NEUTROPHILS AND THE REGULATION OF MATRIX METALLOPROTEINASES IN TUBERCULOSIS

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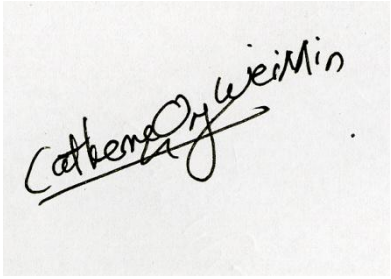
"Reach high, for stars lie hidden in your soul. Dream deep, for every dream precedes the goal." – Mother Teresa

DECLARATION OF AUTHENTICITY

I hereby declare that the following work presented in this thesis is my own.



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ABSTRACT

Tuberculosis (TB) continues to kill globally and this is compounded by increasing drug-resistance. Neutrophil influx and tissue destruction are hallmarks of TB and this is detrimental to the host. A matrix degrading phenotype causes tissue damage in TB whereby the activity of matrix metalloproteinases (MMPs) is unopposed by their specific tissue inhibitors (TIMPs). The hypothesis is that neutrophils have a key role in causing MMP-dependent tissue destruction in human TB. The aims are to investigate MMP and TIMP secretion from *M.tuberculosis* (*M.tb)* infected neutrophils, neutrophil networks regulating MMP secretion and MMP and TIMP activity regulation in neutrophils *in vivo.*

Using an *in-vitro* model, healthy primary human neutrophils were infected *in vitro* with *M.tb* or stimulated with conditioned media from *M.tb*-infected monocytes (CoMTB). MMP-8/-9 and TIMP-1/-2 secretion were analysed by Luminex array and zymography. Intracellular signalling pathways were investigated by phosphoarray, western blot and inhibitors. Gene expression was studied by real-time PCR. Neutrophil extracellular traps (NETS) were examined by immunofluorescence. DQ collagen degradation was examined by confocal microscopy and quantitative fluorescence assay. These studies were extended to investigate MMP-8 and -9 secretion from patients with AMPK mutation to evaluate if AMPK regulates neutrophil MMP secretion. Induced sputum samples from 108 patients and controls were analyzed. Immunohistochemistry of human TB lung specimens was performed.

Neutrophil MMP-8 and -9 secretion are up-regulated 3 and 5 fold by *M.tb* respectively while CoMTB stimulation caused a 2 and 3 fold increase in MMP-8 and -9 (all $p<0.001$). The MAPK, PI3-kinase and AMPK pathway regulate neutrophil MMP secretion in CoMTB-stimulated neutrophils but not *M.tb*-infected cells. *M.tb*-infected

neutrophils degrade collagen, which is abolished by the MMP inhibitor doxycycline. *M.tb*-driven NET formation is associated with MMP-8 and -9. MMP-8 and -9 concentrations are elevated in induced sputum of TB patients compared to controls (both $p<0.001$) and correlate with neutrophil markers myeloperoxidase and neutrophil gelatinase associated lipocalin. MMP-8 concentration correlated with clinical TB severity score ($r=0.55$, $p<0.0001$) and CXR score ($r=0.52$, $p<0.0001$). Induced sputum from TB patients has increased collagenase activity which is suppressed by MMP-8 neutralisation. MMP-8 secretion is decreased in AMPK patients with CoMTB stimulation. Immunohistochemistry of human TB lung specimens confirmed cellular findings.

M.tb drives neutrophil MMP-8/-9 gene expression and secretion following direct infection and in monocyte-dependent networks. Pathways regulating neutrophil MMP secretion are stimulus specific. *In vivo* findings underscore the crucial role that neutrophils have in tissue destruction in TB.

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CHAPTER 1 - INTRODUCTION

1. *The tuberculosis epidemic*

In the Mortality Bills, pneumonia is an easy second, to tuberculosis; indeed in many cities the death rate is now higher and it has become, to use the phrase of Bunyan 'the captain of the men of death.'

— Sir William Osler, 1904 [1]

Tuberculosis (TB) is one of the oldest diseases that afflicts mankind and has been discovered in human remains more than 9000 years old [2]. Active TB often manifests itself by symptoms of fever, weight loss, loss of appetite and should the lungs be affected, cough and haemoptysis. Nevertheless, any organ can be affected by the pathogen including the brain, pericardium, bone, reproductive system, urogenital system and the abdomen. Active TB can be highly contagious, in contrast with its latent stage where the tubercle bacilli lie dormant and individuals asymptomatic. One would have expected that mankind would have found a cure to such an ancient disease that has beleaguered humans since the beginning of history, but the converse is true and TB continues to plague.

TB was declared a global health emergency by the World Health Organisation in 1993 and continues to be one of the world‘s leading killers. It is estimated that one third of the world‘s population is infected with the causative organism, *Mycobacterium tuberculosis(M.tb)* [3]. Twenty years after the global health emergency was declared the incidence of TB is still in epidemic proportions with 8.7 million new cases in 2011 and 1.4 million deaths [4]. Although 95% of TB cases arise from middle and low-income

countries [4], affluent nations are similarly afflicted. London has been disgracefully declared the ‗TB capital of Europe' [5] and this can be attributed to the high immigrant population [5]. Moreover, Multi-drug resistant (MDR) and Extensively drug- resistant (XDR) TB incidences are rising [6] with very limited therapies available to treat these diseases. This is reminiscent of the early 1900s when standard TB chemotherapy was not yet available and patients were either prescribed long stays at TB sanatoriums with or without surgery. A variety of reasons including limited access to healthcare resources, lack of culture and susceptibility testing and inappropriate use of first and second-line drugs have led to the emergence of XDR-TB which are refractory not only to isoniazid and rifampicin but also to at least one fluoroquinolone and at least one injectable second-line drug. XDR-TB has now been found in 77 countries, including the UK and US [7]. More alarmingly, totally-drug resistant TB has been found in Iran in 2009 [8] and Mumbai in 2011 [9] and South Africa in 2013 [10] which leaves patients the bleak prospect of either major surgery or death.

1.1 The compounding effect of HIV on TB

Contributing to the challenging situation is the rise in the number of people living with HIV. HIV is a well recognised risk factor for contracting TB with at least one-third of the 34 million people with HIV having latent TB and 1.1 million having active HIV-associated TB [4]. HIV leads to an increased risk of developing rapidly progressive primary TB following exposure as well as an increased risk of reactivation of latent TB infection to active disease [11]. TB can be an early manifestation of HIV infection, but the risk of developing TB and contracting disseminated TB increases as the CD4 count decreases. It is known that people living with HIV are more likely to succumb to TB as

patients with HIV may not be diagnosed with TB or vice versa until the disease is far too advanced. Diagnosing TB among people living with HIV is particularly challenging because this population that is infected with pulmonary TB frequently have negative sputum smears and up to a third may have completely normal chest radiographs [12]. Additionally, TB in people living with HIV often occurs in extraplumonary sites, evading traditional diagnostic tests. Between 8-26% of patients die in the first year of anti-retroviral treatment [13] and both diagnosed and undiagnosed TB are recognised as major causes of this mortality. Autopsy studies conducted in sub-Saharan Africa before and during the anti-retroviral therapy (ART) era in adult HIV-infected hospitalised patients discovered that TB, specifically disseminated TB was responsible for 40-50% of deaths [14-17]. Correspondingly, TB patients diagnosed with HIV and not receiving ART have a high risk of mortality, with death occurring early during the course of anti-TB treatment [18] and the risk increasing as the CD4 count decreases [4, 19, 20]. Furthermore, people living with HIV are more susceptible in contracting drug-resistant TB [21-23] which confers a higher mortality. TB and HIV thus act synergistically within a population to cause excess morbidity and mortality.

1.2 Risk factors for TB

HIV can be said to be globally the singular most important risk factor for contracting TB. Other risk factors can be broadly categorised to those who have epidemiologic risk factors to *M.tb*, and those who are immunosuppressed. Since TB is spread through aerosols, those who are in close contact with patients with active TB or certain high-risk populations such as homeless people, injection drug users, and people working with people at high risk for TB in institutions such as hospitals, homeless shelters,

prisons and nursing homes can become infected. Certain members of these groups require TB prophylaxis when the situation is warranted to prevent progression of latent to active TB.

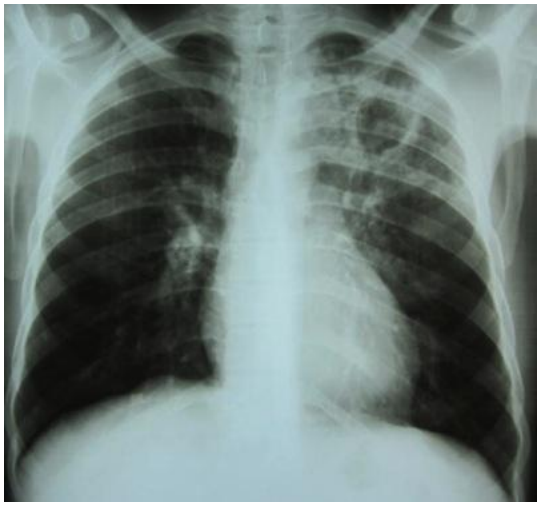
Diabetes mellitus has been recognised to be a moderate risk factor for active pulmonary TB as well as a predictor of poor treatment outcomes and reduced survival. Most of these observations arise from case-control studies [24, 25]. The mechanisms by which diabetes mellitus increases TB risk are not well understood. Several early studies reported reduced pro-inflammatory cytokines in murine models of diabetes mellitus after infection with *M.tb*. Others found that cytokine responses were consistent with a Th2-phenotype [26, 27] although more recently Th1 cytokines have been implicated [28, 29]. Patients with chronic renal disease especially those requiring dialysis are 6 to 25 times more likely to develop TB than the general population due to impaired cell-mediated immunity. They often have atypical clinical presentations, often presenting with extrapulmonary TB and thus resulting in a delay in accurate diagnosis [30, 31]. Another group at risk are patients who receive immunosuppressive therapy which are increasingly being used in this age of medical therapy. These include those on long term steroids as well as anti-TNFα agents. The risk of re-activating latent TB infection has been well-recognised in those using anti-TNFα therapies [32]. These agents include eternercept, infliximab and adalimumab. Multiple factors are thought to be involved in reactivation such as the down-regulation of IFN-γ responses, down-regulation of CD8 T cells and disorganised structure or functioning of tuberculous granulomas [33-35].

Given the multiple risk factors, what are the prevention strategies against TB? The Bacillus Calmette Guerin (BCG) vaccine offers 70-80% efficacy against severe forms of

tuberculosis in childhood, particularly TB meningitis in infancy and miliary TB [36, 37]. However when given later in adult life, the efficacy against TB varies in different part of the world for reasons which are not fully understood [36]. Uncertainty remains about how long the protection afforded by BCG vaccination will last [38]. Moreover, BCG exists as a live attenuated vaccine which precludes those with an already impaired immune system from receiving it lest they face the risk of contracting disseminated BCG. Thus, there is a requirement for a better vaccine to prevent contracting TB.

1.3 Current diagnostic tools for active TB

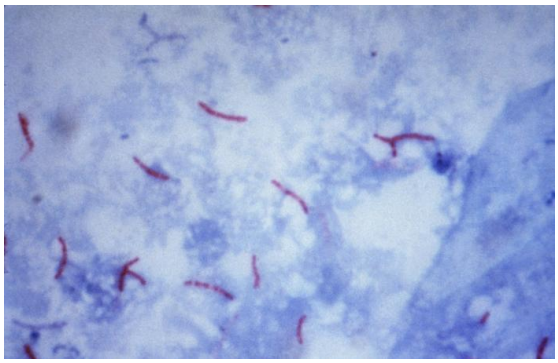
The current routine diagnostic tests for patients suspected of having active TB have their limitations. Chest radiographs are most often used, and characteristic lesions such as cavitation, infiltrates especially in the upper lobes of the lungs (akin to the one pictured below) would lead one to highly suspect the disease.



Adapted from sharinginhealth.ca.

http://www.sharinginhealth.ca/pathogens/bacteria/mycobacterium_tuberculosis.html

Sputum smear microscopy (pictured below) is used to detect acid-fast bacilli (AFB) in sputum specimens by Ziehl-Neelsen (Z-N) or fluorescence staining. It is a cost-effective tool for diagnosing and monitoring the progress of TB treatment especially in developing countries. However there are multiple drawbacks such as difficulty of obtaining sputum samples especially in very young children and the elderly who have impaired cough responses. Furthermore there is low sensitivity in detection especially in HIV-infected patients and the consequent false negative results. It has been estimated that the sensitivity is approximately 70% in a recent systematic review [39] and can be as low as 35% in some settings with high rates of HIV coinfection [40]. Occasionally, there may be false positive results with non-tuberculous mycobacteria.



Adapted from http://pathmicro.med.sc.edu/infectious%20disease/mycobacterial%20diseases.htm

Ziehl-Neelson positive stain for *Mycobacterium tuberculosis* in sputum

Sputum culture is the gold standard for TB diagnosis with excellent sensitivity and specificity. This traditional method of inoculating specimens onto solid medium such as Lowenstein-Jensen (LJ) or 7H10/7H11 media is however slow, requiring 10-14 days for

liquid culture and 3-4 weeks for solid culture weeks to diagnose the infection and further more time to ascertain the drug susceptibility patterns. Drug sensitivity testing nonetheless is not routinely available in all labs and is sorely lacking especially in resource poor settings where drug-resistant TB is prevalent and where it is needed most. Altogether, this translates to a delay in the initiation of appropriate therapy.

Technological advances have brought about incremental improvements in TB diagnostics since the 1980s. The introduction of the automated liquid culture systems such as the Bactec 960 MGIT allowed the detection of *M.tb* in a few days compared to conventional culture methods. These rapid automated systems have increased sensitivity of isolation of *M.tb* from clinical samples, decreasing the time to obtain positive culture results considerably to 9-10 days [41]. Automated systems additionally permit a higher throughput of specimens for testing. There are also several alternative, non-commercial, culture-based methods such as the microscopic observation drug susceptibility (MODS) assay, the nitrate reductase assay (NRA), colorimetric redox indicator (CRI) method which all have been approved by WHO to be used in the interim measure before leading to the eventual use of automated liquid culture.

Nucleic acid amplification tests (NAAT) for the diagnosis of TB and/or detection of drug resistance have also been approved by WHO [41]. The Gen-Probe assay for *M.tb* complex is a rapid detection, nucleic acid amplification test and results can be rapidly obtained in two hours. Furthermore, it has a high sensitivity and specificity of more than 90% [42]. The disadvantage is that it requires a positive culture which can take several days, and labs in resource poor settings would not be able to afford this technology. Line-probe assays such as HAIN Genotype MTBDR plus and the microfluidic PCR device

(GenExpert/Cepheid) allow for both the fast detection of *M.tb* as well as the simultaneous detection of drug resistance patterns. The HAIN test can be used both on culture-based isolates and directly on smear positive sputum samples from patients with pulmonary TB. This assay has the advantage of identifying mutations in the rpoB gene (rifampicin resistance) and concurrently mutations in the katG gene (high-level isoniazid resistance) and the inhA gene (low-level isoniazid resistance) [41]. More recently, WHO has endorsed the GeneXpert MTB/RIF in 2010 which uses a semi-nested real-time PCR reaction to amplify *M.tb* specific sequence of the rpoB gene which is then probed with molecular beacons for mutations within the rifampin-resistance determining region [43, 44]. The sensitivity of a single direct MTB/RIF assay in culture-confirmed *M.tb* was 92.2% [41] and provided results from unprocessed sputum in less than 2 hours. However, approximately 60% of patients with TB worldwide present in resource-poor settings [45] where diagnostic tests based on sophisticated instruments such as Xpert MTB/RIF will not be feasible. Such novel technologies are expensive and additionally neglect testing other anti-tuberculous drugs. There is thus a need for cheap, sensitive and specific diagnostic tests that can simulataneously detect drug-resistance and can be readily delivered at the point of care.

1.4 Anti-tuberculous chemotherapy

One of the key arms that contribute to the strategy in controlling the TB epidemic is the development of new drugs. The advances in the treatment of TB began with the advent of streptomycin in 1944 followed by a whole range of highly effective anti-tuberculosis therapies with rifampicin being the last discovery of that era in 1957. With the availability of these drugs, TB was largely removed from the public perception as a feared and deadly

contagious disease. Yet, in the latter part of the 20th century a prolonged period of neglect of quality control program implementation, research and failure to develop new diagnostic tools led to persistently high TB incidence rates. The flagrant misuse of anti-tuberculous drugs coupled with patient non-adherence led to the emergence of drug-resistant TB. Highly publicised nosocomial outbreaks in the 1990s of multidrug-resistant tuberculosis (MDR-TB) in New York City and California prisons which led to mortality even among healthcare workers brought TB back onto the radar screen. Despite this, most of the world still relies on drugs discovered 50 years ago. The current standard treatment of drug-susceptible TB comprises multiple drugs for a minimum duration of six months. These consist of rifampicin/isoniazid/ethambutol/pyrazinamide for 2 months and then rifampicin/isoniazid for the remaining months. This prolonged course of polypharmacy has contributed to high rates of non-compliance with the development of drug-resistant TB. Nonetheless with the emergence of drug resistance, there was a renewal of efforts and promises in the development of new drugs for chemotherapy of TB. There is now a long list of new molecules of potential interest for TB treatment that is beyond pre-clinical development: ATP synthase inhibitor TMC 207, nitroimidazopyrans PA-824 and OPC-67683, oxazolidinones PNU-100480 and AZD-5847, trehalose monophosphate transferase inhibitor SQ109 and benzothiazinones BTZ043. Despite the hurdles of developing a new drug, and more so in developing a new drug regimen, a degree of optimism is allowed in that the US Food and Drug Administration has recently approved bedaquiline (TMC 207) in December 2012 for the treatment of drug-resistant tuberculosis [46]. However further evaluation needs to be done to determine if bedaquiline can truly shorten treatment in drug-susceptible TB cases.

A well-recognised phenomenon that occurs with TB treatment is TB paradoxical reaction, a clinical, often accompanied by radiological worsening of pre-existing TB lesions or the development of new lesions during treatment. It is unpredictable in its timing, occurring as soon as a few days of initiation of anti-tuberculous treatment to many months after. It also varies in its duration and severity. In addition it is a diagnosis of exclusion, as factors such as treatment failure, drug-resistance or infections need to be simultaneously considered. Most of these occurrences frequently run a self-limiting course however occasionally patient deterioration and death will occur. The majority of such paradoxical reactions have complicated the treatment of TB lymph nodes, CNS-TB and TB pericarditis. A large case series reported approximately 30% enlargement of lymph nodes while on anti-tuberculous treatment [47]. Corticosteroids were often prescribed for those with severe systemic manifestation which can lead to an improvement by shortening the duration of symptoms [48, 49]. Certainly, steroid adjunctive therapy has been advocated in most national guidelines when treating CNS-TB and TB pericarditis [50, 51] as it is well-established that the administration of steroids improves patient morbidity and mortality outcome. In rare circumstance, anti-TNFα therapy has been prescribed for patients refractory to steroids in CNS-TB [52, 53] in an attempt to dampen the over-robust host inflammatory reaction. There has furthermore been an increase in TB paradoxical reaction in the context of HIV coinfection, more commonly known as TB-associated immune reconstitution inflammatory syndrome (IRIS). This frequently occurs in the setting of initiating anti-retroviral therapy soon after anti-tuberculous therapy. The underlying mechanism for this is unclear although there was a suggestion that a rapid reduction in HIV plasma viral load may be associated with TB-IRIS [54]. Like TB

paradoxical reactions in HIV negative individuals, TB-IRIS can be life-threatening and steroids prescribed with severe manifestations.

In summary, there have been advances in anti-tuberculous therapy with a considerable number of new drugs in the drug discovery pipeline. Treatment duration for TB however still requires a minimum of 6 months with multiple drugs, and emergence of drug-resistant disease underscore the lack of highly effective anti-tuberculous therapy. There is still a lack of appreciation of the mechanisms behind host-inflammatory reactions that accompanies the initiation of anti-tuberculous therapy and at times such reactions are life-threatening, refractory even to steroids. As such, there is still a pressing need for new and adjunctive therapy to prevent and treat the active disease with a truly shortened course so as to halt the transmission of *M. tb*, stem the rising rates of drug-resistance and modulate host-pathogen responses. To achieve this, it is imperative to understand the underlying mechanisms that cause pathology in TB infection.

2. *The concept of tuberculosis pathogenesis*

Tuberculosis is transmitted primarily through the aerosol route where aerosolised droplets from an infected host can remain airbourne for several hours. The mycobacterium is inhaled and establishes infection in the lungs of a new host. The nature and importance of the host immune responses, specifically the cell-mediated host immune responses, is recognised to be key in the pathogenesis of tuberculosis [55, 56]. The important role of macrophages as effector cells in host defence against *M.tb* was initially identified from animal studies [57-60]. It is commonly believed that the mycobacteria are first phagocytosed by alveolar macrophages. It is widely assumed that *M.tb* persists in

immature phagosomes by avoiding their maturation into phagolysosomes and acidification [61, 62]. This partially depends on the interference by *M.tb* of small GTPases termed Rabs, which control almost every aspect of intracellular trafficking in mammalian cells. Mycobacterial manipulation of Rab recruitment and function has a cascade of downstream effects, involving Rab-interacting partners and effectors such as phosphatidylinositol-3-kinase (PI_3K) which modifies membrane-associated lipids, specifically phosphatidylinositol (PI) by phosphorylating PI into phosphatidylinositol 3-phosphate (PI_3P). PI_3P serves as a tag for the recruitment of factors that execute conversion of phagosomal organelles into phagolysosome [62]. *M.tb* phagosomes do not acidify properly as it does not acquire the late endosome Rab7 [63] which brings about recruitment of the vacoular proton ATPase responsible for phagosomal acidification and proteolytic activity of a phagosome. This results in the host mistaking that the *M.tb* phagosome is static and allows *M.tb* to survive in the phagosome. It has also been reported that after a prolonged incubation time *in vitro*, *M.tb* escapes into the cytosol where it can apparently replicate robustly [64].

By expanding its population, *M.tb* then invades the adjacent epithelial layer. This results in a localised inflammatory response that leads to the recruitment of mononuclear cells from nearby blood vessels which in turn provides new host cells for the growing mycobacterial population [65]. These cells set the stage for the formation of the caseating granuloma which is pathognomonic of TB. Initially the granuloma is an amorphous mass of neutrophils, monocytes and macrophages. Thereafter, the macrophages differentiate into specialised subtypes such as multinucleated giant cells, foamy macrophages and epitheliod histiocytes. Animal experiments then established that lymphocytes were the

primary component of the cellular response [66, 67]. With the arrival of lymphocytes, the granuloma acquires a more organised structure. The center becomes surrounded by lymphocytes that may be enclosed by fibroblasts which demarcates the peripheral structure of the granuloma and may aid in the containment of the infection. The appearance of *M.tb*-specific lymphocytes heralds the end of the phase of rapid bacterial replication and the onset of a ‗containment' stage. Histology of infected tissues from patients with active TB reveal granulomas in all states of development from containment to active disease implying that the fate of each granuloma is not determined systemically but locally [65]. It is thought that ultimately the granuloma ruptures, spilling out abundant amounts of viable infectious bacilli into the respiratory airways [68] Our understanding of the pathogenesis of tuberculosis supports a framework which includes the initial response to inhaled organisms, granuloma formation with the establishment of a state of latent infection, and reactivation of the latent infection triggered by a relative immunosuppressed state in the host resulting in active disease.

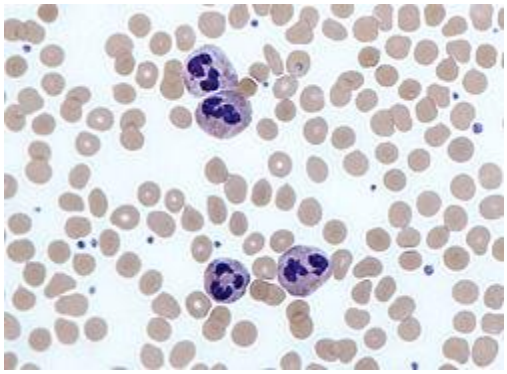
3. *The significance of the neutrophil*

Neutrophils have been traditionally viewed as short-lived effector cells of the innate immune system, having limited capacity for biosynthetic activity and playing a key role in resistance against extracellular pathogens and in acute inflammation. They are professional phagocytic cells, release lytic enzymes from their granules and generate reactive oxygen intermediates with antimicrobial potential. However, this limited view was challenged by a demonstration that neutrophils survive longer than initially suggested, up to 5.4 days [69]. Neutrophils can also be induced to express genes encoding key inflammatory mediators including complement components, Fc receptors, chemokines and

cytokines [70]. Furthermore, new evidence suggests that neutrophils can also produce anti-inflammatory molecules and factors that can promote the resolution of inflammation [71]. Recent evidence underscored the *de novo* induction of microRNAs that may be part of the crucial regulatory circuit which regulate neutrophil gene expression [72]. Neutrophils have thus emerged as a crucial component of the effector and regulatory circuits of the innate and adaptive immune systems [73] which has thus led to a renewed interest in their biology.

3.1 Neutrophil morphology

Microscopic examination of the mature neutrophil reveals two striking features: a single multilobed nucleus and a dense granular appearance of the cytoplasm. The nucleus comprises two to four segments and within this organelle the chromatin is coarsely clumped.



Blood film with Giemsa staining showing the multilobed nucleus of neutrophils with their dense granular cytoplasm

It is now appreciated that the mature neutrophil does perform active transcription [74, 75] although rates of biosynthesis are lower than those in monocytes. The most abundant organelles within the cytoplasm are the granules, which are membrane-bound organelles containing an array of antimicrobial proteins. Three major types have been identified: azurophil (primary), specific (secondary) and gelatinase-containing (tertiary) granules. Azurophils largely contain proteins and peptides directed towards microbial killing and digestion. Their contents include myeloperoxidase (MPO) and three main serine proteinases that are cathepsin G, elastase and proteinase 3. Specific granules contain neutrophil collagenase (MMP-8), lactoferrin, and neutrophil gelatinase-associated lipocalin and about two thirds of cellular lysozyme [76]. Gelatinase granules as its name suggests, contain gelatinase B (also known as MMP-9) in the absence of lactoferrin. Lastly, secretory vesicles contain serum albumin [77] and provide a valuable reservoir of membrane components, reassociating with the plasma membrane and in so doing replenishing membranes that have been consumed during phagocytosis. Neutrophil granules are formed sequentially during granulocytic differentiation in the bone marrow and there is a distinction in their mobilisation, with the granules that were formed latest being the ones to be released earliest [78]. Apart from containing degradative enzymes that may be extracellularly secreted from the neutrophil or else discharged into phagocytic vesicles, the membranes of these granules and vesicles contain important molecules including receptors (eg. CR1, CR3, CD11b/CD18) and cytochrome b of the NADPH oxidase. A summary of neutrophil granule proteins profiled at the protein level and their location is summarised in **Table 1**.

	Azurophil granules (Primary granules)	Specific granules (Secondary granules)	Gelatinase granule (Tertiary granule)	Secretory vesicles
Membrane proteins				
Adhesion molecules	N.A	CD11b/CD18, CD66, CD67		
Antibacterial proteins	N.A	Gp91phox/p22phox	Gp91phox/p22phox	Gp91phox/p22phox
Proteases	N.A	N.A	MMP25	MMP25
Receptors	N.A	uPA	N.A	CD35, CD14, CD16; C1q-R, IFN-αR1, fMLPR
Others	CD63, CD68, presenilin	SNAP-23, VAMP-2, stomatin	SNAP-23, VAMP-2, Nramp1	SNAP-23, VAMP-2, Nramp1, alkaline phosphatase, DAF, CD10, CD13
Matrix proteins				
Proteases	*Elastase*, cathepsin G, proteinase-3	*Collagenase (MMP-8),* uPA	*Gelatinase (MMP-9),* Arginase 1	N.A
Antibacterial proteins	Defensins (including human neutrophil peptides 1-3), BPI, *MPO*, lysozyme	hCAP18, *NGAL*, B12BP, lysozyme, lactoferrin, haptoglobin, pentraxin 3, prodefensin	lysozyme	N.A
Others	Sialidase, azurocidin (heparin-binding protein), β-glucuronidase	SLPI, orosomucoid, heparanase, β2-microglobulin, CRISP3	β2- microglobulin, CRISP3	N.A

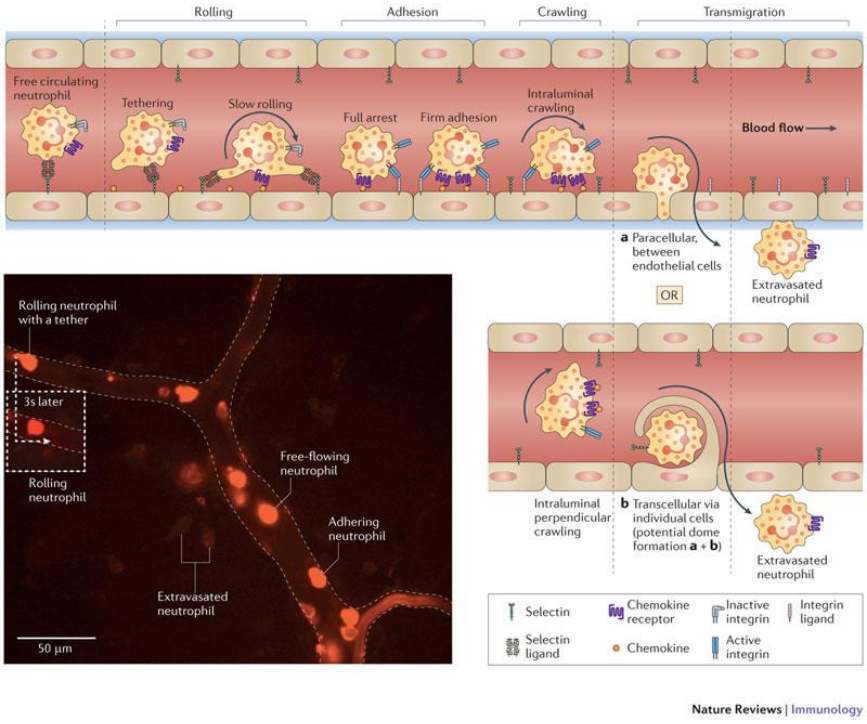
B12BP, vitamin 12 binding protein; BPI, bactericidal permeability-increasing protein; CRISP, cysteine-rich secretory protein; DAF, decay-accelerating factor; hCAP, human cathelicidin antimicrobial protein; MPO, myeloperoxidase; NGAL, neutrophil gelatinase associated lipocalin; Nramp1, Natural resistance-associated macrophage protein 1; SLPI, secretory leukocyte peptidase inhibitor; SNAP, Synaptosomal-associated protein; uPA, urokinase plasminogen activator; VAMP, vesicle-associated membrane protein

Modified from Borregaard et al [79]

Table 1: Neutrophil granule proteins. Proteins mentioned in this thesis have been italicised.

3.2 Neutrophils in innate immunity

Neutrophils are highly mobile phagocytic cells and need to cross the vascular endothelial wall to arrive at the portal of entry of pathogens. Migration occurs predominantly at postcapillary venules where the wall vessel is rather thin. The initial attachment of neutrophils to endothelial cells is determined by the expression of integrins on their luminal surface and this mediates neutrophil rolling and firm adhesion (see picture below) [80]. Neutrophils then undertake a transcellular or paracellular route for transmigration and thereafter cross the endothelial lining, migrating through the basal membrane. They are highly equipped with proteases capable of breaking down basement membrane collagens and laminin including neutrophil elastase, MMP-8, MMP-9 and membrane attached matrix metalloproteinase MT6-MMP.



Adapted from Kolaczkowska and Kubes, Nat Rev Immunol 2013

Once they have migrated into tissues, they are more active as phagocytic cells than those neutrophils circulating in the blood [81], activating gene expression that results in the secretion of chemokines including IL-8 and attracting more of their kind. Upon encounter with a pathogen these cells activate NADPH oxidase, generating reactive oxygen intermediates in an attempt to kill the pathogen. The initiation of NADPH oxidase coincides with degranulation with a lag phase of approximately 20 seconds [82]. Degranulation occurs with gelatinase granules being mobilised first, before specific granules. Azurophil granules can only be partially mobilised [83]. The process of phagocytosis is dependent on the Fcγ receptors which initiate the extension of pseudopods that surround and ultimately entrap the pathogen [84]. Once engulfed, the abundant cytoplasmic granules which are bactericidal are discharged into the phagosome containing the microbe. Changes in cystosolic calcium are required for granular fusion with phagosomes in neutrophils [85]. It is also believed that protein kinases such as the Src family of protein tyrosine kinases are implicated in phagosome maturation [86].

Further to the formation of the mature phagosome, microbes which also have been termed pathogen-associated molecular patterns (PAMPs) interact with pattern-recognition receptors (PRRs) including toll-like receptors (TLRs). TLRs 1,2 and 4-10 are expressed in human neutrophils [87, 88]. Activation of neutrophil TLRs especially TLRs-2 and -4 activate signal transduction pathways that eventually enhances cell vibility [89], facilitate adhesion[90], phagocytosis [87], enhance release of cytokines and chemokines [87] and promote degranulation[91].

Cross-talk occurs between components of the innate immune system and neutrophils. CCR2, the receptor for CC chemokine ligand 2 (JE, MCP-1) are highly expressed in circulating monocytes [92] and are critical not only for monocyte influx but also early accumulation of neutrophils [93]. Furthermore, components of neutrophil secretion such as LL-37 and heparin-binding protein can recruit inflammatory monocytes [94]. It has been shown that macrophages acquire neutrophil anti-microbial granule proteins when they engulf apoptotic neutrophils, providing a co-ordinated defence strategy involving different innate immune cells against invading pathogens [95]. For example, neutrophil granule proteins heparin-binding protein and the defensin human neutrophil peptides 1-3 (HNP1-3) triggers secretion of macrophage TNF-α and IFN-γ, concurrently thereby enhancing phagocytosis of *Staphylococcus aureus* [96]. There are thus considerable intercellular networks and crosstalk involving neutrophils.

3.3 Neutrophil extracellular traps (NETs)

Neutrophil extracellular traps are a distinct anti-microbial activity that has been recently described [97]. They are a meshwork of chromatin fibers that contain granule-derived antimicrobial peptides and enzymes extruded by neutrophils and are triggered when encountering microbes, or upon stimulation with Phorbol-12-myristate-13-acetate (PMA). The NET scaffold consists of chromatin fibers and comprises DNA and histones [97]. Mass spectrometry has identified an array of neutrophil granule proteins some of which possess anti-microbial properties associated with NETs [98]. The mechanism of how NETs are formed, termed NETosis, has been a subject of intense interest and 2 models have been proposed. The first proposes a form of active cell death, characterised by the dissolving nuclear and granular membranes, with nuclear contents decondensing

into the cytoplasm followed by plasma membrane rupture, and subsequent release of decondensed chromatin with granular contents into the extracellular space [97, 99]. The second proposed model involves the release of mitochondrial DNA with serine protease extrusion from intact neutrophils and is not associated with cell death [100]. There have also been *in vivo* studies demonstrating that neutrophils are live, mobile and anuclear after NET formation has occurred [101]. A vast array of micro-organisms ranging from viruses, bacteria, fungi and protozoa can trigger NET formation which serves to entrap and prevent further dissemination of pathogens. NETs are however double-edged swords. They have been described as a source of autoantigens and immunostimulatory proteins and have been discovered to have crucial roles in inflammatory diseases such as systemic lupus erythematosis, vasculitis, gout and even thrombotic microangiopathies [102, 103]. The biological significance of NETs is just beginning to be revealed.

3.4 Neutrophils in adaptive immunity

Phagocytosis of bacteria simultaneously triggers the transcription of neutrophil genes encoding numerous chemokines and cytokines including IL-1α, IL-1β, IL-6, IL-8, IL-12β, IL-15, IL-18, CCL2 (MIP1α), CCL3 (MIP1β) , TNFα, VEGF and oncostatin M [71, 104-106]. Further to recruiting more neutrophils and subsequently modulating neutrophil functions, these molecules coordinate early responses of monocytes, macrophages, dendritic cells, natural killer (NK) cells and lymphocytes [71], serving as a bridge between the innate and adaptive immune responses [71]. More recent studies have demonstrated that human neutrophils are a main source of cytokines such as B cell-activating factor (BAFF) [107, 108] and a proliferation ligand APRIL [107, 109] that are critical for the survival, maturation and differentiation of B cells. The initial evidence that

neutrophils can interact with dendritic cells (DCs) s from investigations demonstrating that supernatants of cultured murine neutrophils stimulated with *Toxoplasma gondii* and this resulted in the maturation of bone marrow-derived DCs and their production of IL-12 and TNFα [110] while DCs in neutrophil-depleted mice infected with *T. gondii* had suppressed IL-12 and TNFα secretion. Human neutrophils can modulate NK cell activation status, survival, cytotoxic activity and IFNγ secretion by the generation of reactive oxygen intermediates or the release of granule components [111, 112]. Additionally, crosstalk between human neutrophils and NK cells is present. Culture of neutrophils with NK cells or NK cell-derived soluble factors such as GM-CSF and IFNγ induces neutrophil survival, expression of activation markers, priming of reactive oxygen intermediates [113, 114]. On a similar note, human neutrophils can also crosstalk with T-cells. Activated neutrophils can attract Th1 and Th17 cells to sites of inflammation with the release of CCL2, CXCL9 and CXCL10 and CCL20 [115]. Likewise, activated CD4 and CD8 T cells including Th17 secrete cytokines such as IFNγ, GM-CSF and TNFα that modulate neutrophil survival and expression of activation markers [115, 116].

3.5 The significance of neutrophils in TB

While it is accepted that neutrophils play an important role in the first line of defence against intracellular pathogens, the role of neutrophils in the pathogenesis of tuberculosis remains controversial. This is partly due to a substantial body of literature that is derived from animal models, presenting us with a contradictory picture of the importance of neutrophils in tuberculosis. Clinical studies and experimental infections have shown that acute pulmonary tuberculosis is accompanied by an influx of neutrophils

[117-121] and neutrophils are present in the early stages of TB infection [122-124]. It is well recognised that good quality sputum from patients with mycobacterial infections are neutrophil rich with 92% of sputum that contains neutrophils being smear and culture positive [125]. Furthermore, human studies show that neutrophils play a role in the risks of contracting TB with lower peripheral blood neutrophil counts [126], can be used to diagnose active TB from other infectious diseases [127] and when present in large numbers reflect a worse disease prognosis [128]. They are the predominant phagocytic cells in patients with pulmonary tuberculosis [121] and are found in sites of alveolar inflammation [129]. Neutrophil depletion *ex vivo* impaired the ability of whole blood to restrict mycobacterial growth via depletion of secreted anti-microbial peptides [126]. Conversely, severity of disease seemingly correlates with the degree of neutrophilia in humans: neutrophils are abundant in cavitatory pulmonary TB [118], and a high proportion of peripheral blood neutrophils is associated with poor prognosis and delayed sputum culture conversion [128, 130]. In murine TB models, a predominant neutrophilic response is frequently seen in genetically susceptible strains [131], in mice with impaired immune functions [132] as well as in infections with virulent compared to their attenuated counterpart [123]. Necrosis has been induced in the granulomata of mice with established *M.tb* infection by stimulating neutrophil influx using nasal LPS [133].

3.6 Neutrophil response to *M. tuberculosis*

Neutrophils contribute to the early defence against mycobacteria, as mice depleted of neutrophils have an increased bacterial load [134]. They are rapidly recruited to the site of *M.tb* infection and engulf the microbe [135]. Following the encounter with *M. tuberculosis*, neutrophils are activated, undergo a respiratory burst with release of superoxide radicals [136] and have an increased phagocytic activity [137], a process which is calcium dependent and mediated by annexins [138]. Opsonisation appears to enhance neutrophil phagocytosis of *M.tb* [138]. The pathogen is killed or inactivated upon fusion of the neutrophil phagosome with lysosomal compartments and specialised cytoplasmic granules that contain a vast arsenal of antimicrobial effector molecules. α-defensins (HNP 1-3) [126], myeloperoxidase [139], neutrophil peptides cathelicidin LL-37 and lipocalin-2 [126] have been demonstrated to have microbicidal activity or restrict growth of *M.tb*. In addition to their direct microbicidal role, neutrophils secrete chemokines and cytokines such as TNF-α , IL-8 [140], GRO-α [141] and MIP-1α [142] on exposure to *M.tb* to attract and instruct other immune cells. *M.tb* also induces subcellular changes on infected neutrophils, leading to neutrophil-extracellular traps (NETs) which ensnare mycobacteria [143]. In one of the final stages of the neutrophil-microbe encounter, *M.tb* activates caspase-3 thereby causing apoptosis in human neutrophils [33]. They may also undergo necrotic cell death which is deemed less desirable as this may prevent the cross-presentation of antigens to antigen-presenting cells via apoptotic vesicles [144]. Furthermore, the loss of plasma membrane allows the release of proteolytic enzymes and the antimicrobial arsenal which are likely to be detrimental to innocent bystanding host cells.

3.7 Neutrophil networks in the immune response against *M.tb*

There is increasing evidence that neutrophils play a role in coordinating both the host innate and adaptive immune response against *M.tb*. The phagocytosis of *M.tb*-induced apoptotic neutrophils markedly increased the production of proinflammatory cytokine TNF-α by human macrophages [145, 146] and results in decreased viability of intracellular *M. tuberculosis* [95]. IL-10 secreted by mycobacteria-stimulated murine neutrophils controlled the inflammatory response of dendritic cells, monocytes and macrophages in the murine lungs [147]. Similarly, phagocytosis of *M.tb*-induced apoptotic neutrophils by dendritic cells leads to lymphoproliferation [144]. Neutrophils also assist dendritic cells to cross-present mycobacterial antigens to T cells [148]. *In vitro*, dendritic cells that acquire mycobacterial antigens from apoptotic neutrophils migrate more efficiently compared to directly infected dendritic cells [149]. Recent studies have shown that recruitment of neutrophils and inflammatory monocytes occurs early in TB infection and precede the rise of IL-12 with consequent accumulation of adaptive T cells [122]. Conversely, defects in T-cell immunity, primarily in patients with HIV, affects neutrophil functions towards *M.tb* including that of decreased respiratory burst [150], decreased chemotaxis [151] and impaired degranulation [152].

4. *Matrix metalloproteinases*

The matrix metalloproteinases (MMPs) currently comprises 25 related but distinct family of zinc-containing proteases of which 24 are found in mammals. They share structural domains but differ in substrate specificity, cellular sources and inducibility. They are sub-classified on the basis of substrate specificity, such as the collagenases, gelatinases, stromelysins, matrilysin and elastase [153]. Their characteristics and substrates are detailed in **Table 2**. MMPs have a flexible proline-rich hinge region and a carboxy (C)-terminal hemopexin-like domain, which functions in substrate recognition. They are often the enzymes responsible for the turnover, degradation, catabolism and destruction of the extracellular matrix (ECM). They are secreted or anchored to the cell surface, thereby confining their catalytic activity to membrane proteins and proteins in the secretory pathway or extracellular space. Similar to all secreted proteinases, the catalytic activity of MMPs is regulated at four points — gene expression, compartmentalization (i.e pericellular accumulation of enzymes), pro-enzyme (or zymogen) activation and enzyme inactivation — and is further controlled by substrate availability and affinity. MMPs have specific inhibitors, the Tissue Inhibitors of MMPs (TIMPs) that bind non-covalently in a 1:1 manner to inactivate them [154].

Enzyme	Common name	Substrates	Activated by	Activator of
Collagenases				
MMP-1	Collagenase-1 (Interstitial collagenase, fibroblast collagenase)	Collagen I, II, III (III>I), VII, VIII, X, gelatin, aggrecan, versican, proteoglycan link protein, L-selectin, entactin, tenascin, serpins, α2-macroglobulin, Latent TNF	MMP-3, -10, plasmin, kallikrine, chymase	MMP-2
MMP-8	Collagenase-2 (Neutrophil collagenase)	Collagen I, II, III (I>III), VII, VIII, X, gelatin, aggrecan, fibronectin, laminin, serpins, α2-macroglobulin	MMP-3, -10, plasmin	ND
MMP-13	Collagenase-3 (Rat collagenase)	Collagen I, II, III (II>I or III), IV, IX, X, XIV, gelatin, aggrecan, perlecan, fibronectin, laminin, tenascin, fibrillin, serpins	MMP-2, -3, -10, -14, -15, plasmin	MMP-2, -9
Gelatinases				
MMP-2	Gelatinase A (72-kDa gelatinase)	Gelatin, collagen I, IV, V, VII, X, XI, XIV, aggrecan, versican, proteoglycan link protein, fibronectin, laminin, laminin-5, fibrillin, elastin, vitronectin, α2-macroglobulin, latent TNF	MMP-1, -7, -13, -14, -15, -16, -24	MMP-9, -13
MMP-9	Gelatinase B (92-kDa gelatinase)	Gelatin, Collagen IV, V, VII, X, XIV, aggrecan, versican, proteoglycan link protein, fibronectin, elastin, vitronectin, α1-antitrypsin, α2-macroglobulin, latent TNF, latent TGF-β1, latent VEGF, fibrin, NG2 proteoglycan	MMP-2, -3, -13, plasmin	ND
Stromelysins				
MMP-3	Stromelysin-1 (Transin-1)	Collagen III, IV, V, IX, X, gelatin, versican, aggrecan, perlecan, fibronectin, laminin, tenascin, fibrillin, latent TGF-β	Plasmin, kallikrein, tryptase, elastase, cathepsin G	MMP-1, -8, -9, -13
MMP-10	Stromelysin-2	Collagen III, IV, V, gelatin, nidogen, aggrecan, fibronectin, elastin	Plasmin, kallikrein, elastase, cathepsin G	MMP-1, -7, -8, -9

MMP-11	Stromelysin-3	α1-proteinase inhibitor, fibronectin, laminin	Furin	ND
Membrane-type MMPs				
MMP-14	MT1-MMP	Collagen I, II, III, gelatin, aggrecan, fibronectin, laminin, tenascin, vitronectin, fibrillin	Plasmin, furin	MMP-2, -13
MMP-15	MT2-MMP	Aggrecan, fibronectin, laminin, fibrin	ND	MMP-2, -13
MMP-16	MT3-MMP	Gelatin, casein, fibrin, Syndecan-1	ND	MMP-2
MMP-17	MT4-MMP	Gelatin, latent TNF	ND	MMP-2
MMP-24	MT5-MMP	ND	ND	MMP-2
MMP-25	MT6-MMP (Leukolysin)	ND	ND	ND
Other MMPs				
MMP-7	Matrilysin	Collagen IV, gelatin, aggrecan, fibronectin, laminin, elastin, vitronectin, Pro-α defensins FAS ligand, Latent TNF, Syndecan-1, E-cadherin, Elastin	MMP-3, plasmin	MMP-2
MMP-12	Metalloelastase	Collagen IV, gelatin, aggrecan, fibronectin, laminin, fibrillin, elastin, vitronectin, latent TNF, α1-antitrypsin	ND	ND
MMP-19		Gelatin	trypsin	ND
MMP-20	Enamelysin	Amelogenin	ND	ND
MMP-21		ND	ND	ND
MMP-22		ND	ND	ND
MMP-23	CA-MMP	ND	ND	ND
MMP-26	Endomelase	ND		
MMP-27		ND		
MMP-28	Epilysin	ND		

ND, Not determined.

Modified from Parks et al[153], Kahari et al [155], Chandler et al [156] and Ashworth et al [157].

Table 2: Matrix metalloproteinases, substrates, endogenous activators and their activating capacity.

Physiological processes such as cell migration, embryonic development, reproduction and tissue remodelling involve MMPs. MMP-1 is required for the repair of skin wounds, altering the migratory substratum that consists of Type I collagen [158]. Similary, MMP-7 is expressed by wound-edge epithelial cells in mucosal tissues and is required for re-epithelialisation. This presumably happens with the shedding of E-cadherin, loosening cell-to-cell contacts thereby facilitating cell migration [159]. MMP-7 also activates intestinal pro-α-defensins in murine models, and due to the lack of mature active α-defensins, MMP-7 deficient mice have impaired ability to mount a response towards pathogenic enteric organisms [160].

Specific MMPs control chemokine activity and this is mediated by MMP cleavage of these molecules resulting in enhancement, inactivation or antagonism of chemokine activaties. CCL7 (MCP-3) is a substrate of MMP-2, losing its chemokine activity when cleaved [161]. On the same note, MMP-1, -3, -13 and -14 cleave MCP-1, MCP-2 and MCP-4 resulting in antagonist factors [161]. In contrast, the processing of IL-8 by MMP-9 markedly increases its chemotactic activity [162, 163]. Similarly, ADAM-17, a member of the disintegrin family of metalloproteinases (ADAMs) cleave pro-TNFα into active TNFα [164, 165]. Several other MMPs including MMP-1, -2, -3, -9 and -17 can also process pro-TNF to its active form *in vitro* [165, 166].

On the other hand, MMPs are also implicated in many disease processes involving abnormal matrix turnover, such as arthritis, tumour invasion and atherosclerosis. Studies have confirmed the critical role of MMPs, specifically the collagenases as mediators of arthritic diseases [167], demonstrating that rheumatoid synovial tissue invading and destroying collagen. As a result, there is a focus of therapies that specifically target these

enzymes. The association of matrix breakdown and cancer is well-established and destruction of the basement membrane is a hallmark of malignancy. This activity was the attributed to specific MMPs when Type IV collagenases/gelatinases MMP-2 and MMP-9 were cloned and when the correlation between MMP-3 expression and tumour progression in mouse skin was shown [168]. The original concept of MMPs breaking down basement membranes to allow tumour cells to metastasise was challenged when it was shown that TIMP-1 expressing tumour cells can breach blood vessel endothelium and leave the circulation efficiently but cannot grow once they reach a secondary site [169]. Subsequently, it was realised that MMP inhibition may not necessarily prevent escape of the tumour cells but it can be useful to keep small metastatic lesions in a dormant state. The interest in MMPs as a therapeutic target for cancer increased when MMPs were further shown to contribute to tumour angiogenesis and consequently affected the growth potential of primary and metastatic lesions [170, 171].

MMPs were a prime target in the 1990s for drug development [172]. Batimastat was the first MMP inhibitor to be tested in humans [173] with the drug administered through intraperitoneal and intrapleural administration to treat oncological disease. Batimastat was rapidly replaced by Marimastat, a related compound which can administered through the oral route. Nevertheless, it was soon reported that sustained treatment with Marimastat caused severe arthralgia commencing with the hands and spreading through the extremeties. In an attempt to create specific inhibitors, those interested in oncological applications designed MMP inhibitors selective for gelatinases but with limited activity against collagenases. Conversely, collagenases were considered the optimum target for arthritis. By the 2000s, the pendulum had swung the other way

when multiple clinical trials demonstrated that oncology patients administered MMP inhibitor faired worse in terms of survival benefit than their placebo-treated counterparts [174] . Further investigations are required to be done before concluding that MMP inhibitors do not have any beneficial effect.

One disease of which the role of MMPs is well understood is periodontitis. Inflammatory cytokines elicited by bacteria result in considerable tissue destruction by MMPs and loss of teeth. This can be prevented by treating with doxycycline which inhibits MMP activity and also seem to decrease MMP expression. Doxycyline is the only MMP inhibitor licensed by the US Food and Drug Aministration [175].

Other avenues in which MMP inhibitors have been investigated as therapeutic targets include corneal ulcers [176] as well as Batimastat-coated stent in the setting of restenosis after coronary angioplasty [177]. In animal models, MMP inhibitors could be effective in diseases such as multiple sclerosis, glomerulonephritis, emphysema, aortic aneurysm, bacterial meningitis and graft-versus-host disease [177]. Further evaluations are required before it can be truly ascertained that MMP inhibitors are beneficial in these context in humans.

4.1 Matrix metalloproteinases in tuberculosis

As *M. tuberculosis* establishes infection, the local cellular organization of the surrounding tissues is modified to facilitate leucocyte infiltration and initiation of granuloma formation. MMPs are likely to play a role in the tissue remodelling and destruction during the process [178]. Infection by *M.tb* has been shown to induce the

secretion of MMP-1, -2, -7 and -9 [179-181] as well as a decrease in expression of TIMP-1, -2 and -3 [154, 182] from peripheral blood mononuclear cells and human airway epithelial cells. MMP-9 has been demonstrated to be involved in macrophage recruitment and granuloma development in an *in vivo* mouse model of tuberculosis [183]. Studies of *M.tb* infection in MMP-9-deficient mice revealed that host MMP-9 is required for full mycobacterial proliferation, as these mice showed reduced bacterial burden and reduced lung macrophage recruitment compared with wild type. In addition, the MMP-9 knockout mice displayed reduced ability to produce well-formed granulomas. Volkman et al similarly demonstrated using *Mycobacterium marinum* infection of zebrafishes that MMP-9 morpholinos (MMP-9 knockdown) transgenic zebrafish have reduced granuloma formation [184].

Our group has previously shown in two separate studies that MMPs are elevated in the induced sputum samples of TB patients. Elkington et al had compared patients with respiratory symptoms with TB patients and found that MMP-1 and -3 were increased [185]. TB patients with more extensive disease on the chest radiograph also had a higher MMP-1 in their sputum. Using MMP-1 knock-in transgenic mice, since mice do no express an orthologue of human MMP-1, it was demonstrated that there was more matrix destruction in such mice compared to wild type mice [185]. Walker et al had shown when comparing a cohort of HIV positive and negative patients that MMP-1, -2, -3 and -8 were elevated in their induced sputum [186]. It was found MMP-1, -2, -8 and -9 were significantly lower in the induced sputum from patients with advanced HIV-TB co-infection than HIV-negative patients with TB. In the same paper, doxycycline was shown to reduce MMP-1, -3 and TNFα secretion. At the same time, doxycyline inhibited *M.tb*

growth *in vitro* as well as in guinea pigs. These two studies supported the theory that MMPs are critical in inducing tissue destruction in TB infection.

In previously published work from our group, strong immunohistochemical staining of MMP-9 staining was found in monocytic cells at the centre of granuloma in *M.tb*-infected patient lymph nodes with minimal TIMP-1 staining, indicating MMP-9 activity is unopposed *in vivo* [187]. Similar findings were found in the cerebrospinal fluid (CSF) of patients with TB meningitis [188] where MMP-9 activity was unrestricted by TIMP-1. Compared with other forms of meningitis, the MMP-9 per leukocyte ratio was significantly increased and associated with signs of tissue damage, such as neurological deficit, confusion, unconsciousness and death. Monocyte-dependent networks have also been found to upregulate MMP-9 secretion from astrocytes [189] and correlate *in vivo* with increased immunohistochemical staining of MMP-9 in astrocytes [190]. Furthermore, in response to infection with tuberculosis, MMP secretion has also been found to be upregulated in other cells including fibroblasts [191], microglial [192], and multinucleate giant cells [193].

4.2 Matrix metalloproteinases in neutrophils

Neutrophils secrete MMP-8 (neutrophil collagenase) and MMP-9 (gelatinase B), which are present in the secondary and tertiary granules respectively. These two MMPs are released extracellularly following exposure to bacteria [194, 195], and mycobacterial antigens [196].

MMP-8 is an interstitial collagenase that degrades Type I, II and III collagen and cleaves chemokines such as IP-10 and MCP-1 [197]. It had been shown that when it comes to degradation of Type I collagen, the main structural of protein in human lungs as well as other organs in the human body [198], it degrades Type I collagen more potently than MMP-1 or MMP-13 [199-201]. It plays a crucial role in the disassembly of cell junction components and cell adhesion in bacterial meningitis [202].

Neutrophil collagenases have been implicated in diseased states and severity. MMP-8 knockout mice when subjected to high-pressure injurious ventilation had decreased neutrophil infiltration, lower levels of IFN-γ and significant increases in anti-inflammatory cytokines IL-4 and IL-10. Similar results were demonstrated when wild-type mice were treated with an MMP-8 inhibitor [203]. Neutrophil MMP-8 has been found to be elevated in the bronchoalveolar lavage fluid of patients with bronchiectasis [204]. Sepper et al utilised 100 μM of doxycycline and its differential inhibition of MMP-1(IC50 = 280 μM) and MMP-8 (IC50 = 26 μM) at this dose and found that interstitial collagenases originated from neutrophils and correlated with disease severity [204]. Furthermore, non-healing wounds in humans were found to predominantly have active MMP-8 while healing wounds have MMP-8 which are in their inactive form [205].

MMP-9 is able to cleave gelatin (denatured collagen) and type IV collagen, one of the main components of the basement membrane which also constitute the blood-brain barrier (BBB). In addition, MMP-9 is also able to clip myelin compounds such as myelin basic protein (MBP) in humans, which are epitopes for T cells [206, 207], and may play a role in antigen presentation and T-cell activation. The major neutrophil chemoattractant, IL-8, is processed by MMP-9 and results in a truncation variant with increased activity

[208]. This results in an efficient amplification of neutrophil influx to combat infections. MMP-9 is thus not only an effector but also a regulator of leukocyte function. MMP-9 also has a role for the anti-microbial activity of neutrophil elastase. With the activation of neutrophils, MMP-9 secretion degrades and neutralise serine protease inhibitor α1-antiproteinase, a potent inhibitor of neutrophil elastase [209].

Similar to MMP-8, MMP-9 has been implicated in various diseased states but the data has been conflicting. MMP-9 knock-out mice interestingly developed more severe lung injury when subjected to high-pressure ventilation compared to wild-type mice, with the effects correlating with an increased cell count and myeloperoxidase activity in the bronchoalveolar lavage fluid in the knock-out mice. This has been attributed to an increase in IL-1β and IL-4 in the MMP-9 knockout mice [210]. This differed from clinical studies. In patients with viral meningitis, MMP-9 concentrations were found to be elevated compared to controls and is associated with CSF neutrophil count [211, 212]. CSF MMP-8, -9 and TIMP-1 are also raised in patients with bacterial meningitis and raised MMP-9 is associated with long-term complications such as hearing impairment and focal epilepsy [213]. It is thus unclear if MMP-9 might be protective or harmful and this maybe attributed to the divergences in animal models.

4.3 Neutrophils and MMPs in TB

There is limited literature describing neutrophil MMPs in TB. In TB meningitis, CSF MMP-9 is markedly elevated [188, 214, 215] where it is well-recognised that there is loss of integrity of the blood-brain-barrier. CSF MMP-9 activity supersedes that of bacterial and viral meningitis and this persists even when corrected for the total CSF

leukocyte number [188]. TIMP-1, which binds to MMP-9 was not raised in the CSF of patients with TB meningitis compared with those in bacterial meningitis and normal controls. Furthermore, patients with CNS tissue injury, as manifested by unconsciousness scored with a Glasgow coma score of less than 11, confusion or focal neutrological deficit had significant higher levels of MMP-9/CSF leukocyte compared with other patients. Moreover, MMP-9/leukocyte ratios were higher in patients who died compared to survivors [188]. In a separate study, it was found that CSF MMP-9 in patients with TB meningitis correlated with absolute neutrophil count [215]. The administration of dexamethasone, an adjuvant treatment for patients with TB meningitis which improves prognosis and neurological outcomes [216], decreased the concentrations of MMP-9 in the CSF at day 5 of treatment of TB meningitis. There was also a similar but less marked effect of dexamethasone on MMP-8 concentrations. MMP-8 and -9 as well as MMP-3 fell significantly over the course of 9 months of anti-tuberculous treatment. This suggests that neutrophils may play a central role in the early pathogenesis of TB meningitis.

5. *Hypothesis and experimental aims*

With the background that neutrophils correlate with CSF MMP-9 and that a high MMP-9/leukocyte ratio worsens the prognosis in patients with TB meningitis, my hypothesis is that in human *M.tb* infection, neutrophils have a central role in the development of a matrix degrading phenotype. The specific aims of my project are to investigate:

- MMP and TIMP secretion from *M.tb*-infected neutrophils
- Neutrophil networks regulating MMP gene expression
- MMP and TIMP activity and regulation in neutrophils *in vivo*

.

CHAPTER 2 - MATERIALS AND METHODS

1. *M. tuberculosis culture*

M. tuberculosis H37Rv Pasteur (a kind gift of Dr R. Wilkinson, Imperial College London) was cultured in Middlebrook 7H9 medium supplemented with 10% ADC enrichment medium, 0.2% glycerol and 0.02% Tween 80 with agitation at 10 rpm. Culture growth was monitored with a Biowave cell density meter (WPA, Cambridge) and *M.tb* sub-cultured when the optical density exceeded 1.00. For infection experiments, culture at mid log growth at an optical density of 0.50 – 0.70 were used, which corresponded to 1 x 10^8 – 2 x 10^8 colony forming units (CFU) per ml. Optical density was correlated with CFU by performing colony counts in triplicate on Middlebrook 7H11 agar supplemented with OADC enrichment medium and 0.5% glycerol. The amebocyte lysate assay (Associates of Cape Cod, East Falmouth, MA) was used to assay the endotoxin level of the *M.tb* culture and this was found to be less than 0.3ng/ml lipopolysaccharide. The growth of luminescent *M.tb* H37Rv LuxG13 [217] (a kind gift of Dr Nuria Andreu, Imperial College London) was also used in some experiments and these were cultured with the addition of kanamycin at 25μg/ml. Luminescence was measured with Glomax 20/20 luminometer (Promega). In experiments assessing *M. tuberculosis* growth with neutrophil derived MMP-8/-9 (Enzo Lifesciences, Exeter, UK), CoNCont or CoNTB, H37Rv was grown with the respective agent diluted with 7H9 at an initial optical density of 0.1, and growth monitored using the cell density meter. At specified time points, colony counts were performed on Middlebrook 7H11 agar. This was done by taking 20μl of H37Rv in 7H9 and doing serial 1:10 dilutions and plating 20 μL of each dilution onto 7H11. The plates were incubated at 37^{o}C for 2-6 weeks before being read.

2. *TB supernatant production*

To control for the presence of TB antigens such as lipoarabinomannan, mycolic acid and ESAT-6 in experiments, TB supernatant (TB supp) was used. This was made when *M.tb* growth reached an optical density of 1 and adding 70 µL of *M.tb* to 3 mls of 7H9, and gives the equivalent of TB antigens present in CoMTB at a 1:5 dilution (CoMTB is described in Section 6 of this chapter). The suspension was centrifuged, 0.2 µm sterile filtered (Nalgene, Hereford, U.K) and frozen at -20 ^{o}C for further use.

3. *Neutrophil isolation*

Blood from healthy volunteers (Outer West London REC reference: 09/H0709/46) were drawn in preservative-free heparin and mixed with equal volumes of 3% dextran/0.9% sodium chloride. The suspension was incubated in an upright position for 20 minutes until a clearly defined interface was discerned. The leukocyte-rich plasma was pipetted and centrifuged at 250 x g at 5^{o}C for 10 minutes. The cell pellet was resuspended with 0.9% sodium chloride and layered onto Ficoll-Paque (GE Healthcare, Little Chalfont, UK) and centrifuged at 400 x g at 20^{o}C for 40 minutes. Thereafter, the saline layer and Ficoll were aspirated, leaving the neutrophil/erythrocyte pellet. The pellet was subjected to 30 seconds of hypotonic lysis with 0.2% sodium chloride and restoration of isotonicity with 1.6% sodium chloride. The suspension was centrifuged at 250 x g at 5^{o}C for 6 minutes, and the cycle is repeated 3 times. The final neutrophil pellet was resuspended in RPMI 1640 supplemented with 10% FCS. Neutrophil purity was assessed by means of flow cytometry and staining with mouse anti-human CD66c-PE (phyco-erythrin, PE; BD Biosciences, Oxford, UK) and analysed on a BD FACSCalibur flow cytometer using CellQuest (BD, Mountain View,

California). Neutrophil purity was over 98%. (**Figure 1**). Viability was consistently >99% by trypan blue viability assay.

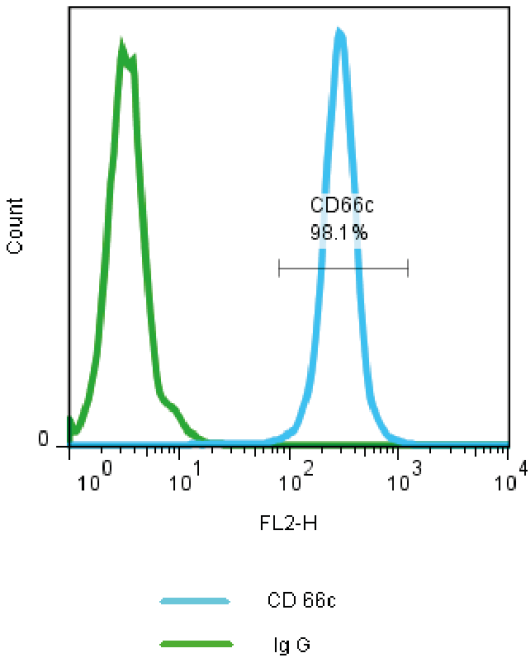


Figure 1: FACS analysis of neutrophil preparations. Cells isolated were labelled with CD66c-PE or isotype control and 10 000 events were gated.

Initially, there was a concern that neutrophils isolated using this protocol would be activated as control neutrophils were secreting substantial amounts of MMP-8 and 9 at time = 0 (data not shown). A trip to the University of Sheffield to Professor Moira Whyte's neutrophil laboratory was made to learn neutrophil isolation using Optiprep followed by Magnetic Activated Cell Sorting (MACS), and neutrophil supernatant from this method at time = 0 was also collected. Baseline MMP-8, -9 and IL-8 as a marker for neutrophil activation from the 2 techniques were analysed, and were found to be comparable (**Figure 2**) and so the Ficoll-Hypaque hypotonic lysis method was used to isolate neutrophils.

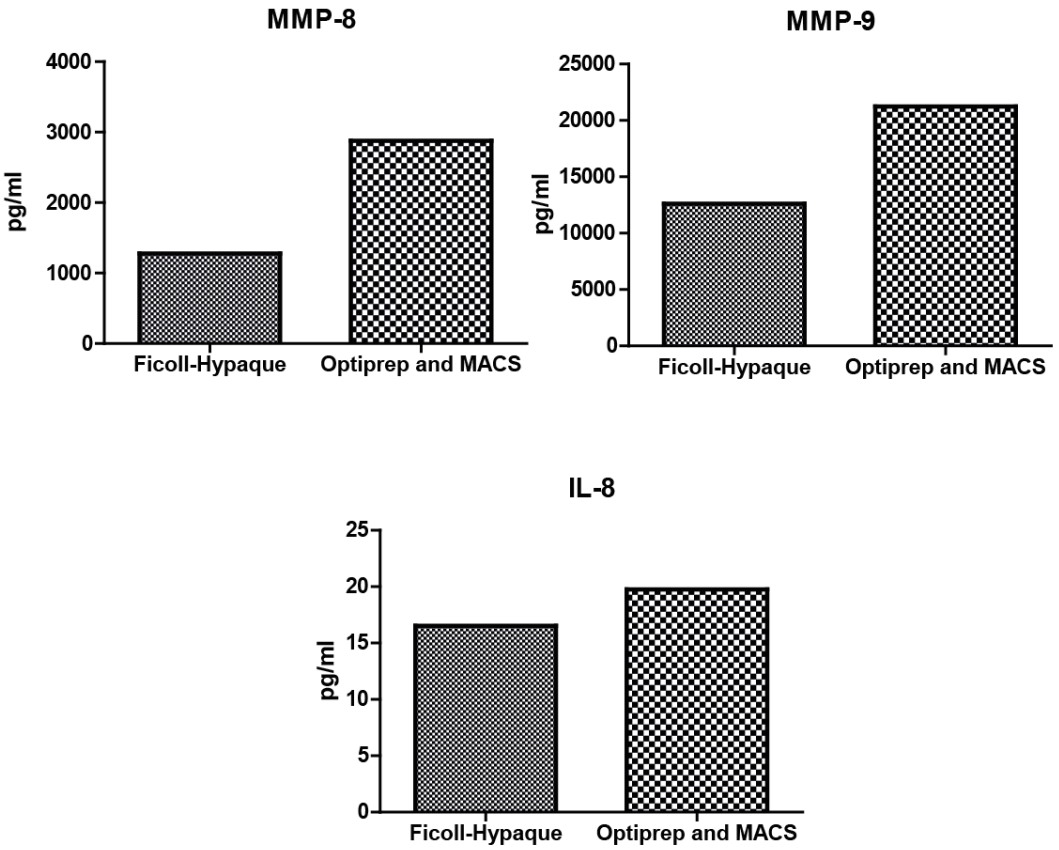


Figure 2: MMP-8, -9 and IL-8 secretion from neutrophils from two different isolation protocols. 7.5 x 10^5 neutrophils were extracted using each protocol and supernatants at time = 0 was collected and analysed. Experiment was done once with a single sample.

4. *Neutrophil infection protocol*

Neutrophils were infected immediately after isolation using *M.tb* at an OD of 0.5-0.7 to provide the correct multiplicity of infection (MOI). Initially, infection of neutrophils was done using *M.tb* in 7H9. Preliminary experiments demonstrated that neutrophils inherently release more MMPs with 7H9 compared to the PBS control (Gibco, Invitrogen, UK) (**Figure 3**), and so after this, infection of neutrophils was done with *M.tb* resuspended in PBS. This may be due to the effect of L-glutamate since it has been shown that L-glutamate also upregulates MMPs from astrocytes [218]. Neutrophils were also found to secrete more MMPs with normal polystyrene tissue culture plates (data not shown) and after discussing with the neutrophil lab at University of Sheffield, subsequent experiments were done in 96-well flat bottom PVC plates (VWR, Leicestershire, UK) or polypropylene eppendorfs which resolved this problem.

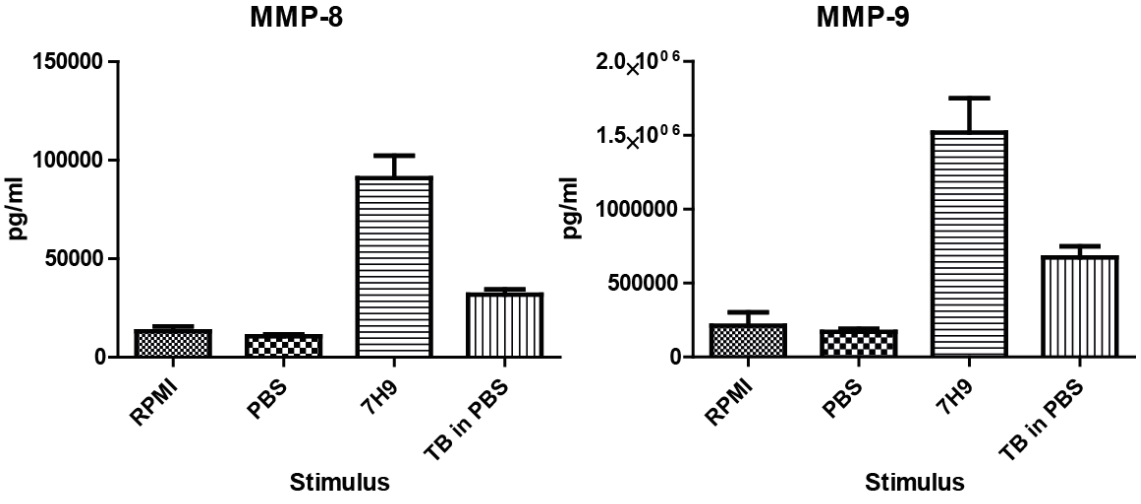


Figure 3: Effect of growth media on MMP-8 and -9 secretion from neutrophils. 1 x 10^6 neutrophils were incubated with the stated media or *M.tb* MOI of 1 for 4 hours. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 2 independent experiments.

The effect of *M.tb* infection versus concentration of neutrophils demonstrated a dose response in MMP secretion (data not shown). It was decided that a final concentration of 5 x 10^6 cells / ml should be used to reflect normal human physiology, and is within the concentration range often used in literature for neutrophil infection and stimulation experiments. After infecting neutrophils with *M.tb* at an MOI of 1 for 4 hours, cell culture medium was collected and filtered through 0.2-µm sterile-filters (Nalgene, Hereford, U.K). Conditioned medium from *M. tuberculosis* infected neutrophils was termed CoNTB, and conditioned medium from uninfected neutrophils was termed CoNCont.

In inhibition experiments, neutrophils were preincubated for 30 minutes with p38 inhibitor SB 203580 (Calbiochem), ERK inhibitor PD 98049 (Calbiochem), PI3 kinase inhibitor LY294002 (Enzo Life Sciences, Exeter, UK), Wortmannin (Sigma-Aldrich), NF-kB inhibitor Helenalin (Sigma-Aldrich), IKK_2 inhibitor SC-514 (Enzo Life Sciences), AMPK activator AICAR (Sigma-Aldrich), AMPK inhibitor Compound C (Merck Biochemicals), Rapamycin (Sigma-Alrich), Akt VII inhibitor (Calbiochem) and dexamathasone (Sigma-Aldrich).

5. *Monocyte purification and maturation into macrophages*

Monocytes were isolated from single donor Component Donation Cones from the National Blood Transfusion Service, Colindale. The leukocytes were mixed 50:50 with Hanks' Balanced Salt Solution (HBSS), layered onto Ficoll Paque (GE Healthcare, Little Chalfont, UK) and centrifuged at 480 x g for 30 minutes. The mononuclear cell layer was removed and then washed a total of five times in HBSS, spinning down the cell pellet at 308 x g after each wash. Total monocytes were calculated by counting the

number of adherent cells in a Neubauer counting chamber after incubation for 5 minutes at 37^{o}. Monocytes were plated in at 250,000 monocytes /cm^{2} diluted in RPMI 1640. After one hour, non-adherent cells were removed by washing three times with HBSS, then the media was replaced with RPMI 1640 supplemented with 2mM glutamine and 10μg/ml ampicillin. Monocyte purity was assessed using 2 colour flow cytometry for anti-CD3 (fluorescein isothiocyanate, FITC) and anti-CD14 (phyco erythrin, PE) and analysed on a BD FACSCalibur flow cytometer. Monocyte purity was over 95%, with less than 5% cells CD3 positive. For maturation into monocyte derived macrophages (MDM's), monocytes were cultured in RPMI 1640 supplemented with 2mM glutamine, 10% fetal calf serum (FCS), 10μg/ml ampicillin and 20ng/ml macrophage colony stimulating factor (M-CSF, R&D, Abingdon, UK). After 4 days in the growth factor containing media, the media was changed to RPMI with 10% FCS but no growth factors, and 24 hours later the experiment was commenced.

6. *Monocyte infection protocol*

Monocytes were infected immediately after the completion of adhesion purification, using *M.tb* at OD of 0.6 at multiplicity of infection (MOI) of 1. Trypan blue exclusion was used to compare cell viability in uninfected and infected monocytes at 24 hours. No difference in trypan blue uptake was observed. At 24 hours, cell culture medium was collected and filtered through 0.2-μm sterile-filters (Nalgene, Hereford, U.K.). Conditioned medium from *M. tuberculosis* infected monocytes was termed CoMTB, and conditioned medium from uninfected monocytes was termed CoMCont. Each batch of CoMTB and CoMCont was tested on A549 adenocarcinoma cell lines (ECACC) and the MMP-1 concentration secreted from the cells was measured. CoMTB which had previously been tested before was used as a positive

internal control for comparison. CoMTB contains minimal MMPs in part due to the sterile filtration process [219] and also verified with MMP-8 and -9 ELISAs (see Figure 8A).

7. *Sterilisation of cell culture supernatants*

Following experiments involving *M.tb*, samples required removal of *M.tb* by filtration before analysis. Supernatants are centrifuged at 12 000 x g for 5 minutes, and subsequently transferred to 0.2 μm PVDF centrifugal filter units (Millipore, Watford, UK) whereupon a further centrifugation at 12 000 x g for 1 minute was undertaken.

8. *Astrocyte and microglial cell culture*

Human astrocytoma cell lines U87-MG, U373-MG Uppsala and U251 MG (ECACC nos. 89081402, 08061901, and 09063001 respectively) were maintained in Eagle‘s MEM supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, and 10 μg/ml ampicillin according to the supplier‘s instructions. All experiments were performed in serum-free medium before passage 15.

Human CHME3 microglial cells (used with permission of Marc Tardieu, Paris, France, and kindly provided by Nicola Woodroofe, Sheffield Hallam University, Sheffield, U.K.) were maintained in DMEM with 10% FCS, (Biosera, Ringmer, U.K.) and 3 mM glutamine. Cells were seeded at 35– 50,000 cells per cm^2 for experiments, which were performed in Macrophage Serum-Free Medium (Invitrogen).

9. *MMP-8 and -9 ELISA*

MMP-8 and 9 concentrations in cell culture medium were measured by Duoset ELISA Development System (R&D Systems) according to the manufacturer's instructions. ELISA plates were coated with 100 µL of capture antibody at 2.0 µg/ml and 1.0 µg/ml respectively overnight at room temperature, then washed three times with PBS / 0.05% Tween. Free binding sites were then blocked for 1 hour with PBS / 1% BSA at room temperature. Standards (serial dilutions 4000 to 62.5 pg/ml for MMP-8 and serial dilutions 2000 to 31.25 pg/ml for MMP-9) and samples (1 in 30 and 1 in 300 for MMP-8 in PBS / 1% BSA; 1 in 300 and 1 in 3000 for MMP-9) were added to the appropriate wells and incubated for 2 hours at room temperature. After 3 washes, the detection antibody (50 ng/ml MMP-8; 200ng/ml MMP-9) was added for 2 hours at room temperature, 3 further washes were performed, then 100µl streptavidin-HRP (1 in 200 dilution) was incubated with each well for 20 minutes, before washing again. 100µl substrate solution containing tetramethylbenzidine (Sigma-Aldrich) was then added per well and the plate incubated for 1-5 minutes. The reaction was then stopped with 50 µL per well of 2M sulphuric acid and the plate read at 450nm using a microplate reader (µQuant, Biotek Instruments, UK).

10. *TIMP-1 and 2 ELISA*

TIMP-1 and 2 concentrations were measured using the Duoset ELISA Development System (R&D Systems) according to the manufacturer's instructions using protocols similar to those for MMP ELISAs. ELISA plates were coated with 100µl of capture antibody at 2.0µg/ml overnight at room temperature, then washed three times with PBS/ 0.05 % Tween 20. Free binding sites were blocked for 1 hour with 1% BSA in PBS at room temperature. After 3 washes, samples (1 in 3 and 1 in 30

dilutions, made in the appropriate culture medium) and standards (serial dilutions of 2000 pg/ml to 31.2 pg/ml) were added and incubated at room temperature for 2 hours then washed three times. 100µl of biotinylated detection antibody (50 ng/ml for TIMP-1 and 2) was added to each well, incubated for 2 hours at room temperature, then washed 3 times. After 20 minutes with 100µl Streptavidin horseradish peroxidise and 3 further washes, 100 µL of substrate reagent was added to each well. When the highest two standards neared an equal colour change, the reaction was stopped with 2M sulphuric acid and optical density was read at 450nm.

11. *Human myeloperoxidase (MPO) ELISA*

The human MPO Quantikine ELISA kit (R&D Systems) was performed according to manufacturer‘s instructions. 100 µL of assay diluent RD1-62 was added per well. Standards were diluted and commencing with a highest concentration of 100 ng/ml with the lowest at 1.56 ng/ml. 50µL of standards and samples were added per well and this was covered and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 rpm. The plate was washed three times with Wash Buffer. 200µL of MPO conjugate was added to each well and the plate incubated for a further hour on the shaker at room temperature. The plate was washed three times, prior to the addition of 200µL of substrate solution and incubated for 20 minutes in the dark. 50 µL of stop solution was added to each well, and the optical density was read using a microplate reader set to 450nm. The lower limit for detection for MPO is 0.1ng/ml.

12. *Human neutrophil gelatinase-associated lipocalin (NGAL) ELISA*

The human NGAL ELISA kit (Bioporto Diagnostics, Denmark) was performed according to manufacturers' instructions. Standards commenced at the highest concentration of 1000 pg/ml down to the lowest of 10 pg/ml. 100 µL of standard and samples were added and incubated for 1 hour at room temperature on a shaking platform set at 200 per minute. The wells were washed three times before dispensing 100 µL of biotynylated NGAL antibody and incubated for 1 hour at room temperature. The plate was washed and then 100 µL of HRP-streptavidin conjugate added and incubated for another hour. After washing 100 µL of TMB substrate (Sigma Aldrich) was added into each well for 10 minutes in the dark, and 100 µL of stop solution was added. The wells were read at 450 nm (reference wave-length 650 nm). The lower limit for detection NGAL is 1.6 pg/ml respectively.

13. *Gelatin zymography*

Substrate zymography analyses total potential enzymatic activity by separating proteins on a substrate-embedded gel under non-reducing conditions, then facilitating re-constitution of the tertiary structure of the enzyme, followed by incubation to allow enzymatic degradation of the substrate. Breakdown products diffuse out of the gel. Staining with Coomassie blue then reveals areas devoid of protein as a light band on a dark background [220]. Both pro-enzymes and active forms are demonstrated as the pro-enzymes become activated during the process.

Cell culture supernatants with 5x loading buffer (0.25M Tris pH 6.8, 50% glycerol, 5% SDS, bromophenol blue) were run on an 11% acrylamide gel impregnated with 0.1% gelatin (Sigma, Poole, UK) at 180V for approximately 3 ½ hours (buffer 25mM Tris,

190mM glycine, 0.1% SDS). Standards of 2 ng MMP-9 (Oncogene, Nottingham, UK) were loaded on each gel. Once the marker had reached the end of the gel, the gel was washed in 2.5% Triton X for 1 hour, then rinsed twice in low salt collagenase buffer (55mM Tris base, 200mM sodium chloride, 5mM calcium chloride, 0.02% Brij, pH 7.6) prior to incubation at 37^{o} for 16 hours in collagenase buffer. Areas of gelatinolytic activity were revealed by a single step stain-destain method using 0.02% Coomassie Blue (Pharmacia, Sweden) in 1:3:6 acetic acid : methanol : water. Densitometric image analysis was performed using Scion Image version Beta.4.0.2. A central problem to zymography is gel to gel variation, so to perform statistical analysis comparing samples between gels each densitometric value was divided by the MMP-9 standard value on the gel. Wherever possible, all samples for comparison were run on the same gel to minimise this source of variability, and all samples from the same experiment were run concurrently.

14. *Western blotting*

Pelleted neutrophils infected with *M.tb* or stimulated with CoMTB at a set time point were mixed 1:1 with loading buffer (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 0.06M Tris pH 6.8, Bromophenol blue) before they were stored at -80^{o}C for further analysis. Samples are then heat denatured at 90^{o} for 5 minutes and incubated on ice for 1 minute. 8 µL of each sample were then run on at 200V to the end of the NuPAGE® 4-12% Bis-Tris gels (Invitrogen, Paisley, UK) with MES (for protein <60kDa) or MOPS (for protein >60kDa) SDS Running buffer (Invitrogen, UK). A Rainbow Molecular weight marker (GE Healthcare) was run on each gel. Protein was then transferred onto a nitrocellulose membrane (GE Healthcare) at 400mA for 1.5 hours (buffer: 20% methanol, 20mM Tris base, 150mM Glycine, 0.1% SDS). The

membrane was then immersed in blocking buffer (1 x TBS, 5% skim milk, 0.1% Tween-20) for 1 hour. Primary antibody was diluted in 5% BSA/0.1% Tween in TBS with the dilutions listed in **Table 3** and incubated overnight at 4^{o}C with agitation. After three washes in PBS with 1% Tween (5 minutes each), secondary antibody was added for one hour diluted in blocking buffer. Blots were then washed three times (5 minutes each). ECL Substrate Reagent (Amersham) was added according to manufacturer's instructions and luminescence was demonstrated by exposing the membrane to Hyperfilm ECL (Amersham).

Antibody	Manufacturer	Dilution
Primary antibody		
Mouse anti-human Beta-actin	Sigma-Aldrich	1 in 5 000
Mouse anti-human MMP-8	Abcam	1 in 10 000
Mouse anti-human MMP-9	Abcam	1 in 10 000
Rabbit anti-human GAPDH	Abcam	1 in 2 500
Rabbit anti-human histone 2B	Abcam	1 in 20 000
Rabbit anti-human citrulline H3	Abcam	1 in 5000
Rabbit anti-human phospho-Akt	Cell Signaling Technology	1 in 1 000
Rabbit anti-human phospho-AMPKα1/2(T172)	Cell Signaling Technology	1 in 1 000
Rabbit anti-human phospho-ERK	Cell Signaling Technology	1 in 1 000
Rabbit anti-human phospho-JNK	Cell Signaling Technology	1 in 1 000
Rabbit anti-human phospho-p38	Cell Signaling Technology	1 in 1 000
Rabbit anti-human phospho-p70S6k (T229)	Abcam	1 in 500
Rabbit anti-human phospho-Tuberin (S1387)	Cell Signaling Technology	1 in 2000
Rabbit anti-human total Akt	Cell Signaling Technology	1 in 1 000
Rabbit anti-human total AMPKα	Cell Signaling Technology	1 in 1 000
Rabbit anti-human total ERK	Cell Signaling Technology	1 in 1 000
Rabbit anti-human total JNK	Cell Signaling Technology	1 in 1 000
Rabbit anti-human total p38	Cell Signaling Technology	1 in 1 000
Secondary antibody		
Goat anti-mouse IgG (H+L)	Jackson Immunoresearch laboratories, Baltimore, USA	1 in 5000
Goat anti-rabbit HRP linked	Cell Signaling Technology	1 in 2000

Table 3. Primary and secondary antibodies used for western blotting

15. *Human Phospho-kinase Array*

The Proteome Profiler Human Phospho-kinase array kit (R&D Systems, Abingdon) which detects 45 phosphorylated proteins was performed according to the manufacturer's protocol. Thirty minutes after neutrophils were stimulated with CoMTB, the cells were pelleted and lysed in lysis buffer (R&D Systems) before storing at -80^{o}C for further analysis. Total protein concentration was measured by Bradford assay (Bio-Rad) and equal total protein was loaded onto each array. Capture and control antibodies have been spotted in duplicate on the supplied nitrocellulose membranes and these were blocked with array buffer. Cellular extracts are diluted and incubated overnight with the Human Phospho-Kinase Array at 4^{o}C on a rocking platform. The array was washed three times (10 minutes per wash) followed by incubation with a cocktail of biotinylated detection antibodies. Three washes of 10 minutes each were further done followed by the addition of Streptavidin-HRP. After a third wash cycle, chemilumiscence was demonstrated by addition of ECL Substrate Reagent (Amersham) and exposing the arrays to Hyperfilm ECL (Amersham). Densitometric analysis of each array was performed using Scion Image version Beta.4.0.2. The positive signals seen on the developed film were identified by placing the transparency overlay on the array image and aligning it with the three pairs of positive control spots in the corners of each membrane. The location of controls and capture antibodies were correlated with those in the appendix of the handbook provided.

16. *Flow cytometry*

To assess neutrophil viability with CoMCont and CoMTB stimulation, cells were stained with Annexin V-FITC and propidium iodide (eBioscience, Affymetrix, California, USA) according to manufacturer's instructions. 5 x 10^5 cells were washed with PBS, resuspended in 195 µL of 1 x binding buffer with 5 µL of Annexin V-FITC added and incubated for 10 minutes in the dark at room temperature. The cells were subsequently washed in 1 x binding buffer before resuspending in 190 µL of 1 x binding buffer and 10 µL of propidium iodide at 20 µg/m was added. 50, 000 events were collected in the gate of live cells (excluding debris in the FSC versus SSC dot plot) and analysed on BD FACSCalibur flow cytometer using CellQuest (BD, Mountain View, California). Data was analysed using FlowJo 7.6.5.

17. *RNA extraction*

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Crawley, UK) as described by the manufacturer. 2 x 10^6 neutrophils were suspended at a final concentration of 5 x 10^6 cells/ml. At the relevant time points, cells were pelleted, supernatants removed, and the cell pellet was lysed with 350 µL of Buffer RLT and homogenised using the Qiashredder (Qiagen) by spinning at full speed for 2 minutes. A volume of 70% ethanol was added to the homogenized lysate, and 700 µL of the sample was transferred to an RNeasy spin column before centrifuging for 15 seconds at 12 000 x g. 350 µL of Buffer RW1 was added to the RNeasy spin column, centrifuged and the flow-through discarded. Genomic DNA was eliminated by the addition of 80 µL of RNase-free DNase I digestion (Qiagen) for 15 minutes, before the addition of 350 µL of Buffer RW1 to the RNeasy spin column and centrifuge. Following that, 500 µL of Buffer RPE was added to the RNeasy spin column and centrifuged for 2 times. The

RNA was then eluted by the addition of 30 µL of RNase-free water. Total RNA was quantified by spectrophotometry (Nanodrop ND 1000, Thermo Scientific).

18. *Real-time Polymerase Chain Reaction*

As neutrophils have only a small amount of RNA of approximately 1 µg/ 10^7 cells [221], quantitative real-time RT-PCR was performed using the OneStep RT- PCR master mix (Qiagen, Crawley, UK) according to the manufacturer's instruction using a Stratagene Mx3000P platform. This allows between 1 pg to 2 µg of RNA to be transcribed. 5 – 10 µg per sample was used for MMP-8 and -9 gene amplification. The RT-PCR cycling conditions included 50 °C for 30 min, 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min. VIC-labelled beta-actin control reagent (Applied Biosystems) and HEX-labelled GAPDH (Sigma-Aldrich, Dorset, UK) were analyzed in parallel. MMP-8 (catalogue no. Hs00233972), AMPKα1 (catalogue no. Hs01562308_m1) and AMPKα2 (catalogue no. Hs00178903_m1) primer and probe mixes were obtained from Applied Biosystems. MMP-9 and GAPDH primers and probes are described in **Table 4**.

MMP-9	
Forward primer	5'-AGGCGCTCATGTACCCTATGTAC-3'
Reverse primer	5'- GCCGTGGCTCAGGTTCA-3'
Probe	5'-FAM-CATCCGGCACCTCTATGGTCCTCG-TAMRA-3';
GAPDH	
Forward primer	5'- CGCTTCGCTCTCTGCTCCT-3'
Reverse primer	5'- CGACCAAATCCGTTGACTCC-3
Probe	5'-HEX-CGTCGCCAGCCGAGCCACAT-TAMRA-3'

Table 4: MMP-9 and GAPDH primer and probe sequences.

Initial samples were run in individually to determine the optimal efficiencies of both gene of interest before multiplexing was attempted. MMP-8 and 9 reaction efficiencies were 83.1% and 81.5% which were comparable to multiplex reactions (up to 96.1% and 104.3% respectively). Subsequently, RT-PCR reactions were run in multiplex. To accurately determine the quantitative change in RNA levels, standard curves were prepared from plasmids as described in the proceeding section; plasmids standards were diluted in TE buffer and made from 5 x 10^7 copies/ml in serial 1 in 10 dilutions down to 5 x 10^2 copies/ml. These standards were subjected to real-time PCR as above. Standard curves for C_T vs input RNA were generated, and relative levels of starting RNA in each sample were determined. To account for differences in the amount of total RNA, the results of each MMP member were normalized to GAPDH levels from the same sample.

19. *Construction of plasmid standards for real-time PCR*

Plasmid standards were constructed for MMP-1, -3, -7, -8, -9, -10, -14, 18s, beta-actin, GAPDH, AMPKα1 and AMPKα2. PCR was first done for all the genes using historical samples with primer and probe sequences listed in **Table 4** and **Table 5**, using 2x SYBR Green mix (Sigma-Aldrich) with a thermal profile of activation step of 95^{o}C for 10 minutes, followed by 40 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 60^{o}C for 30 seconds and extension at 72^{o}C for 30 seconds.

MMP-1 (Sigma-Aldrich)	
Forward primer	5‘- AAGATGAAAGGTGGACCAACAATT -3‘
Reverse primer	5‘ -CCAAGAGAATGGCCGAGTTC -3‘
Probe	5‘- FAM-CAGAGAGTACAACTTACATCGTGTTGCGGCTC-TAMRA -3‘
MMP-7 (Sigma-Aldrich)	
Forward primer	5‘-CTTTGCGCGAGGAGCTCA -3‘
Rerverse primer	5‘-CAGGCGCAAAGGCATGA -3‘
Probe	5‘- FAM-CCATTTGATGGGCCAGGAAACACG-TAMRA -3‘
MMP-10 (Sigma-Aldrich)	
Forward primer	5‘- GGACCTGGGCTTTATGGAGATATT -3‘
Reverse primer	5‘- CCCAGGGAGTGGCCAAG -3‘
Probe	5‘- FAM- GAAGATGCATCAGGCACCAATTTATTC-TAMRA -3
18s	(Applied Biosystems, catalogue no. 4308329)
Beta-actin	(Applied Biosystems, catalogue no. 4310881E)
MMP-3	(Applied Biosystems, catalogue no. Hs00968305_m1)
MMP-8	(Applied Biosystems, catalogue no. Hs00233972_m1)
MMP-14	(Applied Biosystems, catalogue no. Hs00237119_m1)

Table 5: Primer and probe sequences of gene of interests and housekeeping genes.

PCR products were confirmed by running samples with EZ Vision (Amresco, Ohio, USA) on a 2% agarose gel in 1 x TAE buffer at 120V for 30-60 minutes with a DNA ladder (Invitrogen, Paisley, UK) and visualising under a UV transilluminator.

2 plasmids were created for general use: one contained MMP-1, -3 and -7 and the other containing MMP-8, -9, -10, -14, GAPDH, beta-actin and 18s. PCR products from individual genes were blunt ended using pfu polymerase (Promega, Wisconsin, USA) by the addition of 1Ü of enzyme with 60 µL of PCR product and incubating for 10 minutes at 72^{o}C. These were then precipitated with equal volumes of PEG and placed on ice for 10 minutes. The pellet was then washed with ethanol and resuspended with 30 µL of nuclease free water. The mix was kinased by adding 1 µL of T4 kinase (Promega, Wisconsin, USA), 2 µL of each PCR product to be ligated and the mix was

incubated at 37°C for 10 minutes. Thereafter, 1 µL of T4 ligase (Promega) was added with ligation buffer and incubated at room temperature for 15 minutes. RT-PCR was done to obtain the ligated product using SYBR Green and the relevant primers. The size of the product was confirmed by running on 2% agarose gel.

Once the PCR products were ligated as required, they were cloned using the TOPO TA cloning kit(Invitrogen, Paiseley, UK) according to manufacturer's instructions. 4 µL of fresh PCR product was added to 1 µL of salt solution and 1µL of TOPO vector to make up the cloning reaction. The reaction was incubated for 5 minutes at room temperature and subsequently placed on ice. TOP10 competent *E.coli* were thawed on ice, and 2 µL of cloning reaction was added to the cells and incubated on ice for 5 minutes. The cells were heat-shocked for 30 seconds at 42°C and then immediately transferred to ice. 250 µL of S.O.C. medium was added and incubated at 37°C on a horizontal shaker. After 1 hour, the mixture was added to LB broth containing 50 µg/ml ampicillin and incubated overnight at 37°C. An aliquot was plated onto LB agar with 50 µg/ml ampicillin the following day, colonies subsequently selected, and grown in LB broth with ampicillin. To confirm that the colonies contained the required gene, 50 µL of cells in broth were pelleted, resuspended in 50 µL of nuclease free water and heated at 95°C for 5 minutes to lyse the cells. The cells were then pelleted and the supernatant collected. PCR was done with 2 µL of supernatant with the relevant primer and probe mix and when positive, the appropriate clone was grown overnight. Plasmids were extracted using GenElute™ HP plasmid Miniprep kit (Sigma-Aldrich), and diluted to the desired concentration.

20. *siRNA mediated gene silencing/knockdown*

Neutrophil siRNA gene silencing was performed using the Deliver X siRNA transfection kit (Panomics, France) according to manufacturer's instructions. Briefly, 5 μM siRNAs (AMPKα1, AMPKα2, GAPDH and non-targeting siRNA, Dharmacon, USA) were prepared in Buffer-1, with the transfection reagent prepared in Buffer-2 before mixing in a 1:1 ratio. This was incubated at 37^{o}C for 20 minutes. Neutrophils were pelleted and resuspended with 300 μL of working siRNA/siRNA transfection reagent complex and incubated for 5 minutes. 300 μl of serum-free media was added and incubated for 4 hours and thereafter, 1ml of complete growth media was further added. Stimulation with CoMTB occurred immediately, or after a rest overnight. All experiments were done with non-targeting siRNA. At the end of the experiment, cell viability was assessed using a flow cytometer (FACSCalibur, BD Biosciences, USA) registering 10 000 events staining for cells positive for propidium iodide (Invitrogen, Paiseley, UK). Cells were then processed as for RNA extraction.

21. *MMP bead array*

MMP-8 and -9 concentrations were analyzed by Fluorokine multianalyte profiling kit according to the manufacturer's protocol (R&D Systems, Abingdon, U.K.) on the Luminex platform (Bio-Rad, Hemel Hempstead, U.K.). Samples were diluted 1 in 5 (non-neutrophil samples) or 1 in 50 (neutrophil samples) with calibrator diluent. Standard were reconstituted according to manufacturer's instructions and serial 1 in 3 dilutions were made for a 7 point standard. The filter bottom plate was pre-wetted with 100 μL of wash buffer, 17 μL each of MMP-8 and -9 beads were diluted with Microparticle Diluent, and 50 μL were added into each well. 50 μL of standard and samples were loaded onto the plate, incubated at room temperature for 2 hours on a

horizontal microplate shaker set at 500 rpm. The microplate was washed three times, 50 µL of diluted Biotin Antibody cocktail was added and the plate was incubated for 1 hour on the shaker. After 3 washes, 50 µL of streptavidin-PE was added, incubated for 30 minutes and further washed 3 times. The microparticles were resuspended with 85µL of wash buffer, incubated for 2 minutes and read using the Luminex analyzer. The minimum level of detection for MMP-8 was 110 pg/ml and MMP-9 was 65 pg/ml. When analyzing MMP secretion from monocytes, the Luminex analyzer was set at high sensitivity.

22. *Cytokine bead array*

Analysis of cytokine concentrations were analyzed using the Cytokine Human 30-plex panel (Invitrogen, Paisley, UK) according to manufacturer‘s instructions. This panel comprise IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40⁄p70), IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, Eotaxin, RANTES, MCP-1, VEGF, G-CSF, EGF, FGF-basic, and HGF. Samples were diluted 1 in 5 with RPMI. Standard was reconstituted according to manufacturer‘s instructions and serial 1 in 3 dilutions were made for a 7 point standard. The assay wells were pre-wet by adding 200 µL of Wash Solution and incubated 15-30 seconds at room temperature prior to aspirating the Wash Solution using the vacuum manifold. 25 µL of Bead Solution were added into each well, 200 µL of Wash Solution were added to each well and the washing step repeated. 50 µL Incubation Buffer were pipette into each well. 100 µL of standard were added to the wells designated for standard. For wells designated for samples, 50 µL of Assay Diluent followed by 50 µL samples were loaded onto the plate, covered with foil and incubated at room temperature for 2 hours on a horizontal microplate shaker set at 500 rpm. The

microplate was washed three times with 200 µL of Wash Solution each using the vacuum manifold, 100 µL of diluted Biotin Detector Antibody was added to each well and the plate was incubated for 1 hour on the shaker at room temperature. After 3 washes, 100 µL of streptavidin-PE was added, incubated for 30 minutes at room temperature and further washed 3 times. 100 µL of Wash Solution was added to each well and the plate was incubated for 2 minutes on the shaker at room temperature. The plate was read using the Luminex instrument (Bio-Rad).

23. *DQ collagen assay*

Type I and IV collagen degradation were assessed using the EnzChek ® Gelatinase/Collagenase Assay kit (Molecular Probes, Leiden, Netherlands) and performed according to manufacturer's instructions. In brief, samples diluted in 1 x reaction buffer (1 in 5 for neutrophil supernatants; 1 in 10 for induced sputum samples) were activated with 4-amino-phenyl mercuric acetate (APMA) for 1 hour at 37^{o}C. 80µL of 1x reaction buffer or inhibitor (doxycyline hyclate, Sigma-Aldrich; Neutrophil elastase inhibitor N-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone, Sigma-Aldrich; Mouse anti-human MMP-1, R&D Systems; Goat anti-human MMP-8, R&D Systems; Mouse anti-human MMP-9, Calbiochem, UK) were added into a 96 well black fluorescence plate with 20µL of DQ collagen at a final concentration of 25µg/ml. 100µL of activated samples was subsequently added, and fluorescence was measured at specified times using a fluorometer (FLUOstar Galaxy, BMG Labtechnologies, Germany) using a 10% gain setting.

24. *Isolation and quantification of neutrophil extracellular traps (NETS)*

For experiments involving NETS, human neutrophils were infected with *M.tb* at an MOI of 10 or stimulated with CoMTB at 1:5 dilution over 4 hours in 96 well plates. A final concentration of 20nM PMA was used as a positive control. 50µL of micrococcal nuclease (Fermentas), a non processive nuclease that cuts DNA at linker sites, at a final concentration of 5U/ml was added in each well for 10 minutes at 37^{o}C, after which EDTA at a final concentration of 5mM was used to halt the reaction. Supernatants were collected, sterile filtered throught 0.2 µm Anopore membrane and kept at 4^{o}C until further use. NETS were quantified using QuantiT PicoGreen (Invitrogen, Paisley, UK) according to manufacturer's instructions. In brief, a standard was made using serial 1 in 10 dilutions of Lambda DNA commencing from 2 µg/ml to 2 ng/ml diluted in 1 x TE buffer. 50µL of samples and standards were loaded onto 96 well black plates, and 50 µL PicoGreen at a 1 in 200 dilution were added in each well. The plate was then incubated at room temperature for 5 minutes, and then read using the fluorometer (FLUOstar Galaxy, BMG lab technologies, Germany).

25. *Co-immunoprecipitation*

To determine if NETS were associated with neutrophil MMPs, co-immunoprecipitation using Dynabeads Protein G (Invitrogen, Paiseley, UK) and the Dynabeads Co-immunopreciptation kit (Invitrogen) were used according to manufacturer's instructions. For the former, 50 µL of Dynabeads in suspension were transferred to an eppendorf microcentrifuge tube, placed onto a magnet and suspending liquid removed. 1 – 10 µg of desired antibody was added with antibody binding and

washing buffer to make up to a final volume of 200 µl. This was incubated with rotation for 10 minutes at room temperature, and thereafter the buffer was removed. When crosslinking of the antibody to the bead was desired, the antibody-coupled beads were washed twice in conjugation buffer (20 mM sodium phosphate, 0.15M sodium chloride, pH 7-9) and resuspended in 5mM BS3 solution. This was incubated for 30 minutes with rotation, and crosslinking was terminated with 12.5µl of quenching buffer (1M Tris HCl, pH 7.5). Between 100-1000 µL of sample were added to the antibody-coupled beads, incubated at room temperature for 10 minutes prior to washing 3-6 times with washing buffer. The supernatant was then removed and 20 µl of elution buffer with 10 µl premixed LDS sample buffer (Invitrogen) and sample reducing buffer were added. The sample was frozen as an antigen-bead complex at -80^{o}C until analysis whereupon the beads will be emoved using a magnet. When the Dynabeads Co-immunoprecipitation kit was used, the appropriate quantity of Dynabeads M-270 Epoxy was weighed out, washed with 1 ml of C1 buffer, placed onto a magnet and supernatant removed. The appropriate volume of antibody (5-7 µg Ab/ mg Dynabeads) with C1 were added to the beads. The appropriate volume of C2 was added and the mixture was incubated on a roller at 37^{o}C overnight. The next day, the supernatant was removed using a magnet and the beads were washed with HB wash, LB wash, twice with SB wash, and resuspended in the same volume of SB as was the total coupling reaction volume. 1.5 mg of antibody coupled Dynabeads was used for each coimmunoprecipitation and was resuspended with the sample and incubated on a roller at 4^{o}C for 10-30 minutes. The Dynabeads were washed three times with extraction buffer (1 x IP solution, 100mM NaCl, protease inhibitor (Sigma) and a final wash with last wash buffer (1 x LWB, 0.02% Tween 20). The bead suspension was transferred to a clean tube, supernatant removed, and finally resuspended in 60 µL of EB and

incubated on a roller for 5 minutes at room temperature. The supernatant was then transferred into another clean tube and frozen at -80^{o}C until analysis.

26. *Patient recruitment and data collection*

Please see Chapter 6 for specific methodology for the analysis of induced sputum samples from TB patients and healthy controls.

AMPK patients and control. This study received the Institutional Review Board approval from Central London Research Ethics Committee (REC 11/LO/0913). The cardiology specialty consultation service of The Heart Hospital, University College London identified patients who were clinically diagnosed to have cardiomyopathy, and confirmed via genotyping to have AMPKγ2 mutation. Neutrophil isolation was done concurrently from patient and from a healthy volunteer and stimulated at the same time.

27. *Immunofluorescence*

Permanox chamber slides (Nunc Labtech, Thermo Fisher Scientific) were coated with 0.1 mg/ml fibrinogen with or without 25 μg/ml of DQ collagen for 30 minutes and then aspirated. Thereafter neutrophils were added and stimulated with CoMTB or infected with *M.tb* for the specified period before media was removed. Samples were then fixed with 4% paraformaldehyde for 30 minutes. Cells were washed twice with PBS, and permeabilised with 0.5% saponin for 10 minutes. Following permeabilisation, cells were washed twice with PBS before blocking with 10% human AB serum with 2.5% BSA and 0.05% saponin for 1 hour. Primary antibodies at the stated dilution as in **Table 6** were added overnight. Blocking buffer alone was used as a control. These were then aspirated and the chamber slides were washed twice again

with PBS prior to the addition of secondary antibodies as stated in **Table 6** for 1 hour. After 2 more washes, the chambers were removed from the slide, and Fluoroshield Mounting medium with DAPI (Abcam) was added prior application of the coverslip. Images were captured using Leica confocal microscope (Leica TCS SP5) and processed using Leica LAS AF Lite 2.6.0 (Leica Microsystems, Germany) and Image J 1.43U (NIH, USA).

Antibody	Manufacturer	Dilution
Primary antibody		
Mouse anti-human MMP-8	Abcam	1 in 200
Mouse anti-human MMP-9	Abcam	1 in 500
Rabbit anti-Mycobacterium tuberculosis	Abcam	1 in 50
Rabbit anti-human Rab 5	Abcam	1 in 250
Sheep anti-human histone 2B	Abcam	1 in 1000
Secondary antibody		
Donkey anti-Sheep IgG DyLight 488	Abcam	1 in 1000
Goat anti-mouse IgM DyLight 549	Abcam	1 in 1000
Goat anti-rabbit IgG Cy5	Abcam	1 in 1000

Table 6: Primary and secondary antibodies used for immunofluorescence.

28. *Immunohistochemistry*

Immunohistochemistry of lung biopsy specimens were done in collaboration with Dr Joanna Porter and Jenny Paterson at University College London. Ethical consent for the study of anonymized paraffin-embedded sections from histopathology was obtained from the Hammersmith Hospitals Research Ethics Committee in accordance with The Human Tissue Act 2004. Five non immunosuppressed patients with biopsy proven pulmonary *M.tb* infection were analysed. The positive controls were breast (MMP-9) and colon (AMPK) tumours from 10 patients and inflammed

appendix (MMP-8). Negative controls were performed using the appropriate isotype control antibodies. Three-µm thick sections were immunostained for MMP-8 (Novus Biologicals, NBP1-85576, rabbit polyclonal; 1/250) and phospho-AMPK alpha 1 and 2 (T172) (Abcam, ab51110, rabbit polyclonal; 1/8000) with heat induced epitope retrieval using a citrate-based solution (pH6.0) for 30 minutes; MMP-9 (Millipore, MAB3309, mouse monoclonal, clone 56-2A4; 1/8000) with heat induced epitope retrieval using an EDTA-based solution (pH9.0) for 20 minutes; and Neutrophil Elastase (Dako, M0752, mouse monoclonal, clone NP57; 1/10) with epitope retrieval performed by enzyme digestion using Bond Enzyme Pretreatment Kit. All antibodies were incubated for 15 minutes at room temperature. All immunohistochemistry was performed using the Leica Bond-III automated platform and associated ancillary reagents (all Leica Biosystems, Newcastle Upon Tyne, UK). The antibodies were detected using the Bond Polymer Refine Detection System and Bond DAB Enhancer according the manufacturer‘s instructions

Analysis of CNS-TB brain biopsy specimens were done in collaboration with Dr Federico Roncaroli and Mrs Poonam Singh at the Division of Brain Sciences. Tissue samples were provided by the Imperial College Healthcare Tissue Bank. Ethical permission for this study was obtained from the Hammersmith Hospitals Research Ethics Committee. Five immunocompetent patients with biopsy proven CNS *M.tb* infection were analysed. Haematoxylin-eosin (HE) stained sections and paraffin blocks were retrieved from the archives of the Histopathology at Charing Cross Hospital. Samples were anonymyzed for the purpose of this study. The original HE-stained sections were reviewed by an experienced neuropathologist (FR) and the most representative samples were chosen for this project. The cases were investigated using immunoperoxidase method with antibodies directed against MMP-8 (Abcam; 1/400), MMP-9 (Abcam; 1/200), neutrophil elastase (Dako, clone NP57; 1/100) and CD15 (BD Science, clone Leu-M1; 1/100). Sections of acute appendicitis were used as positive

control. Immunostains with omission of the primary antibody were performed as negative controls. Five-micron sections were cut from each block, dewaxed in xylene and rehydrated in decreasing alcohols to distilled water. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% hydrogen peroxide for 30 minutes. For antigen retrieval, sections were steamed for 20 minutes in 0.01 M citrate, pH 6.5 and then gently cooled in water. In order to block nonspecific binding of the primary antibody, sections were incubated with 10% normal goat serum for 10 minutes (Vector Laboratories, Burlingame, California). The primary antibodies were applied overnight at 4^{o}C. After incubation, they were washed for three times in PBS for 10 minutes each. Staining was visualised using the Vecta stain Elite ABC kit (Vector Laboratories) following the manufacturer instructions. The reaction product was visualised with 2 ng/ml 3,39-diaminobenzidine and 0.0075% hydrogen peroxide in PBS.

29. *Statistics*

Multiple intervention experiments were compared with one-way ANOVA followed by Tukey‘s post-test correction, using GraphPad Prism Version 5.04 for Windows (GraphPad Software). A p – value of less than 0.05 was taken as statistically significant. For secretion analysis, experiments were all performed in triplicate on at least two occasions, while RNA analysis was performed on triplicate samples on a minimum of 2 separate experiments unless otherwise stated.

CHAPTER 3 – NEUTROPHIL MMP AND TIMP SECRETION AND GENE EXPRESSION IN TB

1. *Introduction*

This chapter is dedicated to characterising neutrophil MMP and TIMP secretion in response to *M. tuberculosis* and CoMTB stimulation at the protein level and at gene expression at mRNA level. One of the key aims of my research is to determine how neutrophil MMPs are secreted in response to *M.tb* as well as in monocyte-dependent networks, the latter because both monocytes and neutrophils are present in the granuloma [222]. This was achieved with the use of CoMTB. Once neutrophil MMP secretion was characterised, I next assessed if the secretion could be modulated by a variety of therapeutic agents including dexamethasone which is used in the context of CNS-TB and TB pericarditis, anti-TNFα which is occasionally used in CNS-TB patients refractory to dexamethasone, as well as the Th2 cytokine IL-4.

To determine if neutrophil MMPs are also detrimental to the host, the decision was made to investigate the effect of neutrophil MMPs secreted with *M.tb* and CoMTB on matrix destruction, in particular Type I collagen as this is the most abundant lung collagen [223]. When matrix destruction becomes extensive organ function becomes compromised, and in the case of the lung, respiratory impairment. Hence it is also crucial to determine if therapies could potentially abrogate matrix destruction and I looked into modulating neutrophil MMPs *in vitro* using the MMP inhibitor doxycyline.

One highly topical phenomenon that has recently been discovered, occurs when neutrophils encounter pathogens and instigate the production of neutrophil extracellular traps [97]. NET production has been described with various pathogens such as

staphylococcal species and streptococcal species, toxoplasmosis, candidal infections and with *M.tb* [143]. NETS are associated with neutrophil granule proteins such as myeloperoxidase, neutrophil elastase as well as histones. I also sought to determine if NETS are associated with neutrophil MMP-8 and -9.

2. *Results*

2.1 Neutrophils secrete MMP-8 and -9 in response to infection with live *M.tb* infection

MMP and TIMP secretion from live *M.tb*-infected neutrophils was investigated. An initial experiment confirmed that MMP-8 and -9 were secreted in high concentrations from neutrophils (**Figure 4**) after 16 hours, with very low concentrations of MMP-2, -3 and -12 (< 170 pg/ml, 12 pg/ml and 34 pg/ml respectively and below the detection limits of the standard curve). These low levels of MMP-2, -3 and -12 may be derived from contaminating mast cells and eosinophils. It was observed that MMP-8 concentrations in *M.tb*-infected neutrophils were lower than control. As this difference could possibly be due to greater neutrophil cell death with *M.tb*, I went on to assess neutrophil survival in the presence of *M.tb* using Trypan blue viability assay. Using a *M.tb* MOI of 1, the Trypan blue assay revealed that the viability of neutrophils infected with *M.tb* declined rapidly after 4 hours (data not shown), and thereafter experiments were done using 4 hour incubation periods. After *M.tb* infection, MMP-8 and -9 concentrations in cell supernatants were increased significantly compared to controls at 4 hours ($p<0.001$) (**Figure 5A and B**). A *M.tb* MOI of 1 resulted in a 1.5 fold increase in MMP-8 (p =NS) and 2 fold increase in MMP-9 ($p < 0.05$), and this increased to 5 fold for MMP-8 and 3.5 fold for MMP-9 with *M.tb* MOI of 10 (both $p <0.01$). Having characterised the basic pattern of MMP-8 and -9 secretion from *M.tb*-infected neutrophils, I next examined TIMP secretion. There was a MOI dose-dependent response in secretion of TIMP-2 with *M.tb* MOI with a significant difference at a *M.tb* MOI of 10 ($p<0.01$). This effect was however absent with no increase for TIMP-1

(**Figure 5C**). In addition, the concentration of neutrophil MMPs are much greater than the concentration of TIMPs, swinging the balance towards matrix degradation.

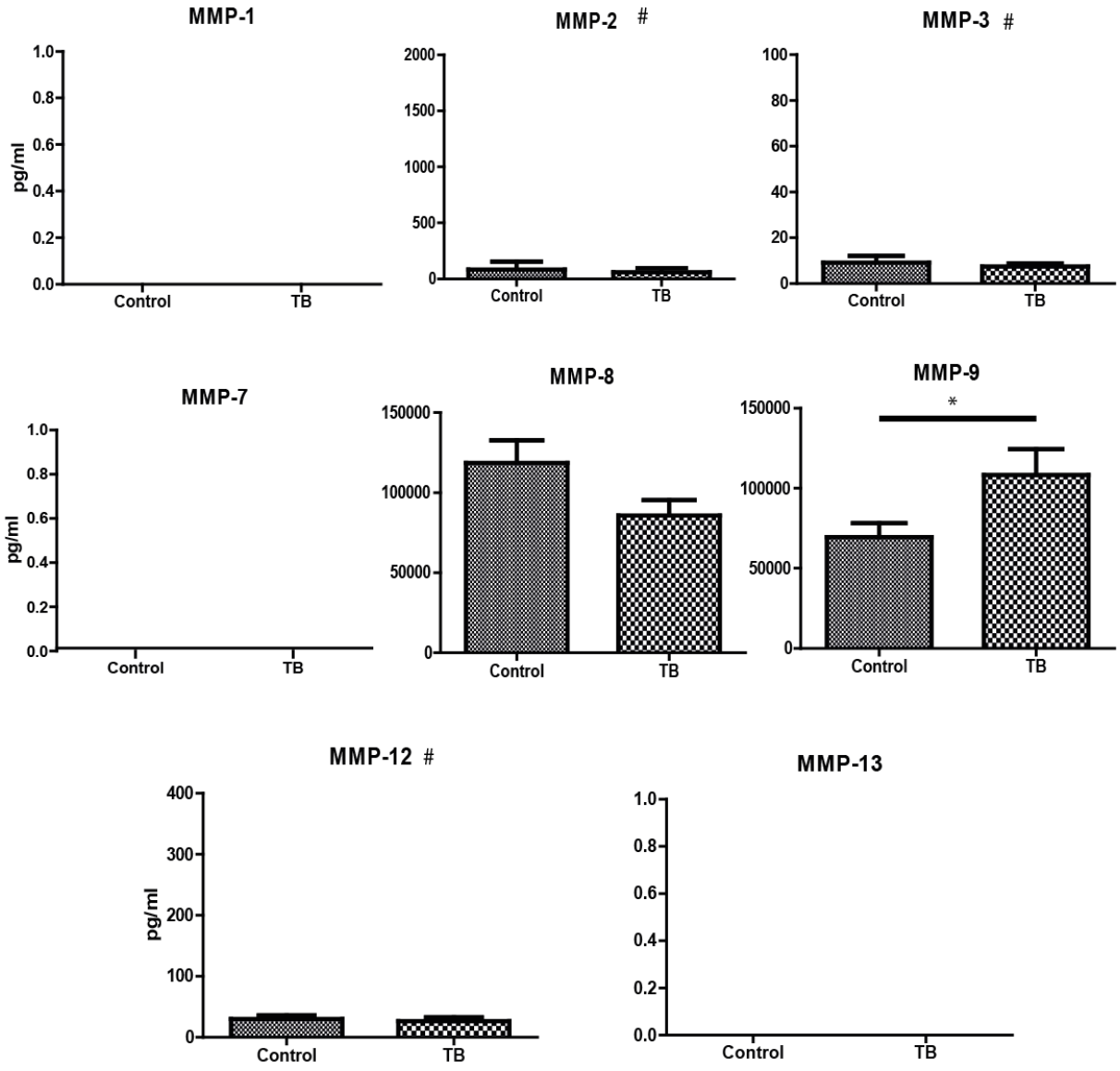


Figure 4: MMP secretion from M.tb-infected neutrophils. 1.25 x 10^6 neutrophils at 1500/μL were infected with an M.tb multiplicity of infection (MOI) of 1 for 16 hours. Bars represent mean +/- SD of an experiment done in triplicate. # values below standard curve, *$p<0.05$

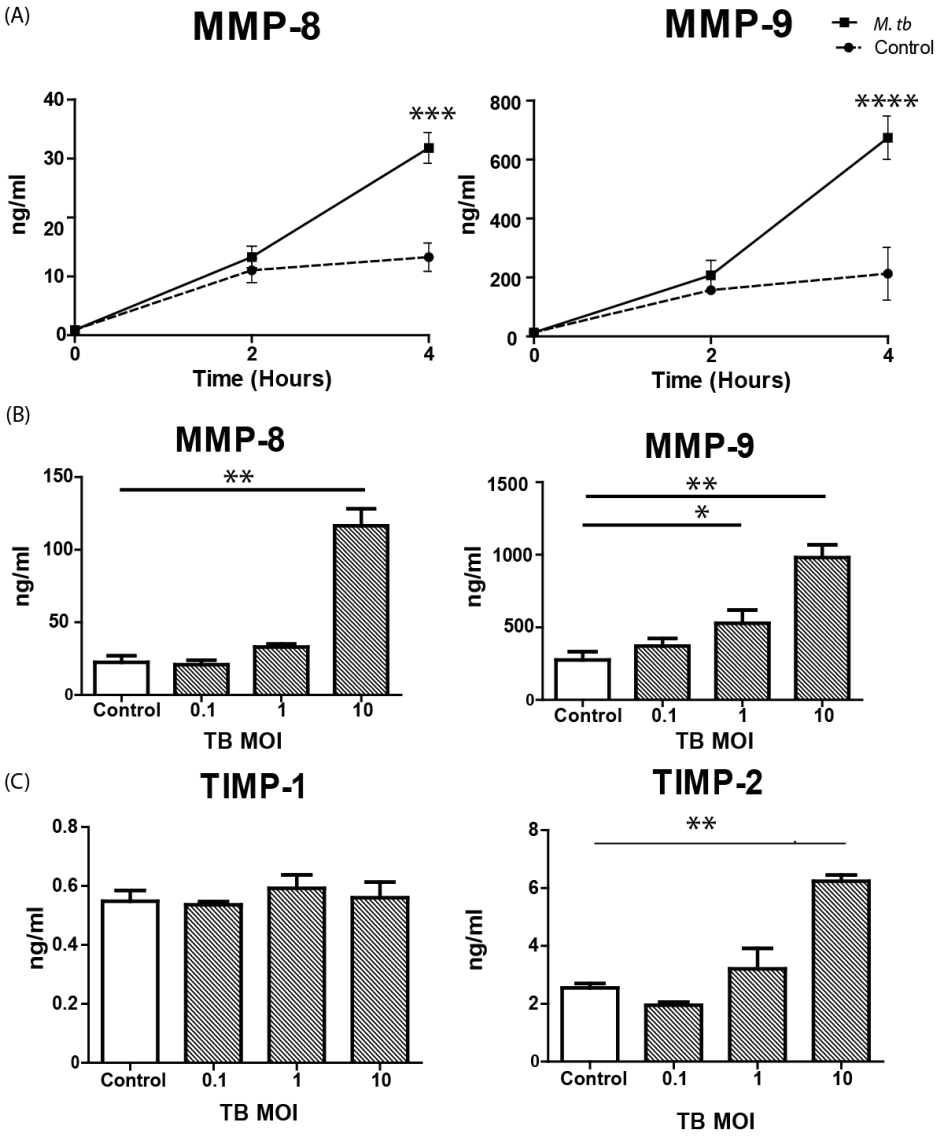


Figure 5: Effect of direct *M. tb* infection on MMP and TIMP secretion from neutrophils. (A) Secretion of MMP-8 and -9 from *M.tb*-infected neutrophils after 4 hours. 7.5 x 10^5 at 5000 / µL neutrophils were infected with *M.tb* at an MOI of 1. Bars represent mean +/- SD of an experiment done in triplicate and is representative of three separate experiments. (B) Dose-response secretion of MMP-8 and -9 from *M. tb* infected neutrophils after 4 hours. 7.5 x 10^5 neutrophils were infected with the stated dose of *M.tb*. Bars represent mean +/- SD of three samples and is representative of 2 separate experiments done in triplicate. (C) TIMP-1 and -2 secretion from *M.tb* infected neutrophils after 4 hours. Bars represent mean +/- SD from three samples from an experiment and is representative of 2 independent experiments. ****p<0.0001, *** p<0.001, **p<0.01, *p<0.05.

2.2 Live *M.tb* infected neutrophils secrete IL-6, IL-8 and MIP-1β

The effect of *M.tb*-infection and the consequential release of cytokines from neutrophils were examined using Luminex array. Using a 30-plex cytokine luminex array as a screening process, uninfected neutrophils and *M.tb*-infected neutrophils cytokine secretion were examined. *M.tb*-infected neutrophils contained significantly more IL-6, IL-8 and MIP-1β compared to controls ($p<0.001$, $p<0.01$ and $P<0.001$ respectively). All other cytokines (IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-7, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, GM-CSF, HGF, MCP-1, MIP-1α, IP-10, MIG, Eotaxin, RANTES, MCP-1, VEGF, G-CSF, EGF, FGF-basic, and HGF) were either below the standard curve or not secreted in significantly different amounts compared to controls (**Figure 6**).

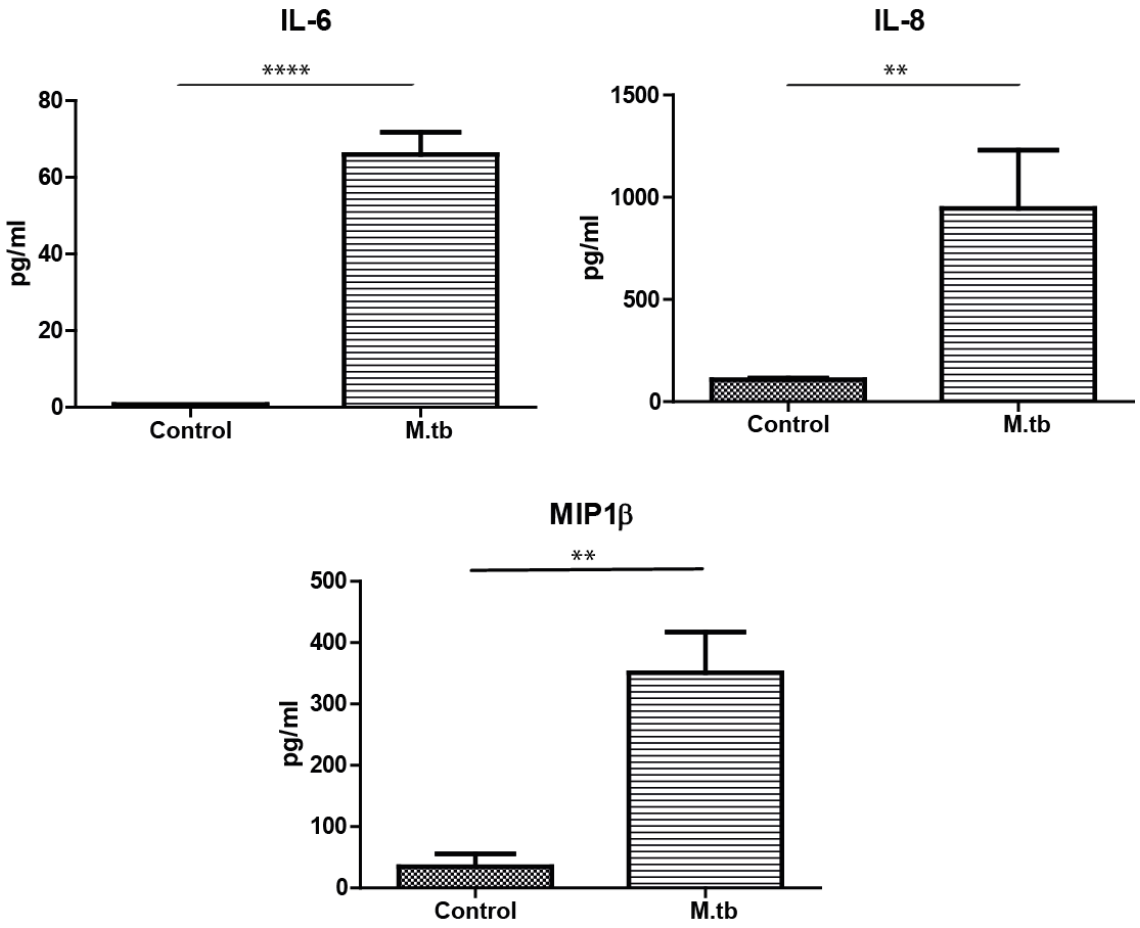


Figure 6: Cytokine secretion from *M.tb*-infected stimulated neutrophils. 7.5 x 10^5 neutrophils were stimulated with *M.tb* MOI of 1 for 4 hours. Bars represent mean +/- SD of an experiment done in triplicate. *p<0.05, **p<0.01, ***p<0.001.

2.3 CoMTB stimulated neutrophils secrete MMP-8 and -9

Having determined the pattern of MMP, TIMP and cytokine secretion from neutrophils infected with the whole live organism, I then set out to examine the role of monocyte-dependent networks on neutrophil function by stimulating them with conditioned media from *M.tb*-infected monocytes (CoMTB). Initial experiments demonstrated a dose–response effect from CoMTB stimulation after 6 hours (**Figure 7**). CoMTB at a dilution factor of 1:5 resulted in the greatest concentration of MMP-8 and -9 secretion from neutrophils and subsequently, a 1:5 dilution was used to stimulate neutrophils in all subsequent experiments.

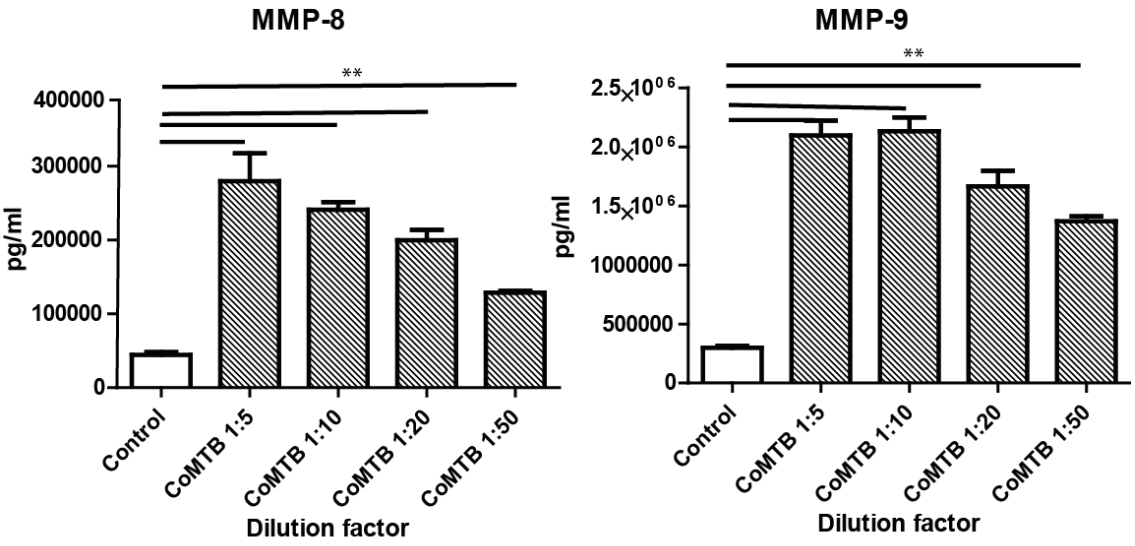


Figure 7: Effect of various dilution factors of CoMTB on neutrophils. 7.5 x 10^5 neutrophils were stimulated with the stated dilutions of CoMTB over 4 hours. Bars represent mean +/- SD of an experiment done in triplicate. **p <0.01 by one-way Anova.

CoMTB stimulation of neutrophils resulted in a 2-fold rise in MMP-8 secretion ($p<0.001$) and a 3-fold rise in MMP-9 secretion ($p<0.001$) compared to CoMCont (conditioned media from uninfected monocytes) (**Figure 8A**). This effect is not due to the sole presence of TB antigens, as stimulation with TB supernatant (TB supp) only caused a 1.5 fold increase in MMP-8 and an approximate 2-fold rise in MMP-9 concentration. Similar data were seen using gelatin zymography which detects potentially active forms of MMP-9 (**Figure 8B**). Next, the effect of CoMTB on neutrophil TIMP-1 and TIMP-2 secretion was examined. There was a 2.5 fold increase in TIMP-2 secretion from CoMTB stimulated neutrophils compared to CoMCont ($p<0.001$), but no significant increase in TIMP-1 secretion (**Figure 8C**). This concurs with the results seen when neutrophils were infected with live *M.tb* (section 2.1). As previously found, the concentration of MMPs secreted is sufficient to overwhelm TIMP activity bringing the milieu towards a matrix degrading phenotype.

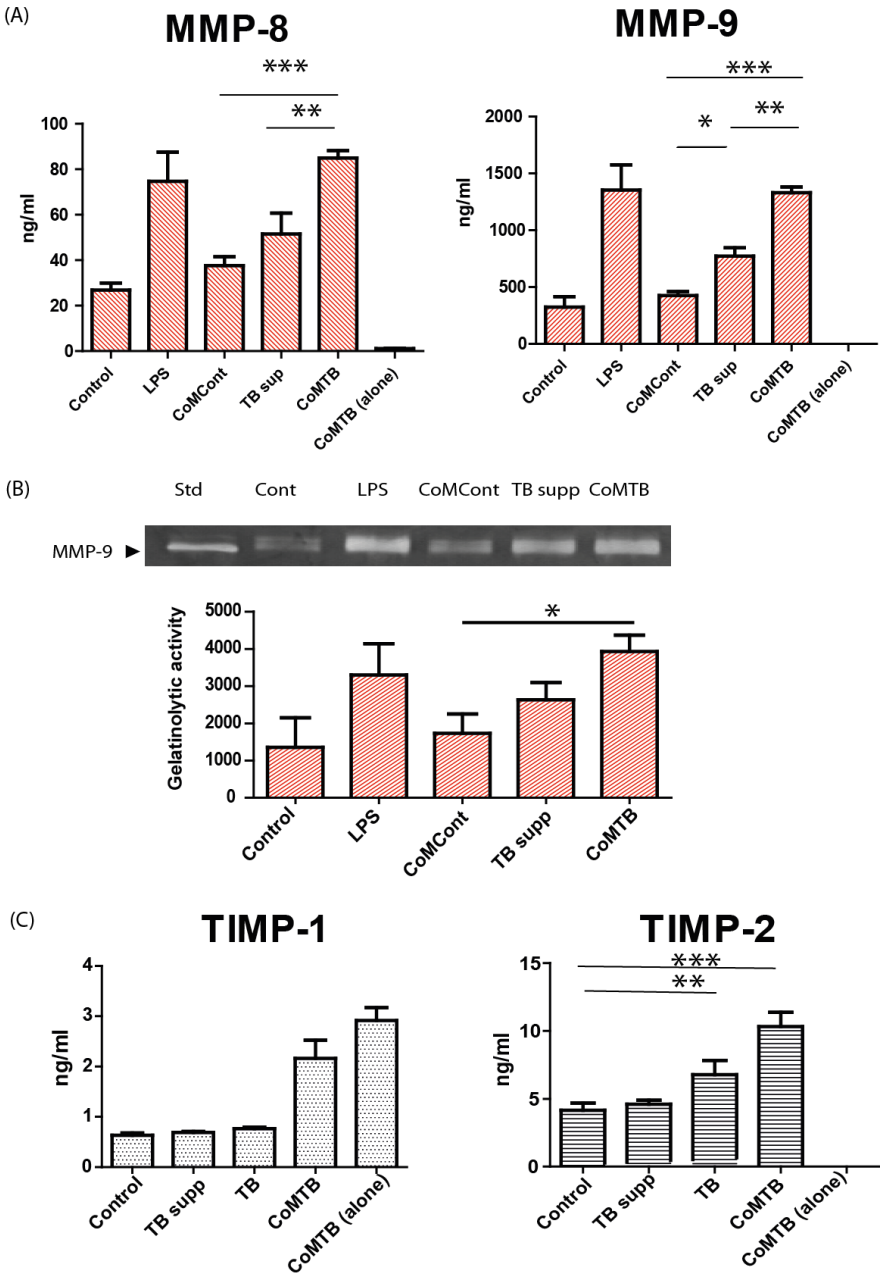


Figure 8: Effect of CoMTB and TB supernatant on neutrophil MMP and TIMP secretion. 7.5 x 10^5 neutrophils were stimulated over 4 hours with CoMCont and CoMTB both at a 1:5 dilution. (A) ELISA readout. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 3 separate experiments. *p<0.5, **p<0.01, ***p<0.001. (B) Gelatin zymography with corresponding densitometric analysis. Samples were diluted 1 in 20 before running the zymogram and is representative of 3 separate experiments. Bars represent mean +/- SD of an experiment done in triplicate. *p<0.05. (C) Secretion of TIMP-1 and 2. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 2 separate experiments. **p<0.01, ***p<0.001

2.4 CoMTB stimulated neutrophils show increased MMP-8 and -9 gene expression

I next investigated the gene expression of MMP-8 and -9 from 3 different donors. Real time-PCR analysis was performed using GAPDH as a medium copy number reference gene as it was more reliable than the high copy number ribosomal 18s RNA reference gene (data not shown). The initial experiments from 3 different donors demonstrated that there was 2.3 fold up-regulation of MMP-8 ($p<0.001$) and 3.1 fold up-regulation of MMP-9 at 4 hours ($p<0.01$) (**Figure 9A**). A literature search on MMP-8 and 9 revealed that gene expression experiments in neutrophils were normally reported at the 24 hour time point [224, 225] Hence, I repeated the experiment over a 24 hour period and determined neutrophil viability. This demonstrated that the gene expression of MMP-8 and 9 was increased to 2.0 fold and 1.9 fold respectively at 6 hours. At 24 hours, MMP-8 and 9 gene expression was increased to 3.1 fold ($p<0.05$) and 8.2 fold respectively ($p<0.01$) (**Figure 9B**). In addition, there was a corresponding increase in protein secretion of MMP-8 and 9 at six and twenty-four hours respectively ($p<0.01$) (**Figure 9C**). Annexin V and propidium iodide staining revealed that there was comparable neutrophil viability at 24 hours in both CoMCont and CoMTB-stimulated cells (**Figure 9D**). I further set out to ascertained if the total MMP-8 and -9 (i.e sum of extracellular and intracellular) increased over time with CoMTB stimulation. To do this, neutrophils were stimulated with CoMCont or CoMTB and at stated time points, neutrophil supernatants (extracellular MMPs) were harvested and cells lysed with RIPA buffer to determine intracellular MMPs. In CoMTB stimulated neutrophils, the majority of MMP-8 remained intracellular, whilst the majority of MMP-9 was secreted (extracellular) (**Figure 10A and B**). Total MMP-9 was increased

over time with CoMTB whilst the total MMP-8 seemed to be decreased with CoMTB stimulation although this was not statistically significant (**Figure 10C**).

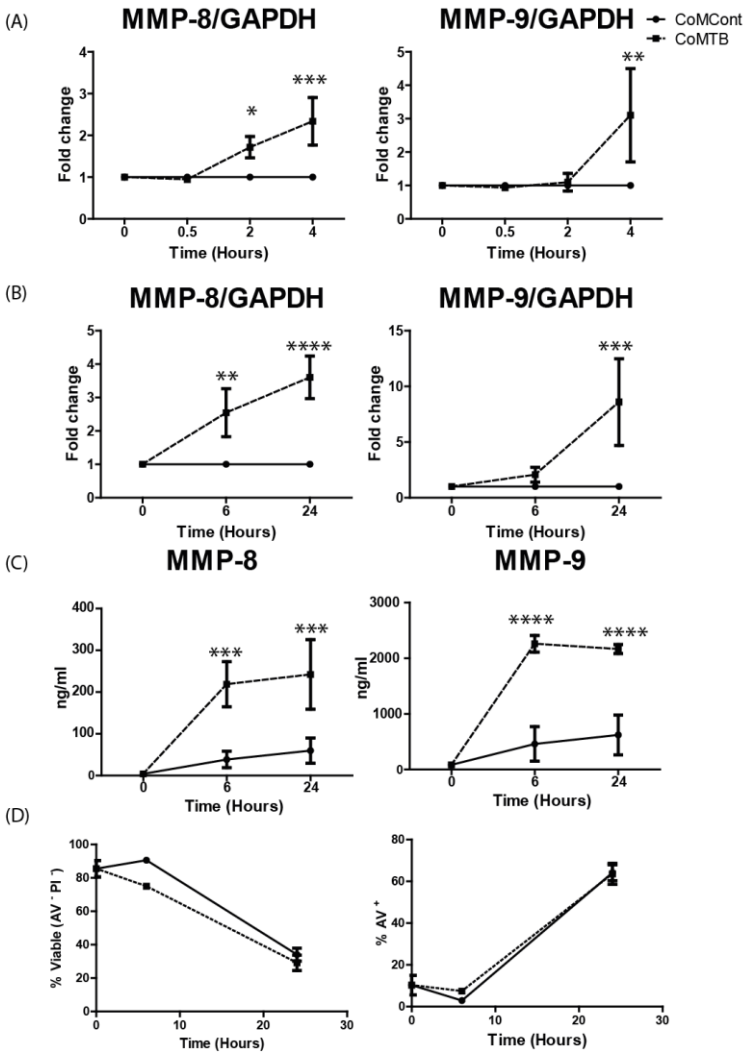


Figure 9: Kinetics of MMP-8 and -9 gene expression and secretion in CoMCont or CoMTB stimulated neutrophils. 2 x 10^6 neutrophils were stimulated with 1:5 dilution CoMCont or CoMTB for the specified duration. (A & B) Gene expression from 0-4 hours and 0-24 hours. Bars represent mean +/- SEM of 3 donors. (C) MMP-8 and -9 secretion from 0-24 hours. Bars represent mean +/- SEM of 3 donors which were used in (B). (D) Viable cells and proportion of apoptotic neutrophils over time. 5 x 10^5 neutrophils were stimulated with 1:5 dilution of CoMCont or CoMTB and stained with Annexin V and Propidium iodide. Bars represent mean +/- SEM of 3 donors. p=NS.*p<0.5, **p<0.01, ***p<0.001, ****p<0.0001 compared to CoMCont.

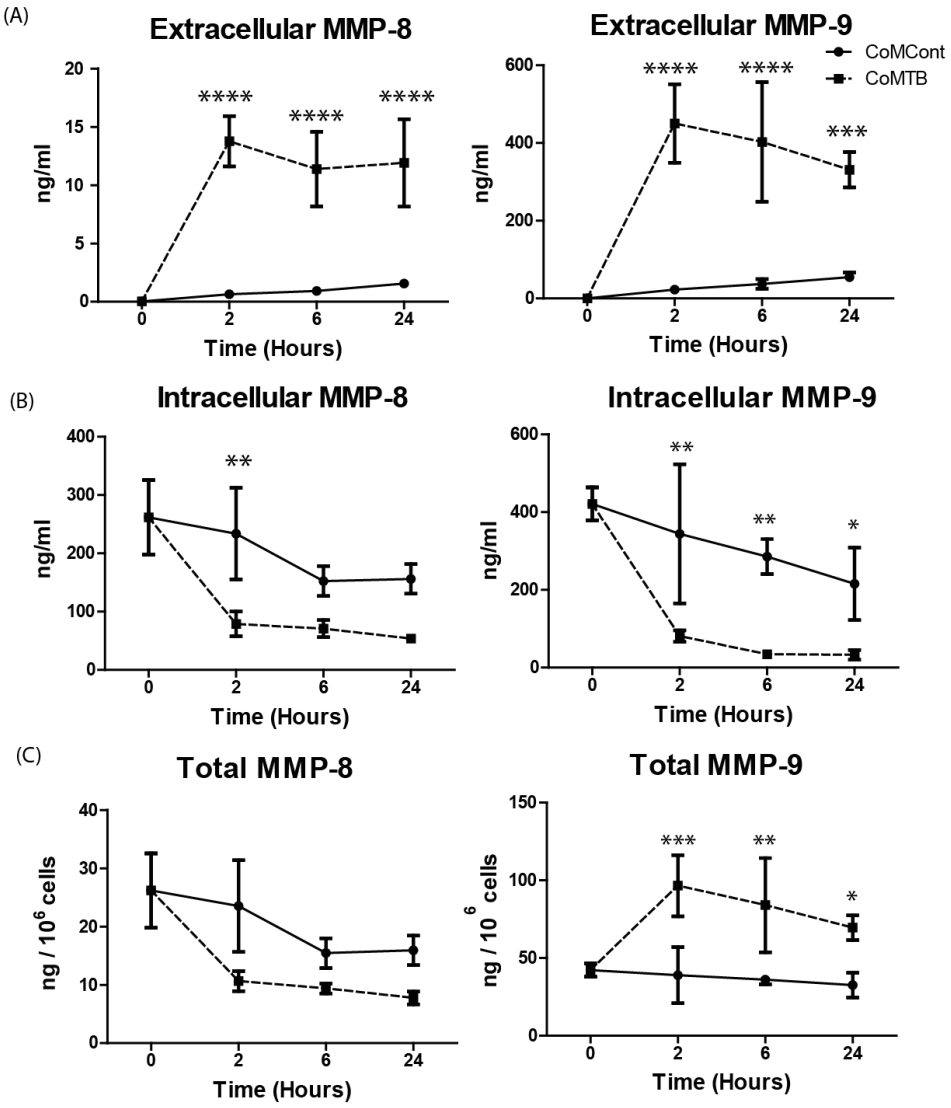


Figure 10: Kinetics of MMP-8/-9 from CoMTB stimulated neutrophils. (A) Extracellular MMP-8/-9 from 1.5 x 10^6 neutrophils (B) Intracellular MMP-8/-9. Neutrophils were lysed with 150µL of whole cell lysis buffer at the stated time points and lysate analysed with MMP-8/-9 using luminex array. (C) Total MMP-8/-9 / 10^6 neutrophils. The sum of extracellular MMPs and intracellular MMPs. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 3 separate experiments.

2.5 CoMTB-stimulated neutrophils show increased IL-8, IL-1RA, IL-12, TNF-α and MCP-1 protein secretion

The secretion of cytokines from CoMTB stimulated neutrophils was examined using a 30-plex cytokine luminex array. The cytokines concentrations present in CoMTB alone were taken as a baseline. What was most significant was that CoMTB stimulated neutrophils secreted significantly greater IL-8 concentrations (6.5 fold) compared to CoMTB (alone) ($p<0.0001$) (**Figure 11**). There was slightly higher IL-1RA ($p<0.05$), IL-12 ($p<0.05$), TNF-α ($p<0.05$), and MCP-1 concentrations ($p<0.001$) compared to CoMTB (alone). All other cytokines concentrations were below that of the standard curve or undetectable.

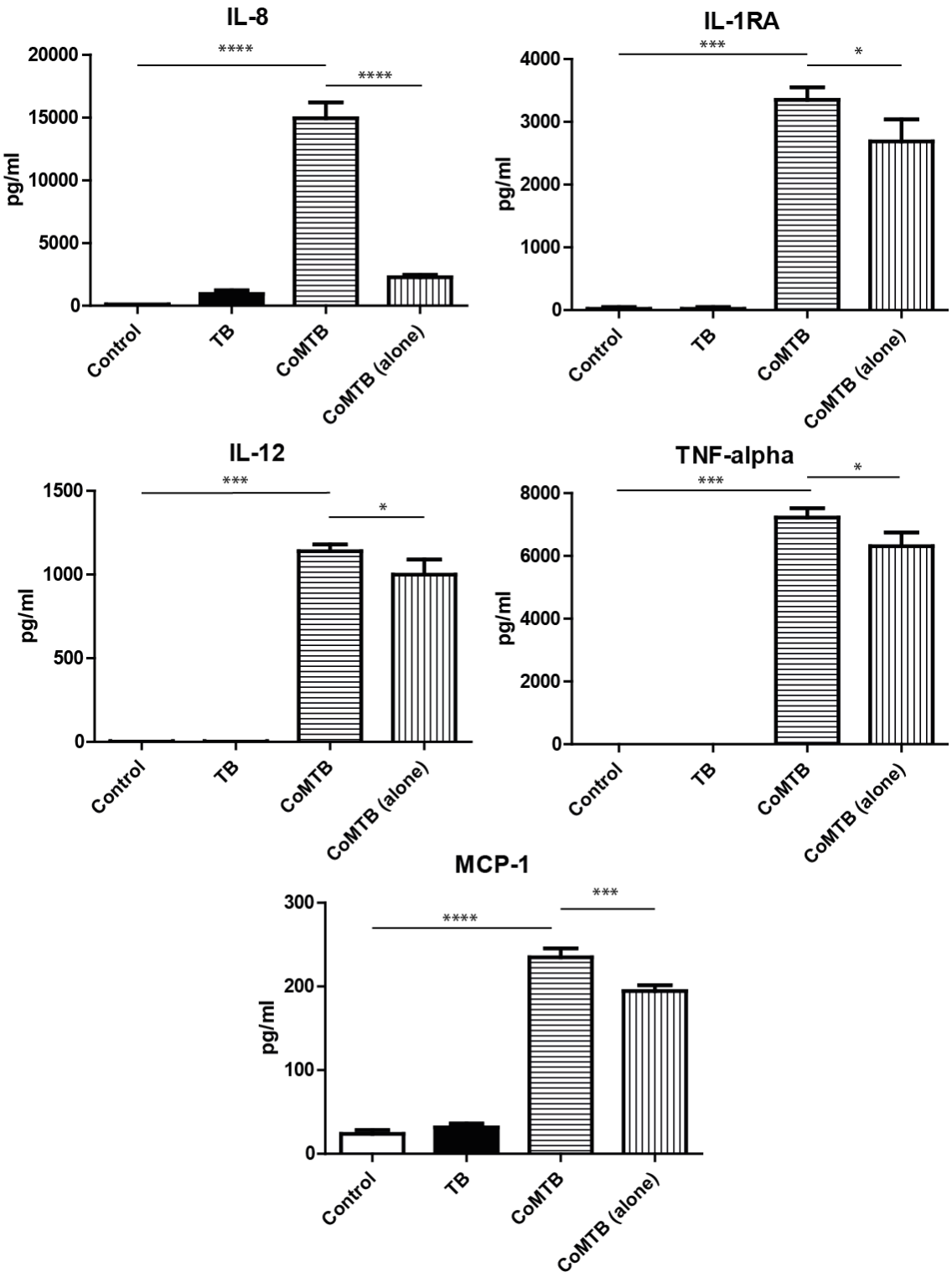


Figure 11: Cytokines secreted from CoMTB stimulated neutrophils. 7.5 x 10^5 neutrophils were infected with *M.tb* MOI of 1 or stimulated with CoMTB at a 1:5 dilution for 4 hours. Bars represent mean +/- SD of an experiment done in triplicate. *p<0.05, ***p<0.001, ****p<0.0001.

The cytokine luminex also determined the approximate amounts of chemokines and cytokines present in CoMTB at 1:5 dilution and this is listed in **Table 7.** Only cytokines and chemokines relevant to this project have been listed.

Cytokine/chemokine	**Concentration (pg/ml)**
TNF-α	6,300
IL-1β	4,300
IL-4	undetectable
IL-6	15,000
IL-8	2,200
IL-10	undetectable
IL-12	1,000
IL-13	65
MIP-1α	23,000
MIP-1β	5,400
MCP-1	194
IFN-γ	600
IL-1RA	2,700
GM-CSF	42

Table 7: Cytokines and chemokines present in CoMTB.

2.6 The effect of TB treatment immune modulators: Dexamethasone and anti-TNFα inhibit MMP-8/-9 secretion in CoMTB stimulated neutrophils

Dexamethasone is used in patients with CNS-TB as an immunomodulatory therapy. Occasionally even with dexamethasone, patients fail to improve and remain in a comatosed state. There are increasing reports that anti-TNFα therapy has improved patient outcomes in such scenarios [52]. I therefore set out to determine if dexamethasone and anti-TNFα would modulate neutrophil MMPs in this context. The dose of dexamethasone used was similar to that used in clinical trials of patients with CNS-TB [216], whilst that of anti-TNFα neutralising antibody was determined to be the amount required to neutralise TNFα in CoMTB. Dexamethasone inhibited MMP-8 and -9 secretion in *M.tb*-infected neutrophils and MMP-8 but not MMP-9 in CoMTB stimulated neutrophils (**Figure 12A and B**). With TNFα neutralisation of CoMTB, neutrophil MMP-8 and -9 secretion was suppressed in a dose-dependent manner ($p<0.001$ with MMP-8 and $p<0.01$ with MMP-9) with MMP-9 completely suppressed at a dose of 5μg/ml of antibody (**Figure 13A**). Gene expression of MMP-8 and -9 was similarly reduced with TNFα neutralisation ($p<0.01$ for MMP-8 and $p<0.001$ for MMP-9 both at anti-TNFα of 5μg/ml) (**Figure 13B**). TIMP-2 secretion is suppressed as well in a dose-dependent manner but TIMP-1 secretion was unaffected.

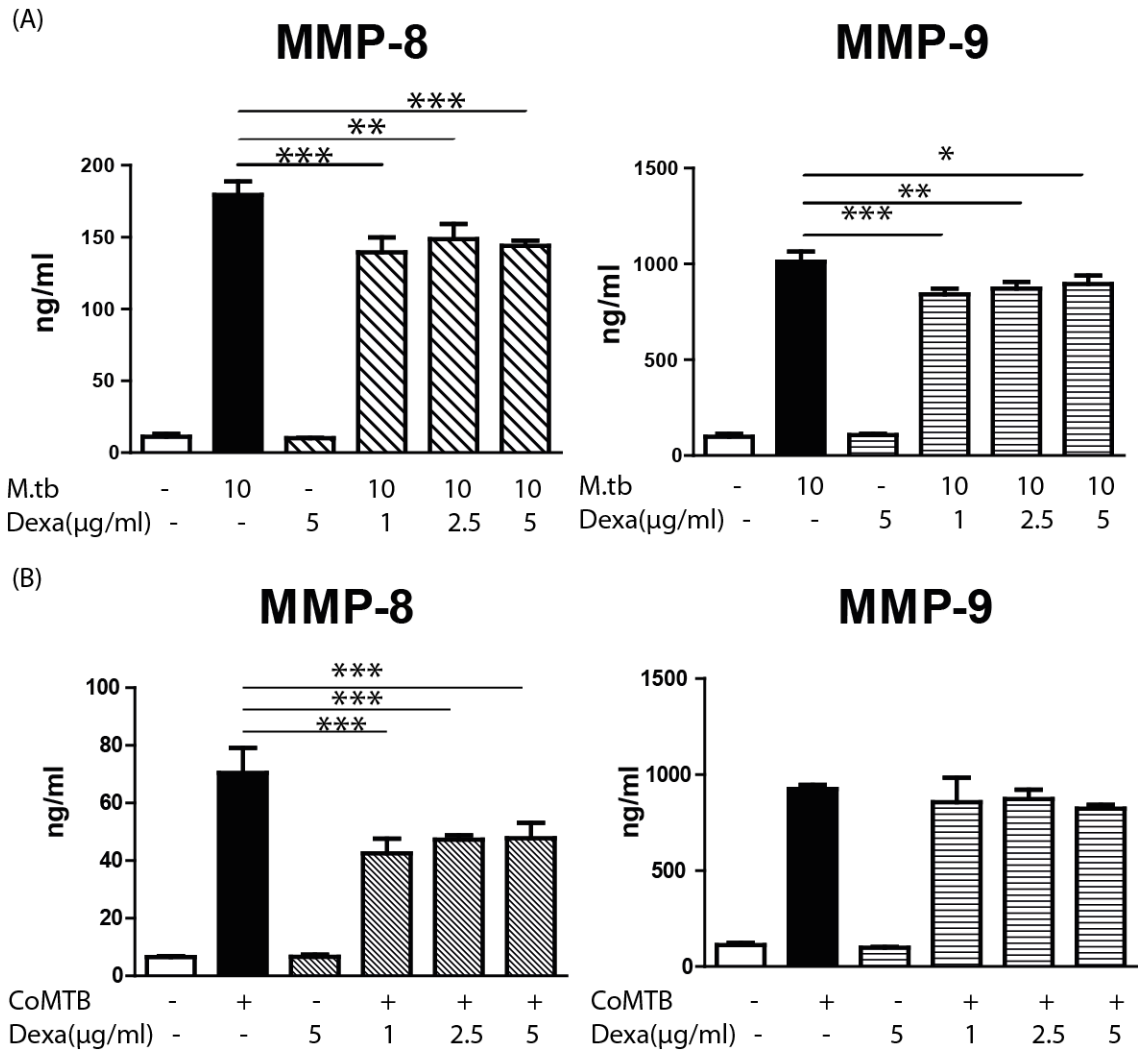


Figure 12: Dexamethasone inhibits neutrophil MMP secretion. 7.5 x 10^5 neutrophils were pre-incubated for 30 minutes with the specified concentrations of dexamethasone and subsequently stimulated. (A) *M.tb* infection. Neutrophils were infected with *M.tb* at an MOI of 10 for 4 hours. (B) CoMTB stimulation. Neutrophils were stimulated with CoMTB at 1:5 dilution for 4 hours. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 2 independent experiments. *p<0.05, **p<0.01, *** p<0.001.

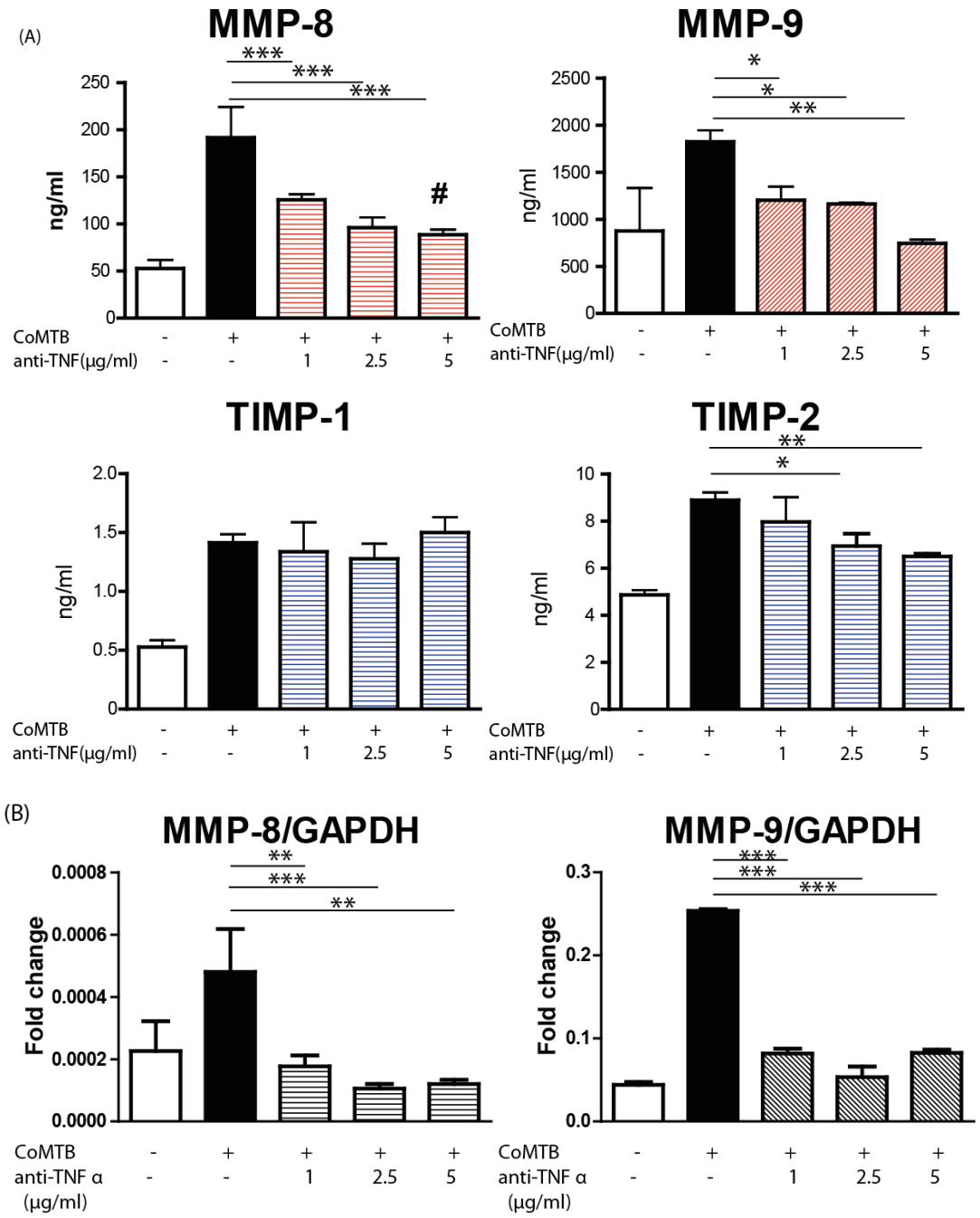


Figure 13: Anti-TNFα inhibits MMP-8/-9 secretion and gene expression in CoMTB stimulated neutrophils. CoMTB was pre-incubated for 2 hours with anti-TNFα and a 1:5 dilution was subsequently used to stimulate neutrophils for 4 hours. (A) MMP-8 /9 and TIMP-1/-2. (B) MMP-8/-9 gene expression. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 2 experiments. *p<0.05, **p<0.01, ***p<0.001, #p<0.05 compared to control media only.

2.7 The effect of host TB immune modulators: IL-4 inhibits neutrophil MMP-9 secretion at low concentrations in CoMTB-stimulated cells

IL-4 is the principle Th2 cytokine increased in TB patients compared to normal subjects [226]. In addition, studies in murine models have indicated that IL-4 presence in lung lesions coincided with pneumonia and necrosis [227]. IL-4 also suppresses MMP expression in human fibroblasts [228]. I thus sought to determine if IL-4 would augment neutrophil MMP secretion. Interestingly, at lower concentrations of IL-4, MMP-9 but not MMP-8 secretion is suppressed, while there seem to be an increase of MMP-9 with increasing concentrations of IL-4. Conversely, TIMP-1 but not TIMP-2 secretion was decreased with increasing IL-4 concentration (**Figure 14**). It has been previously reported that there is increased neutrophil phagocytosis with increasing IL-4 (15 ng/ml) with concurrent enhanced neutrophil chemotactic migration [229] and further assessment would be required to determine if IL-4 truly affects neutrophil degranulation.

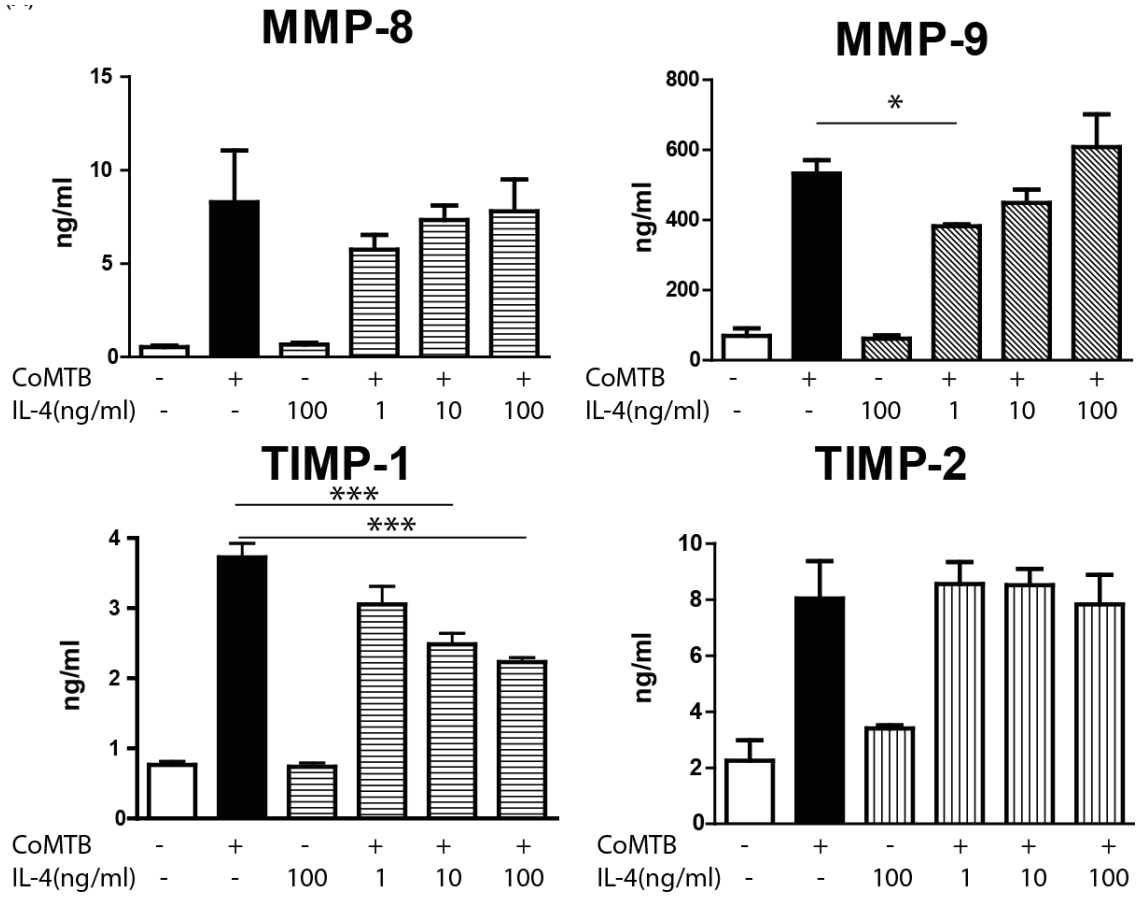


Figure 14: Effect of IL-4 on CoMTB stimulated neutrophils. 7.5 x 10^5 neutrophils were pre-incubated for 30 minutes with IL-4 prior to stimulation with CoMTB at 1:5 dilution for 4 hours. Bars represent mean+/- SD of an experiment done in triplicate. *p<0.05, ***p<0.001.

2.8 *M.tb* and CoMTB stimulated neutrophils degrade collagen

Tissue destruction is a predominant feature of TB, and the question is if neutrophils play a role. Type I DQ collagen which emits fluorescence when degraded was therefore used to investigate collagenase activity in an *in vitro* neutrophil model. When neutrophils plated onto collagen-coated coverslips were stimulated with CoMTB (**Figure 15**) or *M.tb* (**Figure 16**), there was a resultant increase in fluorescence of DQ collagen as detected by confocal microscopy. A similar increase in collagenase activity was demonstrated using a quantitative fluorescence assay in both *M.tb* and CoMTB stimulated neutrophils (**Figure 17A).** Collagenase activity was abolished with doxycyline, an MMP inhibitor (**Figure 17B**).

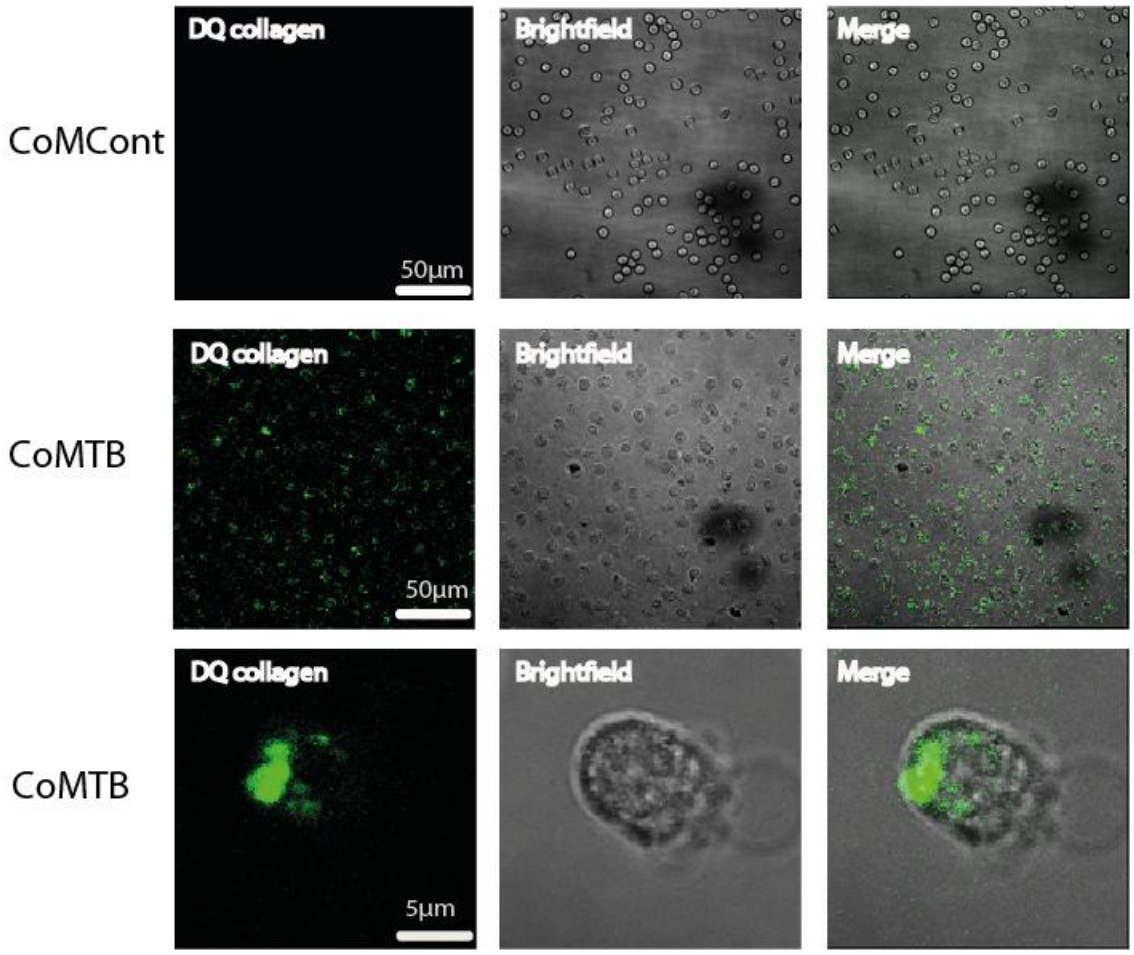


Figure 15: CoMTB stimulated neutrophils degrade Type 1 collagen. Coverslips were coated with 25 µg/ml DQ Collagen Type 1 for 1 hour prior to the addition of 5 x 10^5 neutrophils stimulated with the respective agents in a 1:5 dilution for 4 hours. Images representative of 2 independent experiments.

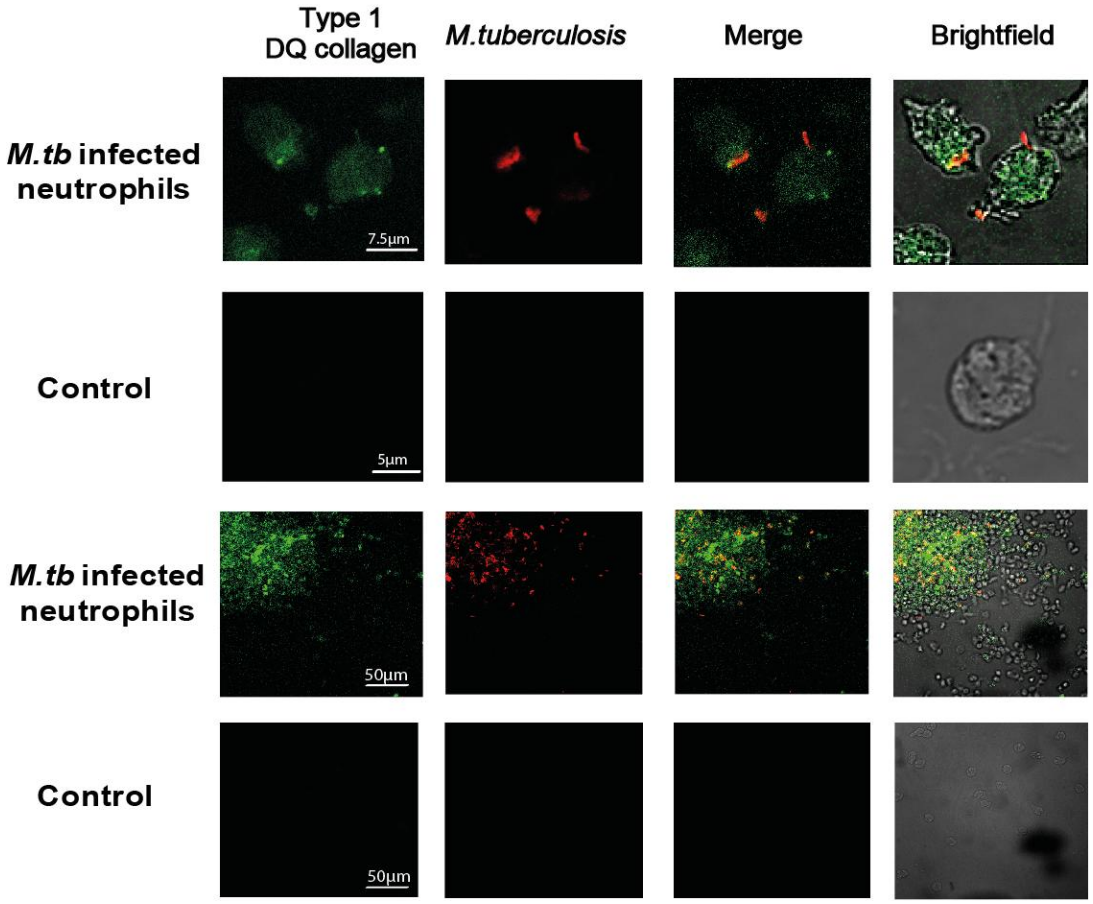


Figure 16: *M.tuberculosis* infected neutrophils degrade Type 1 collagen. Coverslips were coated with 25 µg/ml DQ Collagen Type 1 prior to the addition of 5 x 10^5 neutrophils infected with *M.tb* MOI 10 for 4 hours. Data representative of 2 independent experiments.

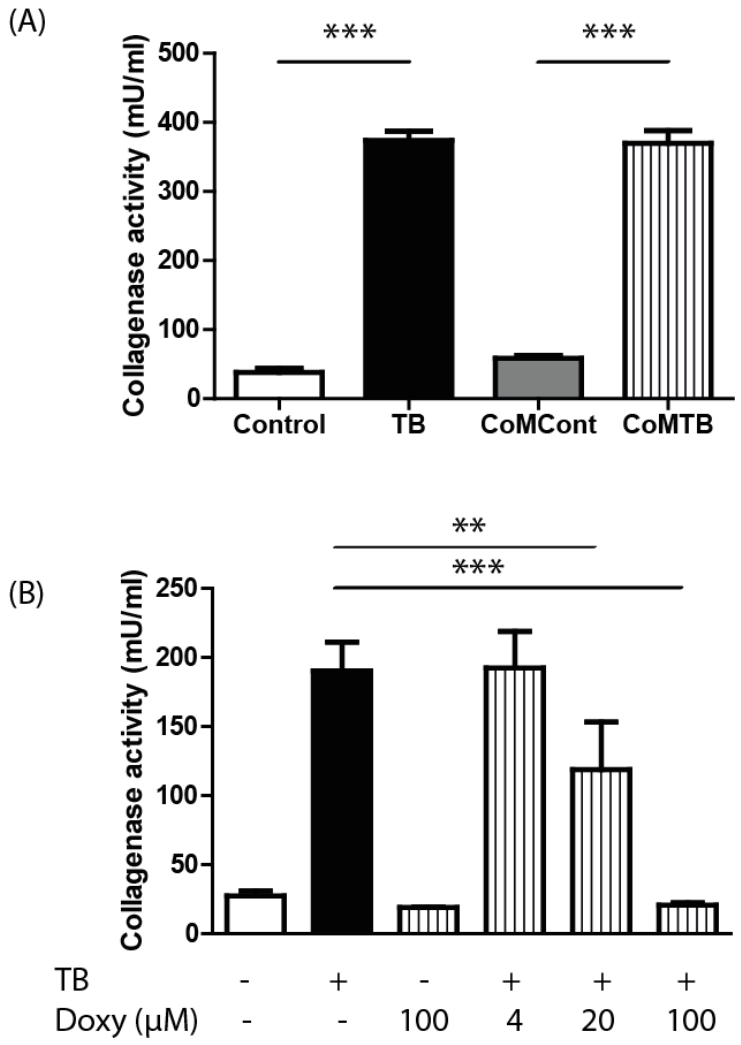


Figure 17: *M.tb* infected neutrophils and CoMTB stimulated neutrophils degrade Type 1 collagen. 7.5 x 10^5 neutrophils were infected with *M.tb* (MOI 10) or stimulated with CoMTB at 1:5 dilution for 4 hours and the supernatant was incubated with 25 μg/ml Type 1 DQ collagen for 24 hours. (A) Collagenase activity of supernatants from *M.tb* and CoMTB stimulated neutrophils. (B) Doxycyline (Doxy) inhibits collagenase activity from *M.tb*-infected neutrophils. Doxycyline and neutrophil supernatants were added concurrently into the assay without pre-incubation. Bars represent mean +/- SD of an experiment in triplicate and is representative of 2 independent experiments. **p<0.01, *** p<0.001.

2.9 Neutrophil extracellular traps are produced following M.tb infection

It had previously been reported that neutrophils produce NETS after exporsure to *M.tb* and I wanted to confirm this with my experimental set-up, using 20 nM of PMA as a positive control. NET production following treatment with *M.tb* was demonstrated by QuantiT Picogreen but minimal with CoMTB (**Figure 18A**). Immunofluorescence (**Figure 18B**) clearly showed the mycobacterium trapped by the DAPI-stained DNA of the NETs. In addition, the NETS were associated with MMP-8 and -9 (**Figure 18C and D**). Both PMA and *M.tb* induced sheet-like NETS which were digested by DNAse (**Figure 19**). I also wanted to determine if the proteins present in *M.tb* cell walls induced NETs rather than *M.tb* secreted antigens. To do this, I utilised UV-killed *M.tb* and this induced NETS as well (**Figure 20**), confirming that it was proteins present in the mycobacterium cell wall responsible for this phenomenon.

NETS are associated with various neutrophil granular proteins such as neutrophil elastase and myeloperoxidase. I wanted to assess if MMP-8/-9 are strongly associated with NETS, as early immunofluorescence studies had demonstrated the presence of these 2 MMPs To further confirm these observations, I attempted co-immunoprecipitation of MMP-8/-9 for NET associated histones (H2B). Initial co-immunopreciptation experiments were done on NETS produced by *M.tb*-infected neutrophils. However, I found that the process of sterile filterisation removed a considerable amount of NETs, notably genomic DNA (data not shown). I decided to optimise the protocol using PMA to stimulate the neutrophils to produce NETS and use these for co-immunoprecipitation studies prior to switching the stimulus to *M.tb*. However, co-immunoprecipitation with protein G Dynabeads using MMP-8/-9 antibody pull-down demonstrated non-specific binding of NET associated histone and H2B was

present even with IgG isotype control both on agarose gel (**Figure 21A**) and on Western blot (**Figure 21B**). I attempted to optimise co-immunoprecipitation by blocking the beads with 5% BSA, preclearing the supernatant with Dynabeads or IgG bound-Dynabeads for 10 minutes to overnight but each experimental attempt still yielded histone proteins with IgG isotype control pulldown. I also tried a different kit (Dynabeads Co-immunoprecipitation Kit, Invitrogen, Paisley, UK) but without any success. Co-immunoprecipitation was attempted over a period of 3 months and eventually it was abandoned.

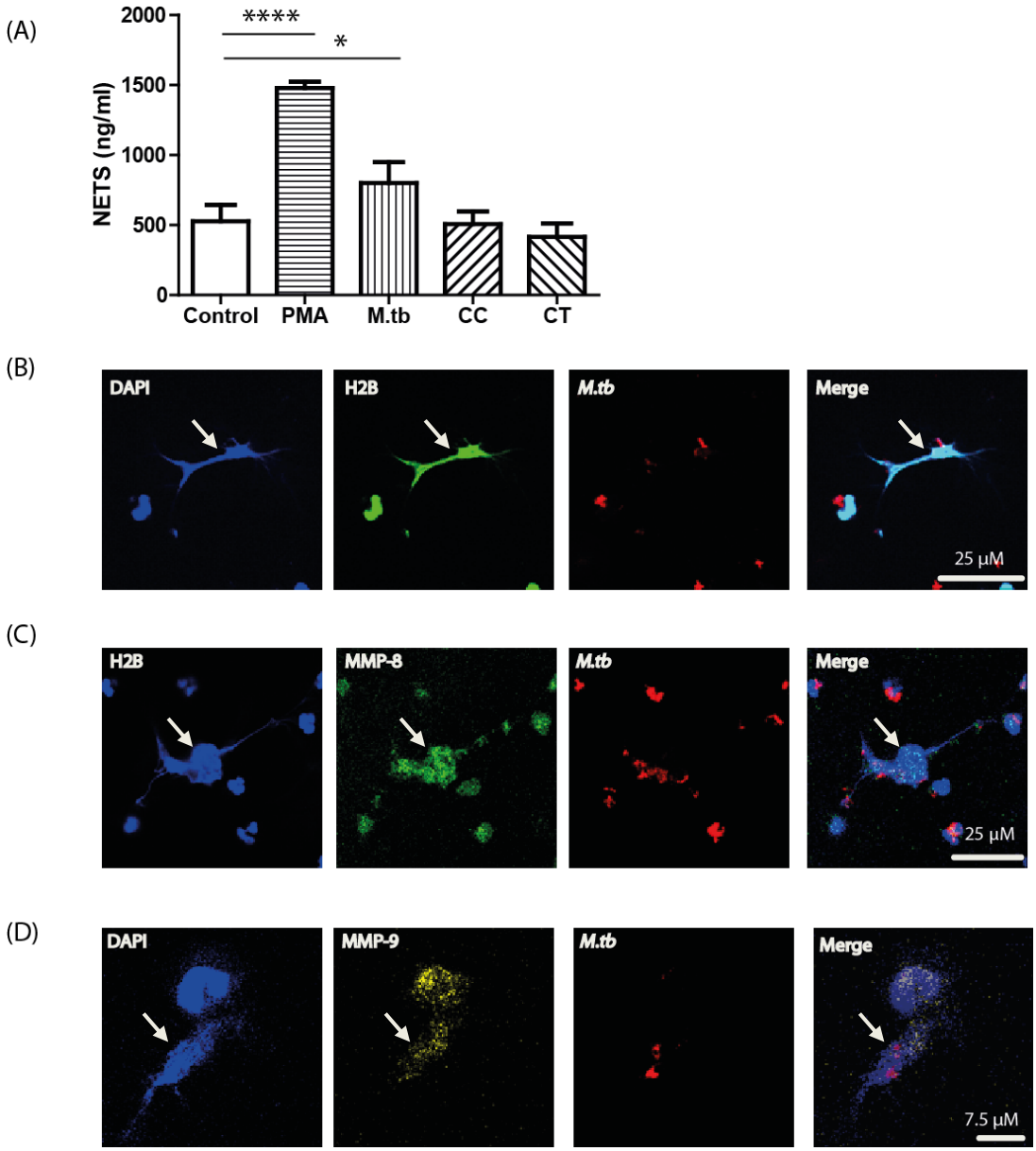


Figure 18: *M.tb* induces NET formation. (A) Quantification of NETS using PMA as a positive control. 5 x 10^5 neutrophils were stimulated with 20nM of PMA, *M.tb* (MOI of 10), CC (CoMCont) or CoMTB (CT) for 4 hours, digested with 5U/ml micrococcal nuclease, supernatants sterile filtered and analysed using Picogreen DNA quantification kit. Bars represent mean +/- SD of an experiment in triplicate and is representative of 3 independent experiments. *p<0.05, **** p<0.0001 (B-D) Immunofluorescence demonstrate association of MMP-8/-9 with NETS. 5 x 10^5 neutrophils were incubated with *M.tb* (MOI of 10) for 4 hours and were stained for DNA with DAPI, histone 2B (H2B), *M.tb*, MMP-8 and -9. White arrows highlight NETS. All immunoflurescence experiments were performed at least in 2 separate occasions.

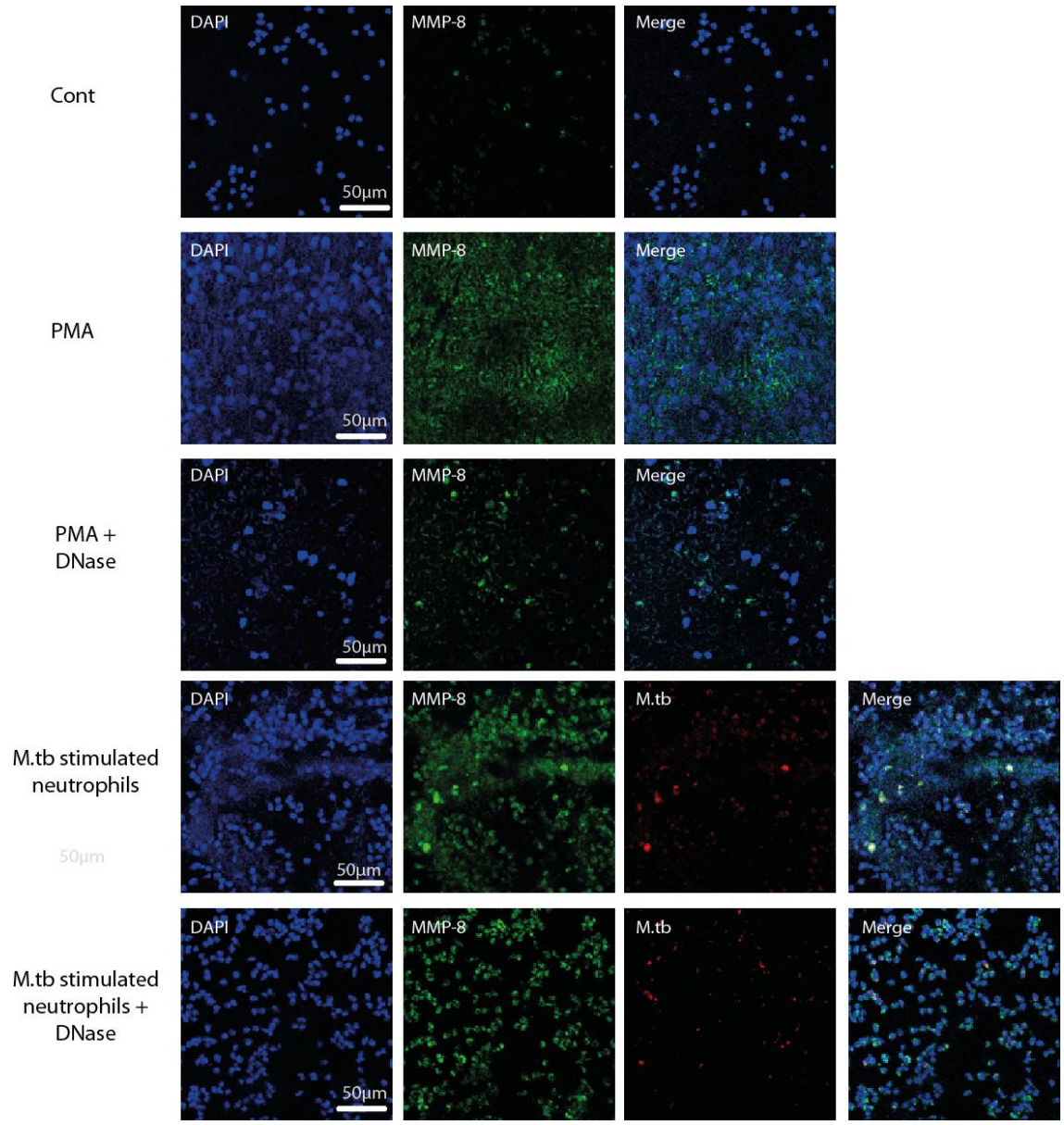


Figure 19: *M.tb* induces NET formation which is digested by DNAse. 5 x 10^5 neutrophils were stimulated with 20 nM of PMA or *M.tb* (MOI of 10) for 4 hours, and 10U/ml of DNAse or media subsequently were added for 20 minutes. Immunofluorescence experiments were done at least twice.

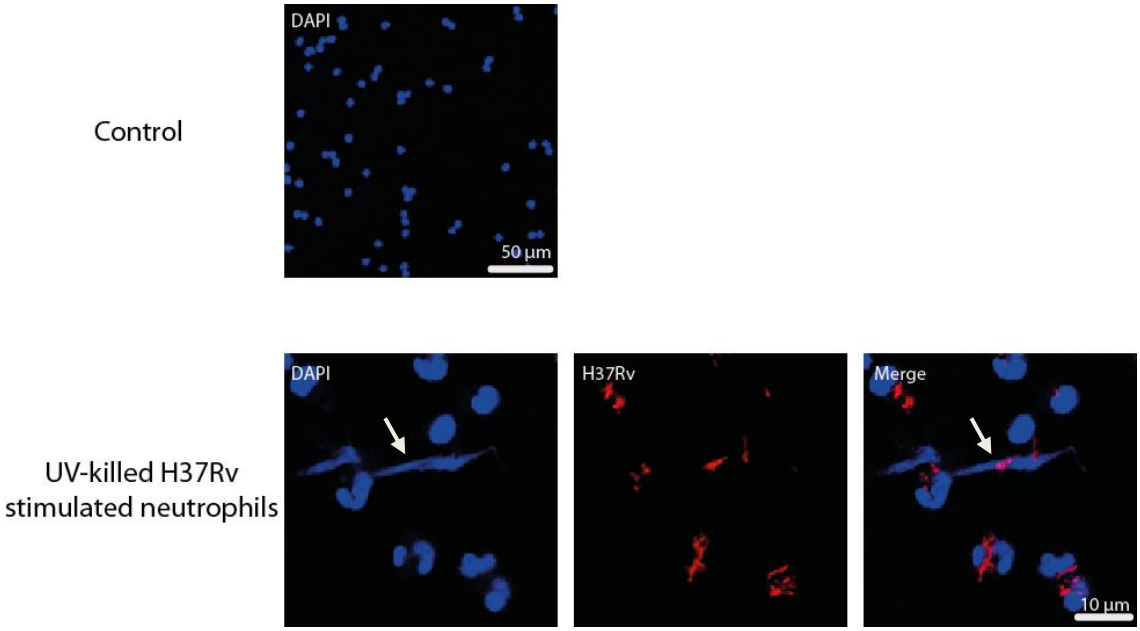


Figure 20: UV-killed *M.tb* induces NET production. H37Rv were subjected to UV exposure for 90 minutes. 5 x 10^5 neutrophils were stimulated with UV-killed *M.tb* (MOI 10) for 4 hours. Slides were stained for Auramine-rhodamine and DAPI. White arrows indicate NETs trapping *M.tb*.

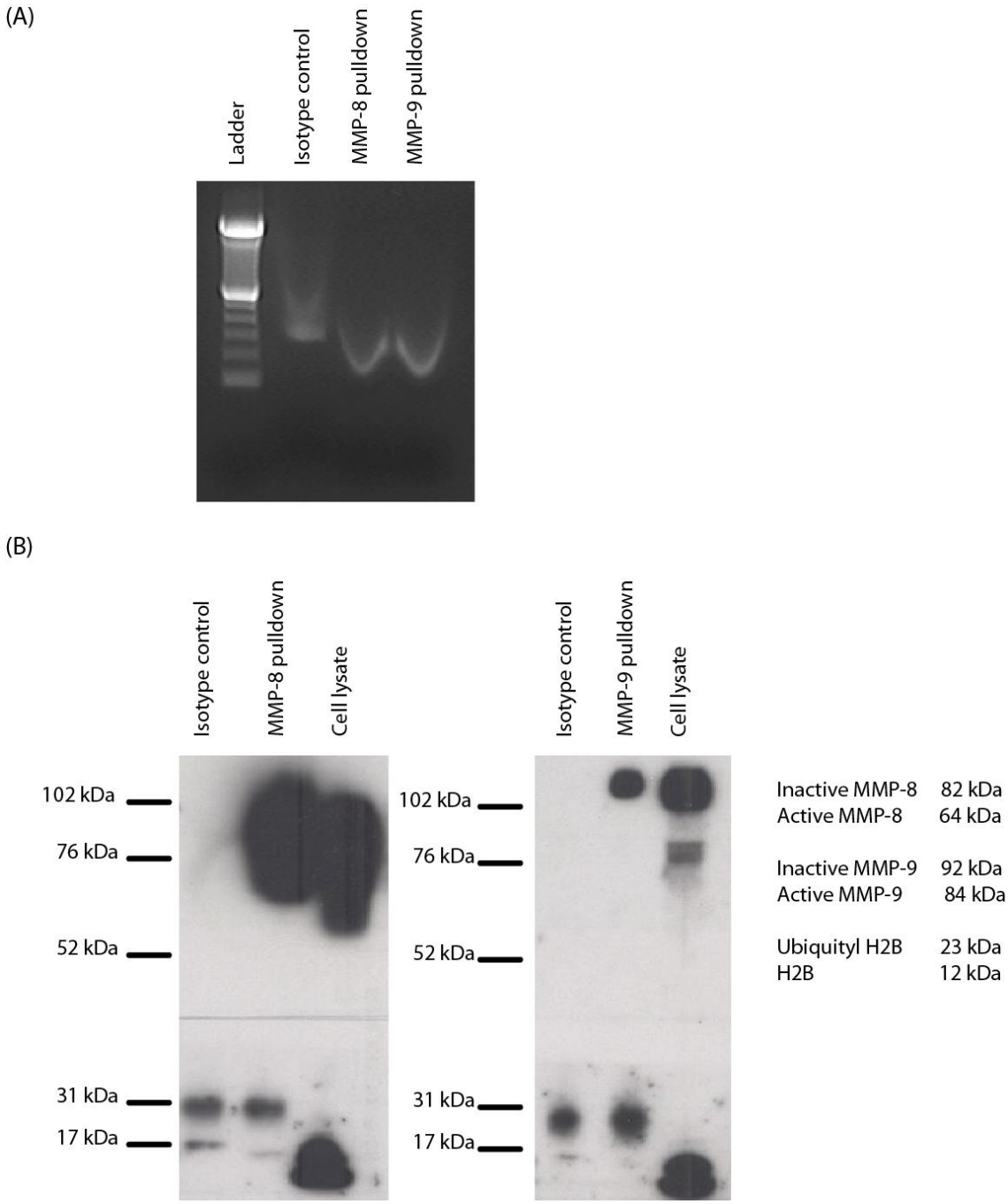


Figure 21: Co-immunoprecipitation of MMP-8/-9 for NETS. (A) 4 x 10^6 neutrophils were infected with *M.tb* (MOI 10) for 4 hours and supernatant sterile filtered. Co-immunoprecipitation was performed using Protein G Dynabeads using MMP-8 and MMP-9 antibodies. The eluted supernatant was ran on a 2% agarose gel. (B) 4 x 10^6 neutrophils were stimulated with 20 nM PMA for 4 hours. Co-immunoprecipitation was performed using Protein G Dynabeads using MMP-8 and -9 antibodies and eluted proteins were immunoblotted.

3. *Discussion*

Neutrophils secrete a wide range of enzymes and proteins in response to pathogens, including myeloperoxidase, neutrophil elastase, α-defensins, as well as MMPs. I have shown in my biological set-up that neutrophils secrete MMP-8 and -9 and demonstrated that *M.tb* induces a highly significant up-regulation of secretion of MMP-8/-9 from neutrophils in a dose-dependent mechanism and over time. This was not associated with an increase in TIMP-1, but there was a modest dose-dependent increase in TIMP-2. The phenomenon of MMPs from neutrophils being secreted in response to a stimulus is not unique to *M.tb*. It had been shown that LPS stimulated MMP-9 secretion from neutrophils *in vitro* and TNF-α, IL-8, and gram-negative and gram-positive infections similarly induced MMP-9 secretion from whole blood and this was likely to be derived from neutrophils [230]. Similarly, bacteria such as Actinobacillus species also induced neutrophil MMP-8 secretion [231]. I have found from my experiments that there is a high MMP to TIMP ratio from neutrophils and a shift in the balance towards degradation of the extracellular matrix, resulting in tissue destruction. This complements clinical findings, where MMP-9 concentrations are elevated and unrestricted by TIMP-1 in patients with TB meningitis [188] where disruption of the blood-brain-barrier occurs. Elevated MMP-9 in the cerebrospinal fluid is correlated with focal neurological damage and death [188, 213] and correlates significantly with the absolute neutrophil count in the CSF of patients with TB meningitis [215].

Neutrophils and monocytes are among the first cells to arrive when *M. tuberculosis* invades the host so as to phagocytose and eliminate the pathogen [121, 122]. It is likely that neutrophils and monocytes cross-prime each other during this

initial process. A key finding in my research is that conditioned media from *M.tb*-infected monocytes (CoMTB) upregulates MMP-8 and -9 protein secretion massively with an associated rise in gene expression in neutrophils. Additionally, there is further up-regulation in secretion of the chemokines IL-1RA, IL-8, IL-12, TNF-α, and MCP-1, all of which are key cytokines produced as a result of the host immune response in TB [232-236]. The most marked increase is seen with IL-8 secretion, and this is possibly due to MMP-9 cleaving IL-8 into a more active truncated form, instigating a feed-forward mechanism of IL-8 production.

One interesting observation was that with CoMTB stimulation, total MMP-8 seemed to decrease, albeit not statistically significant. This may be due to binding of MMP-8 to TIMP-2 and thus not detected by the luminex array, which only detects pro-MMP-8, mature MMP-8 and MMP-8 complexed to TIMP-1. Another possibility would be that the the active MMP-8 can also cleave itself when activated [237], and the smaller sized MMP-8 would also not be detected by the luminex array.

The cytokine multiplex studies performed on *M.tb*-infected neutrophils revealed that secretion of IL-6, IL-8 and MIP-1β are upregulated. IL-6 has been found to have a critical role in driving T-cell responses in mycobacterial infections [238], and IL-6 mRNA is significantly induced post-TB infection [122]. IL-8 is a well-known neutrophil attracting chemokine, and is present in the early host response to *M. tuberculosis* infection [141, 239]. It causes a feed-forward mechanism in neutrophils, with consequential release of more IL-8 and TNF-α [232]. MMP-9 processes IL-8, and results in a truncation variant of IL-8 with increased activity [162], while IL-8 stimulation of neutrophils in turn results in MMP-9 release [208]. In addition, MMP-9 can convert pro-TNFα to its active form [165]. However, it is surprising that minimal

amounts of TNF-α were found in *M.tb* infected neutrophils in my experiments, as it is well-documented that *M.tb* infection causes large amounts of TNF-α to be secreted from host neutrophils [240]. It is likely that there is a step-wise release of TNF-α from neutrophils after they are primed with IL-8 which has not been detected in the short time-frame of 4 hours in my experiments. MIP-1β induces the activation and proliferation of T-cells [241] and of macrophages [242]. MIP-1β from neutrophils also induces the transmigration of dendritic cells from the intravascular space into inflammatory tissues [243]. The secretion of IL-6, IL-8 and MIP-1β from *M.tb* infected neutrophils suggests that neutrophils play an important role in the early human response after infection with tuberculosis and help orchestrate the host innate and adaptive immunity.

In the treatment of TB, adjunctive therapies in the form of dexamethasone and anti-TNFα have been used. The former treatment has been used for CNS-TB and TB pericarditis and the latter for patients with CNS-TB refractory to dexamethasone [52, 53]. The mechanism as to how these two agents aid in treatment and improve patient outcome is not fully known. I have found that dexamethasone decreases neutrophil MMPs both in *M.tb* infection as well as in CoMTB stimulated neutrophils within 4 hours, which may lead to less tissue destruction and host inflammation. This supplements previous findings. In murine studies, glucocorticoids in the form of intraperitoneal dexamethasone decreased the amount of MMP-9 activity in the BAL fluid of mice given intranasal LPS and decreased neutrophilic inflammation [244]. In addition, glucocorticoids decreased the BAL MMP-9 concentration and gene expression in healthy controls but suppression was less pronounced in patients with severe asthma [245]. Steroids in the form of methylprednisolone and hydrocortisone also decreases

neutrophil degranulation [246]. It is likely that dexamethasone similarly suppresses neutrophil degranulation and therefore the release of neutrophil MMP-8 and -9. The use of anti-TNFα therapy in cases of CNS-TB refractory to dexamethasone has received much interest, since it is widely known that anti-TNFα therapy by itself is a risk to TB reactivation. Additionally, withdrawal of anti-TNF therapy is a recognised precipitating cause of TB paradoxical reaction in a case series [247]. One possible mechanism of how anti-TNFα therapy may reduce host inflammation in such cases is that anti-TNFα decreases neutrophil MMP secretion by blocking the stimulatory effect of TNFα. This effect may also occur in other cells and the consequential suppressed MMP secretion decreases tissue destruction which in turn improves host outcome in the context of CNS-TB.

The literature regarding IL-4 in TB is rather conflicting. It is the main Th2 cytokine increased in TB patients compared to normal subjects [226]. In addition, studies in murine models have indicated that IL-4 presence in lung lesions coincides with pneumonia and necrosis [227]. IL-4 also suppresses MMP expression in human fibroblasts [228]. I thus sought to determine if IL-4 would augment neutrophil MMP secretion. Interestingly, at lower concentrations of IL-4, MMP-9 but not MMP-8 secretion is suppressed, while there seem to be an increase of MMP-9 with increasing concentrations of IL-4. Conversely, TIMP-1 but not TIMP-2 secretion was decreased with increasing IL-4 concentration. This could be associated with the increased neutrophil phagocytosis and chemotaxis with IL-4 at higher concentrations [229].

Interstitial collagen fibrils are resistant to breakdown by most proteinases and only interstitial collagenases MMP-1, MMP-8 and MMP-13 can degrade the intact triple-helical collagen into one-quarter and three-quarter fragments. MMP-1 and -13

preferentially cleave type III and II collagen respectively [200]. MMP-8 cleaves Type I collagen more potently than MMP-1 [200, 201] and this suggests that MMP-8 (neutrophil collagenase) may be the main collagenase responsible for Type I collagen degradation. Neutrophils are a major source of MMP-8, but other sources include macrophages, endothelial cells, smooth muscle cells [248] and fibrocytes [249] among others. I have focused on the extracellular matrix Type I collagen as it is the most abundant collagen in the human lung [250, 251]. Collagen is the most abundant protein in the lung, comprising 20% of dry weight of the adult human lung [252, 253]. Type I collagen is distributed through the lung including the tracheobronchial tree, blood vessels and alveolar interstitium. When tissue destruction occurs in TB, the structural support that is provided by the extracellular matrix will be eroded and together with cell death this will lead to cavity formation and areas of necrosis. I have demonstrated that both *M.tb*-infected and CoMTB-stimulated neutrophils degrade Type I collagen and introduction of an MMP inhibitor, doxycyline abolished the collagenase activity from neutrophils. I have chosen doxycycline as this is the only licensed MMP inhibitor by the Food and Drug Administration. These observations support my hypothesis that neutrophil MMPs, particularly neutrophil MMP-8, are critical for tissue destruction.

NETs are produced when neutrophils encounter pathogens to assist in trapping and subsequent phagocytosis of pathogens. This phenomenon was first described by Brinkman et al [97] who showed that NETs also help kill bacteria such as *Shigella spp.* and *S.aureus* and digestion with DNAse abrogated the killing effect of NETS. NETS however do not have any *M.tb* killing effects [143]. NETS are comprised of many neutrophil granule proteins including myeloperoxidase, neutrophil elastase, cathepsin G as well as histone proteins (H1, H2A, H2B, H3, H4) as demonstrated by

immunofluorescence [97]. I have demonstrated that NETS are produced by neutrophils with *M.tb* infection in my system and this is compatible with previous finding [143]. I have shown that MMP-8 and -9 are associated with NETS by immunofluorescence. This association is likely to be non-specific because in immunoprecipitation-Western blot experiments, NET protein histone 2B is present even in the isotype control antibody pulldown. The presence of DNA bands when running both the isotype control pulldown and MMP-8 and -9 pulldown on agarose gels confirms the non-specific association despite my extensive attempts to optimise the immunoprecipitation technique. These ranged from preclearing the NET supernatant overnight, decreasing the amount of starting supernatant and using paraformaldehyde to cross-link MMPs to NETS. One possible explanation about this non-specific association is that NETS are inherently _sticky‘ so as to trap pathogens. Additionally, Protein G a component of the immunoprecipitation kit is derived from streptococcal species and NETS are naturally associated with Protein G when the two come into contact. Interestingly during my extensive literature search of immunoprecipitaion, I did not come across any reports describing immunoprecipitation of NET associated proteins. Most techniques to determine NET components including the first by Brinkman et al [97] utilised immunofluorescence suggesting that immunoprecipitation of NET components is technically challenging. One possible method to be used if this line of enquiry is to be further pursued will be mass spectrometry but the NET purification process needs to be stringent but not so overly harsh that it might destroy NETS itself.

In summary the MMP phenotype when neutrophils are infected with *M.tb* and stimulated with CoMTB have been described with MMP-8/-9 secretion vastly increased with minimal TIMP secretion, leading towards a proteolytic environment with

consequential tissue destruction as demonstrated by collagen degradation in DQ collagen assays. Dexamethasone, anti-TNFα, doxycycline and low-dose IL-4 conversely decreased MMP secretion. NETs are induced with *M.tb* infection, both with live H37Rv and UV-killed organisms but not by CoMTB stimulation. Interestingly, neutrophil MMP-8 and -9 seem to be associated with NETS in immunofluorescence studies but this has not been confirmed by immunoprecipitation.

CHAPTER 4 – EFFECT OF CROSSTALK OF M.TB INFECTED NEUTROPHILS ON OTHER CELLS AND ON *M.TUBERCULOSIS* GROWTH DYNAMICS

1. *Introduction*

In the previous chapter, I had described the MMP phenotype when neutrophils encounter *M.tb* both in direct infection and in monocyte-dependent networks. This chapter aims to address the effects of these encounters on other cells as well as on *M.tb* growth.

Neutrophils are among the first immune cells to arrive at the site of infection. They are professional phagocytic cells and also orchestrate the body‘s defence system. In addition to the production of NETS that was described in the previous chapter, they concurrently secrete a wide arsenal of antimicrobial agents such as reactive oxygen species, human neutrophil peptides 1-3, cathelicidin LL-37 and lipocalin-2 in the attempt to kill or restrict the growth of pathogens. Furthermore, they secrete chemokines and cytokines to set off a cascade of events following their recruitment to inflamed or infected tissues. Human neutrophils are a major source of cytokines such as BAFF [108] and APRIL [254] for the survival, maturation and differentiation of B cells. They may also engage in cellular crosstalk between macrophages [255], dendritic cells [256], natural killer cells [112], lymphocytes [115] and mesenchymal stem cells [257].

Given the considerable amount of cellular crosstalk that can potentially happen with neutrophils, I was interested if the conditioned media from *M.tb*-infected neutrophils (CoNTB) would modulate the MMP secretion from other cells. I looked

into the effects of CoNTB on monocytes, monocyte-derived macrophages and because of the neurological involvement of TB, several astrocytic cells lines, and the microglial cell line CHME3. CoNTB was made by infecting neutrophils with *M.tb* MOI of 1 as this is thought to represent a physiological state of infection outside the pulmonary cavity (eg. CNS) where the bacillary burden would be lower. I also investigated if conditioned media from *M.tb*-infected astrocytic cell line (CoATB) would affect neutrophil MMP secretion.

Since neutrophils secrete an armamentarium of antimicrobial peptides, I was interested if CoNTB and neutrophil MMP-8/-9 would restrict or inhibit *M.tb* growth. As it had been demonstrated that neutrophil cathelicidin LL-37 and lipocalin-2 restricted *M.tb* growth [126] and that these compounds are present in CoNTB, the hypothesis was that CoNTB would therefore modulate *M.tb* growth. In addition to using the conventional optical density method of quantifying *M.tb* growth in 7H9 liquid broth and quantifying colony forming units on 7H11 agar, I utilised luminescent *M.tb* H37Rv luxG13 [217] [258] and measured luminescence as a readout of *M.tb* growth.

2. *Results*

2.1 Effect of CoNTB on monocytes

As neutrophils and monocytes are among the first to arrive at the site of infection, the effect of neutrophil crosstalk with monocytes was investigated. This was done by stimulating peripheral monocytes with CoNTB. A Luminex array run at high sensitivity demonstrated that only MMP-7 was upregulated but this was not statistically significant (**Figure 22**).

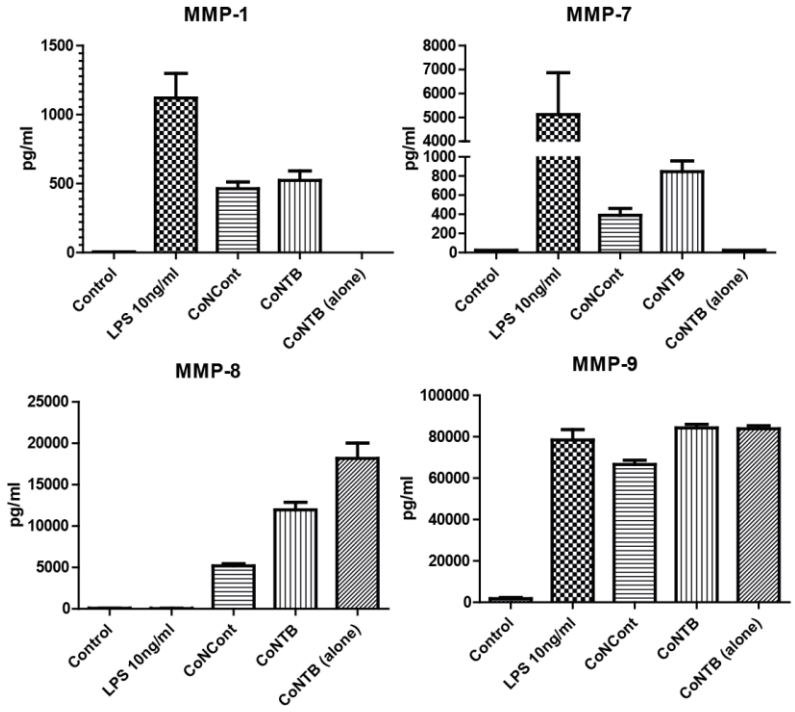


Figure 22: Effect of CoNTB on monocytes. CoNTB and CoNCont was made by stimulating 7.5 x 10^5 neutrophils with *M.tb* at MOI of 1 or the equivalent volume of PBS for 4 hours and sterile filtered. 5 x 10^5 monocytes were stimulated with CoNCont or CoNTB at a 1:5 dilution for 72 hours with 10 ng/ml of LPS as a positive control. MMP-2, -3, -12, and -13 concentrations were undetectable. Bars represent mean +/- SD of an experiment done in triplicate. p=NS for data.

2.2 Effect of CoNTB on monocyte-derived macrophages

Macrophages are known to play key roles in TB pathogenesis. I next investigated neutrophil interaction with monocyte derived macrophage (MDMs) by using CoNTB to stimulate them. There was no significant upregulation of any MMPs from MDMs. (**Figure 23**).

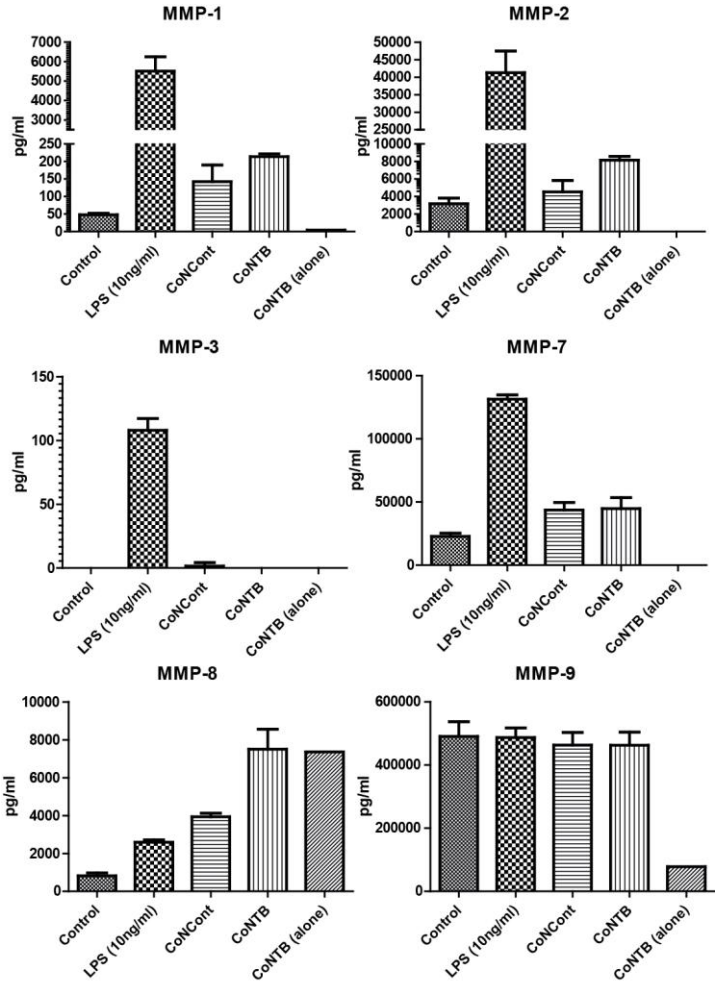


Figure 23: Effect of CoNTB on MDMs. CoNTB and CoNCont was made by stimulating 7.5 x 10^5 neutrophils with *M.tb* at MOI of 1 or the equivalent volume of PBS for 4 hours and sterile filtered. 5 x 10^5 MDMs were stimulated with CoNCont or CoNTB at a 1:5 dilution for 72 hours with 10 ng/ml of LPS as a positive control. MMP-12 and -13 concentrations were undetectable. Bars represent mean +/- SD of an experiment done in triplicate. p=NS for data.

2.3 Effect of CoNTB on astrocytes

To further investigate neutrophil networking effects, astrocyte cell lines were examined. These cells are part of the blood-brain-barrier and are affected in CNS-TB [189, 259]. Since neutrophils counts are raised in CNS-TB [215] I examined whether astrocytes would be affected by neutrophil-dependent networks. I looked at stimulating the astrocyte cell lines U87MG, U373MG Uppsala and U251MG (from the Health Protection Agency Culture Collections) with CoNTB. Experiments showed that most of the MMP-9 detected originated from the CoNTB only control (**Figure 24A**) with MMP-9 secretion unaffected when astrocytes were stimulated with CoNTB. MMP-3 secretion from astrocytes was also minimal. The cells however appeared stressed (data not shown). The experiment was repeated using CoNTB filtered through a 0.2 μM Anopore filter which decreased MMPs in the filtrate [219] (**Figure 24B**) in the hope of decreasing the MMP background but there was no significant increase seen in MMP-9 secretion from astrocytes. In addition, PMA and CoMTB, both proven to be positive controls of MMP-9 secretion from astrocytes, repeatedly produced minimal MMP-9 secretion on gelatin zymograms and ELISAs (data not shown) from all the astrocyte cell lines. More than 5 experiments were repeated with similar results. Hence, the investigation of CoNTB on astrocytes was halted.

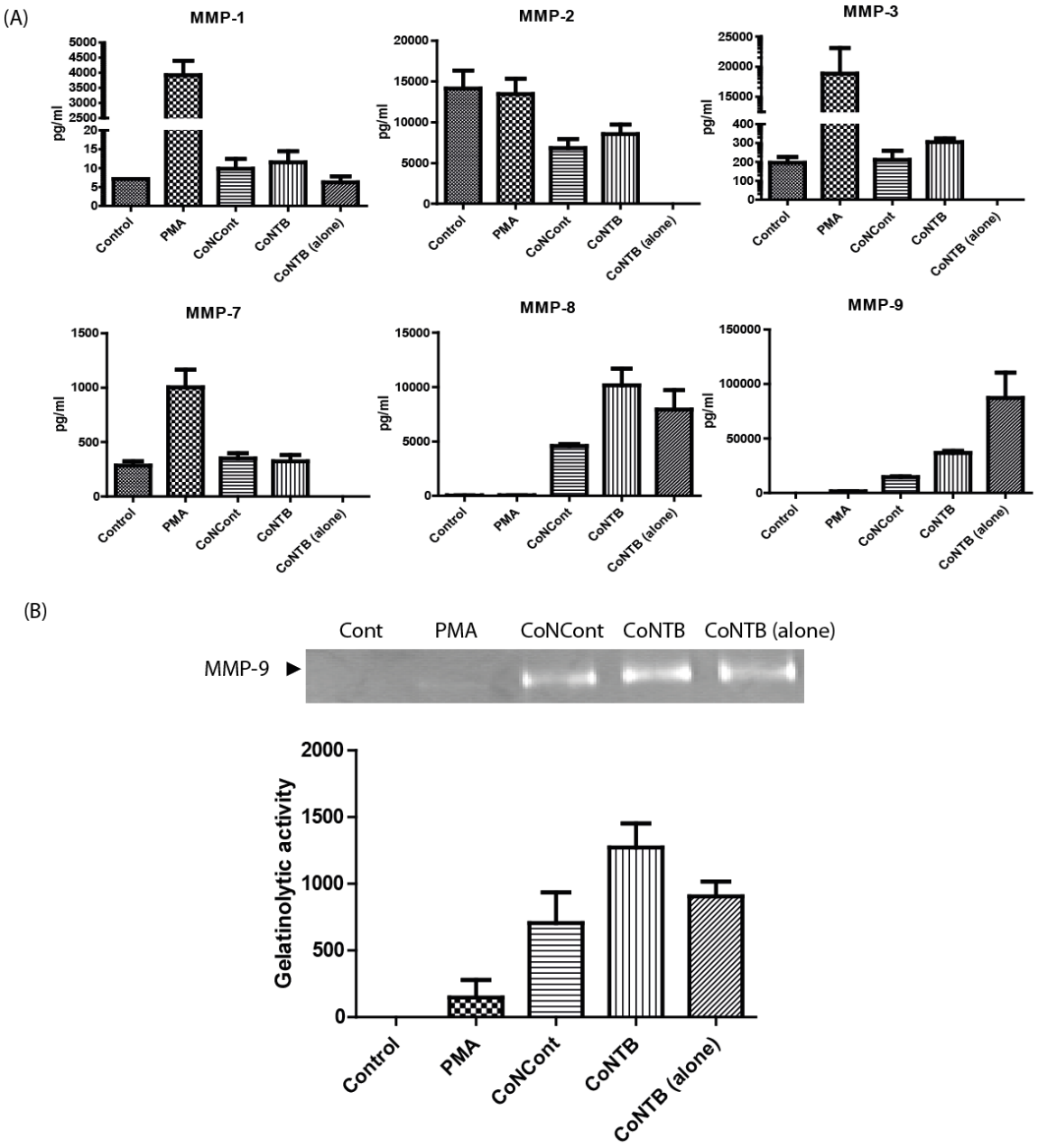


Figure 24: Effect of CoNTB on U251MG astrocytes. CoNTB and CoNCont were made by stimulating 7.5 x 10^5 neutrophils with *M.tb* at MOI of 1 or the equivalent volume of PBS for 4 hours and sterile filtered. Astrocytes were stimulated with CoNCont or CoNTB at a 1:5 dilution for 72 hours with 100ng/ml of PMA as a positive control. (A) Luminex readout. MMP-12 and -13 concentrations were undetectable. Bars represent mean +/- SD of an experiment done in triplicate. p=NS for data. (B) Gelatin zymography and corresponding densitometric analysis. Bars represent mean +/- SD of an experiment done in triplicate. p=NS for data. Similar results were obtained for U373MG Uppsala and U87MG astrocytic cell lines.

2.4 Effect of CoNTB on CHME3 microglial cell line

As microglial are resident macrophages in the CNS, I used the microglial cell line CHME3 to ascertain the effects of neutrophil networking. A Luminex run did not reveal any significant changes in MMP secretion from CHME3 (**Figure 25**).

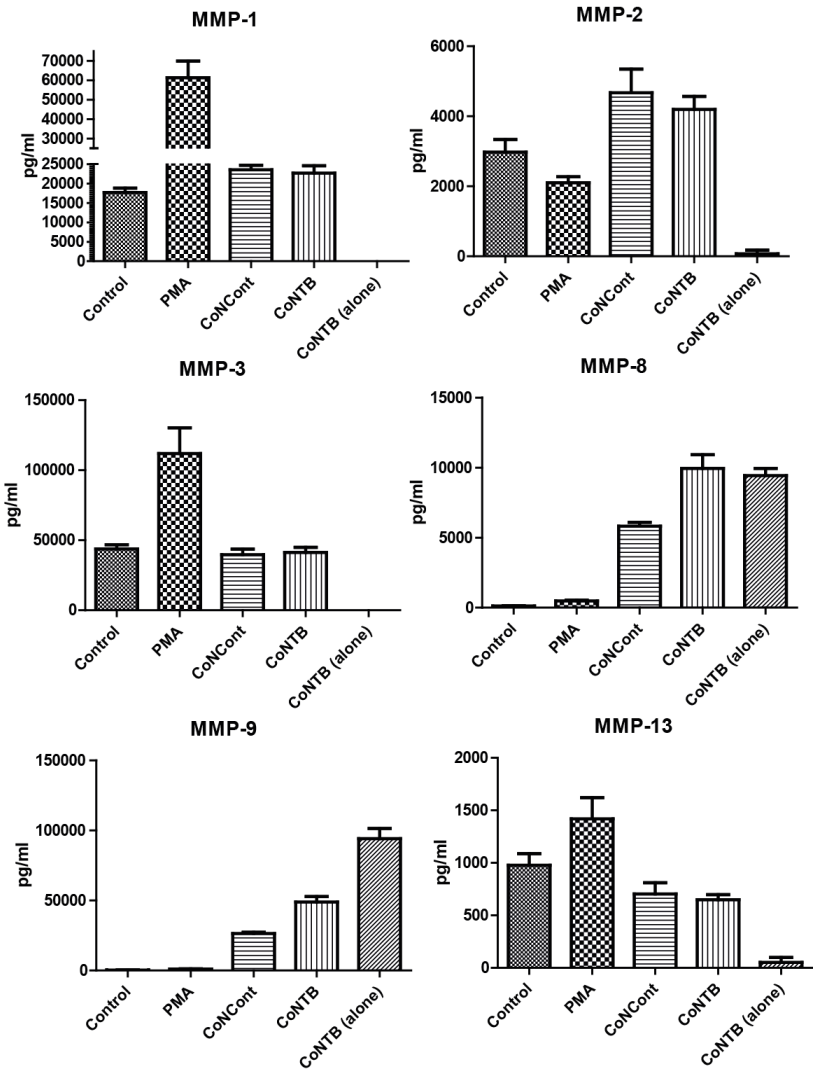


Figure 25: Effect of CoNTB on CHME3 microglial cells. CoNTB and CoNCont was made by stimulating 7.5 x 10^5neutrophils with *M.tb* at MOI of 1 or the equivalent volume of PBS for 4 hours and sterile filtered. CHME3 cells were stimulated with CoNTB at 1:5 dilution for 72 hours with 100 ng/ml of PMA as a positive control. MMP-7 and -12 concentrations were undetectable. Bars represent mean +/- SD of an experiment done in triplicate. p=NS for data.

2.5 Effect of CoATB on neutrophils

The network effects of *M.tb*-infected astrocytes on neutrophils were investigated. However, the experiment showed that there was no increase in MMP-8 and -9 from neutrophils stimulated with Conditioned media from *M.tb*-infected astrocyte (CoATB) (**Figure 26**).

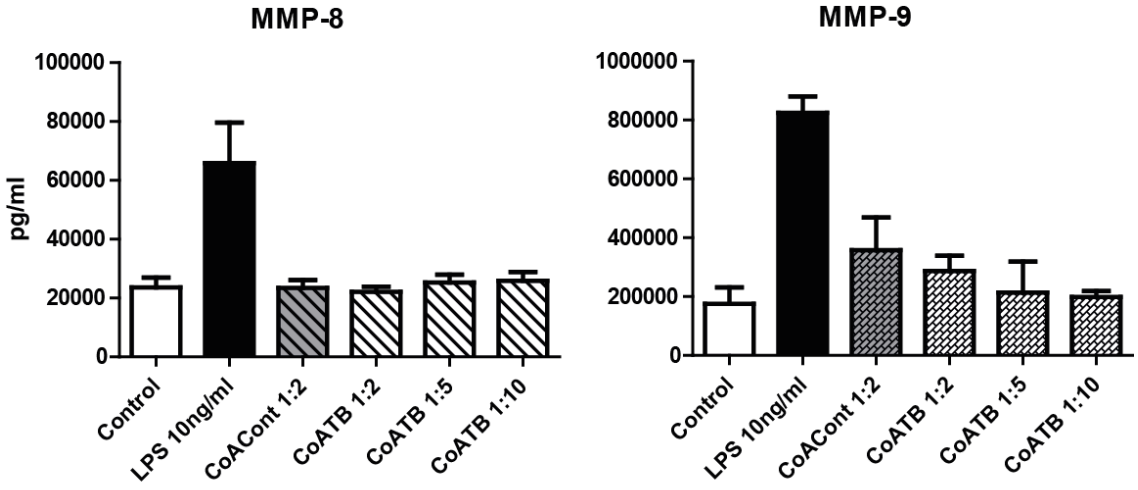


Figure 26: Effect of CoACont and CoATB on neutrophils. U373MG Uppsala was infected with *M.tb* MOI of 10 or stimulated with the equivalent volume of PBS for 72 hours. Supernatants were filtered through a 0.2 μM Anopore membrane and termed Conditioned media from *M.tb*-infected astrocytes (CoATB). Uninfected astrocytes were similarly treated and termed CoACont. Neutrophils were stimulated with CoACont or CoATB at different dilutions for 4 hours. Bars represent mean +/- SD of an experiment done in triplicate. p=NS for data.

2.6 Effect of neutrophil MMP-8/-9 on *M.tb* growth

Certain neutrophil antimicrobial peptides such as HNP 1-3 and lipocalin-2 are mycobactericidal [126]. I wanted to determine if neutrophil MMP-8 and -9 can also restrict or inhibit *M.tb* growth. Purified MMP-8 and -9 from human neutrophil granulocytes were obtained from Enzo Life Sciences and the equivalent concentrations of MMP-8 at 250 ng/ml or MMP-9 at 1μg/ml which are in supernatants from neutrophils infected with *M.tb* at an MOI of 10 were used. These were added to H37Rv in 7H9 broth and growth monitored using optical density and by plating H37Rv onto 7H11 agar after 7 days. Neutrophil MMP-8/-9 however did not affect *M.tb* growth as there was no change in optical density or by CFU (**Figure 27**).

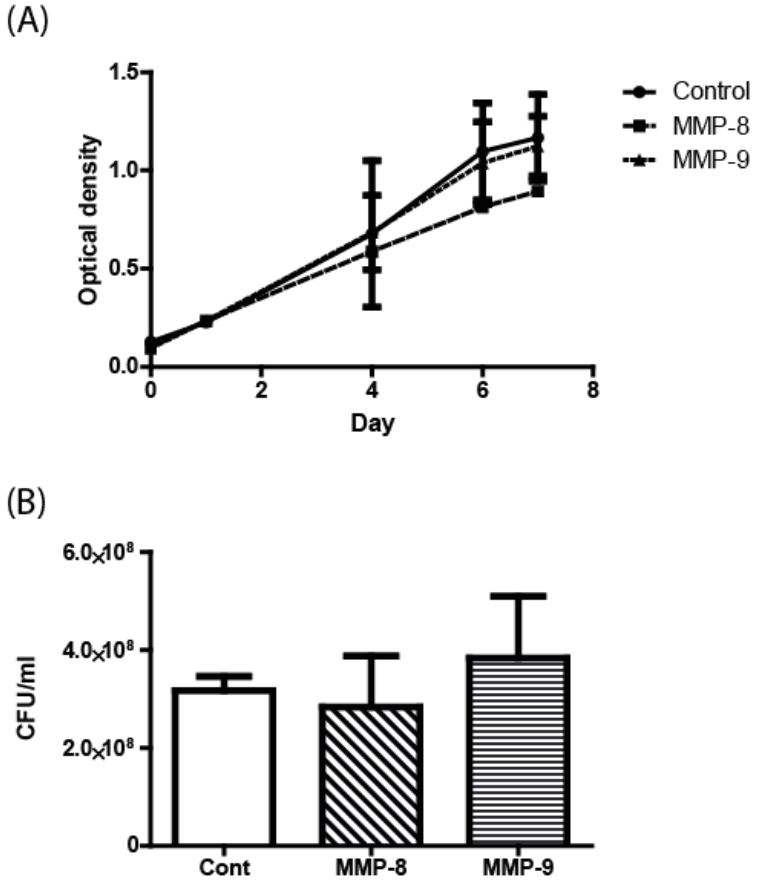


Figure 27: Neutrophil MMP-8 and -9 on *M.tb* growth. A final concentration of 250 ng/ml MMP-8 or 1 μg/ml MMP-9 and H37Rv at initial optical density of 0.1 were used in a total volume of 1.2mls. Control was the buffer (50mM Tris, 200mM NaCl, 5mM $CaCl_2$, 1μM ZnCl2, 0.05% NaN_3, 0.05% Brij-35) in which MMPs were suspended in. Control and MMP-8/-9 were desalted to remove NaN_3 using spin columns prior to addition to H37Rv in 7H9. (A) Optical density over time. (B) CFU/ml. H37Rv was plated out at Day 7 onto 7H11 agar and plates were read at 2 weeks. p=NS for data. Bars represent mean +/- SD of conditions in triplicate and the experiment was representative of 3 independent experiments.

2.7 Effect of CoNTB and CoNCont on *M.tb* growth

Since purified neutrophil MMP-8 and -9 did not seem to have any effect of the growth of H37Rv, I wanted to determine if CoNTB which are reported to contain a range of neutrophil antimicrobial peptides in addition to MMP-8/-9 would modulate *M.tb* growth. It appeared that the addition of CoNTB at 1:3 dilution to H37Rv conversely seemed to increase the growth of H37Rv (**Figure 28**). CoNTB at 1:2 dilution did not have reproducible results on H37Rv growth in 2 separate occasions (data not shown).

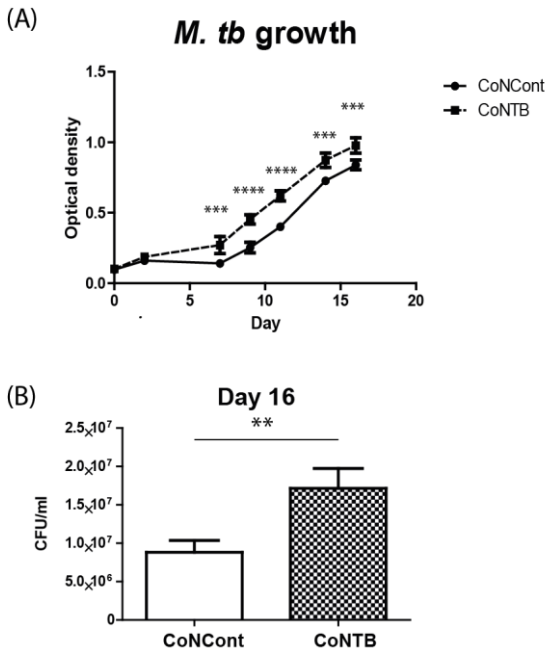


Figure 28: Effect of CoNTB on H37Rv growth. 4 x 10^6 neutrophils were infected with *M.tb* at an MOI of 10 or the equivalent volume of PBS for 4 hours and sterile filtered to make CoNTB and CoNCont respectively. These were added in a 1:3 dilution to H37Rv in 7H9 at a starting optical density of 0.1 to a total volume of 1.2mls. (A) Optical density over time. (B) CFU/ml. H37Rv were plated out at Day 16. Plates were incubated for 2 weeks before colonies were counted. Bars represent mean +/- SD of conditions in triplicate and is representative of 2 independent experiments. ** $p<0.01$, *** $p < 0.001$, ****, $p<0.0001$.

I was not convinced that the addition of CoNTB would increase the growth of *M.tb*, and was concerned that the results I obtained were artifactual. I thus decided that another way would be to measure the luminescence of H37Rv lux G13[217]. H37Rv lux G13 had been used to measure anti-tuberculous drugs in vitro [258] and would be another method to assess if CoNTB truly affected *M.tb* growth. With the incubation of H37Rv luxG13 with CoNTB, CoNCont and media (RPMI), there was a trend towards an increased *M.tb* growth with CoNTB although this was not statistically significant between the different conditions (**Figure 29**).

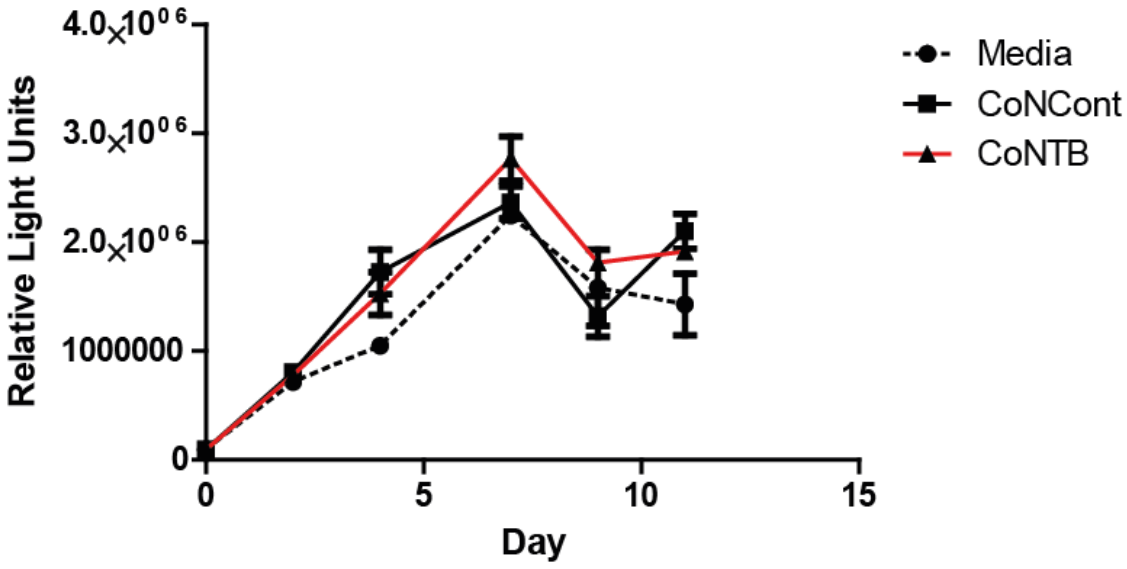


Figure 29: Growth of H37Rv luxG13. 4 x 10^6 neutrophils were infected with *M.tb* at an MOI of 10 or the equivalent volume of PBS for 4 hours and sterile filtered to make CoNTB and CoNCont respectively. These or RPMI(media) were added in a 1:3 dilution to H37Rv luxG13 in 7H9 at a starting optical density of 0.1 to a total volume of 1.2mls. Bars represent mean +/- SD of conditions in triplicate and is representative of experiments done in 2 separate occasions. P = NS for data.

3. *Discussion*

In this chapter, I have investigated the networking effects of neutrophils on cells such as monocytes, macrophages, astrocytes and microglial cells. There was a surprising lack of effect on the MMP secretion from the above cell types that were investigated, although the macrophages and astrocytes appeared stressed when stimulated with CoNTB. One possible reason was that an MOI of 1 of *M.tb* used to make CoNTB may not be sufficient to elicit an increase in MMP secretion from other cells, and a higher MOI such as 10 may be required. However, neutrophils also secrete a large amount of MMP-8 and -9 even when infected with *M.tb* MOI of 1, making it technically challenging to detect a relatively smaller increase in MMP-8 and -9 concentrations from both monocytes and macrophages and MMP-9 from astrocytes, although other MMPs from these cells were not affected. Filtering CoNTB with Anopore filters to remove some neutrophil MMPs from CoNTB did not appear to help in this situation compared to CoMTB where it minimised the MMPs [219], as this is probably due to the larger amounts of MMP-8/-9 present in CoNTB. In comparison, CoMTB had smaller quantities of MMPs and filtration further reduced the concentration of MMPs to a minimum. Thus, it was easier to demonstrate the effect of CoMTB on other cell types and these have been shown in primary airway epithelial cells[180], astrocytes[190], fibroblasts[191] and microglial cells [260]. An alternative readout would be to evaluate the cytokines and chemokines secreted from these cells when stimulated with CoNTB.

I had looked at potential crosstalk between astrocytes and neutrophils, and there was a lack of MMP secretion from neutrophils when stimulated with CoATB from U373MG Uppsala. One limitation was that I did not evaluate the effect of CoATB

made from other astrocyte cells lines. It is quite likely however that there will also be lack of neutrophil MMP response with these other CoATB since these astrocytic cell lines are similarly derived from malignant glioblastoma astrocytomas.

MMPs are well-known for their role in extracellular matrix turnover but there is limited literature if they possess any pathogen killing effect. Houghton et al [261] described MMP-12 $^{-/-}$ mice exhibiting impaired bacterial clearance and had increased mortality when challenged with both Gram-negative and Gram-positive bacteria at macrophage-rich portals of entry, namely the peritoneum and lung. Additionally, they have shown in *in vitro* studies that full-length recombinant human MMP-12 exhibited dose-dependent inhibition of *S.aureus*. I thus wanted to determine if neutrophil MMP-8 and -9 would also have any bactericidal, specifically mycobactericidal effects. However, incubation of H37Rv with purified MMP-8 and -9 did not show any effects on *M.tb* growth. This however does not mean that MMP-8 and -9 do not have any pathogen killing effects as other pathogens have not been tested.

It was interesting to note that CoNTB seemed to increase *M.tb* growth using the readouts of optical density and growth on agar. The results of CoNTB on luminescent *M.tb* although not statistically significant showed a trend towards increase as well. When neutrophils are infected with *M.tb*, they secrete antimicrobial peptides such as human neutrophil peptide 1-3 and lipocalin-2 which have both been shown to inhibit *M.tb* growth [126], so I was surprised that *M.tb* growth was conversely increased when CoNTB was added to H37Rv in 7H9. It had also been shown that human neutrophils exhibited potent intracellular killing activity on *M.tb in vitro* [262] which is independent of oxidative mechanisms. One possibility is that neutrophils secrete some growth factors for *M.tb* and this would need further investigation, perhaps using mass

spectrometry to identify various components of CoNTB, and testing each individual component on *M.tb* growth.

In summary, I had shown a lack of networking effects from neutrophils on monocytes, macrophages, astrocytes and microglial cells in terms of increasing MMP secretion from these cells. There was no astrocyte cross-talk to neutrophils when astrocytes were infected with *M.tb*. Human MMP-8 and -9 do not have mycobactericidal effects and there seemed to be an increase in growth of *M.tb* when cultured with conditioned media of *M.tb* infected neutrophils (CoNTB). With the neutrophil MMP phenotype defined with *M.tb* exposure and the effects of neutrophil cross-talk on other cells explored, the mechanisms regulating neutrophil MMP secretion will be discussed in the next chapter.

CHAPTER 5 – INTRACELLULAR SIGNALLING PATHWAYS AND TRANSCRIPTION FACTORS REGULATING NEUTROPHIL MMP SECRETION

1. *Introduction*

The secretion of MMPs from cells is regulated by a few key pathways. The MAP kinase and the PI3 kinase have been well-described to regulate MMP secretion from macrophages[181], microglial [192] and astrocytes [189] among other cells. However, it is crucial to note that these cells unlike neutrophils synthesise most of their MMPs when gene expression is induced. Neutrophils conversely store majority of MMP-8 and -9 in their secondary and tertiary granules respectively in their pro-form although a small amount of gene expression is involved as described in Chapter 3. The question is which pathways are regulating the release of neutrophil MMPs in TB. Identifying these pathways are essential as they are potential points in which therapeutic interventions can be made to modulate MMP secretion, altering the effects that result from excessive MMP secretion and matrix destruction.

In this chapter, I shall delineate the various pathways and transcription factors that regulate neutrophil MMP secretion both in the context of direct *M.tb* infection as well as in monocyte-dependent networks when neutrophils are stimulated by CoMTB. This is done by western blotting, phosphoarrays and pharmacological inhibitors. In addition, I spent 6 months attempting siRNA on neutrophils which proved to be technically difficult to transfect. Lastly, I have also analysed the neutrophil MMP secretion from a rare cohort of patients with AMPKγ_2 mutation with resultant functional AMPK deficiency and compared this with healthy controls in the same experimental setting.

2. *Results*

2.1 Role of MAP-kinase and PI3-kinase pathways in *M.tb* infected neutrophils

The Mitogen-activated protein kinase pathway and Phosphoinositide 3-kinase pathway have been implicated in regulating MMP secretion from neutrophils. [194, 208, 263] To examine the effect *M.tb* has on these pathways, I investigated the phosphorylation of p38, ERK and the Akt substrate of the PI3-kinase pathway of control versus *M.tb*-infected neutrophils over time. The kinetics experiment demonstrated that p38 was phosphorylated at time = 0 for both control and *M.tb*-infected neutrophils probably due to activation from the neutrophil extraction process. Phosphorylation was subsequently absent in control cells but present through 120 minutes in *M.tb*-infected neutrophils. Phospho-ERK is present from 15 minutes, peaks at 30 minutes and declines by 120 minutes, while Phospho-Akt starts and peaks at 15 minute and declines by 120 minutes in *M.tb*-infected neutrophils. ERK and Akt phosphorylation was not detected in control cultures (**Figure 30**).

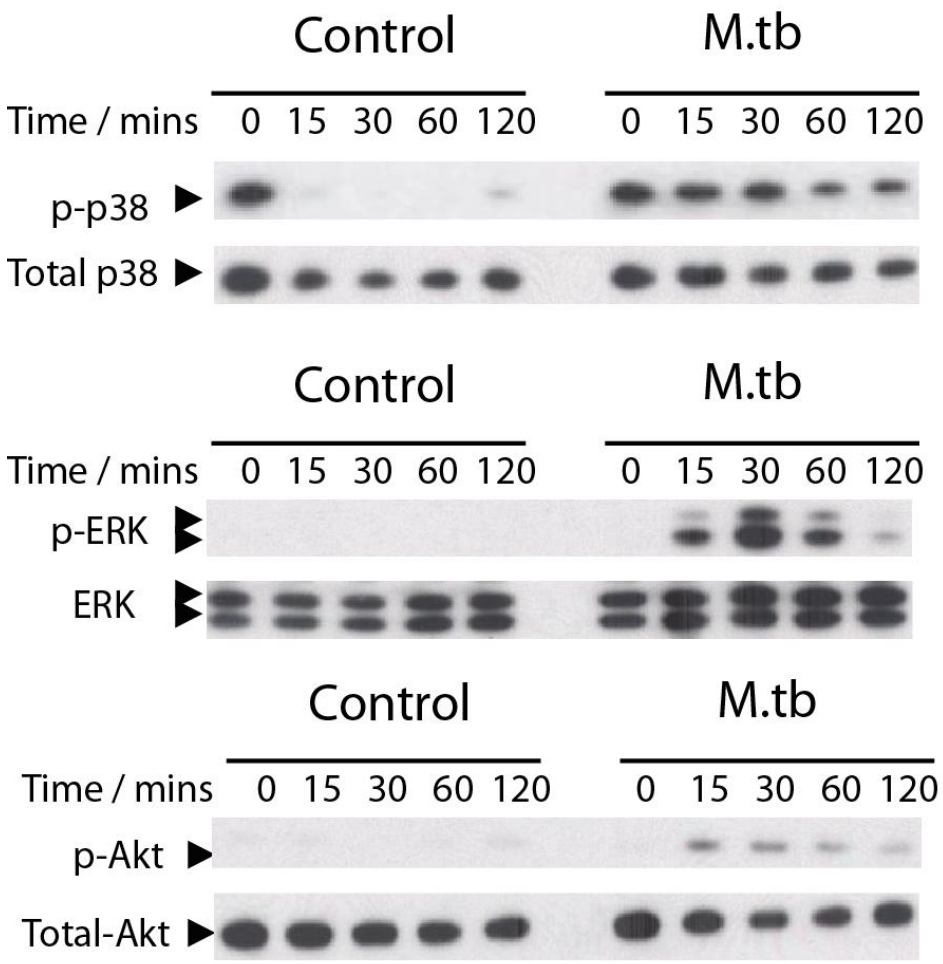


Figure 30: Phosphorylation kinetics of p38, ERK and Akt with control (PBS) and *M.tb* infection in neutrophils. 2 x 10^6 neutrophils were incubated with either PBS or TB MOI of 10 and at specified time points, cells were pelleted and lysed with SDS sample buffer. Data is representative of 2 separate experiments.

As the MAP-kinase and PI3-kinase pathways are activated in neutrophils in response to *M.tb* infection, the question is if they are of any functional significance. To investigate this, I utilised 3 chemical inhibitors: the p38 inhibitor SB 203580, the ERK inhibitor PD98059, and the PI3-kinase inhibitor LY294002 and investigated their effects on MMP-8 and -9 secretion on *M.tb*-infected neutrophils. A TB MOI of 10 was used in the experiments to accentuate the secretion of MMP-8 and -9 so as to demonstrate a dose-dependent suppression if any. No inhibitory effect was demonstrated with the three chemical inhibitors (**Figure 31**) even when the neutrophils were pre-incubated with the inhibitors for 60 rather than 30 minutes (data not shown). Neutrophils were also incubated with DMSO, a commonly used solvent to dissolve chemical inhibitors on a separate occasion and this did not have any effect on neutrophil MMP secretion in the context of *M.tb* infection and CoMTB stimulation (data not shown).

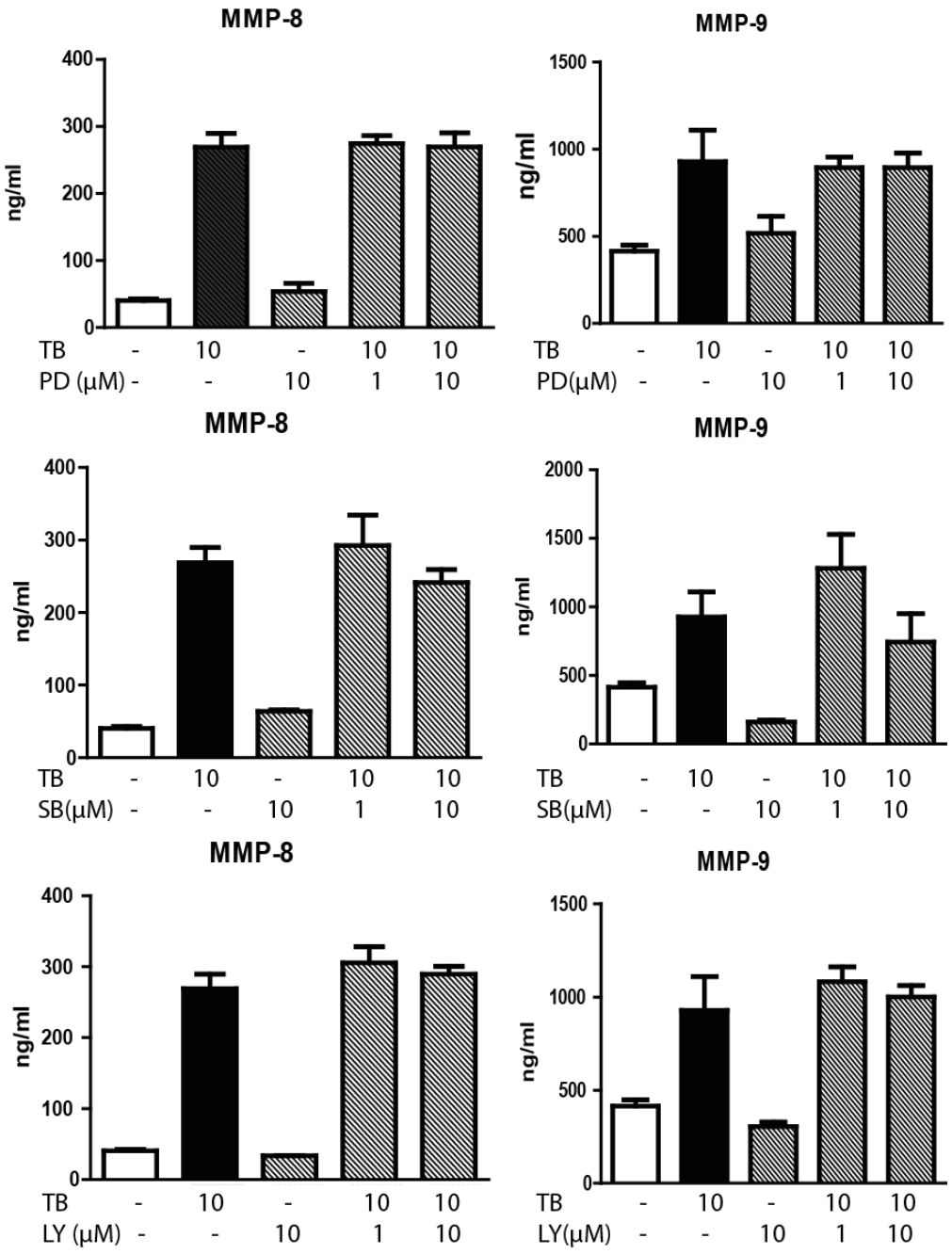


Figure 31: Effects of ERK inhibitor, p38 inhibitor and PI3 kinase inhibitor on *M.tb*-infected neutrophils. 7.5 x 10^5 neutrophils were re-suspended with media, 1 μM or 10 μM of the respective inhibitor and pre-incubated for 30 minutes before infected with *M. tuberculosis* at an MOI of 10 for 4 hours. Supernatants were analysed using luminex array as described in methods. Bars represent mean +/- SD of three samples and is representative of 2 separate experiments done in triplicate. p = NS for data shown.

2.2 Role of NF-kB in *M.tb*-infected neutrophils

Next, the role of NF-kB on MMP-8 and -9 secretion from neutrophils was investigated with the use of 2 inhibitors: Helenalin, an NF-kB inhibitor which targets the p65 subunit, and SC-514, an inhibitor of IKK_2, up-stream of NF-kB. Helenalin demonstrated a significant dose-dependent suppression of both MMP-8 and -9 to baseline from *M.tb*-infected neutrophils with maximal suppression at a concentration of 100 μM ($p<0.001$ for both MMP-8 and 9) (**Figure 32**). Interestingly, inhibition of *M.tb*-infected neutrophils with SC-514 did not show any effect on MMP-8 and MMP-9 secretion. The viability of neutrophils in both experiments were >90% by trypan blue.

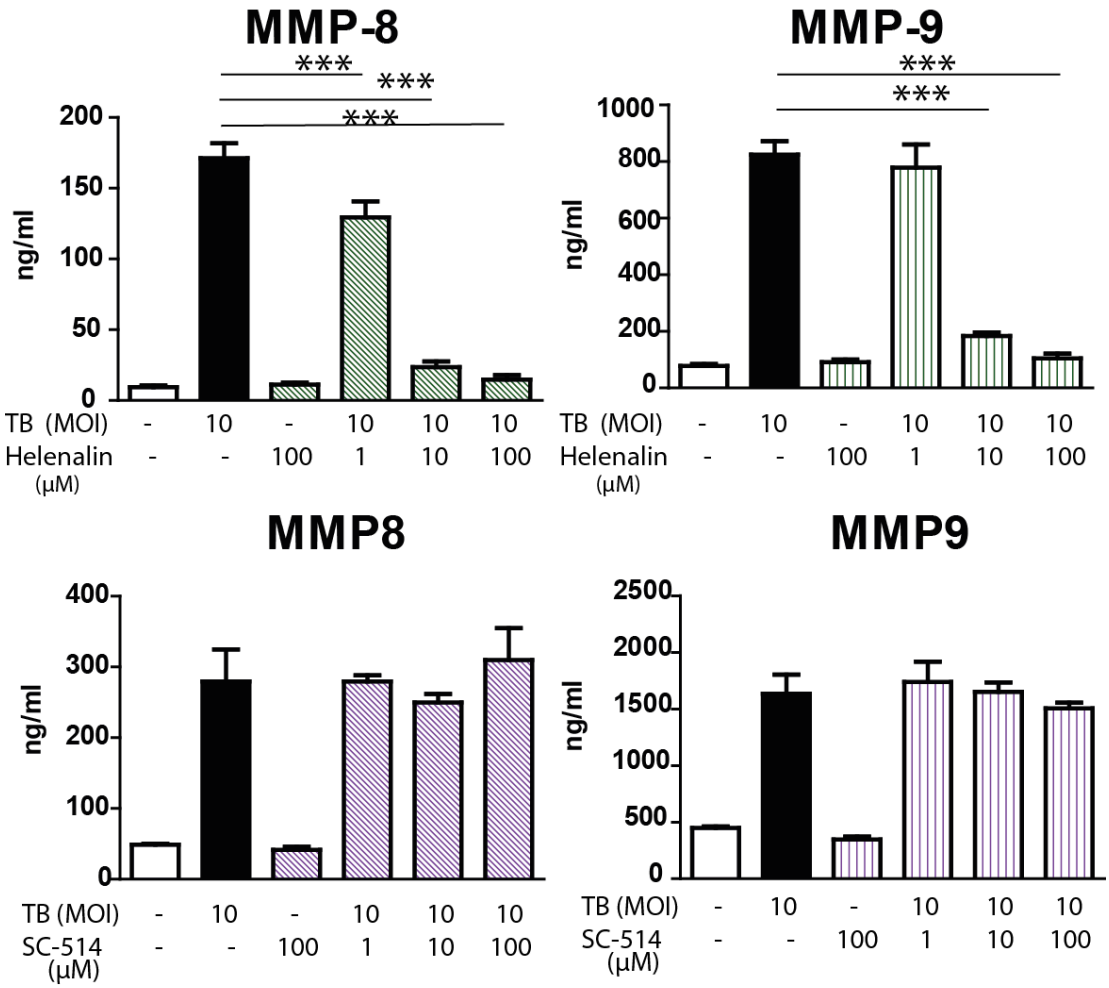


Figure 32: NF-kB inhibitors on *M.tb*-infected neutrophils. 7.5 x 10^5 neutrophils were resuspended with media, 1 μM, 10 μM, or 100 μM of the inhibitors, pre-incubated for 30 minutes and subsequently infected with a TB MOI of 10 for 4 hours. Supernatant were analysed using luminex array. (A) Effect of Helenalin on *M.tb*-infected neutrophils. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 2 separate experiments. ***p<0.001. (B) Effect of IKK2 inhibitor SC-514 on *M.tb*-infected neutrophils. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 2 independent experiments. *p<0.05, **p<0.01.

2.3 Role of MAP-kinase and PI3-kinase in CoMTB stimulated neutrophils

Since the MAP-kinase and PI3-kinase pathways in neutrophils are activated with *M.tb* infection, I hypothesized that these pathways would similarly be activated with CoMTB stimulation. I examined the phosphorylation of the p38, ERK, JNK, and Akt pathways by western blotting (**Figure 33**). With CoMTB stimulation, phospho-p38, which was present at time =0, peaked at 15 minutes and remained phosphorylated through 120 minutes. Phospho-ERK, phospho-JNK and phospho-Akt appeared at 15 minutes and subsided by 120 minutes. However, I faced difficulty in demonstrating the presence of total-JNK due to the high background signal after the membrane was stripped. This attempt was made on three occasions, and using bovine serum albumin instead of skimmed milk during blocking of the membrane in the possible event that it was a component of milk that may have caused non-specific binding of the primary antibody. However, the use of BSA did not improve the quality of the blot. Attempts at using beta-actin as a substitute for protein loading was also not successful (data not shown).

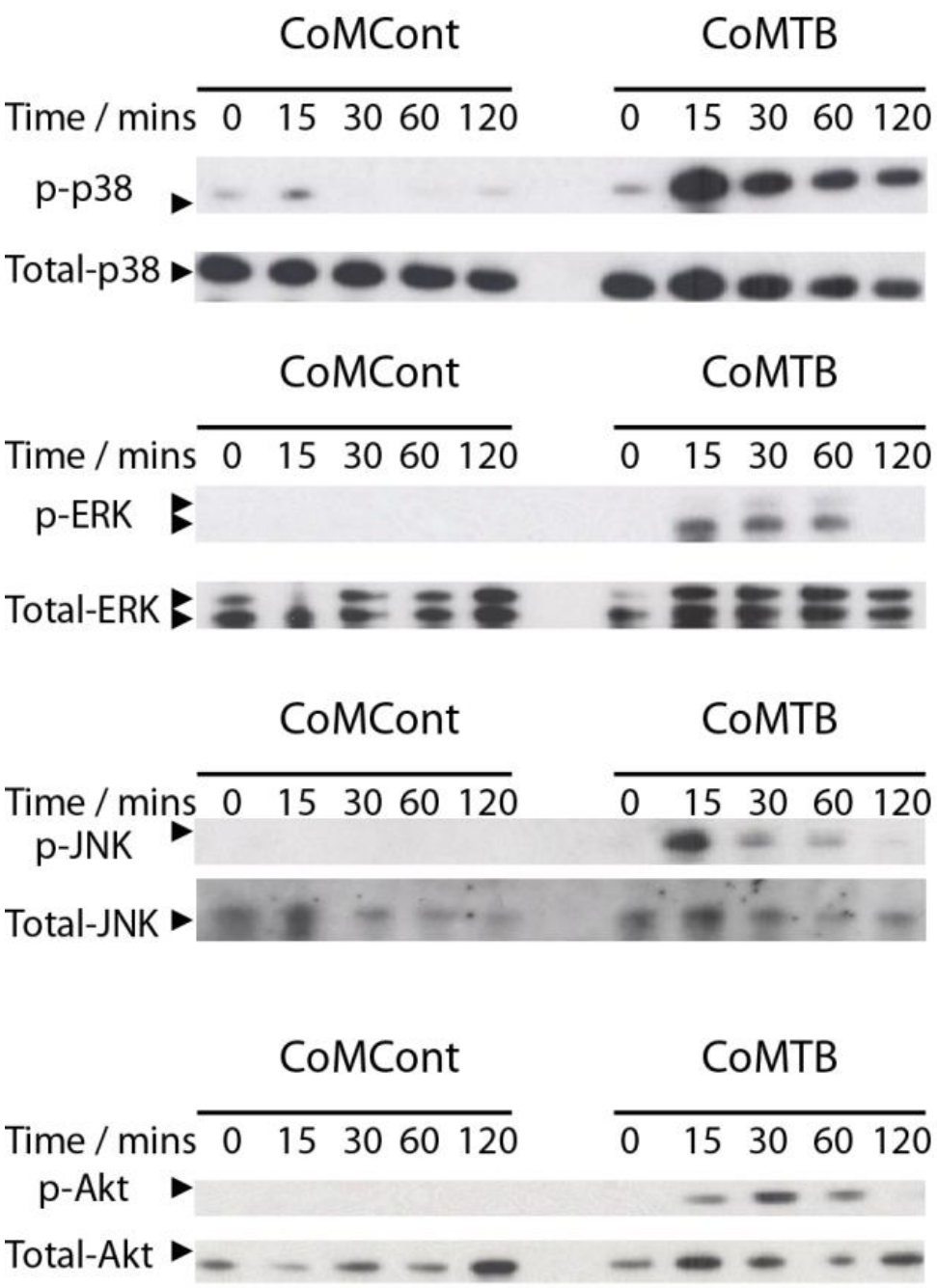


Figure 33: Phosphorylation kinetics of CoMTB-stimulated neutrophils. 2 x 10^6 neutrophils were stimulated with CoMCont or CoMTB at 1:5 dilution and at the specified time points cells were pelleted and lysed with SDS lysis buffer. Data is representative of 4 independent experiments.

Similarly, since the MAP-kinase and the PI3 kinase pathways were activated, the question is whether these pathways actually regulate neutrophil MMP secretion with CoMTB. I went on to investigate the effect of inhibition of these pathways using p38 inhibitor SB 203580, ERK inhibitor PD 98059, PI3 kinase inhibitors LY 294002 and Wortmannin. There was a dose-dependent suppression of MMP-8 and 9 secretion which was statistically significant at 10 μM of PD 98059 ($p<0.01$ and $p<0.05$ respectively) and 10 μM of SB 203580 ($p< 0.01$ and $p< 0.001$ respectively) (**Figure 34A**). There was also a dose-dependent suppression of MMP-9 secretion with LY 294002 at 10 μM ($p<0.01$), but this effect was not observed for MMP-8 (**Figure 34B**). Wortmannin did not suppress MMP-8 and 9 secretion from CoMTB stimulated neutrophils (**Figure 34C**) possibly as it has other non-specific, off-target actions. [264, 265]

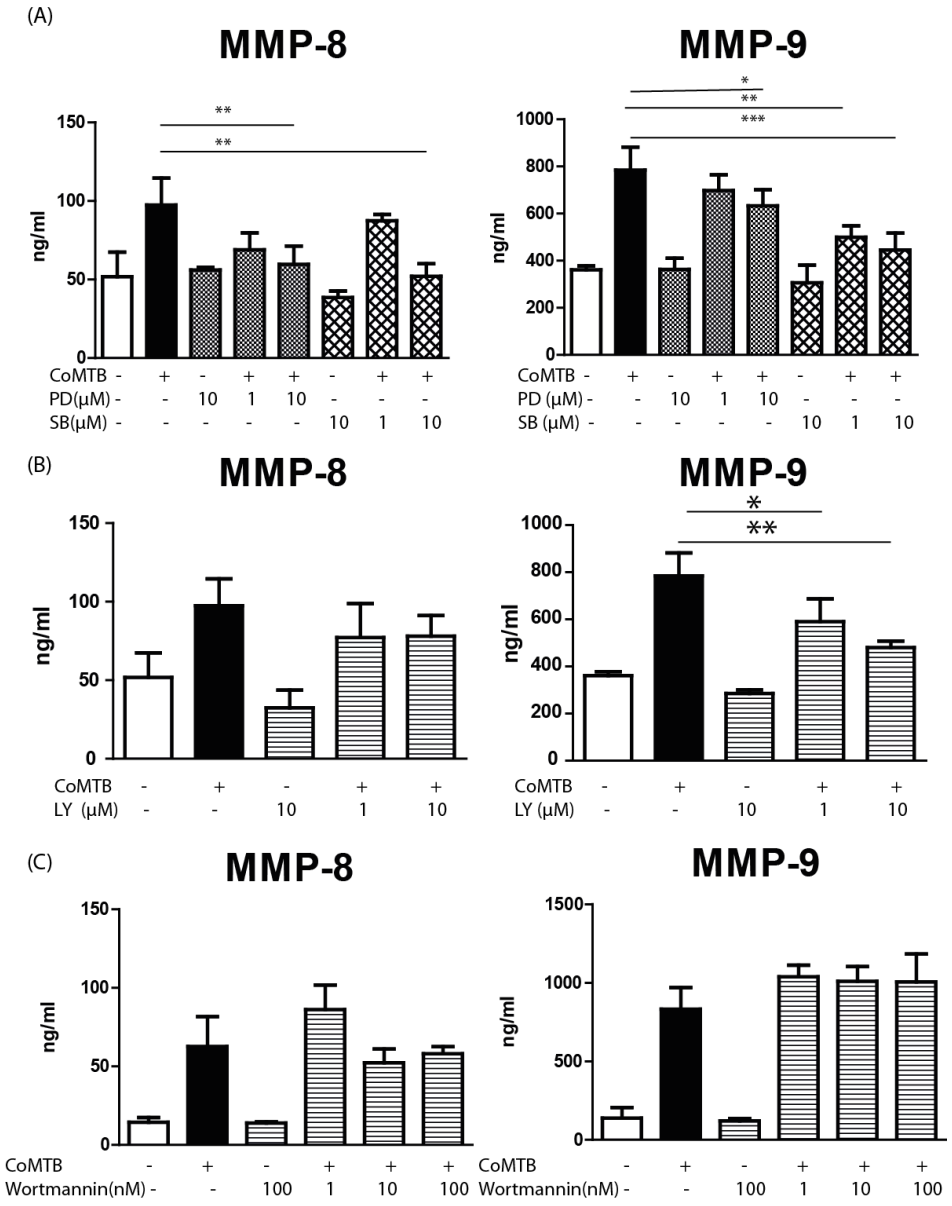


Figure 34: Effect of MAP-kinase and PI3-kinase inhibitors. 7.5 x 10^5 neutrophils were pre-incubated with inhibitor for 30 minutes and subsequently stimulated with CoMTB at 1:5 dilution for 4 hours. Supernatants were analysed using luminex array. (A) Effect of ERK inhibitor PD 98049 and p38 inhibitor SB 203580 on CoMTB stimulated neutrophils. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 2 separate experiments. *p<0.05, **p<0.01, ***p<0.001. (B) Effect of PI3 kinase inhibitor LY 294002 on CoMTB stimulated neutrophils. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 2 separate experiments. *p<0.05, **p<0.01. (C) Effect of Wortmannin on CoMTB stimulated neutrophils. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 2 separate experiments. p=NS for data.

2.4 Role of NF-kB in CoMTB stimulated neutrophils

Next, I investigated the role of NF-kB in CoMTB stimulated neutrophils using the p65 subunit inhibitor Helenalin and IKK2 inhibitor SC-514. There was a significant dose-dependent suppression of MMP-8 and -9 back to baseline at the maximum concentration of 100μM of Helenalin ($p < 0.001$ for both) (**Figure 35**). SC-514 also demonstrated a significant dose-dependent suppression of MMP-8 and 9 in CoMTB stimulated neutrophils ($p < 0.001$).

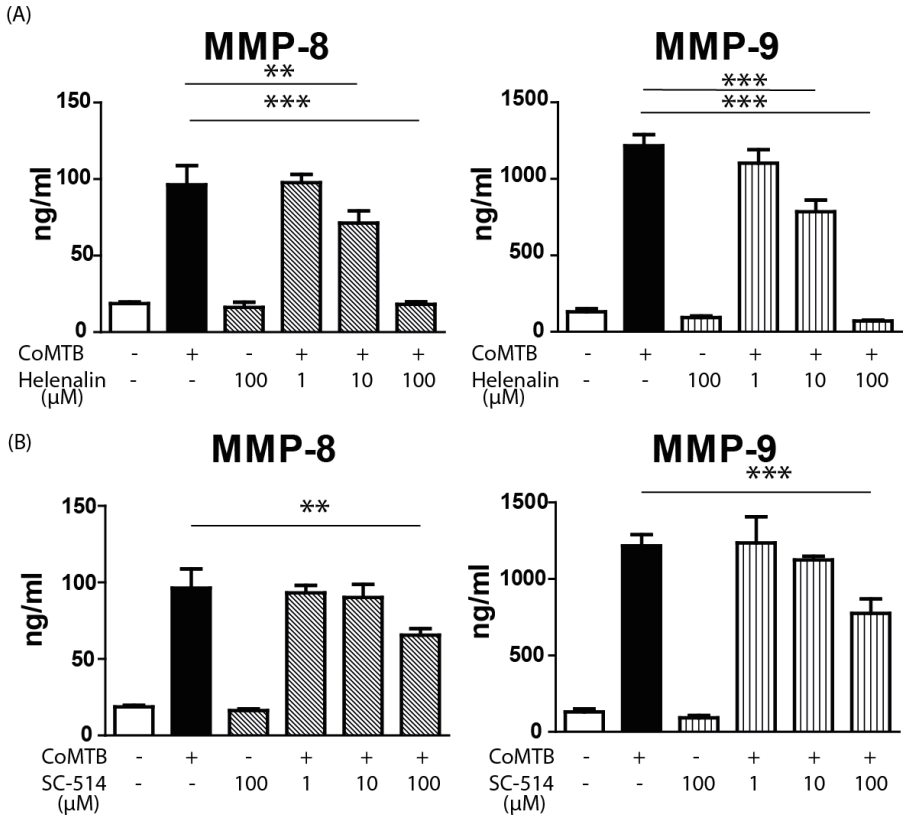


Figure 35: Effect of NF-kB inhibitors on CoMTB stimulated neutrophils. 7.5 x 10^5 neutrophils were pre-incubated with the respective inhibitor for 30 minutes and subsequently stimulated with CoMTB at 1:5 dilution for 4 hours. Supernatants were analysed using luminex array. (A) Helenalin on CoMTB stimulated neutrophils. (B) SC-514 on CoMTB stimulated neutrophils. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 2 independent experiments each. **p<0.01,***p<0.001.

2.5 MAP-kinase, PI3kinase, AMPK and STAT pathways are activated in CoMTB stimulated neutrophils

To dissect other pathways that may be activated in CoMTB-stimulated neutrophils, I used the Human Proteome Profiler Phospho-kinase Array kits. 5 donors on 5 separate occasions were used for this. The blots confirmed that the MAP kinase and PI3 kinase pathways are activated with CoMTB stimulation (**Figure 36**). ERK1/2 which is a component of the MAPK pathway and p53 which is upregulated in apoptosis was found to be phosphorylated from all donors. In addition, members of the AMPK pathway (Akt, AMPKα2, p70 S6 kinase), the master regulator of energy homeostasis, and STAT pathways (STAT1, STAT 5b and STAT 6) which play critical roles in cytokine and chemokine secretion were also found to be consistently phosphorylated.

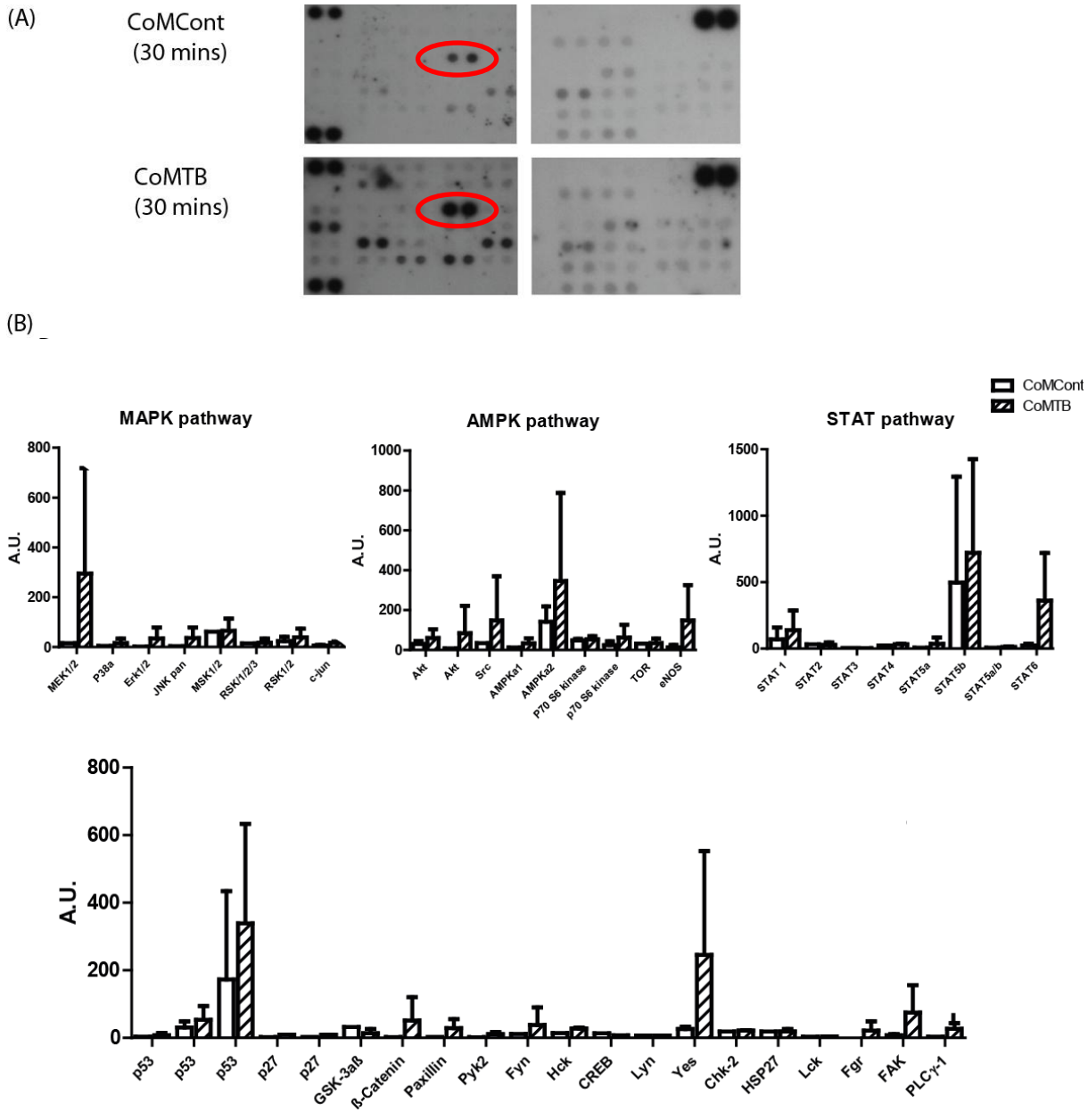


Figure 36: Phosphokinase array. 2.5 x 10^6 neutrophils were stimulated with CoMTB or CoMCont at 1:5 dilution and cells were lysed after 30 minutes. The amount of protein was quantified using a Bradford assay, and equal amounts of cell lysates were added onto the membranes overnight. Blots were developed using ECL. (A) Representative array from one donor. The red circles denotes AMPKα2. (B) Densitometric analysis. Bars represent mean +/- SEM of values from arrays of 5 donors.

2.6 AMPK regulates MMP secretion in CoMTB-stimulated neutrophils

The Human Proteome Profiler Phospho-kinase Array kits had shown that AMPKα2 was phosphorylated consistently. 5‘ Adenosine monophosphate-activated protein kinase is activated when there is increased AMP:ATP ratio, hence regulating cellular energy homeostasis, and is involved in various metabolic pathways including regulating cellular uptake of glucose, steroid synthesis and biogenesis of GLUT-4 [266, 267]. Activation of AMPK has also been shown to decrease neutrophil pro-inflammatory activity and decrease TNF- α [268]. I first investigated phosphorylation of AMPKα2 in CoMTB stimulated neutrophils. The primary antibody from Cell Signalling UK was available only for phospho-AMPKα1/2 (T172) and not for phospho-AMPKα2 alone and other manufacturers do not have a phospho-AMPKα2 (T172) antibody. Nevertheless, Western blotting showed that AMPKα1/2 is phosphorylated from 0 minutes through 120 minutes and confirmed that AMPKα was activated (**Figure 37A**). I next investigated the possible role that AMPK may have in regulating MMP-8 and -9 in CoMTB stimulated neutrophils. I used AMPK inhibitor Compound C and AMPK activator AICAR on CoMTB-stimulated neutrophils using doses that have been previously described[268, 269]. There was a significant dose-dependent suppression of both MMP-8 and -9 with Compound C maximally at a concentration of 100 ($p<0.001$ and $p<0.01$ respectively) (**Figure 37B**). TIMP-2 was similarly suppressed at a dose-dependent manner maximally at 100 μM of Compound C ($p<0.01$) (**Figure 37C**). Ideally, AMPK inhibition should be done with another chemical inhibitor to determine that the suppression of neutrophil MMP was not a non-specific effect of Compound C but there is no other inhibitor available at the time of writing this thesis. There was no effect of AMPK activation with AICAR on neutrophil MMP secretion (**Figure 38**).

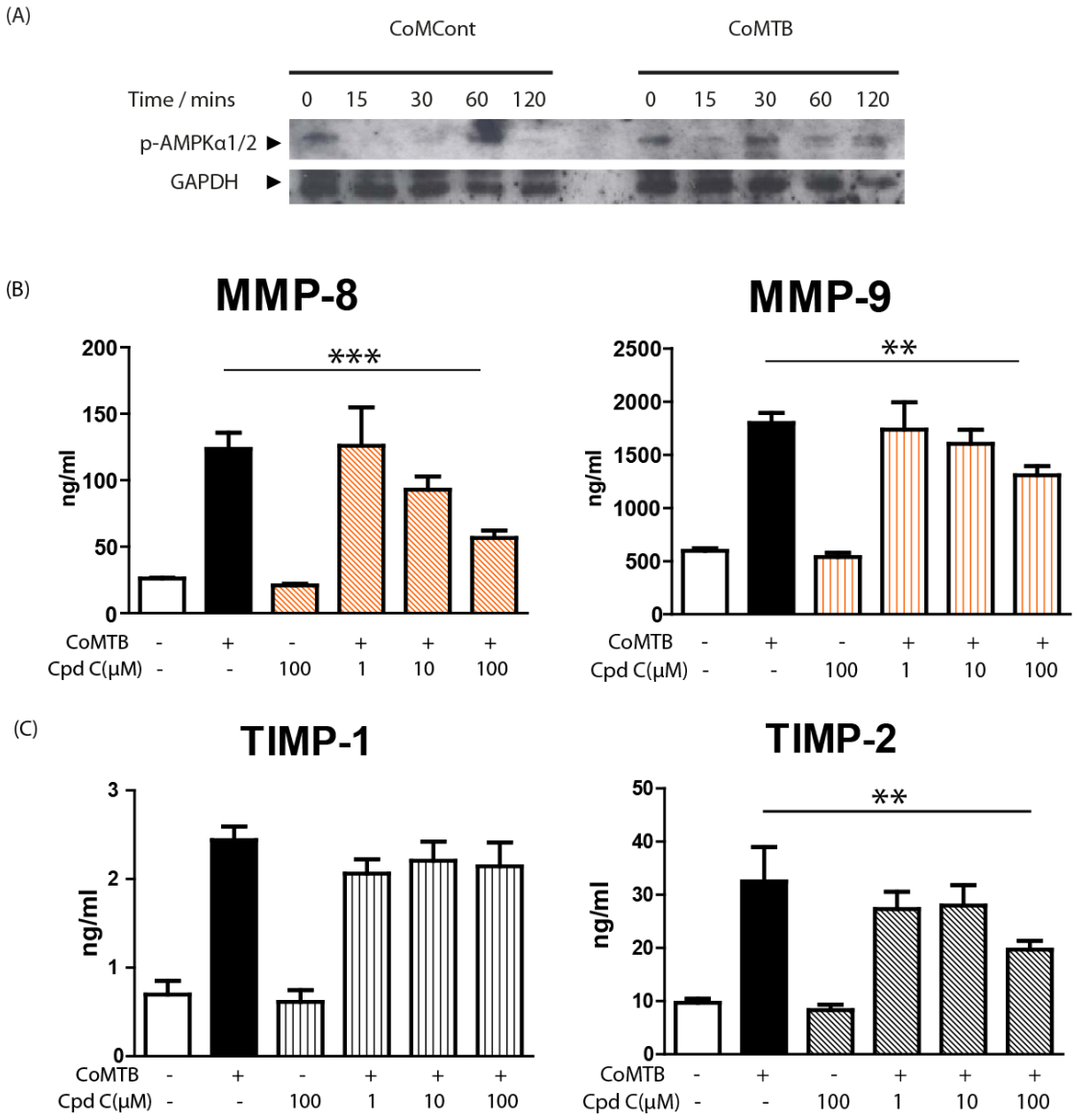


Figure 37: AMPK regulates neutrophil MMP secretion in CoMTB-stimulated neutrophils. (A) Phosphorylation kinetics AMPKα1/2 with CoMCont and CoMTB. 2 x 10^6 neutrophils were stimulated with CoMCont or CoMTB at 1:5 dilution and at specific time points cells were lysed. Data representative of 2 separate experiments. (B) MMP-8/9 secretion in CoMTB-stimulated neutrophils pretreated with Compound C. 7.5 x 10^5 neutrophils were pre-incubated for 30 minutes with Compound C (Cpd C) before stimulating for 4 hours with CoMTB at 1:5 dilution. Supernantants were analysed by luminex array. (C) TIMP-1/2 secretion. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 2 independent experiments. **p<0.01, ***p<0.001.

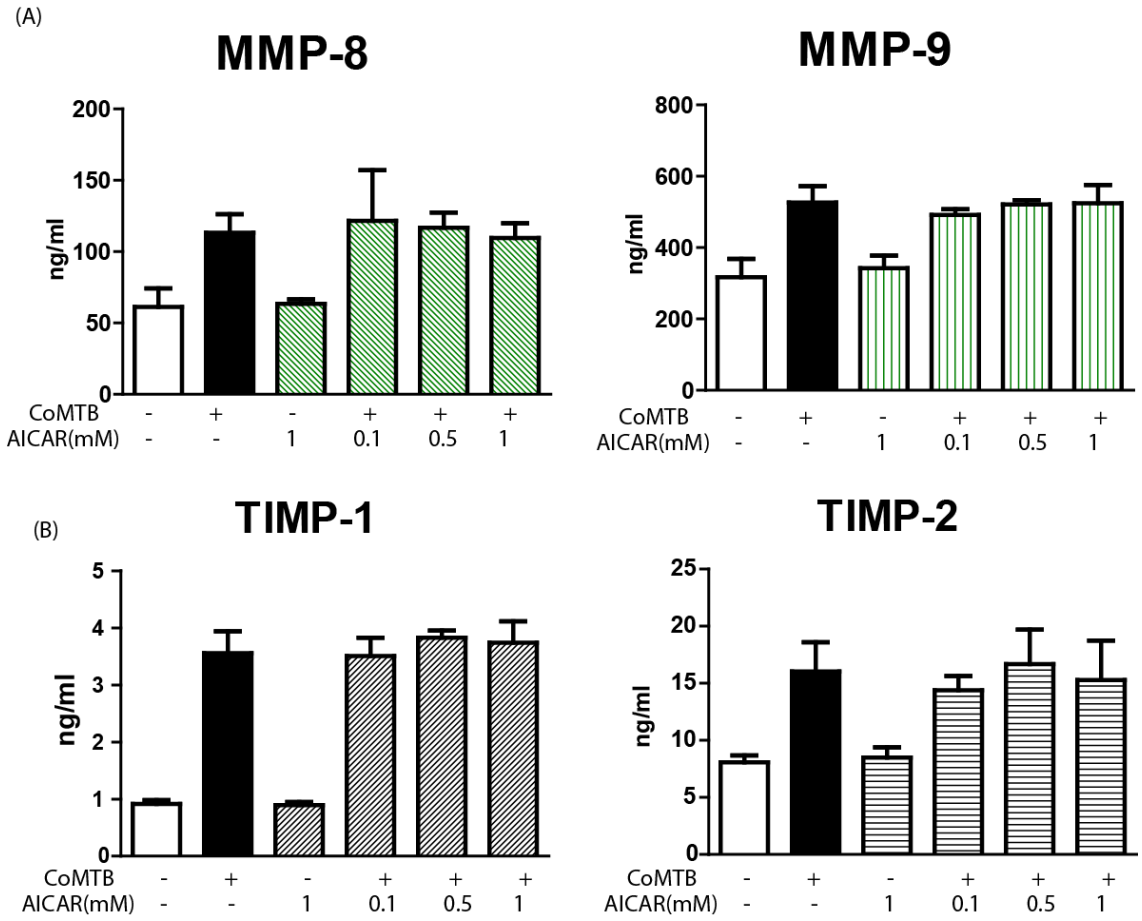
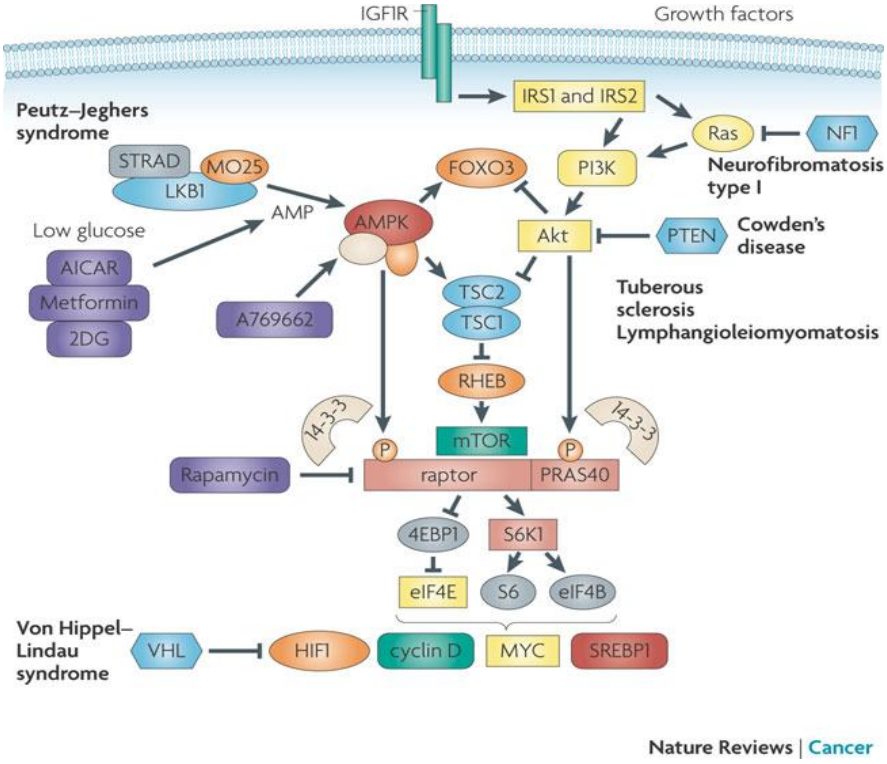


Figure 38: AMPK activation on neutrophil MMPs. 7.5 x 10^5 neutrophils were pre-incubated for 30 minutes with AICAR before stimulating for 4 hours with CoMTB at 1:5 dilution. Supernantants were analysed by luminex array. (A) MMP-8 and -9 (B) TIMP-1/-2. Bars represent mean +/- SD of an experiment done in triplicate and is representative of 2 independent experiments. P=NS for data.

2.7 Neutrophil MMP secretion is independent of the mTOR/p70 S6 kinase pathway in CoMTB stimulated neutrophils

As the mTOR and p70S6 kinase (identified as S6K1 below) pathway is downstream of the AMPK pathway (see adaption), I next evaluated if this pathway might be regulating neutrophil MMP secretion.



Adapted from *Sheckleford et al Nature Reviews Cancer 2009*

I first evaluated if p70S6 kinase was activated with CoMTB. Western blotting confirmed that p70S6 kinase (T229) was phosphorylated with CoMTB commencing from 15 minutes to 240 minutes (**Figure 39A**). Next, I used rapamycin in an attempt to inhibit mTOR and to determine if the mTOR pathway would regulate neutrophil MMP secretion. Rapamycin did not affect neutrophil MMP secretion with CoMTB stimulation (**Figure 39B**).

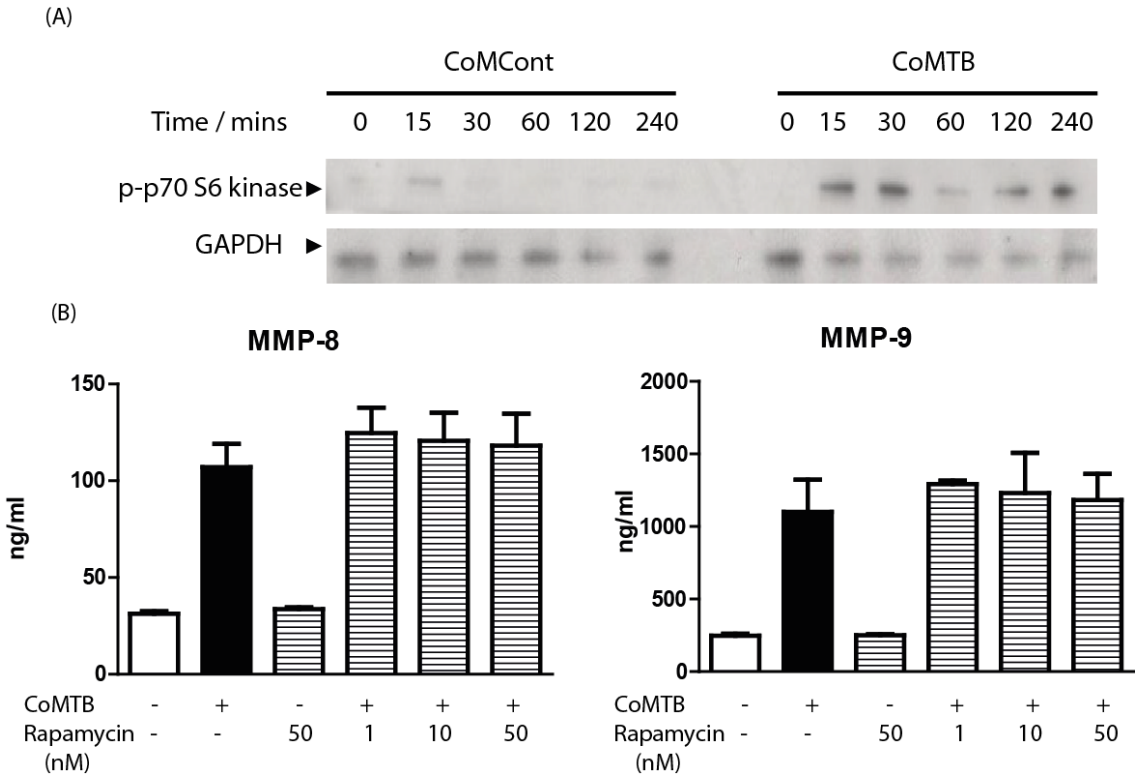


Figure 39: Neutrophil MMP secretion is independent of the mTOR/p70S6 kinase pathway. (A) Kinetics of p70S6 kinase phosphorylation with CoMCont and CoMTB. 2 x 10^6 neutrophils were stimulated with 1:5 CoMCont or CoMTB and lysed with SDS lysis buffer at specified time points. Data representative of 2 separate experiments. (B) Rapamycin does not affect neutrophil MMP-8/-9 secretion. 7.5 x 10^5 neutrophils were incubated with rapamycin for 30 minutes and subsequently stimulated with 1:5 CoMTB for 4 hours. Bars represent mean +/- SD of triplicates and is representative of 2 independent experiments. P=NS for data.

2.8 MMP-8/9 secretion is independent of the AMPK pathway in *M.tb*-infected neutrophils

Given that the AMPK pathway is regulating neutrophil MMP secretion in the context of CoMTB stimulation, I wanted to determine if this would be a similar situation when neutrophils are infected with *M.tb*. I ascertained that phospho-AMPKα1/2 was present in *M.tb* infection and commenced from 15 minutes through to 120 minutes (**Figure 40A**). The AMPK inhibitor Compound C did not affect neutrophil MMP secretion but did inhibit TIMP-2 ($p<0.01$) (**Figure 40B and C**). Turning my attention to AMPK activation in *M.tb* infected neutrophils, activation with AICAR did not affect neutrophil MMP or TIMP secretion (**Figure 41**) which was similar to the context of CoMTB stimulation.

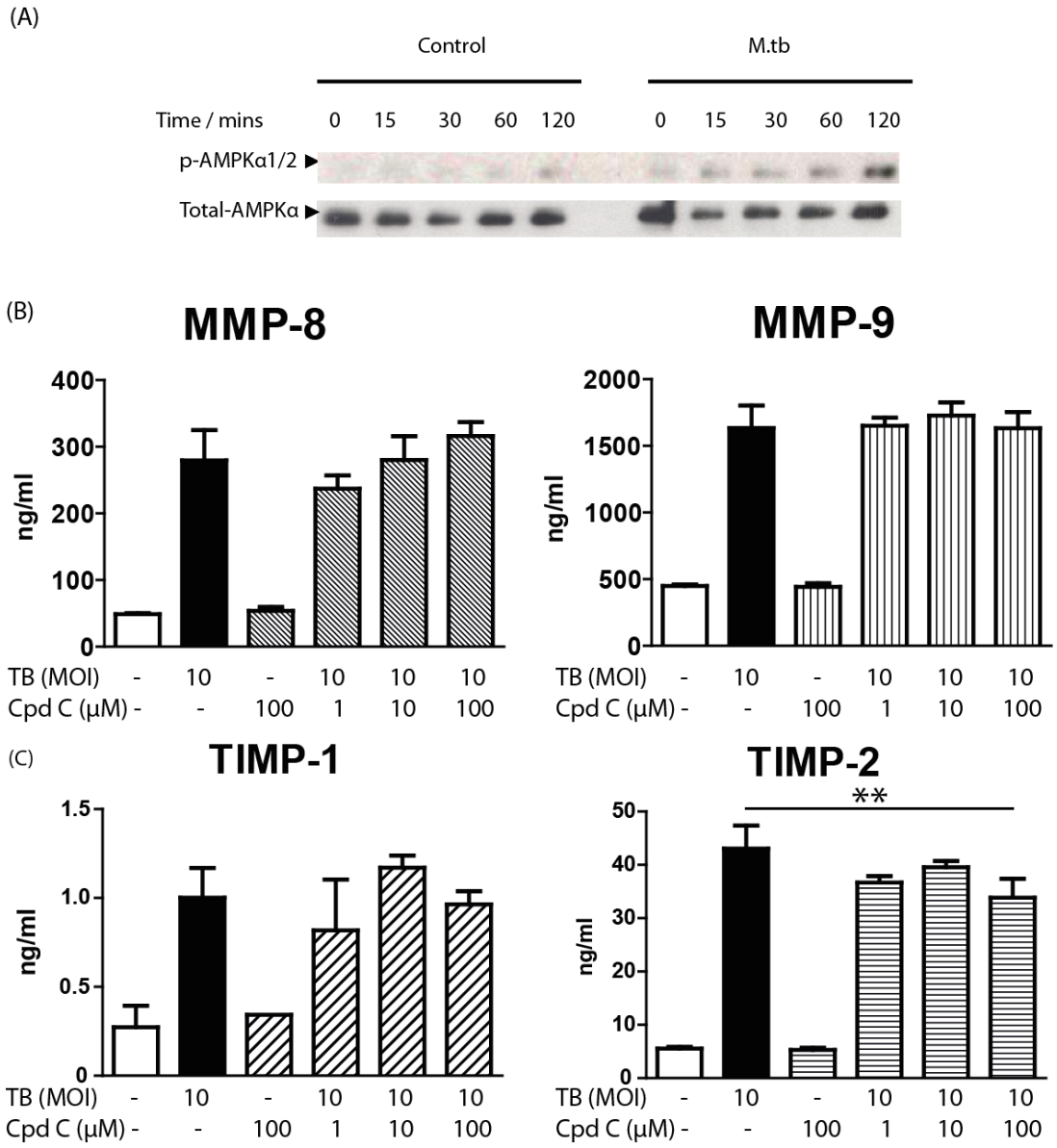


Figure 40: AMPK pathway is activated but do not regulate MMP-8/9 secretion in *M.tb* infection. (A) Kinetics of AMPKα phosphorylation with PBS and *M.tb*. 2 x 10^6 neutrophils were infected with *M.tb* MOI of 10 and at specified points cells were lysed and sterile filtered. Data representative of 2 independent experiments. (B and C) MMP-8/-9 and TIMP-1/-2 secretion in neutrophils treated with Compound C (Cpd C) in *M.tb* infection. 7.5 x 10^5 neutrophils were preincubated with Cpd C for 30 minutes prior to infection with *M.tb* MOI of 10 for 4 hours. Bars represent mean +/- SD of samples in triplicate and is representative of 2 separate experiments. P=NS.

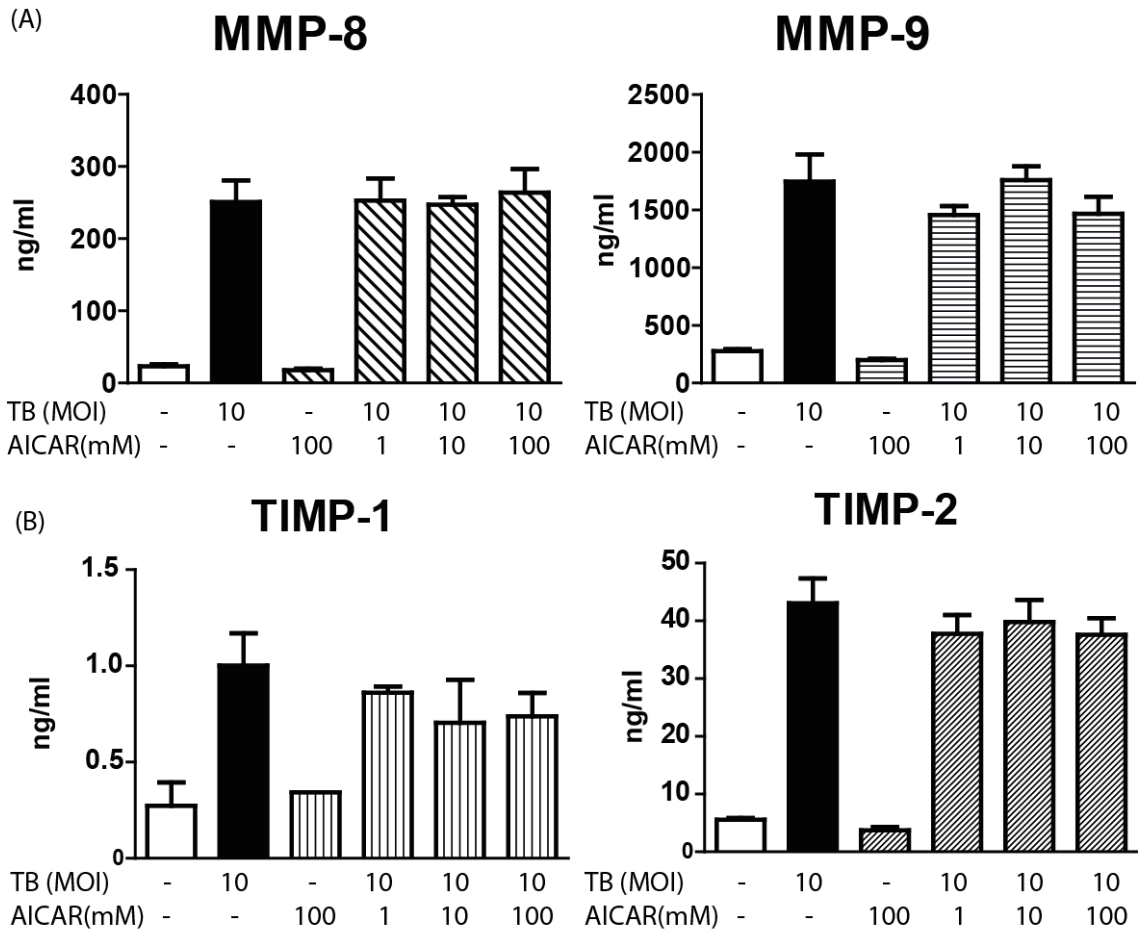


Figure 41: AMPK activation does not affect neutrophil MMP secretion in *M.tb* infection. 7.5 x 10^5 neutrophils were preincubated with AICAR for 30 minutes prior to infection with *M.tb* MOI of 10 for 4 hours. Supernatants were analysed using luminex array. (A) MMP-8 and -9. (B) TIMP-1 and -2. Bars represent mean +/- SD of conditions in triplicate and is representative of 2 independent experiments. P=NS for data.

2.9 siRNA attempt on neutrophils

One criticism on the use of pharmacological inhibitors is that they may have non-specific properties and the suppression of MMP secretion, if any, may be due to these non-specific properties that they possess. To circumvent this, I attempted siRNA on human neutrophils. The main bulk of literature on the use of this technology has been mainly reported on neutrophil differentiated HL-60 (human promyelocytic leukemia cells), but the use of the technology has been increasingly reported in primary human neutrophils [270, 271], and hence I thought this would be a feasible approach. I used the Deliver X siRNA kit (Panomics, France) which utilised a novel "MPG" delivery technology which uses virus-derived amphipathic peptides that directly interact with nucleic acid cargos to form nanoparticles (150-200nm) capable of diffusing through plasma membranes and releasing their contents inside the cell [272]. The mechanism of entry is receptor-independent, involves MPG/lipid membrane interactions and avoids the endocytic pathway thus preventing degradation of nucleic acid cargos. This method was chosen in preference to other technology as there was a publication substantiating its use in primary human neutrophils [273]. Lipid-based technology such as lipofectamine has low transfection efficiency in neutrophils and require cell-sorting to select for transfected cells (personal communication with Dr R Mittal, Division of Infectious Diseases, Children's Hospital Los Angeles). Furthermore, techniques that involve electro or nucleoporation inherently causes degranulation in primary human neutrophils [274] since the process permeabilises the plasma membrane which would undesirable in my experiments since MMP secretion would be affected. Initial experiments to silence GAPDH to assess transfection efficiency appeared successful with gene knockdown of more than 70% (**Figure 42**),

and neutrophil viability using propidium iodide staining was reasonable. However AMPKα1 and α2 gene silencing were inconsistent with CoMTB stimulation despite varying the time of stimulation after transfection from immediate to letting the transfected cells rest overnight and stimulating them the following morning. Eventually, I reverted to confirm if GAPDH protein was decreased with gene silencing using western blotting as I was doubtful whether neutrophils were successfully transfected but GAPDH protein suppression was not detected (data not shown). siRNA was thus abandoned after 6 months.

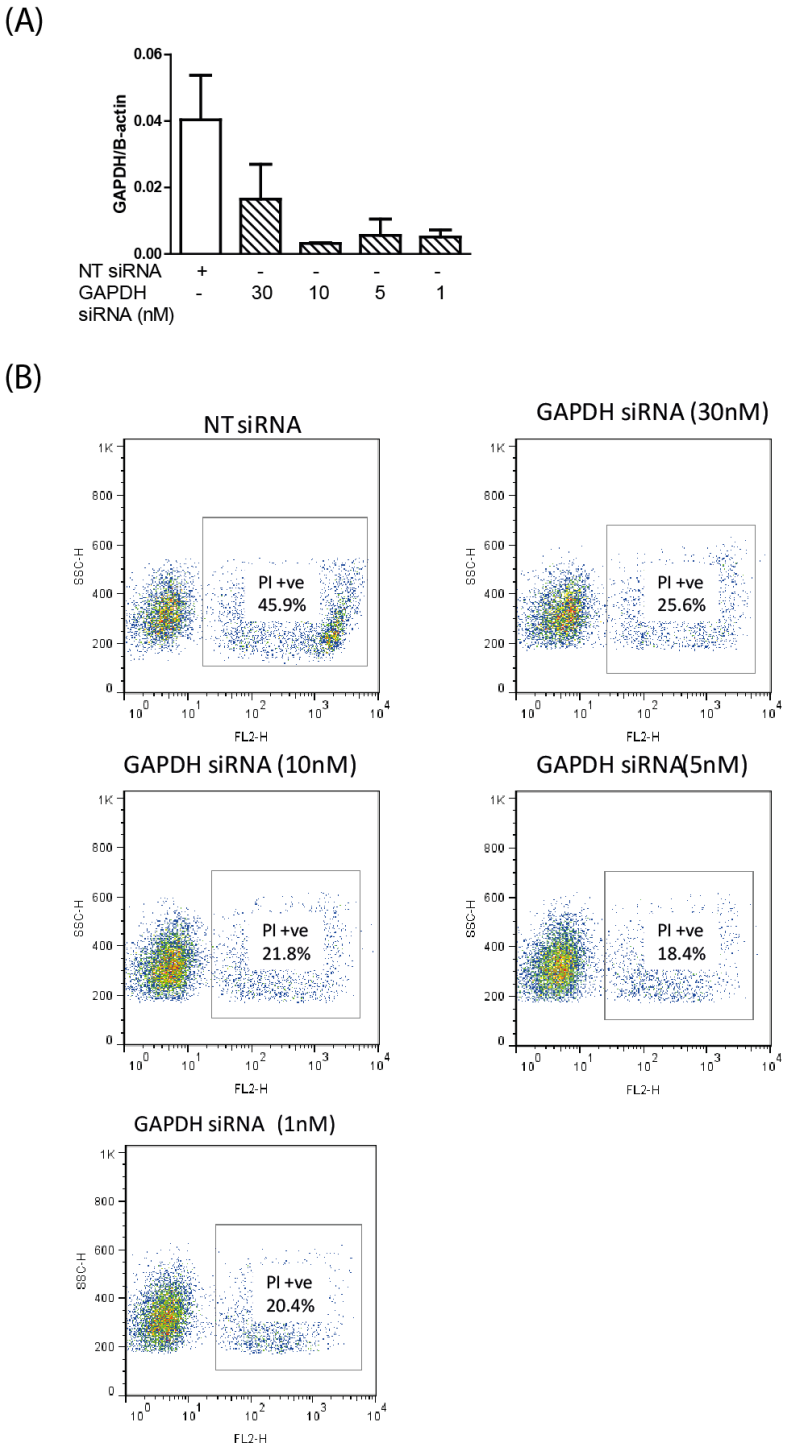


Figure 42: GAPDH silencing in human neutrophils. Primary human neutrophils were transfected with non-targeting siRNA (NT-siRNA) or GAPDH siRNA for 4 hours before cells were lysed and RNA extracted. (A) Real-time PCR GAPDH normalised to beta-actin. Bars represent mean +/- SD of samples in duplicate. (B) Neutrophil viability by FACS. Neutrophils were stained with 20 μg/ml propidium iodide and 10 000 events were gated.

2.10 Neutrophil MMPs are decreased with CoMTB stimulation in patients with AMPK mutation

I next looked at the neutrophil MMP secretion from a rare group of patients with AMPK mutations. This was done in collaboration with the Department of Cardiology at The Heart Hospital which has 3 of such patients. These patients present with cardiomyopathy as well as cardiac arrhythmias and have been genotyped to have AMPKγ2 mutations [275]. They have dysfunctional AMPK phosphorylation [276] and defects in activation of the α1β1γ2 complexes by AMP [277, 278]. Neutrophils from healthy volunteers were also stimulated at the same experimental setting. There were thus 3 sets of AMPK and healthy controls (S1-S3) and their details are in **Table 8**.

With CoMTB stimulation, neutrophil MMP-8 and -9 was decreased in 2 out of 3 of the AMPK patients compared to healthy volunteers (**Figure 43A, B and C**). The pooled data showed that MMP-8 was decreased with CoMTB stimulation ($p<0.01$) and MMP-9 showed a trend towards suppression ($p = 0.093$) (**Figure 43D**). In the context of *M.tb* infection, both neutrophil MMP-8 and -9 secretion was unaffected in the pooled data. I next evaluated the AMPKα activation and was surprised to note that the AMPK patients had increased basal activity of AMPKα1/2 (T172) phosphorylation (**Figure 43E**). The p70S6 kinase phosphorylation was similar in both healthy and AMPK patients. Attempts to probe for phospho-TSC which is also downstream of AMPK as well as AMPKγ2 were unsuccessful (data not shown) as there were no signal for the 2 proteins.

Set	Healthy		AMPK gene mutation patients		
	Age	Gender	Age	Gender	Disease manifestation
S1	25	M	47	M	Hypertrophic cardiomyopathy with permanent pacemaker
S2	32	M	55	M	Hypertrophic cardiomyopathy with implantable cardio-defibrillator, myopathy
S3	32	F	40	F	Hypertrophic cardiomyopathy with implantable cardio-defibrillator

P= NS for age in the groups by Mann-Whitney.

Table 8: Demographics of healthy controls and patients with AMPK gene mutation.

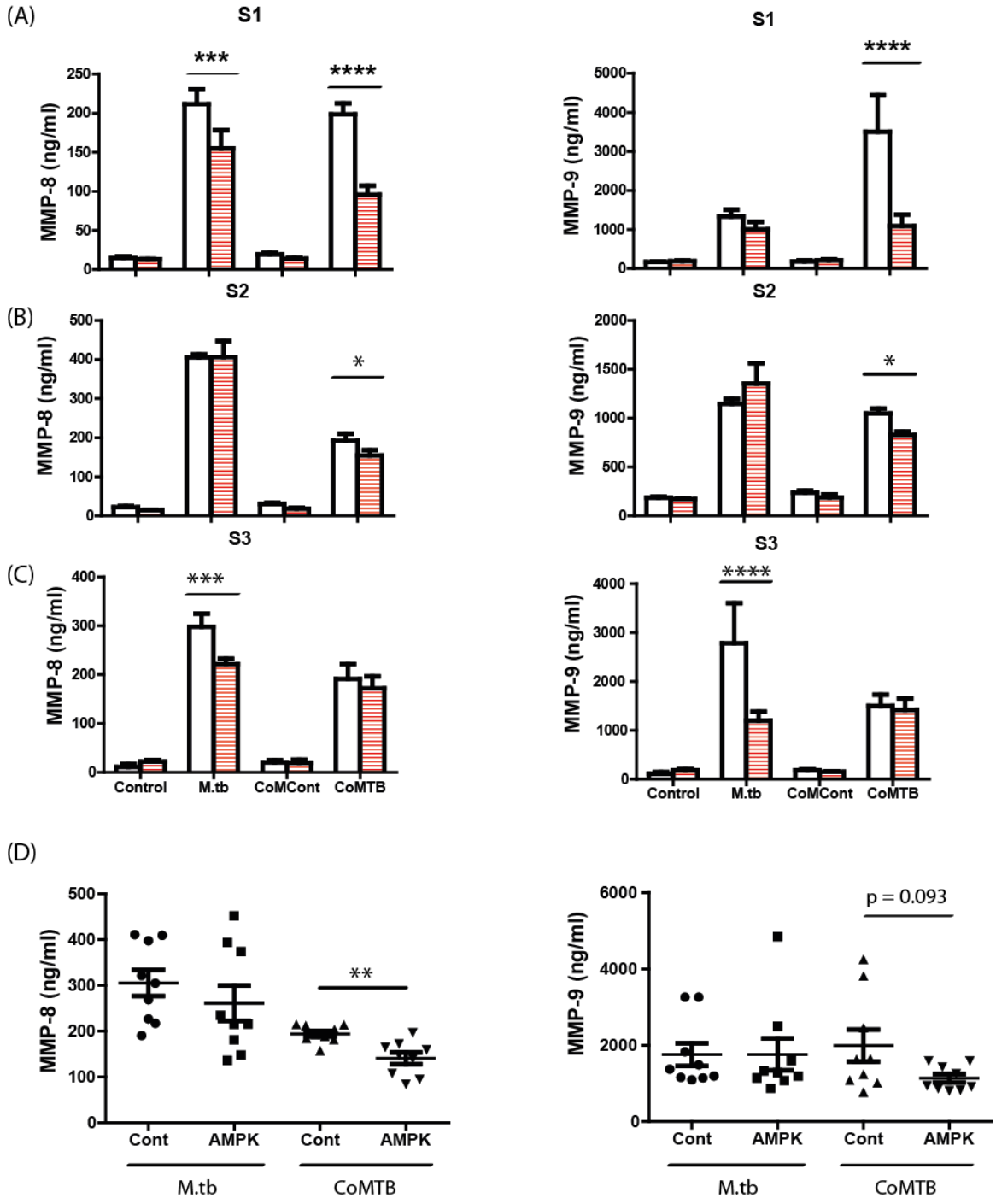


Figure 43: Neutrophil MMP secretion is decreased with CoMTB stimulation in AMPK patients. (A-C) Individual sets S1-3 of healthy volunteers and AMPK patients. 7.5 x 10^5 neutrophils with infected with *M.tb* MOI 10 or CoMCont/CoMTB 1:5 dilution for 4 hours and supernatants were analysed using luminex array. Open bars denote healthy control and red bars denote AMPK deficient patient. Bars represent mean +/- SD of triplicates. (D) Pooled data from 3 healthy-patient sets. Each point represents an individual sample.

(E)

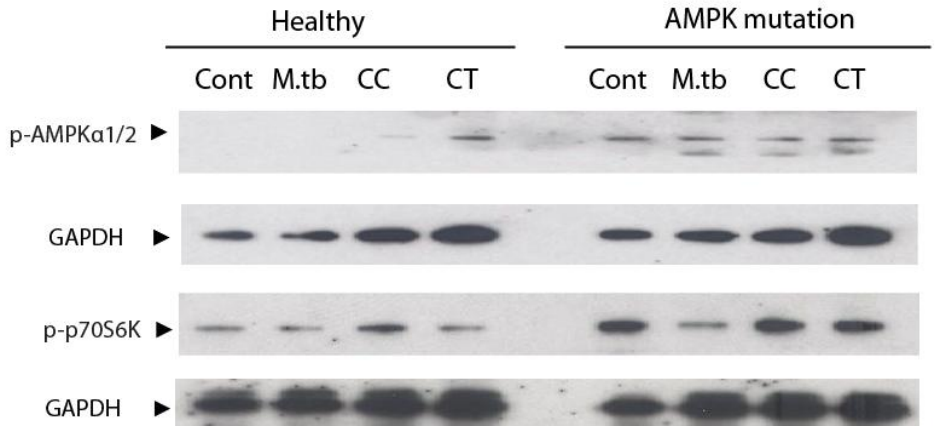
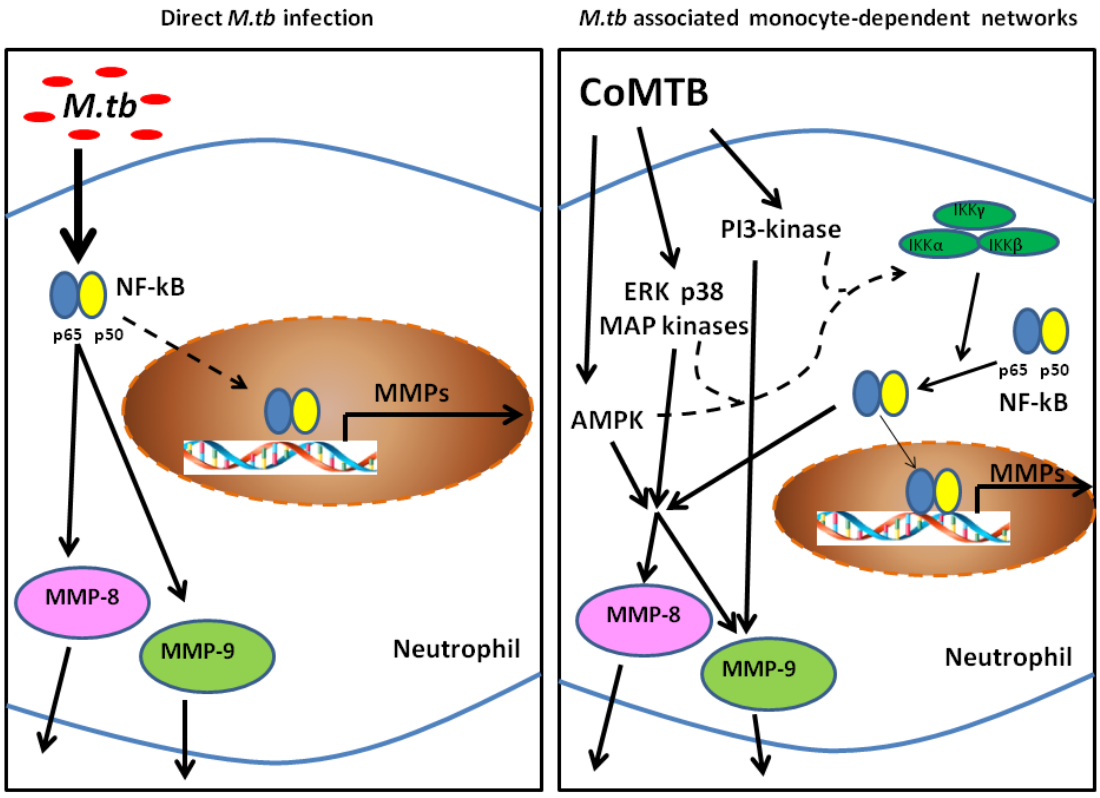


Figure 43 (continued): (E) Phospho-AMPKα1/2 and phospho-p70S6k in healthy and AMPK patient. 2 x 10^6 neutrophils were infected with *M.tb* MOI 10 or CoMCont/CoMTB 1:5 for 30 minutes and cells were lysed. Data representative of the 3 healthy-patient sets.

3. *Discussion*

In summary, I have shown that neutrophil MMP secretion is stimulus specific, with *M.tb* infection and CoMTB stimulation driving different intracellular signalling pathways (see diagram below) resulting in MMP-8 and -9 secretion.



I have established that the p38/ERK MAP kinases, PI3-kinase and the AMPK pathways are activated when neutrophils are infected with *M.tb*. However, MMP-8 and -9 secretion are not suppressed with chemical inhibitors (PD 98059, SB 203580, LY 294002 and Compound C) in *M.tb*-infected neutrophils. One possible explanation is the dose of *M.tb* used at MOI of 10 simply overwhelmed the system and caused massive unregulated secretion of the MMPs. Another explanation is that the secretion of MMPs in *M.tb*-infected neutrophils is regulated by a different pathway, independent of the MAP-kinase, PI3-kinase and AMPK pathway. Thirdly, the pathways could still be regulating neutrophil MMP secretion but this was not demonstrated in my set-up. This

can be due to the requirement of using a much higher dose of inhibitors or a different inhibitor that act on the same path. Regardless, intracellular signalling paths involve activation of NF-kB which can be suppressed by the p65 subunit inhibitor Helenalin. Interestingly IKK2 inhibitor SC-514 did not affect the neutrophil MMP secretion in this context. The NF-kB pathway comprises of the canonical and non-canonical pathways. It is the latter that is independent of IKK2 [279] and it might be that the NF-kB non-canonical pathway regulates neutrophil MMP secretion in *M.tb* infection.

Intracellular signalling pathways that regulate MMP secretion from CoMTB-stimulated neutrophils include the ERK/p38 MAP kinase and the PI3-kinase pathways and differs from the *M.tb*-infected neutrophils. It is interesting that MMP-9 secretion but not MMP-8 is regulated by the PI_3-kinase pathway. This suggests that the secretion of MMP-8 and -9 is regulated by different mechanisms. MMP-8 is stored in the secondary (specific) granules and MMP-9 is stored in tertiary (gelatinase) granules in neutrophils in their pro-forms. Recent studies suggest profound differences in the regulation of tertiary granules versus that of primary and secondary granules in neutrophils, [280, 281] and this may be the case for MMP-8 and -9 secretion. The finding that MMP-9 secretion is also regulated by PI3-kinase in neutrophils is consistent with literature [282]. With regards to transcription factors, herein also lies a difference from *M.tb* infected neutrophils. IKK2 inhibitor SC-514 was able to suppress the neutrophil MMP secretion with CoMTB stimulation and this suggests that neutrophil MMP secretion in this context was dependent both on the p65 subunit and IKK2 pathway, namely the canonical pathway.

The phosphokinase array further confirmed that the MAP kinase is activated with CoMTB stimulation. In addition AMPK, STAT, p53 and Yes were shown to be

activated. AMPK plays a vast role in cellular signalling including cellular energy homeostasis, carbohydrate and lipid metabolism, cell growth and apoptosis, and protein synthesis. It is activated by metabolic stresses such as hypoxia, ischemia, and low glucose when there is an increased AMP:ATP ratio. It is a heterotrimeric complex that comprises the alpha catalytic subunits and the β and γ regulatory subunits. Phosphorylation of Thr 172 in the activation loop of AMPK is required for AMPK activation and this was detected in the phosphoarray. STAT 5b and 6 were also activated. In neutrophils, STAT 5b is known to be activated by GM-CSF [283] which is present in CoMTB. STAT 6 is activated by IL-4 [284] and IL-13 [285], the former which is undetectable in CoMTB and the latter in small amounts (**Table 7**). It would have been ideal to determine if the STAT pathways do regulate neutrophil MMP secretion but this was hampered by the inavailability of a specific STAT inhibitor, and that siRNA is technically challenging in neutrophils. The p53 pathway is a well-known regulator of cell apoptosis but neutrophils are terminally differentiated cells and so it is interesting that this is activated with CoMTB stimulation. One possible explanation is that p53 regulates NF-kB activity in neutrophils as LPS treated $p53^{-/-}$ neutrophils demonstrated increased NF-kB DNA binding compared to $p53^{+/+}$ neutrophils [286]. Lastly, Yes is a member of the src family of kinases and is activated when neutrophils degranulate [287] and is involved in neutrophil signal transduction[288].

I have demonstrated in my *in vitro* experiments that AMPK regulates MMP-8 and -9 secretion in CoMTB stimulated neutrophils. Inhibition of AMPK with Compound C caused a dose-dependent suppression of the MMPs. Ideally, a second specific AMPK inhibitor should be used to confim this but there was none available currently. Furthermore, siRNA attempts were not successful. Although I observed gene

silencing with GAPDH that was used as a positive gene knockdown, this did not translate into protein silencing in neutrophils. Otherwise, siRNA would have been a useful technology to confirm that neutrophil MMP secretion was regulated by AMPK.

An often used method in immunology to investigate a particular pathway or protein is by utilising a genetic approach with transgenic animals. I studied the role of AMPK using humans with AMPK γ2 mutation. AMPKα mutations have yet to be described in man. Even though the subunit of interest is different, it had been shown that these patients have defects in activation of the α1β1γ2 complexes by AMP [277, 278]. All three patients have decreased neutrophil MMP secretion compared to healthy volunteers whose neutrophils were taken in the same experimental setting. This is likely due to their inherent genotype and phenotype but a possible confounding factor is that these patients are also on medication which may have affected neutrophil MMP secretion. This confounding factor however cannot be removed. There is nevertheless consensus between the *in vitr*o findings and that from the AMPK patients. Neutrophil MMP secretion was not affected in *M.tb*-infection with AMPK inhibition with Compound C nor in the AMPK patients (pooled data). Furthermore in CoMTB stimulation, AMPK inhibitor Compound C and in AMPK patients MMP-8 secretion was suppressed more than MMP-9. Ideally there should have been more healthy-patient sets to further confirm but we only have access to these AMPK patients in London. I have chosen to extract neutrophils from healthy volunteers at the same time to minimise factors such as environmental temperature or extraction processes in a different setting which may introduce variability to neutrophil MMP secretion. It is known that variations in temperature modify the effects of proinflammatory cytokines on cells [289]. Furthermore, neutrophil apoptosis varies with temperature with

apoptosis suppressed at temperatures lower than 15^{o}C [290]and thus if there is difference in environmental temperatures for 2 experiments, neutrophil degranulation which inherently depends on viability of cells will be different.

It is interesting to note that the basal AMPKα phosphorylation is increased in these patients although this has been previously reported [276]. The current consensus appears that in such patients basal activity of AMPK is increased which reduced the sensitivity of the protein to AMP [291]. Investigation of mTOR/p70S6 kinase pathway which is downstream of the AMPK pathway showed that neutrophil MMP secretion was independent of it. Inhibition with rapamycin did not affect neutrophil MMP secretion. Furthermore, the phosphorylation of p70S6 kinase in the AMPK patients was similar to healthy controls.

The AMPK pathway is a novel pathway in the context of infection. Upon activation, AMPK increases cellular energy levels by inhibiting anabolic energy consuming pathways such as fatty acid synthesis and protein synthesis and stimulate energy producing catabolic pathways (fatty acid oxidation, glucose transport, etc). Hypothalamic AMPK activation reverses cancer anorexia by blocking the expression of interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) in rats [292], while AMPK inhibition induces feeding-independent weight loss in thyrotoxic rats [293]. It is possible that AMPK may play a role in the anorexia and weight loss seen in TB patients who are in a state of catabolism. AMPK also functions in inflammatory conditions and infectious diseases. Activation of AMPK has been shown to decrease neutrophil pro-inflammatory activity and decrease TNF-α in a murine model of acute lung injury [268]. Thus one would have expected that AMPK activation and not inhibition would

have decreased neutrophil MMP secretion but the reverse was found in my experiments.

AMPK has a role in mycobacterial infections. Mycobacterial antigens activates autophagy in monocytes by activating AMPK [294]. Trials on the use of cilostazol, a phosphodiesterase (PDE_3) inhibitor that also activates/phosphorylate AMPK[295] have been done in tuberculosis and have been shown to shorten TB therapy in murine models by improving sterilisation of mouse lungs one month earlier than the standard 6 month TB drug regimen [296]. Furthermore, cilostazol decreases TNF-α in mouse lungs compared to untreated mice and acts as an immunomodulatory agent. The AMPK pathway may be a potential therapeutic point for interventions to modulate the host response and improve outcome.

Together, neutrophil MMP secretion in *M.tb* infection is dependent on transcription factor NF-kB but I have been unable to demonstrate inhibition of neutrophil MMPs with MAPK, PI3kinase and AMPK inhibitors. These pathways however may still be regulating neutrophil MMP secretion in direct *M.tb* infection. Conversely with CoMTB stimulation, I have demonstrated inhibition of neutrophil MMPs with the respective pathway inhibitors and confirmed that MMP secretion is dependent on the MAPK, PI3kinase, AMPK pathways and on the canonical pathway of NF-kB but independent of the mTOR/p70S6 kinase pathway. *In vitro* and human data support that the AMPK pathway regulates neutrophil MMP secretion in monocyte-dependent networks in TB. The AMPK pathway is a novel and potential point in which therapeutic interventions may be made to improve outcome in TB infections.

CHAPTER 6 – THE POTENTIAL ROLE OF NEUTROPHIL MMPS IN TB PATIENTS: ANALYSIS OF INDUCED SPUTUM SAMPLES FROM A COHORT OF PERUVIAN TB PATIENTS

1. *Introduction*

In the previous chapters, I described the upregulation of neutrophil MMPs in both *M.tb* infection as well as monocyte-dependent networks, and explored the mechanisms regulating neutrophil MMP secretion. To ascertain what I found *in vitro* of the neutrophil matrix degrading phenotype would translate to patients, I investigated the induced sputum samples from a group of healthy controls and TB patients from Peru. This was done in collaboration with Dr Cesar Ugarte-Gil from the Universidad Peruana Cayetano Heredia in Lima who shipped over the samples and was part of a longitudinal study assessing MMP secretion with anti-tuberculous treatment [297].

In TB patients, neutrophils are the predominant phagocytic cells in their respiratory secretions [121] and in murine models their accumulation has been shown to be detrimental to the host [298]. I wanted to determine if neutrophils played a role in TB associated tissue destruction in man. I first assessed markers of neutrophil activation in the respiratory secretions of TB patients and determined if these correlate with induced sputum MMP-8 and -9. I further investigated if the neutrophil MMPs would correspond to the severity of presentation and disease. Our group previously published data showing that in a smaller group of patients comprising of 32 patients with respiratory symptoms as controls and 33 TB patients that MMP-1 and -3 are upregulated in TB patients compared to controls [185] but MMP-8 and -9 were not found to be elevated. An important difference from my current study is that the controls in that study had diseases such as pneumonia and underlying cancer which

may inevitably raise neutrophil MMPs. Hence it is crucial to evaluate how the induced sputum MMPs in TB patients would compare to healthy controls.

Lastly, I looked at immunohistochemistry staining of both human TB lung specimens as well as CNS-TB from patients in London to investigate neutrophil MMP-8 and -9 *in vivo*. This was done in collaboration with Dr Jo Porter at UCL and Dr Federico Roncaroli at the Neuropathology Unit at Charing Cross Hospital.

2. *Methods*

Study recruitment

This study received the Institutional Review Board approval from Universidad Peruana Cayetano Heredia (Lima, Peru) and the Regional Health Department in East Lima. A subset of 108 TB and control patients were randomly selected from the original cohort of 137. TB patients were recruited at the time of diagnosis prior to starting TB therapy and were smear and/or culture positive, had no prior history of TB or TB treatment and were HIV negative. Healthy controls were recruited from the same healthcare facilities and were recruited among the relatives who were accompanying pregnant women, children for routine medical visit or patients with non-related TB disease. They were age ≥ 18 years, did not have symptoms associated with TB, no known TB contact and had a normal chest radiograph and a negative sputum TB culture. All subjects underwent HIV testing and chest radiography.

TB score

To evaluate the severity of the disease, we used the TB Score [299] which is a clinical score that evaluated the following: cough, haemoptysis, dyspnoea, chest pain, night sweats, conjunctival pallor, tachycardia, axillary temperature above 37^{o}C, body mass index and middle upper arm circumference (MUAC).

CXR score

Chest radiographs were scored for degree of pulmonary infiltration with Image J 1.43U (NIH, USA) using the formula: **(Area of TB consolidation/Total lung area) x (Mean Absorbance of TB consolidation/Mean lung absorbance) x 100%**

Induced sputum analysis

Induced sputum of at least 3 mls was obtained from TB patients and healthy controls and the samples were sterile filtered using a 0.2μm Durapore membrane (Millipore). Total protein concentrations were measured using Bradford assay (Sigma-Aldrich, St Louis, MO, USA). Concentrations of MMP-1, -2, -3, -7, -8, -9 were analysed by Luminex multiplex array (R&D Systems, Minneapolis, MN, USA). TIMP-1 and TIMP-2 were analyzed by ELISA kit (R&D Systems, Minneapolis, MN, USA). The former two analyses were performed by Dr Cesar Ugarte-Gil. Human myeloperoxidase ELISA (R&D Systems) and human NGAL ELISA(Bioporto Diagnostics, Denmark) and DQ collagenase assay were analysed as described in Chapter 2 – Materials and Methods.

Analysis of NETS

Induced sputum samples were diluted 1:2 with TE buffer and extracellular DNA was quantified using QuantiT Picogreen (Invitrogen, Paisley,UK) as previously described in Chapter 2. This was normalised to total protein concentration. For detection of H3 citrulline, an induced sputum sample with a protein content of 10μg was acetone precipitated overnight using a ratio of 1:4 protein to acetone volume ratio. The precipitate was spun 10, 000g for 10 minutes at 4^{o}C, supernatant decanted and the residual acetone was left to evaporate for 5 minutes at room temperature. The protein pellet was subsequently washed with 1 ml of 20 mM Tris HCl 80% acetone, spun at 10, 000g for 5 minutes and solubilised with 10 μL of SDS loading buffer. This was evaluated using western blotting as previously described.

Confocal microscopy

Permanox chamber slides (Nunc Labtech, Thermo Fisher Scientific) were coated with 0.1 mg/ml fibrinogen with 25 µg/ml of Type I DQ collagen for 30 minutes and then aspirated. Induced sputum were diluted 1:10 with 1x reaction buffer and activated with activated with 4-amino-phenyl mercuric acetate (APMA) (Sigma-Aldrich) for 1 hour at 37^{o}C and this was added to the collagen coated slide. The slide was incubated for 24 hours and fluorescent images were captured using Leica confocal microscope (Leica TCS SP5).

Statistical analysis

Continuous variables were compared with Mann-Whitney while categorical variables were analysed using Fisher‘s exact tests. Multiple intervention experiments were compared with one-way ANOVA followed by Tukey‘s post-test correction, using GraphPad Prism Version 5.04 for Windows (GraphPad Software). A $p-$ value of less than 0.05 was taken as statistically significant using a two-tailed test.

3. *Results*

3.1 Neutrophil MMP-8 and -9 are significantly raised in patients with TB and correlate with markers of neutrophil activation

I initially evaluated neutrophil myeloperoxidase (MPO) and neutrophil gelatinase associated lipocalin (NGAL) *in vitro* to assess their suitability as markers of neutrophil activation. An experiment was done assessing of the effect of *M.tb* added together with CoMTB on neutrophil MMP secretion. This demonstrated that the effects of stimulating *M.tb* with CoMTB were additive on neutrophil MMP-8 and -9 secretion (**Figure 44)**. Both MPO and NGAL concentrations correlated strongly with MMP-8 and -9 ($r > 0.8$, $p<0.0001$ for all), confirming their suitability as surrogate neutrophil markers for the clinical specimens (**Figure 45**). In the induced sputum samples, MMP-1,-2, -3, -8 and -9 are significantly elevated in TB patients[297]. Of note, MMP-8 had the highest concentrations and MMP-9 the second highest of all the MMPs (MMP-8 $p<0.0001$ and MMP-9 = 0.0007) (**Table 9** and **Figure 46A**). In addition, MPO and NGAL were also significantly elevated ($p<0.0001$ and $p<0.01$ respectively) (**Figure 46B and C**). When analysed, MMP-8 and MMP-9 concentrations strongly correlated with those of MPO and NGAL with a Spearman's correlation coefficient of above 0.5 ($p<0.0001$ for all) (**Figure 46D to G**). Correlation analysis revealed that MPO and NGAL concentrations correlated the strongest with MMP-8 (MPO with MMP-8, $r=0.8259$, $p<0.0001$; NGAL with MMP-8, $r=0.6833$, $p<0.0001$) (**Table 10**), which is highly suggestive that MMP-8 in the induced sputum in TB patients is derived from neutrophils.

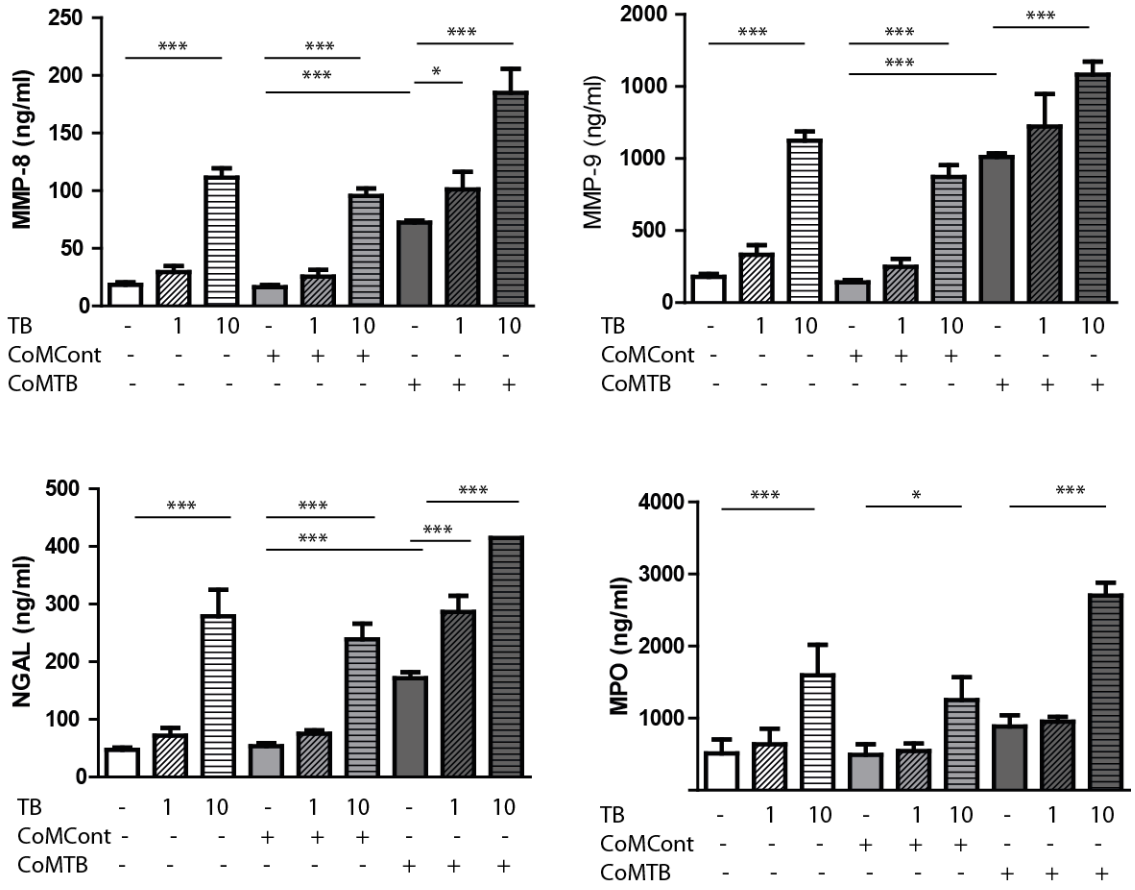


Figure 44: Effect of *M.tb* with CoMTB on neutrophils *in vitro*. 7.5 x 10^5 neutrophils were incubated with the respective stimuli for 4 hours and MMP-8, -9, NGAL and MPO were measured. Bars represent mean +/- SD of experiment done in triplicate and is representative of 2 independent experiments.

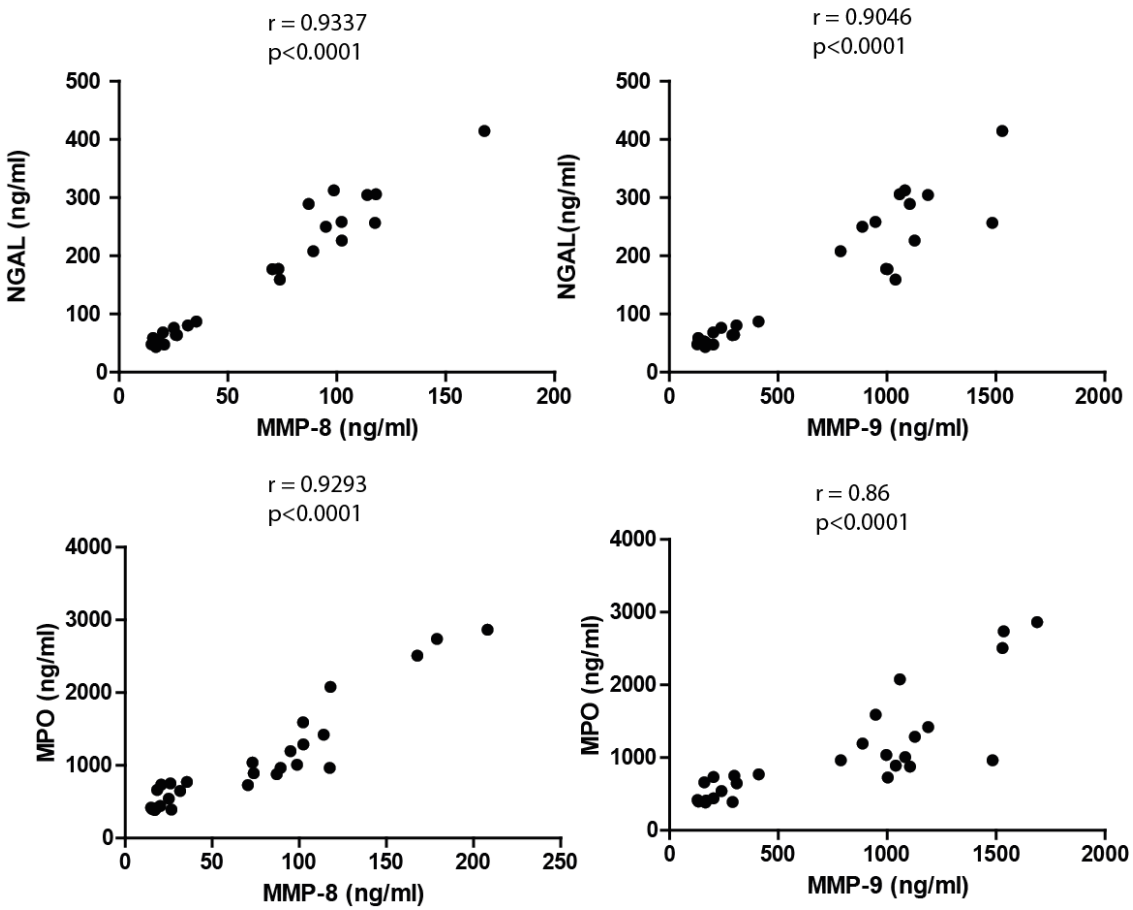


Figure 45: Effect of *M.tb* with CoMTB on neutrophils *in vitro* – correlation analysis. Correlation of NGAL and MPO with MMP-8, and NGAL and MPO with MMP-9 from Fig. 43. Correlation was done using Spearman's coefficient. *p<0.05, ***p<0.001.

Variable[a]	Control	TB	P value[b]
Number	57	51	
Sex (M:F) n	11 : 46	32 : 19	<0.0001
Age (years)	28 (18-62)	25 (18-72)	0.1571
BMI	25.1 (18.5-37.5)	20.9 (11.8-27.9)	<0.0001
Pulse (per min)	76 (61-104)	96 (64-139)	<0.0001
MMP-1 (pg/ml)[c]	285.9 (132.4-776.6)	1394 (406-4759)	<0.0001
MMP-2 (pg/ml)	1065 (384.3-2737)	4329 (1858-9012)	<0.0001
MMP-3 (pg/ml)	952.6(311.4-2366)	11237 (3861-31330)	<0.0001
MMP-7 (pg/ml)	39425 (23548-54187)	36930 (29441-46905)	0.5629
MMP-8 (pg/ml)	89114 (21956-268483)	772962 (338088-1590000)	<0.0001
MMP-9 (pg/ml)	8840 (250.6-53291)	74733 (8334-651907)	0.0007
TIMP-1 (pg/ml)	16582 (7997-39908)	91890 (32587-179832)	<0.0001
TIMP-2 (pg/ml)	2975 (1421-6171)	10878 (5429-18839)	<0.0001

a. Values are median (Interquartile range)
b. Comparison of continuous variables using Mann-Whitney U and categorical variables using Fisher‘s exact.
c. All MMP and TIMP values are normalised to total protein content.

Table 9: Demographic data, MMPs and TIMPs of control and TB patients. This was from a subset of 137 control and patients from the original study [297].

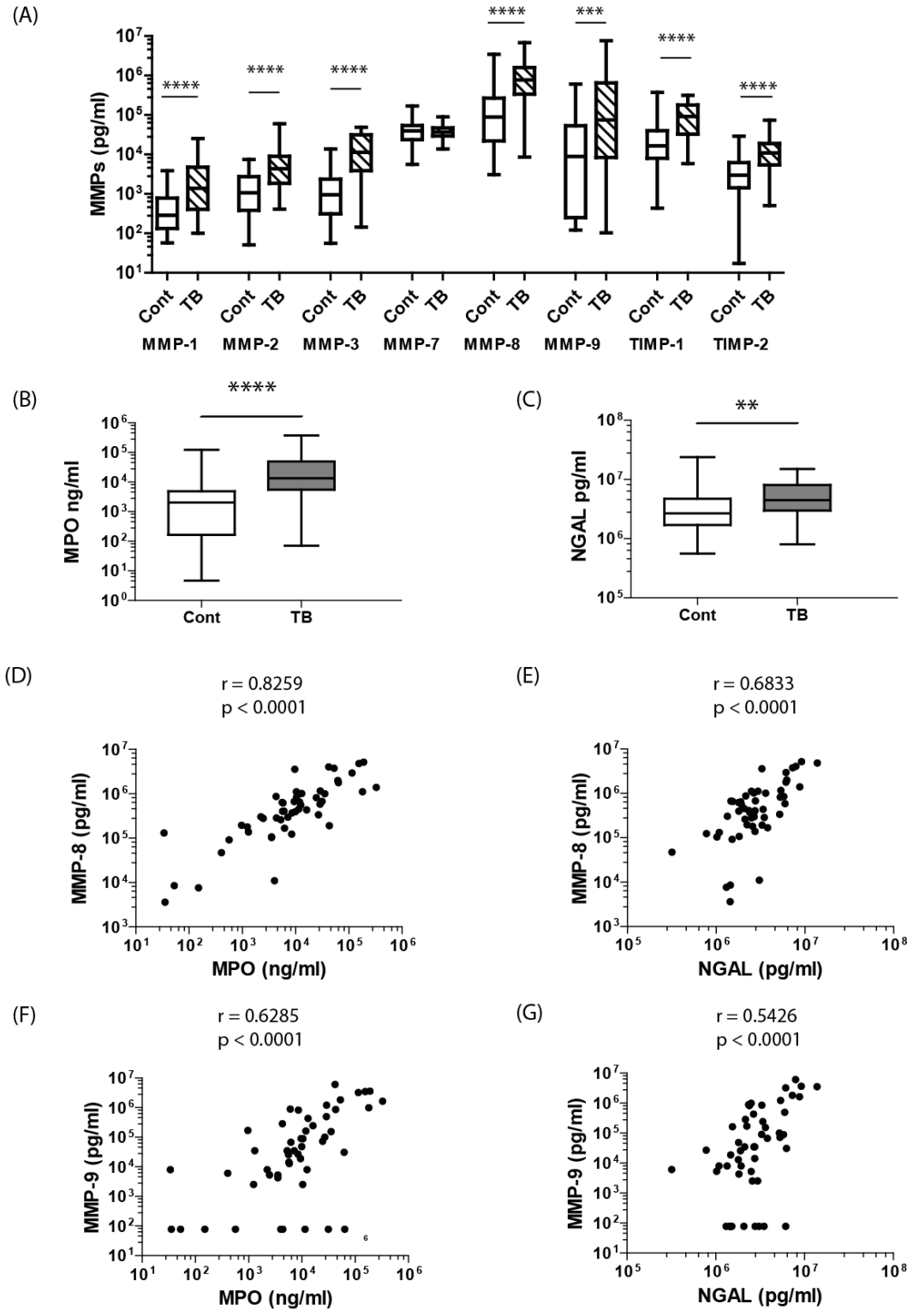


Figure 46: MMP-8 and -9 are raised in TB patients and correlate with markers of neutrophil activation MPO and NGAL. (A) Induced sputum from controls (open bars) and TB patients (hashed bars) were analysed for MMP-1, -2, -3, -7, -8, -9 and TIMP-1 and -2. (B and C) MPO and NGAL from control and TB patients. (D-G) Correlation between MMP-8 and -9 with MPO and NGAL using Spearman's correlation coefficient in TB patients (n=51).

Neutrophil marker	Variable	Correlation[a]	p-value
MPO	MMP-1	0.1051	0.4629
	MMP-2	0.6218	<0.0001
	MMP-3	0.3567	0.0102
	MMP-7	0.2167	0.1267
	MMP-8	0.8259	<0.0001
	MMP-9	0.6285	<0.0001
	NGAL	0.7486	<0.0001
NGAL	MMP-1	0.3382	0.0152
	MMP-2	0.5765	<0.0001
	MMP-3	0.4335	0.0015
	MMP-7	0.2827	0.0444
	MMP-8	0.6833	<0.0001
	MMP-9	0.5426	<0.0001

a. Correlation using Spearman‘s coefficient.
b. n=51 TB patients

Table 10: Comparison of neutrophil markers myeloperoxidase (MPO) and neutrophil gelatinase associated lipocalin (NGAL) with MMPs and TIMPs in TB patients.

3.2 MMP-3 and -8 sputum concentrations strongly correlates with patient TB score and CXR score

I next evaluated if MMP-8 would correlate with clinical severity using the TB score as well as the chest radiograph score. One limitation was that not all the chest radiographs were available. Despite this, MMP-3 and MMP-8 strongly correlated with both TB score (MMP-3, $r = 0.6$, $p<0.0001$; MMP-8, $r =0.55$, $p<0.0001$) and CXR score (MMP-3, $r = 0.59$, $p<0.0001$; MMP-8, $r = 0.52$, $p<0.001$) (**Table 11**).

Clinical Variable	Measured Variable	Correlation coefficient [a]	P value
TB score	MMP-3	0.6077	<0.0001
	MMP-8	0.5566	<0.0001
	MPO	0.4619	<0.0001
	MMP-2	0.4515	<0.0001
	MMP-1	0.4313	<0.0001
	MMP-9	0.3453	0.003
	NGAL	0.2251	0.0192
	MMP-7	-0.04742	NS
CXR score	MMP-3[b]	0.5930	<0.0001
	MMP-8[b]	0.5166	<0.0001
	MMP-2[b]	0.4966	<0.0001
	MPO[c]	0.3873	0.0035
	MMP-1[b]	0.3619	<0.0001
	MMP-9[b]	0.3073	0.0077
	NGAL[c]	0.1452	0.2901
	MMP-7[b]	0.01310	NS

a. Correlation done using Spearman's correlation coefficient. MMP-8/-9, MPO and NGAL are normalised to total protein content.
b. Data available for n=74
c. Data available for n=55

Table 11: Analysis of TB and CXR scores with MMPs, myeloperoxidase (MPO) and neutrophil gelatinase associated lipocalin (NGAL).

3.3 Induced sputum of TB patients have increased collagenase activity which is abrogated with neutralisation by anti-MMP-8 antibodies

I next assessed the collagenolytic activity in the induced sputum of TB patients and control samples. As the main collagen in the lung is Type I [223], the effect of induced sputum on Type I DQ collagen destruction was evaluated. The induced sputum from TB patients had an increased collagen destroying activity compared to healthy controls ($p = 0.02$) (**Figure 47A**). In addition, confocal microscopy revealed patches of fluorescence indicating areas of collagen destruction (**Figure 47B**). When analysed, only the presence of MMP-8 significantly correlated with Type I collagenase activity ($r = 0.6894$, $p=0.0004$) (**Table 12**, **Figure 47C**) indicating that induced sputum MMP-8 was most likely responsible for Type I collagen destruction. When MMP-8 activity was neutralised using an antibody (without pre-incubation), collagenase activity decreased from a mean of 723.8 mU/ml (SEM 254.4 mU/ml) to 528.1 mU/ml (SEM 270 mU/ml) ($p = 0.01$) (**Figure 47D**). In retrospect, a period of pre-incubation of the neutralising MMP-8 antibody with the induced sputum samples may further reduce the collagenase activity.

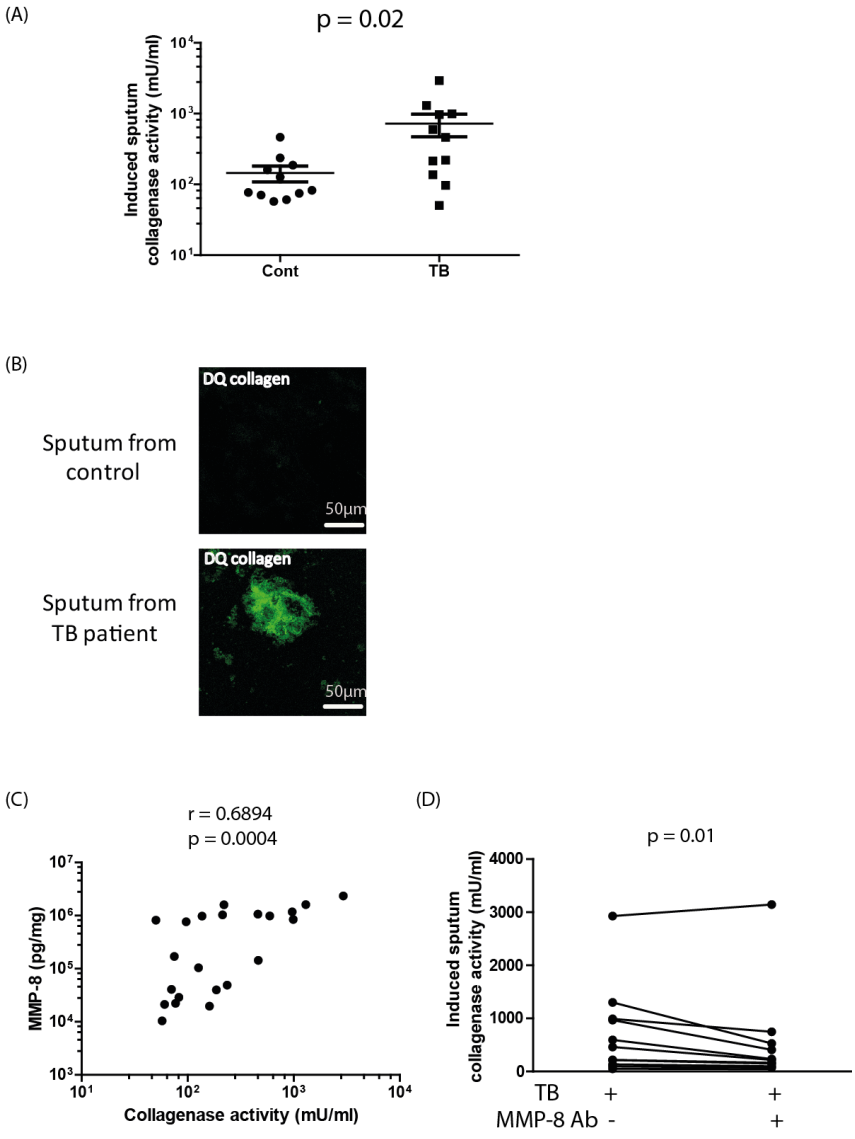


Figure 47: Induced sputum of TB patients have increased capability for collagen destruction. (A) Induced sputum were activated with 2 mM APMA for 1 hour and collagenase activity assessed using Type I DQ collagenase assay. N=11 for both controls and TB patients. (B) Confocal microscopy of induced sputum samples on Type I DQ collagen. Samples were activated with APMA for 1 hour and incubated for 24 hours on DQ collagen. N = 3. (C) Correlation of MMP-8 and collagenase activity. (D) Collagenase activity is neutralised with MMP-8 neutralising antibody. MMP-8 neutralising antibody was added to the DQ collagenase assay without pre-incubation. N = 11. MMP-8 concentrations from the 11 samples were not statistically different from the 51 TB patients by Mann-Whitney.

Measured Variable	Measured Variable	Correlation coefficient [a]	P value
Type 1 collagenase activity	MMP-1	-0.1654	ns
	MMP-2	0.1406	ns
	MMP-3	0.3721	ns
	MMP-7	-0.2072	ns
	MMP-8	0.6894	0.0004
	MMP-9	-0.09656	ns

Correlation using Spearman's correlation coefficient. MMPs have been normalised total protein content. N = 11 controls and 11 TB patients.

Table 12: Comparison of Type 1 collagenase activity versus MMP concentration in induced sputum.

3.4 Induced sputum MMP-1 has minimal collagenase activity in TB patients

The other interstitial collagenases are MMP-1 and MMP-13. I focused on the possibility that MMP-1 activity could also contribute to Type I collagen destruction since MMP-13 concentrations were not detected. First, I performed an *in vitro* analysis of MMP-1 on Type I collagen degradation using purified MMP-1 from fibroblasts. This showed that MMP-1 had minimal collagenase activity compared to neutrophil supernatants which contained the same protein concentration of MMP-8 (**Figure 48A**). I had refrained from using molarity as MMP-8 exists in different molecular weights. The molecular weight of latent neutrophil MMP-8 is 85 kDa while activated MMP-8 is 64 kDa. MMP-1 is 54 kDa. Hence even if MMP-8 concentrations are compared at a molarity level, this was less than MMP-1. In this context, the Type I collagenase activity of MMP-8 was still higher than that of MMP-1. I analysed the collagenase activity of 5 samples and the effects after neutralising MMP-1. There was no significant change from a mean of 106.8 mU/ml (SEM 10 mU/ml) to 102.1 mU/ml (SEM 9.25) (p = NS) (**Figure 48B**). The MMP-1 concentrations of the 5 samples were not significantly differerent from the original group of TB patients.

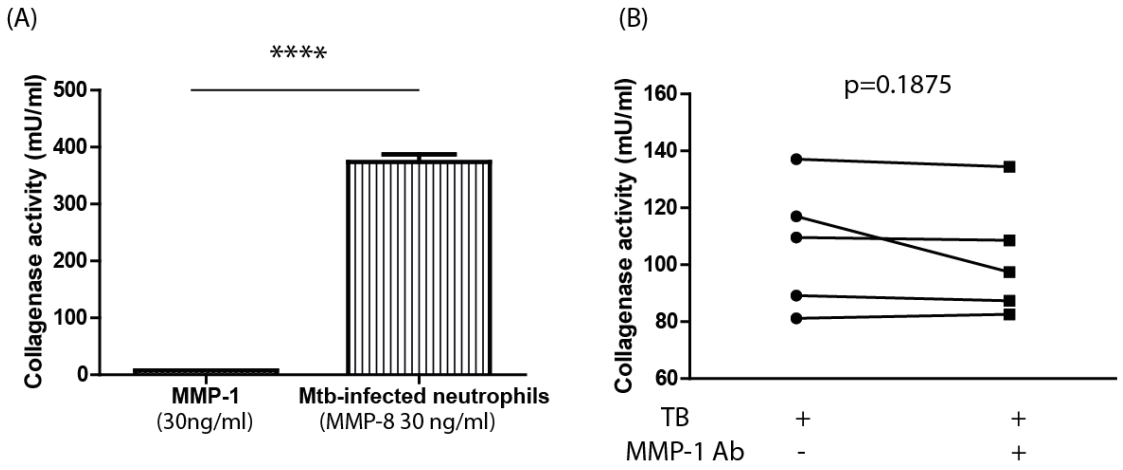


Figure 48: (A) *M.tb* infected neutrophils secreting MMP-8 preferentially degrade Type 1 collagen compared to fibroblast derived MMP-1. Fibroblast MMP-1 and *M.tb*-infected neutrophil supernatants were activated with 2mM APMA for 1 hour and incubated with Type I DQ collagen for 24 hours. **(B) Neutralising MMP-1 antibody in induced sputum of TB patients does not decrease collagenase activity (n=5).** Induced sputum diluted 1 in 10 were activated with 2mM APMA for 1 hour and subsequently incubated with Type I DQ collagen with or without neutralising MMP-1 antibody for 24 hours. MMP-1 concentrations from these 5 samples were not statistically different from the 51 TB patients.

3.5 Components of NETS are present in the induced sputum of TB patients

I next investigated if NETS or their components could be detected in the induced sputum samples. A previous study looking at NETS in the respiratory secretions in mice given pulmonary instillation of LPS and *P.aeruginosa* used confocal microscopy staining for NETS, Picogreen QuantIT kit to detect extracellular DNA and detecting citrulline H3 using western blotting [300], a well-known marker for NETS [301, 302]. Unfortunately the TB induced sputum samples were sterile filtered which would have removed neutrophils and hence made it impossible to perform confocal microscopy to detect NETS. However it was possible to use the latter 2 techniques on the induced sputum samples. I used samples from 10 controls and 10 TB patients. Extracellular DNA was raised at a mean concentration of 2830 ng/mg (SEM 479.7 ng/mg) compared to controls at a mean concentration of 855.8 ng/mg (SEM 241 ng/mg) ($p=0.0021$) (**Figure 49A**). As this could be due to lysed cells, I subsequently looked for and detected citrulline H3 in the induced sputum samples of TB patients but not in controls (**Figure 49B**). I went on to confirm that this was not due to dead cells by comparing PMA stimulated neutrophils which produces NETS to Triton-X lysed neutrophils. The latter did not produce citrulline H3 (**Figure 49C**).

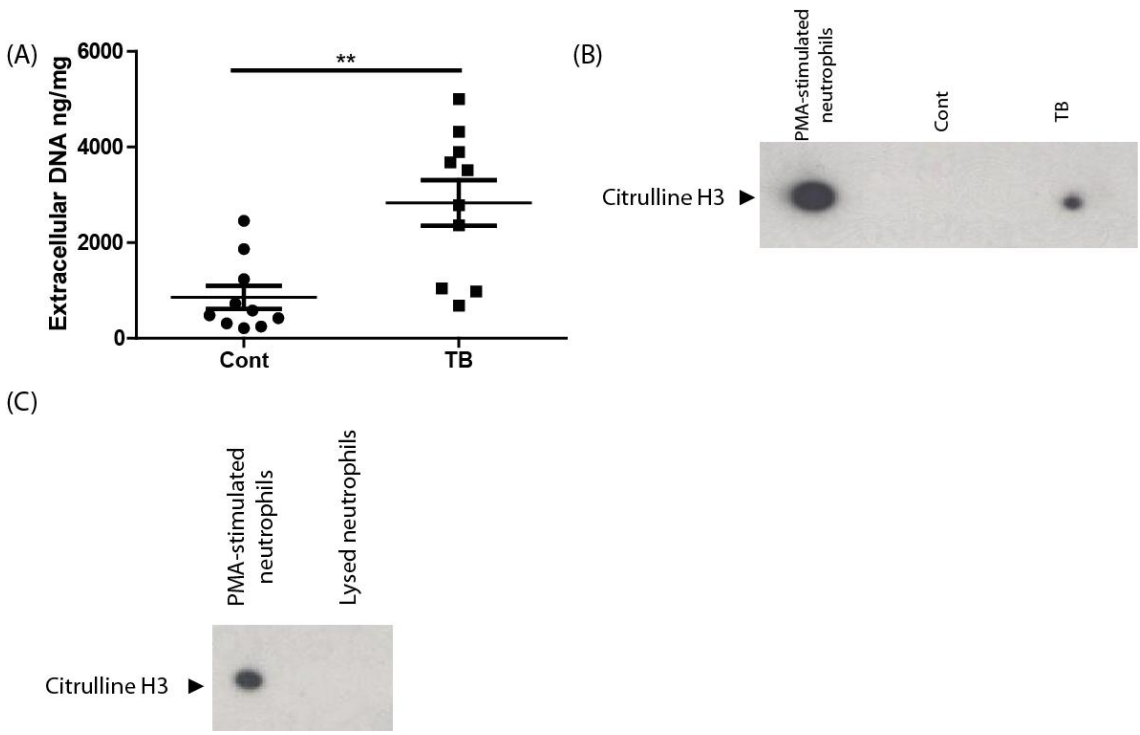


Figure 49: Induced sputum from TB patients contain components of NETS. (A) Induced sputum samples were diluted 1:2 and extracellular DNA was quantified using Picogreen QuantIT (n=10 for both groups). Values were normalised to total protein content by Bradford assay. (B) Citrulline H3 is present in induced sputum of TB patients. 10µg of protein was acetone precipitated and immunoblotted. Blots represent 2 independent experiments. (C) Dead cells do not contain citrulline H3. Supernatants from neutrophils stimulated with 20nM PMA was used as a positive control while supernatants of 0.1% Triton-X lysed neutrophils was used to represent dead cells. 10µg of protein was acetone precipitated and immunoblotted.

3.6 Neutrophils expressing MMP-8 and -9 are present in human lungs and CNS infected with TB

Next, human lung TB sections from the UK were analysed by immunohistochemistry in collaboration with University College London. These patients were confirmed to have pulmonary TB. Neutrophils were present in the inner wall of the pulmonary cavity (**Figure 50A** and **B**) and they stained positive for MMP-8 and MMP-9 (**Figure 50C**) with these stains located within the neutrophil cytoplasm. Phospho-AMPKα1/2 (T172) stained the nucleus of neutrophils indicating that it is predominantly intra-nuclear (**Figure 50C**). Concurrently in collaboration with the neuropathologist unit in Charing Cross Hospital, immunohistochemistry was performed on human TB brain biopsy specimens. This also demonstrated that neutrophils containing MMP-8 and -9 were present in human TB infected brains (**Figure 51**).

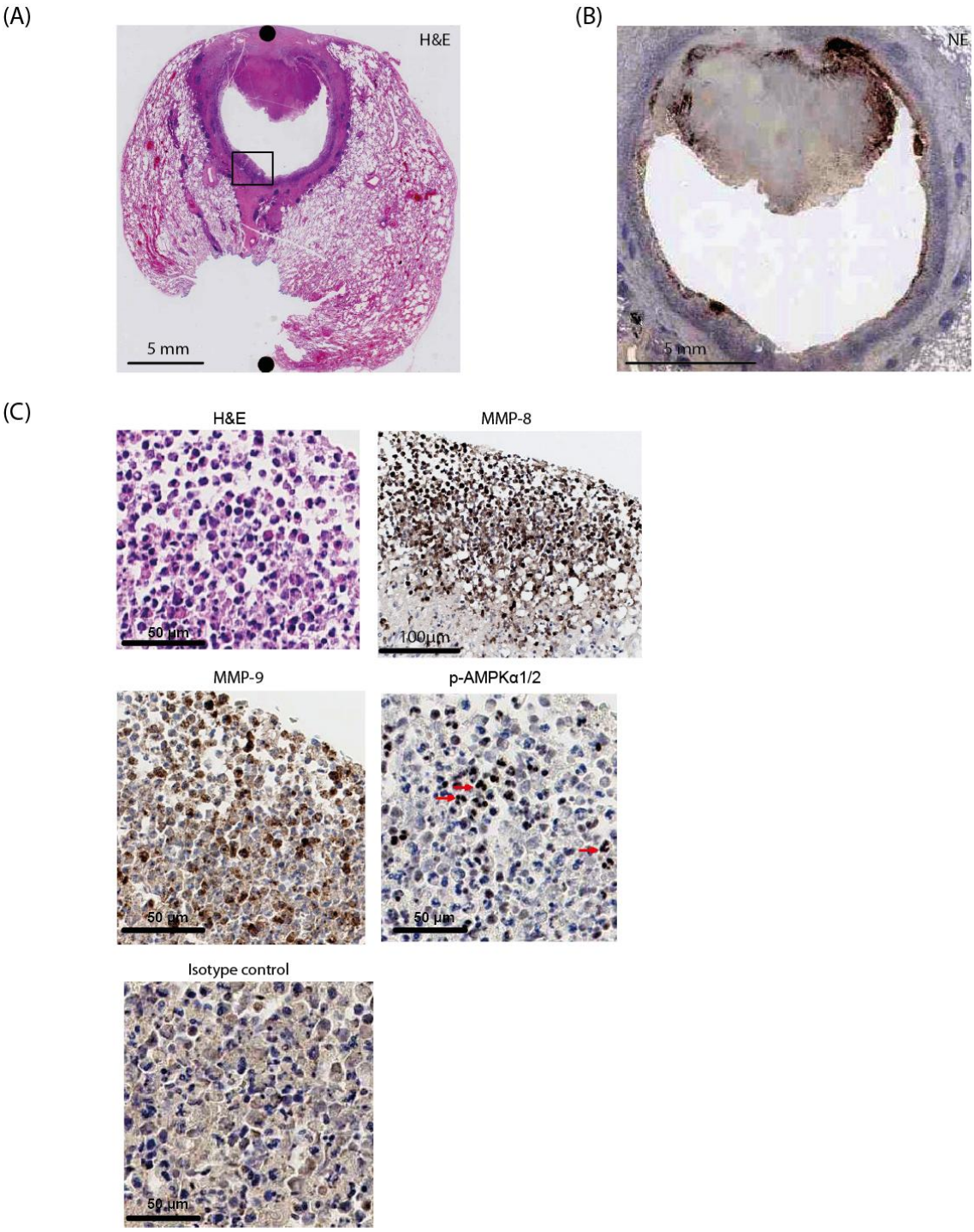


Figure 50: Neutrophils are present in human TB lung. (A) H&E (B) Neutrophil elastase stain (NE) present in the inner wall of the cavity (C) Inset of (A) magnified demonstrating neutrophils stain positive for MMP-8, MMP-9 and phospho-AMPKα1/2(T172) (red arrows) and isotype control antibody.

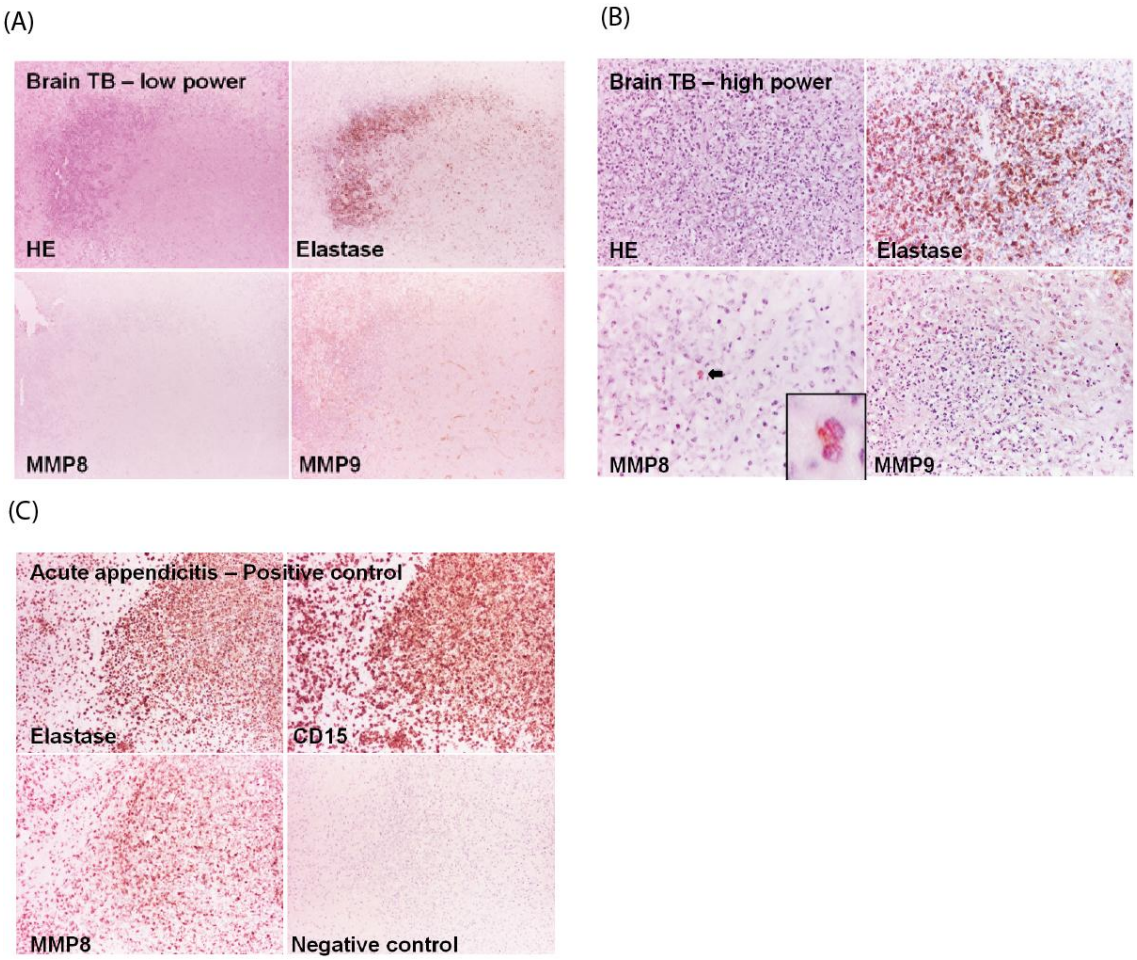


Figure 51: Neutrophils are present in CNS-TB. (A) Low power magnification (4x). (B) High power magnification (10x). Inset (40x). (C) Appropriate positive and negative controls for the antibodies.

4. *Discussion*

This chapter seeks to fulfil the final aim of my project which is to investigate MMP and TIMP activity and their regulation in neutrophils *in vivo*. I have approached this by evaluating the induced sputum samples of TB patients as well as human TB lung and brain specimens. I have found that MMP-8 and -9 concentrations are raised in the induced sputum of TB patients compared to healthy controls and these correlated with the markers of neutrophil activation i.e neutrophil myeloperoxidase and neutrophil gelatinase associated lipocalin. This is the first time that both MMP-8 and -9 concentrations in respiratory secretions have been found to be raised in TB patients. Our group has published on MMPs in respiratory secretions in 2 other TB patient groups [185, 186]. The first compared TB patients with patients with respiratory diseases and found that MMP-1 and -3 but not MMP-8 or -9 were not elevated [185]. Walker et al [186] in a cohort which has both HIV-positive and HIV-negative TB patients demonstrated that MMP-1, -2, -3 and MMP-8 but not -9 were elevated. The 2 groups studied had inherent differences when compared to the group that I used for my project which analysed TB patients and healthy controls in HIV negative subjects and this could account for why both MMP-8 and -9 are raised in this study and not others.

There is no one ideal marker of neutrophil activation and I have chosen to use both neutrophil myeloperoxidase and neutrophil gelatinase associated lipocalin (NGAL) which are secreted in large amounts in activated neutrophils and are commonly used neutrophil markers [303-305]. One limitation is that myeloperoxidase and NGAL are also secreted from other cells (albeit in smaller quantities) such as monocytes [306] and macrophages [307] and hence the decision to use not just one marker but two to increase the specificity to neutrophil origin when correlating induced sputum MMP-8

and -9 to them. Furthermore, I have confirmed in my *in vitro* work prior to testing on clinical samples that both MPO and NGAL concentrations closely correlated with MMP-8 and -9 concentrations. The induced sputum MMP-8 and -9 from TB patients also correlated strongly with MPO and NGAL all with an r value of above 0.5 indicating that the majority of the MMP-8 and -9 present was most likely neutrophil derived. The correlation was stronger with MMP-8 than MMP-9 suggesting that MMP-8 was more neutrophil specific. In addition MMP-8 and -9 have the highest concentrations among all the MMPs in the induced sputum which supports them being predominantly neutrophil derived given that neutrophil influx is a hallmark of active pulmonary TB[121]. Interestingly when correlating MPO and NGAL with the other MMPs, MMP-2 had the second highest r value after MMP-8 (r=0.6218 with MPO and r = 0.5765 with NGAL). MMP-2 is a gelatinase that is secreted by macrophages and Th1 cells [308] and the former is also known to secrete NGAL. It is possible that activated macrophages in the induced sputum of TB patients are responsible for the MMP-2 and MPO/NGAL correlation.

When evaluating tissue destruction using the TB clinical severity score and CXR score, MMP-3 and MMP-8 were found to have the strongest correlations (r values > 0.5). MMP-3 is a stromelysin secreted by activated/infected respiratory epithelial cells and it is likely that the effect of *M. tuberculosis* infection of these cells with or without resultant cell death caused a decline in respiratory function clinically which is also reflected radiographically. It is interesting to note that MMP-8 is also associated with clinical and radiographic severity and this is possibly due to its matrix destructive properties. MMP-8 substrates include Type I, II, III, VIII and X collagen as well as cartilage aggrecan, laminin, fibronectin and proteoglycans[309]. The human lung is

made up of many of such substrates to provide the structural support. The high MMP-8 found in the induced sputum of TB patients thus would result in consequent pulmonary tissue destruction as reflected by the strong clinical and radiographic correlation.

Collagen is resistant to proteolytic degradation and only MMPs are able to initiate collagen degradation [153]. Type I collagen is the main structural protein of the human lung[223] and the question was which collagenase is responsible for its degradation. I have demonstrated that the induced sputum of TB patients have increased collagenase activity and that only MMP-8 concentration correlated with Type I collagen degradation. Furthermore, MMP-8 neutralisation decreased collagen destruction which further supports it being the main collagenase responsible for destroying structural protein in the human lung. The other interstitial collagenases that can degrade Type I collagen are MMP-1 and MMP-13, the former which is in lower concentrations than MMP-8 in the induced sputum and the latter was not detected. It is crucial to note that previous literature had made observations that MMP-8 is more potent in degrading Type I collagen than MMP-1 [200, 201]. My *in vitro* work using fibroblast-derived MMP-1 on DQ collagen degradation showed that there was minimal Type I collagenolytic activity with MMP-1 compared to neutrophil supernatants containing the same concentration of MMP-8 further substantiates this. Furthermore, neutralising MMP-1 in the induced sputum of TB patients did not decrease the collagenolytic activity.

I have demonstrated that components of neutrophil extracellular traps are found in the induced sputum of TB patients by showing an increase in extracellular DNA compared to controls as well as the presence of citrulline H3 a well known marker of NETS [301, 302]. During neutrophil extracellular trap formation, histones undergo

post-translational modification with the enzyme peptidylarginine deiminase 4 (PAD4) converting arginine to citrulline [301]. There is currently no evidence supporting citrullination of H3 occuring during cell death. One other possible cause of increased extracellular DNA is increased cell death in the respiratory secretions in TB patients. However citrulline H3 is not present in the supernatants of lysed neutrophils. Ideally to prove that NETS are truly present in the induced sputum of TB patients, immunofluorescence staining for DNA and neutrophil granule proteins should be done. However this could not be done as the samples have been sterile filtered prior to shipping to our lab.

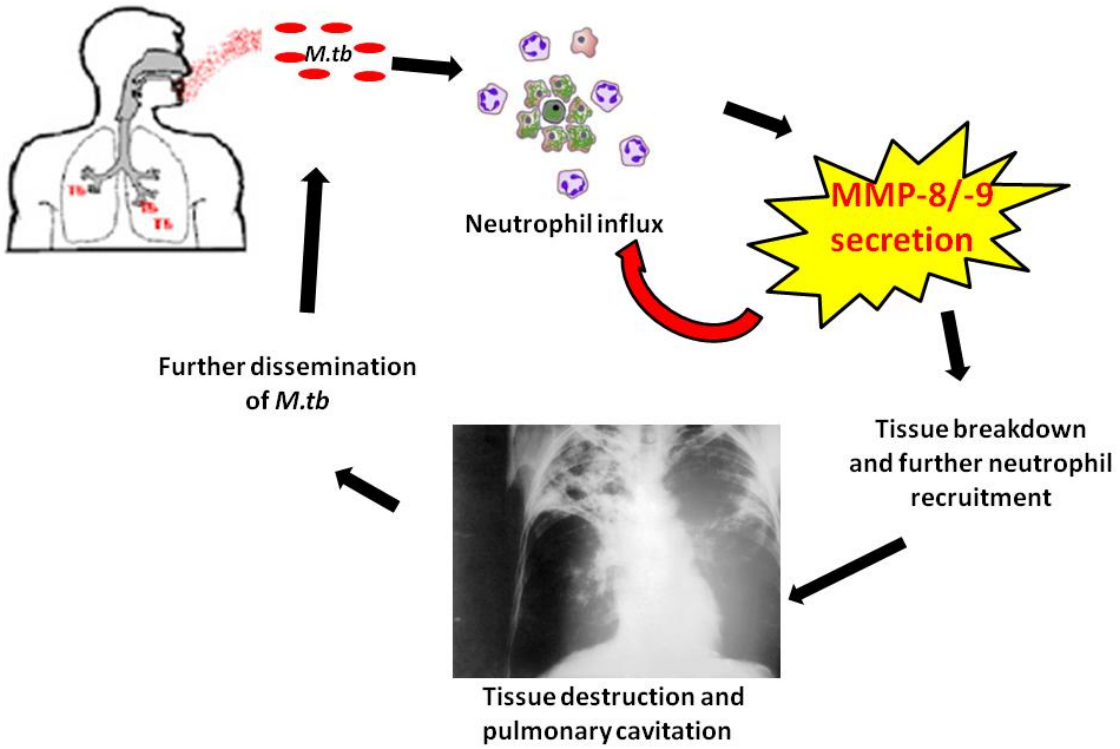
Neutrophils were present along the circumference of the inner wall of the lung cavity in the human TB lung biopsy specimens as indicated by H&E stain by morphology and a positive neutrophil elastase stain. In addition, these neutrophils stain positive for MMP-8 and -9. This suggests that neutrophils are eroding the wall of the cavity. The presence of MMP-8 and -9 staining in the cytoplasm are in keeping with intra-cellular storage of neutrophil MMPs. In addition, phospho-AMPKα is present in the nuclei of the neutrophils in keeping with nuclear translocation of phospho-AMPK consistent with literature [310]. When cells were exposed to stress such as heat, energy depletion and oxidants, the level of phospho-AMPKα accumulates in the nuclei [310] and relocates back into the cytoplasm with removal of the stress and this shuttling is dependent on carrier Crm1. It is likely a similar stress situation occurs in the context of *M.tb* infection resulting in the positive nuclear staining of phospho-AMPK as seen in the lung biopsy specimens. The immunohistochemistry studies of the CNS-TB biopsy specimens also demonstrated staining of neutrophils positive for MMP-8 and -9, underscoring the significance of neutrophils in TB.

In summary, I have shown that neutrophil MMP-8 and -9 are raised significantly in the induced sputum samples of TB patients compared to controls and that the majority of MMP-8 and -9 are most likely neutrophil derived. Induced sputum MMP-8 correlated with TB clinical severity score and CXR score. The induced sputum from TB patients have an increase in MMP-8 dependent Type I collagen destruction and contain components of NETS. MMP-8 and -9 positive neutrophils are present in the inner wall of the cavity in human TB lung biopsy and CNS-TB specimens which support their significant role in tissue destruction

CHAPTER 7 - CONCLUSION

In this chapter, I shall first discuss how my research contributes to the knowledge of the role of neutrophils in TB pathogenesis. I shall then explore the potential interventions that can be made to modulate tissue destruction in TB at an extracellular level as well as at sites of intracellular signalling pathways and transcription factors.

Much of TB pathogenesis has been thought to be attributed to cell-mediated immunity with important roles played by alveolar macrophages and T-cells. My data has elucidated an additional key role that neutrophils play in TB pathogenesis, particularly tissue destruction. The question is however what function they play. It has been previously suggested that neutrophil proteolytic enzymes contribute to cell death as well as dissolution of lung tissues [311, 312]. My results support and expand this theory by demonstrating for the first time that this is in part due to the secretion of neutrophil MMP-8 and -9, which destroy Type I and IV collagen respectively upon *M.tb* infection. Although MMPs particularly MMP-9 are crucial for neutrophil transmigration to the site of infection [313], exposure to *M.tb* results in excessive secretion of neutrophil MMPs with consequent collateral damage. Neutrophils are nevertheless professional phagocytes and with their additional ability to form extracellular traps, they help to contain the dissemination of *M.tb* within the host. It is probably in such a scenario that pulmonary cavitation occurs in TB – the continuous influx of neutrophils causes a vicious cycle of tissue destruction with further influx of neutrophils consequent to the MMPs that they secrete.



Neutrophils causing a vicious cycle of tissue destruction and dissemination of *M.tb*

Pulmonary cavitation arising from tissue destruction is a hallmark of TB infection. Cavitation contributes to the spread of the disease to new hosts and the resultant global success of TB. This is because cavitatory pulmonary TB contain a higher mycobacterial burden [314] which frequently necessitates longer anti-tuberculous treatment. In my first chapter, I have demonstrated a dose-dependent increase in neutrophil MMPs with increasing *M.tb* MOI, and this supports the clinical finding that a higher bacterial burden is associated with increased cavitation. The finding in TB lung biopsy specimens of neutrophils lining the inner wall of the TB cavity possibly aiding in the erosion of the pulmonary interstitium in Chapter 6 further

supports this theory. Both direct *M.tb* infection and monocyte-dependent intercellular networks result in neutrophil MMP secretion. It is important however to keep in mind that tissue destruction is not solely dependent on neutrophils. Other cells such as macrophages, monocytes and respiratory epithelial cells also secrete MMPs explaining the general increase of MMPs found in the induced sputum of TB patients compared to controls in Chapter 6 and these together contribute to the breakdown of various components of the pulmonary extracellular matrix and cavity formation.

Further to their role in limiting dissemination of pathogens, neutrophils also kill via oxidative and non-oxidative mechanisms. In the latter case, neutrophils secrete a wide armamentarium of antimicrobial peptides including human neutrophil peptides 1-3, cathelicidin LL-37 and NGAL [126]. On the other hand, I have found that neither CoNTB which would contain all the above antimicrobial peptides, nor MMP-8 /-9 inhibit or kill *M.tb* in Chapter 4. This is possibly due to lower concentrations of such peptides secreted extracellularly and thus the lack of extracellular killing/inhibition against *M.tb*. It is likely that the antimicrobial peptides exert their activity within the intracellular compartment of the phagosome. However it is controversial if neutrophils kill *M.tb*. Corleis et al showed recently that neutrophils phagocytose but cannot kill *M.tb* when incubating both for up to 6 hours by assessing colony counts [315]. Conversely Brown et al demonstrated a reduction of $^{14}CO_2$ as a representation of growth by H37Rv *M.tb* when culturing neutrophils with H37Rv for 60 minutes [316]. Lowe et al similarly showed a reduction of relative light units when luminescent *M.tb* is incubated with neutrophils [317]. Nevertheless, it is probably the difference in the assays used to assess *M.tb* growth that may explain the different observations, and these need further investigation. It has been ascertained by Ramos-Kichik et al [143] that

neutrophil extracellular traps which are bactericidal to *Listeria monocytogenes*, do not kill *M.tb*. NETS prevent the dissemination of mycobacteria and this may set the scene for granuloma formation.

Neutrophils are thought to be essential for early granuloma formation [124]. Depleting neutrophils from mice impaired granuloma formation and neutrophil-mediated regulation of granuloma formation depended on chemokines signalling through CXCR3, especially MIG. Additionally, Volkman et al demonstrated that MMP-9 contributed to nascent granuloma maturation and bacterial growth in *M. marinum* infected zebrafish model, offering a possibility that targeting MMP-9 can hold promise as a host-targeting tuberculosis therapy [184]. There is also emerging data about the role of MMP-8 in TB. Lyadova et al observed that IL-11, MMP-8 and MMP-10 were over-expressed in I/St mice after infection with *M.tb* up to 5 weeks [318] and MMP-8 induction correlated with TB progression and weight loss. Furthermore, they identified lung phagocytes as the major immune cells producing cytokines and chemokines associated with TB progression in mice. In Chapter 6, I found that MMP-8 is increased in the induced sputum samples of TB patients and controls and this is predominantly neutrophil derived. Similar to the murine study aforementioned, MMP-8 correlated with clinical TB severity score and CXR score. Furthermore, the raised collagenolytic activity seen in the induced sputum of TB patients is likely due to MMP-8 as collagenolytic activity is suppressed with MMP-8 neutralisation. Neutrophils thus seem to be a double-edged sword in TB pathogenesis. They both limit the dissemination of *M.tb* and assist in granuloma formation but in so doing cause collateral damage by the secretion of their proteolytic enzymes and consequent tissue destruction.

To limit tissue damage in TB, it is accordingly crucial to modulate MMP secretion. Steroids are frequent adjunctive therapies used especially in the context of TB pericarditis and CNS-TB, where excessive inflammation in restricted spaces of these organs can be potentially lethal. Rarely, anti-TNFα agents have been used in CNS-TB in patients refractory to steroids [52, 53]. I have shown in Chapter 3 that both dexamethasone and anti-TNFα suppress neutrophil MMP-8 and -9 secretion, the latter at the level of gene expression. The inhibition of MMP secretion may explain how dexamethasone and anti-TNF agents act to decrease inflammation and improve the outcome in TB patients. In addition, I have also shown that doxycycline the only FDA licensed MMP inhibitor for use in treating periodontal disease [319], also decreases collagen destruction at an extracellular level in Chapter 3. This can potentially be a new adjunctive therapy to decrease tissue damage. Doxycyline has mycobactericidal effects, has been shown recently to decrease MMPs [186], and also suppresses TNF-α, a key cytokine in the immune response to TB [320]. Randomised control trials will need to be conducted to assess its efficacy as an anti-mycobacterial and immunomudulatory agent.

I have demonstrated in Chapter 5 that the MAP-kinase, PI_3-kinase and the AMPK pathway regulate neutrophil MMP-8 and -9 secretion in CoMTB stimulated neutrophils but not in *M.tb*-infected neutrophils. This indicates that neutrophil MMP secretion is stimulus specific. *M.tb* infection induces NET formation as well as neutrophil cell death while CoMTB stimulation results in degranulation of neutrophils and this may explain the divergence. Ideally to target tissue destruction in TB, an agent needs to suppress MMP secretion when neutrophils are exposed to both stimuli. I have shown that the NF-kB inhibitor Helenalin suppresses neutrophil MMP secretion both in

direct *M.tb* infection as well as CoMTB stimulation. Thus NF-kB may potentially be a target for therapeutic intervention in TB. Already, oncology clinical trials on NF-kB inhibitors are being carried out and some agents which have NF-kB inhibitory activities such as thalidomide and bortezomib (Velcade ®) are being routinely used in haematological malignancies like multiple myeloma. Animal and human trials need to be conducted to ascertain their efficacy in the context of TB, but considerations also need to be given to their side effect profile as well as drug costs.

In summary, I have shown that neutrophils contribute to TB pathology both in pulmonary and CNS-TB. They limit the dissemination of *M.tb* infection by phagocytosis and by production of neutrophil extracellular traps. Concurrently, infection with *M.tb* and stimulation by monocyte-dependent networks result in neutrophil MMP-8 and -9 secretion with consequent matrix destruction. Adjunctive TB therapies that decrease host inflammation such as dexamethasone and anti-TNFα suppress neutrophil MMP secretion and provides an insight into how these agent may act. Targeting neutrophil MMP-8 and -9 may decrease tissue destruction and potentially improve patient outcome.

CHAPTER 8 - FUTURE DIRECTIONS

In this chapter, I shall first discuss directions of research looking at evaluating further the pathogenesis of TB. I shall then discuss potential therapies that can improve patient outcome.

Hitherto, most TB research on treatment have concentrated on understanding and attacking the organism itself with much less attention being paid to the host response and the way that TB has exploited and evaded host immune control. In this thesis I have set out to partially redress this imbalance of knowledge. The role of neutrophils in immune responses to combat infectious diseases is well known and studied. Conversely very little is known about the role that these cells can play in advancing the disease by causing cavitation and lung destruction. What, for instance, delineates the ability of the host to form granulomas and effectively _contain' the disease (albeit in a latent state) and full-blown cavitation with bacterial dissemination? The presence of neutrophils in both these scenarios, particularly the latter is indicative of an active role of neutrophils in both of these processes. Although my data has shown a role that neutrophils and MMPs play in tissue destruction, one must bear in mind that there are also other proteases from other cells that contribute to tissue destruction in TB. There are a total of 553 human proteases encompassing threonine, aspartic, cysteine, metallo and serine proteases[321]. An in depth analysis of how these proteases and the role that they have in human TB cavitation is warranted. Ideally, human infected samples need to be analysed and compared with controls to determine the significance of these proteases, the role that they have in tissue destruction, mycobactericidal activities if any, cellular source of the proteases and if they would be amenable to

interventions that can reduce tissue damage. Additional work, be it *in vitro* and/or animal models need to be performed to substantiate the findings.

As host inflammation is a critical component in TB, investigations into how immune cells contribute to phenomenons such as paradoxical reactions, thrombosis and immune reactivation inflammatory syndrome (IRIS) in the context of co-infection with HIV is essential. Some of these immune reactions may be fatal even when the appropriate anti-tuberculous therapy is initiated. It is consequently important to determine the cause of such immune phenomena and how components such as neutrophil activity and functions can be regulated or controlled so as not to act to the detriment of the host. Conradie et al reported that in addition to NK cells, the proportion of neutrophils and IL-8 were significantly raised in patients undergoing TB treatment and then initiated anti-retroviral therapy as well as patients with TB-IRIS compared to HIV patients [322]. Furthermore, it has been described that neutrophil extracellular traps cause thrombosis [323] and even thrombotic microangiopathies [103]. Accordingly NETS may play a role in CNS-TB associated cerebrovascular events. Animal and human studies need to be further conducted to explore the pathogenesis of such inflammatory reactions.

The prolonged anti-tuberculous treatment of a minimum of 6 months combined with the need to take multiple drugs to treat TB contributes to non-adherence, treatment failure and emergence of drug resistance. Should a shortened treatment regimen be devised, this would improve adherence to drugs, decrease the incidence of treatment failure and consequently tackle the global problem of rising incidence of drug-resistant TB. We thus need an anti-tuberculous agent that has good tissue penetration and possess immunomodulatory activities to circumvent TB paradoxical reaction that is

frequently seen. There are several new drugs in the drug-discovery pipeline among them bedaquiline (TMC207), PA824, and OPC67683 which has the potential to shorten TB therapy. Bedaquiline has already been approved by the US FDA for treatment of drug-resistant TB [46] and has the potential in combination with other agents to deliver a shortened drug regime for drug-susceptible TB. Steroids are often administered in TB paradoxical reactions but some cases may prove refractory. It is thus necessary to evaluate other potential immunomudulatory agents as an adjunctive therapy and these include MMP inhibitors such as doxycycline, anti-TNFα agents, or NF-kB inhibitors. The ideal immunomodulatory agent should be cheap, easily administered by a convenient route with minimal side effects. Anti-TNFα agents are notoriously known for their potential to reactivate TB [324, 325] and are expensive. NF-kB inhibitors such as thalidomide and bortezumib have severe side effects. One might consider the use of MMP inhibitors such as doxycyline but considerations for tissue penetration need to be bourne in mind when evaluating the potential as an immunomodulatory agent. In the context of the lung, inhibitors could for example be delivered by nebulisation or by an inhaler akin to the approach used to deliver liposomal amphotericin B for aspergillosis. In a situation where long-term therapy is necessary as is the case for TB treatment, slow more localised (i.e non-systemic) release may help to address some of the drug compliance issues. This can potentially lead to a scenario whereby it is not possible to fully eradicate the organism in the host but by manipulating the immune system such therapies would severely restrict the ability of the pathogen to infect new hosts. However as there is no ideal animal model to study TB infection [326] let alone TB paradoxical reactions, studies evaluating the drug efficiency in such circumstances would need to be conducted in humans in a multi-center setting.

Finally, in the days before anti-TB drugs were available, TB patients were sent to TB sanatoriums for treatment where fresh air, plenty of rest and sunshine were prescribed for at least 6 months. In more severe pulmonary TB cases, surgical therapy in the form of lobectomy, thoracoplasty, phrenicotomy, plombage or even pneumonectomy would be performed to in the hope that this would kill *M.tb* and cure the patient. This has been attributed to the role that oxygen plays in innate immunity. The role of hypoxia in the human immune system has been the subject of increasing interest. Neutrophils delay apoptosis in hypoxic or anoxic conditions [327, 328] prolonging inflammation. Intermittent hypoxia in healthy adults has been shown to activate neutrophils, increase circulating complement and immunoglobulins [329]. It has been demonstrated as well that hypoxia exposure in humans increased the chemotaxis of neutrophils to fMLP, the phagocytosis of neutrophils to *E.coli* and the release of neutrophil oxidant products [330]. Prelimnary *in vitro* investigations had shown that hypoxia increased MMP secretion from both neutrophils and macrophages (unpublished data), and so it would be interesting to assess if hyperoxia does the converse in inhibiting MMP secretion both *in vitro* and in animal models. If proven true, a possibility would be to subject TB patients to oxygen as an adjunctive therapy in a randomised control trial and assess if this would modify their time to culture conversion, effects on paradoxical reactions, and monitor inflammatory markers, chemokines and cytokines.

In summary, future research efforts need to be directed towards understanding the human-*M.tb* interaction. Efforts need to be placed into dissecting the key pathways regulating inflammation as well as identifying agents and modalities that can improve patient outcome. This is crucial especially in this age of increasing drug-resistance

where even totally-drug resistant TB has emerged, returning us back to the era when standard chemotherapy was unavailable. We have to reverse the looming possibility of TB becoming once again ‗the captain of the men of death'.

BIBLIOGRAPHY

1. Osler, W., *Medicine in the Nineteenth Century*. Aequanimitas with Other Addresses to Medical Students, Nurses and Practitioners of Medicine 1904, London,: H. K. Lewis.

2. Hershkovitz, I., et al., *Detection and molecular characterization of 9,000-year-old Mycobacterium tuberculosis from a Neolithic settlement in the Eastern Mediterranean.* PLoS One, 2008. **3**(10): p. e3426.

3. Dye, C., et al., *Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project.* JAMA, 1999. **282**(7): p. 677-86.

4. WHO, *Global Tuberculosis Report 2012.* 2012.

5. Zumla, A., *The white plague returns to London--with a vengeance.* Lancet, 2011. **377**(9759): p. 10-1.

6. *Multidrug and extensively drug-resistant TB (M/XDR-TB) 2010* SURVEILLANCE AND RESPONSE 2010; Available from: http://whqlibdoc.who.int/publications/2010/9789241599191_eng.pdf.

7. WHO, *Global map and information on XDR-TB.* 2011.

8. Velayati, A.A., et al., *Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in iran.* Chest, 2009. **136**(2): p. 420-5.

9. Udwadia, Z.F., et al., *Totally drug-resistant tuberculosis in India.* Clin Infect Dis, 2012. **54**(4): p. 579-81.

10. Klopper, M., Warren, R.M, Hayes, C., van Pittius, N.C.G., Streicher, E.M., Muller, B., Sirgel, F.A., Chabula-Nxiweni, M., Hoosain, E., Coetzee, G., van Helden, P.D., Victor T.C., Trollip, A.P., *Emergence and Spread of Extensively and Totally Drug-Resistant Tuberculosis, South Africa.* Emerg Infect Dis, 2013. **19**(3).

11. Lawn, S.D., R. Wood, and R.J. Wilkinson, *Changing concepts of "latent tuberculosis infection" in patients living with HIV infection.* Clin Dev Immunol, 2011. **2011**.

12. Cain, K.P., et al., *An algorithm for tuberculosis screening and diagnosis in people with HIV.* N Engl J Med, 2010. **362**(8): p. 707-16.

13. Lawn, S.D., et al., *Early mortality among adults accessing antiretroviral treatment programmes in sub-Saharan Africa.* AIDS, 2008. **22**(15): p. 1897-908.

14. Lucas, S.B., et al., *Contribution of tuberculosis to slim disease in Africa.* BMJ, 1994. **308**(6943): p. 1531-3.

15. Rana, F.S., et al., *Autopsy study of HIV-1-positive and HIV-1-negative adult medical patients in Nairobi, Kenya.* J Acquir Immune Defic Syndr, 2000. **24**(1): p. 23-9.

16. Ansari, N.A., et al., *Pathology and causes of death in a group of 128 predominantly HIV-positive patients in Botswana, 1997-1998.* Int J Tuberc Lung Dis, 2002. **6**(1): p. 55-63.

17. Cohen, T., et al., *The prevalence and drug sensitivity of tuberculosis among patients dying in hospital in KwaZulu-Natal, South Africa: a postmortem study.* PLoS Med, 2010. **7**(6): p. e1000296.

18. Harries, A.D., et al., *High early death rate in tuberculosis patients in Malawi.* Int J Tuberc Lung Dis, 2001. **5**(11): p. 1000-5.

19. Ackah, A.N., et al., *Response to treatment, mortality, and CD4 lymphocyte counts in HIV-infected persons with tuberculosis in Abidjan, Cote d'Ivoire.* Lancet, 1995. **345**(8950): p. 607-10.

20. Perriens, J.H., et al., *Pulmonary tuberculosis in HIV-infected patients in Zaire. A controlled trial of treatment for either 6 or 12 months.* N Engl J Med, 1995. **332**(12): p. 779-84.

21. Wells, C.D., et al., *HIV infection and multidrug-resistant tuberculosis: the perfect storm.* J Infect Dis, 2007. **196 Suppl 1**: p. S86-107.

22. Gandhi, N.R., et al., *Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa.* Lancet, 2006. **368**(9547): p. 1575-80.

23. Dubrovina, I., et al., *Drug-resistant tuberculosis and HIV in Ukraine: a threatening convergence of two epidemics?* Int J Tuberc Lung Dis, 2008. **12**(7): p. 756-62.

24. Jeon, C.Y., et al., *Bi-directional screening for tuberculosis and diabetes: a systematic review.* Trop Med Int Health, 2010. **15**(11): p. 1300-14.

25. Jeon, C.Y. and M.B. Murray, *Diabetes mellitus increases the risk of active tuberculosis: a systematic review of 13 observational studies.* PLoS Med, 2008. **5**(7): p. e152.

26. Al-Attiyah, R.J. and A.S. Mustafa, *Mycobacterial antigen-induced T helper type 1 (Th1) and Th2 reactivity of peripheral blood mononuclear cells from diabetic and non-diabetic tuberculosis patients and Mycobacterium bovis bacilli Calmette-Guerin (BCG)-vaccinated healthy subjects.* Clin Exp Immunol, 2009. **158**(1): p. 64-73.

27. Stalenhoef, J.E., et al., *The role of interferon-gamma in the increased tuberculosis risk in type 2 diabetes mellitus.* Eur J Clin Microbiol Infect Dis, 2008. **27**(2): p. 97-103.

28. Restrepo, B.I., et al., *Tuberculosis in poorly controlled type 2 diabetes: altered cytokine expression in peripheral white blood cells.* Clin Infect Dis, 2008. **47**(5): p. 634-41.

29. Vallerskog, T., G.W. Martens, and H. Kornfeld, *Diabetic mice display a delayed adaptive immune response to Mycobacterium tuberculosis.* J Immunol, 2010. **184**(11): p. 6275-82.

30. Abdelrahman, M., A.K. Sinha, and A. Karkar, *Tuberculosis in end-stage renal disease patients on hemodialysis.* Hemodial Int, 2006. **10**(4): p. 360-4.

31. Dervisoglu, E., A. Yilmaz, and E. Sengul, *The spectrum of tuberculosis in dialysis patients.* Scand J Infect Dis, 2006. **38**(11-12): p. 1040-4.

32. Keane, J., et al., *Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent.* N Engl J Med, 2001. **345**(15): p. 1098-104.

33. Bruns, H., et al., *Anti-TNF immunotherapy reduces CD8+ T cell-mediated antimicrobial activity against Mycobacterium tuberculosis in humans.* J Clin Invest, 2009. **119**(5): p. 1167-77.

34. Saliu, O.Y., et al., *Tumor-necrosis-factor blockers: differential effects on mycobacterial immunity.* J Infect Dis, 2006. **194**(4): p. 486-92.

35. Chakravarty, S.D., et al., *Tumor necrosis factor blockade in chronic murine tuberculosis enhances granulomatous inflammation and disorganizes granulomas in the lungs.* Infect Immun, 2008. **76**(3): p. 916-26.

36. Rodrigues, L.C., V.K. Diwan, and J.G. Wheeler, *Protective effect of BCG against tuberculous meningitis and miliary tuberculosis: a meta-analysis.* Int J Epidemiol, 1993. **22**(6): p. 1154-8.

37. Trunz, B.B., P. Fine, and C. Dye, *Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness.* Lancet, 2006. **367**(9517): p. 1173-80.

38. Sterne, J.A., L.C. Rodrigues, and I.N. Guedes, *Does the efficacy of BCG decline with time since vaccination?* Int J Tuberc Lung Dis, 1998. **2**(3): p. 200-7.

39. Steingart, K.R., et al., *Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review.* Lancet Infect Dis, 2006. **6**(9): p. 570-81.

40. Corbett, E.L., et al., *The growing burden of tuberculosis: global trends and interactions with the HIV epidemic.* Arch Intern Med, 2003. **163**(9): p. 1009-21.

41. WHO, *Tuberculosis: Diagnostic technology landscape* 2012.

42. Ling, D.I., et al., *Commercial nucleic-acid amplification tests for diagnosis of pulmonary tuberculosis in respiratory specimens: meta-analysis and meta-regression.* PLoS One, 2008. **3**(2): p. e1536.

43. El-Hajj, H.H., et al., *Detection of rifampin resistance in Mycobacterium tuberculosis in a single tube with molecular beacons.* J Clin Microbiol, 2001. **39**(11): p. 4131-7.

44. Piatek, A.S., et al., *Molecular beacon sequence analysis for detecting drug resistance in Mycobacterium tuberculosis.* Nat Biotechnol, 1998. **16**(4): p. 359-63.

45. WHO, *Diagnostics for tuberculosis - global demand and market potential. Geneva: WHO/TDR/FIND, 2006.* . 2006.

46. FDA, U.S., *FDA News Release.* 2012.

47. Campbell, I.A. and A.J. Dyson, *Lymph node tuberculosis: a comparison of various methods of treatment.* Tubercle, 1977. **58**(4): p. 171-9.

48. Breen, R.A., et al., *Paradoxical reactions during tuberculosis treatment in patients with and without HIV co-infection.* Thorax, 2004. **59**(8): p. 704-7.

49. Hawkey, C.R., et al., *Characterization and management of paradoxical upgrading reactions in HIV-uninfected patients with lymph node tuberculosis.* Clin Infect Dis, 2005. **40**(9): p. 1368-71.

50. ATS/IDSA, *Treatment of tuberculosis: American Thoracic Society, CDC and Infectious Diseases Society of America.* 2003.

51. NICE, *Tuberculosis: Clinical diagnosis and management of tuberculosis, and measures for its prevention and control.* 2011.

52. Blackmore, T.K., et al., *Therapeutic use of infliximab in tuberculosis to control severe paradoxical reaction of the brain and lymph nodes.* Clin Infect Dis, 2008. **47**(10): p. e83-5.

53. Wallis, R.S., C. van Vuuren, and S. Potgieter, *Adalimumab treatment of life-threatening tuberculosis.* Clin Infect Dis, 2009. **48**(10): p. 1429-32.

54. Navas, E., et al., *Paradoxical reactions of tuberculosis in patients with the acquired immunodeficiency syndrome who are treated with highly active antiretroviral therapy.* Arch Intern Med, 2002. **162**(1): p. 97-9.

55. Chase, M.W., *The cellular transfer of cutaneous hypersensitivity.* J Bacteriol, 1946. **51**: p. 643.

56. Lawrence, H.S., *The cellular transfer of cutaneous hypersensitivity to tuberculin in man.* Proc Soc Exp Biol Med, 1949. **71**(4): p. 516-22.

57. Lurie, M.B., *Studies on the Mechanism of Immunity in Tuberculosis : The Fate of Tubercle Bacilli Ingested by Mononuclear Phagocytes Derived from Normal and Immunized Animals.* J Exp Med, 1942. **75**(3): p. 247-68.

58. Lurie, M.B., *Studies on the Mechanism of Immunity in Tuberculosis : The Mobilization of Mononuclear Phagocytes in Normal and Immunized Animals and Their Relative Capacities for Division and Phagocytosis.* J Exp Med, 1939. **69**(4): p. 579-99.

59. Suter, E., *Multiplication of tubercle bacilli within mononuclear phagocytes in tissue cultures derived from normal animals and animals vaccinated with BCG.* J Exp Med, 1953. **97**(2): p. 235-45.

60. Patterson, R.J. and G.P. Youmans, *Demonstration in tissue culture of lymphocyte-mediated immunity to tuberculosis.* Infect Immun, 1970. **1**(6): p. 600-3.

61. Sturgill-Koszycki, S., et al., *Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase.* Science, 1994. **263**(5147): p. 678-81.

62. Deretic, V., *Autophagy, an immunologic magic bullet: Mycobacterium tuberculosis phagosome maturation block and how to bypass it.* Future Microbiol, 2008. **3**(5): p. 517-24.

63. Via, L.E., et al., *Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7.* J Biol Chem, 1997. **272**(20): p. 13326-31.

64. van der Wel, N., et al., *M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells.* Cell, 2007. **129**(7): p. 1287-98.

65. Russell, D.G., C.E. Barry, 3rd, and J.L. Flynn, *Tuberculosis: what we don't know can, and does, hurt us.* Science, 2010. **328**(5980): p. 852-6.

66. Wesslen, T., *Passive transfer of tuberculin hypersensitivity by viable lymphocytes from the thoracic duct.* Acta Tuberc Scand, 1952. **26**(1-2): p. 38-53.

67. Coe, J.E., J.D. Feldman, and S. Lee, *Immunologic competence of thoracic duct cells. I. Delayed hypersensitivity.* J Exp Med, 1966. **123**(2): p. 267-81.

68. Kaplan, G., et al., *Mycobacterium tuberculosis growth at the cavity surface: a microenvironment with failed immunity.* Infect Immun, 2003. **71**(12): p. 7099-108.

69. Pillay, J., et al., *In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days.* Blood, 2010. **116**(4): p. 625-7.

70. Cassatella, M.A., *Neutrophil-derived proteins: selling cytokines by the pound.* Adv Immunol, 1999. **73**: p. 369-509.

71. Mantovani, A., et al., *Neutrophils in the activation and regulation of innate and adaptive immunity.* Nat Rev Immunol, 2011. **11**(8): p. 519-31.

72. Bazzoni, F., et al., *Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals.* Proc Natl Acad Sci U S A, 2009. **106**(13): p. 5282-7.

73. Nathan, C., *Neutrophils and immunity: challenges and opportunities.* Nat Rev Immunol, 2006. **6**(3): p. 173-82.

74. Hager, M., J.B. Cowland, and N. Borregaard, *Neutrophil granules in health and disease.* J Intern Med, 2010. **268**(1): p. 25-34.

75. Mayadas, T.N. and X. Cullere, *Neutrophil beta2 integrins: moderators of life or death decisions.* Trends Immunol, 2005. **26**(7): p. 388-95.

76. Baggiolini, M., J.G. Hirsch, and C. De Duve, *Resolution of granules from rabbit heterophil leukocytes into distinct populations by zonal sedimentation.* J Cell Biol, 1969. **40**(2): p. 529-41.

77. Borregaard, N., et al., *Stimulus-dependent secretion of plasma proteins from human neutrophils.* J Clin Invest, 1992. **90**(1): p. 86-96.

78. Borregaard, N. and J.B. Cowland, *Granules of the human neutrophilic polymorphonuclear leukocyte.* Blood, 1997. **89**(10): p. 3503-21.

79. Borregaard, N., O.E. Sorensen, and K. Theilgaard-Monch, *Neutrophil granules: a library of innate immunity proteins.* Trends Immunol, 2007. **28**(8): p. 340-5.

80. Borregaard, N., *Neutrophils, from marrow to microbes.* Immunity, 2010. **33**(5): p. 657-70.

81. Sorensen, O.E., et al., *Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3.* Blood, 2001. **97**(12): p. 3951-9.

82. Segal, A.W., J. Dorling, and S. Coade, *Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies.* J Cell Biol, 1980. **85**(1): p. 42-59.

83. Sengelov, H., L. Kjeldsen, and N. Borregaard, *Control of exocytosis in early neutrophil activation.* J Immunol, 1993. **150**(4): p. 1535-43.

84. Greenberg, S. and S. Grinstein, *Phagocytosis and innate immunity.* Curr Opin Immunol, 2002. **14**(1): p. 136-45.

85. Jaconi, M.E., et al., *Cytosolic free calcium elevation mediates the phagosome-lysosome fusion during phagocytosis in human neutrophils.* J Cell Biol, 1990. **110**(5): p. 1555-64.

86. Lee, W.L., R.E. Harrison, and S. Grinstein, *Phagocytosis by neutrophils.* Microbes Infect, 2003. **5**(14): p. 1299-306.

87. Hayashi, F., T.K. Means, and A.D. Luster, *Toll-like receptors stimulate human neutrophil function.* Blood, 2003. **102**(7): p. 2660-9.

88. Muzio, M., et al., *Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells.* J Immunol, 2000. **164**(11): p. 5998-6004.

89. Sabroe, I., S.K. Dower, and M.K. Whyte, *The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis.* Clin Infect Dis, 2005. **41 Suppl 7**: p. S421-6.

90. Sabroe, I., et al., *Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span.* J Immunol, 2003. **170**(10): p. 5268-75.

91. Lotz, S., et al., *Highly purified lipoteichoic acid activates neutrophil granulocytes and delays their spontaneous apoptosis via CD14 and TLR2.* J Leukoc Biol, 2004. **75**(3): p. 467-77.

92. Mack, M., et al., *Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice.* J Immunol, 2001. **166**(7): p. 4697-704.

93. Maus, U., et al., *The role of CC chemokine receptor 2 in alveolar monocyte and neutrophil immigration in intact mice.* Am J Respir Crit Care Med, 2002. **166**(3): p. 268-73.

94. Soehnlein, O., et al., *Neutrophil secretion products pave the way for inflammatory monocytes.* Blood, 2008. **112**(4): p. 1461-71.

95. Tan, B.H., et al., *Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens.* J Immunol, 2006. **177**(3): p. 1864-71.

96. Soehnlein, O., et al., *Neutrophil primary granule proteins HBP and HNP1-3 boost bacterial phagocytosis by human and murine macrophages.* J Clin Invest, 2008. **118**(10): p. 3491-502.

97. Brinkmann, V., et al., *Neutrophil extracellular traps kill bacteria.* Science, 2004. **303**(5663): p. 1532-5.

98. Urban, C.F., et al., *Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans.* PLoS Pathog, 2009. **5**(10): p. e1000639.

99. Brinkmann, V., et al., *Neutrophil extracellular traps: how to generate and visualize them.* J Vis Exp, (36).

100. Yousefi, S., et al., *Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps.* Cell Death Differ, 2009. **16**(11): p. 1438-44.

101. Yipp, B.G., et al., *Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo.* Nat Med, 2012. **18**(9): p. 1386-93.

102. Kaplan, M.J. and M. Radic, *Neutrophil extracellular traps: double-edged swords of innate immunity.* J Immunol, 2012. **189**(6): p. 2689-95.

103. Fuchs, T.A., et al., *Circulating DNA and myeloperoxidase indicate disease activity in patients with thrombotic microangiopathies.* Blood, 2012. **120**(6): p. 1157-64.

104. Scapini, P., et al., *The neutrophil as a cellular source of chemokines.* Immunol Rev, 2000. **177**: p. 195-203.

105. Kobayashi, S.D., et al., *Down-regulation of proinflammatory capacity during apoptosis in human polymorphonuclear leukocytes.* J Immunol, 2003. **170**(6): p. 3357-68.

106. Kobayashi, S.D., et al., *Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils.* Proc Natl Acad Sci U S A, 2003. **100**(19): p. 10948-53.

107. Puga, I., et al., *B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen.* Nat Immunol, 2012. **13**(2): p. 170-80.

108. Scapini, P., F. Bazzoni, and M.A. Cassatella, *Regulation of B-cell-activating factor (BAFF)/B lymphocyte stimulator (BLyS) expression in human neutrophils.* Immunol Lett, 2008. **116**(1): p. 1-6.

109. Huard, B., et al., *APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa.* J Clin Invest, 2008. **118**(8): p. 2887-95.

110. Bennouna, S. and E.Y. Denkers, *Microbial antigen triggers rapid mobilization of TNF-alpha to the surface of mouse neutrophils transforming them into inducers of high-level dendritic cell TNF-alpha production.* J Immunol, 2005. **174**(8): p. 4845-51.

111. Costantini, C., et al., *Human neutrophils interact with both 6-sulfo LacNAc+ DC and NK cells to amplify NK-derived IFN{gamma}: role of CD18, ICAM-1, and ICAM-3.* Blood, 2011. **117**(5): p. 1677-86.

112. Costantini, C. and M.A. Cassatella, *The defensive alliance between neutrophils and NK cells as a novel arm of innate immunity.* J Leukoc Biol, 2011. **89**(2): p. 221-33.

113. Costantini, C., et al., *Neutrophil activation and survival are modulated by interaction with NK cells.* Int Immunol, 2010. **22**(10): p. 827-38.

114. Bhatnagar, N., et al., *Cytokine-activated NK cells inhibit PMN apoptosis and preserve their functional capacity.* Blood, 2010. **116**(8): p. 1308-16.

115. Pelletier, M., et al., *Evidence for a cross-talk between human neutrophils and Th17 cells.* Blood, 2010. **115**(2): p. 335-43.

116. Pelletier, M., A. Micheletti, and M.A. Cassatella, *Modulation of human neutrophil survival and antigen expression by activated CD4+ and CD8+ T cells.* J Leukoc Biol, 2010. **88**(6): p. 1163-70.

117. Appelberg, R., *Mycobacterial infection primes T cells and macrophages for enhanced recruitment of neutrophils.* J Leukoc Biol, 1992. **51**(5): p. 472-7.

118. Condos, R., et al., *Local immune responses correlate with presentation and outcome in tuberculosis.* Am J Respir Crit Care Med, 1998. **157**(3 Pt 1): p. 729-35.

119. Lasco, T.M., et al., *Rapid accumulation of eosinophils in lung lesions in guinea pigs infected with Mycobacterium tuberculosis.* Infect Immun, 2004. **72**(2): p. 1147-9.

120. Schluger, N.W. and W.N. Rom, *The host immune response to tuberculosis.* Am J Respir Crit Care Med, 1998. **157**(3 Pt 1): p. 679-91.

121. Eum, S.Y., et al., *Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB.* Chest, 2010. **137**(1): p. 122-8.

122. Kang, D.D., et al., *Profiling early lung immune responses in the mouse model of tuberculosis.* PLoS One, 2011. **6**(1): p. e16161.

123. Abdul-Majid, K.B., et al., *Altered cellular infiltration and cytokine levels during early Mycobacterium tuberculosis sigC mutant infection are associated with late-stage disease attenuation and milder immunopathology in mice.* BMC Microbiol, 2008. **8**: p. 151.

124. Seiler, P., et al., *Early granuloma formation after aerosol Mycobacterium tuberculosis infection is regulated by neutrophils via CXCR3-signaling chemokines.* Eur J Immunol, 2003. **33**(10): p. 2676-86.

125. McCarter, Y.S. and A. Robinson, *Quality evaluation of sputum specimens for mycobacterial culture.* Am J Clin Pathol, 1996. **105**(6): p. 769-73.

126. Martineau, A.R., et al., *Neutrophil-mediated innate immune resistance to mycobacteria.* J Clin Invest, 2007. **117**(7): p. 1988-94.

127. Berry, M.P., et al., *An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis.* Nature, 2010. **466**(7309): p. 973-7.

128. Barnes, P.F., et al., *Predictors of short-term prognosis in patients with pulmonary tuberculosis.* J Infect Dis, 1988. **158**(2): p. 366-71.

129. Law, K., et al., *Increased release of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha by bronchoalveolar cells lavaged from involved sites in pulmonary tuberculosis.* Am J Respir Crit Care Med, 1996. **153**(2): p. 799-804.

130. Martineau, A.R., et al., *High-dose vitamin D(3) during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial.* Lancet, 2011. **377**(9761): p. 242-50.

131. Eruslanov, E.B., et al., *Neutrophil responses to Mycobacterium tuberculosis infection in genetically susceptible and resistant mice.* Infect Immun, 2005. **73**(3): p. 1744-53.

132. Feng, C.G., et al., *NK cell-derived IFN-gamma differentially regulates innate resistance and neutrophil response in T cell-deficient hosts infected with Mycobacterium tuberculosis.* J Immunol, 2006. **177**(10): p. 7086-93.

133. Cardona, P.J., et al., *Towards a 'human-like' model of tuberculosis: intranasal inoculation of LPS induces intragranulomatous lung necrosis in mice infected aerogenically with Mycobacterium tuberculosis.* Scand J Immunol, 2001. **53**(1): p. 65-71.

134. Barrios-Payan, J., et al., *[Neutrophil participation in early control and immune activation during experimental pulmonary tuberculosis].* Gac Med Mex, 2006. **142**(4): p. 273-81.

135. Aleman, M., et al., *Mycobacterium tuberculosis triggers apoptosis in peripheral neutrophils involving toll-like receptor 2 and p38 mitogen protein kinase in tuberculosis patients.* Infect Immun, 2004. **72**(9): p. 5150-8.

136. May, M.E. and P.J. Spagnuolo, *Evidence for activation of a respiratory burst in the interaction of human neutrophils with Mycobacterium tuberculosis.* Infect Immun, 1987. **55**(9): p. 2304-7.

137. Rieger, M., et al., *Immunoprofile studies in patients with pulmonary tuberculosis. III. Study of haemolytic complement in serum and phagocytic activity of blood neutrophils.* Scand J Respir Dis, 1979. **60**(4): p. 172-5.

138. Majeed, M., et al., *Roles of calcium and annexins in phagocytosis and elimination of an attenuated strain of Mycobacterium tuberculosis in human neutrophils.* Microb Pathog, 1998. **24**(5): p. 309-20.

139. Borelli, V., et al., *Myeloperoxidase exerts microbicidal activity against Mycobacterium tuberculosis.* Infect Immun, 1999. **67**(8): p. 4149-52.

140. Aleman, M., et al., *Activation of peripheral blood neutrophils from patients with active advanced tuberculosis.* Clin Immunol, 2001. **100**(1): p. 87-95.

141. Riedel, D.D. and S.H. Kaufmann, *Chemokine secretion by human polymorphonuclear granulocytes after stimulation with Mycobacterium tuberculosis and lipoarabinomannan.* Infect Immun, 1997. **65**(11): p. 4620-3.

142. Kasahara, K., et al., *Expression of chemokines and induction of rapid cell death in human blood neutrophils by Mycobacterium tuberculosis.* J Infect Dis, 1998. **178**(1): p. 127-37.

143. Ramos-Kichik, V., et al., *Neutrophil extracellular traps are induced by Mycobacterium tuberculosis.* Tuberculosis (Edinb), 2009. **89**(1): p. 29-37.

144. Aleman, M., et al., *Spontaneous or Mycobacterium tuberculosis-induced apoptotic neutrophils exert opposite effects on the dendritic cell-mediated immune response.* Eur J Immunol, 2007. **37**(6): p. 1524-37.

145. Perskvist, N., et al., *Mycobacterium tuberculosis promotes apoptosis in human neutrophils by activating caspase-3 and altering expression of Bax/Bcl-xL via an oxygen-dependent pathway.* J Immunol, 2002. **168**(12): p. 6358-65.

146. Persson, Y.A., et al., *Mycobacterium tuberculosis-induced apoptotic neutrophils trigger a pro-inflammatory response in macrophages through release of heat shock protein 72, acting in synergy with the bacteria.* Microbes Infect, 2008. **10**(3): p. 233-40.

147. Zhang, X., et al., *Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils.* Immunity, 2009. **31**(5): p. 761-71.

148. Morel, C., et al., *Mycobacterium bovis BCG-infected neutrophils and dendritic cells cooperate to induce specific T cell responses in humans and mice.* Eur J Immunol, 2008. **38**(2): p. 437-47.

149. Blomgran, R. and J.D. Ernst, *Lung neutrophils facilitate activation of naive antigen-specific CD4+ T cells during Mycobacterium tuberculosis infection.* J Immunol, 2011. **186**(12): p. 7110-9.

150. Fiorenza, G., et al., *Impaired neutrophil function in patients with pulmonary tuberculosis and its normalization in those undergoing specific treatment, except the HIV-coinfected cases.* FEMS Immunol Med Microbiol, 2003. **35**(2): p. 159-64.

151. Meddows-Taylor, S., D.J. Martin, and C.T. Tiemessen, *Altered expression of Fc gammaRIII (CD16) on polymorphonuclear neutrophils from individuals with*

human immunodeficiency virus type 1 disease and pulmonary tuberculosis. Clin Diagn Lab Immunol, 1997. **4**(6): p. 789-91.

152. Meddows-Taylor, S., D.J. Martin, and C.T. Tiemessen, *Impaired interleukin-8-induced degranulation of polymorphonuclear neutrophils from human immunodeficiency virus type 1-infected individuals.* Clin Diagn Lab Immunol, 1999. **6**(3): p. 345-51.

153. Parks, W.C., C.L. Wilson, and Y.S. Lopez-Boado, *Matrix metalloproteinases as modulators of inflammation and innate immunity.* Nat Rev Immunol, 2004. **4**(8): p. 617-29.

154. Brew, K., D. Dinakarpandian, and H. Nagase, *Tissue inhibitors of metalloproteinases: evolution, structure and function.* Biochim Biophys Acta, 2000. **1477**(1-2): p. 267-83.

155. Kahari, V.M. and U. Saarialho-Kere, *Matrix metalloproteinases in skin.* Exp Dermatol, 1997. **6**(5): p. 199-213.

156. Chandler, S., et al., *Matrix metalloproteinases, tumor necrosis factor and multiple sclerosis: an overview.* J Neuroimmunol, 1997. **72**(2): p. 155-61.

157. Ashworth, J.L., et al., *Fibrillin degradation by matrix metalloproteinases: implications for connective tissue remodelling.* Biochem J, 1999. **340 (Pt 1)**: p. 171-81.

158. Pilcher, B.K., et al., *The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix.* J Cell Biol, 1997. **137**(6): p. 1445-57.

159. Dunsmore, S.E., et al., *Matrilysin expression and function in airway epithelium.* J Clin Invest, 1998. **102**(7): p. 1321-31.

160. Wilson, C.L., et al., *Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense.* Science, 1999. **286**(5437): p. 113-7.

161. McQuibban, G.A., et al., *Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo.* Blood, 2002. **100**(4): p. 1160-7.

162. Van den Steen, P.E., et al., *Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact.* Blood, 2000. **96**(8): p. 2673-81.

163. Van Den Steen, P.E., et al., *Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities.* Eur J Biochem, 2003. **270**(18): p. 3739-49.

164. Moss, M.L., et al., *Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha.* Nature, 1997. **385**(6618): p. 733-6.

165. Mohan, M.J., et al., *The tumor necrosis factor-alpha converting enzyme (TACE): a unique metalloproteinase with highly defined substrate selectivity.* Biochemistry, 2002. **41**(30): p. 9462-9.

166. English, W.R., et al., *Membrane type 4 matrix metalloproteinase (MMP17) has tumor necrosis factor-alpha convertase activity but does not activate pro-MMP2.* J Biol Chem, 2000. **275**(19): p. 14046-55.

167. Dayer, J.M., et al., *Collagenase production by rheumatoid synovial cells: stimulation by a human lymphocyte factor.* Science, 1977. **195**(4274): p. 181-3.

168. Liotta, L.A., et al., *Metastatic potential correlates with enzymatic degradation of basement membrane collagen.* Nature, 1980. **284**(5751): p. 67-8.

169. Koop, S., et al., *Overexpression of metalloproteinase inhibitor in B16F10 cells does not affect extravasation but reduces tumor growth.* Cancer Res, 1994. **54**(17): p. 4791-7.

170. Stetler-Stevenson, W.G., *Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention.* J Clin Invest, 1999. **103**(9): p. 1237-41.

171. Stetler-Stevenson, W.G., *The role of matrix metalloproteinases in tumor invasion, metastasis, and angiogenesis.* Surg Oncol Clin N Am, 2001. **10**(2): p. 383-92, x.

172. Whittaker, M. and P. Brown, *Recent advances in matrix metalloproteinase inhibitor research and development.* Curr Opin Drug Discov Devel, 1998. **1**(2): p. 157-64.

173. Brown, P.D., Davidson, A.H., Drummond, A.H, Gearing, A., *Matrix metalloproteinase inhibitors in cancer therapy.* 2001: p. 113-142.

174. Coussens, L.M., B. Fingleton, and L.M. Matrisian, *Matrix metalloproteinase inhibitors and cancer: trials and tribulations.* Science, 2002. **295**(5564): p. 2387-92.

175. Wynn, R.L., *Latest FDA approvals for dentistry.* Gen Dent, 1999. **47**(1): p. 19-22.

176. Galardy, R.E., et al., *Low molecular weight inhibitors in corneal ulceration.* Ann N Y Acad Sci, 1994. **732**: p. 315-23.

177. Brinckerhoff, C.E. and L.M. Matrisian, *Matrix metalloproteinases: a tail of a frog that became a prince.* Nat Rev Mol Cell Biol, 2002. **3**(3): p. 207-14.

178. Elkington, P.T. and J.S. Friedland, *Matrix metalloproteinases in destructive pulmonary pathology.* Thorax, 2006. **61**(3): p. 259-66.

179. Elkington, P.T., et al., *Mycobacterium tuberculosis, but not vaccine BCG, specifically upregulates matrix metalloproteinase-1.* Am J Respir Crit Care Med, 2005. **172**(12): p. 1596-604.

180. Elkington, P.T., et al., *Synergistic up-regulation of epithelial cell matrix metalloproteinase-9 secretion in tuberculosis.* Am J Respir Cell Mol Biol, 2007. **37**(4): p. 431-7.

181. Rand, L., et al., *Matrix metalloproteinase-1 is regulated in tuberculosis by a p38 MAPK-dependent, p-aminosalicylic acid-sensitive signaling cascade.* J Immunol, 2009. **182**(9): p. 5865-72.

182. Friedland, J.S., et al., *Differential regulation of MMP-1/9 and TIMP-1 secretion in human monocytic cells in response to Mycobacterium tuberculosis.* Matrix Biol, 2002. **21**(1): p. 103-10.

183. Taylor, J.L., et al., *Role for matrix metalloproteinase 9 in granuloma formation during pulmonary Mycobacterium tuberculosis infection.* Infect Immun, 2006. **74**(11): p. 6135-44.

184. Volkman, H.E., et al., *Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium.* Science, 2010. **327**(5964): p. 466-9.

185. Elkington, P., et al., *MMP-1 drives immunopathology in human tuberculosis and transgenic mice.* J Clin Invest, 2011. **121**(5): p. 1827-33.

186. Walker, N.F., et al., *Doxycycline and HIV infection suppress tuberculosis-induced matrix metalloproteinases.* Am J Respir Crit Care Med, 2012. **185**(9): p. 989-97.

187. Price, N.M., et al., *Unopposed matrix metalloproteinase-9 expression in human tuberculous granuloma and the role of TNF-alpha-dependent monocyte networks.* J Immunol, 2003. **171**(10): p. 5579-86.

188. Price, N.M., et al., *Identification of a matrix-degrading phenotype in human tuberculosis in vitro and in vivo.* J Immunol, 2001. **166**(6): p. 4223-30.

189. Harris, J.E., et al., *Monocytes infected with Mycobacterium tuberculosis regulate MAP kinase-dependent astrocyte MMP-9 secretion.* J Leukoc Biol, 2007. **81**(2): p. 548-56.

190. Harris, J.E., et al., *Monocyte-astrocyte networks regulate matrix metalloproteinase gene expression and secretion in central nervous system tuberculosis in vitro and in vivo.* J Immunol, 2007. **178**(2): p. 1199-207.

191. O'Kane, C.M., et al., *Monocyte-dependent fibroblast CXCL8 secretion occurs in tuberculosis and limits survival of mycobacteria within macrophages.* J Immunol, 2007. **178**(6): p. 3767-76.

192. Green, J.A., et al., *Mycobacterium tuberculosis upregulates microglial matrix metalloproteinase-1 and -3 expression and secretion via NF-kappaB- and Activator Protein-1-dependent monocyte networks.* J Immunol, 2010. **184**(11): p. 6492-503.

193. Zhu, X.W., et al., *Multinucleate giant cells release functionally unopposed matrix metalloproteinase-9 in vitro and in vivo.* J Infect Dis, 2007. **196**(7): p. 1076-9.

194. Wang, Y.Y., et al., *Peptidoglycan of Staphylococcus aureus induces enhanced levels of matrix metalloproteinase-9 in human blood originating from neutrophils.* Shock, 2005. **24**(3): p. 214-8.

195. Delpino, M.V., et al., *Brucella-infected hepatocytes mediate potentially tissue-damaging immune responses.* J Hepatol, 2010. **53**(1): p. 145-54.

196. Faldt, J., et al., *Priming of human neutrophils by mycobacterial lipoarabinomannans: role of granule mobilisation.* Microbes Infect, 2001. **3**(13): p. 1101-9.

197. Van den Steen, P.E., et al., *Carboxyterminal cleavage of the chemokines MIG and IP-10 by gelatinase B and neutrophil collagenase.* Biochem Biophys Res Commun, 2003. **310**(3): p. 889-96.

198. Laurent, G.J., *Lung collagen: more than scaffolding.* Thorax, 1986. **41**(6): p. 418-28.

199. Knauper, V., et al., *Biochemical characterization of human collagenase-3.* J Biol Chem, 1996. **271**(3): p. 1544-50.

200. Horwitz, A.L., A.J. Hance, and R.G. Crystal, *Granulocyte collagenase: selective digestion of type I relative to type III collagen.* Proc Natl Acad Sci U S A, 1977. **74**(3): p. 897-901.

201. Hasty, K.A., et al., *The collagen substrate specificity of human neutrophil collagenase.* J Biol Chem, 1987. **262**(21): p. 10048-52.

202. Schubert-Unkmeir, A., et al., *Neisseria meningitidis induces brain microvascular endothelial cell detachment from the matrix and cleavage of occludin: a role for MMP-8.* PLoS Pathog, 2010. **6**(4): p. e1000874.

203. Albaiceta, G.M., et al., *Absence or inhibition of matrix metalloproteinase-8 decreases ventilator-induced lung injury.* Am J Respir Cell Mol Biol, 2010. **43**(5): p. 555-63.

204. Sepper, R., et al., *Human neutrophil collagenase (MMP-8), identified in bronchiectasis BAL fluid, correlates with severity of disease.* Chest, 1995. **107**(6): p. 1641-7.

205. Nwomeh, B.C., et al., *MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers.* J Surg Res, 1999. **81**(2): p. 189-95.

206. Opdenakker, G. and J. Van Damme, *Cytokine-regulated proteases in autoimmune diseases.* Immunol Today, 1994. **15**(3): p. 103-7.

207. Proost, P., J. Van Damme, and G. Opdenakker, *Leukocyte gelatinase B cleavage releases encephalitogens from human myelin basic protein.* Biochem Biophys Res Commun, 1993. **192**(3): p. 1175-81.

208. Chakrabarti, S. and K.D. Patel, *Regulation of matrix metalloproteinase-9 release from IL-8-stimulated human neutrophils.* J Leukoc Biol, 2005. **78**(1): p. 279-88.

209. Liu, Z., et al., *The serpin alpha1-proteinase inhibitor is a critical substrate for gelatinase B/MMP-9 in vivo.* Cell, 2000. **102**(5): p. 647-55.

210. Albaiceta, G.M., et al., *Lack of matrix metalloproteinase-9 worsens ventilator-induced lung injury.* Am J Physiol Lung Cell Mol Physiol, 2008. **294**(3): p. L535-43.

211. Kolb, S.A., et al., *Matrix metalloproteinases and tissue inhibitors of metalloproteinases in viral meningitis: upregulation of MMP-9 and TIMP-1 in cerebrospinal fluid.* J Neuroimmunol, 1998. **84**(2): p. 143-50.

212. Sulik, A., M. Wojtkowska, and E. Oldak, *Elevated levels of MMP-9 and TIMP-1 in the cerebrospinal fluid of children with echovirus type 30 and mumps meningitis.* Scand J Immunol, 2008. **68**(3): p. 323-7.

213. Leppert, D., et al., *Matrix metalloproteinase (MMP)-8 and MMP-9 in cerebrospinal fluid during bacterial meningitis: association with blood-brain*

barrier damage and neurological sequelae. Clin Infect Dis, 2000. **31**(1): p. 80-4.

214. Matsuura, E., et al., *Marked increase of matrix metalloproteinase 9 in cerebrospinal fluid of patients with fungal or tuberculous meningoencephalitis.* J Neurol Sci, 2000. **173**(1): p. 45-52.

215. Green, J.A., et al., *Dexamethasone, cerebrospinal fluid matrix metalloproteinase concentrations and clinical outcomes in tuberculous meningitis.* PLoS One, 2009. **4**(9): p. e7277.

216. Thwaites, G.E., et al., *Dexamethasone for the treatment of tuberculous meningitis in adolescents and adults.* N Engl J Med, 2004. **351**(17): p. 1741-51.

217. Andreu, N., et al., *Optimisation of bioluminescent reporters for use with mycobacteria.* PLoS One, 2010. **5**(5): p. e10777.

218. Harris, J.E. and J.S. Friedland, *l-Glutamate in Middlebrook 7H9 culture medium upregulates matrix metalloproteinase-2 secretion from human astrocytoma cells.* J Neurosci Methods, 2008. **173**(2): p. 291-4.

219. Elkington, P.T., J.A. Green, and J.S. Friedland, *Filter sterilization of highly infectious samples to prevent false negative analysis of matrix metalloproteinase activity.* J Immunol Methods, 2006. **309**(1-2): p. 115-9.

220. Leber, T.M. and F.R. Balkwill, *Zymography: a single-step staining method for quantitation of proteolytic activity on substrate gels.* Anal Biochem, 1997. **249**(1): p. 24-8.

221. Tamassia N., C.M., Bazzoni F., ed. *Fast and accurate quantitative analysis of cytokine gene expression in human neutrophils by reverse transcription real-time PCR*. Neutrophil methods and protocols, ed. D.F. Quinn M., Bokoch G. 2007, Humana Press.

222. Antony, V.B., et al., *Bacillus Calmette-Guerin-stimulated neutrophils release chemotaxins for monocytes in rabbit pleural spaces and in vitro.* J Clin Invest, 1985. **76**(4): p. 1514-21.

223. Bateman, E.D., M. Turner-Warwick, and B.C. Adelmann-Grill, *Immunohistochemical study of collagen types in human foetal lung and fibrotic lung disease.* Thorax, 1981. **36**(9): p. 645-53.

224. Shimizu, T., et al., *Effect of tranilast on matrix metalloproteinase production from neutrophils in-vitro.* J Pharm Pharmacol, 2006. **58**(1): p. 91-9.

225. Kanai, K., et al., *Suppression of matrix metalloproteinase-9 production from neutrophils by a macrolide antibiotic, roxithromycin, in vitro.* Mediators Inflamm, 2004. **13**(5-6): p. 313-9.

226. Schauf, V., et al., *Cytokine gene activation and modified responsiveness to interleukin-2 in the blood of tuberculosis patients.* J Infect Dis, 1993. **168**(4): p. 1056-9.

227. Hernandez-Pando, R., et al., *Correlation between the kinetics of Th1, Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis.* Immunology, 1996. **89**(1): p. 26-33.

228. Stewart, D., et al., *Interleukin-4 inhibition of interleukin-1-induced expression of matrix metalloproteinase-3 (MMP-3) is independent of lipoxygenase and PPARgamma activation in human gingival fibroblasts.* BMC Mol Biol, 2007. **8**: p. 12.

229. Bober, L.A., et al., *IL-4 induces neutrophilic maturation of HL-60 cells and activation of human peripheral blood neutrophils.* Clin Exp Immunol, 1995. **99**(1): p. 129-36.

230. Pugin, J., et al., *Human neutrophils secrete gelatinase B in vitro and in vivo in response to endotoxin and proinflammatory mediators.* Am J Respir Cell Mol Biol, 1999. **20**(3): p. 458-64.

231. Claesson, R., et al., *Release and activation of matrix metalloproteinase 8 from human neutrophils triggered by the leukotoxin of Actinobacillus actinomycetemcomitans.* J Periodontal Res, 2002. **37**(5): p. 353-9.

232. Lyons, M.J., T. Yoshimura, and D.N. McMurray, *Interleukin (IL)-8 (CXCL8) induces cytokine expression and superoxide formation by guinea pig neutrophils infected with Mycobacterium tuberculosis.* Tuberculosis (Edinb), 2004. **84**(5): p. 283-92.

233. Cooper, A.M., et al., *The role of interleukin-12 in acquired immunity to Mycobacterium tuberculosis infection.* Immunology, 1995. **84**(3): p. 423-32.

234. Appelberg, R., *Protective role of interferon gamma, tumor necrosis factor alpha and interleukin-6 in Mycobacterium tuberculosis and M. avium infections.* Immunobiology, 1994. **191**(4-5): p. 520-5.

235. Ganachari, M., et al., *Joint effect of MCP-1 genotype GG and MMP-1 genotype 2G/2G increases the likelihood of developing pulmonary tuberculosis in BCG-vaccinated individuals.* PLoS One, 2010. **5**(1): p. e8881.

236. Wright, K.M. and J.S. Friedland, *Regulation of monocyte chemokine and MMP-9 secretion by proinflammatory cytokines in tuberculous osteomyelitis.* J Leukoc Biol, 2004. **75**(6): p. 1086-92.

237. Klein, T. and R. Bischoff, *Physiology and pathophysiology of matrix metalloproteases.* Amino Acids, 2011. **41**(2): p. 271-90.

238. Appelberg, R., et al., *Role of interleukin-6 in the induction of protective T cells during mycobacterial infections in mice.* Immunology, 1994. **82**(3): p. 361-4.

239. Sawant, K.V., et al., *Guinea pig neutrophil-macrophage interactions during infection with Mycobacterium tuberculosis.* Microbes Infect, 2010. **12**(11): p. 828-37.

240. Sawant, K.V. and D.N. McMurray, *Guinea pig neutrophils infected with Mycobacterium tuberculosis produce cytokines which activate alveolar macrophages in noncontact cultures.* Infect Immun, 2007. **75**(4): p. 1870-7.

241. Taub, D.D., et al., *Beta chemokines costimulate lymphocyte cytolysis, proliferation, and lymphokine production.* J Leukoc Biol, 1996. **59**(1): p. 81-9.

242. Fahey, T.J., 3rd, et al., *Macrophage inflammatory protein 1 modulates macrophage function.* J Immunol, 1992. **148**(9): p. 2764-9.

243. Chiba, K., et al., *Neutrophils secrete MIP-1 beta after adhesion to laminin contained in basement membrane of blood vessels.* Br J Haematol, 2004. **127**(5): p. 592-7.

244. Vlahos, R., et al., *Glucocorticosteroids differentially regulate MMP-9 and neutrophil elastase in COPD.* PLoS One, 2012. **7**(3): p. e33277.

245. Cundall, M., et al., *Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids.* J Allergy Clin Immunol, 2003. **112**(6): p. 1064-71.

246. Liu, L., et al., *Rapid non-genomic inhibitory effects of glucocorticoids on human neutrophil degranulation.* Inflamm Res, 2005. **54**(1): p. 37-41.

247. Garcia Vidal, C., et al., *Paradoxical response to antituberculous therapy in infliximab-treated patients with disseminated tuberculosis.* Clin Infect Dis, 2005. **40**(5): p. 756-9.

248. Herman, M.P., et al., *Expression of neutrophil collagenase (matrix metalloproteinase-8) in human atheroma: a novel collagenolytic pathway suggested by transcriptional profiling.* Circulation, 2001. **104**(16): p. 1899-904.

249. Garcia-de-Alba, C., et al., *Expression of matrix metalloproteases by fibrocytes: possible role in migration and homing.* Am J Respir Crit Care Med, 2010. **182**(9): p. 1144-52.

250. Laurent, G.J., et al., *Biochemical and histological changes in pulmonary fibrosis induced in rabbits with intratracheal bleomycin.* Eur J Clin Invest, 1981. **11**(6): p. 441-8.

251. Seyer, J.M., E.T. Hutcheson, and A.H. Kang, *Collagen polymorphism in idiopathic chronic pulmonary fibrosis.* J Clin Invest, 1976. **57**(6): p. 1498-507.

252. Bradley, K.H., S.D. McConnell, and R.G. Crystal, *Lung collagen composition and synthesis. Characterization and changes with age.* J Biol Chem, 1974. **249**(9): p. 2674-83.

253. Pierce, J.A. and J.B. Hocott, *Studies on the collagen and elastin content of the human lung.* J Clin Invest, 1960. **39**: p. 8-14.

254. Gabay, C., et al., *Synovial tissues concentrate secreted APRIL.* Arthritis Res Ther, 2009. **11**(5): p. R144.

255. Soehnlein, O. and L. Lindbom, *Phagocyte partnership during the onset and resolution of inflammation.* Nat Rev Immunol, 2010. **10**(6): p. 427-39.

256. Bennouna, S., et al., *Cross-talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection.* J Immunol, 2003. **171**(11): p. 6052-8.

257. Cassatella, M.A., et al., *Toll-like receptor-3-activated human mesenchymal stromal cells significantly prolong the survival and function of neutrophils.* Stem Cells, 2011. **29**(6): p. 1001-11.

258. Andreu, N., et al., *Rapid measurement of antituberculosis drug activity in vitro and in macrophages using bioluminescence.* J Antimicrob Chemother, 2012. **67**(2): p. 404-14.

259. Abbott, N.J., L. Ronnback, and E. Hansson, *Astrocyte-endothelial interactions at the blood-brain barrier.* Nat Rev Neurosci, 2006. **7**(1): p. 41-53.

260. Green, J.A., et al., *Mycobacterium tuberculosis-infected human monocytes down-regulate microglial MMP-2 secretion in CNS tuberculosis via TNFalpha, NFkappaB, p38 and caspase 8 dependent pathways.* J Neuroinflammation, 2011. **8**: p. 46.

261. Houghton, A.M., et al., *Macrophage elastase kills bacteria within murine macrophages.* Nature, 2009. **460**(7255): p. 637-41.

262. Kisich, K.O., et al., *Tumor necrosis factor alpha stimulates killing of Mycobacterium tuberculosis by human neutrophils.* Infect Immun, 2002. **70**(8): p. 4591-9.

263. Harfi, I., et al., *Regulation of human polymorphonuclear leukocytes functions by the neuropeptide pituitary adenylate cyclase-activating polypeptide after activation of MAPKs.* J Immunol, 2004. **173**(6): p. 4154-63.

264. Ferby, I., et al., *PAF-induced MAPK activation is inhibited by wortmannin in neutrophils and macrophages.* Adv Exp Med Biol, 1996. **416**: p. 321-6.

265. Liu, Y., et al., *Polo-like kinases inhibited by wortmannin. Labeling site and downstream effects.* J Biol Chem, 2007. **282**(4): p. 2505-11.

266. Foretz, M. and B. Viollet, *Regulation of hepatic metabolism by AMPK.* J Hepatol, 2010.

267. Lee, J.O., et al., *Metformin induces Rab4 through AMPK and modulates GLUT4 translocation in skeletal muscle cells.* J Cell Physiol, 2011. **226**(4): p. 974-81.

268. Zhao, X., et al., *Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury.* Am J Physiol Lung Cell Mol Physiol, 2008. **295**(3): p. L497-504.

269. Bae, H.B., et al., *AMP-activated protein kinase enhances the phagocytic ability of macrophages and neutrophils.* FASEB J, 2011. **25**(12): p. 4358-68.

270. Ernens, I., et al., *Adenosine inhibits matrix metalloproteinase-9 secretion by neutrophils: implication of A2a receptor and cAMP/PKA/Ca2+ pathway.* Circ Res, 2006. **99**(6): p. 590-7.

271. Ulrich Schaff, S.S., *Delivery of siRNA by electroporation into primary human neutrophils using the Gene Pulse MXcell System*, in *Gene transfer*. 2008, Bio-Rad.

272. Morris, M.C., et al., *A novel potent strategy for gene delivery using a single peptide vector as a carrier.* Nucleic Acids Res, 1999. **27**(17): p. 3510-7.

273. Irukayama-Tomobe, Y., et al., *Aromatic D-amino acids act as chemoattractant factors for human leukocytes through a G protein-coupled receptor, GPR109B.* Proc Natl Acad Sci U S A, 2009. **106**(10): p. 3930-4.

274. Smolen, J.E. and R.R. Sandborg, *Ca2(+)-induced secretion by electropermeabilized human neutrophils. The roles of Ca2+, nucleotides and protein kinase C.* Biochim Biophys Acta, 1990. **1052**(1): p. 133-42.

275. Murphy, R.T., et al., *Adenosine monophosphate-activated protein kinase disease mimicks hypertrophic cardiomyopathy and Wolff-Parkinson-White syndrome: natural history.* J Am Coll Cardiol, 2005. **45**(6): p. 922-30.

276. Burwinkel, B., et al., *Fatal congenital heart glycogenosis caused by a recurrent activating R531Q mutation in the gamma 2-subunit of AMP-activated protein kinase (PRKAG2), not by phosphorylase kinase deficiency.* Am J Hum Genet, 2005. **76**(6): p. 1034-49.

277. Daniel, T. and D. Carling, *Functional analysis of mutations in the gamma 2 subunit of AMP-activated protein kinase associated with cardiac hypertrophy and Wolff-Parkinson-White syndrome.* J Biol Chem, 2002. **277**(52): p. 51017-24.

278. Scott, J.W., et al., *CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations.* J Clin Invest, 2004. **113**(2): p. 274-84.

279. Oeckinghaus, A., M.S. Hayden, and S. Ghosh, *Crosstalk in NF-kappaB signaling pathways.* Nat Immunol, 2011. **12**(8): p. 695-708.

280. Abdel-Latif, D., M. Steward, and P. Lacy, *Neutrophil primary granule release and maximal superoxide generation depend on Rac2 in a common signalling pathway.* Can J Physiol Pharmacol, 2005. **83**(1): p. 69-75.

281. Takafuji, S., et al., *Matrix metalloproteinase-9 release from human leukocytes.* J Investig Allergol Clin Immunol, 2003. **13**(1): p. 50-5.

282. Nanamori, M., et al., *Regulation of leukocyte degranulation by cGMP-dependent protein kinase and phosphoinositide 3-kinase: potential roles in phosphorylation of target membrane SNARE complex proteins in rat mast cells.* J Immunol, 2007. **178**(1): p. 416-27.

283. Al-Shami, A., W. Mahanna, and P.H. Naccache, *Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. Selective activation of Jak2, Stat3, and Stat5b.* J Biol Chem, 1998. **273**(2): p. 1058-63.

284. Ratthe, C., et al., *Molecular mechanisms involved in interleukin-4-induced human neutrophils: expression and regulation of suppressor of cytokine signaling.* J Leukoc Biol, 2007. **81**(5): p. 1287-96.

285. Lee, P.J., et al., *ERK1/2 mitogen-activated protein kinase selectively mediates IL-13-induced lung inflammation and remodeling in vivo.* J Clin Invest, 2006. **116**(1): p. 163-73.

286. Liu, G., et al., *p53 Attenuates lipopolysaccharide-induced NF-kappaB activation and acute lung injury.* J Immunol, 2009. **182**(8): p. 5063-71.

287. Gaudreault, E., et al., *Involvement of BLT1 endocytosis and Yes kinase activation in leukotriene B4-induced neutrophil degranulation.* J Immunol, 2005. **174**(6): p. 3617-25.

288. Welch, H., C. Mauran, and I.I. Maridonneau-Parini, *Nonreceptor Protein-Tyrosine Kinases in Neutrophil Activation.* Methods, 1996. **9**(3): p. 607-18.

289. Hasday, J.D., et al., *Exposure to febrile temperature modifies endothelial cell response to tumor necrosis factor-alpha.* J Appl Physiol, 2001. **90**(1): p. 90-8.

290. Pryde, J.G., et al., *Temperature-dependent arrest of neutrophil apoptosis. Failure of Bax insertion into mitochondria at 15 degrees C prevents the release of cytochrome c.* J Biol Chem, 2000. **275**(43): p. 33574-84.

291. Arad, M., C.E. Seidman, and J.G. Seidman, *AMP-activated protein kinase in the heart: role during health and disease.* Circ Res, 2007. **100**(4): p. 474-88.

292. Ropelle, E.R., et al., *A central role for neuronal adenosine 5'-monophosphate-activated protein kinase in cancer-induced anorexia.* Endocrinology, 2007. **148**(11): p. 5220-9.

293. Lopez, M., et al., *Hypothalamic AMPK and fatty acid metabolism mediate thyroid regulation of energy balance.* Nat Med, 2010. **16**(9): p. 1001-8.

294. Shin, D.M., et al., *Mycobacterial lipoprotein activates autophagy via TLR2/1/CD14 and a functional vitamin D receptor signalling.* Cell Microbiol, 2010. **12**(11): p. 1648-65.

295. Suzuki, K., et al., *Cilostazol activates AMP-activated protein kinase and restores endothelial function in diabetes.* Am J Hypertens, 2008. **21**(4): p. 451-7.

296. Maiga, M., et al., *Successful shortening of tuberculosis treatment using adjuvant host-directed therapy with FDA-approved phosphodiesterase inhibitors in the mouse model.* PLoS One, 2012. **7**(2): p. e30749.

297. Ugarte-Gil, C.A., et al., *Induced Sputum MMP-1, -3 & -8 Concentrations during Treatment of Tuberculosis.* PLoS One, 2013. **8**(4): p. e61333.

298. Nandi, B. and S.M. Behar, *Regulation of neutrophils by interferon-gamma limits lung inflammation during tuberculosis infection.* J Exp Med, 2011. **208**(11): p. 2251-62.

299. Wejse, C., et al., *TBscore: Signs and symptoms from tuberculosis patients in a low-resource setting have predictive value and may be used to assess clinical course.* Scand J Infect Dis, 2008. **40**(2): p. 111-20.

300. Douda, D.N., et al., *Innate immune collectin surfactant protein D simultaneously binds both neutrophil extracellular traps and carbohydrate ligands and promotes bacterial trapping.* J Immunol, 2011. **187**(4): p. 1856-65.

301. Neeli, I. and M. Radic, *Knotting the NETs: analyzing histone modifications in neutrophil extracellular traps.* Arthritis Res Ther, 2012. **14**(2): p. 115.

302. Brill, A., et al., *Neutrophil extracellular traps promote deep vein thrombosis in mice.* J Thromb Haemost, 2012. **10**(1): p. 136-44.

303. Alfakry, H., et al., *The association of serum neutrophil markers and acute coronary syndrome.* Scand J Immunol, 2012. **76**(2): p. 181-7.

304. Carlson, M., et al., *Human neutrophil lipocalin is a unique marker of neutrophil inflammation in ulcerative colitis and proctitis.* Gut, 2002. **50**(4): p. 501-6.

305. Segura, R.M., et al., *Interleukin-8 and markers of neutrophil degranulation in pleural effusions.* Am J Respir Crit Care Med, 1998. **157**(5 Pt 1): p. 1565-72.

306. Locksley, R.M., C.B. Wilson, and S.J. Klebanoff, *Increased respiratory burst in myeloperoxidase-deficient monocytes.* Blood, 1983. **62**(4): p. 902-9.

307. Jung, M., et al., *Interleukin-10-induced neutrophil gelatinase-associated lipocalin production in macrophages with consequences for tumor growth.* Mol Cell Biol, 2012. **32**(19): p. 3938-48.

308. Abraham, M., et al., *Gelatinases (MMP-2 and MMP-9) are preferentially expressed by Th1 vs. Th2 cells.* J Neuroimmunol, 2005. **163**(1-2): p. 157-64.

309. Van Lint, P. and C. Libert, *Matrix metalloproteinase-8: cleavage can be decisive.* Cytokine Growth Factor Rev, 2006. **17**(4): p. 217-23.

310. Kodiha, M., et al., *Localization of AMP kinase is regulated by stress, cell density, and signaling through the MEK-->ERK1/2 pathway.* Am J Physiol Cell Physiol, 2007. **293**(5): p. C1427-36.

311. Fujie, K., et al., *Release of neutrophil elastase and its role in tissue injury in acute inflammation: effect of the elastase inhibitor, FR134043.* Eur J Pharmacol, 1999. **374**(1): p. 117-25.

312. Lee, W.L. and G.P. Downey, *Neutrophil activation and acute lung injury.* Curr Opin Crit Care, 2001. **7**(1): p. 1-7.

313. Keck, T., et al., *Matrix metalloproteinase-9 promotes neutrophil migration and alveolar capillary leakage in pancreatitis-associated lung injury in the rat.* Gastroenterology, 2002. **122**(1): p. 188-201.

314. Palaci, M., et al., *Cavitary disease and quantitative sputum bacillary load in cases of pulmonary tuberculosis.* J Clin Microbiol, 2007. **45**(12): p. 4064-6.

315. Corleis, B., et al., *Escape of Mycobacterium tuberculosis from oxidative killing by neutrophils.* Cell Microbiol, 2012. **14**(7): p. 1109-21.

316. Brown, A.E., T.J. Holzer, and B.R. Andersen, *Capacity of human neutrophils to kill Mycobacterium tuberculosis.* The Journal of infectious diseases, 1987. **156**(6): p. 985-9.

317. Lowe, D.M., et al., *A novel assay of antimycobacterial activity and phagocytosis by human neutrophils.* Tuberculosis (Edinb), 2013.

318. Lyadova, I.V., et al., *In mice, tuberculosis progression is associated with intensive inflammatory response and the accumulation of Gr-1 cells in the lungs.* PLoS One, 2010. **5**(5): p. e10469.

319. Gapski, R., et al., *Systemic MMP inhibition for periodontal wound repair: results of a multi-centre randomized-controlled clinical trial.* J Clin Periodontol, 2009. **36**(2): p. 149-56.

320. Cooper, A.M., *Cell-mediated immune responses in tuberculosis.* Annu Rev Immunol, 2009. **27**: p. 393-422.

321. Puente, X.S., et al., *Human and mouse proteases: a comparative genomic approach.* Nat Rev Genet, 2003. **4**(7): p. 544-58.

322. Conradie, F., et al., *Natural killer cell activation distinguishes Mycobacterium tuberculosis-mediated immune reconstitution syndrome from chronic HIV and HIV/MTB coinfection.* J Acquir Immune Defic Syndr, 2011. **58**(3): p. 309-18.

323. Fuchs, T.A., et al., *Extracellular DNA traps promote thrombosis.* Proc Natl Acad Sci U S A, 2010. **107**(36): p. 15880-5.

324. Keane, J., *Tumor necrosis factor blockers and reactivation of latent tuberculosis.* Clin Infect Dis, 2004. **39**(3): p. 300-2.

325. Keane, J. and B. Bresnihan, *Tuberculosis reactivation during immunosuppressive therapy in rheumatic diseases: diagnostic and therapeutic strategies.* Curr Opin Rheumatol, 2008. **20**(4): p. 443-9.

326. Helke, K.L., J.L. Mankowski, and Y.C. Manabe, *Animal models of cavitation in pulmonary tuberculosis.* Tuberculosis (Edinb), 2006. **86**(5): p. 337-48.

327. McGovern, N.N., et al., *Hypoxia selectively inhibits respiratory burst activity and killing of Staphylococcus aureus in human neutrophils.* J Immunol, 2011. **186**(1): p. 453-63.

328. Elks, P.M., et al., *Activation of hypoxia-inducible factor-1alpha (Hif-1alpha) delays inflammation resolution by reducing neutrophil apoptosis and reverse migration in a zebrafish inflammation model.* Blood, 2011. **118**(3): p. 712-22.

329. Serebrovskaya, T.V., et al., *Intermittent hypoxia mobilizes hematopoietic progenitors and augments cellular and humoral elements of innate immunity in adult men.* High Alt Med Biol, 2011. **12**(3): p. 243-52.

330. Wang, J.S. and H.C. Liu, *Systemic hypoxia enhances bactericidal activities of human polymorphonuclear leuocytes.* Clin Sci (Lond), 2009. **116**(11): p. 805-17.

APPENDIX 1 – ABBREVIATIONS

AMPK	5‘ Adenosine monophosphate -activated protein kinase
APMA	4-amino-phenyl mercuric acetate
ART	Anti-retroviral therapy
BBB	Blood brain barrier
BSA	Bovine serum albumin
CoATB	Conditioned media from M.tb infected astrocytes
CoACont	Conditioned media from uninfected astrocytes
CoMTB	Conditioned media from M.tb infected monocytes
CoMCont	Conditioned media from uninfected monocytes
CoNTB	Conditioned media from M.tb infected neutrophils
CoNCont	Conditioned media from uninfected neutrophils
C_T	Cycle threshold
DMEM	Dulbecco‘s minimal essential medium
DNA	Deoxyribonucleic acid
ECACC	European Collection of Cell Cultures
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid

ESAT-6	Early secreted antigenic target 6 kDa protein
FACS	Fluorescent activated cell scanning
FCS	Foetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HBSS	Hanks‘ balanced salt solution
HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
MACS	Magnetic activated cell sorting
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
MDM	Monocyte derived macrophage
MDR	Multi-drug resistant
MEM	Minimal essential media
MMPs	Matrix metalloproteinases
MOI	Multiplicity of infection
MPO	myeloperoxidase
M.tb	*Mycobacterium tuberculosis*
NADPH	Nicotinasmie adenine dinucleotide phosphate-oxidase

NEI	Neutrophil elastase inhibitor
NETS	Neutrophil extracellular traps
NGAL	Neutrophil gelatinase-associated lipocalin
OD	Optical density
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PE	Phycoerythrin
PEG	Polyethylene glycol
PI3-kinase	Phosphatidylinositol 3-kinases
PMA	Phorbol-12-myristate-13-acetate
PVC	Polyvinyl chloride
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
siRNA	small interfering ribonucleic acid
TAE	Tris-Acetate-EDTA
TB	Tuberculosis

TB supp	TB supernatant
TBS	Tris-buffered saline
TE	Tris-EDTA
TIMPs	Tissue inhibitors of matrix metalloproteases
TLR	Toll-lie receptors
XDR	Extensively drug resistant

APPENDIX 2 - PUBLICATIONS AND PRESENTATIONS

Published

The hidden costs of ethical research.
BMJ; Epub 28 Jan 2011
Catherine WM Ong, Jon S Friedland.

Submitted

Neutrophils drive AMPK-dependent tissue destruction in human Tuberculosis
Catherine WM Ong, Paul T Elkington, Cesar Ugarte-Gil, Maite T Tome-Esteban, Liku B Tezera, Przemyslaw Pabisiak, Vimal Patel, Robert H Gilman, Joanna C Porter & Jon S Friedland

Co-authored

Mycobacterium tuberculosis-infected human monocytes down-regulate microglial MMP-2 secretion in CNS tuberculosis via TNFα, NFκB, p38 and caspase 8 dependent pathways.
Journal of Neuroinflammation 2011, 8:46
J Green, S Dholakia, K Janczar, **CWM Ong**, R Moores, J Fry, P Elkington, F Roncaroli, P Elkington, J Friedland.

2 other manuscripts and 1 review under preparation

Presentations and Awards

Tissue destruction and cavitation: Neutrophils wield a double-edged sword in human pulmonary tuberculosis
CWM Ong*, P Elkington, C Ugarte-Gil, LB Tezera, Robert Gilman, J Porter, J Friedland
ID week 2013, San Francisco, USA. Oral presentation.
International Investigator Award
British Society for Immunology Travel Award

Neutrophil MMP-8/-9 cause matrix destruction in human TB and are associated with NETs
CWM Ong*, P Elkington, C Ugarte-Gil, LB Tezera, J Porter, J Friedland
Graduate School Summer Research Symposium 2013, Imperial College London

Neutrophil MMP-8/-9 cause matrix destruction in human TB amd are regulated by an AMPK-dependent pathway
CWM Ong*, P Elkington, C Ugarte-Gil, MT Esteban, V Patel, J Porter and J Friedland
Matrix Metalloproteinase Gordon Research Seminar 2013, Lucca (Barga), Italy. Oral Presentation

Neutrophil MMP-8/-9 cause matrix destruction in human TB and are associated with neutrophil extracellular traps
CWM Ong*, P Elkington, C Ugarte-Gil, LB Tezera, J Porter, J Friedland
Annual Academic Trainees Event, 2013, Imperial College London

Neutrophil MMP-8/-9 cause matrix destruction in human TB and are associated with neutrophil extracellular traps
CWM Ong*, P Elkington, C Ugarte-Gil, J Porter, J Friedland
Young Scientist Day, Imperial College London 2013. **2nd Prize for Poster.**

Neutrophil MMP-8/-9 cause matrix destruction in human TB and are associated with neutrophil extracellular traps
CWM Ong*, P Elkington, C Ugarte-Gil, J Porter, J Friedland
Keystone Symposia on Tuberculosis March 2013, Whistler, Canada.
Global Health Travel Award funded by the Bill and Melinda Gates Foundation

Neutrophils cause matrix destruction in human pulmonary Tuberculosis: *In vitro* and *in vivo* evidence from a clinical cohort
CWM Ong*, PT Elkington, C Ugarte-Gil, L Tezera, JC Porter, JS Friedland
European Congress of Immunology, Sept 2012, Glasgow. Oral presentation.
European Federation of Immunological Societies Grant Award.

Neutrophil MMP-8/-9 is increased in tuberculosis and causes matrix destruction
CWM Ong*, P Elkington, C Ugarte-Gil, F Roncaroli, J Friedland.
The Neutrophil in Immunity, 9-12th June 2012, Quebec, Canada.

Neutrophil MMP-8/-9 is increased in tuberculosis: evidence from *in vitro* studies and patients with pulmonary disease
C Ong*, P Elkington, C Ugarte-Gil, F Roncaroli, J Friedland
22nd ECCMID 31st March – 3 April 2012, London. Oral presentation **ECCMID Grant Award.**

Neutrophils drive MMP-8/-9 gene expression and secretion in Tuberculosis by complex mechanism.
CWM Ong*, P Elkington, S Singh, J Friedland
Society of Leukocyte Biology Meeting, Sept 2011, USA. Oral presentation.
American SLB Presidential Award, Prize winner.
British Infection Association Travel Grant Award

Neutrophil matrix metalloproteinase secretion in tuberculosis is regulated by intercellular networks
CWM Ong*, P Elkington, J Friedland
British Infection Association, Spring Meeting, UK, 20th May 2011. Oral Presentation