AZITHROMYCIN SUPPRESSES *P. GINGIVALIS* LPS INDUCED PRO-INFLAMMATORY CYTOKINE AND CHEMOKINE PRODUCTION (IL-6, IL-8, MCP-1 & GRO) BY HUMAN GINGIVAL FIBROBLASTS *IN VITRO*

A thesis submitted to The University of Adelaide in partial fulfilment of the requirements of the Degree of Doctor of Clinical Dentistry (Periodontology)

Dr Catherine Jane Doyle

BDent, BSc (Biomedical Science) (Hons)

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Abstract

Azithromycin is a macrolide antibiotic that is well known for its antibacterial properties, as well as possessing potential anti-inflammatory and immune modulating effects. This antibiotic has therefore been widely used in medicine for treating conditions ranging from inflammatory pulmonary diseases to dermatologic skin conditions. It has also been shown to be an effective antibiotic against most common periodontal pathogens and is used as an adjunct to treat periodontitis, a condition with bacterial aetiology and an inflammatory pathogenesis. Furthermore, periodontal case studies report regeneration of alveolar bone accompanied by significant reductions in inflammation have been achieved with azithromycin. The mechanisms however, by which these are achieved in the periodontium are largely unknown. This study aimed to determine the potential anti-inflammatory effect of azithromycin on cytokine and chemokine production by healthy human gingival fibroblasts (HGFs) that were stimulated by *Porphyromonas gingivalis* lipopolysaccharide (*P. gingivalis* LPS). HGFs were isolated from healthy gingiva collected from three donors. The effects of azithromycin at concentrations ranging from 0.1 μg/mL to 10 μg/mL were tested. Cytokine and chemokine protein levels were assessed using the Luminex® multiplex immunoassay. *P. gingivalis* LPS induced cytokine/chemokine (IL-6, IL-8, MCP-1 and GRO) protein production in HGFs was suppressed by azithromycin at all concentrations tested, and in all three donors. Suppression by azithromycin of IL-6, IL-8, MCP-1 and GRO *P. gingivalis* LPS protein induction in HGF was statistically significant when all donor results were collated ($p<0.05$). This study demonstrates that azithromycin suppresses *P. gingivalis* LPS induced cytokine/chemokine protein production in HGFs, which may explain some of the clinical benefits observed with the adjunctive use of azithromycin in the treatment of periodontitis.

Key words: azithromycin (AZM), periodontitis, human gingival fibroblasts (HGF's), *Porphyromonas gingivalis* lipopolysaccharide (*P. gingivalis* LPS), cytokine, chemokine, interleukin-6 (IL-6), interleukin-8 (IL-8/CXCL-8), Monocyte chemoattractant protein 1 (MCP-1/CCL-2), growth-regulated oncogene (GRO).

Declaration

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Contribution to the Paper	Carried out the majority of the laboratory work, analysed samples, interpreted and analysed data, carried out statistical analysis, and wrote basic manuscript.		
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Name of Co-Author	Dr Tracy Fitzsimmons		
Contribution to the Paper	Helped with development of research project. Instructed and supervised laboratory work, provided direction and guidance, helped with data interpretation and editing of the manuscript.		
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Name of Co-Author	Ceilidh Marchant		
Contribution to the Paper	Instructed and supervised laboratory work, provided direction and guidance, helped with data interpretation and edited manuscript.		
Signature		Date	03/10/13

Name of Co-Author	Dr Kencana Dharmapatni		
Contribution to the Paper	Assisted with statistical analysis, and reviewed manuscript.		
Signature		Date	03/10/2013

Name of Co-Author	Dr Robert Hirsch		
Contribution to the Paper	Reviewed manuscript.		
Signature		Date	3/10/13

Name of Co-Author	Professor Mark Bartold		
Contribution to the Paper	Edited manuscript and provided direction and guidance as head research supervisor.		
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Chapter 1. Literature review on the potential role of azithromycin in the mediation of periodontal inflammation

1.1 Introduction

Azithromycin is a macrolide antibiotic. It is commonly used as an antibiotic against a broad spectrum of bacteria and has good absorption, a long half-life and good tissue distribution. It is widely prescribed for the treatment of tonsillitis, pneumonia, middle ear infection, urinary tract infections, malaria and trachoma. It has recently been suspected that a part of its superior action is via anti-inflammatory pathways, although it is not clear to what extent azithromycin influences inflammation and how strong its action is. As inflammation and the host response to bacteria play an important role in periodontal disease, determining the effect of azithromycin on the host inflammatory response may prove it to be a valuable adjunctive in the treatment periodontal disease.

1.1.1 Aetiology and current treatments for periodontitis

Chronic periodontitis is a common disease of the oral cavity (Burt, 2005, Borrell et al., 2005). Cross-sectional epidemiological studies indicate that about 10–15% of the adult population have ‘advanced periodontitis’, while up to 80% of the population may develop ‘moderate periodontitis’ (Loe et al., 1978, Papapanou et al., 1988, Hugoson et al., 1998, Gannon et al., 2012). An American National Health and Nutrition Examination Survey (NHANES) conducted in 2010 reported that 47% of the sample population had periodontitis, comprising 8.7% with mild, 30% with moderate, and 8.5% with advanced disease. When the sample population was restricted to those 65 years old and above, 64% were found to havemoderate to advanced periodontitis (Eke et al., 2012). It is marked by chronic inflammation of the periodontal tissues that is caused by accumulation of dental plaque. As the plaque matures it changes from Gram positive cocci to Gram negative cocci, rods and spirochetes. Bacteria predominantly associated with the initiation and propagation of periodontal destruction are *Porphyromonas g ingivalis* (*P. gingivalis*), *Treponema denticola* (*T. denticola*) and *Tanerella forsythia* (*T. forsythia*) (Socransky and Haffajee, 2002). It is thought however, that the bulk of tissue destruction occurs as a result of the host response (Birkedal-Hansen, 1993) and is driven by inflammatory processes that are triggered not only by the bacteria but their products (Van Dyke et al., 1993). Neutrophils, macrophages, lymphocytes and fibroblasts are the cells that play the largest role in these inflammatory processes.

The main method of treating periodontal disease thus lies in the removal of bacteria and their products, to prevent the potential disproportionate inflammatory reaction that can occur in a susceptible host, leading to tissue destruction. At present this is still achieved by means of mechanical debridement, which is found to improve both clinical and microbiological parameters (Greenstein, 1992). However, not all patients respond well to conventional periodontal treatment and some may still lose teeth despite both non-surgical and surgical periodontal therapy (Hirschfeld and Wasserman, 1978). This seems to be especially true for those with aggressive and refractory forms of periodontal disease (Drisko, 2001). It has therefore been suggested that in patients with aggressive periodontitis or chronic periodontitis with multiple failing sites, that the use of systemic antibiotics in conjunction with mechanical debridement may be of value (Heitz-Mayfield, 2009).

Although the role of bacterial biofilms in initiating the periodontal disease process is undisputed (Socransky and Haffajee, 1992, Haffajee and Socransky, 1994), the contribution of the host response to the bacterial challenge plays a significant role in the propagation of the disease process (Offenbacher, 1996, Page and Beck, 1997, Page et al., 1997). Therefore, an emerging adjunct to the treatment of periodontitis is host modulation therapy, which is based on the understanding that the tissue destruction experienced in periodontitis is a host-mediated process. Root surface instrumentation and mechanical periodontal therapy target one aspect of the pathogenic process by reducing the bacterial burden, whilst host modulation therapy aims to change the balance between wound healing and destructive aspects of the host response (Altenburg et al., 2011a). At present, the only approved host modulation agent has been subantimicrobial-dose doxycycline (SDD), an inhibitor of tissue matrix metalloproteinases (MMPs) involved in collagen degradation. SDD has been used as an adjunct to subgingival root debridement (SRD) (20 mg twice daily) (Preshaw et al., 2004). The long-term effectiveness of SDD treatment remains controversial (Sgolastra et al., 2011). Other agents such as nonsteroidal anti-inflammatory drugs and bone sparing agents such as bisphosphonates have also been trialled with less success, and unfortunately significant systemic side effects (Williams et al., 1989, Reddy et al., 2003).

1.2 Inflammatory mediators involved in pathogenesis of periodontitis

In periodontal disease, the established lesion has features characteristic of a B cell/plasma response, comprising an increased production of inflammatory cytokines such

as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and prostaglandin E2 (PGE_2) (Gemmell et al., 1997). This leads to connective tissue breakdown, resulting in formation of periodontal pockets and loss of bone. Histologically, there is a predominance of plasma cells within the periodontal connective tissue, demonstrating a B cell adaptive immune response (Seymour and Greenspan, 1979, Seymour et al., 1979). In the advanced periodontal lesion, there is further loss of attachment. Fibroblasts and macrophages produce matrix metalloproteinases (MMPs) (Nishikawa et al., 2002, Cox et al., 2006), by stimulation of cytokines such as IL-1, TNF-α, and PGE_2, which results in degradation of the extracellular matrix, and as the lesion advances alveolar bone loss becomes prevalent.

1.2.1 Specific polypeptide mediators in inflamed periodontal tissues

In inflamed gingiva, the levels of cytokines interleukin (IL)-1α, IL-1β, TNF-α, IL-6, IL-8, transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), keratinocyte growth factor, vascular endothelial growth factor (VEGF), and prostaglandins are altered in inflammatory cells, fibroblasts, and endothelial cells. In particular, IL-1α, IL-1β, TNF-α, IL-6, and IL-8 have increased expression in human gingival fibroblasts from patients with inflammatory gingiva, when compared with healthy gingiva patients (Wang et al., 2003). In addition, a multiplex analysis of cytokine levels in the gingival crevicular fluid of patients with periodontitis who had completed initial periodontal treatment revealed that post-therapy, the levels of a number of cytokines and chemokines (IL-1, IL-2, IL-3, IL-6, IL-7, IL-12p40, CCL5/regulated and normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), and interferon-gamma (IFN-γ) at diseased sites all decreased (Thunell et al., 2010).

P. gingivalis is an important element of dental plaque and is particularly associated with the subgingival microflora of patients with periodontal disease. Socransky et al. (1998), established that the subgingival bacterial species co-aggregate into a number of communities, one group later became known as the “red Socransky complex”, considered to be highly periopathogenic and consisting of *T. forsythia*, *P. gingivalis,* and *T. denticola*. *P. gingivalis* has a variety of virulence factors, such as proteases, hemagglutinins, lipopolysaccharide (LPS), and fimbriae, that interact with periodontal tissues and elicit responses which promote infection, signal immune defenses and drive the destructive inflammatory processes (Lamont and Jenkinson, 1998). In particular, the number of polypeptide pro-inflammatory mediators increases in inflammation, due to their expression being induced by bacterial endotoxins and bacteria, and their virulence factors have been

shown to modulate the cytokine network in periodontal tissues (Genco and Slots, 1984) For example, human gingival fibroblasts exposed to *P. gingivalis* LPS respond by increasing the mRNA and protein levels of the cytokines IL-1β, IL-6, IL-8, and TNF-α, as well as receptors CD14, Toll-like receptor-2 (TLR-2) and TLR-4 (Imatani et al., 2001).

Studies have identified a number of specific cytokines and chemokines that appear to have an active role in periodontal disease progression (Kinane et al., 2011).

1.2.1.1 IL-1

IL-1α is considered a key regulator in the normal process of inflammation, as it induces the expression of genes for cell adhesion molecules, cytokines, chemokines, cyclooxygenase-2, inducible nitric oxide synthase, and MMPs (Dinarello, 2002). IL-1α also activates osteoclasts to induce bone resorption. A significant study conducted by Dayan et al., (2004) showed that transgenic mice overexpressing IL-1α in the basal layer of the oral mucosal epithelium displayed all the clinical features of periodontal disease (including epithelial proliferation and apical migration, loss of attachment, and destruction of cementum and alveolar bone), regardless of the level of bacterial load, implicating IL-1α as a major mediator in periodontal disease. Furthermore, in a study of IL-1β-stimulated human gingival fibroblasts (HGFs), 215 genes were found to be upregulated, including inflammatory cytokines, nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) pathway members, chemokines, transcription factors, MMPs and adhesion molecules (Vardar-Sengul et al., 2009).

1.2.1.2 IL-6

IL-6 shares common signalling pathways with IL-11, leukaemia inhibitory factor and oncostatin M, through the signal transducer glycoprotein 130 (Heinrich et al., 2003). IL-6 is activated by a number of cytokines, including IL-1β and TNF-α, and is produced in a number of immune cells as well as non-immune cells such as fibroblasts (Van Snick, 1990). IL-6 has a number of non-immune activities in the cardiovascular system and the nervous system, and has a critical role in haematopoiesis and the signalling of acute-phase response proteins in hepatocytes (Taylor, 2010). With regards to localised immune responses, IL-6 is predominantly involved in the regulation of proliferation and differentiation of B-cells, dendritic cells (Kishimoto, 2005), and the IL-6 secretion by osteoblasts stimulates osteoclast development and bone resorption (Ishimi et al., 1990). Furthermore, IL-6 has been suggested to play a pivotal role in the balance between IL-17

secreting T-helper cells and regulatory T-cells, by promoting the differentiation of IL-17 secreting T-helper cells, thereby enhancing the immune response (Bettelli et al., 2006, Taylor, 2010). IL-6 is elevated in cells, tissues (Yamazaki et al., 1994) and gingival crevicular fluid from patients with periodontal disease (Lin et al., 2005). Furthermore, IL-6 polymorphisms have been shown to be associated with aggressive periodontitis (Shao et al., 2009).

1.2.1.3 IL-8/CXCL-8

IL-8 (CXCL-8) is considered a chemokine, in that it has a key role in the recruitment of neutrophils and other immune cells. In particular, interactions between bacteria and gingival keratinocytes lead to the upregulation of IL-8 and intercellular adhesion molecule 1 expression in the gingival epithelial layer, with a gradient of expression to encourage neutrophil migration into the epithelial layers of the gingival sulcus (Tonetti, 1997). Furthermore, IL-8 has been shown to be produced by human gingival fibroblasts in response to LPS (Madianos et al., 2005).

1.2.1.4 IL-17 (IL-17A)

IL-17 has been suggested to be involved in the induction of IL-8 in gingival fibroblasts, and to upregulate MMP-1 and MMP-3 (Mahanonda et al., 2008). IL-17A is also able to induce CC chemokine ligand 20 (CCL20) in IL-1β-stimulated human gingival fibroblasts in a concentration dependent manner (Hosokawa et al., 2012). Furthermore, in a mouse model of periodontitis (induced by *P. gingivalis* infection), IL-17 receptor deficient mice had an increased susceptibility to alveolar bone loss, suggesting a protective role of IL-17 in bone turnover (Yu et al., 2008) (Refer to section 1.2.3 for further details on IL-17 and the Th17 axis).

1.2.1.5 MCP-1/CCL-2

Marked expression of monocyte chemoattractant protein 1 (MCP-1/CCL-2) has been observed in gingival tissues of adult periodontitis patients (Yu and Graves, 1995). In addition, MCP-1 gene expression was observed from culture supernatants of human normal gingival tissues and human gingival fibroblasts treated with *P. gingivalis* LPS, with the chemotactic activity increasing in a dose dependent manner (Hanazawa et al., 1993).

These *in vitro* studies indicate that gingival fibroblasts can participate in monocyte recruitment in inflamed gingival tissues through the expression of MCP-1.

1.2.1.6 GRO/CXCL-1

The growth-regulated oncogene α (GROα or CXCL1) is a chemokine associated with both inflammation and cell proliferation. When GROα was originally discovered, it was shown to be a gene associated with cell growth and proliferation, being highly expressed in tumour cells. It has since also been shown to be an important chemoattractant for human neutrophils, stimulating their degranulation. In addition to GROα, two more human GRO genes have been identified and named GROβ and GROγ. Jonsson et al., (2009) found that human periodontal ligament cells and mouse gingival fibroblasts respond to LPS by increasing the production of the chemokine GROα, regulating recruitment of neutrophils to the inflammation process. Up-regulation of GROα expression was observed at mRNA as well as protein levels. Furthermore, this study demonstrated that treatment with the glucocorticoid dexamethasone attenuated the LPS-induced GROα production, and the NF-κB blocker MG 132 fully inhibited LPS-induced GROα.

1.2.1.7 VEGF

Vascular endothelial growth factor (VEGF) is a potent inducer of vascular permeability and angiogenesis. VEGF has been associated with the etiology of periodontitis in its early stages due to neovascularization stimulated by periodontal pathogens, causing swelling and oedema (Suthin et al., 2003). VEGF has been detected in human periodontal tissues, gingival crevicular fluid and in saliva, with the total quantity of VEGF found to be increased in periodontally diseased oral sites, compared to healthy sites (Booth et al., 1998). Prapulla et al. (2007) also found decreased VEGF levels in gingival crevicular fluid in patients with periodontitis after periodontal treatment.

In vitro studies have shown increased VEGF expression in human HGF cultures stimulated with LPS, vesicle, and outer membrane protein from *P. gingivalis* and *A. a.* (Suthin et al., 2003). The polyphenol, resveratrol has been shown to inhibit the production of VEGF by HGFs in response to vesicles and outer membrane protein from periodontopathic bacteria, therefore inhibiting angiogenesis (Nunez et al., 2010).

1.2.2 Th cells in periodontitis

As it is generally accepted that the mechanism of tissue destruction in periodontitis is through the effects on the immune response, an understanding of the T-cell mechanisms in periodontitis is important. The balance between the so-called Th1 and Th2 cells has been considered important in immune regulation. The phenotypic dichotomy of the $CD4^+$ T-cells; the T helper 1 (Th-1) and T helper 2 (Th-2) cells were first described by Mosmann et al. in 1986. T-cells described as Th-1 cells promote cell-mediated immunity by secreting IL-2 and IFN-γ, causing activation of macrophages, natural killer (NK) cells and cytotoxic CD8+ T-cells, like the delayed type sensitivity reaction observed during the development of gingivitis. In addition, Th-1 cells supress B cells and plasma cells. Th-2 cells secrete IL-4, IL-5 and IL-13, and thus induce B cell humoral (antibody mediated) immune responses and mast cell activity, while supressing the T cell response (Mosmann et al., 1986, Mosmann and Coffman, 1989).

As the majority of cells (about 65–70%) in periodontitis lesions are B cells or plasma cells (Berglundh and Donati, 2005), it is reasonable to assume that Th-2 cells and related cytokines dominate over Th-1 cells in periodontitis. Furthermore, due to the fact that the development of gingivitis is identical to the development of a delayed type hypersensitivity (DTH) response as well as progressive chronic periodontitis being fundamentally a B cell lesion, the concept that gingivitis, and hence the stable periodontal lesion, is mediated by Th-1 cells, while on the other hand chronic periodontitis is mediated by Th-2 cells was developed (“Seymour hypothesis”) (Ohlrich et al., 2009). A number of human studies support the notion that Th-1 cells and their cytokines predominate in early/stable lesions, while Th-2 cells are associated with disease progression, consistent with the B cell nature of the progressive lesion stage (Sigusch et al., 1998, Lappin and Campbell, 2000). However, there have also been a number of studies (mainly animal models) that have implicated the increased Th1 responses in chronic periodontitis, while others have shown an involvement of other Th subsets such as the Th17 subset (Berglundh and Donati, 2005, Ohlrich et al., 2009).

1.2.3 The Treg/Th17 axis

T helper 17 cells (Th17) are a subset of T helper cells producing interleukin 17 (IL-17) (Steinman, 2007). They are considered developmentally distinct from Th1 and Th2 cells, and excessive production is thought to play a key role in autoimmune diseases. More

specifically, they are thought to play a role in inflammation and tissue injury in these conditions, as IL-17 induces the secretion of IL-6, IL-8, and PGE_2. Furthermore, IL-17 is also thought to affect osteoclast activity and thereby mediate bone resorption (Ohlrich et al., 2009). Th17 cells can cause autoimmune diseases, however they also serve an important function in anti-microbial immunity at epithelial/mucosal barriers (Korn et al., 2009). They produce cytokines (such as interleukin 22) that stimulate epithelial cells to produce anti-microbial proteins to clear out certain types of microbes (such as *Candida* and *Staphylococcus*). Thus, a severe lack of Th17 cells may leave the host susceptible to opportunistic infections. It remains unclear exactly which cytokines contribute to Th17 formation, but transforming growth factor beta (TGF-β), IL-6, IL-21 and IL-23 have been implicated in mice and humans (Gaffen and Hajishengallis, 2008).

In general, the Th1/Th2 paradigm has offered a productive conceptual framework for investigating the pathogenesis of periodontitis. However, the discovery of the Th17 population considers a potential re-examination of periodontal disease in this context. Interestingly, cytokines characteristic of this subset have been found in inflamed periodontal tissue, suggesting a potential role in pathogenesis (Johnson et al., 2004, Takahashi et al., 2005, Vernal et al., 2005, Lester et al., 2007). IL-17 regulates MMP's and inflammatory cytokines in gingival fibroblasts (Beklen et al., 2007), and *P. gingivalis* can stimulate IL-17 production from T-cells *in vitro* (Oda et al., 2003). In addition to Th17 cells, CD4+CD25+ regulatory cells (Tregs) infiltrate and may play a role in periodontal disease, with immunohistological and gene expression studies showing an increase in Tregs in periodontitis with increased proportions of B cells (Nakajima et al., 2005).

Evidence indicates that Th17 cell development is inhibited by some Th-1 and Th-2 cytokines, but is promoted by TGFβ along with IL-6 and IL-21. TGFβ mediates the suppressive activity of Tregs against both Th-1 and Th-2 cells (Bettelli et al., 2006), whereas IL-6 contributes to the alveolar bone loss induced by *P. gingivalis* (Baker et al., 1999). In contrast, IL-17 receptor-deficient mice are more susceptible to alveolar bone loss induced by *P. gingivalis* (Yu et al., 2007), suggesting a protective role for this cytokine. This is thought to be a potential example of a cytokine "switching" from pro-inflammatory to anti-inflammatory, depending on the severity of the disease (O'Shea et al., 2002). Furthermore, T cells are considered to exhibit "functional plasticity" and this phenomenon is influenced by the cytokine milieu (Bluestone et al., 2009). For example, under the influence of IL-12, Th17 cells can differentiate into Th-1 cells (Korn et al., 2009), while follicular cells which reside in the B-cell follicles of lymph nodes, can potentially secrete a

cytokine profile corresponding to Th-1, Th-2 or Th17 cells with influence from IL-6 and IL-21 (Vogelzang et al., 2008).

There is therefore considerable complexity in the potential involvement of the Th-1, Th-2, Th-17 subsets and their associated cytokines on the pathogenesis of periodontal disease. Further research is therefore required to fully elucidate the potential mechanisms involved.

1.3 Inflammatory cytokine/chemokine biochemical signalling pathways

Cytokines and growth factors cause pathologic alterations via their activation or suppression of signalling proteins and transcription factors, which can lead to a cascade of events causing changes in gene expression and cellular function. Among the signalling proteins/kinases involved, one of the most crucial is the mitogen activated protein kinase (MAPK) cascade. The four major pathways of mitogen-activated protein kinase cascades are: extracellular signal regulated kinase-1 (ERK-1 and 2) activated mainly by growth factors and hormones, c-Jun N-terminal kinase or stress-activated protein kinase (JNK/SAPK), p38, and extracellular signal regulated kinase-5, otherwise called big mitogen-activated protein kinase-1, or BMK-1, with all four of these cascades induced predominantly by environmental stress and inflammatory cytokines (Bartold and Narayanan, 2006).

Gingival epithelial cells, fibroblasts, and inflammatory cells respond to bacterial pathogens by increasing the expression of cytokines, growth factors, and MMPs. In the periodontium, bacterial pathogens constantly interact with the cell surface of gingival epithelial cells, inflammatory cells and, if the bacteria/virulence factors penetrate deep into the tissues, potentially fibroblasts. Specifically, bacterial pathogens are recognised by toll-like receptors (TLRs) that function as pattern recognition receptors and play an essential role in host defence by activating the innate host immune system. The toll-like receptor family of receptors that are membrane bound and span the membrane. They share similar extracellular domains, which include 18-21 leucine-rich repeats and a similar cytoplasmic domain of approximately 200 amino acids. The cytoplasmic domain is similar to the IL-1 receptor. At least 11 toll-like receptors have been described. Among these is TLR-4 which recognises lipopolysaccharide endotoxin from Gram-negative bacteria (Bartold and Narayanan, 2006).

Wang et al., (2001) demonstrated that *P. gingivalis* LPS in gingival fibroblasts induced signalling via TLR-4, and that this induced IL-1 production was inhibited by anti-

TLR-4 antibody. Furthermore, *P. gingivalis* LPS treatment of human gingival fibroblasts activated several intracellular proteins, including protein tyrosine kinases, and up-regulated the expression of IL-1 receptor associated kinase (IRAK), NF-κB and activating protein-1 (AP-1), which once again was suppressed by anti-TLR-4 (Wang et al., 2000, Wang et al., 2001). Watanabe et al (1996) also found that *P. gingivalis* LPS in human gingival fibroblasts activated tyrosine kinase via CD14 (one of the LPS receptors), leading to MCP-1 gene expression through the transcription factor NFκB, and AP-1. Furthermore, AP-1 and NF-κB have also both been shown to regulate the expression of MMPs, TGF-β, keratinocyte growth factor and other molecules in epithelial cells and fibroblasts (Tewari et al., 1996, Hamid et al., 2000).

Overall, studies have found that bacterial LPS of *P. gingivalis* binds to TLR-4 via the co-receptor CD14 and leads to the recruitment of a number of adapter proteins, including myeloid differentiation primary response gene (88) (MyD88), IRAK-1, IRAK-4, Toll interacting protein (TOLLIP), TNF receptor associated factor-6 (TRAF-6) and TAK-1. As a result, several kinases are activated, leading to the activation of transcription factors. The p38 and c-Jun N-terminal kinase MAP kinases and NF-κB are subsequently activated, leading to the induction of IL-1β, TNF-α, IL-6, several growth factors, adhesion molecules and cyclooxygenase products (Darveau et al., 2002, Bartold and Narayanan, 2006). IL-1 is the major cytokine induced in these cells, and both IL-1 and LPS induce MMP-1 and -9 expression through p38, mitogen-activated protein kinase kinase (MEK) (which is part of the ERK/MAPK pathway) and phosphotidylinositide 3-kinase (PI3K) (Chang et al., 2004).

The above evidence demonstrates that the pro-inflammatory effects of cytokines are mediated through intracellular biochemical signalling events that can be initiated by bacteria and their virulence factors, such as *P. gingivalis* LPS, in periodontal tissues (Figure 1.1).

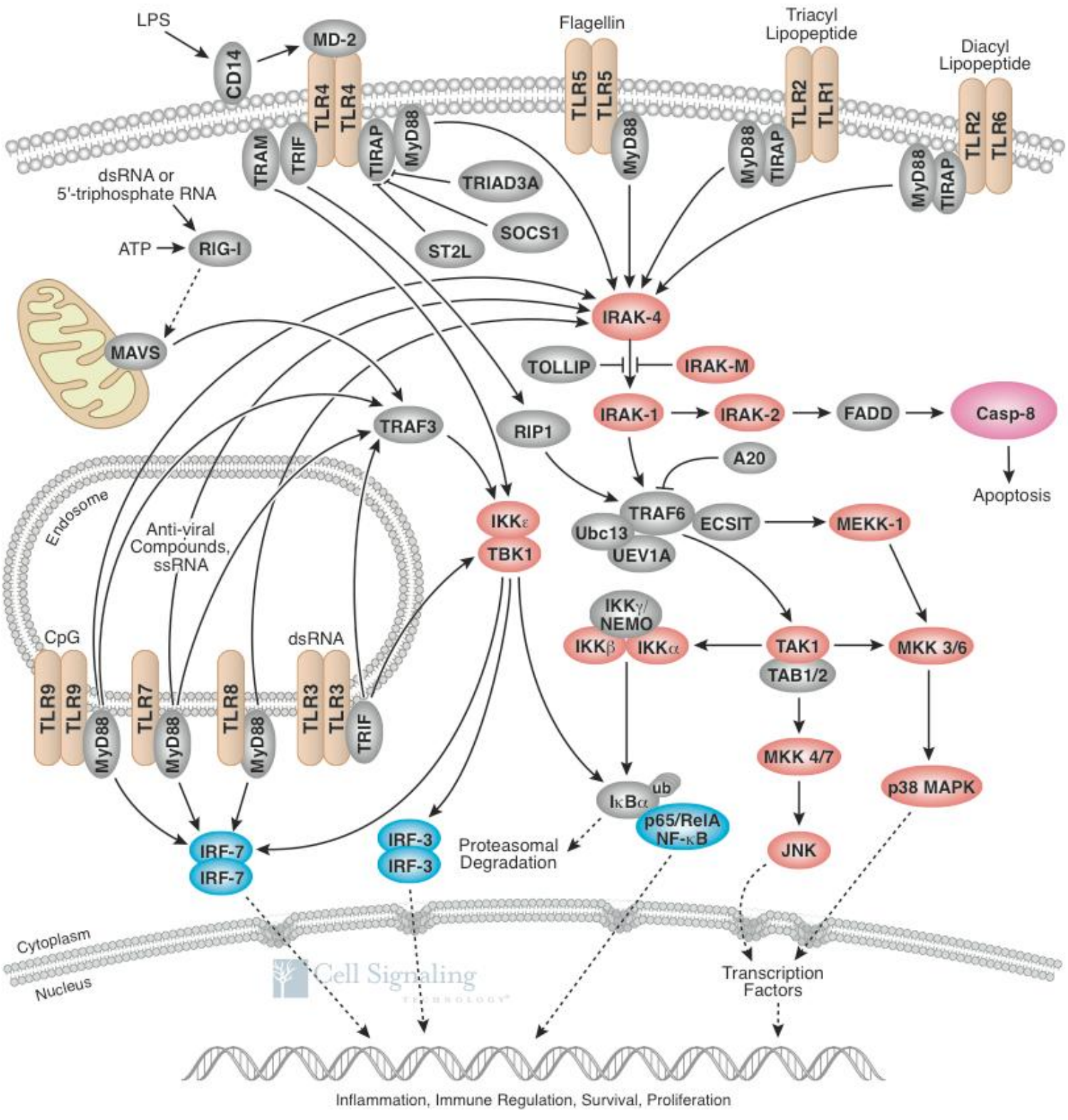

Figure 1.1 Toll-like receptor signalling
Image from Cell Signalling Technology (http://www.cellsignal.com/pathways/nk-kappab-signaling.jsp) Note that *P. gingivalis* LPS signals through TLR-4 (Gram-negative bacteria).

1.4 Antibiotics as an adjunctive treatment for periodontitis

The use of systemic antimicrobials as part of the therapy and management of periodontal diseases has been debated for decades. A clear definition of the type of patient who would benefit from adjunctive antibiotics, as well as the protocols involved have yet to be determined by clinical studies. The American Academy of Periodontology has suggested that potential candidates for adjunctive antibiotic treatment might be patients who are still exhibiting attachment loss after adequate conventional therapy (‘refractory

periodontitis'), patients with aggressive forms of periodontitis, or patients associated with predisposing medical conditions (AAP, 2004). A systematic review of the adjunctive benefits of systemic antimicrobials in the treatment of periodontal disease concluded that in specific clinical situations the adjunctive use of antimicrobial therapy with scaling and root planing (SRP) could be clinically relevant (Herrera et al., 2002). These situations include patients with deep pockets, patients with progressive or "active" disease, with aggressive forms of periodontitis, or those with specific microbiological profiles. However, differences in study methodology and lack of data precluded an adequate and complete pooling of data for a more comprehensive analysis, therefore the authors concluded it would be inappropriate to make definitive and specific recommendations for clinical practice based on the review. In another systematic review on the adjunctive use of antimicrobials with SRP, Haffajee et al., (Haffajee et al., 2003) concluded that despite sufficient data to suggest that antibiotics may help in the treatment of periodontitis, the optimum protocol of use needs to be clearly defined. Herrera et al. (2008) reviewed the literature in the area of potential protocols for the use of antibiotics in periodontitis, concluding that if systemic antimicrobials are indicated in periodontal therapy, they should always be used as an adjunct to mechanical debridement (not used as a monotherapy), and that there is not enough evidence to support their use with periodontal surgery.

A wide range of systemic antibiotics have been used for periodontal treatment, aimed at targeting potential pathogenic bacterial species within the periodontal biofilm. The therapeutic success of an antimicrobial depends on the activity of the antimicrobial agent against the infecting organisms. Periodontitis is a mixed microbial infection, making the choice of antibiotic regimen difficult. Certain antibiotics target specific parts of the subgingival biofilm. For example, metronidazole targets the Gram-negative strict anaerobes from the red and orange complexes (Socransky et al., 1998) such as *Fusobacterium nucleatum* (*F. nucleatum*), *T. for sythia, P. gingivalis* and *T. denticola,* whilst members of the genera *Actinomyces, Streptococcus* and *Capnocytophaga* are minimally affected by metronidazole. Metronidazole also has a limited effect on the species *Aggregatibacter actinomycetemcomitans* (*A. a*), thought to have a key role in the pathogenesis of localized aggressive periodontitis. Amoxicillin has a broader spectrum, lowering counts of Gram negative anaerobes as well as decreasing the counts and proportions of *Actinomyces* species during and after antibiotic therapy (Feres et al., 2001).

Several different antibiotics have been used in the treatment of both chronic and aggressive periodontal disease. The most popular to date are metronidazole (Loesche et al., 1984), penicillins (amoxicillin) (Heitz-Mayfield, 2009), tetracycline (Al-Joburi et al.,

1989) and clindamycin (Brook et al., 2005). These treatments have all been found to produce varying degrees of success.

The most common combination antibiotic regimen reported is metronidazole with amoxicillin (Yek et al., 2010). Research has shown that the combination of metronidazole and amoxicillin three times daily for one week has been shown to be effective against *A. a.* in aggressive periodontitis (van Winkelhoff et al., 1989, van Winkelhoff et al., 1992, Guerrero et al., 2005, Xajigeorgiou et al., 2006), and in some chronic periodontitis studies (Cionca et al., 2009). The combination of metronidazole-amoxicillin therapy for 7 to 8 days can reduce subgingival levels of *P. gingivalis*, although not consistently to below detectable levels (van Winkelhoff et al., 1989, van Winkelhoff et al., 1992). Berglundh et al. (1998) showed that subgingival scaling, together with systemic metronidazole-amoxicillin for 14 days caused an increased suppression of *P. gingivalis* for 12 months compared to SRP without metronidazole-amoxicillin. Furthermore, other studies have also shown statistically significant lower remaining proportions of red complex species with the adjunctive use of amoxicillin and metronidazole (Matarazzo et al., 2008, Mestnik et al., 2010). Compliance can become an issue however, as administration of amoxicillin plus metronidazole requires taking several tablets each day for long periods of time. In addition, the treatment may have potentially unpleasant side effects, such as a disulfiram-like reaction with ethanol (Slots and Ting, 2002).

Recently, attention has also been given to azithromycin, a macrolide antibiotic. A limited number of clinical trials in periodontics have assessed the adjunctive use of this antibiotic to date. Azithromycin appears to be a promising adjunct to conservative periodontal therapy, having not only an antibacterial (bacteriostatic antibiotic) action, but also the potential for host immuno-modulating effects in a number of inflammatory conditions of bacterial aetiology (Culic et al., 2001).

1.5 Therapeutic use of azithromycin in periodontitis

Azithromycin was initially developed for the treatment of bacterial infections of the upper and lower respiratory tracts, skin infections and treatment of uncomplicated *Chlamydia* infections (Hopkins, 1991). However, recent clinical trials have shown potential beneficial clinical and microbiological effects when azithromycin is used as an adjunct to traditional nonsurgical mechanical therapy of both aggressive and chronic periodontitis (Sefton et al., 1996, Sefton, 1999, Smith et al., 2002, Haffajee et al., 2007, Gomi et al., 2007b, Haas et al., 2008, Yashima et al., 2009, Oteo et al., 2010). There have

also been some case reports of alveolar bone regeneration with the administration of azithromycin (Schmidt and Bretz, 2007, Hirsch, 2010). However, there have only been a limited number of clinical studies that have investigated the application of azithromycin in the management of periodontal disease.

1.5.1 Chronic periodontitis

One of the first reports of azithromycin usage as part of treatment for periodontitis found an added reduction in pocket depth when azithromycin was administered in combination with mechanical debridement, with the biggest differences noted in deep pockets when compared to subgingival root planing (SRP) alone (Smith et al., 2002). This study recruited 46 patients who were treated either with azithromycin (500 mg once a day for three days) or placebo, two weeks after the commencement of oral hygiene instruction and mechanical debridement that had been conducted at weeks 0, 1, and 2. The clinical data showed that by week 22, only 5.6% of pockets initially deeper than 5 mm remained above that level in the 23 patients taking azithromycin, compared with 23.3% in the 21 patients taking the placebo. Also at week 22, for pockets initially 4 mm or more, the test group had 26.1% pockets >3 mm deep remaining compared to 44.3% in the control group. There were also fewer pockets failing to improve in probing depth (azithromycin group 6.6%, control group 21.6%), and fewer pockets of initial depth 4 mm or more that continued to bleed on probing in the test group, (46.9%) when compared with the control group (55.6%). Pocket depths initially 4–5 mm or 6–9 mm showed lower mean pocket depths in the patients on azithromycin at weeks 6, 10 and 22 ($p<0.01$).

Other studies have also shown clinical improvements with the adjunctive use of azithromycin. Haffajee et al., (2007), assessed 92 chronic periodontitis patients over a one year period, who were randomly assigned various treatments; mechanical debridement alone, or combined with 500 mg of azithromycin per day for 3 days, 250 mg metronidazole t.i.d. for 14 days, or 20 mg of doxycycline b.i.d. (sub-antimicrobial doses of doxycycline, for host modulation) for 3 months. Results showed that for all treatment groups, clinical improvements were noted over 12 months, with patients who received adjunctive treatment exhibiting greater clinical improvement. Furthermore, sites with initially deeper pocket depths (>6 mm) showed significantly greater pocket depth reduction and greater attachment gain for patients receiving adjunctive metronidazole or azithromycin. Interestingly, 15-39% of subjects in the SDD and mechanical debridement group showed an increase in mean pocket depth, mean attachment level and percentage of sites with

gingival redness, after the 3 month post-therapy monitoring visit (at the time point where the doxycycline administration had ceased). It should be noted that this study showed general clinical improvements beyond that achieved by SRP alone to be quite modest, which can be attributed to the presence of a patient cohort of mainly mild to moderate periodontitis in the test group (Haffajee et al., 2007). Systematic reviews evaluating the effect of systemically administered antibiotics find that antibiotics provide a greater benefit in subjects with advanced periodontal disease and at deeper periodontal sites (Herrera et al., 2002, Haffajee et al., 2003).

In a more recent study, an assessment was made of the effect of azithromycin and mechanical debridement in *P. gingivalis*-positive patients. This study found significantly better clinical improvements in terms of probing pocket depth (PPD) (0.34 mm for placebo and 0.80 mm for the test group) and for mean clinical attachment level (CAL) gain (0.29 for placebo and 0.76 for the test group) after 6 months. *P. gingivalis* microbial detection also decreased in the test group after 1, 3, and 6 months (Oteo et al., 2010). This study however had significant limitations, such as low subject numbers and a particularly low PPD reduction of 0.34 mm with mechanical debridement alone, when compared to general studies assessing average PPD reduction with mechanical debridement (Cobb, 2002).

Systemic azithromycin has also been evaluated in patients with severe chronic periodontitis as an adjunct to full mouth debridement, compared with quadrant debridement without adjunctive antibiotics. Full mouth debridement with adjunctive azithromycin resulted in significantly greater reduction of PPD, bleeding on probing (BOP), gingival index, and microbiological parameters compared to conventional quadrant debridement for up to the 25 weeks of the study's duration (Gomi et al., 2007b). A further non-placebo controlled study was conducted by this group to evaluate whether staged (partial-mouth debridement with azithromycin) or full-mouth debridement with adjunctive azithromycin effects the overall outcomes of treatment. Results demonstrated no difference between partial mouth debridement (conducted within the half-life of the antibiotic) or full-mouth debridement when azithromycin was used adjunctively. Furthermore the treatment groups showed significantly better clinical outcomes than the control group that had conventional debridement and no antibiotic (Yashima et al., 2009).

1.5.1.1 Smokers and effect on chronic periodontitis

Smokers also appear to respond well to azithromycin as an adjunctive treatment with mechanical debridement (Mascarenhas et al., 2005). Similar to non-smoking studies,

this study found that the largest reduction in pocket depth (PD) occurred in deeper pockets treated with non-surgical therapy and adjunctive azithromycin (PD reduction in pockets >6 mm was 3.52 mm in the treatment group and 1.98 mm in the control group). The PD reduction difference was sustained during the 6 month course of the study, but was not statistically significant due to baseline variability between the groups, therefore caution should be taken when interpreting these results. Furthermore, the administration regimen of two 250 mg tablets of azithromycin the first day and one 250 mg tablet each following day for four days used in this study does not correspond to the majority of dosage regimes that other studies use, therefore once again limiting the comparison value to other studies (Mascarenhas et al., 2005).

A different double blinded and randomized placebo controlled pilot study conducted on heavy smokers with moderate to severe chronic periodontitis evaluated the effect of adjunctive treatment of azithromycin with surgical periodontal therapy (apically repositioned flap with osseous recontouring) (Dastoor et al., 2007). Surgical treatment of moderate (PD = 4-6 mm), and deep (PD >6 mm) pockets significantly improved clinical parameters of treated and untreated teeth (CAL gain, PD reduction, and reduction of BOP). The additional adjunctive administration of azithromycin to surgical treatment did not enhance the clinical improvement that was shown with surgery alone. This finding is supported by systematic reviews, finding that the adjunctive use of antibiotics with periodontal surgery does not enhance treatment outcomes (Herrera et al., 2008). Although additional expanded studies are required to confirm these results, Dastoor et al., (2007) also found that at 6 months, only the azithromycin prescribed group had statistically significant gains in overall CAL of non-surgically treated sites. In addition, compared with the control group, the combination of surgery and azithromycin in heavy smokers promoted rapid wound healing and reduced gingival inflammation as recorded by a wound healing and gingival index (Dastoor et al., 2007).

1.5.2 Aggressive periodontitis

Hass et al. (2008) evaluated the use of adjunctive azithromycin with mechanical debridement in the treatment of aggressive periodontitis at 3, 6, 9, and 12 months. PPD and CAL improved significantly from baseline to 12 months in both groups, with the test group showing significantly increased reduction in mean PPD compared to controls (2.88 mm verses 1.85 mm respectively, p=0.025). Also subjects administering azithromycin showed a higher percentage of teeth with attachment gain ≥1 mm. Therefore, overall the adjunctive

use of azithromycin has the potential to improve periodontal health of patients with aggressive periodontitis. This study did however also show that mechanical debridement alone was a very effective treatment modality, with clinical improvements similar to average values obtained in systematic reviews evaluating chronic periodontitis treatment outcomes (Van der Weijden and Timmerman, 2002).

1.5.3 Locally delivered azithromycin

Clinical attachment loss, pocket depth improvements, and an improvement of microbiological parameters has been noted when azithromycin was locally administered at a 0.5 % concentration in a poly(lactic-co-glycolic acid) (PLGA) *in situ* gel, in conjunction with subgingival debridement (Pradeep et al., 2008), compared with SRD alone. However, the differences in both PD (0.4 mm) and CAL (0.47 mm) reduction at three months were very small and not clinically relevant. In addition, this study was not placebo controlled or blinded leading to potential bias.

1.5.4 Microbial effects of azithromycin in periodontal treatment

With respect to microbiological changes in clinical studies with the adjunctive use of azithromycin, Sefton et al. (1996) described reductions in spirochetes and anaerobic 'black pigmented' species at 22 weeks after azithromycin therapy, while Gomi et al. (2007b) found that 'black pigment-producing rods' took longer to return after the adjunctive use of azithromycin with SRD compared to SRD alone. It should be noted that the majority of these studies only evaluated the presence or absence of a few species in a limited number of sites. Also, the reductions in black-pigmented anaerobes may represent a change in the bacterial ecology rather than a true reduction in total bacterial numbers (Gomi et al., 2007b).

Haffajee et al. (2008) systematically evaluated the changes occurring in the microbial profile after azithromycin administration. This was conducted by examining various antibiotic treatment modalities over 3, 6, and 12 months, with analysis of 40 bacterial species in patient samples by checkerboard DNA-DNA hybridization. The study found that all treatments including SRD and azithromycin, metronidazole, and SDD-doxycyline combined with SRD, produced similar reduced counts of red and orange complex bacterial species at all time points. Both antibiotics (azithromycin or metronidazole) significantly reduced counts of red complex species at 2 weeks. However, all adjunctive treatment groups bacterial counts returned to baseline at 12 months. This

transient rapid reduction in pathogenic bacterial species may account for the clinical improvements seen up to 12 months (Haffajee et al., 2008).

1.5.5 Recent clinical studies

The majority of human clinical studies have shown that azithromycin has an additional clinical benefit when compared to subgingival debridement alone, with significant reductions in periodontal pathogens for up to 12 months. However, recently five publications found no additional benefit using azithromycin as an adjunct to mechanical debridement in both severe chronic and aggressive periodontitis (Sampaio et al., 2011, Emingil et al., 2012, Han et al., 2012, Haas et al., 2012).

In a double-blinded, randomized, parallel-designed and placebo controlled clinical trial of 40 patients, no adjunctive clinical benefit was found with azithromycin in the treatment of severe chronic generalized periodontitis (Sampaio et al., 2011). Both control and treatment group showed recolonisation with red complex species over the 12 month course of the study. It should be realised that in this study the clinical benefits of SRD alone in initially deep sites was 3.8 mm, which is considerably above the values reported previously by a comprehensive review or in a meta-analysis (Hung and Douglass, 2002, Cobb, 2002). This could be explained by the exceptional clinical time frame that was used by the clinician for debridement (between 6-12 hours per patient). Thus in the presence of meticulous instrumentation there may be no added benefit of using adjunctive antibiotics in chronic periodontitis. This time frame for instrumentation is not comparable with everyday clinical practice.

Emingil et al., (2012) found in 32 patients with generalized aggressive periodontitis that the group given 500 mg of azithromycin once a day for 3 days presented with a higher percentage of deep pockets that resolved (defined as a probing depth reduction of ≥3 mm from baseline) compared to the placebo (quadrant SRD treatment only) group at one month. However by six months all clinical and microbial parameters improved and crevicular MMP-8 levels decreased to a similar level between placebo or azithromycin treated groups (Emingil et al., 2012). The same research group conducted a similar study in 36 patients with severe generalized chronic periodontitis, with clinical and microbial parameters being similar between the placebo and drug treated groups at 6 months and significant reductions in both groups in MMP-8 levels 2 weeks post treatment (Han et al., 2012). Both studies however, had a short follow up time of 6 months compared with other studies investigating the clinical effects of adjunctive azithromycin in periodontal therapy.

Haas et al. (2012b) evaluated the subgingival microbial outcomes of azithromycin or placebo as adjuncts to mechanical debridement in aggressive periodontitis subjects, finding that both treatments resulted in similar reductions in periodontal pathogens over 12 months. Haas et al. (2012a), also demonstrated similar radiographic bone level gains between groups (azithromycin 0.55 ± 0.10 mm, and placebo 0.42 ± 0.07 mm).

Clearly these studies on the therapeutic effects of azithromycin on periodontitis are heterogeneous. Differences can be found in the dose and timing of azithromycin relative to when mechanical debridement was conducted, inclusion and exclusion criteria (smokers), type of periodontitis and classification method, number of subjects and length of study. Further well designed large randomised controlled studies are required to conclusively evaluate the effect of azithromycin as an adjunctive treatment for periodontitis.

1.5.6 Treatment of gingival enlargement

Interestingly, an additional clinical finding with azithromycin in the gingival tissues is that it can play a role in the reduction of gingival overgrowth that often develops in patients with periodontitis taking cyclosporine-A. When compared to oral hygiene procedures alone, azithromycin with oral hygiene measures was found to significantly reduce cyclosporine induced gingival hyperplasia (Ramalho et al., 2007). In a systematic review, (Clementini et al., 2008) found a general trend in the reduction of cyclosporine induced gingival hyperplasia in studies evaluating azithromycin in combination with calculus and plaque removal, however a well-designed randomized multicenter clinical trial is required to confirm these preliminary studies. Furthermore, there are some researchers who believe azithromycin may also be effective in gingival hyperplasia induced by calcium channel blockers (Fourtounas and Vlachojannis, 2009) and phenytoin (Namazi et al., 2007). A possible mechanism for azithromycin's actions on cyclosporine induced gingival overgrowth was investigated *in vitro*, with azithromycin inhibiting the cyclosporine-induced proliferation of fibroblasts obtained from 10 healthy donors and 7 renal transplant patients displaying cyclosporine A-induced gingival overgrowth (Kim et al., 2008). This was shown to occur in conjunction with halted production of collagen, and activating matrix metalloproteinase (MMP)-2 by gingival fibroblasts.

1.6 Properties of azithromycin

Azithromycin (azalide), is an antibiotic of the macrolide class and is a synthetic derivative of erythromycin (Culic et al., 2001). It differs chemically from erythromycin

and as a result has better absorption, tissue penetration and a much longer half-life and it is also associated with fewer side effects (Addy and Martin, 2004). Erythromycin is limited in its usefulness due to its instability under the acidic conditions of the stomach, poor absorption, low blood concentrations and a reportedly limited spectrum of activity (Wilson and van Boxtel, 1978). The short half-life of erythromycin means that a three to four times daily dosage schedule is required for effective treatment (Zuckerman et al., 2011). In contrast, the azalide structure of azithromycin confers a much improved pharmacokinetic profile with superior acid stability (300 fold) over erythromycin at acid pH (Fiese and Steffen, 1990). Azithromycin differs from erythromycin in that it has a methyl-substituted nitrogen in place of the carbonyl at the 9a position of the aglycone ring, thus blocking the internal dehydration pathway that occurs with erythromycin to cause rapid degradation (Figure 1.2). This inherent acid stability of azithromycin contributes to its higher plasma concentrations found with oral administration (Girard et al., 1987).

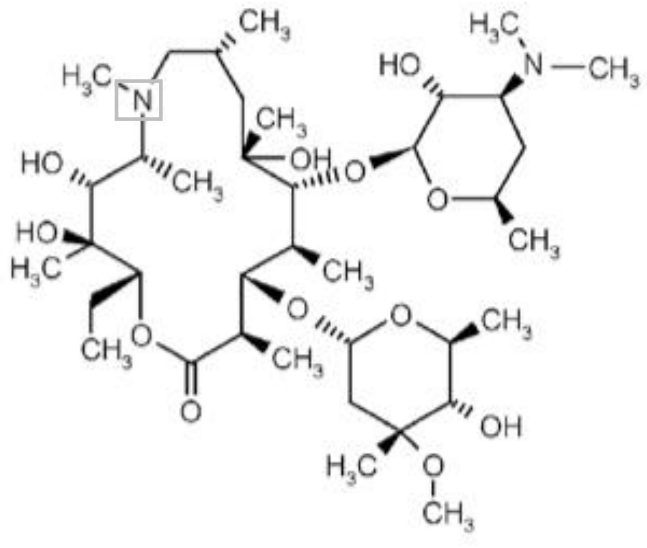

Figure 1.2 Structure of azithromycin
Methyl-substituted nitrogen shown by square (adapted from Culic et al., 2001)

1.6.1 Pharmacokinetic and pharmodynamic properties

As azithromycin is lypophilic (Zuckerman et al., 2011), it can penetrate cells, including tissue fibroblasts and phagocytic cells (macrophages and polymorphonuclear leukocytes) (Bosnar et al., 2005), which results in higher intracellular concentrations compared to serum levels (Gladue et al., 1989) that can be actively released at sites of infection. For example, Gladue and Snider (1990) found exposure of fibroblasts to azithromycin resulted in 27% of the azithromycin remaining in the cells after 48 hours of incubation in drug free media, while the incubation of these fibroblasts with human polymorphonuclear leukocytes (PMNs) resulted in accumulation of azithromycin within the PMNs. Thus, human fibroblasts may act as a reservoir for azithromycin within the

tissues, with the drug being slowly released into the tissues over time (Gladue and Snider, 1990). Following uptake, fibroblasts also have the ability to release the drug to neutrophils for activity against intracellular organisms. Intracellular neutrophil concentrations of up to 226 times greater than extracellular concentrations can be achieved, which persist even in the absence of extracellular antibiotic (Gladue et al., 1989, Bonnet and Van der Auwera, 1992). Owing to these characteristics, azithromycin is found in increased concentrations in tissues with active infection, including the periodontal tissues.

Azithromycin is therefore considered to be delivered to a site of infection by two mechanisms. Firstly, by direct uptake into tissues, which in part is by fibroblasts. Secondly phagocytes deliver the drug to sites of infection where it is released in response to phagocytosis to deliver effective, locally high concentrations of the drug by a biological targeted delivery mechanism (Foulds et al., 1990, Foulds and Johnson, 1993, Lode et al., 1996). Furthermore, azithromycin appears to stimulate neutrophil granulation and phagocytosis associated oxidative burst which may contribute to its antibacterial action (Culic et al., 2002).

A single 500 mg dose of azithromycin produces a serum concentration of 0.4 mg/L (Foulds et al., 1990). However, following the same oral dosage, the tissue concentrations of azithromycin are in the range of 10 to 100 times greater than serum concentrations, and these tissue concentrations persist after serum concentrations decline. Most tissues have concentrations of approximately 3 mg/kg (Foulds et al., 1990). A number of studies have assessed the concentration of azithromycin in gingival tissue. In healthy periodontal tissues the concentration for azithromycin was found to range from a mean of 3.3 mg/kg to 6.3 mg/kg, with the maximum concentration 12 hours after the last dose of 500 mg/day for 3 consecutive days (Blandizzi et al., 1999). Similarly, Malizia et al., (1997) found a mean tissue concentration of 6.47 mg/kg in gingival tissue, with the same administration regime of azithromycin as the previous study. In inflamed periodontal tissue studies the reported concentration of azithromycin has been variable between studies. One study found a mean concentration significantly higher than healthy tissues, with a mean concentration ranging from 11.6 to 3.9 mg/kg over 6.5 days (Blandizzi et al., 1999). Gomi et al., (2007a), however found the mean tissue concentration to be 2.92 μg/g on day four, 1.47 μg/g on day seven, and 0.54 μg/g on day 14, with the same antibiotic regimen of 500 mg once a day for three days. Although this concentration is less than that found in the previous study by Blandizzi et al. (1999), the tissue concentrations were still greater than the minimum inhibitory concentration for 7 days after administration for *P. gingivalis*, and for 14 days for *P. intermedia* and *A. a* (Gomi et al., 2007a). Therefore, azithromycin can be found in

concentrations exceeding Minimal Inhibitory Concentration (MIC) in the periodontal tissues lasting for up to 6.5 (Blandizzi et al., 1999) to 14 days (Gomi et al., 2007a) after administration. In a recent study, azithromycin was shown to have a higher concentration in gingival crevicular fluid (GCF) when compared to serum levels in healthy subjects, and that this amount once again exceeded MIC for *A. a., P. gingivalis,* and *P. intermedia* (Lai et al., 2011). Due to azithromycin's pharmacological properties of long half-life and good tissue penetration, the dose of 500 mg once daily for three days is currently being administered for the adjunctive use in aggressive periodontitis (Haas et al., 2008).

1.6.2 Antimicrobial properties of azithromycin

Azithromycin has a bacteriostatic antimicrobial effect, which is achieved by inhibiting bacterial protein synthesis via interactions with specific ribosomal proteins and the 23S rRNA in the peptidyl transferase centre, interfering with protein elongation (Zuckerman et al., 2011). Azithromycin has good activity against Gram-positive organisms, and when compared with erythromycin a greater spectrum of activity against Gram-negative organisms (Retsema et al., 1987). Azithromycin displays activity *in vitro* against a wide range of organisms such as *St reptococcus pyrogenes, Streptococcus pneumoniae, Haemophilus influenza, Moraxella catarrhalis, Legionella pneumop hila, Neisseria gono rrhoeae, Chlamydophila pneu moniae,* and *Mycoplasma pneumonia* (Zuckerman et al., 2011).

Results from *in vitro* microbial studies have found azithromycin to be highly effective against *P. gingivalis* (Pajukanta et al., 1992, Pajukanta, 1993, Muller et al., 2002) and have modest activity against *A. a.* (Muller et al., 2002). The minimal concentration for azithromycin to inhibit 90% of the bacterial isolates in an overnight incubation (MIC_{90}) has been reported to be 0.094 μg/mL for *P. gingivalis* and 4 μg/mL for *A. a* in a study of patients from the Netherlands (Veloo et al., 2012).

For successful antimicrobial action in periodontitis, an antibiotic must be present in the gingival sulcus and associated tissues at a level that is active against periodontal pathogens. As previously discussed, azithromycin has properties that allow for high concentrations of the drug being available at the site of infection and persisting for up to 6.5 days after administration (Blandizzi et al., 1999). Gingival crevicular fluid samples also have been shown to reach concentrations as high as 8.82 μg/mL (Lai et al., 2011).

Dental plaque can be defined as the microbial community that develops on the tooth structure, embedded in a matrix of bacterial and salivary polymers. The dental plaque

that forms over time results in a structurally and functionally organised species rich microbial biofilm. The biofilm structure of dental plaque protects microorganisms from antibiotics, making periodontitis a particularly difficult disease to treat therapeutically (Marsh et al., 2011). In a biofilm model of *Streptococcus gordonii* and *P. gingivalis*, azithromycin and erythromycin decreased the biofilm and killed bacteria in the biofilm more deeply than other antibiotics such as josamycin and minocycline (Tamura et al., 2008). Maezono et al., (2011) found in a biofilm using four strains of *P. gingivalis*, that submicrobial azithromycin inhibited *P. gingivalis* adherence and ATP levels, while decreasing the density of the biofilm. Importantly, in a recent *in vitro* multi-species biofilm model using concentrations of several antibiotic regimens detectable in gingival crevicular fluid (10 μg/mL for azithromycin), all antibiotics showed that there was no significant reduction in total bacterial loads, but caused a species specific reduction in certain pathogens (*P. gingivalis*, *F. nucleactum*, *Campylobacter rectu s*, and *Streptococcus anginosus*) that may disrupt the biofilm unity (Belibasakis and Thurnheer, 2013). This study supports the clinical principle that antibiotics should not be used as a monotherapy, and justifies the necessity of mechanical debridement (disruption of the biofilm) in periodontal treatment (Herrera et al., 2008).

There is a growing body of evidence suggesting that macrolide antibiotics such as azithromycin possess modes of action that are independent of their antimicrobial properties (Altenburg et al., 2010). These anti-inflammatory or immune modulating effects have led to the widespread use of macrolide antibiotics in the treatment of various chronic inflammatory pulmonary diseases and dermatologic skin conditions that feature damage to the host cells as a result of an exuberant or distorted inflammatory response (Bardazzi et al., 2007, Altenburg et al., 2010, Altenburg et al., 2011a).

Given that periodontitis is a condition with a bacterial aetiology and an inflammatory pathogenesis, a drug with both antimicrobial and anti-inflammatory action may be an ideal adjunct to mechanical debridement. However, to date the mechanisms of possible anti-inflammatory action of azithromycin in the treatment of periodontitis have not been fully investigated.

1.7 Anti-inflammatory/immune modulating effects of azithromycin

The anti-inflammatory or immune modulating properties of azithromycin were first discovered in 1987 when Kudoh et al. reported the dramatic improvements in life expectancy in patients with diffuse panbronchiolitis when treated with erythromycin

(Kudoh et al., 1987). The unexpected success of this treatment was attributed to a potential anti-inflammatory effect, considering that the serum levels of erythromycin in these patients were well below the minimal inhibitory concentrations for detectable pathogens, and erythromycin had a lack of sensitivity to these Gram-negative organisms. Consequently azithromycin has been used for the ongoing management of various chronic inflammatory pulmonary diseases and cystic fibrosis (Amsden, 2005). Azithromycin is thought to exert these immunomodulatory actions through alterations in the innate immune system, influencing the production of cytokines and innate immune cells (particularly neutrophils), as well as influencing the adaptive immune system (Altenburg et al., 2011a).

1.7.1 Effect on cytokines production and transcription factors

Studies conducted in chronic inflammatory pulmonary diseases have found azithromycin to inhibit the synthesis and/or secretion of pro-inflammatory cytokines (Culic et al., 2001). For example, azithromycin has been shown to inhibit the synthesis of reactive oxygen species and the secretion of pro-inflammatory cytokines (especially IL-8, a potent chemotactic agent). It also affects leukocyte adhesion by suppressing concentrations of soluble vascular cell adhesion molecule-1 (Culic et al., 2002). Furthermore, azithromycin reduces IL-1β and GM-CSF in lung tissue (Bosnar et al., 2009) and some of the other macrolides tested could also suppress IL-1β and PGE_2. In a mouse model of septic shock induced by lipopolysaccharide (LPS), it was also noted that azithromycin significantly reduced TNF-α and increased the survival of mice (Ivetic Tkalcevic et al., 2006).

When tested on patients with chronic obstructive pulmonary disease (COPD), azithromycin was associated with a significant decrease in serum neutrophils and basophils as well as C-reactive protein (CRP) and IL-8, although varying results were obtained for IL-8 (Parnham et al., 2005). In addition, in an *in vitro* study testing the effects of a number of macrolides on cells isolated from human steroid-naïve COPD sputum samples, IL-1β, IL-6, IL-10, TNF-α and a number of chemokines showed pronounced concentration-related reductions with the addition of a macrolide, including azithromycin (Marjanovic et al., 2011). Beigelman et al. (2009) also observed the anti-inflammatory effects of azithromycin in an *in vivo* non-infectious model of allergic airway inflammation. In this study, there was a decrease in eosinophils, macrophages, lymphocytes and neutrophils, together with reductions in IL-5, IL-13, and IL-4, although no effect on IL-1β was observed. Furthermore, *in vitro* macrolides (including azithromycin) significantly improve phagocytic and chemotactic properties, as well as decrease the pro-inflammatory cytokines

IL-8, TNF-α and IL-6, as well as inducible nitric oxide (iNOS) in monocytes (Gao et al., 2010), consistent with previously mentioned studies (Parnham et al., 2005, Marjanovic et al., 2011).

The main mechanism by which azithromycin is thought to inhibit pro-inflammatory cytokines is through the effect on several transcription factors involved in the inflammatory response and cytokine production, particularly nuclear factor κB (NF-κB) and the activator protein (AP-1). NF-κB is a ubiquitous rapid acting transcription factor in cells involved in inflammation or immune responses. Activator protein-1 (AP-1) is also a transcription factor that converts extracellular signals in bone and immune cells to change the expression of particular target genes by binding to the AP-1 binding site in the promoter region of the target DNA.

In the presence of azithromycin in cystic fibrosis airway epithelial cells, there was a 50% and 70% reduction of NF-κB and AP-1 in regulating IL-8 promoter transcriptional activity, and accordingly a 40% reduction in IL-8 mRNA and protein expression (Cigana et al., 2006). In tracheal aspirate cells obtained from premature infants and stimulated with TNF-α, NF-κB activation was suppressed by azithromycin with an associated reduction in IL-6 and IL-8 (Aghai et al., 2007). Overall, azithromycin has been shown to reduce cytokine levels via the inhibition of NF-κB in several cell types *in vitro* (Cigana et al., 2006, Aghai et al., 2007, Matsumura et al., 2011). A study has also attempted to compare the potential inhibitory NF-κB action of azithromycin with other anti-inflammatory drugs (hydrocortisone and dexamethasone). Hydrocortisone was approximately four orders of magnitude more potent than azithromycin, while dexamethasone was approximately fourteen times as potent as hydrocortisone (Cheung et al., 2010). Azithromycin also inhibits macrophage IL-12p40 production in macrophages after LPS/IFN-γ stimulation by inhibiting binding of AP-1, and nuclear factor of activated T cells (NFAT) to the DNA binding site of the IL-12p40 promoter (Yamauchi et al., 2009). Bosnar et al. (2011) also found that azithromycin inhibits IL-1β production through AP-1 activation in alveolar macrophages in an *in vivo* mice model of LPS induced pulmonary neutrophilia (Bosnar et al., 2011).

1.7.2 Effect of azithromycin on the innate immune cells

Azithromycin has a range of effects on the function of innate immune cells including polymorphonuclear leukocytes (neutrophils), monocytes, macrophages and dendritic cells.

1.7.2.1 Effect of azithromycin on polymorphonuclear leukocytes (neutrophils)

Polymorphonuclear leukocytes (neutrophils) are cells recruited to the site of infection or injury at the early stages of inflammation due to chemotactic agents. Neutrophils engulf bacteria via phagocytosis and eliminate them by intracellular killing facilitated by two neutrophil processes. These comprise: the release of enzymes such as myeloperoxidase and a variety of proteolytic enzymes from lysosomal granules, and the synthesis and release of superoxide and hydroxyl radicals. The role of neutrophils is to remove and protect the host in the early stages from bacterial infection, however research into the pathogenesis of periodontitis suggests that the destructive aspect of inflammation is through neutrophil-mediated tissue damage and bone resorption (Ryder, 2010).

As discussed in Section 1.6, an important way in which azithromycin is effective in inflamed tissue is the ability of phagocytic cells, particularly neutrophils, to take up azithromycin (Gladue et al., 1989). With the prolonged retention of azithromycin by phagocytic cells, this allows effective delivery and subsequent release of the accumulated drug at sites of infection (Hand and Hand, 2001). In addition to the uptake of azithromycin by neutrophils, there is some evidence that azithromycin may affect neutrophil function by modulating the expression of adhesion molecules, reducing neutrophil chemotaxis, suppressing reduction oxygen species, and induction of cell degranulation and apoptosis (Altenburg et al., 2011a).

In 12 healthy volunteers who received azithromycin (500 mg/day for 3 days), blood samples were taken at various intervals up to 28 days following the last dose in order to evaluate neutrophil activity. An initial neutrophil de-granulating effect was found with enhancement of the oxidative response to a particulate stimulus. These actions were associated with high plasma and neutrophil concentrations. However, after this and up to 28 days after the last dose of azithromycin, a subsequent downregulation in oxidative burst occurred with an increase in apoptosis of neutrophils and a corresponding fall in chemokine and IL-6 concentration (Culic et al., 2002). The acute neutrophil stimulation at the beginning of the study was suggested to facilitate the antibacterial actions of azithromycin, while the delayed anti-inflammatory activity could resolve inflammation. This study also found a decrease in serum concentrations of vascular cell adhesion molecule-1 (VCAM) and E-selectin 24 hours after the last dose of azithromycin and lasting up to 28 days.

Azithromycin was also shown to decrease neutrophil chemotactic factors and reduce neutrophil chemotaxis in other inflammatory or autoimmune conditions. In particular, azithromycin results in a reduction in neutrophilia in patients with bronchiolitis obliterans syndrome after surgery (Verleden et al., 2006), and in patients with cystic fibrosis (Ratjen et al., 2012). It should be noted that these studies prescribed azithromycin to patients for extended periods of time (3 months), compared to the 3 day dose administered in clinical studies in periodontitis patients. The mechanism for the observed reduction in neutrophilia was evaluated in an animal model of intranasal LPS challenge in mice (Bosnar et al., 2009). Azithromycin reduced the neutrophil dominated pulmonary inflammation potentially by the inhibition of GM-CSF and IL-1β production by alveolar macrophages (Bosnar et al., 2009).

1.7.2.2 Effect of azithromycin on monocytes, macrophages, and dendritic cells

Circulating blood monocytes can differentiate into macrophages and dendritic cells and are responsible for antigen presentation, therefore alerting the specific components of the adaptive immune system to injurious stimuli. They also produce cytokines and take part in phagocytosis. In healthy patients treated with a 3 day regimen of 500 mg of azithromycin, high concentrations of the drug were present in neutrophils and monocytes compared with plasma concentrations, and were still detectable in these phagocytes for up to 14 days, enhancing the intracellular antimicrobial action against Staphylococci (Wildfeuer et al., 1996).

Azithromycin has been shown to have an effect on the function and differentiation of monocytes, macrophages, and dendritic cells. In particular azithromycin has been shown to affect the production of cytokines by monocytes. An *in vitro* study evaluating the effect of azithromycin and clarithromycin on cytokine production by LPS stimulated human monocytes found that the production of IL-1α, IL-1β, IL-6, IL-10, GM-CSF and TNF-α were affected (Khan et al., 1999). Of interest in this study was that azithromycin reduced IL-1α and TNF-α in 100% of the donors (five donors), while the response to azithromycin of other cytokine levels varied between donors, suggesting differences in individual responsiveness to the drug.

Macrolide antibiotics also appear to encourage the differentiation of monocytes to macrophages. Erythromycin promoted the differentiation of human monocytes to a macrophage lineage (Keicho et al., 1994). Chemically modified erythromycin A (stronger

promoter activity and no antibacterial action) promoted the differentiation of the THP-1 cell line (derived from patients with monocytic leukemia) (Yoshida et al., 2005).

Macrophages are primarily responsible for the phagocytosis and removal of bacteria, as well as encouraging fibrosis and inflammatory resolution in tissues. Macrophages are activated by bacterial endotoxin and other microbial products, cytokines secreted by sensitized T lymphocytes (in particular IFN-α), various inflammatory mediators, and extracellular matrix proteins (Kumar V, 2007).

There is some evidence that azithromycin is able to modify the activation of macrophages, shifting the phenotype from a classical M1 (inflammatory) phenotype towards the M2 (resolution and repair) phenotype (Navarro-Xavier et al., 2010). In particular, azithromycin has been shown to modulate the classical activation of human monocytes by the inhibition of TLR4-mediated signaling, and by down-regulating NF-κB and STAT1 transcription factors. Azithromycin also enhanced the gene expression of M2 macrophage markers (IL-10 and chemokine (C-C motif) ligand 18 (CCL18) (Vrancic et al., 2012). Azithromycin has also been shown to down-regulate the production of the inflammatory cytokines and chemokines IL-1β, CC-2, and TNF-α by M1-polarised cystic fibrosis alveolar macrophages in a mouse model (Meyer et al., 2009). Finally, azithromycin appears to have a long standing pro-resolution effect on inflammation versus a short term dampening of oedema and leukocyte influx that occurs in the first few hours of an inflammatory process, as tested in a unique aggressive inflammation animal model (10 mg of zymogen to induce peritonitis in mice) (Navarro-Xavier et al., 2010).

Dendritic cells are antigen-presenting cells that are able to initiate the adaptive immune response by activating naïve T lymphocytes. Bacterial products such as LPS, and inflammatory cytokines drive the maturation of dendritic cells, characterized by the up-regulation of major histocompatibility complex (MHC) class II and co-stimulatory molecules CD40, CD80, and CD86. The activation of dendritic cells also results in dendritic cell production of high levels of IL-12, a key cytokine in the induction of T helper type 1 (Th1) responses. In LPS induced dendritic cells derived from murine bone marrow, azithromycin significantly inhibited expression of co-stimulatory molecules and MHC Class II, as well as reducing TLR-4 and therefore inhibiting the NF-κB signaling pathway and IL-12 production. These results suggest that azithromycin inhibits the activation of the adaptive immune system by blocking the interaction between dendritic cells and T lymphocytes (Iwamoto et al., 2011).

1.7.3 Effect of azithromycin on adaptive immune system

Macrolide antibiotics also demonstrate an impact on T cell regulation (Altenburg et al., 2011a). Long term use of erythromycin, roxithromycin, or clarithromycin in patients with diffuse panbronchiolitis has been reported to result in a significant reduction of activated cytotoxic T cells in bronchoalveolar lavage fluid, to levels below normal (Kawakami et al., 1997). *In vitro* studies also suggest that 14- and 15- membered ring macrolides appear to be involved in the amplification of apoptosis of activated lymphocytes (Ishimatsu et al., 2004, Kadota et al., 2005). Macrolide antibiotics also inhibit the release of Th2 cytokines by T-helper cells, with IL-4 and IL-5 cytokine reduction found in healthy donor peripheral blood T cells (Asano et al., 2001).

As previously discussed, dendritic antigen-presentation to T lymphocytes also is inhibited by azithromycin (Iwamoto et al., 2011). Sugiyama et al., (2007) demonstrated that clarithromycin and azithromycin could alter the amounts of co-stimulatory molecules and pro-inflammatory cytokines to produce an overall anti-inflammatory effect in murine bone marrow derived dendritic cells.

1.7.4 Effect of azithromycin in periodontal tissues

Azithromycin is readily distributed in the periodontal tissues, especially in areas of inflammation (Blandizzi et al., 1999), and has been shown to alter cytokine levels in GCF (Ho et al., 2010), thereby being readily available for interactions with the host and pathogen, highlighting a potential anti-inflammatory role for the periodontium. However, there are limited studies that have investigated the anti-inflammatory effects of azithromycin in periodontal connective tissue. In human gingival fibroblasts stimulated with *P. gingivalis* LPS, IL-8 production was shown to be enhanced with azithromycin treatment. This increased production of IL-8 was considered to be a potential anti-inflammatory effect by azithromycin, as it may increase the migration of neutrophils to periodontal tissues and phagocytize the periodontopathic bacteria more efficiently (Kamemoto et al., 2009).

Matsumura et al. (2011), however found that IL-8 expression was inhibited by azithromycin in an oral epithelial cell line, and that this suppression was through the deactivation of the transcription factors NFκB and Rac 1. These results were conducted in KB cells that were thought to be derived from human oral epidermal carcinomas, but have now been known to have arisen from HeLa cell contamination (derived from cervical

carcinoma), and as such are less relevant for studies of the oral cavity (Tribble and Lamont, 2010).

Recently, it was also found that azithromycin inhibited osteoclast resorptive activity *in vitro*, and decreased the expression of the key osteoclast transcription factor Nuclear Factor of Activated T cells (NFATc1) and tumour necrosis factor receptor associated factor-6 (TRAF-6), as well as reducing Integrin β3 and MMP-9 mRNA expression in osteoclasts cultured on dentine (Gannon et al., 2012).

The anti-inflammatory and immunomodulating action of macrolides, and specifically azithromycin, that have been shown *in vitro* provide evidence of the potential for azithromycin to have a beneficial effect in the treatment of periodontal disease through these actions. It is therefore important to clarify the mechanisms of these immune modulating actions of azithromycin in periodontal tissues. This will provide validation of its use clinically as an adjunctive treatment to mechanical debridement for aggressive and refractory forms of chronic periodontitis.

1.8 Common side effects and antibiotic resistance

The presence or absence of adverse events following the adjunctive use of systemic antibiotics appears to be seldom reported within the literature. Most reported adverse effects are minor, and related to gastrointestinal problems such as diarrhoea and nausea. There is however, a potential for serious adverse events to occur with antibiotic use, such as allergic/anaphylactic reactions or pseudomembranous colitis, and patients should be informed of the potential risks, both minor and major, when prescribing systemic antibiotics. Anaphylactic responses to penicillin occur approximately once every 10,000 courses administered, with 10 per cent of these being fatal (Wilson et al., 2007). Furthermore, despite azithromycin being thought to have minimal cardiotoxicity compared with other macrolide antibiotics, a recent cohort study found that during 5 days of azithromycin therapy there was a small absolute increase in cardiovascular deaths, which was most pronounced among patients with a high baseline risk of cardiovascular disease (Ray et al., 2012). Therefore, use of antibiotics should be carefully considered, with a preference given to agents that maximize antimicrobial activity and minimize potential drug interactions and adverse reactions. A thorough medical history should be taken prior to antibiotic prescription.

An increase in microbial resistance following the use of systemic antibiotics as a treatment adjunct in periodontitis has been evaluated in a few studies. Feres et al. (2002)

identified antibiotic-resistant species in subgingival plaque and saliva samples from 20 chronic periodontitis patients treated by scaling and root planing followed by orally administered amoxicillin or metronidazole (14 day course). Results demonstrated an increase in the percentage of resistant subgingival species following antibiotic administration. However, levels returned to baseline after a relatively short period of time (90 days) (Feres et al., 2002).

There has also been the first description of resistance in an oral biofilm, due to horizontal gene transference, reported *in vivo* . *Streptococcus cristaceus* acquired a transposon that conferred doxycycline resistance from a strain of *Streptococcus o ralis*. Both strains were isolated from the subgingival biofilm in patients undergoing doxycycline therapy as part of their periodontal treatment (Warburton et al., 2007). This transfer had been observed previously in non-oral strains, such as two strains of *Staphylococcus aureus* that acquired an operon associated with vancomycin resistance (vanA operon) from *Enterococcus faecalis* strains (Weigel et al., 2003).

In Spain, where systemic antibiotics are readily available over the counter without prescription and widely used in the general population, it has been shown that there was an increase in the microbial resistance patterns of oral bacteria to commonly prescribed antibiotics (higher minimal inhibitory values in Spanish strains of *F. nucleatum* , *P. intermedia*, *M. micros* and *P. gingivalis*, for a number of common antibiotics), compared to the Netherlands where antibiotics use is more restricted (van Winkelhoff et al., 2005). The authors of this study concluded that the higher minimal inhibitory concentrations (MIC) among Spanish bacterial isolates and the higher percentage of resistant periodontal bacterial strains is more likely due to higher antibiotic consumption and poor compliance to the medication in Spain. Similar results were obtained in a recent study comparing antibiotic susceptibility profiles of five periodontal pathogens from Europe with that of a South American country (Colombia), revealing a much higher antibiotic resistance of these pathogens in Colombia (Veloo et al., 2012). These studies underline the importance of responsible use of antimicrobials to prevent the spread of resistant strains of bacteria.

1.9 Compliance

The issue of patient compliance has been infrequently addressed in publications evaluating the effects of systemic antibiotics. Some studies have shown that as little as 20 per cent of patients comply with prescribed antibiotic regimens (Llor et al., 2009). Studies suggest that with the shorter administration regimen of one dose of azithromycin (500mg)

every 24 hours for only three consecutive days may increase patient compliance (Haas et al., 2008).

Compliance in terms of oral hygiene and maintenance care should also be addressed. Kornman et al. (1994) evaluated the impact of plaque control on outcomes following antibiotic therapy in periodontitis treatment, concluding that supragingival plaque control is an essential factor in attaining certain clinical and microbial outcomes following systemic antibiotic therapy in periodontitis.

1.10 Conclusion

Azithromycin is a unique macrolide that shows potential as an adjunct in the treatment of periodontitis, as several clinical studies have demonstrated additional clinical benefits when compared to conventional treatment alone. Azithromycin has good pharmacological properties with a long half-life, and high tissue concentrations at the site of infection. These favourable pharmacokinetic properties of azithromycin allow for administration once a day (500 mg) for short periods of time (3-5 days), therefore facilitating patient compliance to treatment. The drug also has a low incidence of side effects. These favourable characteristics represent numerous advantages over other antibiotics in the treatment of periodontitis.

In addition, increasing research suggests that azithromycin not only has antimicrobial properties, but potential anti-inflammatory and immune modulatory actions as well. *In vitro,* animal models and clinical trials have found that azithromycin has a range of effects including anti-inflammatory and pro-resolving actions, alterations in cytokine and chemokine profiles, and cell modulatory effects on fibroblasts, neutrophils, and monocytes. The anti-inflammatory and immune modulating properties of azithromycin have also been used for many years to treat other chronic inflammatory medical conditions of bacterial origin. Given that periodontitis is a condition with a bacterial aetiology and an inflammatory pathogenesis, a drug with both an antimicrobial and anti-inflammatory action may be an ideal adjunct in the management of periodontal disease. However, to date there has been limited available research on the anti-inflammatory and immune modulatory effects in periodontal tissues.

1.10.1 Study rationale, hypothesis and aims

The study described in Chapter 2 uses an *in vitro* model to assess the effect of azithromycin on the *P. gingivalis* LPS induced pro-inflammatory production of cytokines

and chemokines in human gingival fibroblasts. The rationale for this study is based on a number of findings in the scientific literature. Firstly, resolution of inflammation has been reported in case studies following systemic administration of azithromycin for severe chronic and aggressive forms of periodontitis (Hirsch, 2010). Secondly, azithromycin has been proven to be beneficial in conditions such as panbronchiolitis and cystic fibrosis, presumably due to an anti-inflammatory mechanism of action in addition to its direct antimicrobial action, with increasing studies supporting this concept (Culic et al., 2001, Altenburg et al., 2011b). Human gingival fibroblasts were chosen as the periodontal cell for investigation as, in addition to playing an important role in the remodelling of the periodontal soft tissue, gingival fibroblasts act as regulators of the cytokine network in periodontal tissues, producing cytokines when stimulated by inflammatory cytokines or bacterial cell components (Takada et al., 1991). The aim of this study is therefore to investigate the effect of azithromycin on pro-inflammatory cytokine and chemokine production by human gingival fibroblasts induced with a periodontal pathogen virulence factor (*P. gingivalis* LPS), to further elucidate the potential anti-inflammatory actions of azithromycin in periodontal tissues. This study hopes to provide further evidence to support the use of azithromycin as a host-modulatory adjunct in the treatment of periodontitis. The null hypothesis is that azithromycin will have no effect on the *P. gingivalis* LPS induction of pro-inflammatory cytokines/chemokines in human gingival fibroblasts.

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Chapter 2. Azithromycin suppresses *P. gingivalis* LPS induced pro-inflammatory cytokine and chemokine production (IL-6, IL-8, MCP-1 & GRO) by human gingival fibroblasts *in vitro*

Doyle CJ[1], Fitzsimmons TR[1], Marchant C[1], Dharmapatni AASSK[2], Hirsch R[1], Bartold PM[1]

[1]Colgate Australian Clinical Dental Research Centre, School of Dentistry, The University of Adelaide, South Australia, 5005, Australia.

[2]Discipline of Anatomy and Pathology, School of Medical Sciences, The University of Adelaide, South Australia, 5005, Australia.

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2.1 Introduction

Periodontitis is a common bacterially induced chronic inflammatory disease of the oral cavity (Burt, 2005, Borrell et al., 2005). Cross-sectional epidemiological studies indicate that 10 to 15% of the adult population have 'advanced periodontitis', while up to 80% of the population may develop 'moderate periodontitis' (Loe et al., 1978, Genco and Slots, 1984, Papapanou et al., 1988, Hugoson et al., 1998). In a recent American National Health and Nutrition Examination Survey (NHANES) in 2010, 47% of the sample population had periodontitis, comprising 8.7% with mild, 30% with moderate, and 8.5% with advanced disease. Furthermore, 64% of the 65 year old or older population had moderate to advanced periodontitis (Eke et al., 2012).

Periodontitis is characterised by chronic inflammation of the periodontal tissues that is initiated by an accumulation of dental plaque. As the plaque matures it changes from Gram-positive cocci to Gram-negative cocci, rods and spirochetes. Socransky et al. (Socransky et al., 1998) established that the subgingival bacterial species co-aggregate into a number of communities. One group, known as the "red Socransky complex", is considered to be highly periopathogenic. This group comprises *Tannerella forsythia* (*T. forsythia*), *Porphyromonas gingivalis* (*P. gingi valis*), and *Treponema denticola* (*T. denticola*). The pathogenesis of the disease is complex however, with the bulk of tissue destruction occurring as a result of the host response to the bacterial challenge (Birkedal-Hansen, 1993). The destruction is driven by inflammatory processes that are triggered not only by the bacteria, but also by their products (Van Dyke et al., 1993). For example, *P. gingivalis* lipopolysaccharide (LPS) can stimulate cells in the periodontal tissues (human gingival fibroblasts) to produce inflammatory mediators (cytokines and chemokines) such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor–alpha (TNF-α) (Imatani et al., 2001). Periodontitis therefore occurs due to an interplay between the pathogens within the subgingival plaque and the host response. This host inflammatory response can be influenced by acquired and environmental risk factors such as diabetes and smoking, as well as a genetic predisposition to the condition (Kornman, 2008).

The inflammatory response to the bacterial challenge is characterized by the infiltration of the tissues by neutrophils, macrophages and lymphocytes, as well as the generation of high concentrations of locally produced cytokines, eicosanoids and destructive mediators such as matrix metalloproteinases (MMP's). It is these mediators that, in susceptible individuals with an exuberant inflammatory response, lead to eventual

periodontal tissue destruction. Clinically, this manifests as gingival inflammation and periodontal pocket formation, leading to loss of connective tissue and alveolar bone around teeth. If left untreated, this ultimately leads to the loss of teeth (Preshaw and Taylor, 2011).

The conventional approach for treating periodontal disease relies on the reduction of the bacterial load by disrupting the biofilm, which in turn reduces the disproportionate inflammatory reaction that may occur in a susceptible host. At present, this is achieved primarily by means of mechanical debridement, which is found to improve both clinical and microbiological parameters (Greenstein, 1992).

A small proportion of patients do not respond well to traditional treatment however, and may still lose teeth (Hirschfeld and Wasserman, 1978, McFall, 1982). This is more frequently observed in patients with aggressive and refractory forms of the disease (Drisko, 2001). It has therefore been suggested that the use of systemic antibiotics in conjunction with mechanical debridement may provide additional clinical benefit in patients with aggressive periodontitis, or chronic periodontitis with multiple failing sites (Herrera et al., 2002, Haffajee et al., 2003, Herrera et al., 2008, Heitz-Mayfield, 2009).

Several different antibiotics have been used in the past to treat aggressive periodontal disease. The most frequently used to date have been penicillin (amoxicillin) (Heitz-Mayfield, 2009), metronidazole (Loesche et al., 1984), tetracycline (Al-Joburi et al., 1989) and clindamycin (Brook et al., 2005). These treatments have all been found to produce varying degrees of success (Slots and Ting, 2002). The most common antibiotic regimen reported is a combination of metronidazole and amoxicillin (Yek et al., 2010). The subgingival biofilm in periodontal lesions comprises both anaerobic and facultative bacteria, so the combination of both drugs covers a broad spectrum of microbiota and has been shown to be an effective adjunct to conventional therapy (van Winkelhoff et al., 1989). Compliance can however become an issue, as administration of amoxicillin and metronidazole requires taking several tablets per day for long periods of time, and has potentially unpleasant side effects such as a disulfiram-like reaction with ethanol, which may further lower compliance (Slots and Ting, 2002).

The contribution of the host response to the bacterial challenge may play a significant role in the propagation of the disease process (Offenbacher, 1996, Page and Beck, 1997, Page et al., 1997), so host modulation therapy may offer another adjunct to the conventional treatment of periodontitis. While conventional periodontal therapy targets one aspect of the pathogenic process by reducing the bacterial burden, host modulation therapy aims to reduce the production of inflammatory mediators by the host in response to the bacterial challenge (Altenburg et al., 2011). At present, the only approved host

modulation agent is subantimicrobial-dose doxycycline (SDD, Periostat®), an inhibitor of tissue matrix metalloproteinases (MMPs), that has been used as an adjunct to subgingival root debridement (SRD). The long-term effectiveness of SDD treatment however remains questionable, due to a lack of well-designed long term studies (Sgolastra et al., 2011). Other agents such as nonsteroidal anti-inflammatory drugs and bone sparing agents (bisphosphonates) have also been trialled with limited success, and have significant systemic side effects (Williams et al., 1989, Reddy et al., 2003).

Azithromycin is a bacteriostatic antimicrobial that inhibits bacterial protein synthesis via interactions with specific ribosomal proteins and the 23S rRNA in the peptidyl transferase centre (Zuckerman et al., 2009). It has been shown to have good activity against Gram-negative microorganisms, (Retsema et al., 1987) including periodontopathogens such as *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* (*A. a*.) (Pajukanta et al., 1992, Pajukanta, 1993, Muller et al., 2002). Clinical benefits of azithromycin as an adjunct to conventional therapy have been demonstrated in clinical trials involving both aggressive and chronic periodontitis (Sefton, 1999, Smith et al., 2002, Haffajee et al., 2007, Gomi et al., 2007b, Haas et al., 2008, Haffajee et al., 2008, Yashima et al., 2009, Oteo et al., 2010). Azithromycin possesses favourable pharmacological properties including long half-life and high tissue concentration at the site of infection. These allow for an easy to follow treatment regimen, comprising once daily administration for three to five days, which may facilitate better patient compliance to treatment (Haas et al., 2008). Furthermore, several case studies report significant reductions in inflammation and alveolar bone regeneration with the adjunctive use of azithromycin in periodontitis (Schmidt and Bretz, 2007, Hirsch, 2010).

In addition to the well-known antibacterial properties of macrolide antibiotics, considerable evidence has emerged in the medical literature showing that 14- (e.g. erythromycin, clarithromycin, and roxithromycin) and 15- (e.g. azithromycin) membered ring macrolides possess modes of actions that are independent to their antimicrobial activity. This was first shown in 1987 by Kudoh et al., who reported a substantial increase in life expectancy and decreased symptoms in patients with diffuse panbronchiolitis when treated with erythromycin. Since this discovery, evidence has convincingly shown that macrolides such as azithromycin also possess anti-inflammatory or immune modulating effects. These additional properties have led to the extensive use of macrolides for various chronic inflammatory pulmonary diseases where an altered inflammatory response results in damage to the host tissues (Altenburg et al., 2011). Considering the exuberant host-immuno-inflammatory response observed in aggressive and refractory forms of periodontal

disease, a drug with both antimicrobial and anti-inflammatory actions such as azithromycin may be an ideal adjunctive treatment. (Kudoh et al., 1987)

To date there have been few studies investigating the anti-inflammatory effects of azithromycin in periodontal connective tissue. In a study of human gingival fibroblasts (HGFs) stimulated with *P. gingivalis* LPS, it was found that pro-inflammatory chemokine interleukin-8 (IL-8) production was enhanced with azithromycin treatment (Kamemoto et al., 2009). Matsumura et al., (2011), however found that IL-8 expression was inhibited by azithromycin in an oral epithelial cell line infected with *A. a* LPS. This suppression was mediated through the deactivation of transcription factors, nuclear factor kappa B (NF-κB) and Rac 1. This inhibition of IL-8 expression was thought to suggest a potential anti-inflammatory effect of azithromycin on human oral epithelial cells, as IL-8 is a neutrophil chemoattractant and activator, which can result in gingival tissue damage by neutrophil degranulation. Furthermore, IL-8 mRNA expression has been shown to correspond to the level of periodontitis severity (Tonetti et al., 1994).

More recently, azithromycin was shown to inhibit osteoclast resorptive activity *in vitro*, decrease the expression of key osteoclast transcription factor Nuclear Factor of Activated T cells (NFATc1) and tumour necrosis factor receptor associated factor-6 (TRAF-6), as well as decrease Integrin β3 and MMP-9 mRNA expression in osteoclasts cultured on dentine (Gannon et al., 2012).

Human gingival fibroblasts play an important role in the remodelling of periodontal tissues, through synthesising extracellular matrix proteins and regulating this synthesis through the production of MMPs that degrade connective tissue. Fibroblasts respond to various inflammatory cytokines and growth factors that regulate the balance between extracellular matrix synthesis and degradation, which can result in the connective tissue destruction found in periodontitis (Takashiba et al., 2003). Furthermore, gingival fibroblasts may be regulated in an autocrine manner because they produce several cytokines and chemokines when stimulated by inflammatory cytokines or bacterial cell components (Takada et al., 1991).

In light of the above evidence we have hypothesized that azithromycin has the capacity to reduce inflammatory cytokine production by human gingival fibroblasts and this may explain some of its clinical properties. The aim of this *in vitro* study was therefore to examine the potential anti-inflammatory effect of azithromycin on *P. gingivalis* LPS induced production of pro-inflammatory cytokines and chemokines in human gingival fibroblasts, in order to better understand the potential application of azithromycin in the adjunctive treatment of periodontitis.

2.2 Materials and methods

Ethics approval was obtained from the University of Adelaide Human Research Ethics Committee, project number: H-57-2003.

2.2.1 Sample collection and cell culture

Healthy gingival tissue was retrieved with informed consent following crown lengthening surgery, as described by Wada et al. (2011), from patients attending the Colgate Australian Clinical Dental Research Centre. Tissues were digested with collagenase (3 mg/mL; Worthington Biochemical, Lakewood, NJ, USA) and dispase (4 mg/mL; Roche Diagnostics, Indianapolis, IN, USA) for 2 hours at 37°C to obtain single cell suspensions. Three age and sex matched donor cell lines were used for these experiments. The cell cultures were maintained in α-minimal essential medium (α-MEM) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/mL of penicillin and 50 μg/mL of streptomycin (all sourced from Gibco®, Life Technologies, Mulgrave, Vic, Australia) and 100 μM L-ascorbate-2-phosphate (Wako Pure Chemical Industries, Richmond, VA, USA) in accordance with Wada et al., (2011). The fibroblasts were routinely grown at 37 °C, 5% CO2 in an air atmosphere. All experiments were performed with cell cultures between passage 2 and 9. (Wada et al., 2011)

2.2.2 *P. gingivalis* LPS and azithromycin treatments

Human gingival fibroblasts (three donors in triplicate culture) were suspended at a concentration of 5×10^4 cells/mL and seeded into 24 well plates (Becton Dickinson, Franklin Lakes, NJ, USA), and incubated for 24 hours. Cells were then washed with Hanks Balanced Salt Solution (Gibco®), followed by incubation in serum free media for 24 hours.

Azithromycin dehydrate (Sigma, Sydney, NSW, Australia) was solubilised in dimethylsulfoxide (DMSO, VWR International Ltd, Poole, England). Serial dilutions of azithromycin in serum free media containing 0.1% DMSO were used to obtain concentrations of 0.1 μg/mL, 1 μg/mL, and 10 μg/mL. Cells were then treated (in triplicate) with *P. gingivalis* LPS (InvivoGen, San Diego, CA, USA) at 1 μg/mL as determined by pilot induction studies, or azithromycin at three concentrations (0.1 μg/mL, 1 μg/mL, and 10 μg/mL), or with a combination of both *P. gingivalis* LPS and

azithromycin at all concentrations. Control cells were treated with 0.1% DMSO. Cells were then incubated for 24 hours. In preliminary studies, cells were treated with and without 0.1% DMSO to ensure cell viability was not affected by DMSO exposure. Cell supernatant was collected and stored at -80 ℃ for further analysis.

2.2.3 WST-1 cell viability assay

The viability of cells cultured in the presence of *P. gingivalis* LPS and azithromycin was assessed using a colorimetric assay (WST-1) (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. Briefly, 100 µl of culture medium containing 5×10^4 cells/mL were seeded into a 96 well plate (Becton Dickinson), and were subjected to control (0.1% DMSO), *P. gingivalis* LPS or *P. gingivalis* LPS plus azithromycin as detailed above. Following 24 hours of treatment and 2 hours after the addition of WST-1 Cell Proliferation Reagent, absorbance (quantification of formazan dye produced by metabolically active cells) was measured at 450 nm using a Power-wave plate reader (Biotek Instruments, Winooski, VT, USA) with KC4 microplate data analysis software (Biotek Instruments).

2.2.4 Multiplex analysis of cytokines and chemokines production by gingival fibroblasts

The concentrations of IL-6, IL-8, MCP-1, and GRO in the cell culture supernatants were determined using a multianalyte profiling kit (Milliplex®MAP, Merck Millipore, MA, USA) according to the manufacturer's protocol. In brief, 25µl of cell culture supernatant was incubated with antibody-coated beads for 24 hours at 4°C. Afterwards, washed beads were incubated with biotinylated detection antibodies for 1 hour at room temperature, then incubated for 30 minutes with streptavidin–phycoerythrin conjugate. After a final wash, the beads were resuspended in a buffer (sheath fluid) and analysed with the Luminex 200™ Analyser (Luminex, Austin, TX, USA). A standard curve was generated using a weighted 5-parameter logistic curve (Dudley et al., 1985, Gottschalk and Dunn, 2005) for each mediator ranging from 3.2 pg/mL to 10,000 pg/mL using xPONENT version 3.1 software (Luminex Corporation). The minimal detectable concentration for each mediator for this assay, in pg/mL, were; IL-6: 0.3, IL-8: 0.2, MCP-1: 0.9, and GRO: 10.1. All standards, samples and controls were assayed in duplicate.

2.2.5 Statistics

Results were expressed as mean and standard error of the mean (SEM) and statistical analyses were performed using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp, released 2011). One-way analysis of variance (ANOVA) and a Post-Hoc Bonferroni test were used to compare differences between control (0.1% DMSO), *P. gingivalis* LPS and azithromycin treated *P. gingivalis* LPS induced fibroblast cytokine/chemokine protein induction for each individual donor, as well as for the combined donor data. Statistical significance was accepted at a p value <0.05.

2.3 Results

2.3.1 Effect of *P. gingivalis* LPS, azithromycin, or combination treatments on HGF cell viability

In order to ensure the proposed experimental conditions did not affect cell viability, WST-1 cell viability assays were performed on HGF's treated with *P. gingivalis* LPS, azithromycin or a combination of both. When all results were combined from each donor, 0.1% DMSO, *P. gingivalis* LPS alone (1 μg/mL), azithromycin at 0.1, 1, and 10 μg/mL, or the combination of *P. gingivalis* LPS and azithromycin at the various concentrations showed no statistical difference in the absorbance of formazan dye compared to untreated (control) cell cultures (p=0.98; Figure 2.1).

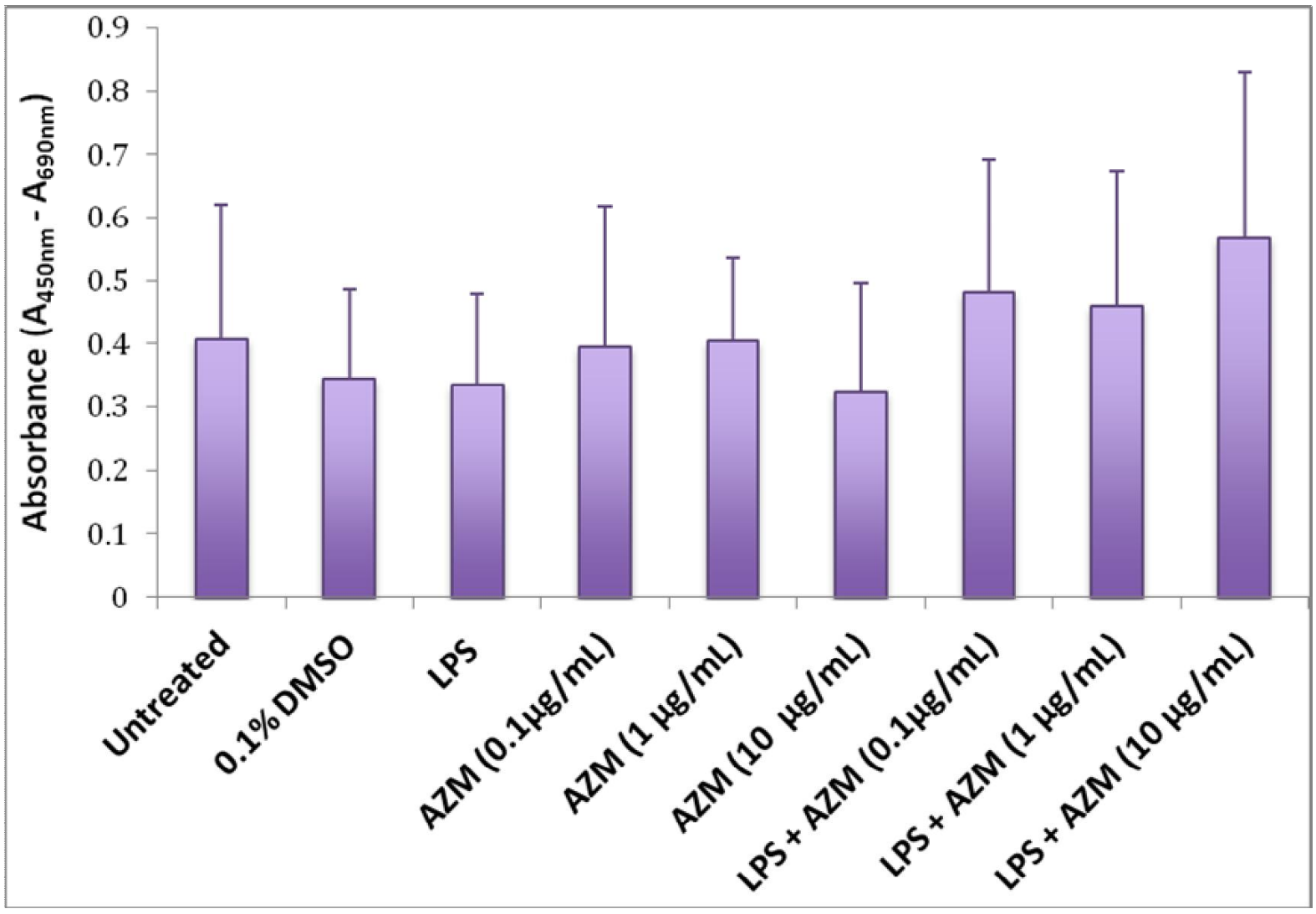

Figure 2.1 Effect of *P. gingivalis* LPS or azithromycin treatment on HGF cell viability
Human gingival fibroblasts were treated for 24 hours with 0.1% DMSO, *P. gingivalis* LPS ([LPS], 1 µg/mL), azithromycin ([AZM] 0.1, 1 10 µg/mL) or a combination of *P. gingivalis* LPS and azithromycin. Cell viability was measured using WST-1 assay with quantification of formazan dye produced by cells [Absorbance (A_{450nm} - A_{690nm})]. Bars represent mean ± SEM of 3 donors with treatments conducted in triplicate (p=0.98).

2.3.2 Effect of azithromycin on *P. gingivalis* LPS-induced GRO production

GROα is a chemokine associated with both inflammation and cell proliferation and is an important chemoattractant for human neutrophils as well as stimulating neutrophil degranulation. When the data from each of the 3 donors were combined, LPS alone significantly increased GRO production to over 400 pg/mL, compared to 17 pg/mL for the control treatment (0.1% DMSO alone, Figure 2.2). There was no effect of AZM alone on GRO production by the cells in the absence of LPS.

The effect of AZM on LPS induced GRO expression was also investigated and all three concentrations of AZM were found to significantly reduce LPS induced GRO production (p<0.05). With increasing doses of AZM (0.1, 1 and 10 µg/mL), LPS induced GRO protein production was reduced by 60, 70 and 72% respectively (Figure 2.2).

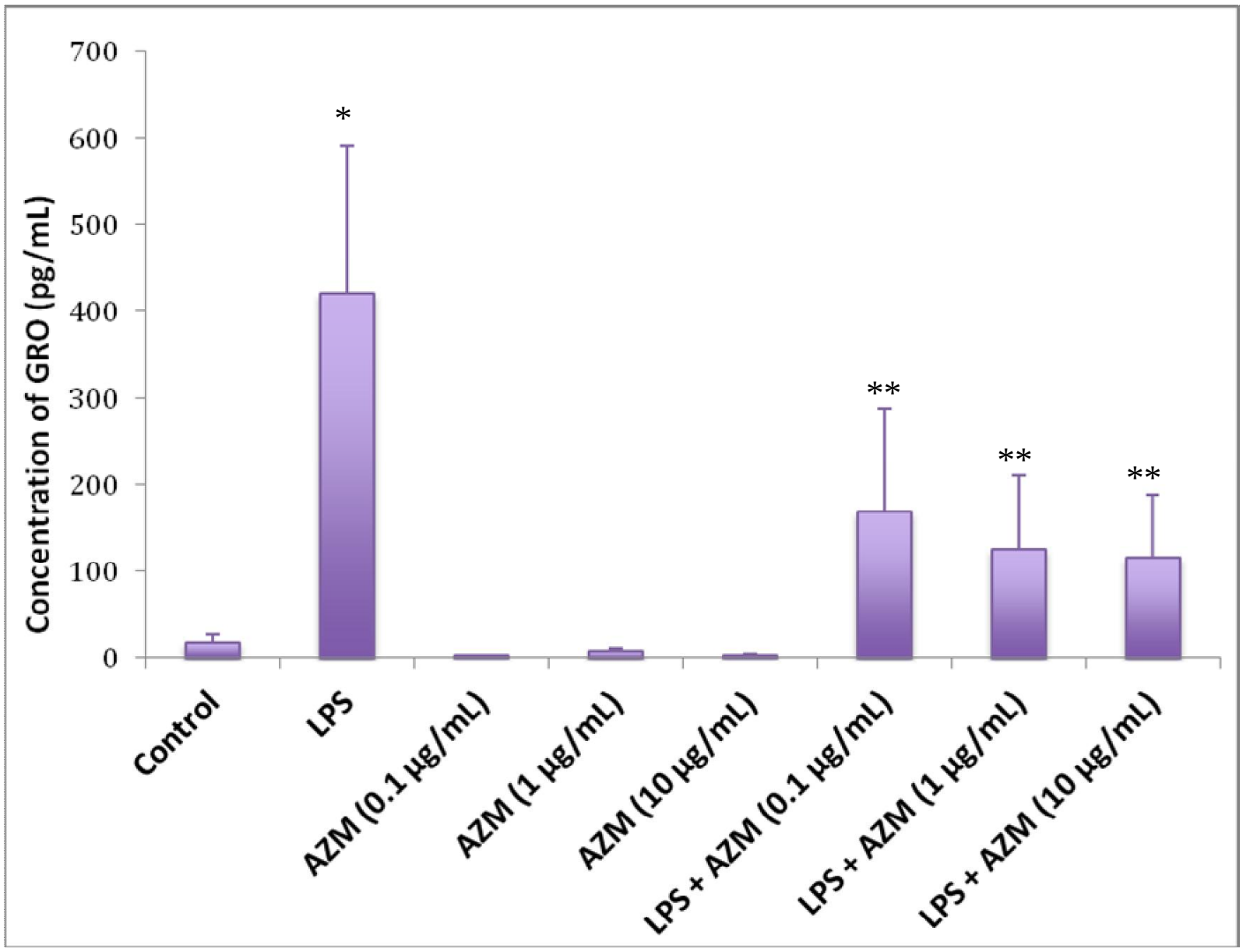

Figure 2.2 Combined donor data demonstrating the effect of a zithromycin on LPS induced GRO production

Human gingival fibroblasts were treated with *P. gingivalis* LPS alone (1 µg/mL), azithromycin (AZM at 0.1, 1 and 10 µg/mL), or a combination of LPS and azithromycin. GRO protein production was measured by multiplex analysis. Bars represent mean ± SEM of all donor experiments combined. Statistical significance was determined using a 1-way ANOVA and Bonferroni Post Hoc Test. *$p<0.05$ compared with control, ** $p<0.05$ compared with LPS.

When the individual donor data were analysed, the baseline production of GRO by HGF's from all 3 donors was minimal (Figure 2.3). Although the response of each of the 3 donors was variable in terms of LPS induction of GRO protein, all responses were statistically significant compared to the control (0.1% DMSO, $p<0.05$). Donor 1 showed the greatest protein induction of 760 pg/mL and Donor 3 showed the least induction of 212 pg/mL.

The combination of *P. gingivalis* LPS and AZM treatment for individual donors significantly reduced GRO protein production for the majority of concentrations tested compared to LPS alone ($p<0.05$; Figure 2.3). Donor 2 and 3 had a comparable average reduction of GRO protein concentration of 83.4% and 82.7% respectively, while Donor 1 had a less significant average reduction of protein induction of 56%.

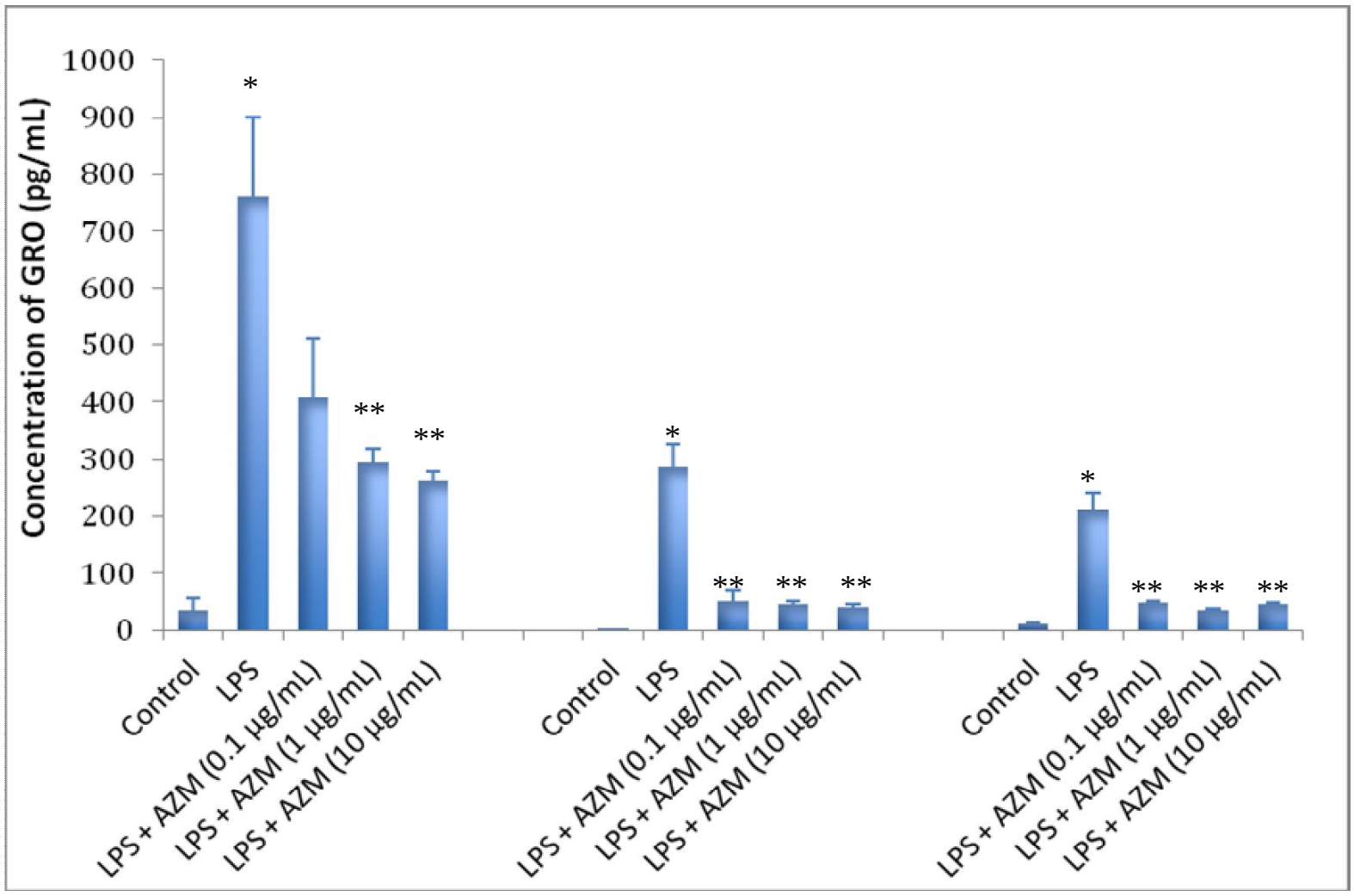

Figure 2.3 Var iability of induced GRO p roduction betwe en donors after treatment with azithromycin

Human gingival fibroblasts were treated with *P. gingivalis* LPS alone (1 µg/mL), or a combination of LPS and azithromycin (AZM, 0.1, 1, or 10 µg/mL). GRO protein production was measured by multiplex analysis. Bars represent mean ± SEM of one experiment performed in triplicate for each donor. Statistical significance was determined using a 1-way ANOVA and Bonferroni Post Hoc Test. *p<0.05 compared with control, **p<0.05 compared to LPS.

2.3.3 Effect of azithromycin on *P. gingivalis* LPS-induced IL-6 production

Given the important role of IL-6 in the regulation of bone resorption and B cell function, the effects of azithromycin treatment on its production induced by *P. gingivalis* LPS were assessed. When the data were combined from each of the 3 donors, LPS alone significantly increased IL-6 production to approximately 450 pg/mL compared to 13 pg/mL for the control treatment (0.1% DMSO alone; Figure 2.4). There was no effect of AZM alone on IL-6 production in the absence of LPS.

The effect of AZM at all three concentrations studied (0.1, 1 and 10 µg/mL) significantly reduced *P. gingivalis* LPS induced IL-6 production by HGFs (p<0.05). AZM reduced IL-6 protein induction by 78 to 86% (Figure 2.4), but there was no specific dose response relationship.

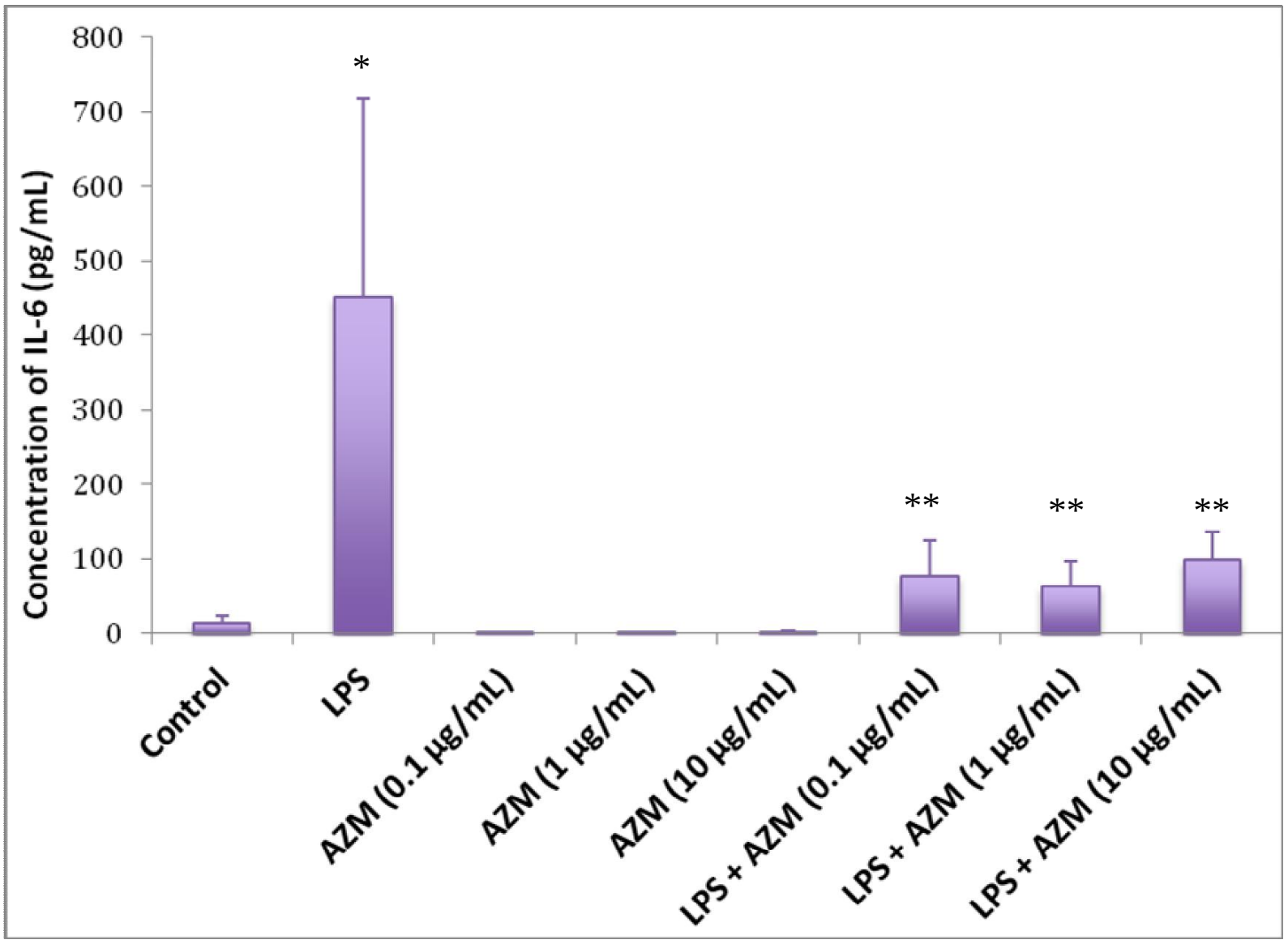

Figure 2.4 Combined donor data demonstrating the effect of azithromycin on LPS induced IL-6 production

Human gingival fibroblasts were treated with *P. gingivalis* LPS alone (1 µg/mL), azithromycin (AZM at 0.1, 1 and 10 µg/mL), or a combination of LPS and azithromycin. IL-6 protein production was measured by multiplex analysis. Bars represent mean ± SEM of all donor experiments combined. Statistical significance was determined using a 1-way ANOVA with Bonferroni Post Hoc Test. *$p<0.05$ compared with control, ** $p< 0.05$ compared with LPS.

The individual donor data from Figure 2.5 shows that the baseline production of IL-6 by HGF's from all 3 donors was minimal. Although the response of each of the 3 donors was variable in terms of LPS induction of IL-6, all responses were significantly increased compared to the control (0.1% DMSO, $p<0.05$). Similar to the data shown for GRO, Donor 1 showed the greatest induction of IL-6 (982 pg/mL), while Donor 3 showed the least induction (133 pg/mL).

When cells were treated with a combination of *P. gingivalis* LPS and AZM, IL-6 production was significantly reduced for all concentrations tested compared to LPS alone ($p<0.05$; Figure 2.5). Reduction of *P. gingivalis* LPS induction of IL-6 ranged from 81 to 86% for Donor 1, 68 to 85% for Donor 2, and 58 to 84% for Donor 3 (Figure 2.5).

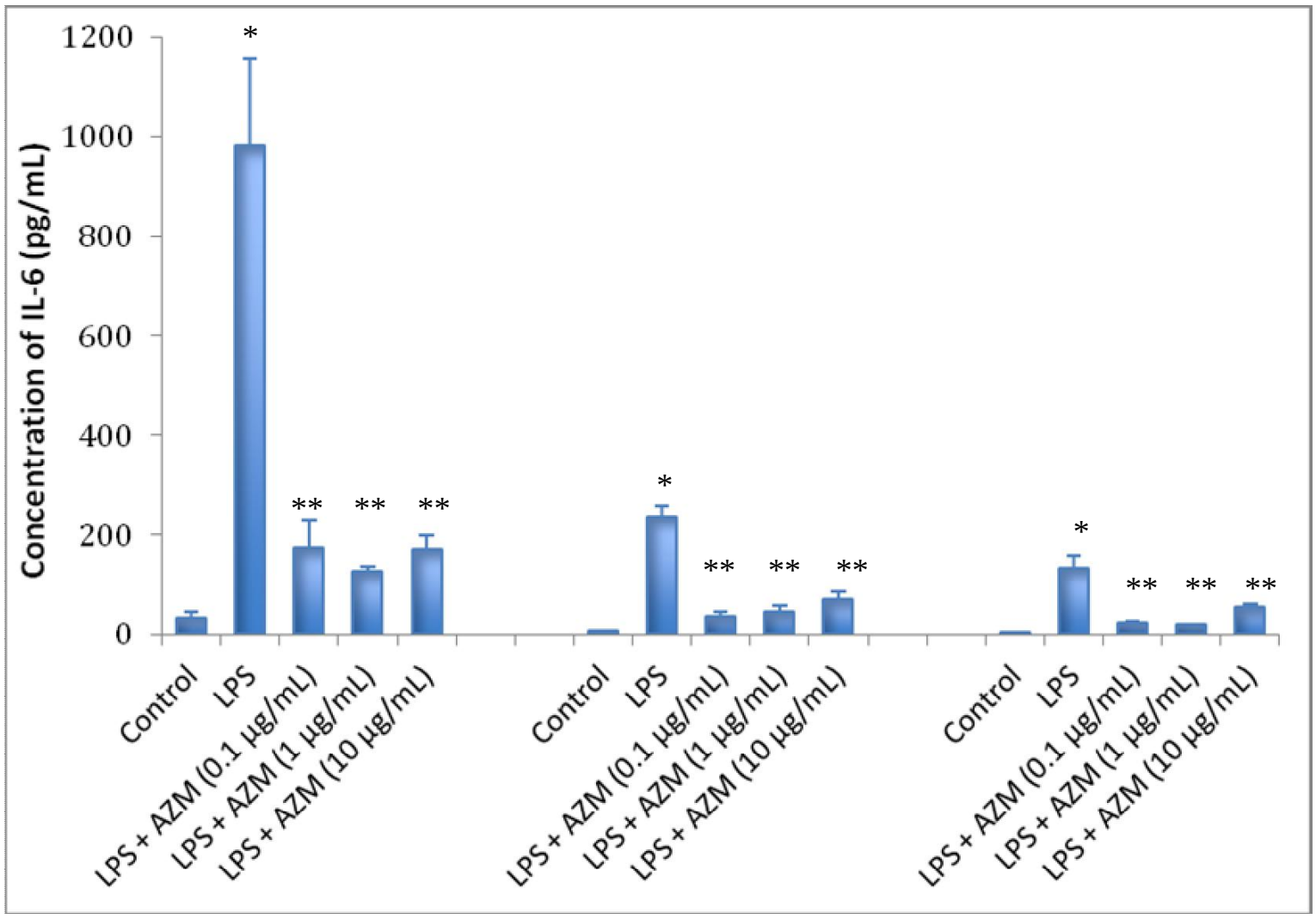

Figure 2.5 Variability of induced IL-6 production between donors after treatment with azithromycin

Human gingival fibroblasts were treated with *P. gingivalis* LPS alone (1 µg/mL), or a combination of LPS and azithromycin (AZM, 0.1, 1, or 10 µg/mL). IL-6 protein production was measured by multiplex analysis. Bars represent mean ± SEM of one experiment performed in triplicate for each donor. Statistical significance was determined using a 1-way ANOVA and Bonferroni Post Hoc Test. *p<0.05 compared with control, **p<0.05 compared to LPS.

2.3.4 Effect of azithromycin on *P. gingivalis* LPS-induced IL-8 production

IL-8 is a chemokine that plays a major role in the attraction and activation of neutrophils and is therefore a key contributor in inflammatory conditions such as periodontitis. We therefore investigated its production by HGF's after azithromycin treatment. When the data were combined from each of the 3 donors, *P. gingivalis* LPS alone significantly increased IL-8 production to approximately 644 pg/mL compared to 12 pg/mL for the control treatment (0.1% DMSO alone, Figure 2.6). There was no effect of AZM alone on IL-8 production by HGF's in the absence of LPS.

The effect of AZM on *P. gingivalis* LPS induced IL-8 production was also investigated. All three concentrations of AZM (0.1 1 and 10 µg/mL) were found to significantly reduce IL-8 production by 75 to 85% (p<0.05, Figure 2.6).

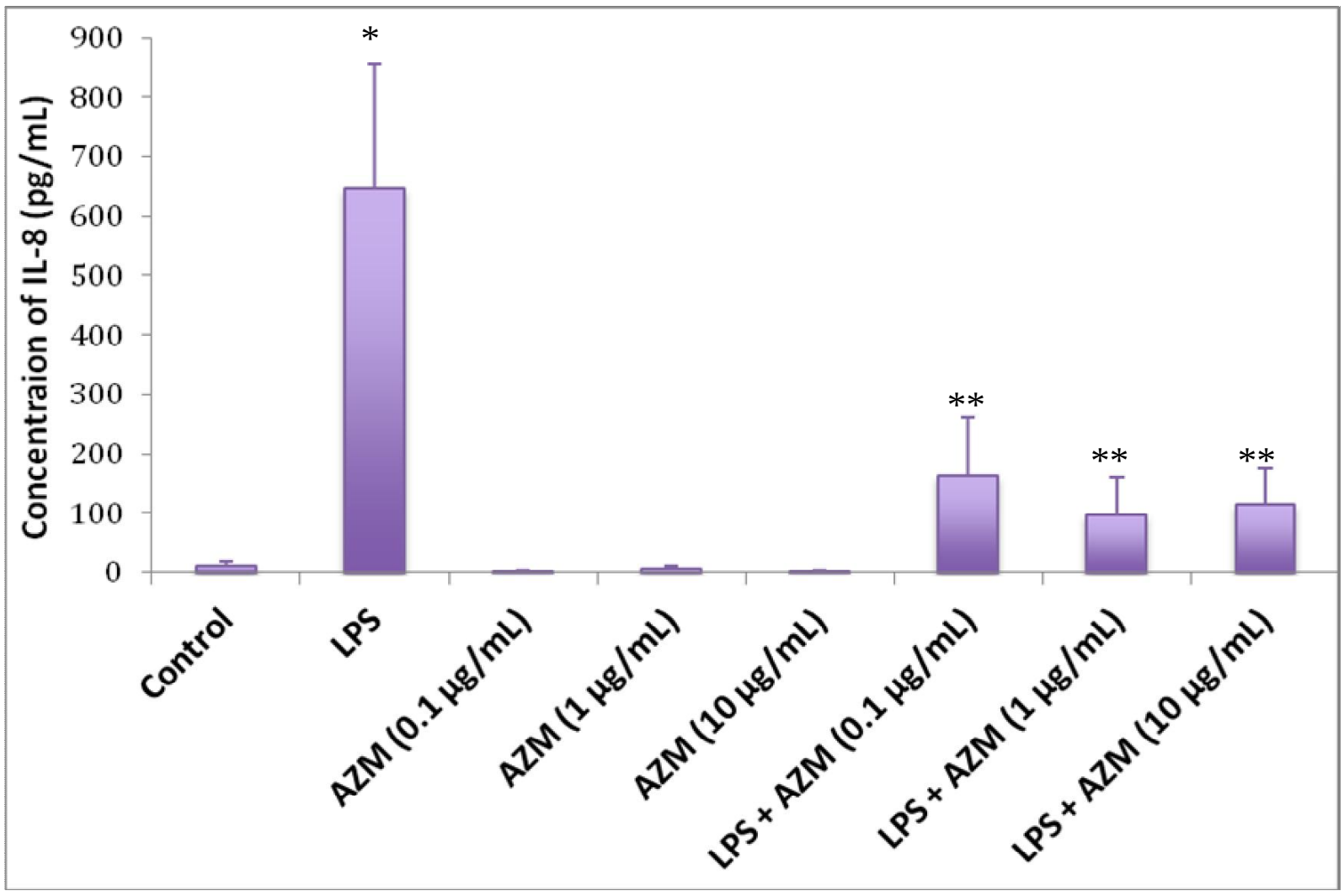

Figure 2.6 Combined donor data demonstrating the effect of a zithromycin on LPS induced IL-8 production

Human gingival fibroblasts were treated with *P. gingivalis* LPS alone (1 µg/mL), azithromycin (AZM at 0.1, 1 and 10 µg/mL), or a combination of LPS and azithromycin. IL-8 protein production was measured by multiplex analysis. Bars represent mean ± SEM of all donor experiments combined. Statistical significance was determined using a 1-way ANOVA with Bonferroni Post Hoc Test. *$p<0.05$ compared with control, ** $p< 0.05$ compared with LPS.

The baseline production of IL-8 by HGF's from all 3 donors was minimal (Figure 2.7). Although the response of each of the 3 donors was variable in terms of LPS induction of IL-8, all responses were significantly increased compared to the control (0.1% DMSO; $p<0.05$). The greatest response was observed by Donor 1 (916 pg/mL), while Donor 3 showed the smallest induction (231 pg/mL).

When cells were treated with a combination of *P. gingivalis* LPS and AZM, the majority of donors showed a significantly reduced IL-8 production for all concentrations tested compared to LPS alone ($p<0.05$, Figure 2.7). Reduction of *P. gingivalis* LPS induction of IL-8 ranged from 62 to 71% for Donor 1, 88 to 93% for Donor 2, and 78 to 90% for Donor 3.

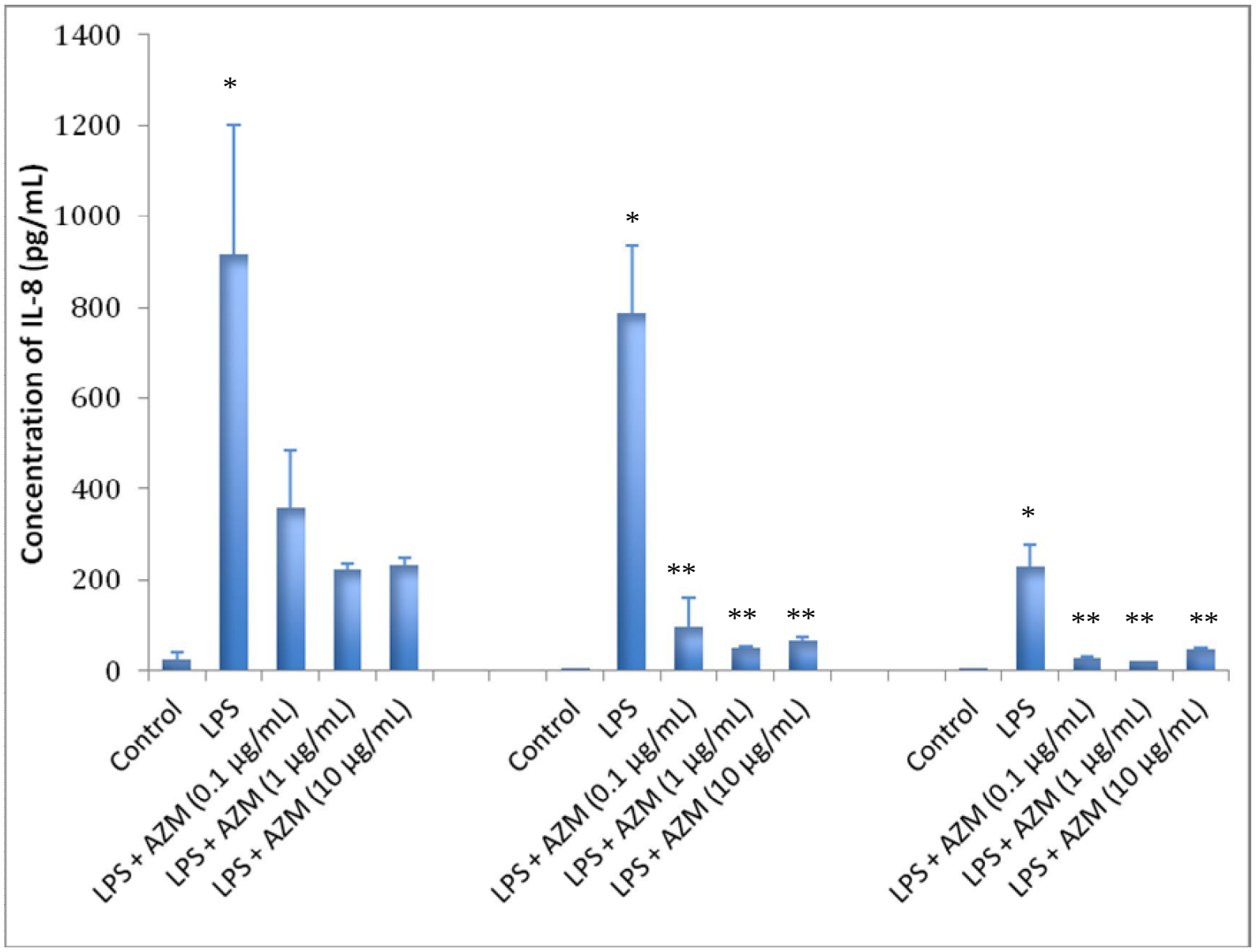

Figure 2.7 Variability of induced IL-8 p roduction b etween donors a fter tre atment with azithromycin

Human gingival fibroblasts were treated with *P. gingivalis* LPS alone (1 µg/mL), or a combination of LPS and azithromycin (AZM, 0.1, 1, or 10 µg/mL). IL-8 protein production was measured by multiplex analysis. Bars represent mean ± SEM of one experiment performed in triplicate for each donor. Statistical significance was determined using a 1-way ANOVA and Bonferroni Post Hoc Test. *p<0.05 compared with control, **p<0.05 compared to LPS.

2.3.5 Effect of azithromycin on *P. gingivalis* LPS-induced MCP-1 production

Increased levels of MCP-1 gene expression have been observed in the gingival tissues of adults with periodontitis compared to healthy subjects, as well as an increase in monocyte chemotactic activity in the crevicular fluid with increasing severity of periodontal disease (Hanazawa et al 1993). The protein induction of MCP-1 was therefore evaluated in this study. When the data were combined from each of the 3 donors, *P. gingivalis* LPS alone significantly increased MCP-1 production to 2702 pg/mL compared with 158 pg/mL for the control treatment (0.1% DMSO alone, Figure 2.8). There was no observed effect of AZM treatment on MCP-1 production by HGF's in the absence of LPS.

The effect of AZM on all three concentrations studied (0.1, 1 and 10 μg/mL) significantly reduced *P. gingivalis* LPS-induced MCP-1 protein production by HGFs ($p<0.05$). With increasing doses of AZM (0.1, 1 and 10 μg/mL), LPS induced MCP-1 protein was reduced by 71, 83 and 87% respectively (Figure 2.8).

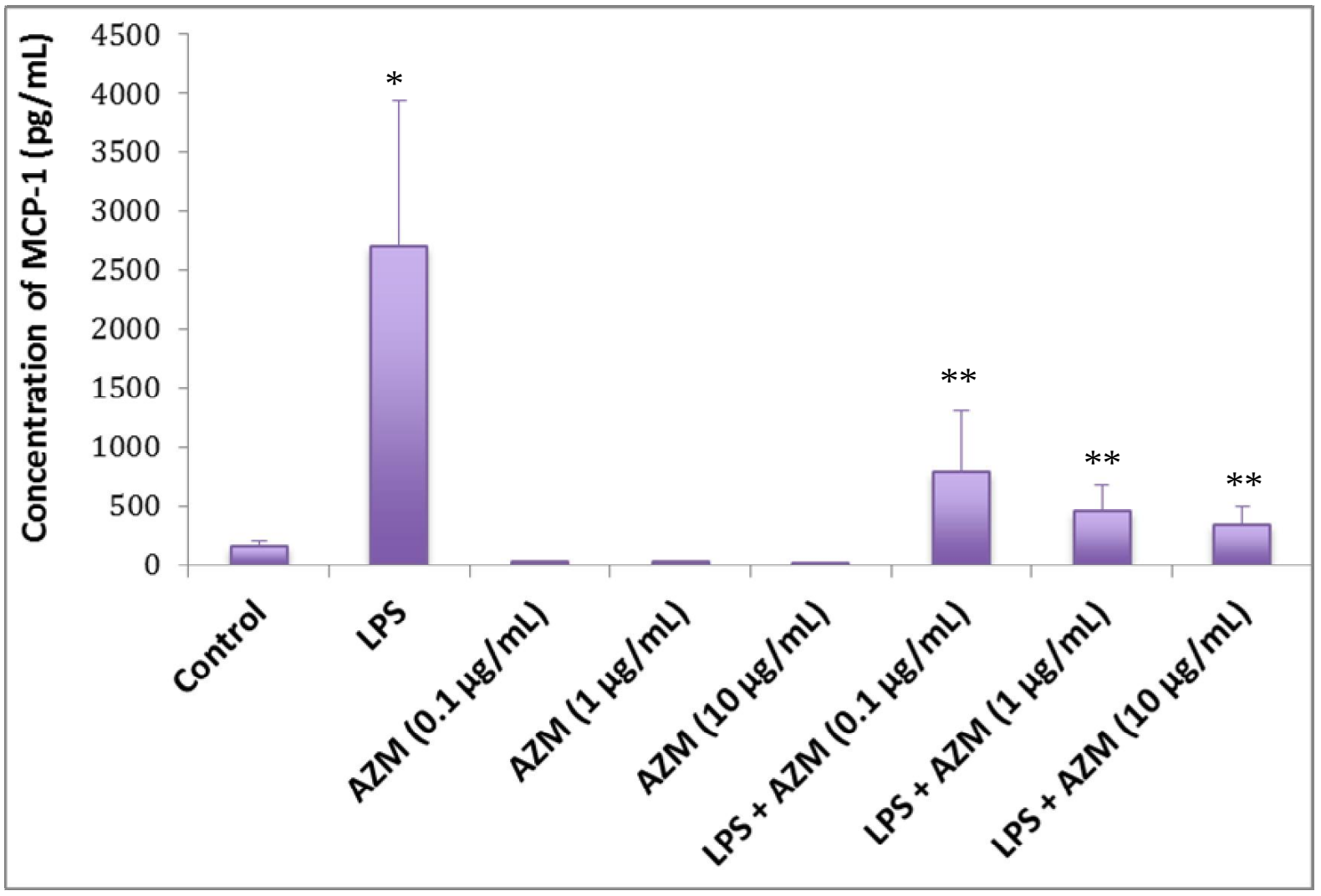

Figure 2.8 Combined donor data demonstrating the effect of a zithromycin on LPS induced MCP-1 production

Human gingival fibroblasts were treated with *P. gingivalis* LPS alone (1 μg/mL), azithromycin (AZM at 0.1, 1 and 10 μg/mL), or a combination of LPS and azithromycin. MCP-1 protein production was measured by multiplex analysis. Bars represent mean ± SEM of all donor experiments combined. Statistical significance was determined using a 1-way ANOVA with Bonferroni Post Hoc Test. *$p<0.05$ compared with control, ** $p<0.05$ compared with LPS.

Figure 2.9 shows that the baseline production of MCP-1 by HGF's without LPS induction was minimal in all 3 donors. Although the LPS induced production of MCP-1 varied in each donor, all responses were significantly increased compared to control (0.1% DMSO, $p<0.05$). Donor 1 showed the greatest induction, increasing MCP-1 production to 4115 pg/mL, while Donor 3 showed the least induction of 253 pg/mL.

When cells were treated with a combination of *P. gingivalis* LPS and AZM, the observed reduction of *P. gingivalis* LPS induced MCP-1 production was variable between donors ranging from 56 to 83% for Donor 1, 91 to 94% for Donor 2, and 17 to 43% for Donor 3 (Figure 2.9). Donor 1 and 2 both had statistically significant reductions in *P.*

gingivalis LPS induced MCP-1 protein production for all AZM concentrations tested ($p<0.05$, Figure 2.9). Donor 3 however, only showed a reduction at the highest concentration of AZM used (10µg/ml).

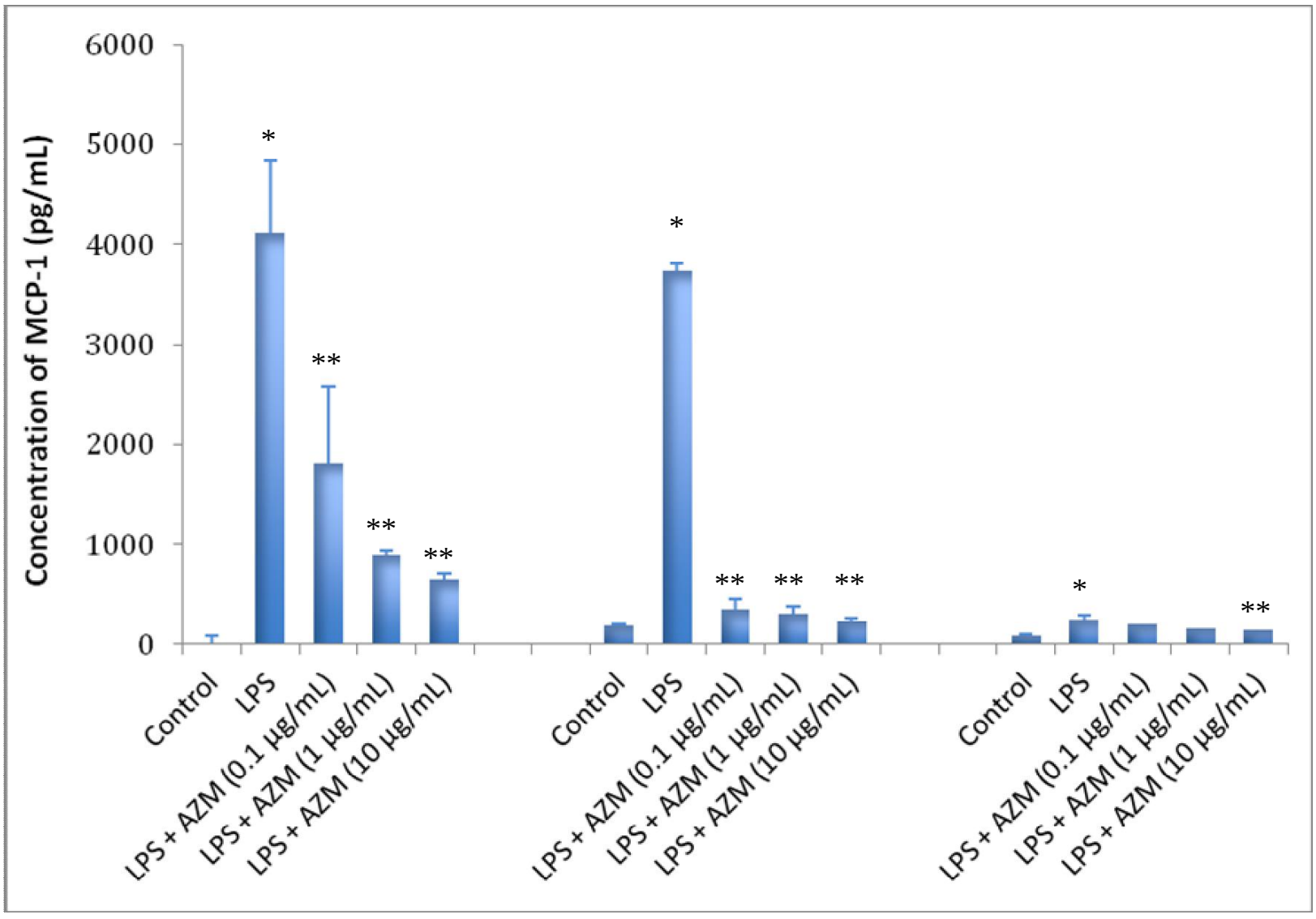

Figure 2.9 Variability of induced MCP-1 production between donors after treatment with azithromycin

Human gingival fibroblasts were treated with *P. gingivalis* LPS alone (1 µg/mL), or a combination of LPS and azithromycin (AZM, 0.1, 1, or 10 µg/mL). MCP-1 protein production was measured by multiplex analysis. Bars represent mean ± SEM of one experiment performed in triplicate for each donor. Statistical significance was determined using a 1-way ANOVA and a Bonferroni Post Hoc Test. *$p<0.05$ compared with control, **$p<0.05$ compared to LPS.

2.4 Discussion

Adjunctive antibiotics are recommended for the treatment of aggressive or refractory forms of periodontal disease (Herrera et al., 2002, Haffajee et al., 2003, Heitz-Mayfield, 2009). The most commonly prescribed is a combination of amoxicillin and metronidazole (van Winkelhoff et al., 1992, Guerrero et al., 2005). Side effects however are common and compliance can be an issue (Guerrero et al., 2007, Cionca et al., 2009). Azithromycin has recently been investigated as an antibiotic adjunct for the treatment of

periodontitis. Its favourable pharmacological properties of long half-life (Fiese and Steffen, 1990) and good tissue penetration at the site of infection (Blandizzi et al., 1999, Gomi et al., 2007a) allow for a more manageable treatment regimen of once daily administration for three to five days.

Preliminary case reports have also suggested that azithromycin can significantly reduce inflammation and inhibit bone resorption in periodontal disease (Schmidt and Bretz, 2007, Hirsch, 2010). Given the extensive and successful long term use of macrolides as anti-inflammatory/immune modulators for various chronic inflammatory conditions (Culic et al., 2001), it is important to determine how azithromycin may be providing an anti-inflammatory action in periodontal tissues. The results of this study demonstrated that azithromycin suppressed the *P. gingivalis* LPS induced pro-inflammatory cytokine/chemokine production in human gingival fibroblasts *in vitr o*, thereby demonstrating an anti-inflammatory property.

Specifically, azithromycin significantly suppressed production of GRO, IL-6, IL-8, and MCP-1 in gingival fibroblast cells that had been exposed to *P. gingivalis* LPS. All azithromycin concentrations used in the current study (0.1, 1 and 10 μg/mL) were effective in reducing cytokine production. These concentrations are comparable with those achieved *in vivo* where a regimen of 500 mg once a day for three days can produce maximal mean tissue concentrations of 2.92 μg/g on the fourth day, that declines to 0.54 μg/g on day 14 (Gomi et al., 2007a).

There are limited studies in the literature assessing the anti-inflammatory effects of azithromycin in periodontal tissues *in vitro*. To the best of our knowledge, our study is the first to demonstrate that azithromycin reduces GRO, IL-6, IL-8, and MCP-1 protein production by HGFs induced by *P. gingivalis* LPS *in vitro*. The present results are similar to the observations of Matsumura et al (Matsumura et al., 2011) who found that azithromycin (1 μg/mL) suppressed IL-8 production by *Escherichia coli* (*E. Coli*) LPS (1 μg/mL) or *A .a.* strain Y4 LPS (10 μg/mL) in an oral epithelial cell line (KB cells). In addition, a clinical study supporting our findings has shown that azithromycin decreases IL-1β, IL-8, TNF-α, and vascular endothelial growth factor (VEGF) in gingival crevicular fluid (GCF) levels for up to 12 days after 3 days of azithromycin intake every 24 hours in periodontally healthy volunteers (Ho et al., 2010).

One study specifically investigating the effect of azithromycin on IL-8 production by human gingival fibroblasts has been identified in the literature. In contrast to our findings, this study found an increased concentration of IL-8 protein when the cells were induced *by P. gingivalis* LPS and treated with azithromycin (Kamemoto et al., 2009). The

cells were seeded at 1×10^4 cells/well, induced with 10 ng/mL of *P. gingivalis* LPS and treated with azithromycin at 0.1, 1, or 10 μg/mL. From this study it was speculated that the increased production of IL-8 by fibroblasts could increase the migration of neutrophils to periodontal tissues and lead to more efficient phagocytosis of periodontal pathogens (Kamemoto et al., 2009). The reasons for the discrepancy in these findings to those of the current study may relate to differences in the research protocols used. In the Kamemoto et al (2009) study, the HGFs were obtained from a single donor. However it should be noted that significant donor variability to *P. gingivalis* LPS virulence factor has been reported (Zhou and Windsor, 2007). Indeed, we also noted donor variability between the three donor cells used in this study. The discrepancy between *in vitro* studies investigating the potential effect of azithromycin on IL-8 may be due to the variation in treatment concentration relative to the number of cells between studies. For example, Kamemoto et al (2009) used five times fewer cells than the current study, and a hundred fold less concentrated LPS, making comparisons between studies difficult. The pharmacological effects of azithromycin on cytokines are very complex and may be dependent on dose, target cells and temporal differences in terms of the host immune-inflammatory response and host modulatory actions (Bartold et al., 2013).

It is plausible that our result of azithromycin mediated fibroblast reduction in chemokines such as IL-8 and GRO may cause a reduction in polymorphonuclear leukocyte chemotaxis and therefore neutrophil recruitment to the site of inflammation. IL-8 is a major pro-inflammatory cytokine that attracts and activates neutrophils as well as T lymphocytes to inflammatory sites, and has increased levels in the GCF of inflamed tissue compared with healthy sites (Tsai et al., 1995). GRO has also been shown to be an important chemoattractant for neutrophils (Jonsson et al., 2009). In other inflammatory or autoimmune conditions, studies have shown the production of chemotactic factors to be reduced by macrolides, and that neutrophil chemotaxis was also reduced (Bosnar et al., 2009, Meyer et al., 2009). Many studies on primary cultures of human bronchial epithelial cells (either stable healthy cells with the addition of LPS, or inflammatory cells) have demonstrated a reduction in IL-8 following azithromycin treatment (Cigana et al., 2006, Murphy et al., 2008, Zimmermann et al., 2009). Furthermore, IL-8 mRNA was shown to be reduced with azithromycin treatment as well as producing a reduction in airway neutrophilia in patients with bronchiolitis obliterans syndrome after lung transplant surgery (Verleden et al., 2006). It should be noted however, that studies in chronic airway diseases generally use long-term azithromycin treatment for up to 3 months, meaning the results may not be directly comparable to the 3 day dose used for the treatment of periodontitis.

Monocyte chemotactic protein-1 (MCP-1) production by HGFs was shown in our study to be significantly induced by *P. gingivalis*. This induction of MCP-1 was greater than the induction of GRO, IL-6, and IL-8 (2702 pg/mL for MCP-1 compared with 419 pg/mL for GRO, 451 pg/mL for IL-6, and 645 pg/mL for IL-8). High monocyte chemotactic activity has been reported in crevicular fluid, and marked MCP-1 gene expression has also been noted in the gingival tissues of adult periodontitis patients (Hanazawa et al., 1993). These results demonstrate that gingival fibroblasts may participate in monocyte recruitment and that this could play an important role in the inflammatory process of periodontitis. The current study found a marked reduction of MCP-1 *P. gingivalis* LPS induction by azithromycin in Donor 1 and 2, both of whom showed a dose dependent response. The strong inhibitory action shown by azithromycin on MCP-1 production in this *in vitro* model may highlight a potential mechanism by which azithromycin is providing resolution of inflammation in periodontal tissues.

IL-6 stimulates the production of acute phase proteins, regulates bone resorption, and induces B-cell maturation, and is therefore involved in the later stages of the inflammatory response (Okada and Murakami, 1998). *In vitro* experiments have shown that gingival fibroblasts produce IL-6 upon stimulation with *P. gingivalis* LPS (Bartold and Haynes, 1991), which in turn activates osteoclasts (Wang and Ohura, 2002). A recent study has found that azithromycin can suppress the bone resorptive ability of human osteoclasts and inhibit osteoclast differentiation from peripheral blood mononuclear cells *in vitro* (Gannon et al., 2012). The current study found that *P. gingivalis* induction of IL-6 was suppressed by azithromycin in HGFs. Such a reduction in production of IL-6 by azithromycin in fibroblasts may explain the potential anti-inflammatory and anti-resorptive activity of azithromycin noted in clinical case reports (Hirsch et al., 2012).

Our study has noted considerable variability in donor cell cytokine/chemokine responsiveness. Donor 1 was shown to be highly responsive to *P. gingivalis* LPS for all cytokines/chemokines tested, while Donor 3 had a much lower responsiveness. This variability may be due to a difference in the passage number of cells between donor experiments. Donor 1 and 2 experiments were conducted between passage 4-6, however the experiment using donor 3 cells was conducted at passage 9, which may have resulted in a reduced fibroblast cell inflammatory response to *P. gingivalis* LPS. Other studies assessing the changes in cellular morphology and cytokine expression due to passage number in healthy human gingival fibroblasts have found that the morphology of the fibroblasts were larger and IL-6 decreased with increasing passage (Kent et al., 1996).

Another possible reason for the observed donor variability is that each donor probably has a different host immune-inflammatory and host modulation state that may be reflective of the individual cytokine/chemokine responses seen in these *in vitro* experiments. Other studies have found a large heterogeneity between individual donors in the inflammatory response (cytokine and chemokine gene expression) to *P. gingivalis* LPS by human gingival fibroblasts (Scheres et al., 2010). Furthermore, significant differences have been observed in the collagenolytic activity between human gingival fibroblast donor cell lines (Zhou and Windsor, 2007). Some studies displayed an 'aggressive' response to *P. gingivalis* culture supernatant with increased MMP-1, -2, and -3 activation and decreased tissue inhibitors of matrix metalloproteinase (TIMP) production, while for other donors the balance of MMP to TIMP production was unaltered. The authors of the study concluded that although it cannot be assumed that the *in vitro* findings reflect the *in vivo* situation, these results might help explain the different susceptibilities and progression of periodontal disease seen in different individuals (Zhou and Windsor, 2007).

In the periodontium, bacterial pathogens interact with the cell surface of gingival epithelial cells, inflammatory cells, and potentially even fibroblasts if the bacteria/virulence factors penetrate deep into the tissues. In particular, bacterial pathogens are recognized by toll-like receptors (TLRs) on the surface of cells that function as pattern recognition receptors to activate the innate host immune system. An increase in the expression of cytokines, growth factors, matrix components and matrix metalloproteinases can occur as a response to LPS through TLRs and activation of intracellular signaling pathways (Bartold and Narayanan, 2006).

P. gingivalis LPS has been shown to bind to the cell surface of HGFs through CD14 and toll-like receptor-4 (TLR-4) to mediate intracellular signaling and up-regulate the expression of a number of cytokines and chemokines (Wang et al., 2001, Wang and Ohura, 2002). In HGFs specifically, it has been demonstrated that *P. gingivalis* LPS activates tyrosine kinase phosphorylation of several intracellular proteins such as ERK1 and ERK2 via CD14, leading to MCP-1 gene expression through the transcription factor NF-κB and activating protein-1 (AP-1) (Watanabe et al., 1996). This activation of intracellular proteins was inhibited by anti-CD14 antibody (Wang et al., 1998). Interestingly, azithromycin appears to inhibit a number of intracellular signaling proteins involved in the production of pro-inflammatory cytokines and chemokines. Azithromycin can inhibit IL-8 production through the inhibition of NF-κB and AP-1 DNA binding in cystic fibrosis airway epithelial cells (Cigana et al., 2006), and inhibits the activation of NF-κB and Rac 1 GTP-binding protein in an oral epithelial cell line (Matsumura et al.,

2011). These findings may provide an insight into how azithromycin reduced chemokine production in the current study, in particular IL-8. To further evaluate the potential mechanism by which azithromycin may be suppressing cytokine/chemokine induction by *P. gingivalis* LPS, future studies should assess cell membrane and intracellular signaling protein levels in the presence of azithromycin in HGFs.

In conclusion, the results of this study support our hypothesis that azithromycin reduces the *in vitro* production of inflammatory cytokine/chemokine production by human gingival fibroblasts exposed to *P. gingivalis* LPS. It is plausible that the noted reduction in synthesis of chemokines by fibroblasts exposed to azithromycin may cause a reduction in polymorphonuclear leukocytes and monocyte chemotaxis and therefore recruitment to the site of inflammation. Whether this represents a significant biological effect with regards to azithromycin's potential anti-inflammatory property remains to be established. Furthermore, whether azithromycin has any other effects on fibroblasts with regards to matrix synthesis and turnover through matrix metalloproteinases and their inhibitors also needs to be investigated in the context of inducing a pro-resolution effect in the treatment of periodontitis that has been observed clinically (Hirsch et al., 2010). Given that periodontitis is a bacterially induced chronic inflammatory disease, showing that azithromycin has anti-inflammatory actions in addition to its antibacterial properties would further support its use as an adjunct to conservative periodontal treatment.

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