Cytokines and Epigenetic Regulation of Matrix Metalloproteinases in Tuberculosis

Thesis submitted for the degree of PhD

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Abstract

Tuberculosis (TB) remains a leading cause of death globally. Some of the morbidity and mortality associated with TB arises from excessive or inappropriate immune activation in response to infection. In pulmonary TB tissue destruction, cavitation and fibrosis drive disease transmission and chronic lung dysfunction. The matrix metalloproteinases (MMPs) are host enzymes controlling extracellular matrix turnover, immune cell recruitment and activation, and they play a key role in the pathology of TB.

This project explores the regulatory mechanisms controlling MMP expression in TB using a tissue culture model of respiratory epithelial cell and macrophage responses to *Mycobacterium tuberculosis* (Mtb). The major human collagenase MMP-1 and its activator MMP-3 are expressed in response to Mtb stimulation. The influence of exogenously added cytokines IL-4, IL-10, IL-13, TGFβ and IFNγ on MMP expression is investigated to examine how immunopathology is driven by acquired immune responses. Epigenetic mechanisms regulating MMP expression in response to Mtb are examined using inhibitors of histone deacetylases (HDACs) and histone acetyltransferases, HDAC siRNA and chromatin immunoprecipitation assays.

Mtb-induced collagenase expression by epithelial cells and macrophages is selectively inhibited by the Th2 cytokines IL-4 and IL-13, but unaffected by IL-10. TGFβ enhances epithelial cell MMP secretion but does not affect macrophage MMP expression. IFNγ has divergent effects, driving epithelial cell MMP secretion but inhibiting MMP-1 and -3 expression in macrophages. The intracellular pathways mediating these effects are explored.

Mtb-driven MMP expression is sensitive to HDAC and HAT inhibition, and altered HDAC expression is observed in Mtb-stimulated cells. Chromatin immunoprecipitation reveals changes in histone acetylation at the MMP-1 promoter in Mtb-stimulated epithelial cells.

In pulmonary TB, production of matrix-degrading MMPs by epithelial cells and macrophages is modulated by Th2 cytokines and IFNγ. Key MMPs implicated in pulmonary immunopathology are subject to epigenetic regulatory mechanisms, which may represent potential targets for selective MMP inhibition.

Table of Contents

Abstract.. 1

Table of Contents... 2

Table of figures 5

Declaration of Originality 8

Copyright Declaration 8

Acknowledgements 9

1 Introduction 10

1.1 Background 10

1.2 The scope of the problem – TB in the 21st Century 11

1.3 The host response to Mycobacterium tuberculosis 12

1.4 The TB granuloma 16

1.5 Matrix metalloproteinases 19

1.6 Models of TB 28

1.7 The role of Interleukin-4 in the immune response to Mtb 32

1.8 The role of Interleukin-13 in the immune response to Mtb 39

1.9 The role of Interleukin-10 in the immune response to Mtb 42

1.10 The role of transforming growth factor-β in the immune response to Mtb 47

1.11 Interferon-γ and the immune response to Mtb 50

1.12 Epigenetic regulation of gene expression 53

1.13 The future for TB research and treatment 63

1.14 Hypothesis and Aims 65

2 Materials & Methods 66

2.1 Cellular model of pulmonary tuberculosis 66

2.2 Materials 67

2.3 Methods 70

3 Interleukin-4 and other anti-inflammatory cytokines influence Mtb-driven MMP expression 85

3.1 Introduction 85

3.2 Interleukin-4 inhibits Mtb-driven MMP expression 87

3.3 Interleukin-13 inhibits Mtb-driven MMP expression 111

3.4 Interleukin-10 does not influence Mtb-driven MMP expression 115

3.5 Transforming growth factor-β1 enhances epithelial cell MMP expression 117

3.6 Discussion 121

4 Interferon-γ modulates Mtb-driven MMP expression 129

4.1 Introduction 129

4.2 Results 130

4.3 Discussion 146

5 The effects of HDAC and HAT inhibition on MMP expression 149

5.1 Introduction 149

5.2 Results 151

5.3 Discussion 178

6 Mtb alters HDAC expression in macrophages and epithelial cells.......................... 186

6.1 Introduction 186

6.2 Results 187

6.3 Discussion 198

7 Summary and future research 201

8 Bibliography 204

9 Abbreviations.. 222

Table of figures

Figure 1. IL-4 inhibits the secretion of MMP-1 by Mtb-infected MDMs from different blood donors ... 88

Figure 2. IL-4 selectively inhibits the secretion of MMP-1 and -3 by Mtb-infected MDMs ... 90

Figure 3. IL-4 acts pre-transcriptionally to inhibit MDM MMP expression ... 92

Figure 4. IL-4 inhibits Mtb-driven secretion of pro-inflammatory chemokines and cytokines by MDMs ... 94

Figure 5. IL-4 does not affect the ability of MDMs to control H37Rv infection ... 96

Figure 6. IL-4 inhibits MMP-1 and -3 secretion by CoMTb-stimulated epithelial cells ... 98

Figure 7. Transient transfection of respiratory epithelial cells with plasmid constructs of the MMP-1 and -3 promoters ... 101

Figure 8. MMP-1 promoter activity is unaltered in the presence of IL-4 but the AP-1 consensus binding site at -1950 bp is necessary for promoter activation by CoMTb ... 103

Figure 9. CoMTb-treatment of NHBEs drives AP-1 DNA binding activity which is impaired in the presence of IL-4 ... 105

Figure 10. CoMTb-driven MAPK phosphorylation is not altered in IL-4-treated A549s ... 107

Figure 11. Stat 6 is phosphorylated in response to IL-4 treatment of NHBEs or MDMs ... 108

Figure 12. IL-4 reduces RNA Pol II binding and histone H4 acetylation at the MMP-1 promoter in CoMTb-stimulated A549s ... 110

Figure 13. IL-13 inhibits CoMTb-driven MMP secretion by respiratory epithelial cells ... 112

Figure 14. IL-13 selectively inhibits MMP-1 and -3 secretion by Mtb-infected MDMs ... 114

Figure 15. IL-10 does not affect Mtb-induced MMP secretion by MDMs or respiratory epithelial cells ... 116

Figure 16. Transforming growth factor beta (TGFβ) enhances respiratory epithelial cell MMP-1 and -3 secretion in response to CoMTb stimulation ... 118

Figure 17. Transforming growth factor beta (TGFβ) does not significantly affect Mtb-driven MMP secretion by MDMs ... 120

Figure 18. IFNγ selectively inhibits MMP-1 and -3 secretion by Mtb-infected MDMs 131

Figure 19. IFNγ-treatment does not affect Mtb-driven upregulation of MMP-1, -3 and -7 mRNA in MDMs 132

Figure 20. IFNγ inhibits MDM MMP secretion in response to UV-killed Mtb....................... 134

Figure 21. IFNγ does not affect the ability of MDMs to control H37Rv infection 135

Figure 22. IFNγ enhances Mtb-driven MDM secretion of IL-6, TNFα and IL-12, and induces secretion of IL-10, IP-10 and MCP-1 137

Figure 23. IFNγ signaling phosphoarray 139

Figure 24. Stat 1 and 3 and AKT are phosphorylated in Mtb-infected MDMs treated with IFNγ 141

Figure 25. IFNγ alters respiratory epithelial cell MMP secretion in response to CoMTb 143

Figure 26. Stat 1 and 3 phosphorylation in IFNγ- and CoMTb-stimulated NHBEs.............. 145

Figure 27. TSA inhibits CoMTb-driven MMP-1 and -3 expression in NHBEs 152

Figure 28. CBHA inhibits CoMTb-driven MMP-1 and -3 secretion by NHBEs.................... 155

Figure 29. MS-275 enhances CoMTb-driven MMP-1 and -3 secretion by NHBEs 155

Figure 30. TSA inhibits MDM MMP-1, -3 and -7 secretion in response to Mtb infection..... 157

Figure 31. CBHA inhibits MDM MMP-1 and -3 and enhances MMP-7 secretion in response to Mtb infection 159

Figure 32. MS-275 selectively inhibits MMP-1 and -3 secretion by Mtb-stimulated MDMs 161

Figure 33. AA inhibits Mtb-driven MMP-1 and -3 secretion in MDMs and NHBEs 163

Figure 34. HATi II selectively inhibits Mtb-driven MMP-1 and -3 secretion in MDMs 165

Figure 35. HATi II inhibits Mtb-driven increases in MMP-1 and -3 mRNA in Mtb-infected MDMs but AA does not 166

Figure 36. Silencing HDAC1 expression in NHBEs does not affect CoMTb-driven MMP-1 & -3 expression 169

Figure 37. Silencing HDAC2 expression in NHBEs does not affect CoMTb-driven MMP-1 & -3 expression 171

Figure 38. Silencing HDAC3 expression in NHBEs does not affect CoMTb-driven MMP-1 & -3 expression 173
Figure 39. CoMTb stimulation drives increased histone H4 at the proximal MMP-1 promoter in A549s........ 175
Figure 40. CoMTb-driven MMP-1 expression is associated with increased RNA Pol II binding and increased histone H3 and H4 acetylation of the MMP-1 promoter region. 177
Figure 41. Class I HDAC mRNA expression is repressed and HDAC4 mRNA increased in Mtb-infected MDMs........ 188
Figure 42. HDAC class I and II protein expression in Mtb-infected MDMs........ 190
Figure 43. HDAC class I and II mRNA in NHBEs is not significantly altered by CoMTb stimulation........ 192
Figure 44. HDAC class I and II protein expression in CoMTb-stimulated NHBEs........ 194
Figure 45. HDAC and HAT activity is unchanged by Mtb infection of MDMs 197

Declaration of Originality

I declare that the work contained in this thesis is my own, and that the work of others is appropriately attributed and referenced where relevant.

Rachel Moores

April 2013

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Acknowledgements

I would like to thank my supervisor, Professor Jon Friedland for his support and encouragement over the seven years since I originally began working with him as an Academic Clinical Fellow. Other members of the Friedland group have contributed significantly with helpful ideas, discussion, and technical support. I would particularly like to acknowledge the help of Paul Elkington, Lucinda Rand and Justin Green in the early years. I am grateful to Shivani Singh who optimized the siRNA transfection protocols for epithelial cells. Greg Brook from the IRDB provided invaluable technical assistance while optimizing the ChIP methodology, and advised on ChIP primer design and data analysis.

Two MSc students co-supervised by Professor Friedland and I contributed to the experimental work presented here. Frans Schutgens performed some of the HDAC inhibitor experiments in epithelial cells in 2009, and Arthur Si's project in 2010 investigated the pathways mediating the effects of interferon-γ in macrophages.

I am extremely grateful to the Wellcome Trust for funding my (prolonged) Clinical Training Fellowship, and the Mason Medical Foundation for additional funding for consumables without which the chromatin immunoprecipitation work would not have been possible.

Finally, I thank my husband, Guy, without whom this project would never have been completed, my daughter Alice for helping me to keep it all in perspective, and number 2, who provided the ultimate thesis deadline.

1 Introduction

1.1 Background

Tuberculosis (TB) is an ancient disease of humanity, caused by the bacterial pathogen *Mycobacterium tuberculosis* (Mtb). Phylogenetic analysis suggests that host and pathogen have co-evolved for millennia, and exist in a finely poised balance, with clinically significant disease occurring in only a minority of those infected [1]. It is estimated that almost one third of the global population have been exposed to Mtb [2], with 125,000 new infections occurring daily. Transmission of the infection to new hosts must be a highly efficient process, given that only a tiny percentage of infected individuals have active disease at any one time, and it is believed that the infectious dose is only a few bacilli.

Despite the identification of the causative organism by Robert Koch in 1882, and the advent of successful antibiotic treatment with the development of streptomycin and para-aminosalicylate (PAS) in the 1940s [3], and subsequently the addition of isoniazid and rifampicin in the 1950's and 60's, TB continues to plague human society. The BCG vaccine was first used in 1921 and is the most widely administered vaccine in the world, yet it provides variable protection against disease, and fails to adequately protect against the most common manifestations of the disease, namely pulmonary and lymph node disease in adults [4, 5]. In the 21st century, efforts to combat the resurgence of TB are hampered by a number of factors: increasing drug-resistance, largely due to the failure of standardised treatment programmes, the HIV/AIDS pandemic, and the lack of rapid, accurate and affordable TB diagnostics, which leads to missed opportunities to detect and treat latent infections and early clinical disease.

In the past efforts to improve TB control concentrated on aspects of TB management such as reliable access to drugs and facilities to make a diagnosis through sputum microscopy. Whilst important, these interventions fail to address the problems associated with the rapid

emergence of multi-drug resistant (MDR-TB) and extremely-drug resistant (XDR-TB) strains over the last 30 years, which are not identifiable without access to culture facilities. Over-reliance on sputum smear microscopy for diagnosis also fails to identify smear-negative and extra-pulmonary disease which occur with increased frequency in children and in the context of HIV infection, where the annual risk of TB reactivation is up to 10%, compared to an estimated lifetime risk of 5-10% of reactivation in HIV-negative individuals [6].

1.2 The scope of the problem – TB in the 21st Century

The World Health Organisation (WHO) declared TB a "global emergency" in 1993, following an alarming increase in cases over several years after decades of steady decline. The STOP TB Campaign followed in 2006, setting targets for the next decade. Other initiatives, including the Global Alliance for TB Drug Development, the Foundation for Innovative New Diagnostics (FIND) and the TB Vaccine Initiative (TBVI) as well as the Bill and Melinda Gates Foundation have massively increased investment in TB research since 2000. The latest incidence and mortality statistics suggest that the Millennium Development Goals of halving TB prevalence and mortality by 2015 will be met, but it is estimated that in 2011 there were 8.7 million new cases (of whom 13% were HIV-positive) and 1.4 million deaths, including over 400,000 deaths in HIV-positive individuals, and thus TB continues to be a major public health challenge from a global perspective [7].

In the UK, TB is primarily a disease affecting those born overseas, predominantly in the Indian subcontinent and Sub-Saharan Africa. 8,963 cases were notified to the Health Protection Agency (HPA) in 2011, with 74% occurring in people born outside the UK [8]. The overall incidence in the UK is 14.4 cases per 100,000 population, but only 4.1 per 100,000 in the UK-born population. Therefore the majority of cases in the UK are probably the result of reactivation of disease acquired abroad, but there is also evidence of ongoing transmission within the UK. Disease is heavily concentrated in urban areas, with London accounting for

39% of cases in 2011, and further clusters in other cities with large immigrant populations. Drug-resistance is an increasing problem, with 7.6% of isolates displaying isoniazid-resistance and a significant rise in MDR-TB isolates to 1.6% of the total over the last decade. By the end of 2011 there had been 24 cases of XDR-TB in the UK, with 6 new cases notified in 2011 [8]. Although the scale of the TB problem in the UK is different to that faced by many developing countries, many of the challenges are the same: delayed diagnosis, long turn-around time for drug sensitivity testing, HIV co-infection, difficulty in tracing and treating latently infected individuals, and late recognition of drug-resistant disease.

Successfully addressing these issues required investment in novel technologies for diagnosis and the identification of new antibiotics, but also an improved understanding of the key immune parameters required for successful host elimination or containment of Mtb infection. As yet the factors determining the outcome of exposure to Mtb are not fully understood, and therefore it is not possible to accurately predict the outcome of latent infection, or to design a novel TB vaccine specifically targeted to confer sterilizing immunity.

1.3 The host response to Mycobacterium tuberculosis

The nature of the immune response to Mtb in an infected individual is a key determinant of the outcome of infection. Studies in highly endemic areas suggest that 10-20% of highly-exposed individuals do not acquire T-cell responses to Mtb, and therefore presumably are able to eliminate Mtb infection at a very early stage of infection. The host factors required for this apparent protection have not been completely defined [9]. Host factors are believed to be more important in determining outcome than pathogen factors since Mtb is a slow-growing organism which displays limited variability within and between infected individuals, while as already stated, there is a wide variation in the outcome of human exposure to infection even between genetically related individuals within the same environment. There is, however, recent evidence to suggest that variation in the immune responses induced by

different modern lineages of Mtb does occur, with different patterns of TNFα, IL-12p40 and IL-10 production in response to highly virulent Beijing-type strains reported [10-12] (also reviewed in [13]).

Although human genetic studies have identified some susceptibility loci for TB, overall inherited susceptibility to TB is rare, and accounts for little of the total burden of disease. This fits with the hypothesis that Mtb has exerted selection pressure on human populations for many generations, through a prolonged co-existence. However, rare familial defects in the IFNγ/Stat1/IL-12 pathways which predispose to severe TB and disease due to non-pathogenic mycobacteria have been described, and provide evidence of the protection afforded by these responses (reviewed in [14]). Polymorphisms in genes such as Toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) [15], the chemokine CCL1 [16] and key mediators of innate immune responses such as the mannose receptor, DC-SIGN, and the chemokines MCP-1, CCL5 and CXCL-10 have been reported that are associated with an increased risk of disease (reviewed in [17]).

The primary site of Mtb infection is the lung, and this is also the source of onward transmission to new hosts. The alveolar epithelium and resident patrolling alveolar macrophages are the first cells to encounter the pathogen, which is deposited in the small airways via inhaled droplets and aerosolised secretions from an infectious contact. Bacteria enter macrophages and dendritic cells by phagocytosis and via interactions with complement receptors, Fc receptors and mannose receptors [18-20]. Initially, bacterial replication at the site of infection appears to be relatively unrestrained, with increasing numbers of bacteria despite infected macrophages recruiting other cells by their production of pro-inflammatory chemokines and cytokines such as TNFα, IL-1α and -1β, CCL2 and CXCL10. The thick acid-fast coating of the bacteria contains potent TLR (Toll-like receptor) agonists which also generate a pro-inflammatory response from uninfected cells [21]. Monocytes, neutrophils, dendritic cells (DCs), NK T cells, and subsequently CD4+ and CD8+ effector T cells are recruited to the site of infection.

Priming of antigen-specific T cells responses is relatively delayed in Mtb infection compared to other pathogens, and this represents one of many immune evasion strategies, perhaps allowing bacterial numbers to reach a critical point before the onset of specific immune responses [22]. After a delay of several weeks, bacterial antigens are transported to local and regional lymph nodes by professional antigen-presenting cells such as DCs, where Mtb antigens are presented on Class II MHC and prime CD4+ T cell responses. Although this is a necessary step in activating acquired immune recognition of the pathogen, there is evidence that Mtb-infected DCs and possibly also neutrophils may act as a "Trojan horse", conveying viable mycobacteria to the lymph nodes from where they may disseminate [23]. At this stage haematogenous dissemination may also occur, although this is rare in immune-competent adults.

There is convincing evidence from both human disease and animal models that TNFα and IFNγ responses are necessary for control of infection. The amplification of the initial inflammatory response, which is mainly driven by TNFα, is modulated by the development of Mtb-specific acquired immune responses, specifically the expansion of IFNγ-producing CD4+ Th1 T cell clones. The acquisition of a sufficient population of Mtb-specific T cells is associated with the development of a delayed-type hypersensitivity reaction to Mtb antigens, as utilised in the tuberculin sensitivity test (TST). This is apparent 1-2 months after the initial exposure to infection. IFNγ-responses to mycobacterial antigens in Mtb-exposed patients are tested in the recently developed IFNγ-release diagnostic assays (IGRAs), neatly demonstrating that production of IFNγ may be necessary, but is not sufficient for protection from TB disease. IFNγ activates macrophage effector mechanisms involved in killing intracellular bacteria, for example by enhancing the production of reactive oxygen and nitrogen species and promoting autophagy over necrosis [24]. One result of the activation of acquired immune responses is the generation of cytotoxic CD8+ T cells that are able to directly lyse infected mononuclear phagocytes.

To date, research in TB immunology has concentrated on the protective role of macrophages and IFNγ-producing T cells. However, it is apparent that other cells, including Th17 cells and IL-17-producing γδ T cells also play a role in the response to Mtb, and these are now the subject of intense study [25]. One of the roles of Th17 cells appears to be the recruitment of neutrophils, and over-activation of this pathway is associated with intense inflammation and immunopathology [26]. The importance of neutrophils has also been neglected, although recent research identifying a neutrophil/type I interferon gene expression signature as a marker of active tuberculosis which resolves with successful treatment has refocused attention on their role [27]. Neutrophils can be infected with Mtb, and prominent neutrophil responses to Mtb infection are associated with susceptibility to disease in mice, and with poor prognosis in man [28, 29].

Mtb is able to survive within human macrophages by subverting a number of bactericidal effector mechanisms. A hallmark feature of pathogenic mycobacteria is their ability to arrest the maturation and acidification of phagosomes by fusion with lysosomes [30], and bacteria deficient in this ability are more effectively eliminated by macrophages. Bacteria and bacterial components are also able to escape the phagosome and reside in the more hospitable cytoplasmic environment [31]. Mtb inhibits antigen presentation, autophagy and apoptosis, which all promote successful elimination of viable bacteria, and promotes cell death by necrosis which permits the spread of bacteria to uninfected cells which phagocytose debris from necrotic cells (reviewed in [9]).

One hypothesis is that active TB arises as a consequence of Th1 responses being undermined by over-exuberant Th2 responses which allow bacterial persistence or dissemination. The original concept of distinct Th1/Th2 subsets was proposed in the 1980's, and based on the cytokine secretion profiles of stimulated helper T cells in mice [32]. Naïve T cells differentiated in the presence of IL-12 became IFNγ-producing Th1 cells, while culturing naïve T cells with IL-4 generated Th2 cells producing IL-4, -5 and -13. As discussed above, IFNγ-production is recognized as a critical effector of antimycobacterial immunity,

and so the concept that the “protective” Th1 response can be opposed by excessive Th2 responses has been widely studied.

The example of leprosy is often cited as a paradigm of the distinction between Th1 and Th2 responses to infection. In leprosy a spectrum of immune responses to the pathogen are associated with a range of clinically and histopathologically different disease manifestations. In paucibacillary tuberculoid leprosy, lesions are dominated by Th2 cytokine production, whereas in multibacillary lepromatous leprosy Th1 cytokines are found [33]. These observations led to further investigation of the role of Th1/Th2 immune responses in the human response to Mtb infection. In this project, the role of cytokines including IL-4 and IFNγ in regulating MMP responses to Mtb, and thus promoting or restraining TB immunopathology, is investigated, and therefore the relevant evidence for the importance of each of these cytokines is reviewed subsequently.

1.4 The TB granuloma

In the majority of exposed individuals, despite the development of Mtb-specific acquired immune responses, Mtb is able to establish chronic infection. The tuberculous granuloma is the characteristic pathological feature of TB, and is believed to represent a successful host immune response to infection, containing and controlling bacterial growth and dissemination. However, the mature granuloma also offers a relatively protected environment in which Mtb can persist for decades. The TB granuloma is a highly ordered accumulation of bacteria and bacterial debris, leukocytes, new blood vessels and fibroblasts [34]. Caseating granulomas, as characteristically observed in tuberculosis, contain a central necrotic region of cellular debris which can also be demonstrated to contain mycobacterial antigens and lipids, surrounded by activated and infected macrophages, foamy macrophages and multi-nucleate giant cells [35]. The outer mantle of the granuloma comprises numerous lymphocytes and fibroblasts but relatively few mycobacteria [36]. As the granuloma develops,

neovascularisation occurs around this cellular collection, and a thick, fibrous wall forms, composed of extracellular matrix components such as fibrillar collagen. This fibrotic reaction is mediated by the cytokines TGFβ and TNFα. Marginal lymphoid follicle-like structures have also been described, which contain accumulations of B and T cells, as well as viable mycobacteria [37].

Although the histopathological structure of the granuloma was eloquently described over a century ago, there is continuing uncertainty over the factors that determine the outcome of granulomatous inflammation. Within the same host, some granulomas acquire a fibrotic phenotype with relatively little caseation, while others progress to necrosis and caseation. Important insights from the non-human primate model of TB suggest that within an individual host, pulmonary granulomas progress independently of one another to different immunological and histological phenotypes. Even as active TB develops, some granulomas are able to eliminate infection completely [38]. Some of the factors controlling granuloma formation and progression are known, for example from studying the altered pathology seen in TB in HIV-infected individuals. Tuberculosis is the major infectious cause of death in HIV-positive people, and the risk of reactivation of latent disease has been estimated at up to 10% per year [39-41]. The introduction of TNFα antagonists for the treatment of clinical conditions such as rheumatoid arthritis and inflammatory bowel disease had the presaged effect of unmasking latent TB infections, demonstrated the key role of TNFα in maintaining the structural and immunological integrity of the granuloma in order to contain Mtb infection [42].

The received wisdom is that as granulomas caseate, they can “rupture”, releasing debris and infectious material into adjacent airways, producing cavities and promoting local propagation of infection (termed “bronchogenic spread”) as well as onward transmission. However, the caseating centre is encapsulated by the fibrotic component of the granuloma, and therefore cavitation necessitates the degradation of this capsule, which must require protease activity. Furthermore, the hypothesis that cavitation occurs as a consequence of granuloma

breakdown and rupture is predominantly based on historical findings in the rabbit model, which is different to human disease in a number of important aspects. In contrast, post-mortem histological examination of human disease has suggested that in post-primary TB lesions cavities develop from areas of necrotising lipoid pneumonia, rather than arising from organised granulomas [43]. It is believed that reactivation of latent infection involves a breakdown in the mechanisms maintaining bacterial containment within the granuloma, and the immunological factors determining this are unclear, although age, nutritional status, micronutrient deficiency, HIV infection and immunosuppression are all known to be important. Whatever the immunological deficit underlying reactivation of infection, the final common pathway of granuloma breakdown and bacterial dissemination necessitates the destruction of extracellular matrix components.

The pulmonary extracellular matrix is predominantly composed of type I collagen and elastin, forming stable fibrils which support the respiratory epithelium and facilitate gaseous exchange. This highly organized structure is absolutely required for normal pulmonary function, with collagen providing strength, and elastin the distensibility necessary to accommodate ventilation of the lungs without damage [44]. These proteins are highly resistant to protease degradation, and the matrix metalloproteinases (MMPs) are the only host proteases capable of degrading the pulmonary extracellular matrix at neutral pH. Pulmonary fibrosis and scarring are common consequences of Mtb infection, supporting the hypothesis that Mtb infection drives pathological dysregulation of pulmonary extracellular matrix turnover. MMP activity may also be involved in the disruption to the normal pulmonary architecture that occurs in the generation of TB granulomas and cavities, and in the breakdown of organized granulomas that occurs in TB reactivation.

1.5 Matrix metalloproteinases

The MMPs are a family of structurally related zinc-dependent endopeptidases originally identified through their role in tadpole metamorphosis [45]. The family comprises 25 members in vertebrates and 24 enzymes expressed in mammals [46]. They have numerous physiological roles in embryogenesis, wound healing and cell-cell signaling, and critical roles in infection including leukocyte recruitment, chemokine and cytokine modification and activation of anti-microbial peptides such as defensins (reviewed in [47]).

The minimal structure of the MMPs includes a pro-domain, catalytic domain and the haemopexin-like domain. The enzyme's active site contains a zinc ion ligated to three highly conserved histidine residues. The pro-domain, a NH_2-terminal region approximately 80 amino acids in length, occupies the active site in the pro-enzyme form. The pro-enzyme is activated by the cysteine switch, which exposes the catalytic-site zinc ion [48]. The haemopexin-like domain confers substrate specificity.

MMPs are sub-classified by their substrate specificity into the collagenases, gelatinases, stromelysins and matrilysins, as well as the membrane-type and other MMPs. However, their substrate specificity is relative rather than absolute, and in practice substrate specificity is probably a function of selective expression of different MMPs, TIMPs and substrate molecules in different tissues, as well as compartmentalisation of MMP activity [49, 50]. In addition to degrading matrix components, they also mediate cell-matrix and cell-cell signalling by their activity on cytokines, chemokines and cell surface receptors.

A degree of functional overlap in the physiological roles of the different MMPs is implied by the relatively normal phenotype of single-MMP-knockout mice, with the exception of MMP-14 (MTP-1)$^{(-/-)}$ which has a lethal phenotype. MMP-knockout mice have phenotypes revealed by injury and infection, demonstrating the essential role of these proteins in inflammation and tissue repair [51].

1.5.1 Regulation of MMP expression

The production of these potently destructive enzymes is tightly controlled, predominantly at the level of transcription. In addition to transcriptional regulation, MMP activity is also controlled by secretion as pro-enzymes which require activation. With the exception of neutrophil MMP-8 and -9, MMPs are not stored intracellularly, but rather are synthesized on demand. They are co-secreted with their endogenous antagonists, the Tissue Inhibitors of Metalloproteinases (TIMPs), a family of four structurally related proteins which bind MMPs in a 1:1 stoichiometry and prevent enzymatic activity. Under physiological conditions, TIMPs are normally produced in excess of MMPs, but their secretion is less inducible than that of many MMPs. MMP activity is also non-specifically inhibited by α_2-macroglobulin, and binding of α_2-macroglobulin to MMPs in the plasma results in rapid uptake and degradation by circulating phagocytes [48].

A further level of control of MMP activity arises from the synthesis of the proteins in a pro-form in which the catalytic site is occupied by the pro-domain. This can be removed enzymatically by cleavage by other MMPs or modified by interactions with reactive oxygen and nitrogen species or plasmin. MMP activity is also compartmentalized by binding to the cell surface or extracellular matrix, for example MMP-1 binds to $\alpha_2\beta_1$integrin and MMP-9 binds CD44 [49].

A number of the human MMP genes, including MMP-1, -3, -7, -8, -10 and -13, are encoded on chromosome 11q, and have similar gene sequences which are thought to have arisen by duplication of an ancestral gene [49]. The MMP gene promoters are divided into three groups with similar structures. The group 1 MMPs (including MMP-1, -3 and -9) are the most complex, with a TATA box at -30 base pairs (bp), and an AP-1 binding site at -70 bp. Expression from these promoters is inducible, for example by pro-inflammatory cytokines. The group 2 MMPs (including MMP-8 and -11) also have a TATA box, but no proximal AP-1 binding site, while the group 3 MMPs (including MMP-2) have multiple transcriptional start sites, no TATA box, and are constitutively expressed [52]. Thus the pattern of MMP

expression is both cell- and stimulus-specific, depending on the expression of different receptors and transcription factors.

The AP-1 binding sites of the group 1 promoters render their expression inducible by both cytokines and growth factors, including TNFα and TGF-β. In addition, the MMP-9 promoter contains a consensus NF-κB binding site at -600 bp and therefore expression is inducible via TNFα [53, 54]. There are no consensus NF-κB binding sites in the MMP-1 and -3 promoters, and yet their expression is induced by NF-κB, either via other intermediate transcription factors, or by signalling through non-canonical NF-κB sites [55, 56].

An additional level of control of MMP gene expression has only more recently been appreciated, with the increasing awareness of the role of epigenetic modifications in the regulation of eukaryotic gene expression. This will be discussed further in Chapter 1.12.

1.5.2 MMPs as regulators of inflammatory and immune responses

1.5.2.1 Chemokine processing

MMPs are involved in the processing of chemokines in several ways: by proteolytic inactivation of active forms, for example for CXCL4, -9, -10 and -12, modifications that increase chemotactic power, for example IL-8, and the generation of antagonistic derivatives, such as an antagonist of MCP-3 (CCL7) (reviewed in [57]). MMPs also modulate chemokine activity by releasing chemokines bound to extracellular matrix or cell surfaces; in MMP-7-deficient mice treated with bleomycin to induce pulmonary fibrosis, neutrophils are trapped in the lung interstitium because MMP-7 is required to cleave syndecan-1 from epithelial cells and release the chemokine KC which drives neutrophil migration [58]. Hence, MMP activity can hone immune responses via both positive and negative feedback.

1.5.2.2 Cytokine processing

MMPs have multiple positive and negative effects on signaling by different cytokines. Several MMPs including MMP-9 are implicated in activating TGFβ from its latent complex to the active form [59]. The inactive IL-1β precursor can be activated by MMP-2, -3 and -9,

which in turn also degrade the active form of IL-1β [60, 61]. In vitro MMP-1, -2, -3 and -9 are capable of activating TNFα from its pro-form, although in vivo the related protease ADAM-17 is thought to be the major activator of TNFα [62].

1.5.2.3 Control of cell migration

MMPs facilitate cell migration by degrading extracellular matrix components, by altering cell surface receptors and surface molecules such as integrins, and by generating chemokine gradients to direct cell migration. Extracellular matrix fragments generated through MMP proteolysis can also have chemotactic properties, for example elastin fragments generated by MMP-12 are chemo attractant to neutrophils and monocytes and implicated in the development of emphysema [63, 64].

MMP-3 deficient mice display reduced neutrophil recruitment to the airways in models of acute lung injury [65]. MMP-12 is required for macrophage influx in mouse models of emphysema [66], and MMP-14 is required for monocyte migration [67]. The zebrafish model of TB implicates epithelial cell MMP-9 expression in the recruitment of naïve macrophages to the site of infection [68]. MMP-9 is a marker of disease severity in TB meningitis, and is thought to facilitate leukocyte transmigration by degrading dystroglycan in the blood-brain barrier [69]. Dendritic cell migration is also dependent on MMP-9, and migratory capacity is reduced by exposure to TIMP-1 [70].

1.5.2.4 Activation of antimicrobial peptides

MMP-7 activates α-defensins in the gut, and in MMP-7-deficient mice bacterial clearance from the intestine is impaired [71]. MMP-7 may play a similar role in the airway: its expression in airway epithelial cells has been shown to be up-regulated in cystic fibrosis [72], and bacterial products have been demonstrated to drive MMP-7 expression by cultured airway epithelial cells [73].

1.5.3 MMPs in tuberculosis

In epidemiological studies, MMP-1 gene polymorphisms have been reported to be associated with susceptibility to pulmonary TB. The -1607 bp 1G MMP-1 genotype was reported by a Taiwanese group to be associated with increased risk of endobronchial TB, and the subsequent development of stenotic lesions [74]. The same group found that this genotype was associated with increased risk of post-treatment pulmonary fibrosis in TB patients, and identified increased IL-1β-induced MMP-1 expression as a possible mechanism to explain this phenomenon [75]. The 2G/2G genotype (which is associated with increased MMP-1 expression as a result of the creation of a binding site for the Ets transcription factor in the MMP-1 promoter), in combination with a particular MCP-1 genotype, has been associated with significantly increased risk of developing pulmonary TB in South American populations [76].

Early in vitro evidence of the involvement of MMPs in human TB came with the finding that mycobacterial lipoarabinomannan (LAM) up-regulated MMP-1 and -9 mRNA in the human monocytic cell line THP-1 [77]. MMP-9 was selectively up-regulated by Mtb infection [69] and conditioned media from Mtb-infected monocytes (CoMTb) stimulated MMP-9 secretion by cultured primary monocytes [78]. MMP-1 and -7 expression in primary human macrophages was induced by Mtb in a p38 and prostaglandin-dependent manner [79]. Macrophage up-regulation of MMP-1 was Mtb-specific and did not occur in cells infected with *M. bovis* BCG [80], suggesting that induction of host MMP expression is a feature of virulent mycobacteria. This is supported by the more recent finding that in the zebrafish model of mycobacterial disease using *M. marinum*, a virulent strain was a more potent inducer of MMP gene expression than an attenuated strain [81].

In pulmonary TB, where the extracellular matrix composition predicts that collagenase activity must be implicated in cell recruitment and granuloma formation, epithelial cells adjacent to granulomas express high levels of MMP-1, emphasizing the neglected role of stromal cells in modulating tissue remodeling and immune responses [82]. In a tissue culture

model of pulmonary TB, stimulation of normal human bronchial epithelial cells (NHBEs) with CoMTb drove MMP-1 and MMP-9 secretion; whereas direct infection with H37Rv did not, emphasizing the importance of cellular networks [83]. Previous work from our group has also demonstrated that matrix-degrading MMPs are selectively produced in response to CoMTb, with expression of the opposing TIMPs inhibited by Mtb stimulation [82, 84].

A recent study found that in HIV/TB co-infected patients MMP concentrations in induced sputum were reduced compared to HIV-negative TB patients. MMPs correlated with clinical markers of tissue damage, while cytokine concentrations did not, supporting the aetiological role for MMPs in pulmonary immunopathology [85]. MMP-9 activity in serum has previously been found to correlate with disease severity in TB patients [86].

The mouse orthologue of MMP-1 (McolA) is not an efficient collagenase and is not expressed in lung tissue [87, 88], and this may be important in explaining the differences between the pathology observed in mouse models and human disease. To investigate the role of MMP-1 in tissue destruction, transgenic mice expressing the human MMP-1 gene were infected with Mtb. MMP-1 expression in the transgenic mice increased in response to Mtb infection, and this was associated with alveolar destruction and significantly greater collagen breakdown than in the controls [89]. The guinea pig does express a functional collagenase, and in this model of TB, doxycycline (used at MMP-inhibiting rather than antibacterial doses) reduced pulmonary bacterial load in Mtb-infected animals in a dose-dependent manner compared to untreated animals [85]. Transcriptomic studies in the macaque model of TB, which closely mirrors the histological findings of human disease, showed that MMP-1 expression (and expression of several other MMPs including -9 and -14) was highly up-regulated four weeks following Mtb infection [90]. Thus evidence from diverse animal models of TB, as well as from human patients and in vitro experiments, supports the hypothesis that excessive MMP activity is a critical effector of tissue damage and immunopathology in TB.

There is good evidence that the gelatinase MMP-9 is involved in the pathogenesis of central nervous system (CNS) TB. MMP-9 activity in cerebrospinal fluid (CSF) from patients with TB meningitis was increased and unopposed by TIMPs, and correlated with the severity of neurological disability [69]. Astrocytes contribute to MMP-9 production in CNS TB, and this is in part driven by IL-1β, TNFα and IFNγ in a p38/ERK/JNK-dependent manner ([91, 92]. CoMTb-stimulated astrocyte MMP-9 secretion involved NF-κB activation and was suppressed by dexamethasone, which is widely used to reduce morbidity and mortality in TB meningitis [93].

Other MMPs are also implicated in CNS disease; microglial CoMTb-stimulated MMP-1 and -3 secretion was significantly inhibited by dexamethasone, and again TNFα and IL-1β were necessary but not sufficient to up-regulate MMP-1 and -3 secretion. NF-κB and AP-1 transcription factors were implicated in the control of MMP expression [94]. In addition, MMP-1 and -3 expression has been identified in CNS TB granulomas by immunohistochemistry [94].

In addition to its role in CNS disease, MMP-9 is emerging as a key mediator of granuloma formation. In the zebrafish model of TB, expression of MMP-9 by epithelial cells at the site of infection has been shown to play a key role in recruiting uninfected macrophages to the nascent granuloma, and when MMP-9 expression was disrupted, granuloma formation and bacterial growth were attenuated [68]. In human disease, MMP-9 was also detectable by immunohistochemistry in monocytic cells contained in lymph node TB granulomas [78]. Early studies demonstrated that Mtb infection or mycobacterial LAM induced MMP-9 expression in mouse models of TB [95, 96]. In MMP-9-knockout mice macrophage recruitment and granuloma formation in response to Mtb are disrupted [97]. In Mtb-infected mice, a non-specific MMP inhibitor was shown to decrease leukocyte recruitment and granuloma size in the lungs, but also reduced bacteraemia, underlining the subtle balance between a sufficient inflammatory response and excessive tissue destruction [98, 99]. However, the non-specific

MMP inhibitor (BB-94) used in this study would also inhibit ADAM-17 (TACE) and therefore some of the effect observed may be due to inhibition of TNFα activity.

The different roles played by different MMPs in the pathogenesis of TB are beginning to emerge. Polymorphisms in MMP-1, the major collagenase, are associated with pulmonary disease, in particular its fibrotic complications, and MMP inhibition or overexpression in animal and tissue cultures models alter the immunopathology observed. MMP-7, an elastase, is produced by Mtb-infected mononuclear cells and plays a key role in cell migration and chemokine/cytokine signalling. MMP-9 expression is key to the pathogenesis of CNS disease, and is implicated in the recruitment of uninfected cells in the zebrafish granuloma model. MMP-9 deficient mice also display impaired granuloma formation and cellular recruitment, and MMP-9 is present in human TB granulomas.

These findings raise the possibility that therapies targeted against excessive MMP activation may benefit certain sub-groups of patients but may be harmful to others. The “Goldilocks hypothesis” of infectious disease suggests that disease can result from either inadequate or excessive inflammatory and cytokine responses to an insult. This is recognised in the use of corticosteroids for CNS TB, where the addition of steroids to standard TB treatment improves disease outcome for a subset of patients with excessive inflammatory (and possibly MMP) responses to Mtb infection, but increases mortality for the group of patients with inadequate immune activation in response to infection [100, 101].

The implication of results in CNS TB suggesting that either insufficient or over-exuberant inflammation and cytokine responses to Mtb disease in this context is that phenotyping or genotyping individual patients’ immune responses at the time of diagnosis may be necessary to provide optimal therapy – the concept of personalised medicine. This may extend to the possible translational use of MMP inhibitors as therapies for infectious diseases, as there are potentially harmful as well as beneficial effects of such strategies.

The composition of the pulmonary extracellular matrix, predominantly comprising type I collagen and elastin, implies that collagenase and elastase activity is likely to be important in the pathogenesis of pulmonary TB. As described above, the major human collagenase is MMP-1, which can be activated by the stromelysin MMP-3. These MMPs and the elastase MMP-7 are all produced by MDMs in response to Mtb infection, while epithelial cells stimulated by intercellular networking effects produce MMP-1, -3 and -9. These MMPs will be the primary focus of investigation in this project.

1.5.4 MMPs in other pulmonary diseases

Indirect evidence from other disease processes such as asthma and chronic obstructive pulmonary disease (COPD) also implicate MMPs in the pathogenesis of lung disease. Transgenic mice expressing human MMP-1 develop spontaneous airspace enlargement analogous to emphysema [102], and TIMP-3 deficiency produces a similar phenotype [103]. Mice deficient in MMP-12 are resistant to cigarette smoke-induced airways disease [66], and in cigarette-smoking guinea pigs inflammation and emphysematous change are reduced by MMP inhibition [104, 105]. In human studies, alveolar macrophages from COPD patients spontaneously produce more MMP-1 and -9 in ex vivo culture systems than macrophages from controls, implying a chronic hyper-responsive state which might result in excessive inflammatory remodeling in response to infection or tissue damage [106].

1.6 Models of TB

Homo sapiens is the definitive host of Mtb, but many other species can be infected by the pathogen or related mycobacterial species under laboratory conditions. Other species of mycobacteria cause naturally occurring disease in some animals and it is therefore possible to use laboratory animals to model and investigate various aspects of human TB. Much of our knowledge of the immunological events in TB has been obtained in such model systems, but there are disadvantages to each of the potential animal systems, and these should be considered when reviewing the experimental evidence obtained.

1.6.1 Studying TB pathogenesis in human subjects

Human studies frequently concentrate on surrogate markers of local immune responses, for example assessing whole blood or PBMC cytokine responses to mycobacterial antigens in human patients compared to healthy controls. However, there is evidence that the nature of the innate and acquired immune response within the lung is significantly different to systemic responses, and may not be adequately modelled by studying the responses of circulating leukocytes [107, 108]. TB is essentially a disease of the human respiratory system. Extrapulmonary and disseminated disease occur with variable frequency according to the genetic susceptibility, age and immune status of the host, and the strain of Mtb concerned, but manifestations such as TB meningitis and lymph node TB are a “dead-end” for the pathogen, as transmission of the infection will not occur from these sites. Bacterial virulence factors and immune evasion mechanisms are likely to be targeted towards improving survival and persistence in the pulmonary environment, and therefore it is essential to improve our understanding of the local processes occurring in the lung during human infection.

It is difficult to perform extensive investigations of pulmonary immune responses in human subjects, although broncho-alveolar lavage (BAL) is relatively frequently performed for diagnostic purposes and provides a useful insight into processes occurring at the alveolar

surface. However, parenchymal granulomas are inaccessible to such techniques, and the risks and technical difficulties of lung biopsy mean that it is rarely performed, so human lung tissue is usually only obtained as surgical specimens from patients with long-standing, destructive TB disease or MDR-TB, or at post-mortem examination.

Cohort studies of inadvertently exposed individuals, and tracing contacts of infectious TB patients permit the study of some aspects of TB immunology by examining the correlates of protection from or susceptibility to disease, but these are limited by ethical considerations restricting the use of invasive investigations or interventions. Commonly, TST conversion, or more recently IGRA positivity, are used to assess whether or not exposed individuals have acquired infection, and they can then be followed to monitor the evolution of disease. The low rate of progression to active disease in such healthy populations means that extremely large cohorts of exposed individuals are likely to be needed in order to recruit adequate numbers of TB cases within the timeframe of most research studies.

1.6.2 Animal models of tuberculosis

Animal models have the powerful advantage of allowing manipulation of various features of the host and pathogen, but human disease differs from each of the animal models described. However, much of the evidence to be presented here regarding the role of different cytokines in TB immunity has been obtained in such model systems.

Well-characterised inbred laboratory mouse strains have been extensively used in the study of TB. The relatively low cost and the availability of reagents and knockout strains of mice make this an attractive model for human disease. Mice are susceptible to infection with Mtb but also to non-virulent strains such as *M. bovis* BCG, and the natural history of the infection is different to that observed in most humans. The low-dose inhaled infection model most closely corresponds to what is believed to occur in humans.

When infected with $<10^2$ bacteria by inhalation, mice develop a progressive pulmonary infection with increasing bacterial numbers over 4 weeks, with dissemination of infection to

lymph nodes, liver and spleen after around 2 weeks [109, 110]. Progressive pathology occurs which is eventually lethal [111]. This chronic phase of infection is not representative of latent TB in humans as although bacterial numbers stabilise [112], there is continuing inflammation, detectable pathology and a high number of viable bacteria in multiple organs. A major failing of the mouse model is that the "granulomatous" inflammation observed is quite different to that in man. Accumulations of monocytic and lymphocytic cells are observed at the site of infection, but do not form organised granulomas as observed in human pathology, and fibrosis, caseation and cavitation do not occur. Importantly, there is also some evidence that murine "granulomas" are not hypoxic to the same degree as those occurring in human disease [34, 113]. Therefore other models are required to investigate the critical events that determine granuloma formation, stability and progression in human TB.

Interest in the rabbit model has been revived recently despite the greater expense of this model and the relative dearth of immunological reagents compared to the mouse. Rabbits are relatively resistant to infection with standard laboratory strains of Mtb, but are highly susceptible to infection with *M. bovis*, which produces pulmonary pathology more similar to that of humans than mice (reviewed in [114]). Caseous necrosis and cavitation do occur, and therefore the rabbit model may be useful in defining the key regulators of granuloma stability in man, and in investigating the role of MMPs in TB immunopathology [115, 116].

Mtb causes a progressive, fatal infection in guinea pigs, with few bacteria required to establish infection. Organised granulomas are formed, but fail to contain the infection, and no latent stage of infection occurs. They therefore represent a model of extreme susceptibility to Mtb [117, 118]. A major disadvantage of the guinea pig model is the deficit of immunological reagents available.

Primates provide a good model for human TB in terms of the pathology produced, but practical, ethical and cost considerations limit the use of primates for TB research. Macaques develop either active disease or a latent infection following low dose respiratory challenge, which is immunologically and pathologically analogous to human disease [119].

This represents the best available system for the investigation of latent human TB. Non-human primates also have the important advantage that co-infection experiments with Simian Immunodeficiency Virus (SIV) can be undertaken which allow the effects of HIV infection in humans to be modelled.

M. marinum is a natural pathogen of fish species, and causes granulomatous inflammation with caseous necrosis. Transparent zebrafish (*Danio rerio*) embryos provide a relatively simple, short-term model of the cellular events involved in granuloma formation, in which cellular events can be directly observed in real time [120]. A number of startling insights have already been obtained from such studies, including the observation that superinfecting mycobacteria preferentially home to the sites of pre-established granulomas [121], and the important role of MMP-9 expression by epithelial cells in recruiting uninfected cells to the nascent granuloma [68].

1.6.3 Tissue culture models of TB

As described above, there are limitations to studying TB in both human patients and animal models. These approaches can be complemented by tissue culture models using primary human cells or cell lines, which allow the study of responses of individual cell types to Mtb infection, and the examination of intercellular interactions. These relatively simple systems can be used to define the contribution made by different leukocyte populations and stromal cells to the complex events observed at a whole-organism level. In this project, studying the control of MMP expression in the context of pulmonary TB, primary human monocytes, macrophages and respiratory epithelial cells have been used to model the role of these cells in the immunopathology of pulmonary TB.

1.7 The role of Interleukin-4 in the immune response to Mtb

The hypothesis underlying this research project is that cytokines produced by the acquired immune response to Mtb will modulate the activity of the innate immune response, in particular the production of matrix-degrading MMPs. The role of important anti-inflammatory and Th2 cytokines is investigated as they are potential negative regulators of MMP activity.

The Th2 cytokine IL-4 drives naïve T cell differentiation towards the Th2 phenotype and also has diverse effects on other immune cells such as monocytes and macrophages. It is produced by a variety of cells, classically Th2 cells and mast cells, but also NK cells, some antigen-presenting cells and eosinophils. Human alveolar macrophages have also been shown to be capable of expressing IL-4 [122]. The related cytokine IL-13 is 30% homologous with IL-4 and both can signal through the IL-4Rα subunit and activate Stat 6, but the two have distinct roles and are non-redundant in their actions. IL-4 also has Jak/Stat-independent effects, for example influencing cellular lipid metabolism through Peroxisome Proliferator-activated Receptor (PPAR) γ and δ signalling [123].

IL-4 is involved in B cell switching to IgE production, and induces T cell proliferation [124]. IL-4 down-regulates the production of IL-12 and other cytokines and chemokines by monocytes and macrophages, thereby inhibiting Th1 differentiation of naïve T lymphocytes. IL-4 also inhibits IFNγ production by CD4+ T-cells [125]. Alternatively activated macrophages (those exposed to IL-4 and or IL-13) are intimately involved in the formation of granulomatous reactions in parasitic infection (reviewed in [126]). IL-4 plays a key physiological role in wound healing and fibrosis, and therefore would be expected to affect the expression of matrix components and MMPs in TB.

Epigenetic mechanisms are involved in maintaining the Th2 phenotype in T cells, and alternative activation of macrophages, and therefore the effects of IL-4 on MMP expression in the context of Mtb infection may also involve epigenetic changes [127, 128]. This will be discussed further in Chapter 1.12.

1.7.1 IL-4 in human TB

In early studies comparing TB patients with various control groups, many authors failed to identify significant differences in Th2 or IL-4 responses. For example, Zhang et al identified depressed IFNγ responses to killed Mtb in treatment-naive TB patients, but found no difference in IL-12 production or in IL-4, -13 or -10. Following successful TB treatment, patients showed increased IFNγ responses compared to the initial samples [129]. In a similar study in which PBMCs were stimulated with live H37Ra Mtb IL-4 was also undetectable. In lymph nodes from TB patients IL-4 mRNA was suppressed relative to healthy controls, and the authors concluded there was no evidence of increased Th2 responses in the blood or at the site of disease in TB patients to account for the observed suppression of Th1 responses [130].

However, later studies, as described below, were able to detect differences in Th1 and Th2 responses in TB patients. These results can in part be explained by technical difficulties in assaying IL-4, including its short half-life and low copy numbers of IL-4 mRNA in clinical samples, as well as the fact that IL-4 is active at low concentrations (reviewed in [131]). Hence, more recent work employing more sensitive assays has been able to demonstrate changes in IL-4 responses when previously no differences were detected.

IL-4 and IL-13 mRNA were found to be elevated in unstimulated PBMCs from recently diagnosed TB patients versus healthy tuberculin-positive contacts [132]. In addition, IL-4 production by CD4+ T cells stimulated with lysed H37Rv was increased in TB patients, whereas IL-2 and IFNγ secretion were reduced in patients compared to controls [133].

The identification of a splice variant of IL-4 lacking exon 2, IL-4δ2, which antagonises some of the effects of IL-4, added a further level of complexity to these studies [134]. Examination of cytokine mRNA in PBMCs from TB patients, latently infected community controls and healthcare workers found decreased IFNγ and IL-12 mRNA in active versus latent infection [135]. There was no difference in IL-4 expression, but elevated expression of IL-4δ2 was

significantly associated with latent infection. They concluded that IL-4δ2 expression, and thus suppression of IL-4 activity, is a correlate of a protective immune response to Mtb.

A further study examined whole blood cytokine mRNA and found ratios of IFNγ/IL-4 and IL-4δ2/IL-4 were lower in TB patients at diagnosis, but increased with successful treatment. Conversely in a subset of household contacts who developed TB symptoms during follow-up levels of IL-4 and IL-4δ2 mRNA rose and the IFNγ/IL-4 ratio fell compared to their initial samples [136]. In South African HIV-negative TB patients Roberts et al found increased IL-4 expression in BCG-stimulated whole blood from newly diagnosed patients compared to treated patients and latently infected controls, but no difference in IL-4δ2 expression [137].

Several studies have identified positive correlations between Th2 cytokine responses and markers of disease severity in different patient populations. In one study IL-4 and -13 mRNA correlated positively with radiological scoring of disease extent, and IL-4δ2 mRNA copy number also correlated with IL-4 expression and disease severity [132]. A UK study of Th1/Th2 factors found elevated IL-4 and IL-4δ2 mRNA in both blood and BAL of TB patients compared to controls [138]. Again, radiological scoring of pulmonary disease severity correlated with IL-4/IFNγ ratio in the blood. However, in this cohort IL-4 mRNA remained elevated at the end of treatment, while IL-4δ2 mRNA was increased in the post-treatment samples. A separate study also noted a correlation between IL-4 production by CD4+ and CD8+ cells and radiographic abnormalities, finding a strong correlation with the presence of cavities in an Indonesian cohort [139].

In a longitudinal study of Portuguese healthcare workers occupationally exposed to Mtb, Ordway et al [140] found increased proportions of IL-4-positive CD8+ and γδT cells in those individuals who went on to develop clinical TB. This study is important as it demonstrates that the altered IFNγ/IL-4 balance associated with TB disease is apparent before the onset of clinical or radiological evidence of infection, thus supporting the hypothesis that these changes have an aetiological role in the breakdown of host control of latent infection.

In South African HIV-negative TB patients increased expression of IL-4 mRNA after one week of TB treatment was a marker of rapid response to treatment, while in slow responders (defined by persistent culture positivity at 8 weeks) IL-4 mRNA was reduced at 1 week but IL-4δ2 mRNA was increased [141]. This finding contrasts with the studies suggesting IL-4δ2 is a correlate of protective immunity. A follow-on from this study identified a panel of potential biomarkers in plasma, including IL-10 and IL-13, associated with rapid or delayed response to TB treatment [142].

In a small cohort of Indian TB patients absolute regulatory T cell (Treg) numbers were increased in TB patients, while Tregs positively correlated with bacterial burden and negatively correlated with IFNγ/IL-4 ratio [143]. Treg numbers fell with successful TB treatment, and this was accompanied by a restoration of Mtb-specific IFNγ responses. They concluded that Tregs may actively suppress antimycobacterial immunity in TB patients.

Globally, helminth and other parasitic infections are extremely common and may generate strong systemic Th2 responses. Given the frequency with which intestinal parasitic infestations would therefore be expected to coexist with exposure to Mtb, these are likely to be an important factor in the outcome of exposure, particularly in children and in developing countries. In patients co-infected with filaria and Mtb, decreased mycobacterial-specific Th1 and Th17 responses have been demonstrated, suggesting that helminth infections do impair acquired immunity to Mtb [144]. Indeed, treatment of chronic parasitic infections has recently been proposed as a potential strategy to improve TB control in Africa [145].

In summary, studies of human cytokine responses in TB patients suggest that in patients with active TB, IL-4 responses are elevated compared to IFNγ production, and that this is associated with radiographic abnormalities including the presence of cavitation. IL-4/IFNγ ratios become elevated before the onset of clinical disease, and seem to be at least partially reversed by successful treatment, such that ratios of IL-4, IL-4δ2 and IFNγ may in fact be plausible biomarkers of early treatment success or failure. The association of IL-4 responses

to Mtb with the extent of radiographic abnormalities fits with an impact on fibrosis and matrix turnover which may involve altered MMP activity.

1.7.2 IL-4 in animal models of TB

Mtb infection induces arginase 1 expression, a marker of alternative activation, in murine macrophages, and it is thought that this constitutes an immune evasion mechanism as arginase 1 competes with inducible nitric oxide synthase (iNOS), a key effector of mycobacterial killing, for the substrate molecule L-arginine [146]. Therefore both IL-4 treatment and Mtb infection may impair NO-dependent effector mechanisms in murine macrophages.

In order to investigate the role of IL-4 in controlling TB, the ability of IL-4 knock-out mice to control infection with H37Rv Mtb was assessed. There was no difference in lung, liver or spleen cfus over 80 days [147]. This was interpreted as inconsistent with the hypothesis that an excessive Th2 response limits IL-12/IFNγ-mediated killing of Mtb and results in persistent infection. C57BL/6 IL-4 knockout mice displayed no significant reduction in their ability to clear infection with *M. bovis* BCG [148]. A criticism of these studies is that these mice were not deficient in IL-13 production, which may have partially compensated for the lack of IL-4 in this model. Also, IL-4 is thought to play a lesser role in the C57BL/6 mouse model of TB compared for example to the BALB/c mouse in which there is an expansion of IL-4 responses as infection progresses, and this may explain the lack of effect seen in this study [149].

A further study using BALB/c mice with deletions in Stat 6, IL-4Rα or both IL-4 and IL-13 showed that defects in Th2 immunity were not associated with improved control of respiratory infection with H37Rv [150]. Stationary phase cfus were higher in the Stat 6 $^{-/-}$ mice, but all the mutant mice were able to control Mtb growth in stationary phase. IFNγ responses were enhanced in the IL-4/IL-13 $^{-/-}$ mice compared to wildtype but this did not result in improved control of Mtb infection.

In contrast, when IL-4$^{-/-}$ BALB/c mice were infected with a reduced-virulence isolate of H37Rv, decreased bacterial counts and increased pulmonary inflammation at early timepoints, and increased TNFα, IFNγ, decreased pulmonary fibrosis and increased granuloma size at later timepoints were observed [151]. The authors concluded that IL-4 has an important role in restraining Th1 responses and promoting fibrosis in this model. This correlates with the findings of Buccheri et al who used an IL-4-neutralising antibody in BALB/c mice infected intranasally with H37Rv. Decreased bacterial counts in the lung and spleen were found at 8 weeks post-infection, with increased granulomatous inflammation and inflammatory mediators in the lung when IL-4 was absent. In IL-4$^{(-/-)}$ mice, reconstitution with recombinant IL-4 resulted in bacterial counts similar to wild-type mice [152]. In an attempt to model the effects of IL-4-neutralising immunotherapy in human TB, IL-4 neutralising antibody was administered to BALB/c mice with established H37Rv infections, and this resulted in a significant and sustained decrease in bacterial numbers in both lung and spleen [153]. These studies collectively suggest that IL-4 plays distinct roles at different stages of the progression of disease.

The C57BL/6 mouse model with low-dose Mtb infection (Erdman strain) was also used to investigate the role of IL-4 in chronic pulmonary inflammation [154]. The M1 macrophage phenotype (iNOS^{high}, arginase^{low}) predominated in the early stages of Mtb infection (day 21) with a change to a M2-skewed phenotype (iNOS^{low}, arginase^{high}) at day 35-60. Aerosolised administration of IL-4 recruited macrophages but not lymphocytes into the airways. Macrophage phenotypes within granulomas remained M1-skewed throughout the experimental time-course, while macrophages in the surrounding uninfected tissue acquired the M2 phenotype. These results are particularly interesting as they emphasis the local compartmentalisation of immune responses, demonstrating differences between the microenvironment of the TB granuloma and adjacent uninvolved areas of the same tissue. This also underlines the difficulty of extrapolating from results obtained from peripheral blood or BAL samples to the microenvironment of the granuloma.

The effect of helminth infections on immune responses to Mtb was examined using a mouse nematode infection model with co-infection with Erdman strain Mtb [155]. A transient increase in bacterial burden occurred in the lung in nematode-coinfected mice, with a concomitant increase in the number of IL-4-secreting cells. There was increased expression of markers of alternative activation of macrophages, such as arginase-1, and these changes in the nematode coinfection model required intact IL-4Rα signalling.

Thus, studies in the BALB/c mouse model of tuberculosis broadly support the hypothesis that the development of a Th2/IL-4 response to Mtb exposure impairs microbicidal Th1 effector mechanisms, reduces inflammation and promotes fibrosis. However, differences in the nature of the pathological process observed in these mouse models compared to human disease predicate caution in interpreting these results as showing a detrimental role for IL-4 in human disease.

1.8 The role of Interleukin-13 in the immune response to Mtb

In addition to IL-4, IL-13 is also produced by Th2 cells in response to Mtb infection. IL-13 is a classical Th2 cytokine produced by cells including Th2 CD4+ lymphocytes and mast cells [156, 157]. The IL-13 receptor comprises the IL-13Rα1 component, which binds IL-13, and the IL-4Rα subunit which is recruited once IL-13 is bound. Intracellular signaling through the JAK/STAT6 pathway is common to both IL-4 and -13, and hence there is a degree of overlap between the functions of IL-4 and -13. IL-13 inhibits macrophage activation and the secretion of pro-inflammatory cytokines, upregulates MHC class II expression on B cells and monocytes, and induces class switching in B cells [158]. IL-4 and IL-13 inhibit autophagy in macrophages [159], and this may be another mechanism impairing effective mycobacterial immunity.

IL-13 has been shown to have a key role in hepatic fibrosis in mouse models of Schistosomiasis, and in pulmonary granuloma models using *Schistosoma mansoni* eggs IL-13 and IL-4 are both required for the formation of granulomas (discussed in [160]). IL-13 has also been shown to have effects on stromal cells, such as fibroblasts and epithelial cells, influencing extracellular matrix remodeling [158]. These findings suggest that IL-13 might play an important role in granuloma formation and maintenance in TB, as well as in the excessive fibrotic reaction which can occur in this disease. Therefore, we hypothesized that IL-13 might regulate Mtb-induced MMP expression.

1.8.1 IL-13 in human TB

Studies of IL-13 responses to mycobacterial antigens in vitro have produced conflicting results. In the first published study of this kind PBMCs from TB patients and controls were stimulated with Mtb and no difference in IL-13 mRNA was identified [129]. However, in a subsequent study of unstimulated PBMCs, IL-13 mRNA was found to be 10-fold higher in TB patients than in healthy TST-positive controls [132]. Sutherland et al found that enhanced IL-

13 responses to Mtb antigen stimulation could be used to distinguish TB cases and TST-positive household contacts from TST-negative contacts [161]. A Korean group found a similar phenotype in whole blood stimulated with Mtb antigen; IL-13 production in response to antigen stimulation was increased in TB cases and those with latent TB infection compared to healthy controls [162]. In a small cohort of children with active and latent TB versus healthy controls, Mtb antigen induced IL-13 expression in all the Mtb-infected and none of the uninfected children, with significantly higher IL-13 secretion in the latently infected compared to active disease group [163]. It is interesting to note that in these studies IL-13 responses appear to correlate only with Mtb infection, and not with the presence of active disease, in contrast to the findings for IL-4.

When different clinical strains of Mtb were used to infect PBMCs, greater up-regulation of IL-4 and -13 was observed with a more virulent Beijing-type strain than with CDC1551, a strain associated with a low rate of clinical disease. Interestingly, the same difference was found when monocytes were stimulated with preparations of mycobacterial lipid from these strains, suggesting a difference in lipid immunogenicity between the two strains [10]. These results suggest that induction of Th2-type cytokine responses could represent a virulence mechanism in Mtb.

1.8.2 IL-13 in animal models of TB

In comparison with the extensive studies investigating the role of IL-4 in the mouse model of TB, relatively little is known about the role of IL-13. As previously discussed, IL-4/ -13 double knockout mice were not more resistant to infection with H37Rv, despite demonstrable increases in pulmonary IFNγ and iNOS expression in the knockout animals, while lack of Stat 6 signaling did result in increased bacterial burden [150]. Administration of IL-13 neutralizing antibodies to BALBc mice with established systemic H37Rv infection significantly reduced lung and spleen cfus for a sustained period after the treatment ended, although to a lesser extent than neutralization of IL-4 [153].

When murine peritoneal macrophages were cultured with H37Ra and H37Rv strains of Mtb, both were found to induce TNFα secretion equally, but there was greater induction of IL-10 and -13 expression by the virulent H37Rv strain [164]. These results correspond with the findings of Manca et al in the human studies described above [10].

1.9 The role of Interleukin-10 in the immune response to Mtb

IL-10 is the classical anti-inflammatory or regulatory cytokine, produced by monocytes and macrophages, as well as Th2 lymphocytes, B cells and Tregs [165, 166]. IL-10 inhibits macrophage functions such as the production of IL-1β, IL-8 and TNFα and expression of class II MHC molecules [167-170]. Macrophages are a major source of autocrine IL-10 in TB; phagocytosis of Mtb induces IL-10 expression in monocytic cells [171] and mycobacterial LAM also induces IL-10 secretion by dendritic cells [172]. IL-10 has been shown to block phagosome maturation, allowing bacterial survival within infected macrophages [173]. In addition to its effects on macrophages, IL-10 inhibits T cell proliferation and Th1 differentiation [174]. Mice deficient in IL-10 develop a spontaneous colitis similar to Crohn's disease, and exhibit exaggerated Th1 responses to bacterial infections [175, 176]. Thus, the normal function of IL-10 is to act as a brake on the immune response. IL-10 signals via the IL-10 receptor complex (IL-10R) which in turn signals through the Jak/Stat pathway and Suppressor of Cytokine Signaling (SOCS) 1 and 3 [177, 178].

In light of the role of IL-10 as a negative regulator of macrophage-driven inflammation, we hypothesized that IL-10 might also inhibit Mtb-driven MMP activity.

1.9.1 IL-10 in human TB

Elevated serum IL-10 concentrations have been reported in patients with active TB disease, compared to healthy contacts and controls [179], and IL-10 fell with successful TB treatment [180, 181]. Other studies demonstrated increased IL-10 mRNA and protein expression in BAL and pleural fluid from TB patients compared to patients with other lung diseases and healthy controls [182, 183]. The allele 2 polymorphism of the human gene SLC11A1 increases monocyte IL-10 production in response to LPS, and has been associated with increased susceptibility to TB disease in one study [184].

Ex vivo studies of IL-10 production by antigen-stimulated whole blood or PBMCs from TB patients compared to various control groups, consistently show increased IL-10 responses to mycobacterial antigen in TB patients compared to healthy controls [135, 185, 186]. When whole blood IFNγ and IL-10 responses to mycobacterial antigens were studied in a large cohort of household contacts of index TB patients in Pakistan, elevated IL-10 responses and failure of expansion of IFNγ responses over time characterised the small group of household contacts who went on to develop active TB over the next two years [187]. These findings support the hypothesis that overproduction of IL-10 in response to Mtb may inhibit efficient Th1 response and play an aetiological role in TB pathogenesis.

A recent study investigating the role of regulatory T cells in TB also found that IL-10 production by antigen-stimulated PBMCs declined during successful TB treatment and correlated with the decline in Treg numbers [143]. In this cohort persistence of Tregs correlated with MDR-TB, which may explain the findings of another study, where TNFα secretion by Mtb antigen-stimulated PBMCs from MDR-TB patients was depressed compared to newly diagnosed TB patients. TNFα secretion was enhanced in the presence of an IL-10 neutralising antibody, indicating that Treg-derived IL-10 may mediate suppression of TNFα responses in MDR-TB patients [188]. A subsequent study showed that whole blood antigen-stimulated IL-10 production was elevated in MDR-TB patients compared to those with drug-susceptible disease, while TNFα secretion was suppressed [189]. In a study of HIV-infected TB patients with the immune reconstitution syndrome (HIV-IRIS) a significantly reduced ratio of IFNγ/IL-10 secretion by PBMCs in response to heat killed H37Rv was reported in patients with MDR-TB as opposed to sensitive isolates. This was postulated to be related to differences in bacterial burden and Treg numbers in patients on ineffective therapy [190].

Human studies suggest that TB patients have elevated levels of IL-10 expression in the blood and at the site of disease, and that leukocytes from TB patients display enhanced

ability to produce IL-10 in response to mycobacteria or mycobacterial antigen in vitro. IL-10 responses correlate positively with both disease severity and with the presence of MDR-TB, and seem to decline with successful TB treatment.

1.9.2 IL-10 in animal models of TB

C57BL/6 IL-10 knockout mice displayed no differences in clinical disease or bacterial load compared to wild type mice when infected with H37Rv [147]. Another group found no difference in the ability of C57BL/6 IL-10 knockout mice to eliminate infection with *M. bovis* BCG administered intranasally [148]. More recently IL-10 mRNA was shown to be detectable in the lung from day 10 onwards following low-dose H37Rv infection in C57BL/6 mice. In IL-10 knockout mice IL-2, IFNγ and iNOS mRNAs were more highly expressed, but again no improvement in bacterial control was observed compared to control mice [191]. These results collectively suggest that an IL-10 response is generated by Mtb infection in the C57BL/6 mouse strain which does have an inhibitory effect on Th1 responses, but not at the expense of bacterial control.

A further study using low-dose aerosol infection in C57BL/6 IL-10 knockout mice, also found no difference in bacterial numbers at four months post-infection, but identified significant histological and cytokine differences, with increased numbers of IFNγ- and IL-12-producing T cells and increased alveolitis. The IL-10 knockout mice became moribund 6 months post-infection with overwhelming pulmonary inflammation, elevated bacterial numbers and increased levels of TNFα and TGFβ in the lung [192]. Another group reported a different phenotype in IL-10 knockout mice on several different backgrounds, with decreased pulmonary bacterial burden, increased CD4+ T cell infiltrate, and enhanced IFNγ and IL-17 in the absence of IL-10 [193]. Together these results suggest that the absence of IL-10 permits uncontrolled inflammation which eventually results in compromised respiratory function, while compensatory overexpression of TGFβ by macrophages may inhibit Th1-mediated bacterial killing.

In contrast IL-10 knockout mice demonstrated improved early control of _M. bovis_ BCG which was associated with increased macrophage production of IFNγ, prostaglandins and NO. The authors concluded that IL-10 inhibits early bacterial clearance by inhibiting macrophage effector mechanisms [194]. Similarly, IL-10 knockout mice eliminated _M. bovis_ BCG infection more rapidly and showed increased macrophage recruitment into granulomas with greater expression of TNFα and iNOS in hepatic granulomas compared to wildtype mice [195].

Overall, these studies suggest that in the absence of IL-10, control of virulent H37Rv Mtb is not improved, and indeed lack of IL-10 may in fact lead to unrestrained inflammation which results in death from cachexia and respiratory failure if the infection model is taken out to beyond 4 months. The studies using _M. bovis_ BCG appear to demonstrate improved bacterial clearance in the absence of IL-10, possibly as a result of increased macrophage recruitment and activation.

Over-expression of IL-10 in murine macrophages resulted in higher bacterial load and earlier death from Mtb infection than in the wildtype group, with reduced reactive nitrogen species production and macrophage activation in the transgenic mice [196]. IL-10 over-expression in T cells also increased susceptibility to infection with _M. bovis_ BCG despite apparently normal Th1 responses [194]. C57BL/6 mice over-expressing IL-10 under the control of the IL-2 promoter displayed increased bacterial numbers and decreased TNFα, IL-12 and IFNγ responses in the reactivation phase of the infection, indicating that IL-10 undermined late control of bacterial replication [197]. Hence, over-expression of IL-10 in the mouse model does seem to result in increased susceptibility to Mtb infection.

Different inbred mouse strains display differing susceptibility to infection with Mtb and other mycobacterial species. Roque et al attempted to explain some of the inconsistencies seen in mouse studies of the role of IL-10 in TB by comparing BALBc and C57BL/6 mice. BALBc mice produce significantly more IL-10 in response to Mtb, and are also more susceptible to _M. avium_ infection. An IL-10 monoclonal antibody reduced BALBc susceptibility to _M. avium_,

but did not have an effect in C57BL/6 mice [198]. Therefore as for IL-4, the choice of mouse strain may be an important determinant of the outcome of the IL-10 knock-out/overexpression studies described above.

The CBA/J mouse strain is susceptible to Mtb infection, displaying increased bacterial burden and early mortality compared to C57BL/6 mice, for example. High levels of IL-10 expression in the lungs of Mtb-infected CBA/J mice have been reported. In this mouse model treatment with an IL-10 receptor antibody in established chronic phase infection resulted in stabilization of bacterial numbers, so it is possible that the propensity to develop an excessive IL-10 response to Mtb might underlie the susceptibility of this strain to disease [199].

1.10 The role of transforming growth factor-β in the immune response to Mtb

Transforming growth factor-β (TGFβ) is a family of related proteins first described for their ability to transform cultured cells [200]. However, this group of cytokines is now known to have diverse actions on a wide range of cell types, including fibroblasts, epithelial cells and leukocytes. It plays a key role in tissue healing and wound fibrosis, and is known to have regulatory effects on MMP expression by multiple cell types, and therefore we wished to investigate its role in regulating Mtb-driven MMP expression.

TGFβ_1 is the major TGF isoform produced by monocytes, and it is both chemo-attractant to monocytes and macrophages, and induces its own expression by these cells. Dendritic cells also produce TGFβ upon phagocytosis of apoptotic cells [201]. Its expression is known to be regulated by the AP-1 (activator protein 1) family of transcription factors [202]. TGFβ is synthesized as a latent complex comprising a TGFβ homodimer and the Latency Associated Peptide (LAP), which then associates with Latent TGFβ Binding Protein (LTBP) for secretion from the cell. It therefore requires activation before exerting its biological effects in vivo. Several mechanisms for activation of TGFβ exist, which include cleavage by MMP-2 and -9 as well as modification of the LAP by reactive oxygen species or low pH [59].

TGFβ signals through TGFβ receptors I and II (TGFβRI and II) on the cell surface. The activated receptors interact with and phosphorylate cytoplasmic receptor-associated Smad proteins (reviewed in [203]). These then associate with Smad4 to form heterodimers which can be translocated to the nucleus and function as transcription factors either alone or in association with other transcription factors such as AP-1 [204].

TGFβ has both pro- and anti-inflammatory effects, modulating macrophage activation and phagocytosis and suppressing expression of TNFα and IL-1β, but also down-regulating its own receptor, inhibiting production of reactive oxygen and nitrogen species, and inhibiting

CD4+ T cell proliferation, IFNγ production and NK cell activity. The functions of TGFβ with respect to macrophage activity were reviewed by Wahl et al [205]. More recently, TGFβ in combination with IL-6 has been found to be key to the differentiation of Th17 cells [206], which are now believed to play an important role in anti-mycobacterial immunity [207].

TGFβ has extensive effects on extracellular matrix turnover and is critical to wound healing; it is chemoattractant to fibroblasts and induces the production of collagen and other matrix proteins [208, 209]. TGFβ has also been shown to affect MMP expression in a variety of cell types, including monocytes [210]. TGFβ is implicated in the pathogenesis of diseases involving dysregulation of extracellular matrix turnover, such as idiopathic pulmonary fibrosis and sarcoidosis (reviewed in [211]). These findings suggest that exposure to TGFβ may modify epithelial cell and macrophage MMP expression in response to Mtb.

1.10.1 TGFβ in human TB

TGFβ expression was induced in human monocytes in vitro by stimulation with preparations of mycobacterial LAM [212]. Monocytes infected with H37Ra in vitro also expressed TGFβ, and neutralizing TGFβ in the culture medium improved the monocytes' ability to control intracellular replication of Mtb. Conversely, the addition of exogenous TGFβ to the culture system decreased phagocytosis of Mtb and enhanced the capacity of the bacteria to replicate intracellularly [213].

Monocytes from TB patients displayed increased spontaneous production of TGFβ, and TGFβ protein was detectable in giant cells and epithelioid cells in TB granulomas [214]. Cells isolated from BAL fluid from TB patients expressed increased TGFβR mRNA and bioactive TGFβ protein compared to patients with other lung pathology or healthy controls [215]. In another study, TGFβ concentrations were elevated in induced sputum from TB patients and fell after 30 days of TB treatment [183].

Ex vivo studies of PPD-stimulated PBMCs from Pakistani TB patients compared to healthy household contacts found increased TGFβ and decreased IFNγ synthesis in patients. In

vitro, anti-TGFβ antibodies restored lymphocyte proliferation and IFNγ production in response to PPD in TB patients but not in contacts [216]. When PBMCs from TB patients were cultured with Mtb, TGFβ production was found to correlate with a clinical grading of disease severity [217]. Low numbers of CD8+ T cells in lymph nodes from children with TB lymphadenitis correlated with high levels of TGFβ and regulatory T cells, consistent with local immunosuppression at the site of infection [218].

Thus human studies suggest that Mtb infection drives expression of TGFβ by monocytes and macrophages both locally and systemically, and that this may impair macrophage-mediated mycobacterial killing as well as suppressing Th1-mediated immune responses and promoting regulatory T cell responses. There is some evidence to suggest that TGFβ responses correlate with disease extent, and that they regress with successful treatment.

1.10.2 TGFβ in animal models of TB

In mice infected with H37Rv, elevated levels of TGFβ were detected by immunohistochemistry as immunopathology advanced, with the appearance of foamy macrophages and fibrosis [219]. In keeping with the in vitro studies using human cells, in a guinea pig model of TB pleurisy involving intrapleural injection of killed Mtb, neutralization of TGFβ resulted in decreased neutrophil infiltrate, increased TNFα and enhanced lymphocyte proliferation to PPD stimulation [220]. Mtb-infected mice treated with asymmetrical liposomes which mimic apoptotic bodies and induce TGFβ expression showed improved control of bacterial growth and reduced TNFα and IFNγ expression [221].

1.11 Interferon-γ and the immune response to Mtb

In contrast to IL-4, IL-10 and IL-13, which are proposed to inhibit effective immunity to Mtb infection, IFNγ is a critical effector of antimycobacterial immunity. Early studies with IFNγ-deficient mice demonstrated its key role in controlling infection, and humans with rare genetic defects in the IFNγ/IL-12 pathway are susceptible to severe Mycobacterial infections [222, 223]. Acquired defects in IFNγ function such as the presence of neutralizing auto-antibodies to the cytokine also result in increased susceptibility to TB [224].

IFNγ is produced by NK cells and T cells: early in Mtb infection NK cells and NK T cells are the major source of the cytokine, but as the acquired immune response develops it is produced by CD8+ T cells and Th1 CD4+ cells. IFNγ secretion defines the Th1 T cell subset, and expands Th1 responses by positive feedback. Its effects are primarily mediated via the Interferon-γ Receptor heterodimeric complex (IFNGR) and the JAK/STAT signaling pathway (reviewed in [225]). IFNγ can also be produced by other cell types; for example examination of surgically excised granulomas showed expression of IFNγ mRNA in CD68+ macrophage-like cells [226]. Indeed, in vitro evidence suggests that macrophages auto-activate Mtb-killing mechanisms through the production of small amounts of IFNγ in response to infection [227].

IFNγ produced either by T cells or by infected macrophages themselves activates mycobacterial killing mechanisms in macrophages, for example inducing the expression of iNOS and enabling the production of reactive nitrogen species, and increasing antigen presentation on MHC classes I and II (reviewed in [225]). IFNγ promotes Th1 differentiation of naïve T cells by increasing the expression of the transcription factor T bet. It also inhibits CD4+ T cell production of IL-17, reducing neutrophil recruitment and independently reducing neutrophil survival, thus displaying immune regulatory functions [26].

The importance of IFNγ in controlling Mtb infection is emphasized by the description of several mycobacterial immune evasion mechanisms which target IFNγ-mediated killing. Mtb

interferes with some of the activating effects of IFNγ on macrophages, possibly via effects on STAT1/p300/CREB signaling [228]. Mycobacterial cell wall components have also been shown to impair macrophage responsiveness to the effects of IFNγ [229].

1.11.1 IFNγ in human TB

In pulmonary TB patients BAL fluid IFNγ concentrations correlated with disease severity and treatment response [230]. In peripheral blood, the ratio of IFNγ to IL-10 production in response to mycobacterial antigen correlated with disease severity in TB patients [231]. Another study found that IFNγ responses increased with successful TB treatment [143].

An early trial of aerosolized IFNγ in addition to usual treatment for cavitatory MDR-TB reported improved bacterial burden and smear conversion, as well as decreased cavity size three months after treatment [232]. A clinical trial of nebulized or subcutaneous IFNγ treatment in addition to standard directly-observed therapy found that only nebulized IFNγ improved the rate of Mtb clearance from sputum. The lack of benefit observed with systemic treatment highlights the importance of considering the local immune response to Mtb as well as responses observed systemically in peripheral blood [233]. A recent systematic review of adjuvant IFNγ treatment for TB found that the existing evidence suggested a positive effect on treatment outcomes without significant adverse effects, but concluded that larger scale trials were needed [234].

1.11.2 IFNγ in animal models of TB

Mice with disruption of the IFNγ gene displayed extreme susceptibility to infection with Mtb, developing rapidly progressive tissue inflammation and necrosis with high bacterial burdens and early death compared to wild type strains [235, 236]. IL-12 knockout mice which are unable to develop IFNγ responses to mycobacterial infections showed decreased lymphocyte recruitment to the site of infection and were unable to control Mtb infection [237].

Elegant experiments using chimeric C57BL/6 mice with IFNγ-unresponsive lung epithelial cells emphasize the important effects of IFNγ on stromal cells. These mice displayed increased neutrophilic inflammation, IL-17 and bacterial numbers, and early mortality compared to wild type mice following infection with a low dose of H37Rv [238]. Therefore although IFNγ is predominantly produced by cells of the acquired immune system, and its dominant effects in immunity to mycobacterial infection appear to be via actions on macrophages and T cells, additional actions on epithelial cells and other stromal cells may also be important.

1.12 Epigenetic regulation of gene expression

The other major aspect of the regulation of MMP activity in the context of TB to be investigated in this project is the contribution of epigenetic mechanisms to the control of MMP expression. The term epigenetics was originally coined by Conrad Waddington in 1942 as “the branch of biology which studies the causal interactions between genes and their products, which brings the phenotype into being” [239]. A more recent definition is “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states” [240]. The term “epigenetic regulation” therefore encompasses all chromosomal modifications that alter gene expression without altering the nucleotide sequence of the DNA. Within the eukaryotic nucleus, DNA is compressed and packaged into chromatin in nucleosomes, which are composed of 146 base pairs of DNA complexed with octamers of histones. Each histone octamer comprises two subunits each of histone H2A, H2B, H3 and H4. The histone proteins contain globular domains and negatively charged tails which are subject to extensive post-translational modification, for example by acetylation, methylation, phosphorylation, ubiquitinylation and sumoylation. These dynamic modifications are currently the subject of intensive study, with acetylation of highly conserved lysine residues in histone tails being the best characterized. Acetylation of histones H3 and H4 is associated with active transcription from the corresponding genes, an idea which was first proposed in 1964 [241], but in recent years there has been an increasing appreciation of the role of these epigenetic modifications in regulating eukaryotic gene expression [242].

An extensive literature is developing on the role such epigenetic mechanisms play in the modulation of inflammation and immunity, but there are few publications dealing specifically with TB. This is an important area of research because Mtb is a highly successful pathogen with well-developed immune evasion strategies, which include a host of mechanisms that undermine the evolution of effective sterilizing immunity. It is possible that studying the interaction between Mtb and the human immune system will identify novel regulatory

mechanisms controlling the immune response, and could also identify potential therapeutic targets for TB treatment.

The subject of epigenetic regulation of MMP expression has been investigated in the context of cancer biology, where MMPs (in particular MMP-9) are important determinants of metastatic potential. Similar epigenetic mechanisms may operate in infection, and may be amenable to manipulation using agents originally developed for cancer chemotherapy. In this project, regulation of MMP expression in epithelial cells and macrophages stimulated by Mtb has been investigated, concentrating on the role of changes in histone acetylation status in modifying MMP gene expression. The key proteins involved in this process are the histone acetyltransferases and deacetylases.

1.12.1 The histone acetyltransferases and deacetylases

Histone acetylation is carried out by the histone acetyltransferases (HATs), evolutionarily conserved proteins which are typically composed of multiple subunits. These proteins were originally identified and classified in yeasts, into nuclear HATs (type A) which are involved in transcription-related acetylation of histone within chromatin, and cytoplasmic HATs (type B) which are responsible for acetylation of free histones, predominantly H4, which is diacetylated prior to the nuclear assembly of new chromatin [243]. The eukaryotic HATs are classified according to the structure of their catalytic domains into the GNAT (Gcn5-related acetyltransferases) family, the MYST (Monocytic leukaemia zinc finger protein (MOZ), Ybf2/Sas3, Sas2 and HIV Tat protein) family and an orphan class of proteins which have functional HAT enzyme activity but no consensus HAT catalytic sequence, such as p300/CBP [244]. PCAF (p300/CBP-associated factor) is a member of the GNAT family, as is HAT1, which is highly conserved between species. A number of other proteins, including transcription factors such as Activating Transcription Factor 2 (ATF2), have also been reported to have HAT activity. Although the individual HATs do not have unique substrates, the formation of protein complexes between HATs and different transcription factors and

enhancers can confer specificity through the presence of chromatin-binding domains in the non-catalytic proteins.

Histone acetylation is associated with increased transcriptional activity, while deacetylation is often associated with transcriptional silencing. Acetylation of lysine residues in the histone protein alters its electrical charge and results in altered conformation of the chromatin from the “closed” or resting state, to the “open” and transcriptionally active state. In this conformation TATA-box binding protein (TBP), transcription factors and RNA Polymerase II can be recruited. However, acetylation coexists with other histone modifications, which can modify its effects, for example sumoylation inhibits acetylation of H3, while methylation of CpG islands marks chromatin for HDAC activity [245]. Equally, the availability of a lysine residue for these other covalent modifications is also controlled by HDACs and HATs as they are mutually exclusive. For example, in yeast deacetylation of lysine 9 of histone H3 (H3K9) marks it for methylation and is associated with the formation of heterochromatin.

Histone deacetylases (HDACs) remove acetyl groups from target lysine residues on histones, and therefore the balance of activity between the two families of enzymes determines the acetylation status of chromatin. Genes encoding 18 different HDACs have been identified in the human genome, and the corresponding proteins are divided into four classes. The class I HDACs (1, 2, 3 and 8) contain a nuclear localization sequence, and are ubiquitously expressed, whereas class II HDACs are selectively expressed in certain tissues and shuttle between the nucleus and the cytoplasm depending on their activation state (reviewed in [246]). Class IIa comprises HDAC4, 5, 7 and 9, which can act as transcriptional activators, while HDAC6 and 10 form Class IIb. The class III HDACs are the sirtuins, of which Sirt1 is the only member with strong HDAC activity, and these require NAD+ as a co-factor. HDAC11 is the only member of Class IV.

Changes in HDAC expression occur with cell differentiation, for example HDAC5 is upregulated in macrophages compared to monocytes. It appears that, in yeast at least,

different HDACs preferentially act on distinct target genes and lysine residues, although there is overlap in their functions [247].

1.12.2 HDAC inhibitors

Experiments using HDAC inhibitors suggest that the normal function of HDACs is to repress the expression of inflammatory genes. For example, in the A549 airway epithelial cell line trichostatin A (TSA) enhanced expression of pro-inflammatory cytokines such as IL-8 in response to IL-1β stimulation [248]. HDACs have also been shown to regulate macrophage functions such as TLR- and interferon-signaling and cytokine expression [249].

Several classes of naturally occurring and synthetic inhibitors of HDACs and HATs have been identified and characterized, and these have varying degrees of specificity. For example, the classical HDAC inhibitor (HDACi) trichostatin A (TSA) is relatively non-selective, while sodium butyrate and the benzamide derivative MS-275 are selective for class I HDACs at low concentrations. Butyrate is not a specific inhibitor as it has other biochemical activities. TSA has an EC_{50} of less than 10 nM for HDACs 1-7, while MS-275 has an EC_{50} of 181 nM for HDAC1 but its EC_{50} for HDAC2 and 3 is greater than 1 μM [250, 251]. The bipolar hybrid compound m-Carboxycinnamic Acid bis-Hydroxamide (CBHA), which is structurally similar to suberoylanilide hydroxamic acid (SAHA), is another potent HDAC inhibitor which has been reported to inhibit HDAC1 and 3 activity in vitro [252]. CBHA at concentrations between 0.1 and 2.0 μM has been shown to induce histone H3 hyperacetylation in pleural mesothelioma cells [253]. Gene expression profiling indicates that HAT/HDAC activity regulates only 2-10% of all genes, and of those whose expression is affected by histone acetylation approximately half are upregulated and half are repressed by treatment of cells with HDACi [254]. HDACi can also induce cell cycle arrest, apoptosis or differentiation.

1.12.3 BET inhibitors

Since this project began, a novel class of specific small molecule inhibitors has been developed, the BET (bromodomain and extra terminal domain) inhibitors, which mimic the

structure of acetylated lysine residues on histone proteins and thus competitively interfere with the recognition of histone acetylation by the BET proteins, which are involved in the assembly of acetylation-dependent chromatin complexes permitting gene transcription. Their effects are therefore similar to that of highly specific HDAC inhibition, and they can be used to examine the role of changes in histone acetylation in controlling gene expression. Bromodomain BET proteins such as BRD4 are known to preferentially bind to the acetylated forms of histone H3 and H4 [255].

Diverse clinical applications for these drugs are being explored: the BET inhibitor JQ1 blocks spermatogenesis via its effect on BRDT and therefore is being studied as a reversible male contraceptive [256]. It has also been proposed as a possible novel class of anti-HIV therapies [257], and anti-leukaemic effects have been reported in vitro and in vivo [258, 259]. Agents such as I-BET have been demonstrated to inhibit LPS-driven inflammatory gene expression in macrophages [260], and JQ1 or BRD siRNA inhibit macrophage inflammatory responses such as expression of IL-6 and TNFα, and can rescue mice from LPS-induced shock [261]. It would have been useful to test the effects of these highly selective compounds, which have been studied in some human lung adenocarcinoma cell lines [262], in this tissue culture model of TB.

1.12.4 HAT inhibitors

In comparison with the several classes of HDAC inhibitors available, relatively few specific HAT inhibiting compounds have been identified. Anacardic acid, a small organic molecule which occurs naturally as a component of cashew nut shell oil, inhibits p300/CBP and p300/CBP-associated factor (PCAF) HAT activity. It appears to be relatively selective for PCAF activity, inhibiting this by 50% at 5 μM, but inhibits the activity of both p300 and PCAF by more than 90% at 15 μM [263]. Anacardic acid has a number of other important activities, including antimicrobial activity against Gram positive bacteria, and inhibition of prostaglandin synthesis and lipoxygenase. A recent publication reports that anacardic acid also directly

inhibits MMP-2 and -9 gelatinolytic activity by binding to the active site of the enzyme. Effects on other MMPs, for example MMP-1 and -3 were not described [264].

HAT inhibitor II, (hereafter referred to as HATi II) whose chemical name is 2,6-*bis*-(3-Bromo-4-hydroxybenzylidene)cyclohexanone ($C_{20}H_{16}Br_2O_3$) is a *bis*-arylidene cyclohexanone compound that acts as a p300/CBP-selective HATi with IC_{50} 5 μM, while affecting PCAF only at higher concentrations. This inhibitor was selected in order to compare the relative effects of inhibiting PCAF and p300/CBP.

In addition to the actions on histones for which they are named, numerous other non-histone targets of the HATs and HDACs have been identified. Indeed, phylogenetic analysis implies that the HDACs pre-date histones in evolutionary terms [265], and therefore their histone-modifying role must have developed subsequent to another conserved intracellular function. HDAC 1 and 3 enhance the stability of the transcription factor Hypoxia-Inducible Factor-1α (HIF-1α) and thus potentiate its activity [266]. Complex actions on the components of the NF-κB family of transcription factors (reviewed in [267]) and the Jak/STAT cytokine signaling pathway have also been described. HDAC3 can deacetylate the RelA subunit of NF-κB, which promotes its binding by IκB-α and thus its nuclear export [268]. In mouse fibroblasts TIMP-1 expression was inhibited by TSA, but this treatment also blocked ERK, JNK and Akt phosphorylation in response to TGFβ stimulation [269]. The activity of the MAP kinase pathway is also influenced by HAT-mediated acetylation of mitogen-activated protein kinase phosphatase-1 (MKP-1), a negative regulator of MAPK signaling [270]. The diversity of these effects, which are not yet completely understood, contribute to the difficulty in predicting the effects of HDAC or HAT inhibition on expression of individual genes in particular cell types in response to different stimuli. Low doses of HDAC inhibitors have anti-inflammatory properties, for example the HDACi vorinostat inhibited IL-1β secretion by PBMCs [271]. Therefore HDACi may have useful immunomodulatory effects in a variety of conditions.

1.12.5 Epigenetic regulation in inflammation and infection

Epigenetic changes are key determinants of cell differentiation, and therefore underpin the diversity of phenotypes acquired by immune cells in the course of development and during responses to pathogens. It is clear that processes such as determination of T cell fates and differentiation to Th1 versus Th2 cell phenotype involves epigenetic mechanisms [272]. Th2 cell fate is governed by epigenetic regulation of IL-4 gene expression [128], and in Th17 cells changes in histone H3 acetylation and methylation affecting gene expression from the IL-17 locus have been described [273].

In the context of dynamic responses to infection or injury, antigen-presenting capacity is regulated by epigenetic modifications influencing MHC Class II expression [274], while changes in histone methylation at the IL-12 promoter have been shown to mediate DC dysfunction after severe sepsis (reviewed in [275]). As epigenetic changes can persist through mitosis and therefore persist through generations of somatic cells, the observation that immune responses alter with ageing may in part be explicable through epigenetic change. It is known that overall levels of genomic methylation decline with age, and it has been postulated that such changes contribute to observations such as T cell senescence [276]. Changes in macrophage function with age, for example reduced MHC Class II expression, reduced phagocytosis and reduced expression of cytokines such as TNFα have also been reported, and epigenetic mechanisms are implicated in these changes [277-279]. Macrophages and TNFα are both known to play a fundamental role in defence against mycobacterial disease, and increasing host age increases the risk of reactivation of latent TB, so such changes offer one explanation for this increased susceptibility.

Studies of histone acetylation in inflammatory diseases such as asthma and rheumatoid arthritis in humans provide evidence to support the hypothesis that pathological inflammation may involve alterations in the balance of histone acetylation and deacetylation. Increased total HAT activity in nuclear extracts from alveolar macrophages from asthmatic patients has been reported, and this was accompanied by a selective reduction in levels of HDAC1

expression [280]. These changes were restricted to the pulmonary environment, with no alteration in HAT activity in peripheral blood mononuclear cells from the same individuals. Similarly, in chronic obstructive pulmonary disease (COPD), reduced HDAC expression and activity have been demonstrated [281].

Macrophages repeatedly stimulated with LPS become tolerized to the pro-inflammatory effects of LPS, but continue to upregulate expression of other genes with antimicrobial effects. The two subsets of genes display distinct patterns of histone acetylation and methylation, implying that tolerance is mediated by epigenetic changes allowing differential gene expression [282]. This is a powerful finding, as if the previous experience of a cell shapes its future responses to stimulation, this constitutes a form of immunological memory occurring in cells of the "innate" immune response. Thus sustained and heritable epigenetic alterations in cells of the innate immune system may represent one aspect of the recently described concept of "trained immunity" [283]. Such developments may be critical in understanding the interaction between different conditions such as diabetes or helminth infection with TB, where one pre-existing condition affects the host response to infection with a pathogen. The emerging field of epigenetic changes induced by bacterial infections has recently been reviewed [284].

In keeping with these findings, epigenetic mechanisms have been described that maintain the alternatively-activated macrophage phenotype in response to IL-4 treatment [127]. In mouse dendritic cells stimulated with a TLR ligand, IL-12 p40 expression was TSA-sensitive and accompanied by increased histone acetylation of the IL-2 p40 promoter region, but IL-1β and TNFα expression were not HDACi-sensitive [285]. HDAC inhibition suppressed chemokine, cytokine and pattern recognition receptor expression in TLR-agonist-treated murine macrophages. HDACi conferred protection against toxic shock in animal models, but increased susceptibility to bacterial infection [286].

Specific examples have been described of pathogens which subvert host immune responses by epigenetically influencing the expression of host genes. This is a prominent feature of

viruses which are able to maintain latent infection long-term, such as Epstein-Barr virus (EBV), but also occurs in chronic infection with some bacterial pathogens. *Helicobacter pylori* (H. pylori)-associated gastritis confers an increased risk of gastric cancer, and H. pylori infection has been correlated with CpG island methylation in biopsy samples from human subjects. This in turn correlated with risk of cancer progression and is postulated to occur via induction of IL-1β and other inflammatory mediators in the context of chronic infection (reviewed in [287]).

TLR signaling also produces changes in HDAC expression and activity. Changes in HDAC gene expression occur in LPS-treated bone-marrow-derived macrophages (BMDM) with early increases in CBP/p300/p/CAF transcripts and later suppression of class II HDAC mRNAs, while treatment with TSA differentially affected expression of different chemokines, increasing CXCL2 and reducing CCL2 and 7 [288].

Thus epigenetic mechanisms provide an additional level of complexity to the regulation of immune responses, allowing pathogen-exposed cells to orchestrate coordinated changes in gene expression.

1.12.6 Epigenetic regulation in TB

In addition to the evidence described above from other models of inflammation and infection, further specific mechanisms may operate in TB. Mtb utilizes multiple strategies to subvert the host immune response, and some of these strategies have been shown to involve alterations in the acetylation of target host genes in infected cells. For example Mtb inhibits the responsiveness of infected macrophages to IFNγ, preventing the induction of class II MHC expression by a mechanism which is sensitive to HDAC inhibition. In THP-1 cells histone acetylation at the HLA-DR promoter is increased by IFNγ, but this was impaired in Mtb-infected cells [289]. This effect was gene-specific, with other interferon-responsive genes being unaffected, and exemplifies the selective mechanisms employed by the pathogen to evade effective macrophage activation and killing.

It has also been suggested that susceptibility to TB disease, which is incompletely explained by the genetic and environmental factors described to date, may be influenced by epigenomic variation between hosts [290].

In a macaque model of early granuloma formation, the early inflammatory response involved suppression of HDAC7 and 10 and SIRT4 in conjunction with strong upregulation of IFNγ and MMP-1 and -7, which was reversed at a later time point when MMPs, pro-inflammatory cytokines and chemokines were downregulated and HDAC expression was increased [90]. Changes in the equilibrium between the key determinants of histone acetylation appear to occur in the context of Mtb infection.

1.13 The future for TB research and treatment

The stated aim of the WHO TB control programme is the eradication of TB by the year 2050, a target which at present appears hopelessly optimistic [7]. Current research priorities are the development of more effective vaccines, more rapid near-patient diagnostics and the identification of novel TB drugs, both to shorten standard TB treatment and to improve the outcome of infection with drug-resistant Mtb. An improved understanding of the factors determining the outcome of Mtb infection, the key correlates of effective immunity, and the factors driving immunopathology in both HIV-positive and –negative patients will assist in designing suitable vaccines, and in developing adjuvant immunotherapies to be used alongside antibiotics in selected cases.

The increasing understanding of the immune correlates of active disease, latency and successfully treated TB over the last 20 years have led to discussion of the possibility of immunotherapy as an adjunct to antibiotics in TB treatment. This has of course already been trialled in treating some mycobacterial infections in individuals with inherited susceptibility due to IL-12/IFNγ pathway defects, where cytokine replacement therapy appears to have improved outcome for selected patients [222, 223].

The subject of TB immunotherapy has recently been reviewed [291]. The cost of recombinant cytokine treatment, or the use of neutralising antibodies, is likely to be high relative to the drug costs of standard TB treatment, and therefore immunotherapy would probably be reserved for cases of XDR and MDR-TB where the cost would be offset by increased likelihood of successful treatment, the high cost of treatment with second and third-line TB drugs, including intravenous therapy, and the expense associated with inpatient treatment and monitoring.

Broadly, immunotherapy might aim to enhance favourable immune responses, for example by stimulating microbicidal effector mechanisms with IFNγ or IL-2, by therapeutic vaccination with selected mycobacterial antigens, or by countering immune exhaustion. Alternatively,

immunosuppression with agents such as anti-TNFα, corticosteroids or thalidomide might reduce pathology associated with chronic non-sterilising inflammation. IL-4 has been mooted as a potential target of immunotherapy either with neutralising antibodies or therapeutic vaccines intended to shift the balance of responses away from IL-4 production (reviewed in [131]).

A recent review of the role of IL-4/IL-13 in the pathogenesis of asthma discusses numerous compounds, such as soluble IL-4R, IL-4-neutralising antibodies and Stat 6 signalling inhibitors which are in development or in early-stage clinical trials for the treatment of refractory asthma [292]. Depending on the outcome of such studies, candidate drugs that are cost-effective and safe may subsequently be identified for trials in TB.

Trials of exogenous recombinant IFNγ as an adjunct to antibiotics have already been undertaken, and show some promise in the limited circumstances studied, reviewed in [234]. However, as animal models and the example of anti-TNFα treatment in human subjects latently infected with Mtb attest, interfering with cytokine signalling can produce dramatic and unexpected effects.

Much of the morbidity and mortality from TB can be attributed to the host response to the infection, rather than to the bacterium itself. MMPs are implicated in most TB immunopathology, and represent attractive targets for therapy, not least because considerable research in the oncology field has already been performed. A broad-spectrum MMP inhibitor (for example doxycycline, which also has bactericidal activity against Mtb) may have additional benefits in the treatment of TB patients if it improves the rate of bacterial clearance whilst also reducing the toxic effects of excessive MMP production [85]. An alternative strategy would be to elucidate the precise roles of the different MMPs in the immunopathology of TB in order to target them more specifically, as reviewed in [293]. This might be achieved using narrow spectrum chemical inhibitors or antibodies, or by targeting the upstream signalling pathways involved in mediating their expression.

1.14 Hypothesis and Aims

Rationale

This project investigates the effect of exogenous Th2 cytokines on MMP expression by macrophages and epithelial cells, with the intention of identifying candidate molecules which might act as brakes on Th1-driven inflammatory responses. The role of Th2 cytokines and the Th1 cytokine IFNγ are therefore compared. Epigenetic regulation of MMP expression is also investigated, with the aim of identifying key control mechanisms which may be amenable to manipulation in order to modulate MMP-driven immunopathology.

The potential translational benefits of these findings would be in developing personalised therapies targeted at reducing MMP-mediated toxicity and pathology, for example in the context of MDR-TB, as well as informing the development of novel TB vaccines which aim to favourably modulate the host immune response to Mtb in order to promote protective immunity.

Hypothesis

The hypothesis investigated in this project is that Mtb-induced MMP expression by cells of the innate immune system, a key driver of tissue destruction and immunopathology in TB, is regulated by cytokines involved in the acquired immune response to Mtb infection as well as by epigenetic mechanisms, specifically histone acetylation and deacetylation.

The specific aims were:

1. To define the effect of treatment with exogenous IL-4, IL-13, IL-10, IFNγ and TGFβ on MMP production by Mtb-infected macrophages and CoMTb-stimulated respiratory epithelial cells and to investigate the signalling pathways mediating these effects.
2. To assess the effects of HDAC and HAT inhibition on Mtb-driven macrophage and epithelial cell MMP expression.
3. To investigate whether Mtb infection or stimulation itself altered the expression of key epigenetic regulators such as HDACs.

2 Materials & Methods

2.1 Cellular model of pulmonary tuberculosis

In this project a well-established tissue culture model of events occurring in pulmonary tuberculosis was employed. The responses of both respiratory epithelial cells and monocyte-derived macrophages to Mtb can be studied, as well as the importance of intercellular networks.

Primary normal human bronchial epithelial cells (NHBEs) and the alveolar carcinoma cell line A549s are stimulated with conditioned medium from Mtb H37Rv-infected primary human monocytes (CoMTb), to mimic the effects of Mtb infection on adjacent uninfected stromal cells. Monocyte-derived macrophages (MDMs) are infected with the virulent laboratory strain of Mtb H37Rv. Previous work from our group has demonstrated that CoMTb potently induces expression of MMP-1, -3 and -9 by NHBEs in a time- and dose-dependent manner [82, 83]. Mtb-infection of MDMs induces expression of a number of MMPs, including MMP-1, -3 and -7 [79, 80]. This is represented in Figure 1, below.

CoMTb contains both host-derived cytokines and chemokines such as TNFα, IL-6 and IL-1β, and pathogen-derived antigens and lipids (Pathogen–Associated Molecular Patterns or PAMPs) which drive host cell responses [83]. Unpublished data show that the key activating components of CoMTb are resistant to up to 5 freeze-thaw cycles (Dr Justin Green, personal communication) and are heat-labile. Previous studies by our group reported that TNFα in CoMTb was the critical mediator of MMP-9 expression by monocytes [78], while TNFα and IL-1β were necessary but not sufficient for CoMTb-driven astrocyte MMP-9 secretion [92]. In respiratory epithelial cells (NHBEs and A549s) CoMTb-driven MMP-9 secretion requires TNFα, and synergistic upregulation is observed with TNFα and soluble MTb antigen but not LAM, but MMP-9 secretion is independent of IL-1 and IL-6 as well as monocyte-derived chemokines such as CCL2, 3 and 5 and CXCL 8 and 12 [83].

In this project, the influence of several cytokines on CoMTb-driven MMP secretion is investigated, and therefore to avoid confounding factors, the concentration of these cytokines in eight representative batches of CoMTb produced in different blood donors was measured (Table 1). The relevant cytokines were present at low levels and variable concentrations (all <1 ng/ml) in the CoMTb batches tested. These results are shown with the permission of Dr Tara Sathymoorthy.

Table 1 Composition of CoMTb

Cytokine	Median concentration (pg/ml)	Maximum concentration observed (pg/ml)
IL-4	undetectable	undetectable
IL-10	12	858
IL-13	55	216
IFNγ	6	450

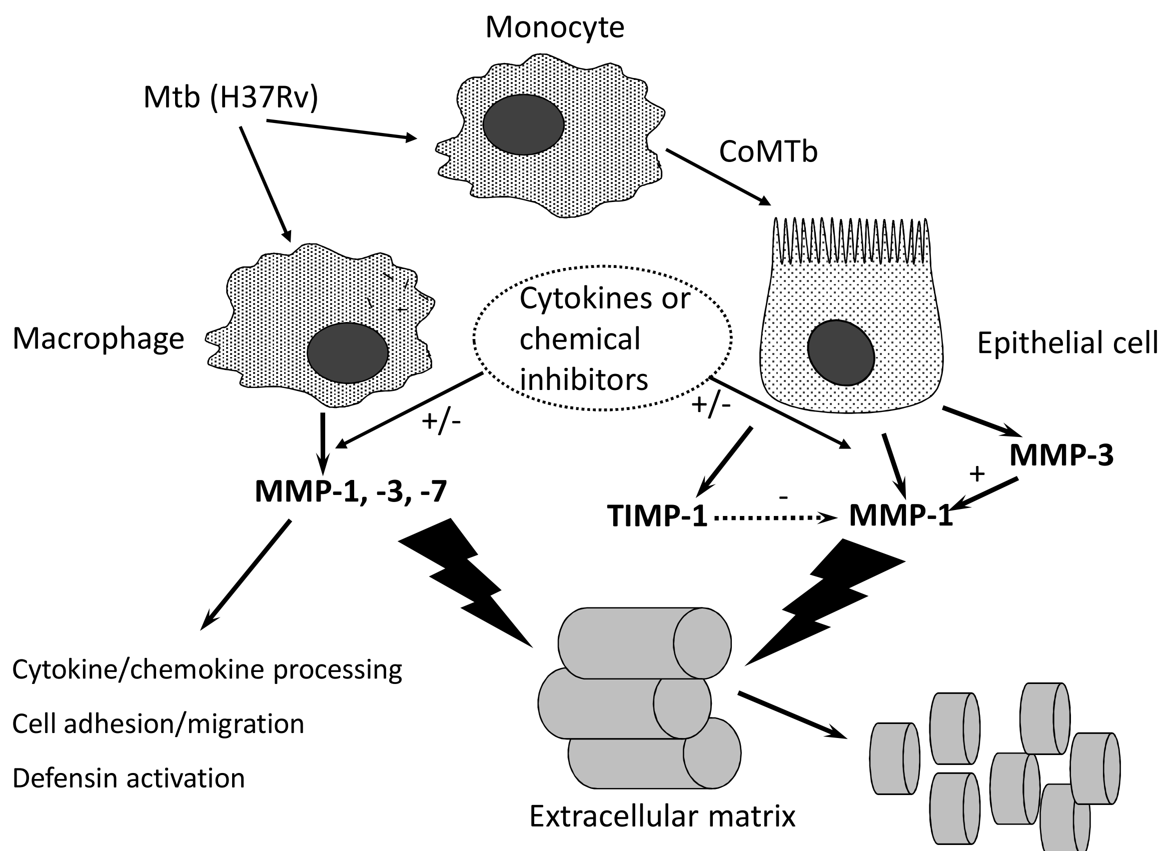


Figure 1. Cellular networks producing MMPs in response to Mtb infection.

2.2 Materials

Tissue culture plastic and consumables were purchased from TPP (Trasadingen, Switzerland) unless otherwise stated. Tissue culture medium was published from Invitrogen (Paisley, UK) unless otherwise stated.

Table 2 Antibodies

Target (technique)	Host	Isotype	Supplier	Cat. No.
Acetyl-Histone H4 (ChIP)	rabbit	polyclonal	Millipore	06-866
Acetyl-histone H3 (ChIP)	rabbit	polyclonal	Millipore	06-599
HDAC1	mouse	IgG1	Cell Signaling	5356
HDAC2	mouse	IgG1	Cell Signaling	5113
HDAC3	mouse	IgG2	Cell Signaling	3949
HDAC4	mouse	IgG2	Cell Signaling	5392
HDAC5	rabbit	IgG	Cell Signaling	2082
HDAC7	rabbit	IgG	Cell Signaling	2882
Rabbit IgG (HRP-linked 2° for Westerns)	goat		Cell Signaling	7074
Mouse IgG (HRP-linked 2° for Westerns)	horse		Cell Signaling	7076
Acetyl-histone H3 (Western)	rabbit		Active Motif	39139
Pan-acetyl histone H4 (Western)	rabbit		Active Motif	39243
Phospho-p38 (Thr 180/Tyr 182)	rabbit		Cell Signaling	9211
Total p38	rabbit		Cell Signaling	9212
Phospho-JNK	rabbit		Cell Signaling	9251
Total JNK	rabbit		Cell Signaling	9252
Phospho-ERK (Thr 202/Tyr 204)	rabbit		Cell Signaling	9101
Total ERK	rabbit		Cell Signaling	9102
Phospho-Stat 6 (Tyr 641)	rabbit	polyclonal	Cell Signaling	9361
Phospho-Akt (Ser 473)	rabbit	IgG	Cell Signaling	4058
Total Akt	rabbit	polyclonal	Cell Signaling	9272
Total Stat 3	rabbit	IgG	Cell Signaling	4904
Phospho-Stat 3 (Ser 727)	rabbit	polyclonal	Cell Signaling	9134
Phospho-Stat 3 (Tyr 705)			Cell Signaling	
Phospho-Stat 1 (Tyr 701)	rabbit	polyclonal	Cell Signaling	9171
Total Stat 1	rabbit	polyclonal	Cell Signaling	9172
β actin	mouse	IgG1	Sigma-Aldrich	A1978

RNA Polymerase II	mouse		Millipore	05-623B
Normal IgG	mouse		Millipore	12-371B
Normal IgG	rabbit		Abcam	ab46540
Anti-mouse IgG	goat	IgG	Jackson Immuno-Research Lab Inc	115-035-062

Table 3 Chemical inhibitors

Name	**Supplier**
Trichostatin A	Sigma Aldrich
m-Carboxycinnamic Acid bis-Hydroxamide (CBHA)	Calbiochem
MS-275	Enzo Life Sciences
Anacardic acid	Calbiochem
Histone Acetyltransferase Inhibitor II	Calbiochem

Table 4 Cytokines

Name	**Supplier**
Recombinant human IFNγ	Peprotech
Recombinant human IL-10	Peprotech
Recombinant human IL-13	Peprotech
Recombinant human IL-4	Peprotech
Recombinant human TGF-β_1	Peprotech
Recombinant human M-CSF	R&D Systems

2.3 Methods

2.3.1 Mycobacterium tuberculosis culture and infection of cultured cells

Mtb strain H37Rv was cultured from frozen stocks stored at -80°C in Middlebrook 7H9 broth (BD Biosciences, Oxford, UK) supplemented with 10% OADC enrichment medium (BD Biosciences), 0.2% glycerol and 0.02% Tween 20 with agitation at 37°C. Growth was monitored by measuring optical density (OD) using a Biowave cell density meter (WPA, Cambridge, UK) and Mtb was sub-cultured weekly at OD 0.9-1.0. Cultures were discarded and replaced with fresh stock every 4 months.

Infection experiments were performed using cultures at mid-log growth, at OD 0.55-0.65, corresponding to 1-2 x 10^8 cfu/ml. Correlation with optical density was checked by performing colony counts in triplicate on Middlebrook 7H11 agar. Cells were infected at a predicted multiplicity of infection (MOI) of 1 unless otherwise stated. Efficiency of infection was not checked by staining for acid fast bacilli.

2.3.2 Epithelial cell culture

A549s

The alveolar carcinoma cell line A549 was cultured in RPMI 1640 supplemented with 2 mM glutamine, 10 μg/ml ampicillin and 10% FCS (Foetal Calf Serum). Cells were trypsinized and sub-cultured twice weekly when more than 90% confluent. For experiments cells were seeded at 4 x 10^4 cells/cm^2 24 hours before the start of the experiment.

NHBEs

Normal human bronchial epithelial cells (NHBEs) (Lonza, Wokingham, UK) were cultured according to the supplier's instructions in bronchial epithelial growth medium (BEGM) supplemented with 52 μg/ml bovine pituitary extract, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 6.5 ng/ml triiodothyronine, 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 0.5 μg/ml recombinant epidermal growth factor, 0.5 μg/ml hydrocortisone and 10 μg/ml ampicillin.

Medium was replaced every 2-3 days. Cells were sub-cultured at 80% confluence and used for experiments at passage 4 or 5.

2.3.3 Monocyte isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from residual Buffy coats or leukocyte cones from healthy blood donors (NHS Blood and Transplant, London, UK) using Ficoll-Paque Plus (GE Healthcare, Little Chalfont, UK) for density gradient centrifugation. Blood residue was diluted to 110 ml with HBSS at room temperature and layered over Ficoll-Paque Plus. After centrifugation at 480 G for 30 minutes at 20°C the cell layer was removed and washed with HBSS 5 times. Cells were pelleted by centrifugation at 300 G for 5 minutes between washes. Monocytes were counted after wash 3 by adherence in a Neubauer haemocytometer with incubation at 37°C for 5 minutes. After wash 5 the cell pellet was resuspended in warmed RPMI 1640 supplemented with 2 mM glutamine and 10 μg/ml ampicillin and plated at 2.5 x 10^5 monocytes/cm^2. Monocytes were purified by adhesion at 37°C and 5% CO_2 for 1 hour prior to 3 washes with warm medium.

2.3.4 Preparation of conditioned medium from Mtb-infected monocytes ((CoMTb)

Monocytes isolated as above were infected with the virulent H37Rv strain of Mtb at MOI 1 in RPMI supplemented with 2 mM glutamine and 10 μg/ml ampicillin only, and incubated at 37°C and 5% CO_2 for 24 hours. The culture medium was then collected, centrifuged at 4000 rpm for 10 minutes in a bench-top centrifuge to remove cellular debris, and then sterilised by passage through a 0.2 μM Anopore syringe filter (Whatman, Brentford, UK). This conditioned medium from Mtb-infected monocytes is hereafter referred to as CoMTb. Paired samples of conditioned medium from uninfected monocytes from the same donor were termed CoMCont and used as a control in some experiments. Once prepared, CoMTb/CoMCont batches were aliquotted and stored at -20°C. Freeze-thaw cycles were limited to 3 or fewer for each aliquot.

2.3.5 Preparation of monocyte-derived macrophages (MDMs)

Monocytes isolated and adhesion-purified as above were incubated in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 10 μg/ml ampicillin and 100 ng/ml recombinant human M-CSF (R&D Systems) for 4 days. Medium was then changed for RPMI without M-CSF and the cells were rested for 24 hours prior to the start of experiments.

2.3.6 Infection of MDMs with Mtb

On day 5 the culture medium was changed for 0.5 ml/cm^2 MSFM and MDMs were infected with H37Rv at an MOI of 1. Culture medium was replaced two hours after infection in order to remove residual extracellular bacteria unless otherwise stated. At the end of experiments cell culture supernatants were centrifuged at 12,000 G for 5 minutes to remove cellular debris. The samples were then sterilised by filtration through a 0.2 μM spin filter (Millipore, Watford, UK).

2.3.7 MMP and TIMP Enzyme-linked Immunosorbant assays (ELISAs)

Duoset ELISAs for MMP-1, MMP-3, MMP-9, TIMP-1 and TIMP-2 were purchased from R&D Systems (UK) and performed according to the supplied protocols. The supplied standard was used to generate a seven-point standard curve which was assayed in duplicate and used to calculate concentrations for the unknown samples.

Briefly, 96-well plates were coated with the primary antibody diluted in PBS overnight at room temperature. After 3 washes in PBS/0.05% Tween the plate was blocked with 1% BSA for 1 hour and then washed 3 times. Diluted samples and standards were incubated in the plate for 2 hours at room temperature, the plate was washed and biotinylated secondary antibody added at the recommended concentration. After 2 hours the plate was washed 3 times and Streptavidin-HRP conjugate added for 20 minutes. The substrate solution 3,3',5,5'-tetramethylbenzidine (Sigma Aldrich) was added and the reaction stopped with 2M H_2SO_4. Absorbance was measured at 450 nm, reference wavelength 540 nm on a μQuant

spectrophotometer (Bio-Tek, Winooski, USA). KCJunior software was used to generate standard curves and to analyse the results.

2.3.8 Luminex multiplex immunoassay

Quantification of MMP-1, -2, -3, -7, -8, -9 and -13 proteins in cell culture supernatants was performed using the Fluorokine MultiAnalyte Profiling MMP Base Kit (R&D Systems, UK) and the Luminex platform Bio-Plex 200 (Bio-Rad, Hemel Hempstead, UK) dual laser analyser. The Invitrogen Human Cytokine 30-Plex Cytokine Panel was used according to the manufacturer's instructions to measure cytokines in cell culture supernatants. For each assay system, cell culture supernatants were incubated with antibody-coated microparticles on a shaking platform for 2 hours. After washing, biotinylated secondary antibody was added for 1 hour. Streptavidin-phycoerythrin was then added before reading the plate on the Luminex® dual laser analyzer. Bio-Plex Manager version 5.0 software was used to construct standard curves and calculate values for unknowns.

2.3.9 Western blotting

Cells were washed in sterile PBS and scraped and homogenized in lysis buffer (62.5 mM Tris, 2% SDS, 10% glycerol, 50 mM DDT with bromophenol blue) prior to storage at -80°C. Samples were denatured by heating to 90°C for 5 mins and then cooled before loading equal volumes onto 4-12% NuPAGE Bis-Tris mini gels (Invitrogen, Paisley, UK). MOPS or MES running buffer with NuPAGE antioxidant (all from Invitrogen) was used according to the molecular weight of the protein of interest. Gels were run at 200 V for 70-90 minutes. Proteins were transferred to Hybond nitrocellulose membrane (GE Healthcare) at 4°C, 30 V, for 1 hour before blocking with 5% skimmed milk protein/TBS/0.1% Tween 20 (block buffer) for 1 hour and overnight incubation with primary antibody diluted in either 5% BSA/TBS/0.1% Tween 20 or block buffer. Membranes were incubated with secondary antibody diluted in block buffer for one hour and then developed using an ECL developing kit (GE Healthcare, Hatfield, UK). Photographic film was exposed to the membranes for different time periods according to the intensity of the signal.

2.3.10 Gelatin Zymography

20 μl aliquots of cell culture supernatants were loaded with 5x loading buffer (0.25M Tris pH 6.8, 50% glycerol, 5% SDS and bromophenol blue) on an 11% acrylamide gel containing 0.1% gelatin. Gels were run for 3.5-4 hours at 180V in running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS) and then washed in 2.5% Triton-X with gentle shaking for 1 hour. The gels were rinsed twice in low-salt collagenase buffer (55 mM Tris base, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij, pH 7.6) and incubated for 16 hours in fresh collagenase buffer at 37˚C. Gelatinolytic activity was detected by staining with 0.02% Coomasie Blue (Pharmacia Biotech) in acetic acid:methanol:water 1:3:6. Densitometric image analysis was performed by digital image acquisition (UVP) followed by band quantification with NIH Image version 1.61 (Scion Image).

2.3.11 Transient transfection with promoter-reporter constructs

MMP-1 promoter constructs were designed and expressed in the pGL3 firefly (*Photinus pyralis*) luciferase expression vector (Promega, Southampton, UK), and were a gift of Professor Ian Clark (University of East Anglia, Norwich, UK).

The full-length wild-type MMP-1 promoter construct (WT) comprised a 4372 base pair (bp) sequence upstream of the MMP-1 transcriptional start site. Deletion constructs ranged in size from 3830 bp to 517 bp. Site-directed mutagenesis was used to disrupt a putative AP-1 binding site at -1949 to -1955 bp (5'-TGA**G**TTA-3' to 5'-TGA**T**TTA-3'). MMP-3 promoter constructs were designed in-house and also cloned into the pGL3 vector. The constructs used were 2183 bp, 1612 bp and 642 bp in length. The PRL-TK control plasmid constitutively expressing *Renilla* luciferase was used to control for transfection efficiency.

A549 cells were cultured in RPMI in 12-well plates and transfected when 60% confluent. The transfection mastermix for each well consisted of 37.6 μl RPMI and 2.4 μl of FuGene 6 (Roche, Lewes, UK) with 0.8 μg plasmid DNA and 0.08 μg control plasmid DNA. 16 hours after transfection the cells were stimulated according to the experimental conditions. 24 hours later cells were washed once in PBS and lysed in passive lysis buffer (Promega).

Luciferase assays were performed using the Promega Dual-Luciferase Reporter Assay kit (Promega) according to the supplied protocol and an L-Max 2 luminometer (Molecular Devices, Sunnydale, CA, USA).

2.3.12 RNA extraction

Cells were washed once with sterile PBS and then lysed in TRI-reagent (Sigma-Aldrich). Samples were stored at -80°C prior to RNA extraction. In early experiments the PureLink RNA mini kit (Invitrogen) with on-column DNase treatment was used to extract total RNA. In later experiments the Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) was used according to the manufacturer's instructions. RNA concentrations and purity were evaluated using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.3.13 cDNA synthesis

For early experiments 1 μg of sample RNA was reverse transcribed using random primers and Superscript III (Invitrogen). Latterly the Quantitect RT Kit (Qiagen, Crawley, UK) with genomic DNA removal was used according to the supplied instructions.

2.3.14 Quantitative Reverse Transcriptase PCR

cDNA was diluted 1:100 in RNase-free water. 18S ribosomal RNA, GAPDH and β-actin were used as reference genes in different experiments. PCR was performed in a 25 μl reaction volume using Brilliant II qPCR mastermix (Agilent, UK) on a Stratagene Mx3000p platform (Stratagene, La Jolla, USA). The thermal profile was 10 min at 95°C, followed by 40 to 45 cycles of 30 seconds at 95°C and 1 min at 60°C. The cycle threshold (Ct) at which amplification entered the exponential phase was determined for each well and analyte. Cts were normalised to Cts for the reference genes which were measured simultaneously for each PCR assay performed.

Table 5 Primers & probes for RT-PCR

Target	Sequence (or catalogue number)	Probe	Supplier
MMP-1	Fwd 5'- AAGATGAAAGGTGGACCAACAATT -3' Rev 5' -CCAAGAGAATGGCCGAGTTC -3' Probe 5'-FAM-CAGAGAGTACAACTTACATCGTGTTGCG GCTC-TAMRA-3'	FAM	Sigma
MMP-3	Hs00968305_m1	FAM	Applied Biosystems
HDAC1	Hs02621185_s1	FAM	Applied Biosystems
HDAC2	Hs00231032_m1	FAM	Applied Biosystems
HDAC3	Hs0018730_m1	FAM	Applied Biosystems
HDAC4	Hs01041638_m1	FAM	Applied Biosystems
HDAC5	Hs00608366_m1	FAM	Applied Biosystems
HDAC8	Hs00218503_m1	FAM	Applied Biosystems
18s	4308329	VIC	Applied Biosystems
GAPDH	Fwd 5'- CGCTTCGCTCTCTGCTCCT-3' Rev 5'- CGACCAAATCCGTTGACTCC-3' Probe 5'-HEX-CGTCGCCAGCCGAGCCACAT-TAMRA-3'	HEX	Sigma
βactin	431088E	VIC	Applied Biosystems

2.3.15 Chromatin Immunoprecipitation assay (ChIP)

ChIP assays were used to investigate transcription factor binding and covalent histone modifications at the MMP-1 promoter. Magna-ChIP kits were purchased from Millipore (Watford, UK) and used according to the manufacturer's instructions.

Cells were cultured in 100mm or 150mm tissue culture dishes until confluent, stimulated according to the relevant experimental conditions, and then fixed and sheared as follows. An additional plate was cultured solely for estimation of cell numbers in order to permit consistent shearing conditions.

Cell fixation

Freshly prepared 18.5% paraformaldehyde solution (Sigma Aldrich) was added to the culture medium to a final concentration of 1% and incubated for 10 minutes at room temperature. 10x glycine solution was added to quench the reaction, and the plates were left for 5 minutes, then transferred onto ice. The culture medium was removed and the plates washed twice with ice-cold PBS before scraping in PBS supplemented with protease inhibitor cocktail (PIC). The cell suspensions were centrifuged at 800 G for 5 minutes, supernatant removed, and pellets resuspended in cell lysis buffer with PIC. The samples were incubated on ice for 15 minutes then centrifuged at 800G for 5 minutes at 4°C. The supernatant was discarded and the pellet resuspended in a volume of nuclear lysis buffer with PIC to give a concentration of 2×10^7 cell equivalents/ml.

Sonication

290 μl aliquots of chromatin were transferred to Snap-Cap microtubes (KBioscience, Hoddesdon, UK) on ice and sonicated with a Covaris S2 ultra-sonicator (KBioscience) using an AFA (adaptive focused acoustics) intensifier. Settings were as follows: temperature 6-8°C, duty cycle 20%, intensity 8, 200 cycles/burst, 30 second cycles for 15 cycles. Validation experiments were performed to verify that these conditions produced DNA fragments of a suitable size for ChIP (400-800bp) (data not shown). After sonication the aliquots were recombined and spun at 14000 G, 4°C for 10 minutes to remove insoluble material. Sheared chromatin was stored at -80°C if necessary prior to proceeding to the immunoprecipitation step.

Immunoprecipitation (IP)

50 μl sheared chromatin per IP were diluted 10-fold with dilution buffer plus PIC and 1% was removed for each sample and reserved at 4°C as “input” control. The IP antibody and 20 μl protein G magnetic beads were added to each tube, followed by overnight incubation at 4°C with rotation. The protein G-antibody-chromatin complexes were then pelleted using a magnetic separator (Magna Grip, Millipore) and washed with 0.5 ml cold buffer in the

following sequence: low salt immune complex wash buffer, high salt immune complex wash buffer, Lithium Chloride immune complex wash buffer, TE buffer.

Elution and reversal of cross-linking

ChIP elution buffer and proteinase K were added to each tube (including "input" controls) and incubated at 62°C for 2 hours with rotation. Samples were then heated to 95°C for 10 minutes, cooled, and the supernatant (eluate) separated from the magnetic beads.

DNA Purification

Genomic DNA fragments were purified from the immunoprecipitated samples using the spin columns and buffers provided in the Magna-ChIP kit as per the supplied instructions. DNA was eluted in 50μl Elution Buffer C and stored at -20°C prior to quantitative PCR.

Quantitative PCR (qPCR)

The PCR mastermix comprised: 10 μl SYBR Green JumpStart Taq Readymix (Sigma), 1 μl each of F and R primers at 10 μM and 6 μl RNase-free water per reaction. 18 μl mastermix was loaded per well with 2 μl undiluted sample DNA. Reactions were performed in triplicate on the Stratagene Mx3000P platform (Stratagene, La Jolla, USA). Custom unlabelled primers were designed in-house and supplied by Sigma (see Table 6 for primer sequences).

The thermal settings were 10 min at 95°C followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C. Finally a cycle of 1 minute at 95°C, 10 seconds at 72°C and 30 seconds at 95°C was performed to allow dissociation curves to be plotted.

The cycle threshold (Ct) at which amplification entered the exponential phase was determined for each well and the "% input" method was used to analyse the results obtained.

Table 6 Primer sequences for Chromatin Immunoprecipitation

Primer pair name	Forward primer (5'-3')	Reverse primer (5'-3')	Product size
MMP-1 transcription start site	TGGGATATTGGAGCAGCAAG	AGCTGTGCATACTGGCCTTT	82 bp
-500 bp MMP-1 promoter	TAAGGGAAGCCATGGTGCTA	AGGTTCCCTTCTGCCTTTGT	65 bp
-2 kbp MMP-1 promoter	TTGCCAGATGGGACAGTGTA	TCAGGAAAGCAGCATGTGAC	123 bp
-4 kbp MMP-1 promoter	CTTGAGGCCAGGAGTTTGAG	ACCACCATGTCCCACTGATT	89 bp

2.3.16 Preparation of nuclear and cytoplasmic extracts

Nuclear and cytoplasmic extracts were prepared from cultured cells using a Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's protocol. Briefly, confluent 60 mm dishes of cells were prepared and treated with the experimental stimulus. At the relevant time-point, plates were washed twice with ice-cold PBS containing phosphatase inhibitors, and then scraped in 2 ml PBS/phosphatase inhibitors, transferred to ice-cold 15 ml Falcon tubes and centrifuged at 700 rpm for 5 minutes at 4°C. The cell pellet was resuspended in 250 μl Hypotonic Buffer and incubated on ice for 15 minutes. 12.5 μl detergent was added, and the samples were vortexed for 10 seconds prior to centrifugation at 14000 G, 4°C for 1 minute. The supernatant was removed to a pre-cooled microtube and frozen at -80°C; this constituted the cytoplasmic extract.

The pellet was then resuspended in 25 μl Complete Lysis Buffer on ice, vortexed and incubated for 30 minutes on ice with agitation at 150 rpm. Samples were then vortexed for 30 seconds, centrifuged at 14000 G for 10 minutes at 4°C and the supernatant (the nuclear extract) was frozen at -80°C. A Bradford assay was used to quantify total protein content in the extracts obtained.

In MDM direct infection experiments using H37Rv, both nuclear and cytoplasmic extracts were sterilised by filtration through a 0.2 μM pore-size spin filter (Millipore, Watford, UK).

2.3.17 Bradford protein quantitation assay

Total protein concentration of cell lysates and nuclear extracts was determined in order to permit equal protein loading in subsequent analyses. Bio-Rad Protein Assay Standard II (Bio-Rad, Hemel Hempstead, UK) was used to generate a standard curve from 1000 to 5 μg/ml in duplicate. 20 μl samples were assayed in duplicate at dilutions intended to allow calculation of protein concentration from the linear part of the standard curve. 200 μl developing reagent (Bio-Rad Dye Reagent) was added per well and absorbance measured at 580 nm on a μQuant spectrophotometer (Bio-Tek, Winooski, USA).

2.3.18 ELISA-based detection of nuclear AP-1 subunit binding

Activation of nuclear AP-1 subunits was assayed using commercially available TransAM kits (Active Motif) which quantify transcription factor binding to immobilised oligonucleotide in a 96-well plate format. The assay measured binding to the AP-1 consensus binding sequence (5' –TGAGTCA– 3'). Primary antibodies were directed against JunB, cJun, JunD, FosB, cFos, Fra-1 and Fra-2.

The supplier's protocol was followed to perform the assay, briefly 2-10 μg of nuclear extract diluted in the buffers provided was incubated per well in duplicate, followed by the addition of the primary detection antibody. Positive and negative controls were included for each assay. HRP-linked secondary antibody was added for one hour prior to incubation with developing solution. 3 washes were performed between each incubation step. Absorbance was measured at 450 nm, reference wavelength 655 nm on a μQuant spectrophotometer (Bio-Tek, Winooski, USA).

2.3.19 Phosphokinase array

The Human Phospho-Kinase Array Kit (R&D Systems) was used to investigate which signalling pathways mediated the effects of cytokines on MMP secretion. MDMs were cultured in 60 mm dishes, pre-incubated with or without cytokines and then infected with M.tb H37Rv MOI 1. After 30 minutes cells were washed with ice-cold PBS and lysed in Lysis

Buffer 6. After 30 minutes incubation on ice the samples were centrifuged at 12000 G for 5 minutes and sterile-filtered prior to storage at -80°C.

Total protein content of the cellular extracts was measured by Bradford assay as described above. Arrays were blocked with Array Buffer 1 for 1 hour. Samples were diluted with Lysis Buffer 6 and Array Buffer 1 to a final concentration of 200 μg/ml total protein. The arrays were incubated overnight with the samples at 4°C on a rocking platform. The following day the membranes were washed three times before adding biotinylated secondary antibody cocktail for 2 hours on a rocking platform at room temperature. After 3 further washes the membranes were incubated with Streptavidin-HRP for 30 minutes. Three more washes were performed, then the membranes were blotted dry and developed using the ECL Plus developing system (GE Healthcare) and exposed to photographic film. Densitometric image analysis was performed by digital image acquisition (UVP) followed by quantification of spot intensity with NIH Image version 1.61 (Scion Image).

2.3.20 Histone deacetylase (HDAC) activity assay

Two different HDAC enzyme activity assays were employed, each of which quantifies total Class I and class II HDAC activity in nuclear extracts. The colorimetric HDAC assay kit (Active Motif) was used according to the supplied instructions. Briefly, nuclear extracts were prepared as above, and total protein concentration measured by Bradford assay. 6 μg protein/well of each sample was loaded in duplicate with Assay Buffer and substrate. A standard curve, blanks and positive controls (HeLa cell nuclear extracts) were run in parallel with the unknown samples. The plate was incubated at 37°C for 4 hours before adding Developing Solution and incubation at room temperature for 15 minutes. Absorbance was then measured at 405 nm on a μQuant spectrophotometer (Bio-Tek, Winooski, USA) and the standard curve used to calculate HDAC activity per μg nuclear extract.

This assay was found to be insufficiently sensitive to detect significant differences in HDAC activity in the nuclear extracts prepared, and the slight yellow colour of the nuclear extracts

imparted by the phosphatase inhibitors used also interfered with the colorimetric detection reaction. Therefore the fluorescent HDAC assay kit (Active Motif) was used in further similar experiments. This assay is reported to be more sensitive and can be performed with smaller quantities of nuclear extract per well (minimum 2 μg/well recommended).

5 μg nuclear extract per well was loaded in duplicate with HDAC Assay Buffer and Substrate. A standard curve was generated in duplicate using the supplied standard at 20 to 0.31 μM. HeLa cell nuclear extract was used as a positive control for the assay. Nuclear extracts were incubated with substrate for 1 hour at 37°C. Developing solution supplemented with Trichostatin A to quench the reaction was then added and fluorescence measured with excitation wavelength 390 nm and emission wavelength 460 nm. The standard curve was used to calculate values for HDAC activity in pmol/μg nuclear extract.

2.3.21 Histone Acetyltransferase (HAT) Assay

Histone acetyltransferase activity in nuclear extracts prepared as above was quantified using the colorimetric HAT assay kit (Millipore) as per the manufacturer's instructions. The principle of the assay is that nuclear extracts containing HAT enzymes are incubated with H4 peptide substrate and acetyl coA as an acetyl-group donor, and the extent of H4 acetylation is then quantified by incubation with a specific anti-acetyl-lysine antibody, followed by streptavidin-conjugated goat anti-rabbit IgG secondary antibody and substrate solution, generating a quantitative colour change. Known amounts of acetylated H3 or H4 peptide were used as positive controls. 10-15 μg nuclear protein was used per reaction, with each sample loaded in duplicate. Samples were incubated in HAT assay buffer with acetyl coA and biotinylated H4 peptide substrates at 30°C for one hour. Wells were washed with TBS and then incubated with anti-acetyl-lysine antibody for 1.5 hours. After 30 mins incubation with secondary antibody the supplied substrate mix was added and incubated for 10 mins before adding 1M H_2SO_4 to stop the reaction and measuring absorbance at 450 nm, reference wavelength 570 nm on a microplate reader.

2.3.22 Transfection of epithelial cells with siRNA

Transfection methodology for post-transcriptional gene silencing using siRNA in NHBEs was previously optimized in our group by Dr Shivani Singh (unpublished data). Her method was used with further validation and modifications for the different siRNAs used.

ON-TARGETplus SMARTpool siRNA oligonucleotides and transfection reagents were purchased from ThermoScientific Dharmacon. These constitute an equimolar mixture of four different double-stranded oligonucleotides targeting the mRNA of interest. siRNA was reconstituted in siRNA 1x Buffer as per the manufacturer's instructions and stored at -80°C. Freeze-thaw cycles were limited to a maximum of five per aliquot.

Previous optimization experiments by Dr Singh achieved a transfection efficiency of greater than 70% with Lipofectamine 2000 transfection reagent at 2.5 μg/ml and siGLO control (measured by FACS). Therefore I performed experiments using Lipofectamine 2000 at this concentration and optimized the siRNA concentration for each new siRNA pool.

NHBEs were cultured in 12-well plates in complete medium and transfected at 60-70% confluence. siRNA was diluted to working concentrations in Optimem and complexed with Lipofectamine 2000 25 μg/ml (also diluted in Optimem) at room temperature for 20 minutes. The culture medium was then changed for 800 μl BEBM (basal medium, without antibiotics) and 200 μl of the siRNA/Lipefectamine complex was added to each well.

After 4 hours each well was washed with 500 μl sterile PBS and fresh BEGM was added. The cells were then rested overnight prior to stimulation with the experimental conditions. For analysis of mRNA expression, cells were lysed and total RNA was extracted 24 hours after stimulation. For analysis of protein expression cell culture supernatants were collected and cells were washed in PBS and lysed in Western lysis buffer 48 hours post-stimulation.

Table 7 Dharmacon siRNA reagents

Target gene/mRNA	ON-TARGETplus SMARTpool catalogue number	Target sequences
HDAC 1	L-003493-00-0005	ACUAUGGUCUCUACCGAAA GCAAGUAUUAUGCUGUUAA CCGGUCAUGUCCAAAGUAA CCACAGCGAUGACUACAUU
HDAC 2	L-003495-00-0005	GUAAGCAGAUGCAUAUAUU GCGGAUAGCUUGUGAUGAA GCAAAGAAAGCUAGAAUUG GAUAACAUGUCUGAGUAUA
HDAC 3	L-003496-00-0005	AAAGCGAUGUGGAGAUUUA GCACCCGCAUCGAGAAUCA GGAAUGCGUUGAAUAUGUC AACAAGAUCUGUGAUAUUG
Non-targeting pool	D-001810-10-05	Negative control siRNA with ≥ 4 mismatches to any human gene

2.3.23 Statistics

Microsoft Excel and GraphPad Prism version 6 software were used to analyse results. Comparisons between two groups were made using the Student t-test (two-tailed with significance set as $p<0.05$ unless otherwise stated). For comparison of three or more groups, one-way ANOVA was used with Tukey's correction for multiple comparisons.

Unless otherwise stated in figure legends, error bars represent standard error of the mean.

3 Interleukin-4 and other anti-inflammatory cytokines influence Mtb-driven MMP expression

3.1 Introduction

IL-4 is a key Th2 cytokine, associated with progression to active disease and increased disease severity in human studies of TB [132, 138], and with increased fibrosis, decreased TNFα responses and altered granuloma formation in mouse models of TB [151, 152]. The closely related cytokine IL-13 has a key role in fibrotic processes, and is known to influence expression of MMPs and matrix components in disease processes such as pulmonary fibrosis and asthma [282, 294]. There is some evidence from animal models of TB to suggest that IL-13 responses to Mtb restrain inflammation and permit bacterial growth.

The results of studies of human TB support the hypothesis that overproduction of IL-10 in response to Mtb may inhibit efficient Th1 responses and promote progression to active disease. A previous study of the effects of IL-10 on MMP expression, which was performed in mice, found that BCG-induced MMP-9 activity was reduced when peritoneal macrophages were pre-incubated with IL-10 [96].

TGFβ is a key cytokine in wound healing and tissue repair processes, modulating extracellular matrix deposition and remodeling. It was therefore expected that exposure to TGFβ would modify the response to Mtb stimulation in this tissue culture model. Previous publications have demonstrated that TGFβ is produced in response to Mtb infection in monocyte/macrophages [212, 213], and that MMP expression can be either increased or suppressed by TGFβ [295, 296]. In stromal cells, TGFβ suppressed fibroblast MMP-1 expression [297] but increased epithelial cell MMP-9 expression [298].

In this chapter, the effect of treating respiratory epithelial cells and monocyte-derived macrophages with these cytokines is investigated, in order to test the hypothesis that they modulate Mtb-driven MMP expression and pulmonary immunopathology.

3.2 Interleukin-4 inhibits Mtb-driven MMP expression

3.2.1 IL-4 inhibits Mtb-driven MMP-1 secretion in MDMs produced from different blood donors

The effect of IL-4 on Mtb-inducible MMP expression in macrophages was investigated. MDMs were treated with IL-4 (1-20 ng/ml) for 2 hours, and then infected with H37Rv. Extracellular bacteria were washed off after 2 hours but IL-4-supplemented culture medium was used throughout the experiment. Supernatants were collected at 72 hours post-infection and MMPs were measured by Luminex. These experiments (and subsequent ones investigating the effects of other cytokines and inhibitors) were performed repeatedly over time using MDMs prepared from different healthy blood donors. Initial experiments suggested that although the trends in MMP secretion were consistent between different donors, the magnitude of the response to Mtb infection and the concentrations of IL-4 required to suppress MMP-1 secretion, for example, were variable between individuals. While not necessarily unexpected, this presented a problem in respect of data presentation, as combining results from different donors was difficult. In Figure 1 I have displayed results from four independent experiments investigating the effect of IL-4 at different concentrations on MMP-1 secretion by Mtb-infected MDMs. In Figure 1a the mean MMP-1 concentrations measured by Luminex are represented, whereas in Figure 1b MMP-1 secretion has been internally normalised for each independent experiment with respect to Mtb alone, which has been set as 1 or 100% expression. These results show that although the absolute values obtained in different individuals were highly variable, the pattern of results was the same.

For this reason, hereafter results from one representative experiment (ie. MDMs prepared from one blood donor) rather than combined results from multiple experiments in different donors will be displayed for clarity.

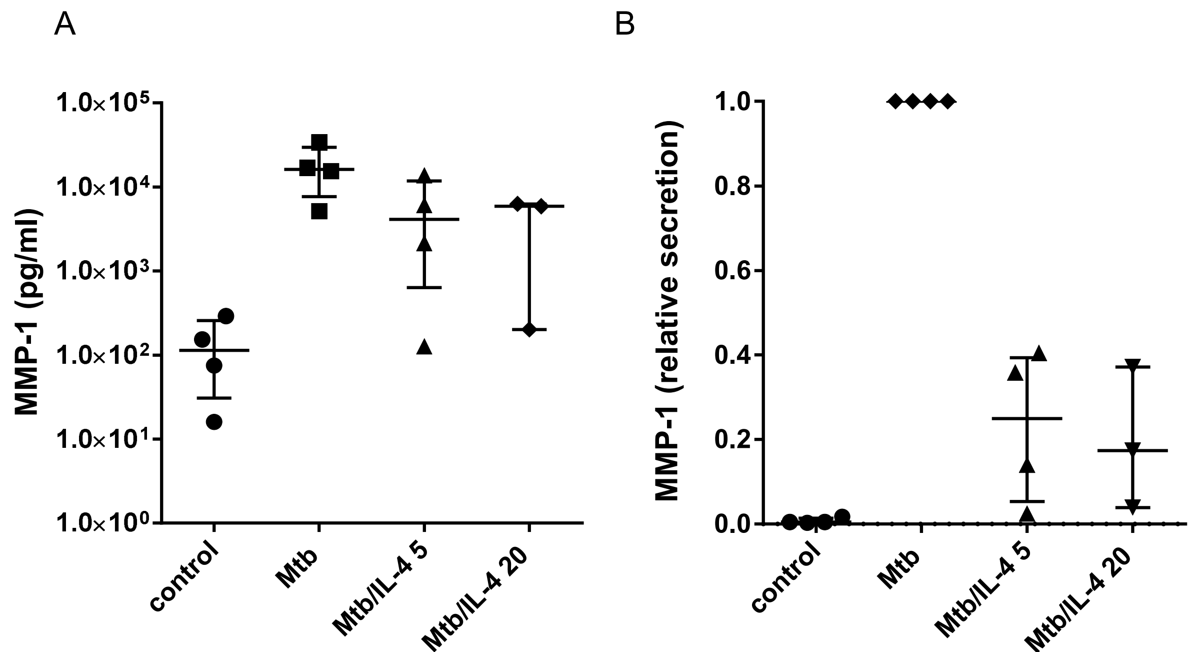


Figure 1. IL-4 inhibits the secretion of MMP-1 by Mtb-infected MDMs from different blood donors

MDMs were pre-incubated with IL-4 at the indicated concentrations for 2 hours and infected with H37Rv (MOI 1). Supernatants were collected at 72 hours and MMP-1 protein secretion quantified by Luminex. The results shown are from four independent experiments performed in triplicate using MDMs from different blood donors. A) MMP-1 secretion from Mtb-infected MDMs is increased in all donors, and Mtb-driven MMP-1 secretion is reduced in the presence of IL-4 (5 or 20 ng/ml). B) The same data presented as relative MMP-1 secretion compared to Mtb-infected MDMs, showing that IL-4 (5 or 20 ng/ml) significantly reduced Mtb-driven MMP-1 secretion in all donors. Each point represents the mean MMP-1 secretion for the condition in one experiment. Bars represent the median and inter-quartile range of the combined results for each condition.

3.2.2 IL-4 selectively inhibits the secretion of MMP-1 and -3 by Mtb-infected MDMs

The effect of IL-4 on Mtb-inducible MMP expression in macrophages was investigated. MDMs were treated with IL-4 (1-20 ng/ml) for 2 hours, and then infected with H37Rv. Extracellular bacteria were washed off after 2 hours but IL-4-supplemented culture medium was used throughout the experiment. Supernatants were collected at 72 hours post-infection and MMP-1, -3 and -7 were measured by Luminex.

As previously shown, Mtb infection typically increased MMP-1 secretion more than 200-fold, from 154 to 33857 pg/ml ($p<0.001$) (Figure 2a). IL-4 (5 ng/ml) inhibited Mtb-driven MMP-1 secretion by 59% ($p<0.01$) while IL-4 (20 ng/ml) inhibited MMP-1 secretion by 83% to 5892 pg/ml ($p<0.01$). MMP-3 was undetectable in supernatants from uninfected cells; the threshold of detection of the Luminex assay is 16 pg/ml. MMP-3 was detectable in supernatants from infected samples cells, and IL-4 (5 ng/ml) suppressed MMP-3 secretion to undetectable levels ($p<0.001$) (Figure 2b). In contrast, MMP-7 secretion, which increased 1.75-fold with Mtb infection ($p<0.01$) was unaffected by co-incubation with IL-4 at concentrations up to 20 ng/ml ($p>0.05$ compared to CoMTb alone). As described in Figure 1 these effects were consistently observed between different blood donors, although there was some variation in the concentration of IL-4 required to suppress MMP-1 and -3 secretion. IL-4 did not inhibit MMP-7 secretion significantly in any donor. The results shown are from one experiment performed in triplicate, and are representative of five independent experiments.

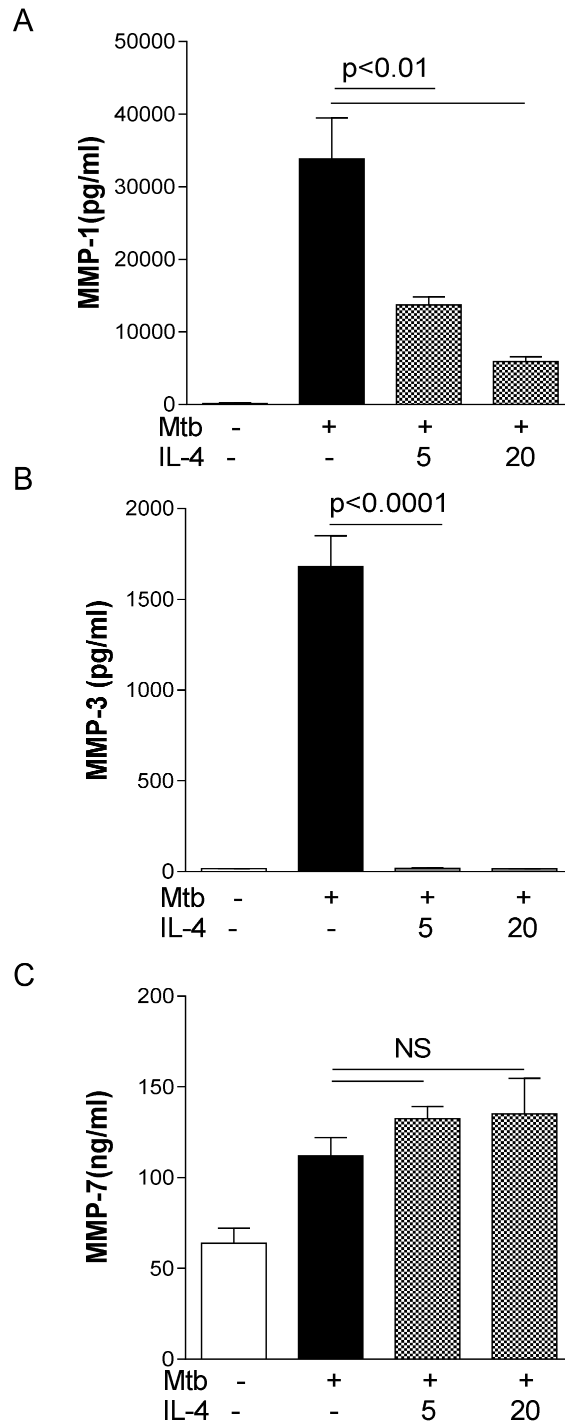


Figure 2. IL-4 selectively inhibits the secretion of MMP-1 and -3 by Mtb-infected MDMs

MDMs were pre-incubated with IL-4 at the indicated concentrations for 2 hours and infected with H37Rv (MOI 1). Supernatants were collected at 72 hours post-infection and MMP-1, -3 and -7 were measured by Luminex. A) Mtb infection increased MMP-1 secretion from 154 to 33857 pg/ml (p<0.001). IL-4 (5 ng/ml) inhibited Mtb-driven MMP-1 secretion by 59% (p<0.01) while IL-4 (20 ng/ml) inhibited MMP-1 secretion by 83% to 5892 pg/ml (p<0.01). B) MMP-3 was undetectable in supernatants from uninfected cells, but present at 1682 pg/ml in infected samples. IL-4 (5 ng/ml) suppressed MMP-3 secretion to undetectable levels (p<0.001) (threshold of detection 16 pg/ml). C) MMP-7 secretion increased 1.75-fold with M.tb infection (p<0.01) but was unaffected by co-incubation with IL-4 at 5 or 20ng/ml (p>0.05).

3.2.3 IL-4 acts pre-transcriptionally to inhibit MDM MMP expression

To investigate the mechanism by which IL-4 acts to inhibit MTb-driven MMP-1 and -3 expression, quantitative PCR was performed. MDMs were pre-incubated with IL-4 20ng/ml for 2 hours and then infected with H37Rv. After 24 hours the cells were lysed and total RNA isolated. RT-PCR was performed and results were quantified relative to a plasmid standard for each gene studied. MMP mRNA was normalised to copy numbers for the reference genes β-actin, 18S ribosomal RNA or GAPDH which were stably expressed between the relevant experimental conditions. MMP-1 mRNA results correlated with the protein secretion data; MMP-1 mRNA was increased 310-fold by Mtb infection, and this was inhibited by 91% in the presence of IL-4 (Figure 3a).

MMP-3 mRNA was undetectable in uninfected cells but increased to 4 copies per 10,000 copies β-actin mRNA in Mtb-infected cells. Pre-incubation with IL-4 resulted in undetectable levels of MMP-3 mRNA 24 hours after Mtb infection. MMP-7 mRNA increased 9-fold with Mtb infection, but was unaffected by the presence of IL-4. The results shown are from one experiment performed in triplicate, and are representative of results from two independent experiments performed in different blood donors.

These findings mirror exactly the observations made in the MMP secretion experiments, and suggest that IL-4 acts pre-transcriptionally to inhibit MMP expression in Mtb-infected MDMs.

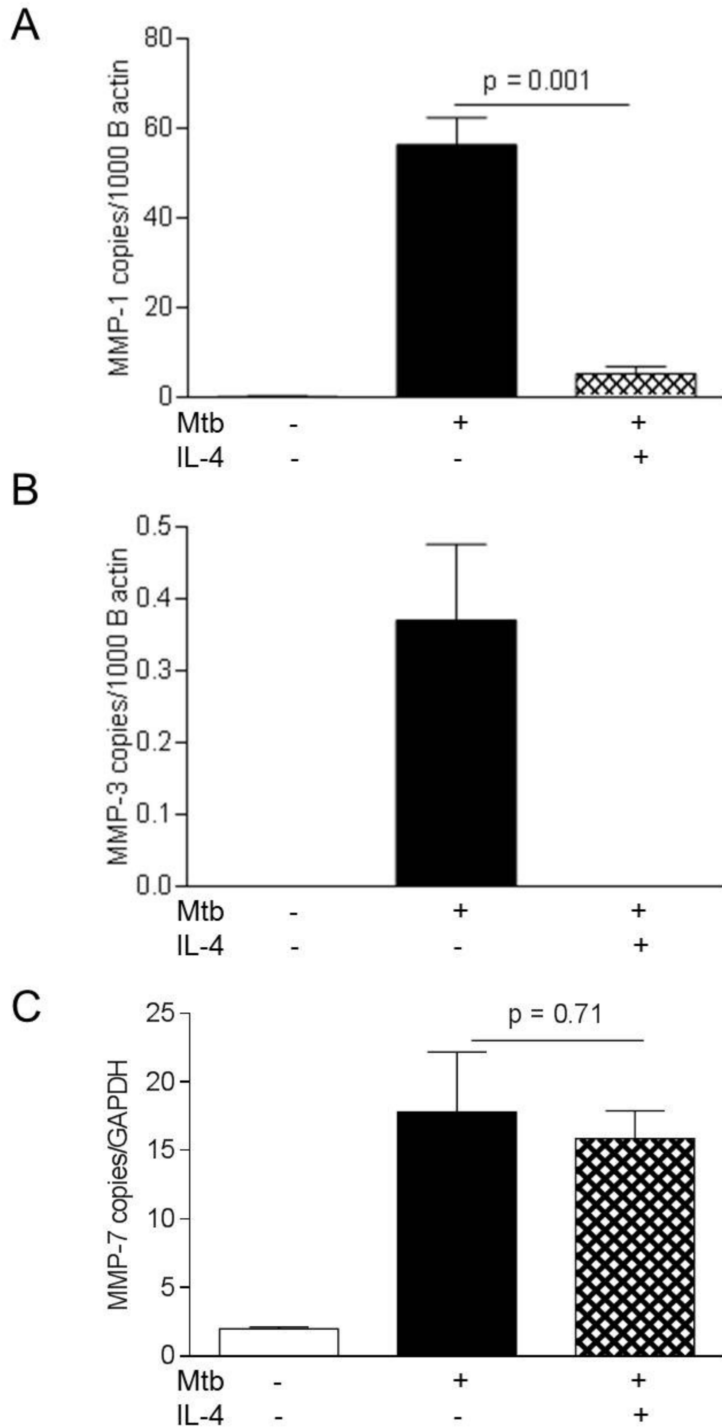


Figure 3. IL-4 acts pre-transcriptionally to inhibit MDM MMP expression

MDMs were pre-incubated with IL-4 (20 ng/ml) for 2 hours and infected with H37Rv (MOI 1). After 24 hours the cells were lysed and total RNA isolated. RT-PCR was performed and results were quantified relative to a plasmid standard. A) MMP-1 mRNA was increased 310-fold by Mtb infection, and this was inhibited by 91% in the presence of IL-4. B) MMP-3 mRNA was undetectable in uninfected cells but increased to 4 copies per 10,000 copies β-actin mRNA in Mtb-infected cells. When cells were pre-incubated with IL-4 MMP-3 mRNA was undetectable. C) MMP-7 mRNA increased 9-fold with Mtb infection, but was unaffected by the presence of IL-4. P values shown are for two-tailed t-test between Mtb alone and Mtb/IL-4.

3.2.4 IL-4 inhibits Mtb-driven secretion of pro-inflammatory chemokines and cytokines by MDMs

As a Th2 anti-inflammatory cytokine, we hypothesized that IL-4 might suppress MMP expression and activity, in addition to its known effects on macrophage phenotype. Having observed reduced macrophage MMP expression in IL-4-treated cells, I therefore went on to investigate whether in this model system other anticipated effects of IL-4 on macrophage activation were observed. A cytokine/chemokine multiplex panel was used to quantify the secretion of 27 analytes in cell culture supernatants from two experiments as performed above. Figure 4 shows the results obtained for selected analytes.

IL-1β, IL-6, TNFα, CCL5 and MIP1α secretion was significantly up-regulated by Mtb, and was inhibited by IL-4 to basal levels ($p>0.05$ vs control). In contrast, IL-12 secretion was increased 48-fold in Mtb-infected samples but was not significantly inhibited by IL-4. Results shown are from one representative experiment of two independent experiments performed in triplicate in different donors.

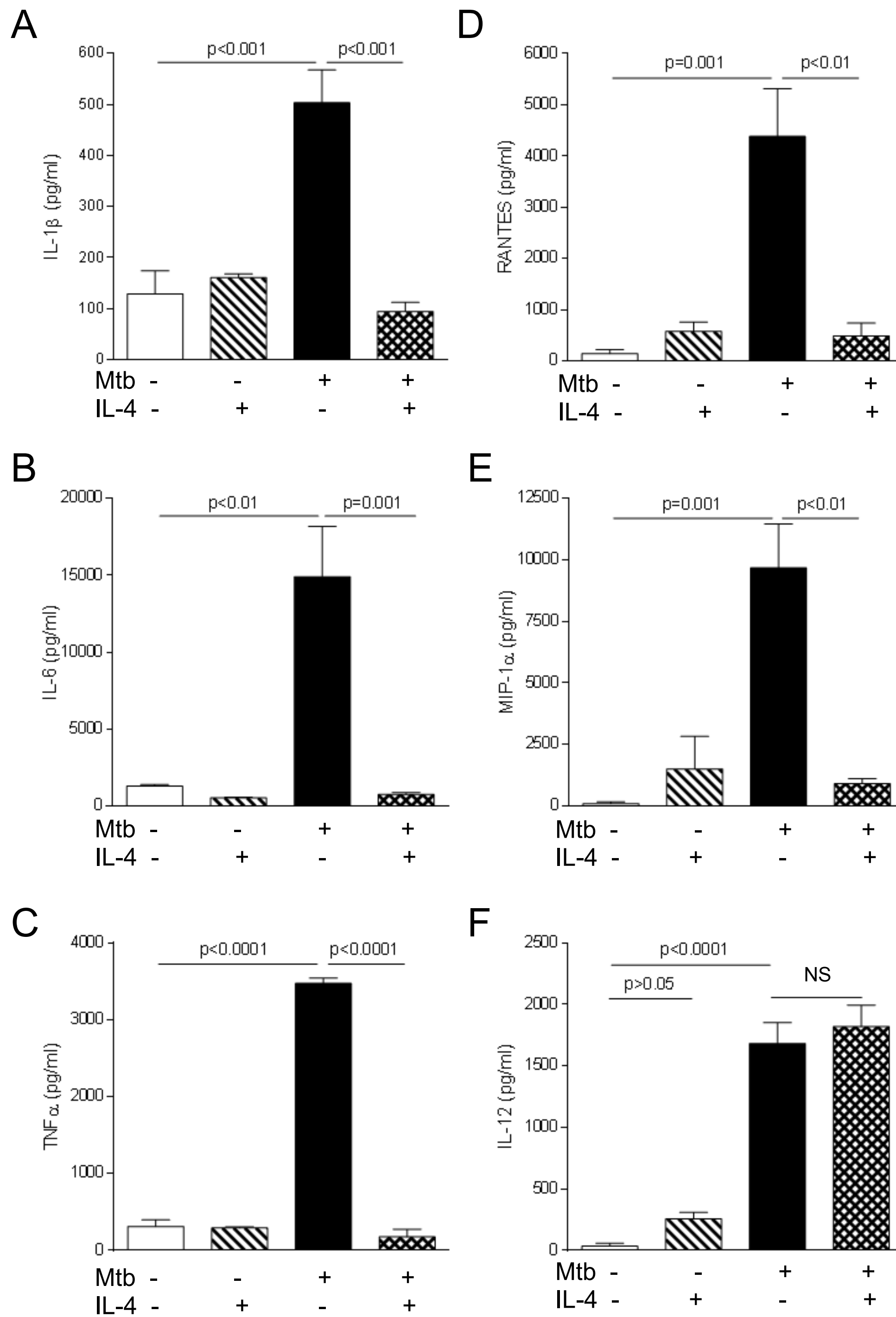


Figure 4. IL-4 inhibits Mtb-driven secretion of pro-inflammatory chemokines and cytokines by MDMs

MDMs were pre-incubated with IL-4 (20 ng/ml) for 2 hours and infected with H37Rv (MOI 1). Supernatants were collected at 72 hours post-infection and chemokine and cytokine concentrations were measured by Luminex. A) IL-1β secretion increased from 129 to 504 pg/ml in Mtb-infected samples ($p<0.001$) and was inhibited by IL-4 to basal levels ($p>0.05$ vs control). B) IL-6 secretion increased from 1293 to 14902 pg/ml in Mtb-infected samples ($p<0.0001$) and was inhibited by IL-4 to basal levels ($p>0.05$ vs control). C) TNFα secretion increased from 158 to 3383 pg/ml in Mtb-infected samples ($p<0.0001$) and was inhibited by IL-4 to basal levels ($p>0.05$ vs control). D) CCL5 (RANTES) secretion increased from 149 to 4387 pg/ml in Mtb-infected samples ($p=0.001$) and was inhibited by IL-4 to 497pg/ml ($p>0.05$ vs control). E) CCL3 (MIP1α) secretion increased from 92 to 9672 pg/ml in Mtb-infected samples ($p=0.001$) and was inhibited by IL-4 to 901 pg/ml ($p>0.05$ vs control). F) IL-12 secretion increased from 35 to 1682 pg/ml in Mtb-infected samples but was not significantly inhibited by IL-4 (1820 pg/ml, $p>0.05$ vs Mtb alone).

3.2.5 IL-4 does not affect the ability of MDMs to control H37Rv infection

IL-4 selectively inhibited macrophage secretion of certain MMPs and cytokines, and therefore I investigated whether this translated into an altered ability of IL-4-treated MDMs to kill intracellular H37Rv. In this model system monocyte-derived macrophages are cultured with Mtb in the absence of other cell types such as cytotoxic T cells or NK cells, and therefore the effects of a single cytokine on macrophage phenotype can more readily be investigated.

MDMs were pre-incubated with IL-4 (20 ng/ml) for 2 hours prior to infection with H37Rv (MOI 1). 72 hours later the infected cells were lysed in 0.1% Triton X and the lysates plated on 7H11 agar for quantification of cfus. No difference in cfus was observed between the IL-4-treated and untreated samples ($p>0.05$) (Figure 5). Results shown are from one experiment performed in triplicate, representative of results from two independent experiments performed in different donors.

This suggests that although IL-4 has significant effects on macrophage production of inflammatory mediators such as cytokines, chemokines and MMPs, over the short timecourse of these experiments it does not impair the ability of MDMs to kill, or control replication of, Mtb. The preserved ability to secrete IL-12 described above in section 3.2.4 may be relevant to this phenomenon.

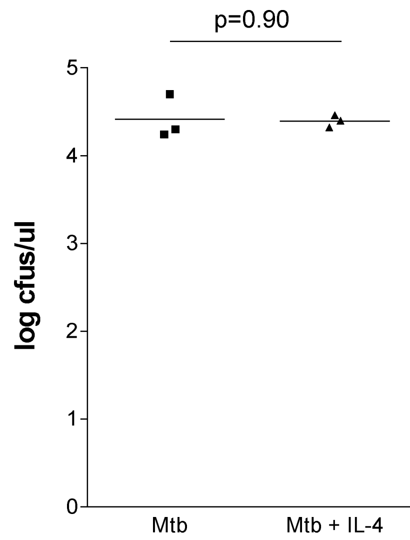


Figure 5. IL-4 does not affect the ability of MDMs to control H37Rv infection

MDMs were pre-incubated with IL-4 (20 ng/ml) for 2 hours prior to infection with H37Rv (MOI 1). Cells were lysed in 0.1% Triton X after 72 hours and plated on 7H11 agar for quantification of colony-forming units (cfus). No difference in cfus was observed between the cytokine treated and untreated samples ($p>0.05$).

3.2.6 IL-4 inhibits MMP secretion by respiratory epithelial cells

Next, I investigated whether IL-4 also affected MMP expression in respiratory epithelial cells. Preliminary experiments to investigate the effect of IL-4 were performed in the A549 cell line. Cells were pre-incubated with IL-4 at a range of concentrations for 2 hours prior to stimulation with CoMTb 1:5 for 72 hours. MMP-3 protein secretion was measured by ELISA.

MMP-3 was undetectable in culture supernatants from unstimulated cells (<16 pg/ml) and was induced by treatment with CoMTb (691 pg/ml). Exposure to IL-4 alone did not induce MMP-3 secretion (data not shown). IL-4 inhibited CoMTb-induced MMP-3 secretion in a concentration-dependent manner between 1 and 20 ng/ml. IL-4 (10 ng/ml) reduced MMP-3 secretion by 37% ($p<0.001$) and IL-4 (20 ng/ml) reduced MMP-3 secretion by 56% ($p<0.0001$) (Figure 6a).

Further experiments were performed in primary NHBEs pre-incubated with IL-4 for 2 hours prior to stimulation with CoMTb 1:5 for 72 hours. MMP-1 and -3 in culture supernatants were measured by ELISA. MMP-9 was measured by Luminex and gelatin zymography. MMP-1 secretion increased from 329 to 4398 pg/ml with CoMTb stimulation, and this was inhibited by 50% in the presence of IL-4 (20 ng/ml) ($p<0.01$) (Figure 6b). MMP-3 secretion from CoMTb-stimulated NHBEs was 2.8–fold higher than basal secretion ($p<0.001$) and was inhibited in a dose-dependent manner by IL-4. IL-4 (20 ng/ml) reduced MMP-3 secretion to basal levels ($p>0.05$ vs control) (Figure 6c). MMP-9 secretion as measured by Luminex increased 10.2-fold with CoMTb stimulation and was reduced by 16% with IL-4 (20 ng/ml) ($p<0.001$) (Figure 6d). Gelatin zymography demonstrated significantly reduced MMP-9 gelatinolytic activity in the same samples (Figure 6e).

Investigation of the effect of IL-4 on TIMP-1 and -2 secretion by CoMTb-stimulated NHBEs showed that TIMP production was relatively unaffected compared to the MMPs studied (data not shown).

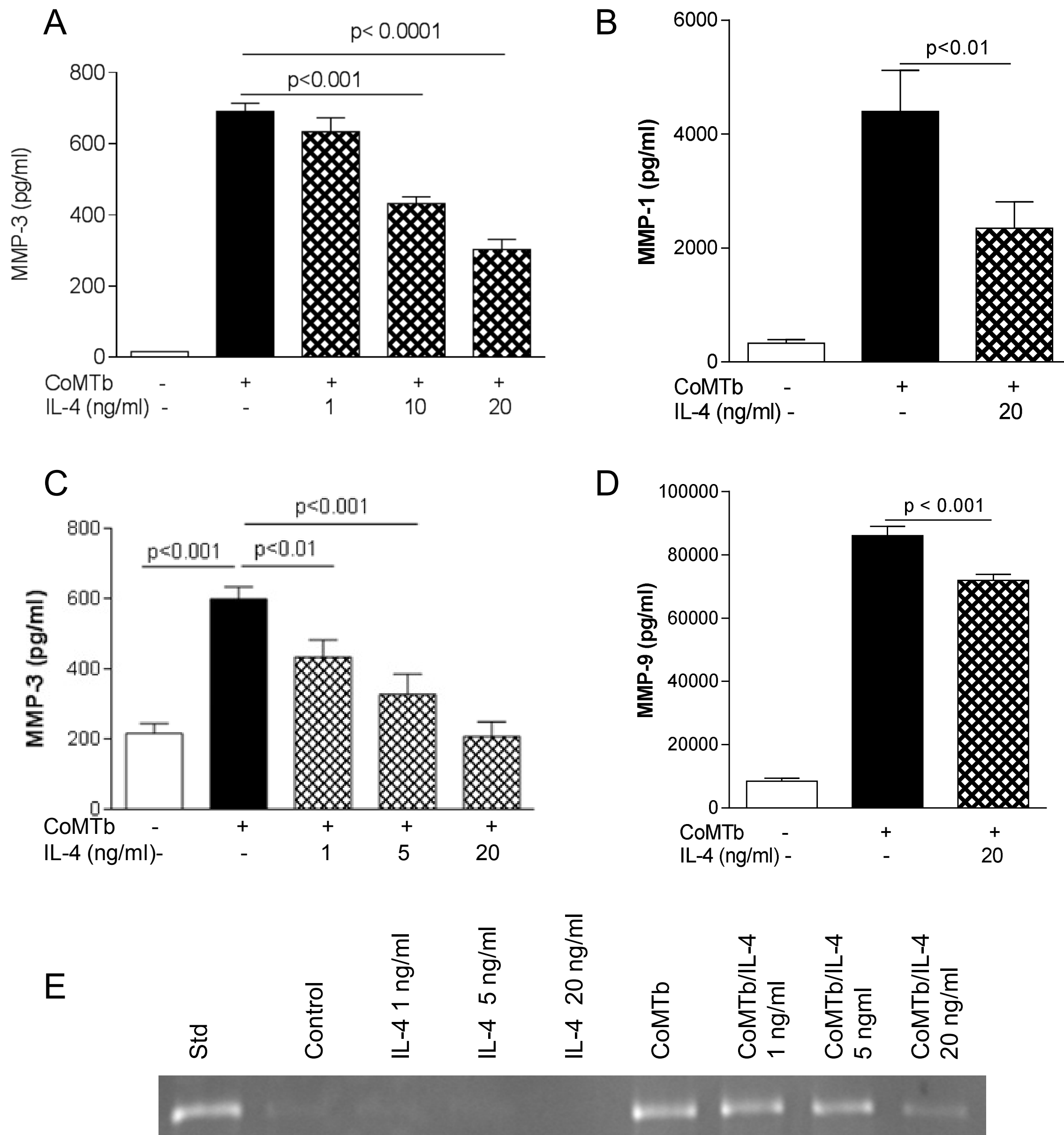


Figure 6. IL-4 inhibits MMP-1 and -3 secretion by CoMTb-stimulated epithelial cells

A549s and NHBEs were pre-incubated with IL-4 at the indicated concentrations for 2 hrs followed by stimulation with CoMTb 1:5 for 72 hrs. MMP-1 and -3 in culture supernatants were measured by ELISA. MMP-9 was measured by Luminex and gelatin zymography. A) MMP-3 secretion by A549 cells was induced by CoMTb (691 pg/ml). IL-4 inhibited CoMTb-induced MMP-3 secretion in a concentration-dependent manner. IL-4 (10 ng/ml) reduced MMP-3 secretion by 37% ($p<0.001$) and IL-4 (20 ng/ml) reduced MMP-3 secretion by 56% compared to CoMTb alone ($p<0.0001$). B) In NHBEs MMP-1 secretion increased from 329 to 4398 pg/ml with CoMTb stimulation, and this was inhibited by 50% in the presence of IL-4 (20 ng/ml) ($p<0.01$). C) MMP-3 secretion from CoMTb-stimulated NHBEs was 2.8–fold higher than basal secretion ($p<0.001$) and was inhibited in a dose-dependent manner by IL-4. IL-4 (20 ng/ml) reduced MMP-3 secretion to basal levels ($p>0.05$ vs control). D) NHBE MMP-9 secretion increased 10.2-fold with CoMTb stimulation and was reduced by 16% with IL-4 (20 ng/ml) ($p<0.001$). E) Gelatin zymography showed significantly reduced MMP-9 gelatinolytic activity with IL-4.

3.2.7 Critical sites situated between 2001 and 2942 bp in the MMP-1 promoter determine CoMTb-inducible activity

In order to investigate the signaling pathways mediating the inhibitory effect of IL-4 on MMP expression, I first examined the regulatory role of different regions of the MMP-1 promoter by transiently transfecting different promoter constructs into epithelial cells. Wild-type and deletion constructs of the MMP-1 promoter were transiently transfected into A549 cells alongside a control plasmid. Figure 7a shows the relative sizes of the constructs used, and the positions of the truncations relative to selected transcription factor binding sites predicted from the nucleotide sequence of the promoter.

MMP-1 wild-type promoter activity increased 3.1-fold with CoMTb compared to control conditions ($p<0.05$). Truncation of the promoter to 3830 bp and 2942 bp resulted in significantly increased CoMTb-stimulated activity (6.2-fold greater and 5.4-fold greater than WT/control respectively), suggesting that this region contains elements involved in restraining promoter activation. Further truncation of the promoter construct to 2001 bp resulted in the loss of inducible activity (2001/CoMTb not significantly different to WT/control), which implies that key activating elements are located in the -2942 to -2001 bp region of the MMP-1 promoter (Figure 7b). Of note, the -2001 bp truncation site is in close proximity to an AP-1 binding site at -1949 to -1955 bp (Figure 7a), although the nucleotide sequence of the AP-1 binding site is not affected. The results shown are from one experiment performed in triplicate, and are representative of results from at least three independent experiments.

3.2.8 MMP-3 promoter activity is reduced step-wise by progressive truncation of the promoter

A smaller range of three short MMP-3 promoter constructs was transfected into A549 cells (Figure 7c). The longest construct (2183 bp) displayed the greatest basal and inducible activity, and CoMTb stimulation increased promoter activity 2.1-fold compared to control conditions ($p<0.001$). Basal activity of the 1612 bp construct was 65% of that of the 2183 bp construct, although the difference was not statistically significant. The promoter activity of the

1612 bp construct increased 1.9-fold with CoMTb compared to control conditions ($p<0.05$). The activity of the shortest promoter construct, measuring 642 bp, was greatly reduced compared to the longer constructs ($p<0.01$) and CoMTb did not induce a significant increase in promoter activity. The results shown are from one experiment performed in triplicate, and are representative of results from two independent experiments.

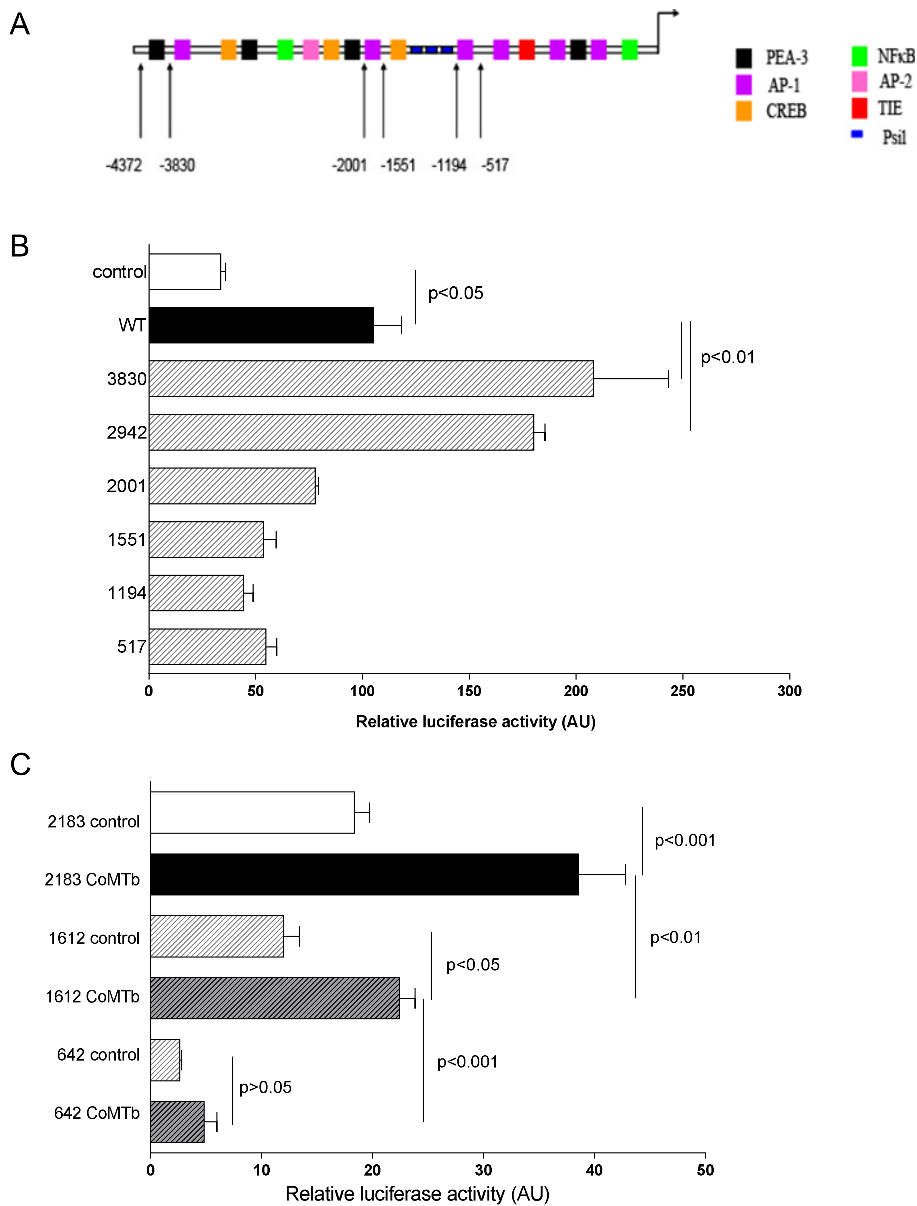


Figure 7. Transient transfection of respiratory epithelial cells with plasmid constructs of the MMP-1 and -3 promoters

A549s were transiently transfected with plasmid constructs of the MMP-1 or -3 promoters and a control plasmid. Cells were then stimulated with CoMTb for 24 hours or treated with control medium. Cells were lysed and a dual luciferase assay was performed. A) Schematic diagram showing the MMP-1 promoter annotated with the location of the truncations and the location of selected transcription factor binding sites. B) MMP-1 wild-type promoter activity was increased 3.1-fold with CoMTb compared to control conditions ($p<0.05$). Truncation of the promoter to 3830 bp and 2942 bp resulted in significantly increased CoMTb-stimulated activity ($p<0.01$ compared to WT/CoMTb) (6.2-fold greater and 5.4-fold greater than WT/control respectively). Further truncation of the promoter construct resulted in loss of inducible activity (activity of 2001 bp to 517 bp constructs not significantly different from WT/control). C) In A549 cells transfected with MMP-3 promoter constructs, CoMTb stimulated significantly increased promoter activity of the 2183 bp and 1612 bp constucts, but not the 642 bp construct. Basal and inducible promoter activity decreased step-wise with truncation of the promoter.

3.2.9 MMP-1 promoter activity is not inhibited by IL-4

Having demonstrated that key regulatory sites situated 2001 to 2942 bp upstream of the MMP-1 transcriptional start site were required for CoMTb-driven promoter activity, I went on to investigate how promoter activity was affected by the presence of IL4. Transient transfection of A549 cells with a wild-type MMP-1 promoter-reporter plasmid construct demonstrated that promoter activity was increased 3.2-fold after 24 hours of CoMTb stimulation ($p<0.01$), and this was unaltered in the presence of IL-4 at a concentration that significantly inhibited MMP-1 secretion ($p>0.05$) (Figure 8a). This finding contrasts with the observation that MMP-1 and -3 mRNA were reduced in IL-4-treated MDMs, and suggests that IL-4 may be influencing mRNA transcription or stability rather than the assembly of the transcriptional start complex at the MMP transcriptional start site. Alternatively, this lack of effect may be due to the nature of this experimental model, as although the plasmid DNA sequence is identical to the chromosomal MMP-1 promoter sequence, higher order structures and chromatin organisation are not reproduced. Therefore, if such factors are critical to the influence of IL-4 on MMP-1 promoter activity, the plasmid reporter-reporter assay will not detect this.

3.2.10 Induction of MMP-1 promoter activity by CoMTb requires a functional AP-1 site at -1950 bp

Transient transfection of A549 cells with a wild-type MMP-1 promoter-reporter plasmid construct and AP-1 mutated constructs demonstrated that the AP-1 site located 1950 bp upstream of the MMP-1 transcriptional start site is necessary for increased promoter activity in response to CoMTb ($p<0.01$) (Figure 8b). Activity of the AP-1-mutated construct was unchanged with CoMTb compared to control conditions ($p>0.05$), indicating that activation of the AP-1 transcription factor family may be a critical final pathway mediating increased MMP-1 transcription in response to CoMTb.

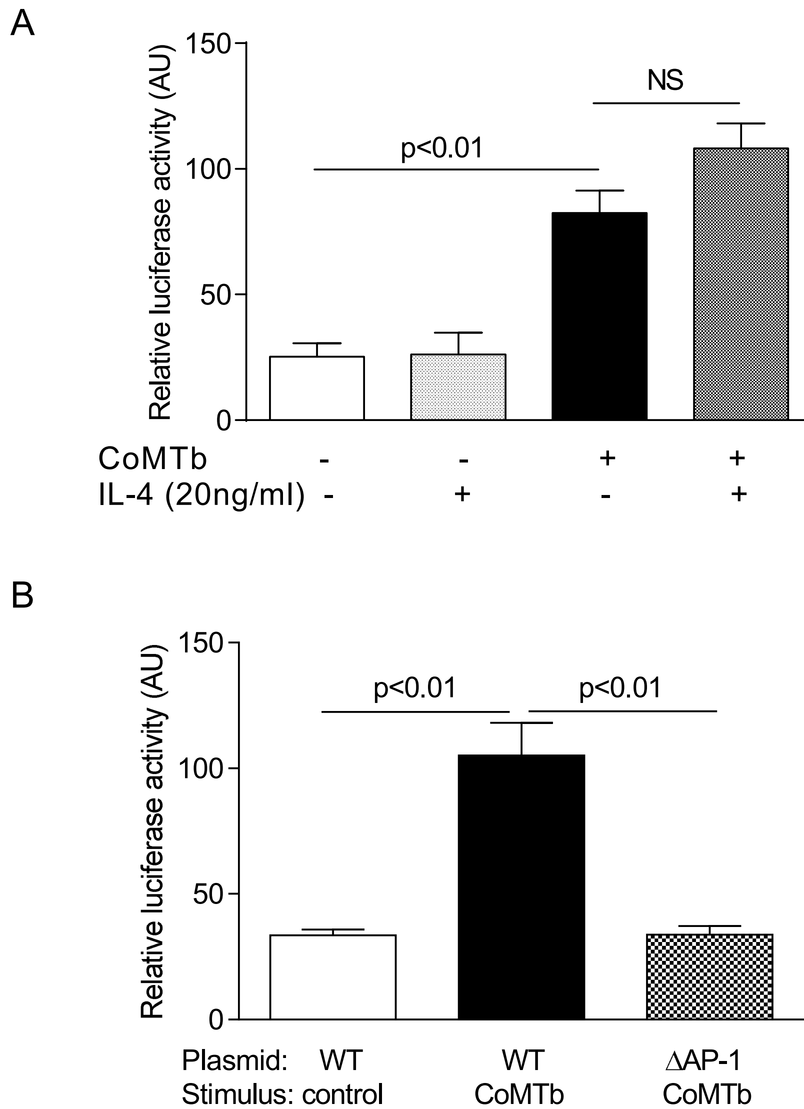


Figure 8. MMP-1 promoter activity is unaltered in the presence of IL-4 but the AP-1 consensus binding site at -1950 bp is necessary for promoter activation by CoMTb

A) Transient transfection of A549 cells with a wild-type MMP-1 promoter-reporter plasmid construct demonstrates promoter activity increases 3.2-fold with CoMTb stimulation ($p<0.01$), and this is unaltered in the presence of IL-4 (20 ng/ml) ($p>0.05$). B) Transient transfection of A549 cells with a wild-type MMP-1 promoter-reporter plasmid construct and AP-1 mutated constructs demonstrates that the AP-1 site is necessary for increased promoter activity in response to CoMTb ($p<0.01$). Results shown are from one representative experiment of four experiments performed in triplicate.

3.2.11 CoMTb-treatment of NHBEs drives AP-1 DNA binding activity which is impaired in the presence of IL-4

As the transient transfection experiments suggested AP-1 signalling might be key to CoMTb-driven MMP-1 expression, AP-1 activity was investigated in CoMTb-stimulated cells. NHBEs were stimulated with CoMTb in the presence or absence of IL-4 (20 ng/ml) for 30 and 60 minutes. Nuclear extracts were prepared and assayed for AP-1 subunit binding to the AP-1 consensus binding sequence using the TransAM AP-1 Family kit.

Binding activity of cJun, JunD, JunB and Fos was increased in the CoMTb-stimulated cells compared to control conditions, and there was reduced binding activity when cells were treated with IL-4 in addition to CoMTb (Figure 9). In contrast, binding activity of the Fra-1 and -2 subunits increased in response to CoMTb but appeared to be unaffected by IL-4. The results shown are from one representative example of two independent experiments which were not performed in triplicate.

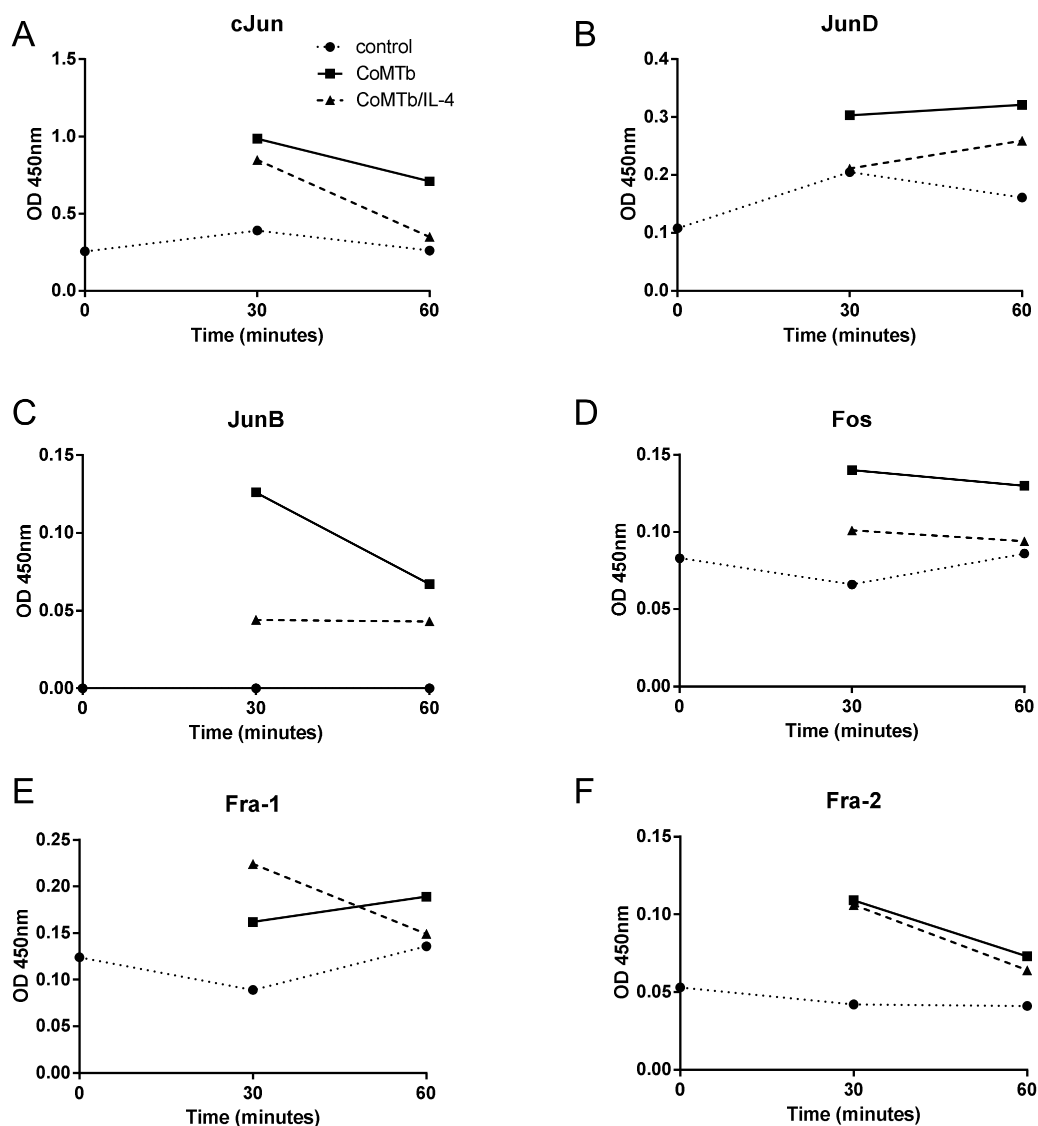


Figure 9. CoMTb-treatment of NHBEs drives AP-1 DNA binding activity which is impaired in the presence of IL-4

NHBEs were stimulated with CoMTb in the presence or absence of IL-4 (20 ng/ml) for 30 or 60 minutes. Nuclear extracts were prepared and assayed for AP-1 subunit binding using the TransAM AP-1 Family kit. A) cJun binding activity increased 3.9-fold over baseline after 30 min CoMTb-stimulation and remained elevated at 60 min. In the presence of IL-4 binding increased 3.4-fold at 30 min but was reduced to control levels at 60 min. B) JunD binding increased 2-fold with CoMTb at 30 and 60 min, but 1.4-fold and 1.7-fold respectively with IL-4/CoMTb combined. C) JunB binding activity was undetectable in control samples, and decreased in CoMTb/IL-4 treated samples compared to CoMTb alone. D) Fos binding activity was increased 1.8-fold at 30 min and 1.6-fold at 60 min with CoMTb compared to control. The presence of IL-4 reduced this to a 1.3-fold and 1.2-fold increase respectively. E) Fra-1 binding activity was increased 1.4 and 1.6-fold with CoMTb compared to control, and similarly increased with CoMTb/IL-4. F) Fra-2 activity was increased 2-fold at 30 min with either CoMTb or CoMTb/IL-4 compared to control.

3.2.12 MAPK activation in response to CoMTb is not altered in the presence of IL-4

MMP-1 expression in response to Mtb has previously been shown to be dependent on p38 Mitogen-Activated Protein Kinase (MAPK) signalling [79]. Mtb infection also affects monocyte and macrophage cytokine and chemokine production by influencing signalling through p38, Extracellular signal-Regulated Kinase (ERK) and c-Jun N-terminal Kinases (JNK) [299-301]. Therefore I investigated whether IL-4 treatment affected activation of the MAPK pathways by CoMTb. A549 cells were pre-incubated with IL-4 (10 ng/ml) for 2 hours and then stimulated for 30 minutes with CoMTb or CoMCont 1:5. The cells were lysed in Western lysis buffer and Western blotting was performed with antibodies to the phosphorylated and total forms of p38, ERK and JNK MAPK.

CoMTb treatment drove phosphorylation of p38, ERK and JNK at 30 minutes, as was expected. IL-4 alone did not alter phosphorylation compared to CoMCont, and CoMTb-driven MAPK phosphorylation was not altered in IL-4-treated cells (Figure 10), suggesting that the suppression of MMP expression observed with IL-4 was not mediated by changes in MAP kinase pathway activation.

3.2.13 Stat 6 is phosphorylated in response to IL-4 treatment of NHBEs or MDMs

As IL-4 did not appear to alter CoMTb-driven MAPK activation, its effect on Stat 6 activation was also examined. NHBEs were treated with CoMTb 1:5 with or without IL-4 (20 ng/ml) for 30 minutes, followed by lysis of the cells and Western blotting with antibodies to phospho-Stat 6. Phospho-Stat 6 was detectable only in the IL-4-treated cells (Figure 11a). A Western blot for β-actin was performed to control for protein loading.

In similar experiments, MDMs were infected with H37Rv (MOI 1) in the presence or absence of IL-4 (20ng/ml). Again, Stat 6 phosphorylation was only detected in the IL-4-treated cells (Figure 11b). No interaction between Mtb- or CoMTb-stimulation of either cell type and the phosphorylation of Stat 6 consequent on IL-4 exposure was observed.

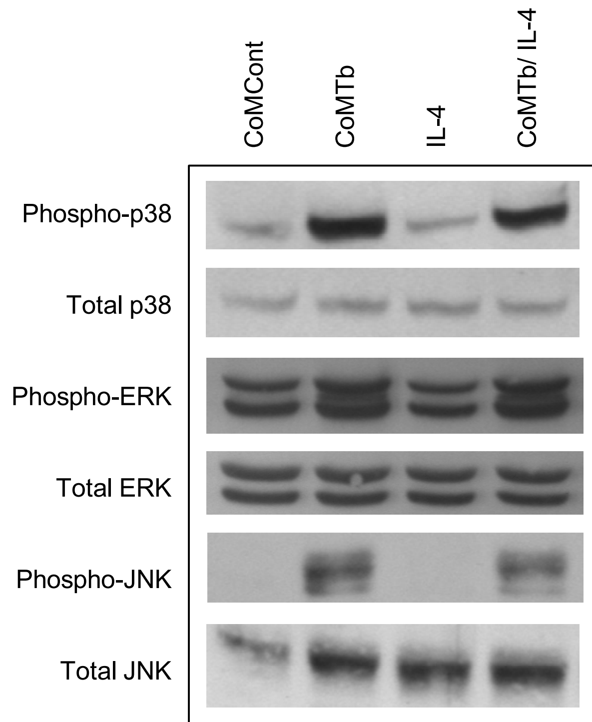


Figure 10. CoMTb-driven MAPK phosphorylation is not altered in IL-4-treated A549s

A549 cells were pre-incubated with IL-4 (10 ng/ml) for 2 hours and stimulated with CoMTb 1:5. Cells were lysed after 30 minutes and Western blotting for total and phospho- p38, ERK and JNK was performed on the lysates. Phospho-p38, ERK and JNK were increased in CoMTb-stimulated cells independently of IL-4 treatment. Total p38, ERK and JNK were unaffected by either IL-4 or CoMTb treatment.

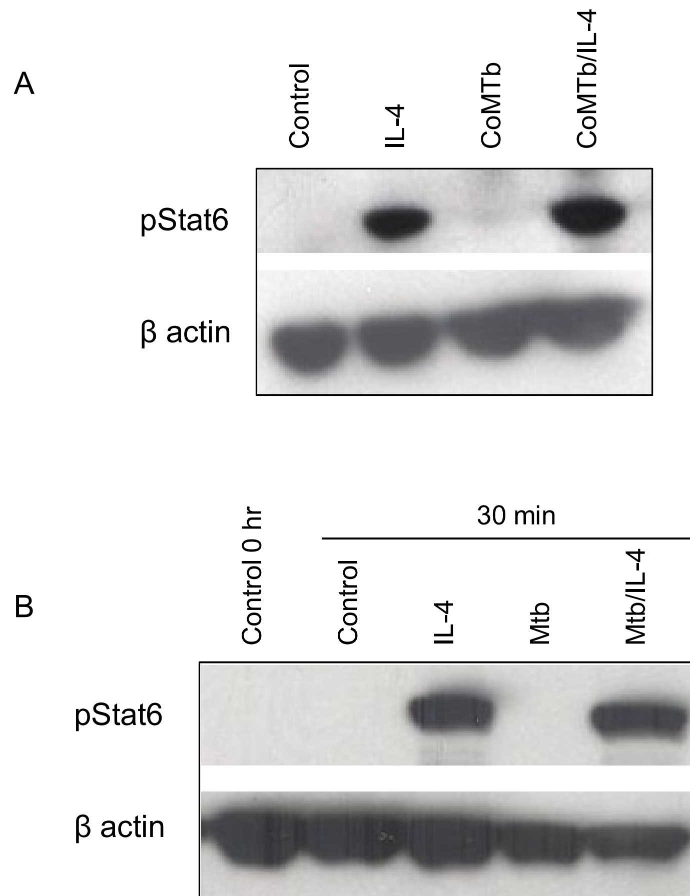


Figure 11. Stat 6 is phosphorylated in response to IL-4 treatment of NHBEs or MDMs

A) NHBEs treated with CoMTb +/- IL-4 (20 ng/ml) for 30 minutes. Phospho-Stat 6 is detectable only in the IL-4-treated cells. B) MDMs infected with H37Rv (MOI 1) in the presence or absence of IL-4 (20 ng/ml). Phospho-Stat 6 is only detected in the IL-4-treated cells. The results shown are representative of two independent experiments, not performed in triplicate.

3.2.14 IL-4 reduces RNA Pol II binding and histone H4 acetylation at the MMP-1 promoter in CoMTb-stimulated A549s

Chromatin immunoprecipitation (ChIP) assays were performed in A549 cells stimulated with CoMTb with or without pre-treatment with IL-4 (20 ng/ml). Timepoints from 1 to 5 hours were investigated. Following fixation and shearing, samples were immunoprecipitated overnight with antibodies directed against RNA Polymerase II (RNA Pol II) or acetyl-histone H4 as well as control IgG. DNA was extracted and PCR performed with primers directed against different areas of the MMP-1 promoter (Figure 12a).

The results shown in Figure 12b show that RNA Pol II association with the MMP-1 promoter was increased under CoMTb-stimulated conditions compared to control, and there was a trend to reduced RNA Pol II binding where the cells had been pre-treated with IL-4. Interestingly, the highest RNA Pol II binding was detected at the -4000 bp promoter site, rather than at the transcriptional start site. There was not sufficient time to investigate this phenomenon further, but it could be explained by the presence of an alternative reading frame, for example for a non-coding RNA molecule at this region, or by the detection of "paused" RNA polymerase molecules. Statistical analysis of the results was not possible as the experiment could not be performed in triplicate and results of different experiments could not be combined due to excessive variability. When acetylation of histone H4 was examined, this appeared to be increased across the promoter region in cells treated with CoMTb, but when IL-4 was also present detection of H4 acetylation was reduced to control levels (Figure 12c).

One of the difficulties encountered in performing these ChIP assays was of variable control IgG results between samples. For this reason, the results shown in Figure 12 have been analysed by the percentage input method, and then normalised to the IgG results for each sample. These results are from one experiment, representative of two independent experiments.

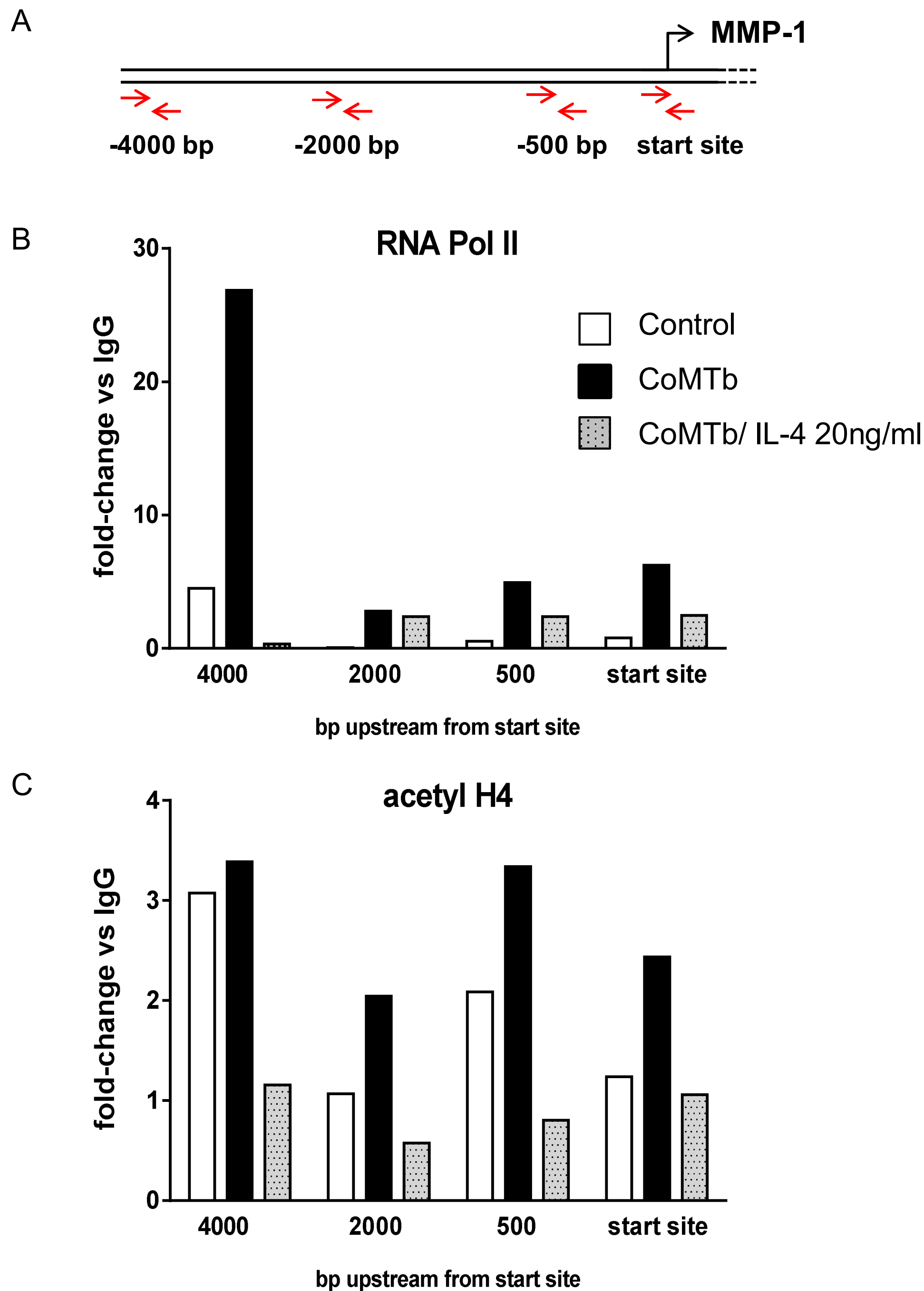


Figure 12. IL-4 reduces RNA Pol II binding and histone H4 acetylation at the MMP-1 promoter in CoMTb-stimulated A549s

ChIP assays were performed on A549 cells treated with CoMTb or control medium for 1 hour with or without pre-treatment with IL-4 (20 ng/ml). Cells were fixed with paraformaldehyde and chromatin was sheared by sonication. Following overnight immunoprecipitation with normal mouse IgG, RNA Pol II or anti-acetyl histone H4 antibody, DNA was extracted and PCR performed with primers directed against different regions of the MMP-1 promoter. A) Diagram showing binding location of ChIP primer pairs used. B) RNA Pol II association with the MMP-1 promoter increases with CoMTb treatment and this appears to be reduced by pre-treatment with IL-4. C) Histone H4 acetylation in the proximal promoter is enhanced in the presence of CoMTb, and reduced to control levels in IL-4 pre-treated cells.

3.3 Interleukin-13 inhibits Mtb-driven MMP expression

3.3.1 IL-13 inhibits CoMTb-driven MMP secretion by respiratory epithelial cells

In preliminary experiments to investigate whether the related Th2 cytokine IL-13 had the same effect on MMP secretion as IL-4, A549s were pre-incubated with IL-13 (1-20 ng/ml) for 2 hours prior to stimulation with CoMTb 1:5 for 72 hours. MMP-1 and -3 in the culture supernatants were quantified by Luminex or ELISA. IL-13 (10 ng/ml) inhibited CoMTb-stimulated MMP-3 secretion by 40% ($p<0.05$) but there was no further inhibition at a higher concentration of 20 ng/ml (Figure 13a).

In NHBEs, early experiments found no significant effect of IL-13 on MMP-3 secretion, but at a higher concentration of 50 ng/ml, MMP-3 secretion was inhibited to basal levels (from 905 to 462 pg/ml) ($p>0.05$ compared to control) (Figure 13b). CoMTb-stimulated MMP-1 secretion by NHBEs was significantly decreased by IL-13 (20 ng/ml) from 1970 to 1448 pg/ml ($p<0.05$) (Figure 13c). The effect of the higher concentration of IL-13 on MMP-1 secretion was not assessed. Results shown are from one experiment performed in triplicate, representative of 3 experiments performed independently. MMP-9 secretion, measured by Luminex, was unaffected by IL-13 (10 ng/ml) (data not shown), and when enzymatic activity was assessed by gelatin zymography no significant difference in MMP-9 gelatinolytic activity was identified with IL-13 concentrations from 5-20 ng/ml (data not shown).

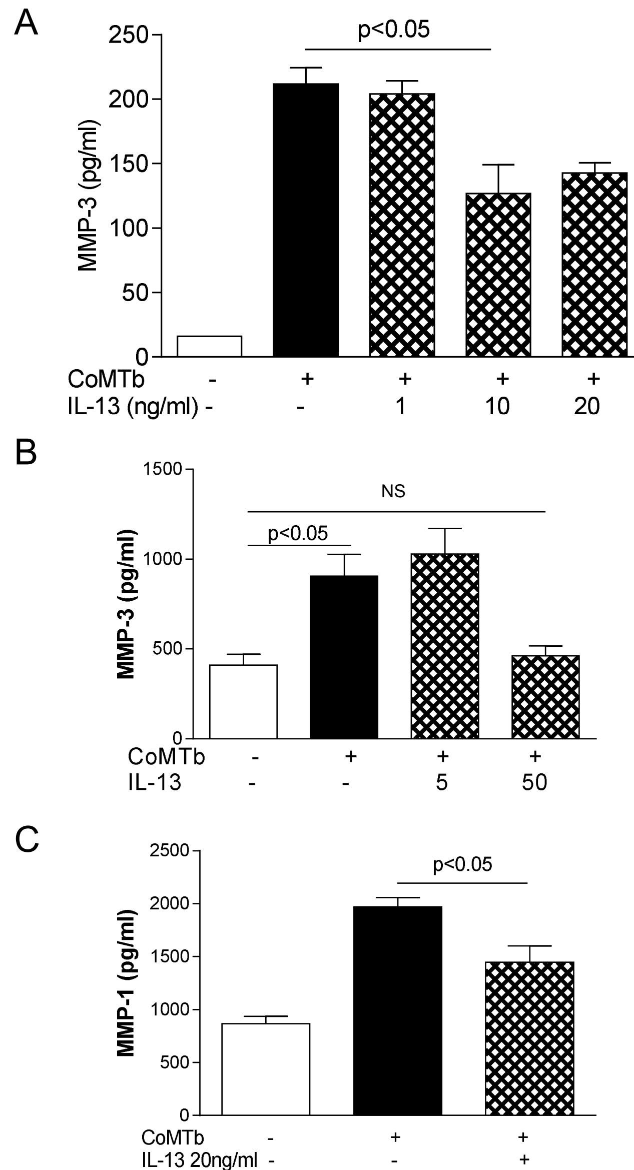


Figure 13. IL-13 inhibits CoMTb-driven MMP secretion by respiratory epithelial cells

A549s and NHBEs were pre-incubated with IL-13 (1-20 ng/ml) for 2 hours prior to stimulation with CoMTb 1:5 for 72 hours. MMP-1 and -3 in the culture supernatants were quantified by Luminex or ELISA. A) IL-13 (10 ng/ml) inhibited CoMTb-stimulated MMP-3 secretion in A549s by 40% ($p<0.05$) but there was no further inhibition at a higher concentration of 20 ng/ml. B) In NHBES, CoMTb-stimulated MMP-3 secretion was decreased by IL-13 (50 ng/ml) from 905 to 462 pg/ml. C) CoMTb-stimulated MMP-1 secretion by NHBEs was decreased by 47% by IL-13 (20 ng/ml) from 1970 to 1448 pg/ml ($p<0.05$).

3.3.2 IL-13 inhibits MMP-1 and -3 but not MMP-7 secretion by Mtb-infected MDMs

As IL-13 had a similar effect on epithelial cell MMP expression to IL-4, its effect on MDM MMP secretion was then investigated. MDMs were pre-incubated with IL-13 (1-20 ng/ml) for 2 hours prior to infection with H37Rv MOI 1. Supernatants were collected 72 hours later and MMP-1, -3 and -7 were quantified by Luminex. Mtb-driven MMP-1 secretion was inhibited by 46% in the presence of IL-13 (5 ng/ml), and this was statistically significant, but there was no further decrease in MMP-1 secretion when IL-13 was used at 20 ng/ml (Figure 14a). Macrophage MMP-3 secretion was significantly reduced by IL-13 (5 ng/ml) from 8286 to 5370 pg/ml ($p<0.01$), and further decreased to 3405 pg/ml ($p<0.001$) with IL-13 (20 ng/ml) (Figure 14b). As was observed in the IL-4 experiments, MMP-7 secretion was significantly increased in Mtb-infected cells, but was unaffected by IL-13 (5 or 20 ng/ml) ($p>0.05$ compared to Mtb alone) (Figure 14c). Results shown are from one experiment performed in triplicate, representative of four independent experiments performed in different donors.

The results seen with IL-13 were similar to those for IL-4 in both cell types, and therefore further investigation of intracellular signaling pathways was not performed for IL-13.

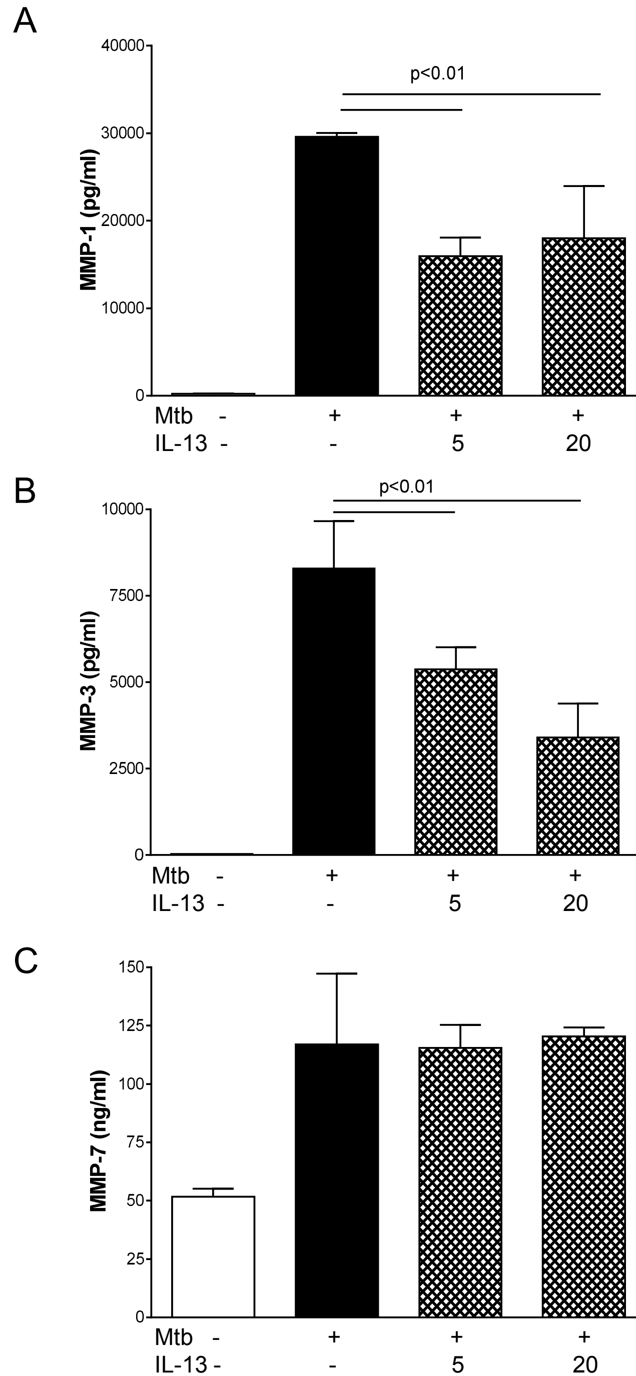


Figure 14. IL-13 selectively inhibits MMP-1 and -3 secretion by Mtb-infected MDMs

MDMs were pre-incubated with IL-13 (1-20 ng/ml) for 2 hours prior to infection with H37Rv MOI 1. Supernatants were collected 72 hours later and MMP-1, -3 and -7 were quantified by Luminex. A) Mtb-driven MMP-1 secretion was inhibited by 46% in the presence of IL-13 (5 ng/ml) ($p<0.01$) but there was no further decrease in MMP-1 when IL-13 was used at 20 ng/ml. B) MMP-3 secretion was significantly reduced by IL-13 (5 ng/ml) (from 8286 to 5370 pg/ml, $p<0.01$) and further decreased to 3405 pg/ml ($p<0.001$) with IL-13 (20 ng/ml). C) MMP-7 secretion was significantly increased in Mtb-infected cells, but was unaffected by IL-13 (5 or 20 ng/ml) ($p>0.05$ compared to Mtb alone).

3.4 Interleukin-10 does not influence Mtb-driven MMP expression

3.4.1 IL-10 does not affect Mtb-driven MDM MMP secretion

In order to investigate the hypothesis that IL-10 would inhibit Mtb-stimulated MMP expression in macrophages, MDMs were pre-incubated with IL-10 (1 to 20 ng/ml) for 2 hours, and then infected with H37Rv. Extracellular bacteria were washed off after 2 hours, but IL-10-supplemented culture medium was used throughout the experiment. Supernatants were collected at 72 hours post-infection and MMP-1, -3 and -7 were measured by Luminex.

Experiments were performed independently in five different donors. In three donors a trend to decreased MMP-1 secretion in the presence of IL-10 was observed, while in the other two MMP-1 secretion was slightly increased when cells were co-incubated with IL-10. In neither case did the changes achieve statistical significance. The results shown are from one of the donors with a trend to decreased MMP secretion and are representative of three donors (Figure 15a). MMP-3 and -7 secretion were also not significantly altered in the presence of IL-10 (Figure 15b,c).

3.4.2 IL-10 does not alter MMP or TIMP-1 secretion by respiratory epithelial cells

Similar experiments were performed in the A549 cell line and primary NHBE respiratory epithelial cells, with similarly negative results (Figure 15d, e, f). IL-10 might also inhibit MMP activity by increasing TIMP expression, but when this was assayed in three independent NHBE experiments, no significant effect of IL-10 on TIMP-1 secretion was found (data not shown). As IL-10 did not significantly affect MMP secretion in any of the cell types studied, further investigation was not pursued.

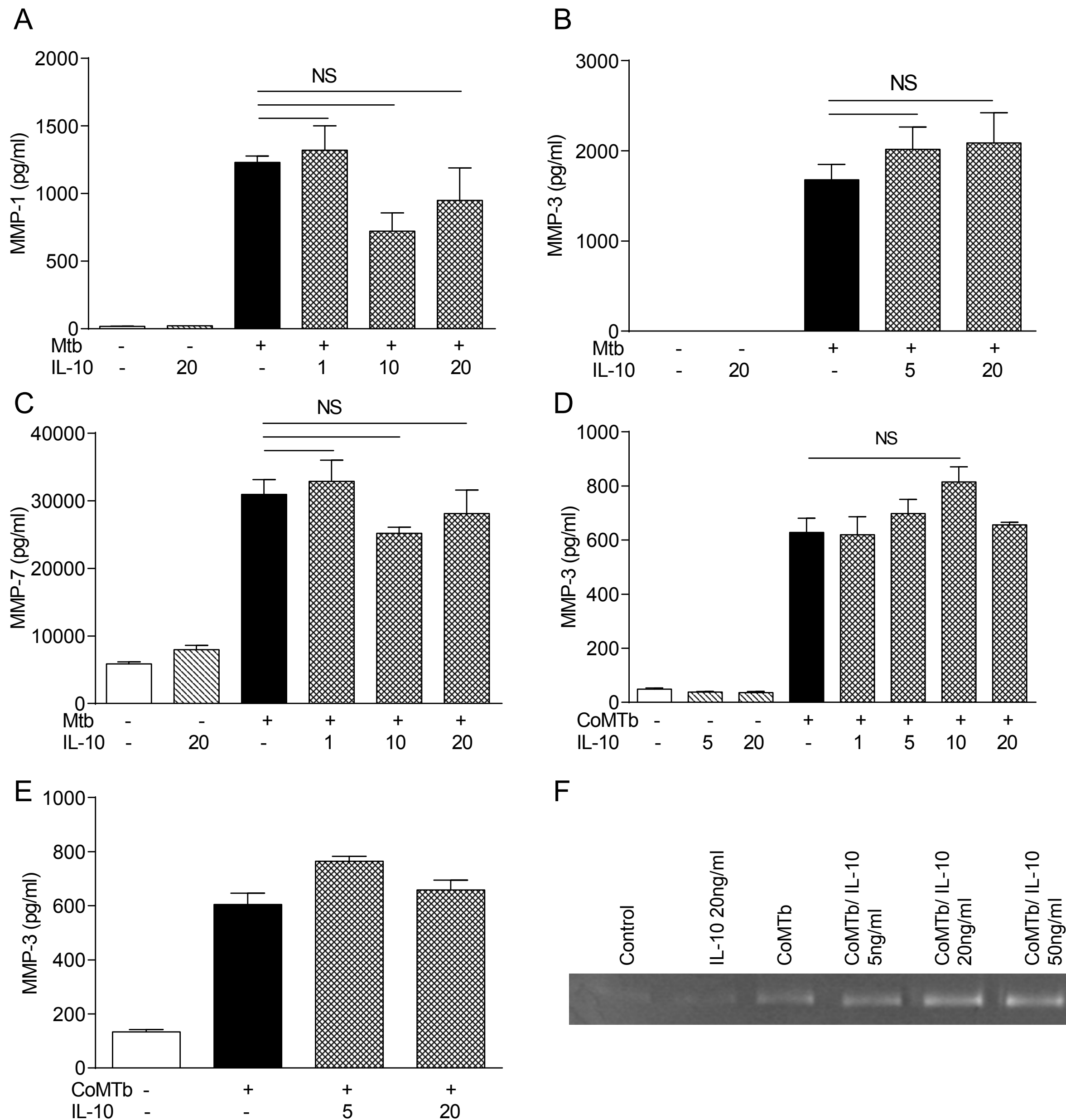


Figure 15. IL-10 does not affect Mtb-induced MMP secretion by MDMs or respiratory epithelial cells

A549s, NHBEs and MDMs were treated with IL-10 (1-20 ng/ml) prior to stimulation with CoMTb or infection with Mtb. Supernatants were collected after 72 hours and MMP-1, -3, -7 and -9 were quantified by ELISA, Luminex or zymography. A) MDM MMP-1 secretion was not significantly altered by IL-10 (1-20 ng/ml). B) MMP-3 secretion by Mtb-infected MDMs was not affected by IL-10 (5-20 ng/ml). C) MDM MMP-7 secretion was not significantly altered by IL-10 (1-20 ng/ml). D) A549 MMP-3 secretion was not affected by IL-10 (1-20 ng/ml) ($p>0.05$ vs CoMTb alone). E) In NHBEs MMP-3 secretion was not reduced in the presence of IL-10 (5-20 ng/ml) ($p>0.05$). F) NHBE CoMTb-induced MMP-9 gelatinolytic activity was not significantly reduced in the presence of IL-10 (5-20 ng/ml). The results shown are from one experiment performed in triplicate, but are representative of at least three independent experiments.

3.5 Transforming growth factor-β1 enhances epithelial cell MMP expression

3.5.1 TGFβ enhances respiratory epithelial cell MMP-1 and -3 secretion in response to CoMTb stimulation

Recombinant human TGFβ protein was used at 1-20 ng/ml in preliminary experiments to assess its effect on CoMTb-stimulated MMP-3 secretion by A549 cells. Supernatants were collected after 72 hours and MMP-3 was measured by ELISA. There was a small increase in MMP-3 secretion in the presence of TGFβ (10 ng/ml), but this increase was not statistically significant. CoMTb stimulation after pre-incubation with TGFβ (20 ng/ml) caused a 13.9-fold synergistic upregulation of MMP-3 secretion compared to CoMTb alone, which was highly statistically significant ($p<0.0001$) (Figure 16a). MMP-1 secretion was not assessed in the A549 experiments.

In NHBEs, TGFβ (20 ng/ml) was a greater stimulus to MMP-1 secretion than CoMTb (11762 vs 1970 pg/ml, $p=0.001$) and the combination of TGFβ and CoMTb increased MMP-1 secretion to 26831 pg/ml ($p<0.0001$ vs either stimulus alone) (Figure 16b). TGFβ (10 ng/ml) doubled MMP-3 secretion compared to control conditions, although this increase was not statistically significant, and also enhanced CoMTb-stimulated MMP-3 secretion, increasing MMP-3 from 1229 pg/ml to 2011 pg/ml ($p<0.0001$) (Figure 16c). Results shown are from one experiment performed in triplicate and are representative of at least 3 independent experiments. MMP-9 and TIMP-1 expression in NHBEs treated with TGFβ was not investigated.

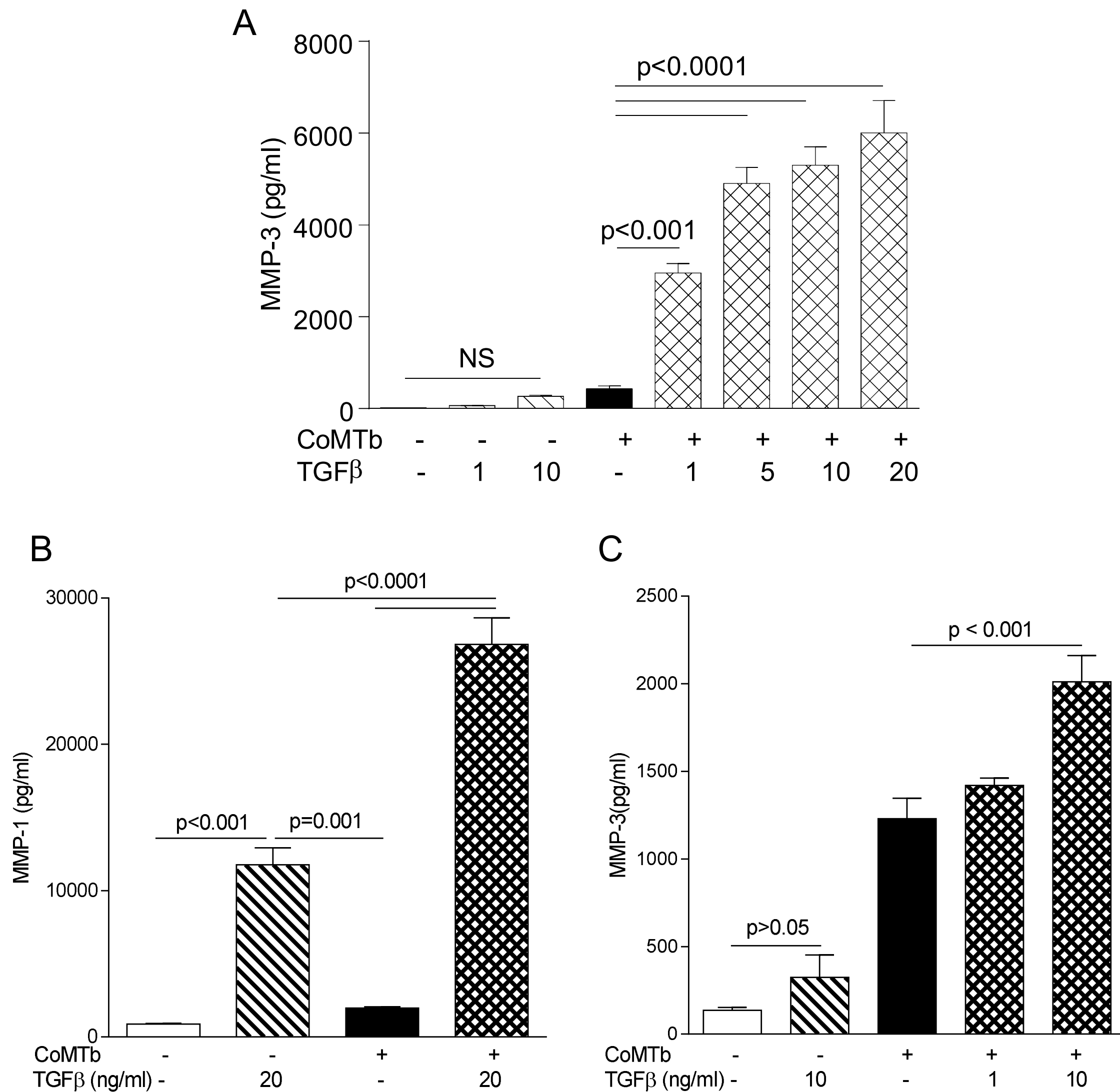


Figure 16. Transforming growth factor beta (TGFβ) enhances respiratory epithelial cell MMP-1 and -3 secretion in response to CoMTb stimulation

A549s and NHBEs were treated with TGFβ (1-20 ng/ml) prior to stimulation with CoMTb. Supernatants were collected after 72 hrs and MMP-1 and -3 were quantified by ELISA. A) In A549 cells CoMTb stimulation combined with TGFβ treatment caused a synergistic upregulation of MMP-3 secretion (MMP-3 secretion with CoMTb/TGFβ (20 ng/ml) was 13.9-fold higher than CoMTb alone, $p<0.0001$). B) In NHBEs, TGFβ (20 ng/ml) was a stronger stimulus to MMP-1 secretion than CoMTb (11762 vs 1970 pg/ml, $p=0.001$) and the combination of TGFβ and CoMTb produced an even greater increase in MMP-1 secretion to 26831 pg/ml ($p<0.0001$ vs either stimulus alone). C) In NHBEs, TGFβ (10 ng/ml) also enhanced CoMTb-stimulated MMP-3 secretion, increasing MMP-3 from 1229 pg/ml to 2011 pg/ml ($p<0.0001$).

3.5.2 TGFβ does not significantly affect Mtb-driven MMP secretion by MDMs

The effect of TGFβ on macrophage MMP expression was investigated in experiments in which MDMs were pre-incubated for 2 hours with TGFβ (0.1-50 ng/ml) prior to infection with H37Rv. The cell culture supernatant was collected after 72 hours and MMP-1, -3 and -7 were quantified by Luminex. The experiments were performed on four separate occasions using different blood donors. In one donor TGFβ treatment resulted in a trend to decreased MMP-1 and -3 secretion which was not statistically significant, whereas in the other three there was a consistent lack of effect. The results shown are from one representative experiment from these three donors. MDM MMP-1 secretion was not affected by TGFβ alone or in combination with Mtb infection (Figure 17a).

MMP-3 was undetectable in uninfected cells treated with TGFβ (10 or 50 ng/ml). In this donor, there was a trend to increased MMP-3 secretion with TGFβ and Mtb infection, which was only statistically significant with TGFβ (10ng/ml) ($p<0.05$) (Figure 17b). This seems biologically implausible, as the results with lower and higher concentrations of cytokine do not correlate with this result. MMP-3 responses were less consistent between donors than MMP-1 secretion, and this phenomenon was only seen in one donor.

Basal MMP-7 secretion by uninfected MDMs increased from 29 to 63 ng/ml with TGFβ (50 ng/ml) ($p<0.05$), but Mtb-induced MMP-7 secretion was not increased by TGFβ over a wide range of concentrations (Figure 17c). This finding was highly consistent in all the four donors studied.

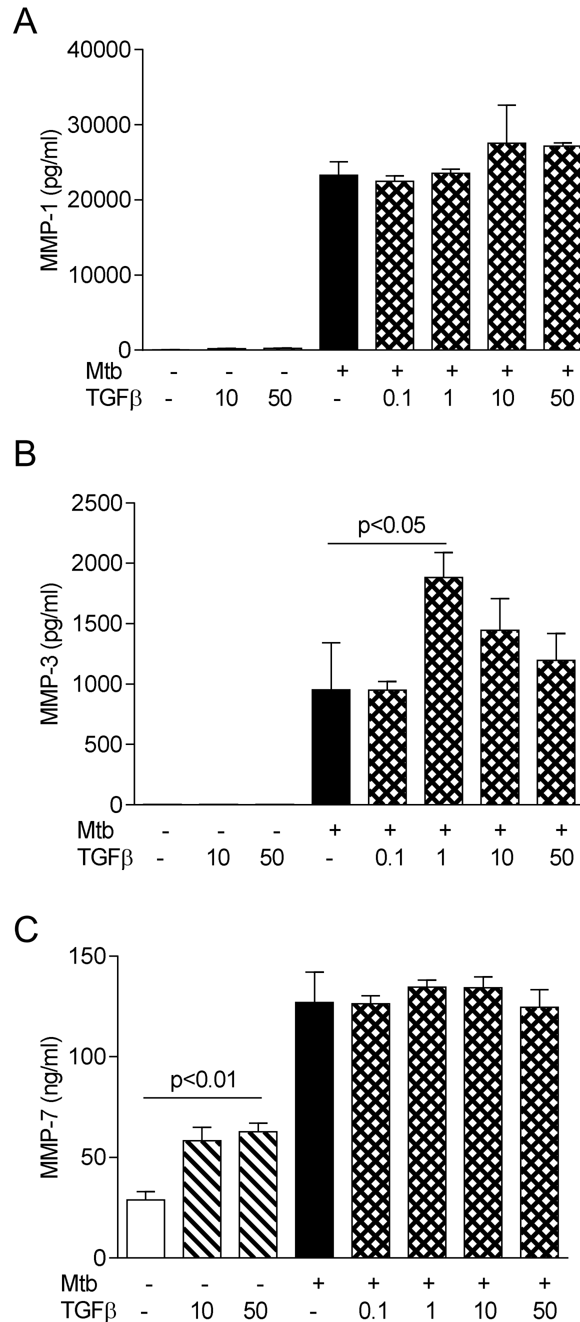


Figure 17. Transforming growth factor beta (TGFβ) does not significantly affect Mtb-driven MMP secretion by MDMs

MDMs were treated with TGFβ(0.1-50 ng/ml) prior to infection with H37RV (MOI 1). Supernatants were collected after 72 hours and MMP-1, -3 and -7 were quantified by Luminex. A) MDM MMP-1 secretion was not affected by TGFβ alone or in combination with Mtb infection. B) MMP-3 was undetectable in uninfected cells treated with TGFβ (10 or 50 ng/ml). There was a trend to increased MMP-3 secretion with TGFβ and Mtb infection, which was only statistically significant with TGFβ (10 ng/ml) ($p<0.05$). C) MMP-7 secretion increased from 29 to 63 ng/ml with TGFβ (50 ng/ml) ($p<0.05$), but Mtb-induced MMP-7 secretion was not further increased by TGFβ (0.1-50 ng/ml) (all $p>0.05$ compared to Mtb alone).

3.6 Discussion

In this chapter the effects of exogenously added T cell cytokines on epithelial cell and macrophage MMP expression have been investigated. Other approaches that could have been taken would include investigating the effect of blocking the effects of these cytokines, for example by incubating cells with blocking monoclonal antibodies to the relevant cytokine, as some of them might have been produced by infected macrophages and acted in an autocrine fashion, or investigating the effect of adding autologous T cells to infected macrophages in tissue culture, in order to investigate their contribution to altering the MMP expression of innate immune cells.

3.6.1 The effect of IL-4 on Mtb-driven MMP expression and activity

The results described here suggest that IL-4 is a potent inhibitor of Mtb-driven MMP expression in epithelial cells and macrophages. In macrophages IL-4 selectively inhibited MMP-1 and -3 secretion, and also inhibited production of the pro-inflammatory cytokines and chemokines IL-1β, IL-6, TNFα and CCL5. Macrophage MMP-7 and IL-12 secretion were not affected by IL-4, and IL-4 exposure did not affect the ability of MDMs to control intracellular H37Rv over a short timeframe. It would have been more informative to check the number of cfus at time zero and then over an extended time course, rather than at a single time point as was done here, as pre-incubation with IL-4 might have affected the efficiency with which bacteria were taken up by macrophages as well as their survival thereafter. This would also have permitted any change in macrophage phenotype over time between the cytokine-treated and untreated cells to be detected.

Previous work by other investigators showed that monocyte MMP-1 secretion could be inhibited with IL-4, and the effect of IL-4 was reversed by exogenous prostaglandin E2 (PGE2). MMP-9 was similarly affected, but TIMP-1 was unchanged [302]. A subsequent publication implicated inducible prostaglandin synthase 2 (PGHS2) as a key mediator of the effects of IL-4 on monocytes. In studies of monocytes isolated from patients treated with

recombinant IL-4, inhibition of PGHS2 gene expression was observed after IL-4 treatment [303]. Yano et al demonstrated that IL-4-mediated inhibition of prostaglandin production occurred in LPS-stimulated alveolar macrophages (AMs) obtained from healthy non-smoking volunteers, but did not investigate MMP expression in this system [304]. Therefore inhibition of PGHS2 is a plausible mechanism which might mediate the observed effect of IL-4 on Mtb-induced MMP expression in macrophages, although I did not investigate this further.

In human alveolar macrophages IL-4 has been reported to inhibit MMP-9 secretion. MMP-1 was not produced by unstimulated cells, but was produced in response to killed *S. aureus*, and this inducible MMP-1 secretion was inhibited in the presence of IL-4 (10 ng/ml) [305]. The phenomenon appears not to be stimulus-specific, as in primary human monocytes, MMP-1 expression induced by combinations of IL-1β, TNFα and GM-CSF was also inhibited by IL-4 (20 ng/ml) [306]. The inhibition of Mtb-induced IL-1β secretion in the presence of IL-4 observed in my experiments could therefore represent a positive feedback mechanism amplifying the effect of IL-4.

In a model of alternatively activated macrophages in which monocytes were cultured for 6 days with IL-4 10ng/ml, MMP-1 and MMP-12 mRNA were increased compared to control cells [307]. These divergent results might be due to genuine differences between monocyte and macrophage responses, or to the effects of differentiating the cells in the presence of IL-4 in this study, as opposed to relatively brief exposure to IL-4 in my experiments and the studies described above.

I found that in contrast to MMP-1 and -3, basal and Mtb-driven MMP-7 expression was unaffected by IL-4 treatment. However, a study by Busiek et al previously reported that constitutive MMP-7 expression by monocyte-derived macrophages was sensitive to inhibition by IL-4 [308].

In murine models of the effect of cytokines on Mtb-driven MMP expression, peritoneal macrophages were exposed to IL-4 prior to infection with *M. bovis* BCG. BCG-induced

MMP-9 gelatinolytic activity was reduced in the presence of IL-4, and, as in my experiments, this was associated with a decrease in TNFα in the culture supernatant [96]. Again, suppression of Mtb-induced TNFα secretion could be a mechanism mediating the effect of IL-4 on MMP expression. In contrast, Kahnert et al used a transcriptional profiling approach and found that MMP-12 mRNA in bone-marrow-derived macrophages increased with IL-4 treatment. However, infection with Erdman strain Mtb did not alter MMP-12 expression [309].

I found that IL-4 inhibited MMP-1 and -3 expression in CoMTb-stimulated epithelial cells. When the underlying mechanisms were investigated, IL-4 did not affect CoMTb-driven activity of a wildtype MMP-1 promoter construct, which contrasts with the finding that MMP-1 and -3 mRNA was reduced in IL-4-treated macrophages. Separate investigations into the key pathways mediating CoMTb-driven MMP-1 secretion identified a key role for the AP-1 site at -1950 bp upstream from the MMP-1 start site (see Figure 8), and this led me to investigate the effects of IL-4 on AP-1 signalling in CoMTb-stimulated cells. The results presented suggest that IL-4 treatment reduces CoMTb-driven activation of Jun and Fos subunits of AP-1, and this may represent one mechanism by which IL-4 exerts its effects. This could be further investigated by using ChIP assays to assess AP-1 subunit binding to the MMP-1 promoter in cells stimulated with CoMTb in the presence or absence of IL-4. Unfortunately there was not sufficient time to perform these experiments. In the preliminary ChIP experiments performed in this chapter, CoMTb treatment drove increased binding of RNA Pol II to the MMP-1 promoter region, particularly a region 4000bp upstream of the transcriptional start site, and also resulted in increased histone H4 acetylation throughout the promoter region. These findings will be further investigated and discussed in Chapter 5.

Many of the intracellular effects of IL-4 are mediated via Stat 6 signalling, and the MAPK pathways are known to be important regulators of MMP expression, therefore Western blots were performed to examine the effect of exposure to CoMTb/Mtb and IL-4 on these signalling pathways. As expected, Stat 6 phosphorylation was observed in IL-4-treated cells, but this was not altered by the presence of CoMTb or Mtb. The question of whether the

observed effect of IL-4 was Stat-dependent or independent could have been addressed using chemical inhibitors of Stat 6 or siRNA silencing of Stat 6 expression. Preliminary experiments with commercially available Stat inhibitors were non-contributory as the inhibitors were found to be toxic to the cells used (data not shown). The investigation of MAPK signalling did not implicate p38, ERK or JNK signalling as mediating the effect of IL-4 on MMP expression.

IL-4 has previously been reported to alter MMP expression in stromal cells. In synovial fibroblasts from arthritis patients, IL-4 inhibited MMP-1 and -3 mRNA and protein expression at doses as low as 0.1 ng/ml, and also reduced IL-1-driven PGE2 production. However, exogenous PGE2 did not reverse the effect of IL-4 on MMP expression. Promoter-reporter transfection using a 2 kbp construct of the MMP-3 promoter in foreskin fibroblasts suggested decreased MMP-3 transcription in the presence of IL-4, but in contrast to my results, IL-4 was not found to affect AP-1 or PEA-3 binding activity [310].

In articular chondrocytes oncostatin M-induced MMP-13 expression was inhibited by IL-4. This was independent of Stat1, p38 and ERK phosphorylation, but was associated with decreased JNK phosphorylation which I did not identify in my experiments [311]. In airway smooth muscle cells IL-4 or IL-13 (20 ng/ml) induced expression of MMP-1 and -3 (but not -2 or -9) mRNA and protein and this effect was not Stat 6-dependent [312]. Thus, the effects of IL-4 on MMP expression appear to be dependent on multiple factors, including cell type, stimulus, duration of exposure to and concentration of the cytokine.

3.6.2 The effect of IL-13 on Mtb-driven MMP expression and activity

In these experiments, IL-13 inhibited Mtb-driven MMP secretion in both epithelial cells and macrophages, and its effects were similar to those of IL-4. However, in NHBEs, a higher concentration of IL-13 (50 ng/ml) was needed to completely inhibit CoMTb-stimulated MMP-3 secretion, and in macrophages greater inhibition of both MMP-1 and -3 secretion was achieved with IL-4 20ng/ml than with IL-13 20ng/ml (83% vs 39% respectively). This correlates with the fact that IL-13 is produced at higher concentrations than IL-4, and higher

concentrations are frequently required to produce a biological effect. No effect of IL-13 on epithelial cell MMP-9 secretion was identified, and this may be because the concentrations of IL-13 used in these experiments were not high enough to produce a statistically significant reduction in MMP-9 activity.

The reported effects of IL-13 on MMP expression vary according to the cell type studied. In human conjunctival fibroblasts IL-13 inhibited MMP-1 expression and increased TIMP-1 and pro-collagen expression [313]. IL-13 also inhibited basal and IL-1β-induced MMP-3 expression, and this required intact IL-4R complex signaling [314]. In contrast, in airway smooth muscle cells IL-13 increased MMP-1 mRNA and protein expression in a Stat 6-independent, Phosphoinositide 3-kinase (PI3K)-dependent manner [315], and in human keratinocytes IL-13 selectively induced MMP-9 expression, and increased cell migration in a collagen matrix [316].

IL-13 is believed to play an important role in the pathogenesis of asthma. In ex vivo studies, airway fibroblasts from asthma patients displayed increased migratory capacity compared to healthy controls in a Matrigel chamber assay when IL-13 was used as the chemoattractant. The IL-13-induced migration required intact MMP and TGFβ activity [317]. Another study showed that IL-13 treatment increased MMP-7 mRNA and activity in the 1HAEO- airway epithelial cell line [318].

IL-13 is known to drive lymphocytic inflammation, alveolar remodeling and fibrosis in the lung. Mice over-expressing IL-13 developed pulmonary fibrosis and this was associated with increased macrophage TGFβ production and MMP-9 mRNA. IL-13-induced fibrosis could be reduced by neutralization of TGFβ, indicating that the pro-fibrotic effects of IL-13 may be mediated at least in part by increased TGFβ synthesis [294]. MMP-9 or MMP-12-deficient mice were found to be resistant to IL-13–induced disease, and MMP-12 was necessary for IL-13-driven macrophage recruitment and MMP-2, -9, -13 and -14 expression [319].

Transgenic expression of IL-13 in the murine lung resulted in increased MMP-2, -9 and -14 expression which was ERK-dependent but Stat 6-independent [320].

Thus IL-13 may inhibit collagenolytic activity mediated by MMP-1 and -3 while promoting cell migration mediated by other MMPs, for example MMP-9. I did not investigate the effect of IL-13 on TGFβ expression in my experiments, but TGFβ mRNA and protein secretion could be measured in IL-13-treated cells to investigate whether the suppression of MMP expression observed involved TGFβ production. This seems unlikely as in the studies where TGFβ was implicated, IL-13 was found to drive, rather than suppress, MMP production. Also, as discussed below, in this experimental model TGFβ was not found to significantly affect macrophage expression of MMP-1, -3 and -7.

3.6.3 The effect of IL-10 on Mtb-driven MMP expression

In this experimental model of pulmonary TB exposure to IL-10 did not significantly affect macrophage or epithelial cell MMP expression, which was unexpected. Different batches of recombinant human IL-10 were purchased, although all were from the same supplier, and none were found to influence MMP expression. It would have been useful to study another well-recognized effect of IL-10 on macrophage phenotype to confirm the biological activity of the reagent, such as inhibition of TNFα secretion in IL-10-treated cells.

There are several publications in the literature reporting a negative influence of IL-10 on MMP expression, including one in which murine peritoneal macrophages were incubated with IL-10 prior to infection with _M. bovis_ BCG. BCG-induced MMP-9 activity was reduced and the effect of IL-10 was found to be mediated by reduced macrophage TNFα production [96]. In alveolar macrophages and primary monocytes IL-10 reduced MMP-9 secretion and increased production of TIMP-1. MMP-1 and -9 expression was induced by stimulation with LPS or killed _S. aureus_, and IL-10 also inhibited this induced MMP expression while enhancing TIMP-1 expresion [321]. Another group found similar effects of IL-10 treatment on monocyte MMP-1 and -9 mRNA and protein expression, and reported that this was reversed

by the addition of exogenous cyclic AMP [322]. In a separate study investigating the regulation of macrophage MMP-7 production, basal and LPS-induced MMP-7 expression by MDMs was also found to be inhibited by IL-10 [308].

In other cell types, similar effects of IL-10 on MMP expression have been identified. In a prostate cancer cell line IL-10 down-regulated MMP-2 and -9 and increased TIMP-1 expression [323]. Cytotrophoblast MMP-9 mRNA and protein expression were reduced on exposure to IL-10, and blocking endogenous IL-10 with a neutralising antibody had the opposite effect [324]. IL-10 was implicated in the impaired placentation observed in the context of CMV infection; CMV drives IL-10 production, blocking MMP-9 activity and therefore cytotrophoblast invasion [325]. In studies of monocyte adhesion to endothelium IL-10 reduced MMP-9 mRNA expression and increased TIMP-1 [326].

In murine models of chronic liver injury administration of IL-10 or gene therapy with transfer of the human IL-10 gene was found to diminish hepatic fibrosis and inhibit MMP-2 expression [327, 328]. IL-10 is cleaved and inactivated by MMP-8, and MMP-8 knockout mice show resistance to bleomycin-induced pulmonary fibrosis which is accompanied by increased levels of IL-10 [329].

In contrast, in a tissue culture model of periodontitis, cardiomyoblasts stimulated with periodontitis pathogens secreted MMP-9, and this was not inhibited by IL-10 [330]. Mouse fetal epithelial cells exposed to mechanical stretch downregulate MMP-9 expression, and this effect was reversed in the presence of IL-10 [331].

The literature suggests that in most cell types IL-10 acts to inhibit MMP expression, and often up-regulates TIMP-1 expression, in accordance with our original hypothesis. This effect was not observed in my experiments. However, the effect of IL-10 appears to be cell- and stimulus-specific, and there are minimal published data on the effect of IL-10 on MMP expression in the context of human TB.

3.6.4 The effect of TGFβ on Mtb-driven MMP expression

TGFβ had different effects on MMP expression in epithelial cells and macrophages. In both A549s and primary epithelial cells, TGFβ was an independent stimulus to MMP-1 and -3 secretion, and also enhanced CoMTb-driven MMP secretion. However, in macrophages no significant effect of TGFβ on MMP expression was observed. This was unexpected, as in previous studies, TGFβ was found to increase the expression of MMP-2 and -9 by monocytes [295], whereas in macrophages, cytokine-induced MMP-12 expression was inhibited by TGFβ, and AP-1 signaling was required for this inhibitory effect [296].

The results of the epithelial cell experiments correlate with a publication reporting that TGFβ increased MMP-2 and -9 expression in a transformed epithelial cell line in a p38-dependent fashion [298]. In contrast, in human fibroblasts and fibroblast cell lines, TGFβ increased TIMP-1 expression and suppressed expression of MMP-1, and AP-1 signaling appeared to mediate both effects [297].

Recombinant human $TGF\beta_1$ was used as the stimulus in my in vitro experiments. This was selected as the dominant form produced by macrophages. However, TGFβ is synthesized as a pro-form in the latent complex, and it may be that some feature of the physiological activation process was not replicated in these experiments using exogenous TGFβ. In both epithelial cells and macrophages, morphological changes in the TGFβ–treated cells were observed which suggest that the recombinant protein did retain some biological activity. As for IL-10, where the results did not support the original hypothesis, it would have been useful to measure another anticipated effect of TGFβ-treatment to confirm the biological activity of the recombinant cytokine employed in these experiments.

4 Interferon-γ modulates Mtb-driven MMP expression

4.1 Introduction

IFNγ is a critical effector of antimycobacterial immunity, activating macrophages and enhancing T cell responses. Defects in IFNγ production and signaling are associated with increased susceptibility to mycobacterial infection [222, 223]. The immune evasion strategies of Mtb include several that subvert IFNγ-induced anti-mycobacterial effector pathways [228, 229]. However, chronic infection and immunopathology in human TB disease develop despite the presence of vigorous IFNγ-responses. IFNγ inhibits neutrophil recruitment and survival, and IFNγ-deficient mice display severe inflammation and tissue necrosis when exposed to Mtb [235, 236]. It has therefore been proposed that a balance exists, whereby production of active IFNγ is required for effective immunity, and to restrain neutrophil-driven inflammation, but excessive IFNγ responses may also be detrimental to the host.

IFNγ has previously been shown to affect MMP expression in a variety of cell types. In macrophages it is reported to inhibit MMP secretion in response to inflammatory stimuli such as cytokines and LPS [308, 332, 333], while the reported effects on stromal cells are variable. We therefore hypothesized that one effect of IFNγ in TB might be to modulate immunopathology by affecting Mtb-driven MMP expression. The effect of IFNγ on the expression of the key MMPs MMP-1, -3, -7 and -9 by Mtb-stimulated macrophages and epithelial cells is investigated in this chapter.

4.2 Results

4.2.1 IFNγ selectively inhibits MMP-1 and -3 secretion by Mtb-infected MDMs

MDMs were pre-incubated with IFNγ (1-10 ng/ml) for 2 hours prior to infection with H37Rv. MMP-1, -3 and –7 in cell culture supernatants collected 72 hours post-infection were quantified by Luminex. MMP-1 and -3 secretion was significantly inhibited in the presence of IFNγ (1 or 10 ng/ml). IFNγ (1 ng/ml) inhibited Mtb-driven MMP-1 secretion by 64% (from 10284 to 3721 pg/ml) and IFNγ (10 ng/ml) inhibited MMP-1 secretion by 90%, to 1005 pg/ml (both $p<0.0001$) (Figure 18a). IFNγ (1ng/ml) reduced MMP-3 secretion by 46% ($p=0.01$), while IFNγ (10 ng/ml) inhibited MMP-3 secretion by 71% ($p<0.001$) (Figure 18b). Basal and Mtb-stimulated MMP-7 secretion were not affected by IFNγ (10 ng/ml) (Figure 18c). The results shown are from one experiment performed in triplicate and are representative of four experiments performed independently in different donors.

4.2.2 IFNγ does not affect Mtb-driven upregulation of MMP-1, -3 or -7 mRNA in MDMs

MDMs were pre-incubated with IFNγ (10 ng/ml) for 2 hours prior to infection with H37Rv. Total RNA was harvested 24 hours post-infection and RT-PCR was performed. MMP-1 mRNA increased 313-fold in Mtb-infected cells and there was no difference observed when cells were also treated with IFNγ (Figure 19a). MMP-3 mRNA was undetectable in uninfected cells but increased to 0.37 copies per 1000 copies β-actin mRNA with Mtb infection. IFNγ (10 ng/ml) did not significantly alter MMP-3 mRNA expression (Figure 19b). MMP-7 mRNA increased 9-fold with Mtb infection and was not affected by pre-treatment with IFNγ (10 ng/ml) (Figure 19c). The results shown are from one experiment performed in triplicate, representative of two independent experiments performed in different donors. These results suggest that IFNγ acts on post-transcriptional mechanisms controlling MMP expression, for example translation, rather than by affecting mRNA transcription in response to Mtb infection.

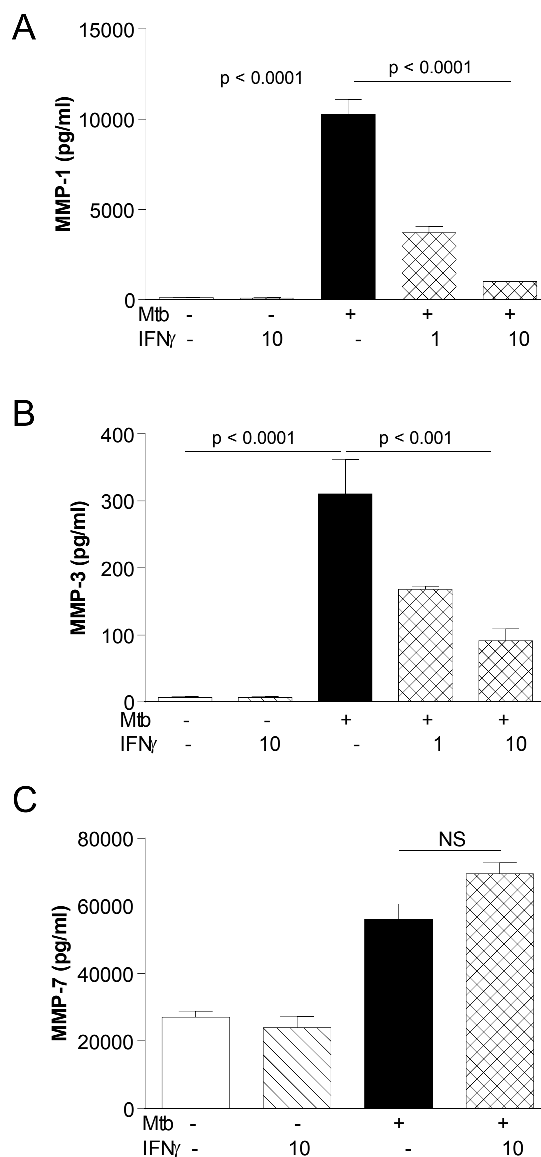


Figure 18. IFNγ selectively inhibits MMP-1 and -3 secretion by Mtb-infected MDMs

MDMs were pre-incubated with IFNγ (1-10 ng/ml) for 2 hours prior to infection with H37Rv. MMP-1, -3 and –7 were assayed by Luminex in cell culture supernatants collected 72 hours post-infection. A) MMP-1 secretion was significantly inhibited in the presence of IFNγ (1 or 10 ng/ml). IFNγ (1 ng/ml) inhibited Mtb-driven MMP-1 secretion by 64% (from 10284 to 3721 pg/ml) and IFNγ (10 ng/ml) inhibited MMP-1 secretion by 90%, to 1005 pg/ml (both p<0.0001). B) Mtb-induced MMP-3 secretion was similarly inhibited in a dose-dependent manner. IFNγ (1 ng/ml) reduced MMP-3 secretion by 46%, while IFNγ (10 ng/ml) produced a 71% inhibition (p=0.01 and p<0.001 respectively). C) Basal and Mtb-stimulated MMP-7 secretion were not affected by IFNγ (10 ng/ml).

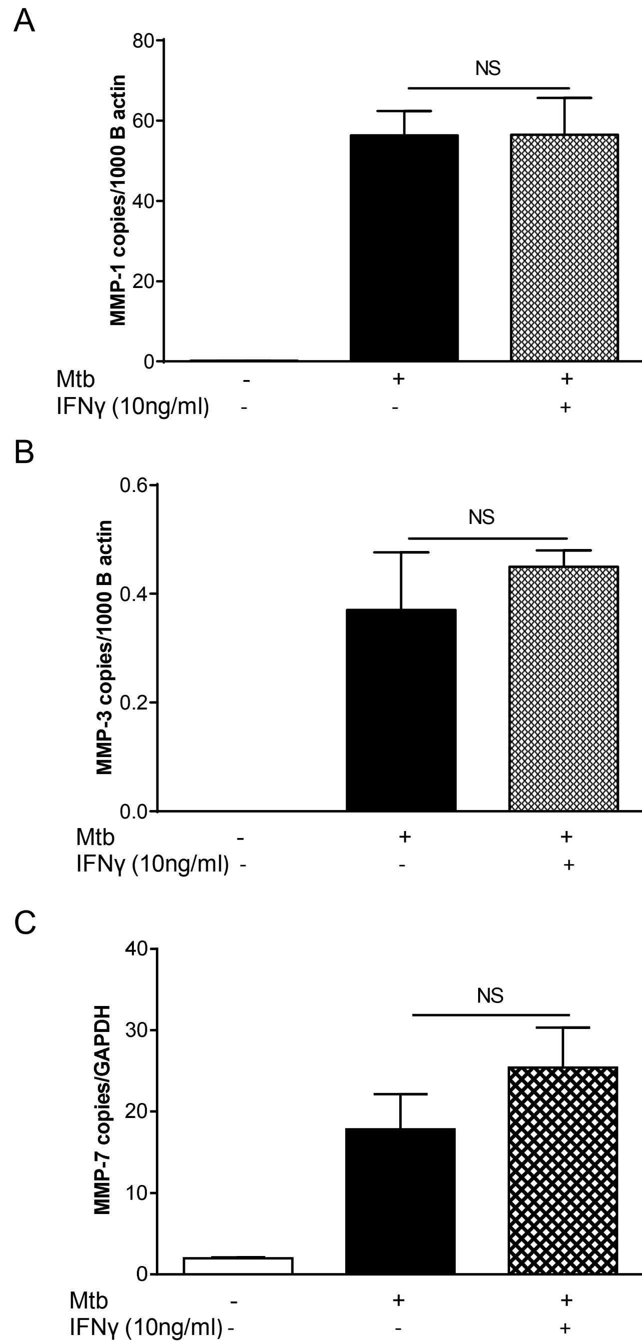


Figure 19. IFNγ-treatment does not affect Mtb-driven upregulation of MMP-1, -3 and -7 mRNA in MDMs

MDMs were pre-incubated with IFNγ (10 ng/ml) for 2 hours prior to infection with H37Rv. Total RNA was harvested 24 hours post-infection and RT-PCR was performed. A) MMP-1 mRNA increased 313-fold in Mtb-infected cells and there was no difference observed in cells pre-treated with IFNγ. B) MMP-3 mRNA was undetectable in uninfected cells but increased to 0.37/1000 copies β-actin mRNA with Mtb infection. IFNγ (10 ng/ml) did not significantly alter MMP-3 mRNA expression. C) MMP-7 mRNA increased 9-fold with Mtb infection and was not affected by pre-treatment with IFNγ (10 ng/ml).

4.2.3 IFNγ inhibits MDM MMP secretion in response to UV-killed Mtb

As IFNγ is a critical activator of mycobacterial killing mechanisms in macrophages, I hypothesized that the reduced MMP response observed in MDMs treated with IFNγ might be a result of more efficient early killing of the infecting bacteria, with a consequent reduction in macrophage activation and magnitude of the inflammatory response generated. In order to investigate whether this was the case, the response to killed mycobacteria and the ability of IFNγ-treated MDMs to control H37Rv were examined.

MDMs were pre-incubated with IFNγ (1 or 10 ng/ml) for 2 hours and then stimulated with ultra-violent light (UV)-killed H37Rv for 72 hours. The killed bacteria were present in the culture medium throughout the experiment. UV-killed Mtb increased MMP-1 secretion 132-fold compared to control conditions and this was inhibited by 95% in the presence of IFNγ (10 ng/ml) ($p<0.0001$) (Figure 20a). MMP-3 secretion increased to 842 pg/ml when cells were treated with UV-killed Mtb, and MMP-3 secretion was 92% inhibited by IFNγ (10 ng/ml) ($p<0.0001$) (Figure 20b). MMP-7 secretion increased 4.2-fold with UV-killed Mtb stimulation, and this increase was 51% inhibited in the presence of IFNγ (10 ng/ml) ($p<0.0001$) (Figure 20c). The results shown are from one experiment performed in triplicate and are representative of two independent experiments in different donors. In these experiments killed Mtb appears to be a stronger stimulus to MMP secretion than infection with live H37Rv. The numbers of UV-killed bacteria used in these studies were equivalent to the number used in the live infection experiments, and so the difference observed is likely to be due to the fact that the killed bacteria were not washed off after two hours, as in the preceding live infection experiments. It would have been preferable to repeat these experiments using only a 2 hour exposure to the UV-killed bacteria, with any remaining extracellular bacteria and debris washed off at the end of this period.

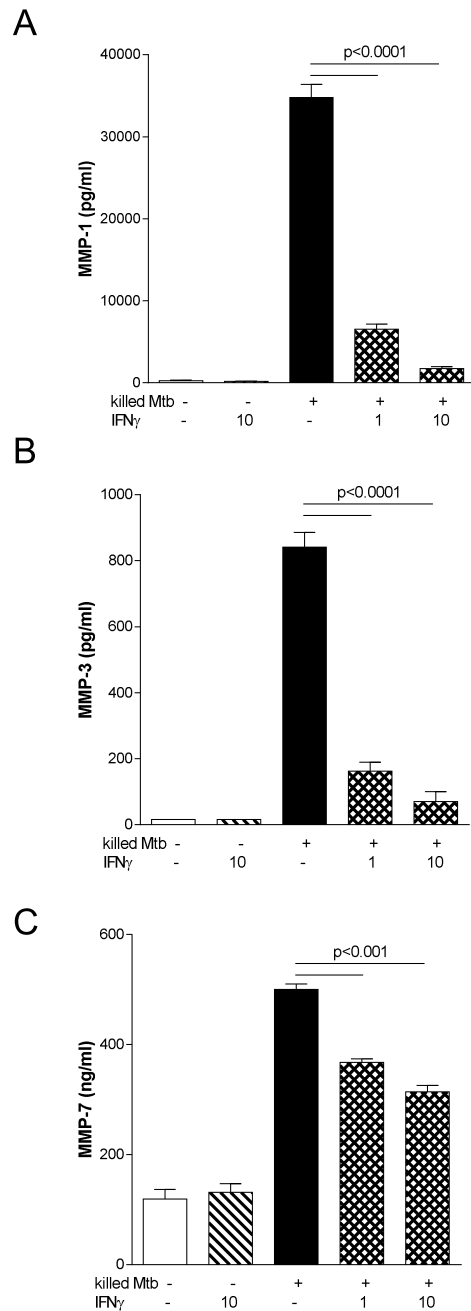


Figure 20. IFNγ inhibits MDM MMP secretion in response to UV-killed Mtb

MDMs were pre-incubated with IFNγ (1 or 10 ng/ml) for 2 hours and then stimulated with UV-killed H37Rv for 72 hours. MMP-1, -3 and –7 were assayed by Luminex in cell culture supernatants. A) UV-killed Mtb increased MMP-1 secretion 132-fold compared to control conditions and this was inhibited by 95% in the presence of IFNγ (10 ng/ml) (p<0.0001). B) MMP-3 was undetectable in supernatants from unstimulated cells but induced at 842 pg/ml by UV-killed Mtb. MMP-3 secretion was 92% inhibited by IFNγ (10 ng/ml) (p<0.0001). C) MMP-7 secretion increased 4.2-fold with UV-killed Mtb stimulation, and this increase was 51% inhibited in the presence of IFNγ (10 ng/ml) (p<0.0001). The results shown are from one experiment performed in triplicate and are representative of two independent experiments in different donors.

4.2.4 IFNγ does not affect the ability of MDMs to control H37Rv infection

MDMs were pre-incubated with IFNγ (20 ng/ml) for 2 hours prior to infection with H37Rv in order to identify any effect of IFNγ-treatment on the ability of MDMs to successfully eliminate Mtb infection. Culture medium was changed after 2 hours to remove non-phagocytosed bacteria. Cells were lysed in 0.1% Triton X after 72 hours and plated on 7H11 agar to quantify intracellular bacterial burden. No difference in cfus was observed between the IFNγ-treated and untreated samples (p=0.96). The results shown in Figure 21 are from one experiment performed in triplicate, representative of results from two independent experiments performed in different donors.

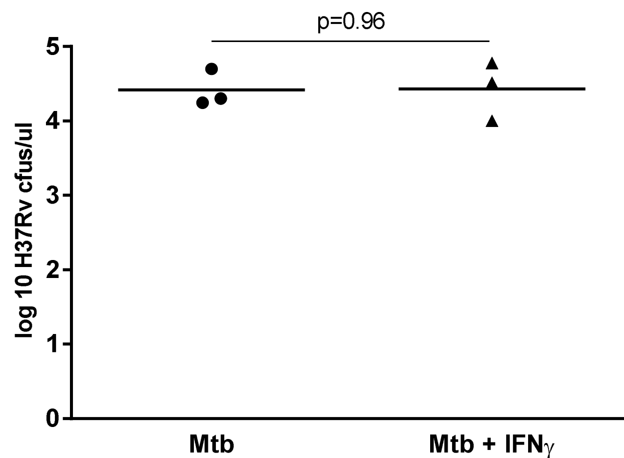


Figure 21. IFNγ does not affect the ability of MDMs to control H37Rv infection

MDMs were pre-incubated with IFNγ (20 ng/ml) for 2 hours prior to infection with H37Rv (MOI 1). Cells were lysed in 0.1% Triton X after 72 hours and plated on 7H11 agar for quantification of cfus. No difference in cfus was observed between the cytokine-treated and untreated samples (p=0.96). Results shown are from one experiment performed in triplicate, representative of results from two independent experiments performed in different donors.

4.2.5 IFNγ enhances Mtb-driven MDM secretion of IL-6, TNFα and IL-12, and induces secretion of IL-10, IP-10 and MCP-1

Chemokine and cytokine concentrations in supernatants from Mtb-infected MDMs with and without IFNγ pre-treatment were measured by Luminex, in order to assess whether IFNγ produced the expected activated macrophage phenotype and drove secretion of pro-inflammatory cytokines and chemokines.

IL-6 secretion increased 3.2-fold with Mtb infection and was further increased in the presence of IFNγ (1 ng/ml) (1240 vs 7407 pg/ml, $p<0.0001$) (Figure 22a). TNFα secretion increased 2.8-fold with Mtb infection, but was 60-fold higher with Mtb/IFNγ (1 ng/ml) than Mtb alone and 160-fold higher with Mtb/IFNγ (10 ng/ml) than Mtb alone (not statistically significant) (Figure 22b). IL-12 secretion increased 2.7-fold with Mtb alone, but 40-fold with Mtb/IFNγ (10 ng/ml) ($p<0.01$ vs Mtb alone) (Figure 22c). IL-10 was undetectable in control and Mtb-infected samples without IFNγ, but was significantly increased in the presence of IFNγ ($p<0.05$) (Figure 22d). IP-10 was present at low concentrations (99 and 192 pg/ml respectively) in control and Mtb-infected samples, but was significantly increased in the presence of IFNγ (10 ng/ml) (Figure 22e). This upregulation was unaffected by the presence of Mtb infection ($p>0.05$ IFNγ (10 ng/ml) alone vs Mtb/IFNγ (10 ng/ml)). MCP-1 secretion was not increased by Mtb infection ($p>0.05$) but increased 2.7-fold with IFNγ (10 ng/ml) ($p=0.001$). IFNγ-driven MCP-1 secretion was significantly reduced in Mtb-infected cells (from 38976 to 24821 pg/ml, $p<0.05$) (Figure 22f). Results shown are from one representative experiment of two independent experiments performed in triplicate in different donors.

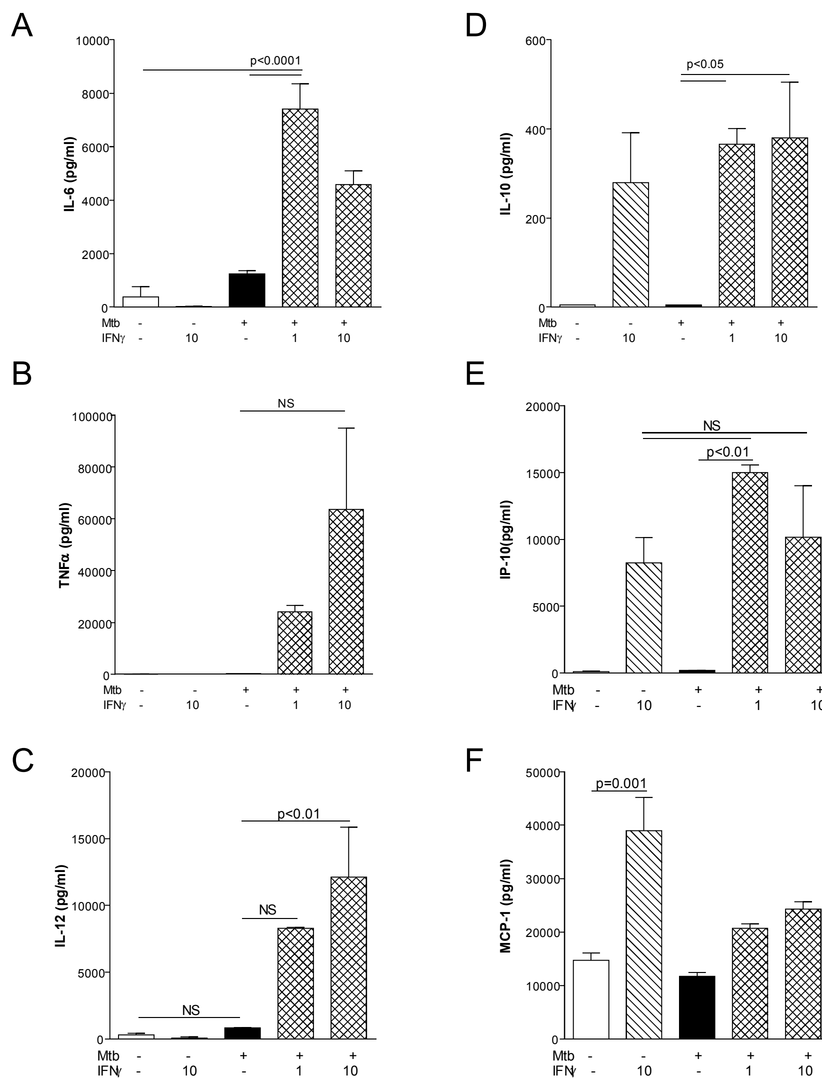


Figure 22. IFNγ enhances Mtb-driven MDM secretion of IL-6, TNFα and IL-12, and induces secretion of IL-10, IP-10 and MCP-1

MDMs were pre-incubated with IFNγ (1 or 10 ng/ml) for 2 hours followed by infection with H37Rv (MOI 1). Supernatants were collected at 72 hours and chemokine and cytokine concentrations were measured by Luminex. A) IL-6 secretion increased 3.2-fold with Mtb infection and was further increased in the presence of IFNγ (1 ng/ml) (1240 vs 7407 pg/ml, $p<0.0001$). B) TNFα secretion increased 2.8-fold with Mtb infection, but was 60-fold higher with Mtb/IFNγ (1 ng/ml) than Mtb alone and 160-fold higher with Mtb/IFNγ (10 ng/ml) than Mtb alone (not statistically significant). C) IL-12 secretion increased 2.7-fold with Mtb alone, but 40-fold with Mtb/IFNγ (10 ng/ml) ($p<0.01$ vs Mtb alone). D) IL-10 was undetectable in control and Mtb-infected samples without IFNγ, but was significantly increased in the presence of IFNγ ($p<0.05$). E) IP-10 was present at low concentrations (99 and 192 pg/ml respectively) in control and Mtb-infected samples, but was significantly increased in the presence of IFNγ (10 ng/m)l. This upregulation was unaffected by the presence of Mtb infection ($p>0.05$ IFNγ (10 ng/ml) alone vs Mtb/IFNγ (10 ng/ml)). F) MCP-1 secretion was not increased by Mtb infection ($p>0.05$) but increased 2.7-fold with IFNγ (10 ng/ml) ($p=0.001$). IFNγ-driven MCP-1 secretion was significantly reduced in Mtb-infected cells (from 38976 to 24821 pg/ml, $p<0.05$). Results shown are from one representative experiment of two independent experiments performed in triplicate in different donors

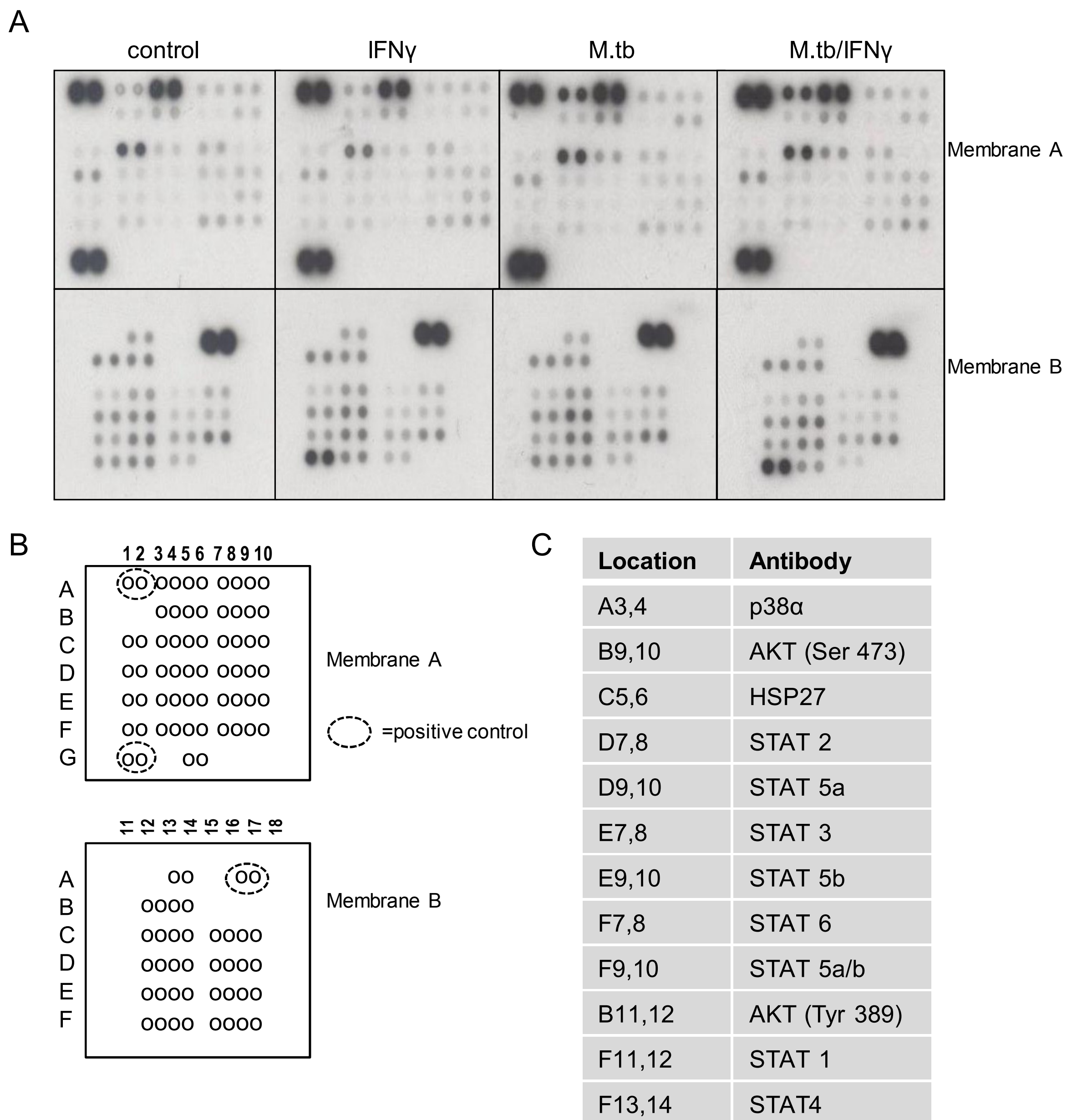
4.2.6 IFNγ signaling in Mtb-infected MDMs: phosphokinase signaling array

An unbiased approach was taken to investigating the potential pathways mediating the observed inhibitory effect of IFNγ on Mtb-driven MMP expression. The aim was to identify signaling pathways that were activated by Mtb but inhibited in the presence of IFNγ, in order to then investigate whether these mediated the effect of IFNγ on MMP expression.

MDMs were infected with H37Rv in the presence or absence of IFNγ (10 ng/ml). After 30 minutes cell lysates were prepared on ice and sterile-filtered for use with the R&D Systems Phospho-Kinase array. The experiment was performed twice in different donors. Densitometry results for each antibody "spot" pair were normalized to the positive and negative controls on each membrane, and the two sets of results obtained were combined to produce the results described. Figure 23a shows one representative set of images of the developed membranes.

Figure 23b shows the layout of the antibody "spots" on the paired membranes. Each antibody is present in two spots, with positive and negative controls on each membrane. The table in Figure 23c lists the spot locations of some of the relevant signaling molecules probed in this assay.

The array results demonstrated that some signaling molecules, such as p38α and HSP27 (Heat Shock Protein 27) were phosphorylated in response to Mtb stimulation, and this was unchanged in the presence of IFNγ. Phosphorylation of p38α increased 3.2-fold with Mtb compared to control conditions ($p<0.05$) and HSP27 phosphorylation increased 2-4-fold. Another group of molecules, including some of the Stats, were phosphorylated in response to IFNγ but unaffected by Mtb. For example, Stat 1 phosphorylation increased 3.4-fold with IFNγ ($p<0.05$). A small number of candidate pathways were identified where Mtb-driven phosphorylation appeared to be reduced in the presence of IFNγ. These included Stat 4 and AKT (densitometric analysis not shown), although the observed changes were small and not statistically significant.



Location	Antibody
A3,4	p38α
B9,10	AKT (Ser 473)
C5,6	HSP27
D7,8	STAT 2
D9,10	STAT 5a
E7,8	STAT 3
E9,10	STAT 5b
F7,8	STAT 6
F9,10	STAT 5a/b
B11,12	AKT (Tyr 389)
F11,12	STAT 1
F13,14	STAT4

Figure 23. IFNγ signaling phosphoarray

MDMs were infected with H37Rv in the presence or absence of IFNγ (10 ng/ml). After 30 minutes cell lysates were prepared on ice and probed for phospho-proteins. A) One representative set of images of the developed membranes for the four experimental conditions. B) Schematic layout of the antibody "spots" on the paired membranes. Each antibody is present in two spots, with positive and negative controls on each membrane. C) Table describing the locations of the antibodies to some of the relevant signaling molecules probed in this assay.

4.2.7 Stat 1 and 3 and AKT are phosphorylated in IFNγ-treated MDMs

Stat and AKT signaling were further investigated by Western blotting. Primary human MDMs were treated with IFNγ (10 ng/ml) with or without infection with H37Rv MOI 1. Samples were lysed after 15 minutes to 4 hours, and Western blotting was performed with antibodies directed against the total and phosphorylated forms of Stat 1 and 3 and AKT.

Phoshorylation of Stat 1 at tyrosine residue 701 (Tyr701) was undetectable in unstimulated cells, but Stat 1 was heavily phosphorylated in IFNγ-treated cells. Mtb infection did not alter IFNγ-driven Stat 1 phosphorylation and Mtb alone did not cause phosphorylation of Stat 1 (Figure 24a). These results correlate well with the results of the phospho-kinase array. Similarly, phosphorylation of Stat 3 at tyrosine residue 705 (Tyr705) and serine 727 (Ser727) was barely detectable in control samples but significantly increased in IFNγ-treated cells. Phosphorylation of Tyr705 did not occur in Mtb-infected cells without IFNγ, but a small increase in Ser727 phosphorylation was observed with Mtb infection alone (Figure 24a). The results shown are from one experiment, not performed in triplicate, but representative of results from three similar experiments.

The results of the phosphokinase signaling array had suggested that phosphorylation of AKT at serine 473 (Ser 473) in Mtb-infected MDMs might be modulated by the presence of IFNγ, and therefore this was further examined by Western blotting for AKT. In this experiment MDMs were first pre-incubated with IFNγ (10 ng/ml) for 2 hours, and subsequently infected with H37Rv. AKT phosphorylation was detected in the 60 minute control sample, although not in 15 or 30 minute controls. Phospho-AKT was increased in the Mtb-infected sample after 60 minutes, and appeared to be increased further when IFNγ was also present (Figure 24b), in contrast to the results obtained in the phosphokinase signaling array as described above. Therefore the possibility that the inhibitory effect of IFNγ on MDM MMP expression was mediated by blocking AKT signaling was not investigated further. This experiment was only performed once.

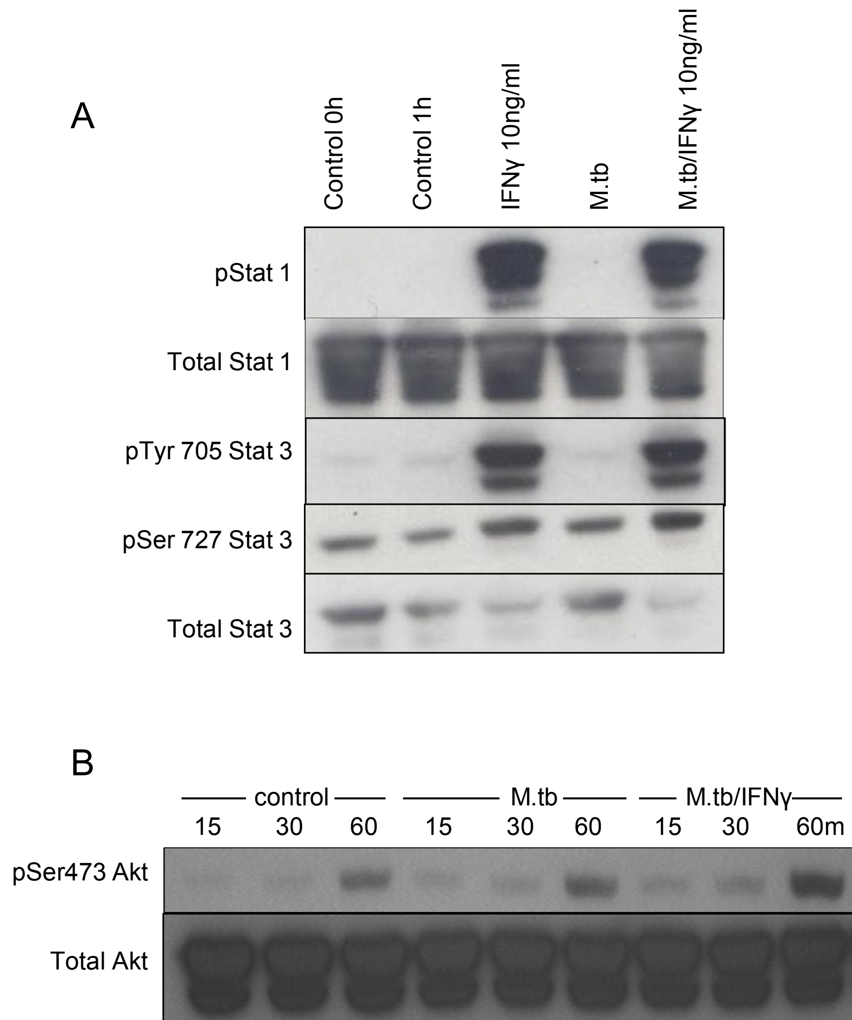


Figure 24. Stat 1 and 3 and AKT are phosphorylated in Mtb-infected MDMs treated with IFNγ

MDMs were infected with H37Rv MOI 1 in the presence of IFNγ (10 ng/ml) or in control medium. Samples were lysed in Western lysis buffer after 15 minutes to 1 hour, and Western blots for total and phosphorylated forms of Stat 1 and 3 and AKT were performed. A) After 1 hour there was no increase in Stat 1 phosphorylation compared to basal levels under control or Mtb-infected conditions. Increased Stat 1 phosphorylation was detected in IFNγ-treated cells. Total Stat 1 was stable across all the experimental conditions. Phosphorylation of Stat 3 at Tyr705 and Ser727 significantly increased in IFNγ-treated cells. Increased phosphorylation of Tyr705 did not occur in with Mtb infection alone, but a small increase in Ser727 phosphorylation was observed with Mtb infection alone. B) Total and phospho-AKT were assayed at 15-60 minutes post-Mtb infection. Total AKT was stable across all the experimental conditions. Phospho-AKT was increased in the Mtb-infected sample after 60 minutes, and appeared to be increased further when IFNγ was also present.

4.2.8 IFNγ alters respiratory epithelial cell MMP expression in response to CoMTb

As IFNγ had been shown to have a profound effect on MDM MMP expression, this was then investigated in respiratory epithelial cells. A549 cells and NHBEs were pre-incubated with IFNγ for 2 hours before stimulation with CoMTb. Supernatants were collected after 72 hours and MMP-1 and -3 were measured by ELISA. In two preliminary experiments using A549 cells, IFNγ significantly inhibited CoMTb-induced MMP-1 secretion (Figure 25a). However, when the experiment was performed in NHBEs, IFNγ (10 ng/ml) was found to be a potent stimulus to MMP-1 secretion, generating similar concentrations of MMP-1 as did CoMTb (Figure 25b). When the two stimuli were combined, an additive effect was observed. In contrast, no significant effect on NHBE MMP-3 secretion was observed (Figure 25d) over the course of several independent experiments.

MMP-1 and -3 mRNA were therefore measured in NHBEs treated with CoMTb with and without IFNγ. The changes in MMP mRNA observed in these experiments were not statistically significant due to the variability observed between replicate samples, but the trends in mRNA supported the secretion data in that there was a small increase in MMP-1 mRNA when cells were treated with both IFNγ and CoMTb (Figure 25c), whereas MMP-3 mRNA was not altered compared to CoMTb alone (Figure 25e).

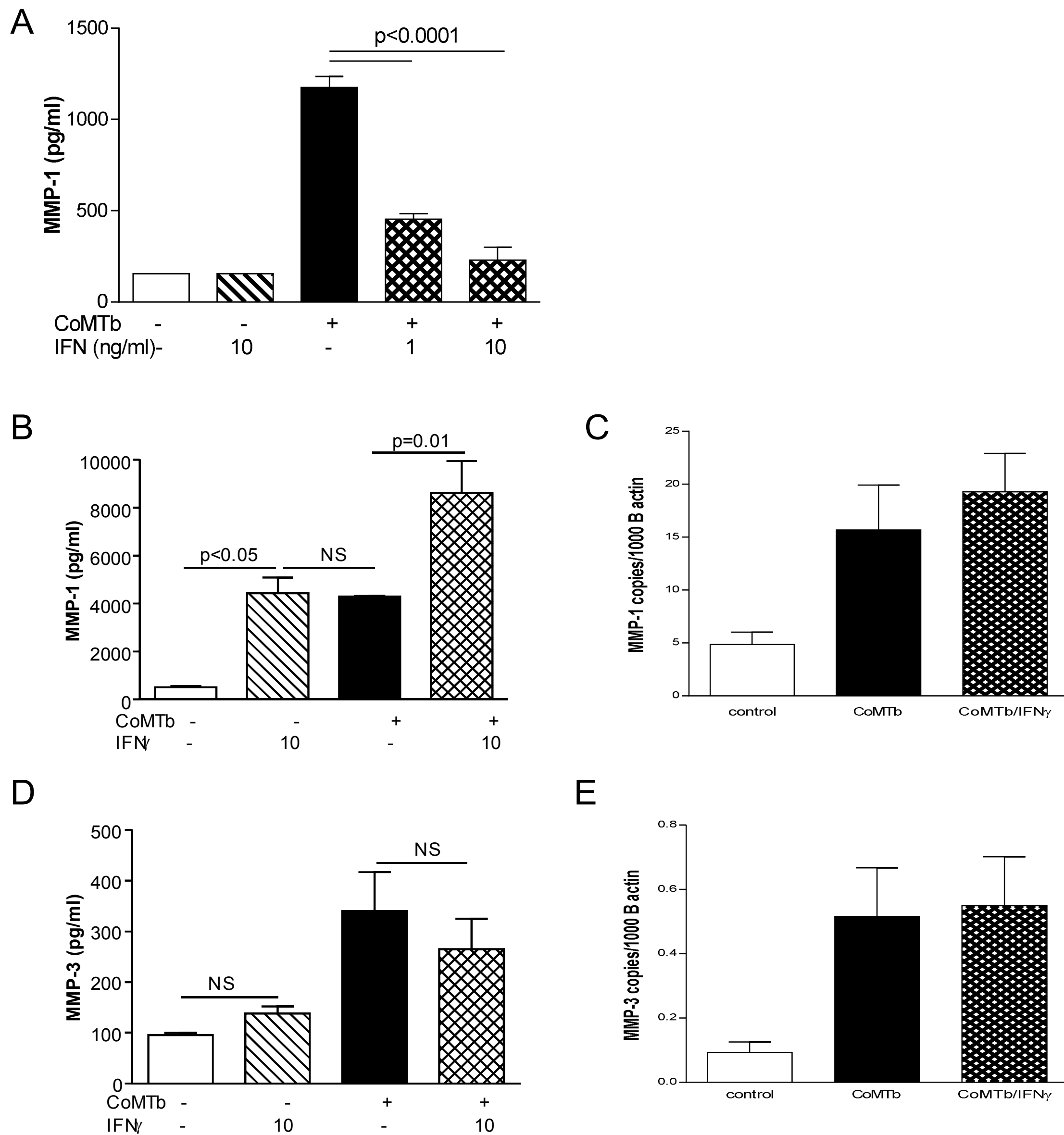


Figure 25. IFNγ alters respiratory epithelial cell MMP secretion in response to CoMTb

A549 cells and NHBEs were pre-incubated with IFNγ for 2 hours before stimulation with CoMTb. For A, B & D supernatants were collected after 72 hours and MMP-1 and -3 were measured by ELISA. For C & E cells were lysed after 24 hours and total RNA was extracted and reverse transcribed for RT-PCR. A) In A549 cells, IFNγ significantly inhibited CoMTb-induced MMP-1 secretion. Experiment performed twice in triplicate. B) IFNγ (10 ng/ml) induced MMP-1 secretion in NHBEs similarly to CoMTb. When the two stimuli were combined, an additive effect was observed. C) MMP-1 mRNA was increased 3.2-fold with CoMTb compared to control conditions, and 4.0-fold with CoMTb and IFNγ combined ($p>0.05$). D) In contrast, no significant effect on NHBE MMP-3 secretion was observed. E) MMP-3 mRNA was increased 5.6-fold with CoMTb compared to control conditions, and 5.9-fold with CoMTb and IFNγ combined ($p>0.05$) MMP secretion results presented are from one experiment performed in triplicate and representative of at least three independent experiments. The RT-PCR results are from one experiment performed in triplicate and representative of two independent experiments.

4.2.9 IFNγ but not CoMTb drives Stat 1 phosphorylation in NHBEs

The intracellular pathways mediating the effects of IFNγ on MMP expression were subsequently investigated. NHBEs were pre-incubated with IFNγ (10 ng/ml) for 2 hours and then stimulated with CoMTb 1:5. Samples were lysed in Western lysis buffer after 1 to 4 hours, and Western blotting was performed with antibodies directed against the phosphorylated forms of Stat 1 and 3. β-actin was used as a loading control.

The results displayed in Figure 26 show that Stat 1 phosphorylation at Tyr701 was specific to IFNγ-treated cells; phospho-Stat 1 was not detected in control samples or CoMTb-stimulated cells at any timepoint. Some basal phosphorylation of Stat 3 at both Tyr705 and Ser727 was detectable in control samples at all timepoints. Stat 3 (Tyr705) phosphorylation was slightly increased by CoMTb alone at the 2 and 4 hour timepoints, but was greatly increased at 1-4 hours in the presence of both CoMTb and IFNγ. Stat 3 (Ser727) phosphorylation was more variable, but appeared to be slightly increased with CoMTb at 2 and 4 hours. Thus IFNγ appears to drive more Stat 1 than Stat 3 phosphorylation in NHBEs, in contrast to the results observed in MDMS where both were activated. This experiment was only performed once.

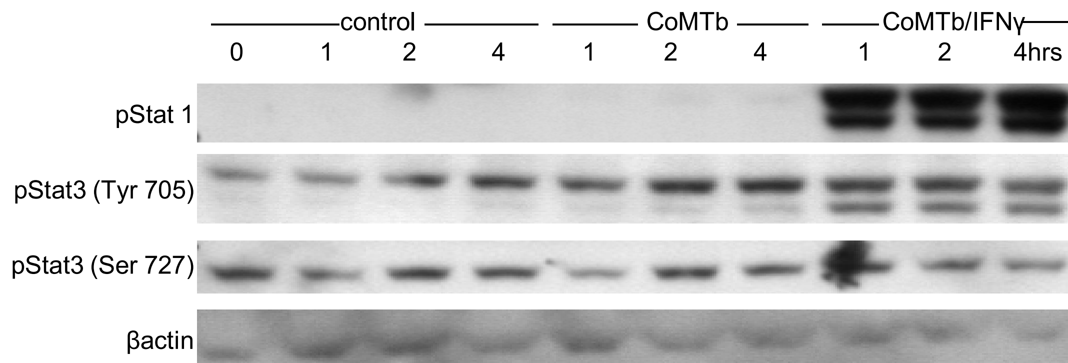


Figure 26. Stat 1 and 3 phosphorylation in IFNγ- and CoMTb-stimulated NHBEs

NHBEs were pre-incubated with IFNγ (10 ng/ml) for 2 hours and then stimulated with CoMTb 1:5. Samples were lysed after 1 to 4 hours and Western blots were performed for total and phosphorylated forms of Stat 1 and 3. Phosphorylation of Stat 1 occurs only in response to IFNγ. Stat 3 phosphorylation at Tyr 705 and Ser 727 was slightly increased by CoMTb and greatly increased by IFNγ.

4.3 Discussion

The results presented demonstrate that IFNγ selectively inhibits Mtb-driven MMP-1 and -3 secretion by macrophages. Although MMP protein secretion was significantly reduced, MMP-1 and -3 gene transcription was up-regulated by Mtb infection despite the presence of IFNγ. IFNγ inhibited MMP secretion in response to UV-killed bacteria as well as live H37Rv, although the results of the experiments with UV-killed bacteria were confounded by an error in experimental design which meant that residual extracellular dead bacteria were not removed. In the presence of IFNγ, Mtb-driven secretion of pro-inflammatory cytokines was enhanced, suggesting that as expected, IFNγ promoted differentiation towards an M1 macrophage phenotype. However, IFNγ also drove increased IL-10 production independently of Mtb infection, and this may represent a negative feedback loop to avoid excessive inflammation in vivo. In these experiments, macrophages secreted IL-10 at a concentration of approximately 0.4 ng/ml, a concentration which did not affect MMP secretion in the experiments described in Chapter 3.4. IFNγ is generally considered to be an “anti-fibrotic” cytokine, acting antagonistically to the Th2 cytokines IL-4 and -13, and therefore it is interesting that in this model, the effects of these “opposing” cytokines on MMP expression were similar.

There is extensive evidence that IFNγ modulates the expression of MMPs and TIMPs by a variety of cell types. These results correspond with previous publications showing reduced expression of MMP-1 and -3 in alveolar macrophages treated with IFNγ [332]. Concavalin A-stimulated MMP-1 expression was also inhibited in IFNγ-treated macrophages [333]. In another study macrophage MMP-7 expression induced by LPS was inhibited by IFNγ, which contrasts with my observation that MMP-7 was unaffected by IFNγ-treatment [308]. MMP-9 expression by MDMs was induced by TNFα/IL-1β and this was inhibited by pre-treatment

with IFNγ [334]. It may be that different intracellular pathways are involved in the induction of MMP expression by these stimuli, and that these in turn are differentially regulated by IFNγ.

The effects of IFNγ were different in A549 cells and NHBEs; in A549s MMP-1 secretion was inhibited by the presence of IFNγ, whereas in NHBEs IFNγ was found to be an independent stimulus to MMP-1 secretion. There are a number of important differences between these two cell types: the A549 cell line has been propagated ex vivo for decades, in addition to being originally derived from an alveolar cell carcinoma. NHBEs are primary human cells, but are obtained from the proximal airways rather than the alveoli. In addition, culturing NHBEs requires tissue culture medium supplemented with growth factors and hormones such as insulin, hydrocortisone and epidermal growth factor, which may themselves influence the cellular response to particular cytokine stimuli.

Previous publications confirm that stromal cells can also be responsive to the effects of IFNγ; for example in arthritis models synovial fibroblasts express MMP-1 and -3 when stimulated with IL-1β, and this can be inhibited by IFNγ [335].

A number of mechanisms have been suggested for the action of IFNγ on MMP expression. Ma et al found that IFNγ-mediated inhibition of astroglioma MMP-9 secretion was Stat 1-dependent [336], and this was also the case in fibrosarcoma cell lines [337]. Further investigation found that Stat 1 associated with CBP in IFNγ-treated cells and decreased its interaction with the MMP-9 promoter, while IFNγ-treatment also resulted in decreased histone H3 and H4 acetylation at the MMP-9 promoter [338]. ATF-1 and the AP-1 transcription factor Fra-1 were implicated in IFNγ inhibition of MMP-1 expression by monocytes and macrophages [339]. The experiments performed here have failed to identify the pathways mediating IFNγ's effect on MMP expression. As expected Stat 1 and 3 were phosphorylated in IFNγ-treated cells, but the phosphokinase array did not identify other candidate pathways for further investigation.

If time had permitted, I would have gone on to investigate whether the observed effects of IFNγ in MDMs were Stat-dependent. Preliminary experiments using THP-1 cells and UV-killed Mtb to try to model this were performed as part of an MSc project by Arthur Si. These were unhelpful as IFNγ did not have the same effect in THP-1 cells as in primary MDMs, and also attempts to inhibit Stat signaling using chemical inhibitors were unsuccessful as the chemicals were toxic to the cells.

I was unable to perform ChIP assays in directly infected primary MDMs due to difficulties in demonstrating that the 1% formaldehyde fixation step in the ChIP protocol was sufficient to completely sterilize the Mtb-infected samples in order to allow them to be removed from the category 3 biocontainment facility. Given the published data suggesting epigenetic changes at the MMP-9 locus in IFNγ-treated cells, I would have liked to go on to investigate changes in histone acetylation and transcription factor binding at the MMP-1 and -3 promoters in IFNγ-treated MDMs [338].

In mice, excessive IFNγ production driven by transgenic expression in the lungs caused emphysematous changes with neutrophil- and macrophage-mediated inflammation and increased expression of MMP-9 and -12 was implicated in this pathology [340]. *M. bovis* BCG-induced MMP-9 expression by murine peritoneal macrophages was suppressed in the presence of IFNγ [96].

Early human trials of adjuvant IFNγ treatment administered at the site of TB disease suggest that there may be benefit in improving bacterial clearance and patient symptoms in cavitatory pulmonary disease [232, 233]. It would be fascinating to study the effect of such treatment on MMP activation in such cases, which could be achieved by measuring MMP activity in BAL or sputum samples, as in [85]. A collaboration with the New York group who carried out the study reported in [232] might allow a retrospective analysis of such parameters in samples from this patient cohort.

5 The effects of HDAC and HAT inhibition on MMP expression

5.1 Introduction

Previous studies by other researchers have demonstrated that the expression of various MMPs is controlled by epigenetic mechanisms, and epigenetic changes at several MMP genes have been described in cytokine-treated cells. In this chapter, the role of histone acetylation and deacetylation in regulating Mtb-driven MMP expression is investigated. Histone acetylation is a dynamic epigenetic mark which usually correlates with transcriptional activation of associated genes.

To investigate whether histone acetylation status affected MMP expression in the context of pulmonary TB, a range of chemical inhibitors were used to inhibit HDAC and HAT activity in epithelial cells and MDMs, and MMP responses to Mtb were examined. The HDAC inhibitors used were Trichostatin A (TSA), MS-275 and CBHA. Two HAT inhibitors, anacardic acid and HAT inhibitor II, were used in epithelial cells and macrophages to determine the effect of inhibiting HAT activity on MMP expression. Initial experiments in A549s were performed to establish the optimal range of concentrations for each inhibitor.

TSA is a non-selective HDACi, and was used at 1-100 ng/ml, which are relatively low concentrations compared to the published literature. CBHA (m-Carboxycinnamic acid bis-Hydroxamide), a hybrid polar compound which inhibits HDACs non-selectively at 1-4 μM, was used to confirm the results obtained with TSA [252]. MS-275 (also known as entinostat), a class I-selective HDACi which is relatively HDAC1-selective at lower concentrations (the EC_{50} for HDAC1 is <0.2 μM compared to >1 μM for HDAC2 and 3) was used at 0.4-10 μM [250].

Apicidin, a relatively HDAC2- and 3-selective HDACi, which has an EC_{50} 100-fold higher for HDAC1, was also used in preliminary experiments with A549 cells. However, this inhibitor did not show a consistent effect on MMP secretion, and caused cell death at higher concentrations (data not shown), therefore further experiments in primary cells were not performed.

SiRNA targeted against the different Class I HDACs was used to further investigate the role of individual HDACs in regulating MMP expression, and ChIP assays were performed to examine changes in histone acetylation occurring at the MMP-1 promoter in CoMTb-stimulated cells.

5.2 Results

5.2.1 TSA inhibits CoMTb-driven MMP-1 and -3 expression in respiratory epithelial cells

NHBEs were pre-incubated with TSA (1-10 ng/ml) for 2 hours prior to stimulation with CoMTb at a 1:5 dilution. For RT-PCR experiments total RNA was collected at 24 hours, for protein secretion experiments supernatants were collected at 72 hours. Cells were treated with TSA continuously throughout the experiment. There was a trend to reduced basal and CoMTb-stimulated MMP-1 secretion in the presence of TSA (10 ng/ml), but these differences were not statistically significant (Figure 27a). MMP-3 was quantified in the same samples, and although TSA did not affect basal MMP-3 secretion, CoMTb-stimulated MMP-3 secretion was significantly reduced in a dose-dependent manner from 2388 pg/ml to 1805 and 685 pg/ml with TSA (1 and 10 ng/ml) respectively (Figure 27b). Basal MMP-3 secretion was not reduced by (TSA 10 ng/ml).

TSA reduced the MMP-1 mRNA response to CoMTb stimulation, but this was not statistically significant (Figure 27c). MMP-3 mRNA increased 4.5-fold with CoMTb stimulation, and this upregulation was significantly inhibited by TSA ($p<0.05$) (Figure 27d). The results suggest that non-selective HDAC inhibition blocks MMP-1 and -3 upregulation in response to CoMTb stimulation of respiratory epithelial cells.

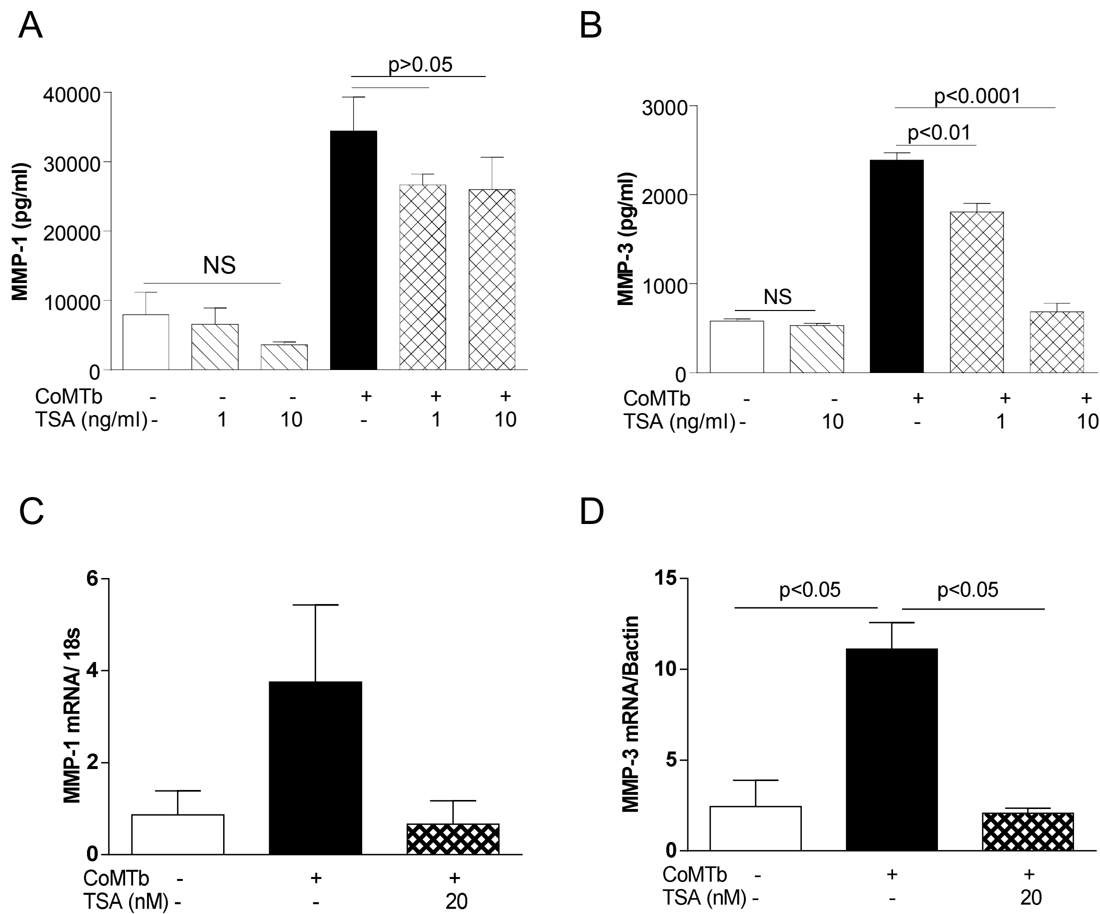


Figure 27. TSA inhibits CoMTb-driven MMP-1 and -3 expression in NHBEs

NHBEs were pre-incubated with TSA at the concentrations indicated for 2 hours prior to stimulation with CoMTb 1:5 for 72 hours (A and B) or 24 hours (C and D). MMP-1 and -3 were measured by ELISA in culture supernatants and MMP-1 and -3 mRNA was quantified by RT-PCR. A) TSA (10 ng/ml) reduced basal MMP-1 secretion by 55% and CoMTb-stimulated MMP-1 secretion by 25% (not statistically significant). B) TSA did not affect basal MMP-3 secretion, but reduced CoMTb-stimulated MMP-3 secretion in a dose-dependent manner from 2388 pg/ml to 1805 or 685 pg/ml (1 and 10 ng/ml respectively). C) MMP-1 mRNA increased 4.3-fold following 24 hours CoMTb stimulation, and this was inhibited in the presence of TSA (20 nM) (not statistically significant). D) MMP-3 mRNA increased 4.5-fold following 24 hours CoMTb stimulation, and this upregulation was inhibited by TSA (20 nM) ($p<0.05$). Results presented for MMP secretion are from one experiment performed in triplicate and representative of at least three independent experiments. The RT-PCR results are from one experiment in duplicate representative of three experiments.

5.2.2 CBHA inhibits CoMTb-driven MMP-1 and -3 secretion by respiratory epithelial cells

An alternative non-selective HDACi CBHA was used at a concentration of 0.1-10 μM in NHBES to confirm the results obtained with TSA. An increase in basal MMP-1 secretion from 156 to 413 pg/ml was observed when NHBEs were treated with CBHA 10 μM, which was not statistically significant. Figure 28a demonstrates that although there was a tendency to reduced MMP-1 secretion in the presence of CBHA, this was only statistically significant for CBHA (5 μM), which reduced CoMTb-driven MMP-1 secretion from 797 to 473 pg/ml ($p<0.05$). It is plausible that the higher concentration of 10 μM may have produced off-target effects, accounting for the U-shaped curve observed.

Basal MMP-3 secretion was not altered by CBHA (1 or 10 μM). CoMTb-driven MMP-3 secretion was inhibited in a dose-dependent manner, and CBHA (1 μM) was sufficient to completely inhibit CoMTb-induced MMP-3 secretion (Figure 28b). This pattern of results for TSA and CBHA, with MMP-3 more readily suppressed by HDACi than MMP-1, suggests that there may be differences in the regulation of these closely-related genes which render MMP-3 more susceptible to HDAC inhibition.

5.2.3 MS-275 enhances CoMTb-driven MMP-1 and -3 secretion by respiratory epithelial cells

NHBEs were subsequently pre-incubated with the class I-selective HDACi MS-275 (1-10 μM), and an opposite effect was observed. Both basal and CoMTb-stimulated MMP-1 secretion were increased in the presence of MS-275 (10 μM), although the increase with MS-275 alone was not statistically significant (Figure 29a). MS-275 (10 μM) also increased basal MMP-3 secretion 3.8-fold but again this was not statistically significant. MS-275 at a lower concentration of 1 μM increased MMP-3 secretion 4.6-fold compared to CoMTb alone (Figure 29b). Thus, a higher concentration which would have inhibited HDAC2 and 3 activity in addition to HDAC1 was required to significantly affect MMP-1 secretion, whereas MMP-3 secretion was enhanced by a concentration of MS-275 which should have preferentially

inhibited HDAC1. The effects observed were not due to cytotoxic effects of the chemical inhibitors, as when cell viability was assessed at the end of the experiment by trypan blue exclusion there was no difference in viability between the control and inhibitor-treated wells (data not shown). The enhancement of MMP-1 and -3 secretion observed with class I HDAC inhibition as compared to non-selective HDACi suggests that the class I HDACs may be key negative regulators of epithelial cell MMP expression.

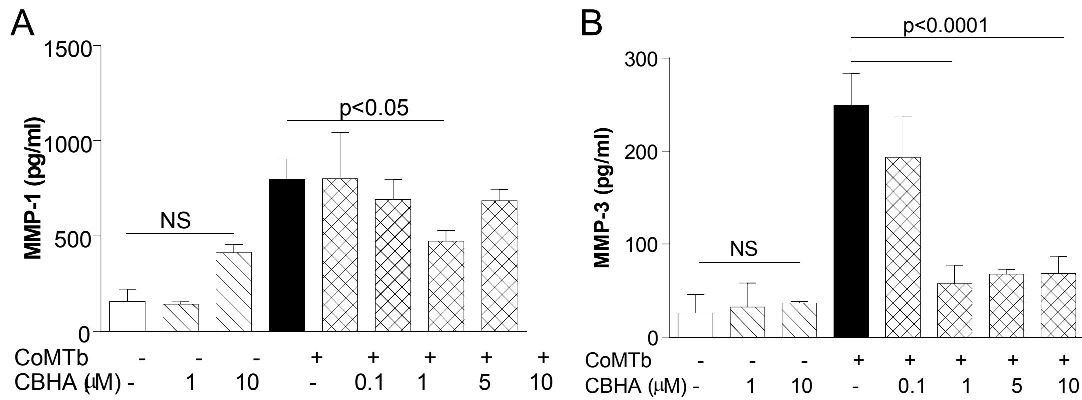


Figure 28. CBHA inhibits CoMTb-driven MMP-1 and -3 secretion by NHBEs

NHBEs were pre-incubated with CBHA (0.1-10 μM) for 2 hours prior to stimulation with CoMTb 1:5 for 72hrs. MMP-1 and -3 were measured by ELISA in culture supernatants. A) Basal MMP-1 secretion increased non-significantly from 156 to 413 pg/ml when NHBEs were treated with CBHA (10 μM). CBHA (5 μM) reduced CoMTb-driven MMP-1 secretion from 797 to 473 pg/ml ($p<0.05$). The reduction in MMP-1 secretion observed at higher and lower concentrations were not statistically significant. B) Basal MMP-3 secretion was not altered by CBHA (1 or 10 μM). CoMTb-driven MMP-3 secretion was inhibited in a dose-dependent manner, with CBHA (1-10 μM) abrogating CoMTb-induced secretion (all $p>0.05$ vs control, and $p<0.0001$ vs CoMTb alone). Results presented are from one experiment performed in triplicate and are representative of two independent experiments.

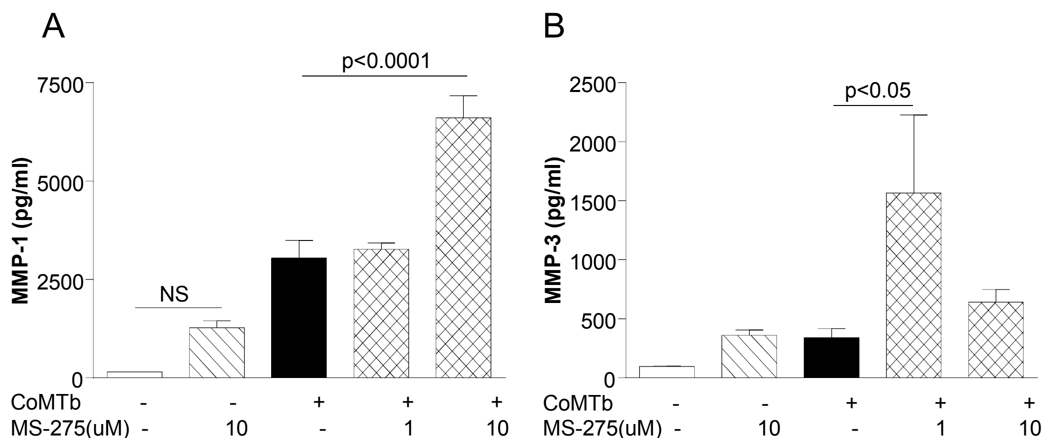


Figure 29. MS-275 enhances CoMTb-driven MMP-1 and -3 secretion by NHBEs

NHBEs were pre-incubated with MS-275 (1-10 μM) for 2 hours prior to stimulation with CoMTb 1:5 for 72 hours. MMP-1 and -3 were measured by ELISA in culture supernatants. A) Basal NHBE MMP-1 secretion increased 8.5-fold in the presence of MS-275 (10 μM) ($p>0.05$). CoMTb-stimulated MMP-1 secretion increased 2.2-fold with MS-275 (10 μM) compared to CoMTb alone ($p<0.0001$). B) MS-275 (10 μM) also increased basal MMP-3 secretion 3.8-fold but this was not statistically significant. MS-275 at a lower concentration of 1 μM significantly enhanced MMP-3 secretion induced by CoMTb (1566 compared to 340 pg/ml, 4.6-fold change, $p<0.05$). Results presented are from one experiment performed in triplicate and representative of two independent experiments.

The effects of these HDACi were then investigated in primary human MDMs, where the concentrations of inhibitors required to demonstrate a significant effect were different. The concentration of TSA was increased to 100 ng/ml rather than 10 ng/ml without a significant effect on cell viability, CBHA was used at a lower concentration of 4 μM, while MS-275 showed effects on MMP secretion at 1 μM, but caused significant cell death at higher concentrations of 5-10 μM.

5.2.4 TSA inhibits MDM MMP-1, -3 and -7 secretion in response to Mtb infection

Preliminary experiments showed that higher concentrations of TSA were needed to inhibit MDM MMP secretion than were used in epithelial cells (data not shown). However, Mtb-driven MMP-1, -3 and -7 secretion was completely inhibited when cells were pre-incubated with TSA (100 ng/ml) and then infected with H37Rv (Figure 30). Inhibition of MMP-1 and -3 secretion was consistent between all the donors studied, but MMP-7 secretion was not suppressed in one of four donors studied. Thus, MDM MMP expression in response to Mtb infection is sensitive to HDAC inhibition.

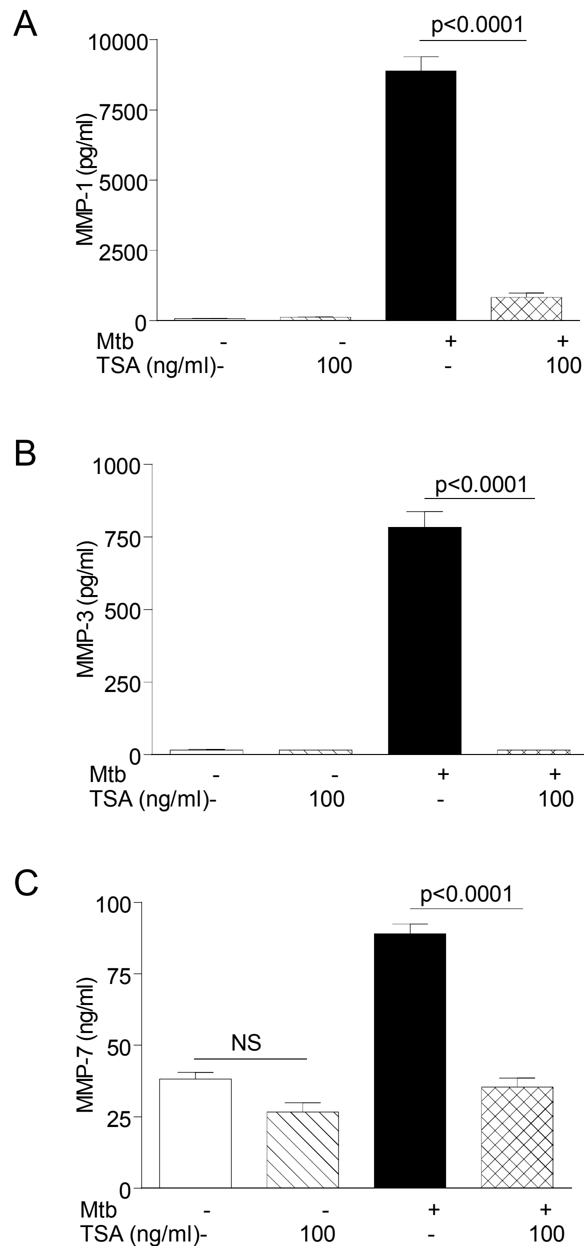


Figure 30. TSA inhibits MDM MMP-1, -3 and -7 secretion in response to Mtb infection

MDMs were pre-incubated with TSA (100 ng/ml) for 2 hours prior to infection with H37Rv (MOI 1). Supernatants were collected after 72 hours and MMP-1, -3 and -7 were measured by Luminex. A) Mtb-driven MMP-1 secretion is completely inhibited by TSA (100 ng/ml) ($p<0.0001$ vs Mtb alone, $p>0.05$ vs TSA alone). B) Mtb-driven MMP-3 secretion is also completely inhibited by TSA (100 ng/ml) ($p<0.0001$ vs Mtb alone, $p>0.05$ vs TSA alone). C) Mtb-driven MMP-7 secretion is completely inhibited by TSA (100 ng/ml) ($p<0.0001$ vs Mtb alone, $p>0.05$ vs TSA alone). Results presented are from one experiment performed in triplicate and representative of three independent experiments in different donors.

5.2.5 CBHA inhibits MDM MMP-1 and -3 and enhances MMP-7 secretion in response to Mtb infection

The results obtained using TSA were compared to those with another non-selective HDACi, CBHA. As for TSA, MMP-1 and -3 secretion was inhibited by CBHA. MDM MMP-1 secretion induced by Mtb infection was inhibited by 77% and MMP-3 secretion was reduced to undetectable levels in the presence of CBHA (4 μM) (Figure 31a & b). CBHA was found to enhance MMP-7 secretion in two of four donors studied, but the effect of CBHA on MMP-7 was not consistent between donors, with no increase in MMP-7 observed in the other two donors (see Figure 31d).

Figure 31c shows that MMP-7 secretion was increased from 29 to 96 ng/ml by CBHA alone ($p=0.0001$), and Mtb-driven MMP-7 secretion was also increased 3-fold from 74 to 225 ng/ml in the presence of CBHA ($p<0.0001$). This was unexpected, and illustrates that the effects of non-selective chemical inhibitors can be unpredictable and difficult to interpret. It may be that the effects of TSA and CBHA on non-HDAC targets are different, and such an off-target effect could mediate the increase in MMP-7 secretion observed with CBHA.

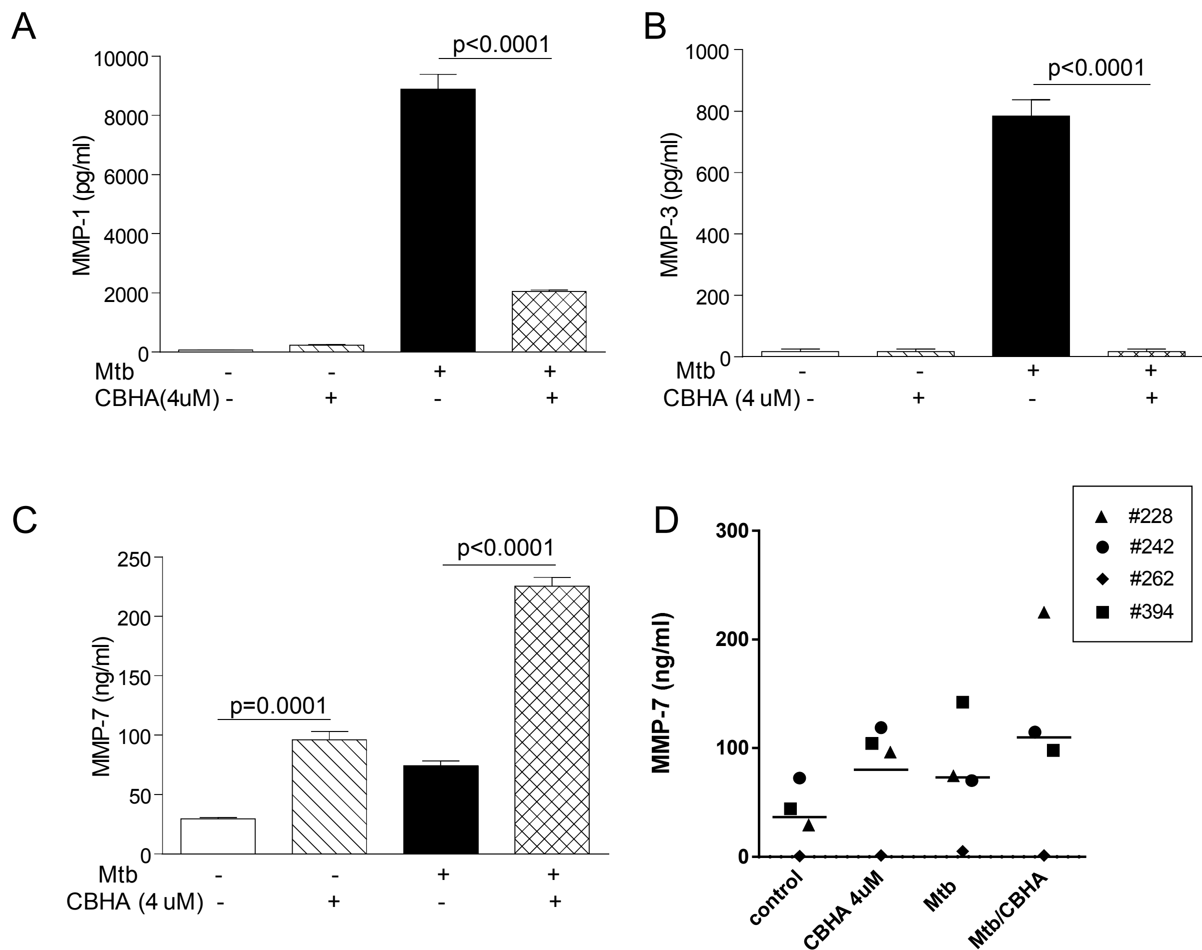


Figure 31. CBHA inhibits MDM MMP-1 and -3 and enhances MMP-7 secretion in response to Mtb infection

MDMs were pre-incubated with CBHA (4 μM) for 2 hours prior to infection with H37Rv (MOI 1). Supernatants were collected after 72 hours and MMP-1, -3 and -7 were measured by Luminex. A) MDM MMP-1 secretion induced by Mtb infection was inhibited by 77% in the presence of CBHA (4 μM) ($p<0.0001$). B) MMP-3 secretion was reduced to basal, undetectable levels by CBHA (4 μM) ($p<0.0001$). C) MMP-7 secretion in uninfected cells was increased from 29 to 96 ng/ml by CBHA alone ($p=0.0001$), and Mtb-driven MMP-7 secretion was also increased 3-fold from 74 to 225 ng/ml in the presence of CBHA ($p<0.0001$). D) Combined results from four independent experiments in triplicate using different donors, demonstrating that in two donors CBHA enhanced Mtb-driven MMP-7 secretion, while in two others MMP-7 secretion did not change significantly compared to Mtb alone. Results presented in A to C are from one experiment performed in triplicate and representative of three independent experiments (two experiments for MMP-7) in different donors.

5.2.6 MS-275 selectively inhibits MMP-1 and -3 secretion by Mtb-stimulated MDMs

As shown in Figure 32, MS-275 (1 μM) was found to be sufficient to inhibit Mtb-driven MMP-1 secretion by 92% and to reduce MMP-3 secretion to undetectable concentrations. However, Mtb-driven MMP-7 secretion was not significantly altered in the presence of MS-275 (1 μM), and higher concentrations could not be used as they were found to cause significant cell death. At a concentration of 1 μM, MS-275 would be expected to predominantly inhibit HDAC1 activity, and therefore these results imply that HDAC1 activity may be necessary for Mtb-driven MMP-1 and -3 expression in MDMs. Again, this result contrasts with the observations made in NHBEs, emphasizing that these epigenetic regulatory mechanisms operate in a cell and stimulus-specific manner.

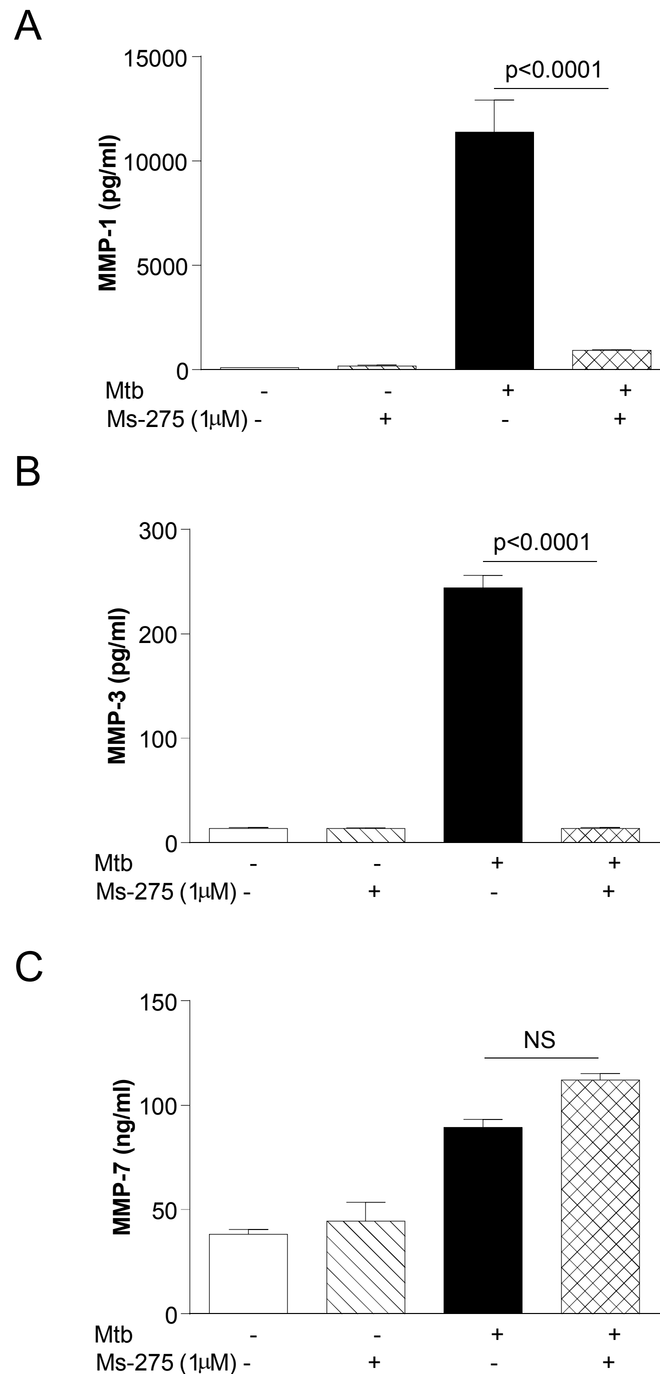


Figure 32. MS-275 selectively inhibits MMP-1 and -3 secretion by Mtb-stimulated MDMs

MDMs were pre-incubated with MS-275 (1 μM) for 2 hours prior to infection with H37Rv (MOI 1). Supernatants were collected after 72 hours and MMP-1, -3 and -7 were measured by Luminex. A) Mtb-driven MMP-1 secretion is inhibited by 92% by MS-275 (1 μM) ($p<0.0001$ vs Mtb alone, $p>0.05$ vs MS-275 alone). B) Mtb-driven MMP-3 secretion is inhibited to undetectable concentrations (<16 pg/ml) by MS-275 (1 μM) ($p<0.0001$ vs Mtb alone). C) Mtb-driven MMP-7 secretion is not significantly altered in the presence of MS-275 (1 μM) ($p>0.05$ vs Mtb alone). Results presented are from one experiment performed in triplicate and representative of four independent experiments in different donors.

Having demonstrated that Mtb-induced MMP expression in both epithelial cells and macrophages was sensitive to HDAC inhibition, the role of HAT activity was then investigated using the chemical HAT inhibitors anacardic acid (AA) and HAT inhibitor II (HATi II). Anacardic acid is closely structurally related to salicylic acid, and has previously been reported to have some antimicrobial activity, including against Mtb, so it was important to assess whether it had an effect on Mtb H37Rv growth or viability before using it in experiments involving direct infection with Mtb. When H37Rv was cultured in broth containing AA at concentrations from 2 to 25 μM, no effect on the rate of mycobacterial growth was observed (Figure 33c). The effect of AA on bacterial growth in the context of intracellular infection was not investigated.

5.2.7 Anacardic acid inhibits Mtb-driven MMP-1 and -3 secretion in MDMs and NHBEs

Anacardic acid (AA) (10 μM) significantly reduced Mtb-stimulated MDM MMP-1 secretion by 74% and Mtb-induced MMP-3 secretion was inhibited by 61% (Figure 33a & b). Lower concentrations of AA did not produce significant inhibition. In NHBEs, MMP-1 secretion was significantly inhibited by a higher concentration of AA (20 μM) (Figure 33d). MMP-3 secretion was also inhibited in the two experiments in which MMP-3 was measured (data not shown).

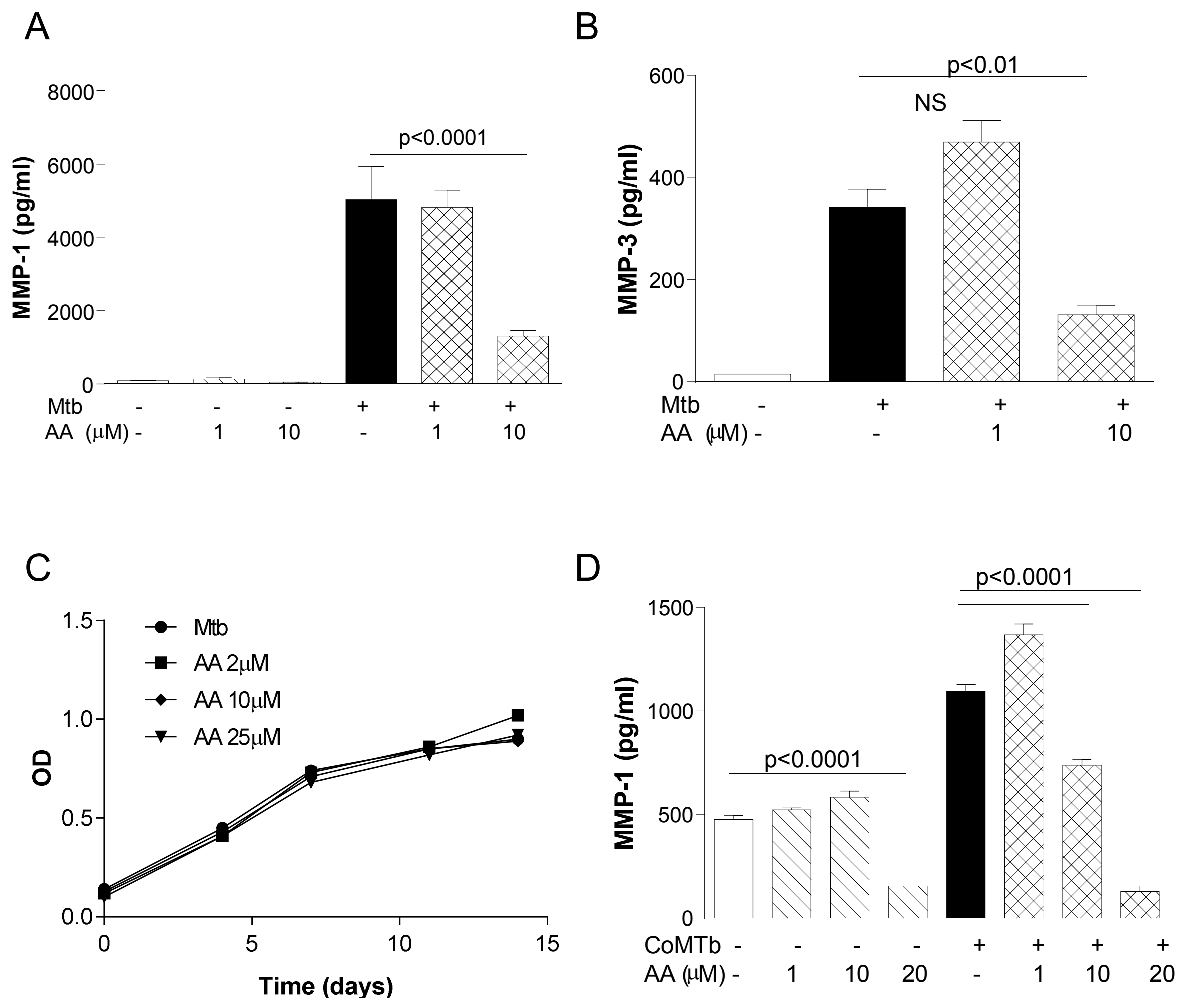


Figure 33. AA inhibits Mtb-driven MMP-1 and -3 secretion in MDMs and NHBEs

In A & B, MDMs were pre-incubated with AA (1 or 10 μM) for 2 hours prior to infection with H37Rv (MOI 1). In C, H37Rv was cultured in the presence of AA (2-25 μM) in order to assess whether it affected bacterial growth. For D, NHBEs were pre-incubated with AA (1-20 μM) for 2 hours prior to CoMTb stimulation. Supernatants were collected after 72 hours and MMP-1 and -3 were measured by ELISA or Luminex. A) AA (10 μM) reduced Mtb-stimulated MDM MMP-1 secretion by 74% (5028 vs 1303 pg/ml, p<0.0001). B) MMP-3 was undetectable in control and AA-treated samples. Mtb-induced MMP-3 secretion was unaffected by AA (1 μM) but inhibited by 61% (342 vs 132 pg/ml) by AA (10 μM) (p<0.01). C) AA (2-25 μM) did not affect the growth of H37Rv as assessed by change in optical density over 14 days. D) In NHBEs, basal MMP-1 secretion is significantly inhibited by AA (20 μM) which also blocks CoMTb-induced MMP-1 secretion (p<0.0001). MMP secretion data presented are from one experiment performed in triplicate and representative of four independent experiments.

5.2.8 HAT inhibitor II selectively inhibits Mtb-driven MMP-1 and -3 secretion in MDMs

An alternative chemical compound, HATi II (10 μM) significantly inhibited MMP-1 secretion by Mtb-stimulated macrophages by 56% (5029 vs 2187 pg/ml) (Figure 34a). HATi II (10 μM) also inhibited Mtb-induced MMP-3 secretion from 1653 to 190 pg/ml (Figure 34b). MMP-7 secretion was found to be unaffected by HATi II at this concentration (Figure 34c). HATi II was not tested in NHBEs due to lack of time to perform the experiments.

5.2.9 HATi II inhibits Mtb-driven increases in MMP-1 and -3 mRNA in Mtb-infected MDMs but anacardic acid does not

As Mtb-driven MMP secretion was found to be sensitive to HAT inhibition, RT-PCR experiments were carried out to examine the effect of these inhibitors on MMP mRNA expression. MDMs were pre-incubated with HATi II (10 μM) or AA (10 μM) for 2 hours prior to infection with H37Rv (MOI 1). Total RNA was extracted after 24 hours and reverse transcribed for RT-PCR.

MMP-1 mRNA was increased 235-fold in Mtb-infected cells and this was inhibited by 62% in HATi II-treated cells ($p<0.05$), but AA at a concentration that had inhibited MMP-1 secretion did not significantly alter MMP-1 mRNA (Figure 35a). MMP-3 mRNA was increased in Mtb-infected cells and there was a trend to reduced MMP-3 mRNA with HATi II which was not statistically significant. Again, no significant change in MMP-3 mRNA with AA was observed despite the fact that MMP-3 secretion had been inhibited by AA (10μM) (Figure 35b). Results presented are from one experiment performed in triplicate and are representative of three independent experiments in different donors.

The HAT inhibition results support the hypothesis that HAT activity is required for inducible expression of MMP-1 and -3 in response to Mtb.

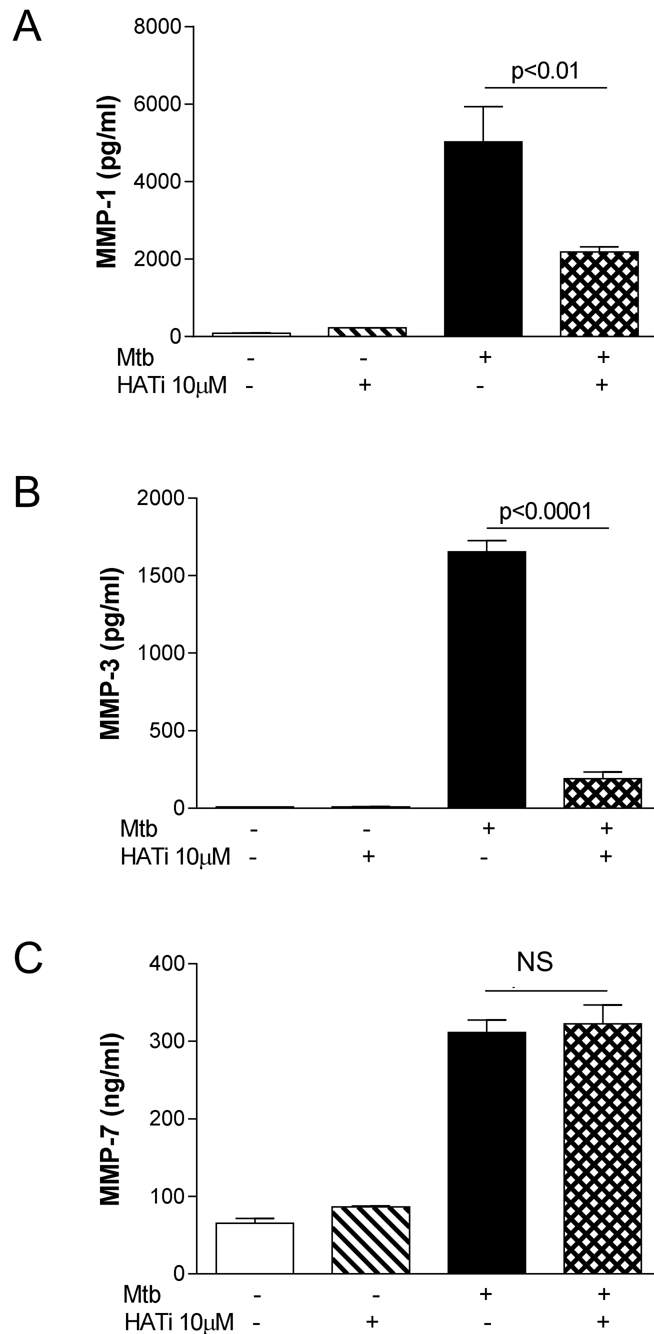


Figure 34. HATi II selectively inhibits Mtb-driven MMP-1 and -3 secretion in MDMs

MDMs were pre-incubated with HATi II (10 μM) for 2 hours prior to infection with H37Rv (MOI 1). Supernatants were collected after 72 hours and MMP-1 and -3 were measured by ELISA or Luminex. A) HATi II (10 μM) inhibits MMP-1 secretion by Mtb-stimulated macrophages by 56% (5029 vs 2187 pg/ml, p<0.01). B) HATi II (10 μM) also inhibits Mtb-induced MMP-3 secretion from 1653 to 190 pg/ml (p<0.0001). C) MMP-7 secretion is not affected by HATi II at this concentration. Results presented are from one experiment performed in triplicate and representative of four independent experiments in different donors.

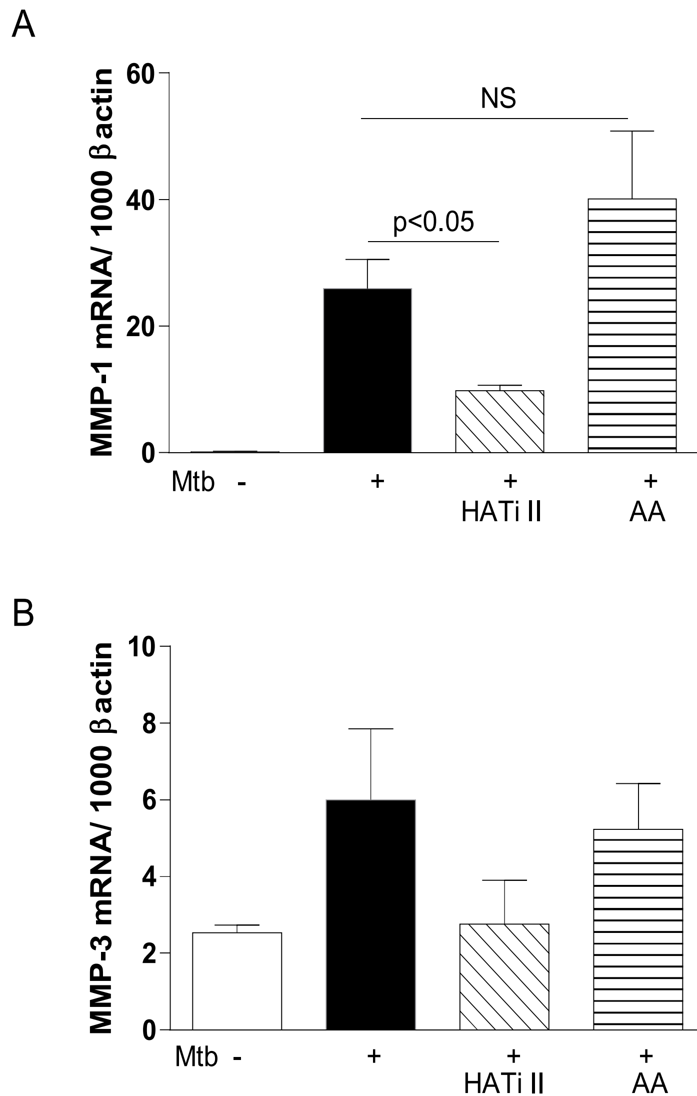


Figure 35. HATi II inhibits Mtb-driven increases in MMP-1 and -3 mRNA in Mtb-infected MDMs but AA does not

MDMs were pre-incubated with HATi II (10 μM) or AA (10 μM) for 2 hours prior to infection with H37Rv (MOI 1). Total RNA was extracted after 24 hours and reverse transcribed for RT-PCR. A) MMP-1 mRNA was increased 235-fold in Mtb-infected cells and this was inhibited by 62% in HATi II-treated cells ($p<0.05$). AA did not significantly alter MMP-1 mRNA. B) MMP-3 mRNA was increased in Mtb-infected cells and there was a trend to reduced MMP-3 mRNA with HATi II which was not statistically significant. No significant change in MMP-3 mRNA with AA was observed. Results presented are from one experiment performed in triplicate and are representative of three independent experiments in different donors. P values are for one-way ANOVA with Tukey's test for multiple comparisons.

5.2.10 SiRNA silencing of Class I HDACs

The chemical inhibition experiments showed that non-selective HDAC inhibition blocked epithelial cell MMP-1 and -3 expression, while MS-275 enhanced MMP secretion. This suggested that different HDACs might have distinct roles in the regulation of MMP expression, and therefore this was investigated by transfection of primary epithelial cells with siRNA targeting different class I HDACs.

A protocol for transfecting NHBEs with Dharmacon Smartpool siRNA and Lipofectamine transfection reagent had previously been optimized in our group by Dr Shivani Singh. This protocol was used with minor modifications alongside commercially available pooled siRNAs (see methods). SiRNA concentrations were reoptimized for each target and gene and protein silencing was verified as described below. Non-targeting siRNA and transfection reagent alone were used as negative controls for non-specific effects on MMP expression.

5.2.11 Silencing HDAC1 expression in NHBEs does not affect CoMTb-driven MMP-1 & -3 expression

NHBEs were transfected with 30 nM non-targeting (NT) or HDAC1 Smartpool siRNA, or treated with lipofectamine alone prior to CoMTb stimulation. HDAC1 mRNA was assayed 24 hours post-transfection to determine the efficiency of siRNA silencing, and MMP-1 and -3 mRNA were also quantified. In separate experiments using a 48 hour timepoint, MMP-1 and -3 were quantified in culture supernatants by ELISA, and cells were lysed for analysis of HDAC1 protein expression by Western blotting.

Initial optimization experiments demonstrated that HDAC1 mRNA was significantly reduced by more than 80% in cells transfected with HDAC1 Smartpool siRNA 30nM compared to NT siRNA or untransfected controls ($p=0.001$ NT vs HDAC1 siRNA) (Figure 36a). Western blotting showed that HDAC1 protein expression was also significantly reduced following transfection with HDAC1 Smartpool siRNA but was unaffected by NT siRNA (Figure 36c).

The Western blot experiment for HDAC1 protein expression was performed once in triplicate.

Further RT-PCR experiments, all using HDAC1 siRNA at 30 nM, showed that HDAC 1 mRNA was consistently reduced by greater than 80% in HDAC1 siRNA-treated compared to NT-treated cells, and HDAC1 mRNA was not affected by CoMTb-stimulation of the cells (Figure 36b). Although HDAC1 expression was effectively suppressed, MMP upregulation in response to CoMTb stimulation was unchanged compared to control conditions. When MMP-1 mRNA was measured, no change in CoMTb-driven MMP-1 expression was observed (Figure 36d). MMP-1 mRNA was assessed in two independent experiments performed in triplicate and the results shown are from one representative experiment. MMP-1 protein secretion was also unchanged: MMP-1 was present at 3204 pg/ml in the CoMTb/HDAC1 siRNA samples, compared to 3746 pg/ml in the CoMTb/NT siRNA samples ($p>0.05$) (Figure 36e). Basal MMP-1 secretion in the transfected but unstimulated cells was also unchanged (NT 312 pg/ml vs HDAC1 243 pg/ml). The protein secretion experiment was performed once in triplicate.

MMP-3 mRNA and protein expression were subsequently measured in the same samples. MMP-3 mRNA was only upregulated 3-fold with CoMTb in these experiments, and this increase was not statistically significant, but no difference in MMP-3 expression was observed between NT and HDAC1 siRNA transfected cells (Figure 36f). MMP-3 protein secretion was also poorly upregulated, from 157 pg/ml in the NT/control conditions to 290 pg/ml with NT siRNA and CoMTb (Figure 36g). Basal MMP-3 secretion was unaffected by HDAC1 siRNA, although the small difference in CoMTb-stimulated MMP-3 secretion with HDAC1 siRNA was statistically significant (CoMTb/HDAC1 siRNA-treated 202 pg/ml vs 290 pg/ml in the CoMTb/NT control, $p<0.01$).

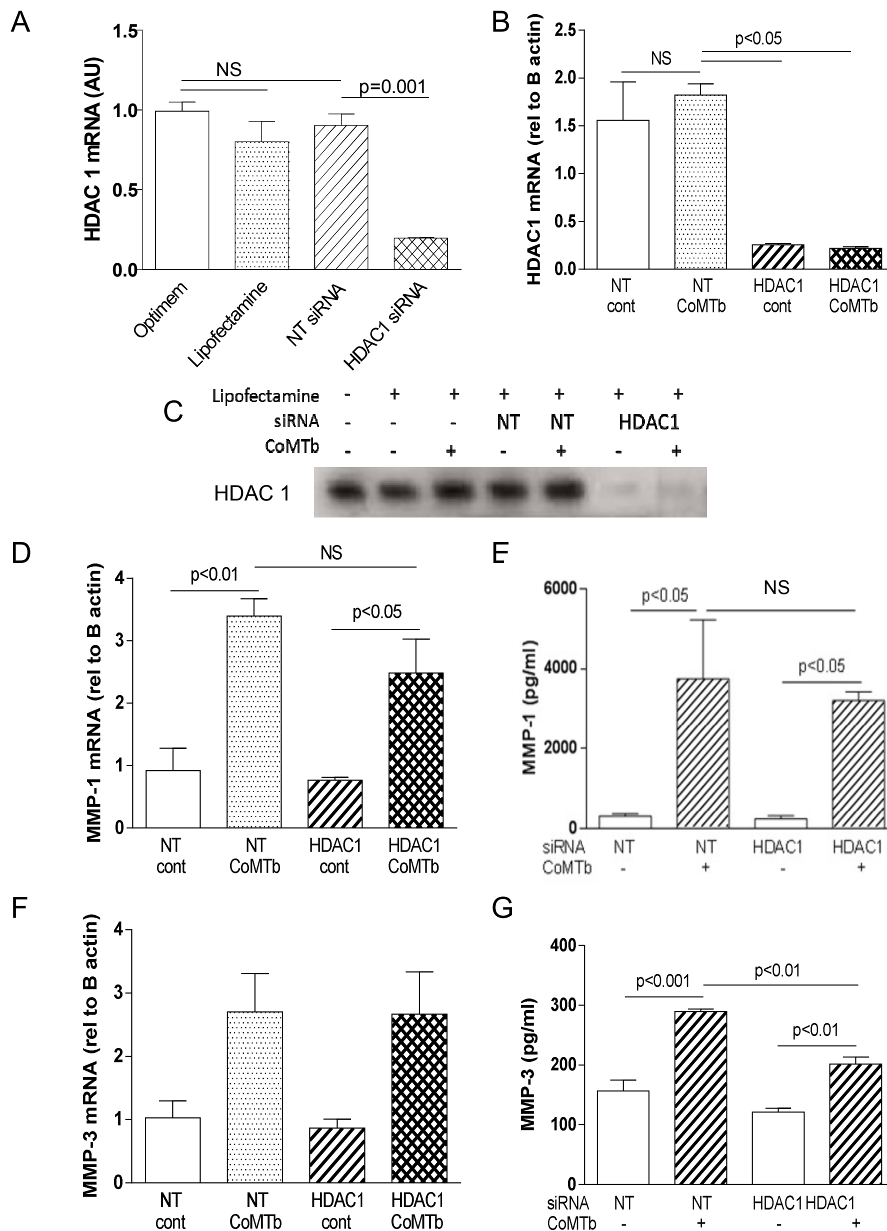


Figure 36. Silencing HDAC1 expression in NHBEs does not affect CoMTb-driven MMP-1 & -3 expression

NHBEs were transfected with NT or HDAC1 siRNA or treated with lipofectamine alone. They were then stimulated with CoMTb 1:5 for 24 or 48 hours. After 24 hours total RNA was extracted for RT-PCR. At 48 hours MMP-1 and -3 in culture supernatants were quantified by ELISA, and cells were lysed for analysis of HDAC1 protein expression by Western blotting. A) HDAC1 mRNA is reduced by 80% in cells transfected with HDAC1 compared to NT siRNA or untransfected controls (p=0.001). B) HDAC1 mRNA is not significantly affected by CoMTb treatment. C) HDAC1 protein expression is greatly reduced following treatment with HDAC1 but not NT siRNA. D) MMP-1 mRNA upregulation in response to CoMTb stimulation is unaffected by silencing of HDAC1 expression. E) Basal and CoMTb-stimulated MMP-1 secretion are not affected by silencing of HDAC1 expression. F) The CoMTb-driven increase in MMP-3 mRNA was not affected by HDAC1 siRNA. G) CoMTb stimulated MMP-3 secretion in both NT and HDAC1 siRNA-transfected cells compared to control conditions, but this was significantly less with HDAC1 siRNA (202 vs 290 pg/ml, p<0.01).

5.2.12 Silencing HDAC2 expression in NHBEs does not inhibit CoMTb-driven MMP-1 & -3 expression

HDAC2 Smartpool siRNA was used at 30 nM as optimized for HDAC1 siRNA, and its effect on NHBE HDAC2 expression was verified at mRNA and protein level. Again, no significant effect on cell viability was detected. Transfection with HDAC2 siRNA reduced HDAC2 mRNA by 88% compared to expression in NT siRNA-treated controls ($p<0.001$) (Figure 37a). Western blots were performed to compare HDAC2 protein expression in HDAC2 and HDAC3 siRNA-transfected cells, and demonstrated a reduction in HDAC2 protein with HDAC2 siRNA, although detectable HDAC2 protein was still present at 48 hours post-transfection (Figure 37b).

Despite the successful knock-down of HDAC2 expression, there was no significant difference in the fold-induction of MMP-1 mRNA in HDAC2-silenced cells stimulated with CoMTb compared to NT-transfected cells (Figure 37c). Similarly MMP-1 secretion increased from 272 pg/ml to 3442 pg/ml by CoMTb in HDAC2-silenced cells ($p<0.01$) and CoMTb-stimulated MMP-1 secretion was not significantly different to that in NT-transfected cells treated with CoMTb (1912 pg/ml, $p>0.05$) (Figure 37d).

MMP-3 mRNA was increased 4.2-fold by CoMTb-stimulation of NT-transfected cells ($p<0.05$), and MMP-3 mRNA was not significantly affected by HDAC2 siRNA treatment (Figure 37e). MMP-3 protein secretion was also measured and was not affected by HDAC2 siRNA (data not shown).

The RT-PCR experiments were performed three times in triplicate and the results shown are from one representative experiment. The protein expression experiment was performed once in triplicate.

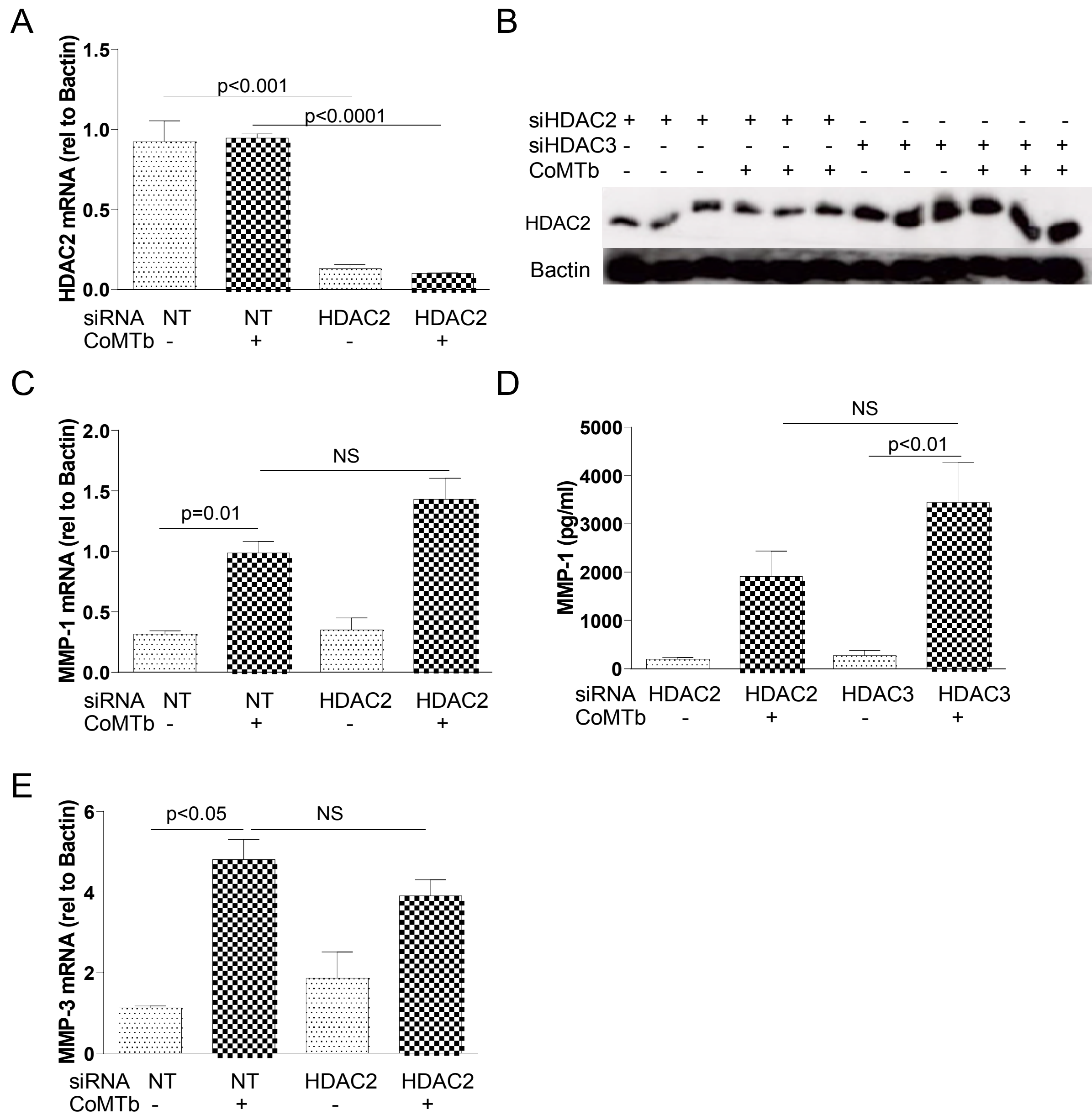


Figure 37. Silencing HDAC2 expression in NHBEs does not affect CoMTb-driven MMP-1 & -3 expression

NHBEs were transfected with HDAC2 or NT siRNA 30 nM and subsequently stimulated with CoMTb 1:5 for 24 or 48 hours. After 24 hours total RNA was extracted for RT-PCR. At 48 hours culture supernatants were collected and MMP-1 secretion quantified by ELISA, and cells were lysed for analysis of HDAC2 protein expression by Western blotting. A) Transfection with HDAC2 Smartpool siRNA reduced HDAC2 mRNA by 88% compared to expression in NT siRNA-treated controls ($p<0.001$). B) 48hrs post-transfection HDAC2 protein expression was reduced in HDAC2 siRNA-transfected cells compared to HDAC3 siRNA-transfected controls. C) MMP-1 mRNA was increased 4.1-fold in HDAC2-silenced cells stimulated with CoMTb compared to a 3.1-fold change in NT-transfected cells ($p>0.05$). D) MMP-1 secretion was increased from 195 pg/ml to 1912 pg/ml by CoMTb in HDAC2-silenced cells ($p<0.01$) and CoMTb-stimulated MMP-1 secretion was not significantly different to that in HDAC3-transfected cells ($p>0.05$). E) MMP-3 mRNA was increased 4.2-fold by CoMTb-stimulation of NT-transfected cells ($p<0.05$), and HDAC2 siRNA did not affect this.

5.2.13 Silencing HDAC3 expression in NHBEs does not inhibit CoMTb-driven MMP-1 & -3 expression

Transfection with HDAC3 siRNA reduced HDAC3 mRNA by 86% compared to NT siRNA-treated controls ($p<0.05$) (Figure 38a). Western blot analysis demonstrated that as with the other Smartpool siRNAs used, HDAC3 protein expression was considerably reduced in the siRNA-transfected cells, although HDAC3 was still detectable (Figure 38b).

Despite effective silencing of HDAC3 expression, MMP expression was unaffected by HDAC3 knockdown (Figure 38c & d). MMP-1 mRNA increased 3.3-fold with CoMTb-stimulation of NT-transfected cells ($p<0.001$) and results were indistinguishable with HDAC3 siRNA treatment. MMP-3 mRNA increased 4.4-fold with CoMTb-stimulation in both NT- and HDAC3 siRNA-transfected cells (both $p<0.001$). CoMTb-driven MMP-1 and -3 secretion were not significantly affected by HDAC3 silencing (data not shown).

The Western blotting and MMP secretion experiment was performed once in triplicate. RT-PCR measurements of HDAC3, MMP-1 and MMP-3 mRNA were performed in triplicate in three independent experiments and the results shown are from one representative experiment in triplicate.

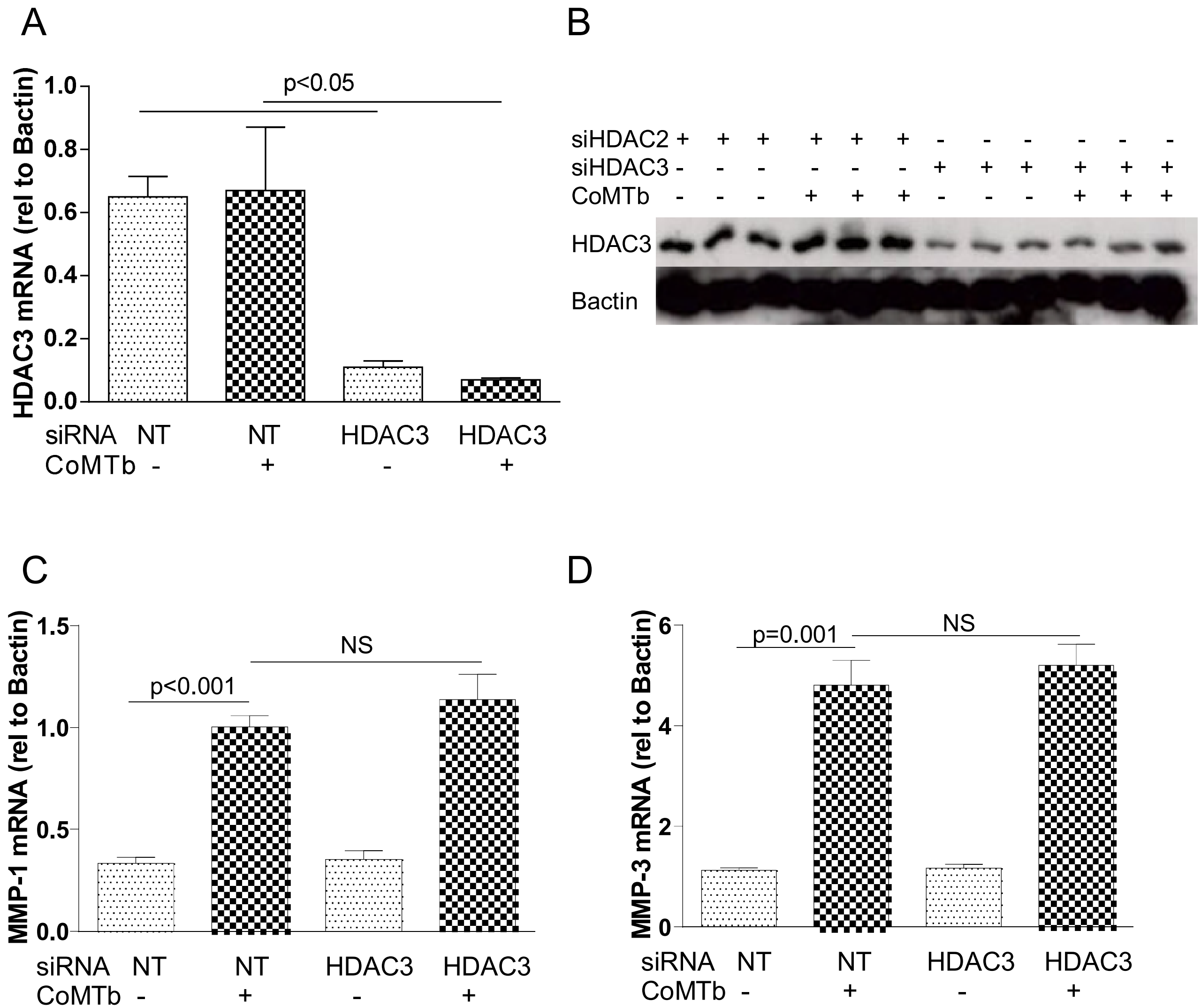


Figure 38. Silencing HDAC3 expression in NHBEs does not affect CoMTb-driven MMP-1 & -3 expression

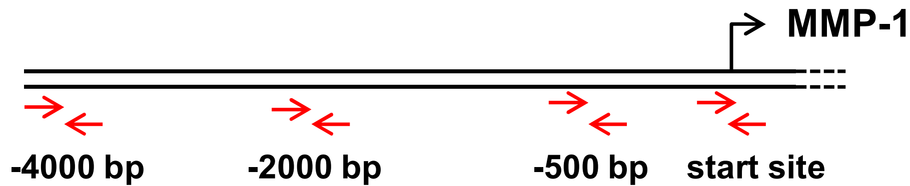
NHBEs were transfected with HDAC3 or NT siRNA 30 nM followed by stimulation with CoMTb for 24 or 48 hours. After 24 hours total RNA was extracted for RT-PCR. At 48 hours culture supernatants were collected and MMP-1 secretion quantified by ELISA, and cells were lysed for analysis of HDAC3 protein expression by Western blotting. A) Transfection with HDAC3 Smartpool siRNA reduced HDAC3 mRNA by 86% compared to NT siRNA-treated controls ($p<0.05$). B) Western blot analysis demonstrated that HDAC3 protein expression was considerably reduced in HDAC3 siRNA-transfected cells compared to HDAC2 siRNA-transfected controls. C) MMP-1 mRNA increased 3.3-fold with CoMTb-stimulation of NT-transfected cells ($p<0.001$) and results were indistinguishable with HDAC3 siRNA. D) MMP-3 mRNA increased 4.4-fold with CoMTb-stimulation in both NT- and HDAC3 siRNA-transfected cells (both $p<0.01$).

5.2.14 CoMTb-driven MMP-1 expression is associated with increased histone acetylation across the MMP-1 promoter region in A549s

The results presented so far suggested that both HDAC and HAT activity were necessary for CoMTb-mediated MMP-1 gene expression, and therefore I went on to examine the histone acetylation status of the MMP-1 promoter region under control and CoMTb-stimulated conditions by chromatin immunoprecipitation (ChIP). Association of RNA Polymerase II (RNA Pol II) with the promoter region was also assessed as a marker of transcriptional activation. These experiments were not performed in triplicate due to the number of cells required per condition. Preliminary experiments in NHBEs established that total cellular acetyl-histone H3 and H4 (as detected by Western blotting) was not altered in CoMTb-stimulated versus control cells. This was assessed at both short (1, 2 and 4 hours post-stimulation) and longer (8-24 hours) time points (data not shown).

Initial experiments in A549 cells suggested marked increases in histone H4 acetylation with CoMTb treatment for 2 hours, but not at earlier timepoints tested (Figure 39b and data not shown). The results shown are from one experiment not performed in triplicate and representative of two independent experiments.

A



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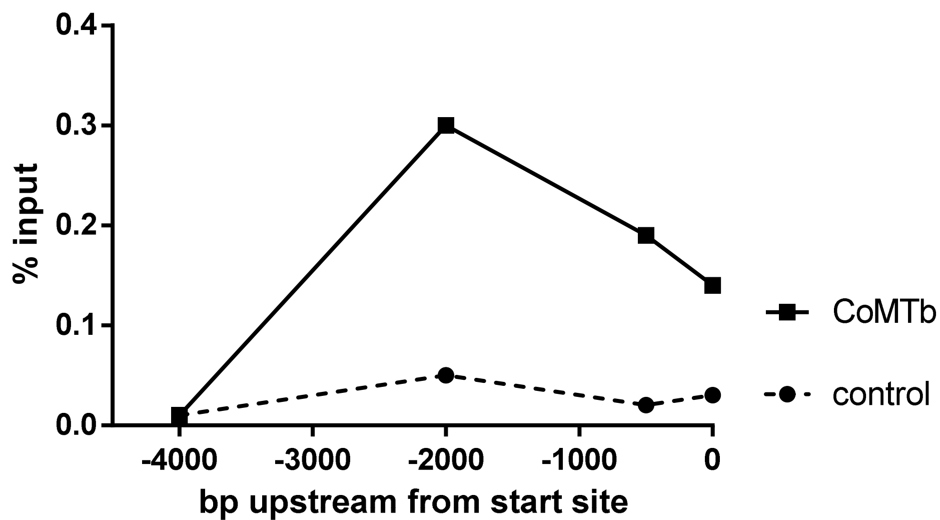

Figure 39. CoMTb stimulation drives increased histone H4 at the proximal MMP-1 promoter in A549s

ChIP assays were performed on A549 cells treated with CoMTb or control medium for 2 hours. Cells were fixed with paraformaldehyde and chromatin was sheared by sonication. Following overnight immunoprecipitation with normal mouse IgG or anti-acetyl histone H4 antibody, DNA was extracted and PCR performed with primers directed against different regions of the MMP-1 promoter (shown in A). Results were normalised to the input control for each sample. B) Low levels of acetyl-H4 are detectable across the MMP-1 promoter in unstimulated A549s, but acetyl-H4 is increased between -2000 bp and the transcriptional start site following 2 hours CoMTb stimulation.

5.2.15 CoMTb-driven MMP-1 expression is associated with increased histone acetylation and RNA Polymerase II binding to the MMP-1 promoter in NHBEs

The ChIP experiments were repeated in NHBEs, to assess whether the same changes in histone H4 acetylation on CoMTb stimulation could be detected in primary cells. Following CoMTb treatment for 1-5 hours, cells were fixed, lysed and sonicated, and immunoprecipitated overnight with antibodies directed against RNA Pol II, acetyl-H3 and acetyl-H4 as well as control normal IgG.

RNA Pol II binding to the MMP-1 promoter was increased 10 to 15-fold in cells treated with CoMTb for 2 hours, but no increase in RNA Pol II association with the promoter was detectable at 1 hour (Figure 40a). Histone H3 acetylation was also increased 2 hours post-stimulation but not after 1 hour, and was approximately 3-fold greater than under control conditions in the proximal promoter, and 5-fold higher than control when measured 2 to 4 kbp upstream of the MMP-1 transcriptional start site (Figure 40b). Acetylated histone H4 after 2 hours of CoMTb-treatment was also increased across the promoter region studied. Detection of histone H4 acetylation was 7-fold higher at -4 kbp, 10-fold higher at -2 kbp and -500 bp, and 6-fold higher than control conditions at the transcriptional start site (Figure 40c). A further experiment performed at a later timepoint (5 hours) suggested that the observed increase in histone H4 acetylation was sustained over this time period (data not shown).

Unfortunately it was not possible to combine the results of different experiments in order to represent them graphically, due to variation in the levels of background IgG binding between samples and experiments. The results shown are from one experiment performed at 1 and 2 hour timepoints, not in triplicate.

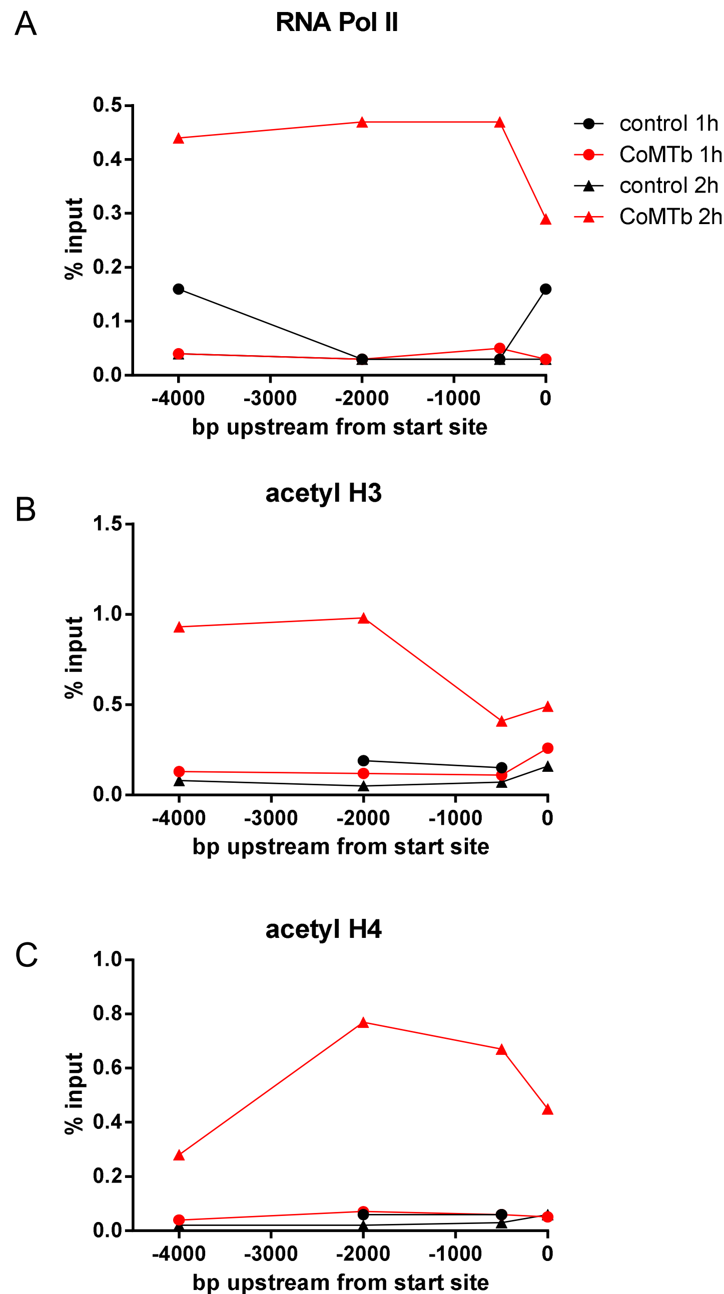

Figure 40. CoMTb-driven MMP-1 expression is associated with increased RNA Pol II binding and increased histone H3 and H4 acetylation of the MMP-1 promoter region.

ChIP assays were performed on NHBEs treated with CoMTb (red) or control medium (black) for 1 (circles) or 2 hours (triangles). A) RNA Pol II association with the MMP-1 promoter was increased 10 to 15-fold in cells treated with CoMTb for 2 hours, but no increase in binding compared to control conditions was detectable at 1 hour. B) Increased histone H3 acetylation was detected 2 hours post-stimulation but not earlier, and was approximately 3-fold greater than under control conditions in the proximal promoter, and 5-fold higher than control 2-4 kbp upstream of the MMP-1 transcriptional start site. C) Detection of acetylated histone H4 after 2 hours of CoMTb-treatment was increased across the promoter region studied. Histone H4 acetylation was 7-fold higher at -4 kbp, 10-fold higher at -2 kbp and -500 bp, and 6-fold higher than control conditions at the transcriptional start site.

5.3 Discussion

The results of the HDAC inhibitor experiments were not as would have been predicted from the theoretical effects of these agents. The HDACs are generally considered to be negative regulators of gene expression, and therefore it was expected that HDAC inhibition would result in a de-repression of MMP expression, as was previously observed in human dermal fibroblasts exposed to ultra-violet (UV) light, where MMP-1 expression was potentiated by the HDAC inhibitors TSA and sodium butyrate [341].

In fact, in my experiments non-selective HDAC inhibition with TSA inhibited Mtb-driven MMP-1 and -3 secretion and mRNA accumulation in both macrophages and primary epithelial cells. This paradoxical effect of TSA on MMP expression has previously been reported in a range of cell types. In an in vitro model of arthritis, chondrocyte collagenase and gelatinase activity was inhibited by either TSA or sodium butyrate. Global transcriptional analysis of MMP, TIMP and ADAMT expression in a chondrosarcoma cell line found that MMP-1, -3 and -7 expression induced by IL-1 were sensitive to HDAC inhibition, but MMP-2 and -9 expression were unaffected [342]. Studies in animal models found that TSA decreased MMP-1, -3 and -13 expression and clinical arthritis [343, 344]. In rats TSA protected against ischaemic retinal degeneration, and in tissue culture studies TSA blocked astrocyte expression of MMP-1 and -3 induced by TNFα [345]. LPS-induced MMP expression by murine BMDM was inhibited by TSA, which also inhibited TLR-induced production of pro-inflammatory cytokines such as IL-12 and TNFα [286]. Studies of MMP-9 expression in the context of cancer have reported contrasting effects of a wide range of HDACi (reviewed in [346]), including reduced MMP-9 expression in cancer cell lines treated with TSA. These contrasting results are attributed to the complex effects of HDACi on the expression of other genes which promote or suppress MMP-9 gene expression, such as Reversion-inducing cysteine-rich protein with kazal motifs (RECK) [346].

The inhibitory effect of TSA on MMP expression in this system was further investigated using the bipolar hybrid HDACi CBHA, in order to assess whether the results observed were a consequence of non-specific effects of TSA. The results obtained were similar to those with TSA; in NHBEs, MMP-1 and -3 secretion was inhibited in a dose-dependent fashion, while in macrophages MMP-1 and -3 were inhibited, with variable effects on MMP-7 which appeared to be a result of inter-donor variability. In some donors HDAC inhibition with CBHA had the predicted effect of enhancing Mtb-stimulated MMP-7 secretion, which was the opposite of the effect observed with TSA.

It would have been useful to study the effect of the HDAC inhibitor sodium valproate in this system, as this is a drug already widely used in clinical practice and therefore would add weight to any possible translational applications of the in vitro research findings. An alternative approach to further elucidating the effects of HDAC inhibition on MMP expression would be to use the novel class of BET drugs. These synthetic compounds interfere with the recognition of acetylated lysine residues on histone proteins by the BET proteins and thus specifically target the expression of many inflammatory genes [260]. These small molecule inhibitors have favourable properties for translational use as drugs, and several, including the BRD4 inhibitor JQ1, are now in clinical trial stages of drug development for cancer chemotherapy [347].

The class I-selective HDACi MS-275 was selected to examine whether the effects of TSA were attributable to inhibition of a particular class of HDACs, or whether they were likely to be due to inhibition of acetylation of other, non-histone substrates. In contrast to the preceding inhibitor experiments, in epithelial cells MS-275 was found to increase both basal and CoMTb-stimulated MMP-1 and -3 secretion. MS-275 is reported to selectively inhibit HDAC1 at lower concentrations, with an effect on HDAC2 and 3 at higher doses. In these experiments, a high concentration which would have affected HDAC2 and 3 activity as well as HDAC1 was required to significantly affect MMP-1 secretion, whereas MMP-3 secretion was enhanced by a concentration of MS-275 which would have preferentially inhibited

HDAC1. The enhancement of MMP-1 and -3 secretion observed with class I HDAC inhibition as compared to non-selective HDACi suggested that the class I HDACs may be key negative regulators of epithelial cell MMP expression, as predicted by the theoretical model of HDAC/HAT action, but that other effects of non-selective HDAC inhibitors, either on other HDACs such as members of the Class II HDACs, or effects on non-histone substrates, have the opposite effect and silence inducible MMP expression when non-selective inhibitors are employed.

Primary macrophages were found to be less resilient to treatment with MS-275, with significant cell death occurring at concentrations of 5 μM and above. However, at the lower concentration of 1 μM, which did not cause cell toxicity, MS-275 significantly inhibited MMP-1 and -3 secretion without affecting MMP-7. The 1 μM concentration of MS-275 is approximately five times the EC_{50} for HDAC1, but below the EC_{50} for HDAC2 and 3, and therefore these results suggest that intact HDAC1 activity may be required for MMP-1 and -3 expression in response to Mtb-stimulation of MDMs. MMP-1 and -3 are highly inducible and frequently co-expressed, and are known to have similar promoter structures, so the differences observed in the effects of HDAC inhibition on these MMPs and MMP-7 are not unexpected.

The intermediate pathways involved in the observed effects of HDACi on MMP expression have not been elucidated. Recently published data investigating the broader effects of HDACi on macrophage antimicrobial defences suggest that HDACi such as TSA and sodium valproate have multiple, diverse effects on macrophage function, for example reducing bacterial phagocytosis, reducing surface expression of TLRs and Macrophage scavenger receptor 1 (Msr1) and reducing generation of ROS and NO in response to infection with bacterial pathogens [348]. Macrophage secretion of pro-inflammatory cytokines such as TNFα, IL-6 and IL-12 were also inhibited. These macrophage functions are important in the response to Mtb infection, and the inhibitory effect of TSA on MMP expression may therefore involve complex effects on the macrophage inflammatory and bactericidal response as well

as, or instead of, direct effects on MMP gene expression as a result of changes in histone acetylation. Such effects could be further investigated in this system by simple experiments investigating the effect of TSA on the ability of macrophages to phagocytose and kill Mtb, as well as their production of pro-inflammatory cytokines and expression of surface markers such as integrins and TLRs. Alternatively, MMP inhibition by HDACi might be mediated by effects on acetylation of transcription factors such as NF-κB and AP-1 or effects on the MAPK pathway, as outlined in Chapter 1.

The effect of HAT inhibition on Mtb-driven MMP expression was investigated in order to determine whether both HDAC and HAT activity were required for MMP secretion. It was expected that HAT inhibition would block CoMTb/Mtb-stimulated expression of MMP-1 and-3. In keeping with this initial hypothesis, anacardic acid inhibited MMP-1 and 3 secretion in both NHBEs and MDMs. These findings correlate with published data in human dermal fibroblasts exposed to UV-light, where anacardic acid inhibited UV-induced MMP-1 expression, as did siRNA-silencing of p300 expression. Increased HAT activity and histone H3 acetylation and decreased HDAC activity preceded changes in MMP-1 mRNA abundance [341].

However, RT-PCR experiments suggested that anacardic acid did not prevent Mtb-driven upregulation of MMP-1 and -3 mRNA in MDMs, at a concentration which had significantly suppressed protein secretion. If the mechanism by which HAT inhibition blocks MMP upregulation is via reduced acetylation of histone proteins at the MMP promoter and start site, then changes in mRNA would be expected as a consequence of decreased promoter accessibility to the transcriptional assembly. This finding is therefore unexplained. As anacardic acid has been reported to display anti-microbial activity, including against mycobacteria, experiments were performed to ascertain that the reduced MMP expression observed in the presence of anacardic acid was not a consequence of reduced bacterial load. These suggested that anacardic acid had no effect on the rate of growth of Mtb H37Rv under cell-free laboratory conditions, but effects on intracellular growth cannot be excluded

on the basis of these results. Anacardic acid is not a specific HAT inhibitor, and it is known to have effects on the prostaglandin and lipoxygenase synthesis pathways. The prostaglandin synthase pathway has previously been shown to regulate MMP-1 secretion by Mtb-infected MDMs and therefore this is an alternative mechanism by which anacardic acid might be affecting MMP expression [79]. This was not investigated further in this system.

A chemically unrelated HAT inhibitor, HATi II, was also used in MDMs to confirm the results obtained with anacardic acid, and displayed similar properties in terms of inhibiting MMP-1 and -3 secretion. However, in this case a significant reduction in MMP-1 and -3 mRNA was observed in the presence of the inhibitor, which is more consistent with the expected mechanism of action of these chemicals.

Other aspects of macrophage and epithelial cell responses to Mtb could also have been studied with respect to the effects of epigenetic manipulation. In this project I restricted my observations to changes in MMP expression, but other characteristics of activated macrophages, such as cytokine and chemokine expression, reactive oxygen and nitrogen species production, and enhanced ability to kill intracellular Mtb could also be investigated. This would be particularly important in going forward with the translational uses of HDAC inhibitors as possible therapies for Mtb-driven immunopathology.

The complex and unexpected results obtained with chemical HDAC inhibition led to a series of experiments intended to elucidate the role of specific HDAC enzymes in the regulation of MMP expression using siRNA. These experiments were performed in NHBE cells, as previous attempts within our group to optimise siRNA methodology in primary human macrophages have been unsatisfactory. The initial hypothesis, following on from the HDACi experiments, was that silencing of one or more class I HDACs would de-repress MMP expression, or that a specific HDAC would be required for CoMTb-driven MMP expression, thus identifying key positive or negative regulators of MMP expression.

The siRNA experiments were successful in the sense that excellent mRNA silencing of greater than 80% was achieved for each of HDAC1, 2 and 3, and this was found to translate into a detectable reduction in HDAC protein in each case. This was achieved without significant cell death in the siRNA-treated cells compared to control conditions. However, in each case, this reduction in HDAC expression did not produce any significant alteration in MMP-1 or -3 mRNA or protein secretion in response to CoMTb. I could have gone on to check for effects on other inducible MMPs, such as MMP-9 or -13, and I would have liked to investigate the effect of silencing HDAC8, the remaining class I HDAC, and also the effect of silencing class II HDACs. Unfortunately there was not sufficient time to perform these further experiments.

In other experimental systems, several different HDACs have been implicated in MMP regulation. Investigation of the mechanisms mediating the observed effects of HDACi in arthritis models identified differences in MMP-1 promoter acetylation in synovial fibroblasts from rheumatoid arthritis (RA) patients compared to osteoarthritis (OA). HDAC4 was implicated in blocking MMP-1 expression [349]. In synovial cartilage from human subjects siRNA silencing of HDAC7 blocked MMP-13 expression [350]. In another study silencing HDAC1 with siRNA in synovial fibroblasts from RA patients enhanced TNFα-induced MMP-1 expression [351]. It therefore seems that different HDACs play specific roles in controlling MMP expression in different cell types and in different pathological conditions.

The results obtained in these experiments may be explained in a number of ways. Firstly, although the silencing of gene expression achieved was efficient and within the expected target range, the HDAC Western blots demonstrated HDAC protein was still detectable 48 hours after siRNA treatment. This may reflect a slow turnover of HDAC proteins under these experimental conditions, or simply that even a low level of HDAC mRNA production can translate into significant protein synthesis. In either case, the residual HDAC expression may be sufficient to fulfill the necessary functions for MMP expression/repression. Alternatively, since in these experiments only one HDAC enzyme was being silenced at a time, there may

be sufficient redundancy of function between the different HDACs that in the absence of one protein, another HDAC is able to perform its usual function. A third explanation would be that our hypothesis that class I HDACs would be key regulators of MMP expression, which was based on the results obtained in the chemical inhibition experiments, was incorrect, and that silencing of other HDACs, such as HDAC8 or the Class IIa HDACs would have had a significant effect on MMP-1 and -3 expression.

Studies undertaken in several different models have previously demonstrated clear phenotypes in HDAC-deficient cells, findings which argue against the hypothesis that my results can be explained by redundancy of HDAC function. Murine HDAC3-deficient macrophages were found to display a significant deficit in the expression of many inflammatory genes in response to LPS. Part of the deficit was attributable to loss of basal and LPS-inducible expression of IFNα, which generates a cascade of Stat1-dependent gene expression. Inhibition of COX-1 partially reversed the phenotype, suggesting that up-regulation of COX-1 expression contributed to the defective inflammatory gene expression [352]. Equally, in primary human GM-CSF-differentiated macrophages siRNA targeted against HDAC1 and 3 enhanced IL-8 production in response to LPS stimulation, although HDAC2 siRNA did not have the same effect [353]. In contrast, silencing of HDAC2, 3 and 8 expression with siRNA in A549 cells enhanced IL-1β-driven GM-CSF expression, whereas HDAC1 siRNA did not have the same effect [354]. These contrasting results imply that the roles of the different HDACs may be determined by both cell type and stimulus.

ChIP experiments were carried out to further investigate the paradoxical results obtained. As MMP-1 mRNA and protein expression have consistently been demonstrated to be increased by CoMTb stimulation, the hypothesis was that CoMTb treatment of A549s and NHBEs would result in increased H3 and H4 acetylation at the MMP-1 promoter, along with increased binding of RNA Pol II. I had hoped to go on from these preliminary experiments to investigate interactions between specific transcription factors and the MMP-1 promoter, but due to the difficulties encountered in optimising the ChIP methodology, and problems with

the reproducibility of the results obtained, this was not possible. However, the results generated do provide preliminary evidence that histone acetylation is involved in CoMTb-driven MMP expression.

In studies examining MMP-1 expression in glioblastoma cells, dimethylation of K4H3 (Lysine residue 4 of Histone H3) was identified as the earliest epigenetic change, followed by a regional increase in acetylation of H3 and H4 at 1 to 4 hours. Chromatin immunoprecipitation assays also showed binding of the AP-1 subunits cFos and cJun to the MMP-1 promoter in the same timeframe [355]. The AP-1 family of transcription factors is known to be important in MMP gene expression, and the MMP-1 promoter contains multiple AP-1 binding sites, including two proximal sites at -77bp and -186bp. It has been postulated that HDACi-mediated changes in MMP gene expression are mediated via interactions with AP-1 signalling. c-Jun can be acetylated at lysine residue 271, and this modification inhibits transactivation of MMP-1 expression, suggesting that HDACi would repress MMP-1 expression. In addition, HDACi have been reported to suppress c-Jun expression. HDAC3 and c-Jun are reported to form an inhibitory complex occupying the proximal AP-1 site and blocking MMP-1 gene expression. These mechanisms are discussed in [356]. PMA-induced MMP-9 expression in fibrosarcoma cells can be inhibited by IFNβ, and the mechanism of inhibition involves recruitment of HDAC1 to a proximal AP-1 site in the MMP-9 promoter with an associated decrease in H3 acetylation [357]. Taken together with the results presented in Chapter 3 suggesting that AP-1 is a critical activator of MMP-1 and -3 expression in epithelial cells, an inhibitory effect of HDACi on AP-1 signaling would be one alternative explanation of the inhibitory effect of TSA and CBHA on MMP expression observed here.

6 Mtb alters HDAC expression in macrophages and epithelial cells

6.1 Introduction

The Class I HDACs are believed to be ubiquitously expressed, while Class II expression is tissue-specific. Previous investigations by other groups have shown that HDAC expression may be altered by exposure to inflammatory or infectious stimuli, and therefore I hypothesized that Mtb stimulation would drive changes in HDAC expression at mRNA and protein levels. The results presented in Chapter 5 support the hypothesis that epigenetic mechanisms regulate MMP expression in the context of TB, and so I was interested to investigate whether these factors were a target of immune evasion by Mtb. Research into the pathogenesis of other inflammatory disorders such as asthma and COPD suggest that the balance of HAT and HDAC activity is also affected by chronic respiratory disease, and therefore HDAC and HAT activity assays were used to investigate the hypothesis that Mtb would alter cellular HDAC or HAT activity in this experimental model of pulmonary TB.

6.2 Results

6.2.1 Class I HDAC mRNA expression is repressed in Mtb-infected MDMs

MDMs were infected with H37Rv (MOI 1) and total RNA was extracted 24 hours later and reverse transcribed. RT-PCR was performed with primers specific for HDAC1, 2, 3, 4, 5 and 8 and MMP-1 mRNA, and results were normalised to expression of the reference genes β-actin, GAPDH and 18s ribosomal RNA. The results are shown in Figure 41.

HDAC1 mRNA was reduced by 68% in Mtb-infected MDMs compared to uninfected controls ($p<0.05$). HDAC2 mRNA was reduced by 69% in Mtb-infected MDMs compared to uninfected controls ($p=0.01$). HDAC3 mRNA was reduced by 76% in Mtb-infected MDMs compared to uninfected controls ($p<0.01$). HDAC8 mRNA was reduced by 58% in Mtb-infected MDMs compared to uninfected controls ($p<0.05$). The experiment was repeated in five independent donors in triplicate in order to ensure that the reduction observed was consistent and reproducible, since the changes in mRNA were relatively small. The results shown in Figure 41 are from one experiment performed in triplicate and are representative of the overall results.

Expression of the Class II HDACs 4 and 5 was also examined by RT-PCR. HDAC 4 mRNA was increased by 48% in Mtb-infected MDMs compared to uninfected controls ($p<0.05$), while HDAC 5 mRNA was unaltered by Mtb infection (Figure 41e & f). For comparison, MMP-1 mRNA was quantified in the same samples, and as expected MMP-1 mRNA increased 23-fold in Mtb-infected MDMs compared to uninfected controls ($p<0.05$) (Figure 41g). The results shown for HDAC4 and 5 are from one experiment performed in triplicate and are representative of three independent experiments in different donors.

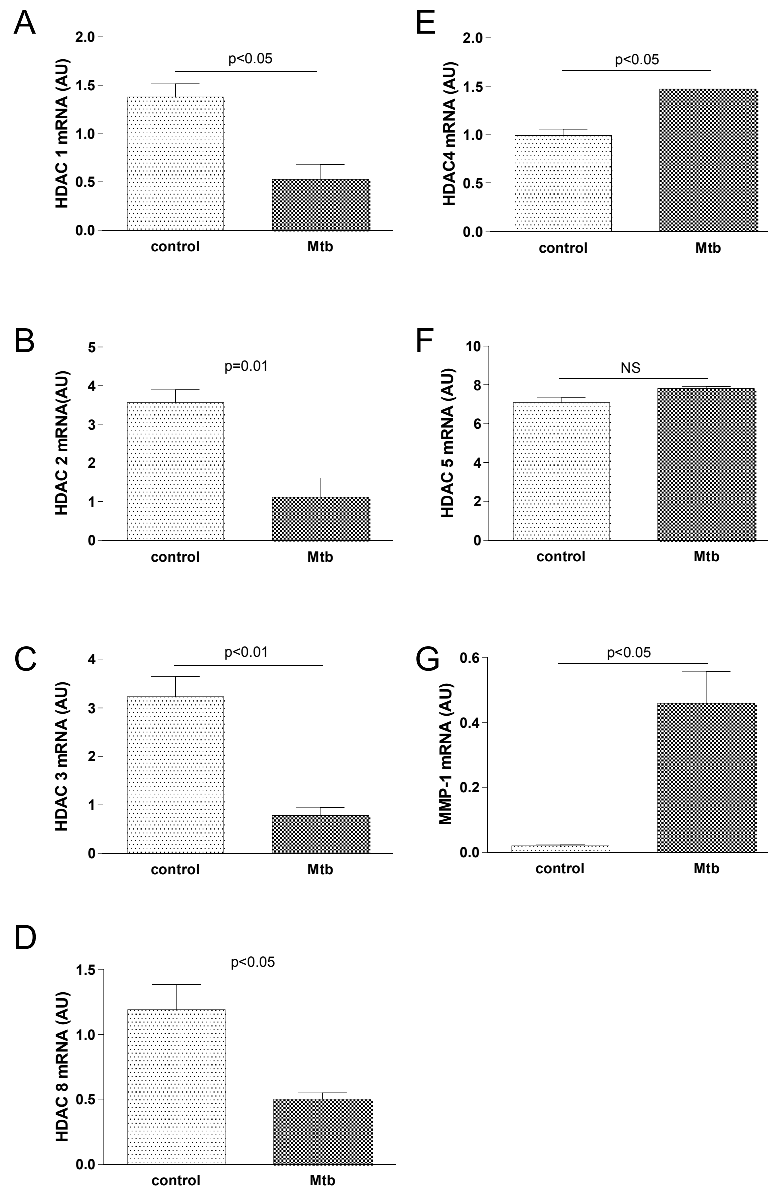

Figure 41. Class I HDAC mRNA expression is repressed and HDAC4 mRNA increased in Mtb-infected MDMs

MDMs were lysed 24 hours post-infection with H37Rv (MOI 1). RT-PCR was performed with primers specific for HDAC1, 2, 3, 4, 5 and 8 and MMP-1 mRNA. A) HDAC1 mRNA was reduced 68% in Mtb-infected MDMs compared to uninfected controls ($p<0.05$). B) HDAC2 mRNA was reduced 69% in Mtb-infected MDMs compared to uninfected controls ($p=0.01$). C) HDAC3 mRNA was reduced 76% in Mtb-infected MDMs compared to uninfected controls ($p<0.01$). D) HDAC8 mRNA was reduced 58% in Mtb-infected MDMs compared to uninfected controls ($p<0.05$). E) HDAC 4 mRNA was increased 48% in Mtb-infected MDMs compared to uninfected controls ($p<0.05$). F) HDAC 5 mRNA in MDMs was unaltered by Mtb infection ($p>0.05$). G) MMP-1 mRNA increased 23-fold in Mtb-infected MDMs compared to uninfected controls ($p<0.05$). Results shown are from one experiment performed in triplicate and are representative of 3 to 5 independent experiments in different donors.

6.2.2 HDAC 1, 2, 3, 5 and 7 are stably expressed in Mtb-infected MDMs, but HDAC 4 increases with Mtb infection

In order to investigate whether the observed changes in HDAC mRNA abundance with Mtb infection translated into altered cellular levels of HDAC proteins, MDMs were infected with H37Rv at MOI 1 for 24 to 72 hours and Western blots for HDACs 1, 2, 3, 4, 5 and 7 were performed on the cell lysates. β-actin was used as a loading control (data not shown). HDAC 8 protein expression was not studied.

The results shown in Figure 42 demonstrate that all the different HDACs assayed were expressed in uninfected MDMs. HDAC 1 and 2 appeared to be the most abundant, and their expression was stable over time and regardless of Mtb infection. HDAC 3 was less abundantly expressed, but was also stably expressed in control and Mtb-infected conditions over the time course studied. HDAC 4, consistent with the mRNA results described above, increased with Mtb infection at 48 and 72 hours post-infection, and this was a consistent finding in three independent experiments. HDAC 5 expression was not altered in response to Mtb infection, but appeared to increase over time in both control and Mtb-infected conditions. HDAC 7 was minimally expressed but did not change over time or with Mtb infection. The results shown are from one experiment and are representative of three independent experiments using different donors.

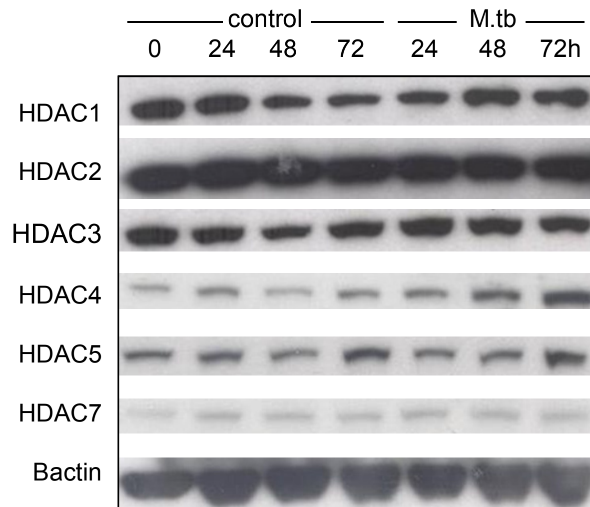

Figure 42. HDAC class I and II protein expression in Mtb-infected MDMs

MDMs were infected with H37Rv at MOI 1 for 24 to 72 hours and Western blots for HDACs 1, 2, 3, 4, 5 and 7 were performed on the cell lysates. HDAC 1, 2, 3, 5 and 7 protein expression was unaffected by Mtb infection over this time course, but HDAC 4 was more abundant in Mtb-infected cells at 48 and 72 hours post-infection.

6.2.3 HDAC class I and II mRNA in NHBEs is not significantly altered by CoMTb stimulation

NHBEs were treated for 24 hours with CoMTb 1:5 or control medium. Total RNA was extracted and reverse transcribed. RT-PCR was performed as before. In comparison with the results obtained in MDMs, the NHBE results were highly variable. Five independent experiments were performed in triplicate, and although both increases and decreases in HDAC mRNA were observed in individual experiments, no clear trend in HDAC expression was observed for any of the class I or IIa HDACs studied.

These NHBE experiments were performed over a long time interval, between March 2010 and July 2012, so the lack of consistency seen may be a consequence of using different lots of NHBEs, and CoMTb produced from different donors. I therefore decided to combine the results of three experiments performed over a shorter time interval, two of which used the same lot of NHBEs and the same batch of CoMTb. Results within each experiment performed in triplicate were normalized to the mean control HDAC expression and expressed as fold-change compared to control. The results of this analysis are shown in Figure 43, and clearly show that CoMTb-stimulation did not significantly alter the abundance of any of the HDAC mRNAs measured at 24 hours compared to control conditions.

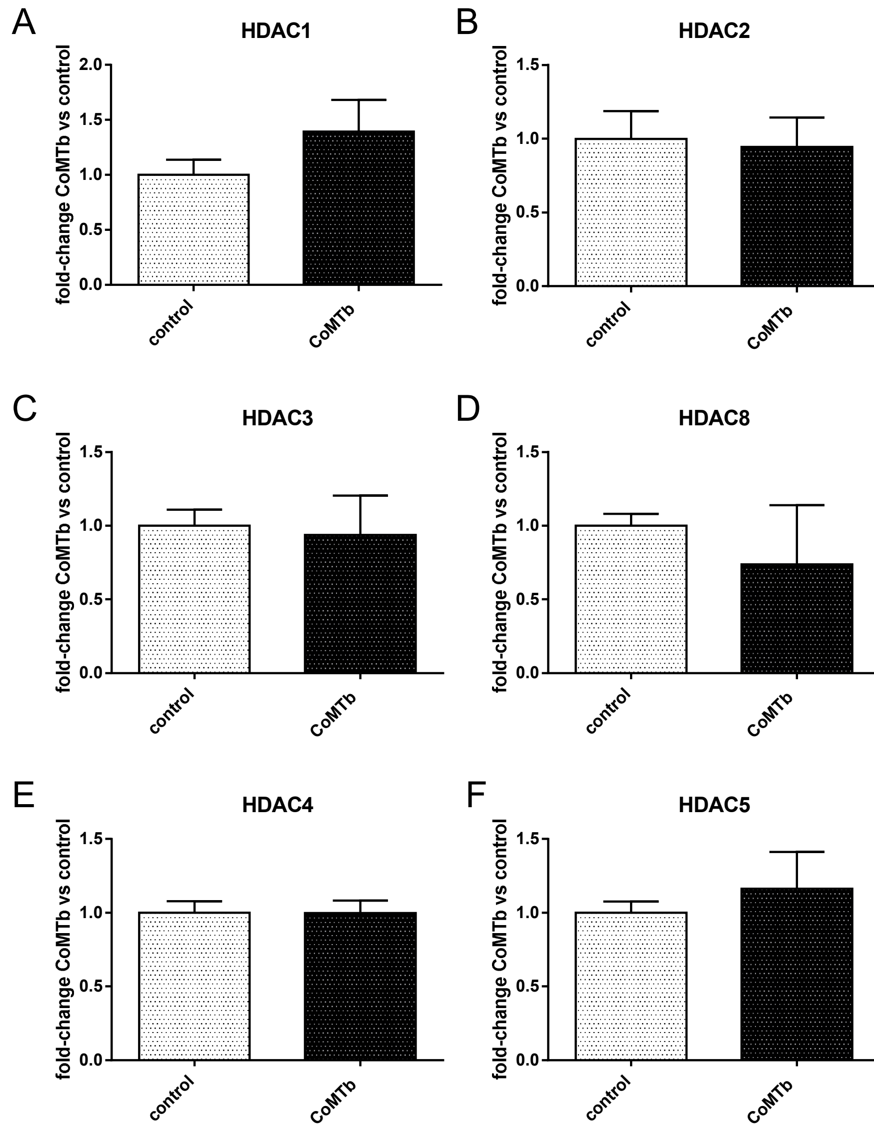

Figure 43. HDAC class I and II mRNA in NHBEs is not significantly altered by CoMTb stimulation

NHBEs were treated for 24 hours with CoMTb 1:5 or control medium. Total RNA was extracted and reverse transcribed. RT-PCR was performed with primers specific for HDAC 1, 2, 3, 4, 5, 8 and the reference genes βactin, GAPDH and 18s. The results shown are pooled results from 3 independent experiments performed in triplicate (2 experiments for HDAC8). No significant change was identified for any of the HDACs measured. A) HDAC1 B) HDAC2 C) HDAC3 D) HDAC8 E) HDAC4 F) HDAC5.

6.2.4 HDAC 1, 2, 5 and 7 protein expression increases in CoMTb-stimulated NHBEs

Western blotting was performed to examine the expression of selected class I and II HDAC proteins in control and CoMTb-stimulated NHBEs from 0 to 72 hours post-stimulation. The results are shown in Figure 44.

Expression of HDAC1 and HDAC2 was increased in CoMTb-stimulated compared to control conditions, and appeared to peak at 48 hours post-stimulation. In contrast, HDAC3 expression was not increased by CoMTb, but increased over time in both control and CoMTb-treated cells (Figure 44a). β-actin expression was stable over time and was unaffected by CoMTb stimulation. The class II HDACs 5 and 7 were barely detectable under control conditions, but were increased after 8 to 72 hours of CoMTb stimulation whereas HDAC4 was expressed constitutively and increased with time independently of CoMTb treatment (Figure 44b). These findings contrast with the mRNA results presented previously.

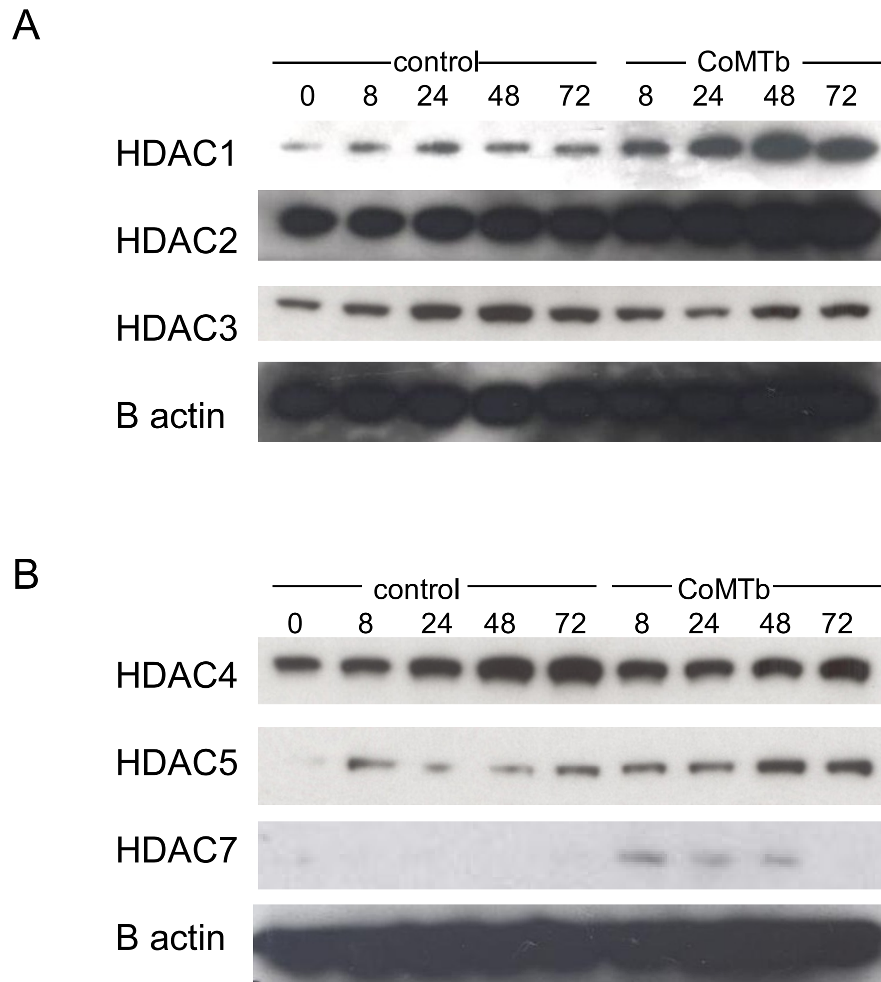

Figure 44. HDAC class I and II protein expression in CoMTb-stimulated NHBEs

Western blotting was performed to examine the expression of HDAC1, 2, 3, 4, 5 and 7 proteins in control and CoMTb-stimulated NHBEs from 0 to 72 hours post-stimulation. A) Class I HDACs: HDAC 1 and 2 expression is increased in response to CoMTb, whereas HDAC 3 increased over time. β-actin expression is stable. B) Class II HDACs: HDACs 5 and 7 are barely detectable under control conditions, but increase after 8 to 72 hours of CoMTb stimulation. HDAC4 is expressed in control conditions and increases with time. β-actin expression is stable.

6.2.5 HDAC and HAT activity is unchanged in Mtb-infected MDMs

Commercially available HDAC activity assays were used to quantify HDAC activity in nuclear extracts from control and Mtb-infected MDMs. The first assay used was a colorimetric assay (Active Motif Colorimetric HDAC assay #56210), and two major problems were encountered with this protocol. Firstly, the assay appeared to be insufficiently sensitive for the samples used, as even when the reaction time was increased to 4 hours, the readouts obtained were only approximately 2-fold higher than the blanks. The amount of nuclear extract loaded per well was increased to 6 μg without improving the results, and higher quantities of protein could not be loaded without preparing extracts from larger plates of cells, which was impractical (60 mm dishes were used). Secondly, the Active Motif nuclear extraction kit used produced nuclear extracts with a slightly yellow colour, due to the buffers employed, and the colorimetric reaction used here generated a yellow product. Therefore, increasing the amount of sample also produced spurious results as it was not possible to adequately account for the background signal.

A second assay was used, with a similar principle of action, but a fluorometric readout (Active Motif Fluorometric HDAC Assay #56200). This was used according to the supplied protocol with 5 μg/well nuclear protein and one hour incubation at 37°C. The results obtained were more satisfactory, with sample optical densities 10 times higher than the blank values, and an excellent standard curve from the supplied reagents. This assay was used to quantify HDAC activity in nuclear extracts from control and Mtb-infected MDMs from two donors on separate occasions. The experiments could not be performed in triplicate for practical reasons, but the assays were run in duplicate. The results shown in Figure 45a are from one experiment, and reveal no convincing change in HDAC activity measured over 6 hours post-infection. It is possible that different timepoints should have been used, and ideally the experiment would have been performed in triplicate to allow some estimation of the margin of error of the measurement.

A HAT activity assay (Millipore) was used to assess changes in total HAT activity in nuclear extracts obtained in similar experiments. 15 μg nuclear protein was loaded per well in order to obtain a sufficient signal, which meant that the assay could not be run in duplicate. The results shown in Figure 45b do not demonstrate any alteration in HAT activity between one and four hours post Mtb-infection.

For both HDAC and HAT activity, similar experiments were performed in NHBEs stimulated with CoMTb. The same problems with assay sensitivity for HDAC activity were encountered, and no significant change in HAT or HDAC activity was identified in two independent experiments for each (data not shown).

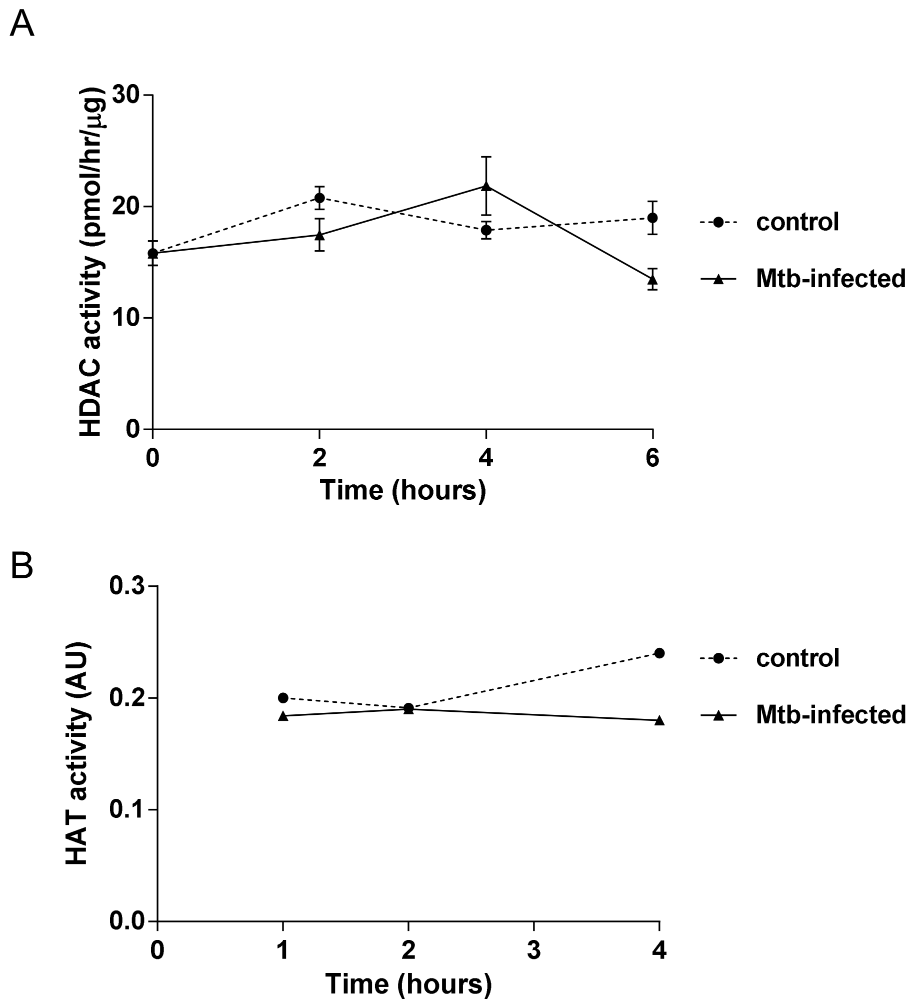

Figure 45. HDAC and HAT activity is unchanged by Mtb infection of MDMs

MDMs were infected with H37Rv at time zero or left uninfected. At each timepoint, cells were lysed and nuclear extracts were prepared. A) HDAC activity was quantified using a substrate-based fluorometric assay. The results shown are from one experiment, with the activity assay performed in duplicate. There is no significant difference between HDAC activity in control and infected samples at any timepoint. B) HAT activity was quantified using an ELISA-type assay. The results displayed show no difference in HAT activity between control and Mtb-infected MDMs between 1 and 4 hours.

6.3 Discussion

The experiments in this chapter were designed to investigate whether stimulation with Mtb in itself altered epigenetic regulation in primary macrophages and epithelial cells. The hypothesis that this could represent a mechanism of immune modulation in response to Mtb was based on recent evidence from other inflammatory disorders, in which chronic changes in HDAC/HAT expression and activity have been described. In asthma patients, chronic inflammation is accompanied by increased HAT and decreased HDAC activity in alveolar macrophages. Since corticosteroids reverse the inhibition of HDAC activity, this may represent one mechanism of their anti-inflammatory action in this disease [280]. Such alterations might be beneficial to the host under some circumstances, for example by damping down inflammatory responses to persistent allergens or inflammatory stimuli, but could also be detrimental, impairing antibacterial responses to pathogens or generating a persistent hyperresponsive state.

I found that in primary human MDMs, Mtb infection suppressed accumulation of class I HDAC mRNA, which could occur either by reduced gene transcription or by reduced mRNA stability. However, this reduction in HDAC mRNA was not reflected by changes in class I HDAC protein expression over 72 hours following Mtb infection. If HDAC protein turnover is slow, as suggested by the HDAC siRNA experiments in Chapter 5, then it may be that a later time point would be required to demonstrate Mtb-driven alterations in protein expression.

In contrast, HDAC 4 mRNA was increased approximately 1.5-fold 24 hours after Mtb infection and a consistent increase in HDAC 4 protein at 48 and 72 hours post-infection was observed, suggesting that the changes in mRNA abundance did translate into increased protein synthesis. Western blotting is only a semi-quantitative technique, as protein detection is dependent on the specificity and affinity of the antibodies employed, but HDAC 4 seems to be much less abundant in unstimulated MDMs compared to the Class I HDACs 1, 2 and 3. The impact of the observed changes in HDAC 4 expression is unknown.

Changes in HDAC expression are known to occur with cell differentiation, for example expression of HDAC5 and 9 is up-regulated in U937-macrophages compared to monocytes [358]. TLR signaling also produces changes in HDAC expression and activity with suppression of class II HDAC mRNAs reported in LPS-treated bone-marrow-derived macrophages [288].

In an in vitro model of cigarette-smoke-induced pathology, there was no change in HDAC1-11 mRNA in GM-CSF differentiated human macrophages treated with smoke, but expression of HDAC3 protein was reduced, and a decrease in HDAC activity without altered HAT activity was detected [353]. There is therefore a precedent in the literature for selective changes in macrophage HDAC expression in response to inflammatory stimuli, although in this case a decrease, rather than an increase in HDAC protein was observed.

The results in primary epithelial cells were markedly different to those obtained in MDMs. In this case, no significant change in HDAC mRNA was found in response to CoMTb stimulation, but increased expression of several class I and class II HDAC proteins was identified in response to CoMTb. The mechanism by which this occurred was not further investigated in the course of these experiments, but might involve, for example, reduced degradation of HDAC proteins under CoMTb-treated conditions.

These preliminary results contrast with published findings in the context of other chronic respiratory disorders. In the A549 respiratory epithelial cell line oxidative stress induced by hydrogen peroxide or cigarette smoke condensate reduced HDAC activity and expression of HDAC2 protein [359]. In rats exposed to cigarette smoke, reduced HDAC2 protein levels and HDAC activity were detected after 3 days of smoke exposure, with no alteration in HDAC1 expression. Reduced HDAC activity was at least in part attributable to covalent modifications of HDAC2 which might have reduced its enzymatic activity or targeted the protein for degradation [360]. These findings suggest that inflammatory stimuli such as cigarette smoke generate a pro-inflammatory epithelial cell phenotype which might produce airway

hyperresponsiveness, whereas my results suggest that in the context of TB, increased HDAC expression might render epithelial cells less responsive to inflammatory mediators.

Studies in models of arthritis have also reported disease-specific changes in HDAC expression; in synovial cartilage from human subjects, increased HDAC7 expression was identified in osteoarthritis, and siRNA silencing of HDAC7 blocked MMP-13 expression [350]. In another study increased expression of HDAC1 and 2 was found in synovial fibroblasts from rheumatoid arthritis patients [351].

The changes in macrophage and epithelial cell HDAC expression identified in response to Mtb led to a series of experiments intended to investigate whether this was reflected in functional changes in measurable HDAC or HAT activity in these cell types. In these experiments I did not detect a change in HAT or HDAC activity in nuclear extracts from Mtb-stimulated cells. There are several possible explanations for this, including a number of practical difficulties with the assays, as discussed in Chapter 2.3.20 and 2.3.21. Possible solutions would include performing an immunoprecipitation step on the nuclear extracts to purify HDAC activity using anti-HDAC antibodies, or using alternative assay formats, such as radionucleotide assays. I also attempted to measure activity at a number of different time points, but these may not have encompassed the time period over which changes in activity actually occurred. For example, the mRNA and Western results suggest that changes in HDAC expression occur over a longer timeframe than was studied. Alternatively, it may be the case that there is genuinely no change in activity under these experimental conditions.

7 Summary and future research

In this tissue culture model of events occurring in pulmonary TB, exposure to IL-4 or IL-13 inhibited the expression of MMP-1 and -3 by Mtb-stimulated macrophages and respiratory epithelial cells, and the effect of IL-4 was more potent than that of IL-13. IL-10 did not affect MMP or TIMP-1 expression in either cell type. TGFβ enhanced MMP secretion by CoMTb-stimulated epithelial cells, but did not affect macrophage expression of MMP-1, -3 or -7.

IFNγ potently and selectively inhibited MMP-1 and -3 secretion by Mtb-infected macrophages, and was also observed to modulate macrophage production of chemokines and cytokines. In contrast, IFNγ increased epithelial cell MMP-1 secretion.

MMP expression in both respiratory epithelial cell and macrophages was sensitive to chemical inhibition of histone acetylases and deacetylases, but silencing the expression of individual class I HDACs in epithelial cells did not affect CoMTb-induced MMP secretion. Preliminary chromatin immunoprecipitation experiments revealed increases in MMP-1 promoter acetylation in CoMTb-stimulated cells which precede increases in MMP-1 mRNA.

Investigation of HDAC expression in response to Mtb suggested that Class I HDAC mRNA is suppressed in Mtb-infected macrophages, while HDAC4 mRNA and protein are increased in infected cells. In epithelial cells no changes in HDAC mRNA after 24 hours CoMTb stimulation were observed, but expression of certain HDAC proteins did increase in CoMTb-treated cells. Despite these observations, changes in HDAC and HAT enzyme activity could not be demonstrated.

The results presented in Chapter 3 showed that IL-4 and the related cytokine IL-13 were able to inhibit Mtb-driven MMP expression in epithelial cells and macrophages. However, preliminary investigations of the signalling pathways mediating the inhibitory effects of IL-4 did not identify the mechanism. I would have liked to go on to examine this further, perhaps by using chemical inhibitors or siRNA to determine whether this phenomenon was Stat 6-

dependent, for example. ChIP methodology could also have been used to study this in more depth, by assessing quantitative and qualitative changes in transcription factor binding at the MMP-1 promoter in IL-4 treated cells.

IL-4 has been proposed as a potential target for immunotherapy as an adjunct to standard treatment for TB. However, the results presented here suggest that one effect of such treatment might be an increase in matrix degradation and inflammation as a result of unrestrained MMP activity, as well as increased macrophage production of cytokines and chemokines such as IL-1β, IL-6, TNFα, CCL5 and MIP1α. Therefore neutralising IL-4 responses might generate a pathological inflammatory reaction to chronic Mtb infection, perhaps akin to the IRIS observed in HIV co-infection. An animal system that adequately models the role of MMPs in human immunopathology would be required to effectively investigate the clinical implications of such a strategy. The mouse model of TB has a number of important differences to the human subject, as discussed in Chapter 1. The experimental model used in [153] has some relevance to this clinical scenario, as IL-4/-13 neutralising antibody was administered to chronically infected mice rather than at the time of inoculation, although MMPs were not studied in this research. A more relevant animal system for investigating the effect of IL-4 inhibition on MMP activity and immunopathology would be the rabbit or primate models, as the pulmonary lesions observed with Mtb infection in these species is more akin to human disease.

In Chapter 4 I have described the effect of IFNγ on Mtb-induced MMP expression, but the mechanism of this was not identified. The cellular effects of IFNγ have been extensively studied elsewhere, and therefore intracellular pathways which might be implicated in this effect can be identified. A preliminary step would be to assess whether IFNγ's activity was Stat-dependent or independent. Initial attempts to examine this were unsuccessful due to the limitations of the chemical inhibitors available at the time, but it would be feasible to investigate this using siRNA targeting Stat 1 and 3.

IFNγ has also been proposed as a target of immunotherapy in TB, and as previously described, small studies have been undertaken in which exogenous recombinant IFNγ was administered to TB patients [232, 233]. It would be fascinating to study the effect of such treatment on MMPs in BAL or sputum, in order to see whether the expected down-regulation of MMP activity that would be predicted from my in vitro findings was observed.

It would have been interesting to assess the effects of HDAC class I siRNA in primary human macrophages. Such primary cells are known to be difficult to transfect, and previous attempts to optimise siRNA methodology in primary monocytes and macrophages in our group had been unsuccessful. Recent unpublished work by other members of the group has demonstrated that siRNA can be successfully transfected into macrophages with lipid-based transfection methods, and therefore these experiments could now be performed. An alternative approach would be to use a cell line such as THP-1 cells as a model of macrophage responses. Studying the effects of siRNA in macrophages as well as epithelial cells would be particularly interesting given the possible translational option of delivering siRNA locally to the lungs to either pulmonary macrophages or alveolar epithelial cells via inhaled microparticles. The HDAC siRNA experiments performed could also be extended to investigate the effect of silencing HDAC8 and the Class II HDACs on MMP expression. The results of the HDAC chemical inhibition experiments I performed showed that MMP-1 and -3 expression in response to Mtb infection was dependent on intact HDAC activity, and in particular low concentrations of the HDAC1-selective inhibitor MS-275 efficiently blocked MMP-1 and -3 expression. I would therefore expect that silencing HDAC1 expression using siRNA might also prevent Mtb-driven MMP expression. A further line of investigation would be to investigate the effects of specific small molecule BET inhibitors such as JQ1 on MMP expression in tissue culture and animal models of TB.

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9 Abbreviations

ADAM	A Disintegrin And Metalloproteinase
AIDS	Acquired Immunodeficiency Syndrome
AP-1	Activator Protein-1
ATF-2	Activating Transcription Factor 2
BAL	Broncho-alveolar lavage
BCG	Bacillus Calmette-Guerin
BEBM	Basal bronchial epithelial cell culture medium
BEGM	Supplemented bronchial epithelial cell culture medium
BET	Bromodomain and extra terminal domain
BMDM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
CBHA	m-Carboxycinnamic Acid bis-Hydroxamide
CBP	CREB-binding protein
CCL	CC chemokine ligand
CD	Cluster of differentiation
cfu	Colony-forming unit
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CoMCont	Conditioned medium from uninfected monocytes
CoMTb	Conditioned medium from M.tb-infected monocytes
COPD	Chronic Obstructive Pulmonary Disease
CREB	cAMP response element-binding protein
CSF	Cerebro-spinal fluid
Ct	Cycle threshold
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbant Assay
ERK	Extracellular signal-regulated Kinase
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FCS	Foetal calf serum
GNAT	Gcn5-related acetyltransferases

HAT	Histone acetyltransferase
HATi II	HAT inhibitor II (2,6-*bis*-(3-Bromo-4-hydroxybenzylidene)cyclohexanone)
HBSS	Hank's buffered saline solution
HDAC	Histone deacetylase
HIF1α	Hypoxia-Inducible Factor-1α
HIV	Human Immunodeficiency Virus
HPA	Health Protection Agency
IFNγ	Interferon-γ
IGRA	IFNγ-release assay
IL-	Interleukin
iNOS	Inducible Nitric oxide synthase
IRIS	Immune Reconstitution Inflammatory Syndrome
IκBα	NF-κB inhibitor α
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LAM	Lipoarabinomannan
LAP	Latency Associated Peptide
LTBP	Latent TGFβ Binding Protein
MAPK	Mitogen activated kinase
MCP-1	Monocyte chemotactic protein-1 (CCL2)
M-CSF	Macrophage colony stimulating factor
MDR-TB	Multidrug-resistant Tuberculosis
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
MOI	Multiplicity of infection
mRNA	Messenger RNA
MSFM	Macrophage serum-free medium
Mtb	Mycobacterium tuberculosis
MYST	Monocytic leukaemia zinc finger protein, Ybf2/Sas3, Sas2 and HIV Tat protein
NF-κB	Nuclear factor kappa B
NHBE	Normal human bronchial epithelial cells
NK	Natural Killer cell
NO	Nitric oxide
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate-buffered saline
PCAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PIC	Protease inhibitor cocktail
PPAR	Proliferator-activated Receptor
PPD	Purified protein derivative
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted (CCL5)
RNA	Ribonucleic acid
RNA Pol II	RNA Polymerase II
RPMI	Rosswell Park Memorial Institute culture medium
SAHA	Suberoylanilide hydroxamic acid
siRNA	Small interfering RNA
siRNA	Small interfering RNA
SIV	Simian Immunodeficiency Virus
SOCS	Suppressor of Cytokine Signaling
STAT	Signal transducers and activators of transcription
TACE	TNFα-Converting Enzyme (ADAM17)
TB	Tuberculosis
TBP	TATA binding protein
TBS	Tris-buffered saline
TGFβ	Transforming growth factor beta
Th	T helper cell
TIMP	Tissue inhibitor of metalloproteinase
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
Treg	Regulatory T cell
TSA	Trichostatin A
TST	Tuberculin sensitivity test
UV	Ultra-violet light
WHO	World Health Organisation
XDR-TB	Extensively drug-resistant Tuberculosis