Modulation of Immunopathogenic Matrix Metalloproteinases in Pulmonary Tuberculosis by T helper-17 cytokines & the PI 3-Kinase pathway

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1. Abstract

*Mycobacterium tuberculosis* (Mtb) kills 1.7 million people annually. The Th1 paradigm does not explain TB-driven cavitation. Current treatment is lengthy with many adverse effects. The IL-23/Th17 axis plays a critical role in early Mtb containment. Respiratory stromal cells are important first-line defence and secrete MMPs. Regulation of Matrix metalloproteinases (MMPs), which are substrate-specific proteases causing extracellular matrix (ECM) degradation/remodelling, was investigated in the context of TB.

Human bronchoalveolar lavage (BAL) samples from 35 well-characterised TB and control patients were analysed for MMPs and Th17 cytokines. TB/Control lung biopsies were stained for MMPs/IL-17. Primary normal human bronchial epithelial cells (NHBEs) and MRC-5 fibroblasts were stimulated with IL-17/IL-22/IL-23, alone and in combination with conditioned medium from Mtb-infected monocytes (CoMTb). Secretion, gene expression, gene silencing and intracellular signalling were investigated by luminex, ELISA, zymography, dual-luciferase promoter-reporter, real time RT-PCR and siRNA transfection.

MMPs were up-regulated in Human TB BALs (p<0.0001). This positively correlated with cavitation score on CXRs. IL-17 and MMP-3 were co-expressed in pneumocytes around granulomas in TB lung biopsies.

CoMTb (but not direct infection) up-regulated secretion and gene expression of MMP-1 (collagenase, p<0.0001), MMP-3 (stromelysin, p<0.001) and MMP-9 (gelatinase, p<0.0001) from NHBEs. MMP-3 protein and promoter activity in MRC-5 fibroblasts was also increased by CoMTb. AKT inhibition suppressed all MMPs (p<0.01) whereas siRNA and chemical inhibition of the proximal PI3Kp110α subunit abrogated MMP-3 only (p<0.001). Distally, p70S6K (mTOR) blockade with rapamycin abrogated TB-driven MMP-1 and MMP-3 (p<0.001). MMP-9 regulation was more complex.

IL-17 independently and also synergistically with CoMTb, augmented MMP-3 secretion/gene expression from NHBEs and MRC-5 fibroblasts in a concentration-dependent manner (peak 8ng/ml, p<0.0001). This was p38-dependent, confirmed by p38-specific siRNA. In contrast, IL-17 down-regulated CoMTb-driven MMP-9 to baseline (p<0.01). Interleukin-22 augmented MMP-3 from fibroblasts in a TB network, but not from NHBEs. IL-23 did not drive MMPs.

MMP-1, MMP-3 and MMP-9 production was also affected by anti-mycobacterial agents in a TB network.

In summary, MMPs are key mediators of tissue damage in human pulmonary TB and are regulated in a cell- and stimulus-specific manner. IL-17 and IL-22 drive MMP-3 from human airway stromal cells. The PI3Kinase/p110α/p70S6K is a crucial target and its immunomodulation (e.g. with rapamycin) has potential as adjunctive therapy to limit tissue destruction and shorten chemotherapy in TB.
I dedicate this to my parents who have blessed me with immeasurable love and support during my research, as always.
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3. Declaration of Originality

I can confirm that the work included in this thesis is my own. The experiments in the chapter on the MMP-modulatory role of antibiotics were contributed to by our BSc students Harriet Gardiner and Andre Kubler, under my supervision in the laboratory. Part of the work on the MRC-5 fibroblasts was undertaken by our MSc student George Maniakis-Grivas, under the joint supervision of Dr Paul Elkington and myself in the laboratory. All these students were finally supervised by Prof Jon S Friedland. The ideas behind various sections of my research were either my own or conveyed to myself for further exploration by Prof Jon S Friedland or Dr Paul Elkington. I have in no shape or form made any attempt to reproduce anyone else’s work, written or practical.

~Shivani Singh  30.07.2011
4. Introduction

A name is the blueprint of the thing we call character. You ask, What's in a name? I answer, Just about everything you do.  ~Morris Mandel.

Consumption, Phthisis, Scrofula, Pott's disease and the White Plague have all been names given to Tuberculosis through history, and Tuberculosis has been true to each one of them. Before the discovery of *Mycobacterium tuberculosis* (Mt), consumption was its household name. It was recognised as a disease that can kill quickly but also one that often takes years to work its damage, ‘A dimple at a time’. We now recognise that dimple as a granuloma. Once again the name has changed but the riddle remains, as does the intrinsic character to deliberately skirt the obvious.

Reference was first made to these ‘dimples’ in modern medical literature by the French physician Rene Theophile Laennec circa 1800. Among other accomplishments, he had invented the stethoscope and his description of pulmonary lesions in patients who had succumbed to Tuberculosis was the beginning of our understanding of its pathology. The description was as follows:

‘Tuberculous matter begins as grey and semi-transparent that little by little becomes yellow, opaque and dense. Then it softens, and slowly acquires a liquidity like pus, and when it is expelled through the airways, it leaves cavities, that we will designate as tuberculous excavations.’

The discovery of Mt by Robert Koch in 1882 and his subsequent work on the pathology it causes, earned him the Nobel Prize for medicine in 1905. It was the beginning of only but an epoch in the history of Tuberculosis, which some have described as the oldest disease known to mankind. Koch had also unknowingly discovered what we now call cell-mediated immunity. He is known to have said of the supernatant from Mt grown in broth as something that would aid the diagnosis of questionable cases in the future, when bacilli can’t be detected in the sputum. The decades following Koch saw several investigators establish the importance of host response in the pathogenesis of Tuberculosis. My PhD focuses on one such host-derived factor that drives tissue destruction in Tuberculosis and thereby allows Mt to persist and spread.
4.1. The Global Burden of Tuberculosis

Despite the enormous strides made by medical research, freedom from the curse of Mtb is a distant dream for mankind. In 2009, the 13th World Health Organization (WHO) estimated 9.4 million incident cases of tuberculosis (TB) in the world (range 8.9–9.9), a rate of 137 cases per 100,000 population. It also estimated 14 million prevalent TB cases and 1.3 million TB deaths in HIV-negative people, with an additional 0.4 million deaths occurring in HIV positive people.


The WHO has developed a new six point Stop TB Strategy to halve the global burden of TB by 2015 by ensuring that all TB patients, including those co-infected with HIV, benefit from universal access to high-quality diagnosis and patient-centred treatment. The major challenges in the achievement of this goal are the emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) strains, lengthy treatment regimens leading to non-compliance, suboptimal health services in the developing world and the lack of an efficacious and predictable vaccine.

TB is also a growing problem in the UK. The number of cases diagnosed has been rising each year. The department of health published a ‘TB action plan toolkit’ in 2007 which estimated that today in the UK, as many people are diagnosed with TB as with HIV.


Approximately 350 people die each year from the disease and parts of the UK are now seeing more than 40 cases of TB per 100,000 population – which puts them on par with some resource poor countries. The incidence of TB correlates strongly with poor socioeconomic status. This variation is further negatively impacted by HIV infection, which also affects some of the poorest geographic areas. Evidence of infection based on skin-test conversion indicates that up to one-third of the world’s population is infected with Mtb and it is estimated that an infected individual has a 5-10% lifetime risk of developing the disease.
4.2. Host Response to Pulmonary TB

4.2.1 Granuloma Formation

The supremely efficient transmission of Mtb is the consequence of a sensitive interplay between the bacterium and the host, encompassing a whole host of events leading to either disease containment or disease dissemination. Mtb has evolved specific strategies and effectors to maximise dissemination. The host initially counters this, not always successfully, at the site of infection. Mtb is a facultative intracellular organism which usually infects the lungs via the aerogenic route. Soon after Mtb enters the macrophage, the host responds by remodelling the site into a cellular mass or ‘tubercle’ or ‘granuloma’, as it is more commonly known. This tubercle contains the initial spread of Mtb but it also provides the bacterium with an ‘immuno-privileged’ site to replicate and evade a potent host immune response and is thereby paradoxically responsible for the immunopathology seen in TB [1].

After the macrophage phagocytoses the bacterium, a localised pro-inflammatory surge of cytokines and chemokines ensues, leading to an influx of mononuclear cells from the neighbouring blood vessels [2]. Studies in mice, human BALs and serum, and retrospective human histological and molecular studies illustrate that TNF-α is the dominant cytokine, and is accompanied with a surge of the chemokines CCL-2, CCL-3, CCL-4, CCL-5, CXCL-8, CXCL-9, CXCL-10, and CXCL-11 [3-6]. These chemokines lead to a recruitment of NK T cells, then CD4+, CD8+, γδ T cells and B cells. A quietening down of this response almost always coincides with the production of IFN-γ. For example, IFN-γ deficient mice are unable to switch off this pro-inflammatory response and suffer from fatal pathology [7]. At this stage, the granuloma can be macroscopically identified as a distinct structure in the lung.
The granuloma consists of infected macrophages at the centre, surrounded by mononuclear cells, lymphocytes and a fibrous cuff of collagen and extracellular matrix components at the periphery [9]. At this stage, there are no signs of disease and the host is not infectious. Containment usually fails when the host immune status changes, generally as a consequence of old age, stress, malnutrition, genetic susceptibility polymorphisms, or HIV co-infection. Following such a change the granuloma becomes avascular, then caseates and ruptures. Thousands of viable infectious bacilli are spilled into the airways. The infectious droplets are known to remain in the atmosphere for several hours after being generated and the infectious dose has been estimated at a single bacterium.
4.2.2 Immune mechanisms related to host protection

Mtb infects phagocytic antigen-presenting cells in the lung, including alveolar macrophages, lung macrophages and dendritic cells. αβ T cell receptor expressing T cells are stimulated by these Mtb-infected dendritic cells in the lymph nodes draining the lung and differentiate into Th1-like CD4+ T cells which among other cytokines, secrete IFN-γ. CD4+ T cells and the major histocompatibility complex (MHC) class II molecules that induce CD4+ T cell responses are central to host protection from Mtb [10]. The critical role of the CD4+ T cells in granuloma formation is clinically evident by the impact of highly active anti-retroviral therapy (HAART) in HIV+ subjects co-infected with Mtb. The reconstitution of adaptive immunity and rise in CD4+ T cell count following HAART is associated with cytokine release, activation of mycobacteria-infected macrophages and significant pathological damage [11]. IFN-γ deficiency causes delayed or absent granuloma formation in both humans and mice [12]. Within the granuloma, IFN-γ stimulates macrophages to kill mycobacteria through a variety of mechanisms, including activation of phagocyte oxidase and inducible nitric synthase to produce reactive oxygen and nitrogen metabolites, and also the up-regulation of p47, the GTPase to stimulate phago-lysosomal fusion [13] [14].

The TNF superfamily is essential for granuloma formation and protection following Mtb infection. In the absence of TNF, there is delay in the initial recruitment of monocytes into infected tissue [15, 16]. Despite normal regulation of T cells, this is followed by a dysregulated inflammatory response, with rapid influx of neutrophils, formation of necrotic lesions, uncontrolled bacterial growth and rapid demise of infected animals. The establishment of chemokine gradients is also crucial for the recruitment and aggregation of inflammatory cells to form granulomas. Multiple studies have demonstrated their indispensable role and their elevated levels in TB [17-20]. Chemokines are discussed in more detail in a later section.

Because of its intracellular location, it was assumed that Mtb is not exposed to antibodies and therefore the antibody immune defence was not considered to be protective. However, at the initial stages of infection, antibodies alone or in conjunction with cytokines, perform
important functions such as prevent entry of Mtb at mucosal surfaces. Passive inoculation with IgA antibodies against α-crystallin, an immuno-dominant mycobacterial antigen, was successful in an experimental mouse model of TB lung infection [21], and the combined administration of IFN-γ and IgA was even more efficacious [22].

4.2.3 Immune mechanisms related to disease dissemination

During the course of active disease, even in the absence of immunosuppressive conditions, several pathogen and host factors can contribute to the down-regulation of protective immunity, permitting disease progression. Mtb uses multiple mechanisms to evade both the innate and the adaptive host immunity such as resistance to innate microbicidal agents and inhibition of antigen presentation.

Mtb factors: Mtb survives inside macrophages by arresting the normal maturation of their phagosomes. Besides having a different morphology, the vacuoles where the bacteria reside present ‘early’ endosomal component markers such as Rab5 and Rab14 GTPases, but not the late markers (e.g. Rab7), indicating that maturation is blocked early on [23]. Another characteristic of the mycobacterial phagosome is restriction of its acidification to pH 6.4 [24, 25]. This strategy adopted by Mtb limits the hostility of the intracellular environment. It has been shown that Mtb-containing vacuoles in infected macrophages isolated directly from BAL fluid of TB patients fail to acidify and do not acquire lysosomal cargo [26]. The mycobacterial phagosome does not physically associate with inducible nitric oxide synthase (iNOS) [27]. As nitric oxide synthase-mediated microbicidal intermediates can be stimulated by cytokines such as TNF-α and IFN-γ, this property of the Mtb phagosome is an additional mechanism by which it overcomes the host defence.

There is rich literature dating back to the 1960’s that has identified a range of mycobacterial cell wall lipids that are central to Mtb’s success [28, 29]. Studies have revealed that the Mtb cell wall components lipoarabinomannan and arabinomannan are released inside infected
cells and trafficked to internal vesicles in lysosomes [30, 31]. These bacterial lipids are morphologically similar to the major histocompatibility complex (MHC) class II-enriched compartment and present in extracellular microvesicular fractions and uninfected cells [32]. As these lipids possess biological properties, their transport to bystander cells expands Mtb’s sphere of influence. Subsequent studies have also shown that similar vesicles can be generated following apoptosis of infected macrophages and dendritic cells [33, 34]. Characterisation of the main lipids have identified them as trehalose mycolates, mycoside B, phosphatidylinositol mannoside, phosphatidylglycerol and phosphatidylethanolamine. Trehalose mycolates (particularly the dimycolates) induce profound recruitment of immune effector cells in vivo, indicating that they are the most potent lipids [35]. Fluorescent tagged lipids were phagocytosed by macrophages and contributed to the generation of a pro-inflammatory response involving TNF-α, IL-1α, IL-6, IL-10, CCL-2 and CXCL-10 [36]. Additionally, protein antigens secreted by multiplying bacteria are also immunogenic, such as early secreted antigenic target 6 (ESAT 6) and culture filtrate protein 10 (CFP 10). Several of these proteins can be presented by the MHC molecules [37-40].

**Host Factors:** Although the Th-1 response is the predominant one in TB immunity, there is evidence that Th-2 T cells have a role as well, albeit generally non-protective. Low antigen loads prime the Th-1 response, while high bacillary load induces a Th-2 response. IL-4 levels tend to be higher in patients living close to the equator, but this is possibly related to simultaneous infections with helminths, or a higher mycobacterium inoculum. IL-4 mRNA is up-regulated in human pulmonary TB and this correlates significantly with serum IgE and the extent of cavitation [41-43]. The presence of IL-4 at later stages of the disease has a direct pathogenic effect as it down-regulates the protective Th-1 response. This balance is related to the pathological effects of TNF-α, such as fever, weight loss and tissue damage. TNF-α can play a paradoxical role in the immunopathology of TB, and whether this response is protective or destructive depends on the prevailing CD4+ T cell cytokine at the site of infection/inflammation. When TNF-α is released into a relatively pure Th-1 inflammatory site, it acts as a supplementary macrophage-activating cytokine and offers protection but in a high IL-4 milieu, it causes damage.
Prostaglandins can de-regulate the protective immune response against TB as PGE-2 is immunosuppressive for T cell mediated immunity. In an experimental mouse model, foamy macrophages from pneumonic areas exhibited strong PGE-2 immuno-staining and the concentrations were 4-fold higher in the late phase [44]. When the prostaglandin production was suppressed, there was reduction in the bacillary load and the pneumonic changes, with a simultaneous increment in granuloma size and expressions of IFN-γ, TNF-α and iNOS.

Adrenal compromise impacts negatively on TB immunity. Reactivation or progression of infection is sensitive to activation of the hypothalamus-pituitary-adrenal axis. In mice reactivation was demonstrated to be due to glucocorticoid release, which reduced macrophage activation and Th-1 T cell activity [45]. Similar cortisol-related changes have been observed in human lymphocytes as well [46].
4.3 Intracellular signalling cascades regulating host response to Mtb

Pattern recognition receptors (PRRs) expressed on macrophages and other leucocytes activate signalling cascades that play a fundamental role in host defence. Well-studied PRRs include the mannose receptor and the toll-like receptor (TLR). Toll-like receptors are key sensors of Mtb and play an important role in innate defence. They are expressed on many cells. TLR2, TLR9 and possibly TLR4 are responsible for recognising Mtb [10]. Several lines of evidence have suggested the protective role of TLR2 in mycobacterial infection. For example, TLR2 has classically been recognised as a principal inducer of TNF-α in Mtb infection [47]. TLR2 signalling mediated by Mtb augments expression of the vitamin D receptor thereby leading to increased expression of antimicrobial peptides [48]. TLR requires the cooperation of a number of signalling adaptors, including myeloid differentiation primary response protein 88, MyD88 [49, 50].

The mitogen-activated protein kinase pathways are induced by mycobacteria via the TLRs. Regulation of the MAPKs is altered in TB patients. Triggering of the MAPKs such as the p38 cascade, leads to activation of several transcription factors including NF-κB, AP-1 and CREB after Mtb infection [51-55]. Previous studies in mice and humans have linked the p38 MAPK pathway to the production of mycobacteria-induced IL-10 as well as other cytokines [56]. It has also been implicated in the arrest of Mtb phagosome maturation, with pharmacological blocking of p38 MAPK activity causing an increase in phagosome acidification [57]. In addition, ERK 1/2 signalling has been shown to increase the expression of TNF-α in human macrophages [58]. Recent studies illustrated that atypical protein kinase C (PKC) ζ played an important role in the upstream regulation of ERK 1/2 regulation and TNF-α expression in response to Mtb from various leukocytic cells [59]. Thus the p38 and ERK pathways play an important regulatory role in Mtb driven host responses and the identification of key components and switch points of these cascades can lead to the revelation of novel targets for TB therapy. Mtb also triggers other intracellular signalling cascades such as the PI3K, this has been discussed in a later section.
4.4 Host genetic susceptibility to TB

Adrian Hill [60] reviewed evidence that susceptibility to TB in humans is a polygenic trait, with increased concordance of the disease in monozygotic twins, and increased susceptibility among inbred populations. Numerous genes that contributed to susceptibility to varying extents in different populations were identified. Genes encoding HLA-DRB1, vitamin D receptor, NRAMP-1, Interferon-γ, cathepsin-Z and complement receptors 1 (CR1 and CD35) have all been implicated in independent studies. In patients with Mendelian Susceptibility to Mycobacterial diseases, key mutations have been identified in the type-1 cytokine pathway, example *IL12B, IL12RB1, IFNCLR1, IFNCLR2* and *STAT1* [61, 62].
4.5 Pulmonary epithelial defence and extracellular matrix degradation in TB immunopathology

4.5.1 Mucosal surfaces: the first line of defence

Mucosal surfaces are often the first interface between the host, the resident microflora and environmental pathogens. It constitutes both a physical barrier and a first line of defence via mechanisms of innate immunity. To counter these impediments, pathogens deploy virulence factors, surface adherence molecules and also induce the host pro-inflammatory response. Conserved structures on pathogens (pathogen associated molecular patterns, PAMPs) are recognised by pattern recognition receptors (Toll-like receptors and NOD-like receptors) of epithelial cells and antigen presenting cells [63, 64]. These host cells are then activated and secrete inflammatory mediators. The epithelium harbours a large variety of such immunologically active cells, including several subsets of T cells, to effectively respond to a plethora of pathogens [65]. Recent studies point towards a new subset of T cells, the Th17 cells, as a key component of mucosal immunity [66] (discussed further in a later section).

The alveolar macrophage is the first cell to engulf Mtb but not the only one. In fact, the number of epithelial cells in the alveoli is 30 times higher than the number of macrophages and thus the likelihood of their being the first cells to be exposed to the bacillus is much higher. The presence of mycobacterial DNA in type II pneumocytes, fibroblasts and endothelial cells was first reported by Hernandez-Pando in necropsy specimens from people who had died of TB [67]. Subsequently, Mtb has been shown to invade type II alveolar epithelial cells [68] through possibly a process of macropinocytosis triggered by an Mtb secretory product [69]. Moreover, epithelial cells are able to contribute to an initial inflammatory environment after infection with Mtb, by the secreting several inflammatory mediators such as CXCL-8 (which reduces Mtb survival within macrophages) [70], the T cell specific CX-C chemokines [71], and also IFN-γ (which has a positive correlation to the expression of inducible NO synthase) [72]. Alveolar epithelial cells can also be antigen-presenting cells [73], and lastly there have been numerous reports that illustrate that upon
infection with Mtb, alveolar epithelial cells secrete and express the important antimicrobial human-β defensins [74, 75].

**4.5.2 The pulmonary extracellular matrix**

Extracellular matrix (ECM) components are critically involved in every stage of lung biology including lung development, normal physiology and acute and chronic disease states [76]. The two basic components of the ECM are the basement membrane, which is a thin network of cross-linked glycoproteins, and the loose fibril-like interstitial matrix [77]. The lung contains a host of interstitial matrix components that provide support to the airways and lung vasculature by virtue of their unique architecture and interaction with pulmonary cells. Collagen type I is the predominant determinant of tensile strength, whereas elastin is essential to lung function at the level of the alveoli. Degradation and turnover of the matrix is therefore a critical process and influences the pathogenesis of many disease states.

The lung remodelling associated with TB has never been convincingly explained. TB causes a wide range of lung ECM pathologies: cavitation, fibrosis, scarring, volume loss and traction bronchiectasis, to name a salient few [78]. Cavitation into airways, with cough-induced aerosol generation is the principle mechanism by which TB is spread. Cavitation may erode the blood vessels with resultant haemoptysis. Poor drug penetration into cavities facilitates latency and selection of drug-resistant strains. Finally, pulmonary cavitation creates an immunologically privileged site that allows Mtb to evade the host immune response and multiply exponentially. This impaired immune surveillance within a cavity is well illustrated by its colonisation with opportunisitic mycobacteria or fungi such as *Aspergillus fumigates*. The importance of the cavity was also well appreciated in the pre-chemotherapy era when all therapies such as phrenic crush, thoracoplasty and plombage aimed to collapse the underlying lung.
Although the exact mechanisms of liquefaction preceding cavitation are unknown, several studies suggest that dysregulation of protease control mechanisms are likely to mediate degradation of the structural components in the lung [79]. The host cytokines and chemokines implicated in anti-Mtb inflammatory response do not have the intrinsic ability to degrade the resilient pulmonary structural collagens, although they can up-regulate protease activity. Release of lysosomal contents and Mtb-derived endopeptidases can only make a small contribution in view of their negligible concentrations. Hence the obvious culpable mediators are host–derived proteases [80]. One such family of proteases are collectively called Matrix Metalloproteinases. They can degrade all components of the extracellular matrix. They are the only proteases that have the ability to degrade collagen at neutral pH [81] and hence have been widely implicated in diseases associated with ECM destruction.
4.6. MMPs—Classification, Modulation & Function in Health and Disease

The discovery of a collagenolytic activity during metamorphosis in tadpoles, secondary to an interstitial collagenase [82] was the cornerstone for the eventual description of a large family of clinically important endopeptidases in man, later called matrix metalloproteinases (MMPs). Further studies showed that beyond their collagen-degrading functions, MMPs degrade and process many other components of the extracellular matrix (ECM). They have been directly implicated in numerous biological and physiological processes from embryo implantation, cell death and necrosis to ductal branching, bone ossification and blood vessel remodelling. MMPs play a primary role in normal tissue maintenance including wound healing and repair, reproductive processes and immune defence [83-87]. As a consequence of such a vast repertoire of physiological functions, altered expression or misregulation of MMP activities in vivo has been associated with the development of a wide range of pathologies, including chronic inflammatory diseases and cancer. Although conventionally speaking, the primary function of MMPs is still believed to be through degradation of the ECM, proteomic and degradomic screens have shown more complex roles for MMPs in the direct regulation of a host of signalling molecules (including almost all the chemokines), in the normal physiology of stromal cells and in the innate immune response by processing non-ECM proteins, growth factors, cytokines, cell receptors, serine proteinase inhibitors and other MMPs [88-90].

4.6.1. Classification and Nomenclature

The MMP family currently comprises of 25 related, but distinct vertebrate gene products, of which 24 are found in mammals [81, 87]. Table 1 adapted from Parks et al [87] (page 23) illustrates all the MMPs found in mammals along with their common names and substrates. MMPs are categorised according to their architectural features and their substrates. The general structural blueprint of MMPs shows three domains: the pro-peptide, the catalytic domain and the hemopexin-like C-terminal domain that is linked to the catalytic domain via
a flexible hinge region. MMPs are initially expressed in an enzymatically inactive state due to the interaction of a cysteine residue in the pro-domain with the zinc in the catalytic site. Only after disruption of this interaction by a mechanism called the cysteine switch (removal of the pro-domain or chemical modification of the cysteine residue) does the enzyme become proteolytically active [91]. MMPs are usually secreted or anchored to the cell surface, thereby confining their activity mainly to the membrane, the secretory pathway or the extracellular space.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Common name</th>
<th>Substrates</th>
</tr>
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<tbody>
<tr>
<td>MMP-1</td>
<td>Collagenase 1</td>
<td>Type I and II fibrillar collagens</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Gelatinase A</td>
<td>CCL7, CXCL12</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Stromelysin 1</td>
<td>E-cadherin, Laminin, type IV collagen, Latent TGF-β</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrilysin</td>
<td>Pro-α-defensin, FAS ligand, Latent TNF, Syndecan-1, Elastin</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Collagenase 2</td>
<td>Mouse CXCL5</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Gelatinase B</td>
<td>Zona occludens 1, α1-antiproteinase, Latent TGF-β1, Latent VEGF</td>
</tr>
<tr>
<td>MMP-10</td>
<td>Stromelysin 2</td>
<td></td>
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<tr>
<td>MMP-11</td>
<td>Stromelysin 3</td>
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<tr>
<td>MMP-12</td>
<td>Metalloelastase</td>
<td>Latent TNF</td>
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<tr>
<td>MMP-13</td>
<td>Collagenase 3</td>
<td>Type I and II fibrillar collagens</td>
</tr>
<tr>
<td>MMP-14</td>
<td>MT1-MMP</td>
<td>ProMMP2, Fibrillar collagens, Fibrin, Syndecan-1</td>
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<tr>
<td>MMP-15</td>
<td>MT2-MMP</td>
<td>Fibrin</td>
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<tr>
<td>MMP-16</td>
<td>MT3-MMP</td>
<td>Fibrin, Syndecan-1</td>
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<td>MMP-17</td>
<td>MT4-MMP</td>
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<tr>
<td>MMP-19</td>
<td>Enamelysin</td>
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<td>MMP-20</td>
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<td>MMP-21</td>
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<td>MMP-22</td>
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<td>MMP-23</td>
<td>CA-MMP</td>
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<td>MMP-24</td>
<td>MT5-MMP</td>
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<tr>
<td>MMP-25</td>
<td>Leukolysin</td>
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<tr>
<td>MMP-26</td>
<td>Endometase</td>
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<td>MMP-27</td>
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<tr>
<td>MMP-28</td>
<td>Epilysin</td>
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</table>

*Table 1, adapted from Parks et al [87]*
Some of the MMP substrates can be localised intracellularly [90]. The earliest intracellular MMP substrates detected were troponin I, lens β-B1 crystallin and αβ crystallin [92-94]. MMP-2 degradation of troponin I is associated with diminished contractive function of the heart, and MMP-9 degradation of the crystallins is associated with multiple sclerosis and cataract.

4.6.2. ADAM (A Disintegrin and Metalloproteinase) and ADAMTS (ADAM with thrombospondin motifs)

Closely related to the MMPs are the ADAM and ADAMTS families of metzincin proteinases. ADAMs fulfil a broad spectrum of roles in fertilisation, embryo development and cancer [95]. Most ADAMs are membrane-anchored and only half of them have proteolytic activity. The ADAMTS are generally secreted and soluble and play a role in ECM assembly, ovulation and cancer.

4.6.3. Regulation of MMP activity

The function of MMPs in vivo depends on the local balance between them and their physiological inhibitors. Substantial energy resources are therefore used up to prevent un-regulated extracellular proteolysis by MMPs. For example high concentrations of the proteinase inhibitors alpha-2-macroglobulin, alpha-1-proteinase inhibitor and alpha-1-chymotrypsin are produced in the liver and released into the plasma [96], where these molecules bind to the active site of MMPs and other proteinases. The proteinase-inhibitor complexes are then recognised by a scavenger receptor and swiftly engulfed by macrophages.

The most important physiological inhibitors of MMP function are the tissue inhibitors of MMPs called TIMPs which are most commonly expressed in tumour sites [97]. TIMP-1, -2, -3 & -4 have been identified to be endogenously expressed in vertebrates and form
stoichiometric 1:1 complexes with active MMPs leading to inhibition of their proteolytic activity. The primary action of TIMPs is to inhibit MMPs, but numerous studies have reported that they have cell-growth-promoting, anti-apoptotic, and antiangiogenic roles.

Another key step in the regulation of MMP activity is the conversion of the zymogen into an active proteolytic enzyme. There are several proteinases that mediate MMP activation, such as plasmin, furin or active MMPs [91]. Once activated the MMP may also launch a negative feedback signal. Additionally, proteolytic activity of MMPs is commonly regulated at the level of gene expression and by compartmentalisation/localisation following their secretion into the extracellular space [87, 89]. The major source of MMPs in tumours is not the cancer cell but the stromal cells infiltrating the tumour [98]. Different types of stromal cells produce a specific set of proteinases and proteinase-inhibitors. The cellular source of MMPs can therefore have an important consequence on their function and activity as well. For instance, neutrophil-derived MMP-9, which does not have a bound TIMP-1, is more volatile [99]. MMPs are also influenced by reactive oxygen species (ROS). These oxidants initially activate MMPs via oxidation of the pro-domain cysteine [100], but eventually in combination with the enzyme myeloperoxidase from inflammatory cells, inactivate them by modification of amino acids in the catalytic domain (mediated by hypochlorous acid) [101].

In mesenchymal and monocytic cells, inflammatory signals such as the cytokines IL-1β, TNF-α, Oncostatin M and RANKL, and microbial products such as LPS are among very potent transcriptional activators of MMP-1, MMP-3, MMP-9, MMP-13 and MMP-14. It is the integration of several pathways through the recruitment of multiple transcription factors that contribute to this. As the first member of the MMP family to be identified and cloned, MMP-1’s gene regulation has been studied in the greatest detail. Unsurprisingly, it is a complex process involving transcriptional and post-transcriptional mechanisms [102]. Studies in human and rabbit genes have established a requirement for the AP-1 and Ets families of transcription factors that bind to their cognate sites just upstream of the TATA box [81]. NF-κB is an important mediator of gene expression in inflammation and immunity, and has been reported by several laboratories to activate both MMP-1 and MMP-9 [103, 104].
The mitogen activated protein kinases (MAPK) are serine threonine protein kinases that activate transcription factors and RNA-modifying proteins in response to growth factors, cell–matrix interactions and inflammatory signals. The MAPKs are divided into the extracellular-regulated kinases (ERK), the stress-activated or p38 protein kinases and the c-Jun N-terminal kinases (JNK). All these MAPKs, in particular ERK and p38 are well known to regulate several MMP genes upon activation by growth factor and inflammatory stimuli[105].

4.6.4. Insights into the role of MMPs in cancer and developmental remodelling: Beyond simply degradation of the ECM

MMPs have been implicated in cancer for more than 40 years and several of them are over-expressed in various carcinomas. The hypothesis that MMP-mediated ECM degradation leads to cancer cell invasion and metastasis has been a guiding principle in MMP research. The discovery that inhibition of MMPs suppresses the invasive potential of tumours in animal studies was implemented into clinical trials rather promptly but failed to improve patient survival [106]. It is now evident that MMP function is more complex than initially envisaged and that these enzymes do more than just degrade physical barriers. Several lines of evidence support an important role for MMPs in angiogenic or lymphangiogenic processes leading to tumour progression. The major MMPs involved in tumour angiogenesis are MMP-2, -9 and -14 and to a lesser extent MMP-1 and -7 [107].

4.6.5. MMPs orchestrate cytokines and chemokines in inflammation, innate immunity and infection

There is increasing evidence implicating MMPs as major regulators of innate and acquired immunity. Several knock-out mice studies show that MMPs play an important role in acute and chronic inflammation [87].
Cytokines: One of the most important pro-inflammatory cytokines is TNF-α, which is expressed as a membrane precursor (pro-TNF-α) on a variety of cells including macrophages and T cells. Conversion of pro-TNF-α into the soluble cytokinetic form requires proteolytic cleavage by ADAM-17, also known as TNF-α converting enzyme (TACE), or by MMPs [108]. Another pro-inflammatory cytokine, interleukin-1β is activated by MMPs-2, -3 and -9, suggesting a tight regulation of IL-1β activity by MMPs [109, 110]. Another example of MMP action in inflammation is the ability of MMP-9 to inactivate IFN-β [111].

Chemokines: A number of studies have shown proteolytic alteration of chemokines by MMPs [84]. MMPs can inactivate, enhance or antagonise chemokines and thereby influence inflammatory responses dependent on chemokine gradients such as regulation of leucocyte trafficking. CCL8/MCP-2 is processed by MMP-1 and MMP-3. MMP-8, -9 and -12 modulate the activity of CXCL11/ I-TAC, a potent Th1 lymphocyte-attracting chemokine [112]. MMP-8 is involved in the generation of chemotactic ECM breakdown fragments and thus regulates neutrophil recruitment to sites of inflammation [113]. N-terminal processing of CXCL8/IL-8 by MMP-9 and MMP-8 causes a ten-fold increase in its chemotactic and pro-angiogenic activity [114]. MMP-8 null mice showed impaired recruitment of neutrophils in an LPS-induced inflammation model [115]. Neutrophils of MMP-7 null mice accumulated in the perivascular region after bleomycin lung injury, unable to infiltrate into the alveolar lumen due to defective cleavage of CXCL1 from syndecan-1 on the endothelial cells and therefore impaired generation of a chemokine gradient [116, 117]. Leukocyte accumulation in the lung parenchyma of allergen-induced asthmatic MMP-2 null mice was correlated with a decrease in free CCL11/ eotaxin level [118].

Innate Immunity and Infection: As innate immunity comprises of mechanisms of defence which do not require previous exposure/ generation of memory, maintenance of intact barriers and secretion of anti-microbial peptides are its paramount properties. Several MMPs contribute to towards a healthy epithelium, in particular MMP-7 [119]. All epithelial tissues that are exposed to the external environment have a high basal level of MMP-7 expression. MMP-7 activates α-defensins and MMP-7 null mice have an impaired ability to clear enteric pathogens such as *E.coli* and *Salmonella* and exhibit impaired airway mucosal wound healing [119] [120]. Other evidence for the role of MMPs in lung innate immunity arises from the
observation that pulmonary surfactant proteins (part of innate host defence) regulate the expression of several MMPs in alveolar macrophages, independent of TNF-α or IL-1β [121]. This also suggests an antimicrobial function of MMPs.

Although it is well-proven that MMP activity is required for protection from various infectious agents, it is also evident that uncontrolled MMP activity can cause infection-related immunopathology. For instance, MMP-14 interacts with the C1q component of the complement system in a non-proteolytic receptor-ligand manner, without inducing C1qr or C1qs, suggesting that this binding may inhibit activation of the complement proteinase cascade [122]. Dengue-infected dendritic cells overproduce MMP-9, and to a lesser extent MMP-2. This is associated with decreased PECAM-1 and VE-cadherin in the vascular endothelium. Since these molecules contribute to the tightness of the intercellular junctions, their deficiency is associated with marked increase in vascular permeability in dengue haemorrhagic fever [123]. High levels of MMP-9 have also been implicated in the pathogenesis of HIV dementia and the associated neuronal cell death, both in human and mice studies[124]. Typically, TIMP-1 levels in the CSF is low. The tat-protein has been shown to up-regulate monocyte MMP-9 secretion [125] and gp120 increases T cell MMP-9 [126].

Another fatal infection that is associated with the overproduction of MMPs is gram negative endotoxaemia. Degradation of type IV collagen leads to breakdown of the endothelial barrier and septic shock/ multiple organ failure ensue. LPS (lipopolysaccharide) from gram negative bacteria upregulates MMP-1, -7 and -9 [127].
4.7. MMPs in Pulmonary diseases and Tuberculosis

4.7.1. MMPs in Pulmonary Diseases

MMPs are critical for the development, repair and immunopathology of various inflammatory and infectious conditions in the lung. They induce morphological changes in the lung, which occur during chronic conditions such as COPD. MMP-12 (macrophage elastase) is important during COPD pathogenesis and increased MMP-12 expression is associated with smoking [128]. An *in vivo* mouse study showed that deletion of MMP-12 abrogated smoking induced inflammation [129]. A recent genetic analysis of human COPD patients showed that the common serine variant at codon 357 of the MMP-12 gene rs652438 is associated with more aggressive matrix degradation and increased disease severity [130]. Although MMP-12 (elastase) has been historically proposed to be the primary enzyme associated with the severity of emphysematous lung damage, analysis of BAL fluids from emphysema has suggested that collagenase activity might be a better indicator of the presence of the disease [131]. MMP-1 was found in type II pneumocytes in emphysema but not in control subjects [132]. Neutrophil-derived MMP-8 levels increased remarkably in patients with non-infectious exacerbation of COPD, compared to stable COPD and healthy controls [133]. Elevated MMP-2/-9 gelatinolytic activity in sputum has been displayed in COPD patients [134] and high levels of MMP-1 and MMP-9 mRNA were also detected in macrophages from COPD patients [131].

MMP-9 was the first MMP to be studied extensively for its role in asthma pathogenesis. Asthma patients have increased MMP-9 levels in sputum, BALF and serum [134]. Endobronchial MMP-9 immunoreactivity on biopsy specimens from asthmatics was closely correlated with asthma severity [135]. On the other hand, MMP-8 seems to play a protective role in asthma, since its deficiency promotes allergen-induced airway inflammation, mainly by delaying clearance of recruited neutrophils [136].
Strong evidences imply that TIMP/ MMP imbalance is an important element of the fibrogenic process. TIMP-1 is known to induce a non-collagenolytic microenvironment and is up-regulated in human pulmonary fibrosis. It was markedly increased in mice lungs, 24 h after the administration of bleomycin [137]. The non-selective MMP inhibitor batimastat reduced the development of bleomycin-induced fibrosis in mice and this was associated with a decrease in TIMP-1 levels [138]. Several MMPs and TIMPs have been found in lung biopsies of patients with sarcoidosis and there was indirect evidence for their involvement in distal airway inflammation [139].

In more acute settings, MMPs were globally up-regulated in acute lung injury and were produced mainly by neutrophils and macrophages [140]. In patients with hospital acquired pneumonia, MMP-8 and MMP-9 levels were elevated and correlated with the state of ventilation and the degree of systemic inflammation [141]. Elevated MMP-1, -8 and -9 expression was found in parapneumonic pleural effusions [142].

Lastly, almost all invasive lung cancers express high levels of MMPs. MMP-9 is elevated in the serum of patients with both small cell and non-small cell lung cancer, thus modifying the natural physiological relationship between MMP-9 and TIMP-1 [143]. Overexpression of MMP-1 is associated with a particularly poor prognosis in lung carcinoma [144].

4.7.2. MMPs drive tissue destruction in Tuberculosis

Chang et al [145] first reported a striking up-regulation of the MMP-9 gene in bronchoalveolar lavage cells of patients with active cavitary tuberculosis. In the same study, Mtb and its major cell wall component LAM stimulated the expression of both MMP-1 and MMP-9 and of the cytokines IL-1β and TNF-α. Mycobacterial infection of murine peritoneal macrophages and BALB/c or SCID mice induced substantial increases in MMP-9 activity [146]. This was regulated by TNF-α and IL-18, as their neutralisation led to reduced MMP
production. BALB/c mice demonstrated a deficit in IL-1 and IL-2 expression and a premature bias towards IL-4 expression, accompanied with delayed granuloma formation and rapid dissemination of TB, when treated with Batimastat (BB-94), a broad spectrum MMP inhibitor [147]. This was reversible upon withdrawal of the drug.

High levels of MMP-9 correlate with disease severity and the presence of granulomas in tuberculous pleurisy [148, 149]. Patients with tuberculous meningitis had higher levels of both MMP-2 and MMP-9 and this correlated with the development of late neurologic complications [150]. The identification of a matrix-degrading phenotype in human TB where MMP-9 activity in monocytic cells was relatively unrestricted by TIMP-1, both in vitro and in vivo by our group (JSF) illustrated it was net MMP activity that was important [151]. Subsequently it was found that Mtb up-regulated MMP-1 from human macrophages but the vaccine strain BCG did not [152]. The role of MMPs in the pathogenesis of TB has been further reviewed in the discussion with my first set of results.
**4.8. Chemokines in Tuberculosis**

**4.8.1. Introduction to Chemokines**

I have briefly discussed here the role of chemokines in TB, in light of my initial investigations to ascertain if they drive MMPs in TB.

Chemokines are small chemoattractant peptides that signal via the G-protein coupled receptors [153, 154]. They are highly basic proteins and although most are secreted, some such as fractalkine are expressed on the cell surface [155]. Chemokines are subdivided into C-, CC-, CX-C or CX-3C groups based on the number of amino acids between the first two cysteines [154]. Their major role as a chemoattractant guides cell migration. Cells follow the signal of increasing chemokine concentration towards the source. This property was first demonstrated in an assay for neutrophils [156] using CXCL-8/IL-8. Pertussis-toxin sensitive G-protein coupled receptor signalling was later found to be required for this effect. This makes them ideally suited for modulation/intervention with small molecule inhibitors and has strong therapeutic potential.

Although historically chemokines were considered to be involved in neutrophil trafficking, it became increasingly apparent that their functions are more widespread. The diverse expression, regulation and receptor binding of each chemokine allows them to control processes such as organogenesis, angiogenesis, hematopoiesis and immune-modulation. Some organisms such as the HIV or Herpes virus have evolved mechanisms to exploit the chemokine system to promote their survival. Dysregulation of chemokines and their receptors has also been implicated in various autoimmune conditions [153].

Chemokines communicate directly to different classes of cytokines and effector leukocytes to orchestrate Th1 and Th2 responses [157]. For example, CCL2-deficient mice do not mount Th2 responses and their T cells do not secrete IL-4, IL-5 or IL-10, although IFN-γ and IL-2 production was unaffected [158]. CCL2 deficient mice are also unable to initiate
immunoglobulin E class switching and were resistant to infection with *Leishmania major*. Additionally, chemokines direct lymphocyte homing in a tissue-selective manner. CCR9 has been recognised as a gut-homing receptor and CCR4 as a skin-homing one [157].

A more recent classification of chemokines into inflammatory and lymphoid types represents a more lucid paradigm [155]. The inflammatory chemokines are produced by several cell types such as endothelial, epithelial and stromal, as well as leukocytes and have distinct kinetics. They attract neutrophils and effector/memory lymphocytes. Classical examples include CXCL-8, RANTES, eotaxin, MIP-1β, MCP-1 and IP-10. Much information regarding the expression and function of inflammatory chemokines in airway diseases has been provided by the ovalbumin-sensitized/challenged allergic airway disease (AAD) model, in which mice are given an intraperitoneal injection of ovalbumin [159]. The study showed chemokine production by a variety of cell types including the alveolar epithelium, endothelium, smooth muscle, alveolar macrophages and infiltrating T lymphocytes. Eotaxin is especially produced in high levels in lungs undergoing allergic inflammation whilst IP-10 is regulated by IFN-γ at sites of delayed type hypersensitivity reactions. Both RANTES and MCP-1 can activate basophils to release histamine and other mediators. Intratracheal administration of MCP-1 has been shown to increase leukotriene-C4 levels in BAL and in pulmonary mast cells [160]. Intra-tracheal instillation of CXCL-8 produces large influxes of neutrophils into the lungs in animal models [161]. CXCL-8 is substantially elevated in BAL from patients with acute lung injury, and expression levels are correlated to a neutrophil influx. Blocking CXCL-8 has been shown to ameliorate the reperfusion-induced neutrophil influx in lungs [162].

The lymphoid chemokines are produced within lymphoid tissues and are involved in maintaining leukocyte traffic and cell compartmentalisation. Examples include B cell attracting chemokine-1 and EBV1 ligand chemokine [155].
4.8.2. Chemokines in TB pathogenesis

Mtb is a strong inducer of chemokines. Chemokines are central to the pathogenesis of TB as they mediate neutrophil influx which is a prerequisite for granuloma formation[163]. Khader et al [164] showed that the chemokines CCL19 and CCL21 were important for the accumulation of Ag-specific IFN-γ producing T cells in the lung, development of the granuloma and the control of mycobacteria. Multinucleate giant cells express and secrete chemokines at the infection site [165]. The role of chemokines has also previously been discussed in the pathogenesis of TB section.

During infection, mycobacteria induce expression of C-C chemokines, also called β chemokines, (namely MCP-1, MIP-1α, MIP-1β, and RANTES) which specifically induce activation and proliferation of T cells and macrophages; and C-X-C chemokines, also called α chemokines, (IP-10 and IL-8) which act on neutrophils [166]. The cell wall components mycobacteria are crucial to this induction, as it has been reported that infection of murine macrophages with dilapidated Mtb resulted in reduced expression of CCL-3 [167]. In another study on CD8+ T cells, it was found that they express CCL-5, granulysin and perforin, thereby providing a host mechanism to attract Mtb-infected macrophages and aid killing of the bacterium [168]. Elevated chemokine levels have been detected in bronchoalveolar lavage fluid, pleural fluid and plasma of TB patients. The levels of IL-8, MCP-1 and RANTES were markedly elevated in the acute phase of pulmonary TB, as compared to those of normal subjects [17]. In vivo studies have shown that tuberculous granuloma formation can be inhibited simply by blocking CXCL-8 [169].

With the emergence of HIV/ Mtb co-infection, a number of studies have addressed the ability of Mtb to enhance HIV infection. The increase of CCR5 and CXCR4 (HIV co-receptors) expression on monocytes in response to Mtb was hypothesized to result in increased susceptibility to HIV infection [170].

The 17q11.2 chromosomal region has been linked to susceptibility to TB and includes genes that encode several chemokines that may contribute to the host’s immunity. The associations
have been summarised in a review by Yim et al [166]. Briefly, CXCL-8 gene polymorphism is associated with susceptibility to human TB, and decreased CXCL-8 secretion occurs in HIV-infected patients with miliary TB. Monocyte chemotactant protein-1, a chemotactant for monocytes and T lymphocytes is key for TB granuloma formation. The GG genotype of MCP-1 -2518 promoter polymorphism produces high concentrations of MCP-1, which inhibits production of IL-12p40 in response to Mtb and promotes active pulmonary TB [171]. In a group of TB infected individuals from Mexico, this polymorphism was five times more prevalent than the uninfecte population. Another study from the same group in Mexicans and Peruvians reported a joint effect between the -2518 MCP-1 genotype GG and the -1607 MMP-1 genotype 2G/2G in consistently increasing the odds of developing TB [172].
4.9. Th-17 Cytokines in TB Immunopathology

4.9.1 Th-17 cells: a third subset of T helper cells

In 1986, the concept of two distinct types of helper T cells was introduced by Mosmann and Coffman [173], which was based on the types of cytokines that T cells preferentially produced when stimulated. When naive T cells were activated in the presence of IL-12, they differentiated into Th1 cells that produced large amounts of IFN-γ, ‘helped’ in activating macrophages and were responsible for host defence against intracellular pathogens. However, culture these naive T cells under IL-4 conditions, and they differentiated into Th2 cells producing IL-4, IL-5 and IL-13, activating eosinophils and participating in host defence against extracellular pathogens.

More recently, transforming growth factor (TGF)-β and IL-6 /IL-21 together were shown to trigger the production of IL-17A from CD4+ T cells, thus designating a third helper T-cell subset known as the Th-17 cells [174, 175]. The primary function of Th-17 cells appears to be the clearance of pathogens that are not adequately handled by Th-1 or Th-2 cells. Interestingly, two opposing cytokines, TGF-β and IL-6 /IL-21 cooperate to induce the differentiation of Th-17 cells. It has been proposed that the unactivated immune system produces TGF-β, which induces the generation of iTreg cells [176]. TGF-β and IL-6 in combination induce Th-17 cells which is dependent on the expression of RORγt via STAT-3 [177]. So an activated / pro-inflammatory immune milieu (marked by the presence of IL-6 or IL-21 for example) induces the differentiation of Th17 cells.

4.9.2. Interleukin-23

IL-23 is a member of the IL-12 family of cytokines and was first described in 2000 as a heterodimer composed of a p19 subunit and the p40 subunit shared with IL-12 [178]. A
connection between IL-23 and the Th-17 cells was established when it was shown that IL-23 promoted the production of IL-17 by activated Th-17 cells [179]. IL-23 is not however, involved in the initial differentiation of Th-17 cells but is essential for the stabilisation and proliferation of naive T cells that have already been committed to a Th-17 lineage.

IL-23 is predominantly produced by activated type 1 macrophages and dendritic cells in peripheral tissues, such as the skin, intestinal mucosa and lungs [180]. Production of IL-23 is stimulated through the activation of toll-like receptors. In humans, high levels of the IL-23 receptor are detected on activated / memory T cells, NK cells, macrophages, dendritic cells and monocytes. It activates the JAK/STAT pathway. IL-23 is a cytokine that bridges the innate and adaptive arms of the immune response and it is produced early in response to a pathogenic/antigenic challenge [180, 181]. It activates and enhances T cell proliferation and also regulates pro-inflammatory cytokines such as IFN-γ. A correlation was shown between the reduction in IFN-γ levels in patients with Salmonellosis / TB and deficiency of the IL-23/IL-12 p40 chain [182] [183].

A number of studies have demonstrated that IL-23 contributes to inflammatory conditions such as inflammatory bowel disease [184]. Up-regulation of local and systemic IL-23 was observed in *H. pylori* induced gastric cancers and ulcers in humans and mice. A 20–fold increase in Th17 cells and IL-17⁺ macrophages was observed in lesions of Crohn’s Disease and was related to increased expression of IL-23 [185]. IL-23 also promotes inflammation in tumours and inhibits infiltration of CD8⁺ T cells into epithelial cancers in murine models [186]. High levels of IL-23 is related to disease severity in multiple sclerosis, rheumatoid arthritis, psoriasis, allergic airway diseases and several other conditions.

Since Th-17 cells are a relatively newly identified subset, we still have a lot to learn about their idiosyncrasies. Th1 cells are important in the control of Mtb proliferation, but despite vaccines having been developed to potentiate the Th-1 response and various IFN-γ immunotherapeutic regimes, improved protection from Mtb is still a distant reality. There are
aspects of the T cell response to TB we need to dissect further, and this will invariably encompass the Th-17 cells.

4.9.3 IL-17: Source and function

IL-17A (commonly referred to as simply IL-17) is the canonical cytokine of the Th-17 cells. In addition to Th17 cells, several other cells are sources of IL-17, including the γδ T cells, natural killer and CD8+ T cells. The gene encoding IL-17 was first described and cloned by Rouvier from a murine cytotoxic T lymphocyte hybridoma cDNA library in an attempt to screen for cytotoxic T lymphocyte-associated transcripts, and was thus named CTLA-8 (cytotoxic T lymphocyte antigen-8) [187]. Subsequently, the T lymphotrophic virus *Herpesvirus saimiri* gene 13 was recognised to have 58% homology with CTLA-8[188]. IL-17 is a pro-inflammatory cytokine and exerts its function mainly on mesenchymal and myeloid cells to induce the expression of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), cytokines, chemokines, antimicrobial peptides and tissue remodelling substances. As IL-17 lacked immediate effects on T and B cells, it was originally thought to be of minimal importance. IL-17 is evolutionarily conserved and the gene existed in species such as molluscs and *Ciona intestinalis*. These species predated the development of adaptive immunity and therefore it could be envisaged that the cytokine likely was bridge between innate and adaptive immunity [189].

When the IL-17 receptor was cloned, it was discovered to be unique compared to other cytokine receptors and although IL-17 had cytokine-like activity, it had no homology to other cytokine families. Thus it represented a new cytokine family. Unlike other cytokine receptors, the IL-17RA subunits are preassembled on the plasma membrane before ligand binding, enabling it to respond rapidly and specifically to its ligand [190]. Human IL-17A is a homodimeric glycoprotein consisting of 155 amino acids and has a molecular weight of about 35kDa. Cloning has revealed that there are an additional five homologous cytokines, termed IL-17B to IL-17F [191]. Amongst these, IL-17F has the highest homology with IL-17A [192].
IL-6 was the first identified gene target of IL-17[188], and since IL-6 is also essential for the de novo differentiation of Th17 cells, there is suggestion herein of a positive feedback loop. In addition to IL-6, IL-17 induces the production of TNF-α, IL-1β, inducible nitric oxide synthase and prostaglandin E2 [193-195]. Ectopic expression of IL-17 causes a strong expansion of neutrophils through G-CSF, and neutralisation of IL-17 is associated with granulopenia and susceptibility to bacterial infections. The chemokines that are the main targets of IL-17 are the CX-C and the CC- chemokines. They potentiate the biological function of IL-17 by attracting neutrophils in vivo. CXCL8 maybe the most important IL-17- induced chemokine in humans [196]. The ability of IL-17 to induce expression of chemokines and cytokines can be enhanced by co-stimulation with TNF-α or IL-1β [197]. IL-17 also synergises with TLR ligands such as lipopolysaccharide.

Elevated levels of IL-17 are found in synovial fluid of patients with rheumatoid arthritis [198]. IL-17 can increase the membrane expression of the NF-κB ligand in osteoblasts, which in turn promotes osteoclastogenesis and subsequent bone destruction [199]. Tissue remodelling mediators such as matrix metalloproteinases (MMP-1, -3, -9 and -13) are targets of IL-17 (discussed in further detail in section on IL-17 and MMPs). Blocking IL-17 signalling disrupts germinal centre formation and reduces humoral responses [200]. In an NF-κB dependent manner, IL-17 can promote human B-cell survival and proliferation [201]

In addition, IL-17 promotes expression of various antimicrobial peptides, β-defensin and some S100 proteins, which act as natural antibiotics in the lung, skin and gut, and is therefore implicated in epithelial/mucosal defence mechanisms [202, 203].
4.9.4 IL-17 in allergic diseases

There is accumulating evidence that IL-17 plays an important role in the development of various allergic diseases, both pulmonary and extrapulmonary. One such very common condition is asthma. Although it is well-accepted now that IL-17 makes little contribution to the induction of Th-2-mediated, eosinophil-dominated atopic asthma, it has clearly been shown to play a key role in neutrophil-dominated non-atopic asthma. OT II transgenic mice exhibited neutrophil—dominated, rather than eosinophil—dominated airway inflammation after OVA inhalation without prior sensitization. Th-1 and Th-17 cytokines were increased in the bronchoalveolar lavage fluid of these mice [204]. IL-17 deficient mice have impaired contact hypersensitivity and IL-17 producing CD8+ T cells are involved in this process [205, 206].

4.9.5 IL-17 in autoimmune conditions

The concept of Th-17 or IL-17 in autoimmunity emerged from studies in two animal models of autoimmune diseases: experimental autoimmune encephalitis (EAE), a well established model for human multiple sclerosis, and collagen-induced arthritis (CIA), a model for human rheumatoid arthritis. IL-23 deficient mice showed dramatically attenuated disease development, indicating that IL-23 rather than IL-12 is critically linked to the pathogenesis of EAE and CIA [207]. Up-regulation of IL-17 producing CD4+ T cells was also observed in the central nervous system in the EAE model. Further studies have shown that neutralisation of IL-17 or IL-17 deficiency rendered mice immune to the induction of EAE or CIA [208-210]. Consistent with these observations, IL-17 expression was up-regulated in the cerebrospinal fluids of patients with multiple sclerosis and in the synovial fluid of patients with rheumatoid arthritis [211, 212]. Recently, a humanised IL-17A antibody, LY2439821, was developed to treat RA [213]. A phase one randomised, double blinded, placebo-controlled study showed clinical improvement in the treatment group.
Both Th-1 and Th-17 are involved in the pathogenesis of inflammatory bowel disease, although their roles requires further elucidation. In humans, Th-17 cells are detected in the gut of Crohn’s disease patients and some of these cells produce IFN-γ in addition to IL-17 [214]. CD161+ CD4+ T cells in the gut produce IL-17A upon stimulation with IL-23, suggesting their involvement in Crohn’s disease [215]. In humans the expression of IL-17, IL-22 and IL-23 is elevated in psoriatic skin [216]. IL-22 producing CD8+ T cells are suggested to be important for the pathogenesis of psoriasis [217]. Polymorphisms in the IL23R gene are associated with psoriasis, suggesting its involvement as well [218].

4.9.6 IL-17 in mucosal host defence against bacteria, viruses and fungi

The earliest study in establishing a critical role for IL-17 in protective immunity against extracellular bacterial infections was using K. pneumoniae in a respiratory infection model [219]. IL-17RA knockout mice showed significant delay in neutrophil recruitment and greater bacteremia. Induction of macrophage inflammatory proteins and G-CSF was vital. IL-17 and IL-22 (discussed later) function synergistically to induce antimicrobial proteins such as human β-defensin-2 and S100 proteins [220] against pathogens at mucosal sites. Using a macaque model of Simian immunodeficiency virus (SIV) to study HIV human disease, it was found that SIV co-infection selectively inhibited Th17 responses elicited by S. typhimurium, probably due to the depletion of CD4+ cells in the ileal mucosa. This allowed dissemination of the bacterium to the mesenteric lymph node [221]. Patients with chronic mucocutaneous candidiasis produced significantly lower levels of IL-17 and IL-22 after antigen stimulation compared to healthy controls, suggesting a Th-17 role in host protection [222]. In a respiratory tract model of fungal infections using P. carinii and A. fumigatus, induction of IL-23 and IL-17 after pathogen challenge was protective, as IL-23 KO mice or neutralisation of the IL-23/IL-17 axis resulted in impaired clearance of the pathogen [223]. IL-17 also functions synergistically with human rhinovirus to induce CXCL-8 from epithelial cells and contributes to the recruitment of inflammatory cells in the lung [224]. Lipocalin-2 is an antimicrobial peptide secreted at high levels by airway epithelial cells at mucosal surfaces in response to IL-17 and IL-22 stimulation [225].
In certain conditions, IL-17 may exacerbate the pathology rather than be protective. The role of IL-17 seems to be dictated by the pathogen and in certain cases can be detrimental to the host. For example, *Helicobacter pylori* infection in the stomach induces a robust IL-17 production by CD4+ and CD8+ T cells and infiltration of neutrophils in the gastric mucosa which leads to gastritis [226]. *Bordetella pertussis* may preferentially induce IL-23 and IL-17 in the respiratory tract, and at the same time inhibit IL-12. This has been associated with severe pathology such as bronchiectasis and widespread inflammation, respiratory distress and cough [227]. Cystic fibrosis, another very common cause of bronchiectasis, is associated with chronic biofilm infection with *Pseudomonas aeruginosa* and elevated IL-17 and IL-22 responses [220].

**4.9.7 IL-17 in malignancy**

Exogenous IL-17 expression *in vivo* can stimulate the secretion of vascular endothelial growth factor (VEGF) and prostaglandin E1 and E2 in murine fibroblasts, thus enhancing tumour angiogenesis [228]. IL-17 can also induce IL-6 and Stat-3 mediated pro-survival and pro-angiogenic genes in tumour cells [229]. In another study, IL-17 was shown to have a direct anti-apoptotic influence on breast cancer cells [230]. However, Th17 cells have also been shown to inhibit tumour development. IL-17 null mice show increased tumour growth and metastasis in the lung and subcutaneous tissues, and this was associated with reduced IFN-γ producing NK and CD8+ T cells [231]. Thus IL-17 plays a multi-factorial role in tumour immunity. The effect depends on the immunogenicity of the tumour, the immune status of the patient and the disease phase. It seems like in the acute phase IL-17 might exert an anti-tumour effect but in the chronic phase the angiogenic effects of IL-17 supersede.

**4.9.8 Interleukin-22**

Interleukin-22 was originally identified as a cytokine induced in CD4+ T cells with 22% homology to IL-10. It was thus first christened IL-10-related T-cell-derived inducible factor [232]. However unlike IL-10, it also has significant pro-inflammatory activities. In addition
to Th17 cells, IL-22 is also expressed by other immune cells such as the CD8+ T cells, NK T cells and γδ T cells [233]. Unlike IL-17 expression, which requires TGF-β and a second inflammatory stimulus, IL-22 expression can be induced solely by IL-6. The IL-22 receptor however is not detectable on any immune cell, instead it is highly expressed within tissues particularly on epithelial cells [234] [235], and primarily activates the Stat3 signalling pathway.

IL-22 is highly expressed during many different chronic inflammatory conditions such as inflammatory bowel disease, psoriasis, and rheumatoid arthritis. In psoriasis, IL-22 stimulated keratinocytes up-regulate the pro-inflammatory S100 family of calcium-binding proteins, and the expression of antimicrobial peptides such as β-defensin [236]. In synergy with IL-17, IL-22 has a pro-inflammatory role in multiple sclerosis and mediates the permeabilisation of the blood brain barrier [237].

Contrary to these roles, IL-22 also has a protective and anti-inflammatory role. It is for example protective during hepatitis. IL-22 deficient mice are highly sensitive to hepatitis and this can be ameliorated by the transfer of IL-22-expressing-Th17 cells [238]. IL-22 promotes innate immunity to bacterial infection by inducing anti-microbial proteins and maintaining the integrity of epithelial barriers [239]. It increases the clonogenic potential of human airway epithelial cells [240]. Neutralisation of IL-22 via antibodies during Gram negative bacterial pneumonia leads to a greater bacterial burden and decrease in host bactericidal activity [220].

**4.9.9 Signal transduction of IL-17**

Precise mechanisms of IL-17 signalling have been difficult to unravel as IL-17 is a unique cytokine and bears no homology to any other cytokine family. While IL-17 has been shown to activate many common pro-inflammatory signalling pathways including NF-κB, MAP Kinases, PI3Kinase and Stats, the proximal receptor signalling remains relatively unclear. Early studies showed that TNF receptor associated factor 6 (TRAF6), a key adaptor in the
TLR and IL-1R signalling cascade, was indispensable for IL-17 triggered NF-κB activation [241]. The next breakthrough was in 2003 when a conserved SEFIR domain, short for SEF (similar expression to fibroblast growth factor genes) and IL-17R, in the cytoplasmic tail of all IL-17 receptors was identified [242]. Deletion or point mutation of this domain in the IL-17 receptor impairs activation of NF-κB. Subsequently ACT1, an activator of the NF-κB pathway was found to contain a SEFIR domain. It is required within minutes after IL-17 stimulation and also contains a TRAF6 binding domain [243]. Deficiency in ACT1 renders cells unresponsive to IL-17A [244], thereby confirming its essential role in downstream signalling of IL-17RA. ACT1 also activates the mitogen activated protein kinase pathway, and together with ERK/ p38 stabilises several mRNAs, especially those encoding pro-inflammatory cytokines and chemokines [245].

A recent observation was that IL-17 can activate JAK1/2 and the PI3K pathways, which coordinate with NF-κB for induction human defence genes (e.g., human defensin 2) in human airway epithelial cells [246]. Another notable feature of IL-17 is its strong cooperative effect with other cytokines in regulating downstream gene expression. It has been shown to synergise with IL-1β, IL-22, IFN-γ, TNF-α, Oncostatin M, CD40 and vitamin D [247]. The mechanisms underlying these synergisms are not fully elucidated but it is in part contributed to via mRNA stabilisation in the presence of other inflammatory mediators.

4.9.10 Th17 cytokines and Tuberculosis

In mycobacterial infection, the production of IL-17 and Th-17 T cell responses largely depend on IL-23. Cooper et al [248] first noted that the combined loss of both IL-12 and IL-23 during Mtb infection led to an increased bacterial burden, reduced IFN-γ production and higher mortality as compared with the loss of IL-12 alone. Antigen presenting cells express IL-12p40 (shared by IL-23 and IL-12) in response to Mtb infection and the pathogen recognition receptors that induce this are the TLRs, NOD2 and Dectin-1 [249] [250]. Stimulation of macrophages with trehalose dimycolate induced IL-12p40, IL-12p70, IL-23, IL-1β, IL-6 and TNF-α production in a MyD88 independent but Mincle dependent manner.
IL-23 mRNA was up-regulated in unfractionated BAL cells from TB patients as compared to controls [252]. It was essential for cessation of bacterial growth and for the establishment of an IL-17 producing CD4+ T cell population in the lung, eventually triggering chemokine production and recruitment of IFN-γ producing CD4+ cells [253]. Pulmonary IL-23 gene delivery and a vaccine adjuvant augmented the expansion of Mtb-specific CD4+ T cells which produced IFN-γ and IL-17 [254, 255], with simultaneous reduction in the mycobacterial burden and inflammation in the lungs. In addition to a robust Th1 response, adults vaccinated with BCG as babies exhibited a strong Mtb-specific Th17 CD4+ T cell response after re-stimulation with PPD [256].

The absence of IL-23 or IL-17 does not significantly impact on the ability of mice to control Mtb following low dose aerosol infection [257, 258], but after high dose intratracheal infection, mice deficient in IL-17 are unable to control Mtb [259]. Although γδ T cells are a major source of IL-17 during mycobacterial infections in mice [260], in humans CD4+ T cells are an important source as well [256, 261]. Neutrophils are important in the early phase of granuloma formation in TB and accumulate along a chemokine gradient. Both IL-23 and IL-17 affect neutrophil homoeostasis and survival in TB. In the absence of IL-17 or when IL-17 was neutralised following aerosol Mtb infection, there was a reduction in the number of neutrophils and also in the bacterial burden [262, 263]. Repetitive BCG vaccination in Mtb-infected mice results in an enhanced IL-17 response along with elevated expression of pro-inflammatory cytokines and enhanced influx of neutrophils [264]. However this response was highly pathological, was abrogated in IL-23p19 null mice and was refractory to the effects of IFN-γ.

It was recently demonstrated that IFN-γ signalling by lung stromal cells is critical to regulate immunopathology following Mtb aerosol infection [265]. The study was on chimeric mice with IFN-γ unresponsive lung stromal cells. The mice exhibited early mortality and increased Mtb burden due to impaired expression of Indoleamine 2,3-dioxygenase, over expression of IL23 / IL-17 and enhanced neutrophilic infiltration in the lung. IFN-γ regulated the IL-17 response in this model [265] and the differentiation of IL-17 producing cells in another study [266], but it was not indispensable for the Th17-mediated immunity. In RAG null mice (which do not have mature T or B lymphocytes) with an intact IL-12/IL-23 axis, both Th17
and Th1 cells were activated and induced significant protection against Mtb. However, the reduction in the bacterial load in recipients of IFN-γ null Th17 cells was associated with significant survival prolongation, as opposed to in recipients of naive IFN-γ T cells [267].

In summary, although the IL-23/IL-17 axis is dispensable for eventual control of Mtb, it has a critical role at the early bacterial containment stage and in vaccine-induced protection.
4.10. PI 3-Kinase pathway in Mtb driven pulmonary events

4.10.1 PI 3-Kinase Pathway Background and Classification

Phosphatidylinositol 3-kinases (PI3K) are a family of lipid kinases that phosphorylate the 3-hydroxyl group of the inositol ring of phosphoinositides. Among the phosphorylated products, phosphatidylinositol 3,4,5-triphosphate (PIP\(_3\)) is generated and serves as a second messenger. Since its discovery in the 1980’s, the PI3K family has been found to have key regulatory roles in many cellular processes such as cell survival, proliferation and differentiation. As major effectors downstream of receptor tyrosine kinases (RTKs) and G-protein coupled receptors, PI3K transduces signals from various growth factors and cytokines to activate several downstream effectors, AKT (a serine-threonine protein kinase, also known as protein kinase B) being the most important one. The tumour suppressor PTEN (phosphatase and tensin homologue) is an important negative regulator of the PI3K signalling pathway. Recent studies have revealed that many components of the PI3K pathway are frequently targeted by germline or somatic mutations and this pathway has therefore become an attractive pharmacological target for modulation [268, 269].

PI3Ks are divided into three classes according to their structural characteristics, sequence homology and substrate specificity [270]. The most commonly studied are the class I enzymes that are activated directly by cell surface receptors. They are heterodimers consisting of a p110 catalytic subunit (α, β, γ, δ) and a p85 regulatory subunit. The former generates PIP\(_3\) whereas the latter mediates receptor binding, activation and localisation of the enzyme. Class 1A PI3Ks are activated by receptor tyrosine kinases whereas Class IB are activated by G protein coupled receptors. Class II PI3K consist of a single catalytic subunit. The specific functions of this family remain unclear but they are known to bind clathrin and localise to coated pits, indicating a function in regulating membrane trafficking and receptor internalisation. Class III PI3K consists of VPS34 (homologue of the yeast vacuolar sorting-associated protein 34). VSP34 functions as a nutrient-regulated lipid kinase that mediates signalling through mammalian target of Rapamycin indicating its potential role in cell growth.
4.10.2 PTEN and downstream effectors (AKT and mTOR-p70S6K pathway)

The cellular levels of PIP₃ are tightly regulated by the opposing activity of PTEN. PTEN is a tumour suppressor and antagonises PI3K activity through its lipid phosphatase activity that reduces the cellular pool of PIP₃. Loss of PTEN can lead to unrestrained signalling by the PI3K pathway, and cause cancer. Germline mutations of PTEN causes various inherited cancer syndromes, including Cowden’s Syndrome and Banyan-Riley-Ruvalcaba’s Syndrome [268] [271]. Somatic loss of the tumour suppressor gene frequently occurs in common human tumours such as gastric, breast and brain, to name a few.

AKT is the most crucial node downstream of the receptor tyrosine kinase - PI3K complex and is widely implicated in disease and health. It is a serine-threonine protein kinase which is expressed in three isoforms AKT1, AKT2 and AKT3 [268]. The three isoforms share a similar structure. The previously elusive PDK2 responsible for phosphorylation of AKT at Ser473 has been identified as mammalian target of rapamycin (mTOR) in the rapamycin-insensitive complex rictor [272, 273]. AKT phosphorylates and inactivates tuberin (TSC2), an inhibitor of mTOR within the mTOR/raptor complex [274]. Inhibition of mTOR stops the protein synthesis machinery due to inactivation of its effector p70-S6Kinase and activation of the eukaryotic initiation factor 4E binding protein 1 (4E-EP1), an inhibitor of translation [274] [275].

Important effectors that AKT activates are GSK-3 (glycogen synthase kinase) and FOXO (forkhead box family of transcription factors). Via these, it regulates a wide array of cellular processes involved in protein synthesis, cell survival, cell proliferation and cellular metabolism [268] [276]. In muscle and fat, AKT promotes glucose uptake by stimulating the membrane translocation of the glucose transporter GLUT4 [270]. AKT also activates glycogen synthase via the activation of GSK-3 [277] and inhibits fatty acid synthesis by activating ATP citrate lyase. In the liver, AKT inhibits gluconeogenesis by blocking FOXO-mediated transcription of gluconeogenic enzymes. AKT signalling inactivates several pro-apoptotic factors that include BAD and procaspase-9, but activates transcription factors that up-regulate anti-apoptotic genes including cyclic-AMP response element-binding protein (CREB). AKT can inactivate p53 contributing to centrosome hyperamplification and
chromosome instability in cancer [271]. It has been implicated in regulating both microtubule dynamics and organisation along with GSK-3 [278].

The figure below adapted from Wickenden et al [276] summarises the salient downstream effects of AKT, although these are much more complex and intertwined than can be demonstrated in one illustration.

![Figure 2, reproduced from Wickenden et al [276]](image-url)
The AKT-independent pathways activated by PI3K include the Bruton tyrosine kinase, the Tec family of non-receptor tyrosine kinases, serum and glucocorticoid regulated kinases (SGKs), ribosomal p70 S6 Kinase (S6K) and regulators of small GTPases that are implicated in cell polarity and migration [276, 279].

**mTOR signalling**

PI3K-related Kinases, that are sometimes referred to as class IV PI3Ks, are a group of protein kinases with a catalytic core structure similar to class I PI3Ks, but they lack the lipid kinase activity. This group includes the mammalian target of rapamycin (mTOR), DNA-dependent protein kinase, ataxia telangiectasia mutated gene product and Rad-3 related gene product [280]. mTOR has now long been a well-validated target for the treatment of cancer.

mTOR resides in close proximity to the plasma membrane in two distinct complexes, and integrates growth stimuli from the environment. These signals can be divided into two classes: growth factors (such as insulin) and nutrients. Additionally, mTOR senses the general availability of substrates for respiration which makes it a key element coupling the growth factor signals with the metabolic activity of the cell [281]. The prototypic mechanism for mTOR regulation is activation by the PI3K/AKT pathway. There are two distinct and mutually exclusive mTOR complexes: each composed of TOR, a common regulatory subunit, and a third subunit that specifies the downstream substrates [282]. These substrate-defining subunits are raptor (mTORC1 complex) and rictor (mTORC2).

Whereas raptor is inhibited by rapamycin, rictor is not affected by the drug. The mTORC2/rictor complex is required for phosphorylation of AKT on Ser473, which then results in increased mTORC1/raptor activity [283].
PI3K/ p70\textsuperscript{S6K} pathway

The presence of raptor in the mTOR complex redirects mTOR towards another downstream target S6K. S6K is a serine/threonine kinase that phosphorylates the 40S ribosomal protein S6, thereby enhancing translation of mRNAs. S6K has two isoforms, α1 (85 kDa) and α2 (70 kDa). S6K activity is nutrient and rapamycin sensitive while AKT is not. It stimulates protein synthesis, ribosomal biogenesis and cell cycle progression [284]. Thyroid hormone stimulated protein synthesis is also mediated via activation of the p70\textsuperscript{S6K} pathway [285].

An intense cross-talk exists between mTOR, AKT and S6K. Constitutive activation of AKT up-regulates the nutrient –S6K branch of the mTOR pathway [274, 275]. In contrast, S6K activity provides a negative feedback on the AKT branch. This has been observed in a variety of conditions and cells [286]. It has, in the light of above, been postulated that AKT up-regulates signalling via the mTOR- S6K pathway which in turn down-regulates AKT. This has been illustrated in the diagram reproduced below reproduced form Pende [287].

*Figure 3, reproduced from Pende et al [287]*
Several studies have established that proliferative signals downstream of PI3K can bifurcate along the AKT/ p70S6K arms. Whereas IGF-1 mediated PI3K/AKT signalling plays a pivotal role in cell survival by inactivating pro-apoptosis and suppressing caspase activation, its stimulation of the PI3K/ p70S6K cascade promotes proliferation [288]. The secretagogue compound 48/80 which is an effective activator of PI3K dependent pathways, causes a differential activation of AKT and p70S6K [289].

S6K has also been shown to trigger a internal feedback loop which acts to inhibit PI3K signalling upon chronic activation of the mTOR-S6K [290]. A number of studies have also shown that S6K can be activated independently of the PI3K/ AKT pathway. For instance, the Ras/MEK/ERK pathway has been found to contribute to the regulation of S6K1 activity via its effect on tuberous sclerosis complex 2 (TSC2) [291]. A constitutively active form of PKCζ enhances the basal activity of S6K to the same levels as after insulin treatment [292].

4.10.3 The PI3K pathway in growth, metabolism, tumorigenesis and host immunity

The PI3K signalling mediates different cellular responses depending on the tissue and the cellular context. These effector functions are usually subunit-specific. Most of our understanding of the PI3K signal transduction is based on studies of class I PI3Ks. The p110α and p110β are ubiquitously expressed, whereas the expression of p110δ and p110γ is mostly restricted to leucocytes. The PI3K p110α isoform performs most of the functions that are commonly assigned to PI3K in the literature. It is frequently mutated in common carcinomas [268, 270]. In contrast, cancer-specific mutations have not been found in the p110β subunit gene.

A chemical array of inhibitors was used to define the PI3K isoforms required for insulin signalling [293] and p110α was found to be the primary insulin responsive PI3K in cultured cells. Compounds targeting p110α block the acute effects of insulin treatment in vivo whereas p110β was dispensable but set a phenotypic threshold for p110α activity. Epithelial Na+ channel-dependent Na+ transport in renal collecting duct cells was blocked by p110α.
inhibition and lacked any dependence on p110β [294]. Inhibition of the Na+ current also blocked serum glucocorticoid kinase, SGK1 and protein kinase B, AKT phosphorylation. In a mouse model of prostate cancer induced by PTEN loss [295], concomitant ablation of p110β, but not p110α, led to decreased AKT phosphorylation and prevented the development of high grade neoplasia. These findings are consistent with the model put forth by Knight et al [293] that p110β generates a basal pool of PIP3 that defines a threshold for p110α activation necessary for signalling. p100β also has kinase-independent roles in endocytosis and cell proliferation [295] [296]. A key role of the p110β subunit isoform was defined by Jackson et al [297] in the formation and stability of integrin adhesion bonds, necessary for shear activation of platelets. In vivo inhibition eliminated occlusive thrombus formation but did not prolong the bleeding time. The study identified p110β as an important new target for antithrombotic therapy.

The p110 γ and δ isoforms share similar patterns of distribution on leukocytic immune cells and they interact closely to accomplish their immunological functions. The p110δ isoform is the main source of PI3K activity following antigen recognition by B cells, T cells and mast cells [298]. Lymphocyte and mast cell antigen receptor-dependent PI3K signalling is compromised in mice in which p110δ has been inactivated by point mutation, gene deletion or small molecule inhibitors. In the thymus, p110γ and P110δ play complementary roles in regulating the transition through key developmental checkpoints of T cells. p110δ regulates the differentiation of peripheral T-helper cells towards a Th1 and Th2 lineage and is also critical for Treg function [299]. p110δ also participates in B cell development, the migration of T cells and NK cells, B and T cell antigen receptor signalling, allergen-induced mast cell degranulation, neutrophil oxidative burst, immune complex mediated macrophage activation and allergic airway inflammation [300] [301]. Blockade of p110δ signalling leads to markedly enhanced B-cell switch to IgE and increased IgE levels in vivo, despite reduced type 2 cytokine production [302]. After ovalbumin inhalation, administration of the selective p110δ inhibitor IC87114 significantly attenuated airway infiltration by inflammatory cells as well as airway hyper-responsiveness. Levels of IL-17, IL-4, IL-5, IL-13, and nuclear factor NF-κB activity were also reduced [303]. Several such studies highlighting the importance of the p110δ isoform as a novel target for therapeutic intervention in asthma are underway [304]. PI3K inhibitors reduce airway smooth muscle proliferation and thereby airway remodelling in asthma.
A significant increase in the genomic signature of the PI3K pathway activation was observed in the cytotologically normal bronchial airway of smokers with lung cancer or with dysplastic lesions [305], suggesting that PI3K is activated in the proximal airway before tumorigenesis. Moreover, PI3K activity decreased in the airways of high-risk smokers who had dysplasia regression after treatment with the chemo-preventative agent myo-inositol.

Several lines of investigation point to the importance of PI3K in the pathogenesis of COPD [306]. PI3K inhibition restored defective HDAC2 expression and activity, the molecule which mediates corticosteroid insensitivity in COPD. When epithelial cells were pre-treated with a PI3K inhibitor, IL-1β-induced MUC2 gene expression and mucin secretion was attenuated [307]. MMP-9 expression, which is associated with COPD pathogenesis, is also regulated by PI3K signalling [308].

The PI3Kinase p110γ and δ partnership exists in airway cells where these two molecules interact to express adhesion receptors [309]. The severity of acute lung injury was significantly reduced in p110γ null mice [310], as were the extent of lung oedema, nuclear translocation of NF-κB, neutrophil recruitment and pulmonary levels of TNF-α and IL-1β. Inhibition of PI3Kγ activity may therefore be effective in curbing excessive neutrophil infiltration in lung injury. PI3Kγ deficient mice also have reduced levels of allergen-induced eosinophilic inflammation in the airways [311] and reduced transcription of fibrogenic markers in response to bleomycin [312].

New layers of complexity in the functioning of both PI3Kγ and δ isoforms keep emerging, and although they profoundly affect the immune cells, other cell and tissues are not beyond their influence. PI3Kγ knockout mice have defective scaffolding function in cAMP homeostasis [313] for example. Inhibition of the p110δ isoform suppressed both constitutive and Fit-3-stimulated AKT activation in acute myeloid leukaemia blast cells, identifying them as a potential therapeutic target in AML [314].
4.10.4 Mtb and the PI3K pathway

Mtb replicates in mycobacterium containing vacuoles (MCVs) that are characterised by the small GTPase Rab5 but not Rab7 and that also exclude the acidifying vacuolar proton ATPase and lysosomal hydrolases [315]. Mtb enters the macrophage in a PI3K independent manner and adopts a dual strategy involving lipid analogues and PI-metabolising enzymes to keep the levels of PIP$_3$ on MCVs low, thus arresting the bactericidal endocytic pathway [316]. To arrest MCV maturation, LAM (lipoarabinomannan) inhibits a calmodulin kinase II-dependent activation of the class III PI3K hVPS34 by blocking a cytosolic Ca release [317, 318]. LAM also interferes with the delivery of vacuolar proton ATPase and acidic hydrolases to MCVs [319, 320]. In summary, by preventing the activation of the Rab5 effector hVLS34, the levels of PIP$_3$ remain low on MCVs and phagosome maturation does not progress. In addition, Mtb secretes PI phosphatases to keep the levels of PIP$_3$ on MCVs low. These include SapM and MptpB [316]. The latter shares an active-site signature with eukaryotic lipid phosphatases such as PTEN.

There have been several studies to delineate the host PI3K pathway mediated response to combat TB infection. Leukotactin-1 (Lkn-1), a novel CC-chemokine is involved in the immune response of macrophages to Mtb. Infection with Mtb increased mRNA expression and secretion of Lkn-1 in a dose-dependent manner [321]. This increase in expression and secretion of Lkn-1 was reduced when the cells were treated with inhibitors of PI3K, PDK1 and AKT. In another study, LAM from virulent species of Mtb stimulated the PI3K-dependent phosphorylation of Bad (a Bcl-2 family proapoptotic protein) in THP-1 cells [322]. This was abrogated in cells transfected with a dominant negative mutant of PI3K. LAM also phosphorylated AKT which was again abrogated in cells transfected with the dominant negative mutant. Sly et al [323] investigated the induction of monocyte antimycobacterial activity after exposure to vitamin D$_3$. Exposure of infected THP-1 cells led to a significant production of the superoxide anion and this was eliminated by chemical inhibition of the PI3K pathway by LY294002 or transfection of the cells with p110α antisense oligonucleotides.
The role of the PI3Kinase pathway in the secretion of TNF-α and IL-10 from primary human monocytes was investigated after stimulation with the PPD antigen [324] and was found that the PI3K/ AKT pathway was essential for the secretion of both IL-10 and TNF-α. Both p38 and ERK were essential for TNF-α production whereas only p38 for IL-10 production. In a similar study looking at the regulatory pathways for IL-12 and TNF-α expression from human monocyte-derived macrophages, a rapid phosphorylation of AKT and ERK was found to be central [325]. As a follow-up to this study, Yang et al [326] investigated the involvement of toll-like receptors and intracellular signalling pathways downstream from PI3K in Mtb-induced AKT activation and IL-23 expression. Both AKT phosphorylation and IL-23 expression were dependent on TLR2 and p70S6K-1 signalling (the latter was confirmed by treatment of the cells with rapamycin). TLR2 signalling was also key in BCG-induced rapid activation of PI3K [327]. BCG induced spreading and polarisation of bone marrow-derived macrophages from TLR-2 expressing mice.
4.11. **MMP immunomodulation as an adjunct to shorten lengthy regimes in Tuberculosis**

4.11.1 **The emergence of Drug Resistant Mtb strains**

The 1950’s and the 1960’s saw a breakthrough period in the battle against TB as most of the current anti-TB chemotherapeutic drugs had been discovered and regimens were in place to cure the disease within 6-9 months. By the late 70’s cure rate for TB had exceeded 95%. As TB began to disappear from the developing world, the impetus for new drug development faltered. The initial success began to quickly wane with the emergence of drug-resistant strains of Mtb. Tuberculosis strains classified as multidrug-resistant (MDR) are those resistant to both of the most potent first-line anti-TB drugs, i.e. rifampicin and isoniazid [328]. Extensively drug-resistant (XDR) strains are resistant to both isoniazid or rifampicin, in addition also to any fluoroquinolone and at least one of the three second-line injectable drugs. With the exception of fluoroquinolones, no new anti-TB drug has been introduced in the past 45 years. Incurable TB is now a distinct threat and is referred to as ‘Totally Drug Resistant TB’ [329].

Treatment of drug-resistant TB becomes more complicated as the resistance profile of the bacterium broadens. Genetic resistance to a TB drug is due to spontaneous chromosomal mutations at a frequency of 1 per $10^6$ to $10^8$ mycobacterial replications. Mobile genetic elements such as plasmids and transposons which mediate drug resistance in other bacterial species do not do so in Mtb. Because chromosomal mutations are unlinked, the likelihood of developing bacillary resistance to three drugs used simultaneously becomes 1 in $10^{18}$ to $10^{20}$. Hence in theory, the chance of drug resistance is virtually non-existent but amplifications of the afore-mentioned genetic mutations through human error results in drug resistant TB. These human errors include monotherapy due to irregular drug supply, inappropriate prescriptions and most importantly poor patient adherence [330] driven principally by a lengthy regime and extensive adverse reactions (e.g. hepatotoxicity, hypersensitivity, neurotoxicity, renal failure) to first-line anti-TB drugs [331]. The sub-optimal use of antituberculous medications creates a selective milieu in the host of drug-resistant mutants,
which can eventually overpower the drug-susceptible population [332]. The chance of this is maximum in monotherapy. Once established in the host, MDR-TB requires a much longer course of treatment and a concomitant increase in costs. The main reason for this is that there is no real substitute for Rifampicin in MDR-TB, the only drug which affects the dormant bacilli and which allowed the duration of antituberculous chemotherapy to be initially reduced from 18-24 to 6-9 months.

4.11.2 Novel Drug Development for TB: Challenges and breakthroughs

Drugs are essential components of anti-TB regimens and high-quality drug candidates are needed before a substantially improved regimen can be identified and developed. Specific and comprehensive drug development approaches have been applied to identify essential Mtb genes, in particular to reveal targets essential for the microbe’s survival [333]. The mycobacterial cell wall has long been a potential target and there have been numerous attempts at inhibiting the synthesis of its constituent polysaccharide and fatty acids. Novel targets are also being explored among mycobacterial genes which encode functional proteins. Some of these protein kinases and phosphatases (STPKs, Serine-Threonine Protein Kinases; PTPs, Protein Tyrosine Phosphatases) are known to cross-talk with the host signalling pathways which inhibit the maturation of the mycobacterial phagolysosome [334] and these kinases could be favourably manipulated. Unfortunately most of these studies have been performed in the mouse which poorly mimics human TB. The lack of a well-accepted model of human TB has been a major obstacle in new drug development.

The cornerstone of antimycobacterial drug development is microbiological proof of efficacy which is measured by minimal inhibitory concentrations of the drug against cultures of Mtb. The biggest challenge is the delineation of sterilising activity in a clinical trial. This encompasses investigating the effect on dormant organisms, the impact on relapses, the penetration into diseased lung, safety during long term use and the role of a new agent within the landscape of existing treatments [335] [336]. Identifying the best drug combination that
shortens the therapy among different drug combinations also presents a challenge, as there are serious limitations /drug interactions in adding a new agent to an established regimen [336].

Worth mentioning is that among all the new anti-TB drugs, moxifloxacin shows great promise. However, second-line anti TB drugs are substantially less effective than first-line drugs/ injectable drugs and some are very heterogeneous, with poor studies in vivo, low efficacy and high toxicity. But there have been a few success stories in the recent past, e.g. the phase 2 trial of TMC-207 [337], a diarylquinoline and an ATP synthase inhibitor discovered from high-throughput screening against M. smegmatis. Results from the initial 2-month treatment phase show that the addition of TMC-207 to a standard treatment for MDR-TB significantly reduced the time to negative culture conversion (p=0.003) and increased the proportion of patients with conversion of sputum culture (48% vs 9%) after 2 months of treatment.

4.11.3 Adjunctive Immunotherapy in TB

Immunotherapy has major potential uses for the treatment of TB, in particular for MDR-TB patients as current therapy for drug resistant TB is sub-optimal and adjunctive immunomodulation may facilitate bacillary clearance, shorten duration of chemotherapy and increase cure rates. Immunotherapy would also down-regulate the host immune response which causes significant morbidity during the early phase of anti-TB chemotherapy, usually in patients with severe disease [338].

That the development of TB is due to failure of immune regulation is well documented. Much of the lung pathology is due to host-mediated immunopathology rather than due to Mtb-derived virulence factors. The disturbance in immune regulation may involve the subversion of a protective Th1 response, the generation of CD8+ cells or mechanisms involving Th2-like cytokines such as TGF-β [339, 340]. Attempts to establish mycobactericidal immunity with
IL-2 [341-343] and IFN-γ [344-347] have been disappointing, although it could be argued that the results from some of these studies could be not believed completely, as most were flawed by a small number of enrolled patients and an experimental design which was not well-controlled. Agents such as steroids, thalidomide and TNF-α have also been studied. Agents that drive an appropriate Th1-type immune response such as \textit{M. vaccae} have not been conclusively efficacious [348, 349] but a recent study of multiple doses of \textit{M. vaccae} in HIV-infected African patients demonstrated a reduction in mycobacteremia in the intervention group [350]. Several other immunomodulatory agents include DNA vaccine encoding HSP65 of \textit{M. leprae} [351], RUTI [352], and anti-IL4 [353] but the value of all these agents remains unclear and well-conducted prospective clinical trials are required.

4.11.4 Immunomodulatory roles of Azithromycin, Moxifloxacin and Rifampicin

For more than 15 years, it has been noted that certain antibiotics have an immunomodulatory role in addition to their anti-infectious qualities. Such a role for Moxifloxacin (an established second line anti-TB drug), Rifampicin (first line anti-TB drug) and Azithromycin (first line anti-atypical mycobacterial drug) has been discussed here.

Macrolides are an old class of antibiotics with an antimicrobial spectrum against mainly gram positive cocci and atypical mycobacteria. At low doses which are not microbicidal, it has been noted to possess immunomodulatory qualities that can help improve pathology in chronic respiratory conditions. For example, before erythromycin was introduced as the main treatment, the 5–year survival rate of diffuse panbronchiolitis (DPB) was <50% [354]. These patients have airway colonisation by \textit{Pseudomonas aeruginosa}, which is the main cause behind the frequent exacerbations. Since this bacterium does not even belong to the antimicrobial spectrum of macrolides, this observation raised numerous questions regarding the non-microbicidal mechanisms of macrolides. The long-term efficacy and safety of Clarithromycin was evaluated in ten patients with DPB over 4 years, and it was observed that pulmonary function improved in most patients within 6 months with no undue adverse effect
In a comparative clinical study of patients with community-acquired pneumonia, those who were treated with Clarithromycin as compared to Amoxicillin demonstrated a considerable decrease in the pro-inflammatory cytokine IL-6 and an increment in IL-10 and IFN-γ [356]. The ability of macrolides to accumulate in host cells, including phagocytes and epithelial cells plays a role in the regulation of cellular functions by these molecules. The non-microbial erythromycin-derivative EM703 decreased bleomycin-induced lung injury in mice by inhibiting both acute inflammation and TGF-β induced collagen synthesis [357]. Consistent with these effects and also suggesting a transcriptional regulatory mechanism, EM703 inhibited type I collagen synthesis in dermal fibroblasts [358]. Azithromycin inhibits IL-8 production and NF-κB and AP-1 binding activities in lung epithelial cells [359], and a suppression of TNF-α protein and mRNA levels was observed in cystic fibrosis airway epithelial cell lines via NF-κB and Sp1 DNA binding [360]. Both Clarithromycin and Azithromycin modulated IL-8 and GM-CSF production by human bronchial epithelial cells via ERK [361] and in cultured primary human tracheal epithelial cells, Erythromycin increased β-defensin-dependent antimicrobial activity of surface fluid [362].

Another class of antibiotics with distinct immunomodulatory properties are the fluoroquinolones. The first findings in this regard were published in the 1980’s. Since then several reports have shown that fluoroquinolones modulate bacterial adherence and colonisation, release of bacterial products, indigenous flora, phagocytosis and intracellular microbial killing, and limit antibacterial activities despite the acidic pH by accumulating intracellularly. Moxifloxacin is an advanced fluoroquinolone that has shown unusual potency against TB. Studies in mice suggested that it could shorten the duration of TB treatment from six months to three or four months [363-366]. In view of these studies, the efficacy of the drug as a first line anti-TB medication is being investigated and initial clinical trials have encouragingly demonstrated that substitution of Isoniazid with Moxifloxacin results in a statistically significant improvement in week -8 culture conversions [367, 368].

Hence, any immunomodulatory function of Moxifloxacin would strongly support its use as a first line anti-TB drug. Moxifloxacin significantly inhibits TNF-α production from human peripheral mononuclear cells stimulated in vitro by LPS [369]. IL-1β, IL-8, IL-6, TNF-α, ERK ½, JNK and NF-κB were also inhibited in studies on human monocytes [370] and cystic
fibrosis epithelial cell line [371]. In a similar study, Ciprofloxacin significantly inhibited TNF-α production from human monocytes with or without the addition of LPS [372]. This was accompanied with the accumulation of cAMP in the monocytes which led to the hypothesis that Ciprofloxacin acts as a phosphodiesterase inhibitor, resulting in augmented protein kinase A activity that in turn inhibits the TNF-α [373].

In the lung, control and Ceftazidime-treated mice developed bronchopneumonia after inoculation of *C. albicans* but Moxifloxacin-treated mice did not. This was accompanied with an inhibition of TNF-α, IL-8 and NF-κB [374]. Moxifloxacin inhibited cytokine-induced MAPKinase and NF-κB activation as well as nitric oxide synthesis in human respiratory epithelial cell lines [375].

Lastly, Rifampicin is a well–established first line anti-TB drug. Suppression of T cell activity by Rifampicin has long been noticed in both patients suffering from tuberculosis and healthy controls [376]. The inhibition of PGE₂ and other arachidonic acid products in a time and dose–dependent manner from alveolar epithelial cells has been postulated to contribute to the efficacy of the drug in TB treatment in a study by Yuhas *et al* [377]. The same group also found that rifampcin augments cytokine-induced nitric oxide production in human alveolar epithelial cells [378] by activation of NF-κB and inhibition of Peroxisome Proliferator-activated receptor-γ [379].

In collaboration with a couple of BSc students in the department, I investigated the effects of the afore-mentioned antibiotics on TB-driven MMP modulation from airway epithelial cells.
5. Hypothesis and Aims

Hypothesis:

Airway stromal cell-derived Matrix Metalloproteinases (MMPs) drive tissue destruction in Pulmonary Tuberculosis.

Aims:

1) Define Pulmonary TB response in vivo- Identify MMPs up-regulated in TB BALF. Correlate with other parameters of severity.

2) Study Airway Epithelial and Fibroblast MMP gene expression and secretion in TB.

3) Investigate interactions of Th17 cytokines, chemokines and antimycobacterial drugs in driving MMPs from airway stromal cells.

4) Delineate transcriptional control and upstream signalling pathways.

5) Identify immunohistochemical co-expressions of key signalling molecules, MMPs and Th17 cytokines in TB Lung Biopsies.
6. Methods

6.1. Materials and Reagents

All standard laboratory reagents were purchased from Sigma (Poole, UK). Cell culture reagents (HBSS, RPMI, glutamine) were purchased from Invitrogen (Paisley, UK). Inhibitors, cytokines and chemokines were purchased from Merck Biosciences/ Peprotech/ Tocris. Table 2 summarises the information.

<table>
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<th>Supplier</th>
<th>Cytokine/ Chemokine/ Antibody/ Inhibitor</th>
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<tr>
<td>Peprotech</td>
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</tr>
<tr>
<td>Merck Chemicals</td>
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</tr>
<tr>
<td>Tocris</td>
<td>Azithromycin, Rifampicin, Moxifloxacin, Levofloxacin, Isoniazid</td>
</tr>
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Table 2

Cytokines, chemokines, antibodies and inhibitors were purchased from Peprotech, Merck and Tocris.
6.2. Mtb Culture

*M. tuberculosis* H37Rv was cultured in Middlebrook 7H9 medium supplemented with 10% enrichment medium, 0.2% glycerol, 0.02% Tween 80 and 2.5µg/ml Amphotericin with agitation at 10rpm. Culture growth was monitored with a Biowave cell density meter and the Mtb was sub-cultured when the optical density exceeded 1.00. For infection experiments, culture at mid log growth at an optical density of 0.60 was used, which corresponded to 1 x 10^8 – 2 x 10^8 colony forming units (CFU) per ml, within a 2-fold error. Optical density was correlated with CFU by performing colony counts in triplicate on Middlebrook 7H11 agar. The amoebocyte lysate assay (Associates of Cape Cod, East Falmouth, MA) was used to assay the endotoxin level of the Mtb culture and this was found to be less than 0.3ng/ml of lipopolysaccharide.

6.3. Airway Epithelial Cell Culture

*Primary Small airway epithelial cells and Normal human bronchial epithelial cells*

Small airway epithelial cells (SAECs) and normal human bronchial epithelial cells (NHBEs) were purchased from Lonza Wokingham, as cryopreserved cells. They were regenerated and maintained according to the supplier’s instructions.

SAECs were maintained and seeded in small airway growth medium containing 13mg/ml bovine pituitary extract, 0.5 mg/ml hydrocortisone, 0.5 µg/ml human recombinant epidermal growth factor, 0.5 mg/ml epinephrine, 10 mg/ml transferrin, 5 mg/ml insulin, 6.5 µg/ml triiodothyronine, 5 mg/ml retinoic acid, 50 mg/ml of gentamicin and 50mg/ml bovine serum albumin. The growth medium was changed every alternate day. To subculture, the medium
was aspirated, the cell monolayer was washed with HEPES solution, trypsin was added for 5 minutes and then neutralized with trypsin neutralising solution. Subculture was done when cells were 70-80% confluent. Cells were resuspended in medium prior to cell counting and seeding.

Normal human bronchial epithelial cells were cultured in bronchial epithelial growth medium supplemented with 52 µg/ml bovine pituitary extract, 0.5µg/ml hydrocortisone, 0.5ng/ml human recombinant epidermal growth factor, 0.5µg/ml epinephrine, 10µg/ml transferrin, 5µg/ml insulin, 0.1ng/ml retinoic acid, 6.5 ng/ml triiodothyronine and 10µg/ml ampicillin, as per manufacturer’s instructions.

All experiments were performed between passage 4 and 5.

**A549 cells**

A549 cells (ECACC 86012804), a human alveolar cell line derived from a lung carcinoma, were maintained in RPMI 1640 supplemented with 2mM glutamine, 10% fetal calf serum (FCS, Biowest, Ringer, E. Sussex) and 10µg/ml ampicillin in T75 flasks and were subcultured every 3-4 days when confluent. Stimulation experiments were performed in RPMI 1640 supplemented with 2mM glutamine and 10µg/ml ampicillin.

**MRC-5 cells**

MRC-5 fibroblasts were grown in Eagle’s medium with 10% FCS. Subculture of MRC-5 fibroblasts was performed when cells were about 70-80% confluent. Adherent cells were washed with PBS and then detached from the surface with 0.25% Trypsin-EDTA solution (GIBCO, Invitrogen). Cells were resuspended in fresh media at the recommended seeding
density (2-4×10^4 cells/cm^2). For experiments, 1-2×10^4 cells/cm^2 were seeded in a 24 well-plate in fresh media with 1% (not 10%) FCS and stimulated at suitable confluence.

6.4. *Mtb* Infection of Epithelial cells

For epithelial cell infections, the media was changed at the start of the experiment and Mtb added at the appropriate MOI. Live bacilli were removed after 2 hours by washing with HBSS once and then fresh culture medium was added.

6.5. Preparation of CoMTb (Conditioned medium from Monocytes infected with Mtb)

This was prepared by directly infecting monocytes with Mtb in RPMI 1640 with 2mM glutamine and 10µg/ml of ampicillin. Monocytes were isolated from a single donor buffy coat. Cell free supernatant was harvested at 24 hours, and Mtb was removed by filtration through the 0.2-µm pore size anapore membrane (whatman). ELISA and Luminex analysis on CoMTb (Conditioned medium from monocytes infected with tuberculosis) has shown that it is rich in cytokines such as TNF-α, IL-1β, IL-6, IL-10 and Oncostatin M (discussed in detail in results chapter, Table 7).

**Figure 4, Preparation of CoMTb**
6.6. Enzyme Linked Immunosorbent Assay

MMP-1, MMP-3 and TIMP-1/-2 levels in cell culture medium were analyzed by ELISA (R&D Systems Duoset ELISA). 96-well plates were coated with 100µl capture antibody (0.8µg/ml) overnight at room temperature (RT). The next day, plates were washed three times with 0.05% Tween in PBS and then blocked with 1% BSA (blocking solution) for an hour. After 3 washes, prepared standards (according to manufacturers’ instructions) and samples (diluted in reagent diluent) were added to the wells and incubated for 2 hours at room temperature (RT). Following 3 washes, the detection antibody (biotinylated – 200ng/ml) was added for 2 hours at RT. Three further washes were performed and then 100µl streptavidin-HRP was added in the wells for 20 minutes. After 3 further washes, 100µl of substrate solution was added for about 5 minutes to obtain a colour change. The reaction was stopped by adding 50µl of H₂SO₄ and the optical density of each well was immediately analysed by using a microplate reader set to 450nm. Reference wavelength was 540 or 570nm. The results were analysed using Excel.

6.7. Gelatin Zymography

Zymographic technique was used to detect MMP-9 by the degradation of gelatin and by its molecular weight. The technique in all forms of zymography are similar except that the substrate differs depending on the type of MMP to be analysed. Proteins are separated in a denaturing (Sodium Dodecyl Sulfate – SDS) polyacrylamide gel electrophoresis (PAGE) containing a specific substrate that is co-polymerised with the acrylamide. After electrophoresis, the gel is washed with Triton X-100 which restores enzyme structure and proteolytic activity. Renatured MMPs in the gel digest the substrate when incubated in appropriate activation buffer (i.e. collagenase buffer). After incubation, the gel is stained with Coomasie Blue and the MMPs are detected as clear white bands against a dark blue background of un-degraded substrate. Finally, the bands are quantified by densitometry using image processing software (i.e. Scion Image).
20µl aliquots of cell culture supernatant and standards of 100pg/µl MMP-9 (Oncogene, Nottingham, UK) were mixed with 5x loading buffer (0.25 M Tris pH 6.8, 50% glycerol, 5% SDS, Bromophenol blue) were run on an 11% acrylamide gel impregnated with 0.1% gelatin (Sigma G2500) at 180V for about 3-4 hours (buffer 25mM Tris, 190mM Glycine, 0.1% SDS). After electrophoresis, the gel was washed for an hour with 2.5% Triton X-100 (Amresco 0694-1L) to remove the SDS. Subsequently, the gel was rinsed twice in 1x low salt collagenase buffer (55mM Tris base, 200mM NaCl, 5mM CaCl₂, 0.02% Brij-35, pH 7.6) prior to incubation at 37°C for 16 hours in fresh collagenase buffer. The next day, gelatinolytic activity was detected by a single step stain/de-stain method using 0.02% Coomasie blue (Pharmacia Biotech, Sweden) in 1:3:6 ratio of acetic acid, methanol and water. Densitometric image analysis was performed by digital image acquisition (UVP) followed by band quantification with NIH Image version 1.61 (Scion Image).

6.8. Phosphowestern Blot Analysis

For signalling molecule analysis, cells were stimulated in a 6 well-plate with the desired stimulus. At specific time points, cells were washed with sterile PBS and lysed with 100µl of SDS sample buffer (62.5mM Tris [pH 6.8], 2% SDS, 10% glycerol, 50mM DTT, and 0.01% Bromophenol blue). Scraped cells were transferred to cold microtubes and frozen at -80°C. 40 µL of sample was mixed with 40µl of loading buffer (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 0.06M Tris pH 6.8, Bromophenol blue for MMP-1 and 62.5mM Tris pH 6.8, 2% SDS, 10% glycerol, 50mM DTT, Bromophenol blue for MMP-3) and heat inactivated at 100°C for ~2 minutes prior to separation on a 10% acrylamide gel at 100V initially, until reaching end of stacking gel (~20 minutes), and then increased to 200V for 3-4 hours. A molecular weight marker (Amersham, Little Chalfont, UK) was run on the gel as well. Electrophoresis was stopped when blue marker reached ~1cm from the bottom. Subsequently, proteins were electro-transferred to a nitrocellulose membrane (Amersham Biosciences) at 400mA for 2 hours (buffer 20% methanol, 20mM Tris base, 150mM Glycine, 0.1% SDS). After transfer, the membrane was blocked for an hour with agitation in 5% milk protein (Marvel, Nestle) mixed with 0.1% Tween-20 (Sigma). The membrane was initially probed with the primary antibody (e.g. phospho-AKT, phospho-p38, phospho-ERK, phospho-p70S6K), by incubating at 4 degree Celsius overnight. The next day the membrane
was washed three times and incubated for an hour with HRP-linked goat anti-rabbit IgG secondary antibody. Luminescence was then detected with the ECL system (Amersham Biosciences) according to manufacturer’s protocol.

6.9. Fluorokine® MAP Assay (Multi-Analyte Profiling)

R&D Systems, Inc. have developed a microparticle based multiplex immunoassay for the accurate quantification of human MMPs and cytokines in various sample types (serum, plasma, bronchoalveolar lavage, cell culture supernatants). Based on the Luminex xMAP technology, this assay provides simultaneous quantification of these MMPs/ cytokines in a single small volume. Specific antibodies are pre-coated onto colour-coded microparticles or beads. Microparticles, standards, and samples are pipetted into the wells and the immobilized antibodies capture the MMP/cytokine of interest. After washing away unbound substances, specific biotinylated antibodies are added and form an antibody-antigen sandwich. Finally, streptavidin phycoerythrin (PE) is added to generate a signal and the beads are resuspended and read using the Luminex® dual laser analyser. One laser classifies the bead and determines the MMP which is being detected and the second laser determines the magnitude of the PE derived signal, which is in direct proportion to the MMP/cytokine bound.

6.10. Promoter-Reporter Assay

Reporter genes are widely used as indicators of gene expression in cells. A reporter gene is linked to a promoter sequence in an expression vector that is transfected into cells. After transfection, the cells are assayed for the presence of the reporter by directly measuring the amount of reporter mRNA, the reporter protein alone or the enzymatic activity of the reporter protein. Promoter-reporter studies were performed using FuGENE® HD Transfection Reagent from Roche Applied Science and Promega’s Dual-Luciferase™ Reporter (DLR™) Assay System.
MMP-3 promoter (1206 base pairs) linked to firefly luciferase in pGL4 basic vector (Promega) and the housekeeping gene thymidine kinase promoter linked to Renilla luciferase in pRL control vector (pRL-TK; Promega) were previously created by a BSc medical student. MRC-5 fibroblasts were grown overnight in a 12 well-plate (3.8 cm²/well) at a recommended seeding density of 100,000 cells/well so the monolayer was 80-90% confluent at the time of transfection. A 6:2 ratio of transfection reagent (37.6 µl/well) to purified plasmid DNA (1.6 µg/well) and pRL-TK (0.16 µg/well) was used according to manufacturer’s instructions. Plasmid DNA purity and concentration had been accurately determined using a 260 nm/280 nm ratio; the optimal ratio being 1.8. Fibroblasts were stimulated with 10% CoMTb and harvested at given time intervals (0, 6, 24 and 48 hours). Cells were washed twice with sterile PBS prior using 100µl of passive lysis buffer from the DLR™ Assay kit (Promega). Luminescence was detected using the same kit with a luminometer. Renilla luciferase activity was used to normalize firefly activity to control for transfection efficiency.

I also attempted to transfect primary airway epithelial cells with the MMP promoters for MMP-1, -3 and -9. Despite numerous optimisation experiments and different transfection reagents that have been developed for efficient plasmid DNA delivery (e.g. Lipofectamine LTX with PLUS™ reagent), this was unsuccessful. Invariably, the primary cells would perish.

6.11. siRNA Transfection

siRNA (small interfering RNA) transfection refers to a mechanism of post-transcriptionally silencing genes using RNA interference. It utilises small double stranded RNAs of 23 to 25 nucleotide length to silence the desired complementary mRNA. It is a highly specific technology, and is associated with very few off-target effects, as compared to chemical inhibitors.

All siRNA oligonucleotides and reagents were purchased from ThermoScientific Dharmacon. The siRNAs were purchased as double stranded, chemically synthesized oligonucleotides in a
smart pool, targeting the transcription products from 4 alleles of the gene of interest. For reconstitution, the siRNA was dissolved in a special buffer, mixed on a shaker for 30 minutes, aliquoted and then stored at -80°C.

Prior to planning the individual experiments, the conditions were optimised using a transfection control and a negative control (non-targeting siRNA). The transfection control, also called siGLO (Green), confirms localisation to the nucleus and optimal delivery of the siRNA in the cell type being investigated. It is an oligonucleotide labelled with a fluorophore on the sense strand. Figure E illustrates 72.45% transfection achieved in NHBEs with siGLO at 30nM, in a 1:1 ratio with lipofectamine.

![Graph showing 72.45% transfection achieved with siGLO](image)

<table>
<thead>
<tr>
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<th>% Total</th>
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<tr>
<td>LR</td>
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**Figure 5.**

72.45% transfection of NHBEs was achieved with siGLO, the transfection control. The graph represents a FACS analysis.

A negative control of non-targeting sequences was incorporated in all experiments. This is important for distinguishing sequence specific silencing from non-specific effects in RNAi experiments. mRNA and protein level of the experimental gene should remain unaffected in the cells transfected with non-targeting siRNA. The cell viability and phenotype should remain comparable to control samples.
The transfection reagent used was Lipofectamine 2000 from Invitrogen. This is a polycationic liposome formulation and mediates siRNA delivery after complexing with the negatively charged nucleic acids. Briefly, 12 well plates were seeded with NHBEs at 150,000 cells per well the day before. It was aimed to have a cell confluence of 60-70% as a higher density causes compromised viability post transfection. Optimem and growth medium without supplements was used for re-constituting the siRNA and the lipofectamine. Lipofectamine was used at 25µg/ml per well, and siRNA at 10-30nM per well. Lipofectamine and siRNA were allowed to complex in a 1:1 ratio (by volume) at room temperature for 20 minutes. The mixture was then dropped on the cells with the basal medium, for 4 hours. After this, the cells were washed and rested for an additional 4 hours. This was very important as omitting this step invariably led to cell death. After resting, the cells were stimulated and samples collected at the end of the experiment.

6.12. RNA extraction, cDNA synthesis and Real time RT-PCR

RNA extraction was performed using the Qiagen RNeasy Minikit. Briefly, cells were plated in a 6 well plate and stimulated upon 70-80% confluence. Lysis was performed with rapid lysis buffer (supplied with the kit) at 24 hours. The sample was then mixed with 70% ethanol, and mRNA extracted as per the manufacturer’s instructions using the supplied RNA extraction reagents and the RNA spin columns. In the final step, RNA was eluted with RNase free water and stored at -80°C.

cDNA synthesis was performed using the Quantitect reverse transcription kit from Qiagen. RNA samples were thawed before quantifying the RNA on the nanodrop machine. Then, gDNA (genomic) wipeout buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer 5x, RT primer mix and RNase-free water were each thawed and mixed as per instructions to constitute the reverse transcription mastermix. Volume of RNA equivalent to 1µg was diluted to 12µl, with RNase-free water. 2 µl gDNA wipeout buffer was added to each sample. Samples were then heated at 42°C for 2 mins. 6µl of the reverse transcription mastermix was
added to each sample, the samples heated again at 42°C for 15 minutes, followed by 95°C for 3 minutes. The cDNA was stored at -20°C.

**Real Time-RT-PCR** (quantitative PCR): A specific gene’s expression is quantified based on its exponential amplification, with sequence-specific primers and probes. The amount of the fluorescence intensity is directly proportional to the amplified product. Quantification is based on this fluorescent signal during the exponential phase of amplification. The first cycle at which the instrument can distinguish the fluorescence as being above the background signal is called the ‘\(C_T\)’ or threshold cycle. This \(C_T\) value can be correlated to the amount of the starting target in the sample.

Quantitative PCR was performed with Brilliant II QPCR master mix (Stratagene) on a Stratagene Mx3000P platform. MMP primers and probes have been described previously [380], and were purchased from Sigma-Aldrich. Each reaction containing 5ng of reverse transcribed RNA in 20µl of primer and probes of interest and the brilliant II mastermix. The \(C_T\) at which amplification entered the exponential phase was determined. A lower \(C_T\) indicated a higher quantity of starting RNA. To determine the relative RNA levels within samples, standard curves were prepared by making five-fold serial dilutions of each sample; these dilutions were subject to real-time PCR as well. Standard curves for \(C_T\) versus input RNA were prepared, and relative levels of starting RNA in each sample was thereby determined.

The results of each MMP (primers and probes purchased from Sigma-Aldrich) was normalized to 3 reference genes viz. GAPDH/ 18S ribosomal RNA/ Cyclophyllin A (primers and probes from Applied Biosystems), for each sample. I had selected these 3 reference genes after a series of experiments that illustrated that their \(C_T\) values remained effectively unchanged under different conditions used to stimulate airway epithelial cells.

Analysis of MMP mRNA expression was first undertaken by the standard curve method, where the MMP and the reference gene \(C_T\)S are extrapolated from the standard curve to
‘quantities’ and expressed in ng/µl. One sample (usually control) was then chosen as the ‘calibrator’ sample and the results expressed in comparative fold changes. All my results have been presented using this method of analysis.

However, I always re-analysed the experiments using the $C_T$ values to assess the levels of gene expression, with a low $C_T$ representing high gene expression.

### 6.13. Immunohistochemistry

Ethical consent for the use of archived lung biopsies was obtained from the Hammersmith Hospitals Research Ethics Committee. Immunohistochemistry was performed on paraffin embedded lung biopsies from 5 patients with culture-proven Mtb infection and 5 non-infected controls. Sections of 4µm thickness were de-waxed in 3 changes of xylene then 3 changes of alcohol and rehydrated in water. Endogenous peroxidase activity was blocked with 0.6% hydrogen peroxide for 15 minutes. Antigen retrieval was performed by microwaving the sections for 20 minutes in citrate buffer (0.01M citrate pH 6.0). Non-specific binding was blocked with 5% normal goat serum for 10 minutes. The primary antibodies were applied in 0.01M PBS / azide / BSA for 1h at room temperature. After 3 rinses in PBS, the antibody was detected with the Menarini non-biotinylated kit according to manufacturer’s instructions. Peroxidase activity was developed with diaminobenzidine (DAB, Menarini). Slides were counterstained with Coles haematoxylin, dehydrated and mounted.

Antibodies for MMP-3 and IL-17 were purchased from Abcam, Cambridge. This work was performed in collaboration with the Histopathology department at the Hammersmith Hospital.

Multiple interventions were analyzed by the One Way Anova followed by Tukey’s multiple comparison, while multiple interventions at two time points were analyzed by a 2-Way Anova followed by Tukey’s multiple comparison. A p value of <0.05 was taken as statistically significant. A p value of less than 0.05 has been illustrated with one star in the figures, p value of 0.01 with two stars, p value of 0.001 with three stars and p value of 0.0001 or smaller with four stars.

Unless otherwise specified, all the error bars represent standard deviations.

All secretion and mRNA expression data shown here are representative of 3 separate experiments. Unless otherwise specified, ‘n’ number for all in-vitro experiments is 3.
RESULTS
7. MMP up-regulation in TB positively correlates with tissue destruction

7.1. Introduction

Animals have long served as tools for the study of TB with mice, rabbits and guinea pigs being the most commonly used ones. However they are not truly reflective of human Mtb infection and the complex disease patterns, progression and manifestations of human TB. Mice are generally resistant to Mtb when compared with rabbits, guinea pigs or even humans. They develop non-caseating granulomas and rather than suppressing bacterial growth to a level consistent with latent infection, manifest a chronic phase of the disease with background fibrosis and slow bacterial growth. The rabbit develops cavitary disease but unfortunately Lurie’s genetically susceptible strain was allowed to die out [381] and the currently available laboratory strains are all relatively resistant to Mtb infection and more susceptible to M. bovis. Also rabbits are very costly to maintain and fewer immunologic reagents exist for them. Lastly, the guinea pig replicates many aspects of human TB but the course of infection consists of a logarithmic phase of bacillary multiplication followed by haematogenous dissemination and quick demise.

Hence a better understanding of TB can be best achieved by studying the disease in humans. This obviously has limitations and warrants much more care in terms of design and safety. Over the last few decades, histological and molecular studies of TB infection in humans have yielded invaluable insights into the progression of the disease. TB BAL studies and post-mortem analyses of lung granulomas have been promising tools.

During my PhD, I collaborated with Nalanda University Hospitals, Patna, India to collect bronchoalveolar lavage samples from patients who were being routinely investigated for respiratory symptoms. To limit user and procedure variability, the procedure was performed between 2 bronchoscopists and flexible bronchoscopes were used. I had hypothesised that
MMPs could be measured in TB BAL as a marker of disease severity, and analysed in conjunction with other clinical parameters such as cavitation on CXRs to predict prognosis.

In collaboration with the medical team, the samples that were AFB smear and Mtb culture positive were stored at -20°C. Along with the clinical samples, demographic, symptomatic, haematologic and radiologic data were also collected by the local team using a standardized questionnaire. Exclusion criteria from the study were as follows:-

a) Previous History of TB (to omit MDR/ XDR cases)
b) Age <18 years
c) Severe Chronic Lung Disease
d) Malignancy
e) Positive HIV status
f) Corticosteroids/ immunosuppressive drugs
g) Mental illness/unable to consent/

Samples were prospectively collected over a year. The final numbers were 17 TB BAL samples and 18 control samples (individuals undergoing bronchoscopy for investigation of other pathology). I visited the centre twice during this period. Prior to despatching them, the samples were centrifuged at the local microbiology laboratory to remove the cellular debris, and then sterile filtered through a 0.2µM Durapore membrane to remove Mtb from the samples. This does not interfere with the levels of MMPs or cytokines. The samples were re-aliquoted and despatched to Imperial College, London via World Courier for further analysis.

Concentrations of MMP-1, -2, -3, -7, -8, -9, -12 and -13, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, TNF-α, IL-17 and IL-23 were measured on the Luminex Multianalyte Profiler. Results between the control and the TB groups were compared and statistically analysed. Correlations were also done between MMP levels, cytokine levels and a CXR score.
I generated a semi-quantitative CXR scoring system with reference to previously published scoring systems [337, 382-385], as follows—

a) 0-3 points based on no cavities, cavities <2 cm, cavities 2-4 cm, and cavities >4cm, respectively in that order.

b) 0-6 points for consolidation in each zone (Right Upper/ Middle/ Lower and the same for the Left Lung).

c) One extra point for bilateral pathology.

The scoring was performed by myself on three different occasions and if different, the final score was averaged. During the course of my analysis, I referred to the control patients as SS/0110/C1 to C18, and the MtB positive patients as SS/0110/P1 to P17.
7.2. Demographic, Haematologic and Radiographic data of an Indian cohort of respiratory symptomatics

<table>
<thead>
<tr>
<th>Demographics, Haematology, CXR Scores and Clinical Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuberculosis(n=17)</td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Male Gender</td>
</tr>
<tr>
<td>Smoker</td>
</tr>
<tr>
<td>Current</td>
</tr>
<tr>
<td>Ex</td>
</tr>
<tr>
<td>Non</td>
</tr>
<tr>
<td>Haematological Markers</td>
</tr>
<tr>
<td>Hb (gm/dl)</td>
</tr>
<tr>
<td>White Cell Count (x10^9 /l)</td>
</tr>
<tr>
<td>Lymphocyte Count (x10^9 /l)</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
</tr>
<tr>
<td>CXR Score (0-10)</td>
</tr>
<tr>
<td>Parenchymal Consolidation (1-6)</td>
</tr>
<tr>
<td>Cavity size (0-3)</td>
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<td>Bilateral Disease (0,1)</td>
</tr>
<tr>
<td>Clinical Features</td>
</tr>
<tr>
<td>Haemoptysis</td>
</tr>
<tr>
<td>Dyspnoea</td>
</tr>
<tr>
<td>Cough</td>
</tr>
<tr>
<td>Weight Loss</td>
</tr>
<tr>
<td>Fever</td>
</tr>
<tr>
<td>Abnormal Respiratory Examination</td>
</tr>
<tr>
<td>Hospitalisation</td>
</tr>
<tr>
<td>TB Contacts</td>
</tr>
</tbody>
</table>

Table 3

The mean age of patients who were positive for Mtb was 34. The Male to Female ratio was 4.6 to 1. None of the patients had a previous history of TB. Smoking patterns in the control and TB groups were similar. An elevated ESR and Lymphocyte count was noted in the majority of TB patients. CXR scores were generated for TB and control patients based on the extent of cavitation and consolidation on CXRs and was significantly higher than the control group.
Diagnoses of Controls | Number
--- | ---
Smoking related Bronchitis/ small airway disease | 3
Pneumonia | 4
Carcinoma/ Lymphoma | 3
Sarcoid | 2
Foreign Body Aspiration | 3
Pulmonary Vasculitis | 2

<table>
<thead>
<tr>
<th>Table 4</th>
</tr>
</thead>
</table>
The above table summarises the diagnoses of the control patients who were confirmed to be negative for Mtb.

The study comprised of 17 subjects who were confirmed to have pulmonary TB and 18 well-matched controls. There was no significant difference between the two groups in age and gender distribution, smoking patterns, respiratory examination and hospitalisation rates (Table 3). Exact duration of symptoms was difficult to ascertain but most subjects had described them for a few months. None of the subjects had a previous history of Tuberculosis or any clinical/ investigative evidence of extrapulmonary TB. Median age of patients in the TB group was 34 (IQR 25.5-46.5). 70% of these patients were male. These parameters were possibly due to a lower age expectancy of the Indian population and the fact that women in the lower socioeconomic strata (most commonly afflicted with infectious diseases such as TB) in India tend to delay seeking medical help. CXR score in the TB group was significantly higher than in the control group. All TB patients had respiratory symptoms but only 8 patients had reported haemoptysis. Most had reported constitutional symptoms such as fever, night sweats and weight loss. Table 4 summarises the diagnoses in the control patients.
7.3. MMP-s are up-regulated in TB BALF and positively correlate with a CXR score of tissue destruction

Figure 6

MMPs -1, -2, -3, -7, -8 and -9 were up-regulated in TB BALF, when compared to control BALF. The above analyses reflect results from 17 TB and 18 control BALs. Scatter dot plots were generated with the median values for each of MMPs profiled. The values in pg/ml are illustrated as a log scale. The Mann-Whitney test was used to generate a two-tailed p value. MMP-12 and MMP-13 were undetectable in these samples.
**Figure 7**

A Spearman ‘r’ value of greater than 0.5 was observed between the TB BALF MMP-3, -7 and -8 concentrations and the CXR destruction score. This represents a moderate level of correlation.
Median MMP-1, MMP-2, MMP-3, MMP-7, MMP-8 and MMP-9 concentrations were significantly up-regulated in patients with TB when compared to the control subjects (Fig. 6 MMP-1 \( p = 0.0003 \), MMP-2 \( p = 0.0004 \), MMP-3 \( p = 0.0002 \), MMP-7 \( p = 0.001 \), MMP-8 \( p = 0.0006 \), and MMP-9 \( p = 0.0068 \)). Since a high CXR score automatically denotes greater tissue destruction and is a marker of immunopathology, a statistically significant correlation \((r>0.5)\) suggests that MMPs are central to this process. Such a correlation was noted for MMP-3, MMP-7 and MMP-8 (Fig. 7).
7.4. **IL-1β, IL-6 and IFN-γ are up-regulated in TB BALF**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control (N=18) (Mean pg/ml +/-SEM)</th>
<th>TB (N=17) (Mean pg/ml +/-SEM)</th>
<th>Two-tailed p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>1.247 ± 0.5399</td>
<td>144.3 ± 46.52</td>
<td>0.0033</td>
</tr>
<tr>
<td>IL-1β</td>
<td>33.02 ± 20.50</td>
<td>1112 ± 439.9</td>
<td>0.0166</td>
</tr>
<tr>
<td>IL-6</td>
<td>49.82 ± 28.34</td>
<td>167.0 ± 39.62</td>
<td>0.0209</td>
</tr>
<tr>
<td>TNF-α</td>
<td>7.209 ± 3.080</td>
<td>22.88 ± 7.104</td>
<td>N/A</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.3278 ± 0.07339</td>
<td>1.399 ± 0.2740</td>
<td>N/A</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.8028 ± 0.3876</td>
<td>0.4900 ± 0.2855</td>
<td>N/A</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.389 ± 0.3745</td>
<td>3.818 ± 1.223</td>
<td>N/A</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.3433 ± 0.1028</td>
<td>1.506 ± 0.3713</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Table 5**

Concentrations of IL-1β, IL-6 and IFN-γ were significantly up-regulated in TB patients as compared to the control group. Mean concentration of IFN-γ in control BALF samples was 1.247 pg/ml ± 0.5399 SEM, as compared to a mean of 144.3 pg/ml ± 46.52 SEM in TB BALF samples, p value=0.0033. Mean concentration for IL-1β was 33.02 pg/ml ± 20.50 SEM in control BALF samples and 1112 pg/ml ± 439.9 SEM in TB BALF samples, p=0.0166. Mean concentration of IL-6 was 49.82 pg/ml ± 28.34 SEM in control BALF samples and 167 pg/ml ± 39.62 in TB samples, p=0.0209. TNF-α, IL-2, IL-4, IL-10 and IL-10 concentrations were not biologically significant.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>MMP-1</th>
<th>MMP-2</th>
<th>MMP-3</th>
<th>MMP-7</th>
<th>MMP-8</th>
<th>MMP-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>ns</td>
<td>0.742</td>
<td>0.5343</td>
<td>ns</td>
<td>0.5786</td>
<td>0.4951</td>
</tr>
<tr>
<td>IL-1β</td>
<td>ns</td>
<td>0.7696</td>
<td>ns</td>
<td>ns</td>
<td>0.7786</td>
<td>ns</td>
</tr>
</tbody>
</table>

**Table 6**

There was a positive correlation between the concentrations of IL-1β and MMP-2 and MMP-8 (Spearman’s R 0.7696 and 0.7786 respectively) and IL-6 and MMP-2, MMP-3, MMP-8 and MMP-9 (Spearman’s R 0.742, 0.5343, 0.5786 and 0.4951 respectively). There was no correlation between the levels of IFN-γ and the MMPs.
Next I analysed the levels of cytokines that have been implicated in the pathogenesis of TB. Although a Th1 response is predominant in TB, a Th2 response is also well-described, and can be a marker of severity [41] [42]. The cytokines measured were IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IFN-γ and TNF-α. Concentrations of IL-1β, IL-6 and IFN-γ were significantly up-regulated in TB patients as compared to the control group (Table 3). Mean concentration of IFN-γ in control BALF samples was 1.247 pg/ml ± 0.5399 SEM, as compared to a mean of 144.3 pg/ml ± 46.52 SEM in TB BALF samples, p value=0.0033. Mean concentration for IL-1β was 33.02 pg/ml ± 20.50 SEM in control BALF samples and 1112 pg/ml ± 439.9 SEM in TB BALF samples, p=0.0166. Mean concentration of IL-6 was 49.82 pg/ml ± 28.34 SEM in control BALF samples and 167 pg/ml ± 39.62 in TB samples, p=0.0209. Concentrations of TNF-α, IL-1β, IL-2, IL-4, IL-10 and IL-13 were not biologically significant in these samples. There was a positive correlation between the concentrations of IL-1β and MMP-2 and MMP-8 (Spearman’s R 0.7696 and 0.7786 respectively) and IL-6 and MMP-2, MMP-3, MMP-8 and MMP-9 (Spearman’s R 0.742, 0.5343, 0.5786 and 0.4951 respectively) (Table 6).
7.5. Discussion

In summary, the preceding set of analyses confirm that MMPs-1, -2, -3, -7, -8 and -9 are elevated in Pulmonary Tuberculosis. MMP-12 and MMP-13 were undetectable in TB BALF. There was significant positive correlation between the MMPs-3, -7 and -8 and a score of parenchymal involvement and cavitation on Chest Xrays. Previous studies from the group have shown elevated MMP-1 and MMP-3 levels in respiratory secretions from TB patients but a small limitation of that study was that some of the samples were induced sputum [386]. This study analysed BALs only from a well-characterised cohort of patients who were undergoing routine bronchoscopies for investigation of respiratory symptoms. This was a strength of the study.

The cytokines that were elevated (IL-1β, IL-6 and IFN-γ) have all been implicated in TB. The roles of IFN-γ have been previously discussed in detail. IL-1β is a pro-inflammatory cytokine that is produced during a myriad infections. Animal that have IL-1β deleted form larger granulomas after infection and are unable to clear the mycobacteria as efficiently as the wildtype mice [387]. There is evidence that susceptibility to clinical disease in an African cohort with Mtb is associated with the IL-1 gene cluster [388]. Studies carried out on emigrated Indians in London showed a relationship between polymorphisms in IL-1β and IL-1RA and the functional ratio of expression/ development of tuberculous pleurisy [389]. Studies have revealed that along with TNF-α and complement C5, IL-6 mediates the initiation and development of the mycobacterial cord factor trehalose -dimycolate and thereby contributes to the granulomatous response [390]. IL-6 played a contributory part in reactive thrombosis and acute phase response in Pulmonary Tuberculosis [391] and levels were increased independent of drug resistance [392]. Mycobacterium tuberculosis H37Rv strain induced monocytic release of IL-6 via the MAPK and this was inhibited by N-acetyl-L-cysteine, a potential anti-TB drug [393].

My observation that soluble IL-17 could not be detected in TB BAL was surprising in view of my cellular results (chapter 8) that have convincingly implicated IL-17 in driving MMPs in
TB. Previous studies that have reported a detectable peripheral blood Th17 response and IL-17 mRNA expression in the lungs of Mtb-infected mice [253, 262, 266]. It has been suggested that low levels of soluble IL-17 in TB is a result of inhibition of Th17 effectors by those of Th1 at the site of TB disease [261]. Murine studies have demonstrated that IFN-γ limits Th-17 lineage formation [394, 395].

IL-17 in BAL and pleural fluid from most subjects, even in the absence of an inhibitory Th1 response, has always been too low or undetectable [256] [396]. A single human study has reported successful detection of IL-17 in BAL fluid from asthmatic subjects, but at a very low concentration of 15pg/ml [397]. These data imply that analysis of cell-associated IL-17 expression in BAL may be more revealing. IL-17 mRNA expression in pleural fluid mononuclear was increased by stimulation with the Mtb peptides ESAT-6 and CFP-10 [261]. This indicated that Mtb-specific Th17 cells were present at the local site of disease. In contrast to IL-17, IL-22 was detected in greater concentrations in BAL fluid form TB patients [256] which suggests that IL-22 producing cells are not influenced by the Th1 polarising conditions. Interestingly, a recent study showed that reduced Th17 response in patients with TB correlates with the down-regulation of IL-6R expression on CD4+ T Cells, and maybe an important mechanism in the development of active TB [398]. Overall, the Th-17 response in TB has emerged to be much more complicated than initially envisaged.
8. IL-17 augments MMP-3 but suppresses MMP-9 in a TB network via the p38 MAP Kinase pathway

8.1. Introduction

Macrophages are the first cells to encounter Mtb in the alveoli and secrete various pro-inflammatory mediators. However, they are not the only source of such mediators. Signalling between stromal cells such as epithelial cells or fibroblasts and inflammatory leukocytes at the granuloma site, often accentuate the host immune response. Additionally, the pulmonary epithelium is the first barrier to Mtb and respiratory epithelial cells cover an alveolar surface area of approximately 70 m².

Previous studies have shown that MMP secretion from normal human bronchial epithelial cells (NHBEs) is up-regulated in a TB network [399, 400]. Conditioned medium from Mtb-infected monocytes (CoMTb) up-regulated epithelial cell MMP-1 promoter activity, gene expression and secretion whereas direct MTb infection did not [399]. MMP-1 up-regulation required synergy between TNF-α and G protein-coupled receptors and was p38 MAP Kinase dependent. Activated p38 localised to the MMP-1 secreting airway epithelial cells in TB patients. In another study, MMP-9 secretion and gene expression was up-regulated by CoMTb from respiratory epithelial cells in a p38 and ERK-dependent but prostaglandin-independent manner [400].

TB granuloma is surrounded by a peripheral rim of fibroblasts. The main function of these fibroblasts has been believed to be physical but recent studies have shown that this was an underestimation. In the lung, fibroblasts can secrete the highest concentration of MMP-1 and cause type IV collagen and proteoglycan degradation via MMP-3 secretion [401]. In vivo fibroblasts can get infected with Mtb without causing local tissue breakdown and remodelling [67]. Fibroblasts also secrete CXCL-8 which limits the growth of Mtb in the lung [402].
The role of Th-17 cytokines, in particular IL-17, in mucosal host defence to TB has emerged in recent years. Although the IL-23/Th-17 axis is dispensable for eventual control of Mtb, it has a critical role at the early bacterial containment stage and in vaccine induced protection. Human bronchial lung epithelial cells express both IL-17R and IL-22R. Treatment of human bronchial epithelial cells with IL-17 induces CXC chemokines, G-CSF and antimicrobial peptides such as human β-defensins [189]. I hypothesized that in a TB network, stromal cell MMP production is up-regulated and can be modified by the Th17 cytokines.

The in vitro TB network that was used in my experiments has been an established model in the Friedland laboratory (Description in Methods chapter). With each batch of CoMTb that is generated, supernatants are also collected from uninfected monocytes (CoMCont) and it is confirmed that CoMCont does not stimulate MMP production from epithelial cells. A comparative analysis of the cytokines and chemokines in CoMCont and CoMTb has been performed several times in our department using the Luminex Bead Multi-Analyte Profiling facility. Monocytes infected with Mtb consistently secrete more cytokines and chemokines that uninfected monocytes. The following table (Table 2) summarises grouped analyses performed by various members of the firm. This was also undertaken to confirm that donor-related variability in the cytokine and chemokine profiles of CoMTb was within statistically acceptable limits.

The figures in my RT-PCR experiments demonstrate relative MMP mRNA normalised to 18S, GAPDH and Cyclophyllin A (3 housekeeping genes selected for NHBEs). This however does not give an indication of the absolute level of gene expression. Therefore PCR $C_T$ data was also used to compare absolute levels of gene expression by the delta-delta $C_T$ method and the results corroborated with the standard curve method analysis. A low $C_T$ represents a high gene expression. For the following set of experiments, the $C_T$ values were as follows:

MMP-1—(a) Control= 27.6±0.7  (b) CoMTb stimulated= 26±0.89

MMP-3—(a) Control= 33.8±1.2  
(b) IL-17 stimulated= 32.1±0.43  
(c) CoMTb stimulated= 32.4±1.33  
(d) IL-17 +CoMTb + SB 203580= 38±0.68  
(e) IL-17 + CoMTb + p38 specific siRNA= 37±0.56
MMP-9—(a) Control = 33±0.38
(b) IL-17 stimulated = 32.5±0.84
(c) CoMTb stimulated = 30±1.2
(d) IL-17 + CoMTb = 33±0.98

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>CoMCont</th>
<th>CoMTb</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean(pg/ml)</td>
<td>SEM</td>
<td>Mean(pg/ml)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>3</td>
<td>0</td>
<td>559.8</td>
</tr>
<tr>
<td>G-CSF</td>
<td>3</td>
<td>0</td>
<td>1178</td>
</tr>
<tr>
<td>IL1-β</td>
<td>131.8</td>
<td>44.5</td>
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</tr>
<tr>
<td>IL-6</td>
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</tr>
<tr>
<td>TNF-α</td>
<td>1</td>
<td>0</td>
<td>26910</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Undetectable</td>
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<td>MCP-1</td>
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<td>MIG</td>
<td>47</td>
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</tr>
<tr>
<td>IL-17</td>
<td>Undetectable</td>
<td>N/A</td>
<td>Undetectable</td>
</tr>
</tbody>
</table>

Table 7

Monocytes infected with Mtb (CoMTb) secreted significantly more GM-CSF, G-CSF, IL1-β, IL-6, TNF-α, IL-12, IL1-RA and chemokines than uninfected monocytes (CoMCont). IL-17 was undetectable in CoMTb. CoMTb has been referred to hereafter as a monocyte-epithelial cell in vitro TB model/TB network.
8.2. Conditioned medium from monocytes infected with Mtb (CoMTb) drives MMP-1, MMP-3 and MMP-9 from Small Airway Epithelial Cells.

**Figure 8**

MMP-1, MMP-3 and MMP-9 secretion from SAECs was augmented in a monocyte-epithelial cell TB network. SAECs were stimulated with CoMTb at a 1 in 5 dilution. Supernatant was analysed at 72 hours by luminex bead multi analyte profiling. **A.B.C.** MMP-1 secretion was increased 4.5 fold from a baseline of 490 pg/ml to 2247 pg/ml (p=0.0084), MMP-3 from 534 pg/ml to 1174 pg/ml (p=0.0049) and MMP-9 from 11446 pg/ml to 119561 pg/ml (p=0.0016). **D.E.F.** There was no significant change in the secretion of MMP-2 or MMP-7 from baseline. MMP-8 secretion was up-regulated from baseline but this was not considered biologically significant.
I first investigated the global secretion profile of all MMPs from SAECs (small airway epithelial cell) in a monocyte-epithelial cell TB network. Previous studies have shown up-regulation of MMP-1 and MMP-9 from human upper airway cells [399, 400].

CoMTb was used at a dilution of 1 in 5 and supernatants were collected at 72 hours as previous studies had shown maximal MMP-1 and -9 secretion from NHBEs at this dilution and time point [399, 400]. All my epithelial cell experiments were performed on primary cells and no cell lines were used. I undertook my initial secretion experiments on small airway epithelial cells (SAECs). I then confirmed that upper airway epithelial cells, also called normal human bronchial epithelial cells (NHBEs) had a similar secretion profile. This was an important finding, as we know from previous studies that the two cell types have different immunological/ inflammatory roles [403]. SAECs are cells from the distal portion of the human respiratory tract in the 0.5-1mm bronchiolar area. This area mainly consists of alveoli and participates in gaseous exchange. NHBEs are cells from the human airways up to and including the terminal bronchioles, but not the respiratory bronchioles. They both stain positively for cytokeratin.

MMP-1, MMP-3 and MMP-9 secretions from SAECs were augmented in a monocyte-epithelial cell TB network. MMP-1 secretion increased 4.5 fold from a baseline of 490 pg/ml to 2247 pg/ml (Fig. 8A, p=0.0084), MMP-3 secretion increased 2.2 fold from 534 pg/ml to 1174 pg/ml (Fig. 8B, p=0.0049) and MMP-9 secretion increased 10.5 fold from 11446 pg/ml to 119561 pg/ml (Fig. 8C, p=0.0016). On trypan blue staining, there was no significant cell death in any of the experiments.

SAECs constitutively secreted MMP-2 at basal levels of ~6000 pg/ml, which was not altered significantly in the TB network (Fig. 8D). MMP-7 was almost undetectable (Fig. 8E). MMP-8 was detectable but not at biologically relevant levels (Fig. 8F). MMP-12 and MMP-13 were undetectable. In summary, secretion of MMP-1, MMP-3 and MMP-9 are significantly augmented in a TB network from SAECs.
8.3. Interleukin-17 has a divergent effect on MMP-3 and MMP-9 secretion from SAECs in a TB network but does not affect TIMPs.

**Figure 9**

IL-17 has a divergent effect on CoMTb-driven MMP-3 and MMP-9 secretion from SAECs. A. MMP-1 secretion at baseline was not altered by IL-17. MMP-2, -7 and -8 secretion after stimulation with CoMTb were insignificantly altered, hence further analysis was not undertaken. B. IL-17 increased the baseline secretion of MMP-3 from SAECs from 534 pg/ml to 1612 pg/ml (p<0.001). CoMTb-driven MMP-3 secretion was augmented four-fold from 1171 pg/ml to 4196 pg/ml (p<0.001). C. CoMTb-driven MMP-9 secretion from SAECs was suppressed by IL-17 from 119561 pg/ml to 7483 pg/ml (p<0.0001). G. IL-17 did not alter TIMP-1 or TIMP-2 secretion from SAECs at baseline or in a TB network.
Next, I investigated the effect of IL-17, the canonical Th17 cytokine, on the secretion of MMPs from SAECs, both alone and in a TB network. There have been previous studies to show that IL-17 can modulate the gene expression and secretion of MMP-1, MMP-3 and MMP-13 from cardiac fibroblasts and synoviocytes, but this was the first study in respiratory epithelial cells. A concentration response experiment illustrated maximal effect of IL-17 on SAECs at 30 ng/ml, hence IL-17 was used at this concentration for subsequent experiments.

IL-17 did not alter MMP-1 secretion from SAECs at baseline, although in a TB network this was augmented from 2247 pg/ml to 3260 pg/ml (Fig. 9A, p<0.01). MMP-2, MMP-7 and MMP-8 secretion had not significantly been altered after stimulation with CoMTb (Fig. 8), hence further statistical analysis was not undertaken.

IL-17 increased baseline MMP-3 secretion from 534 pg/ml to 1612 pg/ml (Fig. 9B, p<0.001) and CoMTb-driven MMP-3 secretion from 1171 pg/ml to 4196 pg/ml (four-fold) (Fig. 9B, p<0.001) in SAECs. There was a divergent effect on the regulation of MMP-9. CoMTb-driven MMP-9 was suppressed by IL-17 from 119561 pg/ml to 7483 pg/ml (Fig. 9C, p<0.0001).

IL-17 did not alter TIMP-1 or TIMP-2 secretion from SAECs at baseline or in a TB network (Fig. 9G, H). This is biologically relevant as it is the MMP/TIMP ratio in vivo that determines extracellular matrix degradation and the final MMP-driven phenotype.
8.4 MMP-3 secretion and gene expression are augmented from NHBEs in a TB network, but not on direct infection.

Figure 10

A.B. IL-17 caused a 3-fold concentration-dependent increment in the baseline MMP-3 secretion (p<0.01) and a 15 fold concentration-dependent increment in the CoMTb-driven MMP-3 secretion to a maximal of 3857 pg/ml (p<0.001). Baseline and CoMTb-driven MMP-3 mRNA expression were also augmented by IL-17. Changes in MMP-3 mRNA levels reflected secretion. C.D. MMP-3 secretion was increasingly augmented over 24-72 hours by IL-17, and mRNA peaked at 24 hours at 4 fold above baseline. E. Direct infection of NHBEs with Mtb did not alter MMP-3 secretion. This remained unchanged in the presence of IL-17.
To investigate the mechanisms underlying MMP-3 up-regulation in an IL-17 and CoMTb network further, I performed direct infection, concentration response, gene expression and kinetics experiments on normal human bronchial epithelial cells.

Mtb at multiplicity of infection from 0.1 to 10 did not alter MMP-3 secretion from NHBEs. Similar results had been observed previously in our lab for MMP-1 and MMP-9 [399, 400]. This was not modified by IL-17 (Fig. 10E).

A concentration response experiment with 1-50 ng/ml of IL-17 showed that maximal MMP-3 secretion was achieved with 5 ng/ml of IL-17 (Fig. 10B). Therefore for other experiments (kinetics, direct infection, intracellular signalling) this concentration was used. Similar to SAECs, IL-17 caused a 3-fold concentration-dependent increment in the baseline NHBE MMP-3 secretion (p<0.01) from 155 pg/ml to 495 pg/ml and a 15 fold concentration-dependent increment in the CoMTb-driven MMP-3 secretion to a maximal of 3857 pg/ml (Fig. 10B, p<0.001). IL-17 dependent increment in the baseline & the CoMTb-driven MMP-3 gene expression reflected secretion (Fig. 10C, p<0.001).

MMP-3 secretion was increasingly augmented over 24-72 hours by stimulation with IL-17 at 5ng/ml (Fig. 10D, p<0.001). IL-17 driven MMP-3 mRNA peaked at 24 hours, 4 fold above baseline (Fig. 10E, p<0.01).
8.5 IL-17 driven MMP-3 up-regulation in a TB network is independent of CXCL-8, MIP-1α, TNF-α and IL-1RA

Figure 11
A. IL-17 drives CXCL-8 from NHBEs, both alone and in a TB network (Fig. 44, p<0.01). B. IL-17 driven MMP-3 secretion from NHBEs is not dependent on CXCL-8. Blocking CXCL-8 with a monoclonal antibody (concentration range 0.1- 30 µg/ml) did not suppress MMP-3 secretion from NHBEs.

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>Mean concentration in NHBEs stimulated with CoMTb only (pg/ml)</th>
<th>Mean concentration in NHBEs stimulated with CoMTb and IL-17 (pg/ml)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>611</td>
<td>752</td>
<td>ns</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>1774</td>
<td>2251</td>
<td>ns</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>5896</td>
<td>5951</td>
<td>ns</td>
</tr>
<tr>
<td>G-CSF</td>
<td>10479</td>
<td>36397</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CXCL-8</td>
<td>178708</td>
<td>409251</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 8
CXCL-8 and G-CSF levels were higher when NHBE’s were stimulated with CoMTb and IL-17, compared to CoMTb alone (p<0.001). TNF-α, IL-1RA and MIP-1α levels were similar in both groups.
To further dissect the IL-17 and CoMTb-driven MMP-3 up-regulation, I undertook an extensive cytokine and chemokine analysis. NHBEs were stimulated with IL-17 alone and in combination with CoMTb. Previous studies in the group have been performed to determine the cytokines/chemokines in CoMTb that drive MMP secretion from NHBEs [400]. These were a series of inhibition/replacement experiments that demonstrated that TNF-α but not IL-1β or IL-6 were necessary for the CoMTb-induced MMP-9 up-regulation. The cytokines and chemokines that I analysed were—

1) Growth Factors- GM-CSF, G-CSF, FGF, VEGF, HGF
2) Cytokines- IL-1β, IL-6, IL-10, TNF-α, IL-1Ra, IFN-α, IFN-γ, IL-13, IL-15, IL-17, IL-12, IL-5, IL-2, IL-7, IL-2R, IL-4
3) Chemokines- RANTES, Eotaxin, MIP-1β, MIP-1α, MCP-1, IP-10, MIG, CXCL-8

The cytokines and chemokines that were significantly up-regulated in the above experiments were TNF-α, IL-1RA, G-CSF, CXCL-8 and MIP-1α. The mean concentration of TNF-α, MIP-1α and IL-1RA were elevated in supernatants from cells stimulated with CoMTb and also from cells stimulated with IL-17 and CoMTb but there was no significant difference between the two groups. However, the concentrations of G-CSF and CXCL-8 were significantly higher in the IL-17+CoMTb group compared to the CoMTb only group. This has been summarised in Table 8.

IL-17 augmented CXCL-8 secretion in NHBEs from a baseline of 12432 pg/ml to 178708 pg/ml (Fig. 11A, p<0.001). This was further augmented to 409251 pg/ml in an IL-17/TB network. I then blocked CXCL-8 with a monoclonal antibody purchased from Peprotech (concentration range 0.1 -30 µg/ml). There was no compromise in cell viability even at the highest concentration of 30 µg/ml. Blocking CXCL-8 did not affect IL-17 driven MMP-3 secretion from NHBEs (Fig 11B). G-CSF levels were also higher in the cells stimulated with IL-17+CoMTb compared to cells stimulated with CoMTb alone. However, I have not performed experiments after blocking G-CSF.
8.6 Interleukin-17 down-regulates baseline and CoMTb-driven MMP-9 secretion and gene expression from NHBEs.

**Figure 12**

A. IL-17 down-regulated CoMTb-driven gelatinolytic activity in a concentration-dependent manner (p<0.001) to baseline levels, i.e. before stimulation with CoMTb. This was maximal at 5ng/ml of IL-17. B. CoMTb-driven MMP-9 gene expression was down-regulated by IL-17 to baseline, with maximal suppression at 5 ng/ml (p<0.001).
Next, I examined whether the down-regulatory effect of IL-17 on MMP-9 secretion in a TB network translated into inhibition of CoMTb-driven gelatinolysis. Previous studies have shown that direct infection of NHBEs with live virulent Mtb at a multiplicity of infection from 0.1 to 20 did not alter MMP-9 secretion [400]. However, stimulation of NHBEs with CoMTb at a 1 in 5 dilution had led to a progressive, concentration–dependent increase in MMP-9 stimulation, to approximately 8 fold at 72 hours, suggesting that host-derived pro-inflamatory factors in CoMTb are required to drive MMP-9 secretion.

IL-17 inhibited the CoMTb-driven gelatinolytic activity in NHBEs (Fig. 12A, p<0.001). This effect was concentration-dependent and maximally driven at 5ng/ml. Cell viability was uncompromised during the course of the experiment. This was important as MMP-9 is extruded from epithelial cells after death/ apoptosis. Densitometric analysis demonstrated that IL-17 abrogated the CoMTb-driven gelatinolytic activity in NHBEs to baseline. Inhibition of gelatinolysis by incubation with 10mM EDTA confirmed that the bands were caused by an MMP.

Next, I investigated whether CoMTb regulates MMP-9 mRNA expression in NHBEs and the role of IL-17 at the level of MMP-9 mRNA expression. MMP-9 mRNA up-regulation by CoMTb reflected secretion, although there was a 5-fold rise from basal levels at 24 hours (Fig. 12B, p<0.001), compared to an 8-fold rise that had been observed in secretion. MMP-9 mRNA kinetics over 6 - 36 hours confirmed highest expression at 24 hours and therefore this time point was used for future mRNA extractions. IL-17 abolished the CoMTb-driven MMP-9 mRNA expression to baseline (Fig. 12B, p<0.001).
Interleukin-22 and Interleukin-23 do not drive MMP-3 or MMP-9 from NHBEs

Figure 13

A.B. IL-23 did not alter MMP-3 or MMP-9 secretion from NHBEs. This was investigated over a biologically significant concentration range of the cytokine and confirmed in a TB network. C.D. IL-22 did not alter MMP-3 or MMP-9 secretion from NHBEs, again investigated over a biologically significant concentration range and confirmed in a TB network.
Another Th-17 cytokine that has attracted a significant scientific attention is IL-22. Although IL-17 and IL-22 have similar immunological profiles, there are significant differences between them. Most importantly IL-22 has a distinct anti-inflammatory property. IL-23 although not a Th17 cytokine per se, supports the expansion of T helper cells that are already committed to a Th17 lineage.

I therefore next investigated the effect of IL-22 and IL-23 on MMP secretion from NHBEs in a TB network. A biologically significant concentration range was used for both cytokines. Supernatants were collected for analysis at 72 hours, and there was no compromise of cell viability.

MMP secretion was unaffected upon stimulation of NHBEs with IL-22 or IL-23 in a TB network.
8.8 CoMTb-driven MMP-3 is augmented by IL-17 and IL-22 in MRC-5 fibroblasts

Figure 14

A. CoMTb caused a concentration-dependent increment in MMP-3 secretion from MRC-5 fibroblasts, maximally at a 1 in 5 dilution (p<0.0001). This was observed at 72 hours. B. MMP-3 wild type promoter activation was observed in response to CoMTb but not upon stimulation with CoMCont at 6 hours. This peaked at 24 hours (p<0.001). C.D. CoMTb-driven MMP-3 level was further augmented from MRC-5 cells by IL-17 (p<0.0001, maximal at 50 ng/ml) and also by IL-22 (p<0.001, maximal at 25 ng/ml).
Next I investigated the MMP secretion profile of another stromal cell, the MRC-5 fibroblast, in a TB network. The initial analysis demonstrated that CoMTb caused a concentration-dependent increase in MMP-3 secretion from MRC-5 cells, maximal at 72 hours (Fig 14A, p<0.0001). Although maximal MMP-3 secretion was observed at a CoMTb dilution of 1 in 5, this was also accompanied with significant cell death, as demonstrated in Figure 15. Hence for subsequent experiments, MRC-5 cells were stimulated with CoMTb at a dilution of 1 in 50. There was significant up-regulation at this concentration as well (p<0.001), and most importantly no cell death was observed (Figure 15).

![Image A](image1.png) ![Image B](image2.png)

**Figure 15**

Increased cell toxicity that was observed when CoMTb was used at a dilution of 1 in 5 (Figure B), was rectified when the CoMTb concentration was reduced to 1 in 50 (Figure A).

To further investigate the regulation of CoMTb-induced MMP-3 regulation from MRC-5 cells, promoter-reporter studies were performed. MRC-5 cells were transfected with wild-type constructs of the MMP-3 promoter (1206 base pairs long) linked to *firefly* luciferase in pGL4 basic vector and the housekeeping gene thymidine kinase promoter linked to *Renilla* luciferase in pRL control vector. Cells were then stimulated with 10% CoMTb. Promoter activation in response to CoMTb was detectable a 6 hours, and peaked at 24 hours, thereafter remaining stable (Fig. 14B, p<0.001)

To investigate the hypothesized role of IL-17 and IL-22 in pulmonary TB, MRC-5 fibroblasts were stimulated with IL-17 alone and in combination with CoMTb. IL-17 up-regulated CoMTb-induced MMP-3 secretion by ~2-fold from ~100 ng/ml to ~200 ng/ml (Fig. 14C, p<0.0001). These experiments therefore demonstrated that similar to airway epithelial cells,
IL-17 synergised with CoMTb to induce MMP-3 secretion from MRC-5 fibroblasts. IL-17 did not affect TIMP-1 secretion (data not shown), which suggests that IL-17 induces an unopposed MMP-3 activity in MRC-5 fibroblasts. Next, MRC-5 fibroblasts were stimulated with IL-22 alone or in combination with CoMTb at a dilution of 1 in 50. Similar to IL-17, IL-22 augmented MMP-3 secretion in a TB network by almost 2-fold (Fig. 14D, p<0.001). Thus in combination with the effects of IL-22 on NHBEs, these results demonstrate that the immuno-modulatory role of IL-22 is cell-specific.
8.9. IL-17 driven MMP-3 regulation in a TB network is p38 MAP Kinase dependent

A. p38 was phosphorylated in NHBEs upon stimulation with IL-17 +/- CoMTb at 30 minutes. There was no change in total p38. B.C. IL-17 driven MMP-3 augmentation in a TB network was inhibited in a concentration-dependent manner by SB203580, both at protein and mRNA level (p<0.001). D.E. IL-17 driven MMP-3 augmentation in a TB network was also inhibited to baseline by siRNA inhibition of p38 in NHBEs (p<0.01), confirming its role.

**Figure 16**

A. p38 was phosphorylated in NHBEs upon stimulation with IL-17 +/- CoMTb at 30 minutes. There was no change in total p38. B.C. IL-17 driven MMP-3 augmentation in a TB network was inhibited in a concentration-dependent manner by SB203580, both at protein and mRNA level (p<0.001). D.E. IL-17 driven MMP-3 augmentation in a TB network was also inhibited to baseline by siRNA inhibition of p38 in NHBEs (p<0.01), confirming its role.
To dissect the signalling cascades that control IL-17 driven MMP regulation from NHBEs, I studied the MAP Kinase pathway with particular emphasis on the p38, ERK and JNK pathways. I first stimulated NHBEs with IL-17 +/- CoMTb and then lysed the cells at 0, 15, 30, 60 and 120 minutes to investigate the phosphorylation of p38, ERK 1/2 and JNK MAP Kinases.

ERK 1/2 and JNK were constitutively phosphorylated in NHBEs, hence further investigation was not undertaken. Phosphorylation of p38 by IL-17 and/or CoMTb peaked at 30 minutes after which it fell to baseline. Figure 16A illustrates peak phosphorylation of p38 in NHBEs at 5 ng/ml of IL-17, both alone and in combination with CoMTb. Phosphorylation of p38 was significantly increased when cells were co-stimulated with IL-17 and CoMTb. Total p38 phosphorylation confirmed equal loading and did not change with different stimuli.

Next, I investigated the effects of chemical and siRNA inhibition of p38 on IL-17 driven MMP-3 production from NHBEs. Cells were first incubated with the p38 specific chemical inhibitor SB203580 for 2 hours prior to stimulation with IL-17 +/- CoMTb. SB203580 suppressed IL-17 driven MMP-3 secretion to baseline (Fig. 16B, p<0.001). This was observed at 2.5µM of SB203580, but was maximal at 10µM. Inhibition of IL-17-dependent MMP-3 mRNA expression was also observed upon incubation with SB203580 and was similar to the decrease in protein levels (Fig. 16C, p<0.001). mRNA was extracted at 24 hours.

Next, I transfected the cells with siRNA directed against the p38 MAP Kinase. As a negative control, cells were also transfected with a non-targeting siRNA (NT siRNA). Firstly, I confirmed knockdown of the phospho p38 in NHBEs by a phosphowestern analysis, and followed this up by confirming knockdown of p38 at the mRNA level. As shown in Fig. 17, significant knockdown was achieved at 10 nM of the specific siRNA and the levels were undetectable with 30 nM.
Figure 17

A. On phospho western analysis of p38 in NHBE’s, CoMTb-mediated activation was abrogated with p38 specific siRNA. A concentration-dependent response was observed. B. Total p38 mRNA levels were suppressed to below baseline when the NHBE’s were incubated with the p38 specific siRNA. A concentration-dependent suppression was again observed.

Transfection with p38 siRNA abolished the IL-17/CoMTb driven MMP-3 secretion by ~50% (Fig. 8D, p<0.01). The suppression was maximal with 30nM of the siRNA. MMP-3 mRNA expression was suppressed to baseline levels. There was no change upon incubation with the non-targeting siRNA (Fig.8E, p<0.01). In summary, the p38 MAP Kinase signalling pathway mediates the IL-17 driven MMP-3 production from NHBEs in a TB network.
8.10. **IL-17 and MMP-3 are both expressed in epithelial cells around TB granulomas.**

**Figure 18**

A. Pulmonary epithelial cells (pneumocytes) and lymphocytes express IL-17 in patients with TB. The figure is representative of lung biopsies from five patients with Mtb infection and five controls. A. Epithelial cells adjacent to the TB granuloma stained strongly for IL-17. C. This was not observed in epithelial cells from control lung. Immuno-reactive cells appear brown. D. Pulmonary epithelial cells adjacent to TB granulomas strongly express MMP-3. The figure is again representative of lung biopsies from five patients with Mtb infection and five controls. E. Minimal staining was observed in normal lung.
Finally, I investigated the expression of IL-17 in 5 TB lung biopsies and 5 control lung biopsies. IL-17 was expressed both in epithelial cells and in lymphocytes, in the TB lung (Fig. 18A, B). Immunoreactivity appears brown and was abundant in the epithelial cells adjacent to the TB granuloma. In contrast very little IL-17 expression was observed in the normal lungs (Fig. 18C).

Pulmonary epithelial cells adjacent to TB granulomas strongly express MMP-3 (Fig. 18D). The figure is again representative of lung biopsies from 5 patients with Mtb infection and 5 controls. Minimal staining was observed in the normal lung (Fig. 18E).
8.11. Discussion

My investigations into the MMP secretion profile of airway epithelial cells in a TB network show for the first time that MMP-1, MMP-3 and MMP-9 are augmented from small airway epithelial cells. This phenomenon had been previously observed in large airway epithelial cells for MMP-1 and MMP-9 [397] [400]. Direct infection of epithelial cells with Mtb did not alter MMP secretion. IL-17 increased baseline and CoMTb-driven MMP-3 secretion and expression but had a dichotomous down-regulatory effect on the regulation of MMP-9. IL-17 also did not alter TIMP-1 or TIMP-2 secretion from epithelial cells at baseline or in a TB network. This is biologically relevant as it is the MMP/TIMP ratio \textit{in vivo} that determines extracellular matrix degradation and the final MMP-driven phenotype. MMP-1 remained unaltered in an IL-17/TB network, over a wide dose response experiment.

Although IL-17 up-regulated CXCL-8 secretion from NHBEs, MMP secretion was independent of this. Other cytokines such as TNF-\(\alpha\) were not triggered by IL-17 from epithelial cells. IL-17 is known to strongly synergise with pro-inflammatory cytokines such as TNF-\(\alpha\), hence it is interesting that it does not induce its production per se.

MMP-3 secretion and promoter activity were also augmented from MRC-5 fibroblasts in a TB network, and this was incremented synergistically by both IL-22 and IL-17. The discrepant cell-specific effects of IL-22 from IL-17 could be explained by crucial differences between the two cytokines. For example, IL-22 signals via Stat3 and unlike IL-17 has a clonogenic effect on human airway epithelial cells [220]. IL-22 and IL-23 do not drive MMP activity/production from airway epithelial cells.

Previous studies from our group have found unopposed TB-driven MMP-1 production from fibroblasts in a STAT3, p38 MAPK and NF-\(\kappa\)B dependent manner[404]. MRC-5 fibroblasts do not express MMP-9, hence this was not analysed. These studies suggest that MMP expression is both cell- and stimulus-specific.
There have been several studies in our group that show that directly infected cells are not the only source of MMPs in TB, and also not necessarily the most important one. Leukocytes at the site of infection interact with epithelial cells and fibroblasts to enhance the production of inflammatory mediators. Our in-house TB model, CoMTb has optimally simulated this interaction in-vitro. CoMTb up-regulated MMP-1 secretion, gene expression and promoter activity from human airway epithelial cells in p38-dependent mechanism. TIMP secretion was concurrently suppressed [399]. Host derived factors such as TNF-α synergised with Mtb to drive MAPK-dependent MMP-9 secretion from airway epithelial cells [400]. Pulmonary epithelial cells are a major source of CXCL-8 in the initial response to TB [70] as are fibroblasts [402]. Oncostatin M was shown to synergise with TNF-α to stimulate MMP-1 and MMP-3 from pulmonary fibroblasts, while simultaneously suppressing TIMPs [405].

Astrocytes produce MMP-9 in CNS TB and in conjunction with monocytes contribute to a matrix degrading phenotype, which is regulated by NF-κB and the MAP Kinases [406] [407]. IFN-γ synergised with IL-1β to up-regulate MMP-9 secretion from astrocytes in CNS TB[408]. Evidence from the zebrafish model of TB demonstrated that ESAT-6 drives epithelial cell MMP-9 to generate a migration gradient for monocytes [409]. Hence, both leukocytic and stromal cells interact closely in TB, and contribute towards MMP production and subsequent matrix degradation. My findings that both IL-17 and MMP-3 were co-expressed in TB lung epithelial cells and lymphocytes reiterate that the cell-cell interactions/networking are important in TB immunopathology. Previous immunohistochemical studies have also been used to localise MMP expression within TB granulomas, and for example, MMP-1, MMP-3 and MMP-9 expression had been demonstrated in the CNS [410] and MMP-1 and MMP-9 in the pulmonary epithelium [399, 400].

Airway epithelial-driven immune mechanisms have an important role as it is the first point of contact for respiratory pathogens. It provides the first physical barrier and the mucociliary elevator is an important mechanism of bacterial clearance. The airway surface fluid also contains several defence factors such as lysozymes, β-defensins and others. Human β-defensins are expressed after infection of airway epithelial cells with Mtb [75] [74]. Other substances elaborated from the respiratory epithelium such as lyophospholipids [411] and oligonucleotides contribute to the host antimycobacterial response by reducing Mtb cytotoxicity, inducing intracellular Mtb killing, and inhibiting phagolysosomal maturation via
phospholipase activation [412]. Epithelial cells can control inflammation as a secondary line of defence by producing factors that attract other immune cells and mediate cross-talk between airway epithelial cells and myofibroblasts to amplify the host immune response [413]. Epithelial cells have a differential secretion profile, depending on various factors such as location in the respiratory tract [403, 414] which has probably evolved to counter location-specific pathogenicity of the organism concerned. Alveolar epithelial, but not upper airway epithelial, networks in pulmonary TB caused AP-1, C/EBPα and NF-κB dependent CCL5 secretion [403].

Epithelial cells also produce significant amounts of MMPs. Although there are 25 MMPs that have been identified in vertebrates, not all are important in the pathogenesis of TB. MMP-1, MMP-3, MMP-7 and MMP-9 have been the most commonly implicated ones thus far. My findings that MMP-1, MMP-3 and MMP-9 are up-regulated from airway epithelial airway cells in TB is therefore comparable with previous studies. In a recent study from our group, MMP-1 was up-regulated in TB patients and a specific MMP-1 inhibitor (Ro32-3555) suppressed Mtb-driven MMP-1 from human monocytes [386].

Briefly, as discussed before, high levels of MMP-9 correlate with disease severity and the presence of granulomas in tuberculous pleurisy. Patients with tuberculous meningitis had higher levels of both MMP-2 and MMP-9 and this correlated with neurologic complications [150]. The ease of detecting and quantifying MMP-9 has led to several studies on the gelatinase. Its multiple functions in different microenvironments are consistent with the multiple locations and cell types producing it. In the normal and in the diseased lung, MMP-9 is produced not just by inflammatory cells such as macrophages and neutrophils but also by resident cells such as bronchial epithelial, clara, smooth muscle and endothelial. Its prodomain can be cleaved by MMP-3, MMP-2 and hypochlorous acid, with MMP-3 possibly the most potent activator of MMP-9 [415]. MMP-9 cleaves denatured collagen and type 4 collagen as well, and this helps lymphocytes, leucocytes and other scavenger cells to enter and leave the lung matrix from the circulation. MMP-9 also modulates other enzymes and cytokines to fine-tune both destruction and repair. There have been studies to suggest that
MMP-9 produced in response to pro-inflammatory stimuli may have a beneficial aspect and should not be indiscriminately inhibited. For instance, MMP-9 can reduce pulmonary oedema in ARDS by improving alveolar epithelial healing [416]. BAL from MMP-9 deficient mice exposed to ozone were more exudative compared to wild type mice [417]. MMP-9 gene deficient mice displayed a reduced resistance to *E.coli* peritonitis, as indicated by enhanced bacterial growth and increased bacterial dissemination [418].

MMP-3, also called a stromelysin, is most abundantly produced from stromal cells. In addition to degrading several ECM components, it can activate both MMP-9 and MMP-1[91] and release several cell surface molecules such as E-cadherins. It has a wide spectrum of substrates such as laminin, type IV collagen and latent TGF-β. Both MMP-1 and MMP-3 levels were increased in respiratory secretions of TB patients but not in symptomatic controls [386].

As MMPs seemed to be attractive cancer targets, drug development programmes were initiated 20 years ago by many companies to therapeutically block the extracellular matrix-degrading activities of MMPs. However this strategy universally failed as MMPs do so much more than simply mediate basement membrane and connective tissue/stromal degradation to facilitate tumour cell migration. Since the MMP inhibitors used in all these trials were broad spectrum inhibitors, they automatically inhibited the anti-tumour effects of MMPs as well, leading to deleterious effects. The beneficial effects of MMPs are commonly referred to as ‘anti-target’ effects, as historically MMPs have been believed to be the ‘bad guys’ (so somewhat of an ‘anti-hero’ concept). Except that blocking some of these ‘anti-target’ effects is anything but beneficial. One important ‘anti-target’ effect is the anti-angiogenic effect of MMPs. What makes this whole conundrum further insoluble is the tissue and cell specificity of these ‘anti-target’ effects, for example MMP-9 can be pro-angiogenic in some tumours but generate anti-tumour fragments in others[96], and MMP-12 has a destructive role in the pathogenesis of COPD but is associated with a better prognosis in several tumours. A recent study reported the association of the 2G allele in the MMP-1 promoter with a favourable prognosis in colorectal cancer [419].
The first milestone in the clinical development of MMP inhibitors occurred in 1992 when British Biotech’s Batimastat (BB-94), a broad spectrum hydroxamic acid derivative based on the structure of collagen, became the first MMP inhibitor to be tested in humans [81]. Batimastat had low water solubility and had to be delivered intraperitoneally or intrapleurally. To overcome this, a new generation of MMP inhibitors was developed which could be delivered orally (Marimastat) but these had to be withdrawn due to extensive musculoskeletal adverse effects. Currently, with more detailed knowledge of the enzyme active site, newer inhibitors are being developed that are more specific in the MMPs that they target [420]. Despite the failure of the MMP inhibitor clinical trials, the total resignation of the idea is not justifiable. As these trials were designed on the basis of incomplete assumptions regarding the diverse functional roles of MMPs, what is required before further exploration, is degradomic analysis to identify the target and anti-target substrates [421].

Another point worth mentioning is that a vast majority of the initial MMP blockade studies had been performed in mice that had been rendered genetically deficient in one of the MMPs. However, many of these null mice have subtle phenotypic changes that resolve with age and hence they cease to be good models of MMP deficiency.

In the context of TB, MMP inhibition would be of relatively short duration, with the intention of limiting immunopathology driven by Mtb itself or its very rapid killing by drugs. Another attractive and promising mechanism to limit MMP activity is to block more upstream pathways that regulate it. This would be more specific and less generalised, hence the likelihood of adverse effects would be limited. The mitogen activated protein kinases (MAPK), in particular ERK and p38 are well known to regulate several MMP genes [105]. Their role in Mtb-driven signal transduction has been previously discussed. Agents like NSAIDS and dexamethasone that modulate the transcription of MMPs can also be explored. For example, para-aminosalicylic acid decreased MMP-1 activity by inhibiting p38 signalling, suggesting the pathway could be a target to reduce tissue destruction in TB [422]. Dexamethasone decreased CSF MMP-9 concentrations early in the treatment of TB meningitis and possibly contributed to a better outcome by this mechanism [423]. I examined the role of p38 in IL-17 driven MMP-3 regulation and found that it was crucial in TB.
Th17 cytokines have been strongly implicated as critical in mucosal immunity. The IL-17 gene has been evolutionarily conserved since species such as molluscs which predated the development of adaptive immunity. Therefore it could be envisaged that the cytokine likely was a bridge between innate and adaptive immunity [189]. Both IL-17 and IL-22 signal to bronchial epithelial cells to regulate diverse functions such as chemokine production, antimicrobial gene expression and bicarbonate secretion [424]. IL-17A and IL-17F were both critical for pulmonary neutrophil recruitment and the elaboration of G-CSF and chemokines from airway epithelial cells [425]. Lipocalin-2 is an antimicrobial peptide secreted at high levels by airway epithelial cells at mucosal surfaces in response to IL-17 and IL-22 [225]. In asthmatics, evidence of an increased glucocorticoid receptor-β expression in epithelial cells following IL-17 stimulation suggests its possible role in the pathogenesis of steroid hyporesponsive asthma [426]. MAPKinase and PI3Kinase pathways regulate the synergy between IL-17 and IL-1β to enhance the CXCL-8 promoter activity, mRNA induction and protein synthesis in human airway smooth muscle cells via AP-1 and NF-κB [427].

Since my project focuses on the role of MMPs in TB, I reviewed the literature for studies on MMP regulation by the Th17 cytokines. In human cardiac fibroblasts, IL-17 induced MMP-1 expression via p38 and ERK dependent AP-1, NF-κB and C/EBP-β activation [428]. Colonic subepithelial myofibroblasts actively secreted MMP-3 in response to IL-17, IL-1β and TNF-α [429]. The role of IL-17 driven MMPs in mediating joint destruction in rheumatoid arthritis has been discussed by Li et al [430]. Briefly MMP-1, -3, -9 and -13 are triggered by IL-17 in articular chondrocytes via the MAP Kinases in NF-κB and AP-1 dependent mechanisms [431-434]. Thus modulating IL-17 to control MMPs could be an option. My findings showed that IL-17 does not activate all MMPs, for example, it down-regulated TB-driven MMP-9. There have been studies to show that in mice for example, the effect is different where IL-17 increases the concentration of MMP-9 [435].
Blocking the pathway for immuno-modulation could be achieved at different levels. Upstream targets could be molecules that control Th17 differentiation such as IL-6, IL-21, IL-1β, TGF-β or IL-23. All-trans-retinoic-acid and its analogues can be used to block IL-17 production in vivo in humans as this will inhibit Th17 cells in favour of Treg cells. Another approach is to use small molecules to inhibit intracellular signalling of IL-17, the key adaptor ACT1 being the best target. More directly, one could target IL-17 itself and its receptors. Several biologics that neutralise IL-17 signalling are now in clinical development, including AIN457 and LY2439821, both monoclonal antibodies. AIN457 has completed phase I/II trials for psoriasis, rheumatoid arthritis and autoimmune uveitis and is in phase II trials for multiple sclerosis, Crohn’s disease and ankylosing spondylitis [436]. LY2439821 is currently in phase II trials for rheumatoid arthritis and psoriasis [213]. A recent observation was that IL-17 can activate JAK1/2 and the PI3K pathways, which coordinate with NF-κB for induction of defence genes (e.g., human defensin 2) in human airway epithelial cells [246].

Another biologically relevant feature of IL-17 is its strong cooperative effect with other cytokines in regulating downstream gene expression. It has been shown to synergise with IL-1β, IL-22, IFN-γ, TNF-α, Oncostatin M, CD40 and vitamin D [247]. The mechanisms underlying these synergisms are not fully elucidated but it is in part contributed to via mRNA stabilisation in the presence of these inflammatory mediators. Inhibitors of TNF-α could be explored with a view to limit the downstream effects of IL-17. We must however keep in mind the limitations of blocking IL-17, as any other cytokine, in clinical therapy. At least in mice, inhibition of IL-17 has been associated with increased mortality from bacterial lung infections.

Monoclonal antibodies against the p40 subunit (common to IL-23 and IL-12) are in phase II clinical trials for patients with Crohn’s disease or psoriasis [437, 438]. The major potential drawback associated with this however, is the simultaneous inhibition of IL-12 due to their common p40 subunit. Similarly, the dual pro-inflammatory and anti-inflammatory nature of IL-22 is the biggest obstacle in development of anti-IL-22 treatment. Recent evidence that Th17 cells are crucial players in the generation of vaccine-induced protective responses
against various pathogens suggests that the incorporation of this knowledge into the design of future vaccines against infectious agents could be an active area of research.

In mycobacterial infection, the production of IL-17 and Th-17 T cell responses largely depends on IL-23. For example, Pulmonary IL-23 gene delivery and a vaccine adjuvant augmented the expansion of Mtb-specific CD4+ T cells which produced IFN-γ and IL-17 [254, 255], with simultaneous reduction in the mycobacterial burden and pulmonary inflammation. IL-22 produced by human NK cells inhibits growth of Mtb by enhancing phagolysosomal fusion [439]. Complexity in the role of Th17 cytokines in TB is associated with IL-22, as IL-22 promotes regeneration and protects against tissue damage. IL-22 expressing CD4+ T cells are distinct from Th17 cells and whereas IL-17 was not found in the BAL of patients, IL-22 levels were elevated [256]. In a TB mouse model study, neutralisation of IL-22 did not have any effect on the lung bacterial burden [440]. Hence IL-17 and IL-22 seem to have distinct roles in TB immunity and disease.

In summary, the preceding set of my investigations show that IL-17 and IL-22 drive specific MMP-3 in a TB network, which can be modulated by the p38 MAP Kinase pathway. The effects are MMP-specific (not global, MMP-3 augmented but MMP-9 downregulated), and also cell-specific. The pathways could be cautiously explored for immunomodulation in TB.
9. PI3K/ p110 α and p70S6K have disparate roles in MMP regulation from airway epithelial cells in TB

9.1. Introduction

Since its discovery in the 1980’s, the PI3Kinase pathway has proven to be important in a number of cellular processes. Its role in the pathogenesis of TB has been discussed previously in the introduction section. For instance, it has been observed that LAM from Mtb promoted macrophage survival by phosphorylating Bad through a PI3K/ AKT pathway [322]. There are also several studies that report cross-talk between the PI3K and the MAP Kinase pathways, [441] [326], the latter having been confirmed beyond any doubt to be crucial in Mtb-driven signalling cascades. I hypothesised that the PI3 Kinase pathway mediated TB-driven MMP production from NHBEs.

I commenced my investigations to support this hypothesis by determining whether AKT, the most proximal and crucial node in the PI3K cascade, was activated in airway epithelial cells in a TB network. Next, I studied the effects of chemical and siRNA mediated inhibition of the pathway at the level of AKT and the proximal p110 catalytic subunit (with LY294002). I concentrated on MMP-1, MMP-3 and MMP-9 as I had previously demonstrated that they were up-regulated by TB in the airway epithelium. Finally, I investigated a more downstream effector of the pathway, the p70S6K.

Supernatants were collected at 72 hours for secretion studies, mRNA extraction was performed at 24 hours and CoMTb was used at a dilution of 1 in 5 with the growth medium. I was always vigilant regarding cell viability as both chemical and siRNA mediated inhibition of signalling pathways frequently compromise this. Experiments in which this was compromised were discarded and re-performed with lower concentration or a new inhibitor.
Again, since my RT-PCR figures demonstrate relative mRNA levels normalised to the reference genes, PCR $C_T$ data was used to compare absolute levels of gene expression by the delta-delta $C_T$ method and the results corroborated with the standard curve method analysis. For the following set of experiments, the $C_T$ values were as follows:-

MMP-1—
(a) Control $= 27.6 \pm 0.83$
(b) CoMTb stimulated $= 26 \pm 1.89$
(c) CoMTb+ LY $= 24.34 \pm 1.23$
(d) CoMTb+ AKT i= 28.5\pm 0.93$
(e) CoMTb+ p110α siRNA $= 22 \pm 0.44$

MMP-3—
(a) Control $= 33.8 \pm 0.49$
(b) CoMTb stimulated $= 32.4 \pm 0.58$
(c) CoMTb+ LY $= 36.78 \pm 1.12$
(d) CoMTb+ AKT i= 35.9\pm 0.51$
(e) CoMTb+ p110α siRNA $= 34 \pm 0.98$

MMP-9—
(a) Control $= 33 \pm 1.4$
(b) CoMTb stimulated $= 30 \pm 1.29$
(c) CoMTb+LY $= 26 \pm 1.67$
(d) CoMTb + AKT i= 32\pm 0.12$
(e) CoMTb+p110α siRNA $= 30.1 \pm 0.26$
9.2. Basal and stimulated phosphorylation of AKT is cell type dependent

**Figure 19**

A. Probing for phospho and total AKT showed that it was not basally phosphorylated in A549 cells. Phospho AKT in control A549 cells was detected at 30, 60 and 120 minutes. In cells stimulated with CoMTb, phospho AKT was nearly undetectable at baseline and peaked at 120 minutes after stimulation. The bar chart is a densitometric analysis of the phosphowestern. Total AKT was detected at baseline and at all time points, in both control and stimulated A549 cells. B. In NHBEs, phospho AKT was constitutively active at 0 minutes. There was no change in the degree/intensity of phosphorylation over 120 minutes, in control or CoMTb-stimulated cells. Total AKT was detected at baseline and throughout the experiment, both in stimulated and control cells.
First, I investigated the activation of AKT in airway epithelial cells.

A549 cells (an airway squamous cancer cell line) were lysed at 0, 30, 60 and 120 minutes, with and without pre-stimulation with CoMTb. The lysates were then probed for phospho and total AKT. AKT was not basally phosphorylated in A549 cells, with phosphorylation being detected in control cells at 30, 60 and 120 minutes. In the CoMTb-stimulated A549 cells, phospho AKT was detectable at 30 minutes and peaked at 120 minutes. Total AKT was detected at baseline and at all time points, in both control and stimulated A549 cells.

In NHBEs, AKT was constitutively active at 0 minutes. There was no change in the degree/intensity of phosphorylation at any time point over 120 minutes, neither in the control nor in the CoMTb-stimulated NHBEs. Total AKT was detected at baseline and throughout the experiment, both in stimulated and control cells.

These results show that the basal activation/phosphorylation of AKT is cell-type dependent. In primary airway epithelial cells it is constitutively phosphorylated.
9.3. **LY and AKTi VIII both suppress CoMTb-driven MMP-3 production in NHBEs**

**Figure 20**

**A.B.** LY294002 suppressed CoMTb-driven MMP-3 secretion and gene expression from NHBEs to baseline (p<0.01) (p<0.001). Suppression was maximal at 10µM. **C.D.** AKT inhibitor VIII suppressed CoMTb-driven MMP-3 production from NHBEs below baseline, maximally at 2.5µM (p<0.01).
To continue my investigation into the PI3K-dependent regulation of MMPs from NHBEs, I commenced with inhibition of the pathway with LY294002. LY294002 is a quercetin analogue and is conventionally used as a first line PI3K inhibitor. The effect of LY294002 on the secretion and gene expression of MMP-1, MMP-3 and MMP-9 were distinct from each other. MMPs differ in cellular production, transcriptional regulation and activation by signalling cascades, hence this was not an unexpected finding. MMP-9 is the most abundant MMP in the lung and there are several reports to suggest that it has a beneficial role in certain conditions [442]. MMP-3, a stromelysin, is strongly expressed in stromal cells and can activate both MMP-1 and MMP-9 [415]. MMP-1 degrades fibrillar collagen which provides tensile strength to the lung ECM.

Along with LY294004, I had also investigated the effects of wortmannin on MMP secretion. There was no change in the MMP profile upon pre-incubation of cells with wortmannin. This was presumably because MMP secretion peaks at 72 hours, whereas wortmannin is ultra-short-acting in its inhibitory effects.

In parallel with LY294002, I used an AKT specific inhibitor, AKT inhibitor VIII. AKT Inhibitor VIII is a cell-permeable quinoxaline compound that selectively and reversibly inhibits AKT1, AKT2 and AKT3 activity. It does not exhibit any inhibitory effect against other AGC family kinases (cAMP dependent, cGMP dependent, Protein Kinase C) in the PI3K cascade. The inhibitor prevails over AKT-mediated resistance to chemotherapeutics and blocks both basal and stimulated phosphorylation/activation of AKT1/AKT2 in cultured cells and in mice. This was important and relevant to my experiments in NHBEs as AKT was basally activated. Cells were pre-incubated with the relevant inhibitor for 2 hours, before being stimulated. There was no cell death at the concentrations included in the analyses. The AKT inhibitor VIII was toxic at and above 3μM, hence those results were not analysed.

LY294002 suppressed the CoMTb-driven MMP-3 secretion and gene expression below baseline. The effect was maximal at 5-10μM of the inhibitor. Both secretion (Fig. 20A, p<0.01) and mRNA expression (Fig. 20B, p<0.001) were suppressed by 100%. The AKT inhibitor VIII had a similar effect on MMP-3 production. Pre-incubation for 2 hours...
abolished MMP-3 secretion (Fig. 20C, p<0.01) and gene expression (Fig. 20D, p<0.01) to baseline. 100% inhibition was observed at 2.5µM. These results show that inhibiting the p110 catalytic subunit (LY 294002) and AKT (AKT inhibitor VIII) in the PI3K pathway has an identical inhibitory effect on the TB-driven MMP-3 secretion and mRNA expression from NHBEs.
9.4. **LY accentuates MMP-1 production from NHBEs but AKTi VIII suppresses MMP-1**

**Figure 21**

**A.** LY294002 augmented CoMTb-driven MMP-1 secretion and gene expression from NHBEs by approximately ~5 fold (p<0.001). This was maximal at 10µM. **C.** AKT inhibitor VIII suppressed CoMTb-driven MMP-1 secretion and gene expression from NHBEs to baseline, maximally at 2.5µM (p<0.001) (p<0.05).
Next I investigated the PI3K regulation of MMP-1 from NHBEs. MMP-1 has been identified as a crucial driver of lung immunopathology in humans and in transgenic mice [386]. LY294002 augmented the CoMTb-driven MMP-1 secretion from NHBEs. The increment was 6-fold and MMP-1 levels peaked at a mean of 5598 pg/ml (Fig. 21A, p<0.001). MMP-1 mRNA was also increased ~6 fold (Fig. 21B, p<0.001) hence confirming that MMP-1 secretory up-regulation by LY294002 was transcription-driven.

Inhibition of AKT by AKT inhibitor VIII suppressed MMP-1 from NHBEs. CoMTb-driven MMP-1 secretion was suppressed by ~50% to a mean of 466 pg/ml (Fig. 21C, p<0.001). Suppression of CoMTb-driven MMP-1 mRNA was proportionate and abolished to baseline (Fig. 21D, p<0.05) by the AKT inhibitor.

In summary, these results show that the PI3K-mediated modulation of MMP-1 is distinct from that MMP-3. The inhibition of the p110 catalytic subunit of the proximal PI3K complex accentuates MMP-1 production. This could be a result of inter-pathway cross-talks [443] or that inhibition of the p110 subunit modifies the PI3K cascade, possibly by alleviating the repressive effect of other prosurvival and growth pathways such as the mTORC1 [444], culminating in MMP-1 increase from NHBEs.
9.5. *LY* accentuates MMP-9 production from NHBEs but AKTi VIII suppresses MMP-9

**Figure 22**

A.B. *LY294002* augmented CoMTb-driven gelatinolysis (p<0.001) and MMP-9 relative mRNA expression (p<0.01) in NHBEs by ~2.5 fold. This was maximal at 10µM. C.D. AKT inhibitor VIII suppressed CoMTb-driven gelatinolysis and MMP-9 mRNA expression in NHBEs to baseline, maximally at 2.5µM (p<0.001) (p<0.05).
Finally in this set of experiments with LY294002 and the AKT inhibitor VIII, I examined the effect of both these inhibitors on the regulation of CoMTb-driven MMP-9 from NHBEs.

I investigated MMP-9 production from NHBEs by performing gelatin zymography, which is a functional assay of MMP-9. Supernatants from cells pre-incubated with LY294002 caused significantly more gelatinolysis, in a concentration-dependent manner (Fig. 22A, p<0.001). The effect was maximal after treatment with 10µM of LY294002. CoMTb-driven MMP-9 mRNA expression was enhanced 3-fold by LY294002 (Fig. 22B, p<0.01).

The effect of AKT inhibition was down-regulatory on the CoMTb-driven gelatinolytic activity in NHBEs (Fig. 22C, p<0.001), suppressing it to baseline. MMP-9 mRNA expression was proportionately suppressed to baseline (Fig. 22D, p<0.05). Maximal suppression was achieved at 2.5µM of AKT inhibitor VIII.

In summary the previous 3 sets of experiments show that the PI3K regulation of MMP-1 and MMP-9 in NHBEs are similar, but divergent for MMP-3. The p110 catalytic subunit has a suppressive effect on MMP-1 and MMP-9, which is alleviated by blocking it. This was not observed for MMP-3.

Blocking AKT has a global suppressive effect on MMP secretion and gene expression. This confirms the crucial role that scientific literature vastly attributes to this proximal node of the PI3K pathway. It is possible that blocking AKT interferes with inter-pathway cross-talk and intrinsic feedback loops, suppressing the production of MMPs globally. From a clinical perspective this would therefore not be a good target in the context of TB, as global suppression of MMPs has never proven efficacious.
9.6. TB-driven MMP-1 and MMP-9 is controlled by the PI3K p110α subunit

**Figure 23**

A.C.E. Inhibition of the PI3K p110α subunit by a specific inhibitor had a similar effect on MMP secretion as LY294002, thus confirming it as the key subunit involved. CoMTb-driven MMP-1 secretion (p<0.001) and CoMTb-driven gelatinolysis (p<0.01) were augmented but CoMTb-driven MMP-3 was suppressed (p<0.001). B.D.F. siRNA mediated inhibition of the PI3K p110α subunit doubled MMP-1 mRNA expression (p<0.05) but suppressed MMP-3 mRNA expression (p<0.05). There was no change in MMP-9 mRNA. Effects were maximal at 30 nM. There was no change with non-targeting siRNA (NT siRNA). These results again confirmed the important role of the p110α subunit.
In order to identify the specific p110 catalytic subunit that is involved in MMP regulation in NHBEs, I next embarked upon specific subunit inhibition. As discussed previously, the proximal PI3K effector has a p85 regulatory subunit and a p110 catalytic subunit. LY294002 inhibits all the isoforms of the p110 catalytic subunit. In order to specifically identify the isoform, I first carried out chemical inhibition and then used siRNA transfection. The γ and δ isoforms are almost exclusively expressed on leukocytic cells, hence I did not investigate them.

Next, I inhibited the p110α subunit, both with a specific chemical inhibitor and with siRNA transfection. This yielded very interesting results that were similar to the inhibition with LY294002. Chemical inhibition of the p110 alpha subunit augmented the CoMTb-driven gelatinolysis and MMP-1 secretion, in a concentration-dependent manner (Fig. 23A, p<0.001). There was a five-fold increase in MMP-1 secretion to 4887 pg/ml, with 10µM of the inhibitor. CoMTb-driven gelatinolysis was also augmented similarly (Fig. 23C, p<0.01). MMP-3 secretion was however, suppressed to baseline (Fig. 23E, p<0.001), without any compromise in cell viability.

I followed up these secretion experiments with transfection of NHBEs with siRNA specific for the p110 alpha subunit. I confirmed knockdown of the p110α catalytic subunit in NHBEs by a phosphowestern analysis, and then confirmed knockdown of the subunit at the mRNA level. As shown in Figure 24, significant knockdown was achieved at 10 nM of the specific siRNA and the levels were undetectable at 30 nM.
A

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B

![Graph showing mRNA fold change](image)

**Figure 24**

A. On phosphopwestern analysis of PI3K p110α in NHBE’s, CoMTb-mediated activation was abrogated with the specific siRNA. A concentration-dependent response was observed. B. Total p110α mRNA levels were suppressed to below baseline when the NHBE’s were incubated with the p110α-specific siRNA. A concentration-dependent suppression was again observed.

Next, I demonstrated that siRNA inhibition of the α subunit knocked down MMP-3 mRNA expression in airway epithelial cells (Fig. 23F, p<0.05). The suppression was approximately 60%. Divergently, MMP-1 mRNA expression was augmented after pre-incubation with the p110α-specific siRNA (Fig. 23B, p<0.05). The increment with 30nM of the siRNA was approximately ~50%. Surprisingly, CoMTb-driven MMP-9 mRNA expression did not alter from baseline (Fig. 23D). This was not the case with MMP-9 secretion. There are a number of reasons which could explain this, for example post-translational modifications, complex
epigenetic mechanisms regulating MMP-9 or complex domain activation of the MMP which is secreted as a complex with TIMP-1.

Finally, I incubated the epithelial cells with the p110 β subunit specific siRNA and as demonstrated in Figure 25, there was no effect on the mRNA expression of any of the MMPs-1, -3 or -9.

**Figure 25**

Upon incubation of NHBE’s with the p110β specific siRNA, no change in the mRNA expression of any of the MMPs-1, -3 or -9 was observed.
9.7. CoMTb phosphorylates the downstream PI3K effector p70\textsuperscript{S6K} in NHBEs

Probing for p70\textsuperscript{S6K} showed that unlike AKT, it was not basally phosphorylated in NHBEs. Upon stimulation with CoMTb, phosphorylation of p70\textsuperscript{S6K} was detected at 30 and 60 minutes. The bar chart is a densitometric analysis of the phosphowestern. Total p70\textsuperscript{S6K} was uniformly detected at all time points, in control and stimulated cells.

\textbf{Figure 26}

Probing for p70\textsuperscript{S6K} showed that unlike AKT, it was not basally phosphorylated in NHBEs. Upon stimulation with CoMTb, phosphorylation of p70\textsuperscript{S6K} was detected at 30 and 60 minutes. The bar chart is a densitometric analysis of the phosphowestern. Total p70\textsuperscript{S6K} was uniformly detected at all time points, in control and stimulated cells.
To further examine the regulation of MMP-1, -3 and -9 in airway epithelial cells, I studied the baseline and CoMTb-stimulated phosphorylation of p70\(^{S6K}\), which is distal to the mTOR complex and part of the internal feedback loop of the PI3K pathway.

The phosphorylation status of p70\(^{S6K}\) was discrepant from that of AKT in primary human bronchial epithelial cells. Phospho and total p70\(^{S6K}\) were probed for in control and CoMTb-stimulated epithelial cells lysates. The cells had been lysed at 0, 30 and 60 minutes after stimulation. There was phosphorylation of p70\(^{S6K}\) at 30 minutes which peaked at 60 minutes in CoMTb-stimulated epithelial cells. p70\(^{S6K}\) was not basally activated.

Total p70\(^{S6K}\) was detected at the same level under all conditions.

**Figure 27**

A. Rapamycin inhibited CoMTb-driven MMP-1 secretion from NHBEs (p<0.001). The response was ~100% at 0.01 µg/ml of rapamycin. B. CoMTb-driven MMP-9 secretion was increased ~3 fold by Rapamycin (p<0.001). There was an incremental concentration response. C. Rapamycin inhibited CoMTb-driven MMP-3 secretion to baseline at 1 µg/ml (p<0.01).
Finally, I inhibited the downstream effector p70S6K in the PI3K pathway with the chemical inhibitor Rapamycin. Rapamycin was isolated from *Streptomyces hygroscopicus* in 1975 and was initially used as an anti-fungal agent. However, its use in clinical practice is now mainly as an immunosuppressant and as an anti-cancer agent. Rapamycin also blocks p70 S6K and is used widely in science as its specific inhibitor.

Rapamycin inhibited MMP-1 and MMP-3 secretion from CoMTb-stimulated NHBEs in a concentration-dependent manner. MMP-1 secretion was suppressed from 1801 pg/ml to 373 pg/ml (Fig. 27A, p<0.001). Similarly, MMP-3 secretion was inhibited from 1213 pg/ml to 186pg/ml (Fig 27C, p<0.01). Progressive inhibition was achieved over a concentration range of 0.01 - 5pg/ml. CoMTb-driven MMP-9 production was augmented by Rapamycin, from 116062 pg/ml to 317300 pg/ml (Fig. 27B, p<0.001).

In summary, the above set of results suggest that the PI3K regulation of TB –driven MMP production from NHBEs is complex. The most straightforward PI3K-mediated regulation is that for MMP-3. Suppression of its secretion and gene expression is observed by blocking the PI3K pathway both proximally, at the AKT node and also more distally at p70S6K.

MMP-1 production is augmented by blocking the pathway at the p110α subunit, but on blocking the pathway more distally or at the level of AKT, this MMP is suppressed. Hence distal blockade of the PI3K pathway (e.g. p70S6K) to curb this MMP would have more therapeutic potential, as blocking the pathway proximally paradoxically augments it.

MMP-9 regulation mechanisms are the most complex. Whilst proximal blockade up-regulates the secretion of the gelatinase, this is not reflected at the level of gene expression. More distal suppression with rapamycin also augments this MMP, with inhibition only being noticed at the AKT junction.
9.9. Discussion

My investigations into the PI3Kinase control of MMP expression from airway epithelial cells showed that the effects of proximal vs distal blockade of the pathway are disparate. I identified that inhibiting AKT had the semblance of an immunosuppressive effect on MMP secretion, as all MMPs were globally suppressed. siRNA mediated inhibition of AKT was unsuccessful, due to poor cell viability. All these findings confirm the importance of this node in PI3K signalling.

I then examined the effect of inhibiting the proximal p110 catalytic subunit of the PI3K pathway. I initially did this with LY294002 and then with isoform specific inhibitors. Inhibition of PTEN (proximally antagonises generation of PIP$_3$) was also attempted with the specific inhibitor bpV-phen (potassium bisperoxo (1,10-phenanthroline) oxovanadate). This was associated with increased cell toxicity and therefore abandoned.

The PI3K mediates different cellular responses depending on the tissue and the cell. These effector functions are usually subunit-specific. The catalytic p110$\alpha$ and p110$\beta$ subunits are ubiquitously expressed, whereas the expression of p110$\delta$ and p110$\gamma$ is mostly restricted to leucocytes. The PI3K p110$\alpha$ isoform performs most of the functions that are commonly assigned to PI3K in the literature. My findings uncovered an important role for the p110$\alpha$ subunit in TB driven MMP production. Pre-incubation with LY294002 suppressed MMP-3 but augmented MMP-1 and MMP-9 from epithelial cells in a TB network. By subunit specific chemical and siRNA inhibition, I identified the p110$\alpha$ subunit as being crucial. Inhibition of this subunit had nearly identical effects to that of LY294002 with respect to augmenting MMP-1 and inhibiting MMP-3. However this was not the case for MMP-9. This could be due to a number of complex reasons. Unlike other MMPs, epigenetic mechanisms contribute significantly to the regulation of MMP-9. Increased promoter methylation suppresses MMP-9, and there is additional synergy between epigenetic mechanisms and transcription factors such as CBP/p300 [445]. The human MMP-9 gene contains NF-κB and Ets binding sites and a TGF-β inhibitory element. Post-translationally, MMP-9 contains a
type V collagen like domain that is highly glycosylated, and which may confer a resistance to degradation. Additionally, MMP-9 is secreted from most cells in a non-covalent complex with TIMP-1 and any active forms are trapped by its circulating inhibitor α2-macroglobulin and removed via scavenger receptors [442].

Specific chemical or siRNA mediated inhibition of the p110β subunit did not affect MMPs.

More downstream, p70S6K blockade suppressed both MMP-1 (which was up-regulated by p110α inhibition) and MMP-3, but not MMP-9. This divergent MMP-1 regulation between p110α and p70S6K could be due to pathway feedback inhibition/pathway cross-talk/ de-repression of a natural inhibitory tendency of the proximal p110 catalytic subunit. Several studies report cross-talks between AKT and p70S6K. Constitutive activation of AKT up-regulates the nutrient –S6K branch of the mTOR pathway [274, 275], but S6K activity provides a negative feedback on the AKT branch. S6K also triggers a feedback loop upon chronic activation, which inhibits PI3K signalling [290]. A number of studies have shown that S6K can be activated independently of the PI3K/ AKT pathway. For instance, the Ras/MEK/ERK pathway has been found to contribute to the regulation of S6K1 activity via its effect on the tuberous sclerosis complex 2 (TSC2) [291]. The PI3K and the Raf-1/MEK/ERK pathways were both synergistically involved in rictor-mediated MMP-9 activity and invasion of glioma tumour cells [446]. mTORC1 inhibition leads to an activation of AKT through abrogation of the feedback inhibition, and this has been proposed as a reason for failure of rapamycin in some epithelial tumours [444]. Another example of pathway cross-talk is the IGF-I regulation of matrix synthesis by chondrocytes [447]. Sustained ERK phosphorylation seen in OA cells or in normal cells under stress inhibits AKT phosphorylation of IRS-1. The balance of PI3K to ERK controls the production of matrix proteins by chondrocytes, PI3K promotes matrix synthesis whilst ERK is inhibitory. There is growing evidence that inhibition of both the PI3K kinase and ERK pathways might be synergistic and therefore more effective [448, 449]. Despite their divergent roles, they both converge on the BH3 family of proteins (regulates apoptosis), and on the mTORC1 signalling pathway (regulates cell growth). Inhibition of PI3K can alleviate other pro-growth and pro-survival pathways.
The first report of a compound that showed an inhibitory effect on the PI3Kinase pathway was the nonspecific kinase inhibitor quercetin [450]. The next inhibitor to be identified was wortmannin, a natural product isolated from *Penicillium wortmannin* and known at that time as an inhibitor of myosin light chain kinase [451]. Wortmannin has been found to be active against all the class I enzymes but there have been problems with its use as it is very short-acting and has IC$_{50}$s in the single digit nanomolar concentrations. In 1994, Vlahos *et al.* [452] developed a quercetin analogue, LY294002, with increased sensitivity towards the p110 catalytic subunits of PI3K. Majority of the PI3K inhibitors that are currently in clinical development inhibit all the catalytic subunit isoforms of class IA PI3Ks, but there are a substantial few that are isoform-specific. It remains unclear whether it would be clinically more effective to use a pan-inhibitor or an isoform specific inhibitor. Blocking all isoforms is undoubtedly related to additional toxicity (notably glucose intolerance and immunosuppression) and there is considerable laboratory evidence that targeting a single isoform may be sufficient, hence developing specific inhibitors is more judicious [268]. Modifications of wortmannin at its active C20 position through opening of its furan ring yielded compounds that increased its half life and also its selectivity for specific isoforms [453]. One such compound is PX-866, which has selectivity for the $\alpha$, $\gamma$ and $\delta$ subunits while inhibiting the $\beta$ isoform at higher concentrations. It has very low selectivity for the mTOR complex. PX-866 is the only irreversible PI3K inhibitor currently being clinically developed.

The most clinically advanced inhibitor of AKT, perifosine, is a lipid–based phosphatidylinositol analogue that targets the pleckstrin homology domain of AKT, and prevents AKT from binding to PIP3 and undergoing translocation [454]. A number of allosteric AKT inhibitors have been developed and show a level of isoform selectivity. AKTi-1/2, a naphthyridinone allosteric dual inhibitor of AKT1 and AKT2, has potent antitumour activity and its analogue is in a phase I trial for locally advanced or metastatic solid tumours [268].

The p110 subunits of PI3K and mTOR share similar structures, and small inhibitors of p110 often inhibit mTOR as well. The obvious key issue again is whether complete inhibition of all p110 isoforms, mTORC1 and mTORC2 would be tolerable. PI-103, a p110α specific
inhibitor was shown to have a potent effect in blocking PI3K signalling in glioma cells [455]. This off-target effect of PI-103 on the mTOR complex opened a new avenue in the search for an effective cancer therapy, and numerous dual PI3K-mTOR inhibitors are being introduced into clinical trials. Although mTOR was only recently defined as a member of the PI3K pathway, it has been previously targeted in clinical practice and was well-tolerated.

Rapamycin, the prototypical mTOR inhibitor, was discovered in 1975 [456]. Sehgal and colleagues isolated a natural product with antifungal and immunosuppressive properties from Streptomyces hygroscopicus, found in a soil sample collected on Easter Island [456]. The drug was named rapamycin for Rapa Nui, the native name for the island. Eventually its structural analysis revealed that it was an analogue of the macrolides and had potent growth inhibitory activity against the development of various tumours and cancers [457]. mTOR signalling was found to mediate human trophoblast through the regulation of MMP-2, MMP-9 and plasminogen activator via the JAK/STAT pathway [458]. Activation of p70^{S6K} stimulated ovarian cellular invasion by augmenting expression and proteolytic activity of MMP-9, but had little effect on MMP-2 [459]. In a model of renal inflammation and fibrosis, Rapamycin attenuated MMP-9 protein and mRNA levels via the NF-κB and AP-1 transcription factors, with a concomitant increment in TIMP levels [460]. In human lung fibroblasts, Rapamycin increased MMP-1 protein and mRNA levels, which was inhibited by inhibition of JNK but not of the other MAPKs [461]. In my experiments with Rapamycin on airway epithelial cells, MMP-9 was augmented and MMP-1 was inhibited, hence confirming that effects of Rapamycin are cell-specific and that it does not globally suppress MMPs. This is mandatory, if its role as an adjunct to TB chemotherapy is to be further explored. In a study on rats with liver cirrhosis, fourteen days of Rapamycin improved liver function with a concomitant decrease in MMP-2 activity and TGF-β expression [462].

Interactions between Mtb and the PI3K had been previously discussed. Briefly, Mtb enters the macrophage in a PI3K independent manner but by preventing the activation of the Rab5 effector, it lowers PIP₃ levels and thereby arrests phagosome maturation. Studies have shown that PI3K inhibition enhances p38 MAPK activation during Mtb exposure [326]. PI3K
mediated MMP control had also been reported, although not in the exact context of TB. Lu et al [463] demonstrated the LPS-driven MMP-9 production from monocytes was dependent on PI3Kinase and NFκB. This was the first time that AKT involvement in monocyte-driven MMP-9 was illustrated. PI3Kγ is as a key factor in TNF-α mediated MMP expression in cardiomyocytes and cardiofibroblasts [464]. PI3K regulated melanoma cell vasculogenic mimicry, via the cooperative interactions of MMP-2, MT1-MMP and laminin [465]. The PI3K/PTEN/AKT/mTOR pathway is activated in hepatocellular carcinoma and drives invasion and metastasis through up-regulation of MMP-9 [466]. Using chemical inhibitors, it was confirmed that the PI3K/AKT pathway was involved in TNF-α induced MMP-9 up-regulation and migration in mouse epidermal cells [467].

To effectively inhibit the PI3K pathway, as any other, it is imperative to understand why previous compounds have failed. Pharmacodynamic assessment of target inhibition after drug treatment is paramount. Imaging techniques might be developed to evaluate the activity of the pathway after inhibition. Insulin resistance, a potential toxicity caused by on-target effects of PI3K inhibitors, might be used an effective marker for inhibition of the pathway [468]. Potential reason for the limited efficacy of single-agent PI3K inhibitors are the presence of signalling feedback loops and cross-talk with other pathways, such as the ERK or p38 MAP Kinases. Eventually the success of pathway inhibition depends on indispensability of the target for the particular disease state and the therapeutic index / selectivity of the drug.

In summary, I have identified that MMP production from airway epithelial cells in TB is controlled by the PI3K pathway. Distal, rather than proximal inhibition of the pathway would be more clinically efficacious as this down-regulates both MMP-1 and MMP-3. More proximal blockade of the pathway at the p110α subunit or the AKT node either augmented more MMPs than it suppressed, or had a global MMP-suppressive effect, which is also not immunologically beneficial. These findings have been summarised in Figure.
Proximal inhibition of the PI3K p110α subunit accentuated MMP-1 and MMP-9 production from airway epithelial cells in a TB network. Distally, Rapamycin (p70S6K inhibitor) suppressed MMP-1 and MMP-3 but accentuated MMP-9. p70S6K inhibits AKT via the mTORC2 complex and this internal feedback affects MMP production. AKT inhibition globally suppressed all MMPs.

In the short term, I intend to correlate the effects of Rapamycin on MMP secretion with mRNA expression, and siRNA transfection of p70S6K. Finally, I will also examine the up-regulation of p70S6K in TB lung biopsies by immunohistochemical studies.
10. Immunomodulatory role of established anti-mycobacterial agents on TB-driven MMPs

10.1. Introduction

I next investigated the immunomodulatory role of antimycobacterial drugs in pulmonary TB. As discussed previously, certain antibiotics have an immunomodulatory role in addition to their anti-infectious qualities. I investigated such a role for Azithromycin (first line anti-atypical mycobacterial drug), Moxifloxacin (an established second line anti-TB drug) and Rifampicin (first line anti-TB drug) in the context of TB-driven MMP regulation. This could potentially be the basis of adding such drugs as an adjunct to shorten the currently very lengthy chemotherapy regime.

Respiratory epithelial cells (NHBEs) were first incubated with the drug for 2 hours. Cell viability was confirmed before proceeding, (and also before collection of the final supernatant). The concentrations used for all the three drugs were calculated on the basis of the volume of distribution of the drugs (human), T ½ of the drug, the area under the curve (AUC) and the peak plasma concentration achieved. This was done with an attempt to study effects at concentrations that are biologically relevant. Subsequently, these concentrations were also confirmed in the literature to have been widely used in experiments.

Levofloxacin was also investigated (data not shown) and had identical effects to Moxifloxacin on the MMP secretion profile of NHBEs. I focussed my investigations on Moxifloxacin as it is an established drug in MDR TB. Amongst the fluoroquinlones, it also has the best sterilising activity and clinical trials in drug susceptible TB have shown that its use is associated with an earlier culture conversion rate.
I also investigated Rifampicin, which has been an established first line anti-TB drug for decades. I surmised that it would be interesting to identify an immuno-modulatory role of the drug, as this would only complement its unique bactericidal effects on non-replicating Mtb, an attribute which has been long believed to be the basis of its indispensability over the decades. Isoniazid did not affect MMP production.

After pre-incubation with the drug, NHBEs were stimulated with CoMTb. I focussed on the MMPs that I had previously identified to be up-regulated from NHBEs in a TB network i.e. MMP-1, MMP-3 and MMP-9. I would like to mention here that the ELISA’s and gelatin zymographies in this section were performed by Andre Kubler and Harriet Kubler who were BSc students I had supervised during my PhD.
**10.2. Azithromycin downregulates MMP-1 and MMP-3 from NHBEs in a TB network.**

**Figure 29**

A.B. Azithromycin suppressed TB-driven MMP-1 secretion by ~50% in a concentration-dependent manner from NHBEs (p<0.0001). MMP-1 mRNA expression was also suppressed to baseline at 30µg/ml, p<0.01. C.D. Azithromycin suppressed both TB-driven MMP-3 secretion (p<0.0001) and mRNA expression (p<0.01) in a concentration-dependent manner.
First, I investigated the effect of Azithromycin on the production of CoMTb-driven MMPs from NHBEs. Cells were pre-incubated with the drug (concentration range 5-50 µg/ml) for 2 hours and then stimulated with CoMTb at a 1 in 5 dilution. MMP-1, MMP-3 and MMP-9 protein and mRNA analysis were performed.

Azithromycin suppressed CoMTb-driven MMP-1 secretion from NHBEs in a concentration dependent manner (Fig. 29A) by ~50%. Levels were not suppressed to baseline but the down-regulation was statistically significant (p<0.0001). Maximal suppression was achieved with 30µg/ml of Azithromycin. Showing a similar trend, Azithromycin also suppressed relative MMP-1 mRNA expression to baseline at a concentration of 30µg/ml (Fig. 29B, p<0.05).

Similarly, Azithromycin suppressed TB-driven MMP-3 secretion (p<0.0001) and mRNA expression (p<0.01) in a concentration-dependent manner. Maximal suppression was observed at 30µg/ml of Azithromycin.

There was no change in MMP-9 production upon incubation with Azithromycin. CoMTb driven gelatinolytic activity / MMP-9 relative mRNA expression were not affected.

As a negative control in each experiment, cells were pre-incubated with Azithromycin alone, without subsequent stimulation with CoMTb. This did not affect the baseline MMP production from the cells.
10.3. Moxifloxacin down-regulates MMPs-1, -3 & -9 from NHBEs in a TB network.

**Figure 30**

A.B. MMP-1 secretion and gene expression were both suppressed to baseline by Moxifloxacin in a TB network, (p<0.0001 and p<0.05 respectively).

C.D. MMP-3 secretion and gene expression were both suppressed to baseline by Moxifloxacin in a TB network, (p<0.0001 for both).

E.F. CoMTb-driven gelatinolyis and mRNA expression were both suppressed upon pre-incubation with Moxifloxacin (p<0.01 and p<0.05 respectively).
Next, I examined the modulation of CoMTb-driven MMP-1, MMP-3 and MMP-9 from NHBEs after pre-incubation with Moxifloxacin. Similar to the macrolide experiments, the effects were investigated over a biologically significant concentration range. A negative control was ensured in every experiment where cells were simply stimulated with Moxifloxacin to rule out baseline changes induced by the drug.

CoMTb-driven MMP-1 secretion and relative mRNA expression were both suppressed to baseline by Moxifloxacin in a TB network, (Fig. 30A/30B, p<0.0001 and p<0.05 respectively). Maximal suppression was achieved with 20µg/ml of the drug. Similarly, MMP-3 secretion and relative mRNA expression was also suppressed to baseline by Moxifloxacin in a TB network, (Fig. 30C/30D, p<0.0001 for both). Lastly, CoMTb-driven gelatinolysis and MMP-9 mRNA expression were both suppressed upon pre-incubation with Moxifloxacin (Fig. 30E/30F, p<0.01 and p<0.05 respectively).

In summary, Moxifloxacin suppresses the secretion of MMP-1, -3 and -9 in a TB network. This would be a useful adjunctive role of the drug when used as an anti-TB agent, for both drug-susceptible and drug-resistant cases. Maximal suppression was achieved with 20µg/ml, which is comparable to the peak plasma concentration at which it exerts its bactericidal effects.
10.4. Rifampicin downregulates MMP-3 from NHBEs in a TB network

**Figure 31**
A.B. Rifampicin had an inhibitory effect on CoMTb-driven MMP-3 secretion (p<0.0001) and mRNA expression (p<0.01) from NHBEs but did not affect the other MMPs.
In my last set of experiments, I examined the effect of Rifampicin on MMP secretion from NHBEs. There was no effect on MMP-1 and MMP-9 in a TB network. However, MMP-3 secretion was suppressed to baseline (Fig. 31A, p<0.0001). Relative MMP-3 mRNA inhibition was comparable, with suppression to baseline as well (Fig. 31B, p<0.01). Maximal effect was observed with 10\(\mu\)g/ml of Rifampicin.
10.5. Moxifloxacin and Azithromycin up-regulate MMP-1 and MMP-3 from MRC-5 fibroblasts in a TB network.

![Graph A](image-url)

**Figure 32**

A. Moxifloxacin up-regulated both MMP-1 and MMP-3 secretion from MRC-5 cells in a TB network (p<0.01). The response was dose-dependent and maximally driven by 10µg/ml of the drug, beyond which there was compromised cell viability. B. In a similar manner, Azithromycin augmented MMP-1 and MMP-3 secretion from MRC-5 cells in a TB network. The increment was dose-dependent and maximal at 10µg/ml (p<0.05).
Finally, in my last set of experiments investigating the MMP-modulatory role of first and second line anti-mycobacterial drugs, I looked at their effect on MRC-5 cells in a TB network. Surprisingly, both Azithromycin and Moxifloxacin augmented MMP-1 and MMP-3 secretion.

The MRC-5 cell line was developed in September 1966 from lung tissue taken from a 14 week foetus aborted for maternal psychiatric reasons. The cell morphology is fibroblast-like.

Upon pre-incubation with Moxifloxacin, there was nearly ~1.8 fold increase in both MMP-1 and MMP-3 secretion from MRC-5 cells (Fig. 32A, p<0.01). A similar increment was observed upon pre-incubation with Azithromycin (Fig. 32B, p<0.05). MMP-9 secretion was not analysed as MRC-5 cells do not produce this MMP. For both the antibiotics, the response was concentration-dependent and maximal at 10µg/ml, beyond which there was compromised cell viability.

These sets of results show that the effects of Moxifloxacin and Azithromycin in a TB network are cell –specific, with disparate effects in airway epithelial cells and fibroblasts.
10.6. Discussion

In summary I have identified an MMP-modulatory role of Moxifloxacin, Azithromycin and Rifampicin, from airway epithelial cells in a TB network. These effects are MMP- and cell-specific. For example, Moxifloxacin suppressed MMP-1 and MMP-3 in airway epithelial cells but augmented them in MRC-5 fibroblasts. Azithromycin, unlike Moxifloxacin, did not suppress MMP-9 production from airway epithelial cells.

The immune system has been described as complex and dynamic but redundant, with numerous non-linear feedback loops, best modelled mathematically as deterministic chaos [469]. Modulation of this system therefore has to be a non-linear cycle of activation and suppression, all with the aim of achieving eventual homeostasis. Immunomodulation is thus a different concept from immunosuppression, that non-selectively dampens the entire system. Additionally, pleomorphism and redundancy of cytokine networks is well-documented. Many cytokines are secreted by more than one cell, and vice versa, but not all cells secrete all the cytokines. Similarly, the effects of different drugs on MMP synthesis is not uniform.

The effect of macrolides on the cells of the innate immune system is not limited to the modulation of pro- and anti-inflammatory mediators (as discussed previously) but also encompasses the effector mechanisms of phagocytosis and the adaptive immune system. Clarithromycin and Azithromycin both increased the in vitro rate of phagocytosis of sequestered apoptotic neutrophils and bronchial epithelial cells by alveolar macrophages [470, 471]. Expression of the CD80 surface marker on dendritic cells isolated from bone marrow of BALB/c mice was primed by Azithromycin or Clarithromycin [472]. Clarithromycin inhibits intracellular production of IL-4 by T-helper lymphocytes leading to an increase of the Th1/Th2 ratio [473].
In airway epithelial cells, Azithromycin changed the location of proteins in cell cultures and induced processing of the tight junction proteins claudin-1, claudin-4 and occludin, thus increasing the trans-epithelial electrical resistance of the epithelium [474]. In a study by Ribeiro et al [475], the effects of Azithromycin on the gene expression of various inflammatory and lipid mediators in the airway epithelium was complex, with the drug preventing up-regulation of MMP-9 for instance, but not affecting CXCL-8. Other macrolides have also been noted to influence MMP production. Suppression of LPS-driven MMP-9 from neutrophils occurred at 5µg/ml of Roxithromycin via the inhibition of NF-κB and AP-1 [476]. Roxithromycin also inhibited TNF-α induced MMP-1 through modulation of the MAPKinase [477]. My findings on the MMP-inhibitory effects of Azithromycin in a TB network are novel. Despite the risk of resistance development, these anti-inflammatory properties of macrolides add a new perspective to the concept of modulating the host’s reaction.

Data on the precise cascade of intracellular processes leading to stimulatory or inhibitory effects of the fluoroquinolones on cytokines, chemokines and other components of the immune system are lacking, but several observations have been made that implicate potential mechanisms. These include—

1) Modulation of intracellular cyclic AMP and phosphodiesterase
2) Direct effect on various kinases such as PKA, PKC and thymidine kinase
3) Effects on transcription factors e.g. c-fos, c-jun and c-myc (AP-1) [478]
4) Interactions with the host topoisomerase II complex [479]
5) Generally a parallel change in the specific mRNA expression [478, 480, 481]

The administration of quinolones to healthy volunteers or to animals has shown that these drugs alone do not exert any measurable immunomodulatory effects. The effect was only elicited in the presence of some kind of additional stimulant or trigger. Drug concentration
was important as well, with the profile of cytokines induced at lower concentrations being very different, and sometimes more efficacious to the host.

Rifampicin is a well-established first line anti-TB drug and is unique in its ability to target non-replicating Mtb. It is also used in prophylactic therapy against Neisseria meningitidis infection for its anti-inflammatory and protein-synthesis-inhibiting properties. In mice model of S. pneumoniae meningitis, mice treated with a single dose of Rifampicin had a lower concentration of lipoteichoic acid and teichoic acid in the CSF than those treated with Ceftriaxone [482]. In a rabbit meningitis model, a similar intervention with Rifampicin prevented the release of pneumolysin [483]. These studies suggest that Rifampicin can modulate the host’s immune response in various disease conditions.

In summary, my results show that first and second line anti-mycobacterial drugs can modulate MMP production from airway epithelial cells in a TB network. In the short term I intend to perform a few more experiments to delineate the signalling pathways involved and determine the effects of the drugs on MMP promoters. I had also investigated the effect of Isoniazid on MMP regulation and the results were all negative.

MMPs can be modulated with the intention to limit immunopathology in TB. There would be the initial benefit of reduced local tissue destruction, followed by better penetration of the chemotherapeutic drugs. These could eventually lead to a much-needed switch from a pro-inflammatory to an anti-inflammatory phenotype and hopefully aid shortening of a lengthy treatment regime [484]. Such an effect could also be beneficial in the scenario of very rapid killing of Mtb when chemotherapy is first commenced.

There are concerns regarding the inclusion of Moxifloxacin as a first line anti-TB drug, as it may fall prey to bacterial resistance. There are groups that advocate the drug should be saved as a second line for MDR TB [485]. The use of Moxifloxacin as an immunomodulator would
only be for short while and hence these important issues would be irrelevant. Doxycycline is the only MMP inhibitor licensed for use in periodontal disease. After oral administration of Doxycycline, mice displayed decreased airway inflammation, decreased airway hyperresponsiveness and a decreased expression of MMP-9 mRNA and protein levels [486, 487]. Taken together, these data suggest that MMPs are a potential therapeutic target as an adjunct to established chemotherapy in TB, and that such treatment can be undertaken with short courses of antibiotics that are already in use for other conditions, hence bypassing the tedium of new drug development.
11. Chemokines do not drive MMPs in TB

11.1. Introduction

I had performed a series of experiments to examine whether the chemokines CXCL-8 (IL-8), CCL-2 (MCP-1) and CCL-5 (RANTES) would drive MMPs from airway epithelial cells in a TB network. All my experiments were negative for this hypothesis and have been summarised briefly in the next few pages.

MMP-1 results have not been mentioned herein as they had been previously analysed by another member of the firm.
11.2. Chemokines +/- TNFα +/- CoMTb do not drive MMPs from NHBEs

**Figure 33**

**A.** Stimulation of NHBEs with chemokines +/- TNF-α did not drive MMP-3. **B.** Stimulation of NHBEs with chemokines in a TB network did not drive MMP-3.
Normal Human Bronchial Epithelial Cells were stimulated with the chemokines CXCL-8 (IL-8), CCL-2 (MCP-1) and CCL-5 (RANTES). 1ng/ml TNFα (as has an autocrine synergistic effect with chemokines) was added to examine any increment in MMP secretion (Fig. 33A).

Another set of experiments was designed with the chemokines in a TB network. Analyses for MMP-3 and MMP-9 were performed.

There was no significant regulation of either MMP-3 or MMP-9 upon stimulation with the afore-mentioned chemokines +/- TNFα OR +/- CoMTb. The experiments on MMP-9 were part of a BSc project and have therefore not been included herein.
11.3. Chemokines +/- TNF-α do not drive MMP-3 or MMP-9 from small airway epithelial cells (SAECs)

**Figure 34 (a)**
Chemokines +/- TNF-α do not drive MMP-3 from SAECs.
**Figure 34 (b)**
Chemokines +/- TNF-α do not drive MMP-9 from SAECs
11.3. Chemokines +/- TNFα do not drive MMP-3 or MMP-9 secretion from SAECs.

SAECs were stimulated with the chemokines MCP-1, RANTES and IL-8 at concentrations of 1, 10 and 100ng/ml with or without 1ng/ml of TNFα. Supernatant was collected at 72 hours and analyzed for MMP-3 (ELISA) and MMP-9 (gelatin zymography).

There was no significant change in the secretion of either MMP-3 or MMP-9.
11.4. Discussion

Chemokines have an important role in the pathogenesis of TB. Amongst other functions they mediate the entry of inflammatory cells at the site of infection and thereby aid in granuloma formation. Chemokines are also associated with genetic susceptibility to TB.

There have been several studies to show that chemokines can modify the secretion and function of MMPs. IL-8 has been reported to induce MMP-9 from neutrophils through the receptor CXCR2, via PKC and ERK 1/2 signalling [488]. RANTES and CXCL-12 increased MMP-9 secretion from primary human monocytes while TIMP-1 secretion was not affected [489]. This was mediated through CCR1. Expression of collagenases, gelatinases and stromelysins by ovarian cancer cells was modulated by CCL-25 in a CCR9 dependent manner [490]. CCL-5 is a potent chemoattractant for immature dendritic cells and under inflammatory conditions, it further enhanced their migration through the basement membrane by rapidly increasing MMP-9 secretion [491]. Several chemokines augmented gelatinase and collagenase activities in cultured synoviocytes from rheumatoid arthritis patients, in a dose and time dependent manner [492]. The up-regulation of MMP activity was significantly abrogated by the presence of anti-IL-1β, but not anti-TNF-α blocking antibodies.

In the light of the above studies and the crucial role of chemokines in TB, I had hypothesized that chemokines could drive MMPs from airway epithelial cells in a TB network. Cells were stimulated with IL-8, MCP-1 or RANTES, alone and in combination with TNF-α/CoMTb. Similar to other experimental analyses, supernatants were collected at 72 hours.

No up-regulation / down-regulation in MMP production from airway epithelial cells was observed.
12. Final Conclusions and Future Directions

The above experiments and analyses demonstrate that MMPs -1,-2,-3,-7,-8 and -9 are elevated in Pulmonary Tuberculosis and that their concentrations correlate positively with a CXR score of parenchymal involvement and cavitation. The clinical study exclusively analysed BALs from a well-characterised cohort of patients who were undergoing routine bronchoscopies for investigation of respiratory symptoms which was a unique strength of the study.

There have been several studies in our group that show that directly infected cells are not the only source of MMPs in TB. Our in house in vitro TB model, CoMTb up-regulated MMP-1 secretion, gene expression and promoter activity from human airway epithelial cells in a p38-dependent mechanism [399]. Host- derived factors such as TNF-α synergised with Mtb to drive MAPK-dependent MMP-9 secretion from airway epithelial cells [400]. Evidence from the zebrafish model of TB has demonstrated that ESAT-6 drives epithelial cell MMP-9 to generate a migration gradient for monocytes [409]. Hence, both leukocytic and stromal cells interact closely in TB, and contribute towards MMP production and subsequent matrix degradation. Airway epithelial-driven immune mechanisms have an important role as it is the first point of contact for respiratory pathogens and provides the first physical barrier. Epithelial cells can control inflammation as a secondary line of defence by producing factors that attract other immune cells and mediate cross-talk between airway epithelial cells and myofibroblasts to amplify the host immune response [413].

My investigations into the MMP secretion profile of airway epithelial cells in a TB network show for the first time that MMP-1, MMP-3 and MMP-9 are augmented from small airway epithelial cells. This phenomenon had been previously observed in large airway epithelial cells for MMP-1 and MMP-9 [397] [400]. The first milestone in the clinical development of
MMP inhibitors occurred in 1992 when British Biotech’s Batimastat (BB-94) became the first MMP inhibitor to be tested in humans [81]. Currently, with more detailed knowledge of the enzyme active site, newer inhibitors are being developed that are more specific in the MMPs that they target and therefore have a better adverse effect profile [420]. In the context of TB, MMP inhibition would be of relatively short duration, with the intention of limiting immunopathology driven by Mtb itself or the host. Another attractive and promising mechanism to limit MMP activity is to block more upstream pathways that regulate it such as the MAPKs [105]. Agents such as para-aminosalicylic acid decrease MMP-1 activity by inhibiting p38 signalling, further suggesting that such pathways could be a target to reduce tissue destruction in TB [422].

Recently, Th17 cytokines have been strongly implicated as critical in mucosal immunity [189]. Both IL-17 and IL-22 signal via the JAK1/2 and PI3K pathways to bronchial epithelial cells to regulate diverse functions such as chemokine production, anti-microbial gene expression and activation of transcription factors [424] [246]. Several biologics that neutralise IL-17 signalling are now in clinical development, including AIN457 and LY2439821, both monoclonal antibodies [436] [213]. Another biologically relevant feature of IL-17 is its strong cooperative effect with other cytokines such as IL-1β, IFN-γ and TNF-α in regulating downstream gene expression [247], via mRNA stabilisation.

In mycobacterial infection, the production of IL-17 and Th-17 T cell responses largely depends on IL-23. For example, Pulmonary IL-23 gene delivery and a vaccine adjuvant augmented the expansion of Mtb-specific CD4+ T cells which produced both IFN-γ and IL-17 [254, 255], with simultaneous reduction in the mycobacterial burden and pulmonary inflammation. Complexity in the role of Th17 cytokines in TB is associated with IL-22, as IL-22 promotes regeneration and protects against tissue damage. IL-22 expressing CD4+ T cells are distinct from Th17 cells and whereas IL-17 was not found in the BAL of patients, IL-22 levels were elevated [256].
In order to delineate the role of Th-17 cytokines in MMP regulaton, I undertook several experiments on airway stromal cells in a TB model. I identified that IL-17 increased baseline and CoMTb-driven MMP-3 secretion and expression but had a dichotomous down-regulatory effect on the regulation of MMP-9. MMP-1 remained unaltered in an IL-17/TB network. Although IL-17 up-regulated CXCL-8 secretion from NHBEs, MMP secretion was independent of this. IL-17 is known to strongly synergise with pro-inflammatory cytokines such as TNF-α, but in my experiments, it did not induce its production per se. MMP-3 secretion and promoter activity were also augmented from MRC-5 fibroblasts in a TB network, and this was incremented synergistically by both IL-22 and IL-17. The discrepant cell-specific effects of IL-22 from IL-17 could be explained by crucial differences between the two cytokines. For example, IL-22 signals via Stat3 and unlike IL-17 has a clonogenic effect on human airway epithelial cells [220]. IL-22 and IL-23 did not drive MMPs from airway epithelial cells. These studies suggest that MMP expression is both cell- and stimulus-specific. I examined the role of p38 in IL-17-driven MMP-3 regulation and found that it was crucial, its inhibition caused a concentration-dependent downregualtion.

That soluble IL-17 could not be detected in TB BAL was in keeping with the finding that low levels of IL-17 in TB are the result of inhibition of Th17 effectors by Th1 effectors at the site of TB disease [261]. IL-17 in BAL and pleural fluid from most subjects, even in the absence of an inhibitory Th1 response, has always been too low or undetectable [256] [396]. A single human study has reported successful detection of IL-17 in BAL fluid from asthmatic subjects, but at a very low concentration of 15pg/ml [397]. These data imply that analysis of cell-associated IL-17 expression in BAL may be more revealing. In contrast to IL-17, IL-22 was detected in greater concentrations in BAL fluid form TB patients [256] which suggests that IL-22 producing cells are not influenced by the Th1 polarising conditions and overall, the Th17 response in TB has emerged to be much more complicated than initially envisaged.

Next I investigated the PI3Kinase pathway mediated control of MMP expression from airway epithelial cells and found that the effects of proximal vs distal blockade of the pathway are disparate. Inhibiting AKT had the semblance of an immunosuppressive effect on MMP
secretion, as all MMPs were globally suppressed. The PI3K mediates subunit-specific cellular responses depending on the tissue and the cell. The catalytic p110α and p110β subunits are ubiquitously expressed, whereas the expression of p110δ and p110γ is mostly restricted to leucocytes. My findings uncovered an important role for the p110α subunit in TB driven MMP production. Chemical and siRNA-mediated inhibition of this subunit had nearly identical effects to that of LY294002 with respect to augmenting MMP-1 and inhibiting MMP-3. However this was not the case for MMP-9.

More downstream, p70^{S6K} blockade suppressed both MMP-1 (which was up-regulated by p110α inhibition) and MMP-3, but not MMP-9. This divergent MMP-1 regulation between p110α and p70^{S6K} could be due to pathway feedback inhibition/ pathway cross-talk/ de-repression of a natural inhibitory tendency of the proximal p110 catalytic subunit. Several studies report cross-talks between AKT and p70^{S6K}. Constitutive activation of AKT up-regulates the nutrient –S6K branch of the mTOR pathway [274, 275], but S6K activity provides a negative feedback on the AKT branch. The p110 subunits of PI3K and mTOR share similar structures, and small inhibitors of p110 often inhibit mTOR as well. Although mTOR was only recently defined as a member of the PI3K pathway, it has been previously targeted in clinical practice and was well-tolerated.

Rapamycin, the prototypical mTOR inhibitor, was discovered in 1975 [456]. Sehgal and colleagues isolated a natural product with antifungal and immunosuppressive properties from Streptomyces hygroscopicus, found in a soil sample collected on Easter Island [456]. Eventually its structural analysis revealed that it was an analogue of the macrolides and had potent growth inhibitory activity against the development of various tumours and cancers [457]. In my experiments with Rapamycin on airway epithelial cells, MMP-9 was augmented and MMP-1 was inhibited, hence confirming that effects of Rapamycin are cell-specific and that it does not globally suppress MMPs. This is imperative, if its role as an adjunct to TB chemotherapy is to be further explored.
Finally, I investigated the MMP-modulatory role of Azithromycin (another macrolide), Moxifloxacin and Rifampicin, from airway epithelial cells in a TB network. The effects were MMP- and cell-specific. For example, Moxifloxacin suppressed MMP-1, MMP-3 & MMP-9 from airway epithelial cells but augmented them in MRC-5 fibroblasts. Azithromycin, unlike Moxifloxacin, did not suppress MMP-9 production from airway epithelial cells but suppressed MMP-1 and MMP-3. Rifampicin is a well-established first line anti-TB drug and is unique in its ability to target non-replicating Mtb. My studies suggest that in addition to this Rifampicin can also modulate the host’s MMP immune response in TB. There are concerns regarding the inclusion of Moxifloxacin as a first line anti-TB drug, as it may fall prey to bacterial resistance and there are groups that advocate the drug should be saved as a second line for MDR TB [485]. The use of Moxifloxacin as an immunomodulator would only be for short while and hence these important issues would be irrelevant.

In summary, the results in my thesis have demonstrated that secretion of MMP-1, -2, -3, -7, -8 and -9 is up-regulated in BAL from TB patients and there was a positive correlation with a CXR tissue destruction score. MMP-3 production from airway epithelial cells is synergistically augmented by IL-17 in a TB network. This is dependent on the p38 MAPKinase signalling cascade. In contrast, IL-17 down-regulates CoMTb-driven MMP-9 to baseline. Interleukin-22 augmented MMP-3 from MRC-5 fibroblasts in a TB network, but IL-23 did not drive MMPs. Both IL-17 and MMP-3 were co-expressed in pulmonary epithelial cells around TB granulomas. Investigation of the PI3Kinase pathway revealed that AKT inhibition suppressed all MMPs but proximal PI3Kp110α subunit blockade inhibited MMP-3 only. However, it paradoxically also led to an increase in MMP-1 and MMP-9. More distally, p70S6K (mTOR) blockade with Rapamycin abolished TB-driven MMP-1 and MMP-3 in epithelial cells. Therefore, the PI3Kinase/p70S6K cascade is crucial in TB and could be targeted, distally rather than proximally, (e.g. with Rapamycin) to limit destructive MMP activity in TB. TB-driven MMP-1, MMP-3 and MMP-9 production were also affected by anti-mycobacterial agents. This could have the potential to be translated into a short course of adjunctive immunotherapy to limit the lengthy durations of current chemotherapy regimes. Although previous studies with MMP inhibition have been unsuccessful, newer more specific
inhibitors are being developed and the idea should not be abandoned. In particular, more upstream regulators such as the MAPKinases and the PI3Kinases are attractive targets.

Taken together, these data suggest that MMPs are a potential therapeutic target as an adjunct to established chemotherapy in TB, and that such treatment can be undertaken with short courses of antibiotics that are already in use for other conditions, hence bypassing the tedium of new drug development. Typically, patients with high levels of MMPs in respiratory secretions could be targeted, as they would be most likely to benefit from their modulation.
13. List of abbreviations

ADAM: A Disintegrin and Metalloproteinase
ADAMTS: ADAM with thrombospondin motif
ATP: Adenosine triphosphate
BALF: Bronchoalveolar lavage fluid
BSA: Bovine serum albumin
cAMP: cyclic adenosine monophosphate
CFP-10: Culture filtrate protein
cGMP: cyclic guanosine monophosphate
CIA: Collagen induced arthritis
COPD: Chronic obstructive pulmonary disease
CoMCont: Conditioned medium from monocytes (control)
CoMTb: Conditioned medium from monocytes infected with Mtb
CSF: Cerebrospinal fluid
CXR: Chest xray
DC-SIGN: Dendritic cell specific ICAM3 grabbing non-integrin
DNA: Deoxyribonucleic acid
EAA: Experimental autoimmune encephalitis
ECM: Extracellular Matrix
EGFR: Epidermal growth factor receptor
ERK: Extracellular-regulated kinases
ESR: Erythrocyte sedimentation rate
ESAT-6: Early secreted antigenic target 6
GTPase: Guanosine triphosphatase
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
HAART: Highly active anti-retroviral therapy
HDAC: Histone deacetylase
HIV: Human immunodeficiency virus
ICAM3: Intercellular adhesion molecule 3
IFN-γ: Interferon-γ
IL: Interleukin
IP-10: Interferon-γ induced protein-10
JAK: Janus kinase
JNK: c-Jun N-terminal kinases
LAM: Lipoarabinomannan
LPS: Lipopolysaccharide
MAP Kinase: Mitogen activated protein kinase
MCV: Mycobacterium containing vacuoles
MCP-1: Monocyte chemotactic protein-1
MDR: Multi drug resistant
MIP-1β: Macrophage inflammatory protein-1β
MHC: Major histocompatibility complex
MMP: Matrix Metalloproteinase
mTOR: mammalian target of rapamycin
Mtbc: Mycobacterium tuberculosis
NHBE: Normal human bronchial epithelial cell
NK: Non killer
NOD: Nucleotide-binding oligomerisation domain
NOS: Nitric oxide synthase
NRAMP: Natural resistance-associated macrophage protein

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffered saline

PECAM-1: Platelet/endothelial cell adhesion molecule

PGE: Prostaglandin E

PH: Pleckstrin homology

PI-3Kinase: Phosphoinositide-3 Kinase

PIP₃: Phosphatidylinositol triphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PPD: Purified protein derivative

PTEN: Phosphatase and tensin homologue

RA: Rheumatoid arthritis

RANTES: regulated upon activation, normal T cell expressed and secreted

ROR-γ: Retinoic acid-related orphan receptor-γ

ROS: Reactive oxygen species

RT-PCR: Reverse transcriptase polymerase chain reaction

SAEC: Small airway epithelial cell

SDS: Sodium dodecyl sulphate

SCID: Severe combined immunodeficiency

siRNA: small interfering RNA

SGK: Serum and glucocorticoid related kinase

STAT: Signal Transducers and Activators of Transcription

TB: Tuberculosis
TGF-β: Transforming growth factor-β
TIMP: Tissue inhibitor of metalloproteinase
TLR: Toll like receptor
TNF-α: Tumour necrosis factor-α
VEGF: Vascular endothelial growth factor
WHO: World health organisation
XDR: Extensively drug resistant
14. Appendices

1) Poster presentations:

a) IL-17 drives MMP-3 but inhibits MMP-9 in a TB network. British Society of Immunology, Glasgow, 2008.

b) Th17 cytokines and stromal cell-derived MMPs drive tissue destruction in pulmonary TB. European Congress of Immunology, Berlin, 2009.

c) The PI3Kinase cascade and Th17 cytokines in Pulmonary TB. Internal Poster Presentations, Imperial College London. 2009.

d) Airway epithelial cell MMP production in TB is controlled by the PI3K/p110α subunit. British Society of Immunology, Liverpool, 2010.

e) Immuno-modulatory role of anti-mycobacterial drugs in Pulmonary TB. British Society of Immunology, Liverpool, 2010.
2) Oral presentations:


3) Manuscripts in Preparation

a) Th17 cytokines drive airway stromal cell-derived MMPs via the p38 MAPKinase in Pulmonary TB.

b) The PI3K/p100α/p70S6K pathway is crucial for MMP control in Pulmonary TB.

c) MMPs are up-regulated in human TB BALF and correlate with lung matrix tissue destruction.

d) Immunomodulatory role of anti-mycobacterial drugs in Pulmonary TB.
15. Bibliography


257. Khader, S.A., et al., IL-23 compensates for the absence of IL-12p70 and is essential for the IL-17 response during tuberculosis but is dispensable for protection and antigen-specific IFN-gamma responses if IL-12p70 is available. J Immunol, 2005. 175(2): p. 788-95.


