INVESTIGATION OF THE POTENTIAL OF SPLEEN TYROSINE KINASE (SYK) AS A TARGET FOR HOST-DIRECTED THERAPY DURING MYCOBACTERIAL INFECTION IN MACROPHAGES

By

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DECLARATION FORM

I, Sephekana Samuel Mohapi, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABBREVIATIONS

BCG	Bacillus Calmette-Guerin
CFUs	Colony forming units
DCs	Dendritic cells
IL	Interleukin
SYK	Spleen tyrosine kinase
ELISA	Enzyme linked immunosorbent assays
FACS	Fluorescently activated cell sorting
Mtb	*Mycobacterium tuberculosis*
MPO	Myeloperoxidase
PCR	Polymerase chain reaction
SYK	Spleen tyrosine kinase
TB	Tuberculosis
TNF	Tumour necrosis factor alpha
WT	Wild type

ABSTRACT

Tuberculosis (TB) is a communicable disease caused by a single infectious agent, *Mycobacterium tuberculosis* (Mtb). TB affects mostly the lungs and despite treatment being available, it still causes long term functional disability due to collateral tissue damage. The TB burden is exacerbated by the lengthy treatment period of 6-12 months which may result in issues of toxicity and poor adherence. Novel therapeutics are therefore urgently needed. Host directed therapies (HDT) are currently a promising way forward for limiting tissue pathology caused by Mtb.

Spleen tyrosine kinase (SYK) plays an important role in innate immune signalling. It is expressed on innate immune cells such as macrophages. Macrophages play a critical role in the pathophysiology of TB. They are the first responders to Mtb infection and are phagocytic cells that engulf and destroy Mtb. They also produce inflammatory cytokines such as TNF and IL-1β. Recent studies have suggested an involvement of SYK in the inflammatory signalling cascade linked to necrotic and caseous regions of granulomas of TB patients. However, it is unclear what role SYK plays in the pathophysiology of TB and whether its inhibition would result in resolution of excessive tissue damage in the lungs.

Our study is based on an *in vitro* infection model of Thp-1 derived macrophages. We differentiated Thp-1 monocytes into macrophages and infected them with BCG or the pathogenic laboratory strain Mtb H37Rv. We then treated infected macrophages with SYK inhibitors; Fostamatinib and Piceatennol, collected supernatants and analyzed cytokine production using enzyme-linked immunosorbent assay (ELISA). Moreover, we also evaluated whether SYK inhibition with Fostamatinib or Piceatennol might affect the intracellular survival of Mtb in macrophages. We also attempted to confirm the reduced expression of SYK at gene and protein level after treating infected cells with Fostamatinib.

Our data showed that Fostamatinib reduced the production of inflammatory cytokines IL-6, IL-1β and TNF-α in macrophages infected with BCG. Similarly, these findings were also observed in macrophages that were infected with Mtb H37Rv, with the exception of IL-1β that was unaltered in macrophages treated with Fostamatinib. Moreover, Fostamatinib reduced the production of anti-inflammatory cytokine IL-10 and the chemokine monocyte chemotactic protein-1 (MCP-1) in macrophages infected with Mtb H37Rv. We observed that Fostamatinib rescued macrophages from cell death induced by both BCG and Mtb H37Rv. Finally, we showed that treatment with Fostamatinib also reduced bacterial loads inside macrophages.

In essence, our study showed that SYK inhibition attenuate Mtb induced inflammatory profile in macrophages and aids in macrophage anti-mycobacterial effects. Further, it suggests that SYK inhibition might be an attractive avenue to explore further as a potential host-directed therapy for TB.

CHAPTER 1: INTRODUCTION

Tuberculosis: a global health concern

Tuberculosis (TB) is a disease caused by the bacterium *Mycobacterium tuberculosis* (Mtb). The disease remains a great global health problem in terms of morbidity and mortality. According to the 2019 Global TB report, in 2018 alone, there was an estimated 10 million new cases of infection, and 1.5 million deaths were reported globally (World Health Organization, 2019), making TB the highest cause of death from a single infectious agent, surpassing HIV/AIDS (Frieden, Brudney and Harries, 2017). It is also the first disease to be declared by World Health Organization (WHO) as a worldwide health emergency (Zaman, 2010).

One-third of the world's population is infected with Mtb; however, about 10% of the infected individuals will advance to active disease in their lifetime while 90% remains latently infected but healthy (Devalraju *et al.*, 2018). A timely diagnosis and treatment of TB with first line drugs is pivotal if most infected people are to be cured; thus, preventing a continual transmission of the disease (World Health Organization, 2019). In addition, new TB cases and ultimately TB-related deaths can be curtailed by decreasing factors that pose a health risk such as smoking, diabetes and HIV infections. Moreover, this can also be achieved by providing treatment to latently infected people (so that they do not progress to active diseases) as well as addressing the principal social determinants of TB infection (i.e., poverty, malnutrition, and housing) (World Health Organization, 2019).

TB is treatable with first line drugs such as rifampicin, isoniazid, ethambutol and pyrazinamide. Resistance to either rifampicin or isoniazid is termed multi-drug resistant TB (MDR-TB), which can be treatable with second-line TB drugs. The latter include fluoroquinolones (ofloxacin, levofloxacin, moxifloxacin, ciprofloxacin), injectable drugs (kanamycin, amikacin and capreomycin) as well as less effective drugs such as ethionamide, prothionamide, cycloserine, terizidone, and p-aminosalicylic acid (Forget and Menzies, 2006). In the event that further resistance occurs, this is termed extensively drug resistant (XDR-TB),which is a more fatal form of the disease. XDR-TB is defined as resistance to rifampicin, isoniazid and at least one of the second line linedrugs, fluoroquinolones or injectable drugs (Günther, 2014).

Drug resistance is therefore one of the major reasons why TB remains a global health threat. The long treatment period of 6-9 months makes it difficult for people to complete, causing poor adherence. This is due to some liver toxicity and unpleasant side effects, amongst other reasons, which results in the emergence of drug resistant strains and a high number of treatment

refractory cases (Brooks *et al.*, 2011). In his 1959 paper, Sir John Crofton wrote: "The greatest disaster that can happen to a patient with tuberculosis is that his organisms become resistant to two or more of the standard drugs." Moreover, he went on to say that it is not only a tragedy that maybefall the patient but also those that may be infected with this resistant strain (Crofton, 1959). Indeed, this has become an unfortunate reality lately as every day, 400 people die from MDR-TB and 80 from XDR-TB related cases respectively (Seung, Keshavjee and Rich, 2015). Thismeans that drug-resistant strains of mycobacteria are very well on their way to becoming the world's most deadly organisms, claiming 1 in 4 of all antimicrobial resistances (Chiang, Centisand Migliori, 2010). This therefore makes one wonder if drug resistant strains will outnumberdrug-susceptible strains in the near future.

Co-infection with HIV increases the mortality rate of TB as HIV significantly increases vulnerability to TB. This is because HIV weakens the immune system by depleting CD4+ T-cells, which are crucial for mediating TB immunity (Mbow et al., 2013). In addition, latently infected people with HIV stand a staggering 800-fold risk of developing active TB disease (World Health Organization, 2016). TB and HIV co-infection requires more medication to treat which may lead to possible hepatotoxicity and side effects. In sub-Saharan Africa, TB is the highest cause of death amongst HIV positive people and close to 20% of global HIV-linked TB deaths are in South Africa alone (Id et al., 2019). Furthermore, there is a need for more effective vaccines for TB. The only approved vaccine is the Bacille Calmette Guerin (BCG), which has been in use for about a century. Although BCG is effective and is estimated to save 120 000 newly-borns per year, it has limitations in that its efficacy is only high in infants and it wanes in adulthood. In addition, there is too much concern around its efficacy. Numerous studies have reported efficacies ranging between 0 and 80%, due to big differences in protection from region to region (Vaudry, 2003). Vaccination is therefore a great tool for TB control and there is a need a vaccine that can extend protection beyond childhood and into adulthood.

Immune responses against *Mycobacterium tuberculosis*

TB affects mostly the lungs; however, about 15% of patients present with extra-pulmonary TB where other organs such as the bones, central nervous system, lymph nodes and joints are affected (Raja, 2004). Initiation of host immunity against Mtb starts after uptake of the pathogen by phagocytes such as alveolar macrophages and dendritic cells (DCs) in the lungs. The interaction between Mtb and host phagocytes results in expression of pro-inflammatory cytokines. In addition, chemokines are also produced which recruit other innate immune cells as well as adaptive immune cells to the site of infection to assist with the clearance of Mtb (Algood, Lin and Flynn, 2005). The recruited immune cells accumulate and surround the infected phagocytes. The resultant aggregates of cells form an immune structure termed the granuloma (figure 1), a hallmark of TB infection. This structure is self-organized and is a combination of both innate and adaptive immune cells such as macrophages, dendritic cells, T and B lymphocytes, and neutrophils. There is also specialized cells such as multinucleated giant cells and epithelioid macrophages (Marino, Linderman and Kirschner, 2011).

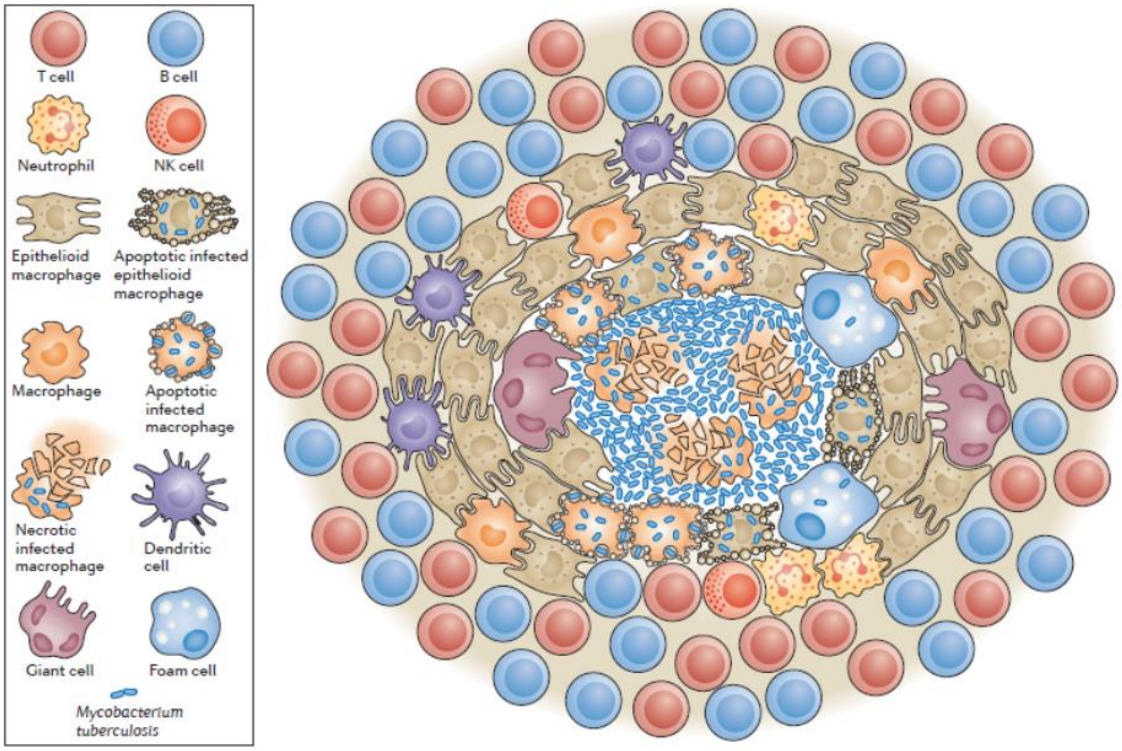

Figure 1. Tuberculous granuloma structure and its cellular constituents. The tuberculous granuloma is a compact highly organized structure of various cell types. Epithelioid cells are specialized macrophages with interlocked cell membrane projections that form tight junctions with each other.

Epithelioid cells form the basis of the granuloma and are said to be phagocytic, however seldom contain any bacteria. Granulomas also include giant multinucleated macrophages and differentiated foamy macrophages, with the former being a fusion of monocytes or macrophages whereas accumulated lipid droplets are found in the latter. Foamy macrophages surround the inner core of the granuloma containing necrotic Mtb-infected dead or dying macrophages in mature TB granulomas. In addition to these specialized cells are other cell types including lymphocytes which form an outer lymphocytic cuff, natural killer (NK) cells, neutrophils, dendritic cells, and fibroblasts which secrete extracellular matrix (ECM). Adapted from (Ramakrishnan, 2012).

A granuloma is thought to keep Mtb contained at the center; however, an imbalanced immune system that is under/hyperactive may lead to remodeling of the granuloma, leading to formation of a caseous granuloma which contains a cheese-like structure composed of necroticmaterial at the center (Vyas and Goswami, 2017). The caseum may further dissociate as the disease progresses leading to cavitation of the granuloma and bacterial dissemination (Ndlovu and Marakalala, 2016). The exact role of the granuloma in TB pathogenesis is complicated asit is thought to be host protective but also possibly detrimental (Ndlovu and Marakalala, 2016;Vyas and Goswami, 2017).

Phagocytes which have taken up the mycobacteria employ several mechanisms to attempt killing of intracellular Mtb. One of these ways is by producing reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). These reactive species are meant to create an environment that is hostile to mycobacteria and help to clear it (Chan *et al.*, 1999). Despite these efforts by phagocytes, Mtb may still persist and replicate inside the host cells, owing to some genes that the pathogen expresses to counter the intracellular hostile conditions. Studies have demonstrated that Mtb can express antioxidant genes such as *noxR1* that can neutralize ROS and RNI and evade the host killing mechanism (Ehrt *et al.*, 1997). In addition, studies have identified some cellular processes such as autophagy as pivotal in defence against intracellular Mtb in macrophages (Gutierrez *et al.*, 2004; Schaible, 2015). However, Mtb possesses evasive strategies to counteract this as well. Mtb can evade the host by impeding phagosome maturation, and therefore block phago-lysosomal fusion, or lysosomal acidification, processes which are essential in its killing (Armstrong and Hart, 1971; Deretic *et al.*, 2006). When the innate immune cells are unable to control the infection, the adaptive immune system must kick in.

Antigen presenting cells such as dendritic cells (DCs) containing intracellular bacteria or antigens migrate to lymph nodes to present to and prime T cells. Activation of CD4+ T cells in a T helper 1 (Th1) response, which involves production of interferon-γ (INF-γ), interleukin-12

(IL-12) and tumor necrosis factor-alpha (TNF-α) (Zeng, Zhang and Chen, 2017). IL-12 also functions as a co-stimulatory cytokine released by infected macrophages. which, alongside antigen presentation, instructs T cells towards a Th-1 response (Hamza, Barnett and Li, 2010). INF-γ helps increase the killing capacity of phagocytes or even increasetheir apoptosis; hence, aid in maintenance of consistent antigen presentation (Herbst, Schaibleand Schneider, 2011). It can also help in recruitment of CD4+ T cells and CD8+ cytotoxic T cells (Russell *et al.*, 2009). In addition, Mtb can be directly killed by cytotoxic lymphocytes such as CD8+ T cells, NK cells or NK T cells, which release effector molecules. These toxic molecules include granulysin, granzymes and perforin which help clear Mtb by lysing infectedcells (Stegelmann *et al.*, 2019). However, the death of infected cells may be detrimental to thehost as it causes the release of cellular constituents and "danger signals" to the extracellular milieu. This triggers recruitment of more inflammatory cells such as neutrophils (Matzinger, 2002).

The role of early recruited neutrophils has been shown to be positive to the host in terms of pathogen clearance. In addition, neutrophils play a protective non-phagocytic role in systemic Mtb infection of mice (Eruslanov *et al.*, 2005). However, increased recruitment of neutrophils is associated with tissue damage due to production of excessive inflammatory cytokines such as TNFα. Increased levels of TNF-α and other inflammatory cytokines such as IL-1β and IL-6 can cause inflammation that is deleterious to host and may result in lung damage (de Melo *et al.*, 2018). Furthermore, TB patients starting antiretroviral treatment (ART) are also susceptible to TB immune reconstitution inflammatory syndrome (TB-IRIS), an inflammatory condition which results in worsening or progression of TB in patients (Walker *et al.*, 2018). This therefore highlights a need for a tightly regulated immune defence system that results in efficient mycobacterial clearance while reducing tissue/cell damage (Torrelles and Schlesinger, 2017). Recent studies have shown that by targeting the host immune responses and cell functions, optimal Mtb clearance may be achieved and therefore this may lead to new avenues in the development of new treatment strategies for TB (Kim and Yang, 2017).

Host-directed therapy against Mtb

The WHO in 2014 announced a very ambitious global TB strategy aimed to reduce TB deaths by 95% and to cut new cases by 90% between 2015 and 2035 (World Health Organization, 2016). In order to achieve these targets, it is important that new diagnostic tools are identified, developed and implemented (World Health Organization, 2016). In addition, there is an immediate need to identify new therapeutic targets to enable development of drugs that can target both drug susceptible and resistant strains of Mtb (Wang *et al.*, 2015). Furthermore, identification of host immune responses involved in granuloma dissociation may also offer an attractive window of opportunity for new drugs that limit tissue pathology by targeting the detrimental immune factors (Kim and Yang, 2017). Molecules involved in these host immune responses could also be drivers of progression from latent infection to active TB disease. Therefore, focusing research on them could help bridge the knowledge gap in the mechanisms and pathways at play in disease progression.

Simply put, host-directed therapy (HDT) means moving from targeting Mtb itself and shifting focus on to the host-pathogen interaction. This is particularly important as Mtb has evolved abilities to hijack host cellular factors for its own persistence and flourishment (Guler and Brombacher, 2015) Additionally, HDT promises to be a way forward to help overcome the challenge of drug resistance. Recent insights into the host-pathogen interface, host innate and acquired immune responses as well as inflammatory pathways involved in the pathogenesis of Mtb have led to discovery of a vast range of HDTs (Zumla *et al.*, 2016). HDTs can be used adjunctly with the standard anti-TB therapy.

Different host directed therapies have different mechanisms of action. They have the ability to alter cellular anti-Mtb mechanisms and may also reduce the sustained local inflammatory pathology of tissues which causes permanent lung damage in about half of survivors of TB (Torrelles and Schlesinger, 2017). Examples of host directed therapies currently employed (Table 1) include the use of autologous bone marrow mesenchymal stromal cells, drugs repurposed fromtreatments of other non-communicable diseases such as arthritis and cancer, immune based therapy such as administration of cytokine and vaccines (Tobin, 2015; Zumla *et al.*, 2016; Machelart *et al.*, 2017).

Table 1. List of host-directed therapeutic agents and their application in tuberculosis treatment.

HOST DIRECTED THERAPEUTIC AGENT	MECHANISM OF ACTION AND BIOLOGICAL SIGNIFICANCE	REFERENCE
Vitamin D	• Enhances innate immune responses. • Promotes autophagy of Mtb infected cells. • Suppresses NF-κB signaling pathways and therefore reduces expression of proinflammatory cytokines and chemokines.	(Liu *et al.*, 2007) (Ralph *et al.*, 2017)
Non-steroidal anti-inflammatory drugs: Ibuprofen, Zileuton and Aspirin	• Increases the LXA4 production and therefore Activates vitamin D-mediated anti-mycobacterial activities. • suppresses excessive inflammation and tissue pathology and reduces bacterial burdens in the lung. • Inhibits COX1 and COX2 and this suppresses prostaglandin H2 production. This Regulates TNF-α production and dampens excessive inflammation.	(Tobin, 2015) (Vilaplana *et al.*, 2013)
Corticosteroid: Prednisone Dexamethasone	• They are antagonists of the glucocorticoid receptor and suppress inflammation by reducing TB immune reconstitution inflammatory syndrome (TB-IRIS). • Dexamethasone is effective in TB meningitis.	(Alzeer and FitzGerald, 1993) (Jubelt, 2006) (Walker *et al.*, 2018)
Statins**:** simvastatin	• reduce cholesterol levels and limit bacterial growth in the body's immune cells. • Downregulates production of pro-inflammatory cytokines which cause tissue damage	(Parihar *et al.*, 2014)
Verapamil	• Blocks calcium and maintains cellular ionic environments and enhances the efficacy of anti-TB drugs.	(Chen *et al.*, 2018)
Cytokine neutralizers: Adalimumab Siltuximab	• Neutralizes excess tissue damaging cytokines such as TNF- α associated with TB infection.	(Bourigault *et al.*, 2013)
Bevacizimub	• Inhibition of angiogenesis (neovascularisation) by neutralizing Vascular endothelial growth factor (VEGF)) in lung granulomas. • Increases penetration of anti-TB drugs into granulomas and also increases oxygen supply.	(Datta *et al.*, 2015)

The spleen tyrosine kinase (SYK)

An immune response is triggered by a signal transduction that occurs inside immune cells such as B cells, T cells, macrophages, and natural killer cells. These cells have receptors such as B cell receptors (BCRs), T cell receptors (TCRs) and Fc receptors (FcRs) collectively called classical immunoreceptors. These receptors share a similar signalling mechanism in that they bind to a transmembrane domain of proteins that have immunoreceptor tyrosine-based activation motifs (ITAMs) (Sanderson *et al.*, 2009). These ITAM motifs are basically a peptide sequence that includes two tyrosine residues that have been separated by 6-12 amino acids residues. Engagement of these receptors quickly leads to the ITAM phosphorylation by SRC kinases which results in recruitment of the spleen tyrosine kinase (SYK). This is the case with BCR or FcR signalling only; however, in the case of TCR, ZAP70, a SYK mammalian homologue restricted to T and NK cell lineages is recruited (Mócsai, Ruland and Tybulewicz, 2016).

SYK is a 72 kDa non receptor tyrosine kinase expressed mostly by haematopoietic cells. It belongs to a family of protein tyrosine kinases, which include ZAP70. Moreover, SYK has an alternatively spliced variant called SYK B, which lacks 23 amino acids within the interdomain B (Sada *et al.*, 2001). SYK and ZAP70 are both non receptor cytoplasmic kinases having a characteristic dual Proto-oncogene tyrosine-protein kinase (SRC) homology 2 (SH2) domains. The main difference is that unlike ZAP70, SYK activation does not heavily rely on phosphorylation by SRC kinases. The two SH2 domains of SYK/ZAP70 are found at the N-terminal region and are connected to linker A (or interdomain A) and are separated from the catalytic region by a linker B (or interdomain B) which is much longer (Sada *et al.*, 2001) (figure 2). The aromatic amino acids that are presentin linker A, linker B, the catalytic domain and the far end C-terminus of SYK interact with each other to form what is called a 'linker–kinase sandwich.' This sandwich helps maintain SYK in an auto-inactivated form (Mócsai, Ruland and Tybulewicz, 2016).

Activation of SYK occurs when the two SH2 domains are engaged by binding of a phosphorylated ITAM or when the linker kinase sandwich become phosphorylated. It is suggested that dual activation of SYK by both initial ITAM binding as well as phosphorylation of the 'linker kinase sandwich' may have some physiological relevance (Mócsai, Ruland and Tybulewicz, 2016). The ITAM binding causes a quick SYK activation while the other proceeding phosphorylation causes a sustained activation and downstream signal transduction even when there is no phosphorylated ITAMs. Worth noting is the fact that SYK is also capable

of auto-phosphorylating itself, resulting in a more durable activation following a temporary ITAM phosphorylation. Furthermore, SYK itself is also capable of directly phosphorylating the ITAMs, implicating it as an initiator of the positive feedback loop at the receptor (Mócsai, Ruland and Tybulewicz, 2016). Negative regulation of SYK requires a critical balance between phosphatases like protein tyrosine phosphatases-6 (PTPN-6) and SYK activities (Rolli *et al.*, 2002)**.** In addition, the Casitas B-lineage lymphoma (CBL) family of ubiquitin ligases such as Cb1 and Cb1-b are known to contain domains that recognize and interact with protein kinases such as the SYK/ZAP-70 family kinases (Rao, Dodge and Band, 2002). This ubiquitination of SYK leads to proteasomal degradation due to a very specific binding of Cbl phospho-tyrosine-binding domain to the phosphorylated tyrosine residue of SYK (Lupher *et al.*, 1998).

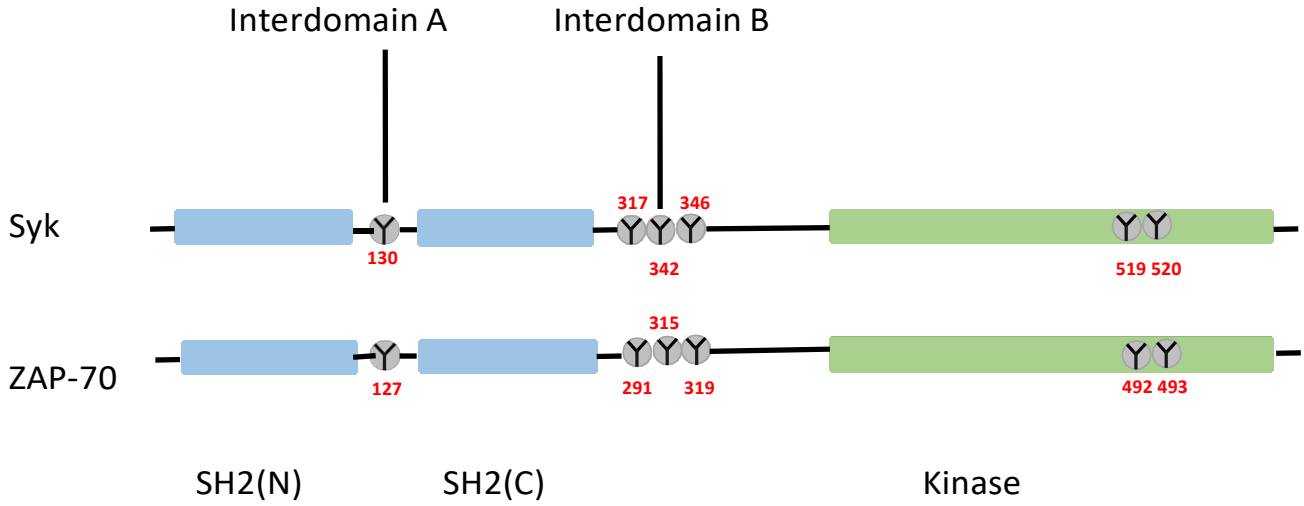

Figure 2. Schematic of the structure of SYK protein kinases family: SYK and ZAP70. The two SRC homology domains (SH2) are depicted in blue, joined by interdomains A and B as labelled. At the far right, shown in green, is the catalytic kinase domain of each protein. The tyrosine residues that get phosphorylated are depicted and numbered in red and are responsible for the protein's enzymatic activity and ability to recruit other downstream proteins. (image adapted from (Shinohara, 2016)

SYK signalling pathway was initially thought to be applicable only to adaptive immune responses; however, recent evidence has shown that there is ITAM based signalling in *Drosophila*, which lacks the adaptive immune response (Mócsai, Ruland and Tybulewicz, 2016), suggesting that SYK also functions in innate immunity. It was also thought that SYK signalling is relevant to only immunity; however, a study by Poole et al., 1997 showed that glycoprotein VI which is a collagen glycoprotein receptor expressed by platelets utilizes this

pathway in mice (Poole *et al.*, 1997). Abtahian et al., 2003 also demonstrated an important role played by SYK in controlling blood and lymphatic vasculature formation when they showed that mice embryos lacking the SYK gene were haemorrhagic and had arteriovenous malformation (Abtahian *et al.*, 2003). Another study showed that SYK was also involved in development of osteoclasts and their functional role in bone resorption (Csete *et al.*, 2019). These studies expanded the role of SYK beyond immune signalling to other diverse developmental roles, showing just how complex this molecule is and why targeting it might explain many mysteries regarding disease pathologies including TB.

C-type lectin receptor (CTLR)-SYK signalling in Mtb immunity.

Mtb needs to be recognised by cells of the immune system so that an appropriate immune response can be mounted. The pathogen or the pathogen associated molecular patterns (PAMPS) are recognised by pathogen recognition receptors (PRRs) that are expressed by innate cells such as alveolar macrophages and dendritic cells (Janeway and Medzhitov, 2002). PAMPs from microbes such as Mtb are evolutionarily conserved and this helps to distinguish host components from those that are foreign (Janeway and Medzhitov, 2002). There are several PRRs that recognize different Mtb PAMPs and these include toll-like receptors (TLRs), C-type lectin receptors (CLRs) and nucleotide-binding and oligomerization domain (NOD)-like receptors (figure 3 depicts some of the CLRs) (Wagener *et al.*, 2018). Upon encounter with a pathogen, a particular PRR or set of PRRs gets activated and induce a subsequent signalling cascade involved in production of soluble mediators such as cytokines and chemokines. Some of these soluble factors may activate adaptive immune cells such as T and B cells and enable the host to control Mtb infection (Vyas and Goswami, 2017).

PRRs recognize different ligands from different pathogens and activate various cellular responses via downstream signalling molecules such as SYK. SYK engages numerous CLRs that recognize Mtb. SYK initiates an antimycobacterial response by attaching to the phosphorylated immunoreceptor tyrosine-based activation motifs (ITAM) intracellularly associated with CLRs (except DECTIN-1 which has a hemITAM motif) (Yi *et al.*, 2014). Furthermore, this SYK-ITAM motif complex associates with a macromolecular complex involving phosphokinase C delta (PKC-δ), CARD9, B cell lymphoma-10 (BCL10) and mucosal associated lymphoid tissue (MALT1) (Strasser *et al.*, 2012; Parihar *et al.*, 2018). This in turn activates nuclear factor kappa beta (NF-κB), which results in subsequent production of

cytokines and chemokines that initiate an adaptive T cell response (Geijtenbeek and Gringhuis, 2009) (figure 3B).

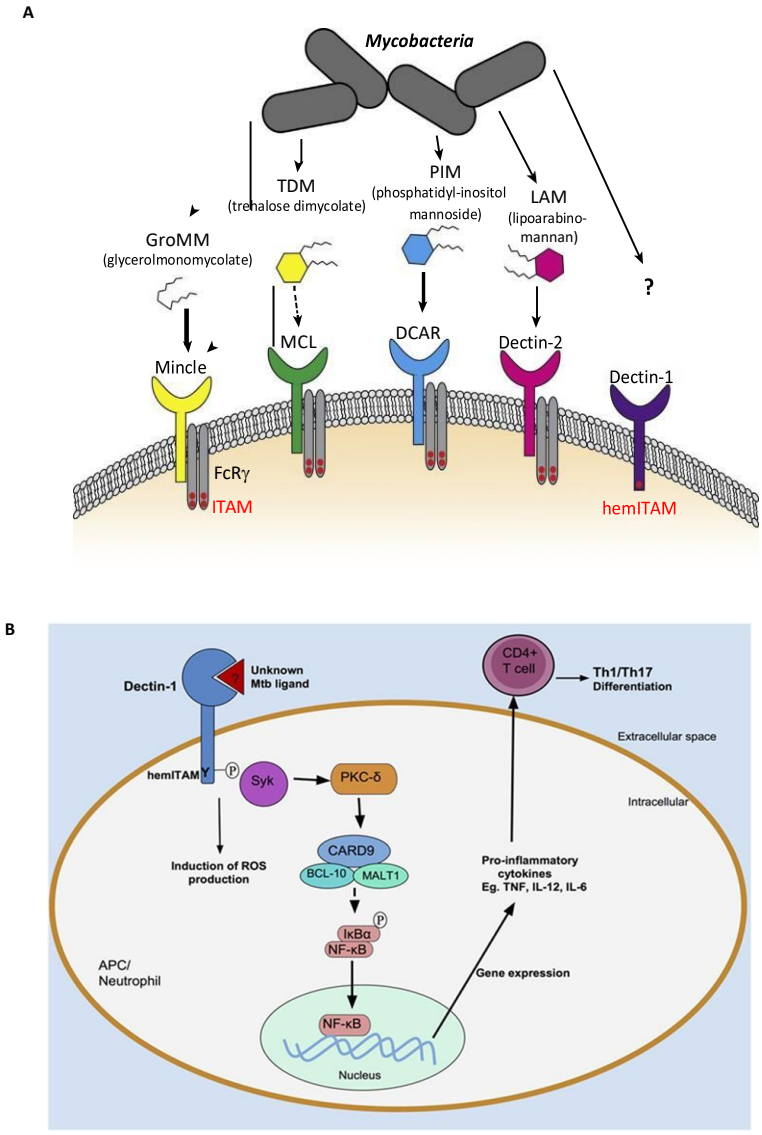

Figure 3. Schematic representation of C-Type Lectin Receptors (CLRs) that recognize mycobacteria. (A) Multi-valent mycobacterial PAMPs (TDM, trehalose-6,6-dimycolate; GroMM, glycerol monomycolate; PIM, phosphatidylinositol mannoside; LAM, lipoarabinomannan) bind to and are recognized by their respective CLRs to initiate an immune response. (B) Mtb recognition by Dectin-1. Dectin-1 leads to activation of the SYK pathway by recognizing an unidentified Mtb ligand. This causes dectin-1 tyrosine phosphorylation at its hemITAM intracellular residue. This phosphorylation creates a docking site for SYK which subsequently associates with CARD9/BCL-10)/MALT1 protein complex, resulting in NF-κB activation. Translocation of NF-κB into the nucleus leads to expression of

pro-inflammatory genes and therefore an induction of an adaptive T cell response. Images adapted from (Ishikawa, Mori and Yamasaki, 2016) (A) and Wagener et al., 2018 (B).

Some of the well characterised CLR signalling via SYK are dectin-1, dectin-2, macrophage inducible Ca^{2+}-dependent lectin (MINCLE) and dendritic cell activating receptor (DCAR), all of which have been implicated in recognition and ligation of mycobacterial PAMPS (Miyake *et al.*, 2010). These PAMPS include trehalose-6,6-dimycolate (TDM) and glycerol monomycolate (GroMM) that are recognized by MINCLE; and phosphatidylinositol mannoside (PIM) and lipoarabinomannan (LAM) which bind to DCAR and dectin-2, respectively (figure 3A).

DECTIN-1 is a glycosylated CLR which possesses an extracellular domain, a transmembrane domain as well as a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM)-like domain. This ITAM-like domain is known as a hemITAM and initiates downstream immune signalling (Marakalala and Ndlovu, 2017). Dectin-1 has been extensively studied as a beta-glucan receptor that is important for antifungal immunity (Rothfuchs *et al.*, 2007; Strasser *et al.*, 2012). Several studies have also implicated it in antimycobacterial immune responses; however, its Mtb ligand remains unknown (Brown, 2006) (figure 3). An *in vitro* study by Rothfuchs et al., 2007 first demonstrated the role of dectin-1 in recognition of Mtb in a SYK dependent manner. They found that treatment of splenic dendritic cells with either laminarin or glucan phosphate, both of which are known to inhibit dectin-1, resulted in a suppression of Mtb-induced IL-12p40 and IL-12p70 (Rothfuchs *et al.*, 2007). In addition, splenic DCs from dectin-1 knock-out mice produced reduced amount of IL-12p40 when compared to their wild type counterparts (Rothfuchs *et al.*, 2007). ToThe authors went on to show that dectin-1 inhibition by laminarin reduced the Mtb-induced phosphorylation of SYK (Rothfuchs *et al.*, 2007). On the other hand, Marakalala et al., did an *in-vivo* study in which they showed that both WT and mice lacking dectin-1 are resistant to Mtb infection in a similar manner. In spite of this, dectin-1 deficient mice had a slightly reduced lung bacterial burden (Marakalala *et al.*, 2011). These findings indicated that Mtb triggers dectin-1 mediated responses in a SYK-dependent manner Despite its impressive pro-inflammatory induction profile *in vitro*, dectin-1 seems to play a dispensable role *in vivo*. The discovery of Dec-1 Mtb ligand will perhaps enable better understanding of the role played by the receptor in interaction with the pathogen. (Ishikawa, Mori and Yamasaki, 2017)

DECTIN-2 is another one of the CLRs that unlike dectin-1, contains an actual ITAM-motif in its cytoplasmic portion. It belongs to the dectin-2 cluster of CLRs alongside MCL and DCAR (Graham and Brown, 2009). The recruitment of ITAM-linked FcRγ results in a SYK-dependent downstream signal cascade via SYK involving CARD-9-BCL10-MALT1 complex. When challenged with ManLam or BCG, bone marrow derived DCs induce pro-inflammatory cytokines such as MIP-2, TNF and IL-6 in a ManLam concentration dependent manner (Yonekawa *et al.*, 2014). However, this cytokine induction was slightly reduced in dectin-2 deficient bone marrow derived DCs infected with BCG (Yonekawa *et al.*, 2014). Interestingly, this study also established the importance of dectin-2-SYK signalling in adaptive immunity. It was shown that T cells from TB patients stimulated with ManLam-activated antigen presenting cells induce a TB specific response (Yonekawa *et al.*, 2014). These T cell mediated effects were diminished by inhibition of dectin-2 with anti-dectin-2 monoclonal antibody (Yonekawa *et al.*, 2014). This highlights a fundamental role played by dectin-2 via SYK.

MINCLE is an inducible CLR expressed on cells of the myeloid lineage. It contains an extracellular recognition domain, a transmembrane domain, and a short cytoplasmic domain. Mincle does not possess an ITAM motif and to initiate cellular responses, its short cytoplasmic tail recruits FcR gamma chain which links to SYK (Yonekawa *et al.*, 2014). Ishikawa et al., identified TDM as a ligand of mincle after showing that heat killed Mtb was able to activate mincle expression (Ishikawa *et al.*, 2009). However, upon de-lipidation of mycobacteria, this activity was abolished and analysis of the lipids identified TDM as the key ligand of mincle (Ishikawa *et al.*, 2009). TDM-activated macrophages induced pro-inflammatory cytokine expression and granuloma formation, all of which were abolished in mincle knock-out mice (Ishikawa *et al.*, 2009). When TDM and its synthetic analogue trehalose-6,6-dibehenate (TDB) are recognized by mincle, they activate antigen presenting cells to express NF-$_{\kappa}\beta$ through the SYK-CARD-9-BCL10 pathway and they can also be Th1/Th17 adjuvants (Schoenen *et al.*, 2010).

The antimycobacterial role played by mincle via SYK is contradictory in several studies. Two findings by Lee et al., and Heitmann et al., were in stark contrast when it came to Mtb clearance in mincle-deficient mice after aerosol infection. Lee et al., reported increased lung bacterial loads in these mice while Heitmann et al., reported no significant differences in not only bacterial loads in lungs of the mice, but also in granuloma formation (Lee *et al.*, 2012; Heitmann *et al.*, 2013). In support of findings by Lee et al., Behler et al. found that mincle-deficient mice were more susceptible to intravenous BCG infection than WT mice (Behler *et*

al., 2012). Moreover, after systemic infection, they also found high burdens of Mtb in liver and spleen as well as a reduced granulomas in mincle-deficient mice compared to WT mice (Behler *et al.*, 2012). The reasons behind these contradictory findings remain unknown; however, different expression of mincle in different cell types and different routes of infection could perhaps help explain these differences (Ishikawa, Mori and Yamasaki, 2017). Furthermore, a very recent study by Bowker et al., studied the correlation between 4 mutations in mincle encoding gene, *CLEC4*, and found that there was no association of this polymorphisms with TB (Bowker *et al.*, 2016). This therefore implies that MINCLE plays a redundant role in antimycobacterial immunity.

Macrophage C-type Lectin (MCL) is a CLR that is predominantly expressed by myeloid cells and it has a sequence that is similar to mincle. It does not contain a signalling motif in its cytoplasmic region and it is coupled to FcRγ, which initiates downstream cellular responses (Miyake *et al.*, 2013). MCL has been shown to be required for positive regulation of mincle expression upon myeloid cell stimulation (Miyake, Masatsugu and Yamasaki, 2015). It achieves this function via a heterodimeric interaction (Miyake, Masatsugu and Yamasaki, 2015). MCL also binds TDM to activate cells and initiate immune signalling via SYK to induce phagocytosis or pro-inflammatory cytokine expression (Miyake *et al.*, 2013). Upon TDM stimulation, MCL-deficient mice showed significantly reduced TDM-induced responses, and this is thought to be due to a suppression of its "co-receptor or partner" MINCLE (Ishikawa, Mori and Yamasaki, 2017).

The importance of MCL in TB immunity was highlighted by a study that showed that mice deficient in this receptor had high mycobacterial burdens, displayed increased pulmonary inflammation and have higher mortality rates during infection than wild type mice (Miyake *et al.*, 2013; Richardson and Williams, 2014). In addition, human MCL polymorphisms were found to be associated with increased predisposition to pulmonary TB (Wilson *et al.*, 2015). Collectively, these studies demonstrated an unprecedented, non-redundant role played by MCL-SYK signalling in immunity to Mtb, both in mice and humans (Wilson *et al.*, 2015).

CARD-9 operates downstream of SYK in the immune response against pathogens such as Mtb amongst others. CARD-9 is an adaptor protein that functions downstream of ITAM associated PRRs. It is expressed in innate cells and is found mainly in tissues such as the liver and lungs (Dorhoi *et al.*, 2010). The role of CARD-9 in antimycobacterial immunity was demonstrated by Dorhoi et al, where CARD-9-deficient mice were shown to succumb rapidly to infection.

with Mtb H37RV strain compared to wild type control mice. Based on the minor roles reported on Mincle and Dectin-1 *in vivo*, it is tempting to speculate that CARD9 most likely plays a role in SYK mediated excessive inflammation caused by Mtb. Additionally, the CARD-9 knockout mice displayed higher lung bacterial burdens, severe tissue damage and necrosis (Dorhoi *et al.*, 2010). Interestingly, CARD-9 seemed to function only in innate responses and not adaptive responses as there was no significant differences in T cell recruitment to the lung between the CARD9-deficient mice and wild type mice (Dorhoi *et al.*, 2010). Therefore, CARD-9 most likely functions in Mtb recognition by merging different signals from numerous PRRs (Marakalala, Graham and Brown, 2010).

PKC-δ is a kinase protein involved in SYK downstream signalling by mediating CARD-9 phosphorylation and organization of the CARD-9-BCL-10 complex (Strasser *et al.*, 2012). During Mtb infection in mice, PKC-δ was shown to mediate macrophage killing abilities and PKC-δ-deficient mice were shown to be more susceptible to Mtb infection and displayed increased lung damage due to dysregulated pro-inflammatory cytokines and high bacterial burdens (Parihar *et al.*, 2017). The CLR-SYK pathway is therefore an attractive pathway to be explored as a possible target for host directed therapy and more work is needed to understand mechanisms underlying its role.

SYK is widely involved in pathology and disease.

Innate immunity is not only triggered by infectious pathogens or antigens but can also be activated by stimuli such as cell death, developmental cues, sterile injury or even sterile inflammation caused by autoimmune disorders (Medzhitov and Janeway, 1997). There is evidence implicating CLR-SYK in detection of danger associated molecular patterns (DAMPS) from damaged cells (Yamasaki *et al.*, 2008). One study identified mincle as one of the receptors expressed by cells activated in the presence of dead cells, and the spliceosome associated protein (SAP130) was identified as a ligand of this receptor (Yamasaki *et al.*, 2008).In addition, when mice were irradiated to cause thymocyte cell death, there was a high thymic infiltration of neutrophils, which could be reduced by inhibition of mincle with its neutralizing antibodies. These suggested that the mincle -SYK pathway could be involved in sensing cell death and subsequent production of inflammatory cytokines to recruit neutrophils to areas of tissue damage (Yamasaki *et al.*, 2008). Another receptor called dendritic cell NK lectin group receptor-1 (DNGR-1) was shown to be highly expressed in the CD8-a+ DC subset and was shown to recognize necrotic cell material and phagocytose them for cross presentation to CD8+ T cell (Sancho *et al.*, 2009)**.** DNGR-1-deficiency was found to not impede the ability of DCs to uptake necrotic cell material but specifically reduced antigen presentation to CD8+ T cells *in vitro* (Cueto, del Fresno and Sancho, 2020). Interestingly, this sensing of necrotic cell material and cross presentation by this subset of DCs was found to be SYK mediated. This suggested an important role for SYK in immune responses against cell injury (Cueto, del Fresno and Sancho, 2020).

SYK is involved in multiple biological processes as well as very diverse roles in immunity so it is highly probable that it could be involved in allergy or autoimmunity (Mócsai, Ruland and Tybulewicz, 2016). Indeed, SYK-deficiency was found to be beneficial and protective against experimental auto-antibody-induced arthritis in mice (Jakus *et al.*, 2010).

SYK plays a key role in immune function and inflammatory processes, making it a target for the development of many small molecule inhibitors. There are over 70 reports outlining small molecule SYK inhibitors that can be used in therapy of a plethora of diseases ranging from arthritis, lymphomas and asthma. A small molecule inhibitor and prodrug known as Fostamatinib (R788) as well as its active metabolite (R406) (figure 4) were shown in early studies to have therapeutic benefits in clinical arthritis and synovitis (Pine *et al.*, 2007). Fostamatinib, under the pharmaceutical name ‘Tavallise’ has now become an FDA-approved drug for the treatment.

of chronic immune thrombocytopenia (CIT). Furthermore, it was shown to have clinically meaningful outcomes when used as treatment for thrombocytopenia purpura (TP), in parallel phase 3 clinical studies (Bussel *et al.*, 2018). The starting median platelet count of the participants was 16 000/μL and median time living with TP was 8.5 years. Stable responses were observed in in 18% of patients on fostamatinib compared to the 2% on placebo (Bussel *et al.*, 2018). In addition, overall responses (defined retrospectively as ≥1 platelet count ≥50 000/μL within the first 12 weeks on treatment) occurred in 43% of patients on fostamatinib vs. 14% on placebo (Bussel *et al.*, 2018).

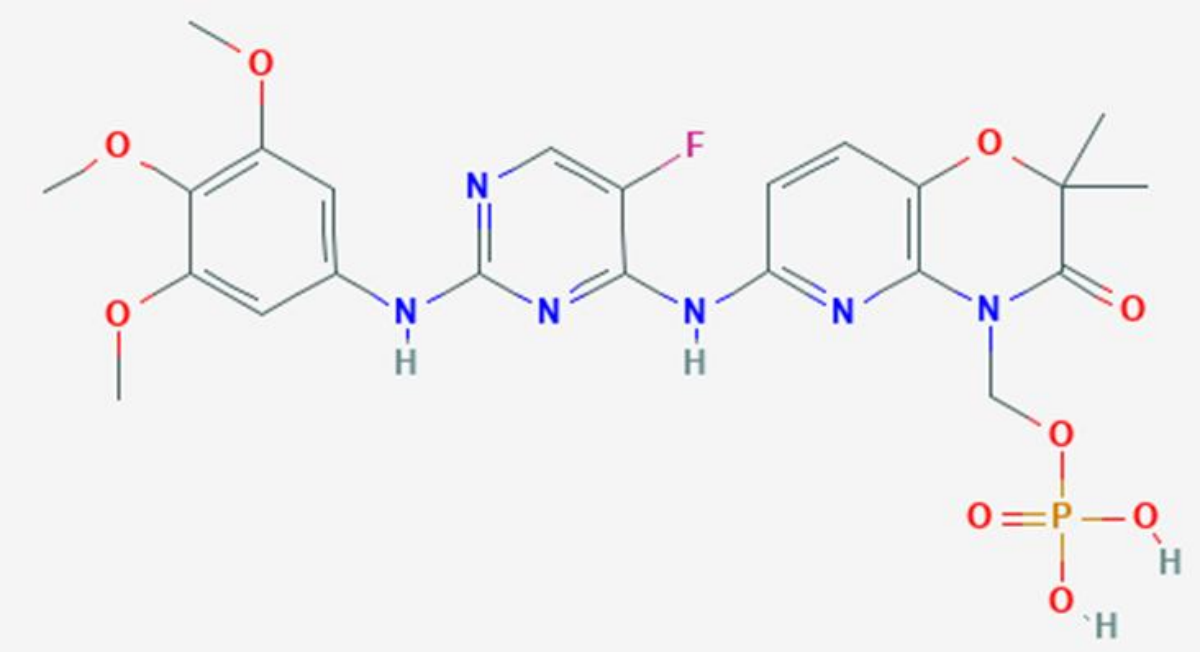

Figure 4. Schematic of the chemical structure of Fostamatinib, (R788); Tavalisse. The international Union of Pure and Applied Chemistry (IUPAC) name of Fostamatinib is **[6-[**[5-fluoro-2-(3,4,5-trimethoxyanilino) pyrimidin-4-yl] amino]-2,2-dimethyl-3-oxopyrido[3,2-b] [1,4] oxazin-4-yl] methyl dihydrogen phosphate. It has a molecular formular of $C_{23}H_{26}FN_6O_9P$ and a molecular weight of 580.5g/mol.

In a collagen induced arthritis model in rats, SYK was found to be overly expressed in synovial tissues, correlating with high inflammatory cell accumulation while it was almost undetectable in arthritic rats treated with fostamatinib (Pine *et al.*, 2007). SYK inhibition also reduced the inflammatory cytokines such as TNF-α IL-1β and IL-6 in the synovial tissue (Pine *et al.*, 2007).

In humans, fostamatinib also had significant therapeutic benefits in a 12-week clinical study of patients with rheumatoid arthritis (Kunwar, Devkota and Ghimire, 2016). The benefits of fostamatinib were observed at least one week after initiation of treatment, indicated by reduced serum levels of IL-6 and matric metalloprotease-3 (MMP-3) (Kunwar, Devkota and Ghimire, 2016). Taken together, these studies elucidated a very important role of SYK in inflammatory disorders.

SYK also plays a role in the development and maintenance of haematological cancers, particularly B cell lymphoma (Fueyo *et al.*, 2018). SYK exogenous overexpression transforms normal human pre-B cells into malignancy, which is likely attributable to the role of SYK in blocking apoptosis and favouring pre-B cell proliferation (Ackermann *et al.*, 2015). Furthermore, SYK has yet again become a target of many studies of liver pathology. Chronic liver diseases such as hepatitis, alcohol-induced liver disease, non-alcoholic fatty liver disease, autoimmune liver diseases and hepatocellular carcinoma are some of the world's leading causes of morbidity and mortality worldwide. In all these studies, SYK was identified to be overexpressed and therefore a potential therapeutic target for these diseases (Kawaratani *et al.*, 2017; Lu *et al.*, 2017; Qu *et al.*, 2018; Wahyu *et al.*, 2018; Wang, 2019).

In summary, SYK is a 72kDa molecule that plays an important role in biological functions and disease. Its ability to engage multiple CLRs such as dectin-1, dectin-2, mincle and MCL as well as downstream molecules such as PKC-δ and CARD-9, all of which have been implicatedin TB pathogenesis, makes it a target of our research.

Rationale

Mycobacterium tuberculosis, has over the years, evolved mechanisms to subvert and escape the host immune system and also exploit host cell factors in order to survive and flourish. This makes Mtb tricky to control and reflects an urgent need for new therapeutic strategies that focuses on targeting the host to control Mtb infection.

Therefore, our research focuses on a host-directed therapeutic approach as one way to solve the problem. We and other researchers have identified SYK as one of the proteins abundant in necrotic and caseous regions of granulomas in the lungs of patients with severe TB (Marakalala *et al.*, 2016; Flores-valdez, 2020). In addition, it is closely linked down-stream with PKC-δ, which was found to be upregulated in whole blood of patients with active TB (Parihar *et al.*, 2017). This suggested that SYK plays a critical role in TB and targeting it may reveal the role it may play in TB pathogenesis.

Hypothesis

We hypothesized that blocking SYK activity with Fostamatinib or Piceatennol will reduce the production of pro-inflammatory cytokines and impair cell death in macrophages infected with either BCG or Mtb H37RV strain.

Aim and objectives

Our study was aimed towards determining the effect of intercepting SYK with pharmaceutical small molecule inhibitors (fostamatinib and piceatannol) in macrophages derived from monocytes and infected with either BCG or Mtb H37Rv.

The study objectives were:

1. To optimize a Thp1 macrophage model of BCG and Mtb infection
2. To determine the cytokine profile of infected macrophages after treatment with either Fostamatinib or Piceatennol.

3. To verify the inhibition of SYK by Fostamatinib and Piceatannol in infected macrophages by Western blot as well as RT-qPCR
4. To analyse and quantify cell death of macrophages that were infected with BCG and treated with Fostamatinib or Piceatennol.
5. To determine the effects of SYK inhibition on the survival of intracellular Mtb by enumerating colony forming units after treatment and infection.

CHAPTER 2: METHODS

Cell culture and differentiation of Thp1 cells

We received Thp-1 cells as a generous gift from Professor Muazzam Jacobs's laboratory at the University of Cape Town. The cells were cultured and maintained in RPMI 1640 media containing 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (complete media), all of which were purchased from Sigma-Aldrich. The cells were grown at 37^{o}C in a humidified incubator with 5% CO_2. All experiments were performed on logarithmically growing cells. Once a stable culture was achieved, the cells were seeded in a 12 or 24-well tissue culture costar plates at a density of 2.5 - 5 × 10^5 cells per well. The monocytes were differentiated to macrophages by the addition of 100nM phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) and the cells were incubated for 48 hours in the incubator. The differentiated cells were washed once with 1× phosphate buffered saline (PBS) and left to recover overnight in complete media.

SYK inhibition and BCG/Mtb infection of macrophages

SYK selective inhibitors, fostamatinib was purchased from MedChem Express and piceatennol was purchased from Sigma-Aldrich (USA). Differentiated macrophages were treated with 20μM of fostamatinib or piceatennol and incubated overnight before infection.

Mycobaterium bovis Bacillus Calmette-Guérin (BCG) Pasteur strain was donated by Professor Muazzam Jacobs's group and was grown to a log phase in sterile 7H9 Middlebrook liquid medium. The media was prepared in accordance with the manufacturer's instructions whereby 2,3g of Middlebrook broth base (Sigma-Aldrich) was mixed with 450ml distilled water and supplemented with 4ml glycerol and 1,25ml of 80% Tween (Merck) and with 10% oleic albumin dextrose-catalase (OADC). All infections were done with bacteria grown to optical density (OD_{600}) between 0.8 and 1. The macrophages were infected at a multiplicity of infection (MOI) of 10. For infection with virulent Mtb H37Rv, the bacteria were cultured for 21 days in sterile 7H9 Middlebrook liquid medium and incubated at 37°C and 5% CO_2. Aliquots (1ml) were then collected and stored at -80°C. In order to determine the concentrationof the frozen vials, the stocks were thawed and passaged 30× through a 29.5G needle to eliminate any clumping. Serial dilutions were then made up to 10-fold and plated on Difco Middlebrook 7H10 agar plates and incubated at 37°C with 5% CO_2 for 21 days. For every

infection, frozen vials of H37Rv with a known concentration were thawed, and vortexed for 1min in beads for homogenous mixing and reduction of clumps. The cells were then infected with Mtb H37Rv at a MOI of 5 or 10. LPS (1μg/ml) was also used as a positive control in some cases.

Enzyme linked immunosorbent assay (ELISA)

Culture supernatants were collected at 24, 48 or 72 hours post infection and stored at -80^{0}C. The supernatants that were collected from the macrophages infected with Mtb H37Rv were double filtered before leaving the Biosafety level 3 facility. Pro- and anti-inflammatory cytokines including TNF-α, IL-1β, IL-6, IL-10, and MCP-1 were quantified by enzyme-linked immunosorbent assay (ELISA) using BD OptEIA™ ELISA kits (BD Bioscience, The Scientific group) according to the manufacturer's instructions. Briefly, 96-well plates were coated with 100μl of specific coating antibody and incubated overnight at 4^{o}C. The plates were washed 7 times with 300μl of wash buffer (1× PBS, 0.05% tween-20). The plates were blocked with 200μl of blocking buffer (10% foetal bovine serum (FBS) in 1X PBS) and incubated at room temperature (RT) for 2 hours. The plate contents were discarded and plates were washed 7 times with wash buffer. The samples (100μl) and serially diluted standards were then added to the wells and incubated for 1 hour at RT and the plate was aspirated and washed 5 times. For detection, 100μl of a working detector solution (specific detection antibody + streptavidin-Horse radish peroxidase (HRP) were added to each well. The plate was then sealed with foil and incubated for 1 hour at room temperature, followed by aspiration and 7 washes at 30 second intervals. Subsequently, 100μl of substrate solution (Tetramethylbendizine (TMB) and hydrogen peroxide) was added to the wells and the plates incubated at room temperature, in the dark, for 30 minutes. Finally, the detection reaction was stopped by addition of 50 μl stop solution (1M HCl) and absorbance was read at 405nm wavelength.

Lactate dehydrogenase assay (LDH)

To determine the amount of cell death of macrophages after infection with mycobacteria, the Pierce™ lactate dehydrogenase (LDH) cytotoxicity assay kit (ThermoFisher Scientific) was used in accordance with the manufacturer's instructions. Briefly, 5 × 10^5 cells were differentiated into macrophages in a 12-well plate and treated with Fostamatinib or piceatennol

for 12 hours. The cells were infected with BCG or Mtb and supernatants were collected after 24-, 48- and 72-hours post-infection. Supernatant (50µl) was added to a 96-well flat bottom plate in triplicates and the reaction mixture (50µl) was added to each sample and mixed by gentle agitation of the plate. The plate was then incubated at RT for 30 mintutes covered in foil and placed in the dark. Finally, the stop solution (50µl) was added to each well and the absorbance was measured at 490nm and 600nm.

Flow cytometry

To determine the mode of cell death, the same SYK inhibition and BCG infection were performed as described above. The infected macrophages were then detached from the culture plate by incubating in 200µl of pre-warmed Accutase solution at 37^{o}C for 15 minutes with gentle agitation of the plate to expedite the detachment. The cells were then added to 500µl FACS buffer-containing tubes (1X PBS,10% FBS, 0.1% NaN3 sodium azide). The tubes were centrifuged at 1000rpm for 5 minutes. The supernatant was discarded and the pellet was subsequently resuspended in 150µl FACS buffer. The cells were then stained with both propidium iodide (10µl) and Annexin-V FITC (1µl) and incubated for 30 minutes at RT. The cells were then acquired on BD LSR Fortessa flow cytometer (BD Biosceinces, Rockford USA) and the data was analysed using FlowJo software (TreeStar).

RNA extraction and quantitative reverse transcriptase -polymerase chain reaction (qRT-PCR)

In order to validate the inhibition of SYK expression by fostamatinib and piceatannol, total RNA was extracted from the infected cells using high pure RNA isolation kit (Roche) according to the manufacturer's instructions. Briefly, treated cells were resuspended in 200µl ice cold 1× PBS and then added to 400µl of lysis/binding buffer, followed by 15 seconds of vortexing. The sample (700µl) was transferred to a high pure filter tube inserted into a collection Eppendorf (filter tube assembly) and centrifuged for 15 seconds at 8000g. The flowthrough in the collection tube was discarded and the the nucleic acid material trapped on the glass fibre fleece was mixed with DNAse I (10µl) and DNAse I incubation buffer (90µl). The reaction mixture was then placed into the filter tube assembly and incubated at RT for 15 minutes followed by addition of 500µl wash buffer I and cenrtrifugation at 8000g for 15

seconds. After discarding the flowthrough, 500µl wash buffer II was added to the nucleic acid material trapped on the glass fibre fleece followed by another 15 minutes centrifugation at 8000g. The wash buffer II step was repeated with 200µl wash buffer II and 2 minutes of centrifugation at 13000g to remove the excess wash buffer. The final eluate in the glass fibre fleece was eluted out with addition of 50µl elution buffer and centrifugation at 8000g for1 minute.

RNA concentrations were measured using Nanodrop 2000 (Thermofischer) at an absorbance of 260 and 280nm where an A260/280 ratio of 1.8 – 2.0 is considered pure. qRT-PCR was conducted using cDNA prepared from reversely transcribed RNA using the Improm II Reverse Transcription kit (Thermofischer) according to the manufacturer's instructions (see table 2 below for reagent quantities).

Table 2. Complementary DNA (cDNA) synthesis reaction mix used for PCR amplification of SYK and PKC-□.

Reagent	Quantity of 1 reaction (µl)	Quantity of 9 reactions (µl)	Quantity of 12.5 reactions (µl)
Nuclease free water	6.1	54.9	76.25
5× reaction buffer	4	36	50
$MgCl_2$	2.4	21.6	30
dNTP mix	1	9	12.5
RNAse inhibitor	0.5	4.5	6.25
Reverse transcriptase	1	9	12.5
RNA quantity added	1µg RNA in 1µl of oligo dT primers and water to make up 5µl reaction mixture		

Complementary DNA (cDNA; 930ng) was loaded into a 96 well plate in the presence of KAPATM SYBR green fast qRT-PCR master mix (KAPA Biosystems) and forward and reverse primers as well as RNAse free water were added and then run on QuantStudio 3 Real-Time PCR System (ThermoFisher) (see tables 3, 4 and 5 for reagent quantities, primer sequences and fluoro-cycling conditions respectively). SYK gene expression was normalized to a house keeping gene glyceraldehyde3-phosphate dehydrogenase (GAPDH).

Table 3. RT-qPCR reaction mix used for PCR amplification of SYK and PKC

Reaction mix (10 µl)	SYK cDNA 1 reaction (µl)	SYK cDNA 21reactions(µl)	PKC- 1 reaction (µl)	PKC- 21 reactions (µl)
SYBR green DNA dye	5	105	5	105
Forward primers	0.2	4.2	0.2	4.2
Reverse primers	0.2	4.2	0.2	4.2
Nuclease feee water	2.6	54.6	2.6	54.6
cDNA	2	42	2	42

Table 4. Sequences of SYK and PKC primers used in the study

gene	foward sequence	reverse sequence
SYK	5’ AAT CGG CAC ACA GGG AAA T 3’	5’ CAT CCG CTC TCC TTT CTC TAA C 3’
PKCδ	5’ TGC GCA TCT CCT TCA ATT CC 3’	5’ ACG GCC TTC ATA GAT GTG GG 3’

Table 5. Amplification conditions for SYK and PKC-□ genes.

Step	Temperature °C	Time (minutes)
1. Initial denaturation	95	11
2. Denaturation	95	1
3. Primer Annealing	60	1
4. Extension	95	2
5. Final Extension	95	5
Steps 2 –4 repeated 35 x		

Protein extraction, quantification and western blot

Infected and treated macrophages were washed twice with 1ml of ice-cold 1× cold PBS. The cells were then lifted with Accutase solution and spun down at 1000rpm for 5 minutes in 5ml of 1× PBS. The supernatant was discarded and the pellet was resuspended in 250µl of RIPA lysis buffer with a cocktail of protease inhibitors both which were purchased at (Sigma-Aldrich SA), followed by incubation for 15 minutes at 4^{o}C. The lysates were then centrifuged at 13 000rcf for 15 minutes at 4^{o}C using Labnet Prism R centrifuge. The protein-containing supernatant was then transferred to a 1.5ml eppendorf tube and stored at -80 ^{o}C until use.

Quantification of the protein was performed using the Pierce™ BCA protein assay kit (ThermoFisher). Briefly, 25µl of - fold serially diluted standards and samples were added to a 96-well plate and 200µl of working reaction was then added to each well and mixed on a shaker for 30 seconds. The plate was then covered with foil and left incubating at 37°C for 30 minutes. The plate was cooled at RT and the optical density (OD) values were measured at 562 nm on a RT-2100C microplate reader. The concentration of the protein samples was then determined from the standard curve.

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Quantified whole cell protein lysates were thawed on ice for 30 minutes and then mixed with loading dye and double distilled water to make up a total loading volume of 30µl containing 10µg of protein in all samples. Samples were denatured for 5 minutes in a heating block at 100^{o}C before being loaded on an 8% SDS-PAGE gel. Proteins were then separated by SDS-PAGE at 100V for 90 minutes. Thereafter, the protein bands were then transferred to nitrocellulose membrane (Hybond ECL, GE Healthcare Life Sciences, Amersham, United Kingdom) at 100V for 1 hour 30 minutes. In order to ensure that equal amounts of protein had indeed been loaded and that the protein transfer had occurred successfully, the membranes were reversibly stained with Ponceau S and the gel was stained with Coomassie blue. After washing off the Ponceau-S with distilled water, the membrane was then blocked with 5% non-fat dry milk (phospho-SYK) or 5% (w/v) non-fat dry milk (SYK or GAPDH) in 0.1% TBS (Tris, NaCl, HCl, deionised water) tween-20 for 1 hour at RT. The membrane was then incubated overnight at 4^{o}C with mouse anti-human SYK/p-SYK or mouse anti-human GAPDH primarymonoclonal antibodies (Cell Signalling Technology) were used to probe for proteins. Membranes were washed for 30 minutes in a series of PBS/Tween wash steps at 5 and 10 minute intervals and incubated with goat anti-mouse antibody conjugated to HRP (1:1000) (Bio-Rad, Hercules, CA, USA) for 1 hour at RT in an orbital shaker. Thereafter, membranes were washed for 30 minutes in a series of PBS/Tween wash steps at 5- and 10-minute intervals. Supersignal West Pico Substrate chemiluminescent reagent (ThermoFisher) was added, and thesignal captured using Biospectrum imaging system and Vision Works LS software (UVP, Cambridge, UK) and/or in the darkroom using various exposure times.

Colony forming units (CFU) count

The infected cells were lysed with 1× Triton-X after 48 hours post-infection and the lysate serially diluted and plated on 7H10 Middlebrook agar plates. The plates were incubated for 21 days to determine the number of colony forming units.

Statistical analysis

Data were analysed using Prism software (GraphPad). Student's *t*-test was used to ascertain statistically significant differences between untreated and treated samples. Data are shown as

the mean ± SEM and represent at least two independent experiments. A $p<0.05$ was considered as statistically significant.

CHAPTER 3: RESULTS

Abundance of SYK protein at tissue level is associated with disease severity.

Previous work in our laboratory has characterized the spatial organization of inflammatory proteomic signatures in granulomas of patients with severe TB-induced lung damage (Marakalala *et al.*, 2016). These patients were treatment-refractory and had undergone surgery to remove parts of their lungs. By analyzing the publicly available proteomics data, we have determined the abundance of SYK in the various regions of each granuloma type. Our analysis showed that SYK expression was predominantly associated with the necrotic regions of caseous and cavitary granulomas, and less abundant in cellular regions of solid granulomas (figure 5A)

More data on the expression of SYK in TB granuloma was acquired from a recent study by Seto *et al.* From this study, data pertaining to human lung granulomas induced by Mtb or *Mycobacterium avium* complex lung disease (MAC-LD) was deposited to Proteome Xchange consortium via jPOST partner repository for public access (dataset identifier PXD014086/JPST000609). Our analysis revealed that SYK expression was higher in caseous regions of six Mtb induced granulomas compared to cellular regions (p=0.0014) (figure 5B). Whereas in the case of of MAC-LD, there was a slight difference in SYK expression in caseous regions compared to cellular regions (p=0.0078) (figure 5C). Taken together, these data suggest that the abundance of SYK expression was associated with exaggerated disease pathology in the lung. Moreover, the increased SYK protein abundance at the site of severe tissue damage suggested that SYK might be a possible host factor contributing to TB disease progression in humans.

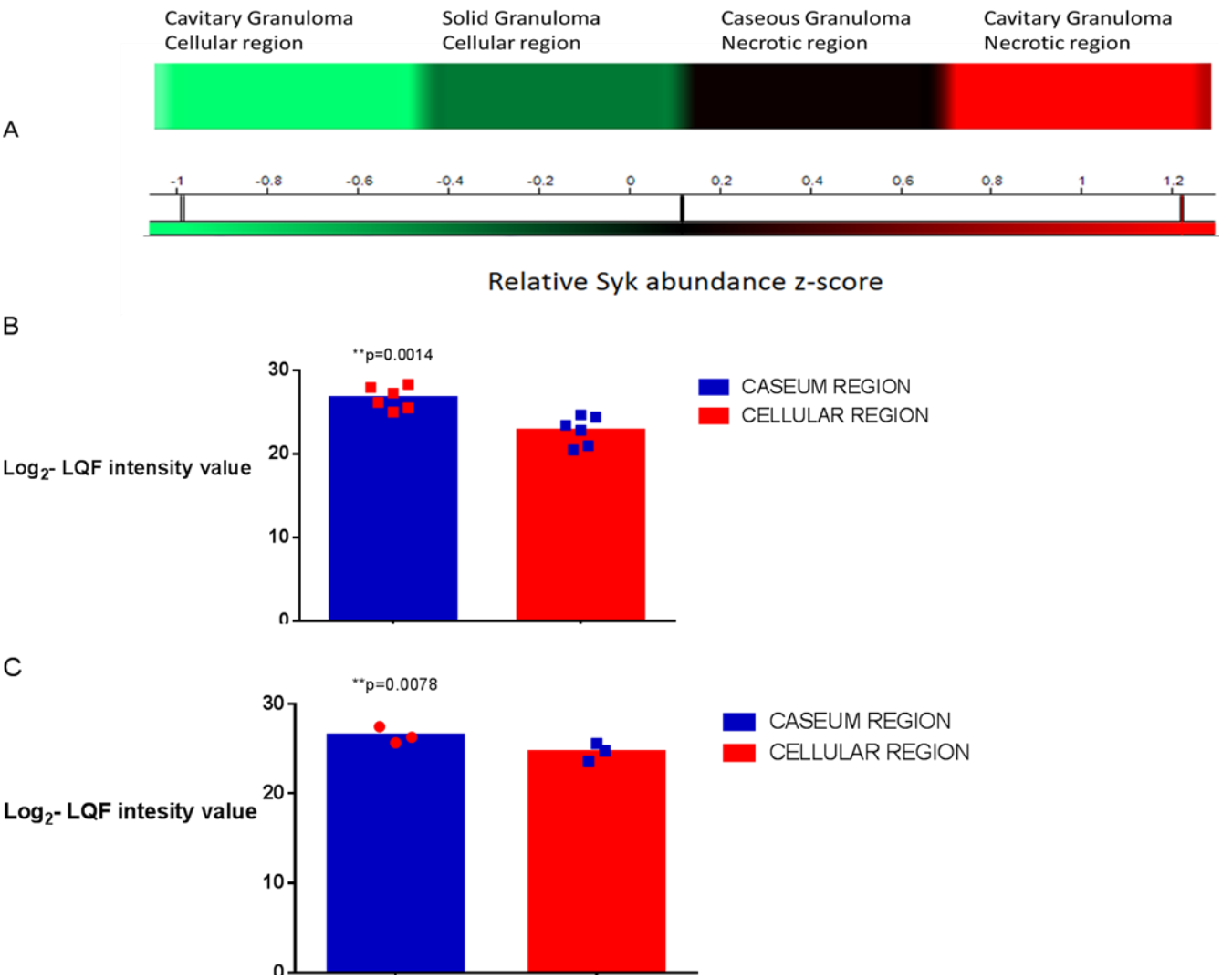

Figure 5. Increased expression of SYK in necrotic regions of caseous granulomas from the lungs of patients with severe TB disease (A) Heat map of Z-score and log2-transformed label-free quantification (LFQ) intensity of SYK in different regions of various granuloma types. Different granulomas had been excised from patients infected with multidrug-resistant Mtb and had undergone lung pneumonectomy (*n*=5) (B-C). Quantitative profiling of SYK protein abundance in Mtb (B) or *Mtb avium* complex lung disease (MAC-LD) (C) in granulomatous sub-compartments of the lung. Log2-transformed LFQ intensity values of SYK in each granuloma region are shown. *n*=6 and *n*=3 for B and C, respectively.

SYK inhibition with Fostamatinib reduced the secretion of pro-inflammatory cytokines in macrophages infected with BCG.

Initially, intracellular pathogens at the site of infection are controlled by innate immune cells such as macrophages. Macrophages respond to mycobacterial infection by producing pro- and anti-inflammatory cytokines which would, as a result, determine the fate of the pathogen. We sought to determine the effect of SYK inhibition on the production of pro-inflammatory cytokines by monocyte-derived macrophages. We infected macrophages with *Mycobacterium bovis* BCG at MOI of 5 or 10 over 48 hours, with or without SYK inhibition with fostamatinib or piceatennol. Supernatants from the macrophage culture were analysed for extracellular TNF-α, IL-1β and IL-6 using the BD OptEIA™ ELISA kits. Untreated and uninfected macrophages produced very little cytokines compared to BCG-infected macrophages, which produced high amounts of cytokines (figure 6A-C). Fostamatinib treatment resulted in a significant reduction of BCG-induced TNF-α and IL-6 production at both day 1 and day 2 (figure 6A-C). The inhibition also resulted in reduction of IL-1β at day 1 and no significant changes were observed at day 2 compared to the untreated and BCG infected macrophages (figure 6B). We also evaluated the BCG induced production of the anti-inflammatory cytokine, IL-10, and found that BCG does not induce detectable amounts of IL-10 (data not shown). These results suggested that SYK plays a key role in production of pro-inflammatory cytokines in macrophages infected with BCG, and that this response can be abrogated through inhibition with fostamatinib. It is therefore important to unearth the implication of this abrogation on cell death as it has been established that excessive pro-inflammatory cytokines can kill cells.

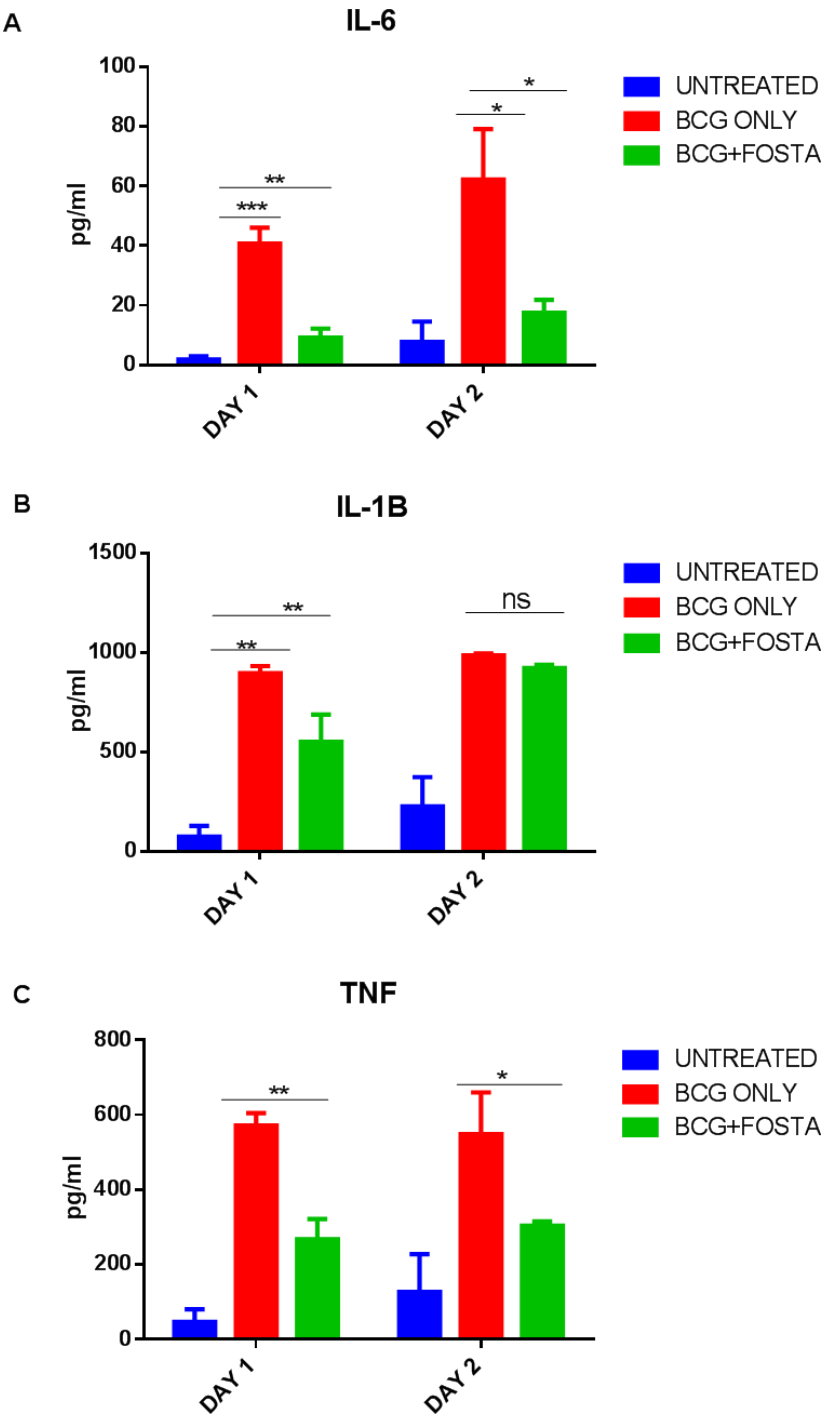

Figure 6. Blockade of spleen tyrosine kinase (SYK) with Fostamatinib reduces BCG-induced production of pro-inflammatory cytokines. Cultured Thp-1 cells were differentiated into macrophages using 100nM PMA for 48 hours. The cells were pre-treated with 20μM fostamatinib overnight and then exposed *in vitro* to live *M. bovis* BCG at the MOI of 10 as indicated. The levels of cytokines in the culture supernatant at 24- and 48-hours post-infection were evaluated using ELISA for (A) IL-6, (B) IL-1β and (C) TNF. The experiment was repeated 3 times and samples were analysed in triplicates. Significant differences (* $p<0.05$) were determined by Student's-*t* tests.

SYK inhibition with Fostamatinib or piceatannol does not directly affect the pro-inflammatory profile of macrophages.

We next wanted to advance our inhibition and infection studies to a pathogenic strain of Mtb (H37Rv), that would have a more clinical relevance, to see if we could recapitulate the same findings we obtained with BCG infection. However, before we could continue, it was imperative to show that the SYK inhibitory drugs, fostamatinib or piceatannol, did not induce any cytokine expression or had any kind of toxicity to the cells. We found that the drugs did not directly induce the production of pro-inflammatory cytokines while LPS significantly induced the expression of TNF and IL-1β compared to untreated cells or cells treated wth SYK inhibitors. (figure 7A & B). As a positive control, LPS was included and its induction of cytokines was very high. This data suggests that fostamatinib and piceatennol did not have a direct effect on the production of the cytokines concerned. This means that all the cytokines were induced solely by infection with BCG.

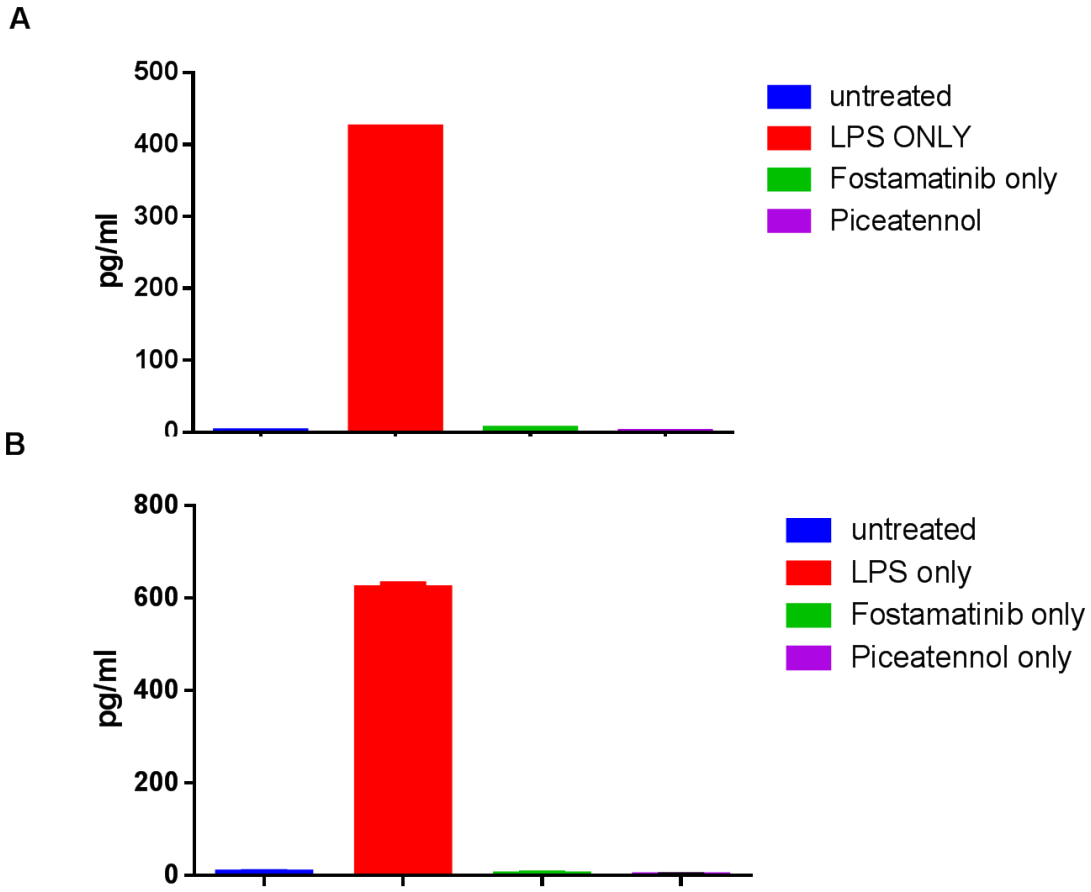

Figure 7. Fostamatinib or piceatennol do not directly induce inflammatory cytokine production. Cultured Thp1 monocytes were differentiated into macrophages in a 24-well plate using 100nM PMA for 48 hours. The cells were pre-treated with 20μM concentrations of fostamatinib or piceatennol overnight. Additionally, 10ng/ml LPS was also added as a positive control. The levels of cytokines in the culture supernatant at 24 hours post-infection were evaluated using ELISA for TNF (A) and IL-1β (B). The data shown is expressed as mean ± SEM and is representative of 2 independent experiments. Significant differences (*p<0.05) were determined using Student's-t tests.

SYK inhibition with fostamatinib rescues macrophage from cell death after infection with BCG

BCG infection of macrophages is associated with the production of pro-inflammatory cytokines, which is a defence mechanism against intracellular pathogens. However, overwhelming inflammatory responses can be detrimental to cells, as it is well established that too much inflammation drives tissue damage and possibly plays a role in the pathogenesis of TB progression (Marakalala *et al.*, 2016). We therefore determined whether inhibition of SYK and the subsequent reduction of pro-inflammatory cytokines during BCG infection would rescue the cells from death. We infected macrophages with BCG and collected supernatant after 48 hours to determine cell death using lactate dehydrogenase (LDH) assay. Interestingly, we found that even though there was some form of spontaneous cell death in untreated cells, there was an increase in cell death upon BCG infection, which was rescued by fostamatinib treatment in a seemingly dosage dependent manner (figure 8). This data suggests that Fostamatinib's ability to reduce BCG-induced inflammatory cytokines could be effecting a reduction in cell death.

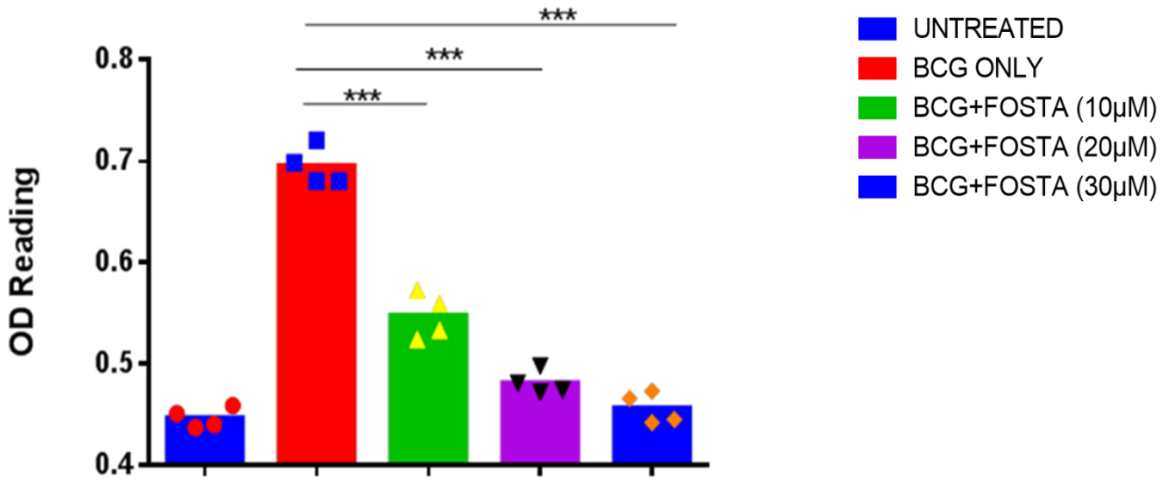

Figure 8. Blockade of spleen tyrosine kinase (SYK) with Fostamatinib reduces BCG-induced macrophage cell death. Cultured Thp1 cells were differentiated into macrophages in a 24-well plate using 100nM PMA for 48 hours. The cells were pre-treated with varying concentrations of fostamatinib overnight and then infected with *Mycobacterium* bovis BCG at the given MOI of 10. The levels of lactose dehydrogenase (LDH) in the culture supernatant at 48 hours post-infection was determined by an LDH assay following the manufacturer's instructions. The data shown are expressed as mean ± SEM. The experiment was repeated 3 times and samples were analysed in triplicates. Significant differences (*p<0.05) were determined using Student's-*t* tests.

SYK inhibition with Fostamatinib reduced secretion of pro- and anti- inflammatory cytokines in macrophages infected with pathogenic Mtb H37Rv strain.

We have demonstrated using a BCG-macrophage infection model that SYK inhibition by fostamatinib reduced expression of pro-inflammatory cytokines by macrophages and that this in turn, rescued the cells from BCG-induced death. We therefore sought to investigate macrophage responses to a more clinically relevant and virulent strain of *Mycobacterium tuberculosis* H37Rv and whether these responses would be dependent on SYK signalling. Thp-1 derived macrophages were treated with fostamatinib overnight, infected with Mtb H37Rv at an MOI of 10 and culture supernatants were collected at day 1 and day 3 after infection. TNF, IL-6 and MCP-1 were significantly increased in macrophages that were infected with Mtb H37Rv compared to untreated cells (figure 9B & C). Interestingly, we found that treating infected cells with fostamatinib significantly reduced the production of TNF, IL-6 and MCP-1 compared to untreated cells (figures 9B, C & E). Moreover, we found no difference in the production of IL-1β both at day 1 and day 3 post infection between infected untreated macrophages and infected macrophages that were treated with fostamatinib (figure 9D). Macrophage infected with Mtb H37Rv significantly upregulated the production of IL-10 and this was significantly abolished by treatment with fostamatinib (figure 9A). It is worth noting that treatment of the cells with fostamatinib alone did not induce expression of any of the cytokines that were analysed (figure 9). Taken together, these results indicate that SYK plays a fundamental role in expression of both pro- and anti-inflammatory cytokines in a response to virulent mycobacterial infection.

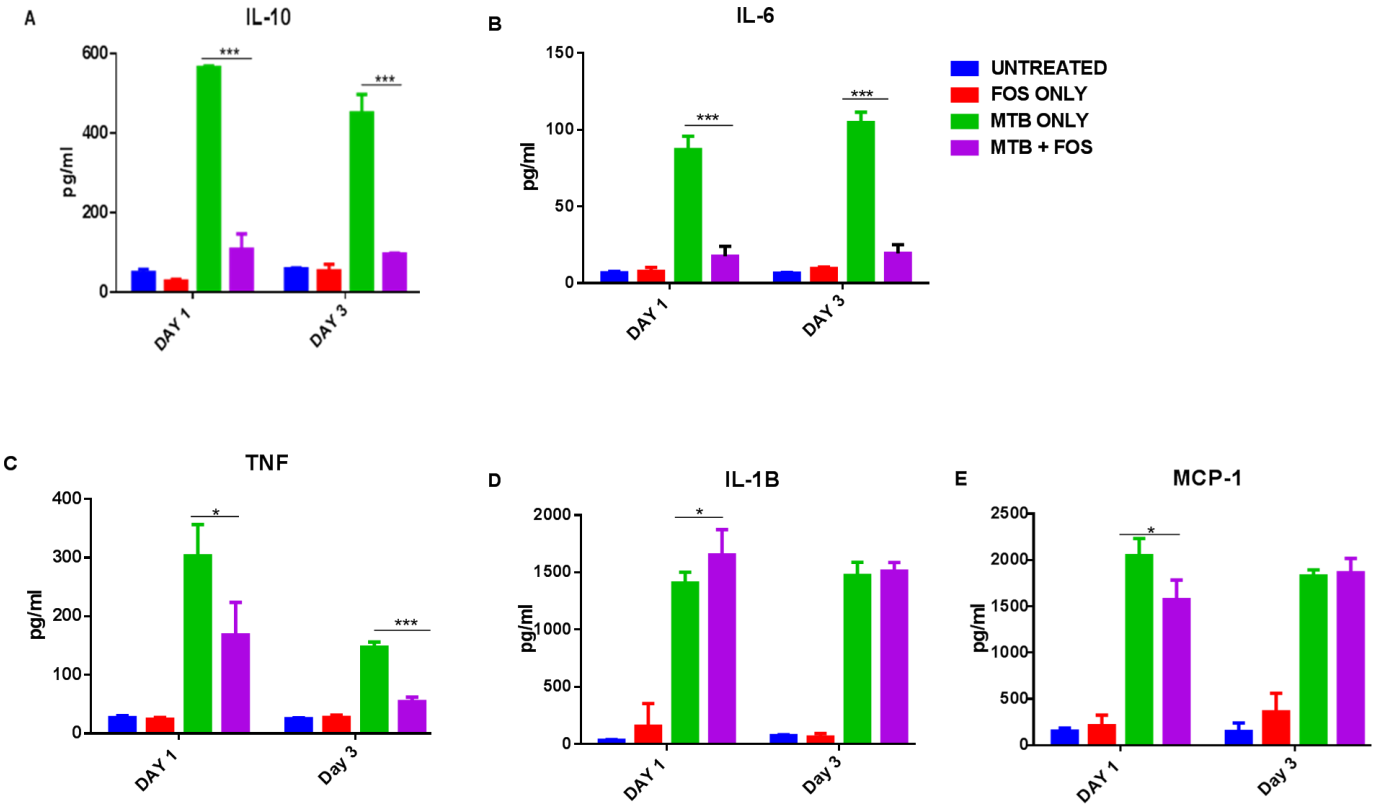

Figure 9. Inhibition of spleen tyrosine kinase (SYK) with Fostamatinib reduces production of pro- and anti-inflammatory cytokines by macrophages infected with Mtb H37Rv. Cultured Thp-1 cells were differentiated into macrophages using 100nm PMA over 48 hours. The cells were pre-treated with 40μM fostamatinib overnight and then exposed *in vitro* to live Mtb H37Rv at the MOI of 10. The levels of cytokines in the culture supernatant at 24- and 72-hours post-infection were evaluated using ELISA for (A) IL-10, (B) IL-6 (C) TNF, (D) IL-1β and (E) MCP-1. The data shown are expressed as mean ± SEM and are representative of 2 independent experiments.Significant differences (* $p<0.05$) were determined by Student's-*t* tests.

SYK inhibition with fostamatinib rescued macrophages from Mtb H37Rv-induced cell death.

We also went further to determine whether inhibition of SYK would also rescue macrophages from Mtb H37Rv induced cell death as we had earlier observed with BCG. Interestingly, we found that fostamatinib significantly reduced cell death as indicated by reduced release of LDH compared to infected and untreated cells at day 1 post infection (figure 10). We observed residual cell death in untreated cells as well as cells that were only treated with fostamatinib as indicated by the detection of LDH (figure 10). In contrast, we found that fostamatinib failed to reduce cell death at day 3 post-infection compared to the untreated and infected macrophages (figure 9). Therefore, we concluded from this data that fostamatinib's ability to reduce inflammatory cytokines could be directly or indirectly linked to the reduction of Mtb H37Rv induced cell death.

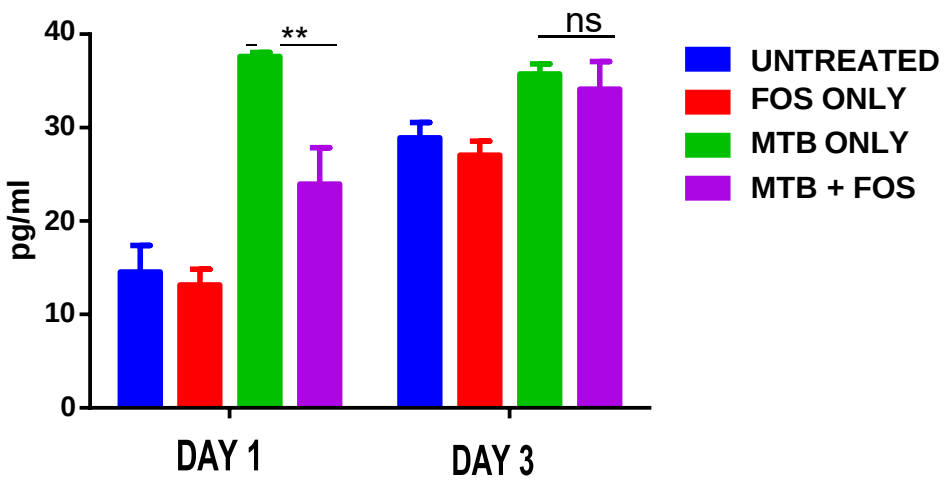

Figure 10. Inhibition of spleen tyrosine kinase (SYK) with Fostamatinib rescued macrophages from Mtb H37Rv induced cell death. Cultured Thp1 cells were differentiated into macrophages in a 24-well plate using 100nM PMA for 48 hours. The cells were pre-treated with 40µM of fostamatinib overnight and then exposed *in vitro* to live Mtb H37Rv at an MOI of 10. The levels of lactate dehydrogenase (LDH) in the culture supernatant at 24 and 72 hours post-infection was determined by an LDH assay following the manufacturer's instructions. The data shown is expressed as mean ± SEM (where n = 3 samples) and is representative of 2 independent experiments. Significant differences (* $p<0.05$) were determined using Student's-*t* tests.

Effects of fostamatinib and piceatennol on the intracellular survival of Mtb.

Up to date, there had been no studies exploring the effect of inhibiting SYK signalling on the intracellular survival of Mtb in macrophages. However, a recent study looking at the potential of protein kinase G (PKnG, a Mtb virulence kinase) inhibitors, identified an active metabolite of fostamatinib R406 as one of the PKnG inhibitors. The study found that R406 could inhibit PKnG and reduce survival of Mtb inside macrophages by increasing lysosomal transfer of bacilli (Kanehiro *et al.*, 2018). We therefore evaluated the effect of blocking the activity of SYK using fostamatinib and piceatannol on the intracellular survival of Mtb in macrophages. The results showed that fostamatinib significantly reduced the growth of Mtb inside macrophages (reduction from 2.23×10^6 to 8.5×10^5 CFU/ml, $p<0.05$) (figure 11). Another inhibitor, piceatannol, had no effect on the intracellular clearance/survival of Mtb (figure 11). These results suggest that SYK inhibition not only plays a role in reduction of macrophage inflammatory responses but may also influence the survival of Mtb in macrophages. It remains to be evaluated whether this is as a result of direct inhibition of Mtb replication by fostamatinib or via regulation of immune responses. In addition, other mechanisms could involve the off target effect of fostamatinib on other kinases.

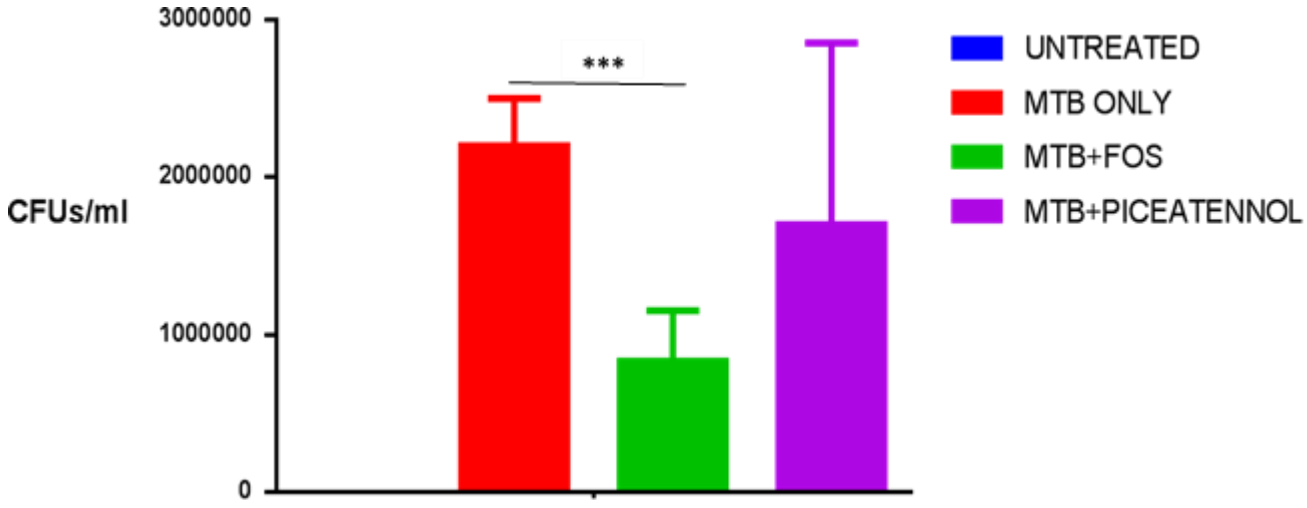

Figure 11. Effects of SYK inhibition on the growth of pathogenic Mycobacterium tuberculosis H37Rv in macrophages. Macrophages were seeded in a 12-well plate (5×10^5 cells/mL) and treated with 20μM fostamatinib or piceatennol. Untreated macrophages under basal conditions (cultured in RPMI and 10% FBS) were included as controls. The cells were infected at an

MOI of 10 after which they were incubated at 37^0C in a 5% CO_2 incubator. After 48 hours, the cells were lysed, and serial dilutions of the lysates were inoculated in triplicates onto Middlebrook 7H10 agar plates. The plates were incubated and enumerated after 21 days. The data is a representation of 2 independent experiments. Significant differences (*p<0.05) were determined by Student's-t tests.

CHAPTER 4: DISCUSSION

Presently, TB remains the top causative agent of mortality amongst all infectious diseases globally with over 1.5 million deaths reported in 2018 alone (World Health Organization, 2019). TB can be managed with antibiotic therapy which is effective and includes first line drugs such as isoniazid, rifampicin, pyrazinamide and ethambutol. Inspite of these available drugs, it is still a challenge to completely eliminate Mtb in some infected individuals because these drugs lack a stronger potency against slow dividing Mtb (Kanehiro *et al.*, 2018)**.** In addition, Mtb has developed many defence mechanism that can help it escape the immune system and flourish inside macrophages (Liu, Liu and Ge, 2017). The rapid rise of multi-drug resistant strains of Mtb is also another issue, aggravating the mortality of TB. Therefore, there is an urgent need for the development of drugs that may target the host in order to boost its antimycobacterial activity while dampening an aggressive inflammatory response.

In this study, we have demonstrated the role played by SYK in response to mycobacterial infection in macrophages. We have shown that SYK is abundant in necrotic regions of caseous granulomas in the lungs that were obtained from treatment refractory patients with severe TB disease. We have also analysed publicly available data from the Proteome Xchange consortium and found that SYK was highly expressed in caseous regions than cellular regions of a granuloma (Seto *et al.*, 2020). Pro-inflammatory signatures are necessary for early control of bacterial replication but could lead to tissue damage if not regulated (Tobin *et al.*, 2011). The question that we therefore wanted to answer was whether SYK inflammatory signalling was behind the pathological inflammation at the site of infection, or its abundance was remnants of robust efforts of the immune system to control the bacteria.

We have used a common, well established model of Thp-1 derived macrophages to evaluate the therapeutic potential of SYK inhibition with fostamatinib, an orally available small molecule inhibitor of SYK. We also used two different strains of mycobacteria: *Mycobacterium bovis* BCG Pasteur as well as the virulent *Mycobacterium tuberculosis* H37Rv to study the effects of SYK inhibition on macrophages after infection. Our data showed that inhibition of SYK with fostamatinib in macrophages infected with BCG reduced the production of pro-inflammatory cytokines TNF, IL-1β and IL-6 and this was also consistent in macrophages infected with Mtb H37Rv with the exception of IL-1β. Fostamatinib failed to reduce IL-βproduction on both day 1 and day 3. In addition, Mtb H37Rv also induced expression of anti-

inflammatory cytokine IL-10 as well as a chemokine MCP-1 both of which were reduced by fostamatinib treatment.

Fostamatinib rescued macrophages from cell death induced by infection with BCG. Similarly, fostamatinib reduced cell death induced by Mtb H37Rv after 24 hours of infection; however, the effect dissipated at day 3 post infection. Lastly, we showed that fostamatinib treatment reduced the intracellular growth of Mtb H37Rv in macrophages.

During Mtb infection in the lungs, early pathogen recognition begins with alveolar macrophages, a critical component of the innate immune system (Cohen *et al.*, 2018). The early Mtb-macrophage interaction is followed by recruitment of monocytes from subtending blood vessels to the site of infection which differentiate into interstitial macrophages (Huang *et al.*, 2018). Additionally, macrophages constitute the highest cell population at the site of pulmonary Mtb infection (D. G. Russell *et al.*, 2009). It is for these reasons that our study attempted to model early events occurring in the lungs by establishing an infection model of monocyte derived macrophages.

Differentiation of Thp1 monocytes into macrophages was induced by the addition of 100nM PMA for 48 hours. We observed that macrophages at this time had fully differentiated and adhered to the plate. The morphology had also changed from small round monocytes to large spindle shaped macrophages (Park *et al.*, 2007). Furthermore, macrophage stability was also great as the cells remained adhered for up to 4 days. Macrophages responses to LPS was also similar to those of studies which showed a high expression of cytokines such as TNF, IL-1β and IL-6 compared to unstimulated macrophages (Chanput, Mes and Wichers, 2014). Secretion of cytokines and chemokines is also another way of recruiting other immune cells following activation of macrophages (Chávez-galán *et al.*, 2016). Similarly to LPS stimulation, BCG also triggered macrophage production of TNF, IL-6 and IL-1β, which can be detected at 24 and 48 hours post-infection. These findings were echoed by other studies that looked at macrophage responses to BCG at low MOI (Chávez-galán *et al.*, 2016).

Many studies have investigated the the effects of host-directed therapeutic agents on the survival of Mtb inside macrophages. (Guerra-de-blas *et al.*, 2019) Like many studies, we evaluated if fostamatinib could affect the growth of Mtb in macrophages. Our data showed a significant reduction in Mtb growth in macrophages treated with fostamatinib.

Cytokines such as TNF and IL-6 play a key role in modulation of macrophage immune responses against mycobacterium. In this study, we realized that mycobacterium-induced pro-

inflammatory cytokines such as IL-6 and TNF were reduced by fostamatinib. Both these cytokines are differentially essential for anti-mycobacterial immunity by mediating inflammation (Martinez, Mehra and Kaushal, 2013). On the other hand, excessive production of these cytokines may also bring about local necrosis in tissue and therefore pathology (Turner *et al.*, 2014).

Similar to our findings was work done by Rothfuchs et al., which showed that SYK inhibition with piceatannol reduced Mtb induced IL-12p40 in splenic dendritic cells. Their results, however, were in contrast with our findings because they also found that SYK inhibition does not affect TNF expression by splenic dendritic cells. It is possible that these differences are due to different cell types whereby they used dendritic cells and we used macrophages. Our data showing fostamatinib reduction of these Mtb induced cytokines is consistent with other findings from other studies that have demonstrated that anti-TNF agents can limit tissue pathology and enhance mycobacterial clearance (M.-L. Bourigault *et al.*, 2013).

However, it is important to note that most studies looking at TNF neutralization were *in vivo* studies while our study was *in vitro*. In addition, these studies looked at TNF induced pathology in the context of lung examinations while we looked at pathology in the context of cell death. The limitations of using cell death as a measure of pathology is that cell death in the context of mycobacteria infection could be beneficial if it is apoptotic, and detrimental if it is necrotic or pyroptotic (Butler *et al.*, 2012). Moreover, studies have shown that virulent strains of Mtb such as H37Rv tend to inhibit apoptosis in macrophages and trigger necrotic cell death (Behar *et al.*, 2011). This is contrary to less pathogenic strains such as BCG which predominantly induces apoptosis, a host protective cell death that reduces Mtb viability (Behar, Divangahi and Remold, 2010).

Our study also aimed to determine whether SYK inhibition could affect the mode by which macrophages die after Mtb infection. Apoptosis is a cell death modality which results in an intact plasma membrane while necrosis is a form of death defined by complete cell lysis (Behar *et al.*, 2011). Apoptosis tends to favour the host against Mtb as it causes infected cells to secrete signals that recruit other phagocytes to clear the infected cells. Necrosis on the other hand is to the detriment of the host as the complete cell lysis allows Mtb to escape and infect more cells. In addition, necrosis leads to release of cell contents that cause excessive inflammation (Henson and Tuder, 2008; Silva, do Vale and dos Santos, 2008)

We had therefore hypothesized that the fostamatinib reduction of pro-inflammatory cytokine by Mtb infected macrophages would favour more apoptotic cell death. Flow cytometry results showed no significant difference in necrosis or apoptosis in fostamatinib treated versus untreated macrophage (Appendix figure 14). Moreover, we encountered a setback during our research whereby there was a high unexplained cell death in untreated control group. Due to time constraints caused by COVID-19, this experiment could not be repeated to investigate this spontaneous cell death.

IL-1β is another cytokine that mediates inflammation and is necessary for host mycobacterial resistance. Research has shown that IL-1β knockout mice are more susceptible to Mtb infection as indicated by increased lung bacterial burdens and high mortality (Krishnan, Robertson and Thwaites, 2013). Our results showed that fostamatinib only reduced IL-1β production on day 1 after infection; however, there was no difference in IL-1β levels between infected macrophages and infected and treated macrophages at day 3 after infection. This implies that SYK pathway is perhaps dispensable for IL-1β production or other innate signalling pathways may compensate for the lack of SYK in the production of IL-1β. The NLRP3 inflammasome for example, is another pathway that has been shown to function differentially between virulent Mtb and attenuated *M. bovis* BCG induced IL-1β production (Dorhoi *et al.*, 2012).

Work by Bourigault et al., has demonstrated the significance of IL-1α and IL-1β to the host in response to both virulent Mtb and BCG. The authors observed that the absence of both these cytokines resulted in uncontrolled infection and excessive lung inflammation (M. Bourigault *et al.*, 2013). Similarly, another study showed that susceptibility of mice to chronic Mtb infection is due to blockade of IL-1α and IL-1β, suggesting that expression of IL-1β is associated with a better disease outcome (Guler *et al.*, 2011). They also showed that the presence of either one of these cytokines can reverse this phenotype. Moreover, IL-1β is even more essential in Mtb infection of macrophages, however, the whole IL-1 pathway is said to be dispensable in the clearance of less pathogenic BCG (M. Bourigault *et al.*, 2013). Perhaps this phenomenon could explain the ability of fostamatinib to reduce IL-1β expression on day 1 only and not in day 2 or during late stage infection with BCG.

These findings pointing to the importance of IL-1β in Mtb infection are however, contradicted by work done by Zhang et al., who demonstrated and defined the links between single nucleotide polymorphisms (SNPs) in the IL-1β gene and the severity of TB disease outcome. They showed that increased IL-1β is associated with pathological inflammation (Zhang *et al.*,

2014). Additionally, research also demonstrated that NLRP3-produced IL-1β increases Mtb induced immunopathology and that this can is controlled by nitric oxide (Mishra *et al.*, 2012).

IL-10 is an anti-inflammatory cytokine largely known for its inhibitory effects on the innate immune cells that are required to control Mtb and on the initiation of immune responses (Redford, Murray and O'Garra, 2011). We reported that fostamatinib significantly reduced the secretion of IL-10 by macrophages infected with Mtb H37Rv, while infection with BCG failed to induce IL-10 production. The role of IL-10 in TB has been much debated, with evidence showing its antimycobacterial effects in limiting tissue pathology (Guler *et al.*, 2011). Some evidence, however, supports its role in establishment of chronic Mtb infection as its production was seen as a mechanism by which Mtb can subvert the host protective response (Das *et al.*, 2014). In our research, the reduction of IL-10 production by macrophages that were infected with Mtb H37RV and treated with fostamatinib was probably beneficial to the host as we observed significantly reduced intracellular Mtb survival in treated macrophages.

Several other studies reported that IL-10 deficiency in mice infected with Mtb resulted in low lung bacterial burdens and enhanced Th-1 responses. Furthermore, human IL-10-expressing mice were more susceptibible to *Mycobacterium avium* infections. (Feng *et al.*, 2002; Redford *et al.*, 2010). These findings were also consistent with our findings of decreased IL-10 and low Mtb burdens in macrophages. However, a study by O'Leary et al., investigated the role of IL-10 in a similar infection model to ours. Their findings contrasted with our speculation of reduced IL-10 being associated with decreased Mtb survival as they reported that blocking IL-10 inhibited phagolysosome maturation and enabled Mtb to flourish intracellularly (O'Leary, O'Sullivan and Keane, 2011). Furthermore, human polymorphism studies in the IL-10 gene have also been shown by several studies to render humans more susceptible to TB (Oral *et al.*, 2006; Oh *et al.*, 2007). These studies have proved to be inconclusive as they had mixed outcomes, which seemed to vary according to geographical factors (Redford, Murray and O'Garra, 2011).

We also attempted to validate the pharmacological inhibition of SYK at two levels: at gene level as well as at protein level using RT-qPCR and Western blot. However, due to time constraints, we were unable to validate this inhibition as Western blot and RT-qPCR experiments failed to yield expected results. In the Western blot, we did not see the expected reduction of the SYK protein band in cells that were treated with fostamatinib compared to the untreated (Appendix figure 12). Similarly, at gene level, our results were inconclusive as we

did not see a reduction in SYK gene expression after treatment of macrophages with fostamatinib (Appendix figure 13). These observations were similar to findings by Rothfuch et al., whose data showed that Mtb infection of splenic dendritic cells did not necessarily affect total SYK expression, but resulted in increased SYK phosphorylation, which could be diminished by SYK inhibition with piceatannol (Rothfuchs *et al.*, 2007). This suggested that fostamatinib could perhap be targeting SYK at the protein phosphorylation level. Future work should determine the effects of fostamatinib on SYK phosphorylation after infection with Mtb.

A study by Kanehiro et al., identified PKnG as another kinase that can be inhibited by R406, an active metabolite of fostamatinib. Similarly, to our study, the authors reported no cytotoxicity of R406 on Thp-1 monocytes at concentrations similar to our fostamtanib concentrations. Moreover, the authors reported that PKnG promoted survival of BCG inside macrophages (Kanehiro *et al.*, 2018). Therefore, R406, a PKnG inhibitor was able to decrease Mtb survival in macrophages. This study was of great importance to our research as it can perhaps explain why we were unable to show inhibition of SYK at protein level. It is possible that fostamatinib had off target effects and its active form targeted other kinases such as PknG. This suggests that perhaps the reduction in intracellular survival of Mtb inside macrophages was due to PKnG inhibition and not necessarily SYK or a synergistic effect of the two. Interestingly, PKnG is one of the mycobacterial protein factors involved in cellular metabolism and escaping lysosomal degradation (Kanehiro *et al.*, 2018; Zhai *et al.*, 2019). It would have been interesting to evaluate whether fostamatinib had a direct effect on survival of Mtb or the reduction in CFUs was due to a different mechanism. However, the former is unlikely to be the case as Yorishama et al., did not find R406 to directly affect BCG survival. Furthermore, since their data suggests that the antimycobacterial activity of R406 is due to its ability to increase phagolysosome fusion, it would have been interesting to also carry out this assay. It is also worth noting that the authors reported a reduction in phagocytotic activity of macrophages treated with high concentrations of R406, adding another layer of complexity to the exact mechanisms of kinase inhibitions on Mtb survival. Future research should look into exact mechanisms underlying fostamatinib facilitated bacterial clearance.

CHAPTER 5: CONCLUSION

We initially hypothesized that SYK plays a key role in disease pathology induced by mycobacteria and that targeting SYK with fostamatinib could reduce expression of mycobacterium induced pro-inflammatory cytokines and cell death, and therefore likely limiting tissue pathology. It is important to note that our hypothesis is based on data that implicated high SYK expression in chronic TB infection of patients whose lungs had undergone pneumonectomy due to disease severity. We speculated that SYK is a driver of disease progression. However, our research study looked at the effects of inhibiting SYK *in vitro* and also studied the effects on this during early infection.

SYK inhibition during early infection resulted in reduction of TNF and IL-6 as well as the anti-inflammatory cytokine IL-10. IL-1β, one of the crucial cytokines in immune responses aimed at Mtb clearance, remained high and uninhibited by fostamatinib. We therefore hypothesize that during early infection of macrophages by Mtb, SYK plays a double-edged sword role by increasing pro-inflammatory cytokines needed to help fight Mtb while also increasing expression of IL-10, in order to try balance out excessive inflammation. However, at such an early stage of infection, IL-10 expression could be detrimental to macrophages as their primary role is pathogen clearance.

The major postulation from this study is that SYK inhibition needs to be balanced to allow enough inflammatory cytokine production that is not damaging while also reducing IL-10 which would otherwise dampen macrophage antimycobacterial activity. Therefore, during early stages of infection, the immune responses to Mtb prevent chronic infection and progression of the disease and this relies heavily on the critical balance between pro-inflammatory and immunoregulatory efforts by macrophages. SYK regulation and inhibition presents such a fundamental host target for a balanced antimycobacterial response.

FUTURE WORK

In future, we need to repeat the flow cytometry experiments with fresh Thp-1 monocytes that have minimal spontaneous cell death in order to determine if SYK inhibition results in apoptosis or necrosis. Moreover, we would need to repeat Western blot in order to determine whether fostamatinib prevents the phosphorylation of SYK after Mtb infection in macrophages. We also intend to explore the effect of fostamatinib treatment alongside first line TB drugs such as rifampicin or isoniazid in order to evaluate whether this combination may have a synergistic effect on clearance of Mtb in macrophages. This would validate SYK as potential adjunctive therapy for TB. We also want to identify which of the CLRs are involvedin the CLR-SYK signalling pathway. Such CLRs can also be intercepted in conjunction with SYK for a possible synergistic anti-inflammatory and anti-mycobacterial effects that we observed. Finally, it would also be interesting to advance our studies to pre-clinical level and look at SYK inhibitors in animal models so as to study the role of SYK in clinical progressionof TB.

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APPENDIX

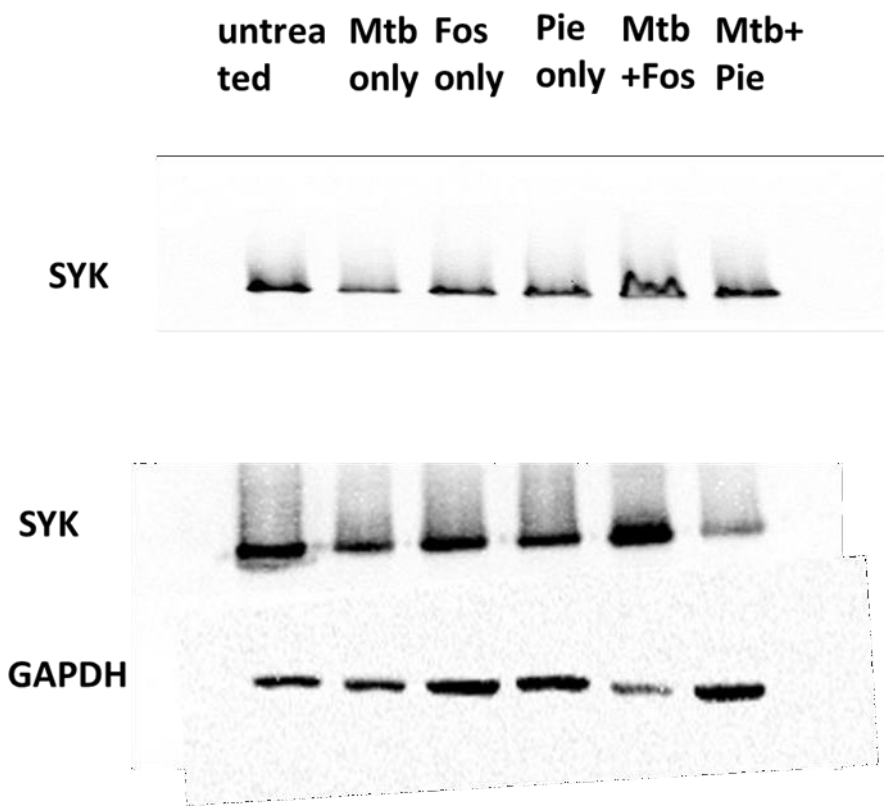

Figure 12. Western blot showing the protein expression of SYK in macrophages after treatment with fostamatinib or piceatannol and infected with Mtb. GAPDH was used as a loading control.

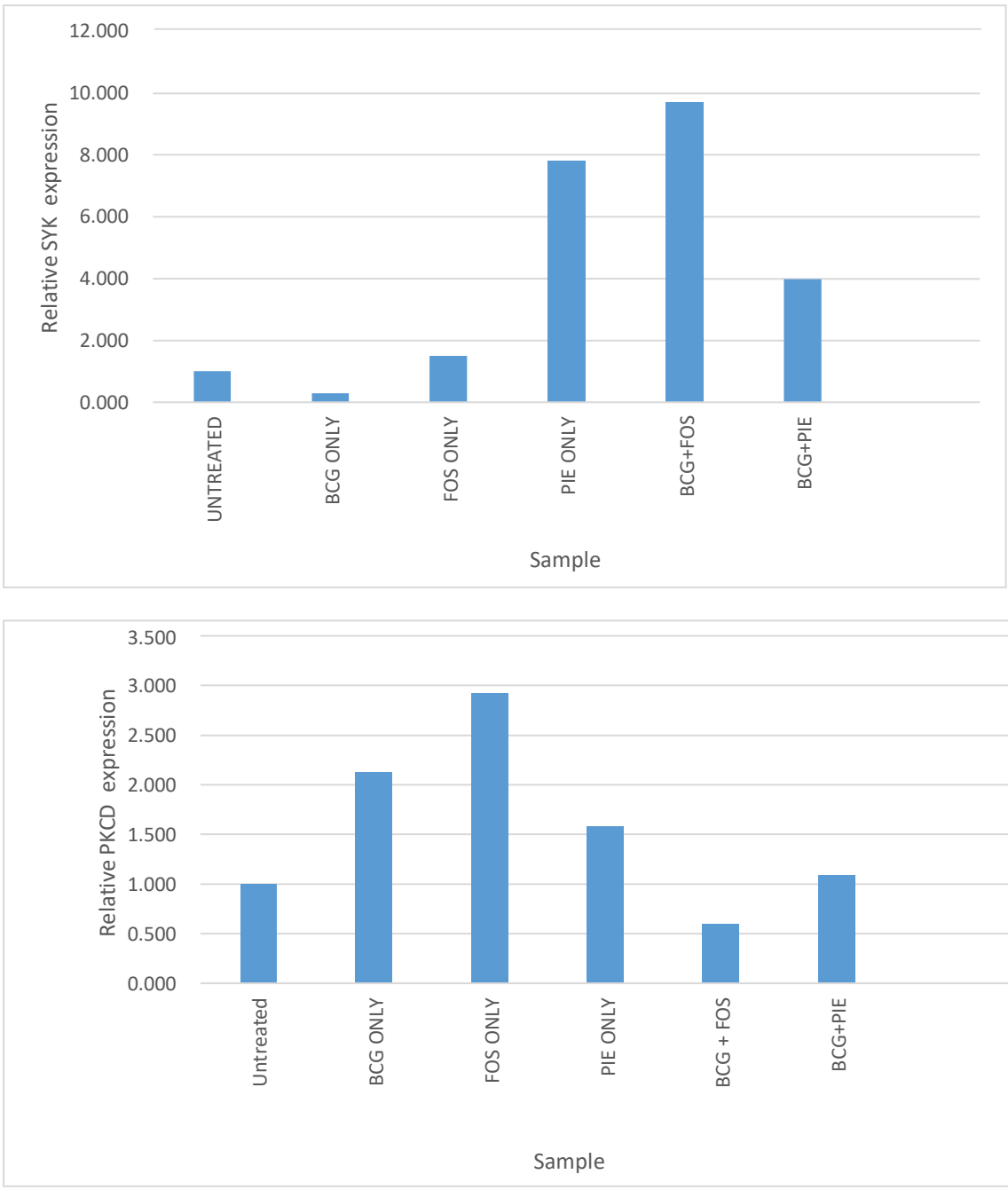

Figure 13. RT-qPCR expression of SYK and PKCδ in mycobacteria-infected macrophages after treatment with fostamatinib/piceatannol. GAPH was used as a house keeping gene on which the genes of interest were normalized.

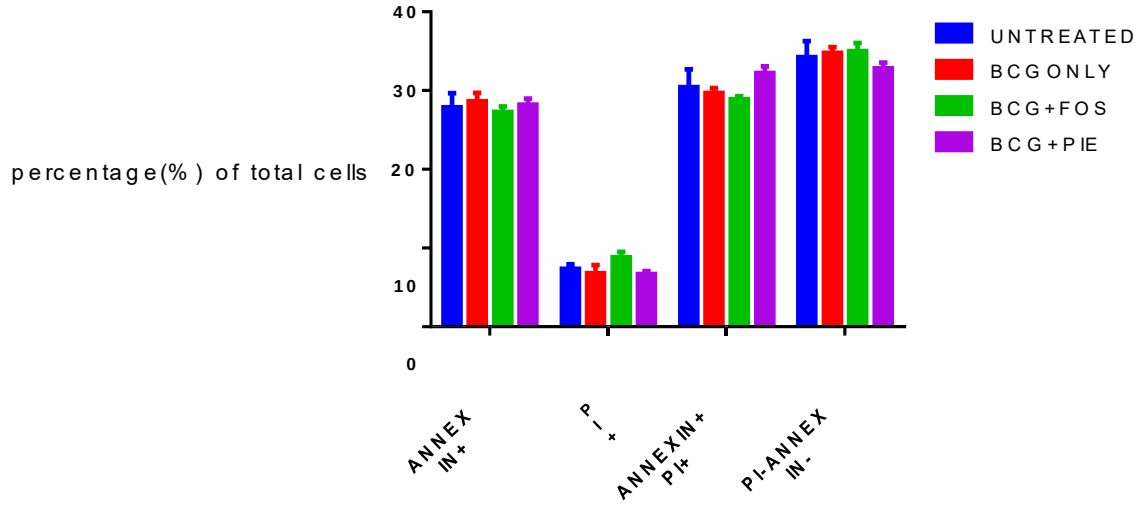

Figure 14. Flow cytometry determination of cell death (apoptosis (annexin+), Necrosis (PI+) or both) after macrophage treatment with Fostamatinib/Piceatannol and infection with BCG.