Evaluation of salivary sirtuin 2 as a novel biomarker for periodontitis.

Christopher Dowson BSc (Hons) MRes

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Abstract

SIRT2 is an NAD-dependent histone deacetylase (HDAC) that is involved in the regulation of gene expression and protein function. This study aimed to evaluate SIRT2 as a salivary biomarker for periodontitis and investigate any association between SIRT2 and the inflammatory processes relevant to periodontitis.

Immune responses were investigated in vitro using THP1 monocytes differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA) and stimulated with TLR2 or TLR4 agonists.

Analysis using qPCR and western blotting showed there were no significant changes to SIRT2 mRNA or intracellular protein expression respectively after stimulation with TLR agonists. Secreted SIRT2 levels measured by ELISA were significantly elevated after stimulation with TLR2 agonists but not after stimulation with TLR4 agonists. TLR agonists had no effect on SIRT2 deacetylation activity in macrophages. Inhibition experiments in macrophages showed that SIRT2 regulates secretion of TNFα, IL-6, IL-8, and IL-1β as measured by ELISA. Multiple regression analysis (ANCOVA) showed that SIRT2 was significantly elevated in periodontitis when accounting for the influence of age but SIRT2 levels did not correlate with clinical measurements of periodontitis such as bleeding on probing and pocket depth. Receiver operating characteristic (ROC) curve analysis showed that salivary SIRT2 could detect periodontitis with a high degree of sensitivity and specificity (AUC 89%).

In summary, SIRT2 levels accurately represent the presence of periodontitis, but do not correlate with clinical measures of periodontitis, which may limit its utility as a diagnostic biomarker. We have demonstrated a novel TLR2-mediated pathway of SIRT2 secretion from THP1-derived macrophages which may explain the elevated levels of SIRT2 present in the saliva of patients with periodontitis but will require further investigation. We have also shown that SIRT2 mediates the secretion of pro-inflammatory cytokines after stimulation with TLR agonists which may be of relevance to the pathogenesis of periodontitis.

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List of Abbreviations

ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AUC	Area under curve
BOP	Bleeding on probing
BSA	Bovine serum albumin
CAL	Clinical attachment loss
CSF1	Colony stimulating factor 1
DAMP	Damage-associated molecular pattern
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
GCF	Gingival crevicular fluid
HDAC	Histone deacetylase
HRP	Horseradish peroxidase
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IL	Interleukin
IFN	Interferon
IRF	Interferon regulatory factors
LOC	Lab-on-a-chip
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1

MED	Mouse embryonic fibroblasts
MGI	Modified gingival index
MMP	Matrix metalloproteinase
NAD	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
NAMPT	Nictoinamide phosphoribosyltransferase
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor κB
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NMN	Nicotinamide mononucleotide
NMNAT	Nicotinamide mononucleotide adenylyltransferase
MyD88	Myeloid differentiation primary response 88
NPX	Normalised protein expression
PAMP	Pathogen-associated molecular pattern
qPCR	Quantitative polymerase chain reaction
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PDL	Programmed death ligand 1
PDLF	Periodontal ligament fibroblast
PDSC	Periodontal ligament stem cell
PEA	Proximity extension assay
PESA	Periodontal epithelial surface area
PISA	Periodontal inflamed surface area

PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear neutrophil
PPD	Probing pocket depth
PRR	Pattern recognition receptor
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
TIMP	Tissue inhibitor of matrix metalloproteinase
TLR	Toll-like receptor
TNFα	Tumour necrosis factor α
TRIF	TIR-domain-containing adapter-inducing interferon-β
VCAM-1	Vascular cell adhesion protein 1

Chapter 1. Introduction

1.1 The Pathogenesis of Periodontitis

Periodontitis is a chronic inflammatory disease affecting the connective tissues that support the teeth. These tissues are collectively referred to as the periodontium and include the gingiva, the periodontal ligament, and the alveolar bone (Figure 1.1). This inflammation is triggered by the formation of plaque, bacterial biofilms on the surface of the teeth, that begin to colonise the gingival sulcus. Traditionally, the "red complex" organisms *Porphyromonas gingivalis*, *Treponema denticola,* and *Tannerella forsythia* were considered the aetiological agents of periodontitis (Hajishengallis and Lamont 2012). However, more recent thinking leans towards polymicrobial synergy and dysbiosis being key to the development of periodontitis. The move away from the "red complex" organisms was partly due to the finding that these organisms are present in healthy individuals and partly due to increasing knowledge of the oral microbiome (Hajishengallis and Lamont 2012; Hajishengallis 2015). An analysis of periodontally healthy controls and subjects with chronic periodontitis detected 596 known bacterial species, and 123 of these were significantly elevated in patients with periodontitis (Griffen *et al.* 2012). This demonstrates the extent of the diversity within the oral microbiome and is at least in part why less emphasis is placed on the role of the "red complex" organisms.

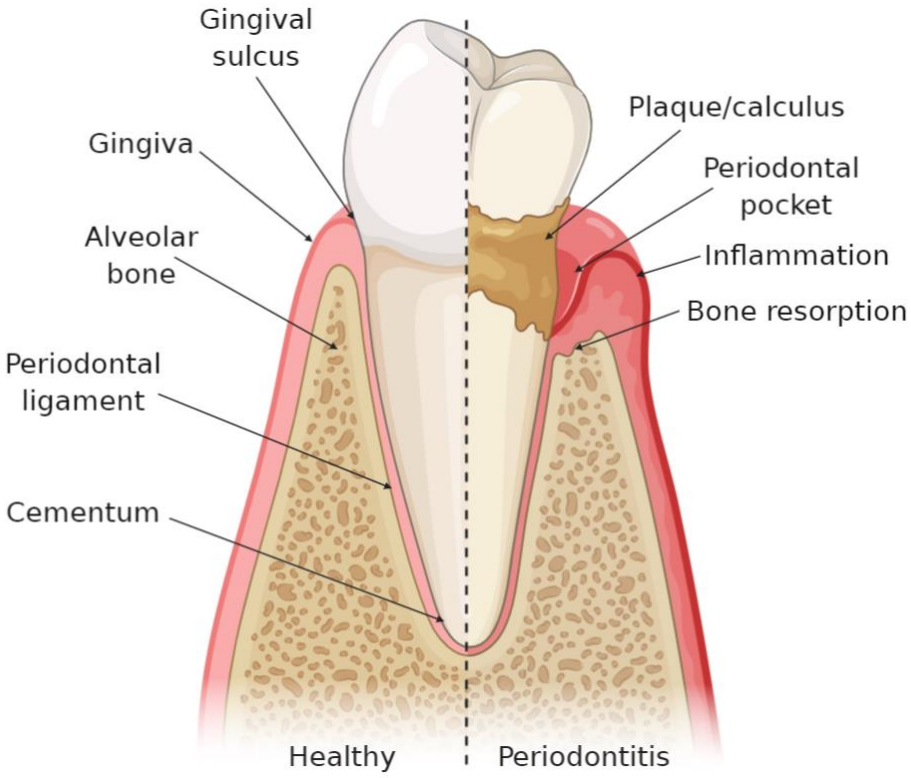


Figure 1.1. The structure of the periodontium.

Consisting of the gingiva, periodontal ligament, cementum and alveolar bone, the periodontium provides support to the teeth. In periodontitis, the buildup of plaque leads to inflammation and the formation of a periodontal pocket which provides more space for plaque formation. Resorption of the alveolar bone can occur due to the chronic state of inflammation. Image adapted in BioRender.

As the inflammation progresses, the integrity and structure of the periodontal tissues are disrupted, leading to the formation of periodontal pockets, which provide further space for bacterial biofilms to occupy (Trindade *et al.* 2014). Disruption to the homeostasis between osteoclasts, cells that degrade bone, and osteoblasts, cells that produce bone, by cytokines such as IL-1, TNFα and IL-6 lead to resorption of the alveolar bone (Liu *et al.* 2010). Additional problems are caused by increased activity of matrix metalloproteinases (MMP), a family of enzymes that degrade the extracellular matrix and basement membrane. These enzymes are usually involved in tissue remodelling and wound healing and are regulated by the tissue inhibitor of matrix metalloproteinases (TIMP) family of protease inhibitors, however, in periodontitis this regulation of activity is disrupted (Cekici *et al.* 2014a).

Neutrophils (Cortes-Vieyra *et al.* 2016), macrophages (Nibali 2015), dendritic cells (Cury *et al.* 2013), fibroblasts (Scheres *et al.* 2011; Baek *et al.* 2013) and epithelial cells (Chen *et al.* 2014) are central to the innate immune response that produces the inflammation seen in periodontitis. Pattern recognition receptors (PRRs) on the surface of these cells detect the presence of microorganisms through the recognition of pathogen-associated molecular patterns (PAMPs). PAMPs are diverse molecules expressed by microbes and contain conserved motifs that are recognised by PRRs. PRRs also recognise damage-associated molecular patterns (DAMPs), which are released when an endogenous cell is damaged. When a PAMP or DAMP is recognised, the PRR begins a signalling cascade that ultimately leads to initiation of the immune response and recruitment of immune cells (Takeuchi and Akira 2010).

One of the key classes of PRRs are the Toll-like receptors (TLRs), a family of 13 proteins that detect bacterial components and stimulate an immune response. TLRs 1, 2, 4, 5, 6 and 10 are found on the surface of the cell, whilst TLRs 3, 7, 8, 9, 11, 12 and 13 are found intracellularly within the endosome (Kawasaki and Kawai 2014). TLR2 and TLR4 are of prime importance in the pathogenesis of periodontitis. The epithelial cells within the oral cavity primarily respond to components of periodontopathic bacteria via TLR2 and TLR4. TLR2 and TLR4 recognise molecules such as peptidoglycan and lipopolysaccharide respectively (AlQallaf *et al.* 2018). There is also evidence that TLR2 and TLR4 expression increases in periodontitis (Rojo-Botello *et al.* 2012; AlQallaf *et al.* 2018). Increased expression of TLR2 and TLR4 was also found to be localised to sites of periodontal disease (Fatemi *et al.* 2013). Perhaps unsurprisingly, there are elevated levels of

TLR2 and TLR4 ligands found in the saliva of patients with periodontitis compared to healthy individuals (Lappin *et al.* 2011). It may also be of importance that the cells found deeper within the periodontium, the osteoclasts, cementoblasts and periodontal ligament fibroblasts only express TLR2 and TLR4, whilst gingival epithelial cells express TLRs 2, 3, 4, 5, 6 and 9 (Hans and Hans 2011), however, the cells of the innate immune system, such as macrophages, dendritic cells and neutrophils are the cells express the most PRRs (Mogensen 2009; Riera Romo *et al.* 2016).

One of the key features of periodontitis is the failure of inflammation to resolve and entering a chronic state (Cekici *et al.* 2014a). However, it remains unclear which mechanisms are responsible for the failure to resolve, and the real cause is likely multifactorial. Evidence points towards contributions from both genetic and environmental risk factors. Risk factors associated with periodontitis include poor oral hygiene, ageing, diabetes mellitus (Preshaw *et al.* 2012), polymorphisms in genes for interleukins IL-1A, IL-1B, IL-6, IL-10 and MMP-3 and 9 (da Silva *et al.* 2017), as well as environmental factors such as smoking (Bergstrom *et al.* 2000). Ultimately, polymicrobial synergy and dysbiosis are the trigger for inflammation, whilst disease progression is subsequently driven by continued activation of the immune system which can be influenced by genetic and environmental factors (Hajishengallis 2014b; Nibali 2015).

1.2 Systemic Effects of Periodontitis

Patients with periodontitis also have increased systemic levels of cytokines such IFN-γ, TNFα, IL-1β, IL-2 and IL-6. Additionally, levels of C reactive protein, coagulation factor and leukocyte counts are also elevated. It is thought that this contributes to systemic inflammation resulting in an increased risk of developing conditions such as cardiovascular disease and diabetes (Taylor *et al.* 2013; Sanz *et al.* 2020). Whilst the reported relative risk varies, several studies have identified increased risk of a first coronary event in patients with clinically diagnosed periodontitis (Dietrich *et al.* 2013). The risk of developing periodontitis is between 2 and 3 times higher in individuals with diabetes (Preshaw and Bissett 2019). Additionally, periodontal intervention can improve glycaemic control for at least 3 months in patients with type 2 diabetes (Nazir 2017; Preshaw *et al.* 2020). Periodontitis can also have significant effects on the patient's quality of life. Increased tooth mobility and tooth loss can affect an individual's nutritional state and can also have a psychological impact (Tonetti et al., 2017).

1.3 Economic Impact of Periodontitis

Additionally, periodontitis is a widespread disease with around 743 million people affected worldwide, a prevalence of 11.2%, making it the sixth most common disease. Additionally, between the years 1990 and 2010, the global burden of periodontitis increased by 57.3%. Consequently, periodontitis has significant economic effects arising from treatment costs and lost work hours, with the global cost being estimated at 54 billion USD/year (Kassebaum *et al.* 2014; Listl *et al.* 2015; Jin *et al.* 2016b; Tonetti *et al.* 2017). Improvements to our ability to diagnose periodontitis earlier and to better manage treatment for the disease could be beneficial in increasing the efficiency with which we manage patients with periodontitis, and reduce the time required for diagnosis (Sorsa *et al.* 2017; Cafiero *et al.* 2021).

1.4 Ageing and Periodontitis

Ageing is another important factor in periodontitis. As an individual ages there is dysregulation of the entire immune system. This ageing of the immune system is termed immunosenescence and leads to irregularities in the initiation and resolution of immune responses and chronic low-grade inflammation (Franceschi *et al.* 2000; Deleidi *et al.* 2015; Ebersole *et al.* 2016). It is also thought that this contributes towards the prevalence of age-related diseases such as cancer, cardiovascular disease and autoimmune conditions (Hajishengallis 2014a; Elibol and Kilic 2018).

Numerous changes occur in both the innate and adaptive immune system with age. Ageing has significant effects on neutrophils, including impaired chemotaxis which has an impact on migration to and from sites of inflammation (Brubaker *et al.* 2013; Shaw *et al.* 2013). Neutrophils from older individual also demonstrate a diminished capacity to phagocytose and reduced lethality to phagocytosed organisms (Simell *et al.* 2011). Aged macrophages also show decreased activity, it is thought that this is due to dysregulation of signalling mechanisms subsequent to TLR stimulation such as p38 mitogen-activated protein kinases (MAPKs) and NF-κB (Helenius *et al.* 1996; Boehmer *et al.* 2005; Chelvarajan *et al.* 2006). Macrophages also display an age-associated increase in IL-6 and IL-8 secretion in response to TLR5 stimulation (Qian *et al.* 2012). Changes to lymphocyte populations have also been observed with age, such as decreases in the percentage and number of both naïve and regulatory T-cells and B-cells. These cells are thought to be important for maintaining a balance between periodontal tissues and the microbiome (Ebersole

et al. 2016). A recent study has shown that the onset of inflammation in the young and old is similar, however, there is a severe impairment of the resolution of inflammation in the elderly. This was found to be due to an impairment to the ability of mononuclear phagocytes, such as monocytes and macrophages, to clear dead cells in a process known as efferocytosis. This leads to a state of chronic inflammation. However, the inhibition of p38 was found to restore efferocytosis capacity and lead to the resolution of inflammation (De Maeyer *et al.* 2020).

This dysregulation contributes towards increasing the susceptibility of older individuals to infection and leads to mortality of infectious diseases being approximately three times higher in aged individuals compared to their younger counterparts (Simon *et al.* 2015)

Resolution of inflammation is delayed in aged mice. Macrophages from aged mice were found to have a lower ability to phagocytose apoptotic polymorphonuclear neutrophils (PMNs) than those from young mice. Phagocytosis of PMNs is an important part of inflammation resolution, and deficiency of this process leads to prolonged inflammation (Arnardottir *et al.* 2014). A more recent study using a dermal model of acute inflammation has shown that there on the onset of inflammation in the young and old is similar, however, there is a severe impairment of the resolution of inflammation in the elderly. This was found to be due to an impairment to the ability of mononuclear phagocytes, such as monocytes and macrophages, to clear dead cells in a process known as efferocytosis. This leads to a state of chronic inflammation. However, the inhibition of p38 was found to restore efferocytosis capacity and lead to the resolution of inflammation (De Maeyer *et al.* 2020).

In a dermal excisional injury model in mice, there was decreased numbers of infiltrating neutrophils in the first 3 days after injury in aged mice. Conversely, macrophage infiltration in aged mice was increased compared to young mice. This was found to be caused by elevated levels of monocyte chemoattractant protein-1 present in the wounds of aged mice. Additionally, macrophages from aged mice were found to possess less phagocytic capacity than those from young mice (Swift *et al.* 2001). Some of these features of immune senescence have been observed when comparing older and younger patients with periodontitis, including altered neutrophil function and increased production of pro-inflammatory cytokines (Preshaw *et al.* 2017). One study reported that older patients with periodontitis demonstrated decreased neutrophil

extracellular trap (NET) formation compared to younger patients. NETs are primarily composed of DNA with some other intracellular proteins and function to neutralise virulence factors of invading microbes and activate complement. This makes them an important component of the innate immune response (Hazeldine *et al.* 2014). In a non-human model of periodontitis, gene expression patterns were identified to distinguish M1 (inflammatory) and M2 (anti-inflammatory) polarised macrophage populations. This information was then used to investigate differences between macrophage polarisation in young healthy primates, older healthy primates and older periodontally diseased primates. M1 gene transcription patterns which included CCL13, CCL19, CCR7 and TLR4, were found to significantly increase with age. The same increase was seen when comparing older healthy primates to older periodontally diseased primates (Gonzalez *et al.* 2015).

The exact mechanisms through which immunosenescence may contribute to development of periodontitis are currently unknown, but statistics demonstrate a clear association between ageing and periodontitis. Individuals over 35 years of age having a significantly increased risk of developing periodontitis. The latest Adult Dental Health Survey reported that 15% of the UK population aged 55 or older has advanced periodontitis (White *et al.* 2012). Data from the Office of National Statistics states that in the UK in 2016, 18% of people were aged 65 or over. They project that this will increase to 23.9% of the population in 2036 (Hayter 2018). Thus, the burden of periodontitis on the NHS is likely to increase as the growing population ages.

1.5 Macrophages in Inflammation and Resolution

Macrophages are cells of the innate immune system that are present in every tissue. These macrophage populations can be tissue resident, derived from the yolk sac and foetal liver during development or monocyte-derived, where circulating monocytes infiltrate the tissue and differentiate into macrophages. Macrophages are involved in tissue homeostasis and repair, as well as responding against pathogens and debris (Watanabe *et al.* 2019).

Activated macrophages have traditionally been classified into two groups based on their phenotype. These phenotypes are referred to as M1 and M2, representing pro- and anti-inflammatory populations respectively (Shapouri-Moghaddam *et al.* 2018; Sima *et al.* 2018). M1 macrophages are pro-inflammatory macrophages, secreting cytokines such as TNFα, IL-1β, IL-6 and chemokines including CXCL9, CXCL10, and CXCL11 (Shapouri-Moghaddam *et al.* 2018). M2

macrophages possess increased phagocytic activity, along with higher expression of scavenging receptors, and mannose and galactose receptors. Additionally, M2 macrophages also exhibit high expression of IL-10, IL-1 receptor antagonist and IL-1 receptor type II (Biswas and Mantovani 2010). IL-10 is an anti-inflammatory cytokine that suppresses the release of immune mediators, antigen expression and phagocytosis by innate immune cells (Kany *et al.* 2019). IL-1 receptor antagonist is a competitive inhibitor or IL-1 signalling (Palomo *et al.* 2015). IL-1 receptor type II is a decoy receptor for IL-1, meaning it can bind to IL-1, but is incapable transducing the signal as it lacks a toll/interleukin-1 receptor (TIR) domain (Shimizu *et al.* 2015). Together, these features of M2 macrophages allow them to fulfil an anti-inflammatory role.

Whilst this categorisation of macrophage polarisation is observed both *in vitro* and *in vivo,* the reality is that rather than being two categorical polarisation states, there is a spectrum of polarisation and any stimulus *in vivo* will likely result in a polarisation state that lies between the two extremes (Mosser and Edwards 2008; Huleihel *et al.* 2017). However, these categorisations are useful in a lab setting in allowing selection of appropriate stimuli and cellular responses for a given experiment.

1.6 Macrophages in Periodontal Disease

Gingival biopsies have shown that there are a greater number of M1 macrophages present in the gingiva of patients with periodontitis when compared against biopsies from healthy individuals, and elevated levels of TNF-α, IFN-γ, IL-6 and IL-12 were observed. Comparatively, biopsies from patients with gingivitis had similar levels of M1 macrophages as healthy individuals, but with elevated levels of TNFα and IL-12 (Zhou *et al.* 2019). This suggests there is an association between the more severe inflammation seen in periodontitis and the number of M1 macrophages present in the gingiva. Cytokines such as TNFα and IL-1β, produced by M1 macrophages are involved in the mediation of osteoclastogenesis both directly and through the recruitment of osteoclast precursors (Sima *et al.* 2019).

Almubarak *et al.* identified an elevation in the ratio of M1/M2 macrophages in periodontally affected sites. Additionally, monocytes and M1 macrophages were found to have increased expression of programmed death-ligand 1 (PDL1), a transmembrane protein that provides an inhibitory signal to T-cells, regulating their activation. This has also been shown to lead to a lack

of resolution during chronic infection. A subset of M1 macrophages were also identified which expressed increased levels of CD47, an immunoglobulin involved in self-recognition and inhibition of phagocytic signalling (Almubarak *et al.* 2020).

Macrophages are also influenced by their surrounding environment. *In vitro,* periodontal ligament stem cells (PDSC) have been found to secrete extracellular vesicles after stimulation with LPS. The extracellular vesicles induced M1 polarisation when added to macrophage cultures. Interestingly, this effect was abolished by treatment of extracellular vesicles with DNase (Kang *et al.* 2018). Co-culture of macrophages with periodontal ligament fibroblasts (PDLF) has been found to decrease TNFα secretion from macrophages but increased their phagocytic activity. Additionally, co-culture resulted in increased IL-6 secretion from PDLFs and induced secretion of IL-10 (Tzach-Nahman *et al.* 2017). In murine models of periodontitis, induction of M2 macrophages has been found to prevent bone loss. This coincided with reduced numbers of osteoclasts, cells which break down bone, present within the alveolar bone (Zhuang *et al.* 2018). Additionally, depletion of macrophages in mice leads to decreased bone resorption during infection with *P. gingivalis*, further highlighting the importance of macrophages in tissue homeostasis and infection (Lam *et al.* 2014).

In a ligature-induced model of periodontitis, it was found that activated monocytes and macrophages were able to circulate in the blood and adhere to endothelial cells within the vasculature where they subsequently induced activation of NF-κB and upregulation vascular cell adhesion protein 1 (VCAM-1) (Miyajima *et al.* 2014). Thus, it appears that macrophages and their circulating predecessors may contribute to the systemic inflammation that can occur in patients with periodontitis.

Macrophages are also one the major producers of the pro-inflammatory cytokine IL-1β. Production is typically initiated in response to PAMPs. IL-1β is first produced in an inactive form named pro-IL-1β, it will then be cleaved into its active form by caspase-1. IL-1β causes typical features of inflammation such as increased blood flow and leukocyte recruitment, but also increases the expression of MMPs (Cheng *et al.* 2020). Additionally, IL-1β upregulates receptor activator of nuclear factor kappa-B ligand (RANKL), leading to an increase in osteoclastogenesis, production of osteoclasts, the cell type responsible for bone resorption (Huynh *et al.* 2017).

1.7 Biomarkers for Periodontitis

A biomarker has been described in joint by the US Food and Drug Administration and the National Institutes of Health as “A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention” (Group 2016). Whilst the World Health Organization defines a biomarker as any “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” (Strimbu and Tavel 2010). These definitions include physiologic, radiographic, histologic and molecular characteristics that are associated with a disease. There is extensive ongoing research into molecular biomarkers as they are broadly quantitative rather than qualitative like radiographic or physiologic biomarkers, meaning there is less room for individual interpretation.

There are distinct types of biomarker: diagnostic biomarkers are used for disease detection and diagnosis. Monitoring biomarkers are used to assess the status of a disease. Pharmacodynamic/response biomarkers are used to assess treatment. Predictive biomarkers are used to predict suitable drug treatments for an individual or any susceptibility to toxicity. Prognostic biomarkers identify the likelihood of disease progression or recurrence. Susceptibility biomarkers are used to assess the risk of an individual developing a certain disease or condition. (Group 2016; Califf 2018)

Currently, there is no molecular biomarker for the diagnosis of periodontitis or for the measurement of disease severity and treatment effectiveness that has achieved widespread clinical usage. Current methods for the diagnosis and monitoring of periodontitis rely on physical examination of the patient (Preshaw 2015). A viable biomarker must prove its usefulness in diagnosing the presence of periodontal disease, reflecting the severity of the disease, allow for monitoring of disease response to treatment, and predict the prognosis/progress of the disease (Ji and Choi 2015). A biomarker for periodontitis could potentially allow for detection of the disease earlier than the current standard of physical examination allows. Currently, periodontitis is diagnosed by assessment of physical parameters such as modified gingival index (MGI), probing pocket depth (PPD), bleeding on probing (BOP), clinical attachment (CAL) loss and radiographic assessment (Preshaw 2015). Periodontal probing involves inserting a small probe into the

periodontal pocket and measuring its depth. Similarly, bleeding on probing is a sign of inflammation of the periodontium. Clinical attachment loss is a measure of the loss of connective tissue attachment to the teeth. This involves measuring the position of the soft tissues of the gingiva relative to the cemento-enamel junction. Radiographic assessment is used to assess loss of the alveolar bone (Taylor 2014; Preshaw 2015).

This type of assessment for the diagnosis of periodontitis has some drawbacks. Firstly, it is a time-consuming process to gather these measurements at affected sites requires both skill and individual interpretation. Additionally, diagnosis in this manner gives only an indication of historic disease severity and may not be reflective of its current severity. This an area where a suitable biomarker would help, with levels of the biomarker correlating to disease severity (Taylor 2014).

Salivary biomarkers for periodontitis are likely to be useful due to the ease with which saliva samples can be collected from patients and its proximity to the site of disease. Saliva is produced by the parotid, submandibular and sublingual glands and by numerous smaller glands. Saliva composition can vary based on the gland it is produced by, however, it is consistently composed of a high percentage of water (>90%) and contains numerous proteins and lipids, as well as carbohydrates, salts, amino, creatine, urea and uric acid. Additionally, saliva will also include gingival crevicular fluid, components derived from the serum, bacteria and bacterial metabolites. There will also be epithelial cells present that have been exfoliated off the inside of the oral cavity (Jaedicke *et al.* 2012; Chojnowska *et al.* 2018). Collection is non-invasive and saliva samples can easily be stored or shipped to locations as needed based on analysis requirements (Kaczor-Urbanowicz *et al.* 2017). Analysis of saliva could also be carried out relatively easily in a point-of-care setting if adequate development is invested into a biosensor or some other rapid test with a low man-power requirement (Sorsa *et al.* 2017; Taylor *et al.* 2019b).

Conversely, there are some challenges presented in the analysis saliva. Levels of any potential analyte may be low and require high assay sensitivity for detection (Srivastava *et al.* 2017). Another challenge presented is degradation of biomarkers due to proteolysis (Thomadaki *et al.* 2011), saliva contains numerous proteases and given time, these proteases can alter the proteomic profile of samples (Al-Tarawneh and Bencharit 2009; Al-Tarawneh *et al.* 2011). Additionally, the lack of a standardised collection method for obtaining saliva samples from

patients can have an impact on measurements of some analytes. In a comparison of collection methods, unstimulated whole saliva, unstimulated sublingual saliva, stimulated whole saliva, stimulated sublingual saliva and stimulated parotid saliva it was concluded that saliva collection methods are not interchangeable and that cross-study comparisons can only be made if the same collection methods were used (Jasim *et al.* 2018).

However, there has also been some investigation into periodontal biomarkers in the blood. Elevated levels of soluble ST2, a member of the interleukin-1 receptor family, and the inflammation-associated C-reactive protein have been observed in the serum of patients with periodontitis (Torrungruang *et al.* 2019). Elevated levels of the microRNAs hsa-miR-664a-3p, hsa-miR-501-5p, and hsa-miR-21-3p have also been observed in the serum of patients with periodontitis (Yoneda *et al.* 2019). A potential biomarker for periodontitis present in the blood or sera of a patient presents more difficulties in obtaining samples, however, may still be of value. Particularly if said biomarker can be used as a measure of the systemic effects of periodontal disease (Romandini *et al.* 2018; Torrungruang *et al.* 2019).

A recent systematic review and subsequent meta-analysis has reported that MMP-8, MMP-9, IL-1β, IL-6 and haemoglobin all demonstrated a good capability to detect periodontitis in systemically healthy individuals (Arias-Bujanda et al., 2020).

MMP-8 has been found to be one of the most promising salivary biomarkers for periodontitis and has been found to be elevated in the saliva of patients with chronic periodontitis (Gupta *et al.* 2015; Rathnayake *et al.* 2015; Rangbulla *et al.* 2017). MMP-8 is a collagenase that is produced by neutrophils, articular chondrocytes, synovial and gingival fibroblasts, epithelial cells, odontoblasts, plasma cells, as well monocytes and macrophages (Sorsa *et al.* 2006). In a recent meta-analysis of 10 studies investigating salivary MMP-8 levels in patients with periodontitis, MMP-8 levels were significantly higher than healthy controls in 8 of these studies (Zhang *et al.* 2018b). Prototype biosensors for MMP-8 have been developed utilising antibodies specific to MMP-8 and surface acoustic wave technology to analyse saliva samples. This method for analysis of saliva samples demonstrated comparable performance to more tradition ELISA methods in distinguishing periodontitis from health but requires only 20 minutes to complete an assay (Taylor *et al.* 2019a). A biosensor like this can be more easily applied into a clinical setting than a

traditional assay and due to the speed and reduced labour involved, allows for a higher total throughput.

Alkaline phosphatase is a membrane-bound glycoprotein that has also shown potential as a biomarker for periodontitis in both saliva and gingival crevicular fluid (Malhotra *et al.* 2010; Dabra and Singh 2012). It is produced by neutrophils, osteoblasts and periodontal ligament fibroblasts and its activity has been demonstrated to reflect periodontal healing/recurrent inflammation phases in chronic periodontitis patients (Perinetti *et al.* 2008). Other biomarkers that have demonstrated effectiveness in discriminating between healthy patients include the cytokines IL-1β, IL-6 and macrophage inflammatory protein-1α (MIP-1α) (Ebersole *et al.* 2015; de Lima *et al.* 2016).

IL-1β has also been investigated as a biomarker for periodontitis. Levels of salivary IL-1β have been found to have a high sensitivity and specificity for diagnosing periodontitis. Additionally, levels of IL-1β decrease after periodontal treatment meaning that it may have potential in assessing treatment effectiveness (Sanchez *et al.* 2013). Other studies have confirmed elevated levels of IL-1β in the saliva of patients with periodontitis that were found to correlate significantly with the clinical parameters of attachment loss, probing depth, bleeding on probing, periodontal index and gingival index (Kaushik *et al.* 2011). Numerous studies investigating IL-1β have been reviewed by Jaedicke *et al.* and concluded that there was substantial evidence of IL-1β being a robust biomarker for periodontitis (Jaedicke *et al.* 2016).

It has been suggested that using a panel of multiple biomarkers may provide the best diagnostic accuracy that can compete with physical examination (Ji and Choi, 2015). One such suggested panel includes measuring levels of salivary *P. gingivalis* in combination with MMP-8 and IL-1β to give a full picture of disease progress (Gursoy et al., 2011, Salminen et al., 2014, Salminen et al., 2015). Evaluation of the ratio of MMP9 to TIMP1 and the ratio of MMP8 and MMP9 to TIMP1 as biomarkers for periodontitis, demonstrated only slightly better discriminatory ability than individual biomarkers (Bostanci et al., 2021). Thus, whilst there may be some benefit to using a panel of biomarkers, diagnostic ability will still be limited by the chosen biomarkers, and benefit can be gained by identifying additional biomarkers for periodontitis.

1.8 Proteomic Analysis to Identify Novel Biomarkers in Periodontitis

Previous work in our group has identified SIRT2 as a candidate biomarker in periodontitis. In this study, saliva was collected from 15 periodontally healthy volunteers and 15 patients with untreated periodontitis. Ninety-two inflammation related proteins were quantified in all saliva samples by proximity extension assays (Olink Proteomics, Uppsala, Sweden) in order to identify candidate biomarkers for periodontitis. The relative levels of individual proteins between health and disease were analysed using Welches t-test adjusted for multiple testing using the False Discovery Rate method. Seventeen of the 92 proteins were found to be significantly elevated in periodontitis (adjusted P value < 0.05) and SIRT2 exhibited the highest relative levels between health and disease (fold change 4.42, adjusted P value 6.28×10^{-5}). It is reasonable to hypothesise that SIRT2 may represent a novel candidate biomarker in periodontitis, but further work is needed to qualify this protein as a biomarker by identifying any possible role in disease pathogenesis.

1.9 The Sirtuin Family of Histone Deacetylases

The acetylation and deacetylation of proteins are important post-translational modifications that can have significant effects on protein interactions and functionality (Duan and Walther 2015). Histone deacetylases (HDACs) are a class of enzymes that remove acetyl groups from ε-N-acetyl lysine amino acid residues. Despite their name, HDACs also exhibit deacetylase activity against non-histone proteins (Harting and Knoll 2010). The acetylation and deacetylation of proteins plays an important role in fine tuning many biological processes. HDACs are divided into subclasses and present in various areas intracellularly (Table 1.1). HDACs of classes I, IIA, IIB and IV are acetyl coenzyme A dependent, whilst class III HDACs are nicotine adenine dinucleotide $(NAD)^+$ dependent and as such, link metabolism and the post-translational modification of proteins (Drazic *et al.* 2016).

Class	Member	Location	Reference
I	HDAC1	Nucleus	(de Ruijter *et al.* 2003)
	HDAC2	Nucleus	
	HDAC3	Nucleus	
	HDAC8	Nucleus	
IIA	HDAC4	Nucleus and cytoplasm	(de Ruijter *et al.* 2003)
	HDAC5	Nucleus and cytoplasm	
	HDAC7	Nucleus and cytoplasm	
	HDAC9	Nucleus and cytoplasm	
IIB	HDAC6	Cytoplasm	(de Ruijter *et al.* 2003)
	HDAC10	Nucleus and cytoplasm	
III	SIRT1	Nucleus and cytoplasm	(Tanno *et al.* 2007)
	SIRT2	Nucleus and cytoplasm	(Vaquero *et al.* 2006)
	SIRT3	Mitochondria	(Huang *et al.* 2010)
	SIRT4	Mitochondria	
	SIRT5	Mitochondria	
	SIRT6	Nucleus	(Mostoslavsky *et al.* 2006)
	SIRT7	Nucleolus	(Ford *et al.* 2006)
IV	HDAC11	Nucleus	(de Ruijter *et al.* 2003)

Table 1.1. List of mammalian histone deacetylases.

The mammalian HDACs, sorted by class, and their intracellular locations.

1.10 The Biological Function of Histone Deacetylases

Deacetylation of histones is an epigenetic modification and is one mechanism through which gene expression can be regulated. Histones are the proteins which organise DNA into structural units, referred to as nucleosomes. These nucleosomes then assemble into chains and form defined chromatin structures. Deacetylation of histones can cause condensation of the chromatin structure which prevents transcription factors and RNA polymerase II-complexes from interacting with the DNA, resulting in a reduction in gene expression (Cantley *et al.* 2016; Drazic *et al.* 2016). Epigenetic modifications are dynamic and can be influenced by environmental factors, including stressors and nutrition (Ions *et al.* 2013; Moosavi and Motevalizadeh Ardekani 2016). Epigenetic changes occur under normal physiological conditions and are involved in the regulation of immune homeostasis (Obata *et al.* 2015), however they can also contribute to disease, with some evidence suggesting they may affect bone resorption in periodontitis (Cantley *et al.* 2011; Cantley *et al.* 2016).

The acetylation state of non-histone proteins can have effects on localisation, as is the case with forkhead box protein O1 (Daitoku *et al.* 2011), a transcription factor which is important in insulin-signalling, gluconeogenesis and apoptosis. Acetylation state can also affect activity and interaction affinity, as with the immune response regulator, NF-κB (Quivy and Van Lint 2004). Acetylation state of cytoskeletal proteins, such as α-, β-, and γ-actin, and also α-tubulin can alter their stability and have an effect on cell motility (Hubbert *et al.* 2002; Zencheck *et al.* 2012).

1.11 The Sirtuin Family and NAD

All HDAC classes, with the exception of class III, are zinc dependent. The class III HDACs are a family NAD-dependent enzymes known as sirtuins. The sirtuin family is composed of seven members that are classified into four groups (I-IV). Sirtuins deacetylate lysine residues, transferring the acetyl group onto ADP-ribose, producing O-acetyl-ADP-ribose and releasing nicotinamide (NAM) as a by-product, which is also a non-competitive inhibitor of sirtuins. As sirtuins are NAD-dependent, they provide a link between the metabolic state of the cell and the posttranslational modification of proteins and are involved in various biological processes including DNA repair, lipid metabolism and inflammation (Dang 2014).

Nicotinamide phosphoribosyltransferase (NAMPT) is involved in the maintenance of intracellular NAD levels by catalysing the reaction of NAM with 5-phosphoribosyl pyrophosphate to form nicotinamide mononucleotide (NMN) which is then adenylated by nicotinamide mononucleotide adenylyltransferases (NMNATs) to synthesise NAD (Garten *et al.* 2015; Lin *et al.* 2016). NAMPT expression can also have effects on cellular function, potentially through regulation of sirtuins. mRNA expression of NAMPT is higher in M1 macrophages than in M2, however, there are higher levels of NAMPT in the supernatant of M2 macrophages. Neutralising extracellular NAMPT, where it is termed visfatin, through the use of specific antibodies has been shown to reduce M2 macrophage polarization levels (Zhang *et al.* 2018a). Visfatin is also found extracellularly within the circulation (Friebe *et al.* 2011; Zhang *et al.* 2018a). Visfatin has been shown to increase the phagocytic activity of THP1-derived macrophages (Yun *et al.* 2014) and is elevated in the plasma and synovial fluid of patients with rheumatoid arthritis (Nowell *et al.* 2006; Otero *et al.* 2006). Thus, there appears to be some relationship between NAMPT/visfatin and immune regulation.

1.12 SIRT2 Biological Function

SIRT2 is predominantly localised within the cytosol but translocates to the nucleus in the G2/M phase of the cell cycle where it regulates chromosomal condensation (Vaquero *et al.* 2006). There are three isoforms for SIRT2 which all exhibit deacetylase activity against α-tubulin (Maxwell *et al.* 2011; Zhang *et al.* 2021a) and contain multiple phosphorylation sites (Nahhas *et al.* 2007). As SIRT2 is NAD-dependent deacetylase, its activity is regulated in part by the availability of NAD, which is in turn regulated by the activity of NAMPT (Figure 1.2). SIRT2 can also be regulated by phosphorylation and acetylation by p300, acetylation leading to reduced activity (Han *et al.* 2008; Liu *et al.* 2019b). During mitosis, the deacetylase activity of SIRT2 preferentially targets histone 4, lysine 16 (H4K16) over other histones. It should be noted that the deacetylation activity of SIRT2 is not limited to histones. Key targets of SIRT2 deacetylation include the p65 subunit of NF-κB (Rothgiesser *et al.* 2010b), the transcription factors FOXO1 (Jing *et al.* 2007) and 3 (Wang *et al.* 2007), and α-tubulin (Skoge *et al.* 2014) with numerous others previously identified (Table 1.2).

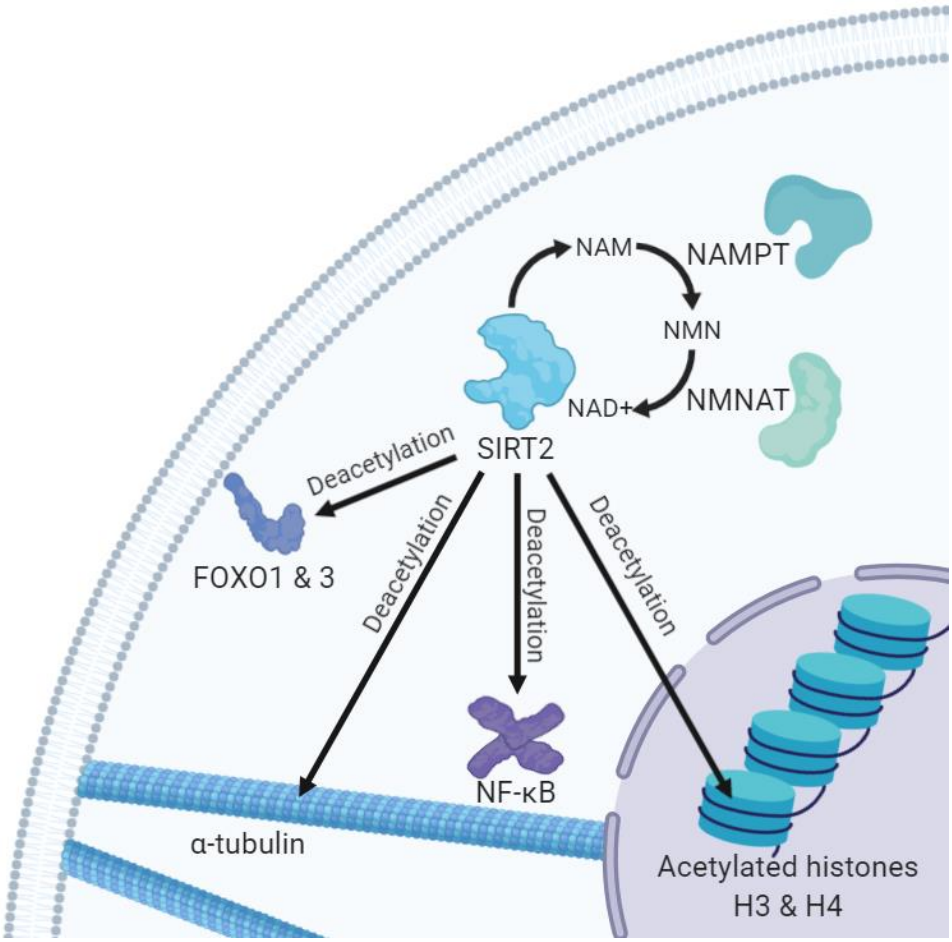


Figure 1.2. Diagram of SIRT2 and its key targets.

SIRT2 activity is limited by NAD availability, which is catalysed from NAM into NMN by the enzyme NAMPT. NMN is then catalysed into NAD by the enzyme NMNAT. SIRT2 utilises NAD to remove acetyl groups from lysine residues within proteins to regulate activity or function.

Substrates	Site	Functions	Reference
PGAM2	K100	Oxidative stress	(Xu *et al.* 2014)
AKT	–	Inactivates GSK-3β	(Dan *et al.* 2012)
G6PD	K403	Oxidative stress	(Wang *et al.* 2014)
MPK-1	–	I/R injury	(Wang *et al.* 2017)
MEK	–	Drug resistance	(Bajpe *et al.* 2015)
23 GKRP	K126	Hepatic glucose uptake	(Watanabe *et al.* 2018)
BubR1	K668	Lifespan	(North *et al.* 2014)
NRF2	K506, K508	Iron homeostasis	(Yang *et al.* 2017)
HIF-1α	K 709	Hypoxic response	(Seo *et al.* 2015)
p65	K310	NF-κB-dependent gene expression	(Rothgiesser *et al.* 2010b)
CNK1	K414	ERK signalling	(Fischer *et al.* 2017)
FOXO1	–	Adipocyte differentiation	(Jing *et al.* 2007)
FOXO3a	–	Oxidative stress	(Wang *et al.* 2007)
Keratin 8	K207	Filament organization	(Snider *et al.* 2013)
TUG	K549	Insulin sensitivity	(Belman *et al.* 2015)
ATG5	–	Mitophagy	(Liu *et al.* 2017a)
α-tubulin	K40	–	(North *et al.* 2003b)
Histone3	K56 K18	- Bacterial infection	(Das *et al.* 2009; Eskandarian *et al.* 2013)
Histone4	K16	Mitosis regulation	(Vaquero *et al.* 2006)

Table 1.2. Important substrates of SIRT2.

A list of notable targets of SIRT2 deacetylation activity and the subsequently regulated biological function. Modified from (Wang *et al.* 2019).

NF-κB is a particularly relevant target of SIRT2, as they are transcription factors that are involved in modulating the expression of immune-related genes that are relevant to periodontitis (Ghafouri-Fard *et al.* 2022), and NF-κB has long been known to be involved in the regulation of inflammation by controlling expression of cytokines and genes that control leukocyte recruitment (Lawrence 2009; Tornatore *et al.* 2012).

There is currently little published information on SIRT2 in the context of periodontal disease, however, from the currently published literature SIRT2 has been shown to be involved in the regulation of immune function and inflammation (Rothgiesser *et al.* 2010b; Ciarlo *et al.* 2017; Zandi *et al.* 2018). Deficiency of SIRT2 has been found to lead to an increase in the phagocytic activity of macrophages (Ciarlo *et al.* 2017). Knockdown of SIRT2 in macrophages has been found to lead to lower levels of lipopolysaccharide (LPS)-induced nitric oxide (NO), inducible nitric oxide synthase (iNOS) and reactive oxygen species (ROS) (Lee *et al.* 2014b). NO and ROS are important components of the immune response, with NO acting as a signalling molecule for both the innate and the adaptive immune response and as an antimicrobial and antiviral effector (Bogdan 2015), whilst ROS has been identified as having a role in secondary signalling after ligation of LPS with TLR4 alongside antimicrobial activity (Kohchi *et al.* 2009).

SIRT2-mediated deacetylation of NF-κB p65 regulates expression of genes such as IL-6 and MMP-9 (Rothgiesser *et al.* 2010b), both of which have associations with periodontitis (Franco *et al.* 2017a; Pan *et al.* 2019a). Macrophages from SIRT2 knockout mice were shown to express lower levels of CD86 when stimulated with IFN-γ than their wild-type counterparts (Lee *et al.* 2014b). CD86 is expressed by antigen-presenting cells and provides a costimulatory signal to T cells required for their activation and survival (Chen and Flies 2013). In a mouse model of collagen-induced arthritis, SIRT2 deficiency was found to cause an increase in the levels of pro-inflammatory cytokines such as IL-1β, IL-6, TNFα, IL-17, IL-33 and monocyte chemoattractant protein 1 (MCP-1) in the serum of arthritic mice (Lin *et al.* 2013b). SIRT2 gene expression has been found to be increased in the gastric epithelium of gastritis patients with *Helicobacter pylori* infection (Zandi *et al.* 2018). As *H. pylori* is also a gram-negative, and therefore LPS-expressing bacteria, there may be a similar effect occurring in periodontitis due to the presence of gram-negative bacteria in the mouth (Noiri *et al.* 2001; Pollanen *et al.* 2013). Thus, from the published

literature discussed here, there is clear evidence for the involvement of SIRT2 in the modulation of the immune response.

With the evidence discussed here that SIRT2 can influence immune system-regulating transcription factors (Fan *et al.* 2010; Tornatore *et al.* 2012), and regulates functional aspects of the immune response such as phagocytosis (Ciarlo *et al.* 2017), there is potential for involvement of SIRT2 in the mechanisms responsible for the development of periodontitis. There is currently a lack of published research investigating any potential involvement of SIRT2 in periodontitis, both direct involvement and how SIRT2 may influence macrophage function relevant to periodontitis. Understanding the how cytokines and the immune response are regulated in periodontitis and in health is of primary importance for development of intervention and new methods of diagnosis.

For this reason, we aim to investigate if SIRT2 could be involved in the immunological processes relevant to periodontitis and if it may qualify as a biomarker for periodontitis.

1.13 Aims

1. To determine whether SIRT2 mRNA and protein expression is regulated by TLR signalling.
    a. qPCR was used to quantify SIRT2 mRNA expression, whilst western blotting was used quantify SIRT2 protein expression.

2. To determine if SIRT2 is present extracellularly in THP1-derived macrophage culture supernatants and is regulated by TLR signalling.
    a. SIRT2 ELISAs were used to analyse macrophage culture supernatants after stimulation TLR2 or TLR4 agonists.
    b. TLR2 and TLR4 inhibitors were used to ensure specificity of our TLR agonists.

3. To assess SIRT2-mediated deacetylation acetylation activity and how it may be regulated by TLR signalling.
    a. Western blotting was used to assess acetylation of the SIRT2 target α-tubulin in THP1-derived macrophages after stimulation TLR2 and TLR4 agonists.

4. To assess how SIRT2 may regulate secretion of pro-inflammatory cytokines in response to TLR signalling.
    a. SIRT2 inhibitors were utilised to interrogate the role of SIRT2 in regulating the secretion of the pro-inflammatory cytokines TNFα, IL-1β, IL-6, IL-8, and IL-12 after stimulation with TLR2 or TLR4 agonists.
5. To further our understanding of the relationship between SIRT2 and periodontitis to determine if SIRT2 may be a viable salivary biomarker for periodontitis.
    a. Analysis of saliva samples to measure SIRT2 levels in the saliva of healthy individuals and patients with periodontitis.
    b. Statistical analysis SIRT2 levels in saliva to determine the ability of SIRT2 levels to discriminate between healthy cases and periodontitis and identify correlations between SIRT2 levels in saliva and periodontal parameters.

Chapter 2. Methods

2.1 Cell culture, Differentiation, and Stimulation

2.1.1 THP1 monocyte culture

THP1 monocytes (European Collection of Authenticated Cell Cultures, Porton Down, UK) were recovered from liquid nitrogen storage and seeded into T75 culture flasks containing 20 ml of RPMI-1640 media (Sigma Aldrich, Gillingham, UK), supplemented with 10% foetal bovine serum (FBS, Sigma Aldrich), 100 U/ml penicillin and 100 µg/ml (Sigma Aldrich). The flasks were then incubated at 37°C, 5% CO_2.

To passage THP1 monocytes, cultures were transferred into sterile 50 ml Falcon tubes (Greiner Bio One, Stonehouse, UK) and centrifuged at 168 g for 5 minutes at 37°C to form a pellet. The supernatant was then discarded, and the pellet was re-suspended in 10 ml of RPMI-1640, before the cell number was then counted using a haemocytometer. An appropriate volume of THP1 suspension, to produce a density of 3.5×10^5 cells/ml, was then added into a T75 culture flask containing 40 ml RPMI-1640. All experiments were conducted using cultures from passage 4 to 10.

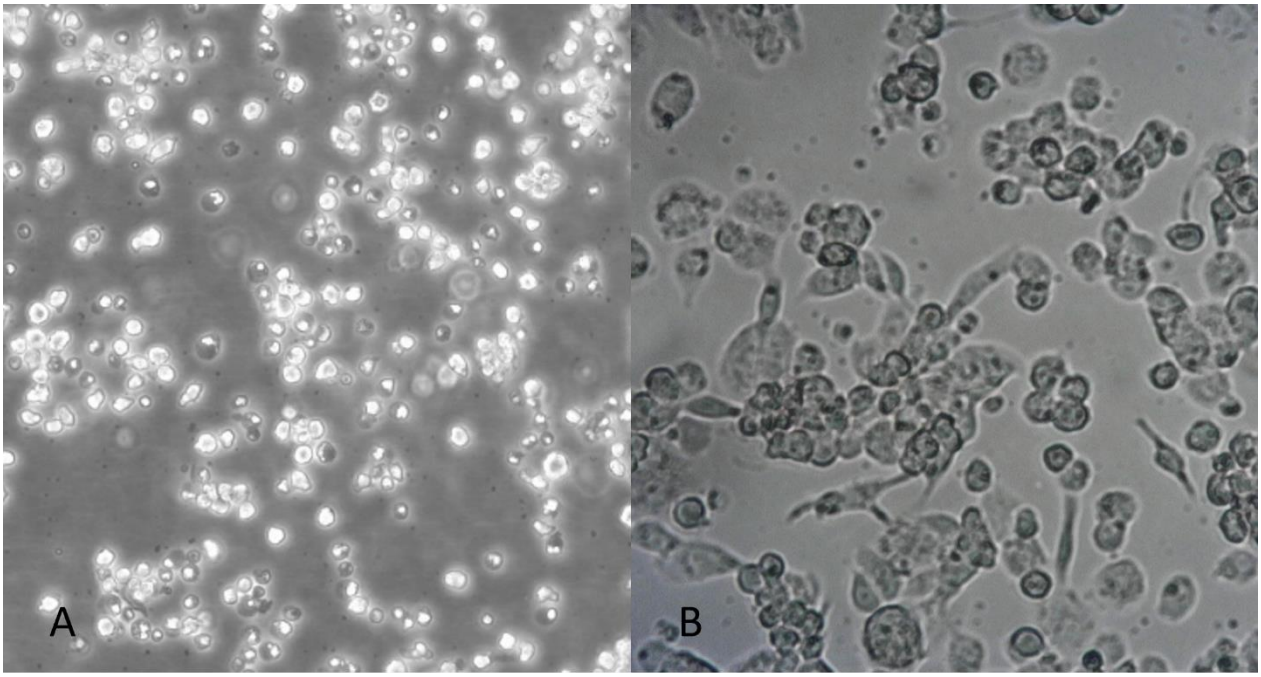


Figure 2.1. THP1 monocytes and THP1-derived macrophages.

(A) THP1 monocytes in their native state prior to differentiation. (B) THP-1 derived macrophages 48 hours after addition of PMA (10 ng/ml). THP1 monocytes grow in suspension and are circular in shape, whilst THP1-derived macrophages become adherent to the bottom of the plate, become more granular, display less regular shape and may become more spindle-like.

2.1.2 Differentiation of THP1 monocytes into macrophages using phorbol 12-myristate 13-acetate (PMA)

THP1 monocytes were counted and seeded at a density of 1 x 10^6 cells per well into 12 well plates containing 2 of serum-free RPMI-1640 in each well (Figure 2.1A). Phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) was added to each well to produce a concentration of 10 ng/ml and left for 48 hours (Figure 2.1B) before the media was replaced with fresh RPMI-1640. Successful differentiation of THP1 monocytes into macrophages can is characterised by changes to cell morphology (Figure 2.1).

2.1.3 Stimulation with *E. coli* and *P. gingivalis* lipopolysaccharide

Lyophilised ultrapure lipopolysaccharide (LPS) from *E. coli* K12 (ATCC 10798) and *P. gingivalis* (ATCC 33277) preparations (InvivoGen, Toulouse, France) were reconstituted in endotoxin free water to a concentration of 1 mg/ml. Ultrapure preparations are certified as being free from contamination with TLR2-activating lipopeptide. Aliquots of these stocks were further diluted prior to use and added into individual wells to give a final concentration of 100 ng/ml.

2.1.4 Stimulation with Pam2CSK4

Lyophilised Pam2CSK4 (InvivoGen) was reconstituted in endotoxin free water to a concentration of 1 mg/ml. Aliquots of these stocks were further diluted prior to use and added into individual wells to give a final concentration of 10 ng/ml.

2.1.5 Stimulation with *B. subtilis* lipoteichoic acid

Lyophilised lipoteichoic acid (LTA) from *B. subtilis* (InvivoGen) was reconstituted in endotoxin free water to a concentration of 2.5 mg/ml. Aliquots of these stocks were further diluted prior to use and added into individual wells to give a final concentration of 100 ng/ml.

2.1.6 Cell lysis for qPCR and western blotting

Media was removed and wells were washed with PBS which was then aspirated. For qPCR, 250 µl of lysis solution supplied in the GenElute™ Mammalian Total RNA Miniprep Kit was added to each well. Wells were then scraped before the contents of the well was aspirated and stored at -80°C until use. For western blotting, 100 µl of ice cold RIPA buffer (Sigma Aldrich) was added into each

well. The plate was then left on ice for 5 minutes before wells were scraped and the contents then aspirated and stored at -80°C until use.

2.2 ELISA Techniques

2.2.1 DuoSet ELISA for TNFα

TNFα secretion by THP1-derived macrophages was quantified using a TNFα DuoSet ELISA kit (Bio-techne, Abingdon, UK). Supernatants were collected from cultures and centrifuged at 15,000 g for 5 minutes at 4°C to remove any debris, then frozen at either -20°C for short term storage or -80°C for long term storage before use. The DuoSet kits were prepared according to the manufacturer's instructions. 96 well plates (R&D Systems) were coated overnight at room temperature with anti-TNFα capture antibody (4 µg/ml). The following day, capture antibody was aspirated and the plate was washed three times in an ELx50 plate washer (BioTek, Swindon, UK) with wash buffer (0.05% Tween20 in PBS). The wells were then blocked with 1% BSA (Sigma Aldrich) in PBS for 1 hour at room temperature before being washed as before. A two-fold serial dilution ranging from 1000 pg/ml to 15.6 pg/ml was prepared using recombinant TNFα standard (supplied in kit), with 1% BSA in PBS as a blank. Standards, blanks and undiluted macrophage culture supernatants were added to the plate in duplicate and incubated for 2 hours at room temperature. The wells were then washed as before and TNFα detection antibody was added (50 ng/ml) and incubated for 2 hours at room temperature before being washed once again. Streptavidin horseradish peroxidase (HRP) was then diluted 1:40 in 1% BSA in PBS and added into each well and left to incubate for 20 minutes in darkness. The wash step was then performed once again, and substrate solution was added and incubated for 20 minutes in darkness. Stop solution (2N H_2SO_4) was then added and the optical density was then measured at a wavelength of 450 nm and 550 nm in a SynergyHT microplate reader (BioTek).

Readings at 550 nm were subtracted from the 450 nm readings. The mean readings of the duplicate standards and blanks were then used to produce a four-parameter blank-corrected logistic curve fit using the curve-fitting algorithms in the Gen5 1.11 software (BioTek) used to operate the plate reader. Sample concentrations were determined from this curve and then the mean of each set of duplicates was calculated.

2.2.2 SimpleStep ELISA for SIRT2

Human SIRT2 SimpleStep ELISA Kits (Abcam, Cambridge, UK) were used according to manufacturer instructions to analyse macrophage culture supernatants. A two-fold serial dilution ranging from 1000 pg/ml to 15.6 pg/ml was prepared using recombinant SIRT2 supplied in the kit. Samples and standards were then added into wells in duplicate, then antibody cocktail was added and left to incubate for an hour. Wells were then aspirated and washed three times with Wash Buffer PT (supplied in kit) and aspirated once more. TMB Substrate was then added to each well and left to incubate for 10 minutes in the dark on a shaker at 400 rpm. Stop solution was then added to each well and agitated briefly before the OD was measured at 450 nm in a SynergyHT microplate reader (BioTek).

The mean readings of the duplicate standards and blanks were then used to produce a four-parameter logistic curve fit. Sample concentrations were determined from this curve and then the mean of each set of duplicates was calculated.

Spike/recovery experiments were conducted using the previously established method to ensure good performance when analysing saliva samples (Jaedicke et al., 2012). The recombinant protein standard supplied with the kit was used to spike saliva samples. Recovery was calculated with the following equation:

$$\text{Recovery \%} = \frac{\text{spiked sample} - \text{unspiked sample}}{\text{amount spiked}} \times 100$$

2.3 Reverse Transcription Quantitative PCR

2.3.1 RNA extraction and reverse transcription

All equipment and work areas were decontaminated using RNaseZap spray (Applied Biosystems, Paisley, UK) before work began. RNA was isolated using GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich). The media was removed from cultures of adherent, THP1-derived macrophages in 12 well plates and cells washed with PBS, then aspirated. Two hundred and fifty microliters of lysis buffer, containing 2-mercaptothanol (1% v/v), was added to each well and agitated to achieve full coverage. This was then left for 2 minutes before being scraped with a disposable cell scraper (Greiner Bio One) and the contents of the well then transferred to sterile

micro-centrifuge tubes and frozen at -80°C until ready for further processing. This lysate was then thawed on ice and transferred into a filtration column, supplied in the GenElute Mammalian Total RNA Miniprep Kit, and processed according to the manufacturer's instructions. RNA was then frozen at -80°C until use.

RNA was reverse transcribed into cDNA using a Precision nanoScript 2 Reverse transcription kit (Primerdesign, Chandler's Ford, UK). The reverse transcription master mix was prepared according to the manufacturer's instructions. The resulting cDNA was then quantified using a QuantiFluor ssDNA quantification kit (Promega, Southampton, UK) according to the manufacturer's instructions.

2.3.2 Quantitative PCR

Primers for PCR amplification were sourced from the literature (Table 2.1). RT-qPCR was carried out using PowerUp SYBR Green Master Mix (Applied Biosystems). DNA (1 ng) was added to each reaction and primers were added at a concentration of 400 nM. The tubes were then sealed, vortexed and centrifuged at 3000 rpm for 30 seconds. The contents of the tubes were transferred to a 96 well MicroAmp optical microplate (Applied Biosystems) and placed in a QuantStudio 3 thermal cycler (Thermo Fisher Scientific) set for 95°C for 10 minutes, then 95°C for 15 seconds and 60°C for 1 minute, repeated for 40 cycles. Controls containing nuclease free water instead of cDNA were prepared in the same manner and used to ensure there was no contamination present within reactions.

To select the optimal reference genes, we determined their stability within our experimental conditions. To do this, samples were collected from stimulation experiments and the CT values of control and stimulated samples were compared. There was no significant change to the Ct values for GNB2L1 and RPL32 after stimulation with TLR agonists, meaning they were suitable for use in our experiments.

Gene	Primer Sequences (5’ – 3’)	Sequence Source
SIRT2	Fwd: CCT CGC CTG CTC ATC AAC A Rev: TCC TCC GAG GCC CAT AAT C	(McGlynn *et al.* 2009)
GNB2L1	Fwd: GGT CAC TCC CAC TTT GTT AG Rev: AGA AGC GGA CAC AAG ACA	(Del Vecchio *et al.* 2009)
HPRT1	Fwd: TGA CCT TGA TTT ATT TTG CAT ACC Rev: CGA GCA AGA CGT TCA GTC CT	(Storch *et al.* 2017)
RPL32	Fwd: GAA GTT CCT GGT CCA CAA CG Rev: GCG ATC TCG GCA CAG TAA G	(Manukyan *et al.* 2015)
GAPDH	Fwd: TGT GGG CAT CAA TGG ATT TGG Rev: ACA CCA TGT ATT CCG GGT CAA T	(Wang *et al.* 2016)

Table 2.1. Primer sequences used in RT-qPCR experiments.

All primers were sourced from the literature and purchased from Thermo Fisher.

To help ensure the validity of our results, all primer sets for reference genes and SIRT2 underwent a melt curve cycle. In this process amplified DNA undergoes a cycle where the temperature from is raised from 65°C to 95°C. As SYBR Green is a dye that only fluoresces when it is intercalated to double stranded DNA, the increasing temperature in the melt cure cycle will cause double stranded amplification products to dissociate, preventing the SYBR Green dye from fluorescing. Different sized amplification products will dissociate at different temperatures, by measuring the fluorescence as the temperature changes, this can be plotted as a graph showing fluorescence against temperature. If only a single peak is present on this graph this is indicative of only one amplification product being present, demonstrating that the amplification was specific (Ririe *et al.* 1997).

Additionally, amplified products were run down an agarose gel to assess amplification of a single product. A 2% agarose gel was made by dissolving 2g of agarose in 100 ml of Tris-acetate-EDTA (TAE) buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA sodium salt dehydrate) and microwaving until boiling. The agarose solution was then left to cool. GelRed (Cambridge BioScience, Cambridge, UK) was added to a final dilution of 1:10,000. The agarose gel was then poured into a gel tray with a comb in place. The gel was then left to set at room temperature for 30 minutes until completely solidified. The gel was then placed into the electrophoresis unit which was filled with TAE buffer until the gel was covered. 5 µl of Hyperladder IV DNA ladder (Bioline, London, UK) was loaded into the first well before 5 µl volume of samples were loaded into the subsequent wells. The gel was then run at 120V until the dye front is approximately 80% of the way down the gel. The gel was then visualised in a G:BOX gel documentation system (Syngene, Cambridge, UK), if a single band at the appropriate position, as indicated by the ladder, is present then specific amplification was achieved (Lee et al., 2012).

The results are presented as fold-change in gene expression of SIRT2 mRNA relative to the reference gene GNB2L1 and RPL32 mRNA ± SD, calculated from threshold cycle (Ct) using $2^{-\Delta\Delta Ct}$ analysis (Livak and Schmittgen 2001). To do this, the following equation was used:

$$\Delta\Delta Ct = (Ct_{\text{Gene of interest}} - Ct_{\text{Housekeeping gene}}) - (\Delta Ct_{\text{Sample}} - \Delta Ct_{\text{Control average}})$$

This was then transformed to fold-change gene expression using the following equation:

$$\text{Fold-change gene expression} = 2\text{^-}(\Delta\Delta Ct)$$

2.4 Bicinchoninic Acid Assay

A bicinchoninic acid assay (BCA) was carried out using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Working reagent was first prepared, mixing BCA reagent A and BCA reagent B in a 50:1 ratio. A seven-point protein standard curve was then prepared, ranging from 4000 µg/ml – 125 ng/ml. 25 µl of these protein standards, blanks and samples were then added into a flat-bottomed 96 well plate (Greiner Bio One) before 175 µl of working reagent was added. This was then left to incubate for 30 minutes at 37°C. Absorbance was then measured at 562nm in a plate reader (BioTek). A four-parameter blank-corrected standard curve was then constructed using the curve-fitting algorithms in the Gen5 1.11 software and sample concentrations were calculated from this curve.

2.5 Western Blotting

2.5.1 SDS-PAGE

Polyacrylamide gels (12%) were cast. Separating gel (12% v/v bis/acrylamide (Sigma Aldrich), 350mM Tris-base pH 8.8, 0.1% w/v SDS, 0.2% w/v ammonium persulphate (APS, Sigma Aldrich), 3% v/v tetramethylethylenediamine (TEMED, Sigma Aldrich) was prepared and allowed to set. Stacking gel (4.5% v/v bis/acrylamide, 125 mM Tris-base pH 6.8, 0.1% w/v SDS, 0.1% APS, 2% v/v TEMED) was then prepared and poured on top of the separating gel and a comb inserted. Once the gel had set, gels were placed in a tank (Mini-PROTEAN Tetra Cell system, Bio-Rad, Watford, UK) and submerged in running buffer (25 mM Tris-base, 200 mM glycine, 1 % w/v SDS). The comb was then removed and 4 µl Chameleon Duo Pre-stained Protein Ladder (LI-COR Biotechnology, Cambridge, UK) loaded. Samples were loaded into the gel to give 30 µg of protein, diluted in equal volumes of loading buffer and boiled at 100 ˚C for 5 minutes. Electrophoresis was then conducted at 120 V until the blur dye front had run off the bottom of the gel.

2.5.2 Blotting and detection

The protein was then transferred from SDS-PAGE gels onto Odyssey nitrocellulose membrane (LI-COR Biotechnology) using a Trans-Blot Turbo Transfer System (Bio-Rad) according to manufacturer's instructions. The standard 30-minute semi-dry transfer protocol was used for transfer. Membranes were then stained with REVERT Total Protein Stain (LI-COR Biotechnology)

and washed according to manufacturer's instructions. Images of membranes were then captured using the 700 nm channel on the Odyssey Fc Imaging System (LI-COR Biotechnology). Membranes were then blocked with Odyssey PBS blocking buffer (LI-COR Biotechnology) for 1 hour. Next, membranes were incubated for 1 hour in primary antibody at the indicated dilutions, (see Table 2.2), in Odyssey PBS blocking buffer with 0.2% Tween20. Membranes were then washed 4 times for 5 minutes each in PBS-Tween20 (0.1%) with agitation. Then membranes were incubated IRDye 680LT Donkey (polyclonal) anti-mouse IgG and IRDye 800CW Goat (polyclonal) anti-Rabbit IgG secondary antibodies (LI-COR Biotechnology) diluted in Odyssey PBS blocking buffer with 0.2% Tween20. After this, membranes were washed again as before, and images were captured using the 700 nm and 800 nm channels on the Odyssey Fc Imaging System (LI-COR Biotechnology).

Target	Dilution	Manufacturer
SIRT2	1:500	Abcam
GNB2L1	1:1000	Santa Cruz
β-actin	1:50000	Proteintech
α-tubulin	1:5000	Abcam
Acetyl (K40) α-tubulin	1:1000	Abcam
NF-κB p65	1:1000	Cell Signalling Technology
Acetyl (K310) NF-κB p65	1:400	Abcam

Table 2.2. Antibodies used for western blotting.

All antibodies were used at dilutions within the range recommended by their manufacturer.

2.5.3 Determination of linear range

Before experimental use, the linear range of detection was determined for each antibody (Figure 2.2). This is the range in which the measured signal from a given antibody scales linearly with the amount of protein originally loaded into the SDS gel. To do this, seven samples were prepared with final amounts of protein ranging from 30 µg down to 0.47 µg and electrophoresed using SDS-PAGE and transferred to nitrocellulose membranes using the previously established method. The signal was then quantified for each band and this value was plotted against protein loaded on a scatter graph. R^2 values were then calculated for each graph. Any concentration range with R^2 values above 0.97 have been deemed suitably linear (Koch *et al.* 2018).

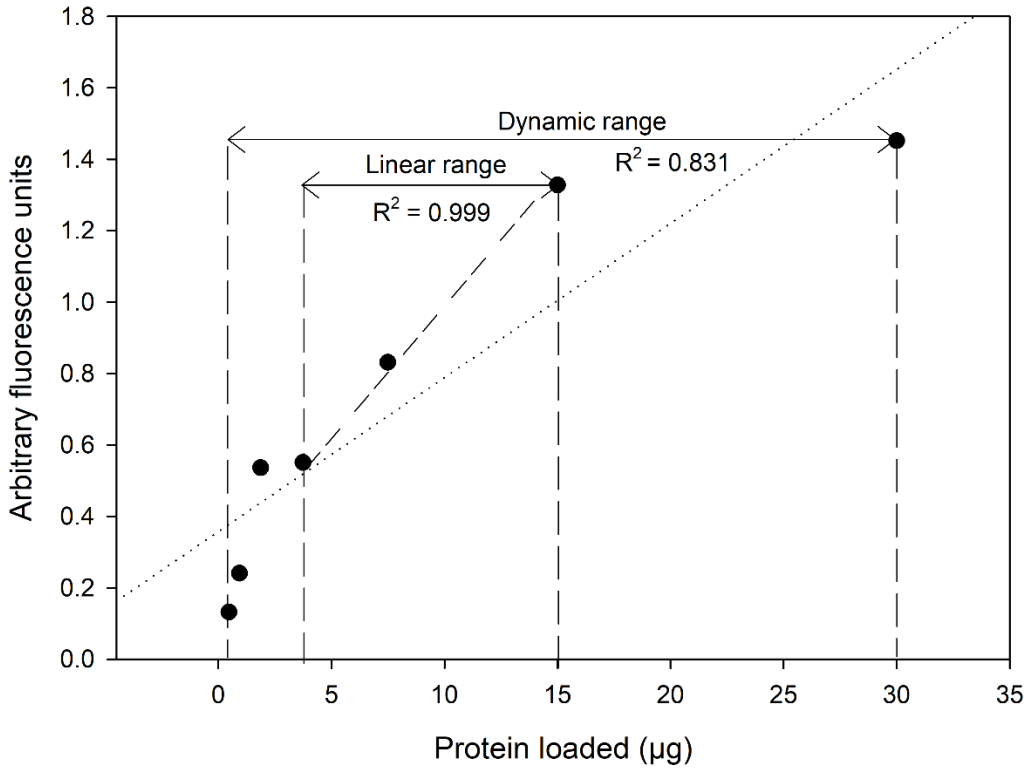


Figure 2.2. Representative plot for determination of linear range of antibodies used in western blot experiments.

R^2 values are shown for both the linear range, where the signals are directly proportional to the concentration of the analyte, and the dynamic range, the range where the response changes when the analyte concentration is changed but the relationship may be non-linear.

2.5.4 Loading normalisation against total protein stain

Signal from REVERT Total Protein Stain was then used to calculate a lane normalisation factor for each lane to correct for variations to protein loading. This was done using the following equation:

$$\text{Lane normalisation factor} = \frac{\text{Total protein stain signal}}{\text{Highest signal value}}$$

Lane normalisation factors were then used to calculate normalised signal for their corresponding lanes with the following equation:

$$\text{Normalised signal} = \frac{\text{Target protein signal}}{\text{Lane normalisation factor}}$$

For western blots analysing the acetylation of α-tubulin, signals were normalised using the method above, then expressed as a relative ratio or acetyl α-tubulin to total α-tubulin with the controls normalised to 1.

2.6 MTT Assay

MTT Cell Proliferation Assay kit (Trevigen, Gaithersburg, MD, USA) was used to measure any change to cell viability or proliferation. Macrophages were differentiated as described previously, media was removed wells and 0.9 ml of fresh culture medium was added into each well. 100 µl of MTT reagent was then added into each well and the plate was returned to the incubator for 4 hours. 1 ml of detergent reagent was then added into each well and was left in the dark overnight. The next day the absorbance in each well was measured at 570 nm in a SynergyHT microplate reader (BioTek).

2.7 Chemical Inhibitors

Prior to use, all inhibitors were evaluated using MTT assays to ensure no cytotoxic effects were present at experimental concentrations.

2.7.1 TLR inhibitors

Inhibitors of TLR2 and TLR4 were used to confirm specificity of TLR agonists. Cells were pre-treated with the TLR2 inhibitor C29 (Abcam) for 0.5 hours at 50 μM in normal growth medium or with the TLR4 inhibitor C34 (Bio-Techne) for 0.5 hours at 10 μM in normal growth medium. Media was replaced with normal growth medium prior to addition of TLR agonists as described previously (Section 2.1).

2.7.2 SIRT2 inhibitors

Inhibitors of SIRT2 activity were used to determine the role of SIRT2 in the regulation of cytokine secretion. FK866 is an inhibitor of NAMPT, leading to reduced levels of NAD and therefore reduced activity of NAD-dependent HDACs. Cells were treated FK866 (Sigma Aldrich) for 24 hours at a concentration of 2 μM in normal growth medium. AK1 is a SIRT2 inhibitor this is cell permeable and targets the nicotinamide binding site of SIRT2. Cells were treated with AK1 (Abcam) for 24 hours at a concentration of 10 μM in normal growth medium. Media was then replaced with normal growth medium before experiments were conducted.

2.8 Collection of Saliva Samples

Three subject groups were recruited as part of this study: patients with chronic periodontitis (N = 65), patients with gingivitis (N = 47), and periodontally healthy volunteers (N = 56). Participants were males or females aged between 18 and 65 and had a minimum of 20 natural teeth (excluding 3rd molars) and were non-smokers. The diagnostic criteria were as follows: healthy participants had mean PPD of ≤ 3 mm in all sites, no sites with interproximal attachment loss, mean modified gingival index (MGI) scores of ≥ 2.0 in ≤ 10% of sites and % BOP scores of ≤ 10%; gingivitis patients had MGI of ≥ 3.0 in ≥ 30% of sites, no sites of interproximal attachment loss, PPD > 4 mm and % BOP scores of ≥ 10%; periodontitis patients had interproximal mean PPD of ≥ 5 mm at ≥ 8 teeth and %BOP scores of ≥ 30%.

Subjects provided written informed consent, the study was conducted at the Dental Clinical Research Facility of Newcastle Dental Hospital (Newcastle upon Tyne Hospitals NHS Foundation Trust) and was fully approved by the National Research Ethics Service North East Newcastle and North Tyneside 1 committee (Ref: 12/NE/0396). Methods were performed in accordance with the relevant guidelines and regulations. Subjects with evidence of infectious or systemic disease, currently undergoing treatment with antibiotics or immunosuppressants were excluded from the study, along with subjects who had smoked within the last 2 years.

Full mouth periodontal clinical indices were recorded, and saliva samples were collected at month 0 (all subjects), 3 months post-treatment (gingivitis and periodontitis patients), and 6 months (periodontitis patients). Periodontal clinical indices were recorded at 6 sites per tooth and included MGI, PPD, gingival recession, CAL and % BOP. PISA and PESA were calculated as previously published (Nesse *et al.* 2008).

Unstimulated saliva samples (3–5 ml) were collected by expectoration into a plastic centrifuge tube and placed on ice immediately after collection, before centrifugation for 15 minutes at 1500 g and at 4 °C. Aliquots were frozen in liquid nitrogen and stored at −80 °C until analysis.

2.9 Statistical Analysis

Normality of data was evaluated using the Shapiro-Wilk test and Levene's test for homogeneity of variance. Normally distributed data was analysed by either Student's t test or ANOVA. Non-normally distributed data was analysed by Mann-Whitney U. Before analysis of saliva samples began, advice was obtained from Dr Kim Pearce, Senior Statistician at Newcastle University Faculty of Medical Sciences. Mann-Whitney U test was used to identify differences to measured periodontal parameters in healthy and periodontitis groups. Kendall's tau b was used to evaluate correlations between SIRT2 and periodontal parameters. ANCOVA was used to interrogate the effect of age on the relationship between SIRT2 levels and periodontitis. Receiver operating characteristic (ROC) curve analysis was used to evaluate the ability of SIRT2 to discriminate between cases of health and cases of periodontitis. All statistical analyses, with the exception of ROC curve analysis, were carried out using SPSS version 24.0.0 (IBM, Portsmouth, UK). P values

of <0.05 were considered statistically significant. ROC curve analysis was carried out using SigmaPlot (Sy stat Software Inc., Berkshire, UK).

Chapter 3. Expression of SIRT2 in THP1-derived Macrophages Stimulated with TLR Agonists

3.1 Introduction

Periodontitis is a chronic inflammatory disease that is triggered by the formation of bacterial biofilms on the surface of the teeth. These bacterial biofilms can elicit a response from the innate immune system by activating TLRs on the surface of host (Ilango *et al.* 2016; Song *et al.* 2017) . TLRs recognise certain microbial components (Hans and Hans 2011), leading to the activation of signalling pathways that ultimately lead to secretion proinflammatory cytokines such as IL-1β (Leifer and Medvedev 2016) and TNFα (Kawasaki and Kawai 2014).

TLR2 and TLR4 agonists were selected as these receptors have been found to be crucial in periodontitis, initiating the immune response upon detection of bacterial components derived from biofilms on the teeth (Mahanonda and Pichyangkul 2007; Cekici *et al.* 2014b). Activation of TLR2 and TLR4 have been shown to cause secretion of TNFα in THP1-derived macrophages (Inoue *et al.* 2018; Liu *et al.* 2018). Given its role as a pro-inflammatory cytokine involved in the immunological processes of periodontitis (Khosravi *et al.* 2013; Pan *et al.* 2019a), TNFα was selected as a readout to confirm successful stimulation with TLR2 and TLR4 agonists.

LPS is an endotoxin that can be derived from the outer membrane of most Gram-negative bacteria. LPS is one of the most widely used TLR4 agonists (Okuda *et al.* 2016). LTA is a component of the cell wall of Gram-positive bacteria (Percy and Grundling 2014) that activates TLR2 (Schroder *et al.* 2003; Oliveira-Nascimento *et al.* 2012).

For these experiments, ultrapure preparations of *E. coli* LPS were purchased. These ultrapure preparations are certified as free from contaminating lipopeptides that would result in activation of TLR2. As there was no ultrapure preparation of *B. subtilis* LTA available for purchase and the only assurances made by the manufacturer are that the level of endotoxin present is under 10 EU/mg, we also purchased a synthetic TLR2 agonist, Pam2CSK4. As Pam2CSK4 is synthetic, it is much less likely to contain any contaminating bacterial components and would allow us to confirm that any effect of our TLR2 agonists was genuine and not caused by any contaminant left over from the purification process.

Macrophages are a critical component of the innate immune system and are important in periodontitis, with evidence suggesting there is disruption to the homeostasis of macrophage populations in areas of the mouth affected by periodontitis (Almubarak *et al.* 2020). Macrophages are involved in the activation of the adaptive immune system and sustained inflammation seen in periodontitis (Sima *et al.* 2018). For my experiments, I utilised a model where THP1 monocytes are differentiated into macrophages using PMA (Park *et al.* 2007a; Daigneault *et al.* 2010). THP1 monocytes are a monocyte-like cell line, derived from the peripheral blood of a patient suffering from acute monocytic leukaemia (Bosshart and Heinzelmann 2016). This cell line has been widely used in research as a model for monocytes and macrophages (Starr *et al.* 2018), providing a method for generating macrophages that do not exhibit the variation seen in macrophages derived from primary human monocytes and are more easily obtained (Forrester *et al.* 2018). PMA activates protein kinase C to initiate the differentiation process which subsequently triggers signalling cascades and results in adoption of a macrophage phenotype (Tsai *et al.* 2016). The duration of differentiation and PMA concentration were selected based on published literature which identified a 48 hour differentiation period with 10 ng/ml of PMA as significantly altering macrophage phenotype (Park *et al.* 2007b; Lund *et al.* 2016) .

SIRT2 is the most highly expressed sirtuin in macrophages (Ciarlo *et al.* 2017) and whilst there are numerous studies investigating the role of SIRT2 in immune function (Lo Sasso *et al.* 2014; Ciarlo *et al.* 2017), there is little research on how SIRT2 itself is affected by TLR agonists and even less on any potential role of SIRT2 in periodontitis. There is currently no published research investigating the specific effect of TLR agonists on SIRT2 mRNA or protein expression in human macrophages. The currently published research has utilised either animal models, or models of bacterial infection where cells are exposed to whole bacteria. For this reason, and because of the elevated levels of SIRT2 we observed in the saliva of patients with periodontitis, we began by investigating the effect of TLR signalling on the mRNA and protein expression of SIRT2 in THP1-derived macrophages. ELISAs for TNF were used to confirm successful stimulation with *E. coli* LPS, *B. subtilis* LTA or Pam2CSK4. qPCR was then used to quantify SIRT2 mRNA expression and western blotting was used to quantify SIRT2 protein expression.

3.2 TNF-α Secretion by THP1-derived Macrophages Stimulated with TLR Agonists

To understand the effect of pro-inflammatory signals on SIRT2 mRNA and protein expression in macrophages, experiments were conducted to confirm that the macrophages would respond to the chosen TLR agonists. This was determined by measuring the levels of secreted TNFα compared to control using ELISA. To optimise the stimulation of THP1-derived macrophages with *E. coli* LPS, Pam2CSK4 and *B. subtilis* LTA, a series of time course experiments were conducted. The short time course consisted of time points at 0.5 hours, 1 hour and 2 hours, whilst the long time course consisted of time points at 4 hours, 8 hours and 24 hours.

A time course experiment was first conducted to assess TNFα secretion from THP1-derived macrophages stimulated with *E. coli* LPS for 0.5 hours, 1 hour or 2 hours (Figure 3.1). TNFα secretion was significantly increased at all time points compared with controls, with the largest increase at the 2-hour time point ($p<0.001$). Smaller, but still significant, increases were seen at the 0.5-hour and 1-hour time points ($p=0.016$ and 0.004 respectively).

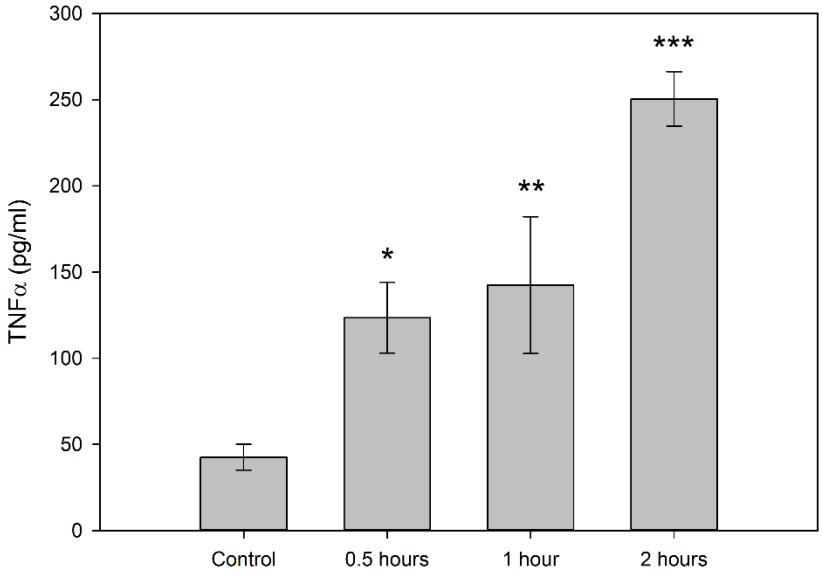


Figure 3.1. Short time course of TNFα secretion from LPS-stimulated macrophages.

TNFα measured by ELISA in supernatants of THP1-derived macrophage cultures stimulated for 0.5 hours, 1 hour or 2 hours with *E. coli* LPS (100 ng/ml). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). * 0.016, ** 0.004, *** <0.001 calculated by one-way ANOVA, followed by Tukey's post-hoc test.

Next, a time course experiment was conducted to assess TNFα secretion from THP1-derived macrophages stimulated Pam2CSK4 at time points of 0.5 hours, 1 hour and 2 hours (Figure 3.2). TNFα secretion was significantly increased at the 2-hour time point (p=0.0015) compared with controls, whilst no significant change was detected at the 0.5- and 1-hour time points.

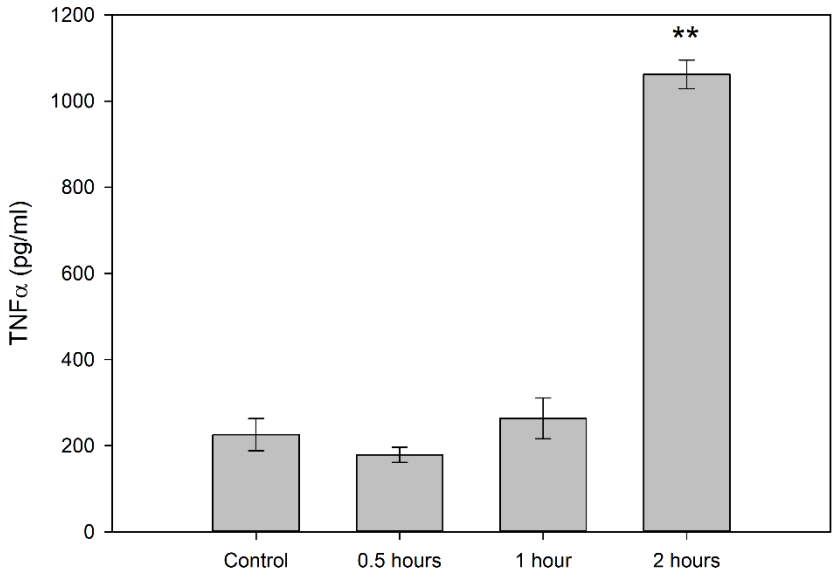


Figure 3.2. Short time course of TNFα secretion from Pam2CSK4-stimulated macrophages.

TNFα measured by ELISA in supernatants of THP1-derived macrophage cultures stimulated for 0.5 hours, 1 hour or 2 hours with Pam2CSK4 (10 ng/ml). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). ** 0.0015 calculated by one-way ANOVA, followed by Tukey's post-hoc test.

Another time course experiment was then conducted to assess TNFα secretion from THP1-derived macrophages stimulated *B. subtilis* LTA at time points of 0.5 hours, 1 hour and 2 hours (Figure 3.3). TNFα secretion was significantly increased at the 2-hour time point (p=0.0029) compared with controls, whilst no significant change was detected at the 0.5- and 1-hour time points.

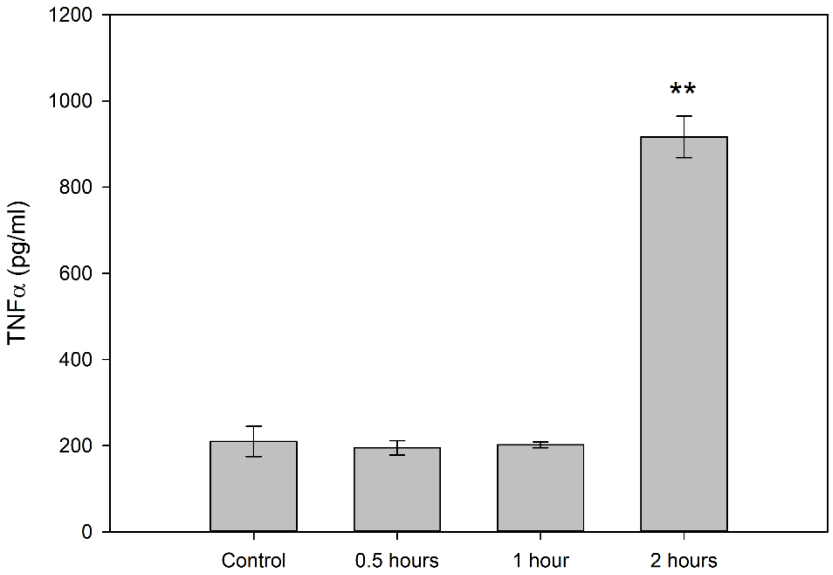


Figure 3.3. Short time course of TNFα secretion from LTA-stimulated macrophages.

TNFα measured by ELISA in supernatants of THP1-derived macrophage cultures stimulated for 0.5 hours, 1 hour or 2 hours with *B. subtilis* LTA (100 ng/ml). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). ** 0.0029, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

Next, time course experiment was conducted to assess TNFα secretion from THP1-derived macrophages stimulated *E. coli* LPS at time points of 4 hours, 8 hours, and 24 hours (Figure 3.4) was conducted. TNFα was most significantly elevated as compared to controls after 4 hours of stimulation ($p<0.001$), with slightly lower levels at 8 hours ($p<0.001$). Twenty-four hours of stimulation produced a smaller, but still significant, increase in TNFα ($p=0.002$). MTT assays were conducted and confirmed that there was no cytotoxic effect of *E. coli* LPS causing the less significant increase in TNFα secretion at 24 hours (see Chapter 3.3).

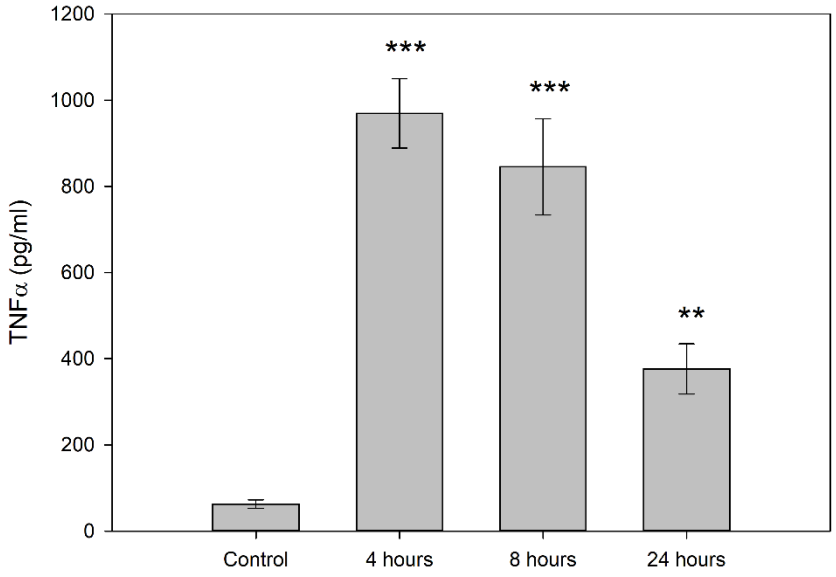


Figure 3.4. Long time course of TNFα secretion from LPS-stimulated macrophages.

TNFα measured by ELISA in supernatant of THP1-derived macrophages stimulated for 4 hours, 8 hours or 24 hours with *E. coli LPS* (100 ng/ml). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). ** 0.002, *** <0.001 calculated by one-way ANOVA, followed by Tukey's post-hoc test.

A time course experiment was conducted to assess TNFα secretion from THP1-derived macrophages stimulated Pam2CSK4 at time points of 4 hours, 8 hours, and 24 hours (Figure 3.5). TNFα was significantly elevated as compared to controls at all time points. Four hours and 8 hours of stimulation produced the most significant increase (both $p<0.001$), with a lesser, but still statistically significant increase seen at 24 hours ($p=0.0017$).

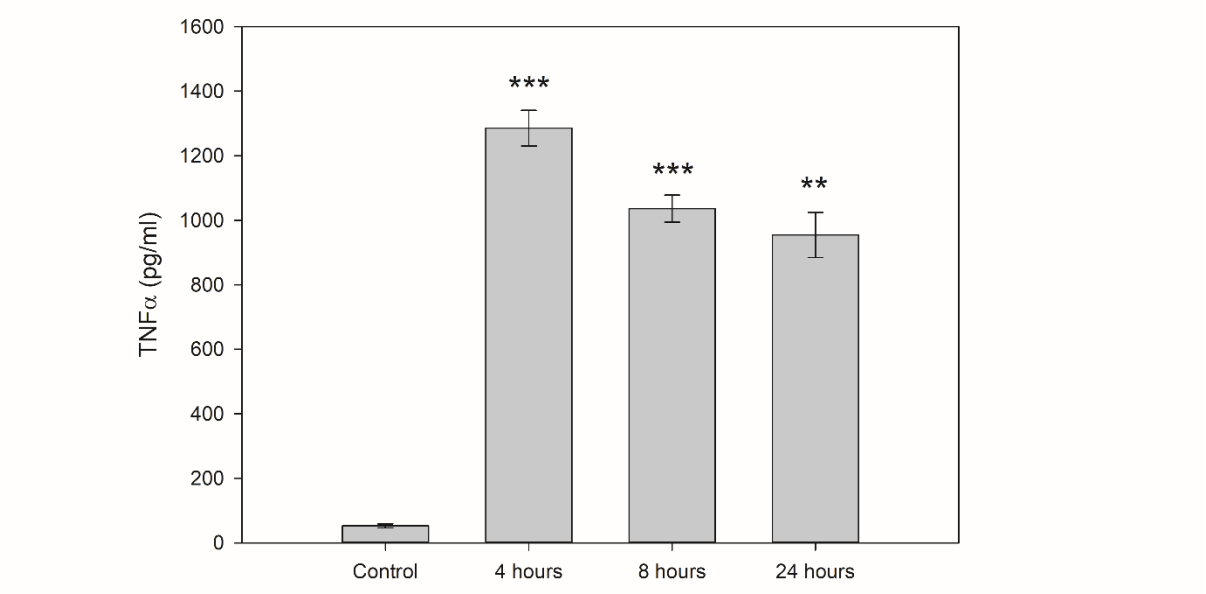


Figure 3.5. Long time course of TNFα secretion from Pam2CSK4-stimulated macrophages.

TNFα measured by ELISA in supernatants of THP1-derived macrophage cultures stimulated for 4 hours, 8 hours or 24 hours with Pam2CSK4 (10 ng/ml). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). ** 0.0017, *** <0.001 calculated by one-way ANOVA, followed by Tukey's post-hoc test.

An additional time course experiment was then conducted to assess TNFα secretion from THP1-derived macrophages stimulated LTA at time points of 4 hours, 8 hours, and 24 hours (Figure 3.6). TNFα was significantly elevated compared to controls at 4, 8 and 24 hours ($p<0.01$, $p<0.01$ and $p<0.001$ respectively).

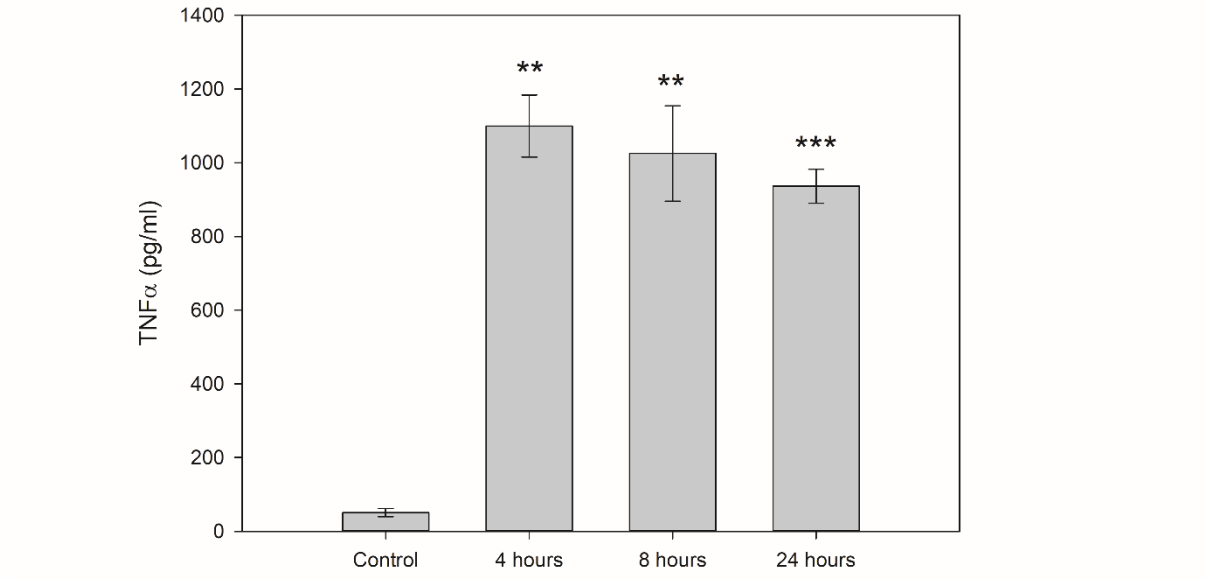


Figure 3.6. Long time course of TNFα secretion from LTA-stimulated macrophages.

TNFα measured by ELISA in supernatant of THP1-derived macrophages stimulated for 4 hours, 8 hours or 24 hours with *B. subtilis* LTA (100 ng/ml). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). ** <0.01, *** <0.001 calculated by one-way ANOVA, followed by Tukey's post-hoc test.

In the next series of experiments, the secretion of TNFα from THP1-deriverd macrophages was examined after 4 hours of stimulation with TLR2 and TLR4 agonists (Figure 3.7). All three stimuli acted to increase TNFα secretion compared with control macrophages. Stimulation of macrophages with *E. coli* LPS produced a statistically significant increase (p=0.002) in TNFα secretion when compared with controls. Pam2CSK4, and *B. subtilis* LTA, also produced statistically significant increases (both p<0.001) in TNFα secretion compared with controls.

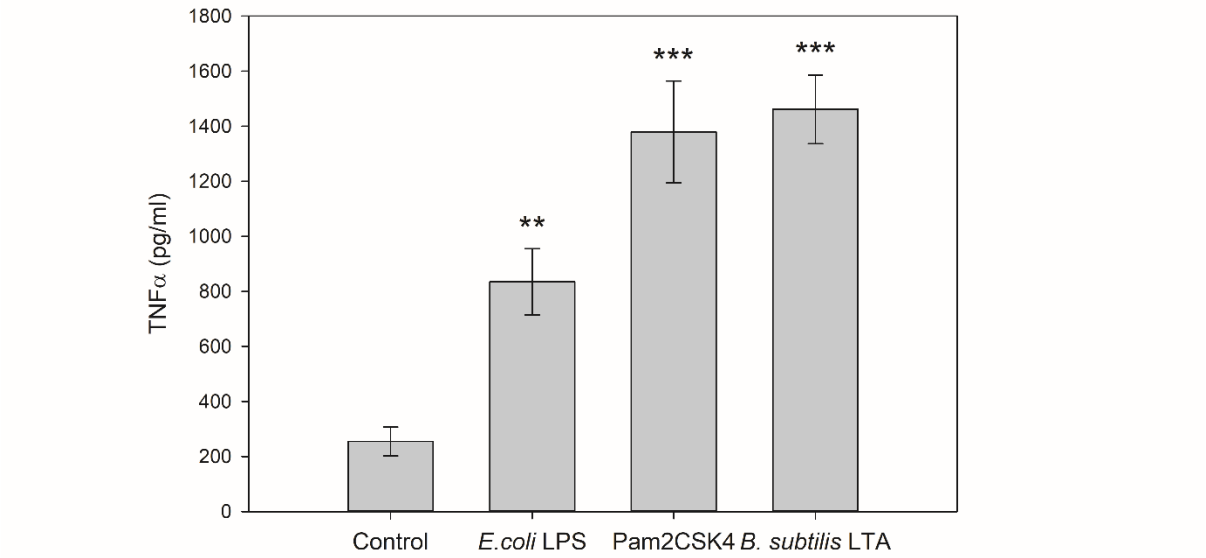


Figure 3.7. TNFα secretion from macrophages stimulated with TLR agonists for 4 hours.

TNFα measured by ELISA in supernatant of THP1-derived macrophages stimulated for 4 hours with *E. coli* LPS (100 ng/ml), Pam2CSK4 (10 ng/ml) or *B. subtilis* LTA (100 ng/ml. Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 3 separate experiments (N=9). ** p=0.002 *** p<0.001, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

Next, the secretion of TNFα from macrophages was examined after 24 hours of stimulation with TLR2 and TLR4 agonists. *E. coli* LPS, Pam2CKS4 and *B.* subtilis LTA acted to increase TNFα secretion from THP1-derived macrophages as compared with control macrophages (Figure 3.8). Stimulation with *E. coli* LPS produced a statistically significant increase (p=0.004) in TNFα secretion when compared with controls. Pam2CSK4 and *B. subtilis* LTA produced even greater statistically significant increases (both p<0.001) in TNFα secretion compared with controls.

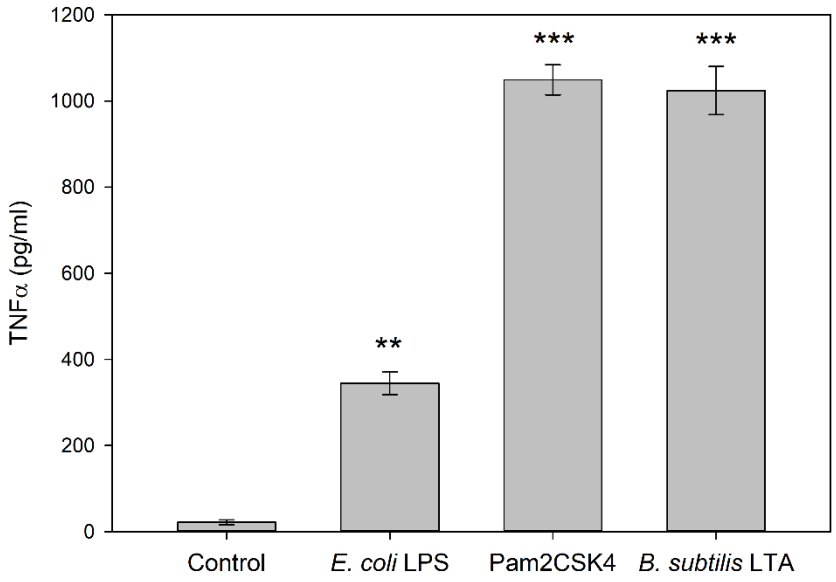


Figure 3.8. TNFα secretion from macrophages stimulated with TLR agonists for 24 hours.

TNFα measured by ELISA in supernatant of THP1-derived macrophages stimulated for 24 hours with *E. coli* LPS (100 ng/ml), Pam2CSK4 (10 ng/ml) or *B. subtilis* LTA (100 ng/ml. Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 3 separate experiments (N=9). ** p=0.004 *** p<0.001, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

From these experiments it was determined that THP1-derived macrophages could be successfully stimulated with *E. coli* LPS, Pam2CSK4 or *B. subtilis* LTA and the subsequent TNFα response could be measured by ELISA. This informed the time points selected for the experiments analysing SIRT2 mRNA expression. The format of experiments in Figures 3.7 and 3.8 was utilised to confirm successful stimulation THP1-derived macrophages in subsequent experiments. Next, the effects of TLR agonists on the viability THP1-derived macrophages were investigated to ensure that there were no toxic or mitogenic effects.

3.3 Viability of THP1-derived Macrophages After Exposure to TLR Agonists

MTT assays were used to determine if the selected TLR agonists had any effect on cell viability. THP1-derived macrophages were stimulated with TLR agonists before being incubated with MTT to produce formazan crystals which were then dissolved, and absorbance is measured. This was compared to a standard curve generated by culturing varying numbers of cells and incubating them with MTT. From this, the number of viable cells in the TLR agonist-stimulated cultures could be calculated.

Stimulation of THP1-derived macrophages with *E. coli* LPS, Pam2CSK4 or *B. subtilis* LTA for 4 hours (Figure 3.9) had no statistically significant effect on cell viability.

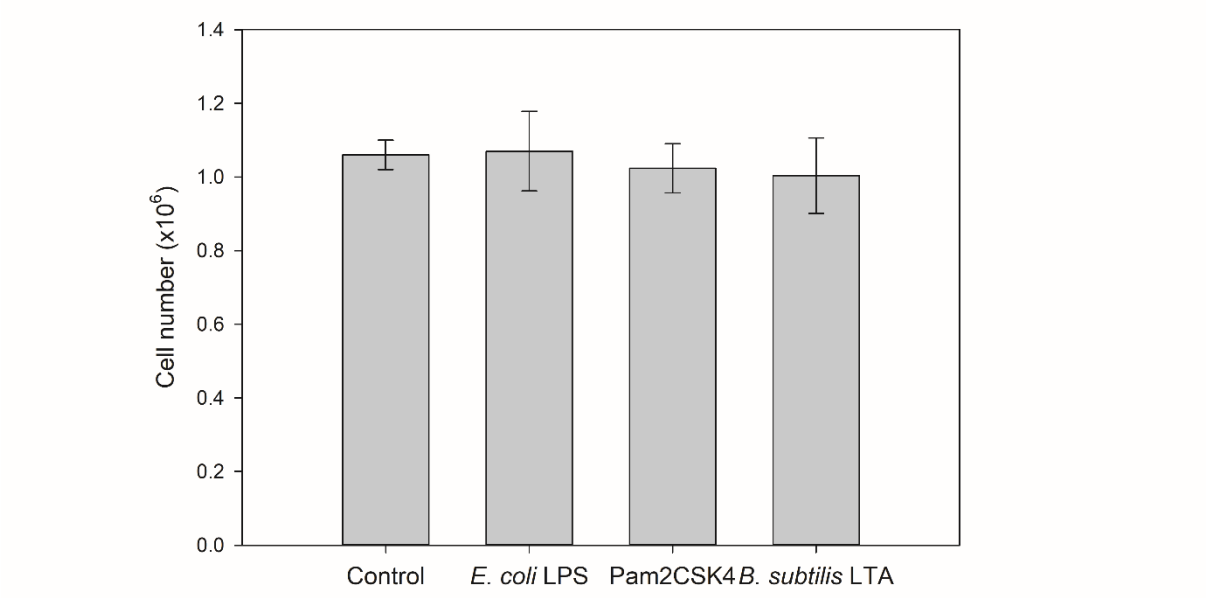


Figure 3.9. Cell number determined by MTT assay with macrophages stimulated with TLR agonists for 4 hours.

THP1-derived macrophages stimulated for 4 hours with *E. coli* LPS (100 ng/ml), Pam2CSK4 (10 ng/ml) or *B. subtilis* LTA (100 ng/ml. Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). Analysed using one-way ANOVA.

Stimulation of THP1-derived macrophages with *E. coli* LPS, Pam2CSK4 or *B. subtilis* LTA for 24 hours (Figure 3.10) had no statistically significant effect on cell viability. After determining that the selected TLR agonists had no significant effect cell viability, their effect on SIRT2 mRNA expression could be investigated.

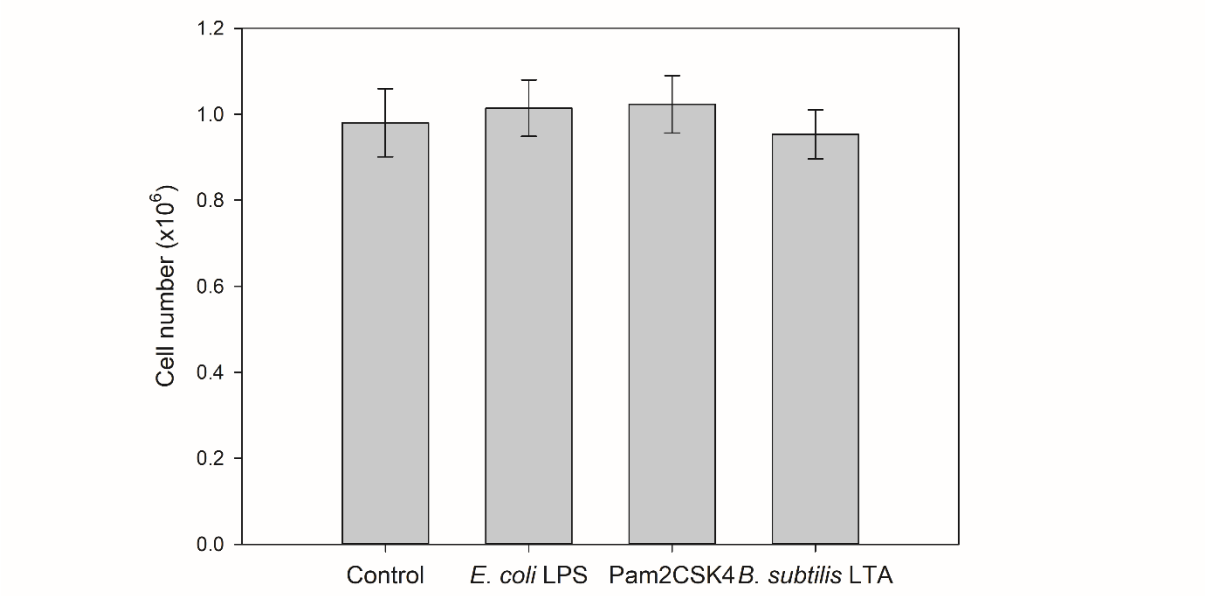


Figure 3.10. Cell number determined by MTT assay with macrophages stimulated with TLR agonists for 24 hours.

THP1-derived macrophages stimulated for 24 hours with *E. coli* LPS (100 ng/ml), Pam2CSK4 (10 ng/ml) or *B. subtilis* LTA (100 ng/ml. Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). Analysed using one-way ANOVA.

3.4 SIRT2 mRNA Expression by THP1-derived Macrophages Stimulated with TLR agonists

To determine the effect of TLR2 and TLR4 agonists on SIRT2 mRNA expression, RNA was extracted from THP1-derived macrophages that had been stimulated or left as controls. ELISAs for TNFα were carried out on supernatants to confirm successful stimulation. The RNA samples were then reverse transcribed into cDNA and used in qPCR experiments.

In order to maximise the chance of detecting any change to SIRT2 mRNA expression we selected 3 different stimulation durations. The stimulation duration investigated was 0.5 hours with the selected TLR agonists. Stimulation of THP1-derived macrophages with *E. coli* LPS, Pam2CSK4 or *B. subtilis* LTA for 0.5 hours (Figure 3.11) had no significant effect on SIRT2 mRNA expression compared with unstimulated controls.

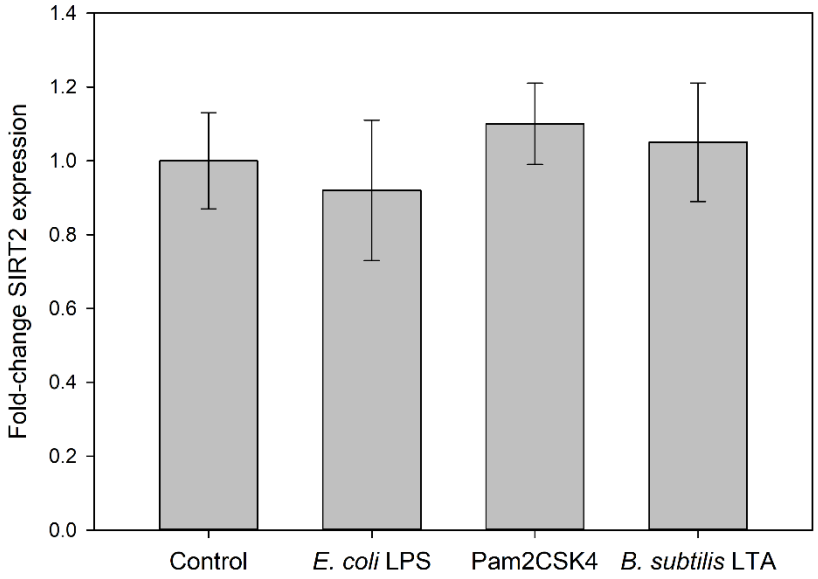


Figure 3.11. SIRT2 mRNA expression in macrophages stimulated with TLR agonists for 0.5 hours.

SIRT2 mRNA expression THP1-derived macrophages stimulated with *E. coli* LPS (100 ng/ml), Pam2CSK4 (10 ng/ml) or *B. subtilis* LTA (100 ng/ml) for 0.5 hours. Cells were lysed and cDNA was generated using reverse transcription. qPCR was then used to assess SIRT2 mRNA expression using $2^{-\Delta\Delta CT}$ against the geometric mean of reference genes GNB2L1 and RPL32. The data shown represents the mean fold change ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). Analysed using one-way ANOVA.

The next experiments utilised a 4-hour stimulation duration. This is the time point at which *E. coli* LPS had the most significant effect on TNFα secretion. qPCR was used on cDNA generated from RNA samples collected from THP1-derived macrophages after 4 hours of stimulation with TLR2 or TLR4 agonists.

Stimulation of THP1-derived macrophages with *E. coli* LPS, Pam2CSK4 or *B. subtilis* LTA for 4 hours (Figure 3.12) had no significant effect on SIRT2 mRNA expression compared with unstimulated controls.

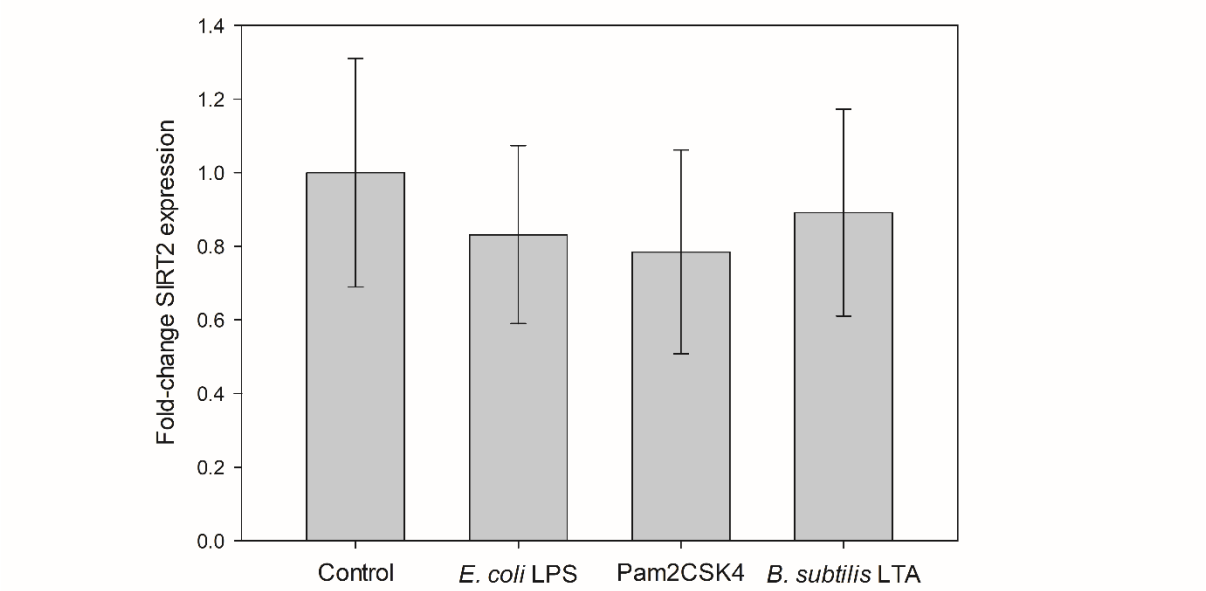


Figure 3.12. SIRT2 mRNA expression in macrophages stimulated with TLR agonists for 4 hours.

SIRT2 mRNA expression THP1-derived macrophages stimulated with *E. coli* LPS (100 ng/ml), Pam2CSK4 (10 ng/ml) or *B. subtilis* LTA (100 ng/ml) for 4 hours. Cells were lysed and cDNA was generated using reverse transcription. qPCR was then used to assess SIRT2 mRNA expression using $2^{-\Delta\Delta CT}$ against the geometric mean of reference genes GNB2L1 and RPL32. The data shown represents the mean fold change ± SD of 3 cultures for each condition obtained in 3 separate experiments (N=9). Analysed using one-way ANOVA.

The next time point selected was a 24-hour stimulation duration. qPCR was again used on cDNA generated from RNA samples collected from THP1-derived macrophages after 24 hours of stimulation with TLR2 or TLR4 agonists.

Stimulation of THP1-derived macrophages with *E. coli* LPS, Pam2CSK4 or *B. subtilis* LTA for 24 hours (Figure 3.13) had no significant effect on SIRT2 mRNA expression compared with unstimulated controls.

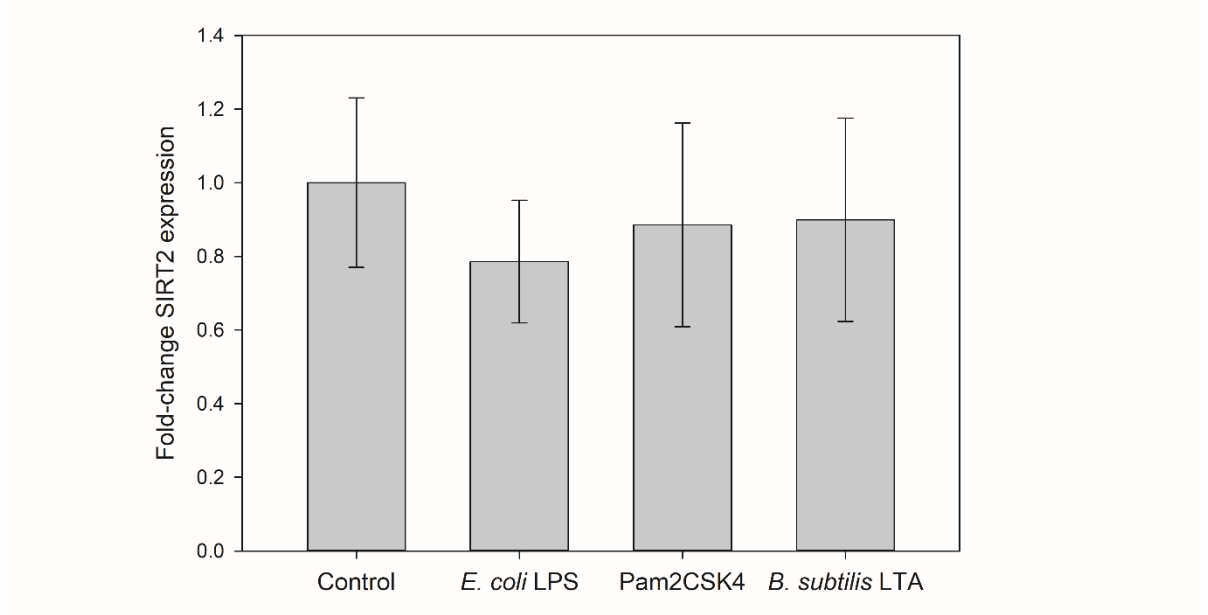


Figure 3.13. SIRT2 mRNA expression in macrophages stimulated with TLR agonists for 24 hours.

SIRT2 mRNA expression THP1-derived macrophages stimulated with *E. coli* LPS (100 ng/ml), Pam2CSK4 (10 ng/ml), or *B. subtilis* LTA (100 ng/ml) for 24 hours. Cells were lysed and cDNA was generated using reverse transcription. qPCR was then used to assess SIRT2 mRNA expression using $2^{-\Delta\Delta CT}$ against the geometric mean of reference genes GNB2L1 and RPL32. The data shown represents the mean fold change ± SD of 3 cultures for each condition obtained in 3 separate experiments (N=9). Analysed using one-way ANOVA.

It was concluded from these experiments that SIRT2 mRNA expression is not affected by stimulation with E. coli LPS, Pam2CSK4 or B. subtilis LTA in THP1-derived macrophages. This finding was consistent across stimulation durations of 0.5 hours, 4 hours, and 24 hours. The next experiments aimed to measure the effects of TLR 2 and TLR 4 agonists on SIRT2 protein expression.

3.5 Optimisation of SIRT2 Protein Detection by Western Blot – Determination of the Linear Range

To investigate if pro-inflammatory stimuli had any effect on SIRT2 protein expression, we used quantitative western blotting with fluorescently labelled antibodies. In order to accurately quantify proteins using western blotting, the linear range must first be determined for each protein. This is the range in which the measured signal correlates linearly with the amount of protein loaded into the gel; this can be determined by performing western blotting on serial dilutions of sample and calculating the coefficient of determination, denoted as R^2. This describes the proportion of the variance in the dependent variable that can be predicted from the independent variable. An R^2 value of >0.97 was deemed suitable for this purpose (Koch *et al.* 2018). Thus, the linear range for SIRT2 detection in cell lysate was determined as between 3.25 µg and 15 µg of total protein with a R^2 value of 0.999 (Figure 3.14). SIRT2 remained detectable even with only 0.47 µg of total protein loaded. With 30 µg of total protein loaded there was a significant reduction in the ratio of signal to protein loaded.

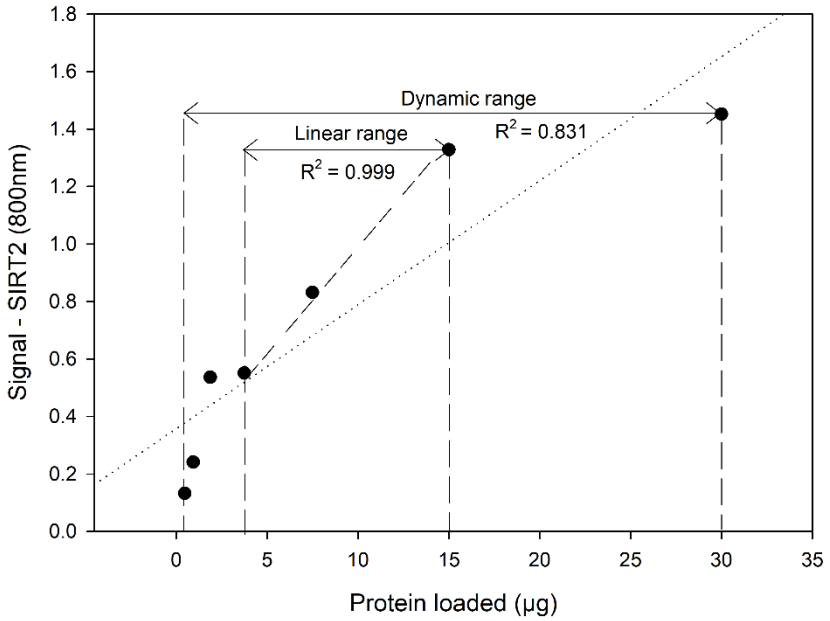


Figure 3.14. Determination of linear range of SIRT2 protein detection.

Western blot to determine linear range for detection of SIRT2. Cell lysate of THP1-derived macrophages was diluted in Laemmli loading buffer to generate a seven-point, two-fold serial dilution of total protein ranging from 30 µg to 0.47 µg. These samples were electrophoresed using SDS-PAGE and transferred to nitrocellulose membranes (section 2.5). The signal at 800 nm was then quantified for each band and this value was plotted against total protein loaded on a scatter graph. The coefficient of determination, denoted as R^2, was then calculated. This describes the range in which the measured signal correlates linearly with the amount of protein loaded into the gel. The data shown represents 1 sample for each plotted point, generated from 3 pooled cultures obtained in 1 experiment (N=1).

3.6 SIRT2 Protein Expression After Stimulation with TLR Agonists

SIRT2 protein expression in THP1-derived macrophages was analysed using quantitative western blotting. THP1-derived macrophages were stimulated with *E. coli* LPS or *B. subtilis* LTA for 4 hours or left untreated as controls, as confirmed by ELISA. Samples that had been treated with Pam2CSK4 were not included in these experiments due to the limitation of the number of lanes available for sample loading in the SDS-PAGE gel and as the behaviour of *B. subtilis* LTA and Pam2CSK4 had been comparable thus far, this was deemed acceptable.

Stimulation of THP1-derived macrophages with *E. coli* LPS or *B. subtilis* LTA for 4 hours (Figure 3.15) had no significant effect on SIRT2 protein expression compared with unstimulated controls.

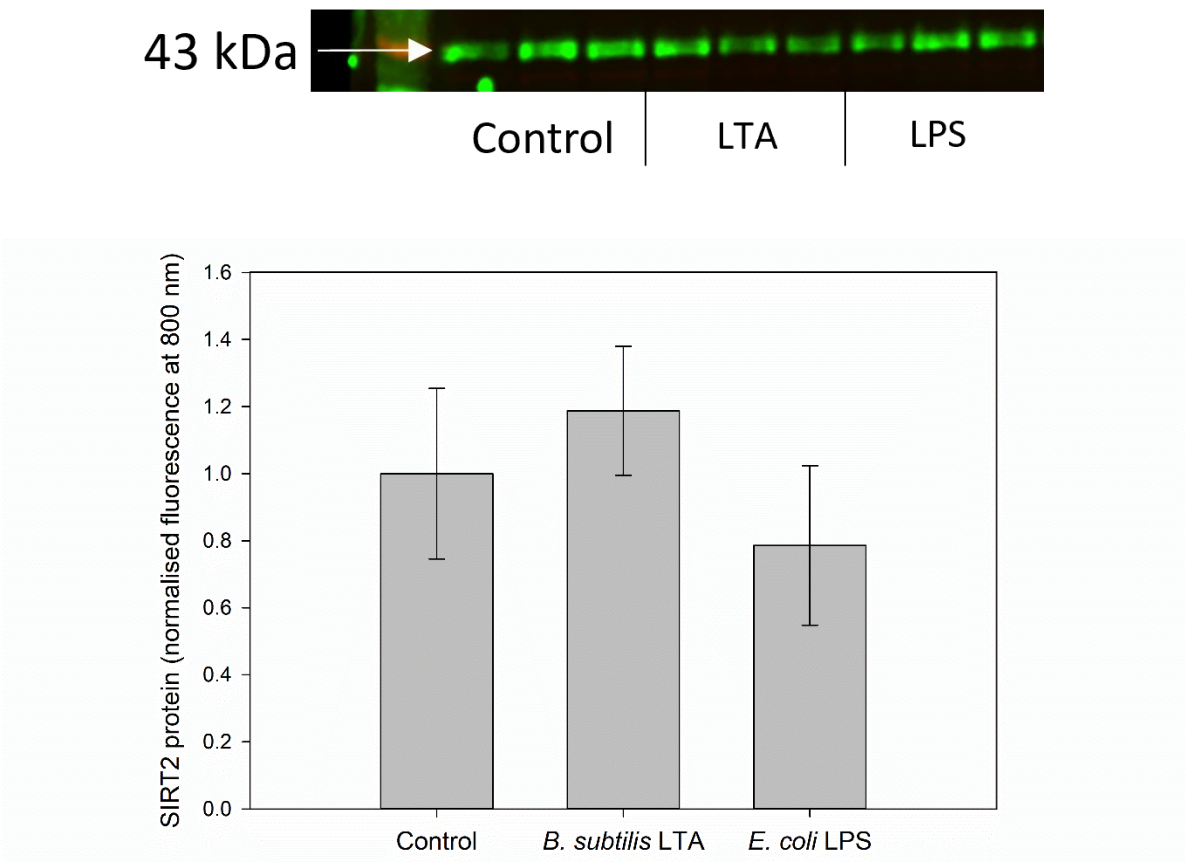


Figure 3.15. SIRT2 protein expression in macrophages stimulated with TLR agonists for 4 hours.

SIRT2 protein expression in THP1-derived macrophages stimulated with *B. subtilis* LTA (100 ng/ml) or *E. coli* LPS (100 ng/ml) for 4 hours. Cell lysates were collected before being used in SDS-PAGE and immunoblotted with a SIRT2-specific antibody. Signals were normalised against a total protein stain to correct for variations in protein loading. Analysed using one-way ANOVA.

Stimulation of THP1-derived macrophages with *E. coli* LPS or *B. subtilis* LTA for 24 hours (Figure 3.16) had no significant effect on SIRT2 protein expression compared with unstimulated controls.

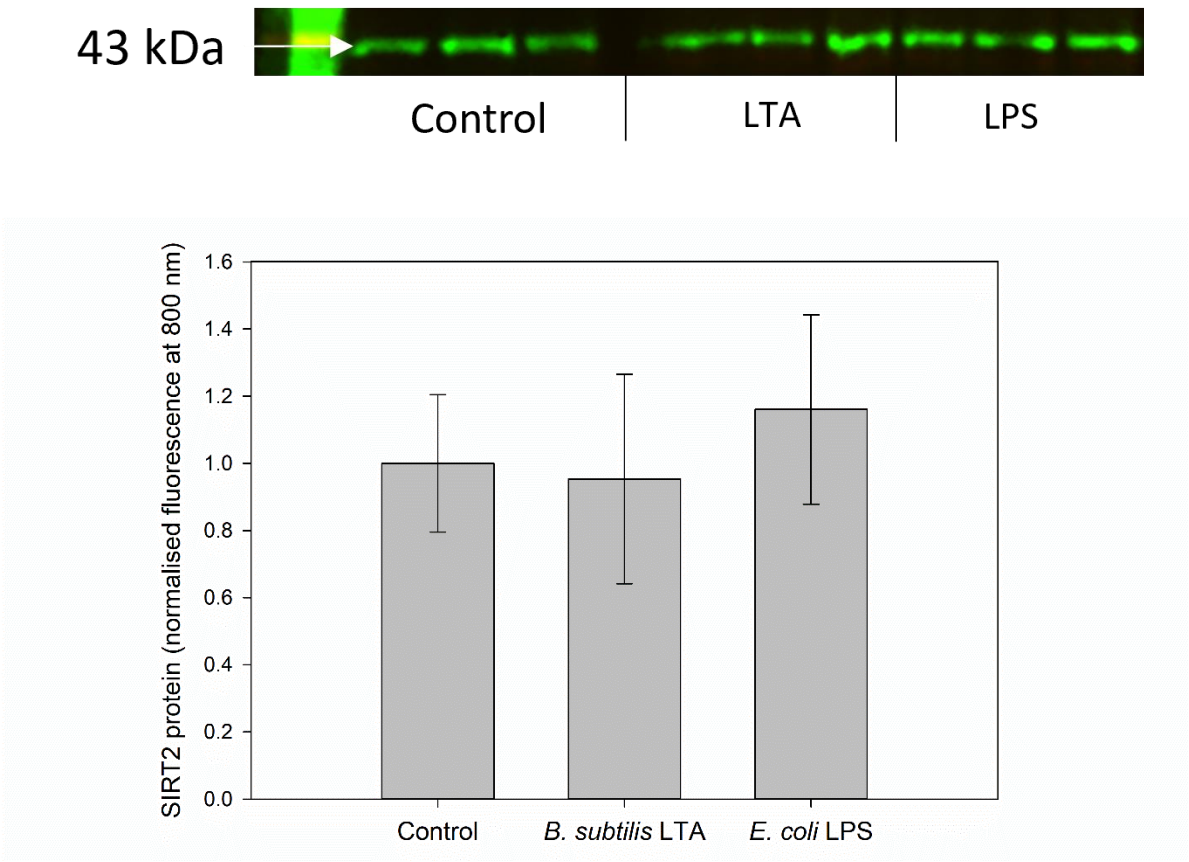


Figure 3.16. SIRT2 protein expression in macrophages stimulated with TLR agonists for 24 hours.

SIRT2 protein expression in THP1-derived macrophages stimulated with *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) for 24 hours. Cell lysates were collected before being used in SDS-PAGE and immunoblotted with a SIRT2-specific antibody. Signal (800 nm) was normalised against a total protein stain to correct for variations in protein loading. Analysed using one-way ANOVA.

From these experiments it was concluded from these experiments that SIRT2 protein expression is not affected by stimulation with *E. coli* LPS or *B. subtilis* LTA in THP1-derived macrophages. This finding was consistent across stimulation durations of 4 hours, and 24 hours.

3.7 Discussion

My first experiments aimed to determine the duration of exposure to *E. coli* LPS required to produce an increase in TNFα in the supernatant of THP1-derived macrophages at levels that would be detectable by ELISA. Experiments were carried out analysing culture supernatant samples from THP1-derived macrophages that had been stimulated for 0.5, 1 or 2 hours with *E. coli* LPS, Pam2CSK4 or *B. subtilis* LTA (Figures 3.1, 3.2 and 3.3) and 4, 8 or 24 hours (Figures 3.4, 3.5 and 3.6). There is a smaller increase to TNFα levels seen at the 24-hour time point (Figure 3.4), which may be due to endotoxin tolerance, the process by which the cellular response to an endotoxin, such as LPS, becomes diminished (Widdrington *et al.* 2018; Liu *et al.* 2019a). It is thought that this phenomenon exists as a control mechanism to prevent excess inflammation (Liu *et al.* 2019a). This response has been observed in THP1 monocytes (Widdrington *et al.* 2018) and THP1-derived macrophages (Foey and Crean 2013), although typically after repeated exposures rather than one. Whilst we cannot say with certainty that this is the explanation, MTT assays showed that there were no cytotoxic effects of LPS at 4 or 24 hours of stimulation (Appendix A), meaning that cytotoxicity can be ruled out as the cause for the lower levels of TNFα.

Once we had established the time span at which we could detect changes to TNFα in the supernatant, we tested the TLR2 agonists Pam2CSK4 and *B. subtilis* LTA at the chosen time points of 4 hours (Figure 3.7) and 24 hours (Figure 3.8). These TLR2 agonists proved to be more potent than *E. coli* LPS in their ability to stimulate TNFα secretion at these two time points.

TLR2 forms heterodimeric pairs with TLR1 or TLR6, with TLR2/1 complexes detecting triacylated peptides and TLR2/6 complexes detecting diacylated peptides. Pam2CSK4 is diacylated and as such acts upon TLR2/6 (Kang *et al.* 2009). There are conflicting reports as to which of these receptor complexes LTA acts upon. It has been reported that LTA acts upon TLR2, with partial dependency on both TLR1, TLR6 and CD14 (Cot *et al.* 2011). Others have reported LTA acts upon TLR2/6 with the required involvement of CD36 (Long *et al.* 2009). Due to LTA being diacylated (Long *et al.* 2009), it seems that TLR2/6 is likely the main receptor complex involved in detection.

Within the literature there are numerous studies that utilise knockout (Lin *et al.* 2013b; Lo Sasso *et al.* 2014) or knockdown (Pereira *et al.* 2018) of SIRT2 to investigate its role, but to my knowledge there are currently no published studies that have looked at how SIRT2 mRNA expression is affected by the TLR signalling pathways. In a mouse model of *M. tuberculosis* infection there was an increase in the expression of SIRT2 was observed in peritoneal macrophages, but this study did not investigate if TLR activation was involved (Bhaskar *et al.* 2020). Changes to SIRT2 mRNA expression have been observed in patients with active rheumatoid arthritis and whilst there is no TLR involvement (Kara *et al.* 2017), it raises the question if there is some other component of the inflammatory response that is responsible for these changes. This question may provide an avenue of research that could be pursued in the future.

We ensured the robustness of western blotting by first determining the linear range of detection where the band intensity of the target protein scales linearly with the amount of total protein loaded (Biosciences 2015; Pillai-Kastoori *et al.* 2020). We used fluorescently labelled secondary antibodies which offer advantages over the more traditional chemiluminescence method of detection as the fluorescent signal is stable and not affected by enzyme/substrate kinetics in the way that chemiluminescent detection is (Ghosh *et al.* 2014). The fluorescent signal from the secondary antibody is normalised against a fluorescent total protein stain, as this is more resistant to variability than a normalising against a single housekeeping protein (Eaton *et al.* 2013; Biosciences 2015; Kirshner and Gibbs 2018). A digital imager was used to detect the fluorescent signals, providing higher sensitivity than traditional film-based methods of visualisation (Khoury *et al.* 2010; Biosciences 2015).

Using western blotting, we observed no change to SIRT2 protein expression after 4 or 24 hours of stimulation with *E. coli* LPS or *B. subtilis* LTA (Figures 3.15 and 3.16). Samples that had been treated with Pam2CSK4 were not included in these experiments due to the limitation of the number of lanes available for sample loading in the SDS-PAGE gel and as the behaviour of *B. subtilis* LTA and Pam2CSK4 had been comparable thus far, this was deemed acceptable. There is little published research looking at the effect TLR activation has on SIRT2 protein expression, but one study did find that 2 days after injection of LPS, SIRT2 protein expression was significantly reduced in the cerebral cortex of mice (Pais *et al.* 2013). Whilst changes in SIRT2 protein expression after stimulation with LPS was not detected, the findings of Pais *et al.* are not

necessarily inconsistent due to the differences between the live mouse model they utilised and the human macrophage model.

There are significant differences between human and murine models of disease. This extends beyond the fact that human models of disease will typically be *in vitro* and utilise only one cell type, whilst murine models of disease able to assess a whole organism (Guvva *et al.* 2017). In addition to this, one study found significant differences in gene expression when comparing multiple human and murine tissues types. In fact, greater gene expression similarities were observed between all examined tissue types in one species than that observed when comparing the same tissue type of humans and mice (Lin *et al.* 2014). There are also differences between the human murine immune system, including B cells, T cells and TLRs, with an absence of TLR7/8 and TLR4 responses in mouse classical monocytes (Mestas and Hughes 2004; Bjornson-Hooper *et al.* 2019). More broadly, a systematic review conducted by Leenaars *et al.* reviewed 121 published studies to evaluate the range of animal-to-human translational success rates. The reported translational success rates ranged between 0% and 100%. As these 121 studies included a wide array of research areas, such as sepsis, oncology, drug toxicity and pharmacokinetics studies, it is perhaps not surprising that the range of success rates is so large. Additionally, the included studies utilised numerous different animal species, which may further increase the range of success rates. Whilst the authors also acknowledge that this systematic review has many limitations due to the quality and quantity of currently published, it still provides an interesting overview of animal-to-human translation success rates (Leenaars *et al.* 2019). Ultimately, these differences do not mean that mouse models of disease should be avoided, but any subsequent findings should not have their translational significance overstated.

The THP1-derived macrophages used in my experiments do not provide a perfect representation of macrophages derived from primary monocytes, but nonetheless this model is generally considered to be a useful tool in translational research. THP1-derived macrophages provide many benefits over their counterparts derived from primary monocytes. They provide a more consistent model for experimentation due to their origin in one individual (Bosshart and Heinzelmann 2016) and they are easily obtainable, particularly when greater quantities are needed (Forrester *et al.* 2018). There are however some downsides to the use of THP1-derived macrophages. Firstly, the process of differentiation from monocyte to macrophage is sensitive to

culture condition such as confluency (Aldo *et al.* 2013), as well as the duration of the differentiation (Daigneault *et al.* 2010). It has been observed that THP1-derived macrophages display lower phagocytic activity (Tedesco *et al.* 2018), and numerous differences have been observed when comparing the surface markers present on THP1-derived macrophages against those on primary monocyte-derived macrophages (Forrester *et al.* 2018; Tedesco *et al.* 2018). PMA treatment of THP1 monocytes induces expression of several genes which expressed by macrophages derived from primary monocytes, but not monocytes themselves. This includes apolipoprotein-E, MMP9 and α2 macroglobulin (Kohro *et al.* 2004). Although it should be noted that conversely, IL-1β gene expression was decreased. In a study investigating *Mycobacterium tuberculosis* infection it was found that the bacterial uptake and host response in THP1-derived macrophages and primary monocyte-derived macrophages were comparable (Madhvi *et al.* 2019), with no significant difference between mRNA expression of TNFα, IL-1β and IFNγ. Overall, THP1-derived macrophages are useful tool in research, as long as care is taken in the protocol for differentiation and their limitations are acknowledged.

Chapter 4. Secretion of SIRT2 by Macrophages

4.1 Introduction

Saliva is an excellent source of candidate biomarkers for periodontitis due to the ease with which it can be collected and because its components reflect the overall state of inflammation in the mouth. Gingival crevicular fluid (GCF), a serum exudate into the gingival crevice, has previously been investigated for many candidate biomarkers for periodontitis due to its high protein concentration, however, biomarker levels in GCF are typically only representative of disease activity at that specific site, meaning GCF would have to be sampled from multiple sites in order to accurately represent overall disease activity (Yoshizawa *et al.* 2013; Taylor 2014). Additionally, the molecular content of the GCF can enter the saliva where it can be detected (Tsuchida *et al.* 2012). Due to our preliminary finding that SIRT2 was elevated in the saliva of patients with periodontitis (Chapter 6, Figure 6.1) we hypothesised that elevated salivary SIRT2 might reflect immune cell activity in the periodontium. Thus, we investigated if SIRT2 could be secreted by macrophages stimulated with TLR agonists, as activation of these TLR signalling pathways leads to the secretion of numerous effector proteins during immune responses to pathogens (Inoue, Niki et al. 2018, Liu, Yin et al. 2018). This would help determine if the elevated levels of SIRT2 in the saliva of patients with periodontitis could be caused by TLR activation of macrophages in the oral tissues including the periodontium. To achieve this, we utilised ELISAs to analyse SIRT2 levels in the supernatants of THP1-derived macrophages stimulated with TLR agonists. ELISA was chosen to due to its sensitivity and high throughput.

The TLR2 agonists Pam2CSK4 and *B. subtilis* LTA and the TLR4 agonist *E. coli* LPS were again utilised to model different TLR signalling pathways due to the importance of these pathways in the pathogenesis of periodontitis (Cekici *et al.* 2014a).

The primary TLR4 agonist of relevance in periodontitis is LPS derived from Gram-negative bacteria, such as *P. gingivalis* and *T. forsythia.* Common TLR2 agonists relevant in periodontitis include LTA and peptidoglycan. LTA is found in the cell wall of Gram-positive bacteria, such as *Staphylococcus aureus* (Fritschi *et al.* 2008) and *Fusobacterium nucleatum* (Han *et al.* 2005), whilst peptidoglycan is found in both Gram-negative and Gram-positive bacteria. Whilst the selected TLR4 agonist, *E. coli* LPS and the selected TLR2 agonists, Pam2CSK4 and *B. subtilis* LTA, are not

derived from sources relevant to periodontitis they still allow for investigation into the TLR2 and TLR4 pathways that are highly relevant to periodontitis.

Macrophage activation leads to the production of pro-inflammatory cytokines and mediators (Mosser and Edwards 2008; El-Zayat *et al.* 2019). TLR2 and TLR4 have been found to be particularly important in periodontitis, initiating the immune response upon detection of bacterial components derived from biofilms on the teeth (Mahanonda and Pichyangkul 2007; Cekici *et al.* 2014a). It is well established that activation of TLR2 and TLR4 signalling pathways cause secretion of TNFα in THP1-derived macrophages (Foster *et al.* 2005), and the sensitivity of macrophages to TLR agonists makes them an ideal cell type for investigations into TLR signalling (Kawasaki and Kawai 2014). Macrophages are involved in the activation of the adaptive immune system and act to sustain the inflammation seen in periodontitis (Sima *et al.* 2018) and there is also evidence suggesting that there is disruption to the homeostasis of macrophage populations in areas of the mouth affected by periodontitis (Almubarak *et al.* 2020).

Previous findings in the literature show that visfatin, the same enzyme that is involved in intracellular NAD production, is found extracellularly and is elevated in the plasma and synovial fluid of patients with rheumatoid arthritis (Nowell *et al.* 2006; Otero *et al.* 2006), and has been linked to macrophage polarisation (Zhang *et al.* 2018a).

I next aimed to determine if SIRT2 might also be present extracellularly, and the effect TLR signalling may have on any extracellular SIRT2, as a potential pathway that may explain the elevated levels of SIRT2 identified in the saliva of patients in our preliminary data. To do this, SIRT2 ELISAs were used to analyse the supernatants of THP1-derived macrophages after stimulation with LPS, LTA or Pam2CSK4. TLR inhibitors were then used to ensure specificity of the TLR agonists against their corresponding receptors.

4.2 SIRT2 Secretion in Response to Stimulation with TLR agonists

As preliminary data showed that SIRT2 was elevated in the saliva of patients with periodontitis, the culture supernatants of THP1-derived macrophages were analysed using ELISA to determine if SIRT2 was present. THP1-derived macrophages were stimulated with the TLR 4 agonist *E. coli* LPS, or the TLR2 agonists Pam2CSK4 and *B. subtilis* LTA for 4 hours and 24 hours. Successful

stimulation of macrophages was confirmed in every culture experiment by TNFα ELISA as in the previous chapter (Chapter 1, Figures 1.7 and 1.8).

After 4 hours of stimulation, ELISA for TNFα confirmed statistically significant increases in the *E. coli* LPS, Pam2CSK4 and *B. subtilis* LTA treated samples (see appendix B). ELISA for SIRT2 (Figure 4.1) showed there was no SIRT2 detectable in the supernatants of the control or *E. coli* LPS-stimulated samples. There was a statistically significant increase in SIRT2 in the supernatants of the Pam2CSK4- and *B. subtilis* LTA-stimulated samples ($p<0.001$) when compared with controls.

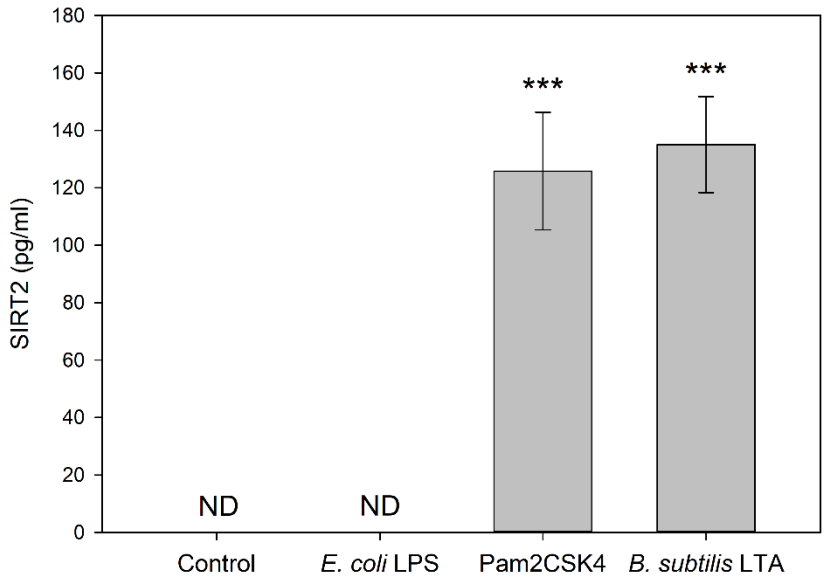


Figure 4.1. SIRT2 secretion from macrophages stimulated with TLR agonists for 4 hours.

SIRT2 measured by ELISA in supernatant of THP1-derived macrophages stimulated for 4 hours with *E. coli* LPS (100 ng/ml), Pam2CSK4 (10 ng/ml) or *B. subtilis* LTA (100 ng/ml. Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). *** $p<0.001$, calculated by one-way ANOVA, followed by Tukey's post-hoc test. ND = no data.

After 24 hours of stimulation (Figure 4.2), levels of SIRT2 were detectable in the control and *E. coli* LPS-stimulated samples. Once again, there was a statistically significant increase in SIRT2 in the Pam2CSK4 and *B. subtilis* LTA-stimulated samples ($p<0.001$) when compared with controls.

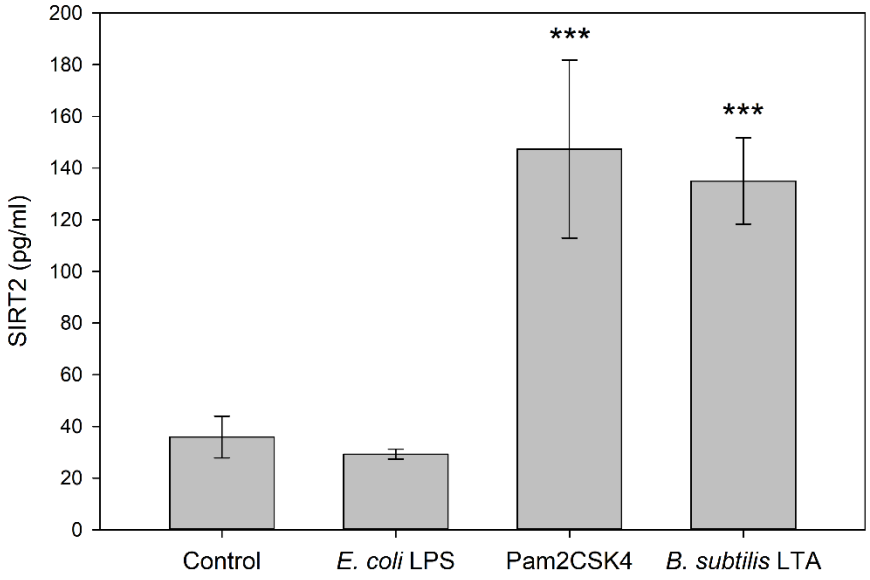


Figure 4.2. SIRT2 secretion from macrophages stimulated with TLR agonists for 24 hours.

SIRT2 measured by ELISA in supernatant of THP1-derived macrophages stimulated for 24 hours with *E. coli* LPS (100 ng/ml), Pam2CSK4 (10 ng/ml) or *B. subtilis* LTA (100 ng/ml. Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). *** $p<0.001$, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

In these experiments it was demonstrated that the stimulation of THP1-derived macrophages with TLR2 agonists leads to a significant increase of SIRT2 levels in the supernatant. The next experiments aimed to confirm that TLR2 signalling was responsible for this effect.

4.3 SIRT2 Secretion in the Presence of TLR2 Inhibitors

Specific small molecule TLR inhibitors were used to determine if the secretion of SIRT2 from THP1-derived macrophages was indeed due to intra-cellular signalling by TLR2 agonists via the canonical TLR2 pathway. First, due to similarities between the subsequent signalling pathways downstream of TLR2 and TLR4 activation, a TLR4 inhibitor was used to ensure there were no off-target effects from the chosen preparations TLR2 agonists. LPS contamination of preparations of other bacterial macromolecules has been known to occur and can result in erroneous data and conclusions (Nerurkar *et al.* 2005; Yang *et al.* 2006; Schwarz *et al.* 2014)

To confirm the selected TLR inhibitors were specifically preventing activation of their corresponding TLRs, THP1-derived macrophages were pre-treated with the TLR4 inhibitor, C34, or left in standard growth medium for 30 minutes before removal of the growth medium and addition of media containing *E. coli* LPS, *B. subtilis* LTA or Pam2CSK4 (see Figure 4.3 for experimental timeline). Controls were left untreated (Figure 4.4).

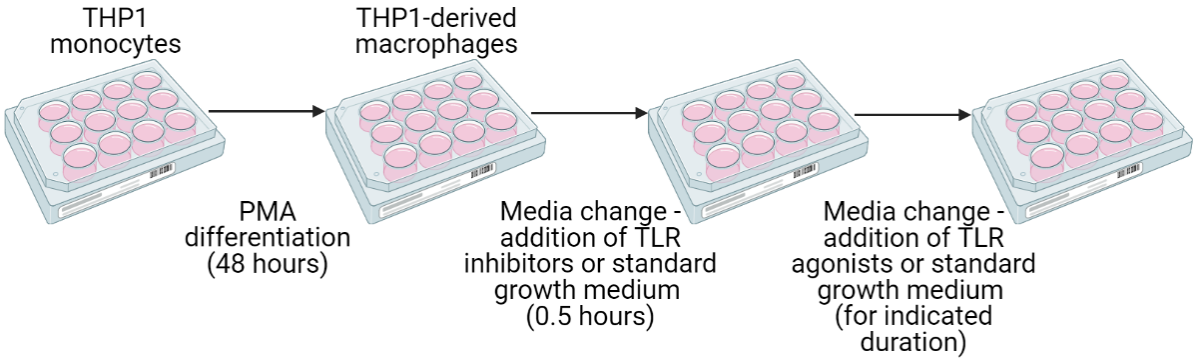


Figure 4.3. Method for differentiation of THP1 monocytes, treated with TLR inhibitors and addition of TLR agonists.

THP1 monocytes were treated with PMA for 48 hours in order to induce differentiation into macrophages. These THP1-derived macrophages then had the growth medium changed to either standard growth media, growth media with the addition of TLR of the described TLR inhibitor for 0.5 hours. The growth media was then changed once more to media containing the described TLR agonist or standard growth media for controls.

Stimulation of macrophages with *E. coli* LPS, *B. subtilis* LTA and Pam2CSK4 (Figure 4.4) resulted in a statistically significant increase in TNFα secretion compared with unstimulated controls ($p<0.001$). In the samples stimulated with *E. coli* LPS, pre-treatment with C34 lead to significantly lower TNFα secretion between the two groups ($p<0.001$). In the samples stimulated with *B. subtilis* LTA and Pam2CSK4, pre-treatment with C34 had no effect on TNFα secretion, confirming that there is no non-specific inhibition of the TLR2 pathway from the C34 TLR4 inhibitor. Additionally, treatment of macrophages with C34 alone had no effect on TNFα secretion compared with controls.

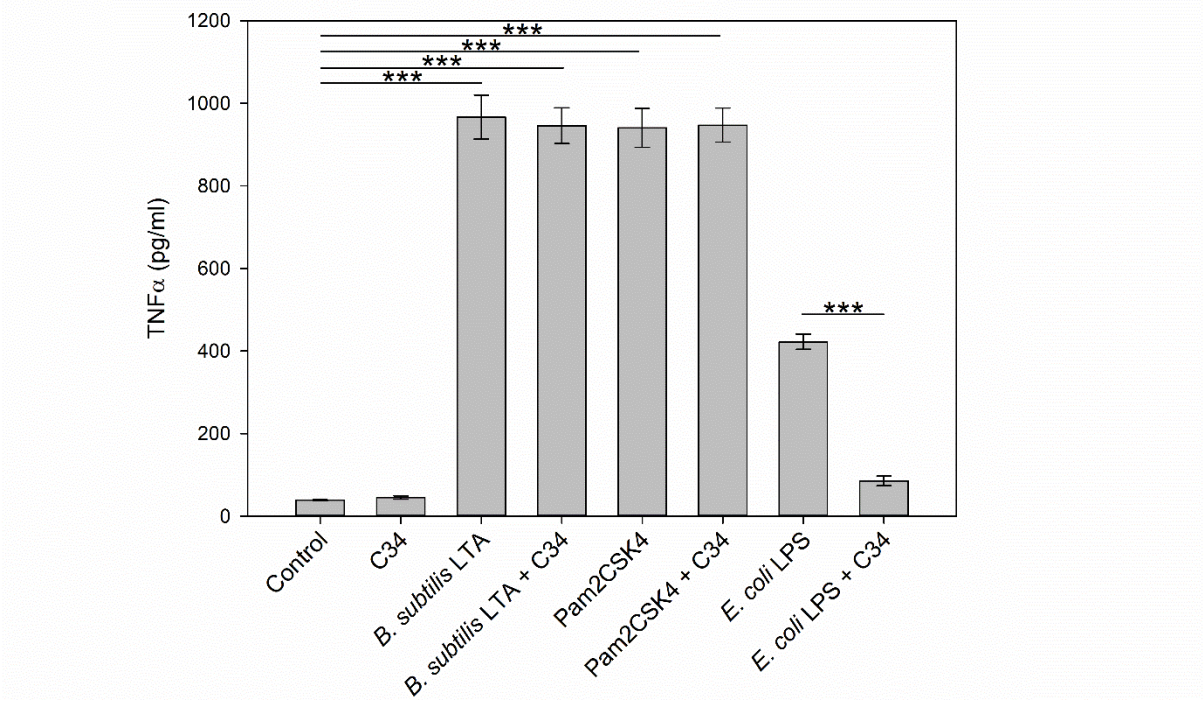


Figure 4.4. TNFα secretion from macrophages stimulated with TLR agonists for 4 hours with or without treatment with a TLR4 inhibitor.

TNFα measured by ELISA in supernatant of THP1-derived macrophages stimulated for 4 hours with *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml.), with or without the TLR4 inhibitor C34 (10 µM pre-treatment for 0.5 hours). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). *** $p<0.001$, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

Next, THP1-derived macrophages were pre-treated with the TLR2 inhibitor, C29, or left in standard growth medium for 30 minutes before removal of the growth medium and addition of media containing *E. coli* LPS, *B. subtilis* LTA or Pam2CSK4 (see Figure 4.3 for experimental timeline). Controls were left untreated (Figure 4.5). *E. coli* LPS was included in these experiments to ensure the specificity of the chosen TLR2 inhibitor. Stimulation of macrophages with *B. subtilis* LTA and Pam2CSK4 resulted in a statistically significant increase in TNFα secretion compared with unstimulated controls ($p<0.001$). However, in samples pre-treated with C29, the secreted TNFα levels were significantly lower than in the samples without pre-treatment ($p<0.001$). Treatment of macrophages with C29 alone had no effect on TNFα secretion compared with controls.

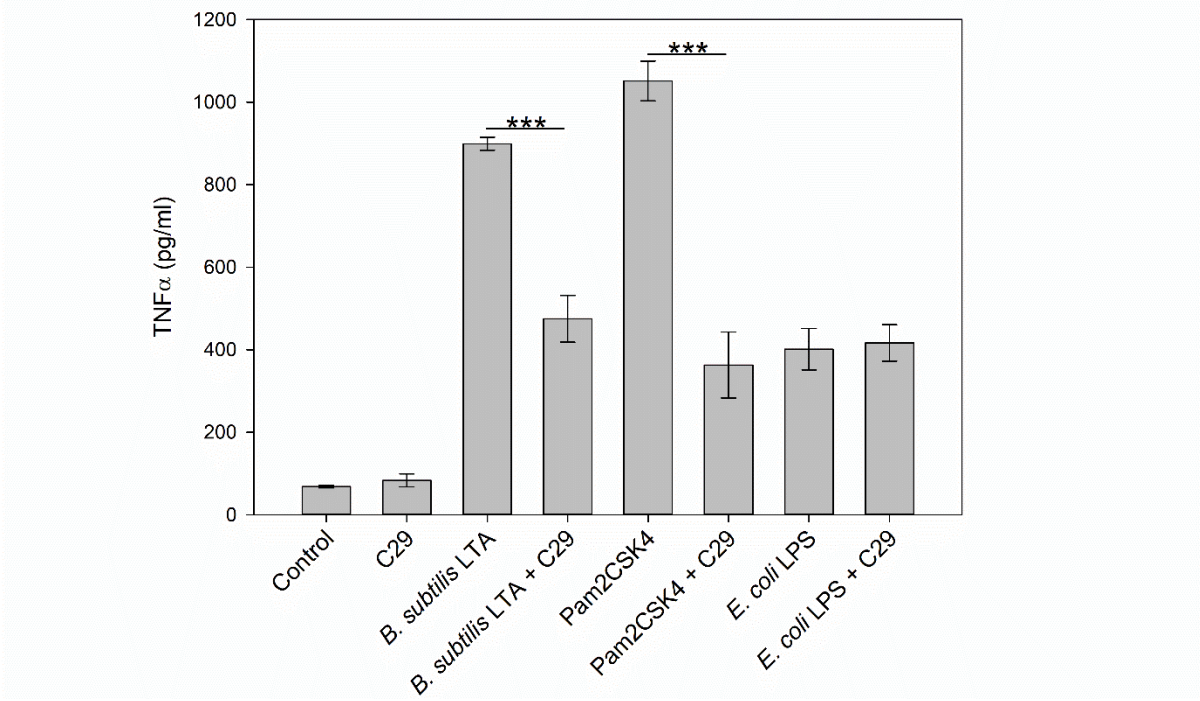


Figure 4.5. TNFα secretion from macrophages stimulated with TLR agonists for 4 hours with or without treatment with a TLR2 inhibitor.

TNFα measured by ELISA in supernatant of THP1-derived macrophages stimulated for 4 hours with *B. subtilis* LTA (100 ng/ml.) or Pam2CSK4 (10 ng/ml), with or without the TLR2 inhibitor C29 (50 µM pre-treatment for 0.5 hours). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). *** $p<0.001$, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

MTT assays were used to determine if the selected TLR inhibitors had any effect on cell viability. As before, THP1-derived macrophages were treated with TLR inhibitors before being incubated with MTT to produce formazan crystals which were then dissolved, and absorbance is measured. By comparing this to a standard curve generated by culturing varying numbers of cells and incubating them with MTT (see Methods 2.7). From this, the number of viable cells could be calculated. Treatment with the TLR4 inhibitor, C34, and the TLR2 inhibitor, C29, for 0.5 hours, in concordance with experimental conditions, had no significant effect on cell viability compared to controls (Figure 4.6). This allowed for the next experiments to take place, utilising these TLR inhibitors to determine if the secretion of SIRT2 could be abrogated.

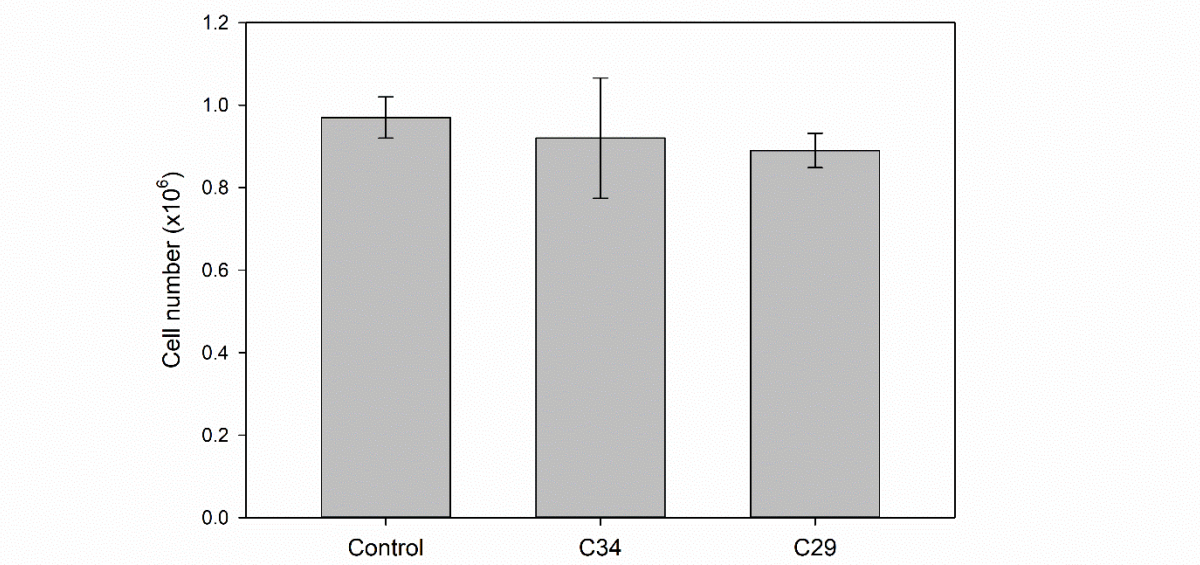


Figure 4.6. Cell number determined by MTT assay with macrophages stimulated with TLR inhibitors for 0.5 hours.

THP1-derived macrophages stimulated for 0.5 hours with the TLR4 inhibitor C34 (10 µM pre-treatment for 0.5 hours) or the TLR2 inhibitor C29 (50 µM pre-treatment for 0.5 hours). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). Analysed using one-way ANOVA.

ELISAs were used to measure SIRT2 in the culture supernatant of THP1-derived macrophages that had been treated with the TLR agonists, *E. coli* LPS, *B. subtilis* LTA and Pam2CSK4 with and without the addition of the TLR2 inhibitor C29 (Figure 4.7). No SIRT2 was detected in the supernatant of the control, C29, *E. coli* LPS or *E. coli* LPS+C29 treated macrophages. The supernatant from *B. subtilis* LTA and Pam2CSK4-treated macrophages had significantly elevated SIRT2 levels compared to controls ($p<0.001$ for both). Pre-treatment of macrophages with C29 resulted in a statistically significantly lower levels of SIRT2 to the levels of SIRT2 following stimulation *B. subtilis* LTA and Pam2CSK4 as compared to cultures without inhibitor ($p<0.01$).

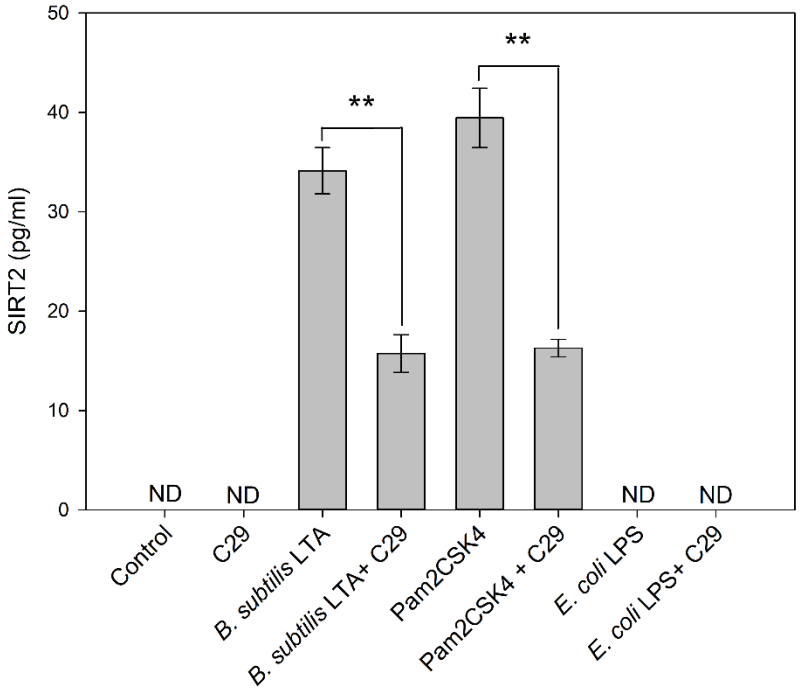


Figure 4.7. SIRT2 secretion from macrophages stimulated with TLR agonists for 4 hours with or without treatment with a TLR2 inhibitor.

SIRT2 measured by ELISA in supernatant of THP1-derived macrophages stimulated for 4 hours with *E. coli* LPS (100 ng/ml), Pam2CSK4 (10 ng/ml) or *B. subtilis* LTA (100 ng/ml), with or without the TLR2 inhibitor C29 (50 µM pre-treatment for 0.5 hours). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). ** $p<0.01$, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

ELISAs were then used to measure SIRT2 in the culture supernatant of THP1-derived macrophages that had been treated with the TLR agonists, *E. coli* LPS, *B. subtilis* LTA and Pam2CSK4 with and without the addition of the TLR4 inhibitor C34 (Figure 4.8). SIRT2 was detected in samples stimulated with LTA and Pam2CSK4. Addition of C34 had no effect on SIRT2 levels. No SIRT2 was detected in the supernatant of control or LPS-stimulated macrophages.

These experiments confirmed that stimulation of THP1-derived macrophages with TLR2 agonists results in increased levels of SIRT2 in the supernatant and that the release of SIRT2 into the supernatant was dependent on TLR2 signalling pathways.

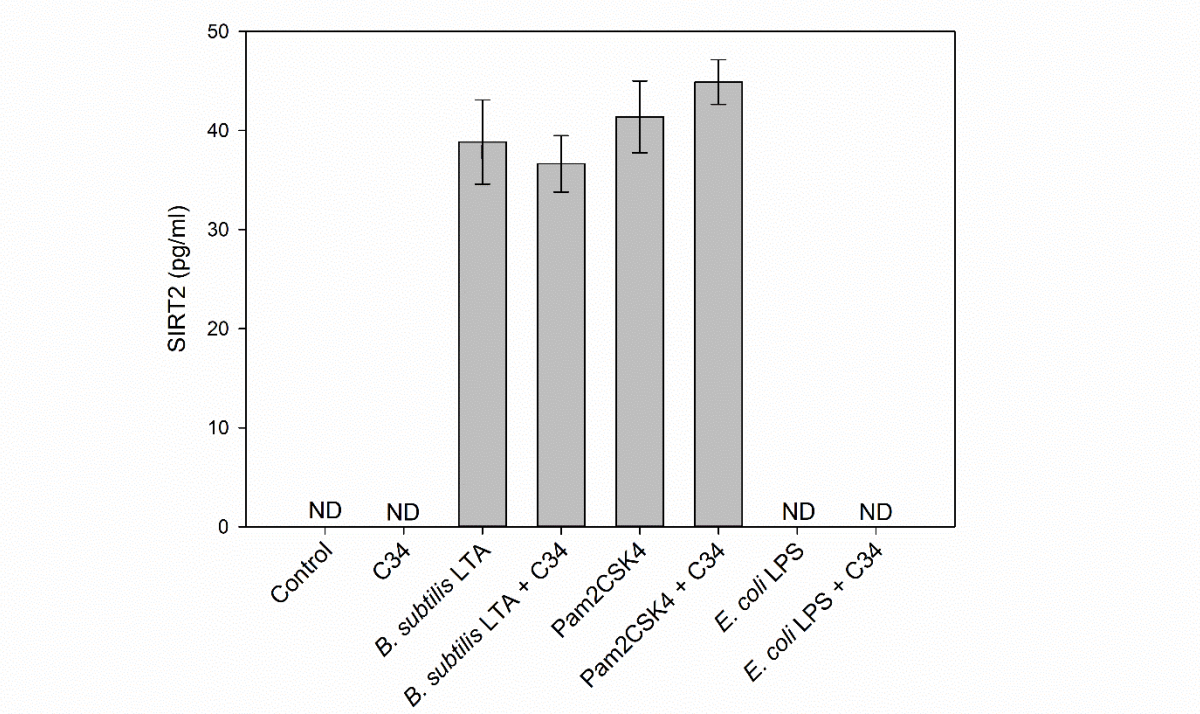


Figure 4.8. SIRT2 secretion from macrophages stimulated with TLR agonists for 4 hours with or without treatment with a TLR4 inhibitor.

SIRT2 measured by ELISA in supernatant of THP1-derived macrophages stimulated for 4 hours with *E. coli* LPS (100 ng/ml), Pam2CSK4 (10 ng/ml) or *B. subtilis* LTA (100 ng/ml), with or without the TLR4 inhibitor C34 (10 µM pre-treatment for 0.5 hours). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). Analysed using one-way ANOVA.

4.4 Discussion

The first experiments shown here (Figure 4.1 and Figure 4.2) aimed to measure SIRT2 in the supernatant of THP1-derived macrophages after stimulation with TLR agonists and in unstimulated controls. After 4 hours of stimulation with TLR2 and TLR4 agonists, there was a statistically significant increase to SIRT2 levels present in the supernatant of *B. subtilis* LTA- and Pam2CSK4-stimulated macrophages, whilst no change was observed to the levels of SIRT2 in the supernatant of *E. coli* LPS-stimulated macrophages (Figure 4.1). The same effect was seen after 24 hours of stimulation with TLR2 and TLR4 agonists, a statistically significant increase in the levels of SIRT2 present in the supernatant of *B. subtilis* LTA- and Pam2CSK4-stimulated macrophages, with no change was observed to the levels of SIRT2 in the supernatant of *E. coli* LPS-stimulated macrophages (Figure 4.2).

The secretion of SIRT2 by macrophages is a novel finding, with no published information on any extracellular function of SIRT2, and no published studies have investigated this. Thus, there is no published information on any potential extracellular targets of SIRT2. SIRT2 is known to be largely located within the cytosol where it acts to deacetylate proteins including α-tubulin, transcription factor FOXO1 and NF-κB p65, it also translocates to the nucleus during mitosis (Dryden *et al.* 2003; Wang *et al.* 2019). There is evidence of involvement of SIRT2 in inhibiting the loading and release of extracellular vesicles, with knockout of SIRT2 leading to increased release of extracellular vesicles (Lee *et al.* 2019), which are important in immune signalling and inflammation by transporting micro RNAs, cytokines and chemokines into the extracellular environment (Buzas *et al.* 2014; Andres *et al.* 2020).

SIRT2 has been found to be elevated in the serum of patients with rheumatoid arthritis who were also suffering from periodontal disease compared to those who were periodontally healthy (Panezai *et al.* 2020), but there was no further investigation into its function or how it came to be present in the serum. Additionally, the enzymatic activity of SIRT2 is NAD-dependent and there is some evidence that NAD is present in the extracellular space (Koch-Nolte *et al.* 2011; Adriouch *et al.* 2012) suggesting that SIRT2 in these compartments may be biologically active, providing there are sufficient levels of this co-factor. There is no published information on NAD levels present in saliva, but levels in the plasma can range from levels below 1 μM up to 10 μM (Kulikova *et al.* 2019), and as the GCF is a serum exudate that has passed through blood vessel walls into the

crevicular space (Taylor 2014; Taylor and Preshaw 2016; Bostanci and Belibasakis 2018), levels of NAD present in the GCF may be similar to that found in the plasma. The GCF can then mix with the saliva and can be the source of certain molecules that may be detected (Tsuchida *et al.* 2012). There is also evidence obtained from experiments in mice that NAD is elevated during acute inflammation (Adriouch *et al.* 2007). Again, there is little published information regarding the NAD levels required for SIRT2 function, however, in vitro 100 nM NAD is sufficient to activate deacetylation activity in SIRT2 (Nielsen *et al.* 2021).

The enzyme nicotinamide phosphoribosyltransferase (NAMPT) is responsible for NAD production intracellularly but can also be found extracellularly where it acts as an adipokine and plays a role in pre–B cell colony formation (Brentano *et al.* 2007; Park *et al.* 2017). Extracellular NAMPT (also known as visfatin) is contained within extracellular vesicles and is internalised into cells whereupon it increases NAD synthesis (Yoshida *et al.* 2019a).

Visfatin levels are elevated in the plasma and synovial fluid of patients with rheumatoid arthritis (Nowell *et al.* 2006; Otero *et al.* 2006). *In vitro* experiments have shown levels of IL-6 and TNFα are elevated in the culture supernatants of human primary monocytes after stimulation with visfatin (Brentano *et al.* 2007), and levels of IL-1β, IL-1Ra, IL-6, IL-10, and TNFα were found to be elevated in the supernatants of peripheral blood mononuclear cells (PBMCs) (Moschen *et al.* 2007). Additionally, there is a report the stimulation of THP1-derived macrophages with visfatin induced production of MMP-9 (Fan *et al.* 2011), one of the most abundant MMPs in periodontal tissue (Franco *et al.* 2017b). Visfatin was also found to increase CD36 expression and increase the phagocytic activity of THP1-derived macrophages (Yun *et al.* 2014).

Published data also shows mRNA and protein expression of NAMPT, the intracellular form of visfatin, is upregulated in monocytes after stimulation with LPS (Schilling *et al.* 2012). There is evidence that visfatin levels are elevated in the gingival crevicular fluid and saliva of patients with periodontitis and gingivitis (Tabari *et al.* 2014; Ozcan *et al.* 2015; Türer *et al.* 2016) suggesting perhaps that visfatin contributes in some way to the inflammation seen in periodontitis, which may impact NAD levels and subsequently SIRT2 deacetylation activity. Intracellularly, SIRT2 is known to regulate NF-κB-dependent gene expression through deacetylation of p65 K310 (Rothgiesser *et al.* 2010a) and ameliorate inflammation in collagen-induced arthritis in mice (Lin

et al. 2013a) and after LPS-stimulation of macrophages (Lee *et al.* 2014a), potentially explaining one way in which visfatin levels contribute to inflammation.

We also determined that secretion of SIRT2 from macrophages is mediated by TLR2 signalling. The use of TLR2 and TLR4 inhibitors confirmed that the secretion of SIRT2 is mediated by TLR2, with no involvement of TLR4. This use of inhibitors demonstrated the specificity of the TLR2 and TLR4 agonists to act upon their intended receptors (Figures 4.4 and 4.5). As previously mentioned, LPS contamination has been known to occur and can result in erroneous data and conclusions (Nerurkar *et al.* 2005; Schwarz *et al.* 2014). By stimulating macrophages that have been pre-treated with a TLR4 inhibitor with TLR2 agonists (Figure 4.4), we have shown that if there was any low-level LPS contamination on the selected agonists, it had no significant effect on my findings. Additionally, lipopeptide contamination has led to erroneous findings, most notably the controversial report of *P. gingivalis* LPS acting on TLR2, with more recent findings suggesting that in reality *P. gingivalis* LPS only acts on TLR4 and activation of TLR2 was due to contaminating lipopeptides (Nativel *et al.* 2017).

It is interesting that the secretion of SIRT2 into the supernatant was only observed in macrophages stimulated with TLR2 agonists and not those stimulated with TLR4 agonists, as the signalling pathways involved share many similarities (see Figure 4.9). Activation of TLR2 in both of its heterodimeric forms, TLR2/1 and TLR2/6, leads to activation of the signalling protein myeloid differentiation primary response 88 (MyD88). Activation of TLR4 also activates MyD88, along with TIR-domain-containing adapter-inducing interferon-β (TRIF). Ultimately both pathways can lead to activation of transcription factors NF-κB and interferon regulatory factors (IRFs) and transcription of numerous genes controlling expression of cytokines and chemokines (Liu *et al.* 2017c; Song *et al.* 2017). It would be interesting to repeat this experiment and utilise TRIF inhibitors to further interrogate the pathways involved. Perhaps the activation of TRIF signalling in conjunction with MyD88 signalling somehow prevents secretion of SIRT2.

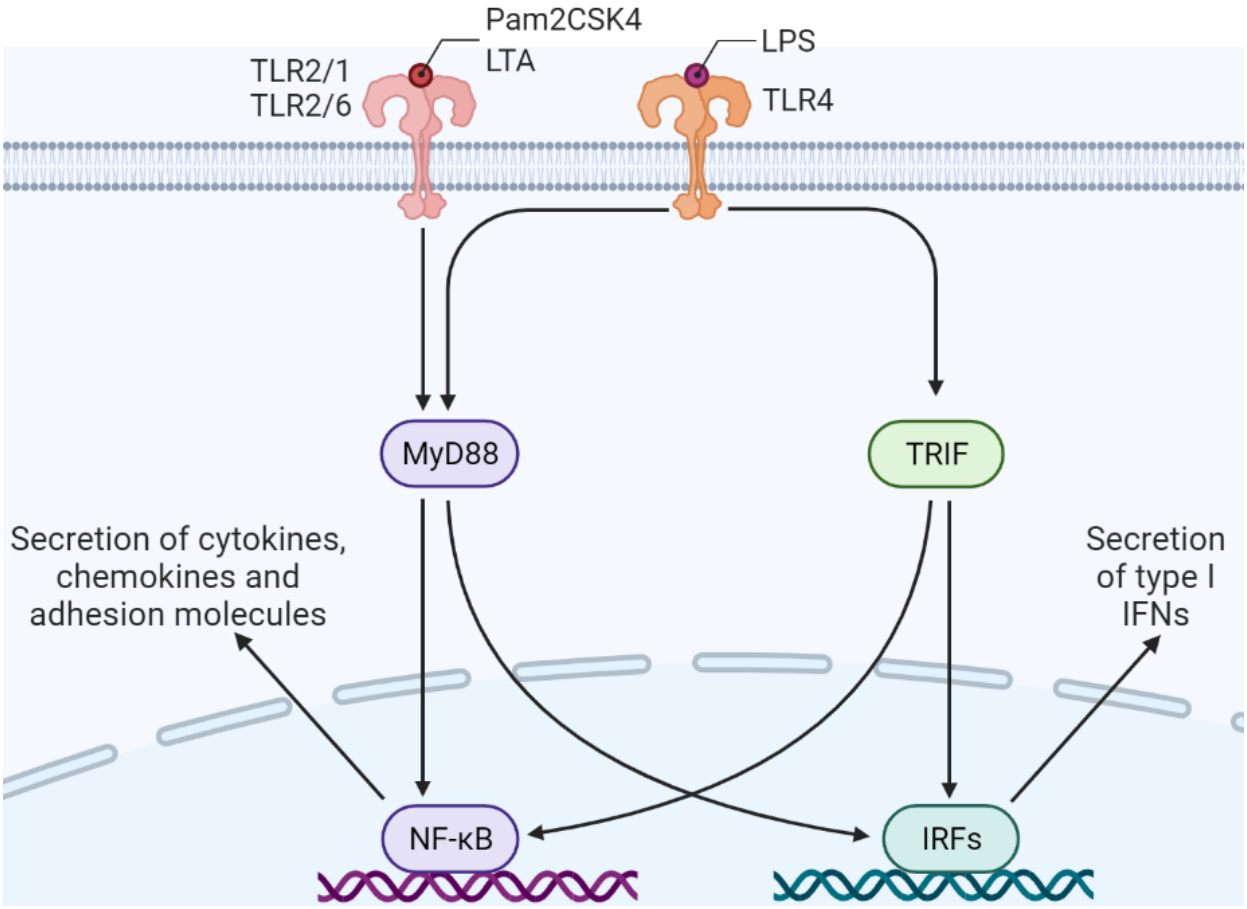


Figure 4.9. TLR2 and TLR4 signalling pathways.

Activation of TLR2/1 and TLR2/6 subsequently activates MyD88, leading to activation of NF-κB. Activation of TLR4 activates both MyD88 and TRIF signalling pathways. Both pathways can lead to activation of transcription factors NF-κB and interferon regulatory factors (IRFs) and transcription of numerous genes controlling expression of cytokines such as IL-1, IL-6, TNFα, and type I interferons (IFNs), chemokines and adhesion molecules (Liu *et al.* 2017c).

We previously utilised MTT assays to determine if there was any toxic effect associated with stimulation with TLR2 agonists (Results chapter 1, figures 9 and 10), so it was clear that the finding of increased SIRT2 in the supernatant of macrophages stimulated with TLR2 agonists was not due to cytotoxicity.

A limitation of this study arises from our preliminary analysis of saliva samples. As previously mentioned, the screening was limited to 15 saliva samples from patients with periodontitis and 15 saliva samples from healthy controls, numbers which clearly have limited statistical power. Also, the screening that identified SIRT2 as being elevated in the saliva of patients with periodontitis was relativistic, meaning the levels of SIRT2 were elevated when compared against the levels in saliva from healthy volunteers, with no absolute quantity being determined. This means it is currently unclear if secretion of SIRT2 from macrophages alone would explain the elevated levels of SIRT2 seen in the saliva of patients with periodontitis. These concerns are addressed in subsequent experiments relating to the quantitative analysis SIRT2 is presented in the Chapter 6.

Chapter 5. Regulation of Cytokine Secretion by SIRT2 in Macrophages

5.1 Introduction

SIRT2 is a NAD-dependent HDAC that removes acetyl groups from lysine residues within proteins (Vaquero *et al.* 2006). SIRT2 has numerous protein targets and SIRT2 deacetylase activity can be determined by measuring the acetylation of one of these targets. As the targets of SIRT2 deacetylase activity are numerous and varied, changes to the acetylation state of a protein can have significant biological consequences, with SIRT2 having been demonstrated to be involved in the regulation of phagocytosis (Ciarlo *et al.* 2017), the expression of iNOS and ROS (Lee *et al.* 2014b), as well as NF-κB-dependent gene expression (Rothgiesser *et al.* 2010b).

Notable targets of SIRT2 include the transcription factors NF-κB p65 (Rothgiesser *et al.* 2010b), FOXO1 (Jing *et al.* 2007) and FOXO3 (Wang *et al.* 2007). Another target of SIRT2 is α-tubulin (Skoge *et al.* 2014), which we selected as a measure of SIRT2 deacetylase activity. The acetylation of α-tubulin is facilitated by α-tubulin N-acetyltransferase and NAD levels have been shown to affect the acetylation of α-tubulin by regulating activity of NAD-dependent HDACs (Skoge *et al.* 2014). SIRT2 can remove acetyl groups from histones and has been show to deacetylate H3K27ac (Jin *et al.* 2016a), a histone modification which has been found to be predictive of gene expression in CD4+ T cells (Karlic *et al.* 2010). Reports suggest that somewhere between 2% and 22% of transcriptionally active genes are regulated by histone acetylation (Peart *et al.* 2005; Rigby *et al.* 2012), whilst proteomic analyses have shown that acetylation of non-histone proteins constitutes a major portion of the acetylome in mammalian cells (Narita *et al.* 2019), thus, understanding the role and the mechanisms that regulate the acetylation of proteins may provide further insight into biological and pathological systems.

We previously observed no changes to the mRNA (Chapter 3.3) or protein (Chapter 3.5) expression of SIRT2 in macrophages stimulated with TLR agonists. However, as the deacetylation of proteins by SIRT2 is an enzymatic reaction, changes to the activity of SIRT2 could arguably be as impactful as changes to mRNA or protein expression would be.

There is evidence that SIRT2 is involved in regulating expression of iNOS and ROS in macrophages after stimulation with LPS, with SIRT2 knockout cells showing suppressed nitric oxide and ROS production (Lee *et al.* 2014b). Lee et al. also found that SIRT2 knockout cells showed decreased

NF-κB activation after stimulation with LPS. However, there is little within the current literature on the role of SIRT2 in regulation of cytokine secretion after TLR activation, with most information coming from murine models, and there is no published information on the effect of TLR activation of SIRT2 deacetylase activity. Whilst the use of animal models is invaluable in translational research (Barré-Sinoussi and Montagutelli 2015), there can be major differences between murine models and human models, despite phylogenetic similarities (Perlman 2016).

Cytokines are powerful regulators of the immune response, and as such are associated with numerous inflammatory conditions, including periodontitis. Key cytokines in the progression of periodontitis include TNFα, IL-1β, (Pan *et al.* 2019a; Pan *et al.* 2019b), IL-6 (Ramadan *et al.* 2020), IL-8 (Finoti *et al.* 2017), and IL-12 (Issaranggun Na Ayuthaya *et al.* 2018). IL-1β has been found to drive inflammation in periodontitis, increasing immune cell chemotaxis and enhancing alveolar bone resorption. IL-6 impairs osteoblast function and increases formation of osteoclasts derived from cells of the monocyte/macrophage family (Tjoa *et al.* 2008). IL-18 influences T-helper cell differentiation, regulating the balance of Th1 and Th2 T cells, and with Th1 cells being associated with bone loss and Th2 cells being considered as protective. TNFα is a crucial proinflammatory cytokine that contributes to periodontitis by regulating apoptosis of gingival fibroblasts and epithelial cells, as well as inhibiting extracellular matrix production in gingival fibroblasts (Pan *et al.* 2019a). IL-6 is elevated in the GCF of patients with periodontitis (Stadler *et al.* 2016) and has been found to involved in bone homeostasis through regulation of RANKL, which can subsequently interact with osteoprotegrin (Wu *et al.* 2017). IL-8 acts as a chemoattractant and activator of neutrophils (Finoti *et al.* 2017). IL-12 is associated with Th1 cells and has been linked to bone resorption in periodontitis (Ramadan *et al.* 2020). The above findings demonstrate the major role cytokines play in regulating inflammation in periodontitis and how they contribute to pathological processes such as bone resorption.

Understanding the regulation of cytokines that drive the progression of periodontitis may provide better insight into the underlying mechanisms of these diseases (Kany *et al.* 2019) and from the literature we know that many cytokines are regulated by acetylation either directly or indirectly through changes to acetylation of transcription factors and histones (Villagra *et al.* 2010).

We therefore aimed to assess the role of SIRT2-mediated deacetylation in regulating the secretion of pro-inflammatory cytokines relevant to periodontitis. To achieve this aim, we developed a western blotting method to assess the acetylation of SIRT2 target proteins, α-tubulin K40 and NF-κB p65. This acted as a readout for SIRT2 activity, which allowed us to assess how TLR2 and TLR4 signalling may affect SIRT2 activity. We then optimised the use of the NAMPT inhibitor FK866 and the SIRT2 inhibitor AK1 to abrogate SIRT2 deacetylase activity. Next, we stimulated THP1-derived macrophages with TLR2 and TLR4 agonists, with or without the SIRT2 inhibitor AK1, allowing us to evaluate α-tubulin acetylation. We could then subsequently collect culture supernatants and assess cytokine secretion by ELISA to gain insight into the role of SIRT2 in regulating expression.

5.2 Optimisation of the Detection of α-tubulin Acetylation by Western Blotting

To assess the potential effect of TLR2 and TLR4 signalling on SIRT2 activity, a western blotting method to measure the acetylation state of SIRT2 target α-tubulin K40 was developed. Quantitative western blotting with primary antibodies against total α-tubulin and acetyl-α-tubulin K40 (Ac-α-tubulin), and fluorescently labelled secondary antibodies were used (Chapter 2.5). For accurate relative quantification of protein acetylation with this method, the linear range of detection was first determined for both antibodies. This is the range in which the measured fluorescent target signal correlates linearly with the amount of protein loaded into the gel. This was determined by blotting serially diluted samples and calculating the coefficient of determination, denoted as R2. An R2 value of >0.97 was deemed suitable (Koch *et al.* 2018). The linear range of detection for total α-tubulin was determined to be between 2.5 µg and 20 µg of total protein, with an R2 of 0.9821 (Figure 5.1). The dynamic range, the range of total protein loading at which total α-tubulin could be detected, lay outside the bounds of protein loaded in this assay.

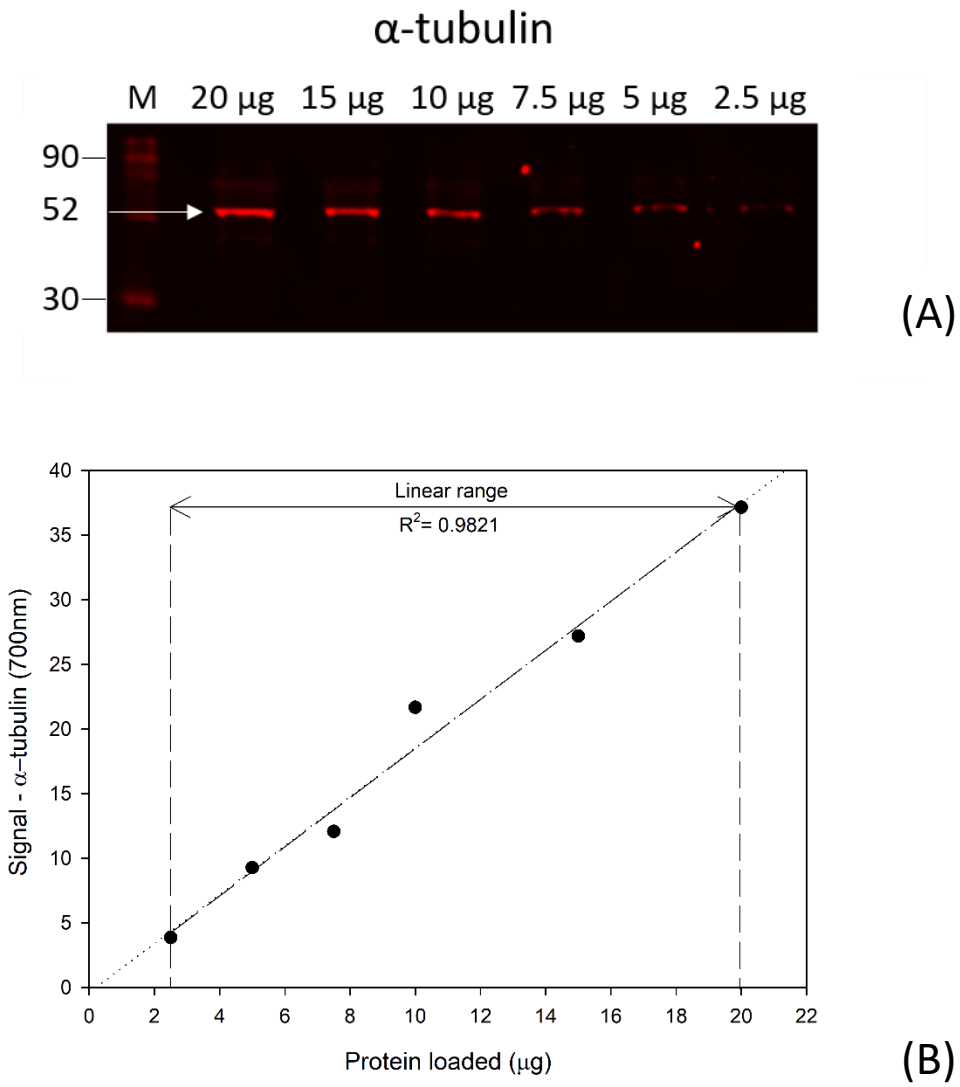


Figure 5.1. Determination of linear range of α-tubulin protein detection.

(A) Western blot to determine linear range for detection of α-tubulin (700 nm). A seven-point, two-fold serial dilution of cell lysate ranging from 20 µg to 2.5 µg was generated (section 2.5). Molecular weight ladder labelled "M" indicates weight in kDa. Predicted molecular weight 52 kDa. (B) Graph showing fluorescent target signal at 700 nm quantified for each band plotted against total protein loaded on a scatter graph. The coefficient of determination was then calculated (R^2=0.9821). The data shown represents 1 sample for each plotted point, generated from 3 pooled cultures obtained in 1 experiment (N=1).

Next, the linear range of detection for Ac-α-tubulin was determined. The linear range of detection for Ac-α-tubulin was found to be between 7.5 µg and 20 µg of total protein, with an R^2 of 0.9903 (Figure 5.2). The dynamic range, the range of total protein loading for Ac-α-tubulin was from 2.5 µg to 20 µg.

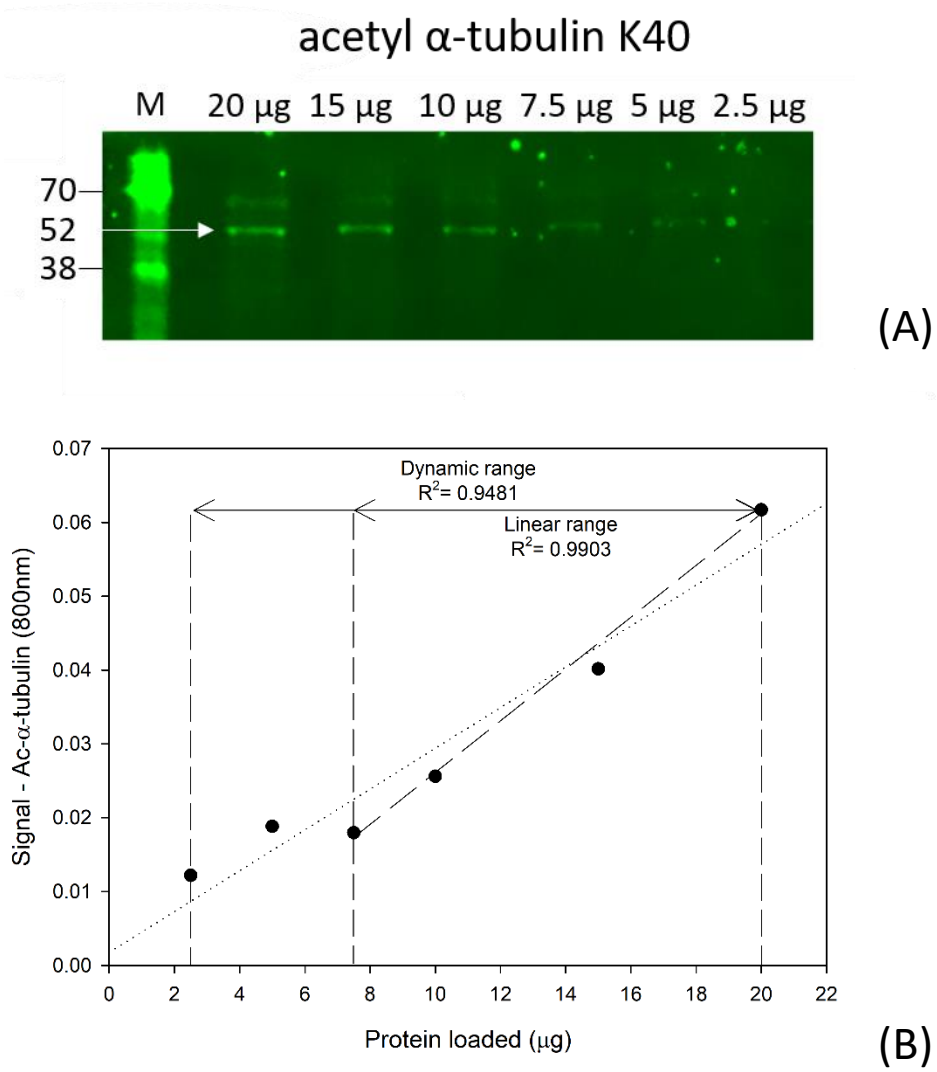


Figure 5.2. Determination of linear range of Ac-α-tubulin protein detection.

(A) Western blot to determine linear range for detection of Ac-α-tubulin (800 nm). A seven-point, two-fold serial dilution of cell lysate ranging from 20 µg to 2.5 µg was generated (section 2.5). Molecular weight ladder labelled "M" indicates weight in kDa. Predicted molecular weight 52 kDa. (B) Graph showing fluorescent target signal at 800 nm quantified for each band plotted against total protein loaded on a scatter graph. The coefficient of determination was then calculated (R^2=0.9903). The data shown represents 1 sample for each plotted point, generated from 3 pooled cultures obtained in 1 experiment (N=1).

From this it was determined that total protein amounts ranging from 7.5 µg to 20 µg could be used for accurate quantitation. This allows for the use of both antibodies simultaneously in western blotting, allowing the ratio of Ac-α-tubulin to total α-tubulin to be calculated from the measured fluorescent target signals using the following:

$$\text{Ratio of } \alpha\text{-tubulin acetylation} = \frac{\text{Ac-}\alpha\text{-tubulin}}{\text{Total } \alpha\text{-tubulin}}$$

The acetylation of α-tubulin was used as a measure of SIRT2 deacetylase activity in THP1-derived macrophages. The acetylation of α-tubulin at lysine 40 was measured using quantitative western blotting with a specific antibody to Ac-α-tubulin K40 and an antibody against total α-tubulin. Fluorescent target signal was normalised against a total protein stain (Methods chapter 2.5.3). The normalised signal (to correct for variations in protein loading) was then used to calculate the ratio of α-tubulin acetylation. The ratio of α-tubulin acetylation in controls was then normalised to 1 and ratio of α-tubulin acetylation in the test groups are plotted relative to this. Data from all western blots for α-tubulin acetylation were processed in the same manner.

THP1-derived macrophages were treated with the NAMPT inhibitor FK866 for 24 hours, in order to deplete intracellular NAD and as a result, reduce NAD-dependent deacetylase activity. Inhibition of NAD-dependent HDACs has been shown to lead to increased acetylation of α-tubulin, whilst inhibition of class I and class II HDACs had no effect (North *et al.* 2003a). It was also demonstrated that SIRT2 is the only NAD-dependent HDAC that is able to target α-tubulin (North *et al.* 2003a). Thus, FK866 was used to establish if changes to the acetylation of α-tubulin could be detected using the quantitative western blot method. To determine FK866 demonstrated any cytotoxic effects, an MTT assay was first carried out.

Treatment of THP1-derived with FK866 for 24 hours resulted in no significant effect of cell viability (Figure 5.3).

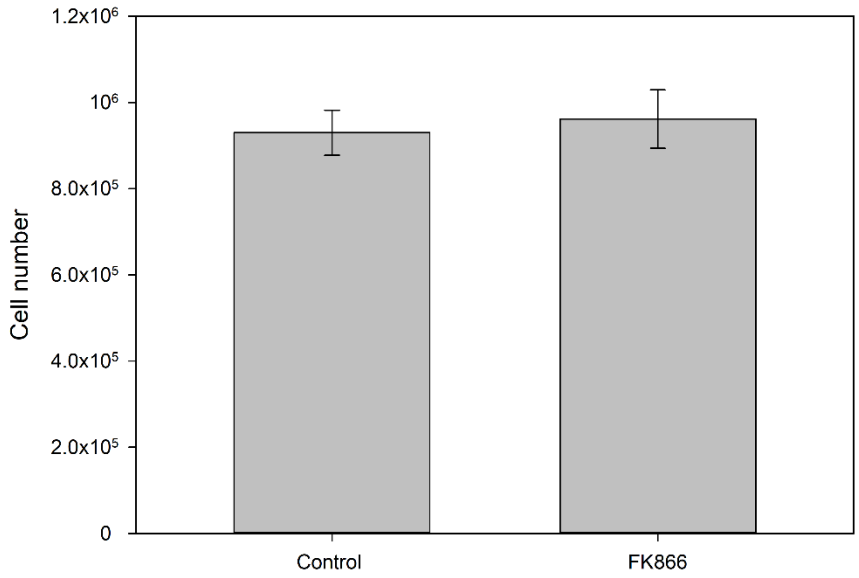


Figure 5.3. Cell number determined by MTT assay with macrophages with the SIRT2 inhibitor FK866 for 24 hours.

THP1-derived macrophages treated for 24 hours with FK866. Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). Analysed by independent T-Test.

Treatment of THP1-derived macrophages with FK866 for 24 hours (Figure 5.4) led to a significant increase of Ac-α-tubulin K40 when compared to untreated controls.

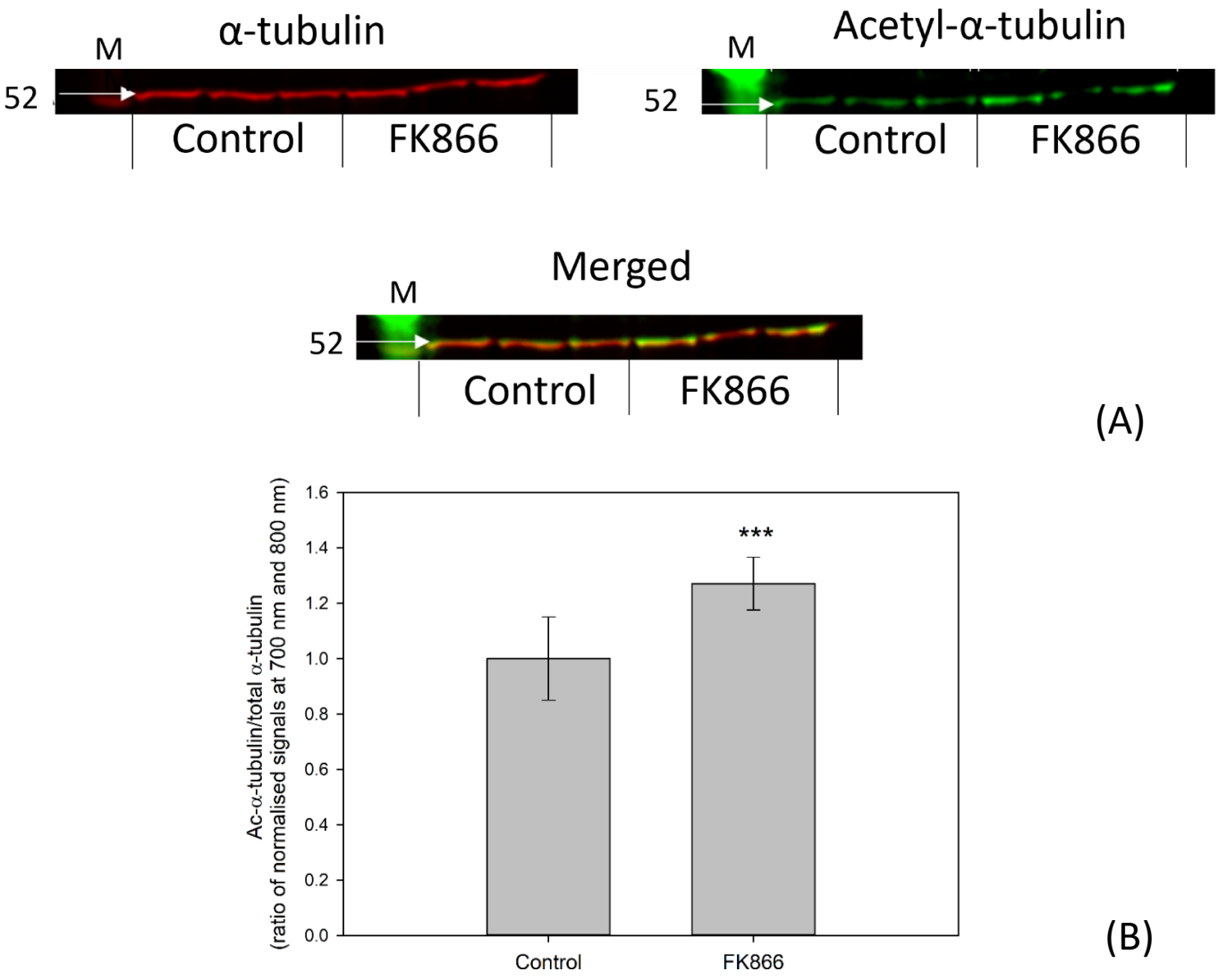


Figure 5.4. Acetylation of α-tubulin in macrophages treated with FK866 for 24 hours.

(A) Representative western blot for α-tubulin (700 nm) and acetyl-α-tubulin K40 (800 nm) in THP1-derived macrophages treated with FK866 (2 µM) for 24 hours. Each lane represents protein lysate from 1 independent culture. Lanes labelled as "C" indicate controls. Molecular weight ladder labelled "M" indicates weight in kDa. Predicted molecular weight 52 kDa. (B) Ratio of acetyl-α-tubulin to total α-tubulin in control cultures and FK866-treated cultures. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). *** $p<0.001$ calculated using independent T-Test.

We next utilised the SIRT2 inhibitor, AK1, to confirm that SIRT2 alone was responsible for any change to acetylation and there was no involvement from any other sirtuin family members. AK1 inhibits SIRT2 activity by interacting with the nicotinamide binding site which also participates in NAD+ binding (Avalos *et al.* 2005; Outeiro *et al.* 2007).

THP1-derived macrophages were treated with AK1 for 24 hours before cell lysates were collected and analysed using the previously established western blotting method with antibodies for total α-tubulin and Ac-α-tubulin to determine the ratio of α-tubulin acetylation.

To determine if AK1 demonstrated any cytotoxic effects, an MTT assay was first carried out. Treatment of THP1-derived with AK1 for 24 hours resulted in no significant effect of cell viability (Figure 5.5).

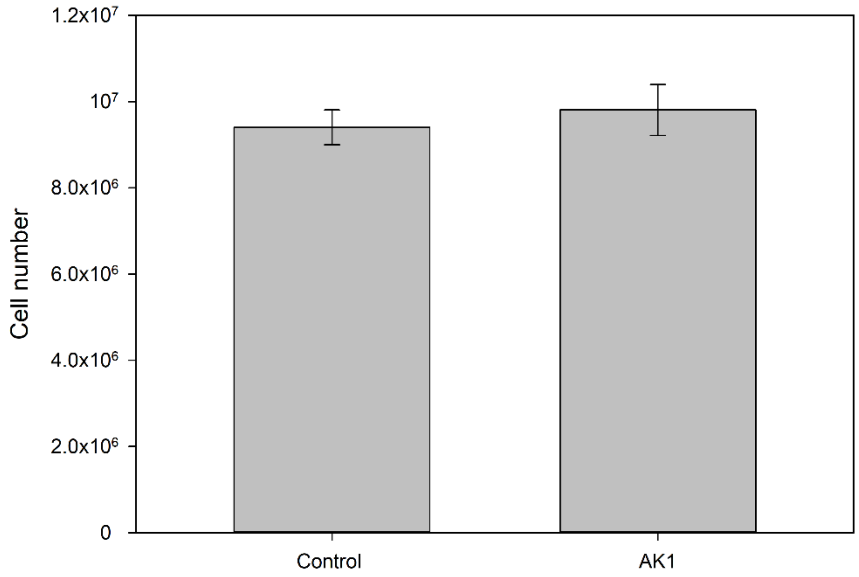


Figure 5.5. Cell number determined by MTT assay with macrophages with the SIRT2 inhibitor AK1 for 24 hours.

THP1-derived macrophages treated for 24 hours with AK1 (10 μM). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). Analysed by Student's t-test.

Treatment of THP1-derived macrophages with AK1 for 24 hours (Figure 5.6) led to a statistically significant increase in the ratio of Ac-α-tubulin to total α-tubulin ($p=0.007$). Confirming that inhibition of SIRT2 leads to increased acetylation of α-tubulin at lysine 40.

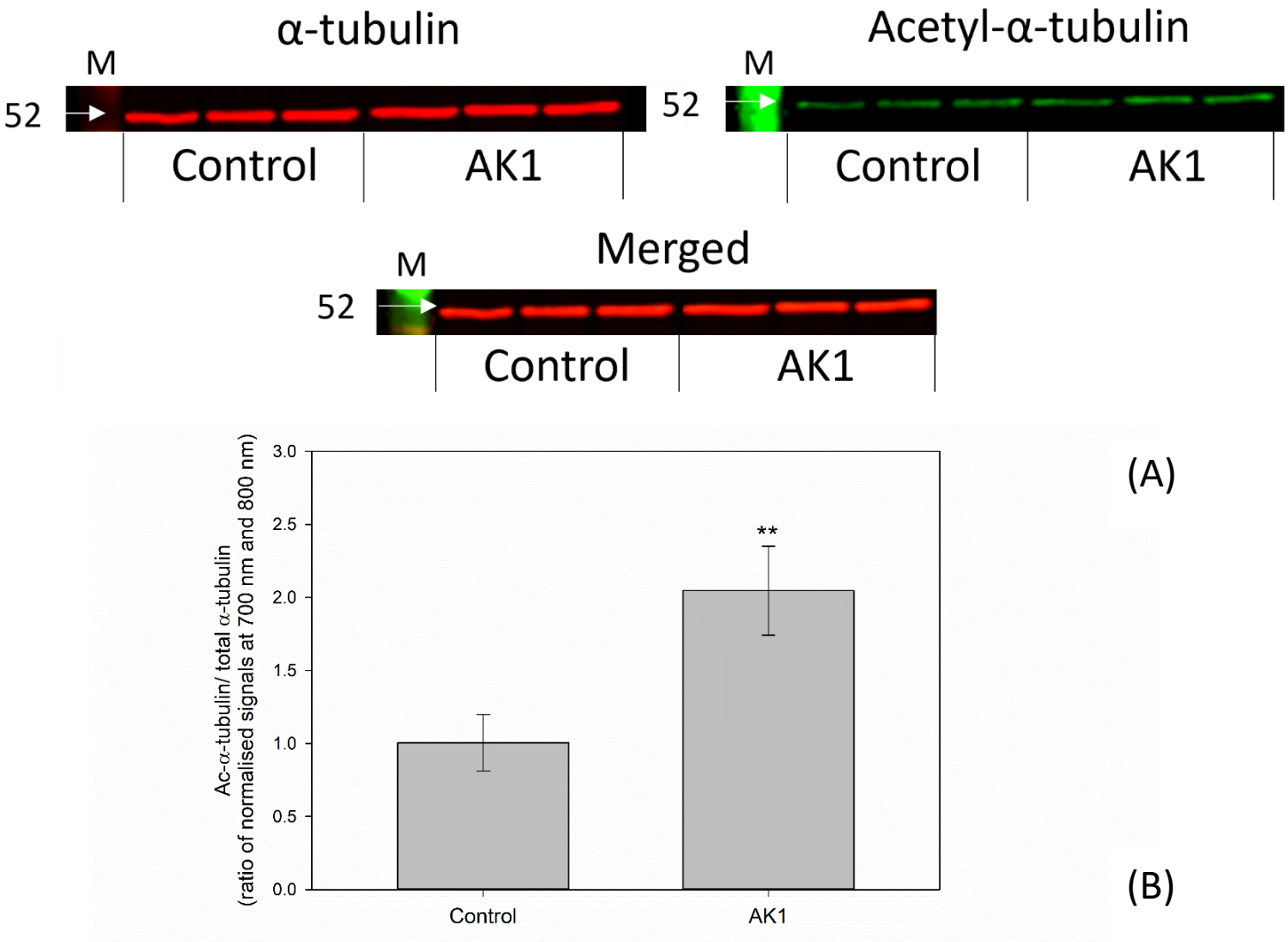


Figure 5.6. Acetylation of α-tubulin in macrophages treated with AK1 for 24 hours.

(A) Representative western blot for α-tubulin (700 nm) and acetyl-α-tubulin K40 (800 nm) in THP1-derived macrophages stimulated with AK1 (10 μM) for 24 hours. Each lane represents protein lysate from 1 independent culture. Molecular weight ladder labelled "M" indicates weight in kDa. Predicted molecular weight 52 kDa. (B) Fluorescent target signals were normalised against a total protein stain to correct for variations in protein loading, and the ratio of acetyl-α-tubulin to total α-tubulin was calculated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). ** p=0.007 calculated using Student's t-test.

5.3 The Influence of TLR2 and TLR4 Agonists on α-tubulin Acetylation

Next, Ac-α-tubulin K40 was measured in THP1-derived macrophages after stimulation with TLR2 and TLR4 agonists for 4 hours. Quantitative western blotting was again used, with an antibody against Ac-α-tubulin K40 and an antibody against total α-tubulin. The fluorescent target signal was normalised against a total protein stain. THP1-derived macrophages were stimulated with *E. coli* LPS or *B. subtilis* LTA for 4 and 24 hours or left untreated as controls. As a matter of routine, stimulation of macrophages with TLR agonists was confirmed by ELISA for TNFα (see chapter 2.3).

Stimulation with *B. subtilis* LTA or *E. coli* LPS for 4 hours (Figure 5.7) led to no statistically significant changes to the ratio of acetyl-α-tubulin K40 to total α-tubulin when compared to unstimulated controls.

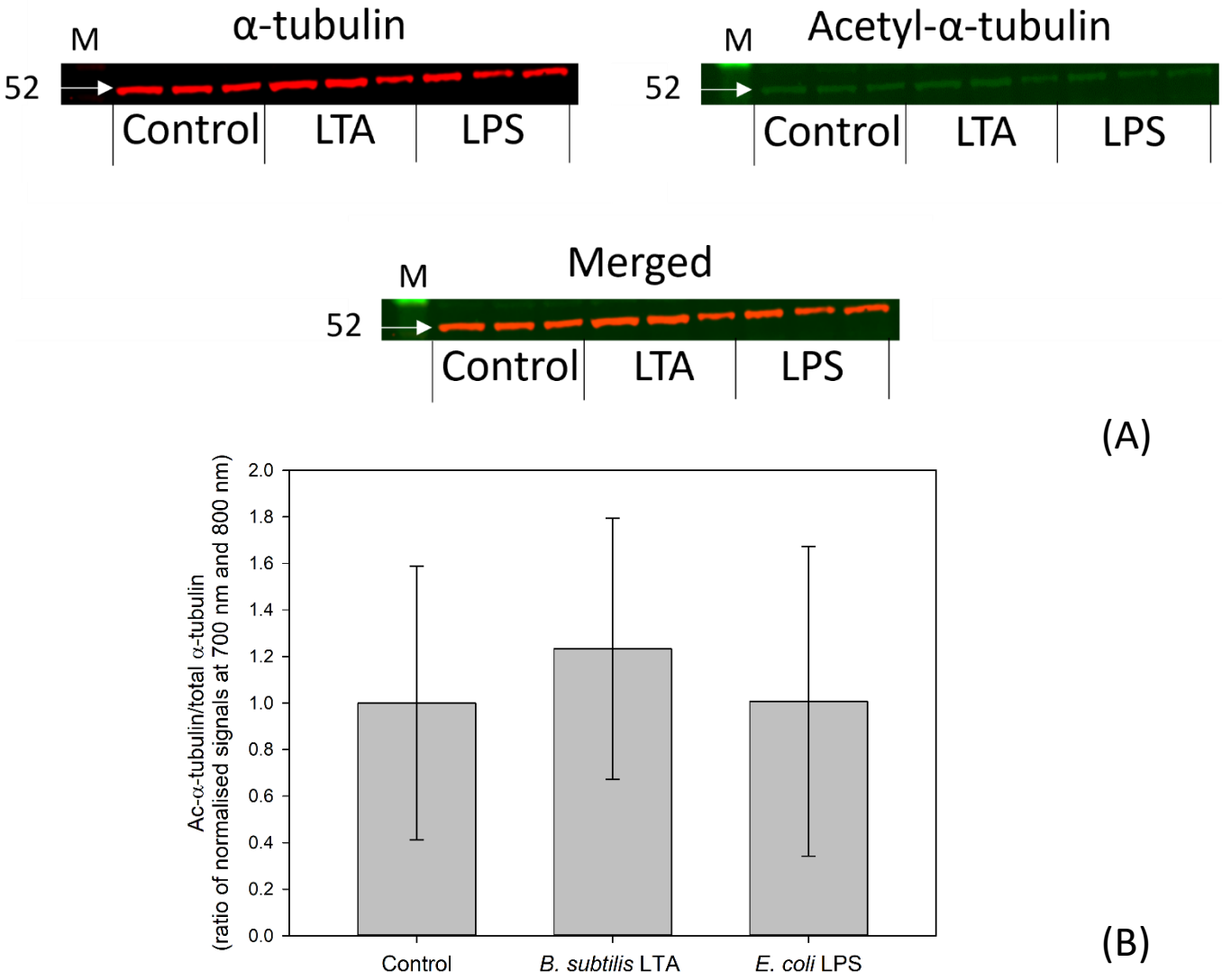


Figure 5.7. Acetylation of α-tubulin in macrophages stimulated with *B. subtilis* LTA or *E. coli* LPS for 4 hours.

(A) Representative western blot for α-tubulin (700 nm) and acetyl-α-tubulin K40 (800 nm) in THP1-derived macrophages stimulated *B. subtilis* LTA (100 ng/ml) or *E. coli* LPS (100 ng/ml) for 4 hours. Each lane represents protein lysate from 1 independent culture. Molecular weight ladder labelled "M" indicates weight in kDa. Predicted molecular weight 52 kDa. (B) Fluorescent target signals were normalised against a total protein stain to correct for variations in protein loading, and the ratio of acetyl-α-tubulin to total α-tubulin was calculated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). Analysed using one-way ANOVA.

Stimulation with *B. subtilis* LTA or *E. coli* LPS for 24 hours (Figure 5.8) also resulted in no statistically significant changes to the ratio of α-tubulin acetylation at K40 compared to unstimulated controls.

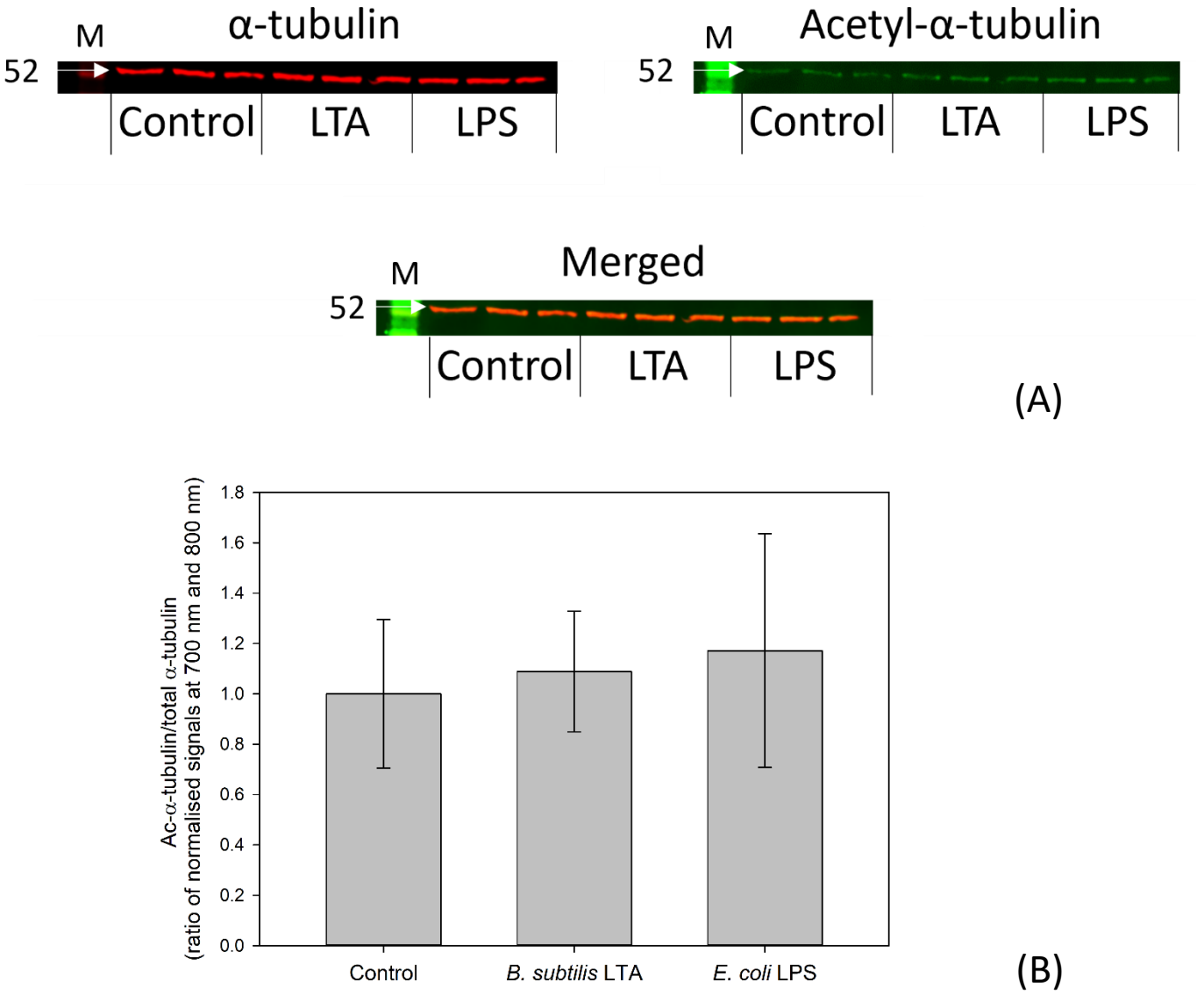


Figure 5.8. Acetylation of α-tubulin in macrophages stimulated with *B. subtilis* LTA or *E. coli* LPS for 24 hours.

(A) Representative western blot for α-tubulin (700 nm) and acetyl-α-tubulin K40 (800 nm) in THP1-derived macrophages stimulated *B. subtilis* LTA (100 ng/ml) or *E. coli* LPS (100 ng/ml) for 24 hours. Each lane represents protein lysate from 1 independent culture. Molecular weight ladder labelled "M" indicates weight in kDa. Predicted molecular weight 52 kDa. (B) Fluorescent target signals were normalised against a total protein stain to correct for variations in protein loading, and the ratio of acetyl-α-tubulin to total α-tubulin was calculated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). Analysed using one-way ANOVA.

5.4 Optimisation of Western Blotting to Measure NF-κB p65 Acetylation

We next utilised antibodies specific against total NF-κB p65 and NF-κB p65 acetylated at lysine 310 (referred to as Ac-NF-κB p65), a target of SIRT2 deacetylation. No other sirtuin family member deacetylates NF-κB p65 at lysine 310 (Rothgiesser *et al.* 2010b). NF-κB is a transcription factor that regulates expression of numerous cytokines, including IL-1, IL-6, IL-8, and TNFα, as well as adhesion molecules and chemokines (Liu *et al.* 2017b). NF-κB p65 is one subunit of the NF-κB transcription factor and increased activation of NF-κB p65 has been associated with many chronic diseases, such as rheumatoid arthritis (Giridharan and Srinivasan 2018).

In order to understand how SIRT2 regulates the acetylation of NF- κB p65, we first attempted to develop a western blotting method to quantify acetylation levels in p65. THP1-derived macrophages were treated with the NAMPT inhibitor FK866 for 24 hours before collecting cell lysates and conducting western blotting to analyse levels of p65 acetylation.

Western blotting of the lysate of THP1-derived macrophages treated with FK866 (Figure 5.9) using antibodies against Ac-NF-κB p65 and total NF-κB p65 failed to produce single bands at the expected molecular weight of 65 kDa on every use. The total NF-κB p65 antibody produced a strong band at approximately 110 kDa and indistinguishable bands at lower molecular weights. The Ac-NF-κB p65 antibody produced 7 bands at varying above and below the predicted molecular weight of 65 kDa. Given the inability of the selected antibodies to produce single bands that could be quantified, NF-κB p65 acetylation was not investigated further.

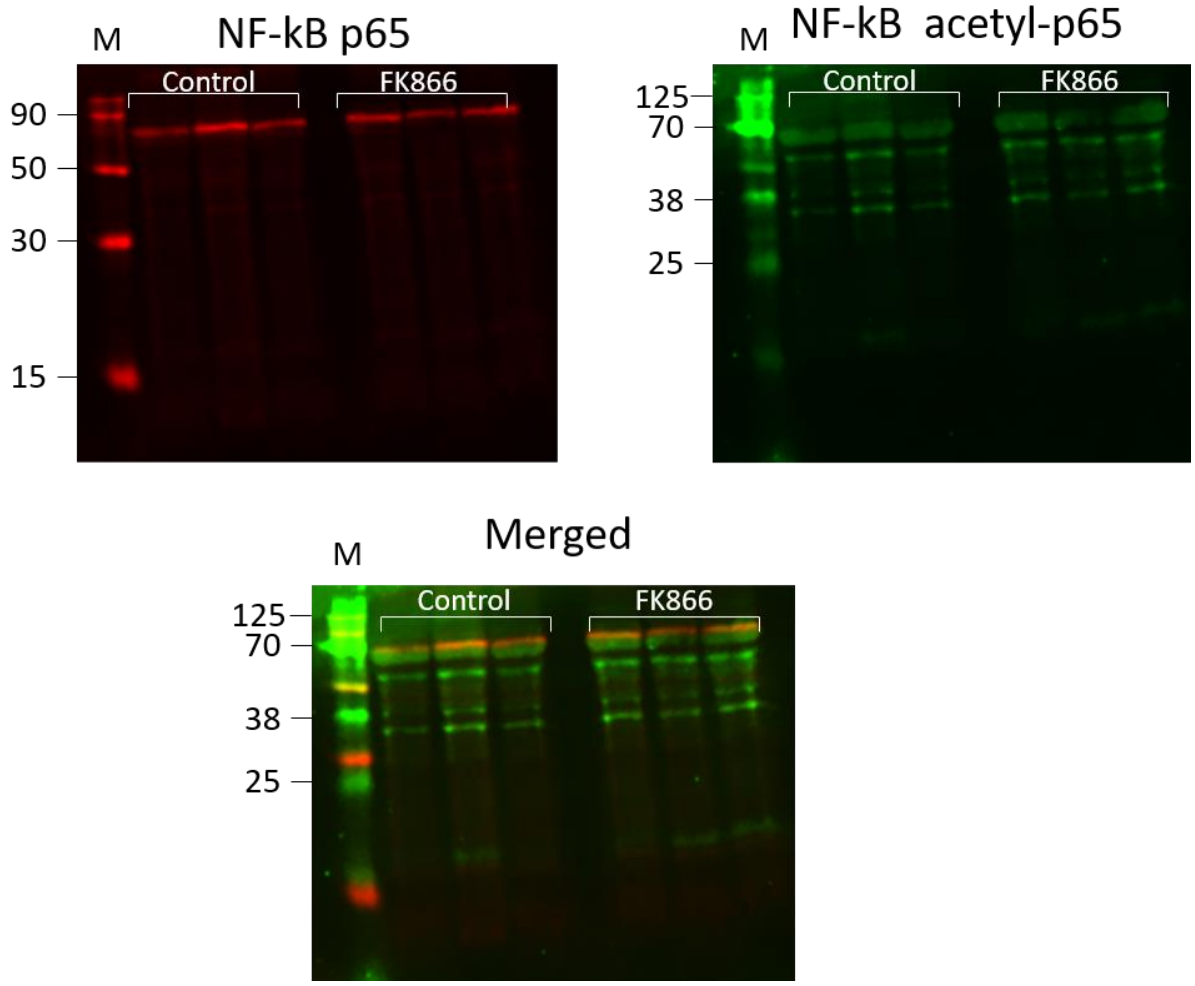


Figure 5.9. Acetylation of NF-κB p65 in macrophages treated with FK866 for 24 hours.

Representative western blot for NF-κB p65 (700 nm) and acetyl- NF-κB p65 (800 nm) in THP1-derived macrophages treated with FK866 (2 μM) for 24 hours. Each lane represents 1 independent culture. Molecular weight ladder labelled "M" indicates weight in kDa. Predicted molecular weight of NF-kB p65 is 65 kDa.

5.5 The Role of SIRT2 in the Regulation of Cytokine Secretion

To investigate the role SIRT2 in the cytokine response to TLR agonists, the SIRT2 inhibitor AK1 was used to abrogate SIRT2 deacetylase activity (Figure 5.6). Culture supernatants could then be analysed by ELISA for proinflammatory cytokines to determine if inhibition of SIRT2 had any effect on cytokine secretion. The cytokines being measured were selected due to their relevance to inflammatory processes and periodontitis and included TNFα, IL-1β, IL-6, IL-8, and IL-12 (Lagdive *et al.* 2013; Pan *et al.* 2019a).

In order to confirm inhibition of acetylation by AK1 in macrophages stimulated by TLR agonists, cells were pre-treated with AK1, or left in standard growth medium for 24 hours before removal of the growth medium and addition of media containing *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) for 0.5, 4 and 24 hours, while controls were left untreated followed by analysis of acetylation of α-tubulin by western blotting (Figures 5.10-5.12). In samples pre-treated with AK1 before stimulation with *E. coli* LPS, a significant increase to the acetylation of α-tubulin was observed at the 0.5 hour, 4 hour and 24 hour time points compared to *E. coli* LPS-stimulated samples that did not receive pre-treatment with AK1 ($p < 0.05$). Samples pre-treated with AK1 before stimulation with *B. subtilis* LTA showed a significant increase in the acetylation of α-tubulin was at the 0.5 hour, 4 hour and 24 hour time points compared to samples that did not receive pre-treatment with AK1 ($p < 0.05$). There was no significant difference between control and TLR agonist-stimulated samples, indicating that the effect of AK1 is independent of TLR signalling, with the exception of the 24 hour cultures.

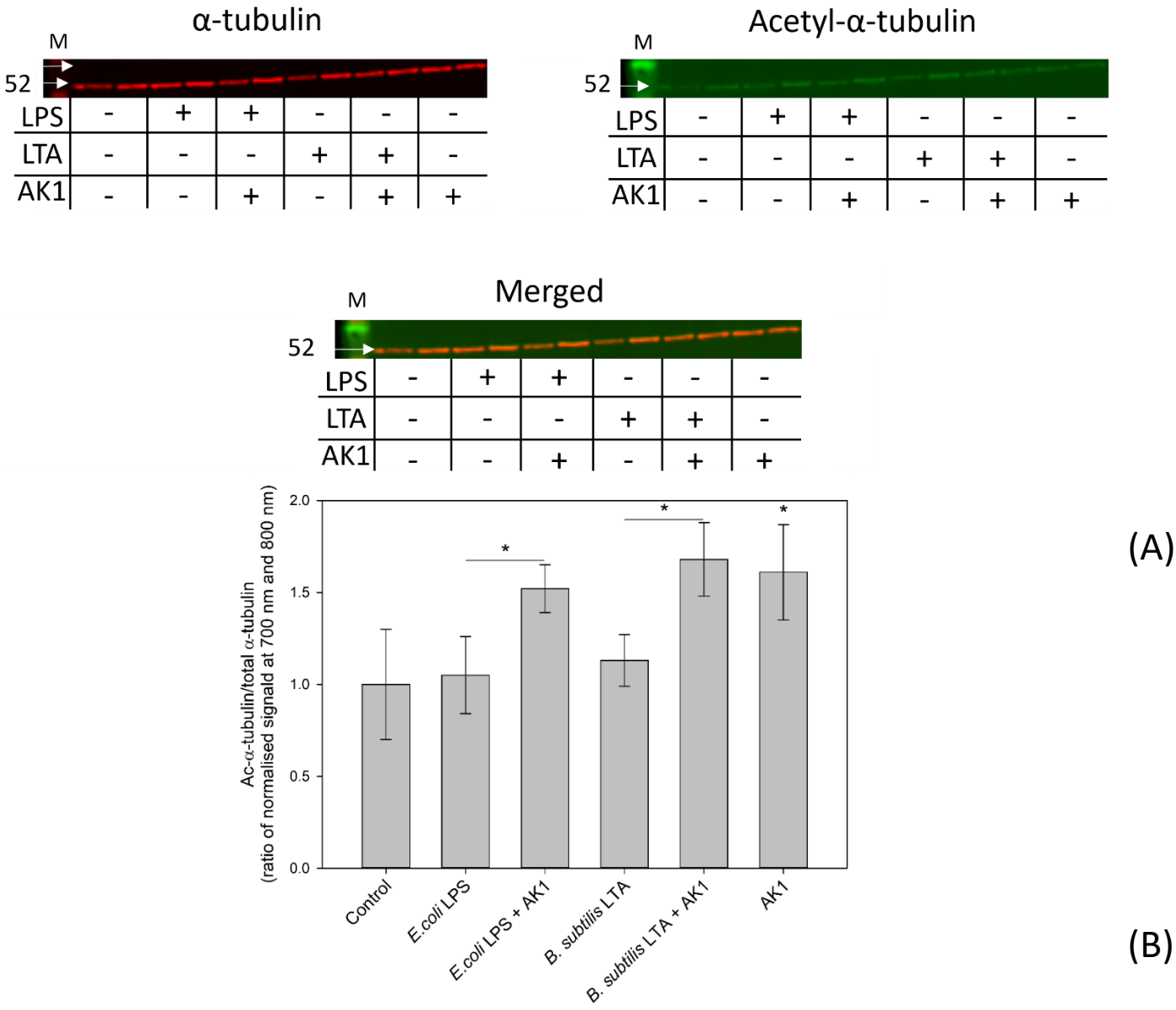


Figure 5.10. Acetylation of α-tubulin in macrophages stimulated with TLR agonists for 0.5 hours with or without AK1 pre-treatment.

(A) Representative western blot for α-tubulin (700 nm) and acetyl-α-tubulin K40 (800 nm) in THP1-derived macrophages stimulated *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) for 0.5 hours, with (labelled LPS+, LTA+ and AK1) or without AK1 pre-treatment (10 µM) for 24 hours. Each lane represents protein lysate from 1 independent culture. Molecular weight ladder labelled "M" indicates weight in kDa. Predicted molecular weight 52 kDa. (B) Fluorescent target signals were normalised against a total protein stain to correct for variations in protein loading. The data shown represents the mean ± SD of 2 cultures for each condition obtained in 3 separate experiments (N=6). * $p<0.05$ calculated by one-way ANOVA, followed by Tukey's post-hoc test.

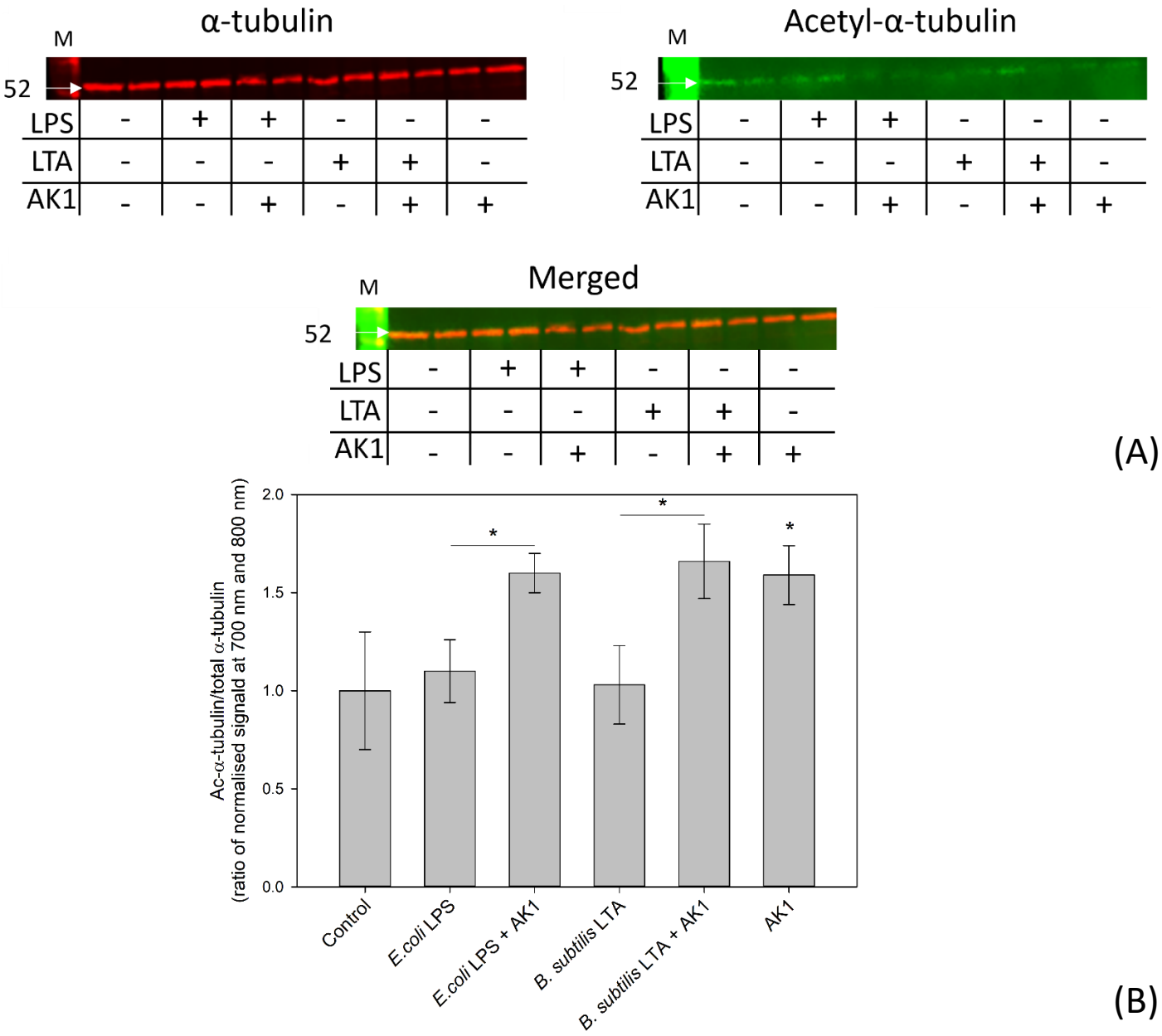
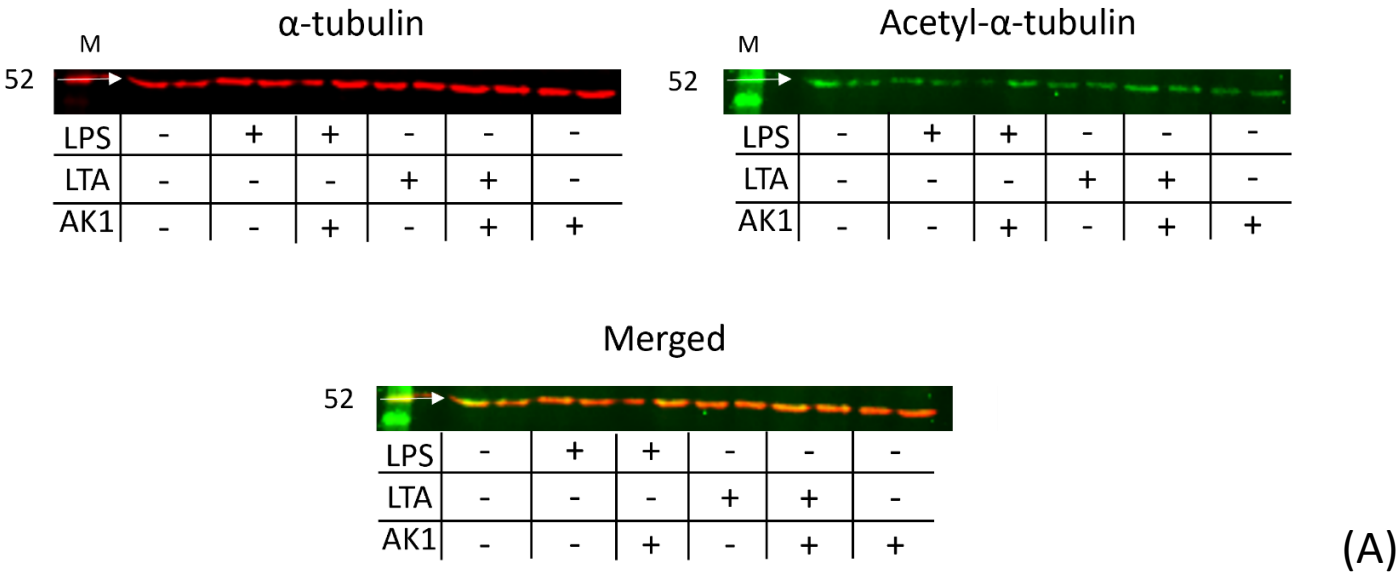
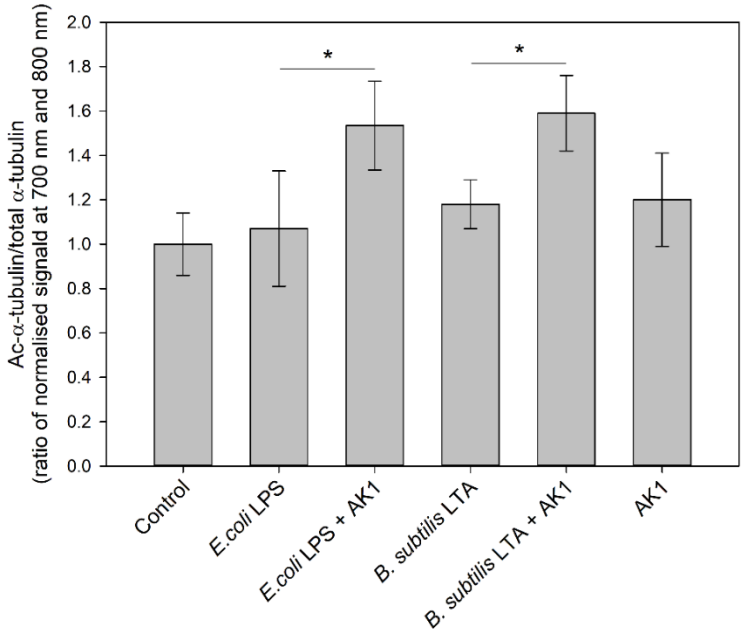


Figure 5.11. Acetylation of α-tubulin in macrophages stimulated with TLR agonists for 4 hours with or without AK1 pre-treatment.

(A) Representative western blot for α-tubulin (700 nm) and acetyl-α-tubulin K40 (800 nm) in THP1-derived macrophages stimulated *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) for 4 hours, with (labelled LPS+, LTA+ and AK1) or without AK1 pre-treatment (10 μM) for 24 hours. Each lane represents protein lysate from 1 independent culture. (B) Fluorescent target signals were normalised against a total protein stain to correct for variations in protein loading. The data shown represents the mean ± SD of 2 cultures for each condition obtained in 3 separate experiments (N=6). * $p<0.05$ calculated by one-way ANOVA, followed by Tukey's post-hoc test.



(A)



(B)

Figure 5.12. Acetylation of α-tubulin in macrophages stimulated with TLR agonists for 24 hours with or without AK1 pre-treatment.

(A) Representative western blot for α-tubulin (700 nm) and acetyl-α-tubulin K40 (800 nm) in THP1-derived macrophages stimulated *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) for 24 hours, with or without AK1 pre-treatment (10 µM) for 24 hours. Each lane represents protein lysate from 1 independent culture. (B) Fluorescent target signals were normalised against a total protein stain to correct for variations in protein loading. The data shown represents the mean ± SD of 2 cultures for each condition obtained in 3 separate experiments (N=6). * $p<0.04$ calculated by one-way ANOVA, followed by Tukey's post-hoc test.

To investigate the role of SIRT2 in the cytokine response to TLR agonists, the SIRT2 inhibitor AK1 was used to abrogate SIRT2 deacetylase activity and measure the effect on cytokine secretion using ELISA (Chapter 2.2). The TLR4 agonist, *E. coli* LPS, and the TLR2 agonist, *B. subtilis* LTA were utilised once again for these experiments. Inhibition of SIRT2 was confirmed using the previously established western blot method to measure the acetylation of α-tubulin before ELISA analysis of supernatants was carried out.

Stimulation of macrophages with *E. coli* LPS or *B. subtilis* LTA for 0.5 hours (Figure 5.13), 4 hours (Figure 5.14) and 24 hours (Figures 5.15) resulted in a statistically significant increase in TNFα secretion compared with unstimulated controls ($p<0.001$). Samples treated with pre-treated with AK1 prior to stimulation with *E. coli* LPS or *B. subtilis* LTA for 0.5 hours or 4 hours showed significantly reduced levels of TNFα compared to samples without pre-treatment ($p<0.001$). No change was caused by treatment with AK1 followed by 24 hours of stimulation with *E. coli* LPS or *B. subtilis* LTA. Treatment of macrophages with AK1 alone had no effect on TNFα secretion compared with controls.

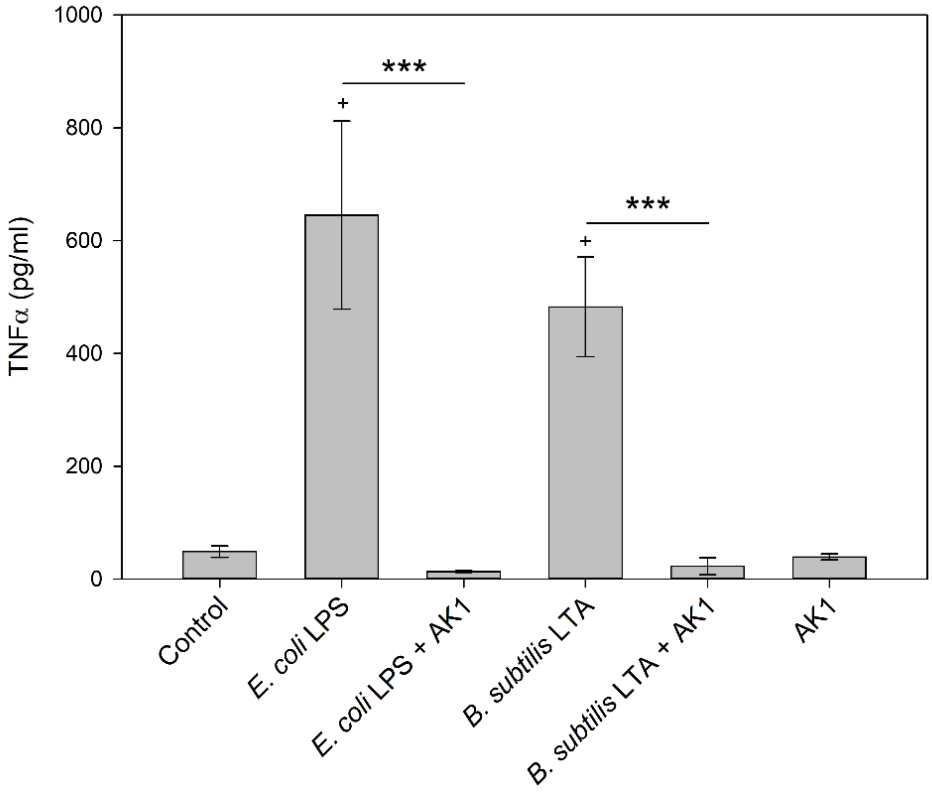


Figure 5.13. TNFα secretion from macrophages stimulated with TLR agonists for 0.5 hours.

TNFα measured by ELISA in supernatant of THP1-derived macrophages stimulated for 0.5 hours with *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) with or without AK1 (10 µM). Controls were unstimulated. The data shown represents the mean ± SD of 2 cultures for each condition obtained in 3 separate experiments (N=6). + indicates $p<0.05$ compared to control, *** indicates $p<0.001$, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

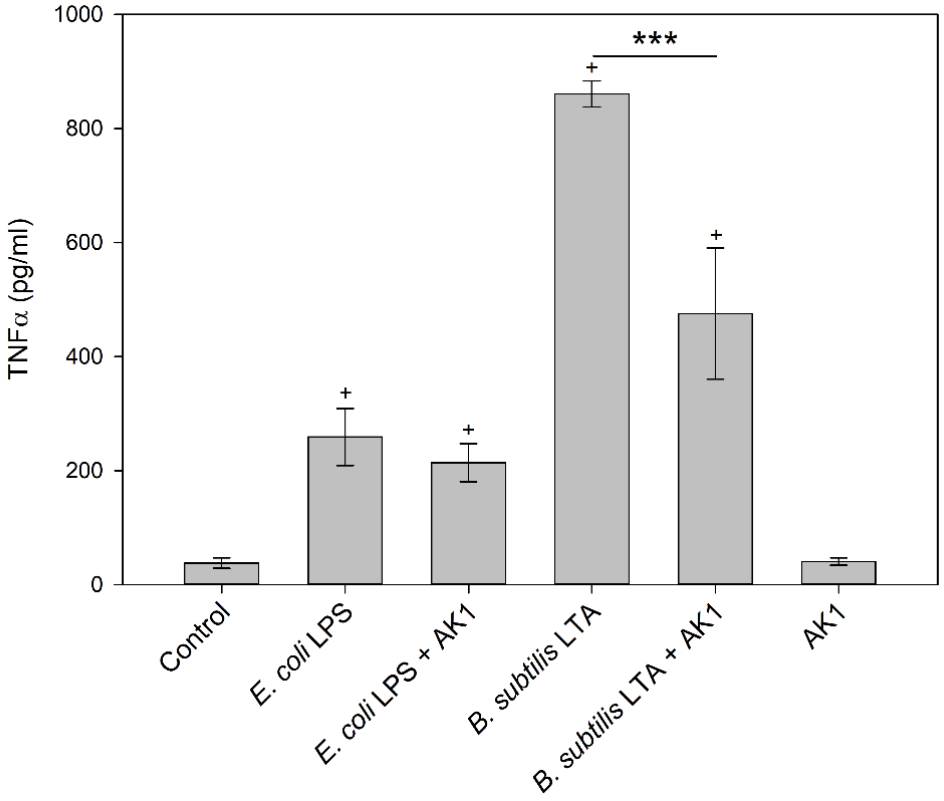


Figure 5.14. TNFα secretion from macrophages stimulated with TLR agonists for 4 hours.

TNFα measured by ELISA in supernatant of THP1-derived macrophages stimulated for 4 hours with *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) with or without AK1 (10 µM). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). + indicates $p<0.05$ compared to control, *** $p<0.001$, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

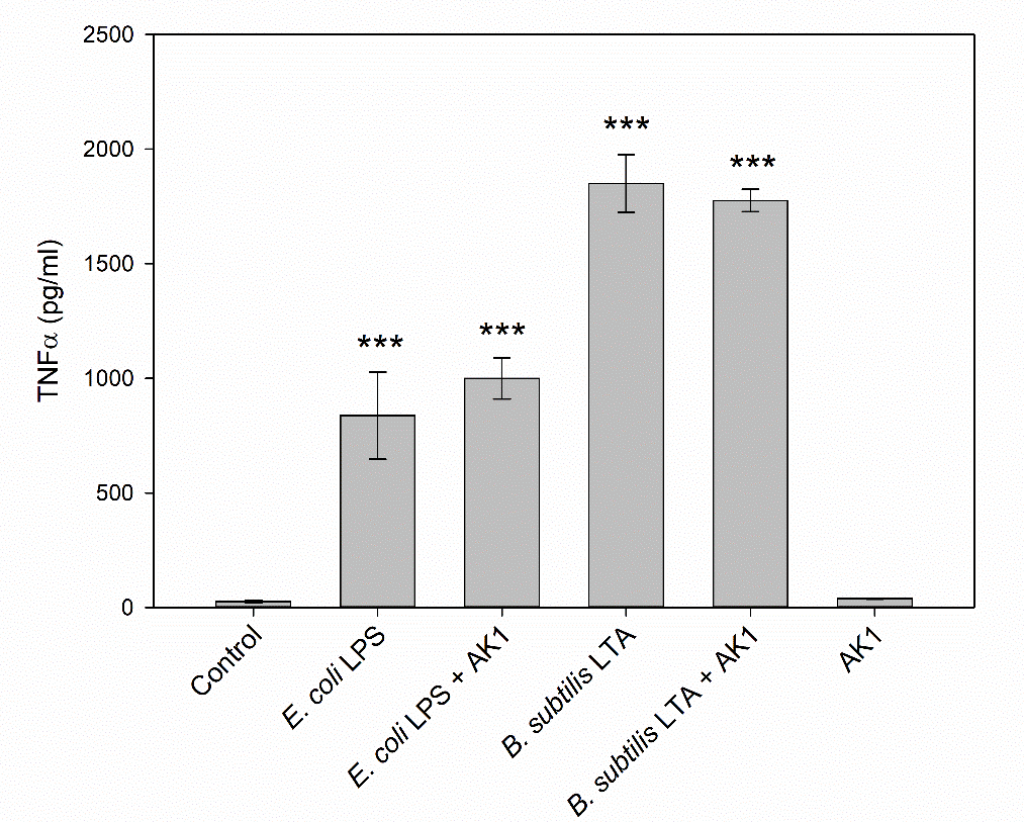


Figure 5.15. TNFα secretion from macrophages stimulated with TLR agonists for 24 hours.

TNFα measured by ELISA in supernatant of THP1-derived macrophages stimulated for 24 hours with *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) with or without AK1 (10 µM). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). *** $p<0.001$, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

At the 0.5-hour and 4-hour time points, no IL-1β was detected in any sample (data not shown). Stimulation of macrophages with *E. coli* LPS and *B. subtilis* LTA for 24 hours (Figure 5.16) resulted in a statistically significant increase in IL-1β secretion compared with unstimulated controls ($p<0.005$ and $p<0.008$ respectively). Pre-treatment with AK1 led to a statistically significant increase in IL-1 β after stimulation with *E. coli* LPS and *B. subtilis* LTA ($p<0.01$ for both). No IL-1β was detectable in the supernatant of control macrophages. Low levels of IL-1β were detected in the supernatant of macrophages treated with AK1 alone, however the concentration bordered the limit of detection for this assay.

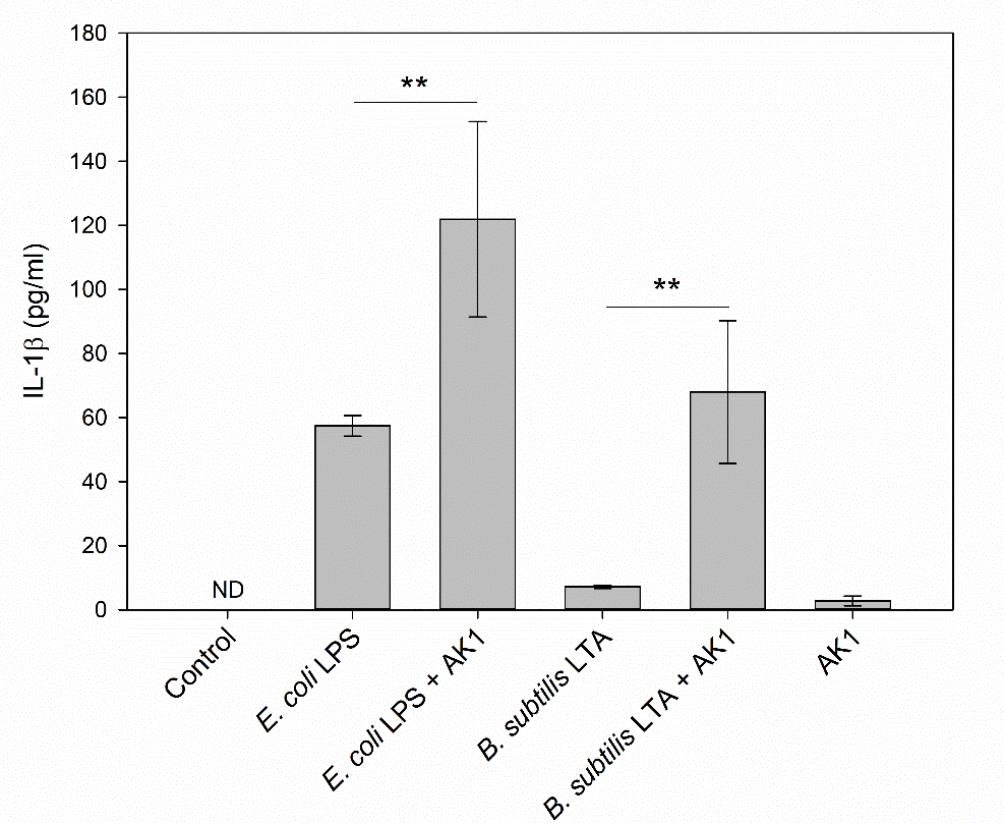


Figure 5.16. IL-1β secretion from macrophages stimulated with TLR agonists for 24 hours.

IL-1β measured by ELISA in supernatant of THP1-derived macrophages stimulated for 24 hours with *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) with or without AK1 (10 µM). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). ** $p<0.01$, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

Stimulation of macrophages with *E. coli* LPS for 0.5 hours, with or without pre-treatment with AK1 (Figure 5.17) resulted in detectable but low levels of IL-6. Pre-treatment with AK1 resulted in no statistically significant change to IL-6 levels and the measured levels were close to the lower limit of detection for this assay. No IL-6 was detected in control, *B. subtilis* LTA, *B. subtilis* LTA + AK1 or AK1 treated samples. At 4 hours (Figure 5.18) and 24 hours (Figure 5.19), IL-6 was detected in all samples. Stimulation with *E.* coli LPS led to statistically significant increase in IL-6 compared to controls. Pre-treatment with AK1 before stimulation with *E.* coli LPS resulted in significantly lower levels of secreted IL-6 ($p<0.001$). There was no statistically significant change to IL-6 levels in samples stimulated with *B. subtilis* LTA*, B. subtilis* LTA + AK1, or AK1 alone when compared with controls.

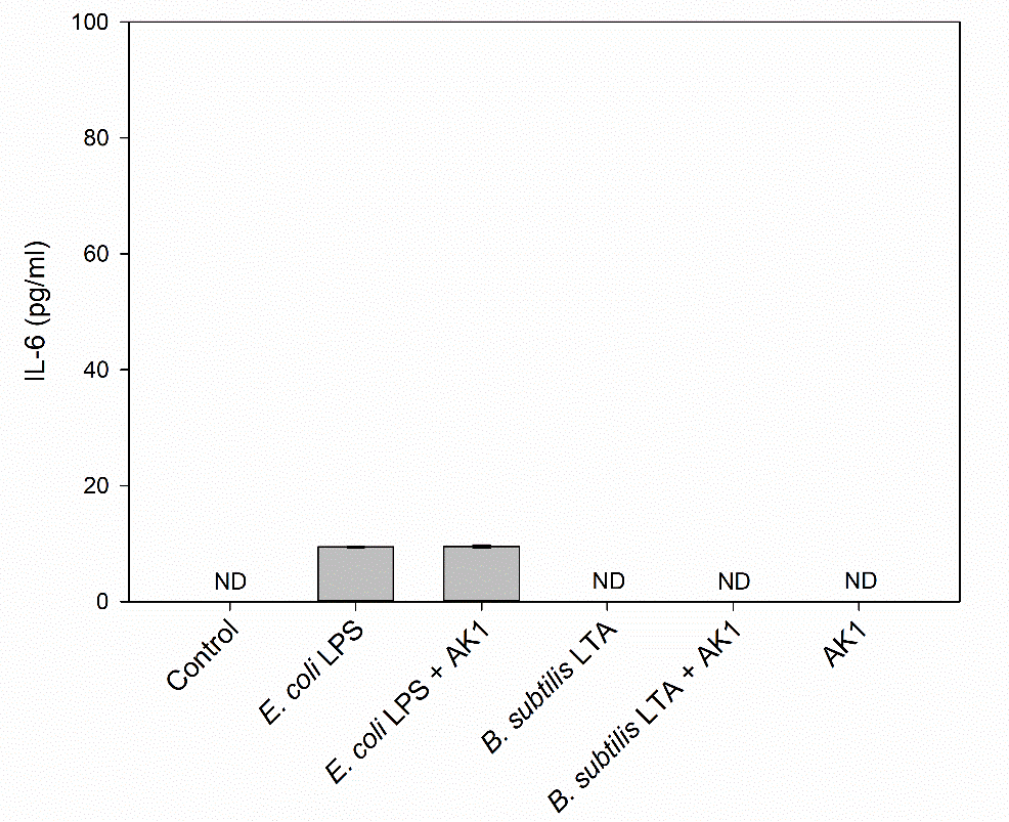


Figure 5.17. IL-6 secretion from macrophages stimulated with TLR agonists for 0.5 hours.

IL-6 measured by ELISA in supernatant of THP1-derived macrophages stimulated for 0.5 hours with *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) with or without AK1 (10 µM). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). Analysed by one-way ANOVA.

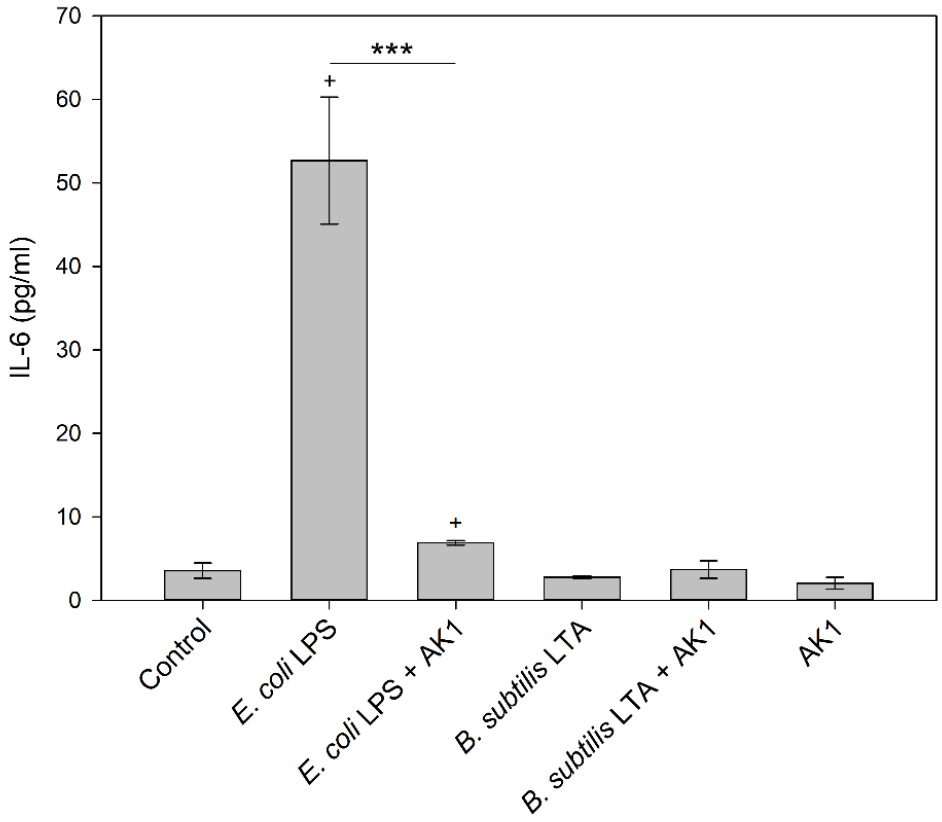


Figure 5.18. IL-6 secretion from macrophages stimulated with TLR agonists for 4 hours.

IL-6 measured by ELISA in supernatant of THP1-derived macrophages stimulated for 4 hours with *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) with or without AK1 (10 µM). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). + indicates $p<0.05$ compared to control, *** $p<0.001$, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

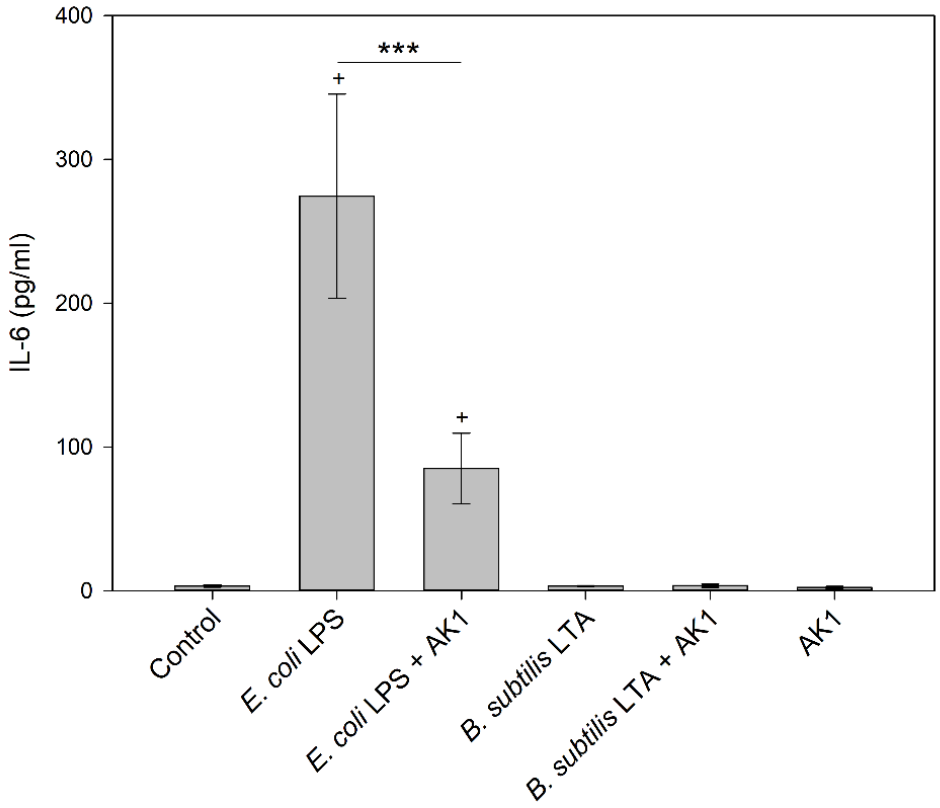


Figure 5.19. IL-6 secretion from macrophages stimulated with TLR agonists for 24 hours.

IL-6 measured by ELISA in supernatant of THP1-derived macrophages stimulated for 24 hours with *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) with or without AK1 (10 µM). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). + indicates $p<0.05$ compared to control, *** $p<0.001$, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

There was no detectable IL-8 in the supernatants of macrophages stimulated with *E. coli* LPS or *B. subtilis* LTA for 0.5 hours, with or without pre-treatment with AK1 and controls. At 4 hours (Figure 5.20) IL-8 was detectable in samples treated with *E. coli* LPS or *B. subtilis* LTA. Macrophages pre-treated with AK1 before stimulation with *E. coli* LPS or *B. subtilis* LTA produced significantly lower levels of IL-8 ($p=0.04$ for both). No IL-8 was detected in controls and samples treated with AK1 alone. At 24 hours (Figure 5.21), IL-8 was detectable in all samples. Stimulation with *E.* coli LPS or *B. subtilis* LTA led to statistically significant increase in IL-8 compared to controls. Pre-treatment with AK1 before stimulation with *E.* coli LPS or *B. subtilis* LTA resulted in comparably lower levels of secreted IL-8 ($p=0.009$ and $p=0.043$ respectively).

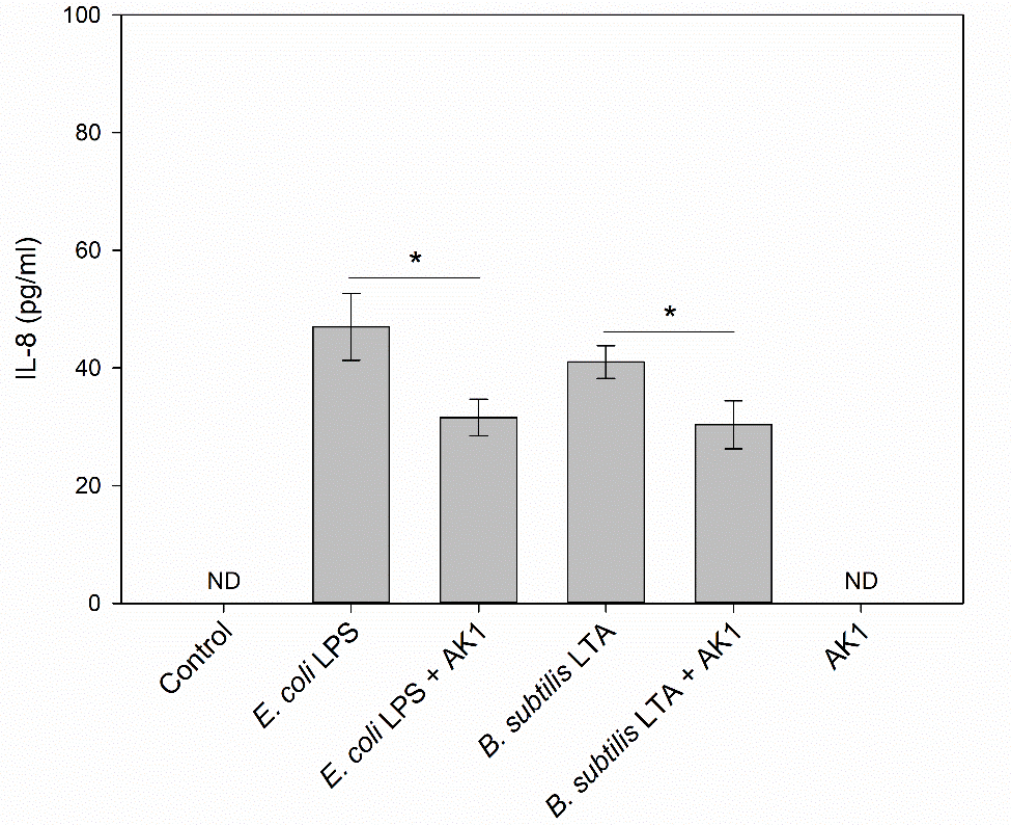


Figure 5.20. IL-8 secretion from macrophages stimulated with TLR agonists for 4 hours.

IL-8 measured by ELISA in supernatant of THP1-derived macrophages stimulated for 4 hours with *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) with or without AK1 (10 µM). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). * p=0.04, calculated by one-way ANOVA, followed by Tukey's post-hoc test.

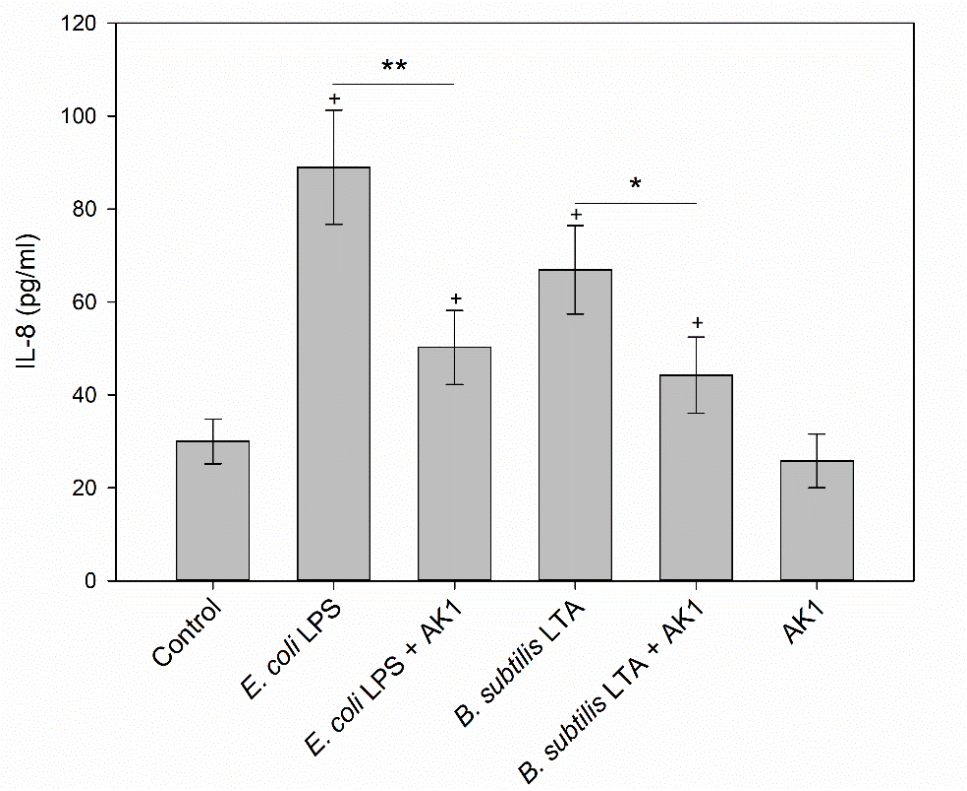


Figure 5.21. IL-8 secretion from macrophages stimulated with TLR agonists for 24 hours.

IL-8 measured by ELISA in supernatant of THP1-derived macrophages stimulated for 24 hours with *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) with or without AK1 (10 µM). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). + indicates $p<0.05$ compared to control, ** $p=0.009$, * $p=0.043$ calculated by one-way ANOVA, followed by Tukey's post-hoc test.

There was no detectable IL-12 in the supernatants of macrophages stimulated with *E. coli* LPS or *B. subtilis* LTA for 0.5 hours, with or without pre-treatment with AK1 and controls. Stimulation of macrophages with *E. coli* LPS and *B. subtilis* LTA for 4 hours, with or without pre-treatment with AK1 (Figure 5.22) resulted in detectable levels of IL-12. Pre-treatment with AK1 had no significant effect on IL-12 secretion when compared to macrophages that were not treated with AK1. No IL-12 was detected in the supernatant of macrophages with treated with AK1 alone or controls. Twenty-four hours of stimulation with *E. coli* LPS and *B. subtilis* LTA with and without pre-treatment with AK1 (Figure 5.23) resulted in a statistically significant increase in IL-12 secretion compared with unstimulated controls ($p<0.001$). Pre-treatment with AK1 had no significant effect on IL-12 secretion when compared to macrophages that were not treated with AK1. Treatment of macrophages with AK1 alone had no effect on IL-12 secretion compared with controls.

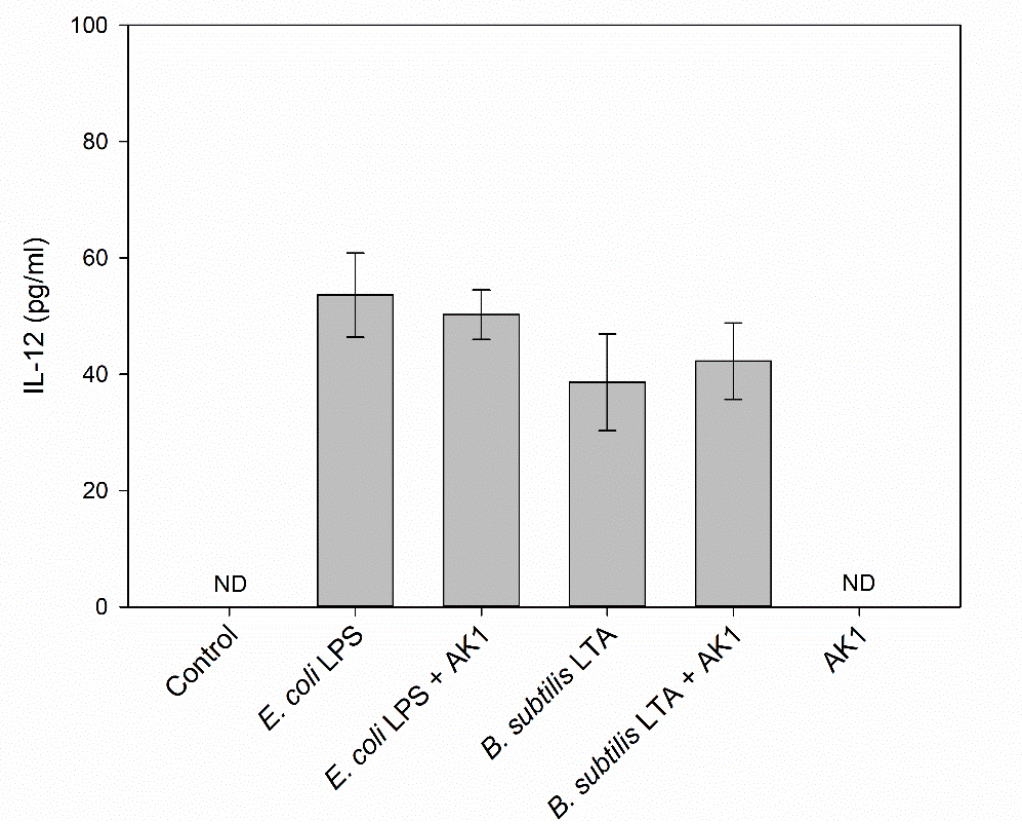


Figure 5.22. IL-12 secretion from macrophages stimulated with TLR agonists for 4 hours. IL-12 measured by ELISA in supernatant of THP1-derived macrophages stimulated for 4 hours with *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) with or without AK1 (10 µM). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). Analysed by one-way ANOVA.

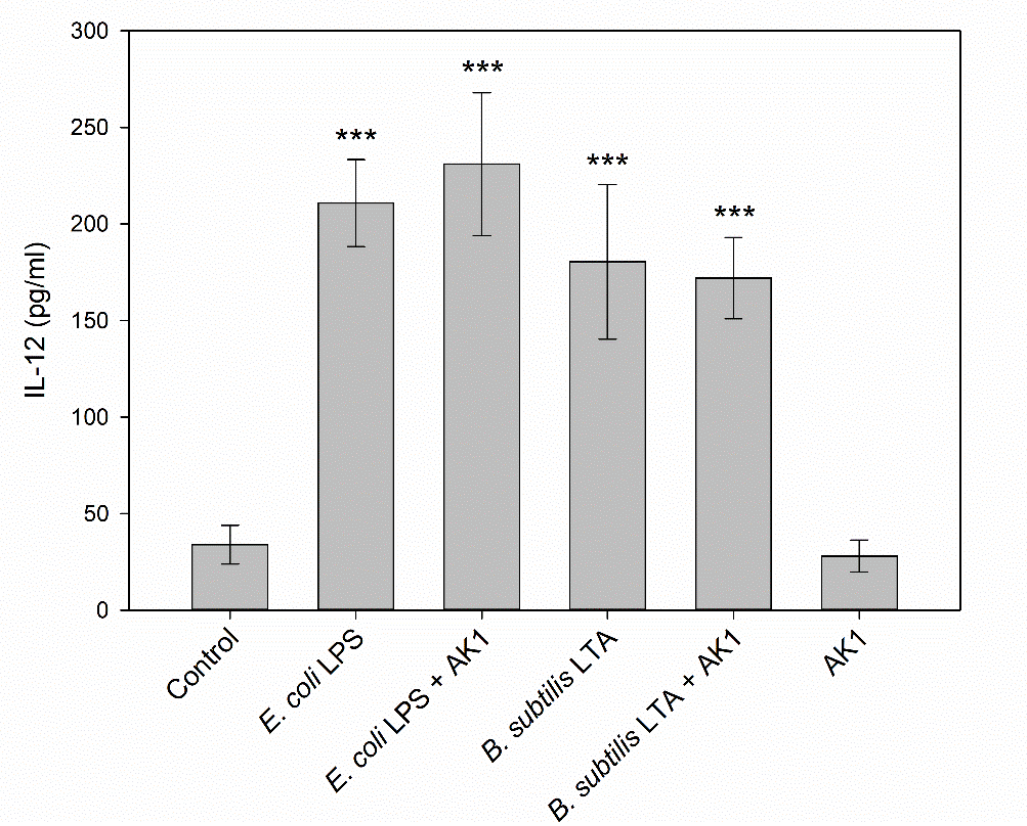


Figure 5.23. IL-12 secretion from macrophages stimulated with TLR agonists for 24 hours.

IL-12 measured by ELISA in supernatant of THP1-derived macrophages stimulated for 24 hours with *E. coli* LPS (100 ng/ml) or *B. subtilis* LTA (100 ng/ml) with or without AK1 (10 µM). Controls were unstimulated. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 2 separate experiments (N=6). *** $p<0.001$ calculated by one-way ANOVA, followed by Tukey's post-hoc test.

5.6 Discussion

We have shown that changes to α-tubulin acetylation can be analysed using quantitative western blotting and that the acetylation of α-tubulin K40 is altered after inhibition of NAMPT using the inhibitor FK866 (Figures 5.4). Thus, FK866 indirectly inhibits SIRT2 activity through depletion of NAMPT-derived NAD (Skoge *et al.* 2014). α-tubulin acetylation is also reduced after direct inhibition of SIRT2 with AK1 (Figures 5.6). Western blotting is a method that has been widely used to assess protein acetylation (North *et al.* 2003a; Rothgiesser *et al.* 2010b; Rigby *et al.* 2012). With this method, we were subsequently able to show that α-tubulin acetylation was not affected by the TLR2 agonist *B. subtilis* LTA or the TLR4 agonist *E. coli* LPS after 4 hours of stimulation (Figure 5.7) or 24 hours of stimulation (Figure 5.8). This may indicate that SIRT2 activity is unaffected by TLR2 and TLR4 activation. Whilst the histone deacetylase HDAC6 can also target acetyl-α-tubulin K40 for deacetylation, it has been demonstrated that HDAC6 has a preference for free tubulin dimers that have not yet assembled into a microtubule structure (Skultetyova *et al.* 2017) and there is evidence that during inflammasome activation in murine macrophages, SIRT2 and not HDAC6 is responsible α-tubulin deacetylation (Misawa *et al.* 2013). Acetyl-α-tubulin K40 has been used in many other studies as a measure of SIRT2 activity (North *et al.* 2003a; Spiegelman *et al.* 2018). There is little in the published literature regarding the effect of TLR2 and TLR4 signalling on SIRT2 activity, however, one study did find that SIRT2 deacetylation of tumour suppressor protein p53 was elevated in extracted rat heart homogenate after administration of LPS, indicating reduced activity of SIRT2. However, this study used a high concentration of LPS, with 12.5 μg/kg/day being administered for 14 days (Katare *et al.* 2020).

We intended to assess changes to acetylation of NF-κB p65, another target of SIRT2 (Yuan, Xu et al. 2016), however we were unable to obtain antibodies that produced single bands, meaning we could not reliably quantify NF-κB p65 (Figure 5.9). The multiple bands observed may be due to the structure of the NF-κB proteins. The NF-κB family of proteins has five members, all of which share a Rel homology domain (Oeckinghaus and Ghosh 2009) and combine into 15 different dimers, which could explain why we observed multiple bands in western blots targeting NF-κB p65, although, as all samples were boiled 100 ˚C for 5 minutes before being loaded into the SDS gel, the dimerisation should have been disrupted. Optimisation of sample boiling, and trials of alternative antibodies may have allowed us to develop a method suitable for the detection of NF-

κB p65, however, as we already had a workable readout of SIRT2 activity in the form of acetyl-α-tubulin K40, it was decided that this would not be pursued further.

We next investigated the role of SIRT2 in regulating the secretion of proinflammatory cytokines in response to TLR agonists. To achieve this, we utilised the SIRT2-specific inhibitor AK1 to reduce SIRT2 deacetylase activity (as shown in Figure 5.6), allowing us to measure the effect of SIRT2 inhibition on cytokine secretion in response TLR2 and TLR4 activation. Western blotting was used to confirm significant differences in the acetylation levels of α-tubulin K40 in controls and macrophages stimulated with LPS or LTA for 0.5, 4, or 24 hours with or without AK1 pre-treatment (Figures 5.10, 5.11 and 5.12). Pre-treatment with AK1 caused a statistically significant increase to α-tubulin K40 acetylation, indicating that the deacetylation activity is not altered by TLR2 or TLR4 agonists. Samples that were treated with AK1 alone and left in plain growth medium for 24 hours (Figure 5.12) showed no significant change to α-tubulin acetylation. It is unclear why no change to α-tubulin acetylation was observed in the 24-hour AK1 alone samples, as significant changes occurred at the 0.5 hour or 4 hour time points. AK1 was selected instead of FK866 due its specificity for SIRT2, as depletion of cellular of cellular NAD+ through the use of FK866 may have resulted in additional unwanted effects within the cell.

My results suggest that SIRT2 is involved in regulating the cytokine response of THP1-derived macrophages to TLR2 and TLR4 agonists. SIRT2 regulates the secretion of TNFα, IL-1β and IL-6 upon TLR2 and TLR4 activation and the secretion of IL-8 upon TLR4 activation. We have also shown that SIRT2 is not involved in regulating the secretion of IL-12 upon activation of TLR2 or TLR4.

Inhibition of SIRT2 (and therefore increased acetylation of its cellular targets) resulted in significantly lower levels of secreted TNFα in response to TLR2 and TLR4 agonists (Figures 5.13 and 5.14), indicating that SIRT2 is involved in regulating the synthesis or secretion of TNFα. After 24 hours of stimulation, SIRT2 inhibition had no effect on TNFα (Figure 5.15) despite measuring increased levels of acetylated α-tubulin K40 in these samples. TNFα is a pro-inflammatory cytokine produced readily by monocytes and macrophages during inflammation where it leads to activation of transcription factors and protein kinases, as well as many other proteins (Idriss and Naismith 2000). TNFα is also able to induce osteoclastic activity and suppress osteoblastic activity,

as well as promote secretion of other pro-inflammatory cytokines (Bostrom *et al.* 1998; Noh *et al.* 2013; Pan *et al.* 2019a) and as such is a contributing factor to bone resorption (Zhao 2017). Activation of TLR2 and TLR4 is widely known to induce TNFα secretion (Inoue *et al.* 2018; Vargas-Hernandez *et al.* 2020).

The mechanism through which SIRT2 regulates the secretion of TNFα is not apparent from these experiments. Transcription of TNFα is regulated on numerous levels and can vary depending upon the triggering stimuli. Interestingly, acetylation of histone H3 and histone H4 at the TNFα promoter region has been correlated with TNFα transcription in monocytes, macrophages, and the T cell lineage (Falvo *et al.* 2010). Additionally, inhibition of non-sirtuin HDACs leads to globally increased acetylation of histones, along with an increased ability to produce TNFα, demonstrating epigenetic regulation of TNFα expression (Sullivan *et al.* 2007). LPS stimulation has been found to increase acetylation of histone 4 at the TNFα promoter and enhancer regions, and histone 3 acetylation at the TNFα enhancer regions (Sullivan *et al.* 2007). This may be relevant as certain lysine residues within histones 3 and 4 can be deacetylated by SIRT2, however, the study in question did not determine if these changes to acetylation occurred on residues which may be targeted by SIRT2. A study in a mouse model of obesity found that acetylation of histone 3 lysine 18, a target of SIRT2, and acetylation of histone 3 lysine 9 was elevated at the *TNFA* gene in obese mice (Mikula *et al.* 2014). As my findings have demonstrated that inhibition of SIRT2, and therefore increased acetylation, led to decreased TNFα secretion, it may not be the action of SIRT2 of on histone acetylation that is regulating TNFα secretion as the literature discussed here suggests that we would see increased secretion.

NF-κB signalling can also lead to TNFα transcription (Collart *et al.* 1990; Page *et al.* 2018; Rothschild *et al.* 2018). As SIRT2 can deacetylate NF-κB p65 at lysine 310, this is another possible method for regulation TNFα secretion. SIRT2 deficiency was found to regulate NF-κB-dependent gene expression in mouse embryonic fibroblasts, demonstrating that SIRT2-mediated changes to acetylation of NF-κB p65 at lysine 310 can regulate gene expression (Rothgiesser *et al.* 2010b), but it remains unclear if this includes TNFα. Acetylation of NF-κB p65 at lysine 310 is required for full transcriptional activity of p65, but does not affect its affinity for DNA binding or the NF-κB-inhibiting protein IκBα (Yeung *et al.* 2004; Calao *et al.* 2008).

Ultimately, the exact mechanism through which inhibition of SIRT2 reduces TNFα secretion remains unclear, however, the specificity of the chosen SIRT2 inhibitor and experiments to confirm there was no cytotoxicity (Figure 5.5) support the finding that SIRT2 does regulate secretion of TNFα after stimulation with TLR2 and TLR4 agonists and that this is mediated by acetylation of target proteins.

In contrast to the data for TNFα (as well as IL-6 and IL-8), inhibition of SIRT2 resulted in significantly elevated levels of secreted IL-1β in response to TLR2 and TLR4 agonists after 24 hours (Figure 5.16). IL-1β is a major pro-inflammatory cytokine which is transcriptionally regulated by NF-κB p65, cJUN, ATF2 and IRF5 (Cohen 2014). IL-1β acts to induce secretion of other pro-inflammatory cytokines and induce the expansion of Th1 and Th2 T-helper cells (Ben-Sasson *et al.* 2009) and IL-1β levels are elevated in in the GCF of patients with periodontitis (Gilowski *et al.* 2014). Whilst we have shown that SIRT2 regulates the secretion of IL-1β, the exact mechanism cannot be determined from the experiments we have conducted here.

In a mouse model of collagen-induced arthritis, SIRT2 was found to regulate expression of IL-1β at the mRNA and protein level through deacetylation of NF-κB p65 lysine 310 and SIRT2 knockout mice displayed increased IL-1β mRNA and protein levels in mice immunised with collagen (Lin *et al.* 2013b), which is consistent with my finding that SIRT2 is a negative regulator of IL-1β expression.

Hyperacetylation of histones induced by the HDAC inhibitor suberoylanilide hydroxamic acid has been found to lead to increased IL-1β expression in macrophages after stimulation with LPS and IFN-γ (Dong *et al.* 2020). Although suberoylanilide hydroxamic acid does not act upon the sirtuin family of enzymes (only class I, II and IV HDACs), these findings do suggest that IL-1β expression can be regulated at the epigenetic level by increasing acetylation and that there are enzymes in addition to SIRT2 that can facilitate this.

The regulation of IL-1β is also complicated by IL-1β being present in the cytosol in its inactive form, termed pro-IL-1β (Netea *et al.* 2010), meaning changes to secretion could be due to increased activation of pro-IL-1β or changes to transcription. One study utilising the non-sirtuin HDAC inhibitor suberoylanilide hydroxamic acid and its derivative ITF2357 found that these inhibitors did not affect the synthesis and intracellular localisation of IL-1β but prevented

exocytosis of secretory lysosomes containing IL-1β by causing disruption to the network of intracellular microtubules (Carta *et al.* 2006). The inhibitors used did not act upon any of the sirtuin family of enzymes, but still demonstrates that secretion of IL-1β can be regulated by the deacetylation of microtubules, something SIRT2 is capable of (North *et al.* 2003a), and may be worthy of further investigation.

We found that stimulation with LPS led to increased secretion of IL-6, with lower levels of secretion observed upon inhibition of SIRT2, whilst stimulation with LTA caused no change to IL-6 secretion compared to controls (Figures 5.18 and 5.19), indicating the SIRT2 is involved in regulating expression of IL-6. IL-6 is a pro-inflammatory cytokine which can activate T and B cells and has been associated with multiple autoimmune diseases (Tanaka *et al.* 2014) and is elevated in the GCF of patients with periodontitis (Pan *et al.* 2019a). It has been reported that stimulation of THP1 monocytes with TLR2 agonists leads to increased secretion of IL-6 (Flynn *et al.* 2019). We did not observe this effect in THP1-derived macrophages, which may be due to differences caused by the differentiation process or alternatively by the different agonists being used, with Flynn, Garbers et al. utilising heat killed *Listeria monocytogenes* as a TLR2 agonist. This method of activating TLR2 is potentially not as specific as utilising a synthetic or purified agonist, as the heat killed *L. monocytogenes* will also contain other components that may activate additional TLRs and other receptors.

SIRT2 has been found to regulate IL-6 expression in mouse embryonic fibroblasts (MEFs). MEFs deficient in SIRT2 were found to express elevated levels of IL-6 mRNA after stimulation with TNFα compared with controls (Rothgiesser *et al.* 2010b). A mouse model of collagen-induced arthritis utilised SIRT2-knockout mice and determined that knockout of SIRT2 led to increased IL-6 secretion (Lin *et al.* 2013b). Trichostatin A, an inhibitor of non-sirtuin HDACs has been found to increase NF-κB p65 acetylation at lysine 310 (Sato *et al.* 2013). NF-κB p65 K310 can also be targeted for deacetylation by SIRT2, meaning there is potential for SIRT2 to also regulate IL-6 secretion via this mechanism, however, my results showed that reduced activity of SIRT2 led to reduced IL-6 secretion, so it is unclear if this is the case.

Secretion of IL-8 from THP1-derived macrophages was found to be regulated by SIRT2 after stimulation with TLR2 and TLR4 agonists, with inhibition of SIRT2 leading to lower levels of IL-8

(Figures 5.20 and 5.21) indicating the SIRT2 is involved in regulating expression of IL-8. IL-8 is elevated in the GCF of patients with periodontitis, with clinical parameters correlating with IL-8 levels (Lagdive *et al.* 2013), and has been shown to facilitate the transit of neutrophils from the gingival tissue into the gingival crevice (Tonetti *et al.* 1998). Both TLR2 and TLR4 stimulation have previously been shown to lead to secretion of IL-8 (Hirao *et al.* 2000; He *et al.* 2013). Changes to histone acetylation have been found to regulate IL-8 expression (Gilmour *et al.* 2003; Angrisano *et al.* 2010). IL-8 expression can also be regulated by NF-κB, including the p65 subunit (Jundi and Greene 2015). There are reports in the published literature that suggest IL-8 levels increase when acetylation of histone 3 and histone 4 is elevated (Gilmour *et al.* 2003; Angrisano *et al.* 2010). This is somewhat contradictory to my finding that inhibition of SIRT2, and therefore increased acetylation, led to reduced secretion of IL-8. Again, due to the differences between my work and the published literature including differing cell types, agonists, and my focus on SIRT2 alone, mean that we cannot draw firm conclusions.

Stimulation with TLR2 and TLR4 agonists resulted in significantly increased levels of secreted IL-12, with inhibition of SIRT2 causing no change to this (Figures 5.22 and 5.23). IL-12 is significantly elevated in periodontitis (Sanchez-Hernandez *et al.* 2011) and while its exact role remains unclear, it has also been associated with rheumatoid arthritis, another disease displaying inflammation-induced bone loss (Issaranggun Na Ayuthaya *et al.* 2018). Whilst IL-12 is involved in the differentiation of naïve T helper cells into the Th1 type (Trinchieri 2003), IL-12 has also been found to induce expression of indoleamine-pyrrole 2,3-dioxygenase (IDO) which reduces T cell functionality (Nelp *et al.* 2018). Levels of IL-12 are significantly higher in the GCF of patients with periodontitis than patients with gingivitis or healthy individuals (Tsai *et al.* 2005), thus it seems that IL-12 may be involved in balancing the immune response in periodontitis. We observed no changes to IL-12 expression after inhibition of SIRT2, however, findings obtained in SIRT2 knockout mice indicate that IL-12 production after stimulation with LPS is lower than controls (Jung *et al.* 2015). NF-κB p65 is involved in transcription of IL-12 (Sanjabi *et al.* 2000; Liu *et al.* 2005) and it has also been established that IL-12 expression can be regulated by histone acetylation mediated by p300 or HDAC1 (Lu *et al.* 2005). Therefore, it is possible that SIRT2 is involved in the secretion of IL-12 after stimulation with TLR2 or TLR4 agonists, but this is not reflected in my findings and further investigation is required.

My results show that SIRT2 is involved in regulating the secretion of IL-1β, IL-8 and TNFα after TLR2 and TLR4 activation and the secretion of IL-6 after TLR2 activation. Despite the similarities between the TLR2 and TLR4 signalling pathways, activation of these pathways can result in different effects, as demonstrated by my results, and supported by the literature (Jones *et al.* 2001; Varadaradjalou *et al.* 2003; Grassin-Delyle *et al.* 2020).

The majority of studies looking at the role of SIRT2 suggest that it acts to limit cytokine secretion, however, there is also evidence to the contrary, where inhibition of SIRT2 decreased the severity of "cytokine storms" in a mouse model of septic shock (Zhao *et al.* 2015; Li *et al.* 2018). Much more investigation is required to fully understand the role of SIRT2 and clarify some of the conflicting findings that exist within the literature, however, the role of SIRT2 in regulating the secretion of proinflammatory cytokines is undoubtedly worthy of further research.

Another consideration is the regulation of secretory pathways by SIRT2. There is evidence that SIRT2 interacts with proteins involved in membrane trafficking and secretory processes, with subsequent knockdown of SIRT2 causing changes to the expression of proteins involved in membrane trafficking and the extracellular matrix protein, tenascin (Budayeva and Cristea 2016). More recently, evidence has been published that SIRT2 regulates cargo-loading and release of extracellular vesicles, with knockout of SIRT2 leading to an increase in the number of extracellular vesicles (Lee *et al.* 2019).

Many of the findings within the currently published literature that have investigated the role of SIRT2 in the regulation of cytokine expression and secretion have utilised SIRT2 knockout animals or cell lines. The process of generating a SIRT2 knockout animal or cell line is undoubtedly useful in research, however, it is much more drastic than chemically inhibiting SIRT2 as we have done. Whilst it is relatively simple to determine if the correct gene has been knocked out, the process of generating a knockout strain can also introduce flanking gene effects and genetic background effects (Wolfer *et al.* 2002; Eisener-Dorman *et al.* 2009). The exact mechanisms of these effects are beyond the scope of discussion here, but put simply, can cause an observable phenotype due to genetic remnants of the knockout generation process (Wolfer *et al.* 2002). Additionally, knockout animal or cell line will have developed with no functioning SIRT2 which may have unknown consequences. SIRT2 knockout mice have been shown to spontaneously develop

tumours (Kim *et al.* 2011) and to suffer from cardiac hypertrophy due to disruption to the transcription factor nuclear factor of activated T-cells (NFAT) (Sarikhani *et al.* 2018). Conversely, the THP1-derived macrophages used in my experiments may not behave in the exact same manner as primary human or murine macrophages. The THP1 cell line is best considered as a simplified model of primary human macrophages that is useful experimentally but may not be entirely representative (Bosshart and Heinzelmann 2016; Tedesco *et al.* 2018). Ultimately, research will require the use of both animal and cellular models to fully understand the role of SIRT2.

Chapter 6. Evaluation of SIRT2 as a Salivary Biomarker for Periodontitis

6.1 Introduction

Preliminary analysis of saliva samples from healthy individuals and patients with periodontitis showed that there were higher levels of SIRT2 in samples from patients with periodontitis. Fifteen saliva samples from healthy individuals and 15 saliva samples from patients with periodontitis were sent to Olink® Proteomics who analysed the samples using proximity extension analysis (PEA) for a panel of 92 inflammation related proteins. Of these 92 proteins, SIRT2 was the most highly elevated in periodontitis.

SIRT2 is known to be involved in the regulation of aspects of the immune response such as phagocytosis (Ciarlo *et al.* 2017), regulation of NF-kB p65-mediated pro-inflammatory gene expression (Rothgiesser *et al.* 2010b), macrophage polarisation (Lo Sasso *et al.* 2014) and regulation of microvascular inflammation in sepsis (Buechler *et al.* 2017). I have also shown SIRT2 is involved in the regulation of cytokine secretion after stimulation with TLR2 and TLR4 agonists (Chapter 5.5), with the secretion of TNFα, IL-1β, IL-6, and IL-8 being regulated by SIRT2, and these cytokines are known to be relevant to periodontitis (Lagdive *et al.* 2013; Pan *et al.* 2019a). I have demonstrated a novel pathway of SIRT2 secretion after stimulation with TLR2 agonists (Chapter 4.2) and whilst it is not yet clear if this pathway is present in other cell types, it may provide an explanation for the elevated levels of SIRT2 observed in the saliva of patients with periodontitis.

PEA has previously been successfully used to analyse saliva samples of patients with oral squamous cell carcinoma and precancerous lesions (Scholtz *et al.* 2020) as well as saliva samples from patients with inflammatory bowel disease (Majster *et al.* 2020). SIRT2 has been analysed by PEA in the serum of patients with systemic lupus erythematosus (Petrackova *et al.* 2017) and those with rheumatoid arthritis (Panezai *et al.* 2020), and was found to be elevated in both diseases which are characterised by inflammation and an immune response targeted against self-antigens, leading to tissue damage and destruction. SIRT2 has been considered as a potential biomarker sepsis and septic shock, where SIRT2 mRNA levels in PBMCs are significantly lower than healthy individuals (Xu *et al.* 2020). Using PEA, SIRT2 in the serum of patients with periodontitis has also been found to correlate with periodontal parameters (Panezai *et al.* 2017). In a very recent study, SIRT2 has also been measured in the saliva of patients with periodontitis

using ELISA. Comparison of SIRT2 levels in the saliva of 16 patients with aggressive periodontitis compared against a healthy control group containing 43 individuals showed that SIRT2 was elevated in the saliva of patients with periodontitis, however, there are some caveats regarding the validity of this study that will be discussed later (Kluknavska *et al.* 2021). Levels of visfatin, the enzyme responsible for NAD production, which subsequently fuels SIRT2 activity, are also elevated in the saliva of patients with periodontitis although the biological significance of this finding and how it may relate to SIRT2 levels in saliva remains unclear (Tabari *et al.* 2014). Taken with the previously discussed findings, it is clear that SIRT2 is involved in regulation of the immune response, but until very recently there was no published evidence directly linking SIRT2 with periodontitis and no studies on the possible role of SIRT2 as a diagnostic biomarker for periodontitis.

Thus, I began with the aim of furthering our understanding of the relationship between SIRT2 and periodontitis to determine if SIRT2 may be a viable salivary biomarker for periodontitis. Our preliminary data had already shown that relative levels of SIRT2 were higher in the saliva of patients with periodontitis, and led to the following aims:

- Evaluate the performance of SIRT2 ELISA kits when analysing saliva samples to determine if this method would allow us to confirm the findings obtained by PEA and obtain absolute quantities of SIRT2 present in saliva.
- Use established ELISA method to expand my investigations to include additional saliva samples from healthy individuals and patients with periodontitis, allowing us to increase the sample number and determine the absolute quantities present in those samples as PEA only measured relative levels of proteins.
- Utilise statistical methods to conduct a more robust analysis of the relationship of SIRT2 and periodontitis to evaluate if SIRT2 levels in saliva are significantly higher in patients with periodontitis whilst controlling for the effect of age.
- Evaluate the diagnostic ability of SIRT2 levels to distinguish between patients with periodontitis and healthy individuals and to investigate the relationship between SIRT2 levels in saliva and periodontal parameters in patients, to identify how SIRT2 levels may relate to clinical measures of disease progress that are currently used for the diagnosis of periodontitis.

6.2 Preliminary Analysis of Salivary SIRT2 Levels in Health and Periodontitis

Prior to the beginning of the study, saliva samples were collected from 56 healthy individuals and 65 patients with periodontitis. All participants were adult males or females aged between 18 and 65 with a minimum of 20 natural teeth (excluding 3rd molars) and were non-smokers. Details of the clinical studies and full methods for saliva collection are described in Methods section 2.8. At the time of saliva collection, clinical measures of periodontal health were also obtained including mean BOP, mean MGI, mean PPD, mean CAL, PESA and PISA, along with participant age.

Fifteen of these saliva samples from the healthy group and 15 saliva samples from the periodontitis group were sent to Olink® Proteomics (olink.com) who analysed these 92 inflammation-related proteins using PEA (Appendix C). The protein targets included in this panel are listed in Appendix C, Table 1. This preliminary study aimed to identify novel candidate biomarkers that may exhibit altered levels in the saliva of patients with periodontitis. PEA is a multiplex assay that provides relative quantification of proteins presented in the log2-transformed arbitrary unit normalised protein expression, meaning normalised protein expression values for different targets cannot be directly compared (Appendix C).

PEA analysis found that from the 92 inflammation-related proteins quantified, SIRT2 was the most highly elevated in saliva samples from periodontitis patients in comparison to similar samples form healthy individuals. Statistical analysis of SIRT2 normalised protein expression showed that SIRT2 was significantly higher ($p<0.001$) in the saliva of patients with periodontitis (Figure 6.1).

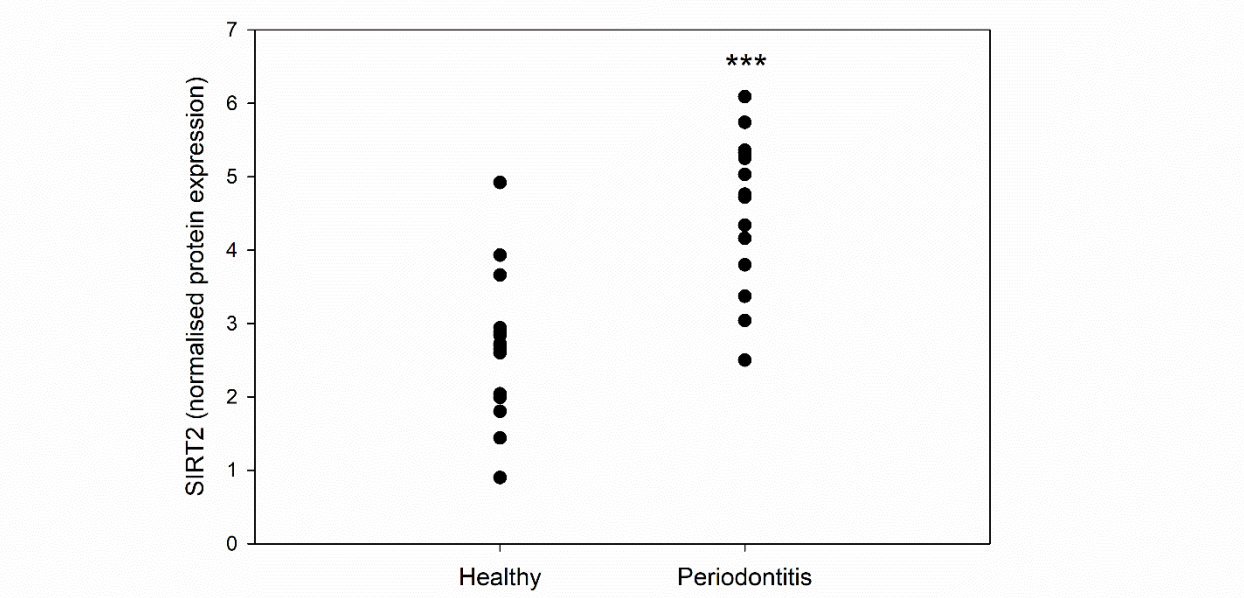


Figure 6.1. SIRT2 normalised protein expression obtained from saliva.

Saliva samples from 15 healthy individuals and 15 patients with periodontitis were analysed for 92 different inflammation-related proteins by proximity extension assay, with SIRT2 being the most highly elevated. *** $p<0.001$ by Student's t test.

6.3 Validation of a SIRT2 ELISA for Saliva Analysis

I first set out to confirm the findings obtained by PEA that SIRT2 is elevated in the saliva of patients with periodontitis (Figure 6.1) before expanding my analysis to include additional saliva samples. To achieve this, I planned to use an ELISA for SIRT2 which would provide several benefits over PEA. Firstly, the use of ELISA would provide absolute quantities of SIRT2 in saliva, whereas PEA only produces relative quantities. Secondly, ELISA provides a more efficient platform for the analysis of additional saliva samples as it analyses the protein of interest alone, rather than including the other 91 proteins that are in the PEA panel and there is no uniplex PEA service is available.

No ELISA kit for SIRT2 was available that was validated for analysis of saliva; thus, the kit produced by Abcam was selected as it was the most extensively utilised kit for analysis of SIRT2 within the published literature (Wei *et al.* 2018; Kluknavska *et al.* 2021). To ensure that my analysis of SIRT2 in additional saliva samples was robust, a spike recovery experiment was first conducted to determine if quantification of SIRT2 in saliva would be accurate using the chosen ELISA kit and to determine if the kit had the required sensitivity to detect the difference in SIRT2 levels detected by PEA (Jaedicke *et al.* 2012).

To conduct a spike recovery experiment, a standard curve was prepared according to the manufacturer's instructions (Method section 2.2.2). The standard curve ranged from 1000 pg/ml to 15.6 pg/ml. The recombinant protein standard supplied with the kit was also used to spike saliva samples. Using the measured level of SIRT2 detected in the spiked saliva samples and unspiked saliva samples, the recovery can be calculated with the following equation:

$$\text{Recovery \%} = \frac{\text{spiked sample} - \text{unspiked sample}}{\text{amount spiked}} \times 100$$

This was done for multiple 2-fold dilutions of saliva, ranging from neat to a 1:4 dilution. The recovery for neat saliva was 50.9%, recovery from a 1:2 dilution was 53.82%, and recovery from a 1:4 dilution was 48.35% a (Figure 6.2). This means that measured levels of SIRT2 in saliva would be approximately 50% lower than the true value. The accepted range for recovery is typically >80% (Jaedicke *et al.* 2012; Andreasson *et al.* 2015).

The low recovery of this ELISA kit indicates that some component of saliva may be interfering with the recovery of the assay and dilution of the saliva did not dilute the interfering component enough to increase assay recovery. This is likely due to differences in the physical and/or biochemical properties of saliva compared to serum or culture supernatants, which are validated for use with the assay. Properties of saliva such as pH, viscosity and proteolytic enzyme content can all affect assay recovery (Jaedicke *et al.* 2016). The low recovery of this ELISA kit means it was unsuitable for my needs and would have resulted in poor accuracy of SIRT2 quantification, which may have influenced statistical analysis and led to incorrect conclusions being drawn.

In the light of the results of these experiments, analysis was not expanded to include addition saliva samples.

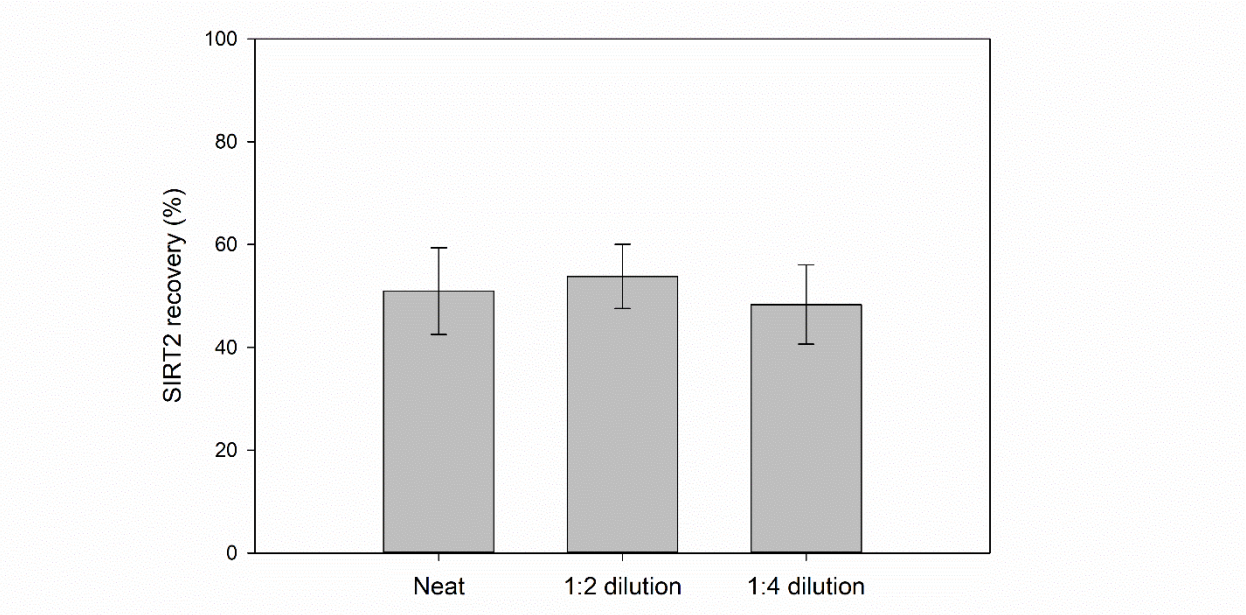


Figure 6.2. SIRT2 recovery from spiked saliva samples measured by ELISA.

Values plotted are mean SIRT2 recovery % ± SD. Three independent samples were spiked to a concentration of 200 pg/ml with recombinant SIRT2 supplied with the ELISA kit. N=3 for each dilution.

6.4 Investigation of the Influence of Age on the Association Between SIRT2 and Periodontitis

Preliminary assessment and descriptive statistics were utilised which identified the average age of participants in the periodontitis group as being higher than the average age of participants in the healthy group (Table 6.3).

The age difference between the healthy and periodontitis groups (Table 6.3) was found to be statistically significant (p=0.002), with mean ages of 29.3 years in the healthy group and 42.5 years in the periodontitis group.

Both the prevalence of periodontitis and its severity are known to increase with age (Lopez *et al.* 2017). Within the literature, age has been widely cited as a confounding factor, with one systematic review finding that 86.3% of evaluated studies included age as confounding factor (Natto *et al.* 2018). For these reasons, I considered age as a confounding factor in statistical analysis going onwards.

Status	Age (years)	SIRT2 (NPX)	BOP (%)	MGI	PPD (mm)	CAL (mm)	PESA (mm^2)	PISA (mm^2)
Healthy	40	3.66	0	0.09	1.46	1.46	778.60	0.00
	27	2.65	-	-	-	-	-	-
	26	2.84	2	0.05	1.54	1.54	792.00	21.90
	37	4.92	0	0.04	1.65	1.65	871.50	0.00
	20	1.80	0	0.43	1.76	1.76	772.70	6.10
	21	2.88	9	0.53	1.74	1.74	910.30	115.20
	23	2.72	9	0.32	1.50	1.50	790.10	79.50
	52	2.70	0	0.60	1.21	1.21	641.20	0.00
	20	2.94	3	0.27	1.52	1.52	821.10	36.20
	21	1.99	-	-	-	-	-	-
	54	2.60	4	0.52	1.77	1.77	927.70	45.60
	20	1.44	0	0.78	1.83	1.83	953.90	0.00
	20	0.90	0	0.37	1.26	1.26	669.00	0.00
	33	3.93	-	-	-	-	-	-
	25	2.04	6	0.25	1.97	1.97	870.00	52.60
Periodontitis	49	5.36	75	2.93	3.45	5.96	2052.10	1557.50
	35	5.74	61	2.78	3.77	4.50	2005.00	1282.80
	51	6.09	48	2.67	2.50	4.10	1356.60	690.00
	44	5.25	57	2.56	3.69	5.35	2050.80	1502.00
	35	4.34	40	2.58	3.48	5.03	1810.70	811.50
	43	5.03	32	2.50	2.35	3.73	1311.40	572.70
	42	5.33	51	2.76	3.19	3.98	1617.90	856.40
	54	3.80	53	2.14	2.63	3.86	1604.40	955.10
	44	3.37	41	2.65	2.97	3.85	1422.10	706.10
	50	3.04	82	2.79	4.32	5.64	2439.50	2078.20
	49	3.37	32	2.80	3.59	5.56	2174.90	1199.10
	46	2.50	48	3.17	3.10	5.37	1776.00	977.60
	30	4.72	85	3.27	3.58	3.69	2046.60	1775.80
	20	4.16	64	2.61	6.99	5.09	2365.70	1515.90
	45	4.76	17	1.88	3.03	4.72	1952.70	489.20

Table 6.2. Measured parameters of 15 healthy individuals and 15 patients with periodontitis included in this study.

SIRT2=SIRT2 normalised protein expression (NPX), BOP=mean bleeding on probing, MGI=mean modified gingival index, PPD=mean probing pocket depth, CAL=mean clinical attachment loss, PESA=periodontal epithelial surface area, PISA=periodontal inflamed surface area.

Healthy			Periodontitis			
	Mean	**SD**		**Mean**	**SD**	**p-value**
Age (years)	29.3	11.6	Age (years)	42.5	9.0	0.0016
SIRT2 (NPX)	2.7	1.0	SIRT2 (NPX)	4.5	1.1	<0.001
BOP (%)	2.8	3.5	BOP (%)	52.4	19.1	<0.001
MGI	0.4	0.2	MGI	2.7	0.3	<0.001
PPD (mm)	1.6	0.2	PPD (mm)	3.5	1.1	<0.001
CAL (mm)	1.6	0.2	CAL (mm)	4.7	0.8	<0.001
PESA (mm^2)	816.5	96.8	PESA (mm^2)	1865.8	349.2	<0.001
PISA (mm^2)	29.8	37.7	PISA (mm^2)	1131.3	473.5	<0.001

Table 6.3. Mean and SD for each periodontal parameter obtained from healthy participants (N=15) and periodontally diseased participants (N=15).

Indicated p-values indicated are comparing the periodontitis cohort with the healthy cohort. The significance threshold was α=0.05. P-values were calculated by Student's t-test for age and SIRT2. P-values for BOP, PPD, CAL, PESA, and PISA were calculated by Mann-Whitney U test due to non-normal distribution.

I next wanted to determine if there was any correlation between SIRT2 normalised protein expression in the saliva of healthy participants and age. Utilising the dataset including SIRT2 normalised protein expression and periodontal parameters from 15 healthy individuals and 15 patients with periodontitis (Table 6.2). Normalised protein expression data generated by PEA is a log2-transformed arbitrary unit, calculated from Ct values, and normalised against internal controls to minimise intra-assay variation and reduce background to approximately zero (Assarsson *et al.* 2014; Olink 2021b).

Due to non-normal distribution and ties when periodontal parameter data was ranked, Kendall's tau b rank correlation analysis was carried out as this method makes adjustments for tied ranks (Ma 2012) and has been found to be slightly more robust than Spearman's rank correlation (Croux and Dehon 2010). Kendall's tau b rank correlation is a non-parametric test based on the number of inversions of the rankings (Howell 2012). A significant p-value indicates that there is a correlation between the two indicated parameters. The correlation coefficient ranges from -1 to +1, with 1 indicating a perfect correlation and – or + indicating whether the correlation is positive or negative.

Kendall's tau b correlation analysis was also conducted on SIRT2 normalised protein expression and age data from healthy participants. Analysis showed there was no significant correlation between SIRT2 and age (correlation coefficient 0.489, p=0.064).

I next wanted to understand the relationship between SIRT2 and periodontal health status in greater detail. To determine if SIRT2 normalised protein expression in saliva was significantly different between healthy and periodontitis groups, analysis of covariance (ANCOVA) was conducted. In regression analyses, multiple independent variables can be included, with one main predictor variable (in this case, periodontal status) and the others as covariates (age). ANCOVA allows for these covariates to be accounted for, allowing us to evaluate the effect of the main independent variable after being adjusted for the effect of the covariate. The assumptions of ANCOVA are as follows: homogeneity of variance should not be violated (i.e. both comparison groups have the same variance), the residuals for both groups should be normally distributed, there should also be homogeneity of regression of regression slopes (Field 2013; Mishra *et al.* 2019).

To ensure that no assumptions of ANCOVA were violated, Levene's test of equality of error variances was conducted and indicated equal variances between group residuals, $F(1, 28)=0.672$, $p=0.42$. Residuals were normally distributed as determined using Shapiro-Wilk test for normality ($p=0.986$). Including the interaction of participant age and group (healthy or periodontitis) in the ANCOVA showed that no assumptions of homogeneity were violated as the p value was not significant ($p=0.175$).

ANCOVA (Table 6.4) showed that there was a significant difference in SIRT2 levels between healthy and periodontitis groups before controlling for age, $F(1, 28)=22.587$, $p<0.001$. There remained a significant difference in SIRT2 levels between groups after controlling for age, $F(1, 26)=5.625$, $p=0.025$, with a partial eta squared value (η_p^2) of 0.178 between healthy and periodontitis groups. The η_p^2 ranges from 0 to 1 and indicates effect size, so a η_p^2 of 0.178 indicates that 17.8% of the variability in SIRT2 levels is accounted for by group classification (healthy or periodontitis).

The effect of the covariate, age, alone had no statistically significant effect on SIRT2 levels, $F(1, 26)=0.348$, $p=0.560$. Thus, the results show that SIRT2 levels are significantly different between healthy individuals and patients with periodontitis and that age has no significant effect on SIRT2 levels in saliva.

Source	Type III Sum of Squares	df	Mean Square	F	p-value	Partial Eta Squared
Corrected Model	26.978[a]	3	8.993	8.710	.000	.501
Intercept	20.511	1	20.511	19.867	.000	.433
Group	5.808	1	5.808	5.625	.025	.178
Age	.360	1	.360	.348	.560	.013
Group * Age	2.006	1	2.006	1.943	.175	.070
Error	26.842	26	1.032	-	-	-
Total	434.527	30	-	-	-	-
Corrected Total	53.820	29	-	-	-	-

Table 6.4. ANCOVA of the relationship between SIRT2 and periodontitis.

ANCOVA of SIRT2 normalised protein expression in saliva of healthy individuals (15) and patients with periodontitis (15), when controlling for the influence of age. a=R^2 0.501 (adjusted R^2 0.444). Threshold for significance was α=0.05.

6.5 Evaluation of Salivary SIRT2 as a Diagnostic Measure for Periodontitis

ROC curves were constructed to investigate the ability of salivary SIRT2 to discriminate between healthy and periodontitis cases. ROC curves plot sensitivity (true positive rate) against 1-specificity (false positive rate). The area under the curve (AUC) is then calculated and can range from 0 (absolute inaccuracy) to 1.0 (perfect diagnostic ability) (Mandrekar 2010). An AUC of 0.5 is indicated by a diagonal line and represents a 50% chance of successful discrimination.

I utilised the SIRT2 normalised protein expression data obtained by PEA (Table 6.2) to carry out ROC curve analysis. The AUC for SIRT2 normalised protein expression in saliva (Figure 6.3) was calculated to be 0.89 (95% confidence interval 0.7677-1.010, p=0.00028), indicating an 89% chance of successfully distinguishing between healthy and periodontitis cases. Mean BOP, mean MGI, and mean PPD each had an AUC calculated to be 1, as they were used to classify cases, they perfectly predict disease in this dataset.

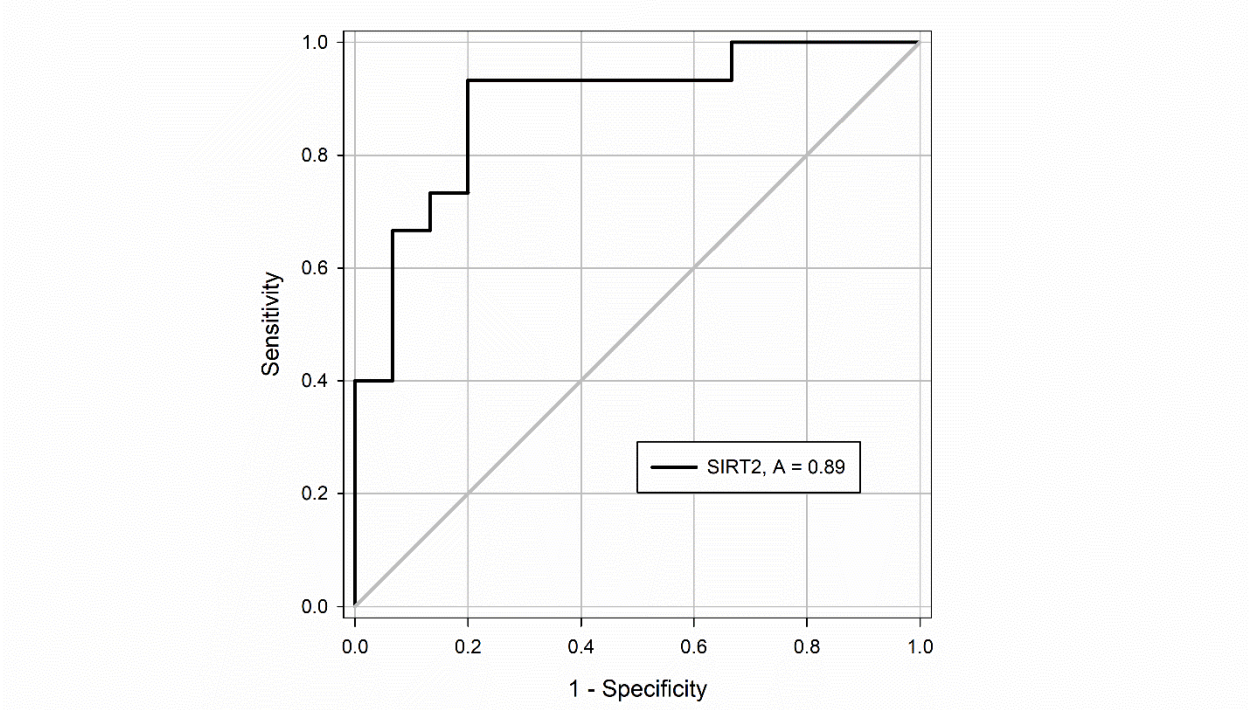


Figure 6.3. ROC curve for SIRT2 normalised protein expression in saliva.

Sensitivity plotted against 1-specificity for salivary SIRT2 normalised protein expression measured by PEA, demonstrating the capacity to discriminate between healthy cases and periodontitis cases. AUC=0.89, 95% confidence interval 0.7677-1.010, p=0.00028. N=30.

6.6 Investigation of the Relationship Between Salivary SIRT2 and Periodontal Parameters

To further understand the relationship between SIRT2 and the periodontal parameters we had previously measured, a correlation analysis was conducted. Again, due to ties when periodontal parameter data was ranked, Kendall's tau b rank correlation analysis was carried out on data obtained from patients with periodontitis. Parameters included were SIRT2 normalised protein expression, mean BOP, mean MGI, mean PPD, mean CAL, PESA and PISA. Missing values were excluded pairwise.

Kendall's tau b correlation analysis of parameters obtained from patients with periodontitis (Table 6.5) indicated that there were no significant correlations between SIRT2 and any of the measured clinical parameters of periodontitis.

		Age	BOP	MGI	PPD	CAL	PESA	PISA
SIRT2	Correlation Coefficient	-0.087	0.058	-0.096	-0.096	-0.153	-0.134	-0.115
	P-value	0.654	0.766	0.620	0.620	0.428	0.488	0.552
	N	15	15	15	15	15	15	15

Table 6.5. Correlation analysis between SIRT2 periodontal parameters.

Kendall's tau b correlation analysis was carried out on the ranked values for SIRT2, and the other clinical parameters obtained from patients with periodontitis, including BOP, MGI, PPD, CAL, PESA and PISA. Threshold for significance was α=0.05.

6.7 Discussion

In this chapter I aimed to investigate the relationship between SIRT2 and periodontitis to determine if it may be a viable salivary biomarker for periodontitis. To the best of my knowledge, my analysis of SIRT2 in saliva is the most thorough to date in terms of statistical analysis methods, looking at levels of SIRT2 in the saliva in periodontitis and healthy individuals, conducting ROC curve analysis to evaluate diagnostic ability and correlation analysis between SIRT2 levels and periodontal parameters.

Utilising the SIRT2 normalised protein expression data obtained by PEA carried out by Olink® Proteomics, analysis using ANCOVA showed that SIRT2 was significantly higher in the samples of periodontitis patients than individuals with periodontal health, even after accounting for age (Chapter 6.4). SIRT2 has previously been associated with ageing, both in the regulation of lifespan (Cosentino and Mostoslavsky 2014) and as a therapeutic target for certain age-related disorders such as Parkinson's disease and Huntington's disease (de Oliveira *et al.* 2012). There were significant age differences between my group of healthy individuals and my group of periodontitis patients. Ideally, in future studies this could be controlled for by age-matching participants in each group during the recruitment process. However, in the present study, ANCOVA showed that age was not associated with SIRT2 levels in the saliva.

ROC curve analysis of salivary SIRT2 detected periodontitis with a sensitivity and specificity (Figure 6.3) indicating that SIRT2 may be a good biomarker for this disease. Bostanci *et al.* evaluated multiple candidate biomarkers for periodontitis in 36 healthy individuals and 60 patients with periodontitis, many of the biomarkers assessed had similar performance to that of SIRT2 in ROC curve analysis (AUC=0.89). The best performing biomarkers evaluated by Bostanci *et al.* were HGF (AUC=0.97), MMP2 (AUC=0.96), MMP9 (AUC=0.95), MMP8 (AUC=0.92), and IL-1β (AUC=0.89) (Bostanci *et al.* 2021). Combinations of biomarkers were also evaluated, with the ratio of MMP9 to TIMP1 (AUC=0.98) and the ratio of MMP8 and MMP9 to TIMP1 (AUC=0.98), demonstrating only slightly better discriminatory ability than individual biomarkers (Bostanci *et al.* 2021). In other studies, IL-1β, IL-6 and MMP-8 have consistently been found to have an AUC >0.904. with the highest AUC being 0.984, indicating that they are highly accurate when discriminating periodontitis from healthy cases (Ebersole *et al.* 2013; Taylor *et al.* 2019a; Balogun *et al.* 2020). In summary, there are numerous biomarkers that have been found to be highly accurate at

discriminating between healthy cases and periodontitis cases, and whilst SIRT2 performs comparably with these biomarkers which are discussed above, it does not outperform them. Additionally, many of these studies did not take age of participants into account, which may mean it is more difficult to compare results directly.

Whilst the ROC curve analysis shows that SIRT2 has sufficient discriminatory ability to be potentially useful for distinguishing patients with periodontitis from those who are periodontally health, Kendall's tau b rank correlation analysis did not identify any correlations between SIRT2 and the measured periodontal parameters in patients with periodontitis (Table 6.4). It is possible that this may indicate that SIRT2 levels in saliva reflect the current disease activity or inflammation, rather than historic disease activity that is reflected by clinical examination and can require time to accumulate (Taylor 2014). In other words, SIRT2 may correlate with other pathways involved in the processes of periodontitis or with other outcomes of the disease. However, given the limited sample size of this study, caution should be shown when interpreting correlation data. Increasing our understanding of the mechanisms that lead to SIRT2 being elevated in the saliva of patients with periodontitis may also help us to better determine its usefulness and better interpret how SIRT2 levels relate to disease processes. Correlation analysis of other biomarkers for periodontitis, such as MMP-8, have previously been found to correlate with certain clinical parameters of periodontitis. There is evidence showing MMP-8 in the saliva correlates with BOP, probing pocket depth (Mauramo *et al.* 2018; Taylor *et al.* 2019a), PISA and PESA (Taylor *et al.* 2019b). Similar associations have been seen of IL-1β and IL-6 with pocket depth and BOP (Ebersole *et al.* 2015; Zhang *et al.* 2021b).

From the literature, it is clear that salivary biomarkers with good ability to discriminate between healthy and periodontitis cases, as well as correlating with disease parameters have been identified and are present in saliva.

My results have demonstrated that SIRT2 is significantly elevated in the saliva patients with periodontitis, regardless of age, whilst ROC curve analysis has demonstrated that salivary SIRT2 is able to accurately discriminate between health and periodontitis to a level comparable of that with other candidate biomarkers for periodontitis. However, due to SIRT2 showing no correlation

with periodontal parameters, its usefulness may be limited until we have a full understanding of what SIRT2 levels in saliva represent at a biological level.

SIRT2 in the serum of patients with periodontitis has been found to correlate with periodontal parameters BOP and marginal bone loss for molars and pre-molars (Panezai *et al.* 2017). It is possible that there are differences in the pathways through which SIRT2 enters the serum and saliva respectively, and for reasons related to the specific mechanisms of these pathways, SIRT2 levels in the serum reflect certain periodontal parameters whilst SIRT2 levels in saliva do not. Alternatively, it is possible I did not identify any correlation between SIRT2 in the saliva and clinical measures of periodontitis due to the limited sample size, whilst the study published by Panezai *et al.* 2017 included samples from 69 patients with periodontitis, providing their analysis with greater resolution than my own.

A study looking at periodontal disease in patients with rheumatoid arthritis did find that, compared to healthy individuals, SIRT2 levels were significantly elevated in the sera of patients suffering from both periodontitis and rheumatoid arthritis, but not in patients with periodontitis alone (Panezai *et al.* 2020), despite correlating with periodontal parameters (Panezai *et al.* 2017). It was also found that patients with both periodontitis and rheumatoid arthritis had significantly elevated serum levels of inflammatory mediators such as IL-18, colony stimulating factor 1 (CSF1), CX3CL1, and CXCL5 (Panezai *et al.* 2020). Taking into consideration the discussed findings from within the literature and my own findings, there is evidence to support the concept that elevated levels of SIRT2 are associated with inflammatory disease, but with more research required to understand the limits in terms of which collection fluid is most suitable (serum or saliva) for analysis of SIRT2 in respect to disease and to understand if there is any biological purpose for the presence of SIRT2 in the serum or saliva.

I initially intended to confirm the findings obtained by PEA that SIRT2 normalised protein expression is elevated in patients with periodontitis (Figure 6.1) using commercially available ELISA kits. As there was no SIRT2 ELISA kit available that was validated for analysis of saliva, I began by evaluating the suitability of the SIRT2 ELISA kit produced by Abcam for saliva analysis. Recovery experiments showed the ELISA kit produced by Abcam did not have acceptable recovery when analysing saliva samples, with 50.9% recovery for neat saliva (Figure 6.2). Acceptable

recovery is typically considered to be between 80-120%. The low recovery is likely due to interfering components present in saliva, or physical properties such as viscosity or pH (Jaedicke *et al.* 2012). I attempted to obtain an alternative kit but only one was available, which was also not validated for use with saliva, and did not arrive due to Covid-related production and shipping delays.

One recently published study has shared findings matching my data showing that SIRT2 is elevated in the saliva of patients with periodontitis (Kluknavska *et al.* 2021), however they used the same SIRT2 ELISA kit produced by Abcam that I have demonstrated is unsuitable for analysis of saliva and did not report conducting any validation of the assay or alterations to the protocol. The protocol used for saliva collection also did not differ from that which we have previously used for saliva collection, and whilst it is possible that the recovery of their samples may have been higher due to natural variations in the components of saliva, the lack of validation adds some question to the accuracy and precision of their measured values of SIRT2. The study conducted by Kluknavska *et al.* included 101 participants who were divided into a control group for healthy individuals, or the appropriate group based on diagnosis of gingivitis, chronic periodontitis, or aggressive periodontitis. Due to the number of groups, the chronic periodontitis group contained 23 individuals, with the control group containing 43 individuals. Comparison of gingivitis (17 participants) and aggressive periodontitis (16 participants) groups with my own data cannot be justified due to the significant differences between these conditions and chronic periodontitis. It was observed by Kluknavska *et al.* that SIRT2 levels were also elevated in the saliva of patients with gingivitis and those with aggressive periodontitis, but chronic periodontitis patients had the greatest elevation compared to healthy controls. No additional investigations were made into any correlations between SIRT2 levels and periodontal parameters or other salivary markers of inflammation (Kluknavska *et al.* 2021).

I have demonstrated the ability of SIRT2 to discriminate between healthy and periodontitis cases in a relatively limited sample (Figure 6.3). To date, no biomarker for periodontitis has moved into routine use for clinical diagnosis, despite multiple candidates having shown promise (Preshaw 2015; Haririan *et al.* 2021). The potential usefulness of a salivary biomarker could be even more valuable if it is able to reflect changes to disease activity as they happen, predict disease outcomes or reflect treatment effectiveness (Cafiero *et al.* 2021). For a biomarker to transition into clinical

use it will need substantial investigation and regulatory qualification from a governing body such as the Medicines and Healthcare products Regulatory Agency, European Medicines Agency or Food and Drug Administration (Sauer *et al.* 2018). It would require an understanding of the relationship between the biomarker levels and disease severity, along with an understanding of the impact that other factors such as age, gender, medical history may have on biomarker levels (Doust 2010). This would then allow for normal reference ranges to be established. An assay would need to be standardised upon to allow for robust measurements to be obtained and to ensure consistent analytical performance. There have been some promising developments for point of care testing methods (Taylor *et al.* 2019a; Steigmann *et al.* 2020) that may make regular use of a biomarker more practical. Additionally, an assessment would likely need to be conducted to determine if using the given biomarker for diagnosis would lead to improved health outcomes and if its use would be cost effective (Doust 2010) and justify the changes to the training and guidance supplied to oral healthcare practitioners.

There is little published information on other members of the sirtuin family of enzymes as biomarkers for periodontitis. One study found that serum levels of SIRT1 increased after periodontal treatment (Caribé *et al.* 2020). The previously discussed study by Kluknavska *et al.* also investigated SIRT1 levels in saliva in gingivitis, chronic periodontitis and aggressive periodontitis and found there were no significant changes to SIRT1 in any group when compared with healthy controls (Kluknavska *et al.* 2021). There are mechanistic links between members of the sirtuin family and ageing, both with longevity (Grabowska *et al.* 2017) and development of age-related disease (Elibol and Kilic 2018). Evidence in mouse models points towards SIRT1 being neuroprotective in Huntington's disease and Parkinson's disease, with some evidence suggesting the SIRT2 may also have neuroprotective effects (Zhao *et al.* 2020). Members of the sirtuin family have been found to be linked to cardiovascular disease and regulation of normal cellular activity within the heart (Chen *et al.* 2012; Zhao *et al.* 2020). Due to NAD-dependency of the sirtuins, they are also linked to diabetes and metabolic disease (Kitada *et al.* 2019; Zhao *et al.* 2020), where they have been targeted therapeutically, particularly SIRT1, SIRT3, and SIRT6 (Huynh *et al.* 2013). The exact relationship between the sirtuin family of enzymes and ageing is still an ongoing area of research with much left to be elucidated that may contribute to our understanding and treatment of age-related diseases.

Systemic markers of inflammation such as TNFα and IL-6 increase with age (Singh and Newman 2011; Reynolds 2014), accompanied by other changes to the immune system that occur with age, such as reductions in the number of naïve T cells and memory B cells (Aiello *et al.* 2019). This has resulted in the concept of "inflammaging", where chronic low-grade inflammation develops with age and contributes to many of age-related disorders such as cardiovascular disease, diabetes mellitus, sarcopenia, dementia, and cancer (Ferrucci and Fabbri 2018). Additionally, an older individual with periodontitis may have more extensive damage to their periodontal tissues simply as a function of disease duration. There are also reports of changes to fibroblast populations and collagen deposition with age, which may alter the balance between collagen deposition and breakdown, along with reduced bone density affecting the alveolar bone (Huttner *et al.* 2009). Age-related changes to fibroblast populations may also contribute to immune dysregulation, with evidence showing increased production of inflammatory mediators in response to LPS in aged gingival fibroblasts (Preshaw *et al.* 2017). Overall, as we increase our understanding of how the immune system changes with age, we reveal more evidence that these changes could contribute to the pathogenesis of periodontitis (Ebersole *et al.* 2016; Preshaw *et al.* 2017). Currently, there is no published research investigating how SIRT2 may contribute to age-related changes to immune system and how this may relate to periodontitis but given the established role of SIRT2 in regulating aspects of the immune response, there is potential for a link to exist, although much more research is required.

Chapter 7. General Discussion

Severe periodontitis affects an estimated 743 million people worldwide, making it the sixth most common disease, with the global burden of periodontitis increasing by 57.3% between the years 1990 and 2010, and more mild forms of periodontitis affecting the majority of adults (Kassebaum *et al.* 2014; Listl *et al.* 2015; Jin *et al.* 2016b; Tonetti *et al.* 2017). Utilisation of a biomarker for periodontitis could significantly improve our ability to identify periodontitis at an early stage and allow for intervention to improve patient outcomes (Steigmann *et al.* 2020). The development of lab-on-a-chip (LOC) technology has greatly improved the ability of a biomarker for periodontitis to be utilised in a clinical setting and would also benefit underserved communities and developing nations (Cafiero *et al.* 2021). Regular treatment has been found to improve tooth retention in patients with periodontitis and biomarker may allow for earlier diagnosis of periodontitis, further increasing treatment effectiveness (Farina *et al.* 2021).

Despite the significant body of evidence supporting biomarkers, such as MMP-8 (Nędzi-Góra *et al.* 2021; Ramenzoni *et al.* 2021; Sorsa *et al.* 2021) and IL-1β (Caldeira *et al.* 2021; Kim *et al.* 2021a) for the diagnosis of periodontitis, no biomarker has yet been adopted for clinical use. Regulatory qualification from a governing body (Sauer *et al.* 2018) and assessment as to whether use of a biomarker for diagnosis would lead to improved health outcomes, as well as proving to be cost effective (Doust 2010) can contribute towards hindering adoption of a clinical biomarker. Thus, it can be said that a candidate biomarker faces additional challenges beyond whether it is able to accurately diagnose disease. However, identifying additional biomarkers for periodontitis remains to be of value, both for aiding diagnosis and identifying additional molecules that may contribute to the disease processes of periodontitis and potential therapeutic targets (Jaedicke *et al.* 2016; Cafiero *et al.* 2021). Modern developments to proteomics such as PEA and mass spectrometry-based methods have greatly increased our capacity to identify new candidate biomarkers (Hartenbach *et al.* 2020; Rizal *et al.* 2020).

The COVID-19 global pandemic resulted in the suspension of routine dental treatment, and it is thought that this has had consequences for the oral health of affected populations (Coulthard *et al.* 2020; Ren *et al.* 2020). It has been suggested that a viable salivary biomarker for periodontitis could provide benefits in situations such as a global pandemic where dental services are

suspended, with the possibility of sample collection being carried out in a socially distanced manner or even from home (Räisänen *et al.* 2020; Sorsa *et al.* 2021). Samples could then be screened for the periodontal biomarker and potentially identify those who have developed periodontitis or have declining periodontal health (Räisänen *et al.* 2020). By directing screening towards those most at risk of developing periodontitis, monitoring of their periodontal health could be maintained whilst also minimising the risk of infection for both patients and dentists, however, such a care pathway would first require a suitable biomarker.

This study has shown that SIRT2 levels are elevated in the saliva of patients with periodontitis and are not influenced by age (Chapter 6.4) and that SIRT2 levels can discriminate between health and disease with a high sensitivity and specificity (Chapter 6.5). However, SIRT2 does not correlate with any periodontal parameters, such as BOP or PPD, in patients with periodontitis (Chapter 6.6). This study has also shown that SIRT2 regulates secretion of pro-inflammatory cytokines in response to TLR signalling (Chapter 5.5) and the SIRT2 is secreted from macrophages after stimulation with TLR2 agonists (Chapter 4.2). Thus, it can be concluded that SIRT2 is a viable diagnostic biomarker for periodontitis and that SIRT2 may contribute to periodontitis through the regulation of pro-inflammatory cytokine secretion and through currently unidentified mechanisms related to the secretion of SIRT2 itself. Increasing our understanding of the role in SIRT2 could provide new opportunities for therapeutic intervention.

In macrophages, deacetylation of NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome is mediated by SIRT2 and using a cellular model of age-associated inflammation it was found that dysregulation of deacetylation of NLRP3 may be an origin for age-associated inflammation (He *et al.* 2020). Whilst in mice, it was found that modulation of SIRT2 and the acetylation of NLRP3 could reverse inflammation in aged mice and also influenced glucose homeostasis (He *et al.* 2020). There is also a known role for SIRT2 in the regulation of macrophage polarisation (Lo Sasso *et al.* 2014) and phagocytic activity (Ciarlo *et al.* 2017). Together, the above roles of SIRT2 may be of relevance to periodontitis where age-related inflammation and a greater number of M1 macrophages are observed (Zhou *et al.* 2019).

To date, no study has investigated SIRT2 activity or tissue expression levels in patients with periodontitis. Given my findings that SIRT2 is involved in regulating the secretion of

proinflammatory cytokines that are relevant to the pathogenesis of periodontitis (Chapter 5.5), it may be possible that alterations to SIRT2 activity or expression could contribute to the dysregulation of the immune response seen in periodontitis (Hajishengallis and Chavakis 2021). Expression of SIRT2 has been found to be lower in the intestine of patients with inflammatory bowel disease which is thought to contribute to dysregulation of epithelial cell proliferation and differentiation (Li *et al.* 2020a). In a murine model of periodontitis, bacterial dysbiosis was found to lead to increased H3 histone acetylation (Martins *et al.* 2016) and the use of HDAC inhibitors has been found to reduce alveolar bone loss (Li *et al.* 2020b), but there is currently no evidence to suggest if there are any changes to SIRT2 expression or activity in periodontitis that may contribute to these processes. Within the literature, there is strong evidence to suggest that SIRT2 is a regulator of pro-inflammatory gene expression, although there is some conflicting evidence regarding whether it induces or inhibits expression (Lin *et al.* 2013b; Lee *et al.* 2014b; Fortuny and Sebastián 2021), and indeed in my findings, we saw inhibition of SIRT2 acting to inhibit secretion of some cytokines, whilst enhancing secretion of others. Further understanding of the role of SIRT2 regulating cytokine secretion may lead to opportunities to target SIRT2 therapeutically in order to regulate the immune response in periodontitis and other diseases. Inhibitors of SIRT2 are widely available, with numerous inhibitors being previously utilised in animal models (Luthi-Carter *et al.* 2010; Chowdhury *et al.* 2020; Yang *et al.* 2020) and inhibitors of SIRT1 have previously been utilised in clinical trials (Carafa *et al.* 2016).

One potential limitation of this study is perhaps the sample size of statistical analysis of SIRT2 in saliva (Chapter 6.3). Analysis of additional samples would have both increased sample size and provided quantitative measurements of SIRT2 levels in saliva. Increases in sample size mean that smaller effect sizes can be detected and increases the probability that a type II error (incorrectly accepting the null hypothesis) will be avoided (Uttley 2019), however as we detected the effect of periodontal health on SIRT2 levels, our sample size was perhaps not a major limitation. Future analysis of SIRT2 levels in the GCF and in serum may also provide some insight into how SIRT2 relates to disease mechanisms and the source of SIRT2 as GCF is a serum transudate, elevated serum levels of SIRT2 could in theory enter the GCF and subsequently elevate levels of SIRT2 in saliva (Subbarao *et al.* 2019; Fatima *et al.* 2021). It is interesting to note that SIRT2 has been found to be elevated in the serum of patients with rheumatoid arthritis who were also suffering from

periodontal disease compared to those with rheumatoid arthritis who were periodontally healthy and those who had only periodontitis (Panezai *et al.* 2020), but no investigations were made regarding levels of SIRT2 in GCF or saliva. In future studies, it may also be worthwhile to measure SIRT2 levels in the saliva of patients with periodontitis before and after treatment to gain a better understanding of how treatment of periodontitis affects SIRT2 levels. Additionally, conducting a longitudinal study to determine if SIRT2 levels in saliva are elevated before the onset of clinical symptoms of periodontitis, which may allow for earlier diagnosis before the presentation of symptoms.

The finding that SIRT2 is secreted from macrophages after stimulation with TLR2 agonists (Chapter 4.2) is novel within the currently published literature. Further investigation is needed, but if this pathway is found to exist *in vivo*, it could provide a pathway that may explain the elevated levels of SIRT2 present in the saliva of patients with periodontitis. Future investigations conducted using primary source monocytes/macrophages would be of value to help determine if this pathway of SIRT2 secretion may also be present *in vivo.* Investigations could also be conducted using additional cell types not of the monocyte/macrophage lineage to determine they also exhibit this pathway of SIRT2 secretion and gingival fibroblasts have been shown to express TLR2, although at lower levels than PBMCs (Moonen *et al.* 2019). If this pathway of SIRT2 secretion is found to exist beyond THP1-derived macrophages, experiments could be conducted *in vivo* to determine if the TLR2-mediated pathway of SIRT2 secretion can produce the elevated levels of SIRT2 seen in the saliva of patients with periodontitis.

When considering the findings of Panezai *et al.* that SIRT2 is elevated in the serum of patients with rheumatoid arthritis and periodontitis, but is not elevated in the serum of individuals who only suffer from one of these diseases (Panezai *et al.* 2020), there was no investigation into how SIRT2 came to be present in the serum, and it is interesting to note that many of the immunological processes involved in rheumatoid arthritis also contribute to periodontitis and there is an observed association between the two diseases (Rodríguez-Lozano *et al.* 2019), so it is worthy of note that the elevated levels of SIRT2 in the serum were only observed in patients with both diseases. SIRT2 has been found to be elevated in the serum of patients with systemic lupus erythematosus and is also elevated in the serum of patients with Parkinson's disease, where it also correlates with α-synuclein (the protein which aggregates to form the Lewy bodies seen in

Parkinson's disease) and was highly accurate at discriminating between health and Parkinson's disease (Singh *et al.* 2019), but again, no pathway was identified that led to elevated levels of SIRT2 in the serum. Ultimately, our understanding of how SIRT2 enters biological fluids such as saliva and serum is limited and increasing our knowledge of this process may lead to insights into disease processes and the immune response.

There is no known extracellular target for SIRT2, thus it is not clear what the biological function of secreted SIRT2 might be. Identifying if the TLR2-mediated pathway of secretion is present in cell types other than macrophages may provide some insight into a potential physiological role. There are three types of acetylation used to post-translationally modify proteins; N-terminal acetylation, lysine acetylation (also known as Nε-acetylation), and O-acetylation (Diallo *et al.* 2019). N-terminal acetylation is irreversible, whilst lysine-acetylation is reversible and is the type of acetylation that is mediated by SIRT2 (Huhtiniemi *et al.* 2010; Diallo *et al.* 2019). O-acetylation is also reversible and remains conserved in humans, but is less prevalent than lysine-acetylation (Diallo *et al.* 2019). Analysis of the human acetylome identified 3600 lysine acetylation sites on 1750 proteins (Choudhary *et al.* 2009; Choudhary and Mann 2020), and whilst not all of these sites will be targets of SIRT2-mediated deacetylation, it illustrates the scope of the investigation required to identify any potential targets of SIRT2 activity. The acetylation of histones is associated with increased gene transcription (Xia *et al.* 2020), whilst acetylation of non-histone proteins regulates protein half-life (Martínez-Balbás *et al.* 2000; Wu *et al.* 2020). Sites of NF-κB p65 polyubiquitination overlap with acetylation sites, meaning that acetylated p65 is resistant to ubiquitination, the process which would usually terminate p65-dependent transcription (Li *et al.* 2012).

In cardiac tissue from patients with aortic stenosis hyperacetylation of H3K27 (a target of SIRT2 deacetylation) was most significantly associated with genes involved in extracellular matrix structure and organisation, such as glycoproteins, collagens and proteoglycans, whilst hypoacetylation was mostly associated with gene expression and RNA processing (Pei *et al.* 2020). From the findings of Pei *et al.* it could perhaps be hypothesised that acetylation may be important in regulating ECM homeostasis in periodontitis also, where homeostasis is disrupted, and ECM degradation occurs (Kim *et al.* 2021b). However, in Parkinson's disease hyperacetylation of H3K27 was instead associated with genes that have previously been implicated in the disease (*SNCA,*

PARK7, and PRKN), whilst hypoacetylation of H3K27 was associated with *PTPRH* (Toker *et al.* 2021). Thus, acetylation state can have differential results based on the tissue and disease in question, and that is when considering acetylation of H3K27 alone, with many other targets of acetylation existing within the human acetylome. In a comparison of three cell lines, 60 to 80% of acetylated proteins and 60 to 75% of acetylation sites in a given cell line were also observed in the other two cell lines (Choudhary *et al.*, 2009), suggesting that it is only a fraction of proteins that are differentially acetylated, but this remains a significant number of possible proteins given the size of the acetylome. From the proteins identified in the acetylome, the highest number of acetylated proteins were associated with the processes of the cell cycle, followed by RNA splicing, and DNA damage repair (Choudhary *et al.*, 2009).

As SIRT2 is NAD-dependent, there is currently no evidence for any extracellular activity of SIRT2. Whilst extracellular NAD is an important signalling molecule (Gasparrini *et al.* 2021), it is currently unclear if extracellular NAD levels would be adequate to allow any function of SIRT2 outside the cell. Visfatin, the enzyme responsible for intracellular production of NAD that fuels SIRT2 activity, is also known to be elevated in the saliva of patients with periodontitis (Tabari *et al.* 2014). The most widely utilised pathway for NAD production in humans is the salvage pathway, where NAM produced by NAD-dependent enzymes is recycled and converted into NMN by visfatin (or NAMPT as it is known intracellularly) before conversion into NAD by the nicotinamide mononucleotide adenylyl transferase enzymes (Xie *et al.* 2020). Both visfatin and nicotinic acid phosphoribosyltransferase, another enzyme involved in NAD synthesis are present extracellularly, but there is currently no evidence to suggest they contribute to any extracellular synthesis of NAD (Gasparrini *et al.* 2021). Visfatin is also present in extracellular vesicles and is internalised into cells where it enhances NAD synthesis (Yoshida *et al.* 2019b), and extracellular vesicles have been found to be elevated in the GCF of patients with periodontitis (Chaparro Padilla *et al.* 2020). It would be interesting to investigate if SIRT2 was also present within these vesicles and internalised into cells alongside visfatin, providing the NAD required for SIRT2 activity. Perhaps supporting this idea, a recently published study has found that oligodendrocytes are able to enhance the metabolic activity of axonal cells through exosomal delivery of SIRT2 (Chamberlain *et al.* 2021). It is interesting to note that SIRT2 is the most highly expressed member of the sirtuin family of enzymes in myeloid cells and highly expressed in macrophages, with only mast cells

expressing higher levels (Ciarlo *et al.* 2017), macrophages may, therefore, be a source of SIRT2 that can be utilised by the nearby cellular environment through extracellular vesicle trafficking.

The role of SIRT2 and its involvement in inflammatory diseases, such as periodontitis, rheumatoid arthritis, and Parkinson's disease is complex and not yet fully understood. This is in part due to nuanced nature of SIRT2 activity and expression that is highly dependent on tissue type and cellular signalling pathways (Sundriyal *et al.* 2017; Chen *et al.* 2021). My findings have significantly contributed to the current knowledge on the role SIRT2 in the immune response and how this may relate to periodontitis. I have also produced robust research evaluating SIRT2 as a biomarker for periodontitis, demonstrating that levels are significantly elevated in the saliva of patients with periodontitis, and can accurately discriminate between instances of periodontal disease and periodontal health. MY findings will help inform future studies investigating the association between SIRT2 and periodontitis. Regardless of its potential as a biomarker, SIRT2 will be an important target future research as my findings, and the literature, have identified a significant role for SIRT2 in the regulation of the immune system, but there is currently a lack of understanding as to how this may contribute to disease pathology.

Appendices

Appendix A. Representative Standard Curves for ELISA

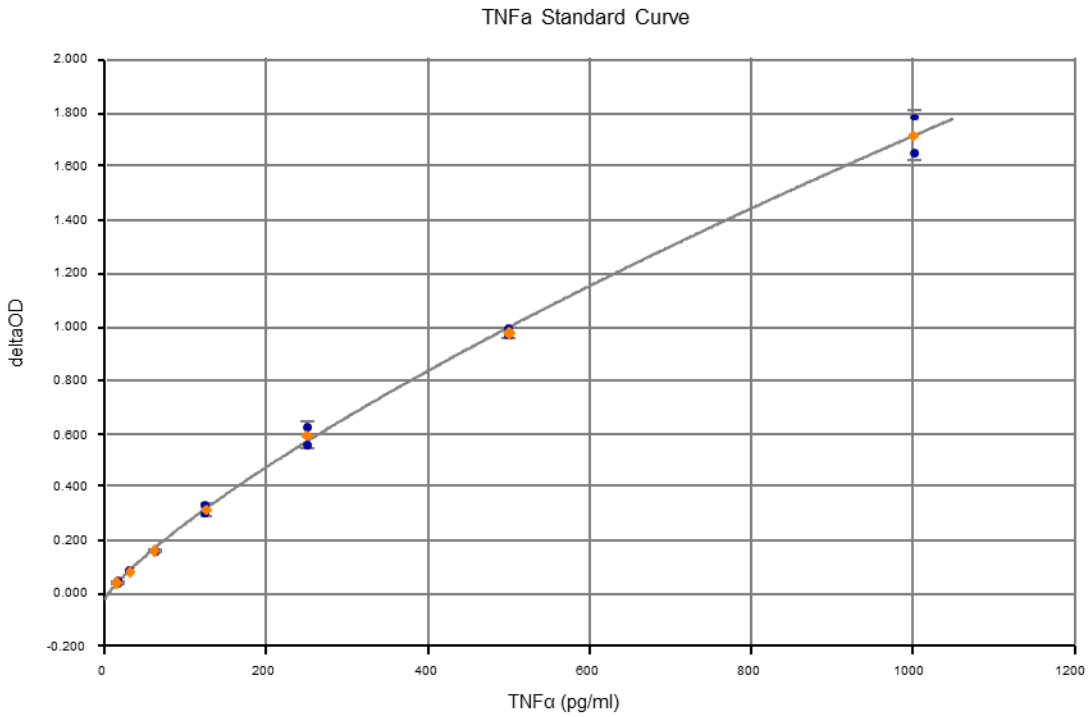


Figure A.1. Representative standard curve for TNFα ELISA.

deltaOD (OD 450 nm–OD 550 nm) was plotted against standard TNFα concentrations (0 – 1000 pg/ml). A 4-parameter curve fit was used to determine the unknown concentration of TNFα in samples. Data points represent triplicates of each concentration.

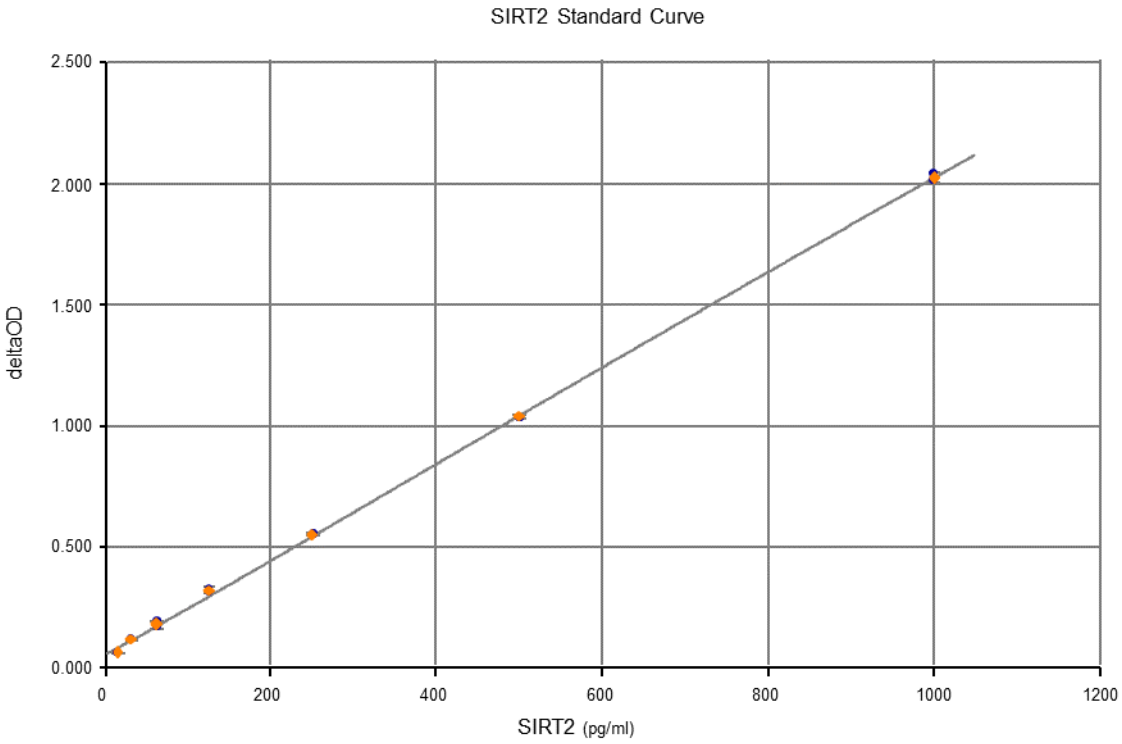


Figure A.2. Representative standard curve for SIRT2 ELISA.

deltaOD (OD 450 nm–OD 550 nm) was plotted against standard SIRT2 concentrations (0 – 1000 pg/ml). A linear trend line was fitted and used to determine the unknown concentration of SIRT2 in samples. Data points represent triplicates of each concentration.

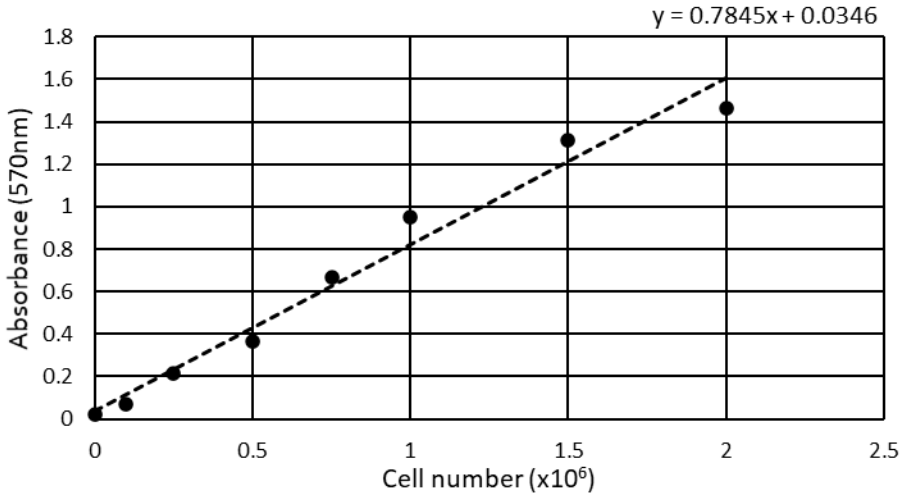


Figure A.3. Representative standard curve for MTT cell viability assay.

Absorbance (570 nm) was plotted against standard cell number (x10^6). A linear trend line was fitted and used to determine the cell number in THP1-derived macrophage cultures. Data points represent the mean of duplicate cultures. The equation of the trend line is shown.

Appendix B. Confirmation of Stimulation with TLR agonists for SIRT2 ELISA and western blotting experiments

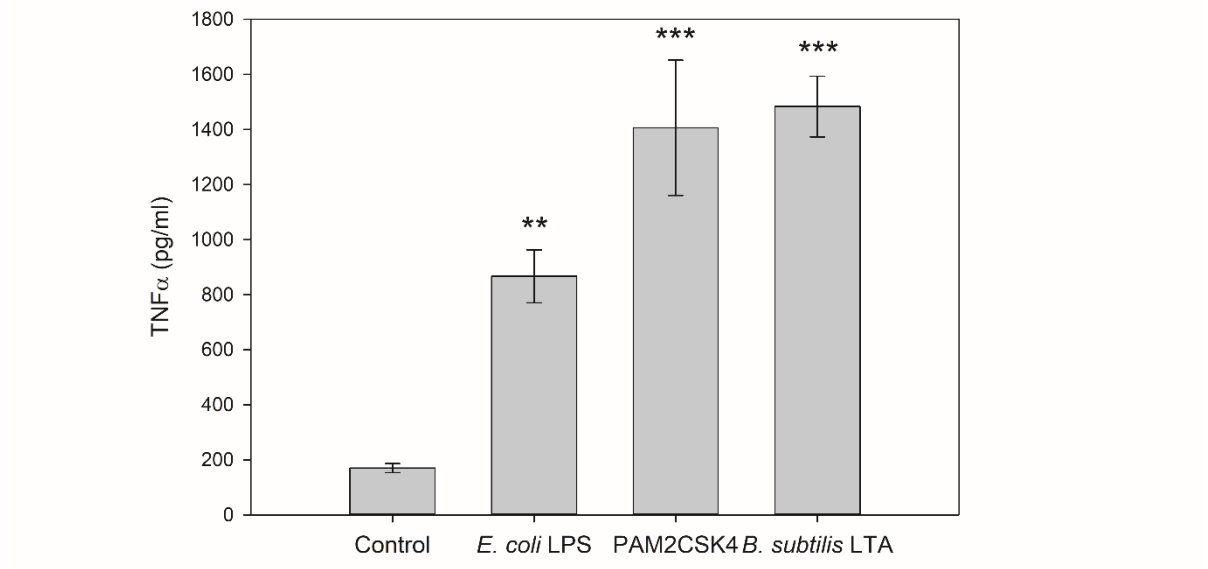


Figure B.1. Confirmation of TLR agonist stimulation of macrophages by measurement of secreted TNFα – 4 hours.

TNFα measured by ELISA in supernatants of THP1-derived macrophages stimulated for 4 hours with *E. coli* LPS (100 ng/ml), Pam2CSK4 (10 ng/ml) or *B. subtilis* LTA (100 ng/ml. Controls were unstimulated. A representative experiment is illustrated. This experiment was performed routinely to confirm macrophage responsiveness to TLR agonists. Cultures that did not show significant increases to secreted TNFα after stimulation with TLR agonists were not analysed for SIRT2 secretion. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). Analysed using one-way ANOVA. *** $p<0.001$, ** $p=0.002$.

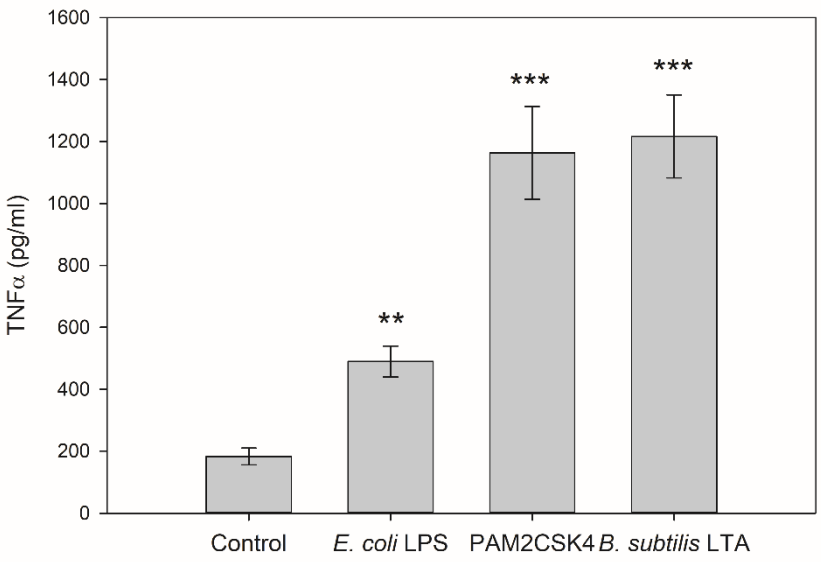


Figure B.2. Confirmation of TLR agonist stimulation of macrophages by measurement of secreted TNFα – 24 hours.

TNFα measured by ELISA in supernatants of THP1-derived macrophages stimulated for 24 hours with *E. coli* LPS (100 ng/ml), Pam2CSK4 (10 ng/ml) or *B. subtilis* LTA (100 ng/ml. Controls were unstimulated. A representative experiment is illustrated. This experiment was performed routinely to confirm macrophage responsiveness to TLR agonists. Cultures that did not show significant increases to secreted TNFα after stimulation with TLR agonists were not analysed for SIRT2 secretion. The data shown represents the mean ± SD of 3 cultures for each condition obtained in 1 experiment (N=3). Analysed using one-way ANOVA. *** $p<0.001$, ** $p=0.029$.

Appendix C. Proximity extension assay

PEA is a multiplex assay that utilises pairs of specific antibodies labelled with oligonucleotides. If the two antibodies for a given protein are bound within close proximity (i.e. bound to the same protein) hybridization will occur between the corresponding oligonucleotide labels which are then extended by DNA polymerization. This process occurs for target proteins and internal controls spiked into samples. The DNA sequences were then utilised, along with the DNA sequence generated by internal controls, in qPCR reactions to generate the relative expression of the target from Ct values, which were then normalised against internal extension controls to give ΔCt values. These ΔCt values were then used to generate ΔΔCt from interplate controls. The equations used were as follows:

ΔCt:

$Ct_{analyte} - Ct_{extension\ control} = \Delta Ct_{analyte}$

ΔΔCt:

$\Delta Ct_{analyte} - \Delta Ct_{interplate\ control} = \Delta\Delta Ct_{analyte}$

The ΔΔCt was then converted into normalised protein expression, which is log2-transformed, using a correction factor generated using negative controls and the following equation:

Correction factor - $\Delta\Delta Ct_{analyte}$ = normalised protein $expression_{analyte}$

As normalised protein expression utilises relative quantification of proteins presented the arbitrary unit normalised protein expression, values for different targets cannot be directly compared. Additional information can be found at the following sources (Assarsson *et al.* 2014; Maalmi *et al.* 2020; Olink 2021b).

Olink inflammation panel targets		
Adenosine deaminase	Artemin	Axin-1
Beta-nerve growth factor	C-C motif chemokine 19	C-C motif chemokine 20
C-C motif chemokine 23	C-C motif chemokine 25	C-C motif chemokine 28
C-C motif chemokine 3	C-C motif chemokine 4	C-C motif chemokine 1
C-X-C motif chemokine 10	C-X-C motif chemokine 11	C-X-C motif chemokine 5
C-X-C motif chemokine 6	C-X-C motif chemokine 9	Caspase-8
CD40L receptor	CUB domain-containing protein 1	Cystatin D
Delta and Notch-like epidermal growth factor-related receptor	Eotaxin	Eukaryotic translation initiation factor 4E-binding protein 1
Fibroblast growth factor 19	Fibroblast growth factor 21	Fibroblast growth factor 23
Fibroblast growth factor 5	Fms-related tyrosine kinase 3 ligand	Fractalkine
Glial cell line-derived neurotrophic factor	Hepatocyte growth factor	Interferon gamma
IL-1α	IL-10	IL-10 receptor subunit alpha
IL-10 receptor subunit beta	IL-12 subunit beta	IL-13
IL-15 receptor subunit alpha	IL-17A	IL-17C
IL-18	IL-18 receptor 1	IL-2
IL-2 receptor subunit beta	IL-20	IL-20 receptor subunit alpha
IL-22 receptor subunit alpha-1	IL-24	IL-33
IL-4	IL-5	IL-6
IL-7	IL-8	Latency-associated peptide transforming growth factor beta-1
Leukaemia inhibitory factor	Leukaemia inhibitory factor receptor	Macrophage colony-stimulating factor 1
MMP-1	MMP-10	Monocyte chemotactic protein 1
Monocyte chemotactic protein 2	Monocyte chemotactic protein 3	Monocyte chemotactic protein 4
Natural killer cell receptor 2B4	Neurotrphin-3	Neurturin
Oncostatin-M	Osteoprotegrin	Programmed cell death ligand 1
S100-A12	Signalling lymphocytic activation molecule	SIRT2
STAM-binding protein	Stem cell factor	Sulfotransferase 1A1
T cell surface glycoprotein CD6	T cell surface glycoprotein CD5	T cell surface glycoprotein CD8 alpha chain
Thymic stromal lymphopoietin	TNFα	TNFβ
TNF-related activation-induced cytokine	TNF-related apoptosis-inducing ligand	Transforming growth factor alpha
TNF superfamily, member 12	TNF	TNF ligand superfamily, member 14
TNF superfamily, member 9	Urokinase-type plasminogen activator	Vascular endothelial growth factor A

Table C.1. List of protein targets included in the Olink Inflammation PEA panel.

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