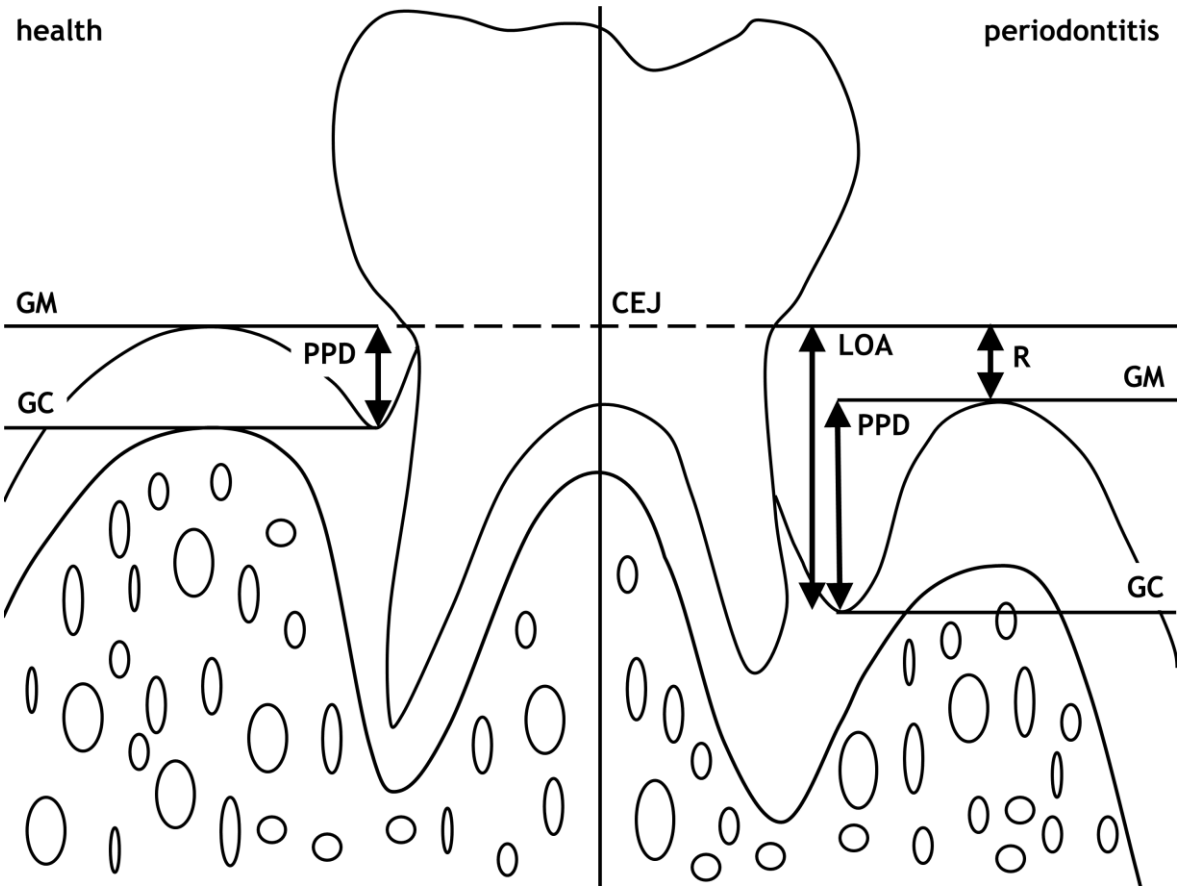

Figure 1.1.1.1. Clinical characterisation of periodontitis. Arrows indicate distances used as clinical parameters of PD. CEJ = cementoenamel junction, GC = gingival crevice GM = gingival margin, PPD = probing pocket depth, LOA = loss of attachment, R = recession. Compared with health, PD is characterised by greater PPD and LOA.

The term 'periodontal disease' can encompass both gingivitis and PD. Gingivitis is reversible inflammation of the gingiva caused by accumulation of dental plaque. Removal of the plaque enables resolution of the inflammation. PD is characterised by irreversible destruction of periodontal tissues, with evidence of persistent inflammation in most cases. Gingivitis may or may not progress to PD. The factors determining progression are complex and not fully understood. In addition to being classified as mild, moderate, or severe, PD can be broadly categorised as either aggressive or chronic. Aggressive PD, which is less common, typically affects younger age groups and progresses rapidly; chronic PD tends to affect patients over the age of 35 and progresses more slowly. Disease may be further described as 'generalised' if the majority of teeth are affected or 'localised' if only a few teeth are affected (Armitage, 2004, Albandar, 2014). The criteria for assigning patients to these different categories of PD have not been strictly standardised and practices can vary greatly between different

clinics, which makes it difficult to review research (Burt, 2005, Savage et al., 2009).

1.1.2 History and epidemiology of periodontitis

Periodontal disease is one of the most common causes of inflammatory bone loss of mankind. It is a worldwide health problem, with the majority of the population experiencing some level of gingival inflammation, and 5-8 % of the population suffering from severe forms of PD (Coventry et al., 2000, Hugoson et al., 2008).

Periodontal disease has affected humans for thousands of years. Data gathered from the dental matter of skeletons indicates that 3,000 years ago almost all British inhabitants over the age of 10 years had gingivitis, and that the prevalence of PD then was similar to the prevalence of PD today (Kerr, 1998).

In the US, analysis of the 2009-2010 National Health and Nutrition Examination Survey suggested the overall prevalence of PD is higher among men (56.4 %) than women (38.4 %). This sexual dimorphism in susceptibility to PD has been also been reported in smaller scale studies elsewhere, and has been attributed to the differential regulation of immune parameters by sex hormones (Shiau and Reynolds, 2010, Thornton-Evans et al., 2013).

Unlike other oral health problems, such as dental caries, the development of PD is not strictly dictated by changes in diet or dental hygiene habits. Many patients suffer PD in spite of good dental hygiene practice. Longitudinal studies of human populations where there is a total lack of dental hygiene, and no access to professional dental care, found that approximately 10 % of people were apparently resistant to PD, 10 % were highly susceptible, and the remainder of the population showed moderate disease progression (Loe et al., 1986, Morris et al., 2001). These observations highlight that PD is a multi-factorial disease. The global distribution of periodontal diseases - including PD - has been found to correlate with low income, poor education, and lack of access to dental care, but these factors are not deterministic (Petersen and Ogawa, 2005).

1.1.3 Treatment of periodontitis

Treatment of PD presently remains focused on physical removal of dental plaque. The main treatment strategies are dental health education, oral hygiene instruction, supra-gingival scaling of teeth, and root surface debridement. In some cases, access to the root surfaces is improved through periodontal surgery (Wang and Greenwell, 2001). Various antimicrobials such as metronidazole, amoxicillin, and chlorhexidine have been used as adjuncts to the physical removal of plaque, but have led to minimal improvements in treatment outcomes (Rooney et al., 2002, Feres et al., 2009, Silva et al., 2011, Soares et al., 2014).

PD patients require long-term maintenance treatment to manage the disease. Although the current approaches to treatment are successful in reducing gingival inflammation and retaining teeth, disease reoccurrence and progression are common (Hujoel et al., 2000, Lorentz et al., 2009, Oliveira Costa et al., 2011, Darcey and Ashley, 2011, Mdala et al., 2013).

The scale of PD prevalence and the intensity of treatment can be gauged in financial terms. The cost to the NHS in Scotland for simple, non-surgical periodontal treatment was just over £23,000,000 in 2010 (ISD Scotland National Statistics). This figure does not include the cost of specialist care or the cost of treating the sequelae of PD (tooth loss).

1.1.4 Risk factors for periodontitis

1.1.4.1 Genetics

Twin studies have indicated that susceptibility to PD is approximately 50 % genetic (Michalowicz et al., 2000, Torres de Heens et al., 2010). Genetic risk factors for PD are generally associated with perturbations of the immune response and bone mineralisation. Candidate gene studies have identified polymorphisms associated with PD located in genes that encode cytokines, cytokine receptors, PRRs, FcRs, MMPs, and the vitamin D receptor (Loos et al., 2003, Laine et al., 2005, Borges et al., 2009, Wang et al., 2009, Karimbux et al.,

2012, Pan et al., 2013). These associations have also been detected within large-scale genome-wide association studies, although none have been found to be significant when analysed in this context (Divaris et al., 2013, Teumer et al., 2013, Shaffer et al., 2014).

As a patient's genome is only partially accountable for their relative risk of PD, studies which combine genome analysis with analysis of other factors, such as the microbiome, may be more likely to shed light on disease pathogenesis. So far, colonisation with PD-associated bacteria has been linked to the chromosomal region 1q42 in a genome-wide study of 1,020 patients (Divaris et al., 2012). This field of 'infectogenomics' is in its infancy and its full potential remains to be explored. Future studies may be able to draw a network of associations between different species of PD-associated bacteria and specific genetic variances in components of the immune system. An earlier study which focused on a limited number of known candidate genes, identified moderate associations between FcR and IL-6 polymorphisms and colonisation with *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* (Nibali et al., 2007, Nibali et al., 2014).

1.1.4.2 Smoking

Among the environmental factors which influence susceptibility to PD, smoking is one of the most potent and most common. According to a National Health and Nutrition Examination Survey, current and former smoking may be responsible for more than half of PD cases in the US (Tomar and Asma, 2000). There is a striking dose-dependent effect, with heavy smokers at greatest risk of PD (Tomar and Asma, 2000).

The nicotine component of cigarettes is known to act as an immunosuppressant (Geng et al., 1995, Geng et al., 1996, Nouri-Shirazi and Guinet, 2003, Kalra et al., 2004). Smoking is associated with reduced production of cytokines and chemokines locally in the gingiva, and reduced rates of phagocytosis by neutrophils (Giannopoulou et al., 2003, Guntsch et al., 2006, Tymkiw et al., 2011, Bondy-Carey et al., 2013, Haytural et al., 2014, Souto et al., 2014a). PD patients with a history of smoking typically have lower antibody titers against

disease-associated bacteria, including *P. gingivalis* (Tangada et al., 1997, Graswinckel et al., 2004, Apatzidou et al., 2005, Giuca et al., 2014).

Numerous studies suggest that smoking promotes colonisation of the oral cavity by PD-associated bacteria (Quinn et al., 1998, Haffajee and Socransky, 2001, Gomes et al., 2006, Mdala et al., 2013, Zambon et al., 1996, Kamma et al., 1999). Potential pathogens have greater opportunities to breach the oral mucosal barrier as toxins in smoke cause tissue damage, and reduce the ability of the host to repair this damage by inhibiting fibroblast growth and collagen production (Zhang et al., 2010, Semlali et al., 2011, Joshi et al., 2014).

1.1.4.3 Obesity

After smoking, obesity is increasingly recognised as the second greatest risk factor for PD. An extensive meta-analysis showed that obesity is associated with a 30-40 % increased risk of PD (Chaffee and Weston, 2010). Obesity is marked by a significant increase in the amount of adipose tissue. Adipose tissue is composed of adipocytes and lymphocytes which actively in regulate metabolism and inflammation through the production of hormones, adipokines, and cytokines (Ouchi et al., 2011, Mraz and Haluzik, 2014). Circulating IL-6 and TNFα concentrations are positively correlated with obesity in patients (Ziccardi et al., 2002, Crowther et al., 2006, Illan-Gomez et al., 2012, Zimmermann et al., 2013). The immunological mechanism linking obesity to PD remains to be fully elucidated. One potential consequence of obesity that could contribute to the destruction of periodontal tissues is the exaggeration or dysregulation of inflammatory responses to bacterial infection (Genoni et al., 2014). Another possibility is that the integrity of the mucosal barrier is compromised in obese individuals, by elevated circulating levels of NAMPT. Extracellular NAMPT can act as both an adipokine, and a cytokine, and has been found to inhibit the regenerative capacity of periodontal ligament cells *in vitro* (Nokhbehsaim et al., 2013).

Obesity may be considered as one component of metabolic syndrome, which also involves insulin resistance, dyslipidemia, and hypertension, all of which have been linked to PD.

1.1.4.4 Insulin resistance and diabetes

Obesity and PD are associated with lower serum levels of the adipokine adiponectin. In obese mice, exogenous delivery of adiponectin reduced *P. gingivalis* infection-induced alveolar bone loss (Zhang et al., 2014b). A decrease in circulating adiponectin causes insulin resistance. Insulin resistance leads to bouts of hyperglycaemia, which can lead to the production of AGEs. AGEs appear to accumulate in the gingiva of diabetic patients (Schmidt et al., 1996). In patients with diabetes, PD is associated with increased insulin resistance and poor glycaemic control, which in turn, exacerbate PD. A five year follow-up study of patients on a periodontal therapy programme reported that progression of PD and tooth loss was significantly higher among diabetic PD patients with poor glycaemic control compared with diabetic PD patients with good glycaemic control or non-diabetic PD patients (Costa et al., 2013). It has been hypothesised that interaction of AGEs with their receptor (RAGE) on endothelial cells, fibroblasts, and leukocytes in the gingiva contributes to the persistent inflammation in PD, and is one of the underlying mechanisms by which diabetes exacerbates PD (Lalla et al., 2000a). Blockade of RAGE has been demonstrated to suppress *P. gingivalis* infection-induced alveolar bone loss in diabetic mice in a dose-dependent manner (Lalla et al., 2000b).

1.1.4.5 Age

Although there are juvenile forms of PD, in the majority of cases, the onset of PD occurs in adulthood. Cross-sectional studies have shown that the prevalence of PD is much higher in older populations compared with the general population, and longitudinal studies have clearly indicated that the risk of developing PD increases with age (Norderyd and Hugoson, 1998, Norderyd et al., 1999, Norderyd et al., 2012, Renvert et al., 2013). In young adults, PD likely manifests as a result of the gradual accumulation of environmental or lifestyle risk factors against a background of genetic predisposition. The increased risk of developing PD in old-age (≥ 65 years) is probably due to age-associated phenomena encompassing a reduction in the capacity of tissues to repair damage and widespread changes in the immune response to bacteria (Ashcroft et al., 1997b, Ashcroft et al., 1997a, Swift et al., 1999, Swift et al., 2001, Ashcroft et al.,

2002). This concept is supported by human studies of experimental gingivitis which involves total abstinence from oral hygiene to enable plaque to accumulate, following the delivery of professional dental care to ensure that all participants have comparable plaque levels at baseline. In groups of young (aged 20-25) and elderly (≥ 60 years) individuals, the latter group developed more severe gingivitis following plaque accumulation (Fransson et al., 1996, Tsalikis et al., 2002). This concurs with observations of mice and non-human primates, which naturally develop periodontal inflammation and bone loss as a product of age, in response to the indigenous oral microbiota (Ebersole et al., 2008, Liang et al., 2010). Further evidence of 'age-altered susceptibility' has been gathered from *in vitro* studies of immune cells and mesenchymal cells (**Table 1.1.4.5.1**). Age-associated changes to both the basal activity of cells, and their response to bacteria have been observed, which may have implications for PD.

Cell type	**Age-associated changes to phenotype and function**	
	Reduced	**Increased**
gingival fibroblasts	• basal IL-6 and IL-8 production	• basal MMP3 and MMP13 production
neutrophils	• signal transduction (TLR2, TLR4, CD14, and CD11b) • chemotaxis (fMLP and GM-CSF) • phagocytosis • microbicidal activity	• stimulus-induced apoptosis
macrophages	• cytokine production • chemotaxis • phagocytosis • intracellular killing	• TLR5 expression
DCs	• antigen presentation • chemotaxis • IL-12 production	• IL-6 and TNFα production • basal expression of co-stimulatory molecules

Table 1.1.4.5.1 Age-associated changes to the phenotype and function of cells. Adapted from Hajishengallis (2014). Data are sourced from a mixture of human and murine *in vitro* studies (Biasi et al., 1996, Wenisch et al., 2000, Butcher et al., 2001, Fulop et al., 2004, Agrawal et al., 2007a, Liang et al., 2009, Panda et al., 2010, Domon et al., 2014).

Telomere length is known to decrease with age, and this has been linked to oxidative stress (von Zglinicki, 2002, Harley et al., 1990). Several studies have found that the telomeres of leukocytes from PD patients were shorter than those of age-matched periodontally healthy controls (Masi et al., 2011, Masi et al.,

2014, Sanders et al., 2015). It is not known whether this sign of aging is a cause or consequence of disease. Oxidative stress is thought to have a role in the immunopathogenesis of PD, and to be one of the ways in which smoking poses a risk factor for PD (Matthews et al., 2012, Akpinar et al., 2013, Hendek et al., 2014).

1.2 *P. gingivalis* and microbial dysbiosis in periodontitis

PD may now be defined as a disease resulting from a dysregulated immune response to a dysbiotic oral microbiome. Formerly, PD was thought to be caused by a handful of 'pathogenic' bacteria; *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* described as the 'red complex' (Socransky et al., 1998). Of the red complex of bacteria, colonisation by one species – *P. gingivalis* – had the strongest positive correlation with the clinical parameters BOP and PPD, and subsequently received a great deal of attention. Presently, it is more widely accepted that *P. gingivalis* and the other red complex members are not 'pathogenic' *per se*, as they do not strictly adhere to Koch's postulates. *P. gingivalis* is often undetectable in PD patients, with as few as 40 % harbouring detectable numbers of *P. gingivalis* in one study (Eick and Pfister, 2002). Meanwhile, *P. gingivalis* can be detected in as many as 25 % periodontally healthy individuals (Griffen et al., 1998). Studies of spouses of PD patients have demonstrated that they are more likely to harbour *P. gingivalis* and other disease-associated bacteria in subgingival plaque and saliva than the general population, but this is not associated with changes to their periodontal health (Von Troil-Linden et al., 1995, von Troil-Linden et al., 1997). Moreover, using mice it has been demonstrated that introduction of *P. gingivalis* to the oral cavity, in the absence of other oral bacteria, is not able to cause disease (Hajishengallis et al., 2011).

A better understanding of how *P. gingivalis* and other putative periodontal pathogens operate within a community of bacteria has emerged with advancements in technology. Socransky *et al.* (1998) studied the relationships between different species of bacteria and between groups of bacteria using checkerboard DNA: DNA hybridisation. This involved isolating the DNA of 40 cultivable species from samples of plaque, and fixing it to a membrane which

was then hybridised with digoxigenin-labelled chromosome probes that could be detected using AP-conjugated anti-digoxigenin antibodies.

A decade later, the Human Oral Microbiome Database was set-up, and now lists over 700 species of bacteria detected in the human oral microbiome, of which approximately 49 % are both named and cultivated. These have all been identified by cloning and sequencing their 16S rRNA genes using CE-based sequencing. Full genome sequences are available for approximately 46 % (315) of the taxa listed. This database was an off-shoot from the NIH-supported Human Microbiome Project which aims to identify all of the major organisms comprising the human microbiome to help establish their role in health and disease (Chen et al., 2010, Dewhirst et al., 2010). Concurrently, the Human Oral Microbe Identification Microarray was developed which enabled analysis of the abundance of 300 different species in dental plaque including those which were uncultivable (Paster et al., 2001). Using these tools, it was revealed that PD is associated with a greater increase in microbial diversity than previously envisaged (Colombo et al., 2009).

The preferred method for compiling a full profile of the oral microbiome is now NGS. Compared with CE-based sequencing, NGS enables multiple, larger DNA fragments to be sequenced in parallel, which in turn increases the level of resolution of bacterial communities, as well as the speed with which the data can be collected (Shendure and Ji, 2008). In addition to sequencing bacterial DNA, NGS provides a platform for the analysis of transcriptomics, and DNA-protein interactions (ChIP-Seq). NGS therefore offers the opportunity to investigate the activities associated with different bacterial biofilms. Typically in phylogenetic studies of the oral microbiome, bacterial DNA is isolated from clinical samples and amplicons of virtually all bacterial 16S genes present are produced by using primers that recognise conserved flanking regions. NGS is then used to sequence regions of 16S rRNA genes which are aligned with those recorded in the Human Oral Microbiome Database to identify different species.

With NGS, the differences and the surprising similarities between the oral microbiota associated with PD and with health have been further characterised. Alongside the previously identified ‘red complex’, novel species have been

identified in the periodontal pockets of PD patients with active disease. One study discovered an abundance of the Gram positive bacterium *Filifactor alocis* in subgingival plaque from PD patients. Surprisingly, this study suggested that the microbial composition associated with PD is generally a mixture of Gram negative and Gram positive bacteria. Previously, Gram negatives were believed to dominate in disease (Griffen et al., 2012).

The relative abundance of over 100 species have been associated with PD, and more than 50 species associated with health. The revelation that changes to the composition of the oral biofilm in PD is due to the rise of pre-existing low-abundance species rather than the invasion and displacement of health-associated species by putative periodontal pathogens is the result of the increased power of detection of low-abundance species conferred by NGS compared to older molecular techniques (Abusleme et al., 2013). The challenge remains to establish which of the bacteria present in PD have an active role in the disease, and which benefit from the micro-environment but contribute little to the stability of the disease-associated biofilm (Griffen et al., 2012, Schwarzberg et al., 2014).

There is substantial variability in the microbiota between different tooth sites in a single patient, and between individuals within the same patient group (Griffen et al., 2012, Abusleme et al., 2013, Ge et al., 2013). It is thought that this variability is influenced by numerous environmental and genetic host factors including the geographical location, race, and smoking status (Nasidze et al., 2009, Ge et al., 2013).

Microbiologists are getting closer to the goal of determining which bacteria or combinations of bacteria in oral biofilms are game-changers in PD pathogenesis following major developments in microarrays, which enable culture-independent linkage of phylogenetic and functional analyses. GeoChip 4.0 and HuMiChip 1.0 contain 83,992 and 36,802 probes respectively, which target key functional genes in various biochemical pathways such as antibiotic resistance; virulence; and the metabolism of amino acids, carbohydrates, lipids, glycan, vitamins, and nucleotides (Tu et al., 2014a, Tu et al., 2014b). Comparison of the metabolic activities of bacteria in plaque samples from patients with PD and periodontally

healthy subjects revealed considerable differences (Li et al., 2014a). Genes enriched in disease-associated bacteria included four which have roles in lysine, alanine and arginine metabolism, and three which are involved in GAG degradation. Conversely, several genes involved in amino acid synthesis and pyrimidine synthesis had a relatively lower abundance in plaque samples from PD patients compared with periodontally healthy patients.

Following an investigation of the murine model of PD, it has emerged that a single disease-associated low-abundance bacterium, *P. gingivalis*, is capable of conducting a massive shift in the oral microbiota. Many studies previously demonstrated that oral inoculation of mice with *P. gingivalis* leads to gingival inflammation and alveolar bone loss characteristic of PD (Baker et al., 1994, Sasaki et al., 2004). Using qRT-PCR, Hajishengallis *et al.* (2011) revealed that introduction of *P. gingivalis* to the murine oral cavity led to an increase in the total cultivable commensal bacterial load (of which *P. gingivalis* itself constituted < 0.01 %) and to alterations in the composition of the oral microbiota compared with uninfected control mice. These quantitative and qualitative changes to the oral biofilm have hence formed the definition of 'microbial dysbiosis' in the context of PD. The ability of *P. gingivalis* to induce microbial dysbiosis is evidently dependent on the interaction between *P. gingivalis* and complement, since it was unable to do so in C3aR and C5aR KO mice, which were resistant to infection-induced alveolar bone loss. The current paradigm is that *P. gingivalis* subverts complement and inhibits phagocytosis by innate immune cells, boosting the survival of an array of bacteria which results in the observed dysbiosis (Wang et al., 2010, Hajishengallis et al., 2011, Liang et al., 2011). GF mice were found to be resistant to *P. gingivalis* infection-induced alveolar bone loss - although the ability of *P. gingivalis* to colonise GF mice was not significantly altered. This indicates that alveolar bone loss in the murine model of PD is dependent on the presence of both *P. gingivalis* and other species of bacteria, and results from the interaction between the host and a dysbiotic oral microbiota.

Understanding the mechanisms behind the development of dysbiosis, the interactions between different bacteria within the dysbiotic biofilm, and the interaction of the dysbiotic community with the host could lead to the

development of interventions which reverse the dysbiotic state (Jenkinson and Lamont, 2005, Curtis, 2014). A small peptide antagonist which blocks the interaction between *P. gingivalis* and *Streptococcus gordonii* reduced *P. gingivalis* colonisation and protected mice from *P. gingivalis* infection-induced alveolar bone loss (Daep et al., 2011). The utility of such a drug in PD patients may be limited however, as there are potentially other disease-associated bacteria which are equally capable of orchestrating dysbiosis. Broader disruption of the dysbiotic biofilm may be required to treat disease and could be achieved by targeting quorum signalling: the non-contact communications between bacteria (Hentzer and Givskov, 2003, Jenkinson and Lamont, 2005).

1.3 Different strains of *P. gingivalis*

Despite the revelation that PD is the product of polymicrobial dysbiosis, *P. gingivalis* continues to be the sole focus of many microbe-host interaction studies in the context of PD. This is partly because *P. gingivalis* possesses an assortment of unique virulence factors, the activity of which benefit not just *P. gingivalis*, but other members of the microbiota.

Around 100 different strains of *P. gingivalis* have been isolated from humans, monkeys, and other animals (Loos et al., 1993, Laine and van Winkelhoff, 1998). A selection of these have become commonly used in the laboratory. These strains vary slightly in the virulence factors they possess, most notably, in the presence and type of polysaccharide capsule, and in the type of fimbriae (**Table 1.3.1**).

Historically, virulence has been assessed through the use of the skin abscess mouse model or the chamber model of infection. The skin abscess model involves subcutaneous inoculation of bacteria into the dorsum. Mice are then monitored daily for the size and ulceration of lesion, body weight, and general health. At the end-point, less than a week after the inoculation, systemic spread of the bacteria is assessed (Ebersole et al., 1995, Katz et al., 1996, Laine and van Winkelhoff, 1998). These virulence studies performed are generally in agreement that the capsulated strains 53977, W50, and W83 are more virulent than the non-capsulated strains 381 and ATCC 33227 as they were associated

with larger lesions and greater lethality. Subsequent studies using the murine model of PD involving oral infection with *P. gingivalis* confirmed that the capsulated strains caused greater alveolar bone loss, with one study indicating that *P. gingivalis* W83 caused the greatest alveolar bone loss (Baker et al., 2000a, Wilensky et al., 2009, Marchesan et al., 2012). For this reason, the W83 strain was selected for use in all the murine studies of PD conducted in the following chapters.

Strain	Capsule ± (serotype)	FimA isoforms (length)	Virulence high/ low
W83	+ (K1)	type IV (very short)	high
W50 (ATCC 53978)	+ (K1)	type IV (very short)	high
HG184	+ (K2)	type II (long)	high
A7A1-28 (ATCC 53977)	+ (K3)	type II (long)	high
ATCC 49417	+ (K4)	type III (long)	high
HG1690	+ (K5)	type II (long)	high
HG1691	+ (K6)	type Ib	high
381	-	type I (very long)	low
ATCC 33227	-	type I (very long)	low

Table 1.3.1. Different strains of *P. gingivalis* isolated from humans. Examples of *P. gingivalis* strains originally isolated from PD patients, some of which are commonly used in the laboratory (Loos et al., 1993, Fujiwara et al., 1993, Sojar et al., 1997, Laine and van Winkelhoff, 1998, Mikolajczyk-Pawlinska et al., 1998, Baker et al., 2000a, Naito et al., 2008, Fabrizi et al., 2013, Kerr et al., 2014, Siddiqui et al., 2014).

Six different capsular serotypes have been identified (K1-6). *P. gingivalis* W83 has the K1 serotype (Laine et al., 1996). Although several *in vitro* studies have reported that K1 and K2 bearing *P. gingivalis* strains have superior resistance to phagocytosis, there is no trend in *P. gingivalis* with a particular capsular serotype being more strongly associated with virulence *in vivo* (Sundqvist et al., 1991, Laine and van Winkelhoff, 1998, d'Empaire et al., 2006, Vernal et al., 2009).

Different forms of fimbriae have been characterised. *P. gingivalis* expresses two distinct types of fimbriae: major (FimA) and minor (Mfa 1). Major fimbriae are classified into six types (type I-V and Ib) based on the isoforms of FimA gene, the key subunit. These different FimA genotypes are associated with different lengths of major fimbriae, although the length is actually regulated by the FimB gene (Nagano et al., 2010). The extremely long length of major fimbriae in *P.*

gingivalis 381 and ATCC 33227 is due to a deficiency in FimB (Nagano et al., 2012).

P. gingivalis strains with type II major fimbriae are most commonly found in the mouths of PD patients, and are more frequently detected in deeper periodontal pockets (Amano et al., 1999, Miura et al., 2005, Zhao et al., 2007, Fabrizi et al., 2013). A selection of *in vitro* studies have indicated that *P. gingivalis* with type II major fimbriae are enhanced in their ability to adhere to and invade epithelial cells (Amano et al., 2004, Nakagawa et al., 2006, Kato et al., 2007). However, not all *P. gingivalis* strains with type II major fimbriae are highly virulent in the skin abscess mouse model (Inaba et al., 2008). The lack of a strong correlation between the type of major fimbriae and virulence observed in the murine abscess model could be due to advantages associated with particular types of major fimbriae being dependent on the context of the oral cavity, as well as other strain-specific features masking the role of major fimbriae in this scenario.

1.4 The host immune response to *P. gingivalis*

A fully-functioning immune system can usually manage the oral microbiome, prevent invasion by potential pathogens, and minimise damage to the host tissues if invasion does occur. *P. gingivalis* contributes to dysbiosis and the development of PD by subverting the host immune response. The following sub-sections of this chapter describe, in detail, the interactions between *P. gingivalis* and components of the innate and adaptive immune system.

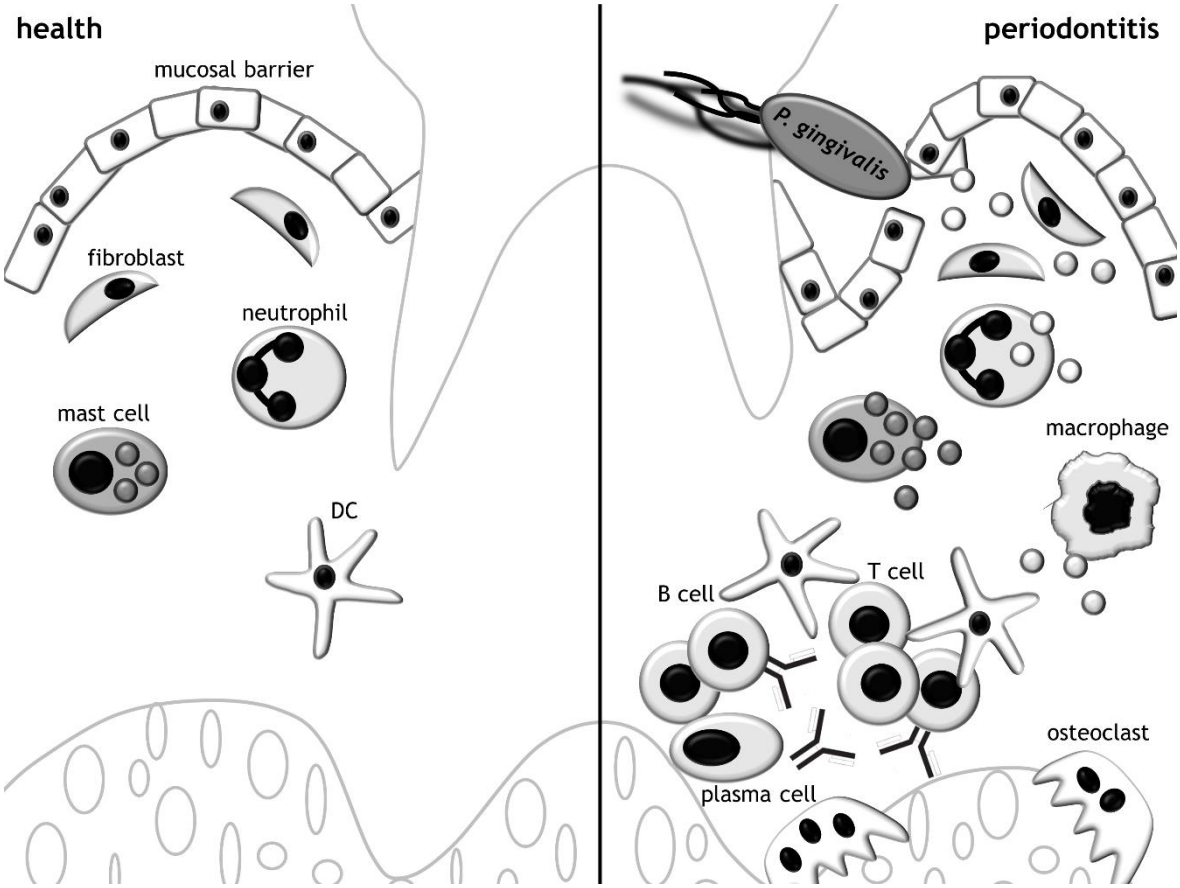

Figure 1.4.1 Summary of the host immune response to *P. gingivalis* in periodontitis. In health, neutrophils migrate through the gingiva. There are a small number of resident mast cells and DCs. In susceptible hosts, *P. gingivalis* colonisation can cause microbial dysbiosis, characterised by an increase in the total biofilm biomass, and a shift in the relative abundance of different species. Interplay between a dysbiotic oral microbiome and a dysregulated immune response leads to chronic inflammation of the gingiva and the development of PD. First an innate immune response is triggered when the oral mucosal barrier is breached by *P. gingivalis*; there is increased influx of neutrophils, activation of mast cells, and recruitment of macrophages. As *P. gingivalis* subverts this innate immune response, it fails to clear the bacteria and inflammation persists. An adaptive immune response is launched following the activation of DCs. Lymphocytes are recruited to the tissue and aggregates of lymphocytes form near DCs. Antibody producing plasma cells appear. Downstream of these activities of the immune system, osteoclast activity is increased, causing resorption of the alveolar bone.

1.4.1 The innate immune response

1.4.1.1 The mucosal barrier

Within the gingival crevice, *P. gingivalis* is able to live in the biofilm, in the GCF, and in and on the GECs that line the crevice (Hajishengallis and Lamont, 2014). In PD patients, there is increased invasion of the gingival tissue by *P. gingivalis*

(Dibart et al., 1998, Colombo et al., 2007). *P. gingivalis* breaches the oral mucosal barrier by two major mechanisms: invasion of GECs, and destruction of GEC junctions.

The FimA subunit of major fimbriae binds to ß1-integrin receptors on the GEC surface (Njoroge et al., 1997, Yilmaz et al., 2002). This then recruits and activates the integrin focal adhesion complex. Simultaneously, *P. gingivalis* secretes the serine phosphatase SerB, which enters cells and activates the actin depolymerising molecule cofilin (Moffatt et al., 2011). As a result, there is transient and localised disruption of the cytoskeleton, which allows the bacterium to move inside the GEC (Yilmaz et al., 2003).

From inside the GECs, *P. gingivalis* degrades junctional proteins, thereby disrupting the bonds between adjacent GECs, and between GECs and the underlying ECM. *P. gingivalis* possesses three cysteine proteinases known as gingipains. Of these, two are arginine-specific (RgpA and RgpB), and one is lysine-specific (Kgp). RgpA and RgpB degrade paxillin, a focal adhesion-associated adaptor protein (Nakagawa et al., 2006). Gingipains are also thought to be responsible for the degradation of other cell adhesion molecules, including integrins and catenins (Hintermann et al., 2002). As a result of this proteolysis, GEC morphology is altered and the wound healing function of GECs is impaired *in vitro* (Hintermann et al., 2002, Nakagawa et al., 2006). Cytochalasin D was found to inhibit *P. gingivalis* degradation of paxillin in GECs, proving that this proteolysis is dependent on cell invasion (Nakagawa et al., 2006).

Whilst inside GECs, *P. gingivalis* SerB dephosphorylates and inactivates NfκB, and induces the expression of suppressive micro-RNAs. This re-programs host cell gene expression, leading to temporary paralysis of chemokine expression (Moffatt and Lamont, 2011, Takeuchi et al., 2013). In mice, the overall expression of CXCL1 (the functional murine ortholog of human IL-8) in the gingiva was significantly reduced at four days post-infection with *P. gingivalis*, but returned to baseline or above basal levels at six weeks post-infection (Hajishengallis et al., 2011).

The gingipains secreted by *P. gingivalis* can rapidly degrade various chemokines secreted by GECs, which may further stall the recruitment of leukocytes. IL-1, IL-6, and IL-8 can all be degraded by Kgp (Stathopoulou et al., 2009). This temporary paralysis of chemokine gene and protein expression is thought to offer *P. gingivalis*, and bystander bacteria, the opportunity to establish colonisation of the gingival tissues, unimpeded by patrolling neutrophils.

1.4.1.2 Neutrophils

In health, co-ordinated gradients of chemokines and adhesion molecules direct the migration of neutrophils to the gingival crevice (Hajishengallis and Hajishengallis, 2014). This migration is not solely regulated by the microbiome since neutrophils are recruited to the gingiva of GF mice, albeit in small numbers (Zenobia et al., 2013). The presence of commensals is associated with increased IL-8 production by GECs, which in turn increases the recruitment of neutrophils (Zenobia et al., 2013). Bacteria-derived products such as fMLP peptide and proteins modified with fMet are also potent chemokines for leukocytes, especially neutrophils (Gallin et al., 1983, Marasco et al., 1984).

Neutrophils roll along vessel walls through a series of weak interactions between endothelial selectins (P- and E- selectins) and neutrophil glycoproteins. IL-8 or microbial products initiate a conformational change in the ß2-integrin LFA-1, which subsequently binds ICAM-1 on endothelial cells with greater affinity to facilitate extravasation. Neutrophils then continue to follow the chemokine gradient as they migrate through the lamina propria of the gingival tissue. Eventually, they cross the GECs lining the gingival crevice to enter the GCF.

In PD, disruption of the chemokine gradients by *P. gingivalis* may delay the recruitment of neutrophils. When neutrophils do reach the gingival crevice, they struggle to phagocytose bacteria within a biofilm, instead undergoing frustrated phagocytosis, whereby the contents of lysosomes and other pro-inflammatory substances are released into the extracellular space, leading to tissue damage (Ryder, 2010, Chapple and Matthews, 2007).

Although undoubtedly a potential source of tissue damage, there is clearly a role for neutrophils in maintaining periodontal health. This is exemplified by the association of congenital conditions which give rise to neutropenia with early-onset PD. One example of this type of condition arises from mutations in the neutrophil elastase gene *ELANE*, which causes the production of misfolded elastase thought to induce apoptosis of neutrophil precursors in the BM (Ye et al., 2011).

Similarly, severe PD develops early on in patients with LAD. LAD refers to a group of inherited disorders in which the normal extravasation of circulating neutrophils to sites of infection or inflammation is inhibited due to defects in the integrins or selectins involved in adhesion to vascular endothelial cells (Waldrop et al., 1987, Deas et al., 2003, Hanna and Etzioni, 2012).

Murine models have confirmed that PD can be exacerbated by a reduction in the number of neutrophils recruited to the gingiva. Mice deficient in LFA-1, P- and E-selectin, or CXCR2 (the chemokine receptor for CXCL1, the functional murine ortholog of IL-8), have a higher oral bacterial load, and apparently increased alveolar bone loss in response to normal flora (Niederman et al., 2001, Hajishengallis et al., 2011, Zenobia et al., 2013).

On the other hand, murine models have also demonstrated that excessive neutrophil recruitment can be disadvantageous in the pathogenesis of PD. Del-1 is an endogenous negative regulator of neutrophil diapedesis which blocks the interaction between LFA-1 and ICAM-1 (Choi et al., 2008). Mice deficient in Del-1 have increased neutrophil infiltrate in the gingiva and spontaneously develop alveolar bone loss (Eskan et al., 2012).

1.4.1.3 Mast cells

Low numbers of mast cells reside in the gingival tissue. They are usually located near small blood vessels beneath the GEC barrier. An increase in mast cells, and specifically, activated mast cells, have been observed in the gingival tissue of PD patients compared with periodontally healthy subjects (Gunhan et al., 1991, Batista et al., 2005, Huang et al., 2012).

Mast cells express FcRs which bind IgE (FcεRI) and IgG ($Fc_{\gamma}RIII$). Tissue resident plasma cells in the gingiva of PD patients, have been reported to frequently produce IgG, but rarely produce IgE (Okada et al., 1983, Ogawa et al., 1989, Marton et al., 1990, Takahashi et al., 1997). Activation of mast cells can also occur downstream of either the classical or alternative complement pathway as mast cells express the C5aR, which binds C5a. *P. gingivalis* is known to selectively inhibit other components of the complement cascade and generate C5a using its gingipains - which proteolytically destroy most complement proteins, but double-up as C5 convertases (Popadiak et al., 2007).

As a result of activation, mast cells undergo degranulation, releasing a vast array of enzymes, cytokines, chemokines, and other inflammatory mediators into the microenvironment. Histamine released by mast cells increases the permeability of blood vessels, assisting the extravasation of leukocytes. MMPs and serine proteases (tryptase and chymase) breakdown ECM, making it easier for leukocytes to migrate through connective tissue (Zhou et al., 2012). Cytokines and chemokines further help to attract and activate neutrophils, macrophages, and lymphocytes (Steinsvoll et al., 2004).

Several of the inflammatory mediators produced by mast cells preferentially support a Th2 biased adaptive immune response by modulating DC function (Mazzoni et al., 2006, Kitawaki et al., 2006). A Th2 response is thought to benefit the survival of the oral bacteria involved in PD, and be detrimental to the host. This is discussed further in section **1.4.2.2**.

1.4.1.4 Macrophages

Macrophages form only 1-2 % of the inflammatory infiltrate in the gingiva of PD patients, but even fewer are found in healthy gingival tissue (Berglundh and Donati, 2005, Jagannathan et al., 2014). The precursors of macrophages, monocytes, can be recruited to sites of infection, by essentially the same mechanism as neutrophils, but they are responsive to a slightly different array of chemokines including MCP-1, MCP-2, MCP-3, MIP-1α, and MIP-1β (Janeway, 2005). Expression of MCP-1 (also known as CCL2) and MCP-3 is increased in the gingiva of PD patients compared with periodontally healthy subjects (Hanazawa

et al., 1993, Garlet et al., 2003, Dezerega et al., 2010). *In vitro* studies indicate that VECs and GECs can secrete these monocyte chemokines in response to oral bacteria (Kusumoto et al., 2004, Maekawa et al., 2010, Peyyala et al., 2012).

Direct ligation of TLR4, and CD14 by bacterial components, or cross-linking of FcRs by bacteria-bound IgG1 and IgG3 would normally lead macrophages to phagocytose a potential pathogen. Like neutrophils, macrophages have several methods of killing engulfed bacteria. Lysosomes filled with enzymes and antimicrobial peptides fuse with the phagocytic vacuole. Added to this mixture are a variety of toxic chemicals including nitric oxide, superoxide, and hydrogen peroxide, which are rapidly synthesised upon phagocytosis (Janeway, 2005).

Paradoxically in PD, the presence of macrophages in the gingiva appears to be more beneficial to *P. gingivalis* than to the host. Depletion of macrophages has been shown to protect mice from alveolar bone loss induced by oral infection with *P. gingivalis* (Lam et al., 2014). *P. gingivalis* manipulates macrophages, promoting inappropriate, inflammatory immune responses and suppressing protective, microbicidal immune responses.

P. gingivalis subverts macrophage function through several strategies. Direct recognition of *P. gingivalis* by macrophages is prevented by the antagonistic effect of *P. gingivalis* LPS on TLR4 (Bainbridge et al., 2002, Coats et al., 2003, Sawada et al., 2007, Zhang et al., 2008), and the degradation of CD14 by *P. gingivalis* gingipains (Wilensky et al., 2014). *P. gingivalis* gingipains also degrade IgG1, IgG3, and the central complement component C3, thereby inhibiting opsonisation and phagocytosis (Vincents et al., 2011, Popadiak et al., 2007).

P. gingivalis gains entry to macrophages by an alternative, safer route, using its major fimbriae to interact with TLR2 and hijack the CR3 complement pathway (Wang et al., 2007). It then subverts several other signalling pathways to enhance its survival inside macrophages, for example, by initiating cross-talk between TLR2 and C5aR. This pathway is augmented by *P. gingivalis* gingipain-generated C5a. C5a activates C5aR, a GPCR. Activation of C5aR synergises with TLR2 activation by *P. gingivalis* to increase intracellular cAMP which activates PKA, which inhibits iNOS. Simultaneously, activation of CR3 by *P. gingivalis*

fimbriae, and of C5aR by C5a, leads to activation of ERKs which inhibits activation of IRF1. IRF1 is a transcription factor which controls expression of cytokines, its inhibition results in suppression of IL-12 and IFN_{γ} production (Wang et al., 2010). The importance of these mechanisms of *P. gingivalis* evasion of host defences has been demonstrated by the resistance of C3 KO mice, C5aR KO mice, and TLR2 KO mice, to *P. gingivalis* infection-induced alveolar bone loss (Burns et al., 2006, Liang et al., 2011, Maekawa et al., 2014). Local inhibition of C5aR has been associated with a reduction in TNFα, IL-1, IL-6, and IL-17 in the gingiva of mice with PD (Abe et al., 2012b). Local inhibition of C3 has similarly been associated with a reduction in pro-inflammatory cytokines in the GCF of non-human primates (Maekawa et al., 2014). These data suggest that these complement pathways are linked to the production of inflammatory cytokines which in turn contribute to alveolar bone loss.

1.4.2 The adaptive immune response

1.4.2.1 Dendritic cells

Different subsets of DCs occupy different regions of the gingival tissue. $CD1a^+$ immature LCs predominantly reside in the gingival epithelium, and $CD83^+$ mature dermal DCs specifically infiltrate the lymphocyte-rich lamina propria. Dermal DCs have been found to increase in number in PD (Jotwani et al., 2001, Cutler and Jotwani, 2006, Cury et al., 2008).

LCs can be derived from $CD14^+$ monocytes, and both LCs and dermal DCs can be derived from $CD34^+$ haematopoietic progenitor cells (Chomarat et al., 2003, Ueno et al., 2007). Little is known about how DCs or DC precursors are recruited specifically to the gingiva. The expression of MCP-1 and MIP-3α positively correlate with increased densities of $CD1a^+$ DCs. (Souto et al., 2014b). MCP-1 binds the chemokine receptor CCR2 and MIP-3α binds CCR6. It is known that *in vitro* and *in situ* immature $CD1a^+$ LCs express high levels of CCR6 and are selectively responsive to MIP-3α, whereas $CD14^+$ precursors of DCs and macrophages are not attracted by MIP-3α. Therefore, it has been speculated that MIP-3α may have an important role in the recruitment of LCs to the gingiva

in health and disease (Dieu et al., 1998, Charbonnier et al., 1999, Cook et al., 2000).

In their immature state, DCs constitutively take up antigens by phagocytosis and non-specific micropinocytosis. Uptake of a potential pathogen and activation of PPRs usually induces maturation of DCs, whereby they undergo phenotypic changes that facilitate their migration toward lymphoid organs and priming of naïve T cells. Mature DCs are less active in taking up and processing antigen, but more efficient at presenting antigen as they increase their surface expression of MHC II and co-stimulatory molecules (Janeway, 2005).

Like macrophages, DCs can be activated by LPS and other PAMPS. The exact set of PPRs expressed by DCs depends on their subtype and location (Geijtenbeek et al., 2004). Epidermal LCs isolated from skin express TLR1, TLR2, TLR3, TLR6, and TLR10, but lack TLR4 and TLR5. In contrast, dermal DCs from skin express TLR2, TLR4, TLR5, and other TLRs which recognise bacterial PAMPs. Therefore, dermal DCs may be more specialised for bacterial recognition (Kadowaki et al., 2001, Ueno et al., 2007). It is not clear which TLRs are expressed by the various DCs in the gingiva, but there is some indication of the CLRs that are expressed: langerin (CD207), macrophage mannose receptor (CD206), and DC-SIGN (CD209) have all been identified. The expression of langerin was reduced in the gingival epithelium in chronic PD, while the expression of macrophage mannose receptor and DC-SIGN is increased in the lamina propria in chronic PD (Jotwani et al., 2001, Jotwani and Cutler, 2003). Unlike TLRs, activation of CLRs often has immunosuppressive effects, and does not directly lead to DC maturation (Cutler and Teng, 2007).

P. gingivalis minor fimbriae subvert the normal DC response via DC-SIGN-mediated internalisation (Zeituni et al., 2009, Zeituni et al., 2010). DC-SIGN$^+$ dermal DCs have been found in greater numbers in the gingiva of PD patients compared with periodontally healthy subjects (Jotwani and Cutler, 2003). This same pathway is used by other pathogens (such as HIV-1 and *Mycobacterium tuberculosis*), which share a common feature of manipulating the adaptive T cell response, and causing chronic infection (van Kooyk and Geijtenbeek, 2003). Compared to macrophages, DCs have less robust bactericidal capabilities,

therefore *P. gingivalis* internalised by DCs may have increased chance of survival.

Cytokine production and migration by DCs are affected by this interaction between bacteria and DC-SIGN. In the gut, the binding of probiotic bacteria to DC-SIGN induces tolerance, characterised by an increase in the generation of IL-10 producing Tregs (Smits et al., 2005). This has led to the hypothesis that *P. gingivalis* may also be attempting to induce a state of tolerance by interacting with DC-SIGN (Cutler and Teng, 2007). An increase in the proportion of Tregs has been observed in the gingiva of PD patients (Cardoso et al., 2008). Numerous studies of the interaction between *P. gingivalis* and DCs report a change in cytokine production, but the nature of this change is variable depending on several experimental parameters. If DCs are stimulated with purified *P. gingivalis* LPS, this tends to increase the production of Th2 cell polarising cytokines (Jotwani et al., 2003, Pulendran et al., 2001). However, there are conflicting reports concerning the cytokine output of DCs stimulated with whole, live *P. gingivalis* bacteria, and these differences may, in part, be strain-dependent (Vernal et al., 2009, Marchesan et al., 2012, Vernal et al., 2014).

Peripheral blood myeloid DCs from PD patients have increased CXCR4 and reduced CCR7 expression compared with periodontally healthy subjects. These changes in chemokine receptor expression can be induced *in vitro* by *P. gingivalis* infection of myeloid DCs. Presumably CCR7-dependent homing of DCs to secondary lymphoid organs is disrupted as a result of reduced CCR7 expression. The proportion of myeloid DCs in circulation, and the *P. gingivalis* carriage rate by these circulating DCs is increased in PD patients compared with periodontally healthy subjects (Carrion et al., 2012, Miles et al., 2014).

1.4.2.2 T cells

T cells form approximately 20 % of the inflammatory infiltrate in the gingiva of PD patients, while few T cells can be found in healthy gingival tissue (Okada et al., 1983, Berglundh and Donati, 2005). The formation of mature DC and $CD4^+$ T cell clusters in the inflamed gingiva suggests that there may be ongoing *in situ* maturation of DCs and priming of T cells in addition to the priming of T cells in

secondary lymphoid organs (Cirrincione et al., 2002, Jotwani and Cutler, 2003, Cutler and Jotwani, 2006).

Numerous different subsets of effector $CD4^+$ T cells have been characterised based upon the transcription factors and cytokines they express, the cytokines they respond to, and their function. Of these, the most-studied in the oral cavity are Th1, Th2, Th17, and Tregs (**Table 1.4.2.2.1**). Th1 responses are usually directed against intracellular and extracellular viral and bacterial infections. Th1 responses primarily support the activities of macrophages, NK cells, and $CD8^+$ T cells, and therefore are synonymous with 'cell mediated immunity' (Janeway, 2005). Th2 responses are generally most effective at dealing with helminth parasites and are associated with high titers of IgM, IgA, and IgE (Else et al., 1993, Pritchard et al., 1995). Th17 responses have only been characterised relatively recently, but appear to be triggered by infections with extracellular pathogens including fungi, bacteria, and parasites (Bettelli et al., 2007). Tregs are responsible for inducing tolerance to harmless stimuli, helping regulate the activities of other $CD4^+$ T cells, and dampening down inflammation following the successful clearance of a pathogen (Piccirillo, 2008).

$CD4^+$ T cell subset	**Transcription factor**	**Polarising cytokines**	**Secreted cytokines**
Th1	T-bet	IL-12	IFN_γ
Th2	Gata-3	IL-4	IL-4 IL-5 IL-13
Th17	$Ror_\gamma t$	IL-1 IL-6 IL-21	IL-6 IL-17 IL-21
Treg	Foxp3	TGFß	IL-10 TGFß

Table 1.4.2.2.1. $CD4^+$ T cell subsets mostifn studied in the oral cavity. (Mosmann et al., 1986, Moser and Murphy, 2000, Sakaguchi, 2000, Hori et al., 2003, Bettelli et al., 2007, Aliahmadi et al., 2009, Chung et al., 2009).

$CD4^+$ T cells may be recruited to the gingiva by MIP-1α and MIP-3α. $CD4^+$ T cells in the gingiva of PD patients have been demonstrated to express the appropriate receptors for these chemokines (CCR5 and CCR6). Moreover, an increase in the availability of MIP-1α has been noted in the gingiva of PD patients, in comparison

to the gingiva of periodontally healthy subjects (Taubman and Kawai, 2001, Hosokawa et al., 2002).

P. gingivalis suppresses GEC gene expression of T cell chemokines CXCL9, CXCL10, and CXCL11 via downregulation of IRF1 and STAT1 transcription factors (Jauregui et al., 2013). These chemokines all induce T cell migration through binding to CXCR3, which is found predominantly on Th1 cells. Meanwhile, *P. gingivalis* gingipains also influence the availability of T cell cytokines including IL-2, which is important for the activation and proliferation of all types of $CD4^+$ T cells (Khalaf and Bengtsson, 2012). Lack of IL-2 signalling can lead to T cell anergy and consequently block TD antibody production by B cells (Macian et al., 2004, Okada et al., 2005). IL-12 is also targeted by *P. gingivalis* gingipains, and this can lead to a reduction in IL-12 induced IFN_γ production by Th1 cells (Yun et al., 2001). It is plausible that *P. gingivalis* defends itself against Th1 cells specifically, because they pose more of a threat to its destruction.
Over 30 years ago, a study of experimental gingivitis found that, comparable with a DTH response, T cells and macrophages infiltrate the gingiva within four to eight days of plaque accumulation (Seymour et al., 1979, Platt et al., 1983, Seymour et al., 1988). As Th1 cells are known to mediate DTH, this histological analysis was interpreted to indicate that Th1 cells are involved in the early stages of PD (Fong and Mosmann, 1989). Within two to three weeks, established gingivitis was distinguished by a predominance of plasma cells (Page and Schroeder, 1976). Similarly, studies of PD patients have revealed that progression of disease is associated with increased infiltration by B cells and plasma cells (Berglundh and Donati, 2005). It has consequently been hypothesised that the initial response to dental plaque is mediated by Th1 cells, and that transition to PD is characterised by switching to a predominantly Th2 polarised adaptive immune response that supports the proliferation of B cells (Gemmell et al., 2007).

This hypothesis has to some degree been supported by murine studies of PD. BALB/c mice are more likely to generate a Th2 type response and are more susceptible to *P. gingivalis* infection-induced alveolar bone loss than C57BL/6 mice (Baker et al., 2000b, Gemmell et al., 2002a). However, extreme polarisation of the adaptive immune response to either Th1 or Th2 has been

found to exacerbate PD in mice. Mice lacking key Th1 cytokines (IL-12, IFN_{γ}, and TNFα) or Th2 cytokine (IL-4 and IL-10) have both been found to develop greater alveolar bone loss than WT mice following infection with *P. gingivalis* (Sasaki et al., 2004, Alayan et al., 2007).

The Th1/Th2 paradigm in PD was revised following the discovery of Th17 cells (Gaffen and Hajishengallis, 2008). Several investigations of T cell transcription factor and cytokine expression have found that a mixture of Tregs, Th1, Th2, and Th17 cells are present in the gingiva of PD patients, with no $CD4^+$ T cell subset forming a majority (Fujihashi et al., 1996, Berglundh et al., 2002a, Ito et al., 2005).

Interestingly, the level of IL-17 in PD patient gingiva has been found to positively correlate with pathology (Johnson et al., 2004, Vernal et al., 2005, Lester et al., 2007). Th17 cells and IL-17 have been associated with elevated levels of RANKL, which promotes osteoclastogenesis (Sato et al., 2006, Moutsopoulos et al., 2012). It has been demonstrated that IL-17 can directly increase the expression of RANKL in periodontal ligament cells (Lin et al., 2014). A role for Th17 cells in mediating pathological bone loss has been well established in murine models of RA (Sato et al., 2006, Komatsu et al., 2014). Furthermore, the proportion of circulating Th17 cells has been found to positively correlate with disease activity in RA patients (Kim et al., 2013, Miao et al., 2014). Consequently, several anti-IL-17 therapies are in clinical trials for application in RA (Martin et al., 2013, Genovese et al., 2013, Genovese et al., 2014). Yet IL-17R-deficient mice are more susceptible to *P. gingivalis* infection-induced alveolar bone loss. This is apparently related to their inability to recruit neutrophils in the early stages of infection (Yu et al., 2007). One explanation put forward for these conflicting results is that the relative protective and destructive effects of some cytokines are context dependent and may differ at different stages of disease (Gaffen and Hajishengallis, 2008).

Tregs have been demonstrated to be capable of reducing inflammation and attenuating PD in murine models (Garlet et al., 2010, Glowacki et al., 2013, Garlet et al., 2014). Nonetheless, excessive or inappropriate activation of Tregs

following exposure to *P. gingivalis* could, hypothetically, stunt the development of an effective Th response.

The overall contribution of $CD4^+$ T cells to PD appears to be pathogenic since $CD4^+$ T cell-deficient mice were found to be protected from *P. gingivalis* infection-induced alveolar bone loss (Baker et al., 2002), as were mice deficient in both T cells and B cells (Baker et al., 1999b). Teasing apart which functions of $CD4^+$ T cells and other leukocytes are pathogenic, and when, remains a challenge.

1.4.2.3 B cells and plasma cells

Together, B cells and plasma cells form approximately 70 % of the inflammatory infiltrate in the gingiva of PD patients (Berglundh and Donati, 2005). Several studies have found that the proportion of B cells and plasma cells increases in association with disease progression, activity or severity (Liljenberg et al., 1994, Lappin et al., 1999, Amunulla et al., 2008, Thorbert-Mros et al., 2014). In comparison, relatively few leukocytes have been found in healthy gingiva, of which only about 5 % are B cells (Gemmell et al., 2002b). Despite their predominance in the established periodontal lesion, the roles of B cells in PD are arguably the least well understood of all the immune cells.

Although aggregates of B cells and T cells have been identified within the gingiva of PD patients, there is no evidence of the formation of GCs (Jotwani et al., 2001, Okada et al., 1983). The basis of the interaction between B cells and T cells in the gingiva has yet to be defined. There have been several reports that B cells in the gingiva of PD patients express the co-stimulatory molecules CD80, CD83, and CD86 (Orima et al., 1999, Mahanonda et al., 2002, Jotwani and Cutler, 2003). Unsurprisingly - given their scarcity - a comparison with B cells in healthy gingival tissue was not made in each case. This has prevented assessment of whether B cells have increased capacity to present antigen or have an activated phenotype in disease.

There is evidence that plasma cells in the gingiva of PD patients are actively producing antibodies. A mixture of antibody classes have detected, indicating

that class-switching occurs. There seems to be a consensus that the majority of antibodies produced belong to the IgG class, followed by IgA, and then IgM (Okada et al., 1983, Ogawa et al., 1989, Takahashi et al., 1997). Up to 22 % of plasma cells in the gingiva of PD patients are producing antibodies which specifically recognise *P. gingivalis* (Mizutani et al., 2014). It is not known whether these locally produced antibodies contribute to those in circulation. The serum anti-*P. gingivalis* antibody titers of PD patients have consistently been found to be higher than those of periodontally healthy individuals (Lopatin and Blackburn, 1992, Benjamin et al., 1997, Takeuchi et al., 2006, Lappin et al., 2013). However, it seems that many of the anti-*P. gingivalis* antibodies produced by hosts are ineffective in promoting clearance of this bacterium. One study showed that out of 18 PD patients with high anti-*P. gingivalis* IgG titers, only three patients had serum antibodies capable of opsonising *P. gingivalis* for phagocytosis by neutrophils (Cutler et al., 1991). In addition, it has been demonstrated that gingipains produced by *P. gingivalis* efficiently destroy opsonising antibodies belonging to the IgG1 and IgG3 subclasses (Vincents et al., 2011).

Accompanying the anti-bacterial humoral response, increased titers of autoantibodies have found in the sera of PD patient, including autoantibodies specific for components of ECM: type I collagen, fibronectin, and laminin (De-Gennaro et al., 2006). Anti-collagen antibodies have also been identified in the gingiva of PD patients (Hirsch et al., 1988, Jonsson et al., 1991, Anusaksathien et al., 1992). These autoantibodies are believed to be involved in the progression of PD and to contribute to more aggressive forms of PD (Anusaksathien et al., 1992, De-Gennaro et al., 2006, Koutouzis et al., 2009).

The production of autoantibodies indicates that B cell function has become dysregulated in PD. There is some evidence that oral bacteria may distinctively and directly regulate B cell function. B cells stimulated with *A. actinomycetemcomitans* increased their expression of RANKL and when transferred to mice orally infected with *A. actinomycetemcomitans*, these B cells exacerbated alveolar bone loss (Han et al., 2006, Han et al., 2009). This indicates that B cell function, particularly their expression of RANKL, may be

altered following interaction with oral bacteria, and subsequently contribute to pathology in PD.

Subsets of mature B cells have been characterised, which may differentially respond to bacterial stimuli (Barr et al., 2007). These are described in more detail in **Chapter 5**. To date, there has been minimal effort to further phenotype the B cells associated with PD in order to determine whether a particular B cell function or subset has a more prominent role in pathology.

1.4.3 Osteoclastogenesis

Osteoclastogenesis is essential for remodelling bone. In health, a balance exists between bone resorption by osteoclasts, and bone formation by osteoblasts. In many chronic inflammatory diseases, the process of osteoclastogenesis becomes dysregulated and this balance is lost, resulting in pathological bone loss.

Osteoclasts are large, multinucleated cells formed by the fusion of precursors of the monocyte-macrophage lineage at or near the bone surface (Kukita et al., 2004, Yagi et al., 2005). M-CSF is essential for the survival and differentiation of pre-osteoclasts (Yoshida et al., 1990). M-CSF up-regulates the expression of RANK by pre-osteoclasts, rendering them permissible to signalling induced by RANKL, a cytokine belonging to the TNF superfamily. In the presence of M-CSF, RANKL is able to induce the differentiation of pre-osteoclasts into osteoclasts via several protein kinase signalling pathways: NFκB, JNK, p38, MAPK, ERK, Src, PKA, and PKC (Darnay et al., 1998, Galibert et al., 1998, Bachmann et al., 1999, Takahashi et al., 1999, Matsumoto et al., 2000, Mizukami et al., 2002, Takayanagi, 2007). During osteoclastogenesis, a number of structural changes take place. The basal membrane of the pre-osteoclast forms a tight junction with the bone surface, creating an external vacuole. Into this space, osteoclasts export hydrogen ions and lytic enzymes such as TRAP and cathepsin K, which resorb the bone (Li et al., 1999). Solubilised components of ECM, as well as calcium and phosphate ions are then processed by the osteoclasts and released into circulation. Not only can RANKL induce osteoclastogenesis in precursor cells, but it can also increase ‘the bone-resorbing activity of mature osteoclasts (Hsu et al., 1999, Burgess et al., 1999).

Mesenchymal cells such as fibroblasts are a primary source of M-CSF and RANKL (Takayanagi et al., 2000, Bloemen et al., 2010). There are three different isoforms of RANKL that cells can produce, one is membrane-bound and the other two are soluble (Ikeda et al., 2001, Suzuki et al., 2004). Activated lymphocytes may contribute to the supply of both cell-surface and sRANKL (Kong et al., 1999a, Han et al., 2009, Belibasakis et al., 2011, Walsh et al., 2013). RANKL-RANK signalling is negatively regulated by OPG, a soluble decoy receptor (Simonet et al., 1997, Yasuda et al., 1999). In gingival tissues, mesenchymal cells are the main source of OPG (Sakata et al., 1999, Crotti et al., 2003). In bone, OPG is additionally provided by B cells (Marusic et al., 2000, Li et al., 2007, Manilay and Zouali, 2014).

There is an increase in the ratio of RANKL to OPG expression and protein in the gingiva of PD patients, which is believed to underpin the observed alveolar bone loss (Liu et al., 2003, Crotti et al., 2003, Wara-aswapati et al., 2007, Giannopoulou et al., 2012). There is some evidence that this increase in RANKL expression may be triggered by the direct interaction of cells with members of the dysbiotic oral microbiota. *In vitro*, *P. gingivalis* induced RANKL expression by mesenchymal cells, and by T cells (Belibasakis et al., 2007, Belibasakis et al., 2011), whilst *A. actinomycetemcomitans* has been shown to induce RANKL expression by B cells (Han et al., 2006, Han et al., 2009). In addition to the direct effects of oral bacteria, the local cytokine milieu in the gingiva may regulate RANK-RANKL interactions and osteoclastogenesis in PD (**Table 1.4.3.1**).

RANKL has a significant role in homeostatic osteoclastogenesis, and many studies of murine PD have indicated that pathological alveolar bone loss induced by *P. gingivalis* is RANKL-dependent (Naito et al., 1999, Han et al., 2006, Jin et al., 2007, Yuan et al., 2011). However, osteoclastogenesis can occur independently of RANKL in the presence of certain cytokine cocktails (Kim et al., 2005, Yago et al., 2009). Furthermore, novel regulators of osteoclastogenesis are continually being identified which can also apparently induce osteoclastogenesis independently of RANKL. One example is SOFAT, the expression of which is increased in PD patient gingiva (Rifas and Weitzmann, 2009) (Jarry et al., 2013).

Cytokine	Effect on osteoclastogenesis	Cellular sources	Cellular targets	Functions
RANKL	activation	• mesenchymal cells • T cells • B cells	• osteoclast precursors • osteoclasts	• induction of osteoclast differentiation • increase activity of osteoclasts
TNFα	activation	• macrophages • Th17 cells	• osteoclast precursors • mesenchymal cells	• induction of RANKL expression by mesenchymal cells • synergy with RANKL
IFN_{γ}	inhibition	• Th1 cells	• osteoclast precursors	• inhibition of RANKL signalling
IL-1α/β	activation	• mesenchymal cells • macrophages	• T cells	• Th17 cell differentiation and survival
IL-4	inhibition	• Th2 cells	• osteoclast precursors	• inhibition of RANKL signalling
IL-6	activation	• mast cells • B cells • Th2 cells • DCs	• T cells • mesenchymal cells	• Th17 cell differentiation • induction of RANKL expression by mesenchymal cells
IL-12	inhibition	• macrophages • DCs	• T cells	• Th1 cell differentiation
IL-17	activation	• Th17 cells	• mesenchymal cells	• induction of RANKL expression by mesenchymal cells
IL-18	inhibition	• macrophages • DCs	• T cells	• Th1 cell differentiation

Table 1.4.3.1. The roles of cytokines in osteoclastogenesis.
Adapted from Takayanagi (2007).

1.5 The association of periodontitis with systemic diseases

PD is a chronic disease associated with profound inflammation locally in the gingiva, and some signs of perturbation to the systemic immune response. Epidemiological evidence indicates that PD is more common, and more likely to be more severe, in patients that have a systemic chronic inflammatory disease such as CVD, diabetes, or RA (Mercado et al., 2001, Bahekar et al., 2007, Humphrey et al., 2008, Chen et al., 2008, de Pablo et al., 2009, Nesse et al., 2010, Smit et al., 2012, Weinspach et al., 2013). PD shares many common risk

factors with these systemic diseases (**Figure 1.5.1**), but this does not fully account for the association between them.

It has been hypothesised that the systemic inflammatory changes that arise in PD may cause or contribute to the inflammation attributed to these other diseases and vice versa. The immunopathology of RA has striking similarities with PD as it involves the destruction of connective tissue and bone, orchestrated by lymphocytes. The potential link between PD and RA is particularly intriguing because RA is an autoimmune disease of unknown aetiology.

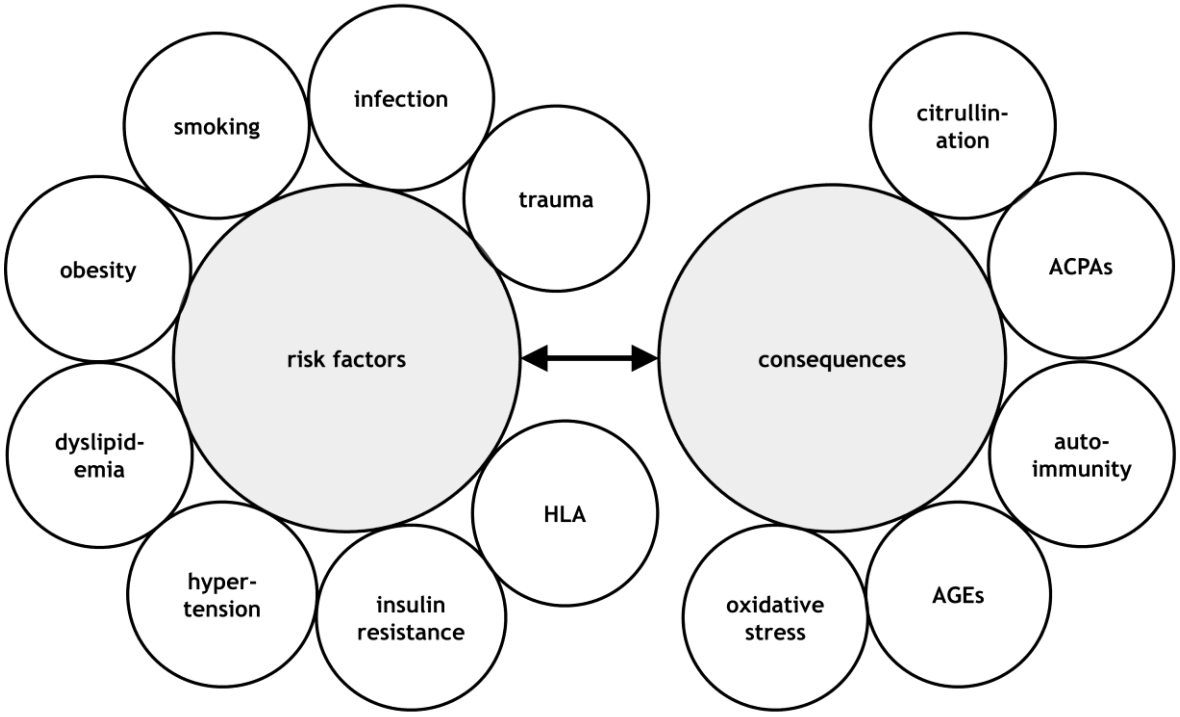

Figure 1.5.1. Common risk factors and consequences of chronic inflammatory diseases: periodontitis, type 2 diabetes, cardiovascular disease and rheumatoid arthritis. A combination of risk factors that may lie at the cause of disease are depicted. Following disease onset, the consequences of disease can drive further inflammation and in this sense, also act as risk factors (Symmons et al., 1997, Genco et al., 2002, Chaffee and Weston, 2010, D'Aiuto et al., 2010, Kallberg et al., 2011, Preshaw and Bissett, 2013, Wesley et al., 2013, Postma et al., 2014). HLA = human leukocyte antigen, AGEs = advanced glycation end products, ACPAs = anti-citrullinated peptide antibodies.

1.6 Rheumatoid arthritis

1.6.1 Clinical characterisation of rheumatoid arthritis

RA is an autoimmune disease characterised by stiffness and swelling of the synovial joints lasting longer than six weeks. It tends to manifest in adults between the ages of 40 and 50. Due to its complex aetiology, there is no single parameter which can be used to conclusively diagnose RA. Since 1987, standardised criteria for clinical classification of RA have been set by the ACR and EULAR. A physical assessment is performed to determine the number of small and large joints involved. X-rays, MRI, and ultrasound scans are used to reveal the extent of damage to the cartilage and bone in the joints. The level of non-specific systemic inflammation is determined by ESR and the concentration of circulating CRP. Serum immunoassays are used to distinguish RA from other forms of arthritis such as osteoarthritis. The anti-IgG autoantibodies known as RF were originally used as the sole indicator of autoimmunity. However, RF have a relatively low specificity of 85 % for RA; they can be found in other autoimmune conditions, and even in healthy individuals (Gran et al., 1984, Hoffman et al., 2005, Nishimura et al., 2007). In 2010, the published RA classification criteria were updated to stipulate the requirement of a second diagnostic test for a different set of autoantibodies: ACPAs (Kay and Upchurch, 2012). In comparison to RF, the standard ACPA assay has a similar sensitivity of 70 % but a much higher specificity of 95 % (Nishimura et al., 2007). Furthermore, ACPAs may be detectable up to 10 years before clinical onset of disease (Nielen et al., 2004, Zendman et al., 2004). These features of ACPAs have led to the concept that ACPA positive and ACPA negative RA represent distinct forms of disease (Daha and Toes, 2011).

There are standardised criteria for monitoring disease progression, response to treatment, and remission. One of these is the DAS28. In accordance with this system, the tenderness and swelling of 28 joints and the patient's perception of disease activity, pain, and physical function are evaluated - usually in conjunction with ESR and CRP (Prevoo et al., 1995). An improvement in symptoms can be determined by calculating the difference between disease activity scores at baseline and follow-up appointments. A patient may meet the

criteria for the ACR20 when there is 20 % improvement in both the tender joint count and the swollen joint count and at least 20 % improvement in three of the five other measures used to determine disease activity (Felson et al., 1995). Similarly, higher thresholds such as the ACR50 or ACR70 may be used to gauge improvement in symptoms, and are typically used as a benchmark in clinical trials (Ma et al., 2014, Ward et al., 2014). A patient is considered to have achieved disease remission if they have just one or no tender or swollen joints, and a CRP level of ≤ 1 mg/dl according to the Boolean-based definition (Felson et al., 2011).

1.6.2 Epidemiology of rheumatoid arthritis

RA affects approximately 0.5 - 1 % of the global population. A very high incidence has been reported in the Pima Indians (5.3 %) and Chippewa Indians (6.8 %), whilst a much lower incidence (0.2-0.3 %) has been reported in China and Japan (Harvey et al., 1981, Del Puente et al., 1989, Shichikawa et al., 1999). In the US and in Europe, the proportion of people affected is in line with the global average (Aho et al., 1998, Cimmino et al., 1998, Simonsson et al., 1999, Carmona et al., 2002, Symmons et al., 2002). Across all regions, it has been reported that RA is around three times more common in women than in men. Twin studies have found that monozygotic twins have a concordance rate of 15 % for RA (Silman et al., 1993). Together these data indicate the involvement of genes in determining development of RA.

The chronic pain and restriction of movement that results from RA can be seriously debilitating, and as a result at least 50 % of patients are unable to work full-time within 10 years of disease onset (Brooks, 1997, Birtane et al., 2008, Walsh and McWilliams, 2012). In 2010, the National Rheumatoid Arthritis Society reported that productivity losses due to RA cost the UK economy approximately £8 billion per year (Bosworth, 2010).

Due to various co-morbidities, the life-expectancy of RA patients is reduced by three to seven years (Wicks et al., 1988, Lassere et al., 2013). The risk of CVD is increased by 48 % in RA patients and almost 40 % of RA patients die from complications of CVD (Dhawan and Quyyumi, 2008, Avina-Zubieta et al., 2012).

1.6.3 Immunopathology of rheumatoid arthritis

Lymphocytes are a prominent feature of the inflammatory infiltrate in the joints. T cells and B cells clearly have important roles in RA as treatments specifically targeting these cells have had some success in patients that were unresponsive to other drugs (Boumans et al., 2012, Keystone et al., 2012, Pieper et al., 2013, Picchianti Diamanti et al., 2014). Murine models have indicated that an increase in Th1 or Th17 cells, and a decrease in Tregs exacerbates RA (Sato et al., 2006, Postigo et al., 2011, Komatsu et al., 2014, Lee et al., 2014). Th1 and Th17 cells contribute to the pathogenesis of RA through the production of inflammatory cytokines and by supporting autoantibody production by B cells. Both in murine models, and in human patients, the titers of autoantibodies have been found to correlate with severity of RA (van Gaalen et al., 2004, Agrawal et al., 2007b, Conigliaro et al., 2011, Patakas et al., 2012).

Histological analysis of synovial tissue samples has revealed that in some cases, the infiltrate is sparse, whereas in others, there are large aggregates of T and B cells, sometimes surrounding clusters of follicular DCs (Yanni et al., 1992, Randen et al., 1995, Thurlings et al., 2008). These more organised aggregates of lymphocytes have been found to exhibit features associated with GCs in lymphoid organs, which has prompted references of tertiary lymphoid organs or ectopic lymphoid organs (Weyand and Goronzy, 2003, Humby et al., 2009). The presence of lymphoid aggregates in synovial tissues has been shown to correlate with increased local expression of cytokines, and elevated concentrations of cytokines in the peripheral blood (Randen et al., 1995, Klimiuk et al., 1997, Thurlings et al., 2008). Although ACPA are produced by tissue-resident plasma cells, no consistent correlation has been found between the presence of ectopic lymphoid structures, and the concentration of RF or ACPAs in synovial fluid or serum (Cantaert et al., 2008, Thurlings et al., 2008, Humby et al., 2009).

1.6.4 Treatment of rheumatoid arthritis

Treatment of RA is focused on preserving function, minimising pain, and reducing inflammation. Research efforts have yielded significant advances in treatment, with a range of drugs now available that target specific cells,

cytokines, and inflammatory pathways. Up to two thirds of patients show some response to one of the currently approved biological therapies (Hyrich et al., 2006, Hetland et al., 2010, Greenberg et al., 2012). However a significant proportion of patients do not respond, and some patients that initially respond later develop resistance to the therapy. Ultimately, 10-50 % of RA patients achieve disease remission (Ma et al., 2010, Scott et al., 2010). The exact figures depend on the criteria used to assess disease activity.

Further developments in treatment are restricted by an incomplete understanding of the causes of RA. PD is one of several potentially modifiable risk factors for RA identified by epidemiological studies. Treatment of PD in RA patients potentially offers a low-risk, simple, and cost-efficient adjunct to the available treatments for RA, and this warrants further investigation.

1.7 The potential immunological link between periodontitis and rheumatoid arthritis

RA patients may be as much as four times more likely to have PD, and are more likely to have more severe PD (Mercado et al., 2001, de Pablo et al., 2008, Smit et al., 2012). The idea PD could cause RA stems from evidence of elevated circulating titers of autoantibodies in PD patients. PD was actually once thought of as an autoimmune disease itself (Anusaksathien et al., 1992, De-Gennaro et al., 2006, Koutouzis et al., 2009).

The majority of the autoantibodies found in PD patients have no specific association with RA. However, there has been one report detailing the detection of antibodies recognising a citrullinated peptide (filaggrin) in PD patient sera (Hendler et al., 2010), and another which claims a cocktail of ACPAs were generally higher in the serum of PD patients compared with periodontally healthy subjects (Molitor, 2009).

Targets of ACPAs include citrullinated forms of various components of connective tissue: vimentin, collagen, fibrinogen, and filaggrin (Burkhardt et al., 2005, Snir et al., 2009, Hansson et al., 2012, Cornillet et al., 2014). The citrullinated peptides recognised by ACPAs have been detected in the synovium

of RA patients and in the gingiva of PD patients (De Rycke et al., 2005, Nesse et al., 2012, Harvey et al., 2013).

Citrullinated peptides can be found in healthy tissues, although less frequently than in inflamed tissues (Vossenaar et al., 2004). Citrullination is a normal post-translational modification of peptides, which, in changing the charge of a positive arginine residue to a neutral citrulline residue, can alter the tertiary structure of a peptide and it's interaction with other peptides. This has a wide range of physiological applications from condensation of chromatin and regulation of gene expression to autophagy and antigen presentation (Leshner et al., 2012, Tanikawa et al., 2012, Ireland and Unanue, 2012).

Citrullination is mediated by a PAD. There are six human PAD isoforms which are expressed by different cells in different anatomical regions. PAD2 and PAD4 are both expressed by fibroblasts and endothelial cells in the gingiva and synovium, and by leukocytes throughout the body (Chang et al., 2005, Foulquier et al., 2007, Harvey et al., 2013). Certain environmental stressors can increase the activity of human PADs. For example, smoking has been associated with increased PAD2 expression and peptide citrullination in the lungs, and smoking status strongly correlates with serum ACPA titers in RA patients (Klareskog et al., 2006, Makrygiannakis et al., 2008).

The citrullination of peptides observed in PD patient gingiva could result from the increased activity of human PADs as a result of inflammation or from the activity of a PAD belonging to *P. gingivalis*, referred to as PPAD. *P. gingivalis* is unique among bacteria in its possession of PPAD. Gingipains produced by *P. gingivalis* cleave peptides at arginine residues, providing substrate to PPAD. PPAD can citrullinate both host and *P. gingivalis* derived peptides at lysine or arginine residues by substituting an amine group for an oxygen moiety and forming ammonia as a bi-product (**Figure 1.7.1**). PPAD will preferentially citrullinate free arginine, and arginine exposed at the end of a peptide, whereas human PADs will citrullinate arginine embedded within a peptide (McGraw et al., 1999).

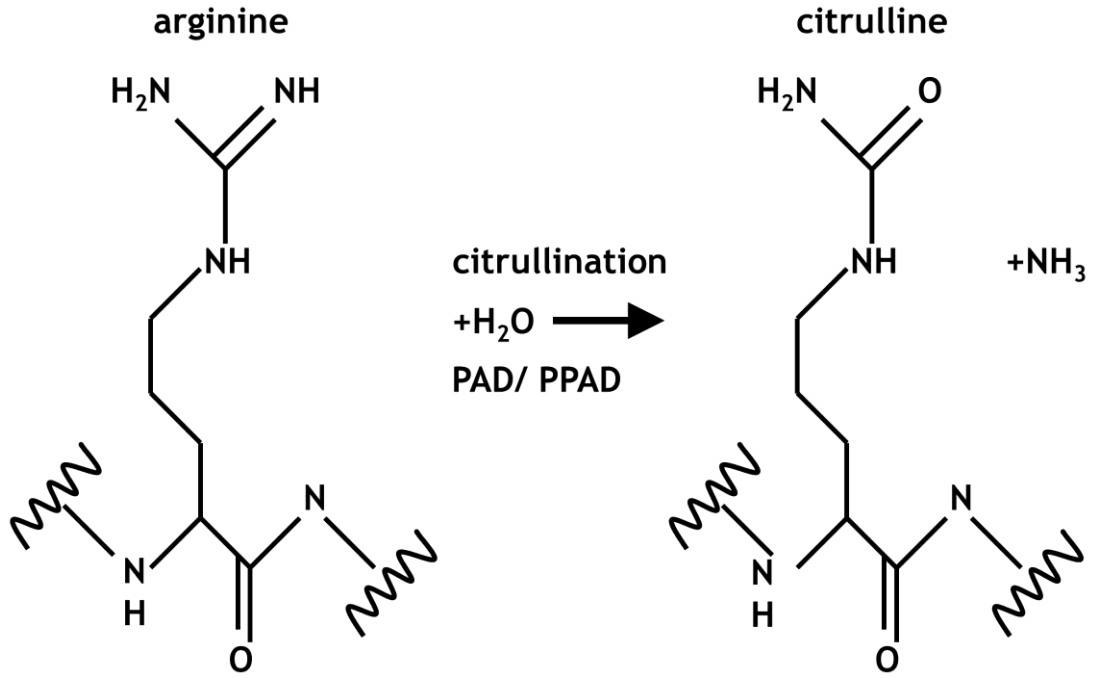

Figure 1.7.1. Citrullination. Host PAD or *P. gingivalis* PPAD enzymes catalyse the substitution of an amine group (=NH) for an oxygen moiety (=O) to convert an arginine residue to a citrulline residue in a peptide. This requires the input of a water molecule ($+H_2O$) and forms ammonia ($+NH_3$) as a bi-product (McGraw et al., 1999).

PPAD is evidently an important virulence factor of *P. gingivalis* since infection with a PPAD-deficient *P. gingivalis* mutant induced less alveolar bone loss than infection with WT *P. gingivalis* in the murine model of PD (Gully et al., 2014). In the initial stages of infection, the production of ammonia by PPAD is thought to help *P. gingivalis* to survive in the acidic environment of the oral cavity by increasing the pH (Marquis et al., 1987, McGraw et al., 1999, Takahashi, 2003). Later, following invasion of the host tissues, citrullination of certain cytokines and chemokines by PPAD is known to inactivate their immune function, and help *P. gingivalis* to evade the host adaptive immune response (Proost et al., 2008, Moelants et al., 2014).

The citrullination of peptides by PPAD is hypothesised to be capable of breaching immune tolerance through two mechanisms: creating *de novo* epitopes and molecular mimicry. Firstly, citrullination of host peptides in a novel way could cause them to be perceived as foreign by the host, which in the inflammatory setting of PD, could stimulate an autoimmune response. Secondly, citrullination of *P. gingivalis* peptides, and the presentation of these alongside PAMPs, could

induce the generation of ACPAs, which are cross-reactive with host peptides that share similar citrullinated epitopes. For example, *P. gingivalis* citrullinated α-enolase shares 51 % sequence homology overall with the human form. This increases to 82 % homology within a specific region, an immunodominant epitope known as CEP-1 (**Table 1.7.1**), and 100 % homology for nine amino acids spanning CEP-1 (Lundberg et al., 2008, Lundberg et al., 2010). It is thought this sequence homology has been conserved in key functional proteins because it enables bacteria to evade the human immune defence (Blank et al., 2007, Wegner et al., 2009). Interestingly, CEP-1 is a major epitope of ACPAs in RA and anti-CEP-1 antibodies are present in around 25 % of RA patients (Fisher et al., 2011, Montes et al., 2012).

Peptide/protein	**Amino acid sequence**
A) *P. gingivalis* CEP-1	K I I G X E I L D S X G N P T V E
B) human CEP-1	K I H A X E I F D S X G N P T V E
C) human REP-1	K I H A R E I F D S R G N P T V E
D) human α-enolase	MSILKIHAREIFDSRGNPTVEVDLFTSKGLFRAAVPSGASTGIY EALELRDNDKTRYMGKGVSKAVEHINKTIAPALVSKKLNVTEQ EKIDKLMIEMDGTENKSKFGANAILGVSLAVCKAGAVEKGVPL YRHIADLAGNSEVILPVPAFNVINGGSHAGNKLAMQEFMILPV GAANFREAMRIGAEVYHNLKNVIKEKYGKDATNVGDEGGFA PNILENKEGLELLKTAIGKAGYTDKVVIGMDVAASEFFRSGKY DLDFKSPDDPSRYISPDQLADLYKSFIKDYPVVSIEDPFDQDDW GAWQKFTASAGIQVVGDDLTVTNPKRIAKAVNEKSCNCLLLK VNQIGSVTESLQACKLAQANGWGVMVSHRSGETEDTFIADLV VGLCTGQIKTGAPCRSERLAKYNQLLRIEEELGSKAKFAGRNF RNPLAK

Table 1.7.1. The amino acid sequence of *P. gingivalis* CEP-1 and human CEP-1 epitopes. A) *P. gingivalis* CEP-1 amino acid sequence and **B)** human CEP-1 amino acid sequence with the nine amino acids sharing 100 % sequence homology shaded in grey. **C)** the sequence of the native, uncitrullinated form of human CEP-1 - human REP-1. **D)** the entire human α-enolase sequence with REP-1 shaded in grey (Lundberg et al., 2008). X = citrulline, R = arginine.

RA is much less common than PD, and not all RA patients have PD. This means even if PD can cause RA, not all PD patients will automatically develop autoimmunity and RA, and that there must be alternative causes of RA. Whether *P. gingivalis* actually induces breach of immune tolerance is likely dictated to a degree by the host immune system, just as whether *P. gingivalis* can cause PD in

the first place is likely dependent on certain weaknesses of the host immune system. Another layer of host-related risk factors comes into play.

RA patient ACPA seropositivity is closely related to the possession of particular HLA alleles on chromosome six (van Gaalen et al., 2004, Snir et al., 2011, Nordang et al., 2013). A number of HLA-DRB1 alleles which dictate the range of peptide-binding specificities of an MHC II molecule predispose people to RA. These alleles all contain a conserved five amino acid stretch, the 'shared epitope' in the third hypervariable region, which forms the peptide-binding pocket. The five amino acids are positively charged, and therefore preferentially bind peptides bearing a neutral citrulline residue rather than a positively charged residue (Gregersen et al., 1987, Ireland and Unanue, 2012). The generation of ACPAs and a sustained autoimmune response in PD patients may depend on these patients having HLA alleles with the 'shared epitope' or an equivalent genetic predisposition. Some studies have shown that the frequency of certain HLA-DRB1 alleles associated with RA is higher among PD patients than in a periodontally healthy population, although reports vary depending on the subtype of PD and the ethnicity of the patient cohort (Ohyama et al., 1996, Bonfil et al., 1999, Reichert et al., 2002, Stein et al., 2003, Jazi et al., 2013).

The two-hit hypothesis was born out of this concept of the cumulative effects of risk factors (Farquharson et al., 2012). In terms of this hypothesis, PD, HLA genotype, and smoking are all potential 'first hits' which lead to loss of immune tolerance to citrullinated peptides and the production of ACPAs (**Figure 1.7.2**). Any factor which subsequently further increases circulating ACPAs presumably increases the likelihood that RA develops. Of the potential 'first hits' indicated, many could also serve as 'second hits'. It is unclear why ACPAs would specifically target the joints and not affect other organs. One thought is that induction of local inflammation and citrullination of peptides in the joints may promote the generation and accumulation of ACPAs in this location.

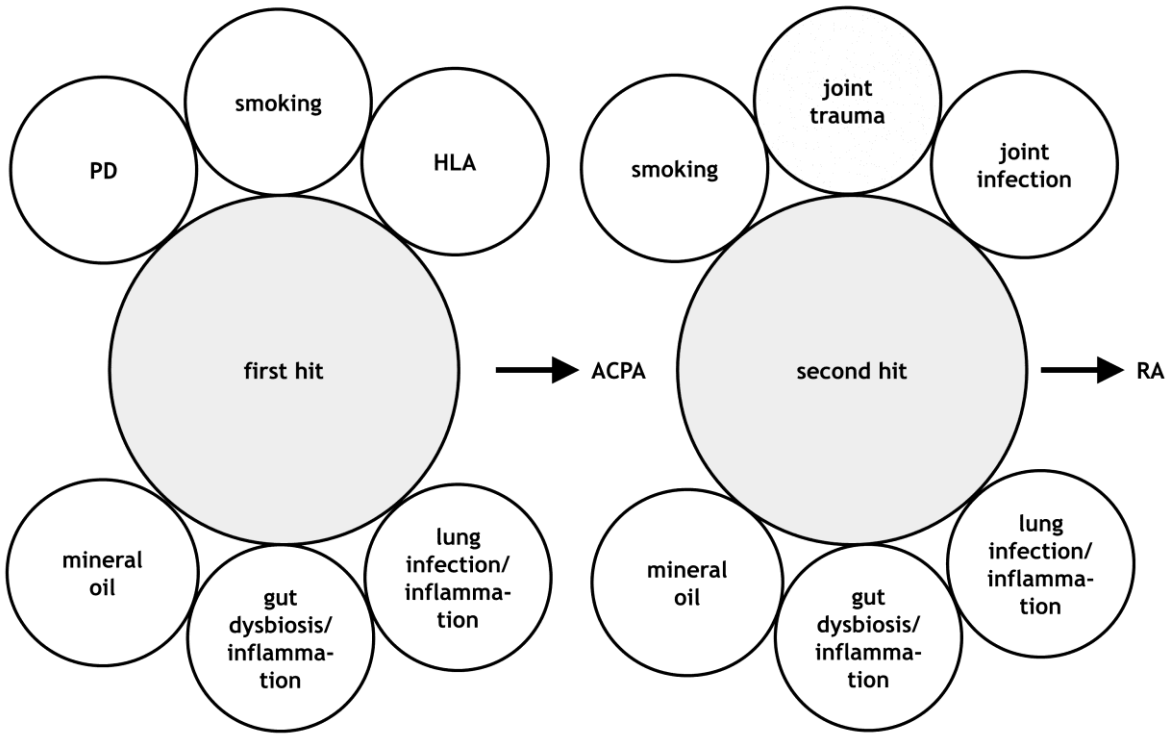

Figure 1.7.2. The two-hit hypothesis. Potential 'first hits' predispose individuals to the production of ACPAs, then a 'second hit' increases ACPA production and triggers the development of RA. (Sverdrup et al., 2005, Klareskog et al., 2006, Makrygiannakis et al., 2008, Mahdi et al., 2009, Scher and Abramson, 2011, Ruiz-Esquide et al., 2012, Brusca et al., 2014, Ytterberg et al., 2014).

1.8 Summary of the general introduction

PD is a chronic inflammatory disease, the aetiology of which has yet to be completely characterised. Microbial dysbiosis is key to the development PD, but the efficacy of current PD treatment, which is focused on managing the oral microbiome, is limited. Patients may benefit from the development and administration of adjunctive therapies which target the damaging inflammatory responses to plaque.

The prevalence of PD is increased in patients with systemic inflammatory diseases, and this association cannot be fully explained by shared risk factors. The relationships between PD and systemic diseases are not well understood, but are potentially mediated by inflammation. RA is a chronic autoimmune disease with comparable immunopathology to PD which has led to the hypothesis that there is an immunological link between these diseases. RA treatment has scope for improvement and modifying risk factors is an appealing avenue.

Treatments that target inflammation in PD could potentially benefit RA patients too. To enable the development of these, a better understanding of the immunopathology of PD and potential mechanisms linking PD to RA is required.

1.9 Aim and objectives

The overall aim of this PhD project was to characterise the role of B cells in the immunopathology of PD. Specific objectives were to:

1) Characterise B cells and plasma cells in the gingiva of PD patients (**Chapter 3**)
2) Characterise the serum antibody response of PD patients (**Chapter 4**)
3) Characterise B cell phenotype and function in the murine model of PD (**Chapter 5**)
4) Assess the contribution of B cells to pathology in the murine model of PD by:
    a) Altering B cell phenotype (**Chapter 6**)
    b) Depleting B cells (**Chapter 7**)

Chapter 2: Methods

2.1 Periodontitis patients

2.1.1 Tissue samples from periodontitis patients and periodontally healthy patients

Samples of healthy gingival tissue (n = 6) were taken from patients undergoing procedures unrelated to periodontal disease, such as exposure of implants or gingivectomy for aesthetic reasons. Samples of diseased gingival tissue (n = 19) were taken from patients diagnosed with chronic PD, who required open flap debridement. These patients had PPD ≥ 5 mm and LOA ≥ 5 mm, which persisted after non-surgical treatment. All samples were obtained from subjects in the Unit of Periodontics at Glasgow Dental Hospital, with written consent. The study was reviewed and approved by the local Research Ethics Committee. Samples of gingival tissue were stored in RNAlater® (Life technologies) at -80 ºC for future extraction of RNA, or in formalin at room temperature for preservation prior to embedding in paraffin for IHC, or in RPMI prior to processing for flow cytometry.

2.1.2 Longitudinal study of periodontitis patients undergoing non-surgical periodontal treatment

This pilot study to investigate the ACPA response in PD patients was designed with Dr Andrea Sherriff (Senior Lecturer in Statistics at the University of Glasgow) and Dr David Lappin (Research Fellow at the Glasgow Dental Hospital and School, University of Glasgow). The samples available to this study were previously collected by Dr Danae Apatzidou to assess the serum antibody response to periodontal pathogens (Apatzidou et al., 2005). The original study was reviewed and approved by the local research ethics committee. Based on the previously generated data on titers of serum antibodies against periodontal bacteria in PD patients compared with healthy participants, it was determined that a group size of approximately 20 patients would be required for the current study. This was based on an effect size of 1 standard deviation, 80 % power, and a significance level of 5 %. As serum was available from 39 PD patients and 36

healthy subjects, it was deemed that sufficient numbers were available for a meaningful study.

All PD patients in the study had been referred to the Unit of Periodontics at Glasgow Dental Hospital for non-surgical treatment of previously untreated chronic PD. The healthy volunteers were of similar age and gender and included a similar proportion of smokers and non-smokers as the PD patient group. All participants had a clinical periodontal examination carried out by the same practitioner, recording the number of teeth, PPD, LOA, and BOP. Patients with PD had a minimum of 18 teeth and at least 2 sites with PPD ≥ 5 mm, LOA ≥ 5 mm, and radiographic evidence of bone loss. Participants were considered to be periodontally healthy if they had no history of PD, no PPD ≥ 2 mm, and no LOA ≥ 2 mm. At the time of recruitment, all participants were systemically healthy and apart from PD, had no other known disease. No participants had received antibiotics 3 months prior to or during the study.

Non-surgical periodontal treatment included detailed oral hygiene instruction, supra-gingival scaling, and root surface debridement using manual and electrical tools, with local anaesthesia. This treatment was carried out by a single clinician during 1 visit or quadrant-by-quadrant at bi-weekly intervals. Patients were re-evaluated 20-26 weeks following the last treatment session and 6 months from baseline. Periodontal treatment was deemed to be successful by reduced PPD, BOP, and plaque scores. Plaque samples were collected from the deepest site in each quadrant before non-surgical periodontal treatment, and at re-evaluation.

Before and after treatment, peripheral venous blood was collected into a glass vacutainer between 9.00 am and 11.00 am to minimise diurnal variations in biochemical parameters. After coagulation, blood was centrifuged at 200 x g and room temperature for 10 minutes. Serum was separated and stored in aliquots at -70 ^{o}C. This study has been previously described in detail (Apatzidou et al., 2005, Lappin et al., 2013).

2.2 Mice

All animals were maintained, and experiments carried out, under standard conditions in accordance with local and UK Home Office regulations (Licence 60/4041, Biological Services, University of Glasgow) and all animal studies are reported according to ARRIVE guidelines (Kilkenny et al., 2010). BALB/c mice (Harlan, UK) and C57BL/6 (Charles River, UK) were housed in standard cages, whereas μMT mice (kindly donated by Prof. Robert Nibbs, University of Glasgow (Kitamura et al., 1991)) were housed in 'barrier' cages. There was a maximum of 5 mice per cage. All mice were maintained on a 12 hour light-dark cycle and received dH_2O and food *ad libitum*. The welfare of the mice was assessed daily. No adverse events occurred during any of the experiments. The number of animals in each experimental group ranged from 4-6. The maximum total number of animals in a single experiment was 22. Experiments were designed with Dr Andrea Sherriff, Senior Lecturer in Statistics at the University of Glasgow. Based on previous data, using clinical disease measures (alveolar bone loss) as our primary outcome, it was estimated that group sizes of 5 were required to have a 80 % power of demonstrating differences at a significance level of 5 % between groups in an unpaired t test.

2.3 *P. gingivalis* growth

Stocks of *P. gingivalis* W83 (ATCC) originating from a human oral infection (isolated in the 1950s by Werner, H. in Germany (Loos et al., 1993)) were stored long-term in 10 % glycerol at -80 °C. Frozen bacteria were applied to Schaedler anaerobic agar (Sigma-Aldrich) supplemented with 10 % FCS and 0.0025 μg/ml Vitamin K (Sigma-Aldrich) following the streak dilution method with sterile pipette tips (Starlab). Bacteria were grown on agar plates for 2-3 days at 37 °C in an anaerobic chamber (Don Whitely, Yorkshire, UK) with 85 % N_2, 5 % H_2 and 10 % CO_2, and then 3-4 colonies of bacteria were collected using sterile loops inoculated into 30 ml of Schaedler anaerobic broth (Sigma-Aldrich) supplemented with 0.0025 μg/ml menadione (Sigma-Aldrich) and 5 % horse blood (TCS biosciences, UK), and incubated for a further 2 days in the anaerobic chamber.

2.4 Murine model of periodontitis

The bacteria were grown as described in section **2.3**. The OD of the planktonic bacteria was measured at 600 nm to determine the number of ml required for the desired total CFU. This was achieved using a previously generated standard curve (kindly provided by Emma Millhouse, Glasgow Dental School). A total CFU of 1 x 10^{10} was needed for infecting 5 mice. The required volume of planktonic bacteria was transferred to a new universal tube and centrifuged at 3,750 rpm for 20 minutes. The majority of the supernatant was poured off and the pellet was re-suspended in the remaining supernatant, then transferred to several 1.5 ml Eppendorf® tubes and centrifuged at 13,400 rpm for 10 minutes. The supernatants were removed and the bacteria were washed twice by re-suspending in 1 ml/tube de-oxygenated PBS, centrifuging at 13,400 rpm for 10 minutes, and removing the supernatant. Finally, approximately 1 x 10^{10} CFU of bacteria were pooled into a single 1.5 ml Eppendorf® tube and re-suspended in 800 µl de-oxygenated 2 % CMC (Sigma-Aldrich) in PBS.

Female mice aged 4-6 weeks were treated with antibiotics (0.08 % sulphamethoxazole, 0.016 % trimethoprim in dH_2O, *ad libitum*) for 10 days, and then rested for 3 days. Mice were then divided into 2 groups. PD was induced in half of the mice by orally infecting with approximately 10^9 CFU *P. gingivalis* W83 in 75 µl 2 % CMC vehicle by gavage into the oral cavity using pipette tips (Starlab). Infections were performed on 3 occasions within 5 days. Control mice were orally inoculated with an equal volume of 2 % CMC only on these occasions. At 1, 2, or 6 weeks post-infection animals were euthanised by increasing concentrations of CO_2 and exsanguination by cardiac puncture to obtain blood. The gingiva were dissected from the maxillae and the maxillae were retained for assessment of alveolar bone level. Inguinal LN, mesenteric LN, dLNs, spleens, and peritoneal fluid were also collected in some experiments.

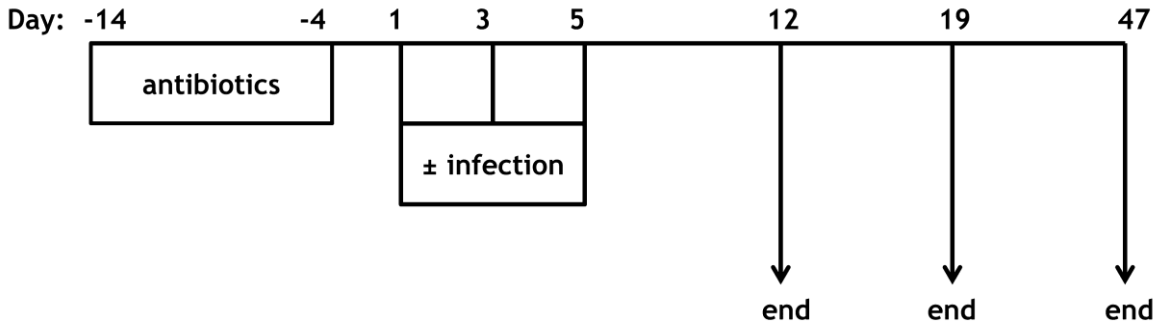

Figure 2.4.1 Timeline of the murine model of periodontitis.

2.5 Murine model of periodontitis with IL-33 treatment

Mice to be infected with *P. gingivalis* and sham-infected mice were divided into 2 sub-groups which either received 0.9 µg recombinant murine IL-33 (BioLegend) or vehicle only (0.1 % BSA in PBS) via intraperitoneal injection. Mice received their first IL-33 injection the day before their first infection with *P. gingivalis*, and the next 2 IL-33 injections on alternate days to the infections (**Figure 2.5.1**). In some experiments, mice were euthanised 1 week post-infection. In other experiments, mice received an additional 6 IL-33 injections over 10 days as indicated (**Figure 2.5.1**), and were euthanised at 6 weeks post-infection.

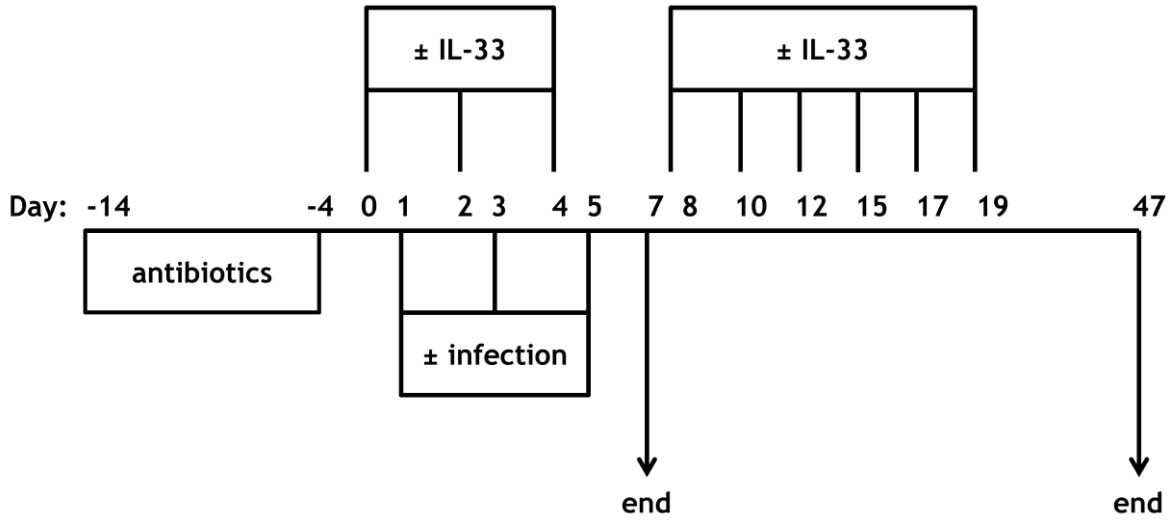

Figure 2.5.1 Timeline of the murine model of periodontitis with IL-33 treatment.

2.6 Dissection of murine gingiva

Gingiva were dissected from the maxillae of mice following a published protocol (Mizraji et al., 2013). In brief, the maxillae were cut away from the head. Incisions were made with a scalpel blade around the gingival tissue, which was stripped away using forceps. The gingiva, and strips of palatal tissue were placed in PBS prior to analysis of cells by flow cytometry, or the gingiva only were stored in RNAlater® for subsequent RNA extraction and analysis of gene expression.

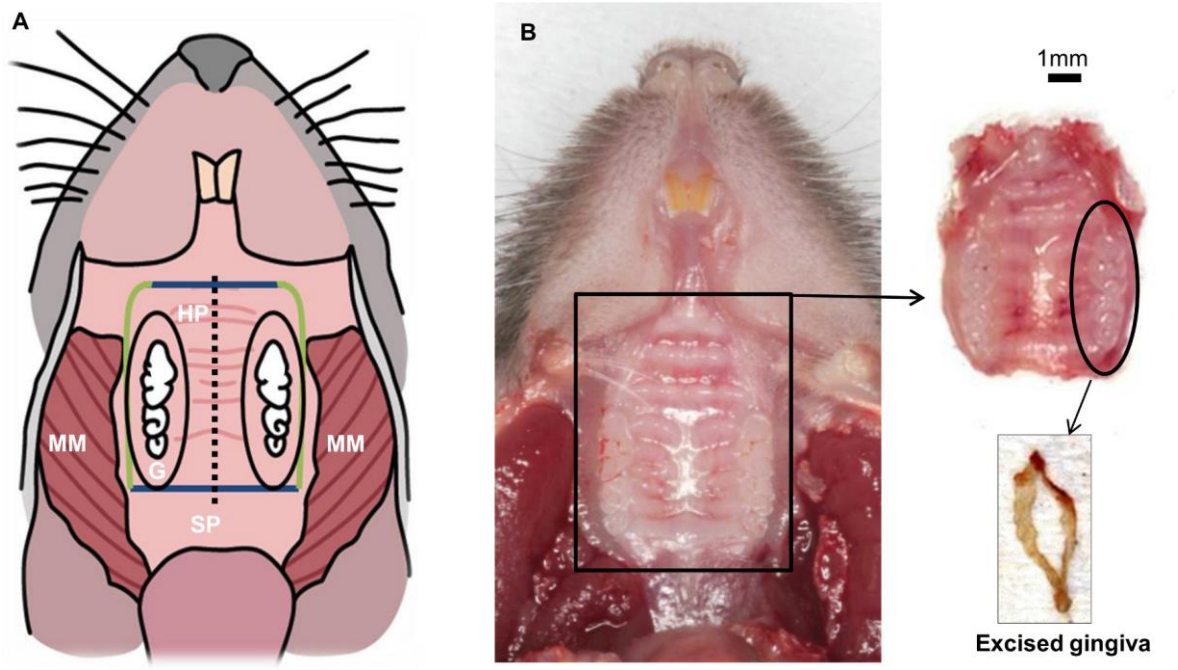

Figure 2.6.1. Dissection of murine gingiva. Images of the inside of the murine oral cavity from Mizraji *et al*. 2013. **A)** cartoon indicating the incisions made to dissect the gingiva (MM = masseter muscle, HP = hard palate, SP = soft palate, G= gingiva). First, the blue and green incisions were made to remove the HP from the oral cavity to improve the access to the gingiva, then the black incisions were made to excise the gingiva from around the teeth. **B)** photographic representation of the dissection.

2.7 Assessment of alveolar bone level in mice

The gingival and palatal tissues were carefully removed by dissection as described in section **2.6**. The left and right sides of the jaw were separated, and the remaining tissue was removed from the bone, by incubation with 2 mg/ml collagenase (Sigma-Aldrich), 50 U/µl DNAse I (Invitrogen) and 1 mg/ml hyaluronidase (Sigma-Aldrich) for 30 minutes at 37 ºC with gentle agitation. The

enzyme reaction was stopped by adding cRPMI and the teeth were washed with distilled water, then incubated with 3 % H_2O_2 at room temperature overnight. The teeth were washed again with dH_2O and incubated with 2 % PFA overnight. The teeth were washed again with dH_2O before finally being stained with 0.5 % methylene blue at room temperature for 30 minutes. The teeth were washed with dH_2O and air-dried prior to imaging. Images were captured at X3.2 magnification using an SZX7 dissection microscope fitted with SC100 camera (Olympus). Maxillae were orientated for measurements by aligning the buccal and palatal tips of the middle cusp of the first (largest) molar. Measurements of the distance between the CEJ and the ABC were made in images using GIMP version 2.8, by referring to the scale bar. This distance was measured on the palatal side of the teeth at 12 points on the left side and 12 points on the right side of the jaw, generating a total of 24 measurements for each mouse (**Figure 2.7.1**). In some cases, measurements of the alveolar bone level were also made independently by Dr Annelie Hellvard and Birth Bergum (Broegelmann Research Laboratory, University of Bergen, Norway) using X-ray micro-CT with OsiriX software (Pixmeo, Switzerland). This method involved measuring the distance between the CEJ and the ABC on the mesial and distal sides of the second molar, on the left and the right sides of the jaw. This measurement was guided by a reference line which indicated the plane of the ABC (**Figure 2.7.2**). All measurements were made blindly. After the measurements were made, the mean distance from the CEJ to the ABC (the alveolar bone level) was calculated for each mouse. The mean value of the alveolar bone level for the whole sham-infected group was subtracted from the mean alveolar bone level of each individual mouse in all groups, including the sham-infected group itself, in order to normalise the data. The mean alveolar bone level of the sham-infected group was consequently 0 mm and the SEM of the sham-infected group was determined from the deviation of normalised measurements of individual sham-infected mice from 0 mm. Values of alveolar bone level > 0 mm were presented as negative and values < 0 mm were presented as positive to indicate the relative loss of alveolar bone for all mice.

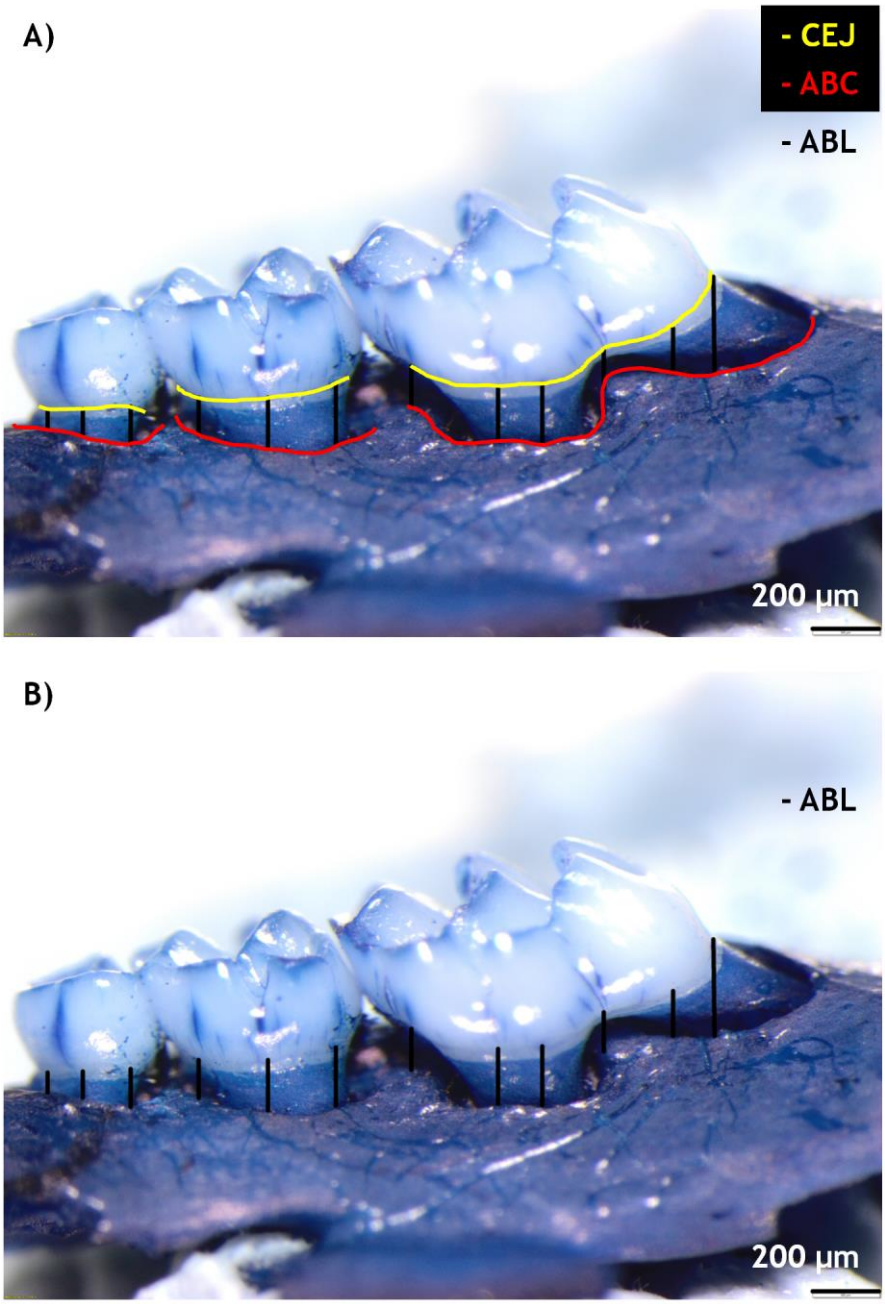

Figure 2.7.1. Assessment of alveolar bone level in mice using a dissection microscope. The maxillary teeth and supporting alveolar bone were stained with methylene blue and images were captured at X3.2 magnification. Measurements of the distance between the CEJ and the ABC were made at 14 points across the lingual side of the teeth to assess the alveolar bone level (ABL) in mice. These measurements were made by referring the scale bar in the bottom-right corner of images, which indicates the length of 200 µm. **A)** the CEJ is highlighted in yellow, the ABC is highlighted in red, and the measurements are shown in black. **B)** the measurements only are shown in black.

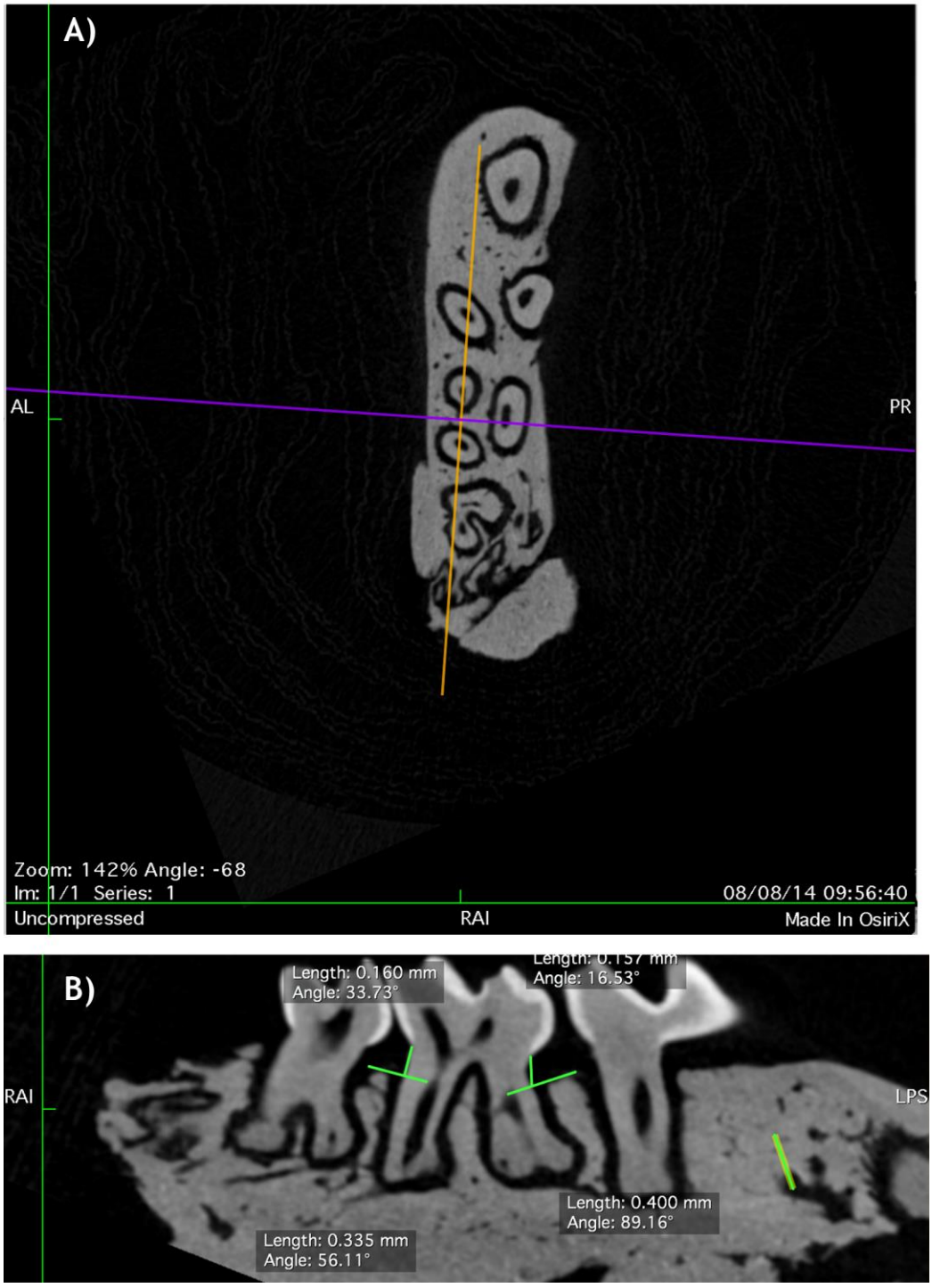

Figure 2.7.2. Assessment of alveolar bone level in mice using X-ray micro-CT. A) the roots of the 3 molars were aligned and the orange line indicated where a cross-section was to be made. **B)** on a cross-sectional image of the 3 molars, the distance between the CEJ (junction between the white enamel and the grey cementum) and the ABC was measured either side of the second molar. This distance is shown by the vertical green lines. Perpendicular to these measurements are guide lines, also green, which rest on the plane of the ABC. In some cases, the angle of these guide lines was somewhat arbitrary due to the degree of bone erosion.

2.8 Extraction of RNA and DNA and analysis of gene expression

2.8.1 RNA extraction and qRT-PCR

Samples of human gingival tissue were retrieved from storage in RNAlater® at -80ºC. Murine gingiva were transferred to an Eppendorf® tube containing 100 µl RNAlater® and RNA extracted later the same day. Murine spleens were collected and transferred to cRPMI (RPMI with 10 % FCS, 1 % Penicillin Streptomycin, and 1 % L-glutamine (Invitrogen)), then dissected into small sections and immediately processed for RNA extraction.

Extraction of total RNA from all tissue samples was carried out using the RNeasy® fibrous tissue kit (Qiagen), according to the manufacturer's instructions. In brief, a maximum of 30 mg tissue was placed in a clean 1.5 ml Eppendorf® tube with 300 µl 1 % ß-mercaptoethanol in buffer RLT. The mixture was homogenised using a cordless motorised homogeniser with disposable pellet pestles (Sigma-Aldrich). Then, 590 µl nfH_2O and 10 µl proteinase K (Qiagen) were added to the homogenous lysate and the mixture was incubated at 55 ºC for 10 minutes. The homogenate was centrifuged at 10,000 rpm for 3 minutes at room temperature. The supernatant (700–900 µl) was transferred into a new 1.5 ml Eppendorf® tube, followed by addition of an equal volume of ethanol (96–100 %). The mixture was gently mixed by pipetting up and down before being transferred onto an RNeasy® spin column placed in a 2 ml collection tube and centrifuged at 10,000 rpm for 15 seconds. The flow-through was discarded and the membrane was washed with 350 µl buffer RW1. The sample was centrifuged at 10,000 rpm for 15 seconds. The flow through was discarded and DNase digestion was performed 'on column' by incubating the membrane with a mixture of 10 µl DNase I stock solution (Qiagen) and 70 µl buffer RDD for 15 minutes at room temperature. Afterwards, the column was washed again with buffer RW1. Next, the column was washed twice with 500 µl buffer RPE. Finally, RNA was eluted from the column by adding 30-50 µl nfH_2O and centrifugation at 10,000 rpm for 1 minute. The RNA was stored at -80 ºC until further use. A NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) was used to assess the concentration and quality of the RNA. Quality was assessed by measuring the ratio of absorbance at 260/280 and 260/230. A 260/280 ratio of around 2.0 and a

260/230 ratio in the range of 1.8-2.2 is generally accepted as 'pure' for RNA. Samples with 260/280 and 260/230 ratios appreciably lower than those in the ideal range were not used as this indicates the presence of protein, phenol, or other contaminants.

Depending on kit availability, reverse transcription was carried out using the either the High Capacity RNA-to-cDNA kit™ or the High Capacity cDNA Reverse Transcription Kit (both Applied Biosystems). A reaction mix was prepared containing 1 µg RNA template (1-9 µl), nfH_2O (0-8 µl), and kit-specific master mix containing manufacturer optimised concentrations of $MgCl_2$, along with dNTPs, random primers, with (RT+) or without (RT-) 1 µl enzyme mix containing manufacturer optimised concentrations of MultiScribe™ MuLV reverse transcriptase enzyme, and recombinant RNase inhibitor protein. RT- reaction mixtures included an additional 1 µl of nfH_2O instead of the enzyme mix. The RNA template was added last to the reaction. Reaction mixtures were prepared in 0.2 ml Eppendorf® tubes on ice. The tubes were sealed, briefly centrifuged, loaded onto a DNA Engine® thermal cycler (BIORAD), and underwent the thermal cycling conditions in **Table 2.8.1.1**.

	High Capacity RNA-to-cDNA Kit™		**High Capacity cDNA Reverse Transcription Kit**	
Step	**Temperature (°C)**	**Time (minutes:seconds)**	**Temperature (°C)**	**Time (minutes:seconds)**
1	25	05:00	25	10:00
2	42	30:00	37	120:00
3	85	05:00	85	05:00

Table 2.8.1.1. Thermal cycling conditions for conversion of mRNA to cDNA using the High Capacity RNA-to-cDNA Kit™ and High Capacity cDNA Reverse Transcription Kit.

Quantitative RT-PCR of cDNA products was performed using TaqMan® reagents (Applied Biosystems). Reactions were prepared in a 96 well plate (Starlab) on ice. All reactions were prepared and measured in duplicate. Each reaction consisted of 1 µl cDNA, 1 µl TaqMan® Primer Probe Assay Mix (Applied Biosystems), 10 µl TaqMan® Universal PCR Master Mix (Applied Biosystems), and 8 µl nfH_2O. The plate was sealed, briefly centrifuged, loaded onto the MxPro3000P qPCR System (Aligent Technologies) or the 7500 Real-Time PCR System (Applied Biosystems), and underwent the thermal cycling conditions in **Table 2.8.1.2**.

Real-time PCR data were analysed using the $2^{-\Delta CT}$ method (Schmittgen & Livak, 2008). Expression of the gene of interest was determined relative to the expression of 18S.

Step	Temperature (°C)	Time (minutes:seconds)
1	95	10:00
2 (x40)	95	00:15
	60	01:00

Table 2.8.1.2. Thermal cycling conditions for qRT-PCR using TaqMan® reagents.

Primer	Species	Supplier	Assay ID	Spans exon
18S rRNA	eukaryote	Applied Biosystems	4352930E	NA
CD19	human	Applied Biosystems	Hs01047410_g1	1-2
FcRL4	human	Applied Biosystems	Hs00972783_m1	10-11
CD19	mouse	Applied Biosystems	mM00515420_m1	4-5
CD4	mouse	Applied Biosystems	mM00442754_m1	5-6

Table 2.8.1.3. TaqMan® primers used in qRT-PCR. NA = not applicable.

2.8.2 Bacterial DNA extraction and PCR

Dr Danae Apatzidou and Dr David Lappin carried out the collection of plaque, extraction of bacterial DNA, and PCR as previously described (Apatzidou et al., 2005, Lappin et al., 2013). Plaque was collected from PD patients and healthy volunteers using a sterile curette and dispersed into 1 ml of PBS. Bacteria were centrifuged at full speed for 10 minutes in a microcentrifuge, then resuspended in 500 µl dH_2O and lysed by incubating at 100 °C for 10 minutes. Carriage of *P. gingivalis* by PD patients was determined by PCR amplification of *P. gingivalis*-specific 16S rRNA sequences. In brief, 10 µl of extracted bacterial DNA was added to a 90 µl reaction mixture containing PCR buffer (10 mM tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton® X-100), 2 U GoTaq® DNA polymerase (Promega), 0.2 mM dNTPs and 50 pM each primer (forward and reverse). Samples were then loaded onto the OmniGene thermal cycler (Hybaid, Teddington, UK) and underwent the thermal cycling conditions in **Table 2.8.2.1**. For analysis, 10 µl each reaction product was added to 1.5 µl gel-loading dye (0.25 % bromophenol blue, 50 % glycerol, 100 mM EDTA pH 8.0), electrophoresed on a 2 % agarose gel containing ethidium bromide (0.5 µg/ml) and visualised and photographed using an ImageMaster video documentation system (Pharmacia Biotech, St. Albans, UK). A 100 bp DNA ladder (Pharmacia Biotech) was used as a

molecular weight marker. The results confirmed whether patients were positive or negative for carriage of *P. gingivalis* but were not quantitative.

Step	Temperature (°C)	Time (minutes:seconds)
1	94	05:00
2 (x35)	94	01:00
	60	01:00
	72	01:30
3	72	10:00

Table 2.8.2.1. Thermal cycling conditions for PCR amplification of *P. gingivalis* 16S in isolates of human dental plaque.

Primer	Supplier	Sequence (5'-3')
P. gingivalis 16S	MWG-Biotech	Forward AGG CAG CTT GCC ATA CTG CG Reverse ACT GTT AGC AAC TAC CGA TGT

Table 2.8.2.2. Primers used for PCR amplification of *P. gingivalis* 16S in isolates of human dental plaque.

Plaque was collected from mice by swabbing the oral cavity using sterile eSwabs™ (Copan, US) containing 1 ml Liquid Amies transport medium. Bacterial DNA was isolated using the MasterPure™ Gram Positive DNA Purification Kit (Epicentre, US), following the manufacturer's instructions with minor modifications. In brief, the oral swabs were vortexed, then 1 ml from each swab was transferred to a 1.5 ml Eppendorf® tube. The bacteria were pelleted by centrifugation at full speed for 10 minutes in a microcentrifuge, and the supernatant was discarded. Bacteria were resuspended in 150 µl/tube TE buffer. After adding 1 µl/tube lysozyme, samples were incubated at 37 °C for 2 hours. A mixture of 1 µl proteinase K in 150 µl Gram Positive Lysis Solution was added to each tube. Samples were incubated at 65-70 °C for 15 minutes with intermittent vortexing, then cooled on ice for 5 minutes. Next, 175 µl/tube of MPC Protein Precipitation Reagent was added, and samples were vortexed for 10 seconds, then centrifuged at full speed for 10 minutes in a microcentrifuge. The supernatant containing the DNA was transferred to a clean tube and then incubated with 1 µl/tube RNase A (5 µg/µl) at 37 °C for 30 minutes. Isopropanol was added 500 µl/tube, and tubes were inverted 30-40 times, then centrifuged at full speed for 10 minutes in a microcentrifuge. The supernatant was removed and the DNA pellet was rinsed with 70 % ethanol. Finally, the DNA was resuspended in 35 µl/sample TE buffer.

Following extraction, 1 µl bacterial DNA was added to a PCR reaction mixture which consisted of 10 µl SYBR® Select Master Mix (Life Technologies) containing manufacturer optimised concentrations of SYBR® GreenER™ dye, AmpliTaq® DNA Polymerase, uracil DNA glycosylase, dNTPs, and ROX passive reference dye in a buffer, 7 µl nfH_2O, and 1 µl each primer (forward and reverse, final concentration 10 µM) as listed in **Table 2.8.2.3**. Reactions were prepared in a 96-well plate (Starlab) on ice. The plate was sealed, briefly centrifuged, loaded onto the MxPro3000P qPCR System (Aligent Technologies) or the 7500 Real-Time PCR System (Applied Biosystems), and underwent the thermal cycling conditions in **Table 2.8.2.4**. *P. gingivalis* 16S could not be detected by this method. The number of CFU present in the 1 ml samples of bacteria was estimated based on the Ct values generated by qRT-PCR with primers recognising a universal 16S sequence detected in the genome of most bacteria (Muyzer et al., 1993). This was achieved using a previously generated standard curve (kindly provided by Lindsay O'Donnell, Glasgow Dental School) of the average CFU and corresponding Ct values of generic 16S for a combination of representative Gram negative and Gram positive bacteria: *Pseudomonas aeruginosa* and *Staphylococcal aureus* (each of which had similar copy numbers of 16S to *P. gingivalis* (Vetrovsky and Baldrian, 2013)).

Primer	Supplier	Sequence (5'-3')
universal 16S	Primer Design	Forward ACT CCT ACG GGA GGC AGC AGT Reverse TAT TAC CGC GGC TGC TGG C
P. gingivalis 16S	Primer Design	Forward GCG CTC AAC GTT CAG CC Reverse CAC GAA TTC GCC TGC

Table 2.8.2.3. Primers used in qRT-PCR of bacterial DNA isolated from mice.

Step	Temperature (°C)	Time (minutes:seconds)
1	50	02:00
2	95	02:00
3 (x40)	95	00:15
	60	01:00

Table 2.8.2.4. Thermal cycling conditions for PCR amplification of bacterial 16S in murine plaque samples.

2.8.3 Murine DNA extraction for anti-dsDNA ELISA

DNA was extracted from the spleens of mice using the DNeasy® Blood and Tissue Kit (Qiagen), according to the manufacturer's instructions. In brief, the tissue was dissected into small fragments weighing < 10 mg each. Individual fragments were transferred to 1.5 ml Eppendorf® tubes and incubated with 180 µl/tube buffer ATL and 20 µl/tube at 56 ºC for 8 hours with intermittent vortexing. After this, 200 µl/tube buffer AL was added and the samples were incubated at 56 ºC for a further 10 minutes. Then, 200 µl/tube 100 % ethanol was added and the samples were vortexed. The samples were transferred into DNeasy® mini spin columns placed in 2 ml collection tubes and centrifuged at 8,000 rpm for 1 minute in a microcentrifuge, then the spin columns placed in new 2 ml collection tubes and 500 µl/tube buffer AW1 was added. The samples were centrifuged at 8,000 rpm for 1 minute then transferred into new collection tubes as before and 500 µl/tube buffer AW2 was added. The samples were centrifuged at 13,400 rpm for 3 minutes and then transferred to clean 1.5 ml Eppendorf® tubes. Finally, the DNA was eluted by adding 200 µl/tube buffer AE, incubating at room temperature for 1 minute, and then centrifuging at 8,000 rpm for 1 minute. A NanoDrop 1000 spectrophotometer was used to assess the concentration and quality of the DNA. Quality was assessed by measuring the ratio of absorbance at 260/280 and 260/230. A 260/280 ratio of around 1.8 and a 260/230 ratio in the range of 1.8-2.2 is generally accepted as 'pure' for DNA. Samples with 260/280 and 260/230 ratios appreciably lower than those in the ideal range were not used as this indicates the presence of protein, phenol, or other contaminants.

2.9 IHC with human gingival tissue

2.9.1 Single IHC staining for light microscopy

Paraffin sections (prepared by staff in the histopathology department at the Veterinary School of Medicine, University of Glasgow) were incubated at 60 ºC for 35 minutes to soften the wax. Sections were deparaffinised and rehydrated by passing through the solutions as indicated in **Table 2.9.1.1**.

Step	Solution	Time (minutes)
1	Xylene	10
2 (x2)	100 % Ethanol	3
3 (x2)	90 % Ethanol	3
4 (x2)	70 % Ethanol	3

Table 2.9.1.1. Steps for deparaffinisation and rehydration of paraffin tissue sections.

Sections were washed with dH_2O, and endogenous peroxidase activity was blocked by incubating sections with 0.5 % H_2O_2 in methanol at room temperature for 30 minutes. Sections were washed with TBST (0.05 % Tween, 20 mM tris, 9 % NaCl, pH 7.6). Epitope retrieval was performed by immersing sections in boiling citrate buffer (0.01 M citrate, pH 6) for 8 minutes. Sections were cooled to room temperature and washed with dH_2O, followed by TBST. Sections were demarcated with ImmEdge™ hydrophobic barrier pen (Vector Laboratories), blocked using the Avidin/Biotin Blocking Kit (Vector Laboratories), then incubated with 100 µl/section of non-specific antibody binding block (2.5 % human serum (Thermo Fisher Scientific), 2.5 % serum of the animal that the secondary antibody was raised in (Vector Laboratories), and 2.5 % avidin D block, in TBST) at room temperature for 30 minutes. Primary antibodies were prepared in primary diluent (2.5 % human serum, 2.5 % serum of the animal that the secondary antibody was raised in, and 2.5 % biotin block, in TBST) as indicated in **Table 2.9.1.2**. Sections were incubated with 100 µl/section primary antibodies at 4 ºC overnight. Sections were then warmed to room temperature and washed in TBST. Biotin-conjugated secondary antibodies were prepared in secondary diluent (2.5 % serum that the secondary antibody was raised in, in TBST) as indicated in **Table 2.9.1.2**. Sections were incubated with 100 µl/section secondary antibodies at room temperature for 30 minutes, then washed with TBST. Sections were incubated with 100 µl/section avidin-biotin complex prepared using the VECTASTAIN® Elite ABC Kit (Vector Laboratories) at room temperature for 30 minutes. The epitopes were visualised with 50 µl/section DAB substrate (Vector Laboratories). Sections were washed with dH_2O, and counterstained with Harris Haematoxylin (Sigma-Aldrich).

Antibody	Isotype	Final concentration	Supplier
mouse anti-human CD19	IgG1	0.83 µg/ml	Dako
goat anti-human CD5	IgG	1 µg/ml	R&D systems
mouse anti-human CD138	IgG1	5.4 mg/ml	Dako
rabbit anti-human IgG	IgG Fab_2 fragment	5.7 mg/ml	Dako
horse anti-mouse IgG	IgG	7.5 µg/ml	Vector Laboratories
rabbit anti-goat IgG	IgG	7.5 µg/ml	Vector Laboratories

Table 2.9.1.2. Primary and secondary antibodies used in IHC.
Final concentrations of antibodies were optimised in preliminary experiments.

Sections were washed with dH_2O, then dehydrated as indicated in **Table 2.9.1.3**, and mounted with histomount® (national diagnostics, US) and glass coverslips (VWR International). Sections were stored at room temperature, protected from light. Images of stained tissue were captured at X20 magnification and stitched together to form a tile scan of the entire section using an EVOS® Cell Imaging System (Life Technologies). The area of tissue containing $CD19^+$ B cells or $CD138^+$ plasma cells was calculated as a percentage of the total area of the tissue section for each patient using ImageJ software (National Institute of Health, US).

Step	Solution	Time (minutes:seconds)
1	70 % Ethanol	00:30
2	90 % Ethanol	01:00
3 (x2)	100 % Ethanol	03:00
4	Xylene	05:00

Table 2.9.1.3. Steps for dehydration of paraffin tissue sections.

2.9.2 Dual IHC staining for light microscopy

Sections were deparaffinised, rehydrated, and epitope retrieval performed as previously described in section **2.9.1**. Sections were stained using the EnVision™ G|2 Doublestain System for Rabbit/Mouse (Dako) according to the manufacturer's instructions (patented biotin-free staining system, all reagents provided by manufacturer at ready-to-use concentrations). In brief, sections were incubated with 100 µl/section dual endogenous enzyme block (0.5 % H_2O_2

and enzyme inhibitors, pH 2) at room temperature for 5 minutes, washed with TBST and incubated with 100 µl/section mouse anti-human CD138 antibody (prepared in TBST as indicated in **Table 2.9.1.2**) at room temperature for 30 minutes. Sections were washed in TBST and incubated with 100 µl/section of polymer-HRP (HRP-conjugated dextran polymer that also carries antibodies to mouse and rabbit antibodies) at room temperature for 10 minutes. Sections were washed in TBST and the CD138 epitopes were visualised with 50 µl/section DAB substrate. Sections were washed in dH_2O, followed by TBST, and incubated with 100 µl/section of double stain block at room temperature for 5 minutes. Sections were washed in TBST, and incubated with the anti-human IgG antibody (prepared in TBST as indicated in **Table 2.9.1.2**) at room temperature for 30 minutes. Sections were washed in TBST and incubated with 100 µl/section LINK polymer (a dextran polymer carrying antibodies to mouse and rabbit antibodies) at room temperature for 10 minutes. Sections were washed with TBST and incubated with 100 µl/section of polymer-AP (AP-conjugated dextran polymer carrying affinity-isolated antibodies) at room temperature for 10 minutes. Sections were washed in TBST and the IgG epitopes were visualised with 50 µl/section of permanent red. Sections were washed in dH_2O and mounted with Faramount Aqueous Mounting Medium (Dako) and glass coverslips. Sections were stored at room temperature, protected from light. Images of $CD138^+$ plasma cell rich areas of tissue were captured at X10 and at X40 magnification using an inverted light microscope fitted with a DP25 digital camera, and Cell B software (Olympus). The total number of $CD138^+$ plasma cells, and the total number of $CD138^+$ IgG^+ plasma cells were counted in 4-5 fields of view at X40 magnification using ImageJ software (National Institute of Health, US). From this, the average percentage of $CD138^+$ plasma cells that were IgG^+ was calculated.

2.9.3 Dual IHC staining for fluorescence microscopy

Sections were deparaffinised, rehydrated, endogenous peroxidase blocked, and epitope retrieval performed as previously described in section **2.9.1**. Sections were washed and non-specific antibody binding blocked (2.5 % human serum, 2.5 % horse serum, and 2.5 % avidin D block, in TBST) as previously described in section **2.9.1**. The first primary antibody, mouse anti-human CD19 was prepared in the appropriate diluent (2.5 % human serum, 2.5 % horse serum, and 2.5 %

biotin block, in TBST) as indicated in **Table 2.9.1.2**. Sections were incubated with 100 µl/section of this antibody at 4 ºC overnight. Sections were warmed to room temperature and washed in TBST. The first secondary antibody, biotin-conjugated horse anti-mouse IgG was prepared in the appropriate diluent (2.5 % horse serum in TBST) as indicated in **Table 2.9.1.2**. Sections were incubated with 100 µl/section of this antibody at room temperature for 30 minutes. SA-conjugated fluorochrome DyLight® 549 (Vector Laboratories) was diluted 1/200 in PBS, Ph8. All subsequent steps were performed with the sections protected from light. Sections were washed in TBST and incubated with 100 µl/section of this fluorochrome at room temperature for 45 minutes. Sections were washed with dH_2O followed by TBST and incubated with non-specific antibody binding block (2.5 % human serum, 2.5 % rabbit serum, and 2.5 % avidin D block, in TBST) a second time, at room temperature, for 30 minutes. The second primary antibody, rabbit anti-human CD5 was prepared in the appropriate diluent (2.5 % human serum, 2.5 % rabbit serum, and 2.5 % biotin block, in TBST) as indicated in **Table 2.9.1.2**. Sections were incubated with 100 µl/section of this antibody at room temperature for 30 minutes. SA-conjugated fluorochrome DyLight® 488 (Vector Laboratories) was diluted 1/200 in PBS, Ph8. Sections were washed in TBST and incubated with 100 µl/section of this fluorochrome at room temperature for 45 minutes. Sections were washed with dH_2O followed by TBST and mounted with VECTASHIELD® mounting media containing DAPI (Vector Laboratories) and glass coverslips. Sections were stored at 4 ºC, protected from light. Images of $CD19^+$ B cell rich regions were captured at X10 and X20 magnification using an M2 epifluorescence microscope fitted with AxioCamMRm and AxioCamMRc cameras, and AxioVision software (Zeiss). The total number of $CD19^+$ B cells, and the total number of $CD19^+$ $CD5^+$ B1a cells were counted in 4-5 fields of view at X20 magnification using ImageJ software. From this, the average percentage of $CD19^+$ plasma cells that were $CD5^+$ B1a cells was calculated.

2.10 Preparation of cells for cell separation, tissue culture, and flow cytometry

2.10.1 Gingiva

Gingiva dissected from groups of 5 mice were placed in 1 ml PBS. Samples of gingival tissue collected from individual PD patients were temporarily stored in 1 ml RPMI (Invitrogen) on ice, then transferred to 1 ml PBS. The human and murine gingival tissues were processed identically. The gingiva were incubated with 200 µl (500 µg)/sample of dispase-high liberase (Roche) at 37 ºC for 30-45 minutes with intermittent pipetting to disrupt the tissue. The enzymatic reaction was stopped by adding 3 ml cRPMI and the tissue suspension was transferred to gentleMACS™ C tubes (Miltenyi). Two 40 second cycles of cellular dissociation were performed using the gentleMACS™ dissociator, program C (Miltenyi). The tubes were briefly centrifuged to remove bubbles, and the cell suspension was passed through a 40 µm cell strainer using the plunger from a 2 ml syringe (BD Bioscience). Excess cRPMI was added to wash the cells which were centrifuged at 400 x g and 4 ºC for 5 minutes. The cells were resuspended in 5 ml cRPMI for counting.

2.10.2 Murine LN and spleen

LN and spleens were harvested and transferred to cRPMI. Single cell suspensions were prepared by passing them through a 40 µm sieve (BD Biosciences) into cRPMI using the plunger of a 5 ml syringe (BD Biosciences). Cell suspensions were washed with cRPMI and were centrifuged at 400 x g for 5 minutes at 4 ºC. Spleen cells were resuspended in 2-5 ml of RBC lysis buffer (eBioscience) and incubated for 5 minutes at 4 ºC. The reaction was stopped by adding excess cRPMI. The cells were centrifuged as before and resuspended in cRPMI for counting.

2.10.4 Murine blood

Approximately 500 µl blood was transferred to a 1.5 ml Eppendorf® tube containing 500 µl Heparin 20 U/ml in PBS (LEO Pharma, Berkshire, UK). Heparinised blood samples were pooled from groups of 5 mice into a 15 ml

universal tube and further PBS added to give a total volume of 15 ml. Blood was layered on top of 15 ml Histopaque® 1083 (Sigma-Aldrich) in a 50 ml universal tube. The blood and Histopaque® were centrifuged at 2,300 rpm for 30 minutes at room temperature, with the brake switched off. The uppermost layer of PBS and blood plasma was discarded. The cellular interface between the PBS and the Histopaque® was aspirated using a pastette, and transferred to a clean 50 ml universal tube. The cells were washed with 30 ml PBS and centrifuged at 250 x g for 10 minutes. The cells were resuspended in 5 ml flow cytometry buffer (0.01 % NaN_3, 2 % FCS in PBS) for a second wash and centrifuged at 250 x g for 10 minutes. The cells were finally resuspended in 5 ml flow cytometry buffer for counting.

2.10.5 Murine peritoneal fluid

Peritoneal washes were performed with 5 ml ice cold PBS with 3 % FCS (Ray and Dittel, 2010). A 27 G needle and syringe were used to inject the wash fluid, and after gentle massage of the peritoneum, a 25 G needle and syringe were used to collect the wash fluid with peritoneal cells which were transferred to universal tubes. Volumes of fluid collected were recorded and cell counts were made. Cells were centrifuged at 1,500 rpm for 8 minutes at 4 ºC prior to resuspension.

2.10.6 Cell counts

All cell counts were performed using a haemocytometer (Neubauer) and BH-2 light microscope (Olympus). Dead cells were excluded on the basis of trypan blue staining.

2.11 Separation of B cells

MACS® separation of B cells was performed according to the manufacturer's instructions (Miltenyi). Cells and reagents were kept ice-cold throughout. All steps were performed under sterile conditions. Cells were resuspended in MACS® buffer (0.5 % FCS, 2 mM EDTA in PBS) 90 µl/10^7 cells, then CD19 MACS® microbeads (Miltenyi) were added 10 µl/10^7 cells, and the mixture was incubated at 4 ºC for 15 minutes. Cells were washed by adding MACS® buffer 1-2

ml/10^7 cells and centrifuging at 350 x g for 5 minutes. Cells were resuspended in MACS® buffer with up to 1 x 10^8 cells/500 µl. MACS® Columns (Miltenyi) were set up in the magnetic field of a MACS® Separator (Miltenyi). MS Columns were used for a maximum total of 2 x 10^8 cells. LS Columns were used for a maximum total of 2 x 10^9 cells. The columns were prepared by rinsing with MACS® buffer (500 µl/MS Column, 3 ml/LS Column), then the cell suspensions were applied. The columns were washed with MACS® buffer (3 x 500 µl/MS Column or 3 x 3 ml/LS Column) and the CD19 negative fraction of cells was collected. These were kept aside to check the efficiency of the process by flow cytometry. Finally the CD19 positive fraction of cells was eluted by adding MACS® buffer to the column (1 ml/MS Column or 5 ml/LS Column), removing the column from the magnetic field and forcing the cells out using the plunger. The CD19 positive fraction of cells was centrifuged at 400 x g for 5 minutes at 4 ºC and resuspended in 5 ml cRPMI, and cell counts were performed. Purity of the CD19 positive fraction of cells was confirmed by flow cytometry.

2.12 Stimulation of cells from murine gingiva and LN

Single cell suspensions were prepared from gingiva and LNs as described in sections **2.10.1** and **2.10.2** respectively. Cells were resuspended in cRPMI at a concentration of 1 x 10^7/ml and aliquoted 1 x 10^6/well in a 24-well flat-bottomed plate (Costar), then topped up with 900 µl/well PMA and ionomycin (10 ng/ml PMA (Sigma), 500 ng/ml ionomycin (Sigma) in cRPMI) or cRPMI only. Cells were incubated at 37 ºC with 5 % CO_2 for 3 days. At the end of this incubation, the media was harvested for analysis by ELISA.

2.13 Stimulation of cells from murine spleen

Spleens were harvested from mice infected with *P. gingivalis* and sham-infected controls at 6 weeks post-infection. Cells were prepared as described in section **2.10.2**, and $CD19^+$ B cells were separated as described in section **2.11.** All cells were plated at a density of 5 x 10^5/well for 3 and 4 day cultures, or 2 x 10^5/well for 1 week cultures, in 96-well round-bottom cell culture plates (Costar). For cultures of mixed lymphocytes, the plates were pre-coated with anti-CD3 antibody (eBioscience) by incubating with 50 µl/well of anti-CD3 antibody (1

μg/ml in PBS) at 37 ºC with 5 % CO_2 for 4 hours, then carefully removing the excess liquid before adding the cells. In some experiments both lymphocytes and purified B cells were additionally cultured with or without 1 μg/well anti-CD40 (eBioscience), 1 μg/well *E. coli* LPS (Sigma-Aldrich), and 1 μg/well *P. gingivalis* LPS (Invivogen) singly or in combination, as indicated in the results, at 37 ºC with 5 % CO_2 for 4 days (**Chapter 5**). In other experiments, both lymphocytes and purified B cells were additionally cultured with 1 μg/well anti-CD40 (eBioscience), 0.2 μg/well anti-IgD (eBioscience), and 2 ng/ well IL-4, with or without 0.5 μg/well *E. coli* LPS (Sigma-Aldrich), 0.5 μg/well *P. gingivalis* LPS (Invivogen), and 4 ng/well IL-33 (BioLegend) singly or in combination, as indicated in the results (**Chapter 6**), at 37 ºC with 5 % CO_2 for 3 days or 1 week.

2.14 Murine osteoclastogenesis assay

To generate pre-osteoclasts, BM cells were flushed out from the 4 rear leg bones of a BALB/c mouse using HBSS (Invitrogen). The BM cells were centrifuged at 380 x g for 5 minutes at 4 ºC and resuspended in 1 ml of RBC lysis buffer for 2 minutes. The lysis was stopped by adding excess HBSS. The BM cells were centrifuged as before, a resuspended at a 1 x 10^6/ml in macrophage media (cRPMI with 25 ng/ml M-CSF (eBioscience)), aliquoted 1 x 10^5/well in a 96-well flat-bottomed plate (Costar), and incubated at 37 ºC with 5 % CO_2 for 1 day. The cells were fed with 100 μl/well osteoclast media (macrophage media with 100 ng/ml sRANKL (Peprotech)) and incubated at 37 ºC with 5 % CO_2 for another 2 days. The pre-osteoclasts had 100 μl/well of their existing media removed and replaced with fresh osteoclast media with $CD19^+$ cells or CD19- cells (separated as described in section **2.11**). These cells were been harvested from the inguinal LN and mesenteric LN of female BALB/c mice 3 days after they had received their final treatment of 80 μl IL-33 (10 μg/ml in PBS with 0.2 % BSA) or vehicle only (n = 3/group) delivered by intraperitoneal injection, within a 4 day period. The pre-osteoclasts were incubated with the $CD19^+$ or CD19- cells at 37 ºC with 5 % CO_2 for 3 days, then 100 μl/well osteoclast media was added and they were incubated at 37 ºC with 5 % CO_2 for 1 more day. The osteoclasts were stained using the acid phosphatase leukocyte staining kit (Sigma-Aldrich) according to the manufacturer's instructions. In brief, all reagents were warmed to room temperature and dH_2O was warmed to 37 ºC. A diazotized fast garnet solution

was made by mixing 200 µl fast garnet base with 200 µl sodium nitrite. After standing for 2 minutes, 200 µl of the diazotized fast garnet solution was added to 9 ml dH_2O 100 µl napthol phosphate, 400 µl acetate, and 200 µl tartrate to make the TRAP stain. A positive control stain was also prepared which lacked tartrate, and a negative control stain was prepared which lacked the napthol phosphate. All staining mixtures were warmed to 37 ^oC before use. Meanwhile, the media and non-adherent cells were removed from the cell culture plate. Cells were fixed with 200 µl/well fixative (26 % citrate, 66 % acetone, 3 % formaldehyde) for 30 seconds, then washed 3 times with 200 µl/well dH_2O. Cells were incubated with 200 µl/well of TRAP stain at 37 ^oC for 1.5 hours, then washed 3 times as before, and air dried. Images were captured at X10 magnification using an inverted light microscope fitted with a Colourview I digital camera, and Cell D software (Olympus).

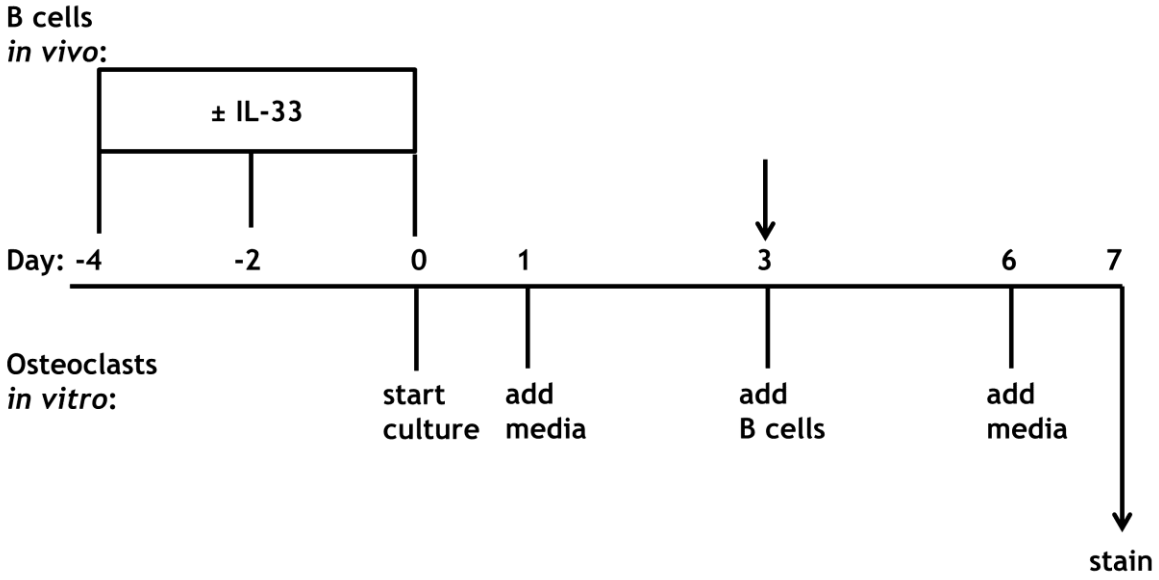

Figure 2.14.1. Timeline of the murine osteoclastogenesis assay

2.15 Flow cytometry

2.15.1 Human gingival cells

Single cell suspensions were prepared from human gingival tissue as described in **2.9.1.** The total live cell count ranged from 5 x 10^5 – 7 x 10^6**.** Cells were centrifuged at 400 x g for 5 minutes at 4 ºC, and resuspended in 600 µl FcR blocking buffer (eBioscience). Cells were incubated at 4 ºC for 20 minutes and split into flow cytometry tubes for the different staining conditions. Antibodies were added (as indicated in **Table 2.16.1.1**.) and cells were incubated at 4 ºC for 30 minutes. Cells were washed twice by centrifuging at 400 x g for 5 minutes at 4 ºC and resuspending in 200 µl flow cytometry buffer (0.01 % NaN_3, 2 % FCS in PBS). Cells were resuspended in 4 % PFA after the second wash and incubated at 4 ºC for 10 minutes. Cells were washed twice as before, resuspended in 200 µl flow cytometry buffer after the last wash, and passed through nitex before proceeding to flow cytometry. Flow cytometry was performed using the CyAn™ (Beckman Coulter) or the MACSQuant® (Miltenyi). Data were subsequently analysed using FlowJo® software (Tree Star Inc., Ashland, US).

Antibody	Isotype	Final concentration	Supplier
CD3 PerCP Cy5.5	Mouse IgG2aκ	0.25 µg/100 µl	eBioscience
CD19 APC	Mouse IgG1κ	1 µg/100 µl	eBioscience
CD138 FITC	Mouse IgG1κ	10 µg/100 µl	BioLegend

Table 2.15.1.1. Anti-human antibodies for flow cytometry.
Final concentrations of antibodies were determined by the manufacturer's recommendations.

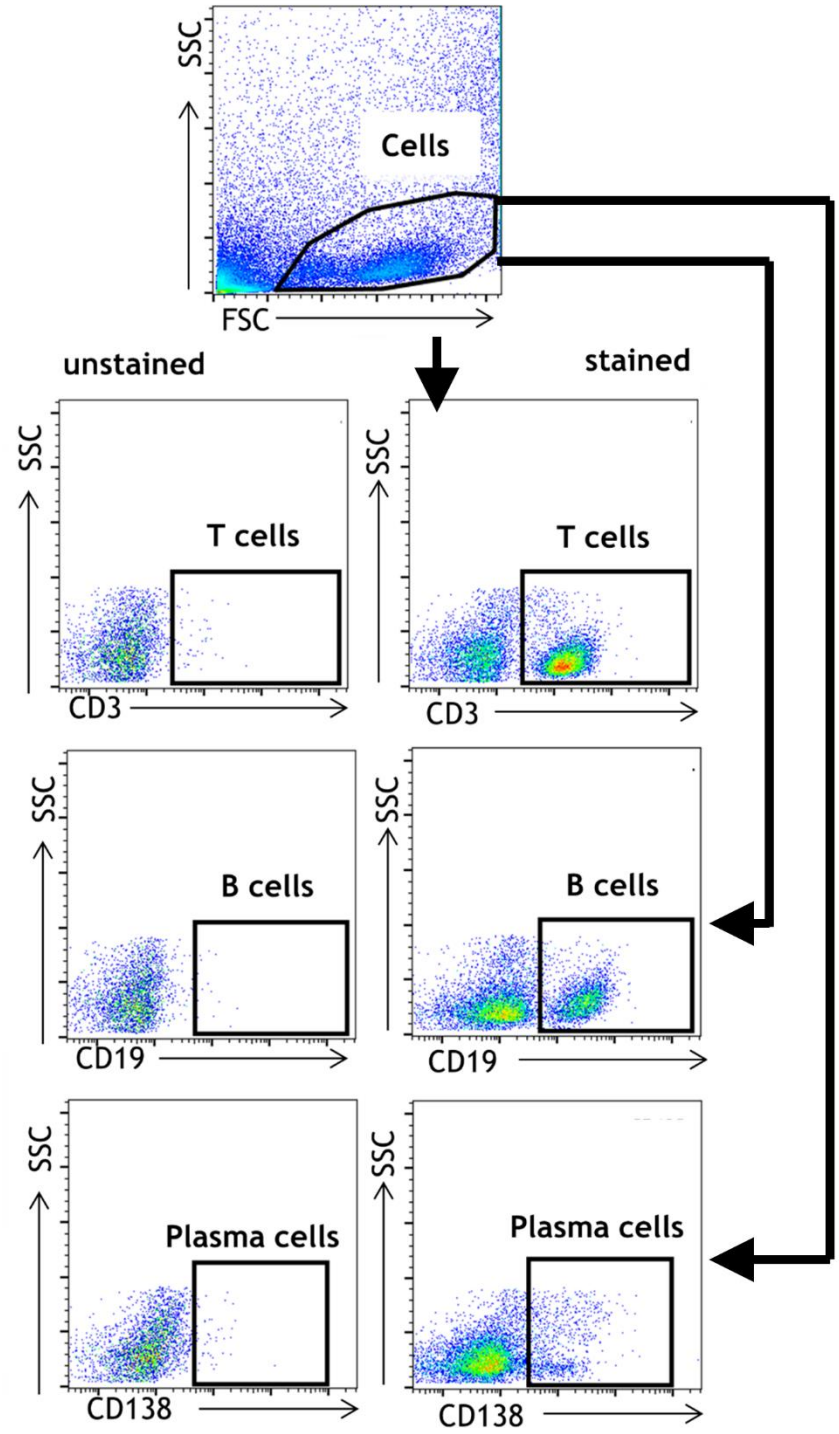

Figure 2.15.1.1. Example of flow cytometry plots of human gingival cells. A population of cells likely to contain lymphocytes, and exclude some debris and dead cells, were identified based on their forward and side scatter properties (top panel). The proportion of cells expressing CD3, CD19, and CD138 was then analysed. Isotype staining was not performed due to insufficient cell numbers in most samples.

2.15.2 Murine cells

Single cell suspensions were prepared as described in **2.10.** Freshly isolated gingiva, LN, and spleen cells were aliquoted up to 1 x 10^6/well of a 96-well round-bottom plate (Costar). Cells from culture were originally plated at a density of 2-5 x 10^5/well in a 96-well round-bottom plate (Costar) and retained in the plate for staining. Freshly isolated blood and peritoneal cells were transferred to flow cytometry tubes. All cells were resuspended in 100 µl/1 x 10^6 cells of FcR blocking buffer (5 % mouse serum in 2.4G2 hybridoma supernatant (containing monoclonal antibodies which block FcRs)) and incubated at 4 ^oC for 15 minutes. Primary antibodies for extracellular staining were prepared in flow cytometry buffer as indicated in **Table 2.15.2.1**. All subsequent steps were performed protected from light. Cells were incubated with the FcR blocking buffer and 100 µl/1 x 10^6 cells of primary antibodies at 4 ^oC for a further 30 minutes. Cells were washed twice with 200 µl/well or tube of flow cytometry buffer and centrifuged at 400 x g and 4 ^oC for 5 minutes.

When viability dye was used, the cells were then washed with 200 µl/well or tube PBS and centrifuged at 400 x g and 4 ^oC for 5 minutes. The viability dye was prepared in PBS as indicated in **Table 2.15.2.1.** Cells were incubated with 100 µl/1 x 10^6 cells of viability dye at 4 ^oC for 30 minutes. This was the final step prior to washing and fixing, which was were both performed in PBS, and the cells were resuspended in PBS for flow cytometry.

When primary antibodies were conjugated to biotin, a secondary antibody conjugated to fluorochrome-labelled SA was required. The secondary antibody was prepared in flow cytometry buffer as indicated in **Table 2.15.2.1**. Cells were incubated with 100 µl/1 x 10^6 cells of secondary antibodies at 4 ^oC for 30 minutes. Cells were washed twice with 200 µl/well or tube of flow cytometry buffer and centrifuged at 400 x g and 4 ^oC for 5 minutes.

Intracellular staining was performed on cells from culture, following incubation with the final surface antibodies. Cells were resuspended in 100 µl/well of fixation-permeabilisation buffer (eBioscience) and incubated at 4 ^oC for 20 minutes, then washed twice with 200 µl/well of permeabilisation-wash buffer

(eBioscience). Intracellular antibodies were prepared in permeabilisation-wash buffer (eBioscience). Cells were incubated with 50 µl/well of intracellular antibodies at 4 °C for 30 minutes. Cells were washed once with 200 µl/well permeabilisation-wash buffer (eBioscience) and centrifuged at 400 x g and 4 °C for 5 minutes. Cells were then washed twice with 200 µl/well or tube of flow cytometry buffer and centrifuged at 400 x g and 4 °C for 5 minutes, and either resuspended in flow cytometry buffer or fixed with 100 µl/well or tube of 4 % PFA at 4 °C for 10 minutes. After fixing, cells were washed twice as before and resuspended in flow cytometry buffer.

All samples were passed through nitex before proceeding to flow cytometry. Flow cytometry was performed using the CyAn™ (Beckman Coulter) or the MACSQuant® (Miltenyi). Data were subsequently analysed using FlowJo® software (Tree Star Inc.). When there were sufficient cell numbers, flow cytometry plots were viewed as pseudocolour density dot plots for analysis (for example **Figure 2.15.2.1**). When there were low cell numbers, flow cytometry plots were viewed as contour zebra plots for analysis (for example **Figure 2.15.2.3**).

Antibody	Clone	Isotype	Final dilution	Final concentration (µg/ml)	Supplier
B220 FITC	RA3-6B2	Rat IgG2a$_\kappa$	1/200	2.5	eBioscience
B220 421	RA3-6B2	Rat IgG2a$_\kappa$	1/400	ND	BioLegend
CD3 PerCP	145-2C11	Hamster IgG1$_\kappa$	1/200	1	BD Bioscience
CD4 PerCP	RM4-5	Rat IgG2a$_\kappa$	1/200	1	eBioscience
CD5 PerCP	53-7.3	Rat IgG2a$_\kappa$	1/300	0.7	BD Bioscience
CD8 FITC	53-6.7	Rat IgG2a$_\kappa$	1/200	2.5	eBioscience
CD19 APC-Cy7	1D3	Rat IgG2a$_\kappa$	1/200	1	BD Bioscience
CD23 Biotin	B3B4	Rat IgG2a$_\kappa$	1/200	2.5	BD Bioscience
CD43 FITC	S7	Rat IgG2a$_\kappa$	1/200	2.5	BD Bioscience
CD44 APC	IM7	Rat IgG2b$_\kappa$	1/200	1	eBioscience
CD45 450	30-F11	Rat IgG2b$_\kappa$	1/400	0.5	eBioscience
CD62L PE	Mel-14	Rat IgG2a$_\kappa$	1/200	1	BD Bioscience
CD69 FITC	H1.2F3	Hamster IgG	1/200	2.5	BD Bioscience
CD86 PE	GL1	Rat IgG2a$_\kappa$	1/200	1	BD Bioscience
CD95 (Fas) PE	Jo2	Hamster IgG2$_{\lambda 2}$	1/200	1	BD Bioscience
CD138 PE	281-2	Rat IgG2a$_\kappa$	1/200	1	BD Bioscience
GL7 FITC	GL7	Rat IgM	1/200	2.5	BD Bioscience
Ki-67 647	16A8	Rat IgG2a$_\kappa$	1/200	2.5	BioLegend
Ki-67 PE-Cy7	SolA15	Rat IgG2a$_\kappa$	1/400	0.5	eBioscience
RANKL 647	IK22-5	Rat IgG2a$_\kappa$	1/100	2	BD Bioscience
viability dye 450	NA	NA	1/1,000	ND	eBioscience
SA PE-Cy7	NA	NA	1/200	1	BD Bioscience

Table 2.15.2.1. Anti-mouse antibodies for flow cytometry.
Final concentrations of antibodies were optimised in preliminary experiments. NA = not applicable, ND = no data available.

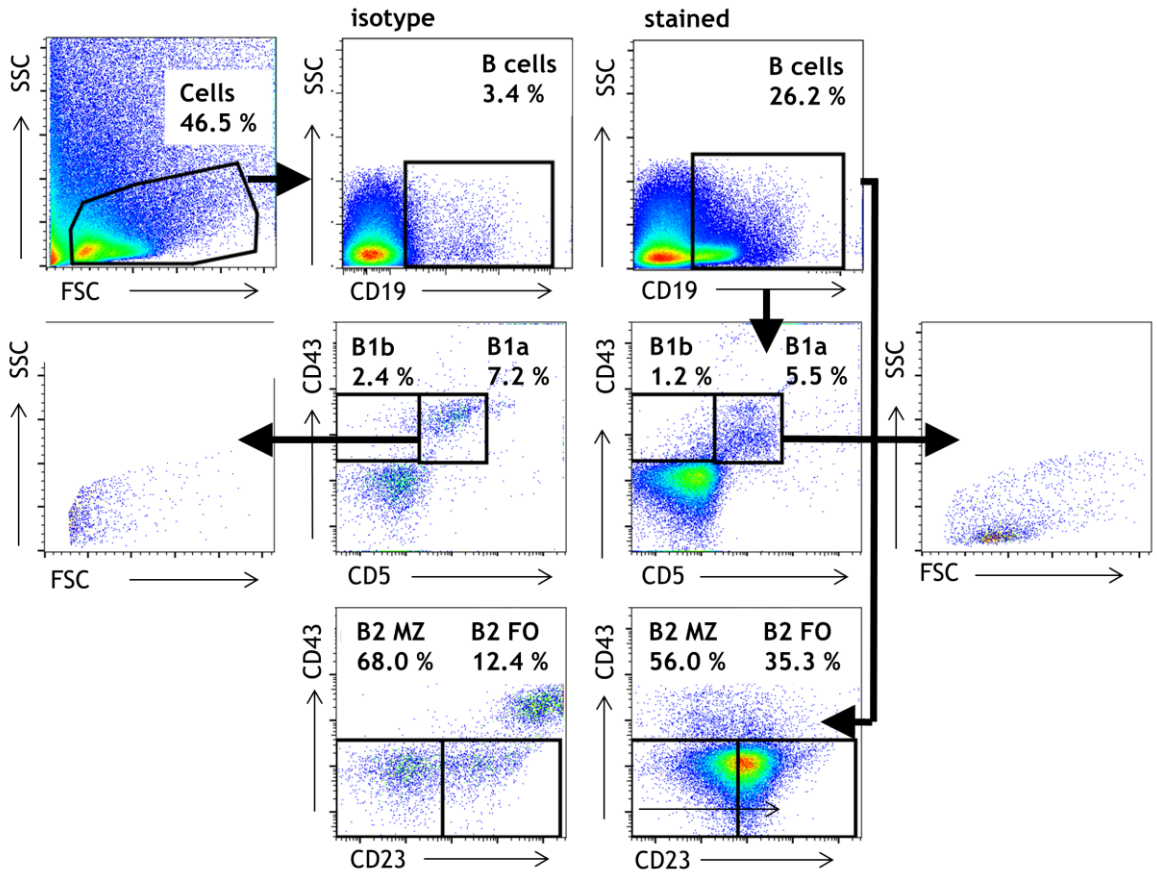

Figure 2.15.2.1. Example of flow cytometry plots of B cell subsets in murine gingiva. A population of cells likely to contain lymphocytes, and exclude some debris and dead cells, were identified based on their forward and side scatter properties (top far-left panel). The proportion of cells expressing CD19 (B cells) was determined, and the proportion of B cells that were $CD43^+$ $CD5^+$ B1a cells, $CD43^+$ $CD5^-$ B1b cells, $CD43^-$ $CD23^-$ B2 MZ cells, and $CD43^-$ $CD23^+$ B2 FO cells was analysed. Stained cells were back-gated to confirm their forward and side scatter properties. Cells that appeared stained in the isotype condition appeared to be mostly dead by forward and side scatter, whereas cells that appeared stained in the stained condition appeared to be lymphocytes.

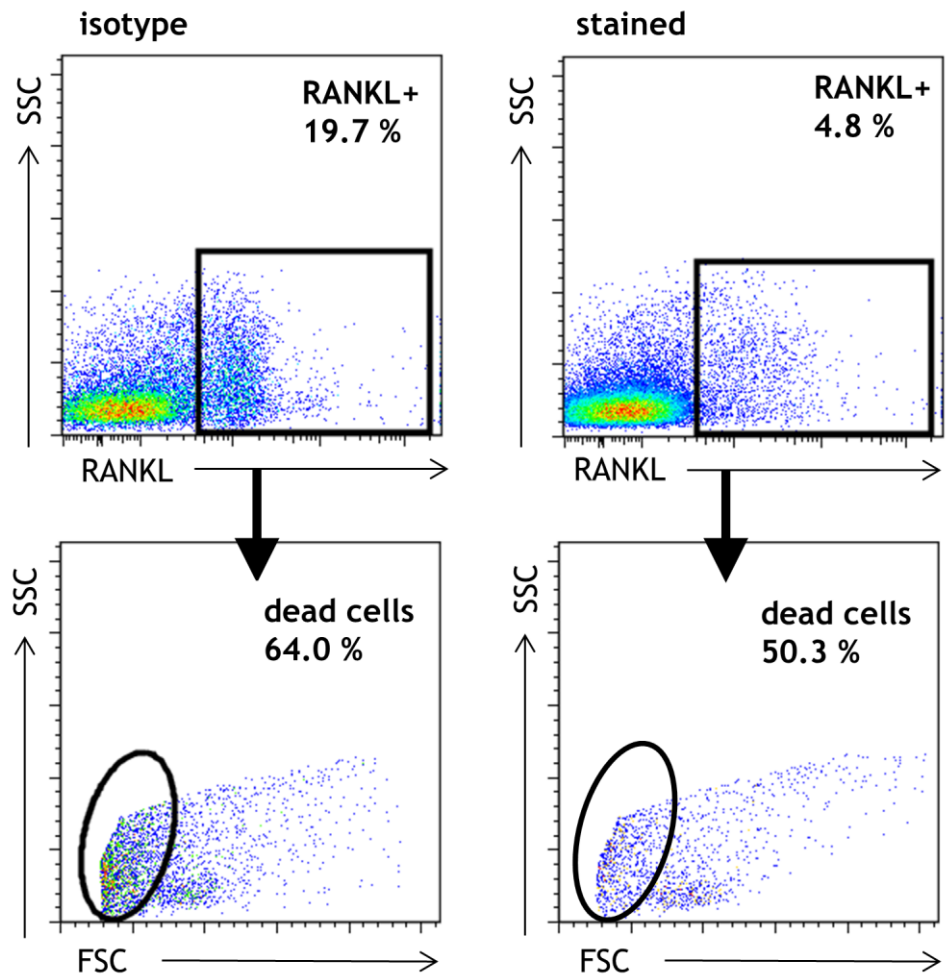

Figure 2.15.2.2. Example of flow cytometry plots of B cell RANKL expression in murine gingiva. The proportion of cells expressing CD19 (B cells) was determined as indicted in **Figure 2.15.2.1**, and the proportion of B cells that were RANKL$^+$ was analysed. The RANKL isotype control appeared to stain a population of cells. However, a high proportion of the cells that appeared stained in the isotype condition appeared to be dead by forward and side scatter compared with the proportion of cells that appeared stained in the stained condition.

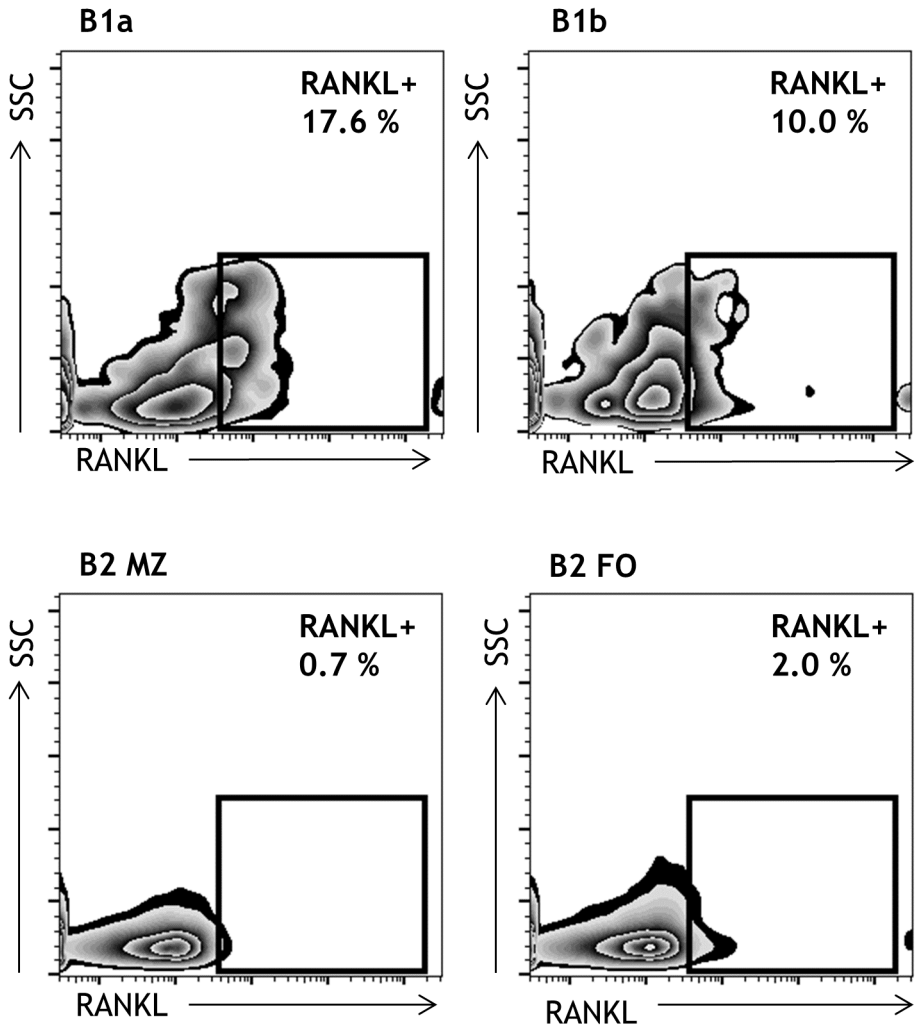

Figure 2.15.2.3. Example of flow cytometry plots of differential B cell RANKL expression in murine gingiva. The proportions of B cell subsets were determined as indicted in **Figure 2.15.2.1**, and RANKL expression by $CD43^+$ $CD5^+$ B1a cells, $CD43^+$ $CD5^-$ B1b cells, $CD43^-$ $CD23^-$ B2 MZ-like cells, and $CD43^-$ $CD23^+$ B2 FO cells was then analysed. RANKL isotype staining on gingival B cells is indicated in **Figure 2.15.2.2.** Flow cytometry plots were viewed as contour zebra plots for this analysis due to low cell numbers.

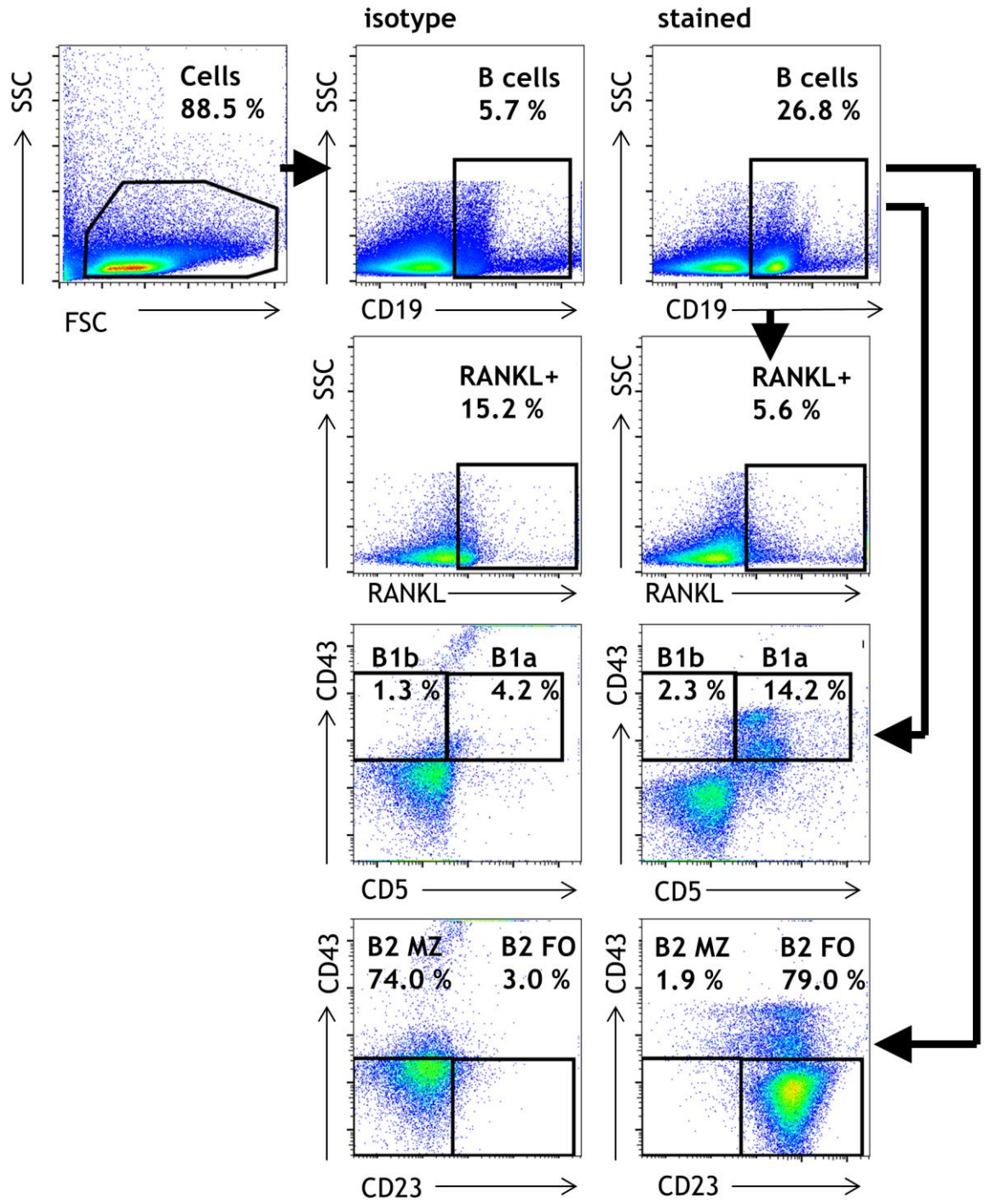

Figure 2.15.2.4. Example of flow cytometry plots of B cell subsets in murine dLNs. A population of cells likely to contain lymphocytes, and exclude some debris and dead cells, were identified based on their forward and side scatter properties (top far-left panel). The proportion of cells expressing CD19 (B cells) was determined, and the proportion of B cells that were $CD43^{+}$ $CD5^{+}$ B1a cells, $CD43^{+}$ $CD5^{-}$ B1b cells, $CD43^{-}$ $CD23^{-}$ B2 MZ-like cells, and $CD43^{-}$ $CD23^{+}$ B2 FO cells was analysed. The RANKL isotype control appeared to stain a higher population of cells than would be reasonably expected, the reason for this is unclear.

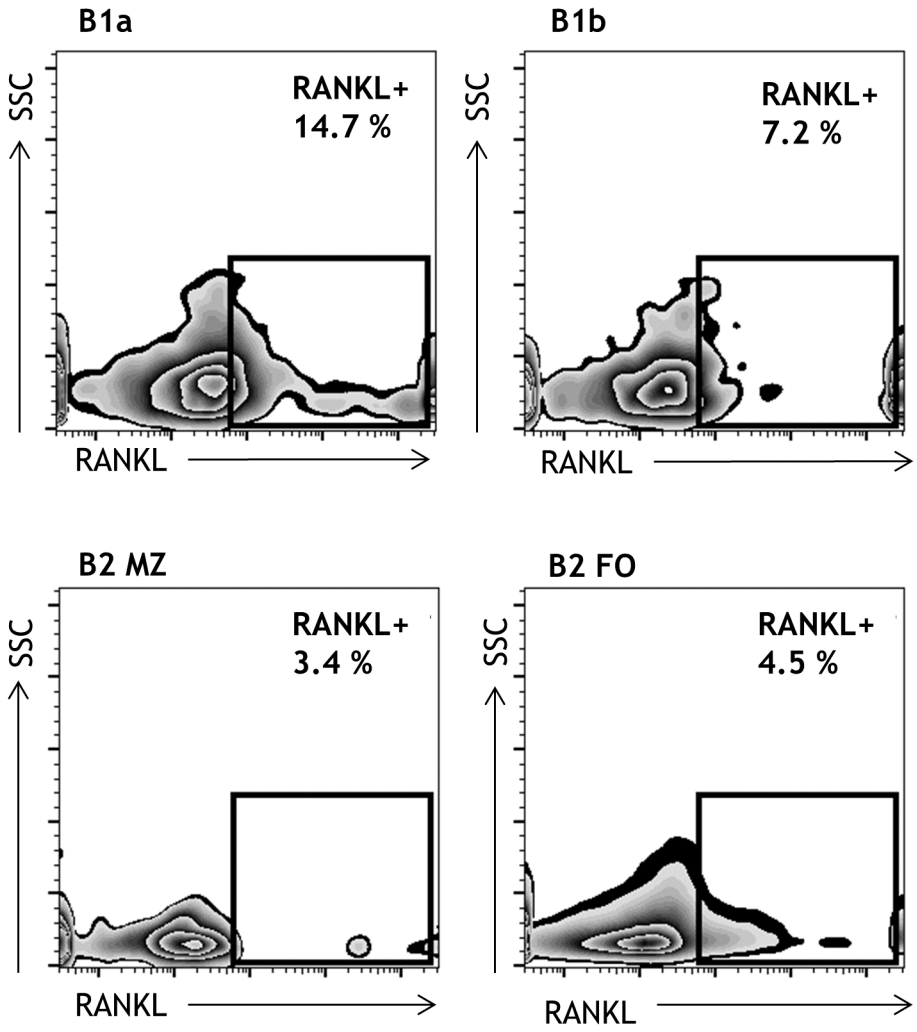

Figure 2.15.2.5. Example of cytometry plots of differential B cell RANKL expression in murine dLNs. The proportions of B cell subsets were determined as indicted in **Figure 2.15.2.4**, and RANKL expression by $CD43^+$ $CD5^+$ B1a cells, $CD43^+$ $CD5^-$ B1b cells, $CD43^-$ $CD23^-$ B2 MZ-like cells, and $CD43^-$ $CD23^+$ B2 FO cells was then analysed. RANKL isotype staining on gingival B cells is indicated in **Figure 2.15.2.4.** Flow cytometry plots were viewed as contour zebra plots for this analysis due to low cell numbers.

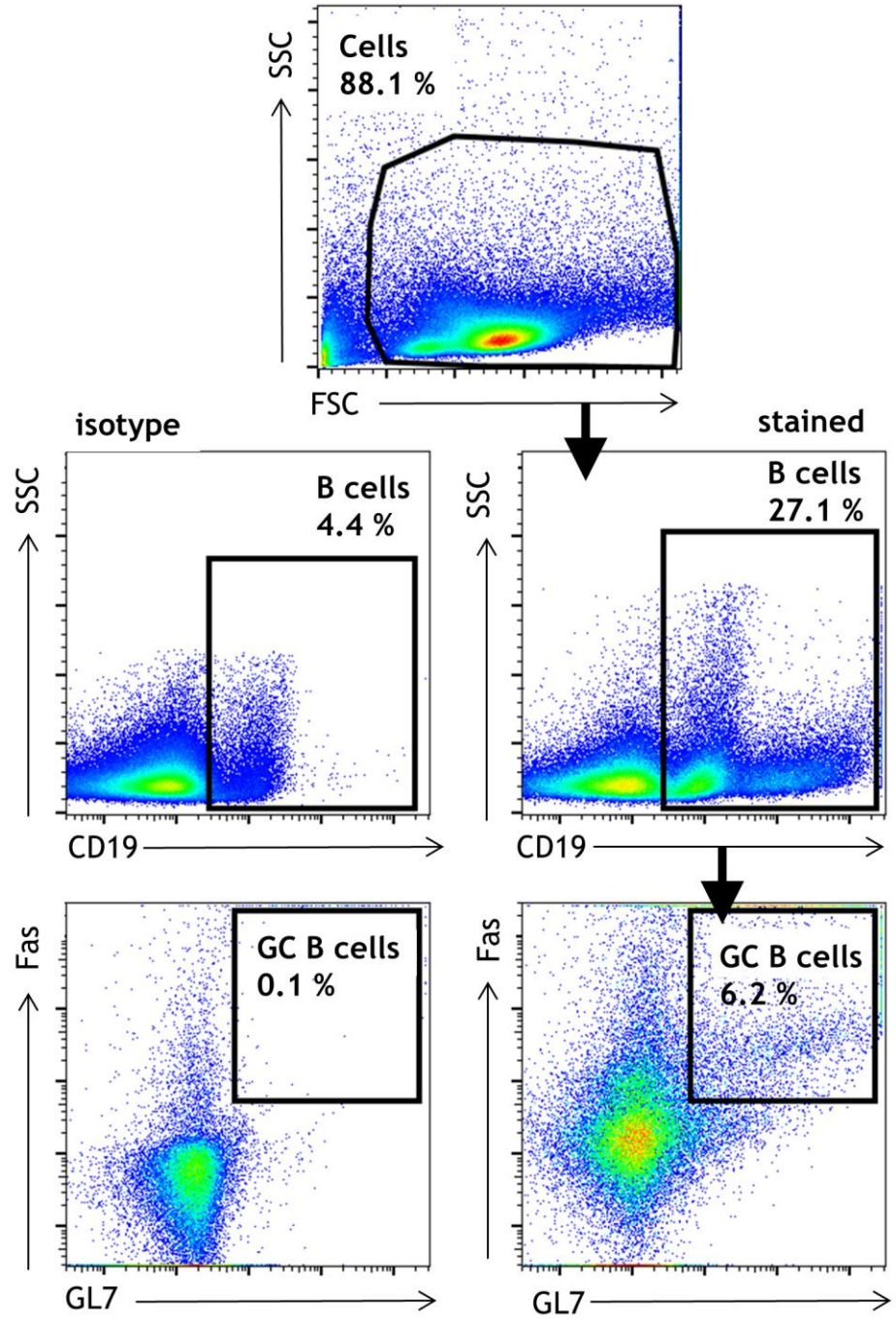

Figure 2.15.2.6. Example of flow cytometry plots of GC B cells in murine dLNs. A population of cells likely to contain lymphocytes, and exclude some debris and dead cells, were identified based on their forward and side scatter properties (top panel). The proportion of cells expressing CD19 (B cells) was determined, and the proportion of B cells that were Fas^+ $GL7^+$ GC B cells was analysed.

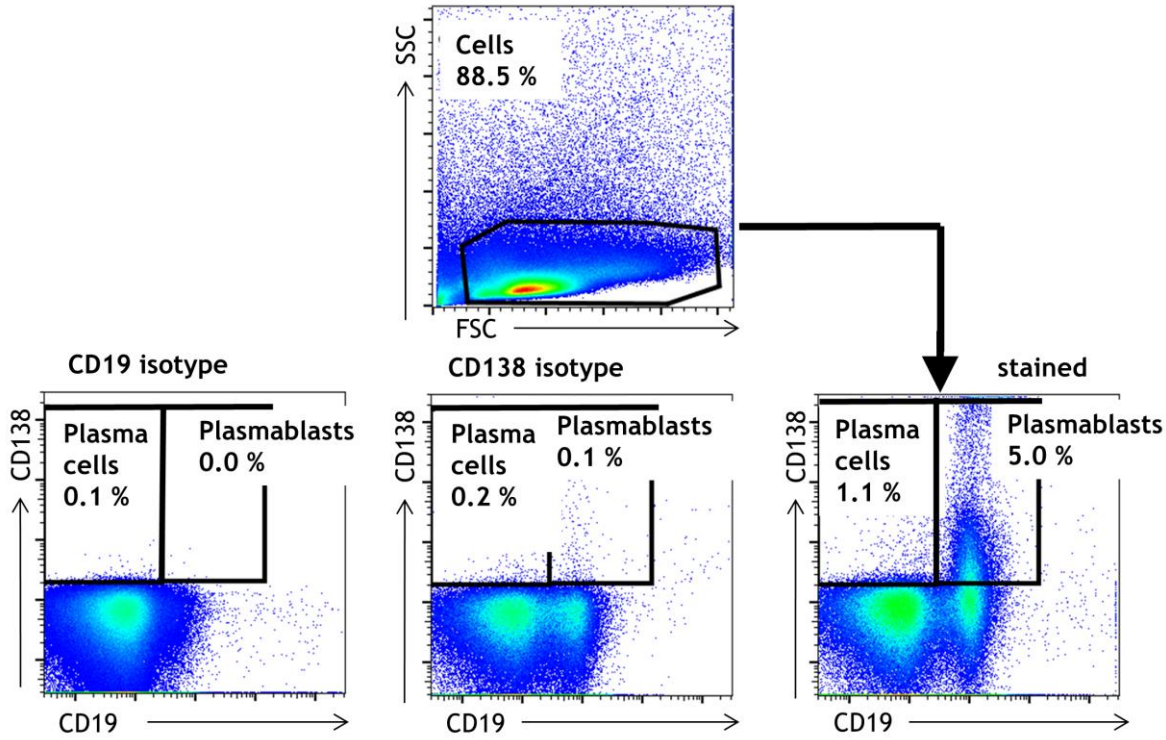

Figure 2.15.2.7. Example of flow cytometry plots of plasmablasts and plasma cells in murine dLNs. A population of cells likely to contain lymphocytes, and exclude some debris and dead cells, were identified based on their forward and side scatter properties (top panel). The proportion of cells that were $CD19^{+}$ $CD138^{+}$ plasmablasts and $CD19^{-}$ $CD138^{+}$ plasma cells was then analysed.

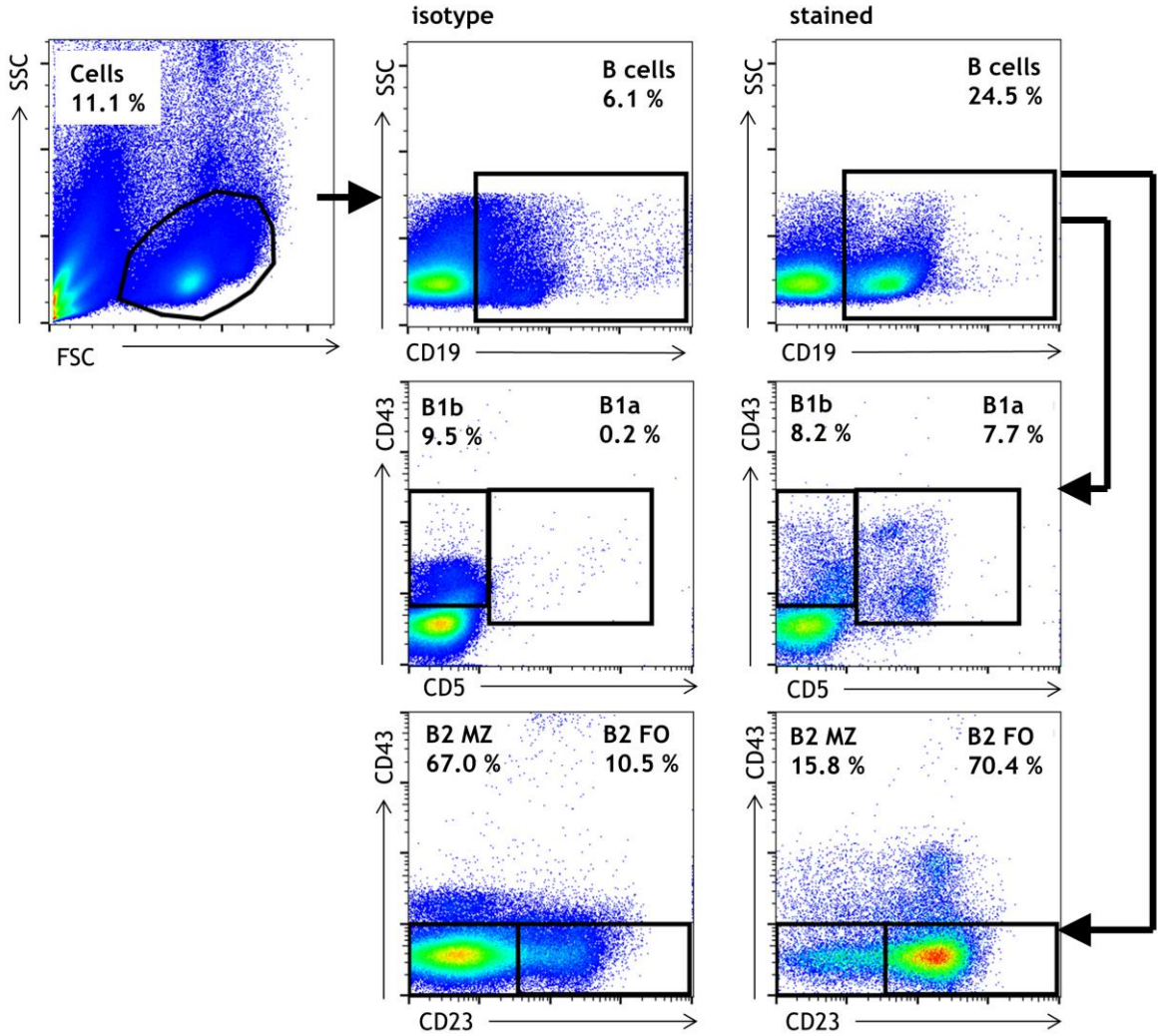

Figure 2.15.2.8. Example of flow cytometry plots of B cell subsets in murine blood. A population of cells likely to contain lymphocytes, and exclude some debris and dead cells, were identified based on their forward and side scatter properties (top far-left panel). The proportion of cells expressing CD19 (B cells) was determined, and the proportion of B cells that were $CD43^{+}$ $CD5^{+}$ B1a cells, $CD43^{+}$ $CD5^{-}$ B1b cells, $CD43^{-}$ $CD23^{-}$ B2 MZ-like cells, and $CD43^{-}$ $CD23^{+}$ B2 FO cells was analysed.

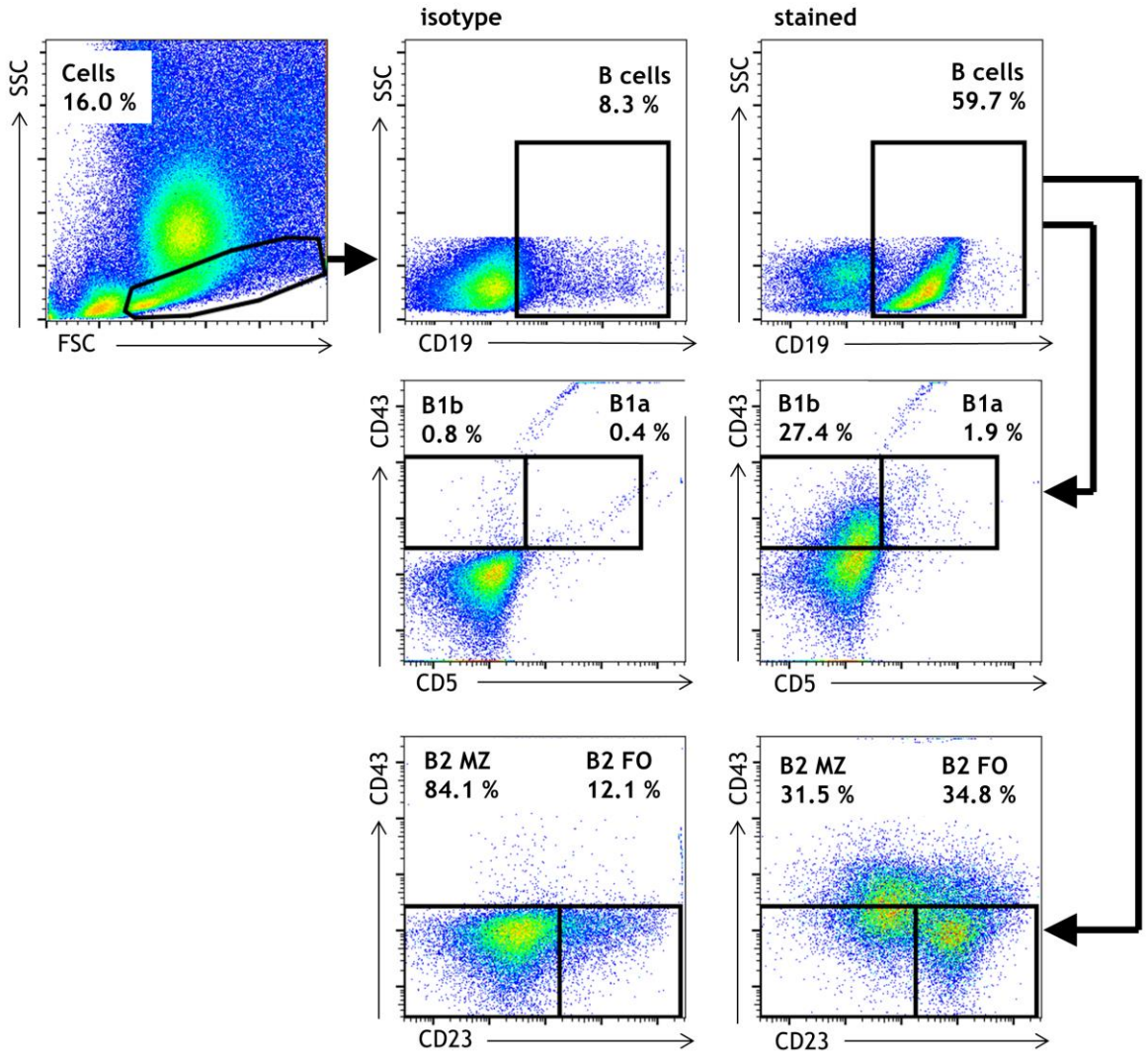

Figure 2.15.2.9. Example of flow cytometry plots of B cell subsets in murine peritoneal fluid. A population of cells likely to contain lymphocytes, and exclude some debris and dead cells, were identified based on their forward and side scatter properties (top far-left panel). The proportion of cells expressing CD19 (B cells) was determined, and the proportion of B cells that were $CD43^+$ $CD5^+$ B1a cells, $CD43^+$ $CD5^-$ B1b cells, $CD43^-$ $CD23^-$ B2 MZ-like cells, and $CD43^-$ $CD23^+$ B2 FO cells was analysed.

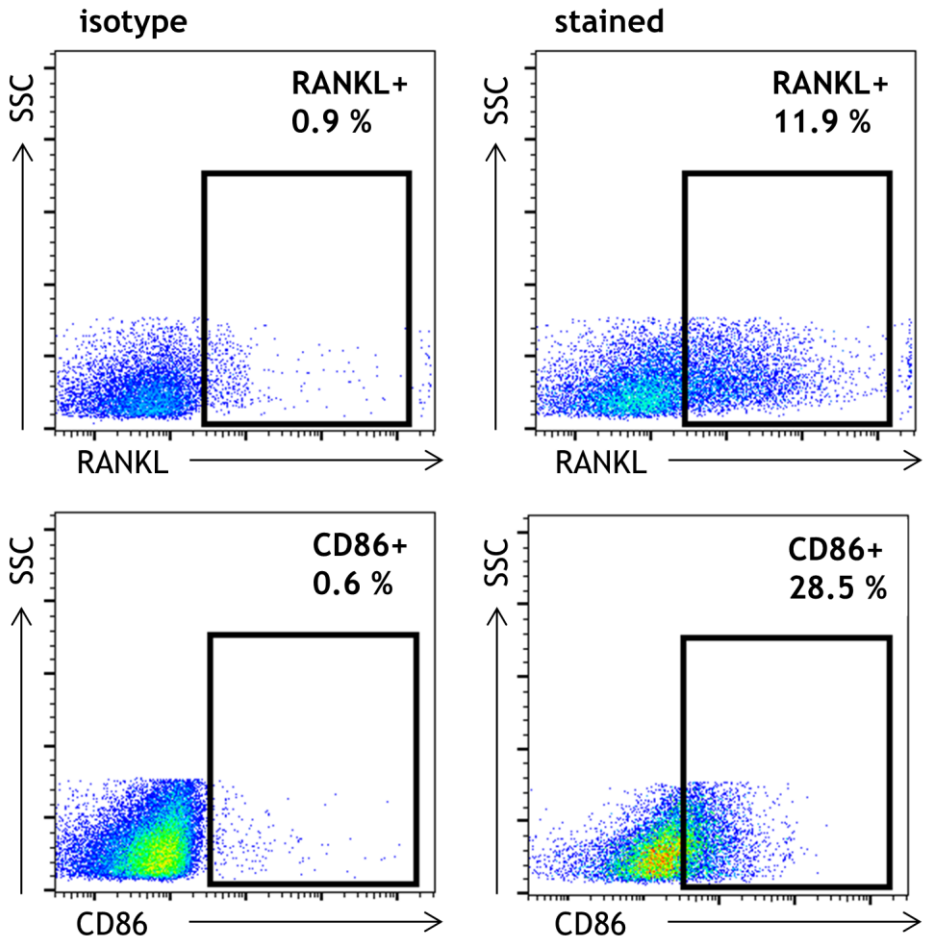

Figure 2.15.2.10. Example of flow cytometry plots of B cell RANKL and CD86 expression in murine peritoneal fluid. Cells that were lymphocytes were identified based on their forward and side scatter properties. The proportion of cells expressing CD19 (B cells) was determined, and the proportion of B cells that were expressing RANKL and CD86 was analysed.

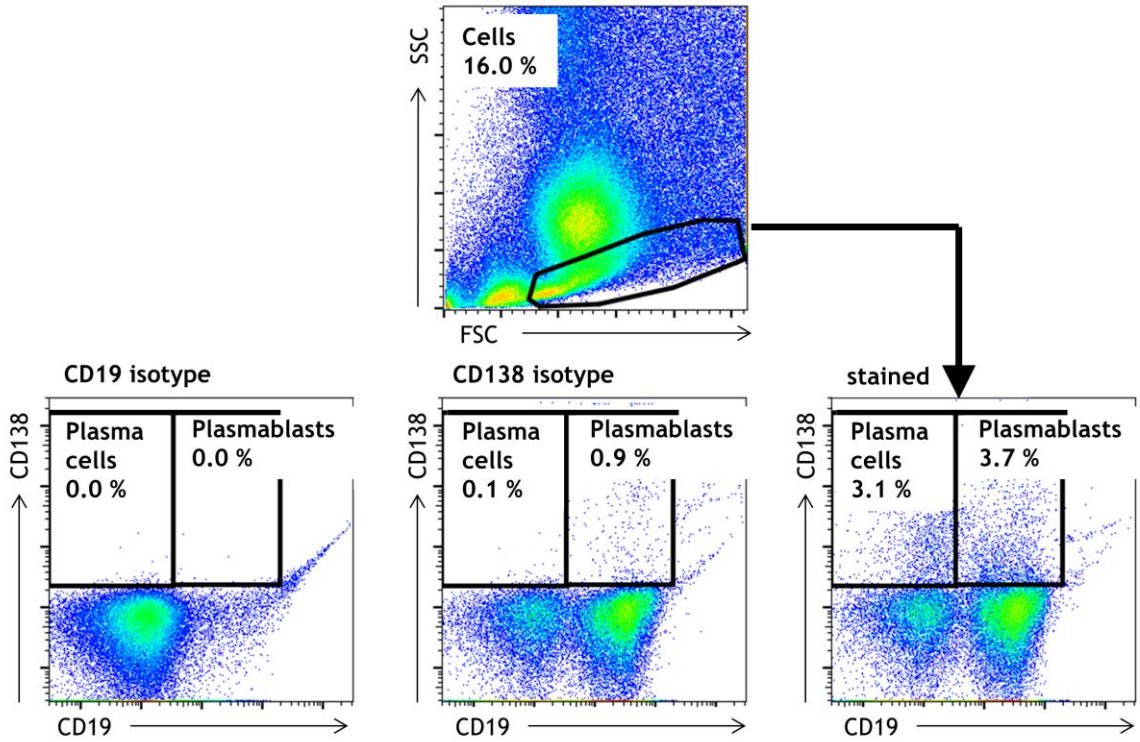

Figure 2.15.2.11. Example of flow cytometry plots of plasmablasts and plasma cells in murine peritoneal fluid. A population of cells likely to contain lymphocytes, and exclude some debris and dead cells, were identified based on their forward and side scatter properties (top panel). The proportion of cells that were $CD19^{+}$ $CD138^{+}$ plasmablasts and $CD19^{-}$ $CD138^{+}$ plasma cells was then analysed.

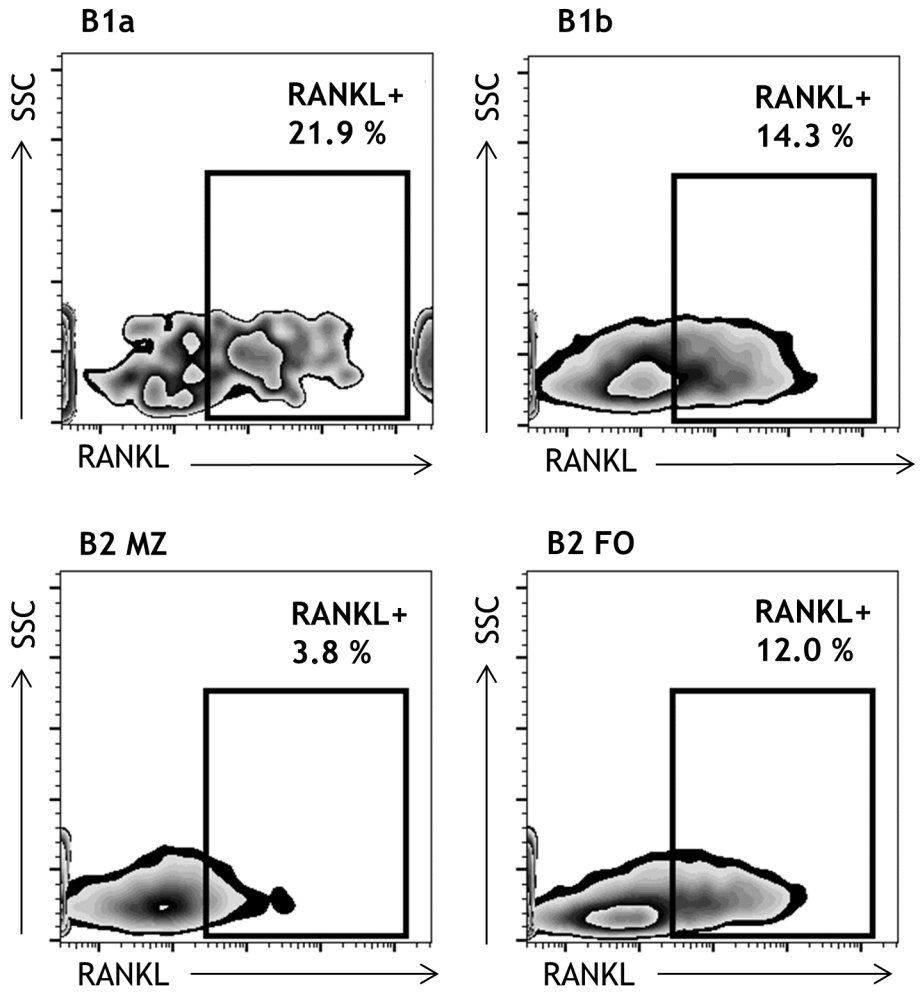

Figure 2.15.2.12. Example of flow cytometry plots of differential B cell RANKL expression in murine peritoneal fluid. The proportions of B cell subsets were determined as indicted in **Figure 2.15.2.9**, and RANKL expression by $CD43^+$ $CD5^+$ B1a cells, $CD43^+$ $CD5^-$ B1b cells, $CD43^-$ $CD23^-$ B2 MZ-like cells, and $CD43^-$ $CD23^+$ B2 FO cells was then analysed. RANKL isotype staining on gingival B cells is indicated in **Figure 2.15.2.10.** Flow cytometry plots were viewed as contour zebra plots for this analysis due to low cell numbers.

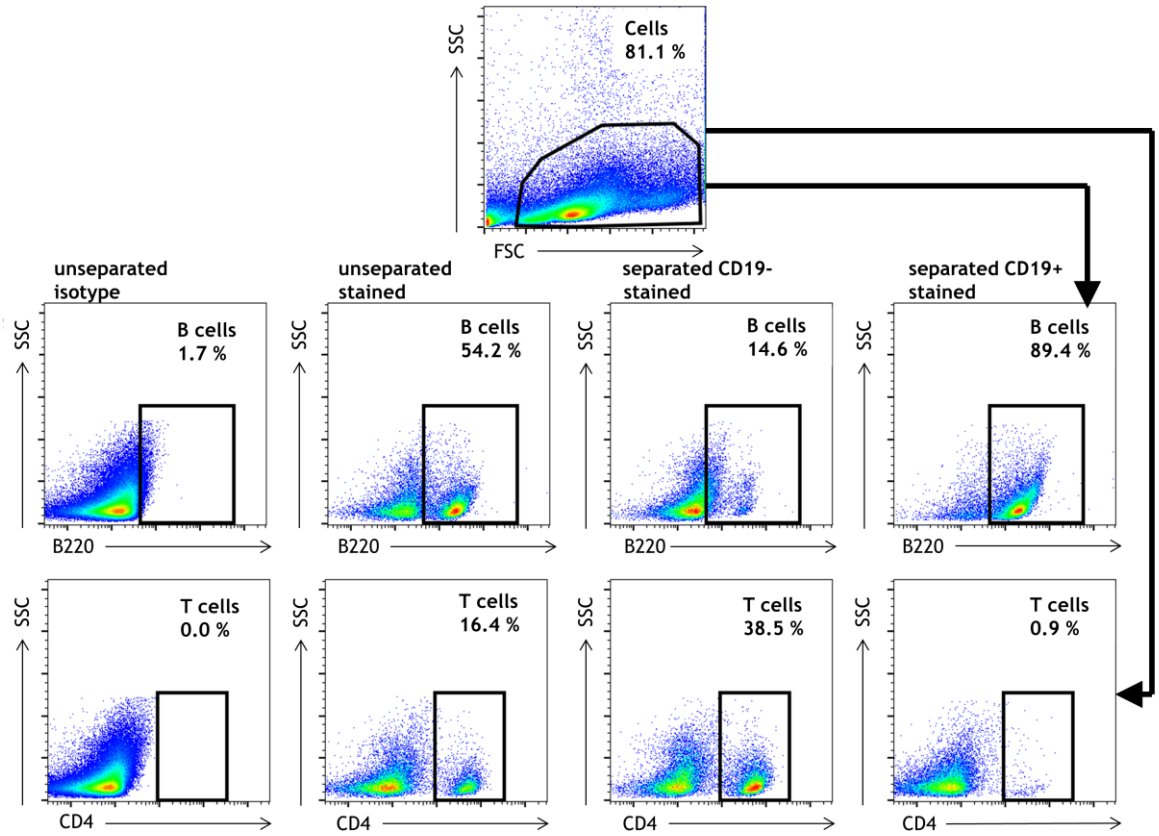

Figure 2.15.2.13. Example of flow cytometry plots of B cell separation. Samples of cells were stained before and after MACS® B cell separation. Cells were separated into CD19 negative and CD19 positive populations. A population of cells likely to contain lymphocytes, and exclude some debris and dead cells, were identified based on their forward and side scatter properties (top panel). The proportion of cells expressing B220 (B cells) and CD4 (T cells) was determined. B cells could not be identified by their expression of CD19 due to interference with the MACS® microbeads.

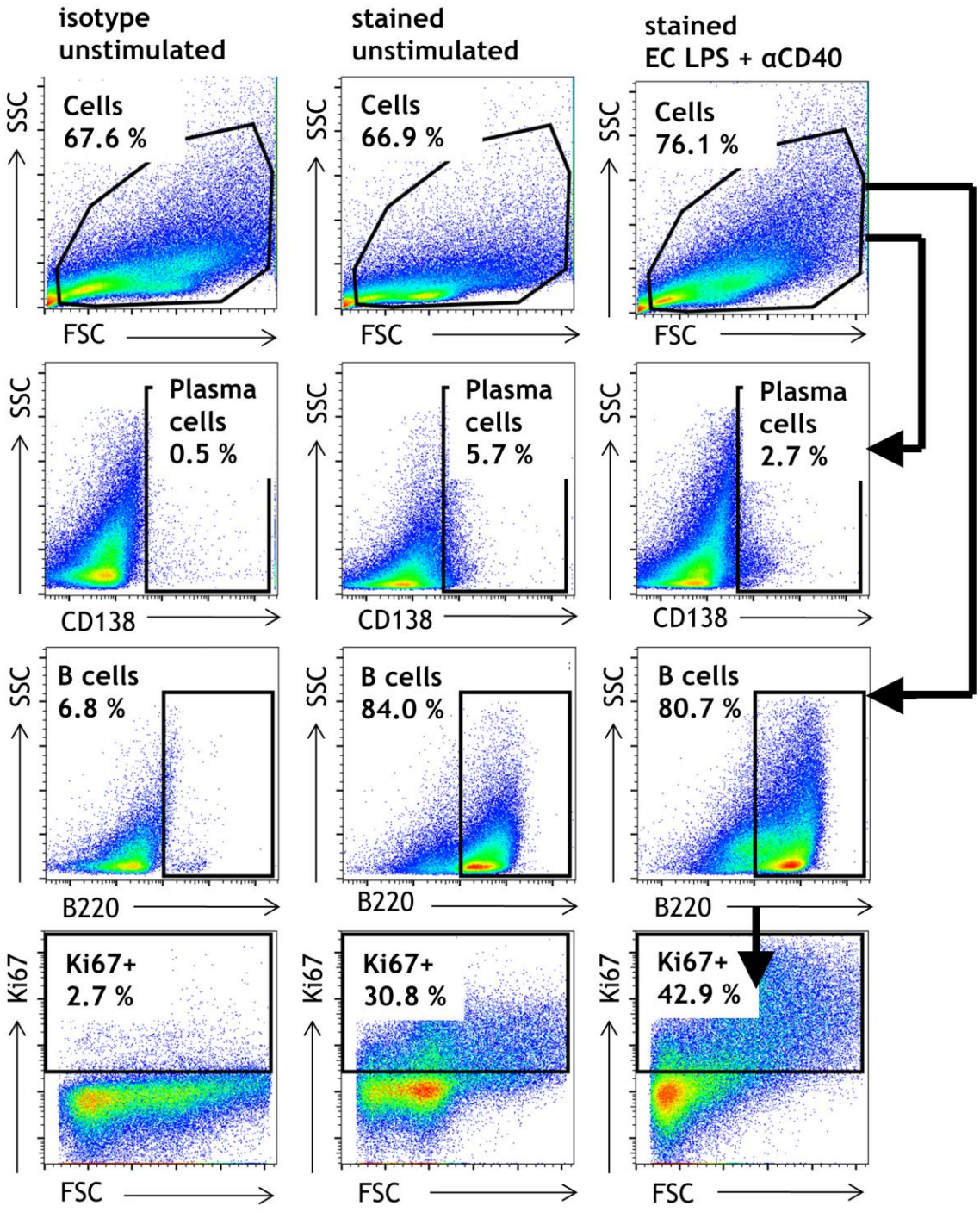

Figure 2.15.2.14. Example of flow cytometry plots of murine splenic B cells after 4 day culture. A population of cells likely to contain lymphocytes, and exclude some debris and dead cells, were identified based on their forward and side scatter properties (top panels). The proportion of cells expressing CD138 (plasma cells) and B220 (B cells) and was determined, and B cell expression of $Ki67^{+}$ was analysed.

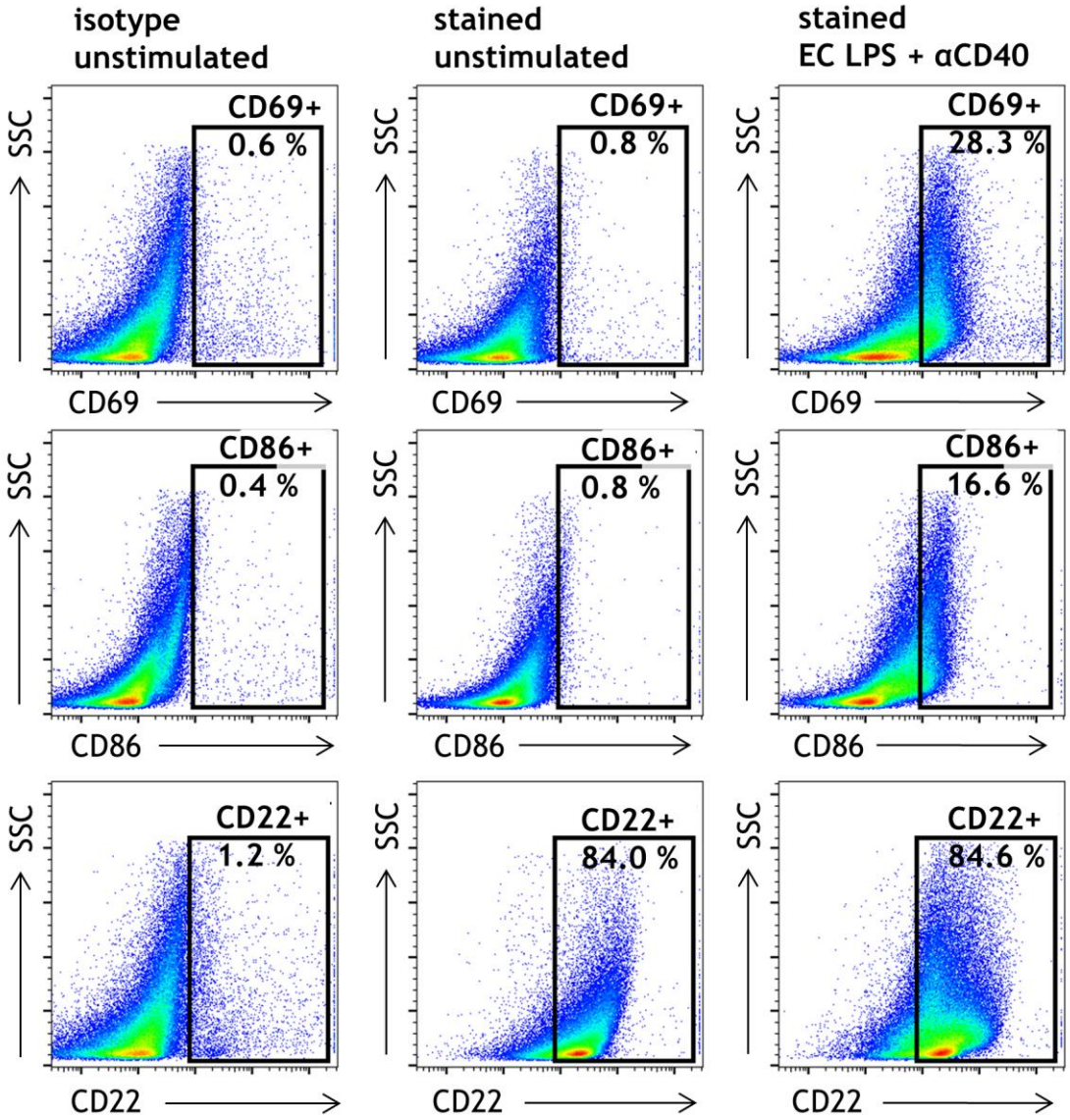

Figure 2.15.2.15. Example of flow cytometry plots of murine splenic B cells after 4 day culture. B cells were identified as indicated in **Figure 2.12.2.14**, and B cell expression of CD69, CD86, and CD22 was analysed.

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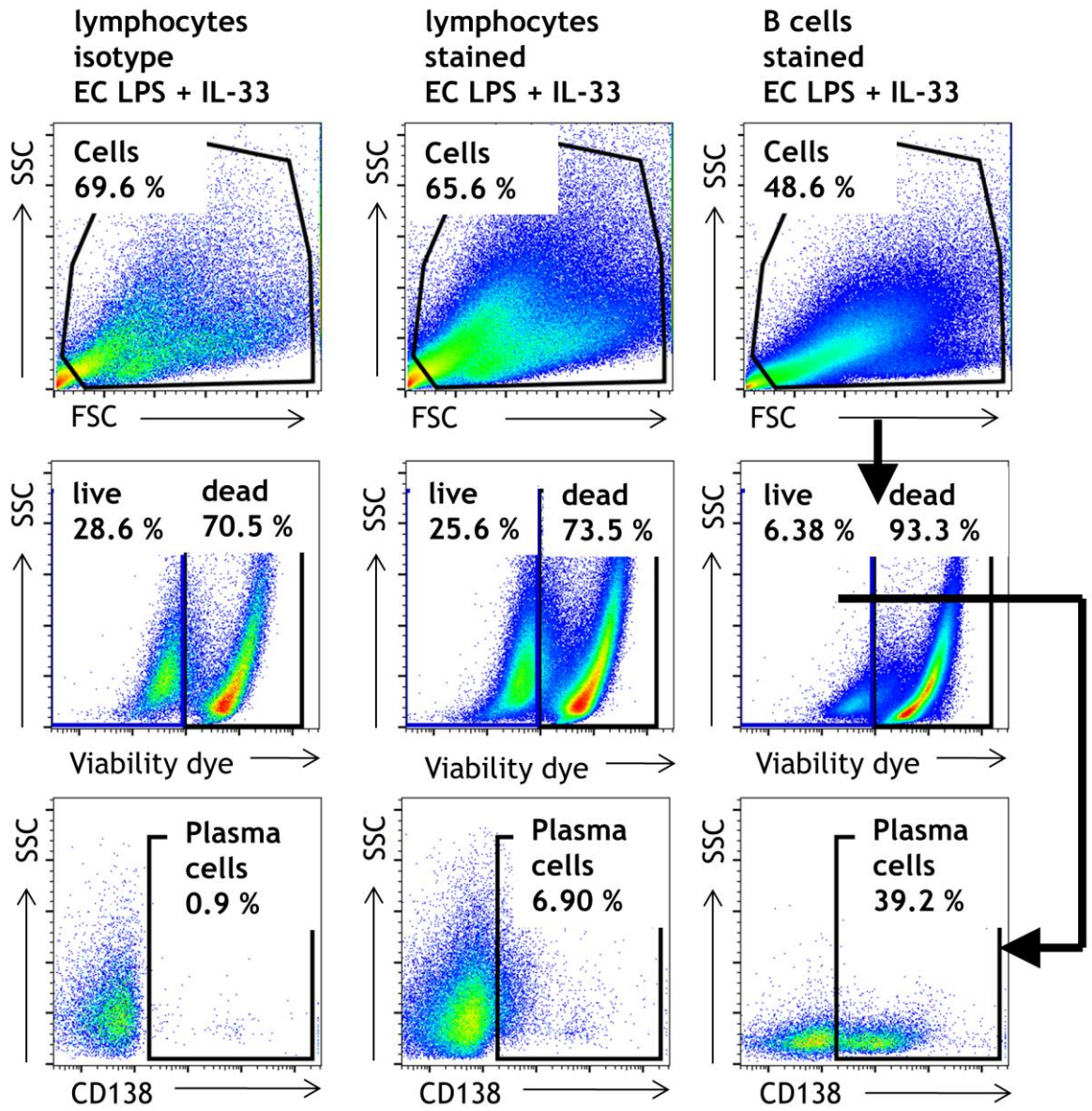

Figure 2.15.2.16. Example of flow cytometry plots of murine lymphocytes after 1 week culture with IL-33. A population of cells likely to contain lymphocytes, and exclude some debris and dead cells, were identified based on their forward and side scatter properties (top panels). Live cells were distinguished by the absence of staining with viability dye (there was no isotype control for this). The proportion of live cells expressing CD138 (plasma cells) was determined.

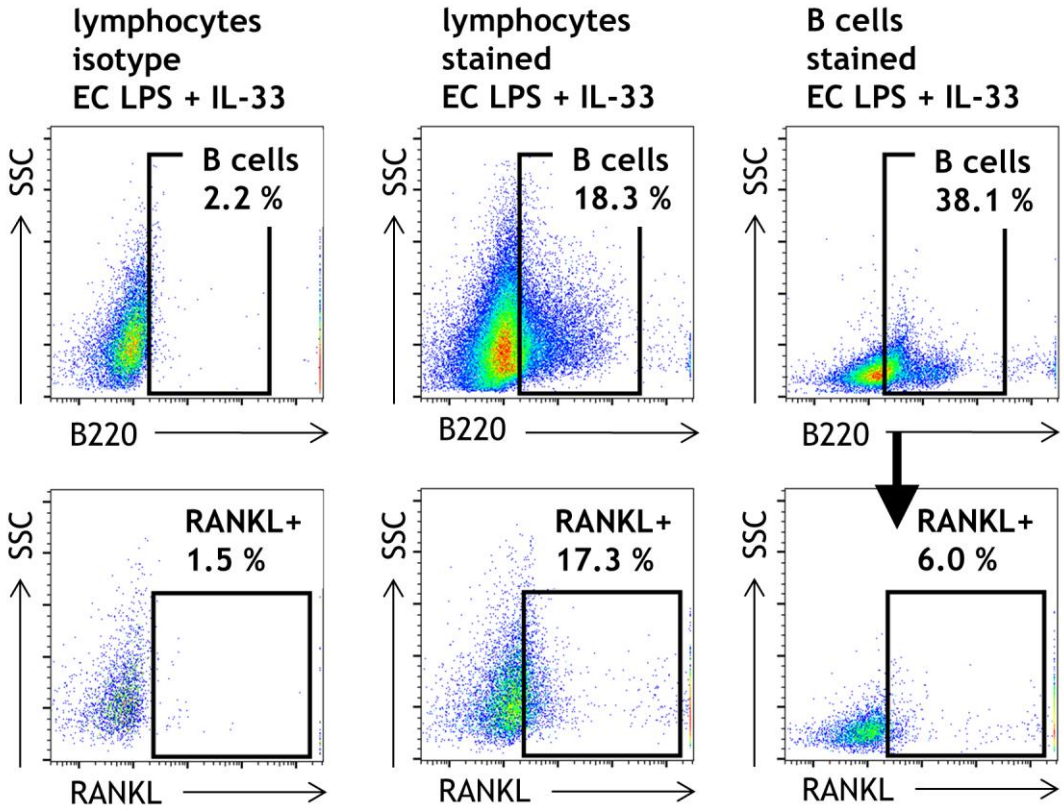

Figure 2.15.2.17. Flow cytometry plots of murine lymphocytes after 1 week culture with IL-33. Live cells were identified as indicated in **Figure 2.15.2.16**. The proportion of live cells expressing B220 (B cells) was determined and B cell expression of RANKL was analysed.

2.16 ELISA

2.16.1 Anti-*P. gingivalis* ELISA

Bacteria were grown as described in section **2.3**. Planktonic bacteria were centrifuged at 3,750 rpm for 20 minutes, the majority of the supernatant was poured off and the pellet was re-suspended in the remaining supernatant, then transferred to several 1.5 ml Eppendorf® tubes and centrifuged at 13,400 rpm for 10 minutes. The supernatant was removed and the bacteria were washed twice by resuspending in 1 ml/tube PBS, centrifuging at 13,400 rpm for 10 minutes and removing the supernatant before being stored at -80 ^{o}C. Frozen stocks of *P. gingivalis* were heat-killed by incubation in a 65 ^{o}C water bath for 30 minutes, then resuspended at 0.02 OD at 600 nm (4 x 10^7 CFU/ml) in carbonate buffer (15 mM Na_2CO_3, 35 mM Na_2 CO_3, pH 9.6). Immunolon IB plates (Fischer Scientific) were incubated with 100 µl/well bacteria in carbonate buffer (15 mM Na_2CO_3, 35 mM Na_2 CO_3, pH 9.6) at 4 ^{o}C overnight. The plates were washed 3 times with 200 µl/well 0.05 % Tween in PBS (PBST) after this and each subsequent step until the substrate was added. The plates were incubated with 200 µl/well blocking buffer (10 % FCS in PBS) at 37 ^{o}C for 1 hour. Serial dilutions of serum ranging from 1/50 to 1/400 were prepared in dilution buffer (0.2 % FCS, 0.05 % Tween in PBS). Samples were prepared and measured in duplicate. The plates were incubated with 50 µl/well of pre-diluted serum samples at 37 ^{o}C for 2 hours, then with 50 µl/well HRP-conjugated goat anti-mouse antibody or biotin-conjugated goat anti-human antibody (prepared in dilution buffer), at 37 ^{o}C for 1 hour. In cases where biotin-conjugated antibody was being used, a further incubation with 100 µl/well ExtraAvidin® peroxidase (Sigma-Aldrich), at 37 ^{o}C for 1 hour was required. Finally, the plates were incubated with 100 µl/well of TMB substrate (Kirkegaard and Perry Laboratories) at room temperature. The reaction was stopped with 50 µl/well 10 % HCl, and absorbance was measured at 450 nm using the MRII plate reader (Dynex Technologies). The mean OD for each dilution of each serum sample was calculated, and the EU for each serum sample was then calculated from the y-intercept of the slope of OD's from 4 serial dilutions. Each EU is equal to the intercept multiplied by 1,000, as previously published (Gmur et al., 1986). The average EU from 'blank' wells (which were not incubated with serum but

otherwise treated the same as the other wells) was subtracted from the EU of the other wells to give the final result.

Antibody	Dilution	Supplier
anti-mouse IgG	1/25,000	Southern Biotech, USA.
anti-mouse IgG1	1/10,000	Southern Biotech.
anti-mouse IgG2a	1/10,000	Southern Biotech.
anti-human IgG	1/10,000	Sigma-Aldrich.

Table 2.16.1. Antibodies used in anti-*P. gingivalis* ELISAs.

2.16.2 Cotinine ELISA

Cotinine ELISAs were carried out by Dr Danae Apatzidou and Dr David Lappin using the serum cotinine assay kit (Cozart Bioscience, Abingdon, UK), according to the manufacturer's instructions, as previously described and published (Apatzidou et al., 2005, Lappin et al., 2013). In brief, 10 µl/well standards and serum samples (neat and 1/5 dilutions) were added to the microplate (pre-coated with anti-cotinine capture antibody). All samples were prepared and measured in duplicate. The microplate was then incubated with 100 µl/well HRP-conjugated cotinine at room temperature for 30 minutes. Cotinine in the samples competed with HRP-conjugated cotinine for binding sites on the capture antibody. The plate was washed 4 times with 200 µl/well wash buffer, then the microplate was incubated with 100 µl/well TMB substrate at room temperature for 30 minutes. The reaction was stopped with 100 µl/well 1M H_2SO_4, and absorbance was measured at 450 nm using the MRII plate reader (Dynex Technologies). The OD's and known concentration units of the 4 standards (0, 10, 25, and 50 ng/ml) were used to make a standard curve, the formula of which was then used to calculate the concentration of cotinine in the samples. The concentration of cotinine was inversely proportional to the OD.

2.16.3 ACPA ELISA

ACPA ELISAs were performed using the DIASTAT anti-CCP kit (AXIS-Shield, UK), according to the manufacturer's instructions. This kit includes a 96-well microtiter plate which is pre-coated with a mixture of 5 (undisclosed) synthetic CCPs and enables the semi-quantitative detection of autoantibodies in sera

which are reactive to these. This ELISA is intended to aid the diagnosis of RA; a positive result in itself is not considered diagnostic. All steps were performed at room temperature. In brief, serum samples, and 'positive' and 'negative' controls (human serum, < 0.1 % NaN_3 (provided with the kit)) were pre-diluted 1/100 with dilution buffer (phosphate buffer, protein stabiliser, 0.1 % NaN_3). All samples were prepared and measured in duplicate. The plates were incubated with 100 µl/well serum samples, standards (human serum, buffer, < 0.1 % NaN_3) and 'positive' and 'negative' controls for 1 hour. After this and subsequent steps, plates were washed 3 times with 200 µl/well wash buffer (borate buffer, 0.05 % NaN_3). The plates were incubated with 100 µl/well AP-conjugated mouse anti-human IgG antibody (pre-diluted in tris buffer, protein stabiliser, < 0.1 % NaN_3) for 30 minutes. The plates were then incubated with 100 µl/well substrate (PMP, Mg^{2+}). The reaction was stopped with 100 µl/well stop solution (NaOH, EDTA, carbonate buffer, pH > 10), and absorbance was measured at 550 mm using an MRII plate reader (Dynex Technologies). The mean OD's of sample duplicates were calculated. The OD's and known concentration units of the 5 standards (0, 2, 8, 30, and 100 EU/ml) were used to generate a standard curve, the formula of which was then used to calculate the concentrations of ACPAs in the samples. The suggested diagnostic cut-off for RA is 5 EU/ml, and the lower limit of detection is 0.05 EU/ml.

2.16.4 CEP-1 and REP-1 ELISA

The CEP-1 and REP-1 ELISAs were carried out by Dr Anne-Marie Quirke and Prof. Patrick Venables at the Kennedy Institute of Rheumatology, University of Oxford. Ninety-six well microtiter plates (Costar) were coated with 100 µl/well CEP-1 or REP-1 peptide (10 µg/ml in carbonate buffer) or carbonate buffer alone overnight at 4 ^{o}C. The plates were washed 3 times with 200 µl/well PBST after this and each subsequent step until the substrate was added. Plates were blocked with 2 % BSA in PBS at room temperature for 3 hours, then incubated with serum samples, diluted 1/100 in RIA buffer (10 mM tris, 1 % BSA, 350 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 10 % FCS) for 1.5 hours. All samples were prepared and measured in duplicate. The plates were then incubated with 100 µl/well AP-conjugated goat anti-human IgG (Jackson laboratories), diluted 1/5,000 in RIA buffer, at room temperature for 1 hour.

Finally, the plates were incubated with AP substrate (Sigma-Aldrich) in the dark, for 30 minutes at room temperature, and absorbance was measured at 405 mm. Control serum was included on all plates to correct for plate-to-plate variation. The value for background OD at 405 nm (wells coated with carbonate buffer alone) was subtracted from the OD value of the patient samples. OD values above 0.1 were considered to be positive. AU for anti-CEP1 antibody titers were determined from a standard curve made from human sera pooled from known positive patients as previously published (Mahdi et al., 2009).

2.16.5 Cytokine ELISAs

IL-6, IL-10, and TNFα ELISAs were performed using Ready-SET-Go!® ELISA kits (eBioscience) according to the manufacturer's instructions. Ninety-six well microtiter plates (Costar) were coated with 100 µl/well of cytokine capture antibody (concentration optimised by manufacturer) diluted in coating buffer and incubated at 4 ^{o}C overnight. All subsequent steps were performed at room temperature. The plates were washed 3 times with 200 µl/well PBST after this and each subsequent step until the substrate was added. The plates were blocked with 200 µl/well assay diluent (provided with the kits) for 1 hour, then incubated with 100 µl/well standards or samples (prepared in assay diluent) for 2 hours, followed by 100 µl/well biotinylated detection antibody (concentration optimised by manufacturer) for 1 hour, then 100 µl/well avidin-HRP for 30 minutes. Finally, 100 µl/well TMB substrate was added. The reaction was stopped with 50 µl/well 10 % HCl and absorbance was measured at 450 nm using an MRII plate reader (Dynex Technologies). The OD's and known concentrations of the standards were used to generate a standard curve, the formula of which was then used to calculate the unknown concentrations of cytokines in the samples. The standards ranged from 4-500 pg/ml for the IL-6 ELISA, 31-4,000 pg/ml for the IL-10 ELISA, and 8-1,000 pg/ml for the TNFα ELISA. In each case, the limit of detection of cytokines by the ELISA was equivalent to the concentration of the lowest standard.

ELISAs for sRANKL were performed using the DuoSet® ELISA kit (R&D systems) according to the manufacturer's instructions. Ninety-six well microtiter plates (Costar) were coated with 100 µl/well goat anti-mouse RANKL capture antibody

at 4 µg/ml in PBS, and incubated at 4 ºC overnight. All subsequent steps were performed at room temperature. The plates were washed 3 times with 200 µl/well PBST after this and each subsequent step until the substrate was added. The plates were blocked with 200 µl/well reagent diluent (1 % BSA in PBS) for 1 hour, then incubated with 100 µl/well standards or samples (prepared in reagent diluent) at room temperature for 1 hour, followed by 100 µl/well biotinylated goat anti-mouse RANKL detection antibody (200 ng/ml in reagent diluent) for 1 hour, and 100 µl/well SA-HRP for 20 minutes. Finally, 100 µl/well TMB substrate was added. The reaction was stopped with 50 µl/well 10 % HCl and absorbance was measured at 450 nm using an MRII plate reader (Dynex Technologies). The OD's and known concentrations of the standards were used to make a standard curve, the formula of which was then used to calculate the concentrations of cytokines in the samples. The standards prepared ranged from 31-4,000 pg/ml. The limit of detection of sRANKL by the ELISA was equivalent to the concentration of the lowest standard.

2.16.6 RF, anti-dsDNA, and anti-type II collagen ELISAs

Ninety-six well microtiter plates (Costar) were coated with 100 µl/well of antigen: 10 µg/ml mouse IgG (Jackson Laboratories) in PBS for RF ELISA; or 5 µg/ml murine DNA (prepared as described in section **2.8.3**) in carbonate buffer (15 mM Na_2CO_3, 35 mM $Na_2 CO_3$, pH 9.6) for anti-dsDNA ELISA; or 4 µg/ml type II murine collagen (Chondrex) in carbonate buffer for anti-type II collagen ELISA, and incubated at 4 ºC overnight. The plates were washed 3 times with PBST after this and each subsequent step until the substrate was added. The plates were incubated with 200 µl/well blocking buffer (10 % PBS, 10 % FCS in dH_2O) at 37 ºC for 1 hour. Serial dilutions of serum ranging from 1/25 to 1/200 were prepared in dilution buffer (0.2 % FCS, 0.05 % Tween in PBS). All samples were prepared and measured in duplicate. The plates were then incubated with 50 µl/well of pre-diluted serum samples at 37 ºC for 2 hours. RF were detected with HRP-conjugated rat anti-mouse IgM antibody (BD Biosciences), diluted 1/1,000 in dilution buffer; anti-dsDNA and anti-type II collagen IgG antibodies were detected with HRP-conjugated goat anti-mouse IgG antibody (Southern Biotech), diluted 1/10,000 in dilution buffer. The plates were incubated with 50 µl/well secondary antibody at 37 ºC for 2 hours. Finally, 100 µl/well TMB substrate was

added. The reaction was stopped with 50 µl/well 10 % HCl, and absorbance was measured at 450 nm using an MRII plate reader (Dynex Technologies). The mean for each dilution of each serum sample was calculated, then the EU for each serum sample was calculated as described in section **2.16.1**.

2.17 Statistics

All statistical analyses were performed using GraphPad Prism® software, version 5 (California, US). The distribution of data was determined using the Kolmogerov-Smirnov normality test. When the means of 2 groups were compared, a Student t test was used if data were normally distributed, or a Mann-Whitney test was used if data were not normally distributed. When the means of more than 2 groups were compared, a One-Way ANOVA with a Tukey post-hoc test was used if data were normally distributed, or a Kruskal-Wallis with Dunn's multiple comparison post-hoc test if data were not normally distributed. When the means of 1 group before and after treatment were compared, a Wilcoxon signed ranks test was used. Correlations between 2 dependent variables measured in 1 group were determined by Spearman's rho. A value of $P \leq 0.05$ was considered to be significant.

Chapter 3: Characterisation of B cells in the gingiva of periodontitis patients

3.1 Introduction

PD results from a dysregulated immune response to a dysbiotic oral biofilm. In order to improve approaches to treatment, a better understanding of the immunopathology of the disease is required. To achieve this, detailed characterisation of the immune cell infiltrate in the gingiva needs to be performed. The main objective of this study was to investigate the proportions and characteristics of B cells and plasma cells in the gingiva of PD patients.

Several studies have reported that plasma cells account for half of the leukocyte infiltrate in the gingiva, and B cells a further 20 % (Berglundh and Donati, 2005). Among these, a few studies found that the combined proportion of B cells and plasma cells was increased in association with PD progression, activity, or severity (Liljenberg et al., 1994, Lappin et al., 1999, Thorbert-Mros et al., 2014). However, the majority of these studies were limited by a low number of PD patient samples, and a lack of healthy control tissue. In cases where healthy gingiva were examined, relatively few leukocytes were found, of which only about 5 % were B cells (Gemmell et al., 2002b). The relative abundance of B cells in the gingiva of PD patients suggests they are involved in disease pathogenesis, but their phenotype and function remains to be fully elucidated.

In order to further characterise B cells in PD patients, studies have previously analysed the proportion of B cells that express CD5. This particular subset of B cells was found to constitute as much as 60 % of the total B cell population in the gingiva and 40-50 % of circulating B cells in PD patients, compared with just 15 % in the gingiva and 15 % circulating in periodontally healthy patients (Berglundh et al., 2002b, Donati et al., 2009a).

These findings are intriguing as $CD5^+$ B cells are thought to belong to the B1a subset of B cells and have been associated with the development of several autoimmune diseases (a more detailed description of the differences which distinguish B1a cells from the other mature B cell subsets is provided in **Chapter 5**).

RA patients have a higher proportion of circulating $CD5^+$ B cells, and the proportion of circulating $CD5^+$ B cells positively correlates with markers of bone resorption (Sowden et al., 1987, Engelmann et al., 2014). Furthermore, $CD5^+$ B cells are a major producer of RF: a class of autoantibodies predominantly of the IgM isotype, which recognise the Fc portion of IgG antibodies, and exhibit a positive correlation with disease activity in RA (Burastero et al., 1988, Nakamura et al., 1988, Harindranath et al., 1991, Mantovani et al., 1993, Vencovsky et al., 2003, Vallbracht et al., 2004).

In PD, B1a cells potentially produce autoantibodies that recognise components of connective tissues. Autoantibodies may contribute to the destruction of periodontal tissue and alveolar bone loss through the formation of immune complexes, and the stimulation of FcR bearing immune cells including innate immune cells and osteoclasts (Seeling et al., 2013). Elevated titers of autoantibodies recognising type I collagen, fibronectin, and laminin have been detected in the sera of PD patients compared with periodontally healthy patients (De-Gennaro et al., 2006). Anti-collagen antibodies have also been identified in the gingiva of PD patients (Anusaksathien et al., 1992), and it has been demonstrated that $CD5^+$ B cells isolated from inflamed gingiva are more proficient than $CD5^-$ B cells in producing anti-collagen antibodies of both IgM and IgG classes (Sugawara et al., 1992). Overall, the majority of antibodies in the GCF and gingiva of PD patients are generally found to belong to the IgG class; IgA is the next most commonly found, and then IgM (Okada et al., 1983, Ogawa et al., 1989, Takahashi et al., 1997). Following confirmation of B cell and plasma cell infiltrate, this study aimed to investigate the proportion of B cells belonging to the B1a subset, and the proportion of plasma cells producing IgG in PD patient gingiva.

3.2 Results

Previous studies have demonstrated increased B cell infiltrate in PD, and initial experiments aimed to confirm these findings. Expression of a B cell–specific gene (*CD19*) was measured by qRT-PCR of cDNA derived from samples of gingival tissue collected from PD patients during periodontal surgery, and periodontally healthy patients (as described in **Chapter 2**, sections **2.1.1** and **2.8.1**). The relative expression of *CD19* was found to be significantly higher in the gingiva of PD patients than in the gingiva of healthy patients (*P = 0.0323, **Figure 3.2.1**).

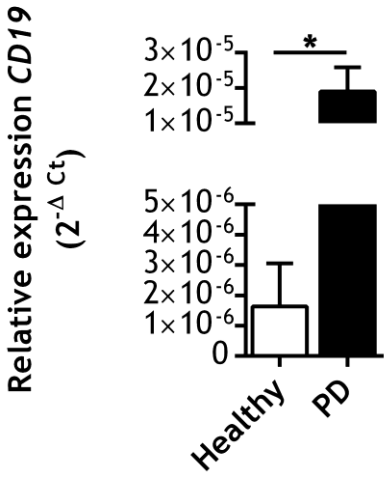

Figure 3.2.1. Relative expression of *CD19* in the gingiva of periodontitis patients. Samples of gingival tissue were collected from 6 PD patients and 6 periodontally healthy patients. With cDNA derived from these tissue samples, the expression of *CD19* relative to the housekeeping gene *18S* was determined by qRT-PCR. Data are shown as mean with SEM. Statistical difference was determined by an unpaired t-test (*P < 0.05).

The relative expression of *CD19* suggested that there was an increased number of B cells, or an increase in *CD19* expression by B cells, in the gingiva of PD patients. To examine the B cell infiltrate at a cellular level, and assess the relative proportion of B cells, plasma cells, and T cells, flow cytometry analysis of CD19, CD138, and CD3 protein expression was performed on cells isolated from freshly collected samples of gingival tissue from four PD patients (as described in **Chapter 2**, sections **2.1.1** and **2.15.1**). In each PD patient sample, $CD19^+$ B cells constituted 20-25 % of lymphocytes. The proportions of $CD138^+$ plasma cells and $CD3^+$ T cells were highly variable between patients. A greater number of PD patient samples would be required for a more comprehensive assessment of these trends. It was not possible to obtain sufficient fresh healthy gingival tissue for this study so a comparison between the cellular composition of tissue from healthy and diseased gingiva could not be made.

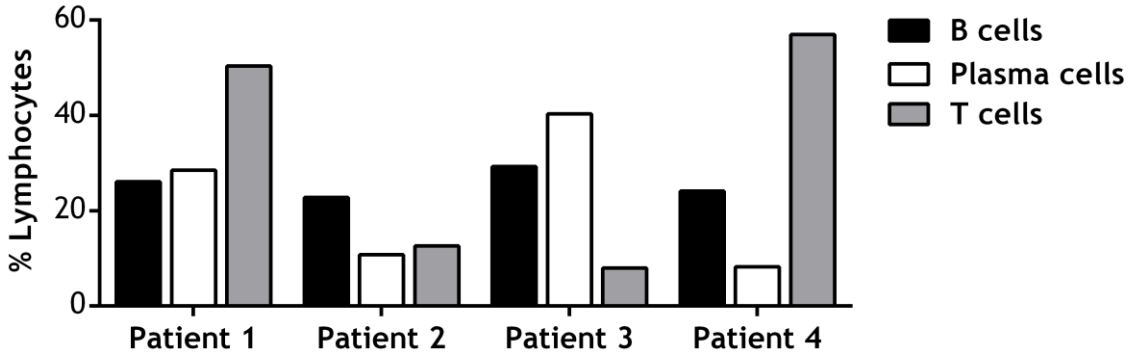

Figure 3.2.2. Proportions of B cells, plasma cells and T cells in the gingiva of periodontitis patients. Samples of gingival tissue were collected from 4 PD patients during periodontal surgery, cells were isolated and analysed by flow cytometry. The data are shown as the percentage of lymphocytes that were $CD19^+$ (B cells), $CD138^+$ (plasma cells), and $CD3^+$ (T cells).

To assess the proportion of $CD19^+$ B cells and $CD138^+$ plasma cells in a greater number of patients, and to investigate the location and distribution of these within the tissue, IHC was performed on paraffin-embedded gingival tissue that had been collected from PD patients during periodontal surgery (as described in **Chapter 2**, sections **2.1.1** and **2.9**). To overcome the potential bias in selecting fields of view and counting positive cells per field in samples of variable size and morphology, whole tissue sections were imaged and analysed. The area of tissue infiltrated by $CD19^+$ B cells and $CD138^+$ plasma cells, relative to the total area of the tissue section, was quantified (**Figure 3.2.3**). Both $CD19^+$ B cells and $CD138^+$ plasma cells were detectible in the sections. However, there was no association between the area of tissue infiltrated by B cells and the area of tissue infiltrated by plasma cells.

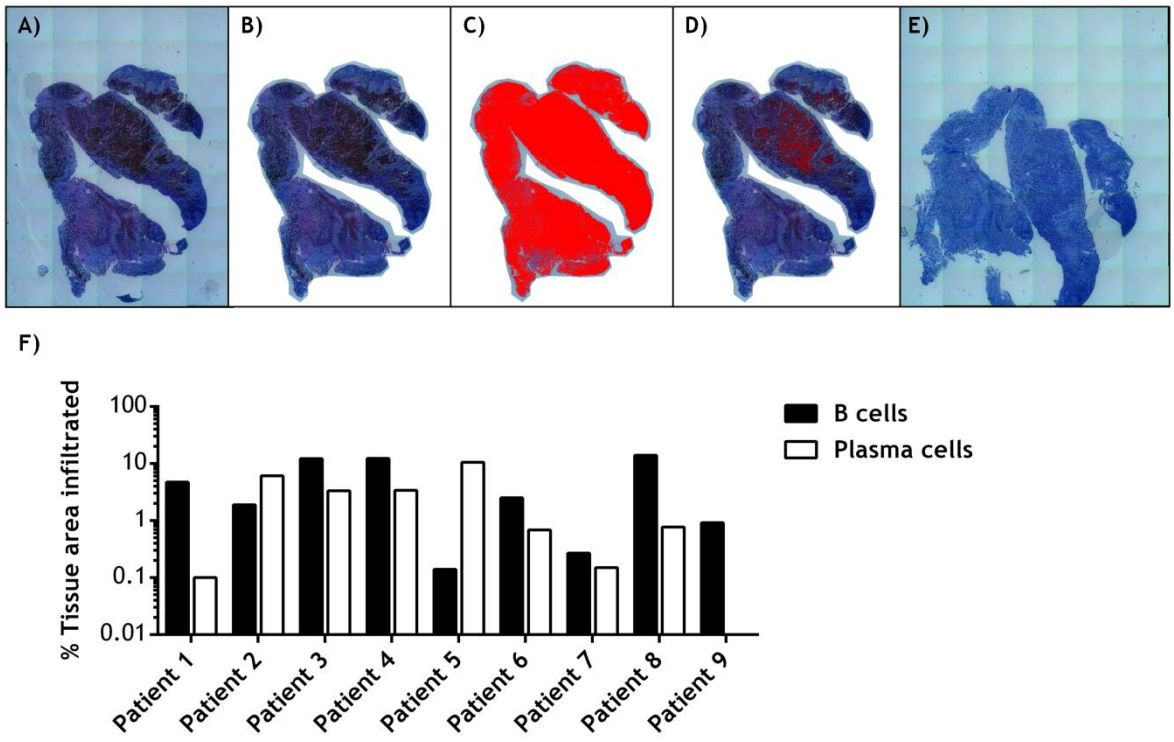

Figure 3.2.3. B cells and plasma cells in the gingiva of periodontitis patients. Samples of gingival tissue from 9 PD patients were collected during periodontal surgery. Samples were embedded in paraffin, then sectioned and stained by IHC. Cell nuclei and cytoplasm were stained blue with haematoxylin. Cells expressing CD19 or CD138 were stained brown with specific antibodies linked to DAB. **A)** images of stained tissue were captured at X20 magnification and stitched together to form a tile scan of the entire section. **B)** the tile scan was edited to remove background. **C)** the area of the entire tissue section was measured (false-coloured red). **D)** the area of tissue containing $CD19^+$ B cells or $CD138^+$ plasma cells was measured (false-coloured red). **E)** a tile scan of a tissue section from the same patient which underwent staining with isotype control antibodies. **F)** the area of tissue containing B cells or plasma cells was calculated as a percentage of the total area of the tissue section for each patient.

To determine whether plasma cells in the gingiva of PD patients were actively producing class-switched antibodies, further staining of paraffin embedded tissue was performed to detect IgG antibodies alongside $CD138^+$ plasma cells (**Figure 3.2.4**). Samples of human tonsil from tonsillitis patients were also stained to validate this method. Out of eight patient samples in which plasma cells could be detected, seven samples had plasma cells which were producing IgG, and in three samples, over 50 % of plasma cells were IgG^+ (**Figure 3.2.4E** and **Table 3.2.1).** This suggests the majority of PD patients have plasma cell infiltrate in the gingiva, and these are likely to produce class-switched antibodies.

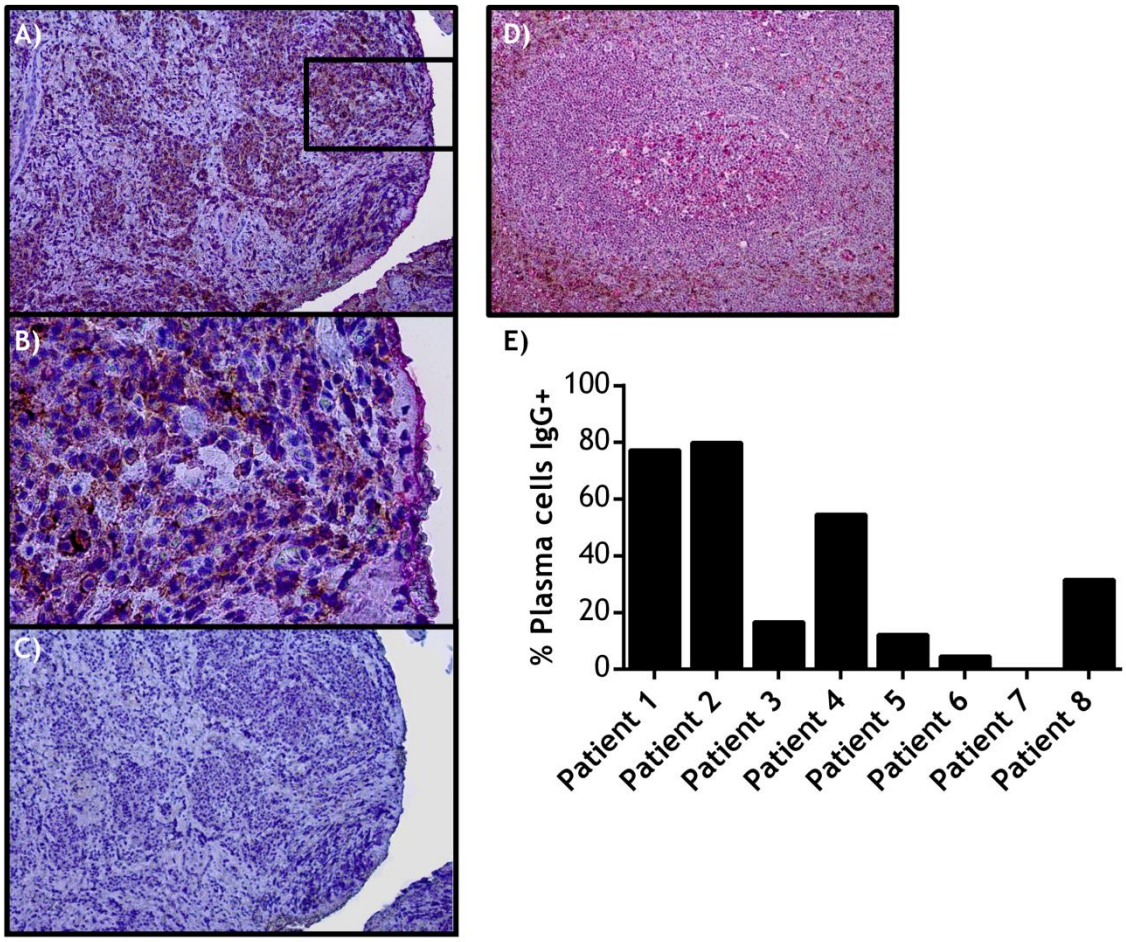

Figure 3.2.4. Plasma cells producing IgG antibodies in the gingiva of periodontitis patients. Samples of gingival tissue were collected from 8 PD patients during periodontal surgery, and processed for IHC. The connective tissue and cell nuclei were stained blue with haematoxylin, CD138 expressing cells were stained brown with specific antibodies linked to DAB and human IgG antibodies were stained pink with specific antibodies linked to permanent red. Representative images of $CD138^+$ IgG^+ plasma cells were captured at **A)** X10 magnification and **B)** X40 magnification. **C)** an image of a tissue section from the same tissue sample stained using isotype control antibodies was captured at X10 magnification. **D)** an image of a tissue section of human tonsil which underwent the same staining procedure was captured at X10 magnification. **E)** the average percentage of $CD138^+$ plasma cells that were IgG^+ in each patient's sample of gingival tissue was calculated from the total number of $CD138^+$ plasma cells, and the number of $CD138^+$ IgG^+ plasma cells, in 4-5 fields of view (X40 magnification) of plasma cell rich regions of tissue.

	Tissue area infiltrated by B cells (%)	Tissue area infiltrated by plasma cells (%)	Plasma cells producing IgG^+ (%)
Patient 1	4.7	0.10	77
Patient 2	1.9	6.1	80
Patient 3	12.3	3.3	17
Patient 4	12.3	3.4	55
Patient 5	0.14	10.6	12
Patient 6	2.5	0.69	5
Patient 7	0.27	0.15	0
Patient 8	14.0	0.77	32
Patient 9	0.92	0.0	NA

Table 3.2.1. Summary of the proportions of B cells, plasma cells and IgG producing plasma cells in the gingiva of periodontitis patients. Data were collated from 9 PD patients. NA = not applicable.

The B cells in the gingiva of PD patients were further characterised by dual staining of CD19 and CD5 to establish the proportion of B cells belonging to the B1a subset (**Figure 3.2.5**). In contrast to previous reports, less than 1 % of B cells in patient samples of gingiva appeared to be $CD5^+$ B1a cells (Berglundh et al., 2002b, Donati et al., 2009a). Of the few $CD5^+$ cells that were observed, the majority were $CD19^-$ and therefore likely to be T cells. As before, samples of human tonsil were stained as a positive control.

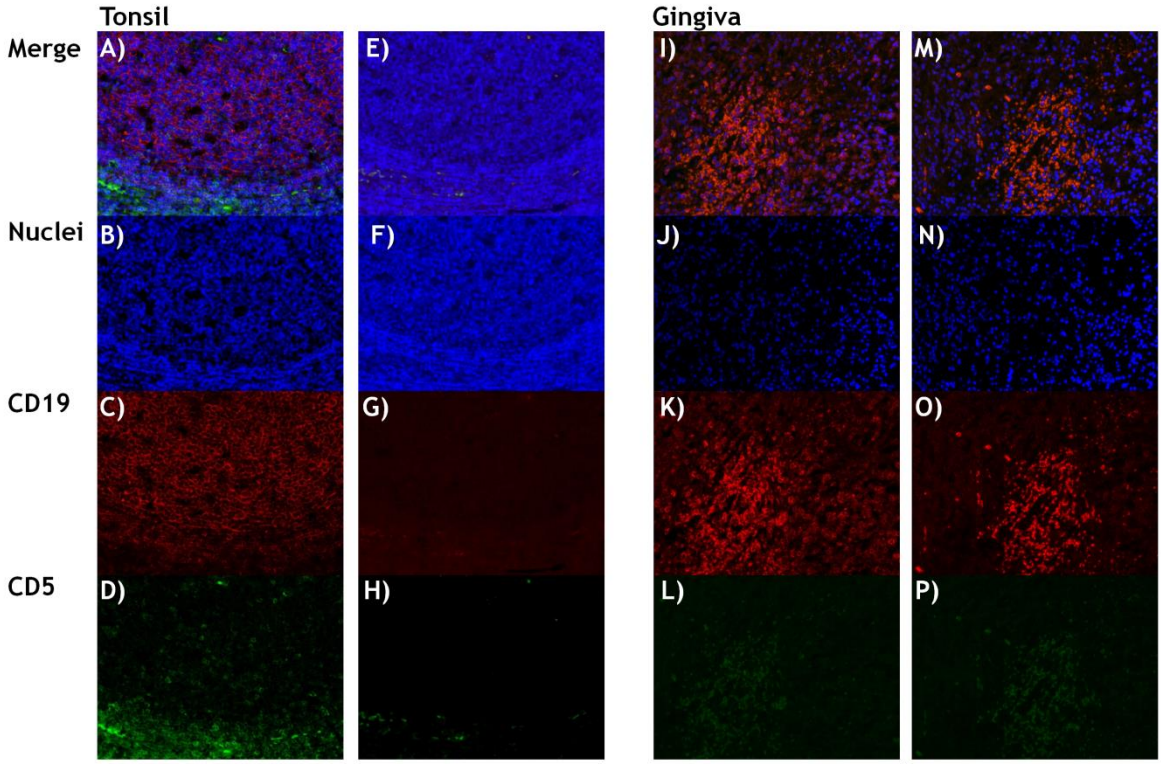

Figure 3.2.5. B1a cells in the gingiva of periodontitis patients. Samples of gingival tissue were collected from 8 PD patients during periodontal surgery and processed for IHC. Cell nuclei were stained blue with DAPI, CD19 expressing cells were stained red with a specific antibody linked to rhodamine, and CD5 expressing cells were stained green with a specific antibody linked to FITC. Images were captured at X20 magnification. **A) - D)** representative images of $CD19^+$ and $CD5^+$ cells in human tonsil which underwent the same staining procedure. **E) - H)** corresponding images of a tissue section from the same human tonsil stained using isotype control antibodies. **I) - L)** representative images of $CD19^+$ and $CD5^+$ cells in one sample of gingival tissue. **M) - P)** corresponding images of a tissue section from the same tissue sample stained using isotype control antibodies. The total number of $CD19^+$ B cells, and the number of $CD19^+$ $CD5^+$ B1a cells were counted in 4-5 fields of view within B cell rich regions of each sample of gingival tissue.

Summary of main results:

- The relative expression of CD19 mRNA was increased in the gingiva of PD patients compared with gingival tissue from periodontally healthy patients
- Variable proportions of B cells, plasma cells, and T cells were detected in the gingiva of PD patients
- Plasma cells producing IgG were detected in the gingiva of the majority of PD patients
- $CD5^+$ B cells formed a very rare subset of B cells in the gingiva of the PD patients

3.3 Discussion

This study successfully confirmed that B cells and plasma cells form a considerable proportion of the immune cell infiltrate in the gingiva. One convenient approach to analysing cells isolated from gingival tissue is flow cytometry. A limitation encountered when performing this analysis was the variability in the size of freshly collected samples. Often, after processing, the number of cells isolated was too low to enable meaningful analysis, particularly of relatively rare cell populations. Whilst some samples were visibly small, other, apparently larger samples, were found to contain either very few lymphocytes, or a high proportion of dead cells. The latter may be associated with the inevitable delay between tissue collection and processing for analysis. Identifying an appropriate control for analysis of cellular infiltrate in diseased gingival tissue is challenging. Healthy gingival tissue contains relatively few immune cells, and there are limitations in the number of donors available, and in the size of samples of healthy gingival tissue which can reasonably be obtained. Alternatively, peripheral blood from the PD patient may be considered as a source of control cells, but this was not available in this study. Hence in this case, the analysis was limited to measuring the relative proportions of B cells, plasma cells, and T cells in the gingiva. A previous study of gingival tissue from six PD patients demonstrated that 15-30 % of mononuclear cells were $CD19^+$ B cells, and 20-40 % were $CD3^+$ T cells by flow cytometry analysis (Lukic et al., 2006). These values are similar to those found in this study, despite slight differences in the way in which the samples were collected and processed.

Embedding gingival tissue in paraffin enabled samples of all shapes and sizes to be analysed by IHC. The irregularity of samples made it impossible to analyse the position of cells within the tissue in reference to known histological features such as rete pegs, as these were not consistently present. Nonetheless, IHC enabled the quantification of the relative area of tissue infiltrated by B cells and plasma cells, and confirmation that plasma cells in the gingiva are a source of IgG. This type of analysis differs from that performed by flow cytometry, in which the proportion of B cells and plasma cells relative to the total population of live cells was calculated. In order to replicate the flow cytometry analysis in IHC, the total number of cells as well as the total number of B cells and plasma

cells in an entire tissue section would need to be counted manually, which is impractical.

The method used to quantify the area of tissue infiltrated by B cells and plasma cells was assisted by computer software (ImageJ) which automatically detected areas of positively stained cells based on a standardised threshold of colour intensity. An advantage of this was that it increased the objectivity of the process of determining which cells were positively stained. Nonetheless this system of quantification is subject to a degree of bias as it assumes that a single section is representative of a whole sample, as well as assuming that a single sample is representative of a patient (which was also a limitation of flow cytometry experiments). The proportion of B cell and plasma cell infiltrate calculated is dependent on the size of the periodontal lesion, and the relative amount of tissue collected from the periodontal lesion and surrounding connective tissue. Preferably, for all experiments, the size of tissue removed from a lesion would have been standardised, and for IHC experiments, B cell and plasma cell infiltrate would have been analysed in a set number sections taken at different depths from each sample.

A different approach to IHC analysis had to be employed to quantify the proportion of plasma cells producing IgG as computer software was not able to detect the difference between single-stained $CD138^+$ IgG^- and dual-stained $CD138^+$ IgG^+ plasma cells. For this reason, the proportion of plasma cells that were producing IgG was calculated from counts of the total number of $CD138^+$ plasma cells within selected plasma cell-rich regions and counts of the total number $CD138^+$ IgG^+ cells. Ideally, a predetermined minimum total number of plasma cells would have been examined in each section, in the region of hundreds, but this was not possible here due to the limited number of patient samples available and the inherent variability in plasma cell number and distribution between samples. The total number of plasma cells counted ranged from 88-379 per section.

Commensurate with previous studies, the production of IgG by tissue-resident plasma cells observed here indicates that it is possible for a specific anti-bacterial or autoreactive humoral response to be triggered at this site, and that

plasma cells here potentially make a significant contribution to the pool of IgG antibodies circulating in saliva, GCF, and blood in PD patients (Okada et al., 1983, Ogawa et al., 1989, Takahashi et al., 1997).

Characterisation of the specificity of the IgG detected in the tissue here would have been enlightening. The full range of components of disease-associated bacteria or components of connective tissue recognised by antibodies in the gingiva has yet to be defined. Robust detection of anti-bacterial antibodies *in situ* has proved challenging. Recently, specific antibody-producing plasma cells were detected in frozen tissue sections using six enzyme-labelled synthetic peptides representing *P. gingivalis*–derived antigens. The binding of these probes was deemed specific as positive signals to antigens were completely abolished following adsorption with an excess of the corresponding unlabelled peptide but not following adsorption with an excess of an unlabelled unrelated peptide (Mizutani et al., 2014). The enzymatic processing of the tissue sections required for the probes to bind, destroyed the CD138 protein on the surface of plasma cells, so that plasma cells could only be co-localised with the probe using the surrogate marker CD79a, an adaptor protein which forms part of the BCR complex.

Somewhat easier to achieve is the phenotyping of B cells by IHC using well-established markers that indicate the activation status of the B cell or which subset it belongs to. Here, CD5 expression by B cells in the gingival tissue of PD patients was investigated. It was found that $CD5^+$ $CD19^+$ B cells were very rare. This was surprising since others have reported that up to 60 % of B cells in the gingiva are $CD5^+$ (Berglundh et al., 2002b, Donati et al., 2009a, Thorbert-Mros et al., 2014). This could be due to differences in the classification of PD, and the way tissue samples were collected or stained. In all the studies reporting high numbers of $CD5^+$ B cells, the patients had advanced, generalised, chronic PD, and relatively large biopsies were collected from diseased sites with PPD ≥ 6 mm (Berglundh et al., 2002b, Donati et al., 2009a) or ≥ 7 mm (Thorbert-Mros et al., 2014). In this study, patients also had chronic PD, and samples were collected from gingiva around teeth with a PPD ≥ 6 mm. However, whether the PD patients had different levels of disease activity is impossible to determine from the available information.

Other B cell features which could have been investigated to obtain insight of their role in PD include the expression of co-stimulatory molecules CD80 and CD86, which provide an indication of the activation status of B cells and their capacity to activate T cells. The expression of CD80 and CD86 by cells in PD patient gingiva has been documented (Orima et al., 1999, Gemmell et al., 2001). One group found elevated B cell expression of CD80 and CD86 in gingival tissue from six PD patients (Mahanonda et al., 2002) when compared with B cells from the peripheral blood of periodontally healthy individuals, assessed by flow cytometry. This may be disputed as a fair comparison as tissue-resident B cells are likely to have a different phenotype to those in circulation, even in health.

The expression of RANKL by B cells in PD patient gingiva could also have been investigated by IHC. RANKL activates pre-osteoclasts to undergo osteoclastogenesis, and osteoclasts breakdown bone. In health, this process is carefully controlled by the negative regulator OPG, a decoy receptor for RANKL (Yasuda et al., 1999). A multitude of studies have shown that the overall expression of RANKL and the ratio of RANKL expression to OPG expression is increased in the gingiva of PD patients (Crotti et al., 2003, Lu et al., 2006, Kawai et al., 2006, Wara-aswapati et al., 2007). Not many studies have examined the cellular sources of RANKL in the gingiva. B cell expression and secretion of RANKL could theoretically contribute to alveolar bone loss in PD as B cells isolated from the peripheral blood of healthy individuals, which are activated to induce RANKL secretion, can enhance osteoclastogenesis by monocytes *in vitro* (Kawai et al., 2006). One study found 90 % of B cells in PD patient gingiva expressed RANKL, compared with 50 % of T cells and 5 % of monocytes (Kawai et al., 2006). As B cell RANKL expression was only determined in 15 PD patients in this particular study, follow-up IHC studies are required to confirm these findings. Co-localisation of RANKL expressing B cells with monocytes and macrophages could additionally be investigated, to determine the likelihood of cell-mediated interaction between these cells in the gingiva. RANKL expressing B cells could also be further characterised to determine if they belong to a particular subset.

FcRL4 expressing B cells form a large proportion of the RANKL expressing B cells in the rheumatoid synovium, and have been identified as pro-inflammatory in

other respects such as in the production of high levels of TNFα relative to other B and T cells (Yeo et al., 2014). FcRL4 is an immunoregulatory molecule expressed by a subset of memory B cells that are typically found in mucosal lymphoid tissues in close proximity to the microbiota and sites of pathogen invasion (Ehrhardt et al., 2005, Sohn et al., 2011). However, FcRL4 expression could not be detected by qRT-PCR of cDNA derived from gingival tissue of six PD patients in this study, although it was detected in human tonsil tissue (data not shown). As an alternative to FcRL4, a broader marker of memory B cells could be selected for assessing differential RANKL expression by B cells in the gingiva by IHC. However, $FcRL4^{+}$ B cells are atypical memory B cells and one of the ways in which they are distinct from other memory B cells is that they lack expression of CD27, which would otherwise be an obvious choice for this purpose (Ehrhardt et al., 2008). There is no single cell-surface protein which could clearly differentiate all memory B cells, from all other B cells or similarly demarcate any other B cell subset. This makes it difficult to pursue this form of analysis by IHC. More complex analysis of B cell RANKL expression could be achieved by flow cytometry, but this would require fresh samples of gingival tissue of adequate size.

Characterising the B cells in the gingiva in health and disease could yield valuable information that helps direct future development of immunological therapies for PD and other diseases characterised by chronic inflammation and bone loss. Targeting very specific subsets of immune cells could be associated with reduced side-effects compared with broad-spectrum therapeutics. For example, Rituximab, which depletes all B cells and is associated with a reduction in the levels of RANKL in synovial fluid, effectively inhibits progressive joint damage in RA patients whom are unresponsive to other treatments, but is also associated with reduced protection following pneumococcal and influenza vaccinations (Boumans et al., 2012, Keystone et al., 2012, Hua et al., 2014).

3.4 Conclusion

This study confirmed that B cells and plasma cells form a substantial proportion of the immune cells in the gingiva of PD patients. As with previous studies, limitations in the number of donors and the size of tissue samples with respect to both PD patients and periodontally healthy individuals prevented more comprehensive analysis. An alternative approach to investigating the roles of B cells in PD is to measure the levels of B cell-derived products - cytokines and antibodies - in PD patients. The production of inflammatory cytokines and autoantibodies are two mechanisms by which B cells may contribute to pathology in PD, and the latter may additionally predispose patients with PD to autoimmunity and the development of RA.

Chapter 4: Influence of periodontitis, *P. gingivalis*, and smoking on systemic anti-citrullinated peptide antibody titers

4.1 Introduction

Evidence pointing towards a central role for B cells and plasma cells in the immunopathology of PD is just one of several features PD shares with RA. The numerous similarities in the aetiology and pathogenesis between these two diseases creates the impression that they are closely connected, but as yet there is no evidence of a causal relationship. In one of the first of its kind, this study sought to make a step towards establishing whether the autoimmunity characteristic of RA begins to emerge in PD patients, and if there is an association with carriage of *P. gingivalis*.

An awareness of the co-incidence of PD with systemic inflammatory diseases has existed for over 50 years (Williams et al., 1960, Liubomorova, 1964). Shared risk factors provide a partial explanation for the increased incidence of PD in patients with diabetes, CVD, and RA (Symmons et al., 1997, Mercado et al., 2001, Genco et al., 2002, Bahekar et al., 2007, Humphrey et al., 2008, Chen et al., 2008, de Pablo et al., 2009, Nesse et al., 2010, Chaffee and Weston, 2010, Kallberg et al., 2011, Smit et al., 2012, Wesley et al., 2013, Preshaw and Bissett, 2013, Weinspach et al., 2013, Postma et al., 2014). As the effects of these risk factors on functions of the immune system are further characterised, and common features in the immunopathology continue to be identified, the concept that there could be immunological interplay between PD and systemic diseases is gaining credibility. In the last decade, there has been some exploration of the hypothesis that PD could cause systemic disease. The potential link between PD and RA is particularly intriguing because RA is an autoimmune disease of unknown aetiology.

RA patients may be four times more likely to have PD, and more likely to have more severe PD (Mercado et al., 2001, de Pablo et al., 2008, Smit et al., 2012). Severity of PD in RA has been found to be associated with serum titers of RF and ACPAs (Dissick et al., 2010). The idea that PD could precede RA stems from evidence of elevated titers of circulating autoantibodies which recognise native

and modified ECM proteins in PD patients. PD was once thought of as an autoimmune disease itself, and these autoantibodies were believed to be involved in the progression of PD and to contribute to more aggressive forms of PD (Anusaksathien et al., 1992, De-Gennaro et al., 2006, Koutouzis et al., 2009).

The majority of the autoantibodies found in PD have no specific association with RA. However, one study has reported the detection of antibodies recognising a citrullinated peptide (filaggrin) in PD patient sera (Hendler et al., 2010), and another study has claimed that a cocktail of ACPAs were higher in the serum of PD patients compared with periodontally healthy controls (Molitor, 2009). ACPAs have a high specificity for RA, are found in 70 % of RA patients, are associated with RA progression, and may be detectable up to 10 years before clinical onset of disease (van Gaalen et al., 2004, Nielen et al., 2004, Zendman et al., 2004). Although ACPA immunoassays are widely used in the diagnosis and monitoring of RA, the triggers of ACPA generation and their role in the immunopathology of RA are unknown. As described in **Chapter 1**, one hypothesis of the immunological link between PD and RA is that the periodontitis-associated bacterium *P. gingivalis* could breach immune tolerance and induce the generation of ACPAs via molecular mimicry of host citrullinated peptides or the creation of novel citrullinated peptides using its gingipains and PPAD.

There are at least 700 species of bacteria in the oral cavity (Chen et al., 2010). *P. gingivalis*, and the antibody response to this bacteria, has received a lot of attention in the study of PD pathogenesis since it was classified as a member of the 'red complex' of bacteria, whose presence is closely associated with clinical parameters of PD (Socransky et al., 1998). Intensive research of *P. gingivalis* has revealed that its impact on oral health is actually dependent on its intricate relationships with other species of bacteria. It has been established that *P. gingivalis* is key to the development of the microbial dysbiosis characteristic of PD, and that in the absence of a bacterial community, *P. gingivalis* is unable to cause alveolar bone loss in mice (Hajishengallis et al., 2011, Hajishengallis et al., 2012).

Once microbial dysbiosis has been established, PD can apparently progress without large numbers of *P. gingivalis* present in dental plaque. *P. gingivalis* is

often undetectable in PD patients, with as few as 40 % harbouring detectable numbers of *P. gingivalis* in one study (Eick and Pfister, 2002). Nonetheless, detection of *P. gingivalis* strongly correlates with disease status since it is less frequently detected in periodontally healthy individuals (Griffen et al., 1998).

Anti-*P. gingivalis* antibodies can be measured in PD patients even when *P. gingivalis* bacteria cannot be detected in the dental plaque. They provide a useful indication that *P. gingivalis* was once present and that it managed to breach the oral mucosal barrier in order to initiate an immune response (Haffajee and Socransky, 1994). Anti-*P. gingivalis* antibody titers have consistently found to be higher in PD patients than in periodontally healthy individuals (Benjamin et al., 1997, Graswinckel et al., 2004, Takeuchi et al., 2006, Miyashita et al., 2012). Moreover, anti-*P. gingivalis* antibody titers in PD patients are often higher than the titers of antibodies against other disease-associated bacteria and some studies show a stronger correlation with clinical symptoms of PD (Apatzidou et al., 2005, Hayman et al., 2011, Ebersole et al., 2014).

Understanding the influence of PD and *P. gingivalis* on parameters of systemic inflammation and autoimmunity is of benefit to both PD and RA patients. PD is one of several potentially modifiable risk factors for RA identified by epidemiological studies. Whether treatment of PD modifies signs of systemic autoimmunity warrants further investigation.

4.2 Results

The patient demographics are outlined in **Table 4.2.1**. Clinical assessment of the oral cavity was performed to confirm the status of periodontally healthy participants and PD patients (**Table 4.2.2**). All PD patients had extensive BOP and PPDs of ≥ 3 mm, with at least two sites that had a PPD of ≥ 5 mm. All periodontally healthy individuals had little or no BOP and PPDs of ≤ 2 mm. Smoking was not associated with a difference in any of these parameters within the healthy group, or the PD group.

Samples of dental plaque and serum were collected from both groups of participants at 'baseline'. PD patients then received a non-surgical periodontal treatment, which included oral hygiene instruction and root surface debridement with local anaesthetic if required. Six months from baseline, clinical assessment and sample collection was repeated for PD patients. Clinical parameters of PD significantly improved after treatment, as expected (**Table 4.2.2**). Periodontally healthy participants did not receive periodontal treatment, and only one batch of samples was collected from them.

Before periodontal treatment, carriage of *P. gingivalis* was determined by PCR of bacterial DNA in the dental plaque (as described in **Chapter 2**, section **2.8.2**). *P. gingivalis* could not be detected in any of the periodontally healthy individuals but was detected in 16 of the 39 PD patients, before periodontal treatment. The remaining PD patients in which *P. gingivalis* could not be detected may not have been harbouring *P. gingivalis* in their dental plaque at the time samples were collected, but may have previously been exposed to *P. gingivalis*, or may have been harbouring such a small number of *P. gingivalis* bacteria that they could not be detected by the methods used (the limit of detection was 10-20 ng *P. gingivalis* dsDNA in the plaque sample).

Before periodontal treatment, PD patients had significantly higher anti-*P. gingivalis* IgG titers than periodontally healthy participants (mean ± SEM: 10,441 ± 2,826 EU PD vs. 64.03 ± 19.36 EU health, **P < 0.001, **Figure 4.2.2A**). When PD patients were sub-categorised based upon whether *P. gingivalis* had been detected dental plaque, it appeared that *P. gingivalis* positive patients had lower anti-*P. gingivalis* IgG titers than *P. gingivalis* negative patients, but this trend was not statistically significant (**Figure 4.2.2B**).

	PD	**Healthy**
Total no.	39	36
Sex M:F	22:17	20:16
Average age (range)	46 (31-70)	43 (30-64)
Non-smokers no.	23	20
Smokers no.	16	16

Table 4.2.1. Demographics of participants.

Periodontitis	Non-smokers	Smokers
PPD before (mm)	4.4 ± 0.6	4.4 ± 0.7
PPD after (mm)	2.5 ± 0.3	2.7 ± 0.3
PPD before vs. after (mm)	1.9 ± 0.6**	1.7 ± 0.5**
LOA before (mm)	4.9 ± 0.8	5.3 ± 1.1
LOA after (mm)	3.7 ± 0.8	4.2 ± 1.1
LOA before vs. after (mm)	1.2 ± 0.5**	1.1 ± 0.5**
BOP before (%)	70.6 ± 18.7	67.3 ± 16.6
BOP after (%)	11.6 ± 5.8	12.1 ± 7.9
BOP before vs. after (%)	59.0 ± 19.8**	55.2 ± 16.7**
Healthy		
PPD (mm)	1.22 ± 0.21	1.32 ± 0.15
LOA (mm)	1.27 ± 0.27	1.41 ± 0.22
BOP (%)	5.32 ± 4.72	2.52 ± 1.07

Table 4.2.2. Clinical assessment of participants.
PPD = probing pocket depth, LOA = loss of attachment, BOP = bleeding on probing. Measurements of PPD (mm), LOA (mm) and BOP (%) were made in healthy participants and in periodontitis (PD) patients before and after treatment. All participants were sub-classified by smoking status. In PD patients, n = 23 non-smokers, n = 16 smokers; in healthy participants, n = 20 non-smokers, n = 16 smokers. Statistical differences between measurements made before and after treatment were determined by Wilcoxon signed ranks test (**P < 0.01).

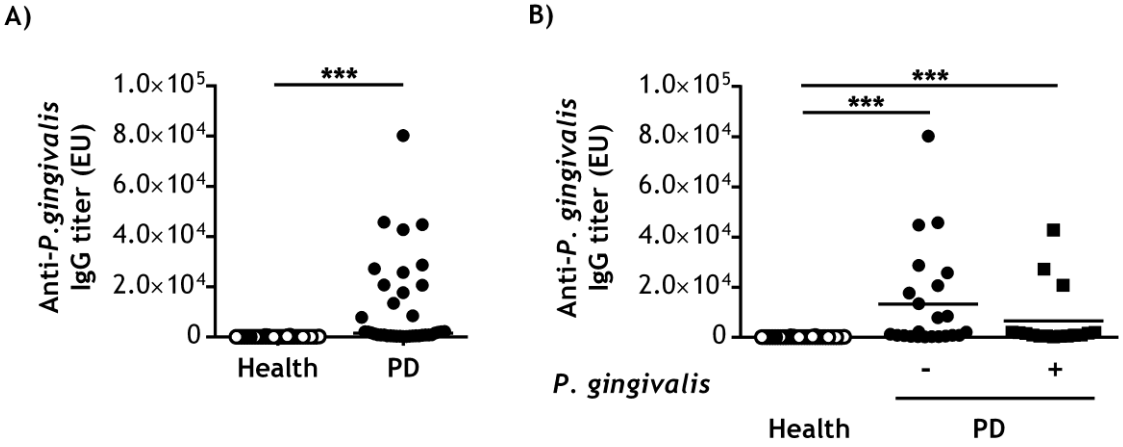

Figure 4.2.1. Serum anti-*P. gingivalis* IgG titers in periodontitis patients before treatment. A) Serum anti-*P. gingivalis* IgG titers in healthy participants, and in periodontitis (PD) patients before treatment. **B)** PD patients were sub-classified by whether *P. gingivalis* could be detected in dental plaque before treatment. In total, n = 36 in the health group, n = 39 in the PD group. Within the PD group, n = 16 *P. gingivalis* positive and n = 23 *P. gingivalis* negative. Statistical differences in **A)** were determined by Mann-Whitney test and in **B)** by Kruskal-Wallis with Dunn's multiple comparison test (***P < 0.001). Experiments were carried out with *Dr David Lappin*.

To investigate whether patients with untreated PD demonstrated evidence of autoimmune responses characteristic of RA, serum ACPA titers were measured (as described in **Chapter 2**, section **2.16.3**). ACPAs are diagnostic of RA and precede the onset of RA (van Gaalen et al., 2004, Nielen et al., 2004, Zendman et al., 2004). Before periodontal treatment, ACPA titers were significantly elevated in PD patients compared with periodontally healthy individuals (1.37 ± 0.23 EU/ml, PD vs. 0.40 ± 0.10 EU/ml, health, $P < 0.001$, **Figure 4.2.2A**). Previous studies have documented an association between ACPAs and anti-*P. gingivalis* antibodies in RA patients (Hitchon et al., 2010, Mikuls et al., 2012). Further analysis revealed that *P. gingivalis* positive PD patients had higher ACPA titers than *P. gingivalis* negative PD patients (2.31 ± 0.48 EU/ml, *P. gingivalis* positive PD vs. 0.71 ± 0.05 EU/ml, *P. gingivalis* negative PD, $^*P < 0.05$, **Figure 4.2.2B**). Three of the *P. gingivalis* positive PD patients had ACPA titers which were above the diagnostic cut-off for RA (5 EU/ml). There was a weak negative correlation between the anti-*P. gingivalis* IgG titers and ACPA titers of PD patients before periodontal treatment (rho = -0.338, $P = 0.0356$, **Figure 4.2.3A**). This correlation had greater significance in *P. gingivalis* negative PD patients (rho = -0.567, $^*P = 0.0048$, **Figure 4.2.3B**), but held no significance in *P. gingivalis* positive PD patients.

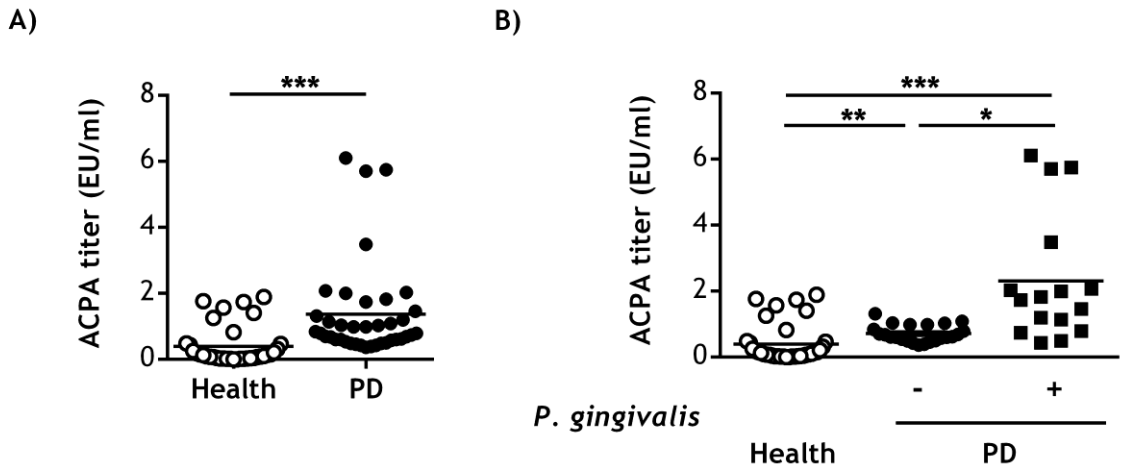

Figure 4.2.2. Serum ACPA titers in periodontitis patients before treatment. **A)** serum ACPA titers were determined in healthy participants and periodontitis (PD) patients before treatment. **B)** PD patients were sub-classified by whether *P. gingivalis* could be detected in dental plaque before treatment. In total, n = 36 in the health group, n = 39 in the PD group. Within the PD group, n = 16 *P. gingivalis* positive and n = 23 *P. gingivalis* negative. Statistical differences in **A)** were determined by Mann-Whitney test and in **B)** by Kruskal-Wallis with Dunn's multiple comparison post-hoc test (*P < 0.05, **P < 0.01, ***P < 0.001). Experiments were carried out with *Dr David Lappin*.

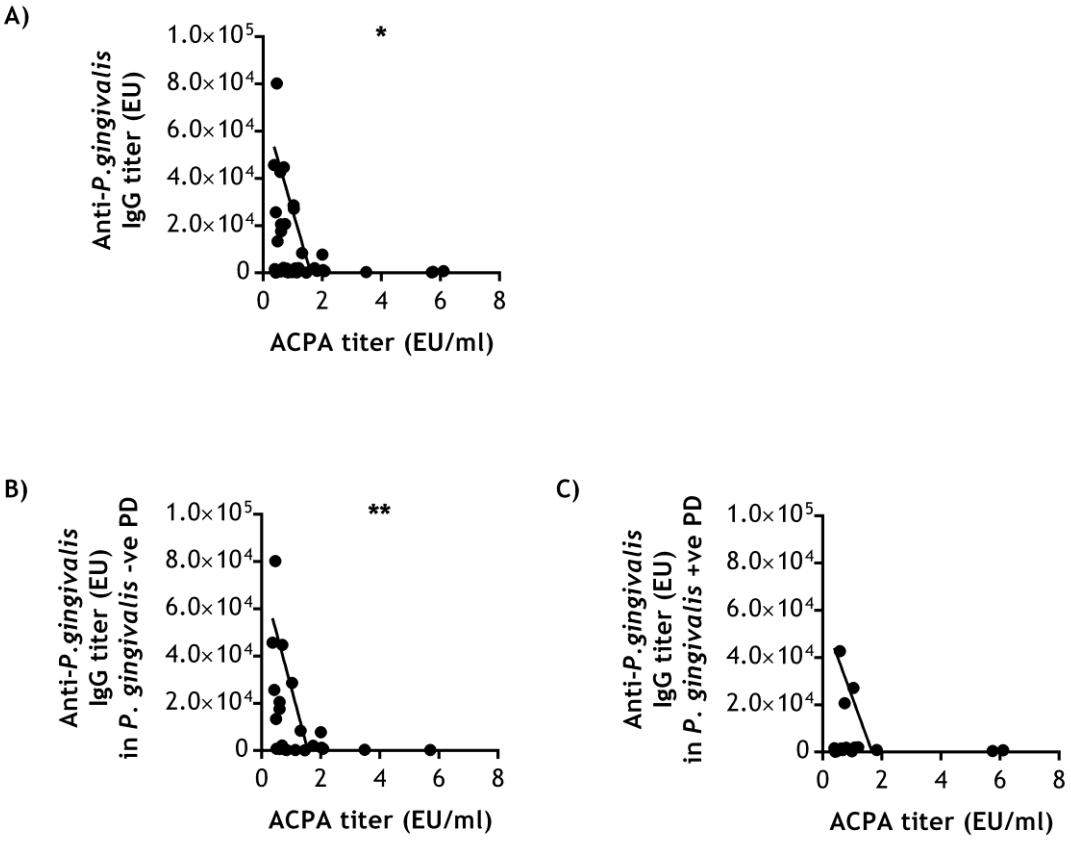

Figure 4.2.3. Serum anti-*P. gingivalis* IgG titers and ACPA titers in periodontitis patients before treatment. The correlation between serum anti-*P. gingivalis* IgG titers and ACPA titers for **A)** all periodontitis (PD) patients, **B)** patients in whom *P. gingivalis* could be detected in dental plaque before periodontal treatment, and **C)** PD patients in whom *P. gingivalis* could not be detected. In total, n = 39 in the PD group. Within the PD group, n = 16 *P. gingivalis* positive and n = 23 *P. gingivalis* negative. A line of best-fit has been drawn on each graph. Statistical correlation was determined by Spearman's rho (*P < 0.05, **P < 0.01). Experiments were carried out with *Dr David Lappin*.

To further probe the specificity of the ACPA response, titers of antibodies recognising CEP-1 - an immunodominant epitope in RA - and the uncitrullinated, native form of CEP-1, REP-1 were measured (as described in **Chapter 2**, section **2.16.4**) (Fisher et al., 2011, Montes et al., 2012). Both titers of anti-CEP-1 and anti-REP-1 antibodies were significantly higher in PD patients compared with healthy participants (7.1 ± 0.46 AU, PD vs. 4.6 ± 0.37 AU, health ***P < 0.001, CEP-1, **Figure 4.2.4A**; 0.11 ± 0.008 OD, PD vs. 0.069 ± 0.007 OD, health, ***P < 0.001, REP-1 **Figure 4.2.4C**), but there was no significant association between anti-CEP-1 or anti-REP-1 antibody titers and carriage of *P. gingivalis* by PD patients. The anti-CEP-1 response strongly correlated with the anti-REP-1 response (rho = 0.762, P < 0.0001), indicating that autoimmunity in PD patients is not restricted to citrullinated epitopes.

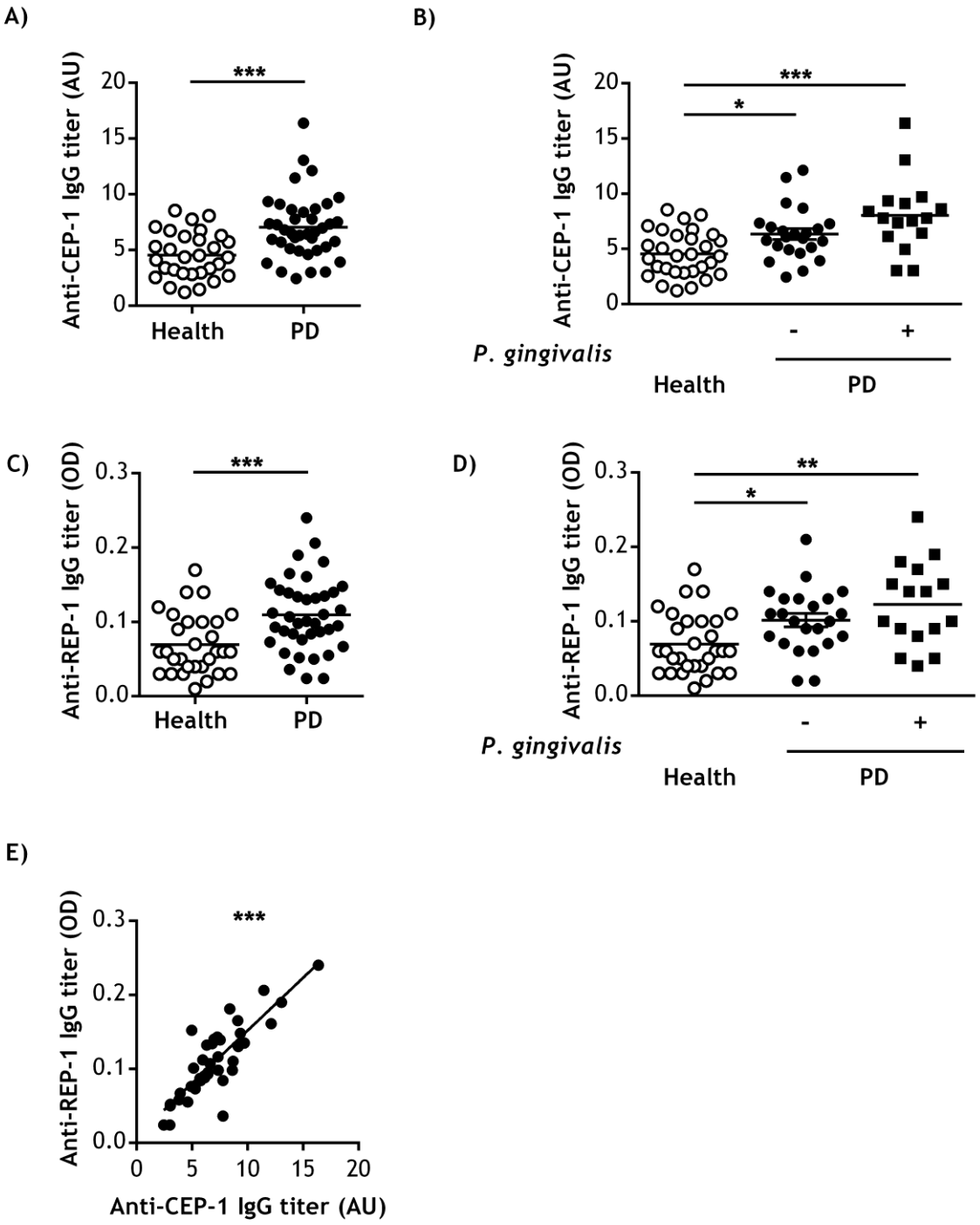

Figure 4.2.4. Serum anti-CEP-1 and anti-REP-1 IgG titers in periodontitis patients before treatment. Serum anti-CEP-1 **(A**, **B**, and **E)** and anti-REP-1 **(C**, **D**, and **E)** IgG titers were determined in healthy participants, and in periodontitis (PD) patients before treatment. **B)** and **D)** PD patients were sub-classified by whether *P. gingivalis* could be detected in dental plaque before treatment. In total, n = 30 in the health group, n = 39 in the PD group. Statistical differences in **A)** and **C)** were determined by Mann-Whitney test and in **B)** and **D)** were determined by Kruskal Wallis with Dunn's multiple comparison test. The correlation between anti-REP-1 and anti-CEP-1 IgG titers in **E)** was analysed by Spearman's rho (*P < 0.05, **P < 0.01, ***P < 0.001). Data were contributed to by *Dr Anne-Marie Quirke* and *Prof. Patrick Venables*.

Smoking was investigated as an independent risk factor for PD and autoimmunity in PD because of a large body of evidence linking smoking with altered innate immune responses to infection in PD, and with citrullination of peptides and ACPA titers in RA (Tangada et al., 1997, Graswinckel et al., 2004, Apatzidou et al., 2005, Guntsch et al., 2006, Tymkiw et al., 2011, Bondy-Carey et al., 2013, Haytural et al., 2014, Souto et al., 2014a, Giuca et al., 2014, Reynisdottir et al., 2014). The smoking history of the participants in this study was determined by a questionnaire and confirmed by a cotinine enzyme immunoassay (as described in **Chapter 2**, section **2.16.2**). Smoking was associated with significantly lower anti-*P. gingivalis* IgG titers in PD patients (2,512 ± 1290 EU, smokers PD vs. 15,956 ± 4,385 EU, non-smokers PD, *P = 0.027, **Figure 4.2.5**), suggesting that smoking suppresses the humoral response to infection with *P. gingivalis*. Smoking and non-smoking PD patients had similar ACPA titers, but in periodontally healthy participants, smoking status had a considerable impact on ACPA titers, with smokers having significantly higher titers than non-smokers (0.74 ± 0.19 EU/ml, health smokers vs. 0.15 ± 047 EU/ml, health non-smokers, **P = 0.0092, **Figure 4.2.6**).

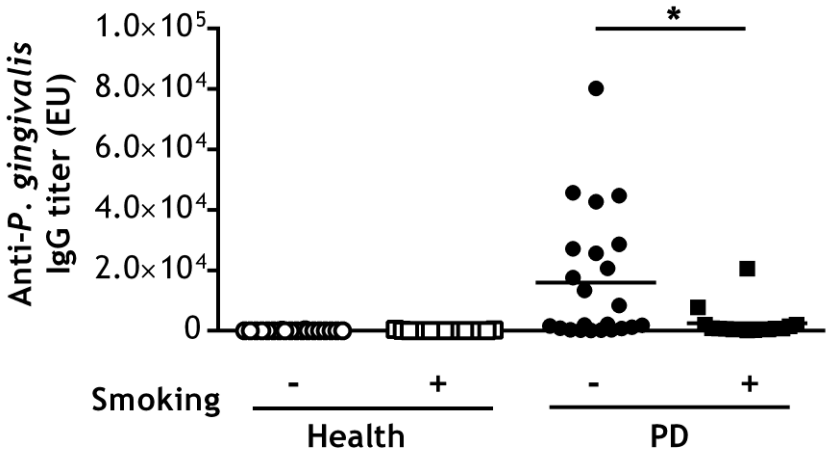

Figure 4.2.5. Serum anti-*P. gingivalis* IgG titers in smoker and non-smoker healthy participants and periodontitis patients before treatment. All participants were sub-classified based upon their smoking status. In total, n = 36 healthy participants, of these n = 20 non-smokers and n = 26 smokers; n = 39 periodontitis (PD) patients, of these n = 23 non-smokers and n = 16 smokers. Statistical differences were determined by Mann-Whitney test (*P < 0.05). Experiments were carried out with *Dr David Lappin*.

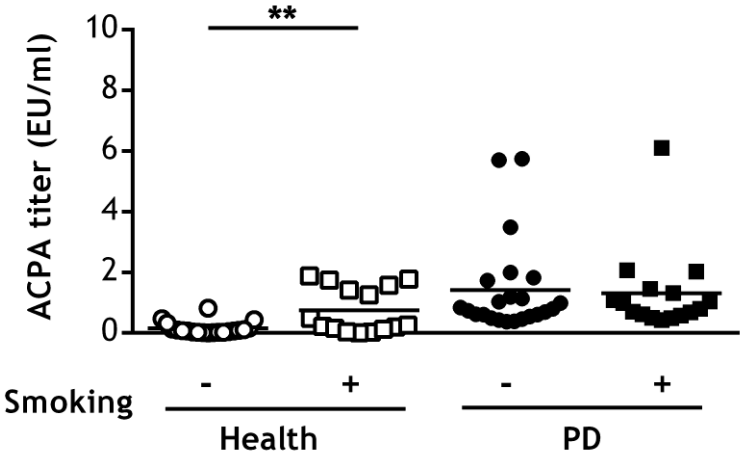

Figure 4.2.6. Serum ACPA titers in smoker and non-smoker healthy participants and periodontitis patients before treatment. All participants were sub-classified based upon their smoking status. In total, n = 36 healthy participants, of these n = 20 non-smokers and n = 26 smokers; n = 39 periodontitis (PD) patients, of these n = 23 non-smokers and n = 16 smokers. Statistical differences were determined by Mann-Whitney test (**P < 0.01). Experiments were carried out with *Dr David Lappin*.

Following treatment, along with the evidence of improvements in clinical parameters (**Table 4.2.2**), there was a reduction in the number of *P. gingivalis* positive PD patients. *P. gingivalis* DNA could only be detected in one PD patient after treatment, compared with 16 patients before treatment. PD patient anti *P. gingivalis* IgG titers showed a trend towards reduction after treatment compared with before treatment, although this did not reach statistical significance (**Figure 4.2.7A**). When PD patients were sub-classified based on whether *P. gingivalis* could be detected before and after treatment, there was no change in the anti-*P. gingivalis* IgG titer in the single patient who was *P. gingivalis* positive both before and after treatment (**Figure 4.2.7B-D**).

In contrast, ACPA titers were reduced in PD patients after treatment (1.37 ± 0.23 EU before vs. 0.95 ± 0.35 EU after, **Figure 4.2.8A**). When PD patients were sub-classified based on carriage of *P. gingivalis* before and after treatment, ACPA titers did not change in PD patients who were *P. gingivalis* negative before treatment (**Figure 4.2.8B**), but ACPA titers were significantly reduced in PD patients who were *P. gingivalis* positive before treatment and *P. gingivalis* negative after treatment (2.33 ± 0.51 EU/ml PD *P. gingivalis* positive before vs. 0.82 ± 0.34 EU/ml PD *P. gingivalis* negative after, **P = 0.0067 **Figure 4.2.8C**).

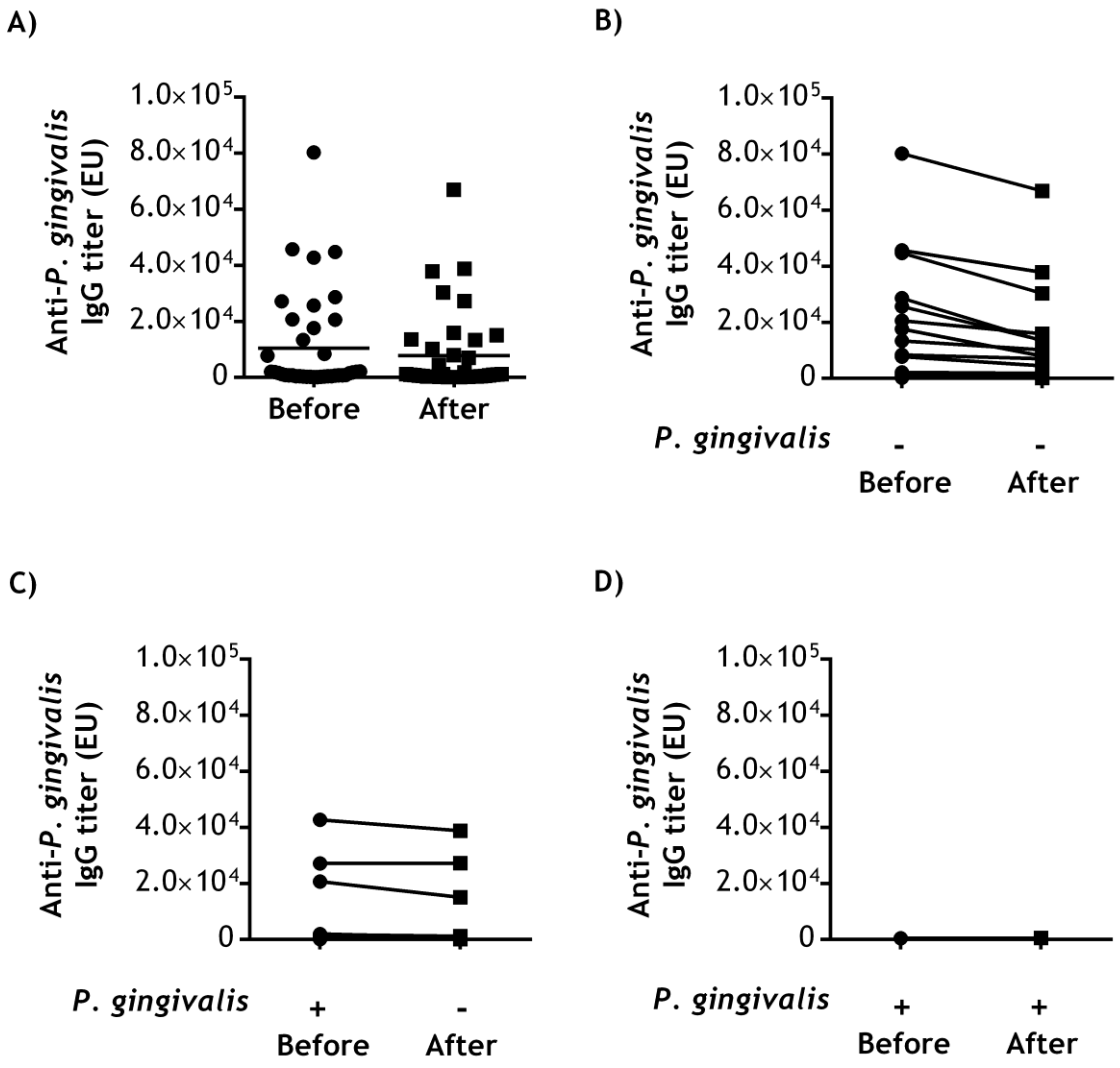

Figure 4.2.7. Serum anti-*P. gingivalis* IgG titers in periodontitis patients before and after treatment. A) serum anti-*P. gingivalis* IgG titers of periodontitis (PD) patients were determined before (circles) and after (squares) periodontal treatment. **B - D)** PD patients were sub-classified based on whether *P. gingivalis* could be detected in dental plaque before or after periodontal treatment. **B)** anti-*P. gingivalis* IgG titers of PD patients that were *P. gingivalis* negative before treatment and remained *P. gingivalis* negative after treatment. **C)** anti-*P. gingivalis* IgG titers of PD patients that were *P. gingivalis* positive before treatment and became *P. gingivalis* negative after treatment. **D)** anti-*P. gingivalis* IgG titer of a PD patient that was *P. gingivalis* positive before treatment and remained *P. gingivalis* positive after treatment. In total, n = 39 PD patients. Before treatment n = 23 *P. gingivalis* negative and n = 16 *P. gingivalis* positive; after treatment, n = 38 *P. gingivalis* negative and n = 1 *P. gingivalis* positive. Data were analysed by the Wilcoxon signed ranks test, but no statistically significant differences were found. Experiments were carried out with *Dr David Lappin*.

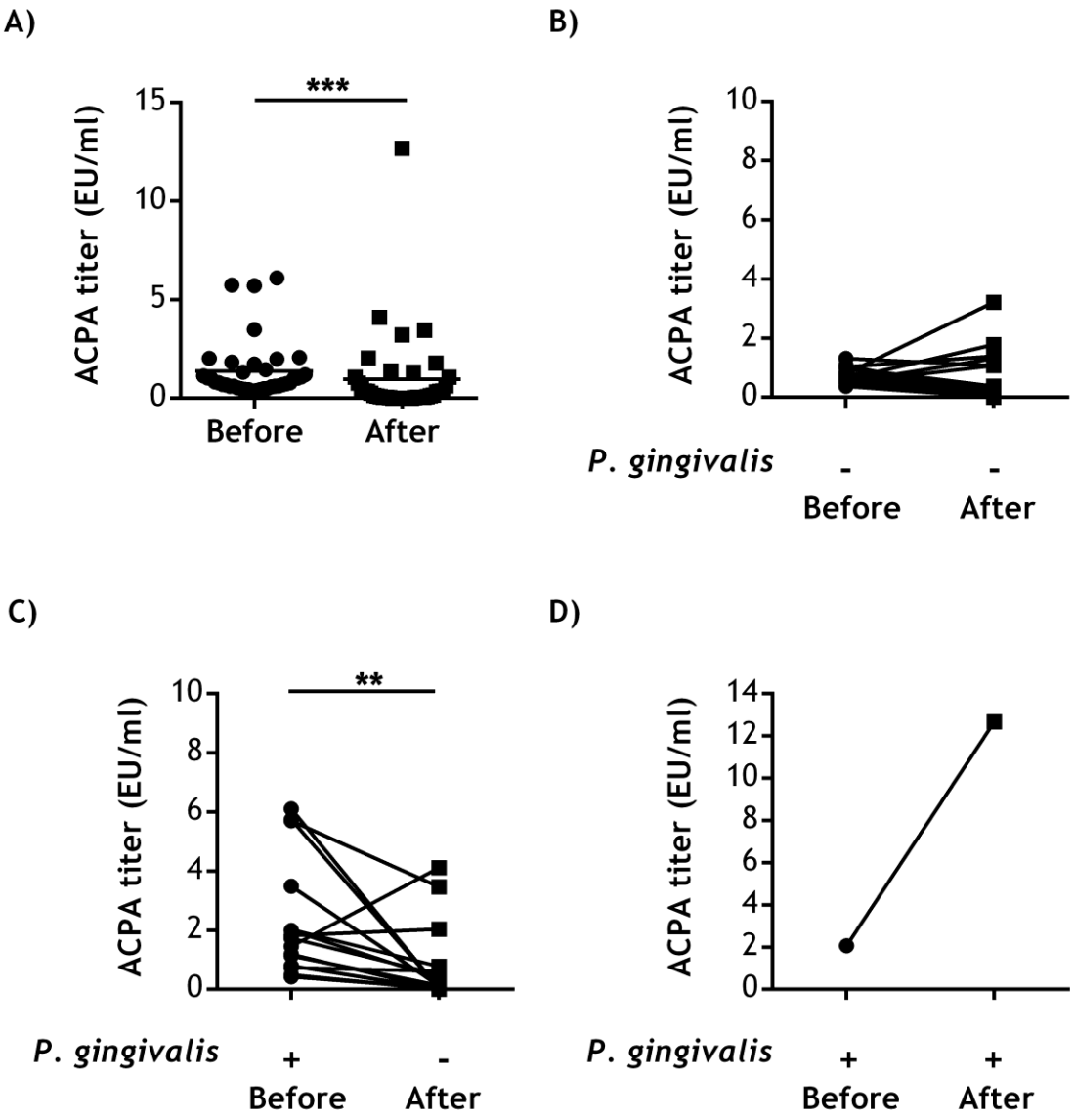

Figure 4.2.8. Serum ACPA titers in periodontitis patients before and after treatment. A) serum ACPA titers of periodontitis (PD) patients were determined before (circles) and after (squares) periodontal treatment. **B – D)** PD patients were sub-classified based on whether *P. gingivalis* could be detected in dental plaque before or after periodontal treatment. **B)** ACPA titers of PD patients that were *P. gingivalis* negative before treatment and remained *P. gingivalis* negative after treatment. **C)** ACPA titers of PD patients that were *P. gingivalis* positive before treatment and became *P. gingivalis* negative after treatment. **D)** ACPA titer of a PD patient that was *P. gingivalis* positive before treatment and remained *P. gingivalis* positive after treatment. In total, n = 39 PD patients, before treatment n = 23 *P. gingivalis* negative and n = 16 *P. gingivalis* positive, after treatment n = 38 *P. gingivalis* negative and n = 1 *P. gingivalis* positive. Statistical differences were determined by the Wilcoxon signed ranks test (**P < 0.01, ***P < 0.001). Experiments were carried out with *Dr David Lappin*.

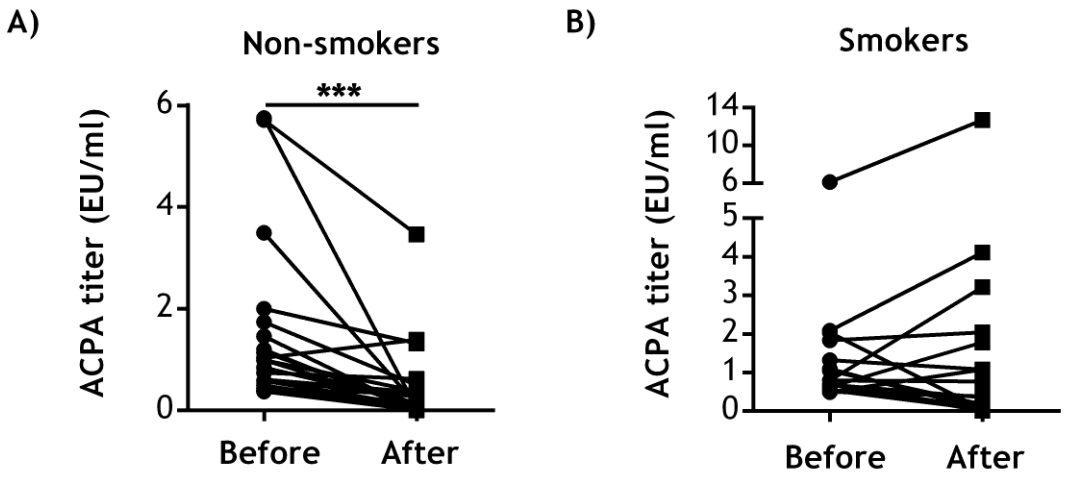

Figure 4.2.9. Serum ACPA titers in smoker and non-smoker periodontitis patients before and after treatment. Serum ACPA titers of periodontitis (PD) patients were determined before (circles) and after (squares) periodontal treatment. All PD patients were sub-classified based on their smoking status. **A)** ACPA titers of non-smokers before and after treatment. **B)** ACPA titers of smokers before and after treatment. In total, n = 39 PD patients, of these n = 23 were non-smokers and n = 16 were smokers. Statistical differences were determined by the Wilcoxon signed ranks test (***P < 0.001). Experiments were carried out with *Dr David Lappin*.

When PD patients were sub-classified based on their smoking status, further trends were revealed with regard to serum ACPA titers. Non-smoker PD patients had a significant reduction in ACPA titers after periodontal treatment (1.39 ± 0.32 EU/ml before vs. 0.41 ± 0.16 EU/ml after, **P < 0.001, **Figure 4.2.9A**), whereas there was no difference in the ACPA titers of smoker PD patients after treatment. Only a single non-smoker, but six smoking patients demonstrated higher ACPA titers after periodontal treatment. This data indicates that whilst periodontal treatment may be effective in reducing ACPA titers in non-smoking PD patients, it is less effective in reducing ACPA titers in smokers.

Summary of main results:

- Serum ACPA titers were higher in PD patients than in healthy participants
- Detection of *P. gingivalis* in dental plaque was associated with higher ACPA titers in PD patients
- Smoking was associated with higher ACPA titers in healthy participants
- Smoking was associated with lower anti-*P. gingivalis* titers in PD patients
- Periodontal treatment was associated with a reduction in ACPA titers in PD patients

4.3 Discussion

The most important finding from this study was that periodontal treatment, and the consequential removal of *P. gingivalis* could reduce ACPA titers in PD patients. The one patient that remained *P. gingivalis* positive after periodontal treatment had an ACPA titer that stayed above the diagnostic cut-off for RA. Subsequently it has been shown that periodontal treatment can reduce both ACPA titers and the DAS28 in RA in patients with underlying PD, although this was in a small study of just 55 patients, the majority of whom were women (Okada et al., 2013). Several other small studies have also found periodontal treatment reduces DAS28 in RA, but have neglected to investigate the effect on ACPA titers (Al-Katma et al., 2007, Ortiz et al., 2009, Biyikoglu et al., 2013, Erciyas et al., 2013). Combined, these data make a strong case that in adjunct with other therapies, periodontal treatment could help to reduce both inflammation and ACPA titers associated with RA, but larger scale studies need to be conducted to prove this.

Before treatment, PD patients had higher ACPA titers than the periodontally healthy participants in this study. Within the PD group, ACPA titers were found to be significantly higher among those that had detectable *P. gingivalis* in their dental plaque before treatment (**Figure 4.2.2B**). Consistent with the majority of studies including microbial analysis, *P. gingivalis* was no longer detectable after treatment, except in one patient (Renvert et al., 1992, Ali et al., 1992, Doungudomdacha et al., 2001, Fujise et al., 2002, Rosalem et al., 2011).

The increasing knowledge of the roles of other bacteria in PD warns against focusing too much on *P. gingivalis* in relation to disease pathology (Hajishengallis and Lamont, 2012). In this study it was appropriate to assess the involvement of *P. gingivalis* in PD, as one of the objectives was to test the hypothesis that *P. gingivalis* may be linked to breach of immune tolerance and the generation of ACPAs in PD. There would presumably have been a shift in the composition of the oral subgingival biofilm after periodontal treatment. The absence of *P. gingivalis* would probably have been associated with a reduction in other disease-associated bacteria – as has been reported elsewhere (Rosalem et al., 2011). However, interpreting the findings of full microbiome analysis in conjunction with the assessment of autoimmunity in PD patients would be challenging, and require a much larger study group.

The idea that the periodontal bacterium *P. gingivalis* is uniquely associated with ACPA titers and possibly triggers the generation for ACPAs has been supported by a study of 284 subjects deemed to be 'at risk' of RA by virtue of having a first-degree relative with RA and having HLA alleles with the 'shared epitope'. In this case, ACPA titers were found to positively correlate with anti-*P. gingivalis* antibody titers but no association was found between ACPAs and antibody titers for other disease-associated bacteria *Prevotella intermedia* and *Fusobacterium nucleatum* (Mikuls et al., 2012). The oral health of participants was not clinically assessed and they were not tested for carriage of *P. gingivalis*. Nonetheless, these results indicate that of these individuals, those who have at some point been exposed to *P. gingivalis* and generated an immune response against the bacterium, are more likely to have higher ACPA titers.

Exactly how and when ACPAs are involved in the pathogenesis of RA is not known. Attempts have been made to characterise the fine specificity of the autoantibody response in PD and in RA to help solve this mystery. The widely used commercial ACPA ELISA presents a limited selection of unidentified synthetic cyclic citrullinated peptides which cannot truly represent the variety citrullinated peptides formed physiologically and therefore can lead to false negative results (Wagner et al., 2014). More frequently, researchers are creating their own libraries of peptides to assess the response to citrullinated and

uncitrullinated autoantigens. One study found no difference in the serum ACPA titers of patients with moderate to advanced PD compared with a periodontally healthy population using the commercial ELISA, but did find increased titers of antibodies specifically recognising CEP-1, REP-1, and unmodified fibrinogen (de Pablo et al., 2013). It was suggested that an ACPA response may emerge after an autoimmune response against uncitrullinated peptides in PD patients that proceed to develop RA. This was supported by a retrospective study of autoantibodies in serum samples collected from RA patients at multiple time-points before the clinical manifestation of the disease (a median of 7.4 years before). In this study, autoantibodies recognising uncitrullinated peptides tended to be detected at earlier time-points than autoantibodies recognising the corresponding citrullinated peptides (Brink et al., 2014). Here, increased titers to both CEP-1 and REP-1 were found in the sera of PD patients, and a positive correlation existed between them. This confirms that an autoimmune response against uncitrullinated peptides tends to accompany the ACPA response, although in this case, it was not possible to distinguish which antibodies were produced first.

If ACPAs are secondary to the production of other autoantibodies in PD, this would refute the idea that *P. gingivalis* induces breach of immune tolerance through the citrullination of peptides. Unravelling the exact order of autoimmune events would require multiple assessments of the specificity of circulating T cells, B cells, and autoantibodies in PD patients from very early on in PD development through to the establishment of chronic PD, and the development of RA. Catching patients in the initial stages of PD is very difficult as many individuals do not find the first signs of disease troubling and do not visit their dentist until there is a very obvious problem. It is also difficult to determine how long patients have suffered with PD before they are clinically diagnosed as some forms of PD progress more rapidly than others. Adding to these complications, the standardised criteria for clinically assessing PD are not universally adopted in studies as those are for RA. It has been proposed that this accounts for many of the inconsistencies between studies of autoimmunity in PD and of PD in RA conducted so far (Dietrich, 2014).

Molecular mimicry between *P. gingivalis* α-enolase can explain the increased titers of anti-REP-1 antibodies in PD patients, but *P. gingivalis* does not possess its own version of other host proteins such as fibrinogen. It is puzzling how *P. gingivalis* could then trigger the production of autoantibodies recognising uncitrullinated epitopes before those recognising citrullinated epitopes in these other targets. It is possible that rapid cleavage of host proteins and production of lots of small citrullinated peptides by *P. gingivalis*' gingipains and PPAD activates T cells which recognise citrulline-containing short amino acid sequences in their primary structure. In some instances T cells could activate B cells which recognise a different epitope of the same peptide in its tertiary structure, which is uncitrullinated. As the autoimmune response persists, and more antigens are exposed in the inflammatory environment, epitope spreading could lead to autoreactivity to the corresponding uncitrullinated forms of peptides. This still cannot fully explain the phenomenon described in the study by Brink et al. (2014), that ACPAs were secondary to other autoantibodies in pre-RA. The periodontal health of the patients in this study was not determined, therefore it is possible they achieved their eventual RA status due to a combination of other risk factors.

One trend that is remarkably consistent across studies of ACPAs in PD and RA is that smoking increases ACPA titers. In this study PD patients that were smokers had similar ACPA titers to non-smokers before treatment; but after treatment, the ACPA titers of non-smokers were significantly reduced, whereas the ACPA titers of smokers were unchanged (**Figure 6.13**). Six smokers actually had higher ACPA titers after treatment, whereas only one non-smoker had a higher ACPA titer after treatment. Numerous others cite smoking as being the major preventable risk factor of ACPA generation and RA. Analysis of data on 1,204 patients from the Swedish Epidemiological Investigation of RA case-control study found a strong dose-dependent relationship between smoking and risk of ACPA positive RA. It was estimated that smoking was responsible for 35 % of cases of ACPA positive RA (Kallberg et al., 2011).

Nicotine has been ruled out as an active component of cigarettes contributing to the association between smoking and ACPAs, as users of smokeless tobacco in Sweden have been shown to have normal ACPA titers, and not to be at increased

risk of RA (Carlens et al., 2010, Jiang et al., 2014). Smokeless tobacco bypasses the lung and delivers nicotine passively across blood vessels in the oral mucosa. This evaluation does not appear to acknowledge the possibility that the lung is more vulnerable than the oral mucosa to the effects of nicotine. It does highlight that there are many other chemical components of smoke that have yet to be independently investigated with regard to their detrimental impact on the immune response.

The immunosuppressive effects of nicotine have been well documented (Geng et al., 1995, Geng et al., 1996, Nouri-Shirazi and Guinet, 2003, Kalra et al., 2004). Other components of smoke may oppose these effects of nicotine. The combination of chemicals renders smokers more susceptible to infection and inflammation of the lungs which ultimately leads to conditions such as emphysema and COPD (Postma et al., 2014). The lungs of smokers have macrophages and neutrophils dispersed throughout the tissue, and have increased numbers of lymphocyte aggregates in the form of BALT (Richmond et al., 1993). It has been hypothesised that the inflammatory environment in the lung induced by smoking promotes the citrullination of peptides and the generation of ACPAs (Perry et al., 2014, Catrina et al., 2014). Citrullination of peptides as a result of increased host PAD2 expression has been observed in bronchoalveolar lavage cells from lungs of smokers (Makrygiannakis et al., 2008). Plasma cells derived from BALT of patients with pulmonary disease or RA are capable of generating ACPAs (Rangel-Moreno et al., 2006). The lung thereby presents a separate route by which smoking can increase the risk of RA, in addition to the effects on the development of PD. This pathway could theoretically account for the slightly elevated ACPA titers observed in the smokers group within the periodontally healthy population in this study, although these patients did not have any kind of pulmonary disease. PD, smoking, and COPD, can be viewed as separate ‘hits’ within the two-hit hypothesis. Individually, they may not cause sufficient inflammation and autoantibody production to cause RA, but in some combination with other ‘hits’, they may contribute to its development (Farquharson et al., 2012).

To explore the associations between *P. gingivalis*, smoking, and the generation of ACPAs further, it would have been useful to assess ACPA production in the

gingival tissue of the PD patients, as the gingiva are directly exposed to both of these factors. However, such samples are generally obtained from PD patients during surgical periodontal treatment, and the patients in this study were not suitable candidates for this. Moreover, the detection of ACPAs in gingival tissue by IHC is fraught with technical issues. This method would require generating a panel of labelled citrullinated peptides to probe the tissue. One approach to generating citrullinated peptides is to incubate the peptides *in vitro* with rabbit skeletal muscle PAD. This approach was employed by Humby et al. (2009) who used biotin-conjugated citrullinated fibrinogen to detect ACPA producing-plasma cells in synovial biopsies from RA patients. The recognition of the citrullinated fibrinogen was compared with the recognition of uncitrullinated fibrinogen by tissue-resident plasma cells to validate that antibodies were specific for the citrullinated form. Ideally in studies where citrullinated peptides are synthesised this way, the purity of the citrullinated peptide would be assessed by mass spectrometry before use – which would not have been feasible in this study. Alternatively, biotinylated, citrullinated peptides can be custom-ordered, but this would require knowledge of the exact position of amino acid residues which are citrullinated in normal physiology that form part of immunodominant epitopes. So far, data gathered on peptides extracted from the rheumatoid synovium have only indicated the overall degree of citrullination of a limited selection of peptides and the regions within these peptides that are most frequently citrullinated (van Beers et al., 2010, De Ceuleneer et al., 2012, Tutturen et al., 2014).

In line with the defined immunosuppressive effects of smoking, here PD patient smokers were found to have lower anti-*P. gingivalis* IgG titers than non-smokers before periodontal treatment (**Figure 4.2.5**). Whether the total anti-*P. gingivalis* IgG titer is indicative of the humoral protection a PD patient possesses is doubtful since the clinical assessment indicated that smokers and non-smokers had similar disease scores before treatment, and both exhibited significant improvement in PPD, LOA, and BOP after periodontal treatment (**Table 4.2.2**). This is in contrast to other reports that at baseline, smokers have greater PPDs and LOA associated with more advanced PD, but reduced BOP due to the damage of blood vessels, and that they benefit less from treatment (Albandar et al., 2000, Calsina et al., 2002, Apatzidou et al., 2005, Gomes et al., 2006).

The improvement in clinical symptoms of PD and apparent absence of *P. gingivalis* observed after treatment was not accompanied by an overall change in the titers of anti-*P. gingivalis* antibodies. Before treatment, anti-*P. gingivalis* IgG titers were higher in PD patients compared with periodontally healthy participants (**Figure 4.2.1A**), and they remained high in the majority of PD patients after treatment (**Figure 4.2.7A**).

Before treatment, *P. gingivalis* DNA could be detected in the dental plaque of 16 out of 39 PD patients (41 %). This proportion is similar to that found by other studies using equivalent methods (Eick and Pfister, 2002). It is possible that *P. gingivalis* may still be present after treatment, but at numbers below the limits of detection. In this study, *P. gingivalis* was enumerated by qPCR of 16S DNA. Other groups have cited the use of different target genes, which have a higher copy number within the genome, such as ISPg1 which has 31 copies, enabling detection of smaller numbers of the bacteria (Hajishengallis et al., 2011). Another possibility is that anti-*P. gingivalis* IgG titers remain high for a long time after treatment in the absence of *P. gingivalis*, since long-lived plasma cells are known to be able to survive months and continue to produce antibody in the absence of antigen (Manz et al., 1997, Manz et al., 1998). It is also possible that the process of treatment temporarily increases the number of *P. gingivalis* in the bloodstream (Lafaurie et al., 2007, Castillo et al., 2011), which could promote the production of new antibodies and mask any loss of antibodies produced by short-lived plasma cells.

Before treatment, anti-*P. gingivalis* IgG titers were highest in *P. gingivalis* negative patients (**Figure 4.2.1B**), which prompts the theory that anti-*P. gingivalis* antibodies could have aided clearance of the bacteria in these patients. Yet despite the large number of studies in which anti-*P. gingivalis* IgG titers have been measured, no consistent correlation with carriage of *P. gingivalis* or treatment outcomes has been identified, and there is no consensus on whether they are protective or destructive (Chen et al., 1991, Mooney et al., 1995, Sakai et al., 2001, Kudo et al., 2012).

Cutler *et al.* (1991) clearly demonstrated how unreliable anti-*P. gingivalis* IgG antibody titers are for predicting the level of protection a patient has against *P.*

gingivalis colonisation when they tested the opsonic capability of anti-*P. gingivalis* IgG antibodies isolated from 18 PD patients. All patients had high anti-*P. gingivalis* IgG titers, but only three patients had antibodies that could promote phagocytosis of *P. gingivalis* by neutrophils. Antibodies of a particular subclass, specificity, avidity, and affinity may be effective in opsonising or trapping *P. gingivalis* – although which antibodies are protective for a particular patient will also be dependent on which strain of *P. gingivalis* they harbour and their FcR repertoire (Dimou et al., 2010).

There are reports that antibodies specific for certain components of *P. gingivalis* such as gingipains are associated with less severe PD or recovery after periodontal treatment (Nguyen et al., 2004, Gibson et al., 2005, Shelburne et al., 2008). This has led to the development of vaccines composed of selected gingipain antigens. Preliminary trials in rodent models indicate such vaccines can effectively protect hosts from alveolar bone loss induced by subsequent oral infection with *P. gingivalis* (Lee et al., 2006, O'Brien-Simpson et al., 2005). As gingipains are one of the major virulence factors of *P. gingivalis*, targeting them is a logical strategy for preventing or treating PD, but no naturally formed antibodies have been shown to actually inhibit gingipains activity (Olsen and Potempa, 2014). It needs be fully established whether specific anti-*P. gingivalis* antibodies can confer protection in PD. The titers of these could then be measured to more accurately assess the impact of risk factors, the success of periodontal treatment, and the relationship between the anti-*P. gingivalis* response and the ACPA response in PD patients.

Ultimately, the sample size of this study limits the extent to which any associations in this study can be interpreted. A total of 39 PD patients and 36 periodontally healthy participants were recruited (**Table 4.1.2**), which is only sufficient for a pilot study. A larger patient cohort would have been desirable, but was not achievable with the following constraints: PD patients had to fulfil the designated criteria for chronic PD, whilst not having any current systemic health problems, and these patients had to attend multiple appointments with the same clinician for clinical assessment, collection of dental plaque and serum samples, and stages of periodontal treatment, all within a set time-frame. A larger number of periodontally healthy participants would also be ideal. This

study could be subject to selection bias as the periodontally healthy participants in the study were Dental School staff; however they had no specific training in oral health care.

The other major limitation of this cross-sectional study is that it provides only a snapshot of the possible immunological link between PD and RA. The ACPA titers observed in PD patients here, although higher than in periodontally healthy controls are generally low compared with those reported in seropositive RA patients. Only three of the 39 PD patients had ACPA titers which were slightly above the diagnostic threshold for RA. Challenging longitudinal studies of PD patients (decades in duration) are required to determine if this level of autoimmunity increases over time and can cause RA.

Some of the limitations faced in this human study could be overcome by utilising murine models of PD. Studies of mice enable greater control of extraneous variables, which minimises variability within groups and reduces the need for larger group sizes. In addition, it is easier to perform longitudinal studies on mice as they naturally have a shorter life-span, therefore systemic complications of PD could theoretically be observed within a relatively short time-frame.

4.4 Conclusion

There is still a great deal of research that needs to be done to establish whether there is a causal relationship between PD and RA, and how the generation of ACPAs is connected to these diseases. This study has helped to confirm earlier findings that ACPA titers are elevated in PD patients and that carriage of *P. gingivalis* and smoking influence the ACPA response. This supports the hypothesis that PD or smoking could provide a 'first hit' in the immunopathological pathway to RA. Furthermore, it has demonstrated that treatment of PD could reduce ACPA titers, which, in addition to preventing tooth loss, may hold promise for the management of RA.88

Chapter 5: Characterisation of B cells in a murine model of periodontitis

5.1 Introduction

In order to elucidate the potential mechanistic links between B cells in the gingiva, alveolar bone loss, and autoimmunity, more detailed phenotyping of B cells locally and systemically at different stages of PD is required. So far, research of B cells in PD patients has been limited in both these, and previous studies, by sample size, sample variation, and the types of samples available. Consequently, a murine model of PD has been established and utilised here to investigate changes in B cell phenotype and cytokine production at early and late stages of disease. In subsequent chapters, the murine model is further utilised to establish whether the B cell response to *P. gingivalis* infection is associated with pathology in PD.

The inflammatory infiltrate in PD lesions includes an abundance of B cells, along with their survival factors: IL-6, APRIL, and BAFF (Gumus et al., 2013a, Gumus et al., 2014). Myriad studies have reported that plasma cells account for half of the leukocyte infiltrate, and B cells a further 20 % (Berglundh and Donati, 2005). These claims were supported by the results in **Chapter 3**. By contrast, relatively few leukocytes have been found in healthy gingiva, of which only about 5 % are B cells (Gemmell et al., 2002b).

The detection of autoantibodies in PD patients and the unresolving nature of chronic PD suggests that the immune response is dysregulated, B cells are abnormally activated, and that this has systemic repercussions (Lappin et al., 2013). Potentially, a minority of B cells are responsible for the majority of B cell-driven immunopathology in PD - including but not limited to - the generation of autoantibodies.

Mature B cells can be divided into four different subsets based upon their signature expression of cell surface markers, size, anatomical location, and specialised functions: B1a cells ($CD19^+$ $CD43^-$ $CD5^+$), B1b cells ($CD19^+$ $CD43^-$ $CD5^-$), B2 MZ cells ($CD19^+$ $CD43^+$ CD23), and B2 FO cells ($CD19^+$ $CD43^+$ $CD23^+$). This is summarised in **Table 5.1.1**.

	B1a	B1b	B2 MZ	B2 FO
Surface markers	$CD19^+$ $CD43^+$ $CD23^-$ $CD5^+$	$CD19^+$ $CD43^+$ $CD23^-$ $CD5^-$	$CD19^+$ $CD43^-$ $CD23^-$ $CD5^-$	$CD19^+$ $CD43^-$ $CD23^+$ $CD5^-$
Main anatomical location	peritoneal and pleural cavities	peritoneal and pleural cavities	spleen and LNs	spleen and LNs
Proportion of total splenic B cell population	2 %	< 1 %	15 %	> 70 %
Main function	production of IgM	production of IgM	phagocytosis and antigen presentation	production of class-switched antibodies

Table 5.1.1. Properties of the major mature B cell subsets.
Adapted from Baumgarth (2011).

The B1 subsets are considered to be innate-like B cells, which respond rapidly to infection and help to bridge the innate and the adaptive immune responses. B1 cells collectively constitute a very small proportion of the total B cell population but evidently make a significant contribution to the immediate defence against microbes as a deficiency in B1a cells is associated with impaired clearance of bacteria and sepsis (Boes et al., 1998, Rauch et al., 2012). B1 cells are rare in the BM, blood, spleen, and LNs, but are enriched in the peritoneal and pleural cavities (Hayakawa et al., 1985, Baumgarth, 2011). They are not trapped at these sites and have been shown to accumulate elsewhere, including the oral cavity, in response to bacterial infection (Donati et al., 2009b, Weber et al., 2014). Their defences against bacteria include enhanced phagocytic activity (Rauch et al., 2012, Gao et al., 2012) and spontaneous secretion of low-specificity, cross-reactive IgM (Kawahara et al., 2003, Alugupalli et al., 2004, Weber et al., 2014). The BCR repertoire of B1 cells has limited diversity, it is more restricted to germline encoded sequences and less permissible to modification (by addition of non-templated nucleotide inserts between the V, D and J segments) than that of the other B cell subsets (Kantor et al., 1997, Tornberg and Holmberg, 1995). Targets of these BCRs typically include repetitive structures associated with pathogens and also molecules of self-origin that are revealed under conditions of stress or tissue damage such as oxidised LDL, annexin IV, and phosphatidylcholine (Nakamura et al., 1988, Kulik et al., 2009). Under normal circumstances, this autoreactive potential is restrained by

inhibitory receptors, but under conditions of stress or injury, the balance between inhibitory and activatory ligands is skewed (Bendelac et al., 2001). CD5, which demarcates the B1a subset, is one example of such an inhibitory receptor which signals via SHP-1 to dampen BCR signalling (Berland and Wortis, 2002).

B2 MZ cells are similar to B1 cells in several respects. True B2 MZ B cells reside in the MZ of the spleen (Kumararatne et al., 1981). Although in humans, something resembling a B2 MZ cell has been described in LNs (Tierens et al., 1999), equivalent reports have not been made in mice (Cerutti et al., 2013). Therefore, B cells which fall into the B2 MZ category on the basis of their expression of cell surface markers, but which are not derived from the spleen, will be referred to as B2 MZ-like here. In the spleen, B2 MZ cells exist in a pre-activated state, enabling them to respond very quickly to blood-borne pathogens. Within four hours of antigen capture *in vivo*, they are capable of antigen presentation and co-stimulation (Martin and Kearney, 2002). B2 MZ cells are the most efficient B cell APC (Attanavanich and Kearney, 2004). They express high levels of TLRs, and activation of these TLRs can boost their expression of co-stimulatory molecules – which may further enhance their ability to present antigen (Barr et al., 2007). Like B1 cells, B2 MZ cells have polyreactive BCRs, which often recognise PAMPs similar to those which bind TLRs, and can rapidly differentiate into plasmablasts secreting IgM and IgG in TI responses to microbial polysaccharide antigen (Guinamard et al., 2000, Macpherson et al., 2000, Martin et al., 2001). Antibodies generated this way can be cross-reactive with host molecules (Bendelac et al., 2001). B2 MZ cells have complex relationships with innate cells including iNK T cells (King et al., 2012) and neutrophils, which modulate their TI responses to antigen (Puga et al., 2012, Cerutti et al., 2013). However, B2 MZ cells are flexible. They can also become involved in antigen-specific TD responses and form GCs, and in this respect they share something in common with B2 FO cells (Song and Cerny, 2003).

B2 FO cells represent the more traditional notion of what a B cell is and does. Forming the majority of B cells in the follicles of secondary lymphoid organs, B2 FO cells are better adapted for engaging in TD responses to antigen. They have differential requirements for activation and are optimally positioned next to T

cell zones to receive this help (Pillai and Cariappa, 2009). Through the TD pathway it typically takes five to seven days to produce antibodies - rather than one to three days by the TI pathway - but the antibody produced is of a higher affinity (Martin et al., 2001, Song and Cerny, 2003).

Individual B cell subsets have been linked to pathology in particular inflammatory diseases. B1a cells have repeatedly been associated with autoimmunity. The production of autoreactive IgM by B1 cells is thought to be central to the development of T1DM and SLE in murine models of these diseases (Murakami et al., 1995, Ito et al., 2004, Enghard et al., 2010, Fletcher et al., 2011, Corte-Real et al., 2012, Lamagna et al., 2014). In patients with RA, B1a cells produce RF, which, along with ACPAs are diagnostic of the disease and correlate with bone erosion (Harindranath et al., 1991, Mantovani et al., 1993, Sokolove et al., 2014, Sun et al., 2014, Hecht et al., 2014). On the other hand, there are many reports that autoreactive IgM produced by B1a cells are protective in murine models of atherosclerosis and inflammatory arthritis, via the clearance of apoptotic cells (Litvack et al., 2011, Notley et al., 2011).

In addition to the aforementioned germline encoded BCR sequences of B1a B cells, there are selective pressures which allow this subset to be autoreactive. Accumulating evidence suggests that there is a positive selection step, after the expression of the BCR by B cell progenitors, that is dependent on BCR specificity and the presence of self-antigen to elicit strong BCR signalling, which preferentially leads to the development of B1 cells rather than B2 cells (Arnold et al., 1994, Lam and Rajewsky, 1999, Hayakawa et al., 1999, Casola et al., 2004). Mature B1 cells in the periphery are in a semi-activated state which seems to lower their threshold for activation by TLR agonists and promotes their production of low-affinity, cross-reactive antibodies in response to these (Fairfax et al., 2007, Genestier et al., 2007, Holodick et al., 2009). Exactly how this pre-activated condition affects responses to BCR stimulation is unclear. This is likely to be influenced by a mixture of signals available in the microenvironment *in vivo*. Peritoneal B1 cells are sub-optimally activated and do not proliferate in response BCR cross-linking in the absence of co-stimulation (Morris and Rothstein, 1993, Chumley et al., 2002). This reaction has been confused with anergy, which is a state of unresponsiveness in optimal stimulatory conditions

associated with a separate series of signalling events (Hippen et al., 2000, Wong et al., 2002, Durand et al., 2009). The altered regulation of responses to BCR signalling, in conjunction with a lack of dependency on survival factors such as BAFF, enables self-reactive B1 cells to persist for a long time (Kyaw et al., 2012).

Since their identification in the 1980's, there has been some interest in the role of B1a cells in PD. One group found the percentage of B1a cells to be greatly increased in PD patients, with 30 % of B cells being $CD5^+$ in PD lesions, and 40-50 % of B cells belonging to the B1a subset in blood samples from PD patients, compared with 15 % in blood samples from healthy people (Berglundh et al., 2002b). Another group found that 60 % of B cells were B1a cells in the gingiva of PD patients, and that the percentage of B1a cells closely correlated with clinical parameters of PD severity, but they did not have healthy gingiva for comparison (Donati et al., 2009a). However, the data presented in **Chapter 3** opposed the findings of these studies, with < 1 % of the large number of $CD19^+$ B cells being $CD5^+$ in samples of gingiva from PD patients. The variability between these and other studies of B1a cells in PD could be due to differences in the clinical assessment of disease and differences in sampling technique. It has been implied that B1a B cells could give rise to autoimmunity in PD, through the production of anti-collagen antibodies (Sugawara et al., 1992), but minimal investigation of the function of B1a cells in PD has taken place.

Aside from the production of autoantibodies, atypical activation of any subset of B cells could contribute to PD pathogenesis by alteration of the cytokine profile. All B cells can produce the inflammatory cytokine IL-6, which is known to promote the growth and differentiation of T cells and B cells as well as enhance osteoclastogenesis (Kurihara et al., 1990). But B cells also have the potential to produce the anti-inflammatory cytokine, IL-10. B cells which produce high levels of IL-10 have been referred to as Bregs. Bregs have been associated with suppression of Th1 and Th17 responses and attenuation of disease in a murine model of arthritis (Fillatreau et al., 2002, Mauri et al., 2003, Mauri and Ehrenstein, 2008, Gray and Gray, 2010, Carter et al., 2012). The relationship between Bregs and the B1a, B1b, B2 MZ, and B2 FO B cell subsets is unclear. Counter-intuitively B1 cells, which are reputed to have a pathological role in

autoimmune diseases, can be a major source of IL-10 (O'Garra et al., 1992, Amel Kashipaz et al., 2003). B1a cells constitutively produce IL-10, and in doing so support their own survival (Balabanian et al., 2002). It is possible that in PD, B cells contribute to pathology through elevated production of IL-6 or that a deficiency of IL-10 producing Bregs allows inflammation to escalate.

RANKL is another cytokine produced by B cells. RANKL belongs to the TNF superfamily, and interacts with its receptor RANK to act as a promoter of osteoclastogenesis. In health, this process is regulated by OPG, a decoy receptor for RANKL (Yasuda et al., 1999). RANKL expression is increased in the gingiva of PD patients, and lymphocytes appear to be a major source of this cytokine (Kawai et al., 2006, Wara-aswapati et al., 2007). In rodent models of PD, RANK-RANKL interactions are essential mediators of pathological alveolar bone loss (Yuan et al., 2011, Han et al., 2013). *In vitro* studies have shown that lymphocyte expression of RANKL can be increased following culture with PD-associated bacteria *A. actinomycetemcomitans* and *P. gingivalis* (Han et al., 2009, Belibasakis et al., 2011). The requirement for antigen specificity in this system was demonstrated in a study in which antigen-experienced *A. actinomycetemcomitans*-specific B cells were adoptively transferred into *A. actinomycetemcomitans*-immunised rats, and exacerbated RANKL-dependent alveolar bone loss (Han et al., 2006, Han et al., 2009). Unfortunately this study was unable to uncouple the contribution of B cell-derived RANKL from other sources of RANKL, and also the contribution of RANKL from other cytokines produced by antigen-experienced B cells. Whether RANKL specifically from B cells has a significant impact on the progress of PD remains to be proven, and if so, whether all B cells are equal in this respect, or if a given subset is more prone to RANKL expression in PD needs to be resolved.

5.2 Results

To first confirm that infection with *P. gingivalis* induced PD in mice, alveolar bone loss was measured in mice orally infected with *P. gingivalis* and sham-infected controls at six weeks post-infection (as described in **Chapter 2**, section **2.7**). Previous studies utilising this model of PD have indicated that significant alveolar bone loss in mice infected with *P. gingivalis* compared with controls can be observed from four weeks post-infection, and continues to progress through to six weeks post-infection (Baker et al., 1999a). Although alveolar bone loss continues beyond this time-point, age itself becomes an influential factor, complicating the interpretation of findings in this model (Liang et al., 2010). Consequently, six weeks post-infection is the most common reference point in the literature, and by assessing parameters of immunopathology at this time-point, these data may be directly compared with those of other studies.

Mice with PD had significant alveolar bone loss relative to the sham controls (mean ± SEM: 0.23 ± 0.01 mm PD vs. 0.15 ± 0.005 mm sham, **Figure 5.2.1**). It was not possible to recover cultivable *P. gingivalis* from the oral cavity or detect *P. gingivalis* by qRT-PCR after one week post-infection (data not shown), therefore serum anti-*P. gingivalis* IgG titers were measured at one, two, and six weeks post-infection to confirm the reproducibility of the adaptive immune response to *P. gingivalis* infection (as described in **Chapter 2**, section **2.16.1**). Anti-*P. gingivalis* IgG titers were significantly elevated at two weeks (201.4 ± 60.45 EU PD vs. 6.23 ± 4.65 EU sham, *P < 0.05, **Figure 5.2.2**) and six weeks (245.2 ± 49.05 EU PD vs. 10.38 ± 2.86 EU sham, ***P < 0.05, **Figure 5.2.2**) post-infection in mice with PD relative to sham controls. However, a degree of variability is apparent in the murine adaptive immune response to *P. gingivalis* infection, with some mice in the PD groups having very low titers of anti-*P. gingivalis* IgG at each time-point. Subsequent assessments focused primarily on the one week and six week time-points post-infection to characterise immunological events preceding and following the onset of the humoral response.

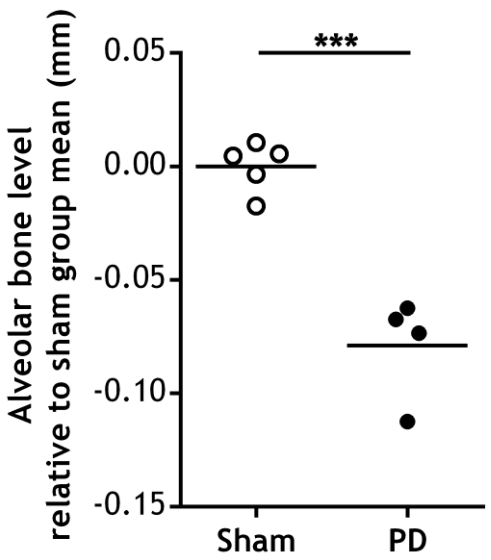

Figure 5.2.1. Alveolar bone level in mice with periodontitis at 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 6 weeks post-infection, the alveolar bone loss was measured. The data are shown as mean per mouse (symbols) and mean for each group of mice (lines) normalised to the sham group mean, n = 4-5 mice/group. Significant difference was determined by unpaired t test (***P < 0.001). Data were contributed to by *Dr John Butcher*.

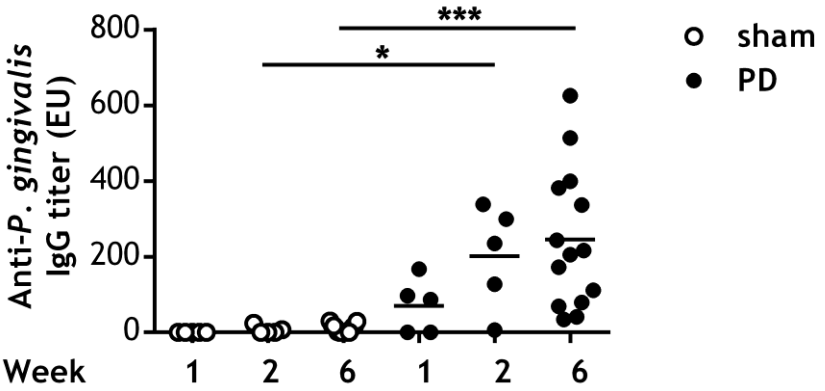

Figure 5.2.2. Anti-*P. gingivalis* IgG titers in mice with periodontitis at 1, 2, and 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 1, 2, and 6 weeks post-infection serum anti-*P. gingivalis* IgG titers were measured by ELISA. At 1 and 2 weeks post-infection, n = 5 mice/ group. At 6 weeks post-infection, data are combined from 3 independent experiments, n = 4-5 mice/group. Significant differences were determined by unpaired t test (*P < 0.05, ***P < 0.001).

Initial experiments sought to confirm that T cells and B cells could be detected in the gingiva of mice. The gingiva of mice infected with *P. gingivalis* and sham-infected mice were harvested at one week and six weeks post-infection. RNA was extracted from each individual mouse's gingiva, then mRNA was reverse transcribed to cDNA, and the relative gene expression of CD4 and CD19 was measured by qRT-PCR (as described in **Chapter 2**, sections **2.6** and **2.8.1**). The concentration of RNA obtained from these small tissue samples was adequate for this purpose in all samples except one (**Table 5.2.1**). The 260/280 ratio indicated that the RNA was consistently of acceptable purity - a 260/280 ratio of ~2.0 is typically accepted as pure for RNA. On the other hand the 260/230 ratio was highly variable and much lower than desirable – a 260/230 ratio between 2.0–2.2 is generally accepted for nucleic acid. This indicated the presence of contaminants which absorb at 230 nm which may be due to carry-over of reagents used in the RNA extraction process. This aspect of RNA quality does not appear to have caused a problem with the qRT-PCR as the amplification curves looked normal (**Figure 5.2.3A**). Controls were included in which the reverse transcriptase enzyme was omitted from the reaction (RT- controls). These controls did not undergo amplification in qRT-PCR of CD4 or CD19, demonstrating that there was little or no contamination of the reaction mixture with genomic DNA. The SD between replicate qRT-PCR reactions was, in the majority of cases < 0.5 Ct's from the mean Ct value, indicating good technical accuracy.

The qRT-PCR data showed that CD4 and CD19 expression could easily be detected in the gingiva, but no significant difference in the expression of either lymphocyte marker was evident between the mouse groups at either time-point (**Figure 5.2.3B** and **5.2.3C**). A high degree of variability within groups was apparent at one week post-infection. With regard to CD19 expression, these results do not mirror the observation in the gingival lesions of human PD patients that CD19 expression was increased compared with periodontally healthy individuals (**Chapter 3**, **Figure 3.2.1**). Potentially, the total number of T cells has been underestimated by investigating CD4 expression rather than CD3 expression, which would also account for CD8 T cells.

Time-point	Sample	RNA ng/ µl	260/280	260/230
1 week	sham 1	325.16	2.07	1.85*
1 week	sham 2	310.53	2.06	2.06
1 week	sham 3	288.31	2.08	2.12
1 week	sham 4	339.02	2.06	2.22
1 week	sham 5	232.11	2.09	1.69*
1 week	PD 1	216.61	2.09	1.78*
1 week	PD 2	303.54	2.06	1.77*
1 week	PD 3	436.11	2.07	2.00
1 week	PD 4	312.45	2.05	2.08
1 week	PD 5	307.39	2.08	1.89*
6 weeks	sham 1	220.26	2.04	1.67*
6 weeks	sham 2	273.30	2.08	1.46*
6 weeks	sham 3	220.16	2.03	1.19*
6 weeks	sham 4	225.50	2.06	1.65*
6 weeks	sham 5	240.12	2.03	1.64*
6 weeks	PD 1	94.47**	2.08	0.77*
6 weeks	PD 2	299.59	2.07	1.34*
6 weeks	PD 3	242.24	2.08	1.26*
6 weeks	PD 4	204.11	2.07	1.45*

Table 5.2.1. Quality of RNA extracted from the gingiva of mice with periodontitis. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 1 and 6 weeks post-infection RNA was extracted from the gingiva. The data shown are values for RNA concentration and relative absorbance at different wavelengths of light. Asterisk * indicates values which were suboptimal for qRT-PCR, ** indicates values which were unusable for qRT-PCR. n = 5 mice/group except group PD at 6 weeks post-infection, n = 4.

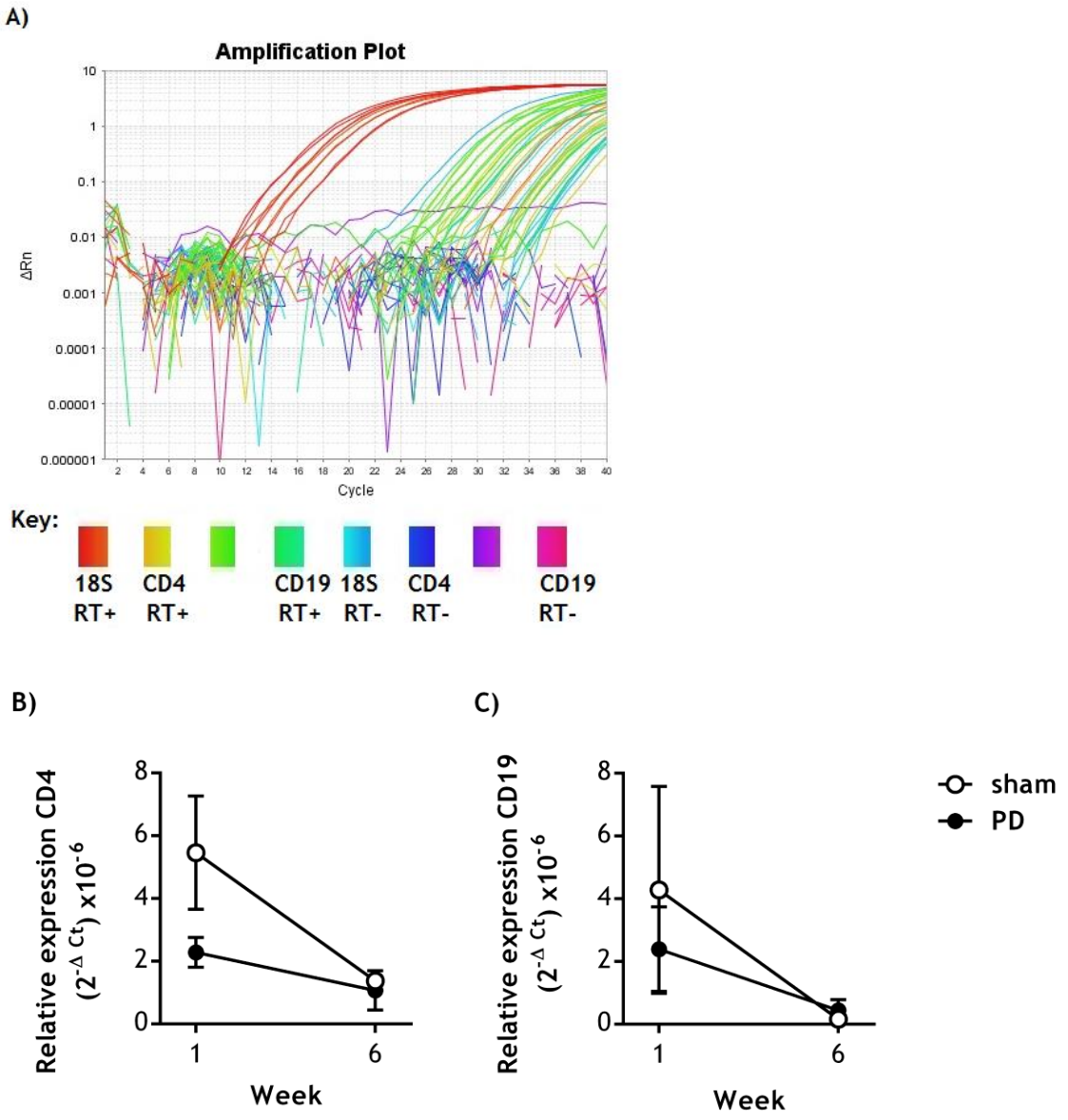

Figure 5.2.3. Relative expression of CD4 and CD19 in the gingiva of mice with periodontitis at 1 and 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 1 and 6 weeks post-infection, RNA was extracted from the gingiva and reverse transcribed to cDNA (RT+) or not (RT-). The expression of 18S, CD4, and CD19 was then measured by qRT-PCR of the cDNA. **A)** amplification curves of 18S, CD4, and CD19 sequences in RT+ and RT- samples. **B**) expression of CD4 relative to 18S. **C**) expression of CD19 relative to 18S. Data are shown as mean with SEM, n = 5 mice/group, except group PD at 6 weeks post-infection, n = 3. Data were analysed by Mann Whitney U test, and no significant differences were found.

To ascertain whether there was a qualitative change in the phenotype of the B cells in mice with PD, he proportions of B cells, GC B cells and plasma cells in the gingiva of mice infected with *P. gingivalis*, and sham controls were analysed by flow cytometry at one week and six weeks post-infection (as described in **Chapter 2**, sections **2.6**, **2.10.1**, and **2.15.2**). In line with the qRT-PCR results in **Figure 5.2.3**, the percentage of B cells in the gingiva of mice with PD was similar to that of sham controls at both time-points (**Figure 5.2.4**). The percentage of plasma cells present was very low (< 1 %) or undetectable in both mice with PD and sham controls (data not shown). These data indicate that there was no increased infiltrate of B cells or increased differentiation of B cells into plasma cells in the gingiva of mice with PD, at the time points investigated, but does not conclusively demonstrate that B cell phenotype is not altered in murine PD. Further experiments were conducted to examine other aspects of B cell phenotype such as activation status and cytokine production.

Throughout the results presented in **Chapter 5**, **Chapter 6**, and **Chapter 7** comparisons between mice with PD and sham controls are made at 1 week or 6 weeks post-infection. These comparisons do not consider that immunological changes have occurred in both groups relative to baseline as a result of age or in sham controls as a result of treatment with CMC. To establish baseline values for each of the parameters investigated, assessments could have additionally been made in sham control mice in untreated mice prior to infection. To investigate the possibility of very early immunological changes, assessments could also have been made at day 0 post-infection. However, such additional assessments could not be justified in line with the UK Home Office legislation to replace, refine, and reduce animal research.

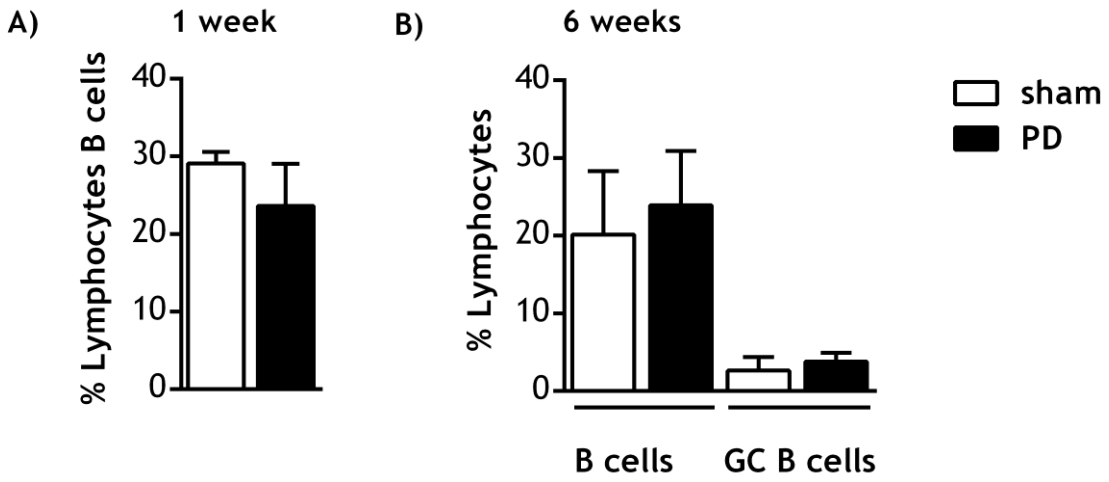

Figure 5.2.4. B cells in the gingiva of mice with periodontitis at 1 and 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 1 week (**A**) and 6 weeks (**B**) post-infection cells were isolated from the gingiva and analysed by flow cytometry. Percentages of B cells are shown as mean with SEM for 3 independent experiments at both time-points. Percentages of GC B cells are shown as mean with SEM for 2 independent experiments at 6 weeks post-infection. Gingiva were pooled from 5 mice/group in each experiment. Data were analysed by Mann Whitney U test and no significant differences were found.

B cells can be stimulated to increase the expression of RANKL (Han et al., 2009), and RANKL has been demonstrated to be a crucial mediator of alveolar bone loss in murine PD (Han et al., 2013). Therefore, B cell expression of RANKL, in the total B cell population and across B cell subsets, was assessed in this model of PD to explore the possibility that B cells could contribute to pathology in PD by this mechanism. No significant difference in B cell expression of RANKL was found in the gingiva at one or six weeks post-infection in mice with PD compared with sham controls (**Figure 5.2.5**).

There were no significant differences in the proportions of B cell subsets in the gingiva of mice with PD compared with sham controls. At one week post-infection there was an increase in the ratio of B2 MZ-like cells to B2 FO cells in the gingiva of mice with PD compared with sham controls. However, as this experiment was only performed once, with pooled samples of gingiva, these data cannot be statistically analysed (**Figure 5.2.5C**). At six weeks post-infection, no differences were observed in the proportion of any of the subsets in the gingiva of mice with PD compared with sham controls. At both time-points, the majority of B cells in the gingiva belonged to the B2 MZ-like or B2 FO cell subsets in both mouse groups (45.57 ± 8.12 % sham, 48.83 ± 9.59 % PD, B2 MZ-like cells; 43.03 ± 8.85 % sham, 39.37 ± 10.75 % PD, B2 FO cells, **Figure 5.2.5D**).

Because RANKL expression by each B cell subset in the gingiva was comparable between mice with PD and sham controls (**Figure 5.2.5E** and **5.2.5F**), these data were collated from the two mouse groups in order to analyse the differential RANKL expression between the B cell subsets. The proportion of B1 cells expressing RANKL was greater than the proportion of B2 cells expressing RANKL. This trend was significant at six weeks, when the data from more than one experiment could be analysed (39.28 ± 3.60 % B1a, mean 38.55 ± 9.60 % B1b, 3.13 ± 0.59 % B2 MZ-like, and 15.80 ± 4.44 % B2 FO, **Figure 5.2.6B**). These results suggest that there are intrinsic differences in the expression of RANKL by different B cell subsets in mice, but these differences do not appear to be altered by infection with *P. gingivalis*.

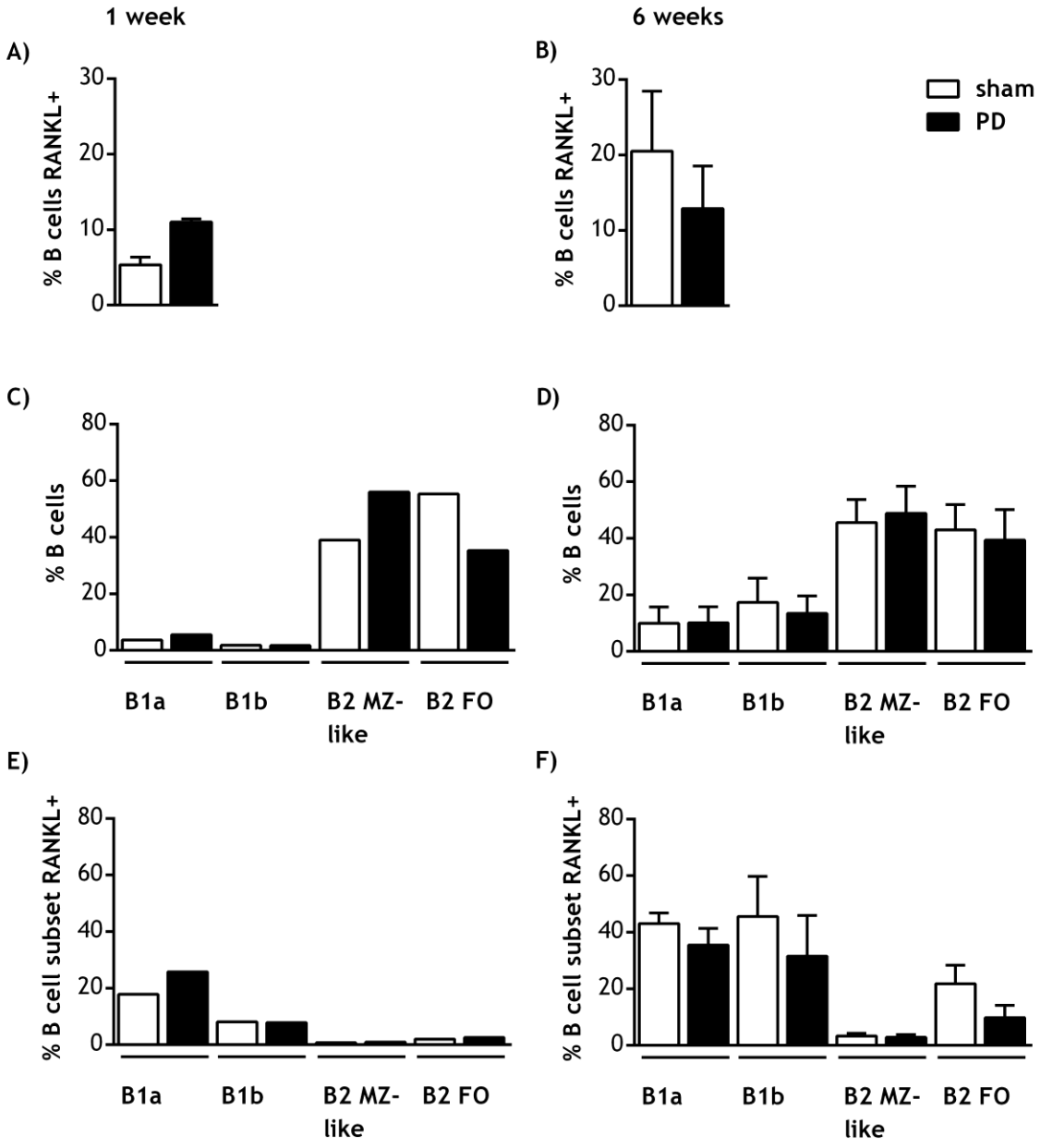

Figure 5.2.5. RANKL expression by B cell subsets in the gingiva of mice with periodontitis at 1 and 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 1 week (**A**, **C**, and **E**) and 6 weeks (**B**, **D**, and **F**) post-infection, cells were isolated from the gingiva and analysed by flow cytometry. Data are shown as mean with SEM for 3 independent experiments (**A**, **B**, **D**, and **F**) or are representative of one experiment (**C** and **E**). Gingiva were pooled from 5 mice/group in each experiment. Data were analysed by Mann-Whitney U test and no significant differences were found. Data were contributed to by Dr *Jennifer Malcolm*.

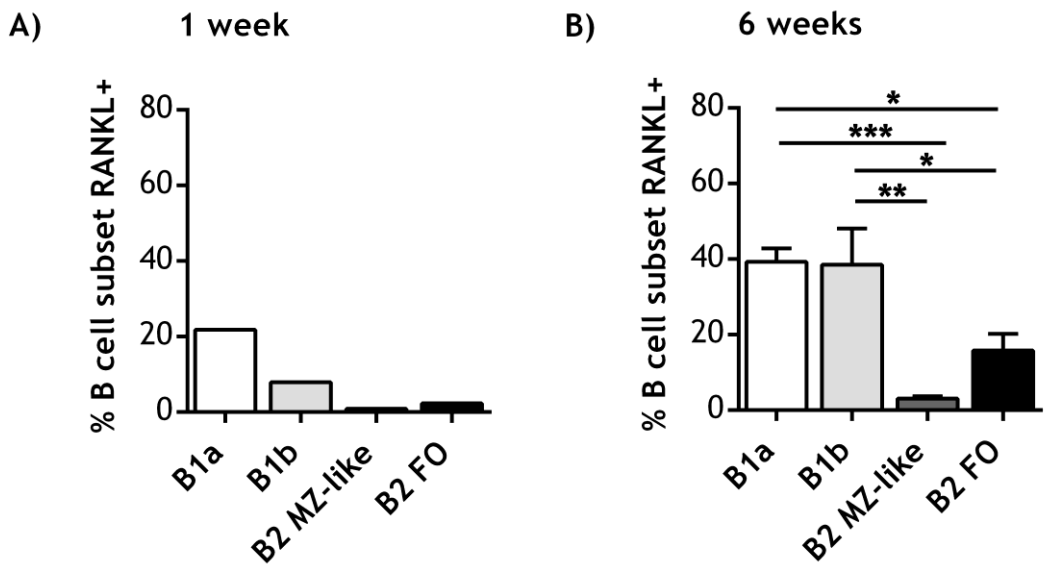

Figure 5.2.6. Differential RANKL expression by B cell subsets in murine gingiva at 1 and 6 weeks. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 1 week (**A**) and 6 weeks (**B**) post-infection, cells were isolated from the gingiva and analysed by flow cytometry. Data from mice with PD and sham control mice have been collated and are shown as mean from 1 experiment in (**A**) and mean with SEM for 3 independent experiments in (**B**). Gingiva were pooled from 5 mice/group in each experiment, giving 2 data-points per experiment. Significant differences in (**B**) were determined by One-Way ANOVA with a Tukey post-hoc test (*P < 0.05, **P < 0.01, ***P < 0.001).

To further investigate whether there were any changes in B cell phenotype as a result of *P. gingivalis* infection, the four superficial cervical LNs were harvested from mice with PD and sham controls at one and six weeks post-infection. It is presumed that in mice, antigen delivered to the oral cavity drains to these LNs which are hence referred to as the dLNs, as it has been shown that OVA delivered sublingually causes the proliferation of OVA-specific T cells in these LNs (Yamazaki et al., 2012). In various other models of bacterial infection or antigen administration, the adaptive immune response is characterised by the activation and proliferation of B cells and their differentiation into GC B cells in the dLNs (Garside et al., 1998, Adams et al., 2003, Maglione et al., 2007). Therefore, total lymphocyte counts were performed, and the proportions of B cells relative to the total lymphocyte population, and the proportion of B cells that had differentiated into GC B cells in the dLNs were analysed by flow cytometry (as described in **Chapter 2**, sections **2.10.2** and **2.15.2**).

Mice with PD exhibited small increases in the numbers and proportions of B cells in the dLNs, which were significant at six weeks post-infection (650,800 ± 173,143 sham vs. 1,551,000 ± 271,777 PD, *P = 0.0235, **Figure 5.2.7B**; mean 20.72 ± 0.89 % sham vs. 23.22 ± 0.20 % PD, *P = 0.0258, **Figure 5.2.7D**). The percentage of B cells that were GC B cells was significantly increased at one week post-infection (2.03 ± 0.47 % sham vs. 6.90 ± 1.30 % PD, **P = 0.0079, **Figure 5.2.7C**), and a moderate increase was sustained at six weeks post-infection (3.35 ± 0.35 % sham vs. 6.89 ± 1.52 % PD, P = 0.0523, **Figure 5.2.7D**). The increased proportion of B cells that were GC B cells in the dLNs of mice with PD compared with sham controls confirmed that there was increased differentiation of B cells into GC B cells in this location as a consequence of infection with *P. gingivalis*.

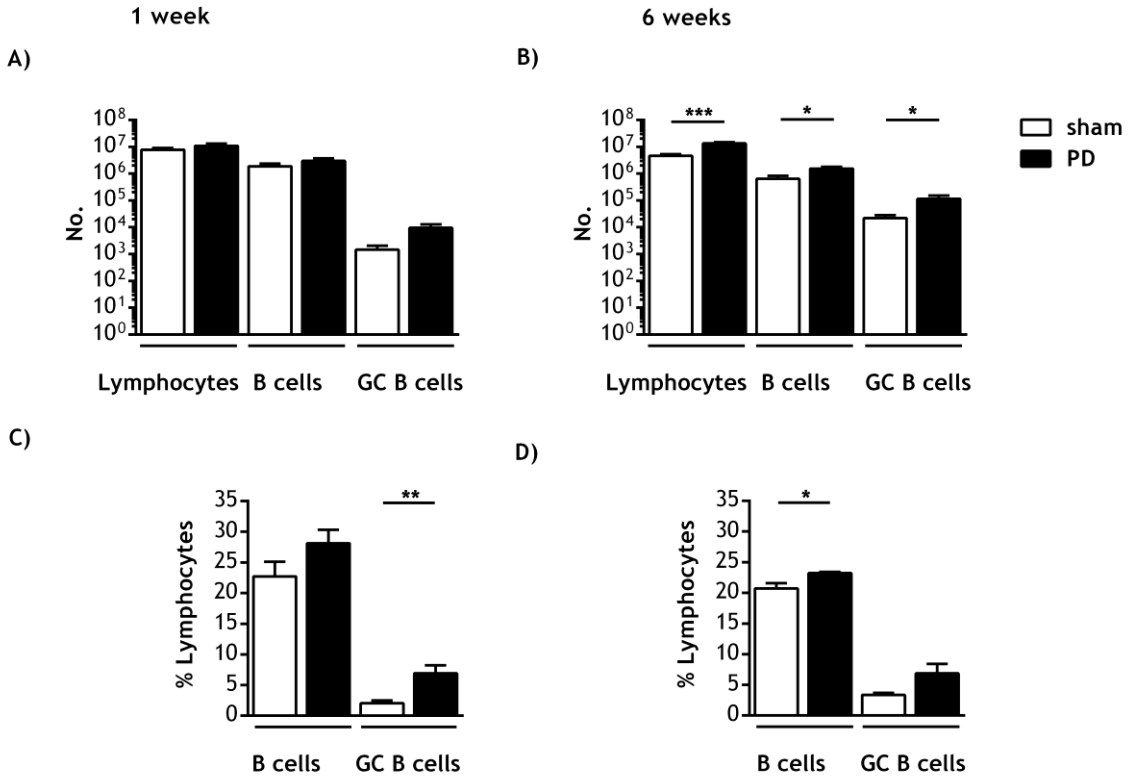

Figure 5.2.7. B cells in the dLNs of mice with PD at 1 and 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 1 week (**A** and **C**) and 6 weeks (**B** and **D**) post-infection cells were isolated from the dLNs and analysed by flow cytometry. Data are shown as mean with SEM for 1 experiment at both time-points, n = 5 mice/group in each experiment. At 6 weeks post-infection data are representative of a total of 3 independent experiments. Significant differences were determined by unpaired t test (*P < 0.05, **P < 0.01, ***P < 0.001). Data were contributed to by *John Butcher*.

B cell RANKL expression was measured in the dLNs by flow cytometry. Differential RANKL expression by the mature B cell subsets (B1a, B1b, B2 MZ-like, and B2 FO), and by GC B cells was analysed. At six weeks post-infection, the proportion of B cells expressing RANKL was slightly increased in the dLNs, but this did not reach statistical significance (5.09 ± 0.70 % sham vs. 7.04 ± 1.50 % PD, P = 0.2513, **Figure 5.2.8B**). As the total number of B cells was increased in the dLNs of these mice, absolute numbers of RANKL expressing B cells were increased in the dLNs of mice with PD. This trend was significant at one week post-infection (107,919 ± 19,654 sham vs. 180,586 ± 17,905 PD, *P = 0.0137, **Figure 5.2.8C**), but whether this represents a meaningful biological difference in the availability of RANKL requires further investigation.

B2 FO cells were the majority B cell subset in the dLNs (**Figure 5.2.8E** and **Figure 5.2.8F**). The composition of B cell subsets in the dLNs was unaffected by PD (**Figure 5.2.8E** and **Figure 5.2.8F**). Mature B cell subsets in the dLNs exhibited differential RANKL expression, but this was also unaffected by PD (**Figure 5.2.8G** and **Figure 5.2.8H**). Data were therefore collated from the two mouse groups in order to increase the statistical power for analysis. The proportion of B1 cells expressing RANKL was significantly greater than the proportion of B2 cells expressing RANKL (12.16 ± 1.08 % B1a, 11.54 ± 0.75 % B1b, 4.86 ± 0.80 % B2 MZ-like, and 3.89 ± 0.28 % B2 FO, one week, **Figure 5.2.9A;** 10.47 ± 0.55 % B1a, 10.98 ± 0.45 % B1b, 2.82 ± 0.35 % B2 MZ-like, and 5.29 ± 0.25 % B2 FO, six weeks, **Figure 5.2.9B**). This pattern in RANKL expression across these four mature B cell subsets in the dLNs is similar to that observed in the gingiva.

RANKL expression by GC B cells was analysed separately from RANKL expression by the four mature B cell subsets. As the GC B cell population was previously shown to change in the dLNs of mice with PD compared with sham controls (**Figure 5.2.7**), data was not collated from the two mouse groups for this. GC B cell RANKL expression was significantly greater than the average RANKL expression by B cells (7.52 ± 0.61 % sham B cells vs. 17.28 ± 1.40 % sham GC B cells, ***P = 0.0002; 14.22 ± 1.70 % PD B cells vs. 19.06 ± 0.81 % PD GC B cells, *P = 0.0333 **Figure 5.2.9C**). There was no significant difference in GC B cell RANKL expression in the dLNs of mice with PD compared with sham controls.

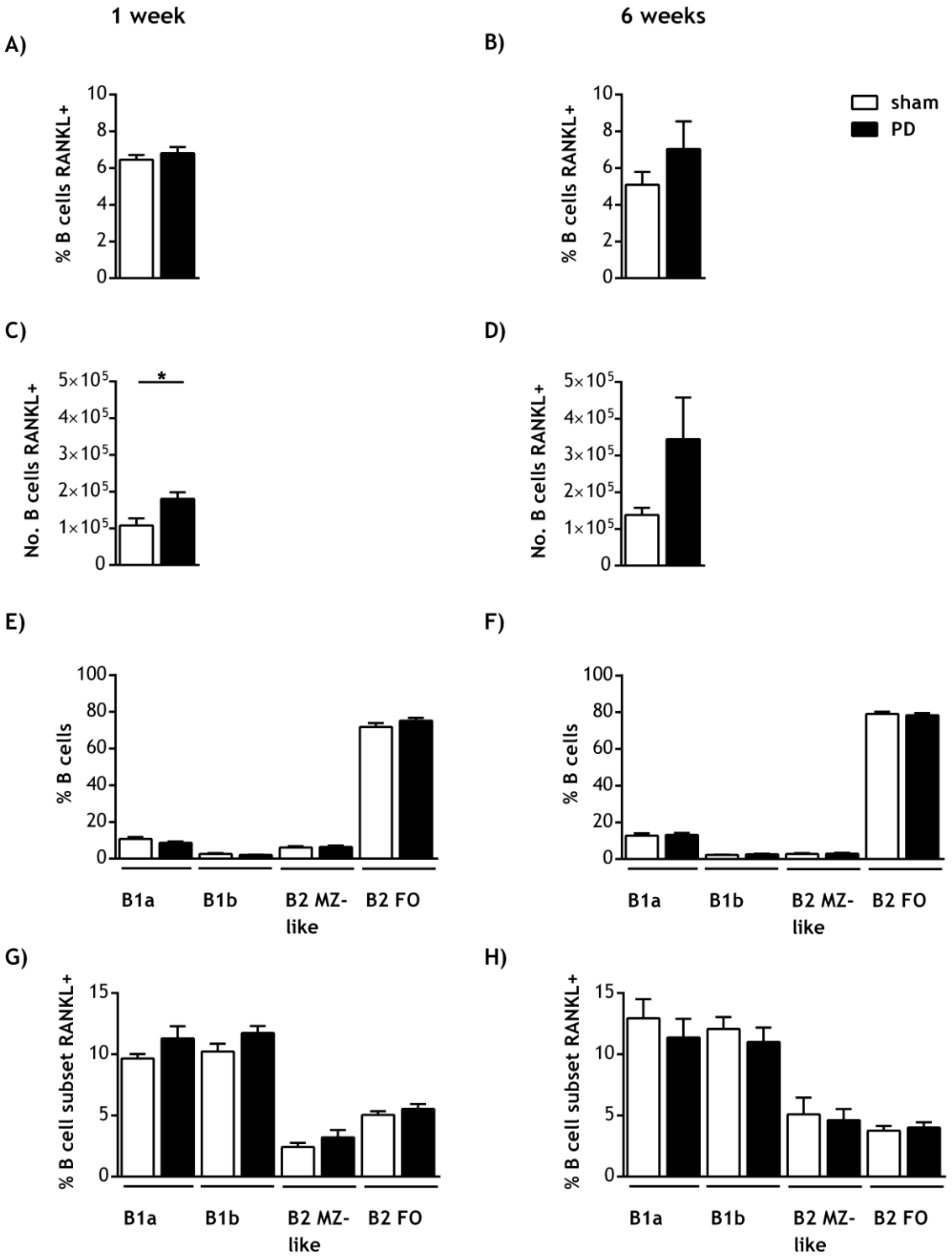

Figure 5.2.8 RANKL expression by B cell subsets in the dLNs of mice with PD at 1 and 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 1 week (**A**, **C**, **E**, and **G**) and 6 weeks (**B**, **D**, **F**, and **H**) post-infection cells were isolated from the dLNs and analysed by flow cytometry. Data are shown as mean with SEM for 2 independent experiments at 1 week or 3 independent experiments at 6 weeks, n = 5 mice/group in each experiment. Significant differences were determined by unpaired t test (*P < 0.05).

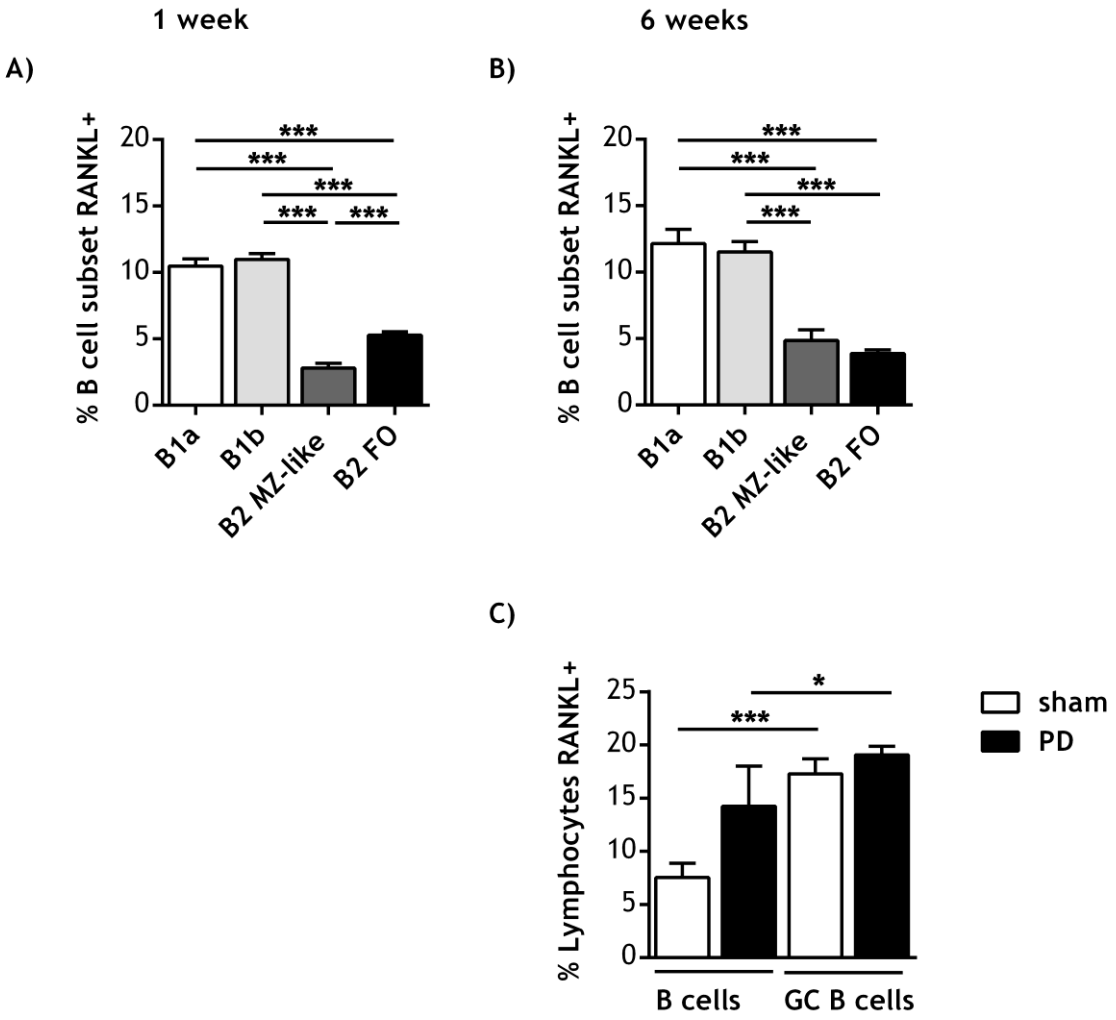

Figure 5.2.9. Differential RANKL expression by B cell subsets in dLNs at 1 and 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 1 week (**A**) and 6 weeks (**B**) post-infection, cells were isolated from the dLNs and analysed by flow cytometry. Data from mice with PD and control mice have been collated in **A)** and **B)**. Data are shown as mean with SEM of 1 experiment in **A**) and **C)** and for 3 independent experiments in **B**), n = 5 mice/group. Significant differences in **A)** and **B)** were determined by One-Way ANOVA with a Tukey post-hoc test, and significant differences in **C)** were determined by unpaired t test (*P < 0.05, **P < 0.01, ***P < 0.001).

RANKL can be expressed in three different isoforms, one is the membrane-bound form, the other two are soluble, secreted forms (Ikeda et al., 2001, Suzuki et al., 2004, Walsh et al., 2013). The membrane-bound form may also be cleaved from the cell surface by MMPs and shed (Lynch et al., 2005). Therefore, an assessment of cell surface RANKL by flow cytometry alone does not provide a complete picture of B cell contribution to RANKL production in PD. To determine if there were any changes in sRANKL production in the gingiva and dLNs, gingival cells and dLN cells were isolated from mice infected with *P. gingivalis* and

controls at one and six weeks post-infection and stimulated for three days with PMA and ionomycin (as described in **Chapter 2**, sections **2.10.1**, **2.10.2**, and **2.12**). PMA and ionomycin were selected as stimulants for this assay as they act broadly and potently on a number of cell types. Heat-killed *P. gingivalis* has previously been found to be ineffective in activating dLN cells from *P. gingivalis* infected mice (data not shown). The concentration of stimulants and the length of stimulation was selected on the basis of previously published methods and preliminary experiments which indicated these conditions were optimal for the induction of sRANKL production by lymphocytes (Fionda et al., 2007). The concentrations of sRANKL and IL-6 in the culture media were measured by ELISA at the end of the stimulation (as described in **Chapter 2**, section **2.16.5**). IL-6 was measured to provide an indication of the activity level of the cells stimulated, and the potential support that was available for B cell growth and differentiation in the tissue of origin (Muraguchi et al., 1988).

Stimulated gingival cells did not produce detectable levels of sRANKL (data not shown). However, stimulated gingival cells did produce high levels of IL-6. Interestingly, IL-6 production was higher by gingival cells isolated from mice with PD compared with sham controls at one week post-infection (**Figure 5.2.10A**), and lower in cells from mice with PD compared with sham controls at six weeks post-infection (**Figure 5.2.10B**), but this experiment was only performed once with pooled samples of gingival cells making it impossible to draw any conclusions. Conversely, high levels of sRANKL and very low levels of IL-6 were detected in the media of stimulated dLN cells from both PD and control mice, but there was no difference in the production of either cytokine between these groups at either time-point (**Figure 5.2.10C - F**). Neither IL-6 nor RANKL cytokine production could be detected when the gingival cells and dLN cells were cultured for three days in media only without stimulants (data not shown). Although the exact contribution of B cells to the production of the IL-6 and sRANKL in health and PD cannot be determined from this study, the data shows that there was no change in total sRANKL potentially available in the gingiva or dLNs.

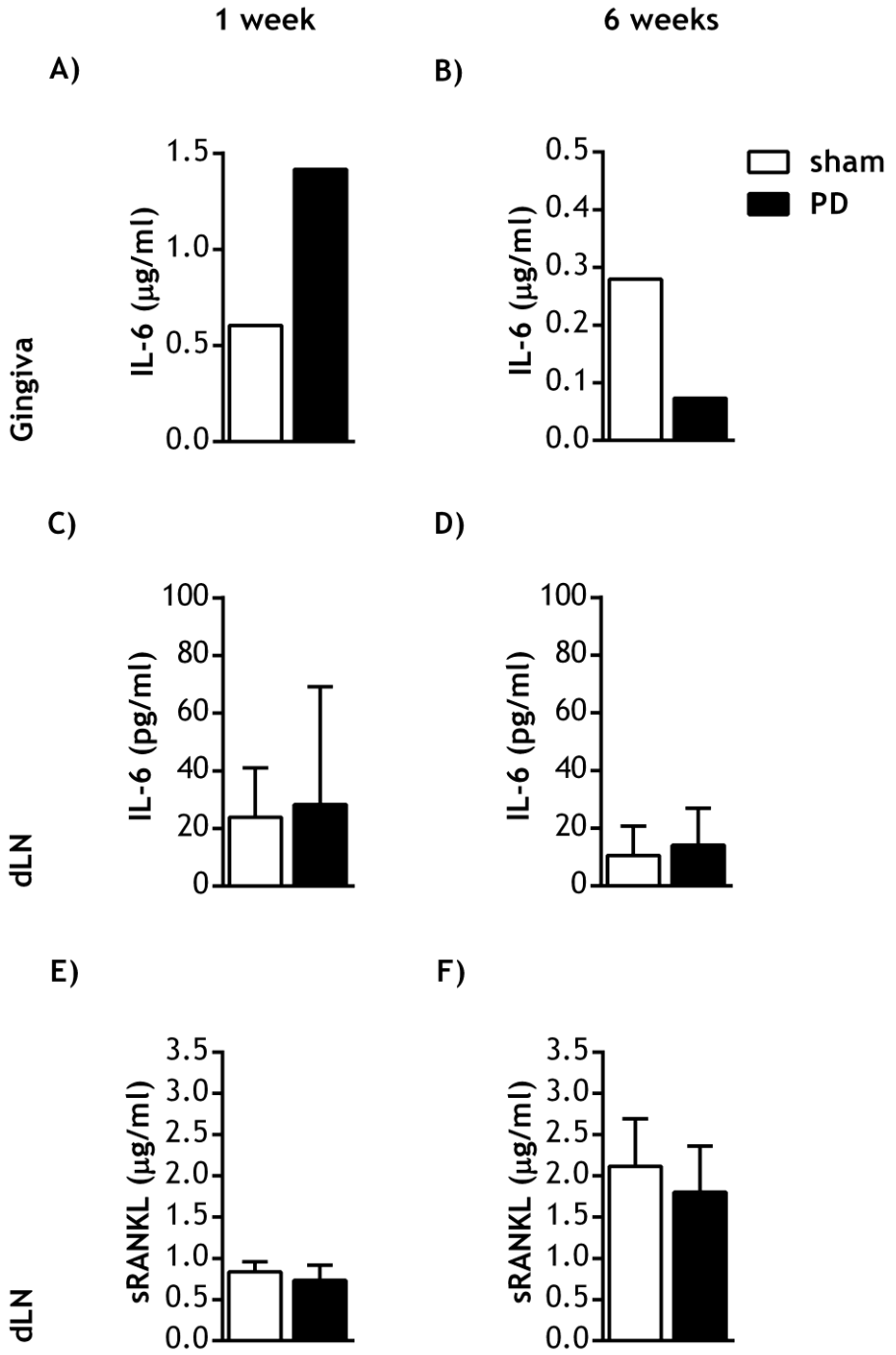

Figure 5.2.10. *In vitro* cytokine production by gingiva and draining lymph node cells from mice with periodontitis at 1 and 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 1 week (**A**, **C**, and **E**) and 6 weeks (**B**, **D**, and **F**) post-infection, cells were isolated from the gingiva (**A** and **B**) and dLNs (**C**, **D, E**, and **F**) and stimulated for three days with PMA (10 ng/ml) and ionomycin (500 ng/ml). At the end of the culture the concentration of sRANKL and IL-6 in the media was measured by ELISA. Gingival cells were pooled from 5 mice/group, whilst dLN cells from each mouse were stimulated separately, n = 5 mice/group. In the supernatants of gingival cell cultures, sRANKL could not be detected. Data shown are mean with SEM for 1 experiment (**A**, **B**, **C**, and **E**) or 2 experiments (**D** and **F**). Data were analysed by unpaired t test and no significant differences were found.

In vitro studies of B cells were carried out to investigate whether B cells from mice infected with *P. gingivalis* respond differently to stimulation compared with B cells from controls. It was anticipated that the nature of the response of B cells to *in vitro* stimulation could be amplified in B cells isolated from mice infected with *P. gingivalis*.

Splenic B cells were selected for the purpose of this investigation for several reasons. Of greatest significance is that inflammatory changes in the spleen have previously been reported in variations of this murine model of PD. In one study, splenocytes harvested from *P. gingivalis* infected mice, and stimulated with *P. gingivalis* antigen for six days, secreted more IFN_{γ} compared with stimulated splenocytes from naïve mice (Aoki-Nonaka et al., 2014). In another study, splenocytes harvested from *P. gingivalis* infected mice, and stimulated with PMA and ionomycin for two days, secreted less IL-10 and more IL-4 compared with stimulated splenocytes from naïve mice (Marchesan et al., 2012). The cellular sources of these cytokines have not been identified, but it has been speculated that T cells are involved in their production. Investigating differences in the behaviour of both T cells and B cells, in secondary lymphoid tissues is a fundamental step in investigating the potential immunological link between PD and systemic inflammatory disease. It has been indicated that the B cell composition of the spleen is altered in mice six weeks post-infection with *P. gingivalis*, with an increase in $CD5^{+}$ B cells reported (Marchesan et al., 2012).

In this study, B cells were purified from the spleens of *P. gingivalis* infected mice and sham controls and stimulated *in vitro*. B cells were harvested at six weeks post-infection to investigate the potential long-term systemic side-effects of murine PD. B cells were cultured with media only, anti-CD40 antibody, *P. gingivalis* LPS, *E. coli* LPS, or a combination of these for four days (as described in **Chapter 2**, sections **2.10.2**, **2.11**, and **2.13**). Proliferation, activation, regulation, and differentiation of B cells into plasma cells was assessed by analysing the expression of Ki67, CD69, CD86, CD22, and CD138 respectively, using flow cytometry (as described in **Chapter 2**, section **2.15.2**). The concentrations of sRANKL, IL-6, and IL-10 in the culture media were measured by ELISA at the end of this stimulation (as described in **Chapter 2**, section **2.16.5**).

P. gingivalis LPS was used in this assay rather than whole heat-killed *P. gingivalis* bacteria because the concentration and purity of *P. gingivalis* LPS could be controlled with greater accuracy. *P. gingivalis* LPS is biochemically distinct from other bacterial LPS and it differentially signals through TLRs, activating TLR2 but having both agonistic and antagonistic effects via TLR4 (Dixon and Darveau, 2005, Diya et al., 2008, Herath et al., 2011, Nebel et al., 2012, Herath et al., 2013). As B cells express both TLR2 and TLR4, they have the capacity to respond to both *P. gingivalis* LPS and *E. coli* LPS and it has been previously demonstrated that *E. coli* LPS induces proliferation and activation of naïve B cells (Genestier et al., 2007, Gururajan et al., 2007, Rubtsov et al., 2008). In addition to establishing whether interaction of B cells with *P. gingivalis* LPS could promote or inhibit the B cell functions measured, culture conditions were set up to test if *P. gingivalis* LPS could oppose or enhance the effect of *E. coli* LPS as a result of differential TLR signalling.

Culture conditions with *E. coli* LPS significantly increased the proliferation, activation, and IL-6 production of B cells relative to corresponding conditions with *P. gingivalis* LPS (**Figure 5.2.11A-C**, **Figure 5.2.12A**, and comparisons 2 and 5 in **Table 5.2.2** and **Table 5.2.3**). *P. gingivalis* LPS had minimal effects on B cell function and did not antagonise the effects of *E. coli* LPS (**Figure 5.2.11, Figure 5.2.12**, and comparisons 3 and 6 in **Table 5.2.2** and **Table 5.2.3**). Splenic B cells from mice infected with *P. gingivalis* generally did not respond very differently to those isolated from sham controls. However, the concentration of IL-6 in the culture media tended to be higher across all culture conditions with B cells from mice with PD, and was significantly greater in conditions where *E. coli* LPS was present (**Figure 5.2.12A** and comparisons 1 – 7, **Table 5.2.4**). In contrast, sRANKL was undetectable in all conditions (data not shown).

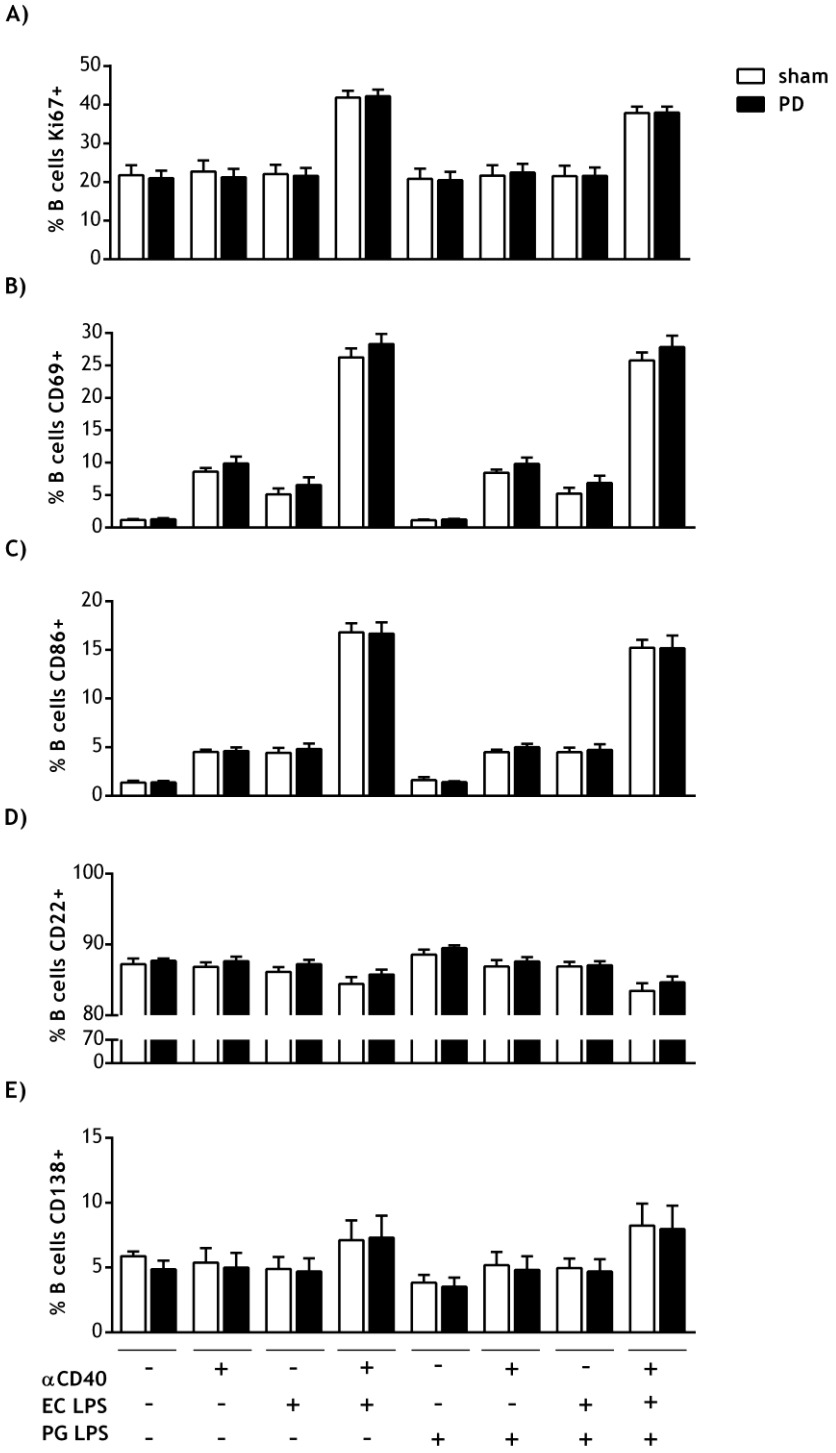

Figure 5.2.11. Phenotype of splenic B cells from mice with periodontitis after 4 day *in vitro* stimulation. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 6 weeks post-infection, B cells were purified (> 85 % $B220^+$) from the spleens and cultured for 4 days with or without anti-CD40 (αCD40), *E. coli* (EC) LPS, *P. gingivalis* (PG) LPS singly or in combination. At the end of the culture B cells were analysed by flow cytometry. Data shown are mean with SEM for 2 independent experiments, n = 5 mice/group. Significant differences between culture conditions within a mouse group were determined by One-Way ANOVA with a Tukey post-hoc test (**Table 5.2.2** and **Table 5.2.3**). Comparisons between mouse groups were made by an unpaired t test and no significant differences were found (**Table 5.2.4**).

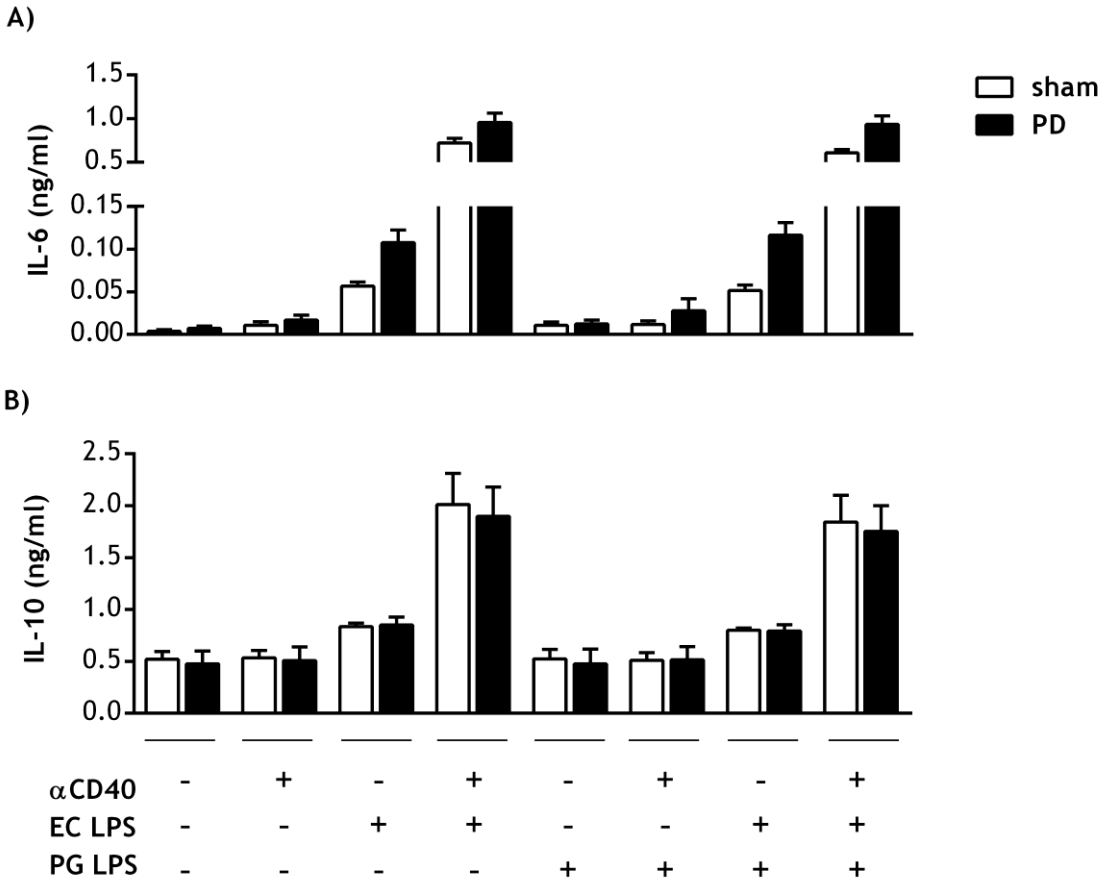

Figure 5.2.12. Cytokine production by splenic B cells from mice with periodontitis after 4 day *in vitro* stimulation. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 6 weeks post-infection, B cells were purified (> 85 % $B220^+$) from the spleens and cultured for 4 days with or without anti-CD40 (αCD40), *E. coli* (EC) LPS, *P. gingivalis* (PG) LPS singly or in combination. At the end of the culture the concentration of IL-6 (**A** and **B**) and IL-10 (**C** and **D**) in the media was measured by ELISA. Data shown are mean with SEM for 2 independent experiments, n = 5 mice/group. Significant differences between culture conditions within a mouse group were determined by One-Way ANOVA with a Tukey post-hoc test (**Table 5.1** and **Table 5.2**). Significant differences between mouse groups were made by an unpaired t test (**Table 5.3**).

Comparison	Ki67		CD69		CD86		CD22		CD138		IL-6		IL-10	
1 A) PG LPS vs. **B)** media	ns	=	ns	=	ns	=	ns	↑	ns	=	ns	↑	ns	=
2 A) PG LPS vs. **B)** EC LPS	ns	=	*	↓	*	↓	ns	↑	ns	↓	ns	↓	ns	↓
3 A) PG LPS + EC LPS vs. **B)** EC LPS	ns	=	ns	=	ns	=	ns	↑	ns	=	ns	=	ns	↓
4 A) PG LPS + αCD40 vs. **B)** αCD40	ns	=	ns	=	ns	=	ns	=	ns	=	ns	=	ns	=
5 A) PG LPS + αCD40 vs. **B)** EC LPS + αCD40	***	↓	***	↓	***	↓	ns	↑	ns	↓	***	↓	***	↓
6 A) PG LPS + EC LPS + αCD40 vs. **B)** EC LPS + αCD40	ns	↓	ns	=	ns	↓	ns	↓	ns	↑	*	↓	ns	↓

Table 5.2.2. Summary of the effect of different stimulants on splenic B cells from sham-infected mice. B cells were purified (> 85 % $B220^+$) from the spleens of sham-infected mice and cultured for 4 days with or without anti-CD40 (αCD40), *E. coli* (EC) LPS, *P. gingivalis* (PG) LPS singly or in combination. At the end of the culture the expression of Ki67, CD69, CD86, CD22, and CD138 by B cells was analysed by flow cytometry, and the concentration of IL-6 and IL-10 in the media was measured by ELISA. Comparisons (listed 1 to 6) were made between different combinations of stimuli (**A** and **B**): those including PG LPS (**A**) with those not including any LPS, or those including PG LPS with those including EC LPS instead (**B**)**.** Significant differences were determined by One-Way ANOVA with a Tukey post-hoc test (ns = not significant, *P < 0.05, **P < 0.01, ***P < 0.001). The direction of the difference between culture conditions has been indicated (= where **A)** is equal to **B)**,↑ where **A)** is greater than **B)**, ↓ where **A)** is less than **B)**).

Comparison	Ki67		CD69		CD86		CD22		CD138		IL-6		IL-10	
1 A) PG LPS vs. **B)** media	ns	=	ns	=	ns	=	ns	=	ns	=	ns	=	ns	=
2 A) PG LPS vs. **B)** EC LPS	ns	=	***	↓	*	↓	ns	=	ns	=	ns	=	ns	=
3 A) PG LPS + EC LPS vs. **B)** EC LPS	ns	=	ns	=	ns	=	ns	=	ns	=	ns	=	ns	=
4 A) PG LPS + αCD40 vs. **B)** αCD40	ns	=	ns	=	ns	=	ns	=	ns	=	ns	=	ns	=
5 A) PG LPS + αCD40 vs. **B)** EC LPS + αCD40	***	↓	***	↓	***	↓	ns	=	ns	=	***	↓	***	↓
6 A) PG LPS + EC LPS + αCD40 vs. **B)** EC LPS + αCD40	ns	=	ns	=	ns	=	ns	=	ns	=	ns	=	ns	=

Table 5.2.3. Summary of the effect of different stimulants on splenic B cells from mice with periodontitis. B cells were purified (> 85 % $B220^+$) from the spleens of mice infected with *P. gingivalis* (PD) and cultured for 4 days with or without anti-CD40 (αCD40), *E. coli* (EC) LPS, *P. gingivalis* (PG) LPS singly or in combination. At the end of the culture the expression of Ki67, CD69, CD86, CD22, and CD138 by B cells was analysed by flow cytometry, and the concentration of IL-6 and IL-10 in the media was measured by ELISA. Comparisons (listed 1 to 6) were made between different combinations of stimuli (**A** and **B**): those including PG LPS (**A**) with those not including any LPS, or those including PG LPS with those including EC LPS instead (**B**)**.** Significant differences were determined by One-Way ANOVA with a Tukey post-hoc test (ns = not significant, *P < 0.05, **P < 0.01, ***P < 0.001). The direction of the difference between culture conditions has been indicated (= where **A)** is equal to **B)**,↑ where **A)** is greater than **B)**, ↓ where **A)** is less than **B)**).

Condition	CD69		IL-6	
1 αCD40	ns, P = 0.299	=	ns, P = 0.425	=
2 PG LPS	ns, P = 0.506	=	ns, P = 0.764	=
3 EC LPS	ns, P = 0.33	=	**P = 0.0042	↑
4 PG LPS + EC LPS	ns, P = 0.25	=	***P = 0.0008	↑
5 PG LPS + αCD40	ns, P = 0.229	=	ns, P = 0.225	=
6 EC LPS + αCD40	ns, P = 0.331	=	ns, P = 0.0599	=
7 PG LPS + EC LPS + αCD40	ns, P = 0.353	=	**P = 0.0054	↑

Table 5.2.4. Summary of differences between stimulated splenic B cells from mice with periodontitis and sham controls. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 6 weeks post-infection, B cells were purified (> 85 % $B220^+$) from the spleens and cultured for 4 days with or without anti-CD40 (αCD40), *E. coli* (EC) LPS, *P. gingivalis* (PG) LPS singly or in combination. At the end of the culture the expression of CD69 by B cells was analysed by flow cytometry, and the concentration of IL-6 in the media was measured by ELISA. Comparisons were made between B cells from different mouse groups that were stimulated in the same conditions (listed 1 to 7). Significant differences were determined by unpaired t test (ns = not significant, **P < 0.01, ***P < 0.001). The trend difference between mouse groups has been indicated (= where PD is equal to sham,↑ where PD is greater than sham, ↓ where PD is less than sham).

The results so far have mainly characterised the nature of the local and regional B cell response to infection with *P. gingivalis* in our murine model. Several studies of PD patients suggest that PD is linked to dysregulation of circulating immune cells, and therefore PD potentially affects parts of the body far removed from the site of infection in the oral cavity (Loos et al., 2000, Berglundh et al., 2002b). To investigate whether murine PD had an impact on the systemic B cell populations at the latter stage of disease, B cell populations in the blood and peritoneal fluid of mice infected with *P. gingivalis* and sham controls were analysed by flow cytometry at six weeks post-infection (as described in **Chapter 2**, sections **2.10.4**, **2.10.5**, and **2.15.2**). The total proportion of B cells and the composition of subsets in these compartments were similar in mice with PD compared with sham controls, except for a small increase in the ratio of B2 MZ-like cells to B2 FO cells in the peritoneal fluid (**Figure 5.2.13**). This suggests that PD does not have a systemic impact on B cell populations in the murine model at this time-point.

It is possible that there are other aspects of B cell phenotype which are altered in murine PD that have not been assessed here. The possibility that any aberrations in B cell phenotype associated with murine PD leads to the production of autoantibodies was explored. Serum was collected from mice with PD and sham controls at one, two, and six weeks post-infection, and titers of anti-dsDNA, anti-type II collagen, and RF autoantibodies were measured by ELISA (as described in **Chapter 2**, section **2.16.6**). No significant differences in any of these autoantibody titers between the mouse groups were observed at one week or six weeks post-infection. At two weeks, there was a significant increase in RF titers in mice with PD relative to sham controls (272.4 ± 40.3 EU PD vs. 154.5 ± 8.2 EU sham, *P = 0.021, **Figure 5.2.14F**) and this was accompanied by a small, but insignificant increase in titers of anti-collagen type II IgG antibodies (144.0 ± 13.5 EU PD vs. 110.1 ± 9.8 EU sham, P = 0.077, **Figure 5.2.14E**). Sera from mice with CIA were included in the assays for comparison, and this contained significantly higher titers of all the autoantibodies measured (data not shown). This suggests that murine PD is not associated with a generalised autoimmune response that is characteristic of murine models of RA.

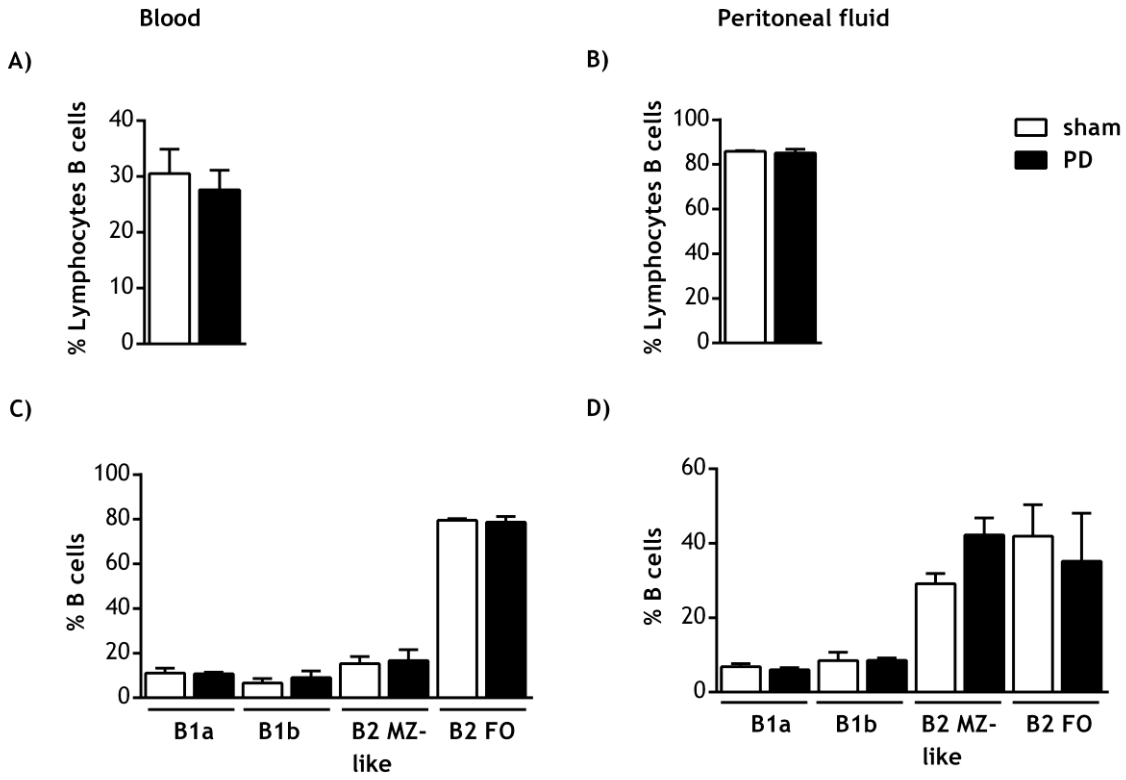

Figure 5.2.13. B cell subsets in blood and peritoneal fluid of mice with periodontitis at 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 6 weeks post-infection, cells were isolated from the blood (**A** and **C**) and peritoneal fluid (**B** and **D**) and analysed by flow cytometry. Data shown are shown as mean with SEM for 3 independent experiments. Samples of blood and peritoneal fluid were pooled from 5 mice/group in each experiment. Data were analysed by Mann-Whitney U test and no significant differences were found.

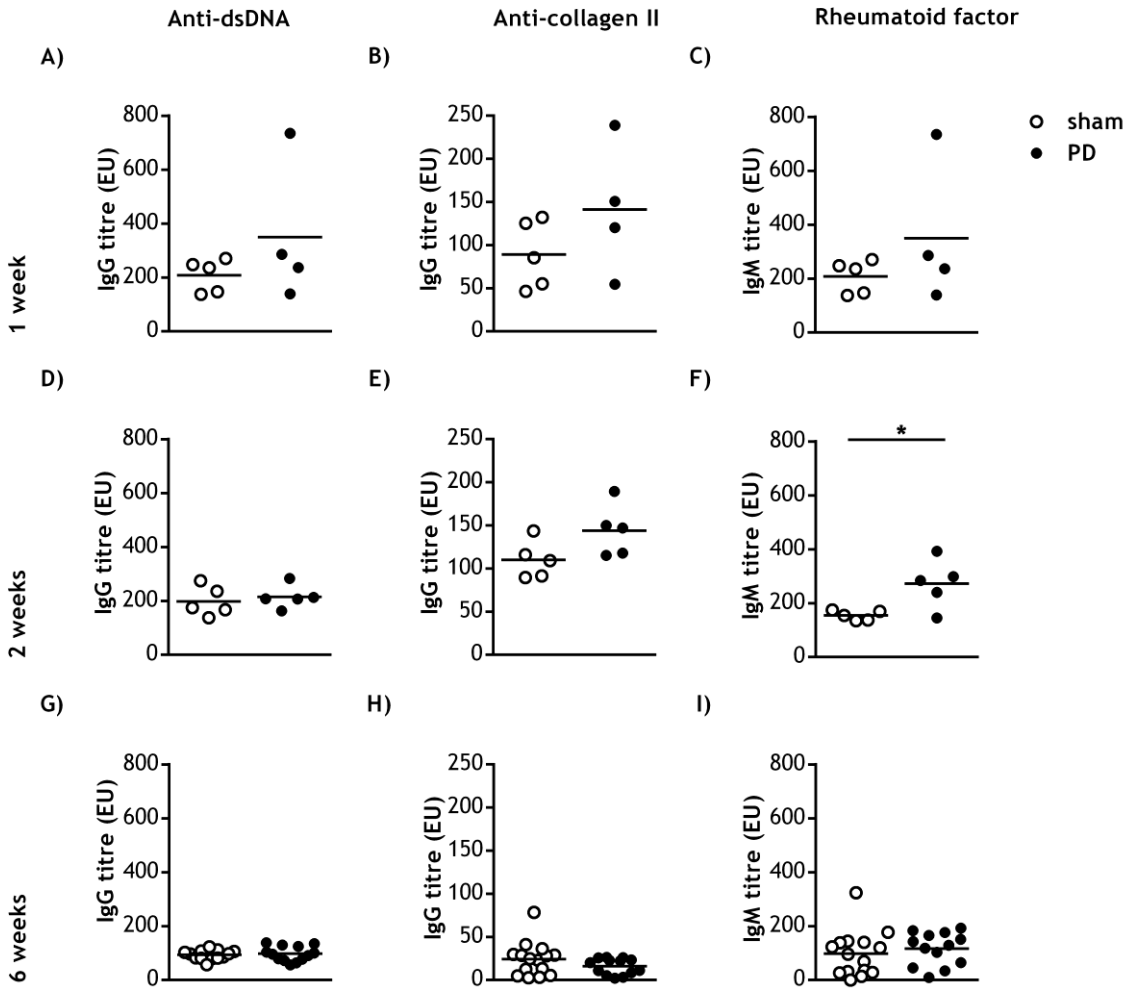

Figure 5.2.14. Serum autoantibody titers in mice with periodontitis at 1, 2, and 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 1, 2, and 6 weeks PI, serum samples were collected and anti-dsDNA IgG, anti-collagen type II IgG, and anti-IgG IgM (RF) antibody titers were measured by ELISA. Data are shown as EU for individual mice (symbols) and mean EU for each group of mice (lines). Data are from 1 experiment at 1 and 2 weeks PI, and from 3 experiments at 6 weeks PI, n = 4-5 mice/group. Significant differences were determined by unpaired t test (*P < 0.05).

Summary of main results:

- The number and percentage of B cells was increased in the dLNs of mice with PD compared with sham controls at six weeks post-infection
- The percentage of GC B cells was increased in the dLNs of mice with PD compared with sham controls at one week post-infection
- RANKL was differentially expressed by the mature B cell subsets (B1a, B1b, B2 MZ-like, and B2 FO) in the gingiva and dLNs of mice but this was unaffected by PD status

5.3 Discussion

Current PD treatment focuses on reducing the bacterial burden in PD patients. Whilst, undoubtedly successful for many patients, partial treatment success and disease recurrence are common, hence a better understanding of the host immune response in PD may yield improvements (Kinane et al., 2011). B cells are abundant in the inflammatory lesions in the gingiva of PD patients and therefore might offer a therapeutic target (Berglundh and Donati, 2005). However, the specific roles of B cells in PD pathogenesis are not fully understood. Extensive analysis of gingival B cell phenotype in PD patients and healthy individuals is difficult due to sample limitations, discussed in **Chapter 3**. Moreover, localised changes in B cells at the initial stages of microbial dysbiosis following *P. gingivalis* infection cannot be characterised in human disease as patients inevitably present following several years of microbial dysbiosis.

Employing a murine model of PD has enabled some of these hurdles to be overcome, but has also presented new challenges. The major advantage of the model was that it relieved many of the restrictions relating to collection of tissue samples. This enabled gingival dissection to be relatively standardised and provided temporal control over sample collection. Repeated experiments could be made at the same stage of disease in infected mice, and the same types of tissue samples could also be taken from healthy, age-matched animals for comparison.

In the murine model used here, *P. gingivalis* was introduced by oral gavage, as this is assumed to be the natural route by which humans would encounter the bacteria. This assumption is based on evidence of its niche requirements for oxygen and nutrients within the oral cavity, its intricate relationships with other oral bacteria, and epidemiological data which implies oral *P. gingivalis* is horizontally transmitted human-to-human (van Steenbergen et al., 1993, Tuite-McDonnell et al., 1997, Asano et al., 2003, Rijnsburger et al., 2007). Although *P. gingivalis* commonly colonises the mouths of humans, it has been noted that *P. gingivalis* is not very good at colonising the oral cavity of rodents, which are not natural hosts. Hence in this murine model the commensal oral bacteria were depleted by antibiotic treatment prior to infection with *P. gingivalis*. Attempts

to induce PD by oral infection with *P. gingivalis* without prior antibiotic treatment did not cause significant alveolar bone loss relative to sham controls (personal communication with Dr John Butcher, University of Glasgow). Antibiotic treatment eliminates any baseline differences in the oral microbiota between mouse groups, and possibly reduces the competition for *P. gingivalis* to colonise. *P. gingivalis* was also delivered in the vehicle of a viscous carbohydrate mixture (2 % CMC in PBS), to help it to adhere to the tooth surface.

Systemic antibiotics disrupt the normal relationship between commensal bacteria and the immune system. This has been well studied in the gut, where commensal bacteria are known to continuously engage with and 'educate' the immune system. For example, a close relationship exists between Gram negative bacteria of the phylum SFB found in the gut and Th17 cell responses. Removal of SFB from the gut through the use of antibiotics or breeding in GF conditions significantly reduces the differentiation of Th17 cells in the lamina propria, and this is associated with a relative increase in the proportion of Tregs (Ivanov et al., 2008). Similarly, depletion of the commensal oral bacteria by antibiotics could be detrimental to the differentiation and maintenance of T cell populations in the oral mucosa. This could have repercussions on the ability of the cells to mount an adaptive immune response to subsequent infection with *P. gingivalis*.

In recent years, the reliance of *P. gingivalis* on other species of bacteria for colonisation of the oral cavity has been unravelled at the molecular level and we have a better understanding of the mechanisms involved (Lamont et al., 2002, Kuboniwa and Lamont, 2010). It has been demonstrated that *P. gingivalis* ATCC 33227 is absolutely dependent on adherence to *Streptococcus gordonii* to cause alveolar bone loss in the murine model of PD (Daep et al., 2011). This, and the work of others, has informed adaptations of the oral inoculation method of inducing murine PD, whereby *P. gingivalis* is delivered in combination with, or shortly after, delivery of a bacterium known to commonly co-inhabit with *P. gingivalis* in the oral cavity - such as *A. actinomycetemcomitans* or *F. nucleatum* (Chen et al., 1996, Polak et al., 2009). However, not all combinations of *P. gingivalis* with other PD-associated bacteria have a synergistic impact on alveolar bone loss. An independent investigation found no significant change in

the disease outcome following dual infection with *P. gingivalis* W83 and *P. intermedia* compared to infection with *P. gingivalis* alone (personal communication with Dr John Butcher, unpublished data, University of Glasgow). Co-infecting mice with *P. gingivalis* and another species of bacteria could actually hinder the ability of this strain to colonise if it is unable to adhere to the other species as there would be increased competition for binding directly to the tooth surface. The interactions of *P. gingivalis* with other oral bacteria can be strain-dependent. *P. gingivalis* W83, used throughout the studies described here can be distinguished by its possession of a polysaccharide capsule, and lack of minor fimbriae. Fimbriae are the major mechanism used by other strains, including ATCC 33227, to bind to the salivary pellicle on the tooth surface and to other species of oral bacteria in a biofilm. It is believed that *P. gingivalis* W83 is consequently better at evading phagocytosis and 'hiding' from the immune system but poorer at colonising the oral cavity compared with *P. gingivalis* ATCC 33227 (Sundqvist et al., 1991, Jotwani and Cutler, 2004, Bostanci and Belibasakis, 2012). Other mechanisms of adherence exist, but are less well characterised. Adhesins are thought to be released in vesicles from *P. gingivalis* which help it to co-aggregate with *P. intermedia in vitro*, but these may not have a significant role *in vivo* (Kamaguch et al., 2001, Kamaguchi et al., 2003).

Alternative routes of *P. gingivalis* infection include the 'airpouch model', which involves injecting *P. gingivalis* into a dorsal subcutaneous pouch (Pouliot et al., 2000), and the 'chamber model' which involves injecting *P. gingivalis* into a subcutaneous surgically implanted metal wire chamber (Genco et al., 1991). Both of these models bypass the oral mucosal barrier and the specific immunological defences associated with this, rendering them less relevant for the study of the adaptive immune response to *P. gingivalis* in PD. The advantage of these models is that they cause substantial histopathology, which can be easily studied and quantified. Consequently, the chamber model has been applied to determine the virulence of different strains of *P. gingivalis* (Graves et al., 2008).

Using the oral gavage murine model, B cell phenotype was characterised in different tissues at different time-points post-infection with *P. gingivalis* and compared with sham controls. Subtle local and regional alterations in B cells

were observed in mice with PD. Interestingly, a small trend increase in the proportion of RANKL expressing B cells – a hallmark of human disease - was observed in the gingiva at one week post-infection (Kawai et al., 2006). It has previously been demonstrated that RANKL is a critical mediator of alveolar bone loss, and that as a source of RANKL, and potentially other osteoclastogenic factors, antigen-experienced B cells can contribute to this process in rodent PD (Han et al., 2006, Yuan et al., 2011, Han et al., 2013). The data presented here have attempted to expand on these findings and for the first time, further classify B cells expressing RANKL in PD. It was found that the majority of them belonged to the B1a and B1b subsets, which constitute a small proportion of the total B cell population in these tissues. The trend in B cell RANKL expression was accompanied by an expansion of the GC B cell population in the dLNs, indicating that differentiation of dLN B cells was enhanced as a result of *P. gingivalis* infection.

Whilst observations of dLN responses in mice were consistent between experiments, achieving reproducible analyses of B cells in the gingiva was challenging. Due to the small size of the gingival tissue, and the complexity of the multi-step processing for flow cytometry, there were a limited, variable, number of cells available. A degree of caution is consequently required when interpreting the results. The analysis of gingival B cells could perhaps be improved by making a few small changes to the protocol. To begin with taking only the gingiva, which is most likely to be inflamed, rather than the gingiva and the palate, could be more revealing of the local immune response. The number of mice per group would have to be increased to compensate for the lower number of cells obtainable per mouse as a result. In subsequent steps, modifying the enzyme cocktail used for digestion and reducing the number of cell transfers between tubes during processing could help to preserve a larger number of cells. B cell phenotype could then be analysed by more sensitive methods. Viable B cells could be separated into subsets using for example a MoFlo™ XDP cell sorter (Beckman Coulter), then the DNA isolated from each subset using a method adapted for recovery from a small number of cells. Finally, a multiplex qRT-PCR could be performed to more accurately analyse the expression of receptors, co-stimulatory molecules, cytokines, RANKL, and other immunomodulators by each subset of B cells.

There was a tendency towards increased IL-6 production, specifically by B cells, in the spleens of mice with PD. Splenic B cells from mice with PD, stimulated with a mixture of *E. coli* LPS and *P. gingivalis* LPS with or without αCD40 produced significantly higher levels of IL-6 than B cells from sham controls, but otherwise exhibited a similar phenotype. These results confirm previous reports that *P. gingivalis* LPS is a poor mitogen for lymphocytes, compared with other bacterial LPS (Gemmell and Seymour, 1992, Petit and Stashenko, 1996, Dixon and Darveau, 2005, Diya et al., 2008, Herath et al., 2011, Jain et al., 2011, Nebel et al., 2012, Herath et al., 2013), which is suspected to stem from the differential interaction of the LPS with TLR2 and TLR4 (Coats et al., 2003, Herath et al., 2013, Jain et al., 2013). Differential expression of TLR2 and TLR4 by B cells relative to monocytes, macrophages, and DCs may explain how *P. gingivalis* LPS can induce a degree of activation in other APCs but apparently not in B cells (Barr et al., 2007, Jones et al., 2010, Yanagita et al., 2012).

Since direct stimulation of B cells with *P. gingivalis* LPS is insufficient to elicit an activated phenotype, the activation of B cells observed *in vivo* must be dependent on various other factors downstream of *P. gingivalis* infection such as contact and non-contact interactions with other immune cells, as well as interactions with a range of bacterial stimuli. This concept could have been further investigated by attempting to recapitulate the hypothetical series of events *in vitro*. For example, epithelial cells and innate immune cells such as neutrophils could have been cultured with a bacterial biofilm containing *P. gingivalis*, then the media from this culture removed and added to a second culture of B cells or a mixture of lymphocytes, supplemented with *P. gingivalis* LPS before analysing B cell phenotype. The indirect and direct effects of *P. gingivalis* on lymphocyte responses to antigen could have additionally been assessed by using B cells from transgenic MD4 mice which specifically recognise HEL, T cells from transgenic DO11.10 mice, which specifically recognise OVA, and creating an OVA-HEL conjugate for this second stage (Goodnow et al., 1988, Murphy et al., 1990, Adams et al., 2003). TLR signals are integrated in both TI and TD B cell responses to antigen, providing *P. gingivalis* LPS with an opportunity to influence this step (Rawlings et al., 2012). Ideally, a transgenic *P. gingivalis* strain expressing OVA or OVA-HEL would be used in this scenario, as

free antigen may be recognised and processed differently to antigen presented by a pathogen (Robson et al., 2008). Unfortunately, attempts to create a transgenic *P. gingivalis* have so far, been unsuccessful. Aside from the difficulties of generating a transgenic *P. gingivalis*, there are several other factors which complicate this *in vitro* system. Firstly, each of the components of the culture systems – the bacteria and the different mammalian cell types – have individual preferences for conditions *in vitro* to survive, and in this respect it would be difficult to establish a fair compromise. Another dilemma is that media which has been 'conditioned' by the secretions of stimulated innate cells would likely contain various inflammatory mediators but also metabolites and other molecules released from dead cells and bacteria which could be toxic to lymphocytes. The time-frame for culturing lymphocytes with conditioned media would have to be optimised to allow sufficient time for the lymphocytes to become activated but not long enough to become overwhelmed and die. As a consequence of these and other practical considerations, a large amount of preparatory experiments would be required to optimise this type of assay.

In conjunction with the data from *in vitro* stimulations of splenic B cells, the lack of significant alterations in B cell populations in blood and peritoneal fluid indicates that oral infection with *P. gingivalis* has limited systemic impact on B cell phenotype. No differences in the proportions of B cell subsets (B1a, B1b, B2 MZ-like, and B2 FO) were observed in the gingiva either, again in contrast to claims from human studies that an increase is observed in B1a cells in these sites (Berglundh et al., 2002b, Donati et al., 2009a, Donati et al., 2009b). The nuances of the human studies referred to may explain these discrepancies. There are only a handful of reports that describe the proportion of $CD5^+$ B cells in PD patients, and the majority of these reports stem from a single research group – hence the patient population used in these studies could skew the results. In some of the studies conducted there was a lack of healthy participants for comparison, which made it difficult to contextualise the findings. These issues aside, direct comparisons between human and murine studies of $CD5^+$ B cells are fundamentally impeded by the differential pattern of CD5 expression between these species. In mice, CD5 expression can be induced in activated B2 cells (Cong et al., 1991), creating the need for additional analysis of CD43 and CD23 to distinguish B1 and B2 subsets. But overall, the

majority of $CD5^+$ B cells in mice belong to the B1a subset, whereas in humans CD5 seems to be expressed more broadly across B cells and does not define a specific population (Carsetti et al., 2004). Other markers have been proposed to better identify 'innate-like' B cell populations that may be equivalent to murine B1a cells, but further research is required to confirm whether any of these truly represents the murine B1a lineage (Baumgarth, 2011). Therefore although CD5 is probably not the most appropriate marker of B1a-like B cells in humans, until suitable alternatives have been identified, it is the best option available.

Another way in which the murine model of PD differed from the human condition was in the absence of autoantibodies. A wide range of autoantibodies have been identified which are increased in the sera of PD patients relative to healthy participants, including anti-collagen antibodies and others targeting components of connective tissue (Anusaksathien et al., 1992, De-Gennaro et al., 2006, Koutouzis et al., 2009). As a result, it was once believed that PD was an autoimmune disease in its own right. The view of PD pathogenesis has since shifted slightly, although there has been renewed interest in the concept that PD could contribute to the development of autoimmune disease, particularly RA, through the generation of autoantibodies. The assessment of autoantibody titers here was not extensive, and has excluded a key class of autoantibodies which have been most closely linked to the development of RA: ACPAs (van Gaalen et al., 2004, Nielen et al., 2004, Zendman et al., 2004). Preliminary experiments carried out indicated that ACPAs could not be detected in the sera of mice with PD or sham controls using components of the commercially available assay intended for use with human samples (personal communication with Dr John Butcher, unpublished data, University of Glasgow).

It is possible that there is no evidence of a systemic impact on B cell phenotype or on the production of autoantibodies in murine PD because it simply does not progress to a chronic inflammatory state in the same way that the human disease does. This may be because the inbred mice used here possess minimal genetic susceptibility and are shielded from the environmental factors, such as smoking, that are known to contribute to human disease (Baker et al., 2000b, Haffajee and Socransky, 2001, Takahashi et al., 2001, Apatzidou et al., 2005, Petersen and Ogawa, 2005, Stabholz et al., 2010). It may be that by six weeks

post-infection, the localised inflammation in the gingiva has resolved, following the generation of an adaptive immune response, and the production of anti-*P. gingivalis* antibodies. In this murine model we are relying solely upon the immune response to *P. gingivalis* to elicit disease. In reality, humans may harbour *P. gingivalis* and not develop PD. It has been shown that up to 25 % of periodontally healthy individuals carry *P. gingivalis* (Griffen et al., 1998).

No other studies using the murine model of PD established by Baker et al. (whereby no more than three inoculations of 10^9 colony forming units of *P. gingivalis* are administered by oral gavage over a period of a week, and in total) in WT BALB/c mice have published evidence of systemic inflammation, specifically in relation to B cells. This leads to the conclusion that such publications have not been made because these parameters have not previously been studied in this model of PD, or because studies performed did not yield positive results.

The search for signs of systemic inflammation tends only to be performed when *P. gingivalis* infection is carried out in conjunction with a model of a systemic disease such as RA, diabetes, or atherosclerosis. Because the primary purpose of these types of experiments is to demonstrate whether *P. gingivalis* exacerbates systemic disease, and there is less interest in the development of PD, the way in which *P. gingivalis* is administered is often quite different from the oral gavage protocol implemented here. In studies where *P. gingivalis* is delivered orally, a much higher number of infections are performed over the time-course, ranging from 10 infections in three weeks to 42 infections in six weeks (Lalla et al., 2003, Maekawa et al., 2011). In other studies, *P. gingivalis* is delivered by an alternative route using the subcutaneous chamber model (Maresz et al., 2013) or intravenous injection (Ashigaki et al., 2013). Intravenous injection of *P. gingivalis* alone is sufficient to cause systemic inflammation, including increasing the concentration of IL-6 in serum (Akamatsu et al., 2011). Arguably, delivering *P. gingivalis* by any of these alternative routes is more artificial. The impact on systemic disease is dependent on exposure to an unusually high bacterial load, and not necessarily at a site this species would normally be encountered. In PD patients, *P. gingivalis* forms an extremely low proportion of the total dental plaque biomass and is not always detectable (Slots and Chen, 1993, Socransky et

al., 1998, Bizzarro et al., 2013). Likewise, in murine studies employing the model of PD established by Baker et al. (1994), *P. gingivalis* is rarely detectable in the oral cavity at the end-point unless highly sensitive qRT-PCR methods are used, which are able to measure in the region of hundreds of bacteria (Hajishengallis et al., 2011).

5.4 Conclusion

In the murine model of PD, subtle changes in B cell phenotype are evident in the gingiva and dLNs. It remains to be established whether B cells contribute to pathology in PD, and if so, by what mechanisms. This particular model of PD does not appear to have a systemic impact on the B cell compartment, and therefore may not be suitable for studies which aim to determine the relative risk of autoimmunity and RA in PD.

Chapter 6: Periodontitis in mice with an altered B cell profile

6.1 Introduction

The murine model of PD described in **Chapter 5** was associated with a significant increase in alveolar bone loss, and with subtle changes to B cell phenotype, mainly localised to the gingiva and dLNs. To discern more clearly, the relationship between changes in B cell phenotype and pathology in PD, there are two strategies which can be implemented using *in vivo* and *in vitro* murine models. The first is to enhance B cell activation and increase B cell numbers. The second is to inhibit B cell activity or deplete B cells. The first strategy was employed in this chapter, and the second in **Chapter 7**.

There are several ways of altering B cell phenotype, which can be thought of in terms of targeting their receptors including the BCR, co-stimulatory molecules, TLRs, and certain IL receptors. The increase in B cell number and expression of RANKL by B cells following *P. gingivalis* infection are among the features which may be enhanced by altering the balance of specific inflammatory and anti-inflammatory cytokines, either by alteration of gene expression of cytokines or their receptors, exogenous delivery of cytokine or exogenous delivery of a cytokine inhibitor.

Amongst the various risk factors associated with chronic PD are numerous genetic polymorphisms and modifications which affect the expression or activity of cytokines, including IL-1, IL-6, IL-18, and TNFα (Holla et al., 2004, Galicia et al., 2006, Ding et al., 2012, Ishida et al., 2012, Karimbux et al., 2012, Deng et al., 2013, Yang et al., 2013, Li et al., 2014b, Zhang et al., 2014a). Clinical improvement in PD is often associated with a reduction in the levels of these cytokines in the gingiva and GCF (Lee et al., 1995, de Campos et al., 2012, Reis et al., 2014).

Many of the cytokines studied in PD can be assigned to one of three categories: those associated with Th1, Th2, or Th17 responses (Mosmann et al., 2005, Chen and O'Shea, 2008). The balance of these Th responses is thought to be central to the pathogenesis of PD, but it has yet to be established if a particular Th

response is more helpful than any other at a particular stage in the course of PD. An immune response strongly skewed towards one of these Th responses can exacerbate PD. Mice that lacked key Th1 cytokines (IL-12, IFN_{γ}, and TNFα) or Th2 cytokines (IL-4 and IL-10) have both been found to develop more severe PD than wild type mice (Sasaki et al., 2004, Alayan et al., 2007). Tregs function to help prevent excessive activation of the Th cells, and maintain a balanced, efficient response. Inhibition of Tregs was demonstrated to increase severity of murine PD (Garlet et al., 2010). Furthermore, recruitment of Tregs to the oral cavity, via exogenous administration of the chemokine CCL22 led to attenuation of murine PD (Glowacki et al., 2013).

Th cells largely dictate the activities of B cells (summarised in **Figure 6.1.1**). Each type of Th response can be further characterised by the consequences on B cell function. Th1 responses are usually directed against intracellular and extracellular viral and bacterial infections. Th1 responses promote B cell production of opsonising antibodies, predominantly of the IgG2a isotype in mice. These help aid the phagocytosis of extracellular pathogens and the destruction of infected cells by ADCC. Th2 responses are generally most effective at dealing with helminth parasites (Else et al., 1993, Pritchard et al., 1995). Th2 responses promote B cell production of neutralising antibodies, predominantly of the IgE and IgG1 isotypes in mice. IgE antibodies help to neutralise toxins released by the pathogen, and direct mast cell degranulation onto the surface of a pathogen. Th2 responses can also lead to an increase in B cell expression of MHC II and antigen presentation (Horsnell et al., 2013). Th17 responses are increasingly being associated with infections by extracellular pathogens, including *P. gingivalis* (Bettelli et al., 2007). Th17 cells produce IL-21 which supports Tfh cells, that in turn provide help to GC B cells in secondary lymphoid organs (Wei et al., 2007). Th17 responses result in the production of IgM, IgG, and IgA antibodies, but not IgE (Annunziato et al., 2007).

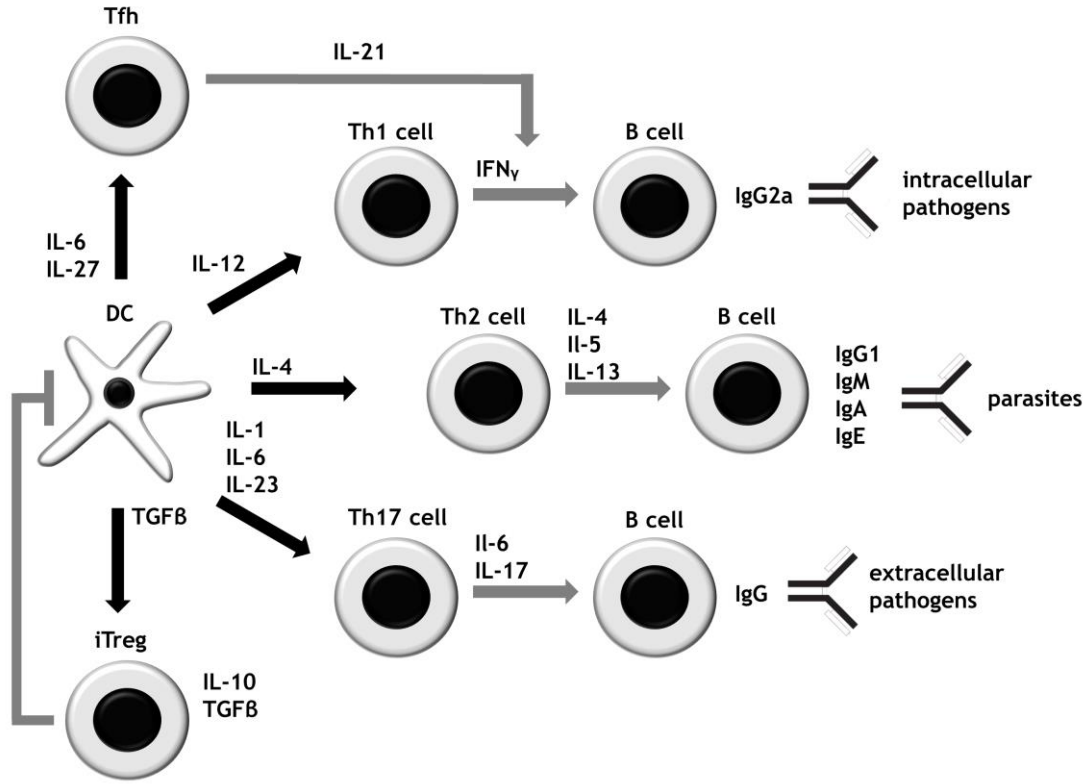

Figure 6.1.1. B cell activity associated with Th1, Th2, and Th17 polarised responses to infection. Cytokine secretion by DCs influences Th cell polarisation following interaction with antigen. In turn, cytokine secretion by polarised Th1, Th2, and Th17 cells influences B cell activity including antibody isotype switching. Tfh cells provide help to cognate GC B cells, supporting the production of high-affinity antibodies. Induction of Treg cell differentiation by DCs creates a negative feedback loop. Tregs suppress the generation and activity of the other Th effector cells (Mosmann et al., 1986, Moser and Murphy, 2000, Breitfeld et al., 2000, Sakaguchi, 2000, Hori et al., 2003, Bettelli et al., 2007, Aliahmadi et al., 2009, Chung et al., 2009, Avery et al., 2010, Gringhuis et al., 2014).

IL-1 increases cytokine production, MHC and co-stimulatory molecule expression by DCs, and acts as a general amplifier of T cell responses (Kruse et al., 2001, Ben-Sasson et al., 2009). IL-1 is especially good at enhancing Th2 and Th17 responses, and is required for full commitment of a naïve T cell to the Th17 lineage as well for the maintenance of Th17 responses (Lichtman et al., 1988, Wilson et al., 2007, Chung et al., 2009, Guo et al., 2009). As a consequence of increasing help from T cells, IL-1 indirectly enhances B cell activation, maturation, and antibody production, but it also directly boosts B cell responses to stimulation (Phillips and Rabson, 1983, Freedman et al., 1988, Rousset et al., 1991, Nakae et al., 2001).

IL-1 has been relatively well studied in the context of PD. IL-1 is produced by GECs and monocytes following stimulation with whole *P. gingivalis* or *P. gingivalis* LPS (Gemmell and Seymour, 1993, Sandros et al., 2000). IL-1 actually refers to two cytokines: IL-1α and IL-1ß. These are produced by distinct genes but share the same receptors and have the same functions (Garlanda et al., 2013). It has been demonstrated that overexpression of IL-1 in the oral epithelium of mice induced spontaneous development of PD (Dayan et al., 2004). It has also been shown that mice deficient in the endogenous IL-1 receptor antagonist are more susceptible to PD (Izawa et al., 2014). Furthermore, the delivery of exogenous IL-1 antagonists led to attenuation of PD in a murine model, and in a primate model of the disease (Delima et al., 2002).

IL-33 is a member of the IL-1 cytokine family, which appears to be involved in various chronic inflammatory diseases including asthma, colitis, and RA (Xu et al., 2008, Prefontaine et al., 2009, Pastorelli et al., 2010, Tang et al., 2013, Sattler et al., 2014). IL-33 is expressed by a range of cells lining mucosal surfaces and the skin, as well as selected immune cells: fibroblasts, endothelial cells, keratinocytes, epithelial cells, dendritic cells, and activated macrophages (Palmer et al., 2008, Kuchler et al., 2008, Moussion et al., 2008). Like IL-1, IL-33 is thought to act as an alarmin – a signal of cellular stress or injury (Cayrol and Girard, 2009, Nile et al., 2010). IL-33 has roles in both intracellular and extracellular signalling, but is only secreted and able to exert extracellular effects following necrosis of the source cell. In living cells, IL-33 is located within the cytoplasm. During apoptosis it is sequestered in the nucleus, where it is cleaved and inactivated.

There are multiple splice variants of the receptor for IL-33. One of these is the soluble decoy receptor sST2, and one is the membrane-bound receptor ST2L. Binding of IL-33 to ST2L and subsequent recruitment of the adaptor IL-1RAP, results in activation of NFκB and MAPK signalling pathways (Chackerian et al., 2007). ST2L was originally described as an orphan receptor, mainly expressed by Th2 cells and mast cells (Moritz et al., 1998, Lohning et al., 1998), but it is now known to be expressed by basophils, eosinophils, NK cells, iNK T cells, DCs, and B cells. As a result, IL-33 has widespread effects on the immune system (**Figure 6.1.2)**.

Analogous to IL-1, IL-33 can enhance both Th2 and Th17 responses. The production of cytokines by Th2 cells is amplified by IL-33 itself whereas Th17 responses are enhanced via IL-33 activation of mast cells, which release an array of inflammatory mediators following degranulation (Schmitz et al., 2005, Komai-Koma et al., 2007, Komai-Koma et al., 2011, Cho et al., 2012). As a result of these effects on Th cells, and the direct interaction of IL-33 with B cells, IL-33 treatment can augment the production of antibodies of most classes. A combination of IL-5 and IL-33 was found to be sufficient to increase the proliferation and production of IgM by the B1 subset of B cells *in vitro*. Both T cells and mast cells are significant sources of IL-5 *in vivo* following IL-33 treatment. These events are thought to account for IL-33 exacerbation of DTH responses (Komai-Koma et al., 2011).

Studies of the role of IL-33 in asthma and RA are especially relevant to PD; RA shares common immunopathological pathways with PD, and asthma, like PD, takes place at a mucosal surface. Increased IgE titers resulting from IL-33 treatment have been linked to increased mast cell activity and exacerbation of airway inflammation in mice, and this is thought to be an underlying mechanism of asthma (Zhiguang et al., 2010, Kobayashi et al., 2013a). Most interestingly, in CIA and CAIA murine models of RA, IL-33 treatment led to elevated titers of anti-collagen IgG antibodies, greater footpad swelling, and higher clinical score in WT mice whilst ST2 KO mice had attenuated disease (Xu et al., 2008, Xu et al., 2010). IL-33 is expressed in the synovial tissue of RA patients, and fibroblasts isolated from the rheumatoid synovium can be stimulated to secrete IL-33 by adding IL-1ß and TNFα (Xu et al., 2008, Tang et al., 2013). Moreover, levels of IL-33 in synovial fluid have been found to positively correlate with titers of RF in RA patients (Tang et al., 2013).

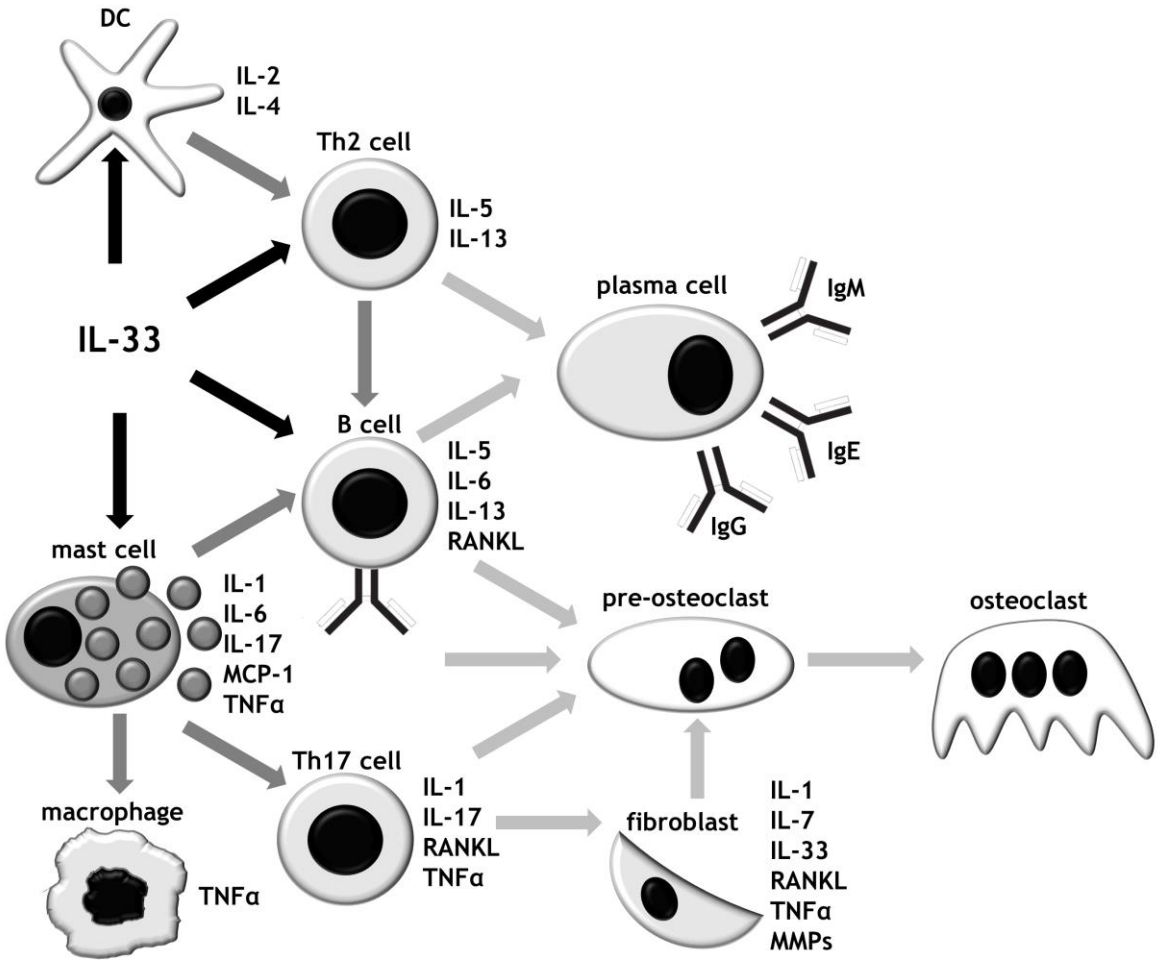

Figure 6.1.2. The IL-33 cytokine-cell network. IL-33 has direct effects on ST2 receptor bearing cells including DCs, mast cells, Th2 cells, and B cells. Activation of this first line of immune cells by IL-33 results in the generation of other cytokines and the activation of a second wave of cells, including plasma cells, macrophages, fibroblasts, and osteoclasts (Barksby et al., 2007, Cherry et al., 2008, Smithgall et al., 2008, Bourgeois et al., 2009, Pecaric-Petkovic et al., 2009, Kroeger et al., 2009, Rank et al., 2009, Nile et al., 2010).

There is some evidence which suggests that oral infection with *P. gingivalis* could result in increased IL-33 gene expression in the gingiva. When stimulated with *P. gingivalis* LPS, a human monoctyte cell line (THP-1) was found to increase IL-33 gene expression (Nile et al., 2010). In addition, preliminary data has indicated that oral keratinocyte cell lines (OKF6 and TERT-2) and primary human GECs stimulated with *P. gingivalis* monospecies biofilms increase IL-33 gene expression, and that IL-33 gene expression and protein are elevated in the gingiva of PD patients *(*Nile et al., personal communication, manuscripts in preparation, University of Glasgow).

Arguing against a role for IL-33 in PD are a handful of small studies of PD patients which found the level of IL-33 protein in the GCF of patients to be reduced or unchanged relative to periodontally healthy participants (Buduneli et al., 2012, Papathanasiou et al., 2013, Kursunlu et al., 2014). Whether IL-33 has a role in PD pathogenesis, and by what mechanism, remains to be fully established.

As IL-33 can strongly influence the adaptive immune response, and enhance certain B cell functions, it seems an appropriate novel tool for further investigating associations between altered B cell phenotype and PD. A series of experiments were therefore conducted to simultaneously determine the impact of IL-33 treatment on alveolar bone loss in the murine model of PD and to assess changes in B cell phenotype associated with this, including the B cell expression of RANKL.

6.2 Results

To determine the effect of IL-33 treatment on pathology in murine PD, mice were infected with *P. gingivalis* or sham-infected and these groups were split further into sub-groups that received either IL-33 or PBS treatment by intraperitoneal injection (as described in **Chapter 2**, section **2.5**). At six weeks post-infection, alveolar bone loss was measured (as described in **Chapter 2**, section **2.7**). Sham-infected mice treated with IL-33 had greater alveolar bone loss than sham-infected untreated mice (mean ± SEM: 0.21 ± 0.009 mm sham vs. 0.24 ± 0.0065 mm sham + IL-33, *P < 0.05, **Figure 6.2.1**), and *P. gingivalis*-infected mice treated with IL-33 had the greatest alveolar bone loss (-0.24 ± 0.0055 mm PD vs. -0.29 ± 0.0053 mm PD + IL-33, ***P < 0.001; -0.24 ± 0.0065 mm sham + IL-33 vs. -0.29 ± 0.0053 mm PD + IL-33, ***P < 0.001, **Figure 6.2.1**). Thus, IL-33 exacerbated alveolar bone loss in murine PD. The next objective was to investigate whether this phenotype was associated with alterations in the local and systemic B cell profile. To begin with, B cell RANKL expression in the gingiva of mice was analysed by flow cytometry (as described in **Chapter 2,** sections **2.6**, **2.10.1**, and **2.15.2**). Unfortunately, due to insufficient resources, not all of the following *in vivo* experiments included a group of IL-33 treated sham-infected controls.

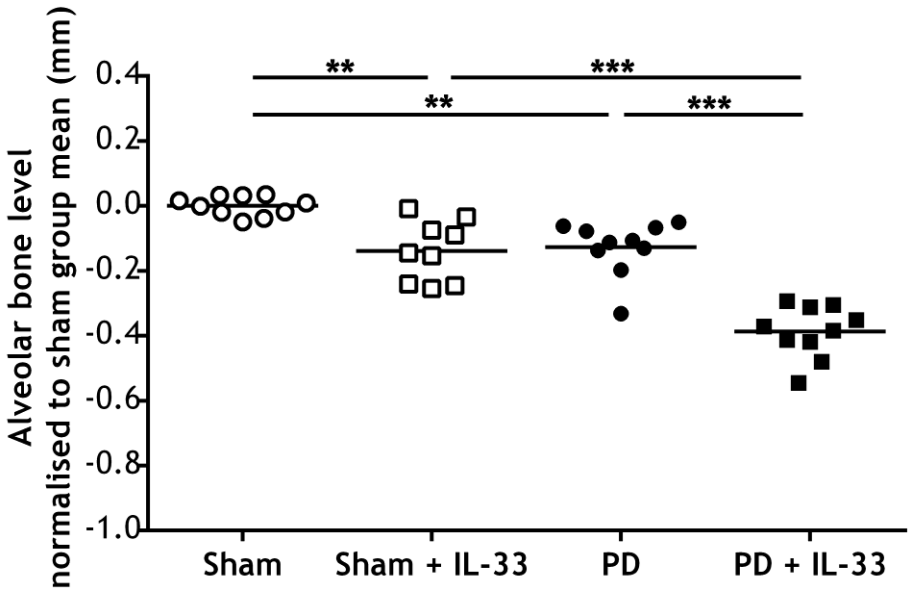

Figure 6.2.1. Alveolar bone level in IL-33 treated mice with periodontitis at 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). Half of the mice with PD and sham controls were treated with IL-33 (PD/ sham + IL-33) and half were treated with PBS. At 6 weeks post-infection, the alveolar bone level was measured. The data are shown as mean per mouse (symbols) and mean for each group of mice (lines) normalised to the sham group mean. The data are combined from 2 experiments, n = 5 mice/group. Significant differences were determined by One-Way ANOVA with a Tukey post-hoc test (*P < 0.05, **P < 0.01, ***P < 0.001). Data provided by *Dr Jennifer Malcolm*.

A number of IL-33-associated changes in B cells were evident in the gingiva at both one and six weeks post-infection. However, none of these trends could be statistically analysed due to the pooling of samples of gingiva within each experiment, and the fact that the data were from just one or two experiments. At one week post-infection there was, surprisingly, a lower proportion of B cells in the gingiva of IL-33 treated mice with PD compared with PBS treated mice with PD (30.45 ± 1.38 % PD vs. 20.95 ± 0.40 % PD + IL-33, **Figure 6.2.2A**). On the other hand, B cell expression of RANKL was increased in IL-33 treated mice and was highest in IL-33 treated mice with PD at one week post-infection (4.39 ± 0.50 % sham vs. 11.30 ± 0.50 % PD; 11.30 ± 0.50 % PD vs. 22.90 ± 2.80 % PD + IL-33, **Figure 6.2.2C**). At six weeks post-infection, B cell RANKL expression in PBS treated mice with PD was reduced relative to PBS treated sham controls, and further reduced in IL-33 treated mice with PD, opposing the trend observed at one week post-infection. Analysis of B cell subsets and differential RANKL expression by B cell subsets unfortunately could not be performed in these studies due to the limited number of gingival cells available and requirements for other ongoing independent studies.

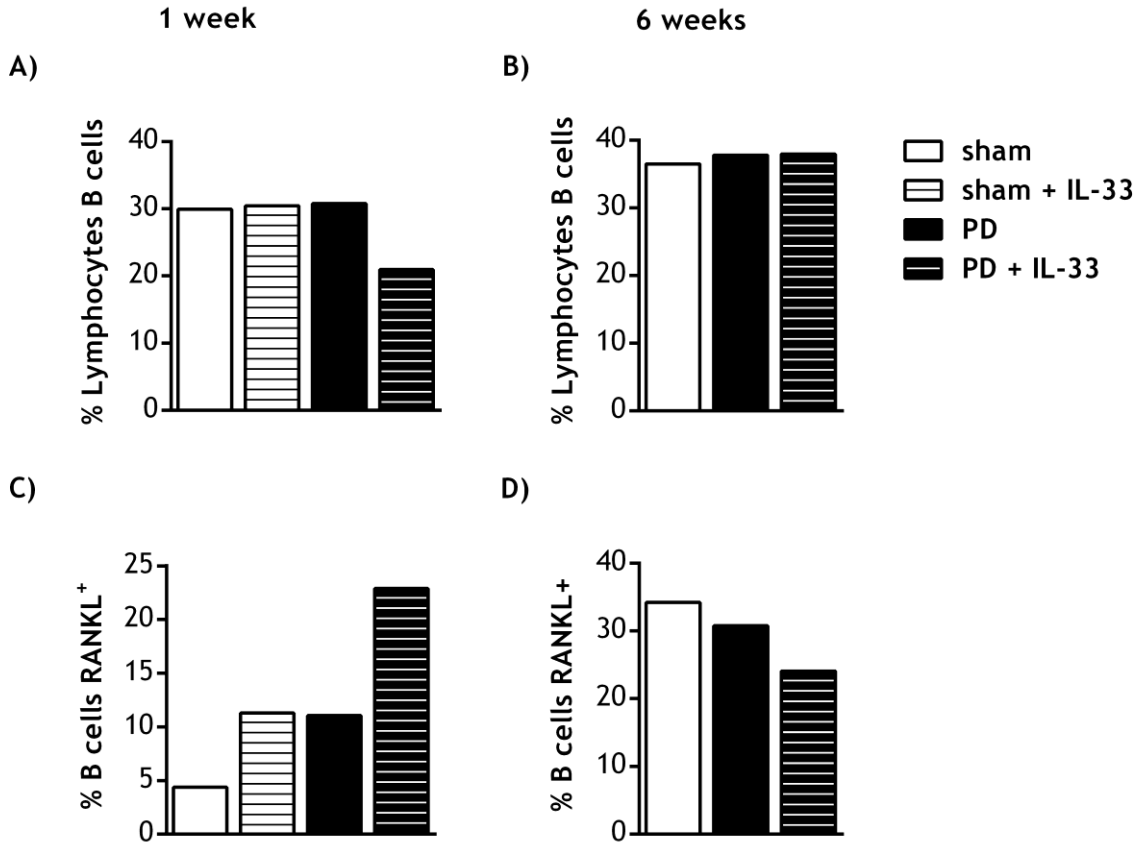

Figure 6.2.2. RANKL expression by B cells in the gingiva of IL-33 treated mice with periodontitis at 1 and 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). Half of the mice with PD and sham controls were treated with IL-33 (PD/sham + IL-33) and half were treated with PBS. At 1 week (**A** and **C**) and 6 weeks (**B** and **D**) post-infection, cells were isolated from the gingiva and analysed by flow cytometry. The data are shown as mean for 2 independent experiments at 1 week, and for 1 experiment at 6 weeks. There was no sham + IL-33 group at 6 weeks. Gingiva were pooled from 4-5 mice/group at 1 week post-infection and 5 mice/group at 6 weeks post-infection. Data contributed to by *Dr Jennifer Malcolm*.

B cell populations in the dLNs were analysed to further probe the impact of IL-33 treatment on the regional immune response to *P. gingivalis* infection (as described in **Chapter 2**, sections **2.10.2** and **2.15.2**). At one week post-infection there was a small increase in the proportion of B cells in the dLNs of PBS treated mice with PD relative to PBS treated sham controls, and a significant increase in the proportion of B cells in the dLNs of IL-33 treated mice with PD relative to PBS treated sham controls (20.82 ± 1.68 % sham vs. 26.98 ± 0.87 % PD; 26.98 ± 0.87 % PD vs. 29.70 ± 1.46 % PD + IL-33; 20.82 ± 1.68 % sham vs. 29.70 ± 1.46 % PD + IL-33, **P < 0.01, **Figure 6.2.3A**). The proportion of B1b cells was significantly increased in IL-33 treated mice with PD relative to PBS treated mice

with PD, and PBS treated sham controls (1.8 ± 0.11 % sham vs. 3.03 ± 0.08 % PD + IL-33, **P < 0.01; 1.99 ± 0.06 % PD vs. 3.03 ± 0.08 % PD + IL-33, *P < 0.05, **Figure 6.2.3C**), and the proportion of plasmablasts was also significantly increased in IL-33 treated mice with PD (7.14 ± 0.26 % sham vs. 11.13 ± 0.35 % PD + IL-33, *P < 0.05; 7.32 ± 0.32 % PD vs. 11.13 ± 0.35 % PD + IL-33, *P < 0.05, **Figure 6.2.3F**). None of these observations of dLNs B cell populations in IL-33 treated mice with PD persisted at six weeks post-infection (**Figure 6.2.4**). The proportion of plasmablasts in the dLNs of IL-33 treated mice with PD was reduced at six weeks post-infection relative to the PBS treated mice with PD, and PBS treated sham controls (4.11 ± 0.44 % sham vs. 3.09 ± 0.19 % PD + IL-33; 4.4-0 ± 0.3 2 % PD vs. 3.09 ± 0.19 % PD + IL-33, *P < 0.05, **Figure 6.2.4F**).

Analogous to observations in the gingiva, at one week post-infection, B cell RANKL expression was increased in the dLNs of IL-33 treated mice with PD relative to PBS treated mice with PD, and PBS treated sham controls (7.14 ± 0.15 % sham vs. 7.69 ± 0.34 % PD; 7.69 ± 0.34 % PD vs.10.98 ± 0.61 % PD + IL-33; 7.14 ± 0.15 % sham vs.10.98 ± 0.61 % PD + IL-33, *P < 0.05, **Figure 6.2.5A**). There were no differences in B cell RANKL expression between the groups at six weeks post-infection (**Figure 6.2.5B**). Nonetheless, due to the differences observed at one week post-infection, differential expression of RANKL by B cell subsets was then analysed in the dLNs of IL-33 treated mice in isolation. At one week post-infection, the B1a and B1b cells had the highest proportions expressing RANKL of the B cell subsets (18.23 ± 1.32 % B1a, 16.28 ± 0.53 % SEM B1b, 6.17 ± 0.79 % B2 MZ-like, 9.15 ± 0.46 % B2 FO, **Figure 6.2.6A**). At six weeks post-infection, all the B cell subsets expressed lower levels of RANKL compared with one week post-infection, and the B1a subset clearly had the highest proportion expressing RANKL (8.73 ± 0.97 % B1a, 4.83 ± 0.24 % B1b, 3.35 ± 0.39 % B2 MZ-like, 4.09 ± 0.15 % B2 FO, **Figure 6.2.6B**). Similar patterns in RANKL expression by B cell subsets were observed in the dLNs of PBS treated mice with PD and PBS treated sham controls (data not shown).

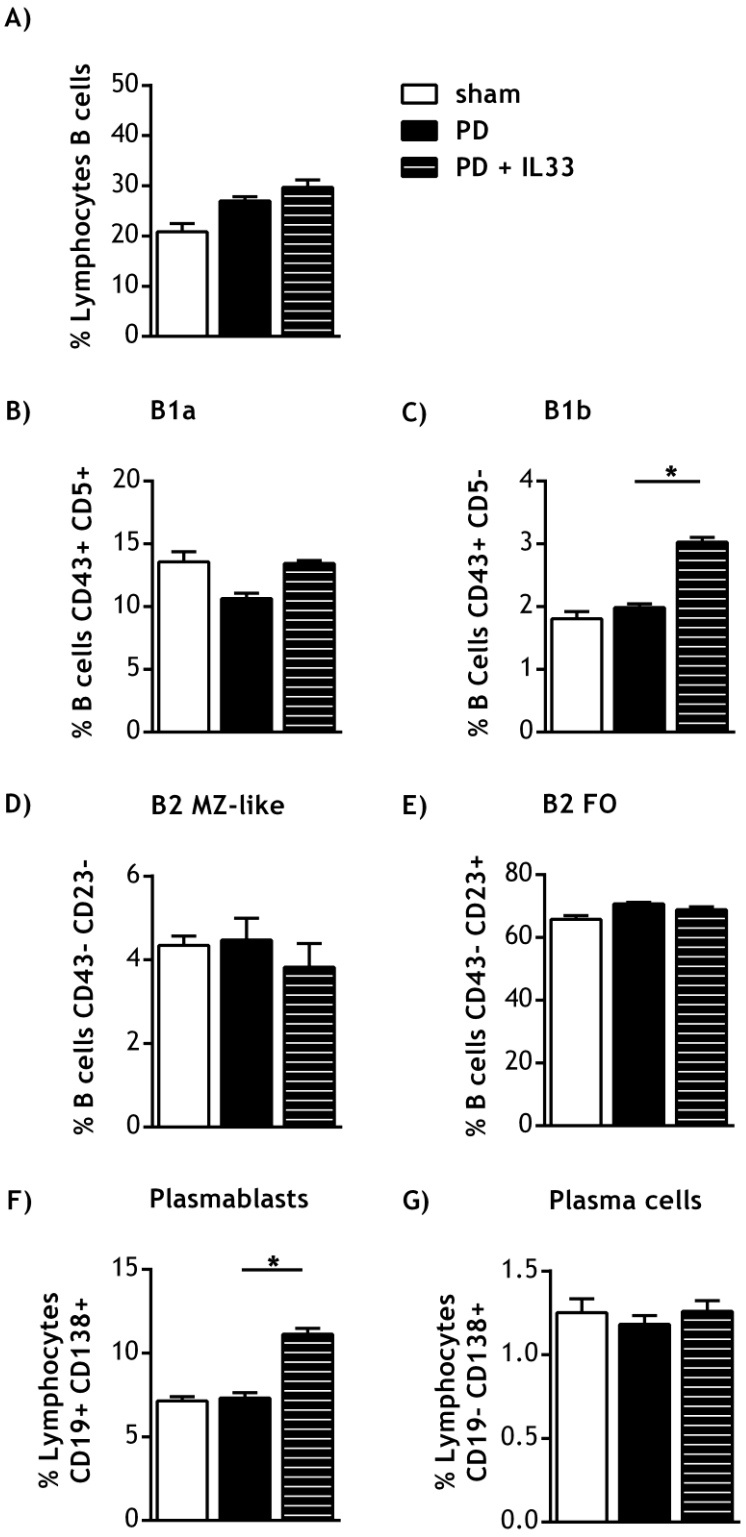

Figure 6.2.3. B cell subsets in the dLNs of IL-33 treated mice with periodontitis at 1 week post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). A group of mice with PD were additionally treated with IL-33 (PD + IL-33). At 1 week post-infection, lymphocytes were isolated from the dLNs and analysed by flow cytometry. The data are shown as mean with SEM, n = 4-5 mice/group. Significant differences were determined by Kruskal-Wallis with Dunn's multiple comparison post-hoc test (*P < 0.05).

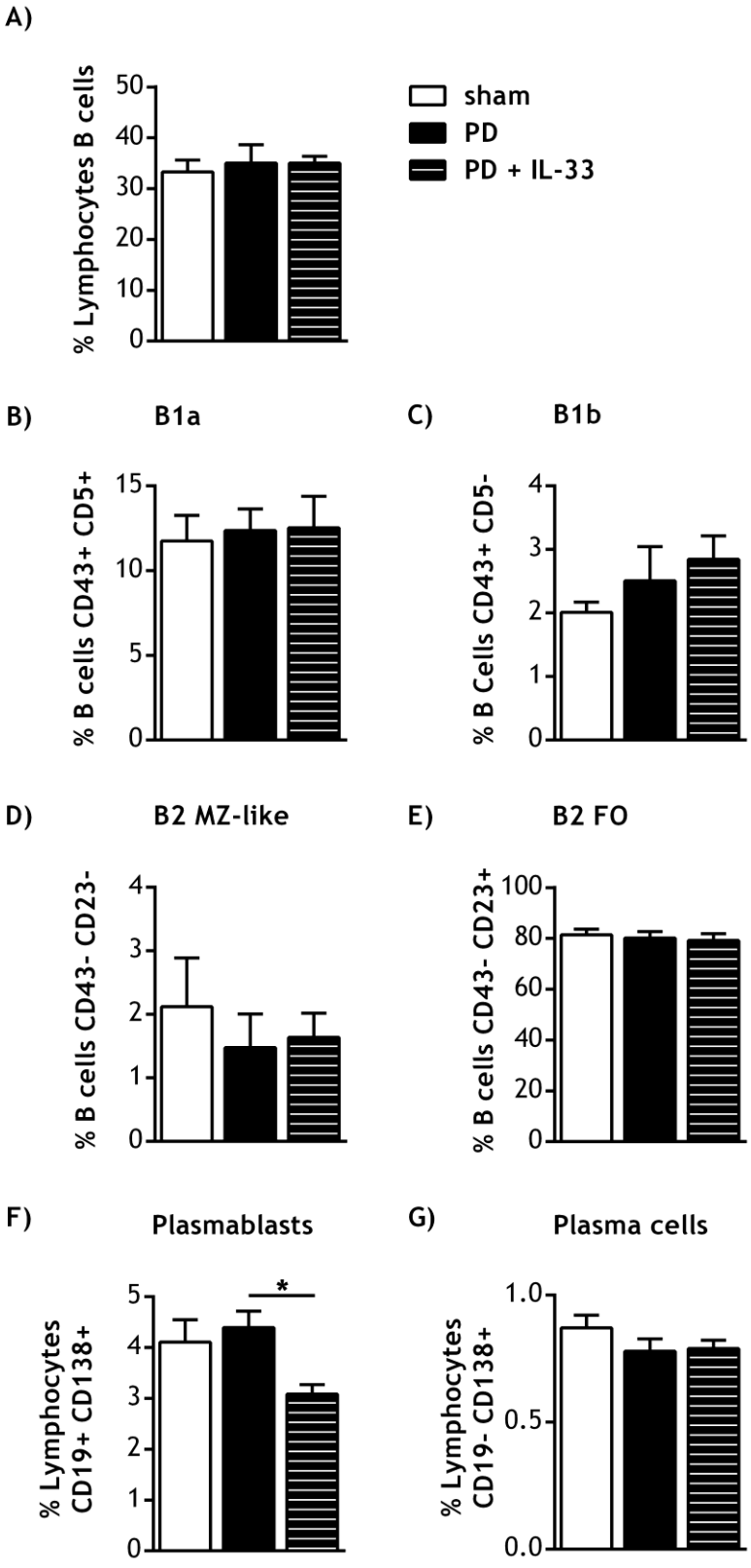

Figure 6.2.4. B cell subsets in the dLNs of IL-33 treated mice with periodontitis at 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). A group of mice with PD were additionally treated with IL-33 (PD + IL-33). At 6 weeks post-infection, lymphocytes were isolated from the dLNs and analysed by flow cytometry. The data are shown as mean with SEM, n = 5 mice/group. Significant differences were determined by One-Way ANOVA with Tukey post-hoc test (*P < 0.05).

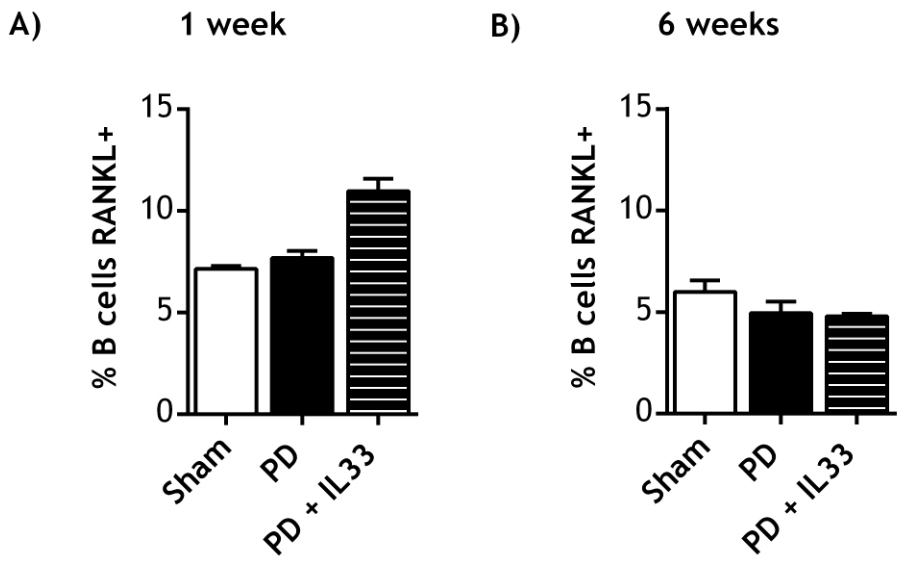

Figure 6.2.5. RANKL expression by B cells in the dLNs of IL-33 treated mice with periodontitis treated at 1 and 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). A group of mice with PD were additionally treated with IL-33 (PD + IL-33). At 1 week (**A**) and 6 weeks (**B**) post-infection, lymphocytes were isolated from the dLNs and analysed by flow cytometry. The data are shown as mean with SEM, n = 4-5 mice/group at 1 week post-infection and n = 5 mice/group at 6 weeks post-infection. Significant differences were determined in (**A**) by Kruskal-Wallis with Dunn's multiple comparison post-hoc test (Sham vs. PD + IL-33 *P < 0.05) and in (**B**) data were analysed by One-Way ANOVA with Tukey post-hoc test, and no significant differences were found.

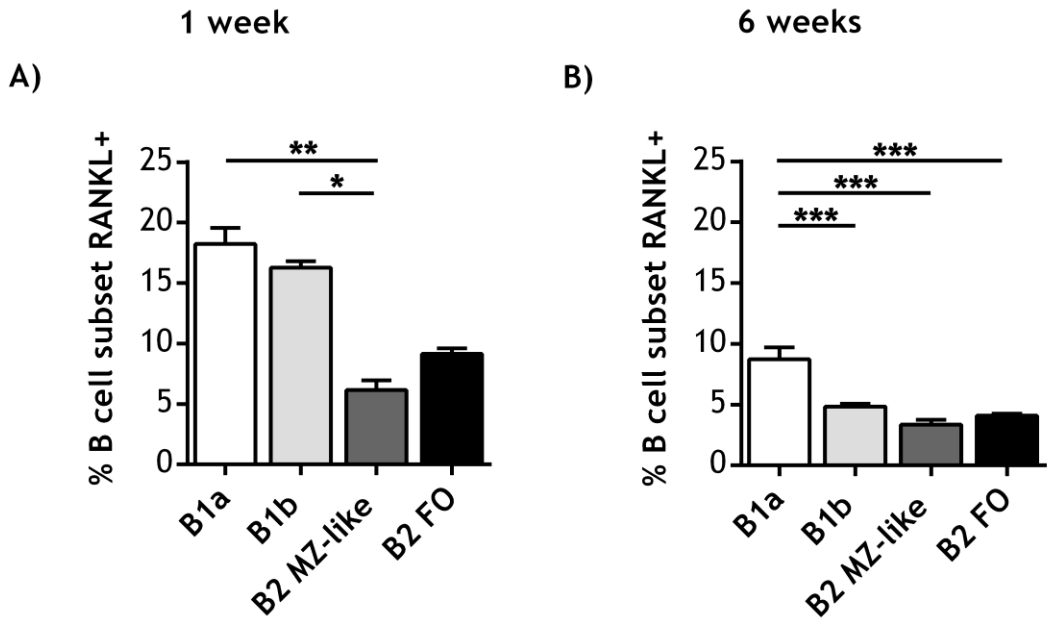

Figure 6.2.6. Differential RANKL expression by B cell subsets in the dLNs of IL-33 treated mice with periodontitis at 1 and 6 weeks post-infection. Mice were infected with *P. gingivalis* and treated with IL-33 (PD + IL-33). At 1 week (**A**) and 6 weeks (**B**) post-infection, lymphocytes were isolated from the dLNs and analysed by flow cytometry. Data are shown as mean with SEM, n = 4-5 mice/group. Significant differences were determined in (**A**) by Kruskal-Wallis with Dunn's multiple comparison post-hoc test and in (**B**) by One-Way ANOVA with Tukey post-hoc test (*P < 0.05, **P < 0.01, ***P < 0.001)**.** Similar patterns in RANKL expression by B cell subsets were observed in the dLNs of PBS treated mice with PD and PBS treated sham controls (data not shown).

In addition to RANKL expressed on the surface of lymphocytes, sRANKL and TNFα can also promote osteoclastogenesis (Lam et al., 2000). Therefore, an *in vitro* lymphocyte stimulation assay was performed to determine if IL-33 treatment affected the production of these cytokines. Lymphocytes from the dLNs and inguinal LNs of IL-33 treated mice with PD, PBS treated mice with PD, and sham controls were stimulated with PMA and ionomycin for three days, and then the concentration of these cytokines were measured in the culture media (as described in **Chapter 2**, sections **2.10.2**, **2.12**, and **2.16.5**). This strong, non-specific stimulation provided an indication of the cytokine secreting potential of lymphocytes *in vivo*. Observations of the dLNs revealed the combined effects of local *P. gingivalis* infection and systemic IL-33 treatment, whereas the inguinal LNs provided insight to systemic effects both *P. gingivalis* infection and IL-33 treatment in LNs which do not directly drain the peritoneal cavity (in which the

potent effects of IL-33 would be more likely to mask any systemic effects of *P. gingivalis* infection).

At one week post-infection, sRANKL production by lymphocytes from the dLNs of PBS treated mice was significantly increased compared with sham controls and an intermediate level was produced by lymphocytes from the dLNs of IL-33 treated mice with PD (81.62 ± 7.23 pg/ml sham vs. 196.3 ± 28.06 ng/ml PD, *P < 0.05, **Figure 6.2.7**). At six weeks post-infection, there was no difference in the production of sRANKL by lymphocytes from the dLNs of the different mouse groups (**Figure 6.2.8A**). There was a trend towards elevated sRANKL production by lymphocytes from the inguinal LNs of IL-33 treated mice with PD compared with PBS treated sham controls, but this did not reach statistical significance (0.055 ± 0.035 ng/ml sham vs. 0.22 ± 0.064 ng/ml PD; 0.055 ± 0.035 ng/ml sham vs. 0.23 ± 0.075 ng/ml PD + IL-33, **Figure 6.2.8B**). Production of IL-6, IL-17, and TNFα, by lymphocytes from the dLNs and inguinal LNs of mice was also analysed at six weeks post-infection, and no significant differences between mouse groups were found at this time-point (data not shown).

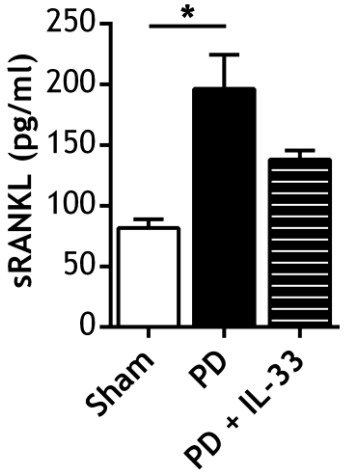

Figure 6.2.7. *In vitro* sRANKL production by lymphocytes from IL-33 treated mice with periodontitis at 1 week post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). A group of PD mice were additionally treated with IL-33 (PD + IL-33). At 1 week post-infection, lymphocytes were isolated from the dLNs of mice and stimulated for three days with PMA (10 ng/ml) and ionomycin (500 ng/ml). The concentration of sRANKL in the culture media was then measured by ELISA. Data are shown as mean with SEM, n = 4-5 mice/group. Significant differences were determined by Kruskal-Wallis with Dunn's multiple comparison post-hoc test (*P < 0.05).

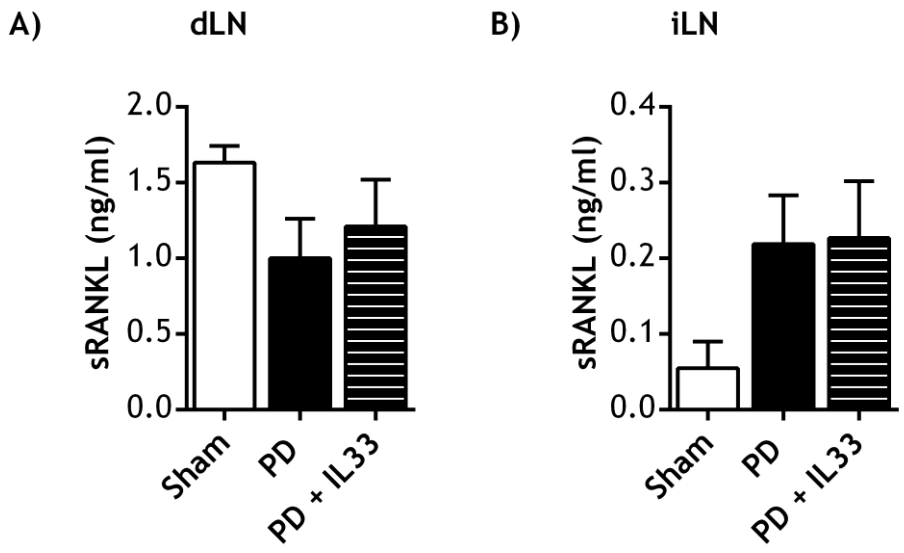

Figure 6.2.8. *In vitro* sRANKL production by lymphocytes from IL-33 treated mice with periodontitis at 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). A group of PD mice were additionally treated with IL-33 (PD + IL-33). At 6 weeks post-infection, lymphocytes were isolated from the dLNs (**A**) and inguinal LNs (**B**) of mice and stimulated for three days with PMA (10 ng/ml) and ionomycin (500 ng/ml). The concentration of sRANKL in the culture media was then measured by ELISA. Data are shown as mean with SEM, n = 4-5 mice/group. Data were analysed by Kruskal-Wallis with Dunn's multiple comparison post-hoc test, and no significant differences were found.

Previously in **Chapter 5**, it was demonstrated that PD alone has no impact on B cell subsets in the peritoneal fluid. However, since the IL-33 treatments were delivered intra-peritoneally, and the peritoneal cavity is a prominent source of B cells, IL-33 induced changes to B cell phenotype were investigated here (as described in **Chapter 2**, sections **2.10.5** and **2.15.2**) (Ray and Dittel, 2010). At one week post-infection, the number of B cells was increased in the peritoneal fluid of IL-33 treated mice, with a significantly increase in IL-33 treated mice with PD compared with PBS treated mice with PD (1,651,000 ± 658,926 PD vs. 6,875,000 ± 737,493 PD + IL-33, *P < 0.05, **Figure 6.2.9A**), although the proportion of B cells as a percentage of total cells was similar across the different groups (**Figure 6.2.9B**). IL-33 treatment was associated with subtle shifts in the proportions of B cell subsets, but none of these trends reached statistical significance. These included reductions in the proportions of B1a cells, B1b cells, and plasmablasts, and an increase in the proportion of B2 MZ-like cells (**Figure 6.2.9C-H**).

In line with earlier observations in the gingiva (**Figure 6.2.2**) and in the dLNs (**Figure 6.2.5**), there was a trend towards increased B cell RANKL expression in the peritoneal fluid of IL-33 treated mice relative to PBS treated mice at one week post-infection (**Figure 6.2.10A**). However, there was no synergistic effect of PD and IL-33 on B RANKL expression. Differential expression of RANKL by B cell subsets was then analysed in the peritoneal fluid of IL-33 treated mice in isolation. As observed in the dLNs (**Figure 6.2.6**), the B1a and B1b subsets formed the majority of RANKL expressing B cells, whilst the B2 MZ-like subset included a comparatively small proportion of RANKL expressing B cells (24.80 ± 4.54 % B1a, 15.66 ± 3.57 % B1b, 6.60 ± 1.11 % B2 MZ-like, 14.11 ± 3.87 % B2 FO, **Figure 6.2.11**). Similar patterns in RANKL expression by B cell subsets were observed in the peritoneal fluid of PBS treated mice with PD and PBS treated sham controls (data not shown).

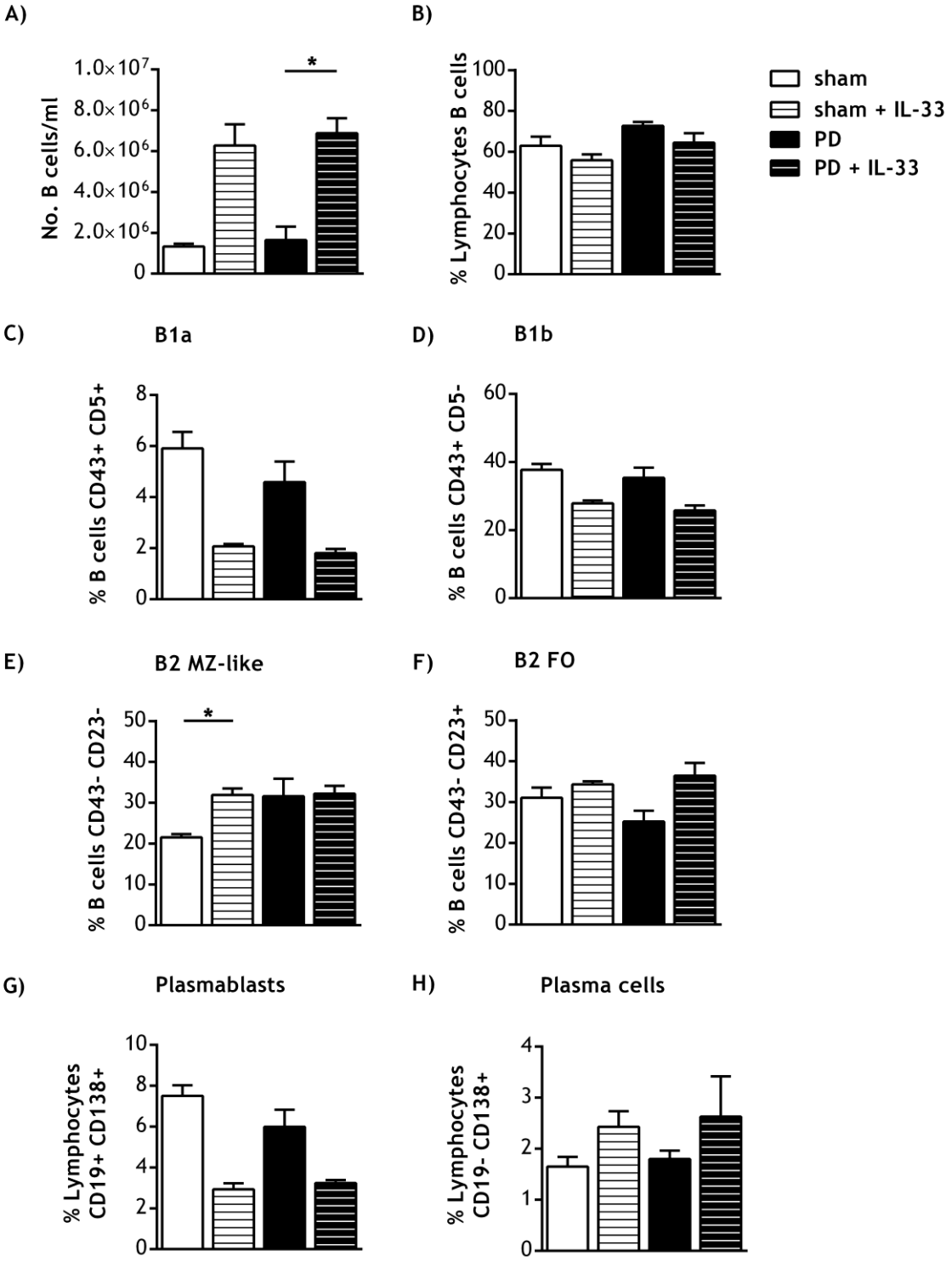

Figure 6.2.9. B cell subsets in the peritoneal fluid of IL-33 treated mice with periodontitis at 1 week post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). Half of the mice with PD and sham controls were treated with IL-33 (PD/sham + IL-33) and half were treated with PBS. At 1 week post-infection, lymphocytes in the peritoneal fluid were analysed by flow cytometry. Data is shown as mean with SEM, n = 4-5 mice/group. Significant differences were determined by Kruskal-Wallis with Dunn's multiple comparison post-hoc test (*P < 0.05).

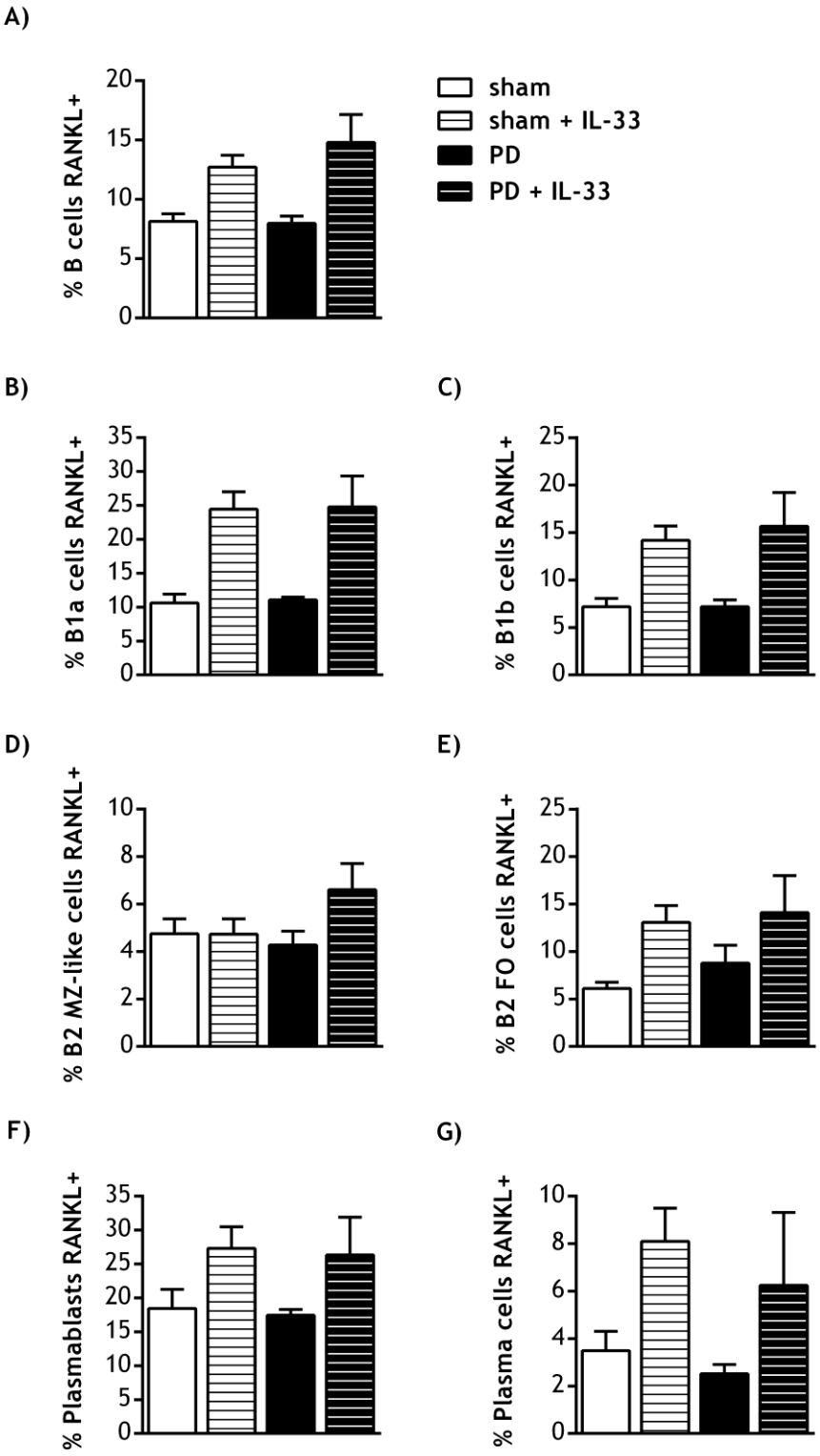

Figure 6.2.10. RANKL expression by B cell subsets in the peritoneal fluid of IL-33 treated mice with periodontitis at 1 week post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). Half of the mice with PD and sham controls were treated with IL-33 (PD/sham + IL-33) and half were treated with PBS. At 1 week post-infection lymphocytes in the peritoneal fluid were analysed by flow cytometry. Data are shown as mean with SEM, n = 4-5 mice/group. Data were analysed by Kruskal-Wallis with Dunn's multiple comparison post-hoc test, and no significant differences were found.

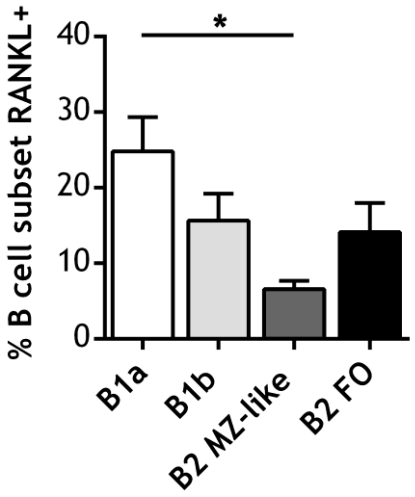

Figure 6.2.11. Differential RANKL expression by B cell subsets in the peritoneal fluid of IL-33 treated mice with periodontitis at 1 week post-infection. Mice were infected with *P. gingivalis* and treated with IL-33. At 1 week post-infection, lymphocytes in the peritoneal fluid were analysed by flow cytometry. Data are shown as mean with SEM, n = 4 mice/group. Significant difference was determined by Kruskal-Wallis with Dunn's multiple comparison post-hoc test (*P < 0.05). Similar patterns in RANKL expression by B cell subsets were observed in the peritoneal fluid of PBS treated mice with PD and PBS treated sham controls (data not shown).

Subtle shifts in B cell populations in the peritoneal fluid associated with IL-33 treatment also existed at six weeks post-infection. The overall proportion of B cells was not altered (**Figure 6.2.12A**), but trends in the proportions of B cell subsets in IL-33 treated mice relative to PBS treated mice included a reduction in the proportion of B1a cells (**Figure 6.2.12B**) and an increase in the proportion of B2 MZ-like cells (**Figure 6.2.12D**). None of these trends in the proportions of B cell subsets reached statistical significance. Data on plasmablasts and plasma cells at this time-point were from pooled samples of peritoneal fluid from one experiment, so statistical analysis could not be performed. RANKL expression by B cell subsets was not analysed at this later time-point due to limited resources. Based on the data from one week post-infection, it was expected that any differences in B cell RANKL expression in the peritoneal fluid of IL-33 treated and PBS treated mice at six weeks post-infection would not reach statistical significance.

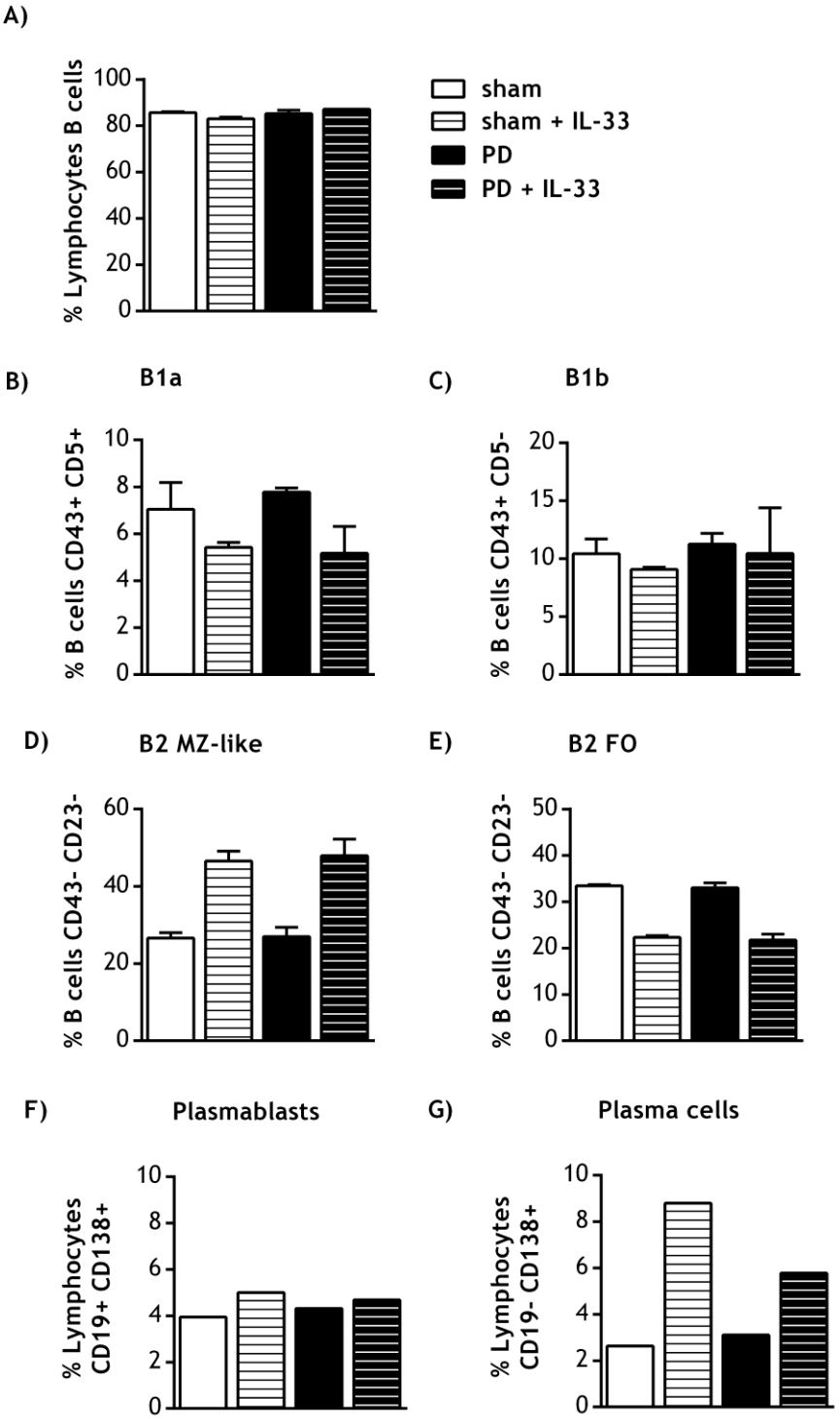

Figure 6.2.12. B cell subsets in the peritoneal fluid of IL-33 treated mice with periodontitis at 6 weeks post-infection. Mice were infected with *P. gingivalis* (PD) or sham-infected (sham). Half of the mice with PD and sham controls were treated with IL-33 (PD/sham + IL-33) and half were treated with PBS. At 6 weeks post-infection, lymphocytes in the peritoneal fluid were analysed by flow cytometry. Data are shown as mean with SEM for 2 independent experiments **(A**, **B**, **C**, **D** and **E)** or 1 experiment **(F** and **G)**. Samples of peritoneal fluid were pooled from 4-5 mice/group. Data in **A - E)** were analysed by Kruskal-Wallis with Dunn's multiple comparison post-hoc test, and no significant differences were found.

Collectively, the data presented demonstrate that IL-33 treatment is associated with a pattern of increased B cell RANKL expression *in vivo* at one week post-infection. Activated B cells can express ST2 receptors and directly react to IL-33, but it is not known whether this interaction directly increases expression of RANKL by B cells or if factors derived from other IL-33 activated cells - such as T cells or stromal cells - are responsible for eliciting this response. To test whether IL-33 could directly induce RANKL expression by activated B cells, whereby purified B cells or lymphocytes were cultured *in vitro* with either media only, or a combination of B cell stimulants; IL-4, anti-CD40, anti-IgD, and LPS from *P. gingivalis* or *E. coli*, in the presence or absence of IL-33. Initially, this culture was set up for three days, then it was repeated for one week (as described in **Chapter 2**, sections **2.10.2**, **2.11**, and **2.13**). B cell phenotype was analysed by flow cytometry and the production of IL-6 and sRANKL was measured by ELISA (as described in **Chapter 2**, sections **2.15.2** and **2.16.5**).

After three days of stimulation, all combinations of LPS and IL-33 apparently resulted in some activation of the B cells in terms of Ki67 expression, Fas and GL7 expression, and RANKL expression. There were significant differences in the proportion of $Ki67^+$ B cells and Fas^+GL7^+ B cells when comparing culture conditions that had both *E. coli* LPS and IL-33 with media control, in both cultures of purified B cells and in cultures of mixed lymphocytes (Ki67: 12.53 ± 0.26 % media vs. 46.03 ± 0.84 % *E. coli* LPS + IL-33, **P < 0.01, **Figure 6.2.13C**; 19.77 ± 1.01 % media vs. 63.83 ± 0.81 % *E. coli* LPS + IL-33, **P < 0.01, **Figure 6.2.13D.** Fas/GL7: 0.65 ± 0.03 % media vs. 19.5 ± 75 % *E. coli* LPS + IL-33, **P < 0.01, **Figure 6.2.13E**; 6.13 ± 0.97 % media vs. 39.8 ± 1.2 % *E. coli* LPS + IL-33, *P < 0.05, **Figure 6.2.13F**). Stimulation of purified B cells with *P. gingivalis* LPS and IL-33 was associated with a significant increase in RANKL expression compared with media control (2.16 ± 0.08 % media vs. 2.98 ± 0.01 % *P. gingivalis* LPS + IL-33, *P < 0.05 **Figure 6.2.13G**).

After one week of stimulation, the overall viability of the cells was very low, and not enough cells in the media only or media with IL-33 conditions survived to perform any meaningful analysis. Lymphocytes were freshly isolated in order to provide 'unstimulated' lymphocytes for comparison. IL-33 treatment appeared to enhance the proliferation of cells, although this is based upon representative

cell counts which cannot be statistically analysed (**Figure 6.2.14A** and **6.2.14B**). In cultures of purified B cells, IL-33 increased the number of cells present when in combination with *E. coli* LPS but not with *P. gingivalis* LPS. In cultures of mixed lymphocytes, IL-33 increased the number of cells present when in combination with either type of LPS. These findings add weight to the *in vivo* observations of increased numbers of B cells in the dLNs and peritoneal fluid of IL-33 treated mice and suggest that IL-33 can augment the proliferation of B cells in response to other stimuli. In cultures of purified B cells, the proportion of viable B cells expressing RANKL was low in all culture conditions (**Figure 6.2.14E**), whereas in cultures of mixed lymphocytes, the proportion of B cells expressing RANKL was relatively high in all culture conditions - compared with unstimulated freshly isolated lymphocytes (**Figure 6.2.14F**). The addition of IL-33 to culture conditions did not alter RANKL expression by purified B cells or by B cells in mixed lymphocyte cultures. The one week period of stimulation was sufficiently long enough to permit the differentiation of B cells into $CD138^+$ plasma cells. A high proportion of viable cells were $CD138^+$ in both cultures of purified B cells and in cultures of mixed lymphocytes relative to samples of unstimulated freshly isolated lymphocytes – but this did not appear to be specifically altered by IL-33 treatment (**Figure 6.2.14G** and **6.2.14H**).

After both three days and one week of cell culture, there was very minimal or no detectable production of IL-6 or sRANKL from either purified B cells or mixed lymphocytes cultured with IL-33 alone or media only. In cultures of purified B cells, there was detectable production of IL-6 following culture with *E. coli* LPS, but this was not significantly altered by the addition of IL-33. In cultures of mixed lymphocytes, IL-6 and sRANKL were produced in response to both *P. gingivalis* LPS, and *E. coli* LPS, but again this was not altered by the addition of IL-33 (**Figure 6.2.15**).

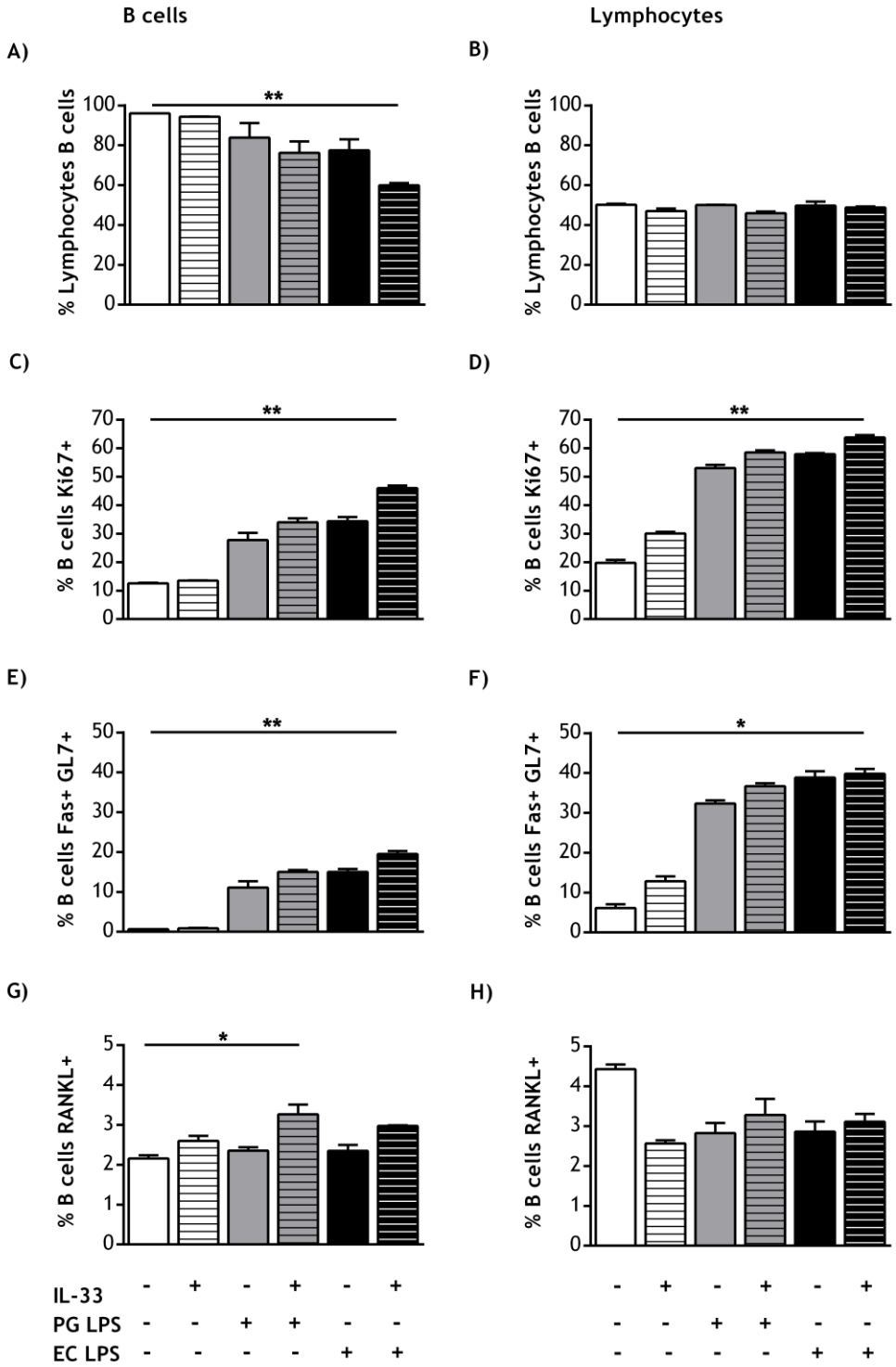

Figure 6.2.13. Phenotype of B cells after 3 day *in vitro* stimulation with *P. gingivalis* LPS and IL-33. B cells were purified (> 90 % $B220^+$) from the spleens and lymphocytes were isolated from LNs of mice. B cells **(A, C, E,** and **G)** and lymphocytes **(B, D, F,** and **H)** were cultured for 3 days in the presence of media only (unstimulated) or B cell stimulants (anti-CD40, anti-IgD, and IL-4) and *P. gingivalis* (PG) LPS or *E. coli* (EC) LPS with or without IL-33. At the end of the culture, the B cells were analysed by flow cytometry. Data are shown as mean with SEM for 3 wells of cells cultured in each condition. Significant differences were determined by Kruskal-Wallis with Dunn's multiple comparison post-hoc test (*P < 0.05, **P < 0.01).

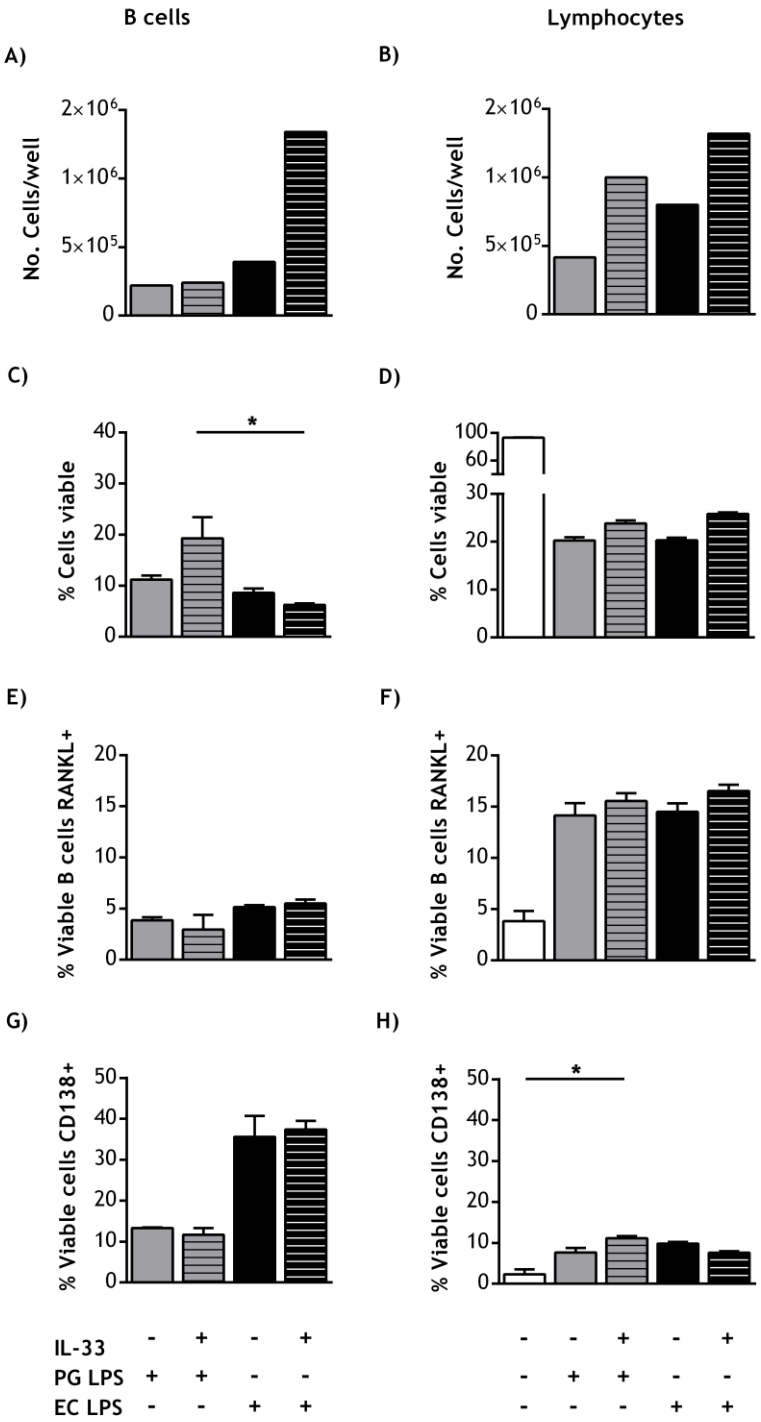

Figure 6.2.14. Phenotype of B cells after 1 week *in vitro* stimulation with *P. gingivalis* LPS and IL-33. B cells were purified (> 85 % $B220^+$) from spleens and lymphocytes were isolated from LNs of mice then cultured for 1 week in the presence of B cell stimulants (anti-CD40, anti-IgD, and IL-4) and *P. gingivalis* (PG) LPS or *E. coli* (EC) LPS with or without IL-33. Lymphocytes were additionally cultured with plate-bound anti-CD3. At the end of the culture, cells were analysed by flow cytometry. Freshly isolated lymphocytes were used as an unstimulated control for comparison. Cell counts in **A**) and **B**) are representative of 1 well. Data in **C – H**) are shown as mean with SEM for 3 wells of cells cultured in each condition, or 2 sets of unstimulated lymphocytes. Significant differences were determined by Kruskal-Wallis with Dunn's multiple comparison post-hoc test (*P < 0.05).

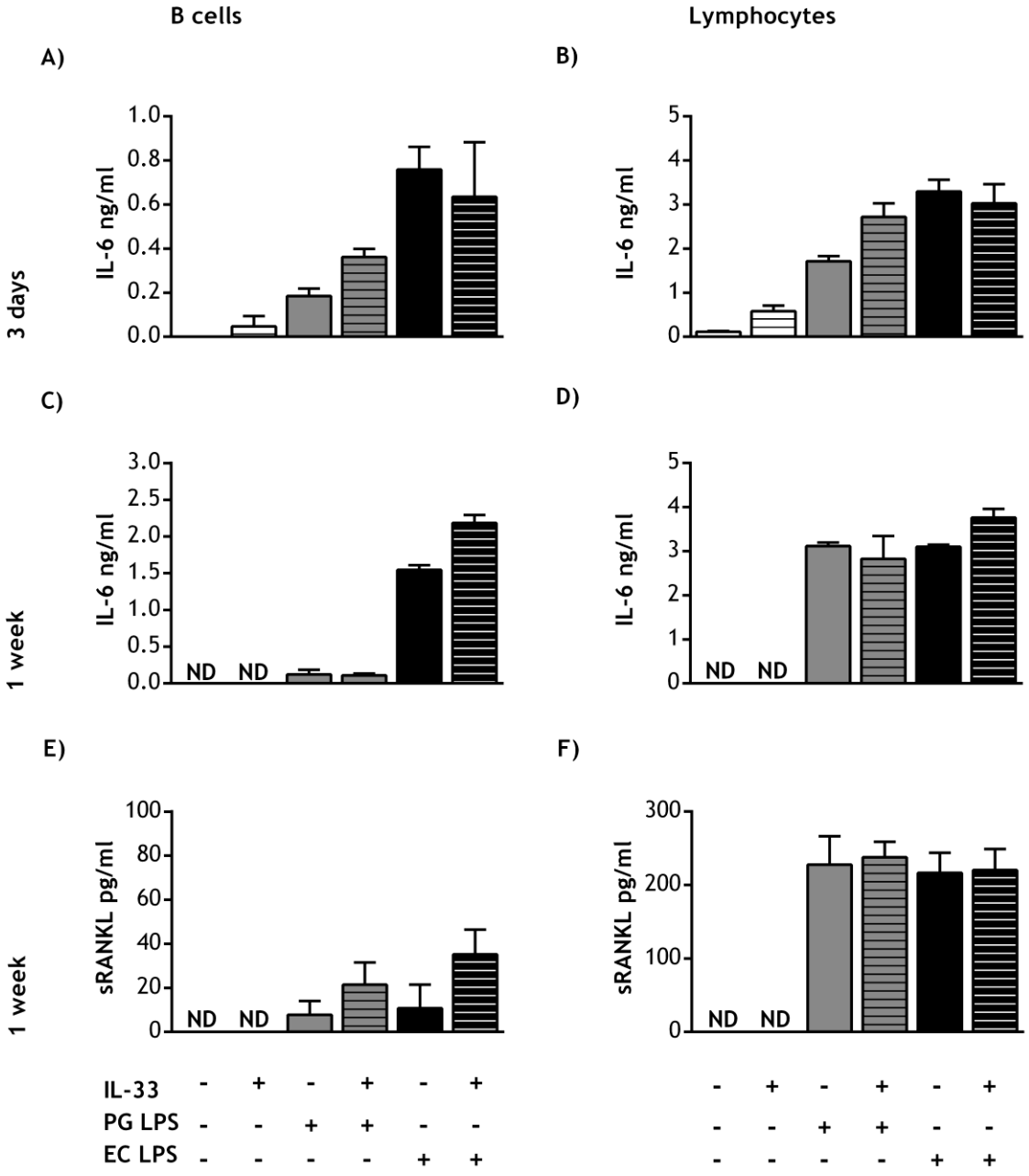

Figure 6.2.15. *In vitro* cytokine production by B cells after 3 day or 1 week stimulation with *P. gingivalis* LPS and IL-33. B cells were purified (> 85 % $B220^{+}$) from the spleens and lymphocytes were isolated from LNs of mice then cultured for 3 days or 1 week in the presence of media only (unstimulated) or B cell stimulants (anti-CD40, anti-IgD, and IL-4) and *P. gingivalis* (PG) LPS or *E. coli* (EC) LPS with or without IL-33. All lymphocytes were additionally cultured with plate-bound anti-CD3. At the end of the culture, the concentration of sRANKL and IL-6 in the culture media was measured by ELISA. Data are shown as mean with SEM for 2 wells of cells cultured in each condition. ND = no data available. Data were analysed by Kruskal-Wallis with Dunn's multiple comparison post-hoc test, and no significant differences were found.

Whilst IL-33 is associated with elevated B cell RANKL expression *in vivo*, *in vitro* studies indicate that IL-33 has limited direct effects on B cells, and that other mediators are involved in the induction of RANKL expression which remain to be identified. There was elevated B cell RANKL expression at one week post infection in IL-33 treated mice with PD, which appeared to correlate with increased alveolar bone loss at six weeks post-infection. As RANKL is known to be a critical mediator of osteoclastogenesis, it was therefore hypothesised that this phenomenon could be contributing to pathology. A second *in vitro* assay was designed to determine whether B cells from IL-33 treated mice could enhance sRANKL mediated osteoclastogenesis or induce osteoclastogenesis in macrophages (as described in **Chapter 2**, sections **2.10.2**, **2.11**, and **2.14**).

Mice received three treatments of IL-33 (0.8 µg) or vehicle control (0.1 % BSA in PBS) by intraperitoneal injection over the course of five days, and one week after the first treatment, B cells were purified from the mesenteric and inguinal LN. The mesenteric LN were selected as they drain the peritoneal cavity, and therefore would be likely to be strongly affected by the IL-33 treatment (Parungo et al., 2007).

Surprisingly, there was no difference in the expression of RANKL by B cells purified from IL-33 treated mice compared with PBS treated mice on this occasion (data not shown). Nonetheless, to determine if other B cell derived factors induced by IL-33 treatment may promote osteoclastogenesis, the purified B cells were added to cultures of bone-marrow derived macrophages or pre-osteoclasts at day three of differentiation. B cells from IL-33 treated mice significantly enhanced osteoclastogenesis in pre-osteoclasts (media 9.5 ± 1.32 vs. 17.19 ± 1.67 IL-33 B cells, *P < 0.05; B cells 8.00 ± 1.63 vs. 17.19 ± 1.67 IL-33 B cells, **P < 0.01, **Figure 6.2.16D**) but were unable to induce osteoclastogenesis in macrophages (data not shown). The exact mechanism behind the enhancement of osteoclastogenesis by B cells from IL-33 treated mice remains unclear. There was no difference in the concentration of sRANKL in the culture media from different conditions, and TNFα and IL-6 were undetectable (data not shown).

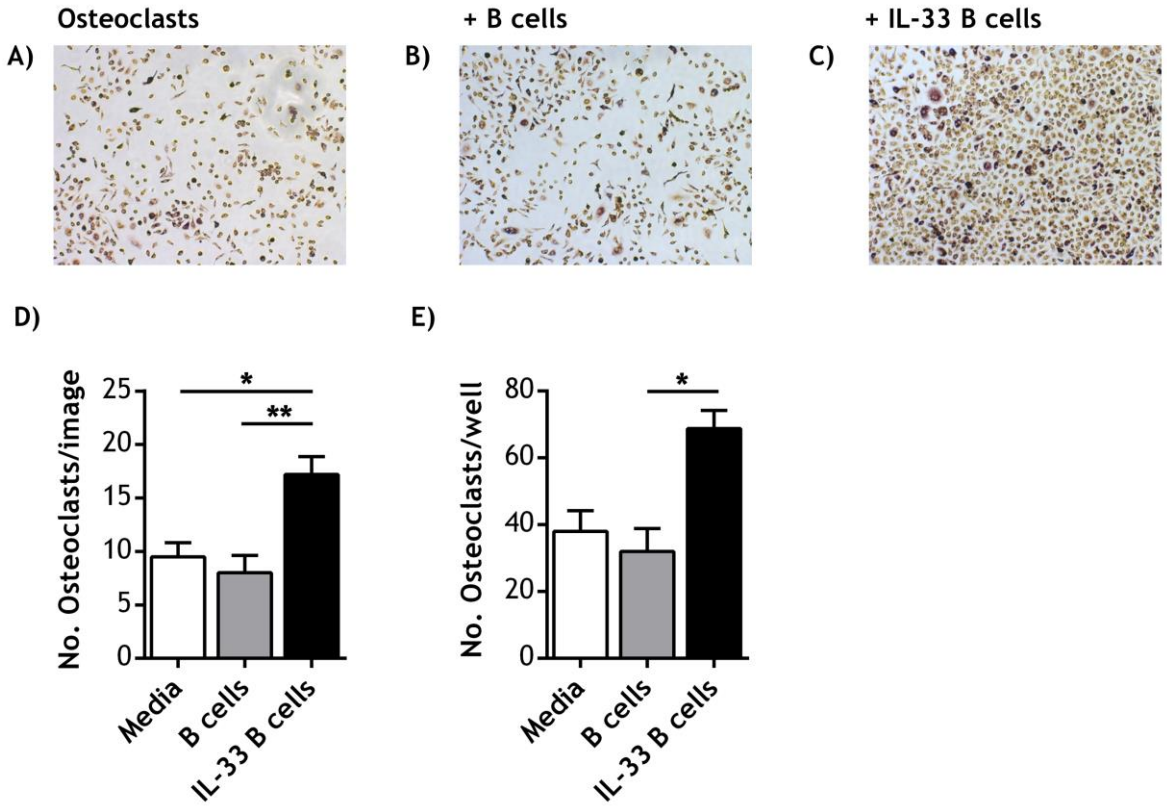

Figure 6.2.16. Osteoclastogenesis in pre-osteoclasts cultured with B cells from IL-33 treated mice. Mice received 3 treatments of IL-33 (0.8 µg) or vehicle control (0.1 % BSA in PBS) via intraperitoneal injection, over the course of 5 days. B cells were purified (> 85 % $CD19^+$) from inguinal and mesenteric LNs of mice treated with IL-33 (IL-33 B cells) and mice treated with vehicle control (B cells), and were added to pre-osteoclasts on day 3 of maturation. At day 7, TRAP staining was performed and the numbers of osteoclasts were counted in 4 images/well at X10 magnification. **A)** Representative image from 1 well of osteoclasts. **B)** Representative image from 1 well of osteoclasts that received B cells from PBS treated mice. **C)** Representative image from 1 well of osteoclasts that received B cells from IL-33 treated mice. **D)** Number of osteoclasts/image. **E)** Total number of osteoclasts/well. Data are shown as mean with SEM for 4 wells of osteoclasts cultured in each condition. Significant differences were determined by Kruskal-Wallis with Dunn's multiple comparison post-hoc test (*P < 0.05, **P < 0.01).

Summary of main results:

- IL-33 exacerbates alveolar bone loss in murine PD
- A higher proportion of B cells from the gingiva and dLNs of IL-33 treated mice with PD express RANKL at one week post-infection compared with PBS treated mice with PD and PBS treated sham controls
- IL-33 is not sufficient to induce B cell RANKL expression *in vitro*
- B cells from IL-33 treated mice enhance osteoclastogenesis *in vitro*

6.3 Discussion

These are the first data which indicate that IL-33 could have an important role in PD by demonstrating that IL-33 treatment exacerbated alveolar bone loss in mice with PD. It is thought that this could be mediated by RANKL. Very recently, IL-33 expression was found to be increased in the gingiva of rodents following induction of PD, and this was associated with increased RANKL expression in the gingiva (Koseoglu et al., 2014). A novel finding among the data presented in this chapter was that IL-33 treatment was associated with increased B cell expression of RANKL in the gingiva, dLNs, and peritoneal fluid at one week post-infection with *P. gingivalis*. IL-33 treatment, however, did not appear to have long-lasting effects on B cell expression of RANKL as no differences were observed in the gingiva, dLNs or peritoneal fluid at six weeks post-infection in IL-33 treated mice compared with PBS treated mice. The concentration of circulating IL-33 was not measured at these time-points but it is likely that by six weeks post-infection the level of exogenous IL-33 in circulation was significantly reduced and therefore no longer able to exert a strong effect.

Whether specifically B cell-derived RANKL expression is an important mediator of alveolar bone loss in IL-33 treated mice with PD, remains to be proven. Studies which validate the overall role of RANKL as a mediator of IL-33 exacerbated alveolar bone loss in murine PD have since been carried out (Malcolm et al., manuscript in press, University of Glasgow). In the first of these studies, an assessment of the overall balance of RANKL and OPG expression in the gingiva was made at one week post-infection, which established that there was not a compensatory increase the expression of OPG which is known to inhibit RANKL activity. In a subsequent study, mice with PD treated with IL-33 and OPG were found to be protected from IL-33 exacerbated alveolar bone loss.

In the results presented here, further experiments were conducted based on the hypothesis that B cell RANKL expression could result from B cell activation under particular circumstances, and that IL-33 could enhance this activation. Ideally, more extensive flow cytometry analysis would have been conducted to determine the activation state of B cells expressing RANKL *in vivo*. The limited number of cells isolated from the gingiva and dLNs made this impossible to

perform alongside B cell subset analysis. Only in the peritoneal fluid was B cell expression of the co-stimulatory molecule CD86 measured to obtain an indication of activation status - and there did not appear to be a strong relationship between IL-33 treatment, CD86 expression, and RANKL expression (data not shown). A relatively high proportion of B cells expressed CD86 in PBS treated sham controls and a comparatively small increase in CD86 expression was observed in mice treated with IL-33. $CD86^+$ B cells were also not significantly enriched for RANKL expression, which suggests that not all $RANKL^+$ B cells are activated by this definition, and that activation does not always result in RANKL expression – or that CD86 was not an appropriate marker for activation in this context. Assessment of additional indicators of activation such as the early activation marker CD69, or the other co-stimulatory molecule CD80, or the CD27 ligand CD70, or various adhesion molecules may have been informative (Lai et al., 1998).

Not all mature B cell subsets appeared to be equally affected by IL-33 treatment. The proportion of certain populations increased or decreased as a result of treatment. Many of the changes were not consistent between tissues or over time, but there was a general increase in the differentiation of B cells towards plasma cells. These observations concur with data from other studies that show IL-33 treatment augments the production of antibodies in mice, which presumably results from an increase in the number and activity of plasma cells (Xu et al., 2008, Xu et al., 2010). In an independent investigation, it was found that the titer of anti-*P. gingivalis* IgA was significantly increased in IL-33 treated mice with PD compared with PBS treated mice with PD; there was no increase in the overall production of anti-*P. gingivalis* IgG antibodies, but the titer of anti-*P. gingivalis* IgG1 was significantly increased. This was reflective of a shift in the Th response towards a predominantly Th2 response in IL-33 treated mice with PD (personal communication with Dr Jennifer Malcolm, manuscript in press, University of Glasgow). Follow-up studies could investigate the differences in functionality of the antibodies produced by IL-33 treated and PBS treated mice with PD, for example, their ability to opsonise bacteria.

It has been proposed that each of the B cell subsets have differential responsiveness to IL-33. B1 cells, particularly B1b cells have been shown to be

more responsive when assessed for proliferation, upregulation of ST2 expression, and IgM secretion following re-stimulation with IL-33 *in vitro*, which is thought to account for their expansion *in vivo* in the peritoneal fluid of IL-33 treated mice (Komai-Koma et al., 2011). In the data presented here, there was a small increase in the proportion of B1b cells in the dLNs at one week post-infection (**Figure 6.2.3C**), but this was less pronounced at six weeks post-infection (**Figure 6.2.4C**). In the peritoneal fluid, there was actually a decrease in the proportion of both B1a and B1b cells at one week post-infection (**Figure 6.2.9C** and **6.2.9D**). These contrasting results may be due to methodological differences with the research conducted by Komai-Koma *et al.* (2011); the dose and time-course of IL-33 treatment differed (they used 2 µg daily for 5-7 days), and B cells were analysed at an earlier time-point, just one day after the last treatment with IL-33. They also used different markers to distinguish the B cell subsets. B1a cells were classified as $CD19^+$ $CD11b^+$ $CD5^+$ and B1b cells as $CD19^+$ $CD11b^+$ CD5-.

Ideally, the differential proliferation, activation, and RANKL expression of distinct B cell subsets in response to IL-33 *in vitro* would have been analysed. It could be the case that only B1 cells respond directly to IL-33 and increase RANKL expression as a result. As B1 cells form a minority of the splenic B cell population this could explain why there was an insubstantial increase in the overall proportion of RANKL expressing B cells in response to IL-33. In view of this, the experiment could be repeated with an alternative source of B cells, such as LN, and with B cell subsets sorted and stimulated separately.

The principal finding from the three day *in vitro* assay was that despite significant activation of stimulated B cells, they did not exhibit an equivalent change in RANKL expression. It is logical that B cells would not automatically increase RANKL expression when activated by the stimuli tested otherwise all immune responses to infection would potentially result in bone erosion, whereas in reality this only occurs in pathological conditions like PD. However, this finding defies a number of claims that induction of RANKL expression in lymphocytes can be achieved using quite crude methods of stimulation for example: whole bacteria (Han et al., 2009, Belibasakis et al., 2011, Reddi et al., 2011), PMA and ionomycin (Fionda et al., 2007), anti-CD3, or anti-CD28 signalling

(Horwood et al., 1999). These mechanisms of stimulation and those implemented here all target the following signalling molecules that are purported to be involved in the induction of RANKL expression: MAPK, p38, PKC, PKA, and calcium (Takami et al., 2000, Oikawa et al., 2007, Reddi et al., 2011, Fujihara et al., 2014). These signalling molecules are central to many different pathways in almost all mammalian cell types. It is not known which adaptor molecules are acting upstream or which transcription factors are acting downstream of these mediators that are more specific to the regulation of RANKL expression. These data indicate that B cell expression of RANKL is more tightly regulated than earlier studies have implied, and highlight the need to fully characterise the signalling pathways involved.

The hypothesis that B cell RANKL expression can result from activation was subsequently modified to acknowledge that this must only occur under particular circumstances which are conducive to pathology. It was then further adapted to encompass the idea that RANKL expression increases with B cell maturation and differentiation following sustained activation. This was partly due to the observation made here that a relatively high proportion of plasmablast and plasma cell populations expressed RANKL *in vivo*. Other studies which informed this hypothesis included one in which an increase in the proportion of IgG expressing plasma cells was observed following a 10 day *in vitro* stimulation of lymphocytes with *A. actinomycetemcomitans*, and several that have found memory B cells are enriched for RANKL expression *in vivo* (Ehrhardt et al., 2005, Ehrhardt et al., 2008, Han et al., 2009).

A problem with performing long-term *in vitro* culture of primary cells is the difficulty in maintaining B cell viability for the entire period. The extremely low viability of the B cells in this case, particularly those in the purified B cell culture, has prevented more extensive analysis of B cell phenotype. One reason for the low viability could be that B cells were hyperactivated at an earlier stage of the culture leading to exhaustion, and death, due to inadequate availability of nutrients and survival factors. Further titration of each of the stimuli should have been performed and the lowest possible concentrations used for the one week culture to reduce the basal level of activation in each of the conditions. This could have not only improved the viability of the cells at the endpoint but

also have helped to distinguish the impact of IL-33 from that of the other stimuli. If a more robust long-term culture system could be established then further questions could be answered regarding the direct and indirect effects of IL-33 on B cell phenotype in the context of infection with *P. gingivalis*.

As well as acquiring a better understanding of the triggers of B cell phenotypic changes in PD and IL-33-exacerbated PD it needs to be demonstrated whether these changes, especially the increase in RANKL expression, make them more likely to cause alveolar bone loss. As a step towards achieving the latter goal, B cells were co-cultured with pre-osteoclasts, and osteoclast formation was assessed by TRAP staining. The B cells used in this assay were isolated from mice treated with IL-33 or PBS control, because IL-33 treatment alone had been shown to increase B cell RANKL expression in preceding experiments whilst attempts to elicit a high level of RANKL expression by B cells *in vitro* were unsuccessful.

In vivo exposure to IL-33 evidently effects B cells in such a way that they enhance osteoclastogenesis. Flow cytometry analysis confirmed that the B cells used in the co-culture were of a high purity and that therefore the effect observed on osteoclastogenesis was not due to contaminating cells. Furthermore, the CD19- fraction was found to inhibit osteoclastogenesis (data not shown). There is a possibility that a fraction of the B cells turned into osteoclasts themselves, as B1 cells have been reported to be capable of doing this (Pugliese et al., 2011). Whether pre-treatment with IL-33 would make B1 cells more likely to turn into osteoclasts is not known. To exclude this possibility, a culture of B cells without pre-osteoclasts but with the M-CSF and sRANKL containing media c ould have been set up.

This preliminary osteoclastogenesis study would ideally have been repeated using B cells isolated from the gingiva or dLNs of mice with IL-33 exacerbated PD which are more likely to be involved in directing alveolar bone loss and which are also likely to have a different phenotype to those B cells derived from peritoneal fluid – the full extent of which has yet to be described. A large number of mice would be required to obtain enough B cells for this from the gingiva – which may in part explain why no-one has done this before.

6.4 Conclusion

It has been demonstrated that IL-33 exacerbates alveolar bone loss in murine PD, and that this is associated with regional and systemic changes in B cell phenotype. Of particular interest is that IL-33 treatment was associated with increased expression of RANKL by B cells in the gingiva and dLNs. This, combined with preliminary data demonstrating that B cells from IL-33 treated mice were capable of enhancing osteoclastogenesis, indicate that B cells potentially have a role in mediating alveolar bone loss in murine PD. Further research is required to characterise the signalling pathways involved in B cell RANKL expression and to determine the relative importance of B cell-derived RANKL in regulating alveolar bone loss in PD. More fundamentally, it is critical to establish whether the net contribution of B cells in PD is pathogenic or protective - since other aspects of B cell behaviour such as the production of anti-*P. gingivalis* antibodies may confer protection in PD.

Chapter 7: Periodontitis in mice with a B cell deficiency

7.1 Introduction

It was shown in **Chapter 5** that the murine model of PD is associated with subtle, localised changes in B cell phenotype. In **Chapter 6** IL-33 was found to exacerbate alveolar bone loss in murine PD, and this was associated with more obvious and widespread changes in B cell phenotype. It is not possible to interpret from these data whether the observed changes in B cell phenotype affected the outcome of PD, whether individual B cell functions are pathogenic or protective, and whether the net contribution of B cells to PD is pathogenic or protective.

An important finding was that B cell RANKL expression in the gingiva and dLNs was increased in PD, and further increased in IL-33 exacerbated PD. RANKL is known to be a critical mediator of alveolar bone loss in PD, but whether B cells are a critical source of RANKL remains to be established (Yuan et al., 2011, Han et al., 2013). Other potential sources of RANKL in the periodontal tissues include fibroblasts, macrophages, and T cells (Kawai et al., 2006, Crotti et al., 2003, Belibasakis et al., 2011). A direct comparison of T cell RANKL expression with B cell RANKL expression in PD has not been made here, but in an independent investigation, it was found that a greater proportion of B cells expressed RANKL compared with T cells in the gingiva and dLNs of both mice with PD and mice with IL-33 exacerbated PD (Malcolm et al., manuscript in press, University of Glasgow). This same trend has also been observed in studies of lymphocytes in lesions of PD patient gingiva (Kawai et al., 2006). Therefore, although B cells may not be the only source of RANKL in PD, they are thought to be a prominent source of RANKL.

Although RANKL is the best characterised, there are other bone-resorptive factors - some of which are also produced by lymphocytes. Activated T cells, for example, are additionally capable of producing the osteoclastogenic cytokine SOFAT. SOFAT can induce osteoclastogenesis independently of RANKL, and potentially has a role in PD as its expression is increased in periodontal lesions (Rifas and Weitzmann, 2009, Jarry et al., 2013).

Various bone protective factors can block or balance the actions of bone resorptive factors. OPG is a soluble decoy receptor which binds RANKL and blocks its interacting with the receptor, RANK. It is the ratio of both these types of factors which regulates bone mass in the steady state. Little is known about whether lymphocytes, specifically those located in the gingiva, are responsible for the production of bone protective factors in health or in PD. Periodontal T cells have been ruled out as a source of OPG in the healthy periodontium, but that appears to be the extent of the knowledge on this subject (Belibasakis et al., 2011).

Other cytokines secreted by B cells may also contribute to PD. B cells are best known for their production of IL-6, which can promote the growth and differentiation of B cells and T cells, especially Th17 cells, as well as enhance osteoclastogenesis (Kurihara et al., 1990, Barr et al., 2012). It has been reported that the expression of IL-6 in gingiva, and the level of IL-6 in GCF and saliva is increased in PD patients compared with healthy individuals (Stefani et al., 2013, Ebersole et al., 2013, Reis et al., 2014). IL-6 KO mice were found to be protected from *P. gingivalis* infection-induced alveolar bone loss, confirming that IL-6 has a pathological role in PD (Baker et al., 1999b). Further validation came from a study in which PD patients, who also had RA, experienced a significant improvement in clinical symptoms of PD after being administered a drug that inhibited IL-6 signalling (Kobayashi et al., 2013b). B cells can also produce large quantities of the anti-inflammatory cytokine IL-10. B cell production of IL-10 in the early stages of infection can delay an effective immune response and clearance of a pathogen, but at later stages of an infection, IL-10 is often integral to the resolution of inflammation, and some argue that B cells are an underappreciated source of IL-10 in these scenarios (Couper et al., 2008, Horikawa et al., 2013, Liu et al., 2014). In murine models of colitis, SLE, and RA it has been demonstrated that B cell production of IL-10 impedes the initiation and promotes attenuation of chronic inflammatory disease caused by breach of immune tolerance and autoimmunity (Fillatreau et al., 2002, Mauri et al., 2003, Matsushita et al., 2008, Carter et al., 2012). In recognition of this role, certain IL-10 producing B cells have been to be referred to as Bregs (Mauri and Ehrenstein, 2008, Gray and Gray, 2010). The extent to which IL-10 production by B cells is determined by their subset lineage and by

the nature of a stimulus, has been debated (Ries et al., 2014). Nonetheless, a defect in this B cell-driven IL-10 feedback mechanism is another potential risk factor for chronic PD in humans. An imbalance between IL-6 and IL-10 is apparent in the early stages of murine PD. From data presented in **Chapter 5**, and published studies, it appears that the tendency for murine gingival cells to produce IL-6 is increased at one week post-infection with *P. gingivalis*, whilst the tendency to produce IL-10 is not changed at this time-point. Later post-infection, the tendency for gingival cells to produce IL-6 falls back to baseline whilst the tendency to produce IL-10 is increased (Kobayashi et al., 2011). As with RANKL, there are several possible cellular candidates that produce IL-6 and IL-10, and the contribution of B cells to the generation of these cytokines in PD may not be significant. Macrophages and mast cells are both capable of producing IL-6, although they are present in much smaller numbers than B cells in the gingiva of PD patients (Berglundh and Donati, 2005). Likewise, Tregs may produce IL-10, and it has been reported that the number of Tregs in the gingiva of mice infected with *P. gingivalis* is increased around four weeks post-infection (Kobayashi et al., 2011). Tregs have also been identified in the gingiva of PD patients, but they constitute a very small proportion of the cells analysed (Cardoso et al., 2009). A direct comparison of the relative contribution of B cells and other leukocytes to the production of these cytokines has yet to be made.

One function which is unique to B cells is the production of antibodies. Antibodies recognising PD-associated bacteria, including *P. gingivalis*, can be measured in PD patient sera, but there has been limited progress in characterising these antibodies further and establishing their ability to confer protection against disease. The generally unresolving nature of chronic PD implies that the ongoing antibody production in a patient with disease is ineffective or insufficient in managing the oral microbiome. Moreover, the anti-bacterial antibody response in PD is often accompanied by increased levels of local and circulating autoantibodies (Anusaksathien et al., 1992, De-Gennaro et al., 2006, Koutouzis et al., 2009, Molitor, 2009, Lappin et al., 2013). It is not known to what extent the kinetics of antibody generation and the properties of the antibodies generated in murine PD resembles the humoral response of PD patients. The studies of murine PD conducted so far have indicated that, in contrast to the human disease, the changes to B cell phenotype are short-lived,

with the majority of changes no longer detectable at six weeks post-infection, and no evidence of an autoantibody response at any time-point.

Performing detailed characterisation of the individual functions of B cells and assessing their relative contribution to PD pathogenesis presents a mammoth task. In order to help direct future investigations, the murine model of PD was performed in B cell-deficient μMT mice, to determine whether the net contribution of B cells to PD is pathogenic or protective. It was expected that B cell-deficient mice would be protected from *P. gingivalis* infection-induced alveolar bone loss if B cells have a predominantly pathogenic role in PD, or that B cell-deficient mice would have exacerbated alveolar bone loss if B cells have a predominantly protective role in PD.

7.2 Results

B cell precursors in μMT mice are unable to express the membrane-bound form of IgM, and are therefore unable to mature in response to BCR signalling. Consequently, μMT mice lack mature B cells and plasma cells (Kitamura et al., 1991). B cell-deficient μMT mice and WT (C57BL/6) mice were infected with *P. gingivalis* or sham-infected and alveolar bone loss was measured at six weeks post-infection (as described in **Chapter 2**, section **2.7**). As demonstrated in previous studies with BALB/c mice, *P. gingivalis*-infected WT mice exhibited alveolar bone loss relative to the WT sham controls (-0.30 ± 0.0079 mm WT sham vs. -0.32 ± 0.0048 mm WT PD, **P = 0.0054, **Figure 7.2.1**). Unexpectedly, μMT sham controls had less alveolar bone than WT sham controls (μMT sham -0.34 ± 0.0048 mm vs. -0.30 ± 0.0079 mm WT sham, ***P = 0.0006, **Figure 7.2.1**) but μMT mice appeared to be protected from the pathological alveolar bone loss associated with *P. gingivalis* infection (μMT sham -0.34 ± 0.0048 mm vs. -0.34 ± 0.0045 mm μMT PD, **Figure 7.2.1**). These data were independently verified by Dr Annelie Hellvard and Birth Bergum (Broegelmann Research Laboratory, University of Bergen, Norway) who performed a blinded assessment of alveolar bone level using X-ray micro-CT (data not shown).

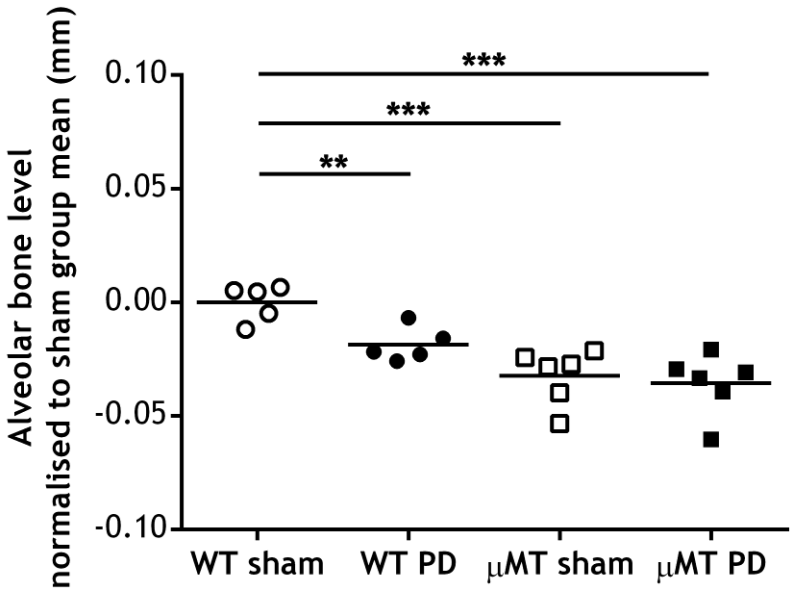

Figure 7.2.1. Alveolar bone level in B cell-deficient μMT mice infected with PD. B cell-deficient (μMT) and wild-type (WT) mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 6 weeks post-infection, the alveolar bone level was measured. Data are shown as mean per mouse (symbols) and mean for each group of mice (lines) relative to the sham group mean, n = 5 mice/group for WT, n = 6 mice/group for μMT. Significant differences were determined by unpaired t test, as indicated on the graph (**P < 0.01, ***P < 0.001).

To validate that μMT mice were B cell-deficient and assess the impact of this on the adaptive immune response to infection with *P. gingivalis*, the lymphocyte composition of the dLNs was analysed by flow cytometry, and serum titers of anti-*P. gingivalis* IgG antibodies were quantified by ELISA (as described in **Chapter 2**, sections **2.10.2**, **2.15.2**, and **2.16.1**). Similar to previous analyses of B cells in the dLNs in murine PD, the total number of B cells was significantly increased in the dLNs of the WT mice with PD group relative to WT sham controls (3,367,820 ± 1,010,000 sham vs. 8,320,240 ± 712,126 PD, ***P < 0.001, **Figure 7.2.2C**), whilst the numbers of B cells present in the dLNs of μMT mice were negligible, irrespective of infection status. T cell populations were also altered in the dLNs of μMT mice. There was a trend towards increased total numbers of $CD4^+$ T cells in μMT mice compared with WT mice, but unlike WT mice with PD, μMT mice with PD did not have a significant increase in the proportion of $CD4^+$ T cells that were $CD44^+$ $CD62L^-$ effector T cells relative to their corresponding sham control group (10.01 ± 0.25 % WT sham vs. 12.46 ± 0.45 % WT PD, *P < 0.001, **Figure 7.2.2F**). These defects of μMT mice translated to an inability to generate class-switched antibodies specific for *P. gingivalis*. As expected, only the WT *P. gingivalis* infected mice had detectable titers of serum anti-*P. gingivalis* IgG (629.62 ± 64.06 EU WT PD vs. 1.73 ± 1.73 EU WT sham, *P < 0.05).

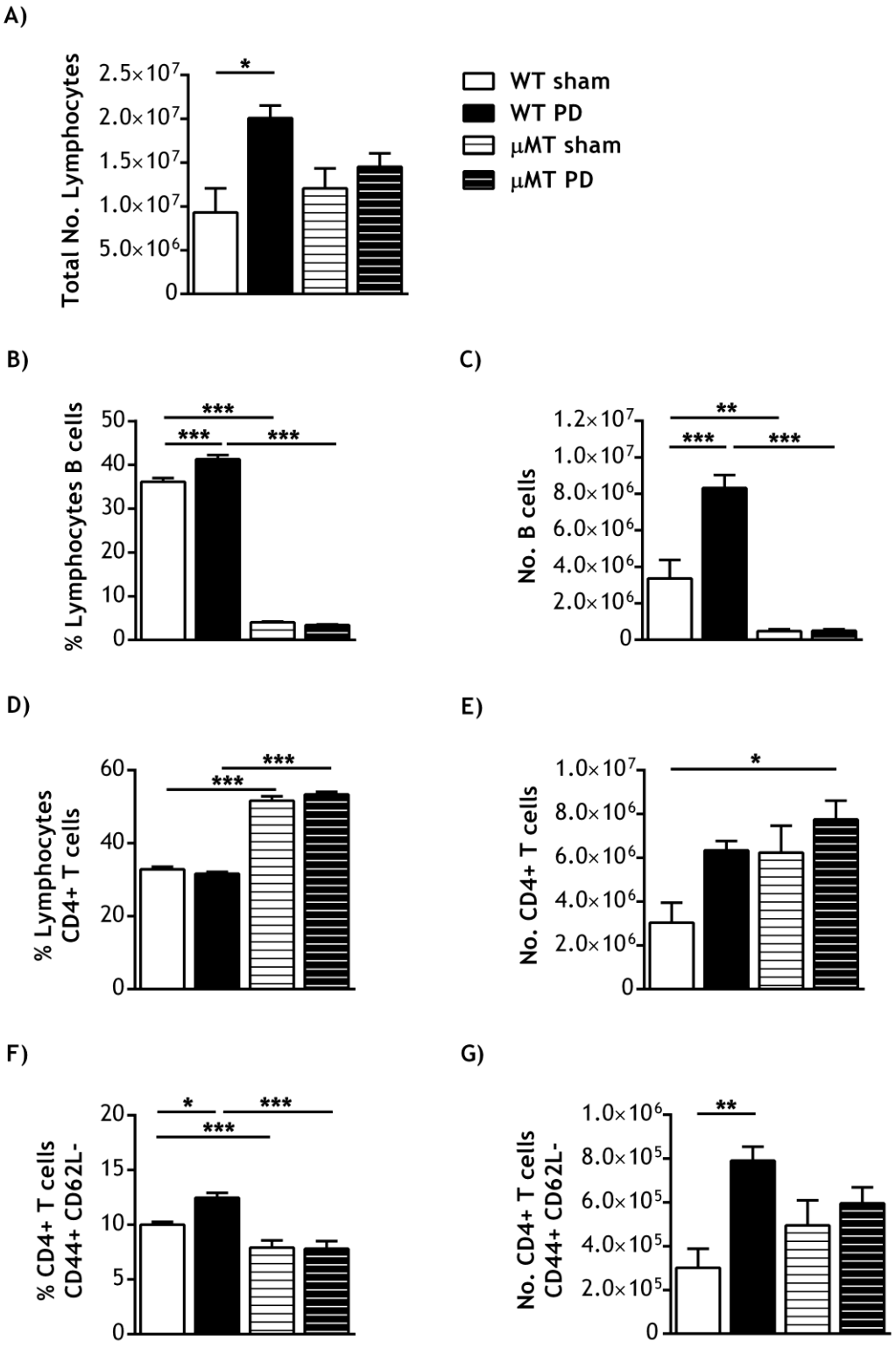

Figure 7.2.2. Lymphocytes in the dLNs of B cell-deficient μMT mice infected with *P. gingivalis*. B cell-deficient (μMT) and wild-type (WT) mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 6 weeks post-infection, lymphocytes were isolated from the dLNs and were analysed by flow cytometry. Data is shown as mean with SEM, n = 5 mice/group for WT, n = 6 mice/group for μMT. Significant differences were determined by One-Way ANOVA with a Tukey post-hoc statistical test (*P < 0.05, **P < 0.01, ***P < 0.001).

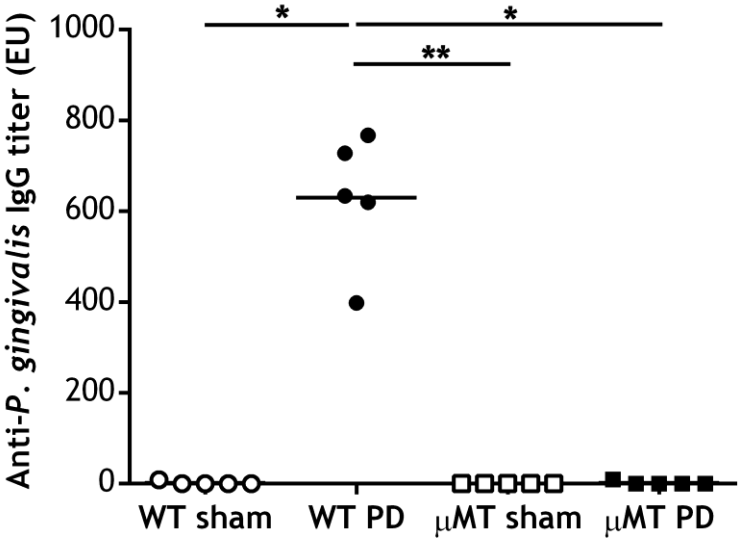

Figure 7.2.3. Serum anti-*P. gingivalis* IgG titers in B cell-deficient μMT mice infected with *P. gingivalis*. B cell-deficient (μMT) and wild-type (WT) mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 6 weeks post-infection, anti-*P. gingivalis* IgG titers in the serum were measured by ELISA. Data are shown as mean with SEM, n = 5 mice/group for WT, n = 6 mice/group for μMT. Significant differences were determined by Kruskal-Wallis with Dunn's multiple comparison post-hoc statistical test (*P < 0.05, **P < 0.01).

As WT and μMT mice with PD were infected with *P. gingivalis* from the same stock, it must be assumed that both groups were successfully infected and that the lack of anti-*P. gingivalis* IgG in the serum of μMT PD mice is due to their B cell deficiency. A *P. gingivalis*-specific 16S sequence could not be detected in the DNA extracted from oral swabs of WT or μMT mice by qRT-PCR, preventing confirmation of successful colonisation. This suggests that there were fewer than 1,000 *P. gingivalis* CFU present in total in each mouse's mouth, as this is the lowest possible detectable number by this method. This inability to detect *P. gingivalis* was unrelated to the late time post-infection, as the same result was achieved with oral swabs from BALB/c mice at one week post-infection. To assess the possibility that the lower level of bone loss in μMT mice was related to differences in the overall bacterial load, the expression of a universal sequence within the 16S gene (found in almost all species of both Gram negative and Gram positive bacteria) was quantified. Interestingly, there were fewer bacteria in the oral swabs from μMT mice than from WT mice (WT sham 20.86 ± 1.12 vs. μMT sham 25.03 ± 0.61; WT PD 20.99 ± 0.71 vs. 25.73 ± 0.71 μMT PD, **P < 0.01, **Figure 7.2.4**). The bacterial load was not affected by infection status in either WT or μMT mice.

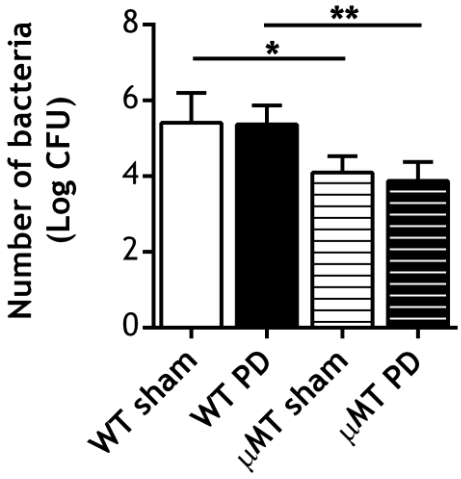

Figure 7.2.4. Oral bacterial load in B cell-deficient µMT mice infected with *P. gingivalis*. B cell-deficient (µMT) and wild-type (WT) mice were infected with *P. gingivalis* (PD) or sham-infected (sham). At 6 weeks post-infection, oral swabs were taken, total bacterial DNA was isolated from these and qRT-PCR of a universal genomic 16S sequence was performed. With the Ct values of 16S amplification the number of bacteria determined from a standard curve of known CFU and Ct values of Gram negative and Gram positive bacteria. Data are shown as mean with SEM, n = 5 mice/group. Significant differences were determined by One-Way ANOVA with a Tukey post-hoc statistical test (*$P < 0.05$, **$P < 0.01$).

Summary of main results:

- B cell-deficient µMT mice were protected from *P. gingivalis* infection-induced alveolar bone loss
- Sham-infected B cell-deficient µMT mice had greater age-associated alveolar bone loss than sham-infected WT controls
- B cell-deficient µMT mice had a lower bacterial load in the oral cavity compared with WT mice

7.3 Discussion

Despite there being a long-standing awareness of the predominance of B cells in the gingiva of PD patients (Berglundh and Donati, 2005), functional studies of B cells in PD have been lacking. Until now, limited use has been made of transgenic mice or modified murine models to investigate, explicitly, the role of B cells in PD. Here, B cell-deficient μMT mice were employed to determine whether or not B cells have an impact on the pathogenesis of PD, and whether their role is predominantly pathogenic or protective.

These data show that B cell-deficient μMT mice were protected from *P. gingivalis* infection-induced alveolar bone loss, and this was associated with an inability to generate class-switched IgG antibodies specific for *P. gingivalis* (**Figure 7.2.1** and **7.2.3**). The WT control mice exhibited relatively modest alveolar bone loss in response to oral infection with *P. gingivalis*. This is likely due to the C57BL/6 background strain of the mice used here. Both these (**Chapter 5**) and other (Baker et al., 2000b, Shusterman et al., 2013) studies indicate that BALB/c mice have greater genetic susceptibility to alveolar bone loss in response to oral infection with *P. gingivalis* compared with C57BL/6 mice. The genes behind this susceptibility are likely to underpin certain aspects of the host immune response. It is known that C57BL/6 mice are prone to making Th1 responses whereas BALB/c mice are prone to making Th2 responses. T cells from C57BL/6 mice preferentially produce IFN_{γ} over IL-4 whereas T cells from BALB/c mice preferentially produce IL-4 over IFN_{γ} (Hsieh et al., 1995). These two mouse strains also have different MHC II haplotypes. C57BL/6 mice have MHC II haplotype *b*, and BALB/c mice have MHC II haplotype *d*. The different array of MHC II alleles within each haplotype could affect the types of *P. gingivalis* antigens which can be presented by APCs, which in turn could determine the efficacy of the adaptive immune response.

B cell-deficient $J^{H}D$ mice may be considered a more desirable choice for the investigation of the impact of B cell deficiency on PD pathogenesis. These mice have a germline deficiency in the J^{H} segments required for rearrangement of the BCR during B cell development. They possess pre-B cells in the BM but lack mature B cells in the periphery and, like μMT mice, are unable to produce antibodies (Chen et al., 1993). Crucially, $J^{H}D$ mice are bred on the PD

susceptible BALB/c background. However, only μMT mice were available for this study.

The data from this study imply that B cell-deficient μMT mice, in addition to being protected from *P. gingivalis* infection-induced alveolar bone loss, had altered dLNs composition, less alveolar bone level at baseline, and reduced bacterial colonisation of the oral cavity as a consequence of the germline defect. Each of these features could have in some way affected the outcome of *P. gingivalis* infection.

B cells have an important role as APCs in the priming of cognate $CD4^+$ T cells and are also required for the maintenance of an effector $CD4^+$ T cell population (Macaulay et al., 1997, Crawford et al., 2006, Mollo et al., 2013). As key producers of lymphotoxin and RANKL, B cells also have an important role in dictating the development and structural remodelling of LN and spleen (Kong et al., 1999b, Tumanov et al., 2002, Tumanov et al., 2003, Tumanov et al., 2004, Kumar et al., 2010, Abe et al., 2012a). The structure of LN in health and inflammation is thought to influence the motility of, and interactions between, T cells and APCs (Bajenoff et al., 2006, Beltman et al., 2007). As a consequence of these pivotal roles of B cells, any method of B cell depletion or inhibition of antigen-specific B cell responses will impede the adaptive $CD4^+$ T cell response to bacterial infection. Therefore, it is not possible to separate the contribution of a reduction in the T cell population to the PD resistance from the contribution of B cell-deficiency. However, both IgD KO mice, and now μMT mice have been shown to be completely protected from *P. gingivalis* infection-induced alveolar bone loss. In comparison, C57BL/6 Tcra mice - which are T cell-deficient due to targeted deletion of the α chain of the TCR - are only partially protected (Baker et al., 2002).

Although *P. gingivalis*-infected μMT mice did not exhibit alveolar bone loss relative to sham-infected μMT mice, both groups of μMT mice had less alveolar bone than their WT counterparts. This could result from the requirement for B cells to regulate basal bone turn over. In the BM, B cells have homeostatic roles in bone growth and repair and are known to be a major source of OPG, with 45 % of total OPG in the BM produced by mature B cells (Marusic et al., 2000, Li et

al., 2007, Manilay and Zouali, 2014). B cell-deficient μMT mice have low levels of OPG in the BM and a correspondingly low BMD, a phenotype that can be rescued by transfer of WT B cells (Li et al., 2007). These features could have been verified here by assessing the relative expression of OPG in the BM of the long bones, the alveolar bone, and in the gingiva by qRT-PCR, and by checking for global defects in the skeleton of μMT mice using high resolution imaging such as micro-CT. It is not obvious how a difference in BMD would affect the alveolar bone level, but it may be the case that there is a trend exists in μMT mice whereby all many bones are shorter or smaller as a result of increased bone turnover.

Another way in which cells of the B cell lineage may maintain alveolar bone level in the steady state is via plasma cell production of salivary IgM and IgA. These low-affinity, cross-reactive antibodies can help to regulate the number and nature of commensal bacteria colonising the tooth surface and prevent non-commensal bacteria from joining the dental biofilm (Kilian et al., 1981, Hajishengallis et al., 1992, Ito et al., 2012, Lonnermark et al., 2012, Brandtzaeg, 2013).

A lower bacterial load was detected in the oral cavity of B cell-deficient μMT mice compared with WT mice (**Figure 5.4**). In contrast to expectations, *P. gingivalis* infection did not appear to change the bacterial load in either B cell-deficient μMT mice or WT mice. Previous research has clearly demonstrated that oral infection of mice with *P. gingivalis* results in an increase in the total number of bacteria (Hajishengallis et al., 2011). The reason behind these contradictory findings is unclear. The key difference in the methodology between the present study and the previous study is that the previous study extracted bacterial DNA from the periodontal tissues rather than from oral swabs. It would be interesting to assess the composition of different species of bacteria within these samples by high-throughput sequencing to establish if there were qualitative differences in the species present in the oral cavity of B cell-deficient μMT mice that related to the difference in alveolar bone level.

Some of the unwanted side-effects of germline B cell-deficiency may be avoided in an inducible model of B cell-deficiency. One example of such a model is the

depletion of B cells in the humanised CD20 mouse strain using the anti-CD20 antibody Rituximab. B cells could be depleted days before infection with *P. gingivalis*. However, this method would still have a short-term impact on the T cell compartment (Misumi and Whitmire, 2014) and there may be additional repercussions that have not yet been published as the mechanism of B cell depletion by Rituximab is not fully understood.

An alternative to depleting all B cells, would be to prevent just the *P. gingivalis*-specific B cell responses by utilising transgenic mice whose B cells can only recognise an unrelated antigen such as MD4 mice whose BCRs all bind HEL with high affinity (Adams et al., 2003). Antigen-specific activation of B cells may be required for the induction of RANKL expression and maximal production of other inflammatory cytokines, as well as the proliferation of GC B cells in the dLNs and the generation of anti-*P. gingivalis* antibodies. The drawback of this method - as with the other models of B cell-deficiency described - is that *P. gingivalis*-specific T cell responses would also be altered.

7.4 Conclusion

This study demonstrated that B cell-deficient μMT mice were protected from *P. gingivalis* infection-induced alveolar bone loss, defining a pathological role for B cells in murine PD. Future studies can aim to unravel which of the B cell functions are responsible for pathology, and if there is a particular B cell subset which is more strongly associated with pathology. This could inform the development of biological therapies in the future for PD patients that exhibit a predominance of B cells in their periodontal lesions.

Chapter 8: General Discussion

The studies presented in this thesis shared the overall aim of acquiring a better understanding of the immunopathology of PD. Based on previous reports indicating that they may have an important role in PD, B cells, plasma cells, and their products were the focus of these studies. There is a growing body of evidence that indicates the immunopathology of PD is linked to the immunopathology of systemic diseases, including RA, although the exact mechanisms have yet to be fully elucidated. Here, the roles of B cells in PD, and their potential roles in linking PD to RA, will be discussed in the context of the new data gathered.

Some of the B cell functions which potentially contribute to PD, including the production of RANKL, IL-6, and autoantibodies are summarised in **Figure 8.1**. The studies performed sought to investigate a number of these. It was confirmed that B cells and IgG producing plasma cells could be found in PD patient gingiva (**Chapter 3**). The main novel finding from the human studies was that titers of ACPAs were higher in the sera of PD patients than in periodontally healthy participants (**Chapter 4**). Following successful non-surgical periodontal treatment, ACPA titers were reduced in PD patients. This suggests a relationship between disease activity and perhaps bacterial burden, and the dysregulated immune response in PD. There is a further implication that the treatment of PD might help to prevent the development of autoimmunity. Assuming that ACPAs play a pathogenic role in RA, it is conceivable that management of PD in patients with RA may provide a low-risk, simple, and cost-effective adjunct to other therapies, by slightly reducing, or altering the on-going ACPA response.

As in previous studies of PD patients, the number and type of patient samples available restricted the investigations conducted here, and larger studies would be required to confirm these findings. To maximise the total size of the study population, future studies could involve collaborations between multiple centres. A wider pool of study participants would potentially enable the assessment of autoantibodies in PD patients whose history of dental health has been well documented. This would mean that PD patients thought to have a similar form of PD, and be at a similar stage of disease, could be grouped

together for analysis. The systemic consequences of PD are likely to relate to the severity and rate of progression or activity of PD. There are established criteria for categorising severity of PD, but these have not been uniformly applied across published studies (Preshaw, 2009). Moreover, there is currently no accepted method of categorising the rate of progression or activity of PD, which adds to the complexity of comparing many small-scale studies.

For assessing the role of a single cell type or cell product in PD, the murine model of PD can provide a convenient alternative to longitudinal studies of PD patients, permitting assessments of immunopathology at different stages of disease. Here, it was employed to perform more detailed assessments of B cells, plasma cells, and antibody responses. As with many disease models, the murine model of PD established by Baker *et al.* (1994) is a far from perfect representation of the human condition. In these studies, BALB/c mice consistently exhibited alveolar bone loss following oral infection with *P. gingivalis*, but the local immune response associated with this appeared to be weak, variable, and self-limiting.

In the gingiva of *P. gingivalis* infected mice there was a small, transient, increase in B cell expression of RANKL, but no change to the overall proportion of B cells present (**Chapter 5**). In the dLNs of *P. gingivalis* infected mice there was an expansion of the B cell population, but no difference in the secretion of IL-6 by cells in this tissue (**Chapter 5**). In contrast, patients with chronic PD suffer persistent inflammation of the periodontal tissues. This is characterised by extensive leukocyte infiltrate, consisting predominantly of B cells, accompanied by elevated levels of IL-6 and other inflammatory cytokines in GCF, saliva, and serum (Berglundh and Donati, 2005, Gumus et al., 2013b). The findings in the gingiva may reflect a true difference in the local immune response in the murine model, or represent a caveat of analysing leukocyte infiltrate in the entire gingiva and palate, instead of a gingival lesion near the worst-affected tooth - as is performed in human studies.

Another way in which observations of the murine model of PD conflicted with reports of PD patients was in the lack of changes to the phenotype of circulating B cell populations or in serum autoantibody titers (Anusaksathien et al., 1992,

De-Gennaro et al., 2006, Koutouzis et al., 2009, Hendler et al., 2010, Lappin et al., 2013). In spite of these differences between PD pathogenesis in mice and humans, preliminary evidence that B cell-deficient μMT mice were protected from *P. gingivalis* infection-induced alveolar bone loss suggests that targeting B cells may be a promising avenue in the development of immunotherapies for severe PD (**Chapter 7**).

Potential follow-up studies pertinent to each set of results presented have been discussed in detail within the preceding chapters. In addition, future studies of the role of B cells - and other components of the immune system - in PD may benefit from adapting the murine model to amplify local inflammation and establish greater chronicity, so that it more closely resembles a form of human PD. Furthermore, animal studies should ideally consider the relevant risk factors of human PD that are likely to alter immune regulation.

Several genetic risk factors for PD are linked to the expression of cytokines (Karimbux et al., 2012, Li et al., 2014b, Zhang et al., 2014a). Altering the balance of cytokines in mice is one way in which the immune response to infection with *P. gingivalis* may be modified. This approach was adopted in **Chapter 6**. As in many *in vivo* studies, the cytokine-axis was modulated by intraperitoneal delivery of recombinant cytokine - in this case IL-33. This method alters the cytokine-axis beyond normal physiological parameters and therefore, the associated results must be interpreted cautiously. An alternative method of disrupting the balance of cytokines would be to generate mice possessing genetic defects that reflect those found in PD patients. For example, specific polymorphisms in the genes encoding IL-1, the IL-1 receptor, and the IL-1 receptor antagonist have been associated with chronic PD (Tai et al., 2002, Lopez et al., 2009, Karimbux et al., 2012, Deng et al., 2013, Hao et al., 2013). It is thought that these defects lead to an increase in IL-1 production or IL-1-mediated signalling (Kornman et al., 1997, Engebretson et al., 1999). Recapturing the subtleties of different combinations of polymorphisms in a murine models is extremely challenging, hence researchers tend to utilise readily available KO mouse strains such as the IL-1 receptor antagonist KO mice, or treat with exogenous cytokine (Izawa et al., 2014).

The two most common environmental risk factors for PD are smoking and obesity (Tomar and Asma, 2000, Chaffee and Weston, 2010). Both of these factors dysregulate the immune response and increase susceptibility to bacterial infection, albeit in different ways. Smoking is associated with the suppression of cytokine, chemokine, and antibody production (Tangada et al., 1997, Graswinckel et al., 2004, Apatzidou et al., 2005, Tymkiw et al., 2011, Giuca et al., 2014, Haytural et al., 2014, Souto et al., 2014a). Obesity on the other hand is associated with systemically elevated levels of cytokines (Ziccardi et al., 2002, Crowther et al., 2006, Illan-Gomez et al., 2012, Zimmermann et al., 2013, Genoni et al., 2014).

The ethics of exposing animals to tobacco smoke are questionable, and, in the context of the current experiments this could not be justified in line with the UK Home Office legislation to replace, refine, and reduce animal research. In comparison, obesity is not considered to be as detrimental to animal welfare, and several obesity models are in widespread use. In some studies, obesity is induced by the administration of a high-fat diet. Other studies utilise strains of mice genetically predisposed to developing a form of metabolic syndrome. Mice with diet-induced obesity have been demonstrated to have greater alveolar bone loss following oral infection with *P. gingivalis*, compared with non-obese controls (Amar et al., 2007). Similarly, Tallyho/JngJ mice - which spontaneously develop hyperlipidemia and hyperglycaemia - exhibit greater alveolar bone loss than WT controls following infection with *P. gingivalis* (Li et al., 2013). Although it has yet to be demonstrated, the periodontal pathology associated with these models of obesity is likely to be associated with an altered inflammatory response in the gingiva. Interestingly, B cell-deficient obese mice are protected from *P. gingivalis* infection-induced alveolar bone loss (Zhu et al., 2014). This supports the concept that B cells have an important, pathological role in PD. Moreover, this indicates that a murine model of PD, exacerbated by obesity, may be useful for further dissecting the mechanisms by which B cells can contribute to pathology in PD.

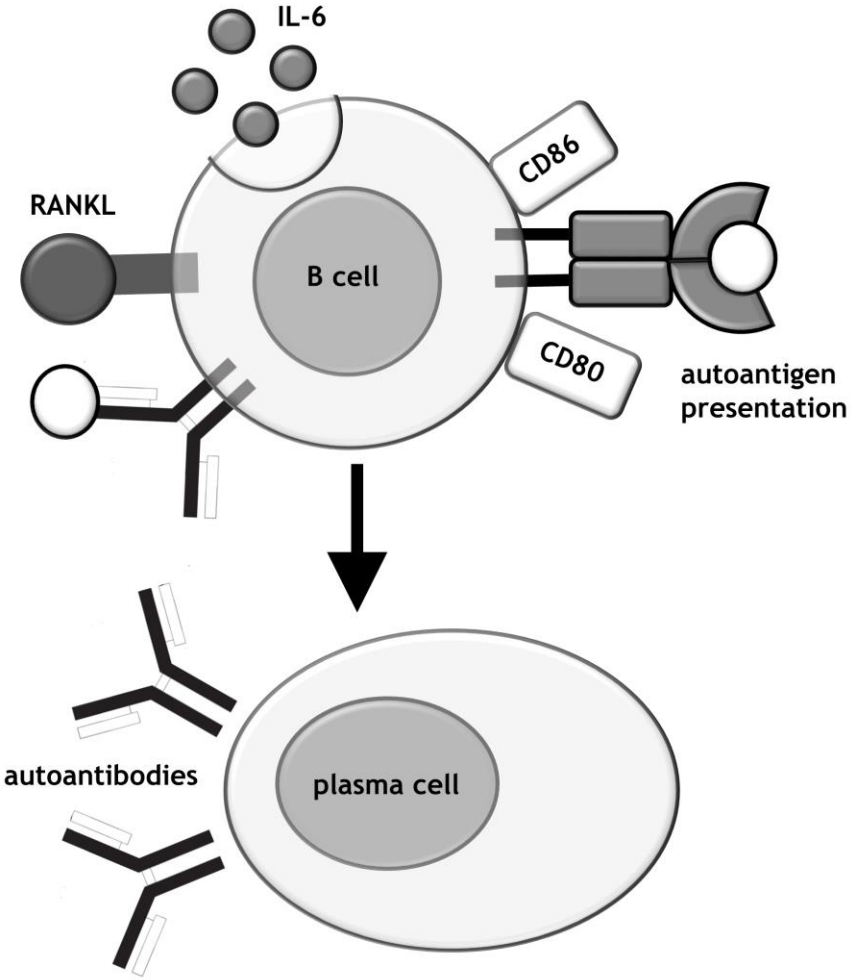

Figure 8.1. B cell and plasma cell functions which may contribute to PD.
B cell production of cytokines and presentation of autoantigens to T cells along with plasma cell production of autoantibodies may contribute to the immunopathogenesis of PD.

With respect to the role of B cells in PD, a number of questions have arisen from the findings in the studies conducted here, which may be addressed by future studies. Firstly, whether B cell-derived RANKL makes a significant contribution to alveolar bone loss in PD could be answered by attempting to induce PD in conditional *CD19 RANKL* KO mice, whose B cells are unable to express RANKL (Onal et al., 2012). The relative contribution of B cell-derived RANKL could be compared with that of T cell-derived RANKL by using conditional *Lck RANKL* KO mice, whose T cells are unable to express RANKL (Fumoto et al., 2014). Similarly, B cell IL-6 KO mice could be employed to establish the relative importance of B cell-derived IL-6 in the immunopathology of PD. This mouse

strain has previously been used to show that IL-6 production by B cells is fundamental in the pathogenesis of EAE (Barr et al., 2012).

More challenging than investigating whether specific B cell functions contribute to alveolar bone loss in PD would be establishing whether individual B cell subsets make a differential contribution to pathology. Therapeutically, more specific targeting of immune components can help to reduce unwanted side-effects of immunosuppression. It was demonstrated that, in both mice with PD and in sham controls, the B1 subsets of mature B cells consistently had a higher proportion of RANKL expressing B cells than the other subsets, in all the tissues analysed (**Chapter 5** and **Chapter 6**). It is possible that subsets which have a higher proportion of RANKL expressing B cells, make a greater contribution to pathology in PD than others. Ideally to explore this possibility, individual subset populations would either be depleted, activated, or enriched *in vivo*. However, it would not be possible to deplete specific subsets of B cells as there is no single surface molecule which could distinguish a given subset from other B cells, as well as from other leukocytes. Equally, it would not be possible to enhance the activation of a specific B cell subset as all B cells respond to broadly the same stimuli, although there may be variations in the strength of the response. Adoptive transfer experiments could be performed to enrich a given B cell subset population in the recipient mouse during the induction of PD. Ensuring that the B cells transferred trafficked to relevant sites such as the gingiva and dLNs would be difficult. Ideally B cells specific for a *P. gingivalis* antigen would be used for this purpose. As previously discussed, a transgenic *P. gingivalis* strain expressing HEL could theoretically be recognised by B cells from MD4 mice (Goodnow et al., 1988, Adams et al., 2003). So far, attempts to create such a bacterial strain have been unsuccessful. An alternative, simpler, approach to answering this question would be to perform *in vitro* assays in which the ability of purified B cell subsets to enhance osteoclastogenesis in pre-osteoclasts is compared. Ultimately, the results from such *in vitro* assays would be of limited value for interpreting the relative contribution of individual B cell subsets *in vivo* as the latter would be greatly determined by the frequency and location of the cells.

Once the contribution of specific B cell functions to the immunopathogenesis of PD have been more clearly established, researchers will be in a better position to investigate the ways in which these B cells potentially contribute to the link between PD and systemic diseases, such as RA. There are three possible routes that could account for the apparent relationship between PD and RA, and these are summarised in **Figure 8.2**. B cells may play a part in each of these scenarios.

One theory proposes that in some patients, PD may precede and perhaps even cause RA. Hypothetically, PD alone could be sufficient to cause RA (Panel 1, **Figure 8.2**). As previously described in **Chapter 1**, this theory is based on the capacity of *P. gingivalis* to citrullinate peptides. This is thought to lead to the generation of ACPAs that precede the onset of RA, and are associated with RA progression (Nielen et al., 2004, van Gaalen et al., 2004, Zendman et al., 2004). However, it is unclear whether the observed level of autoimmunity in PD patients would increase with progression of PD, and whether this does indeed precede the development of RA. Large-scale, longitudinal studies or follow-ups of PD patients are required to assess whether this is the case. It may emerge that although PD tends to precede RA, a second factor is necessary to induce the level of autoimmunity that causes RA. This is essentially, the two-hit hypothesis (Panel 2, **Figure 8.2**). As both PD and RA are complex, multi-factorial diseases, there are several possibilities of what the second 'hit' could be: a second chronic condition such as obesity could augment dysregulation of the immune response (Neumann et al., 2011); smoking may further increase serum ACPA titers (Kallberg et al., 2011); an acute clinical condition such as physical trauma or an aggressive infection could increase inflammation in the joint and/or systemically. Precisely how any of these conditions could render the joints more susceptible to autoimmune attack is as yet unknown. Whilst considering these possibilities, it is also acknowledged that PD may not directly cause, or even modulate, RA. The two conditions may co-exist in a patient as manifestations of immune dysregulation, perhaps exacerbating one another as they share common inflammatory pathways (Panel 3, **Figure 8.2**).

To date, there is no evidence to suggest that the model of PD established by Baker et al. (1999) induces breach of immune tolerance and causes RA in mice (Butcher, 2013), although there are reports that systemic infection with *P.*

gingivalis increases the clinical score in murine models of RA (Bartold et al., 2010, Maresz et al., 2013). Conversely, induction of RA is sufficient to induce pathological alveolar bone loss in mice, and can exacerbate alveolar bone loss associated with murine PD (Park et al., 2011, Queiroz-Junior et al., 2012, Butcher, 2013). One of the major problems with the various murine models of RA is that the majority of them are associated with breach of immune tolerance other than to the specific immunising antigen (Kannan et al., 2005). A second issue is that they are generally not associated with elevated ACPA titers and therefore are not appropriate for investigating the 'citrullination connection' with PD, which is central to the principal hypothesis that PD can cause RA (Lundberg et al., 2008, Lundberg et al., 2010). Moreover, the low levels of ACPAs detected in human patients with PD are not recaptured in the murine model of PD. This may represent an issue with detection (the commercially available ACPA ELISA detects only five human peptides which mice may not respond to), or reflect the lack of chronicity in the murine model, or result from mice simply not producing the same type of immune response and being less predisposed to generating ACPAs than humans. Investigating the relationship between PD and RA in murine models is fraught with complications inherent to each individual model system, which are arguably amplified when the two models are combined.

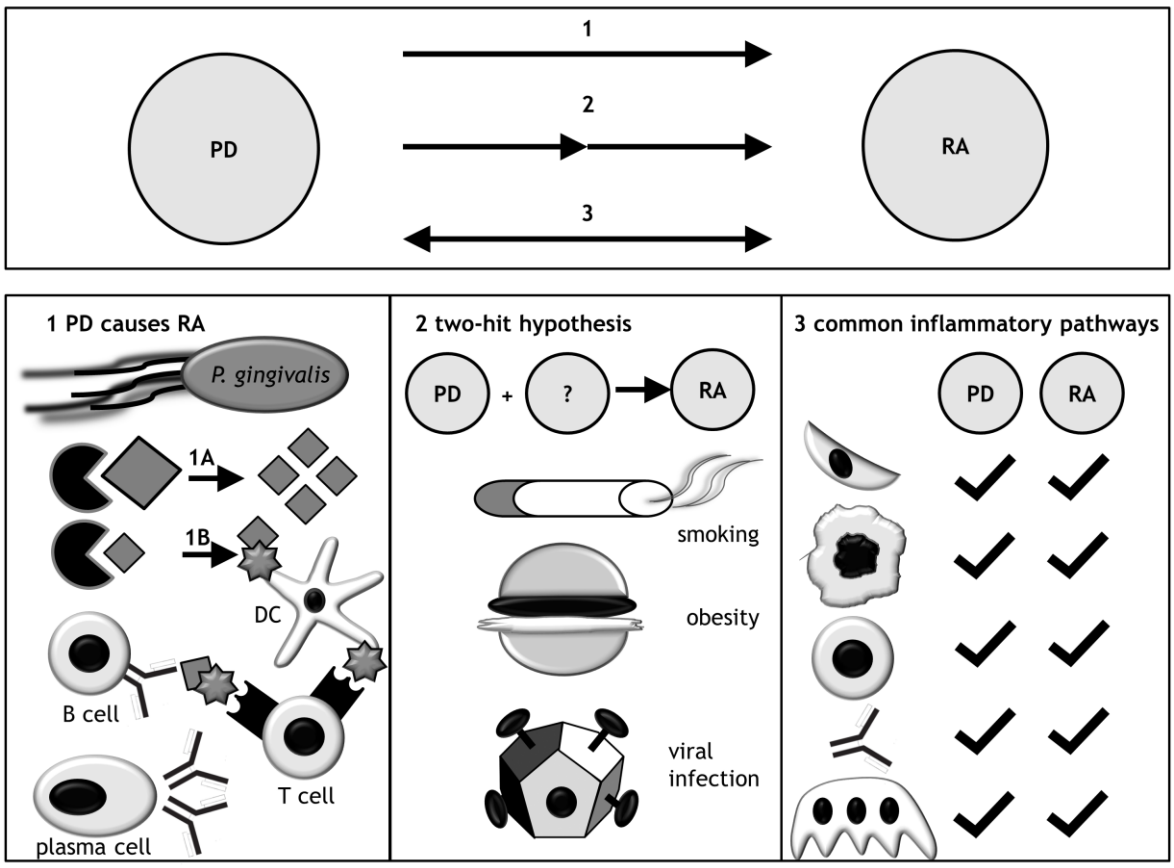

Figure 8.2. Three routes by which periodontitis may be immunologically linked to RA. 1) PD may precede and cause RA by eliciting the generation of ACPAs following the cleavage of proteins (**1A**) and citrullination of peptides (**1B**) by the PD-associated bacterium *P. gingivalis*. **2)** PD may precede but be insufficient to cause RA without a second 'hit', which has yet to be defined (reference). The second 'hit' could be one of a number of risk factors including smoking, obesity, and viral infection. **3)** PD may not cause RA but PD may exacerbate RA and vice versa as a result of common inflammatory pathways (Lundberg et al., 2010, Farquharson et al., 2012).

Conclusions

Whether PD precedes and causes RA through the generation of ACPAs remains unclear. Treatment of PD can lower ACPA titers and thereby potentially reduce the risk of PD patients developing RA, and also potentially help to limit RA progression in patients who already have the condition. B cells clearly have an important role in the immunopathology of PD and may be worthy of therapeutic targeting in severe PD. Further investigations are warranted to elucidate which B cell functions are of greatest importance in this respect. Possibly through its indirect effects on B cells, IL-33 appears to exacerbate PD. Future studies can hopefully confirm whether the IL-33 inflammatory pathway represents a link between PD and RA involving B cells.

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