

**HYPOXIA AND THE MECHANISMS REGULATING
INFLAMMATORY TISSUE DESTRUCTION IN
TUBERCULOSIS**

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**“Learn from yesterday, live for today, hope for tomorrow. The
important thing is to not stop questioning.”**

Albert Einstein, *Relativity: The Special and the General Theory*

DECLARATION OF AUTHENTICITY

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

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ABSTRACT

Mycobacterium tuberculosis (*M.tb*) causes approximately 2 million deaths a year. There have been few new drugs developed to treat the disease and treatment courses are often complex owing to the emergence of drug resistance and the HIV epidemic. It is unknown whether lesions in human tuberculosis (TB) are hypoxic, nor whether this influences disease pathology. Human TB is characterized by extensive lung destruction driven by host MMPs particularly collagenases such as MMP-1. This thesis describes the investigation of hypoxia in patients with the specific tracer [^{18}F]-Fluoromisonidazole (FMISO). [^{18}F]FMISO accumulated in regions of TB consolidation and around pulmonary cavities, demonstrating for the first time severe hypoxia in man. Dynamic imaging and Patlak K_i mapping showed heterogenous levels of oxygenation within and between patients. In *M.tb*-infected human macrophages, hypoxia (1% pO_2) upregulated MMP-1 gene expression 2,800-fold, driving secretion and caseinolytic activity. Dimethyloxalyl glycine (DMOG), a small molecule inhibitor which stabilizes HIF-1 α , similarly upregulated MMP-1. Hypoxia did not affect mycobacterial replication. Hypoxia increased MMP-1 expression in primary respiratory epithelial cells via TB-dependent networks. HIF-1 α and NF- κB regulated increased MMP-1 activity in hypoxia. Furthermore, *M.tb* infection drove HIF-1 α accumulation even in normoxia, and epithelioid macrophages and multi-nucleate giant cells in human TB lung biopsies expressed HIF-1 α . HIF-1 α blockade including by targeted siRNA inhibited TB-driven MMP-1 gene expression and secretion. Human TB lesions are severely hypoxic and *M.tb* directly drives HIF-1 α accumulation, thereby synergistically increasing collagenase activity leading to cavitation and disease transmission.

TABLE OF CONTENTS

DECLARATION OF AUTHENTICITY.....	1
COPYRIGHT DECLARATION.....	2
ABSTRACT.....	3
TABLE OF FIGURES.....	7
ACKNOWLEDGEMENTS.....	12
CHAPTER 1 INTRODUCTION	
1.1 Background.....	14
1.2 TB pathogenicity.....	17
1.3 Clinical features of pulmonary tuberculosis.....	21
1.4 Tuberculosis diagnosis.....	23
1.5 The matrix metalloproteinase (MMP) family.....	26
1.6 MMPs and their role in TB pathogenicity.....	33
1.7 Hypoxia and its role in innate immunity.....	35
1.8 Hypoxia-inducible factor (HIF).....	36
1.9 Hypoxia and tuberculosis.....	43
1.10 <i>In vitro</i> systems of hypoxia.....	48
1.11 Hypoxia and MMPs.....	50
1.12 The radiological manifestations of pulmonary TB.....	51
1.13 Nuclear medicine imaging of TB.....	55
1.14 Assessment of tissue oxygenation.....	55
HYPOTHESIS AND AIMS.....	61

CHAPTER 2 MATERIALS AND METHODS

[¹⁸ F]FMISO PET-CT scans.....	62
[¹⁸ F]FMISO PET-CT data analysis.....	63
Cell culture	
- A549 cells.....	63
- Normal bronchial epithelial cells (NHBE).....	64
- Monocyte purification and maturation into macrophages.....	64
TB culture.....	65
GFP-Tb.....	66
Conditioned media preparation.....	67
Direct infection protocol.....	67
CoMTb experiment protocol.....	68
LDH cytotoxicity assay.....	68
Colony counting.....	69
Generation of hypoxia.....	69
HIF-1 α stabilisation.....	73
Enzyme Linked Immunosorbent Assay (ELISA).....	73
Fluorokine [®] MAP Assay (Luminex xMAP [®] analysis).....	74
Zymography	
- Casein zymography.....	74
- Gelatin zymography.....	74
Anti-HIF-1 α Western blotting.....	76
Transformation of competent <i>E.Coli</i> with MMP-1 promoter constructs.	77
Alkaline Lysis and plasmid DNA purification.....	78
Promoter-reporter assay.....	79
RNA extraction (early experiments).....	80
cDNA synthesis (early experiments).....	80
RNA extraction (2011 onwards).....	81
cDNA synthesis (2011 onwards).....	82

	RT-PCR.....	83
	siRNA.....	83
	Confocal microscopy.....	84
	Immunohistochemistry.....	85
	Statistical analysis.....	85
CHAPTER 3-	INVESTIGATION OF HYPOXIA IN HUMAN PULMONARY TB USING	
	PET-CT.....	86
CHAPTER 4	HYPOXIA AND MMPs IN RESPIRATORY EPITHELIAL CELLS	
	DURING TB INFECTION.....	113
CHAPTER 5	HYPOXIA AND MMPs IN HUMAN MONOCYTE-DERIVED	
	MACROPHAGES.....	142
CHAPTER 6-	HYPOXIA AND THE ROLE OF HIF DURING <i>M.TB</i> INFECTION.....	168
CHAPTER 7	CONCLUSIONS.....	194
CHAPTER 8	FUTURE WORK.....	197
REFERENCES		201
APPENDIX 1	ABBREVIATIONS.....	212
APPENDIX 2	RESULTS OF 30-PLEX ANALYSIS.....	214
APPENDIX 3	PUBLICATIONS AND PRESENTATIONS.....	219

TABLE OF FIGURES

Figure 1: Estimated global incidence of TB

Figure 2: Ziehl-Neelsen stained sputum slide showing acid-fast bacilli

Figure 3: Schematic structure of major MMP groups

Figure 4: Domain structure of human HIF- α and HIF-1 β

Figure 5: Schematic representation of HIF-1 regulation in normoxia and hypoxia

Figure 6: H &E stain of caseous necrosis (G) and PIMO stain (H) in a guinea pig

Figure 7: Plain chest x-ray demonstrating left upper lobe cavity

Figure 8: High resolution CT scan showing apical consolidation and cavitation of pulmonary tuberculosis

Figure 9: Diagram of GFP-expressing M.tb strain

Figure 10: Oxoid hypoxic chamber

Figure 11: Technical drawings of specially commissioned hypoxia workstation

Figure 12: MMP-1 promoter truncation constructs

Figure 13: FACS analysis of siRNA transfection efficiency of primary human MDMs

Figure 14: ¹⁸F-FMISO PET-CT images of right upper lobe lesion in Patient 1

Figure 15: ¹⁸F-FMISO PET-CT images of posterior aspect of left lower lobe lesion in Patient

Figure 16: Time Activity Curves and PET-CT images for Patient 1

Figure 17: Time Activity curves and ¹⁸F-FMISO PET-CT imaging in Patient 2

Figure 18: K_i mapping and TACs for Patient 3

Figure 19: Standardised uptake value (SUV) compared to tissue-to background ratios (TBR)

Figure 20: Standardisation of K_i mapping across all 5 patients

Figure 21: PET-CT Results from Patient 4

Figure 22: PET-CT images from Patient 5

Figure 23: Comparison of K_i images from Patient 2

Figure 24: Schematic demonstrating blockade of HIF by prolyl hydroxylases (PHD1, PHD2 and PHD3) and factor inhibiting HIF (FIH)

Figure 25: Effect of HIF-1 α stabilisation on MMP secretion from CoMTb stimulated A549 cells

Figure 26: Effect of DMOG on TIMP-1 secretion in respiratory epithelial cells

Figure 27: Hypoxia (1% O₂) and effect on MMP-1 and MMP-9 protein secretion from CoMTb stimulated A549 cells

Figure 28: MMP-1 confocal microscopy of CoMTb-stimulated A549 cells in normoxia and hypoxia

Figure 29: Effect of varying degrees of hypoxia (5% and 10% O₂) on MMP-1 secretion from A549 cells

Figure 30: Effect of DMOG on TB networking effects in NHBE cells stimulated with CoMTb

Figure 31: Effect of DMOG on MMP-9 protein secretion and proteolytic activity in NHBE cells

Figure 32: Effect of different oxygen levels on MMP-1 secretion in NHBE cells stimulated with CoMTb

Figure 33: Effect of DMOG and hypoxia on MMP-1 promoter activity in A549 cells

Figure 34: Effect of hypoxia on transcription factors within the MMP-1 promoter

Figure 35: Effect of NFκB chemical inhibition on CoMTb-driven MMP-1 secretion

Figure 36: Effect of DMOG on MMP secretion from MDMs directly infected with *M.tb*

Figure 37: Effect of 1% oxygen on MMP secretion from MDMs directly infected with *M.tb*.

Figure 38: Effect of 1% O₂ on MMP-1 and TIMP-1 activity in MDMs

Figure 39: MMP-1 confocal microscopy of *M.tb* infected MDMs in normoxia and hypoxia

Figure 40: Effect of 1%, 5% and 10% oxygen on MMP-1 protein secretion from *M.tb* infected MDMs

Figure 41: Investigation of the effect of 10% O₂ compared to 1% and 21% O₂ on MMP-1 secretion, cell morphology and cellular pH.

Figure 42: Effect of hypoxia (1% O₂) on phagocytosis of GFP-Tb at 2 hrs

Figure 43: Phagocytosis of GFP-Tb at 8hrs in normoxia

Figure 44: Phagocytosis of GFP-Tb at 24hrs in normoxia

Figure 45: Effect of hypoxia (1% O₂) on intracellular replication of *M.tb* and MMP-1 secretion following “non-live” *M.tb* stimulation of MDMs

Figure 46: Effect of helenalin on MMP-1 secretion in *M.tb*-infected MDMs treated with DMOG or incubated in hypoxia (1% O₂)

Figure 47: HIF-1α western blotting of MDMs directly infected with *M.tb*

Figure 48: Effect of DMOG and hypoxia (1% O₂) on HIF-1α stabilisation in MDMs infected with *M.tb* and BCG

Figure 49: Effect of DMOG on HIF-1α accumulation in respiratory epithelial cells stimulated with CoMTb

Figure 50: Effect of hypoxia (1% O₂) on HIF-2α stabilisation in MDMs infected with *M.tb*

Figure 51: Investigation of effect of hypoxia on prolyl hydroxylase activity in response to *M.tb* infection

Figure 52: Effect of HIF-1α chemical inhibition on MMP-1 secretion in *M.tb*-infected MDMs

Figure 53: HIF-1α knockdown and effect on HIF-1α in *M.tb*-infected MDMs incubated in normoxia and hypoxia

Figure 54: Effect of HIF-1α siRNA on MMP-1 gene expression and protein secretion

Figure 55: Effect of HIF-1α siRNA on MMP-1 secretion in A549 cells

Figure 56: HIF-1α immunohistochemistry

Figure 57: HIF-1α localisation in human TB granulomas

TABLES

Table 1	Summary of major MMP groups and published substrates
Table 2	Patient criteria for recruitment to clinical study
Table 3	Patient demographics, chest radiograph and microbiology results
Table 4	Blood analysis at time of presentation
Table 5	Summary of principle cytokines and chemokines measured in CoMTb and CoMCont
Table 6	Infectious organisms and effect on HIF

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CHAPTER 1: INTRODUCTION

1.1 Background

Tuberculosis (TB) is an infection caused by *Mycobacterium tuberculosis* (*M.tb*). Tuberculosis is a major global health burden and is the second leading cause of death from an infectious disease after the human immunodeficiency virus (HIV). In 2011, 1.4 million people died from TB and there were an estimated 8.7 million new cases of TB globally [1]. It is estimated that 13% of these incident cases is co-infected with HIV [2]. In 1993, TB was declared a global health emergency by the World Health Organisation [1]. This declaration reflects the main global challenges to the control of TB which include insufficient measures to detect, prevent and treat TB, as well as the lengthy antibiotic course required to treat active TB resulting in non-adherence and development of multi-drug (MDR) and extensively-drug (XDR) resistant strains [3].

1.1.1 The global burden of tuberculosis

The global burden of disease caused by TB can be measured in terms of incidence (defined as the number of new and relapsed cases in a set time period, usually one year), prevalence (defined as the number of cases of TB at a given point in time) and mortality (defined as the number of deaths over a defined period, usually one year). The results of these analyses are detailed in the most recent report of the World Health Organisation Global Tuberculosis Report 2012.

In 2011, there were an estimated 8.7 million incident cases of TB (range 8.3-9.0 million) globally. Most of the estimated cases occurred in Asia (59%) and Africa (26%). Despite significant improvements in global surveillance for TB a significant proportion of disease is

under-reported. As such, the exact incidence remains uncertain. Of the estimated 8.7 million incident cases in 2011, 1.0-1.2 million (12-14%) were among people co-infected with HIV. The highest proportion of co-infected cases was in the African region where 39% of TB cases were estimated to be HIV positive [1].

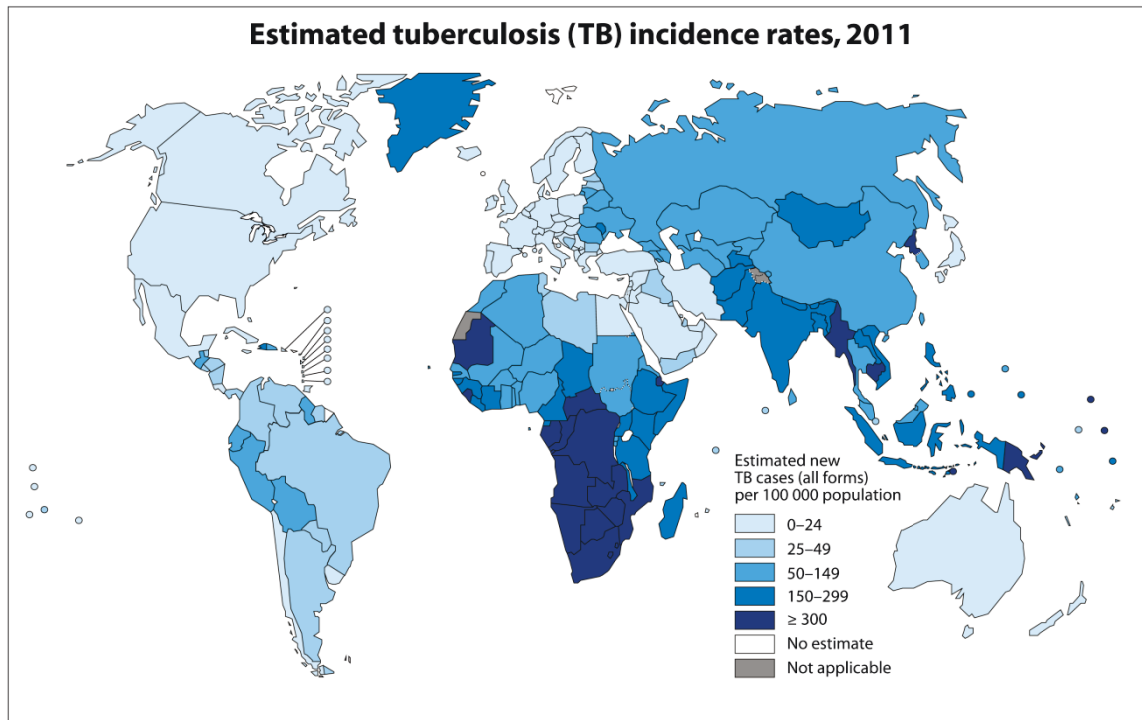


Figure 1: Estimated global incidence of TB (Adapted from Global Tuberculosis Report 2012, WHO)

Globally, incidence rates of TB have remained relatively stable between 1990-2001 and, between 2010 and 2012, the global incidence of disease fell by 2.2% [1]. The latest assessment of what are considered to be High Burden Countries indicates that the incidence of TB is falling in most countries.

The prevalence of TB can only be measured indirectly in most countries but the global prevalence of TB was estimated to be 12 million in 2011 (range, 10-13 million). The prevalence rate has decreased by 36% since 1990. At present many countries do not

accurately record cause of death according the International Classification of Disease (ICD-10). The inclusion of data from China and India in the WHO 2011 report has significantly improved reporting but direct measurement of TB deaths according to this classification are only available for 120 countries. Given these figures only account for approximately 46% of death globally, mortality rates are based predominantly on estimated data. Globally, mortality rates (excluding deaths from HIV positive persons) have fallen by 41% since 1990.

1.1.2 The threat of drug-resistant tuberculosis

Whilst the rates of tuberculosis globally have fallen, the emergence of drug-resistant TB (DR-TB) represents a significant threat to the global control of the disease. In 2011, 3.7% of new cases and 20% of previously treated cases were estimated to have multi-drug resistant TB. Of these cases, 60% were identified to be in India, China and the Russian Federation. Worryingly, the number of reported cases of MDR-TB represented only 19% of the estimated 310 000 cases of MDR-TB reported TB patients with pulmonary TB. Additionally, extensively drug-resistant disease (XDR-TB) has been identified in 84 countries globally. The average proportion of MDR-TB cases with XDR-TB is 9.0% (6.7-11.25) and is increasing particularly in High Burden Countries. It is clear therefore that significant advances are required in order to detect, report and treat drug-resistant disease.

1.1.3 TB in the United Kingdom

Rates of TB within the UK continue to rise with almost 9000 cases reported in 2011. This represents a rate of 14.4 per 100,000 however rates have stabilised since 1995. Of these cases, the majority were reported from urban centres and in individuals from high burden countries and those with social risk factors for developing TB. The incidence of drug-

resistant cases has continued to rise within the UK and in 2011 8.4% of TB cases were resistant to any first-line drug, an increase of 26% compared to 2010. The proportion of isoniazid resistant and multi-drug resistant cases increased in 2011 by 7.6% and 1.6% respectively. With regards to XDR-TB, six cases were reported in 2011 representing 25% of all cases reported since 1995. Whilst rates of TB have started to stabilise in the UK, the trends in drug-resistant disease are concerning. Further and continued efforts are required therefore to reduce the incidence of disease and contain the onset of drug-resistant disease.

1.2 TB pathogenicity

TB is spread from person to person by the inhalation of infection airborne particles containing the *M.tb* bacilli. Upon inhalation, the infectious bacilli are phagocytosed by macrophages which then transit into deeper tissues. There the infected macrophages recruit additional macrophages and other immune cells to form tightly aggregated immune structure called granulomas, the pathological hallmark of TB. The infection can then remain dormant for many years, but with viable bacilli remaining within macrophages. Occasionally (approximately 5% of cases), infection with TB results in an acute symptomatic pulmonary infection or, “primary progressive TB” but most individuals develop asymptomatic or “latent disease” [4].

The exact reason why TB re-activates are not clearly understood but some of the associated risk factors include HIV infection, advanced age, malnutrition, immunosuppression (including corticosteroids, chemotherapy and anti-TNF- α therapies) and other inheritable abnormalities in cytokine production (principally IFN- γ and IL-12). Under such circumstances,

granuloma centres may necrotise spilling viable infectious bacilli into the airways and facilitating their spread. TB can then present in a number of ways. Commonly, this includes pulmonary infection, cervical lymphadenopathy, pleural disease, disease of the central nervous system (CNS) and disseminated infection (miliary TB). Left untreated, TB infection has a 50% mortality rate but with significantly higher rates encountered in CNS and miliary disease.

Whilst it is possible to treat TB using chemotherapy, treatment is both lengthy and complex. Individuals are required to take a combination of 3-4 drugs for a period of at least 6 months which leads to low patient compliance. Furthermore, the emergence of MDR and XDR-TB means that treatment can be extended to 2 years and beyond. There have been no new drug treatments developed for TB in the past 25 years and the currently used vaccine (Bacille Calmette-Guérin or BCG) has been shown to be of limited effectiveness due to high variability in effectiveness (0-80%) [5]. It is clear therefore, that as well as the development of effective vaccines and novel treatment strategies, a greater understanding of the pathogenicity of TB is needed.

1.2.1 TNF

TNF is essential for the control of *M.tb* in humans and in experimental animals [6]. The predominant activities of TNF in immunity to TB are to contribute to the activation of macrophages for killing of intracellular mycobacteria and to modulate apoptosis of infected cells [7]. In particular TNF can act with IFN- γ to activate macrophages for optimal control of *M.tb* growth. Excess TNF also contributes to the immunopathology of *M.tb* [8] indicating that optimal immunity with minimal tissue damage is an important element of the biology of TNF in TB. The critical role of TNF in control of *M.tb* infection in humans has been

illustrated by the increased rate of reactivation of active TB in subjects with latent TB who received anti-TNF therapy for conditions such as rheumatoid arthritis or Crohn's disease [9]. Screening for latent TB infection is now recommended patients being considered for this treatment [10].

1.2.3 IFN- γ

IFN- γ is essential for the control of *M.tb* infection [11]. Whilst IFN- γ is known to exert its effects through broad transcriptional programmes and the induction of cytokines, the effector mechanisms downstream of IFN- γ signalling responsible for the restriction of mycobacterial infection are still to be elucidated. IFN- γ stimulates macrophages to produce reactive oxygen intermediates as well as reactive nitrogen intermediates, both of which have been implicated in intracellular growth inhibition and/or mycobacterial killing. Additionally, IFN- γ stimulates macrophages to increase the expression of MHC class II molecules, thus leading to increased antigen presentation and further amplification of the immune responses. In humans, lack of appropriate IFN- γ responses results in increased susceptibility to severe systemic infection with mycobacterial species that do not usually cause significant disease in immunocompetent individuals [12, 13]

1.2.4 TB granulomas

The granuloma is the hallmark of tuberculosis and is composed of an organised aggregate of macrophages. Macrophages within granulomas exhibit several phenotypes, including mature macrophages, differentiated or epitheloid macrophages, foamy macrophages and multinucleated giant cells (MGCs) which form by fusion of the plasma membrane of multiple macrophages. Traditionally, granuloma formation was believed to be initiated by T

lymphocyte recruitment toward infected macrophages. The ability to visualise granuloma formation in transparent zebrafish infected with *M. marinum* has however provided exciting insights into the possible mechanisms governing granuloma initiation, recruitment and integrity. Research by Davis et al. revealed that macrophages are able to initiate granulomas prior to the appearance of T lymphocytes [14]. Similarly, *in vitro* granulomas models using human peripheral blood cells, confirm that macrophages alone are able to form epithelioid granulomas containing MGCs [15, 16]. After infection of the macrophage, evidence from the zebrafish model demonstrates that bacteria are released in to the extracellular space through the death of the macrophage. Mycobacteria are then rapidly ingested by the surrounding macrophages thereby promoting expansion of the pathogen population. This process is mediated by the EsxA/ESAT-6 protein which induces the expression of MMP-9 to promote the recruitment of uninfected macrophages to the site of infection [17].

In addition to macrophages, human granulomas contain dendritic cells, B cells and T lymphocytes, the latter of which is considered essential for the maintenance of organised granulomas and the control of mycobacterial progression after the acute phase of infection. Evidence from several animal models demonstrates hypoxia as well as an acidic microenvironment within these chronic granulomas. Hypoxia has been shown to induce the gene expression of several hundred genes, under the control of the dosR/dosS regulon, which favour pathogen survival and a shift towards reduced metabolic activity [18]. Because this metabolic adaptation is thought to favour a state of mycobacterial latency, this is an area of great interest particularly with respect to the development of new drugs and vaccines [19]. Interestingly, the discovery of actively replicating subpopulations in some

models of mycobacterial persistence[20] have challenged some of these long-held views of non-replicating persistence.

1.3 Clinical features of pulmonary tuberculosis

Following initial infection with *M.tb*, some individuals progress rapidly to active disease, usually referred to as primary or progressive TB, which is more common in children but also affects adults [21]. The majority of individuals will contain the initial infection and develop latent disease, with latently infected individuals having a 5-10% lifetime risk of developing active TB, termed reactivation or post-primary TB.

1.3.1 Latent TB

The majority of patients infected with *M.tb* (32% of the global population) are asymptomatic and have no clinical evidence of disease. These individuals with latent infection are able to maintain the disease in a quiescent form. This latent state is thought to be maintained by an active immune response in the host initiated by the infecting *M.tb* bacilli, permitting host controlled persistence of the organism [22].

1.3.2 Active TB

Active TB encompasses a heterogenous range of presentations and forms of disease. TB is predominantly a disease of the lungs with pulmonary disease accounting for approximately 70% of cases, although *M.tb* can disseminate to other organs in the body including lymph nodes, bone and meninges, and cause extra-pulmonary disease [21]. Extrapulmonary tuberculosis occurs in 10-43% of patients, depending on race or ethnic background, age,

presence or absence of underlying disease, genotype of the M.tb strain, and immune status [23]. The clinical signs and symptoms of tuberculosis are often non-specific. In adults, up to 5% of cases of active disease may be completely asymptomatic [24] Patients with primary tuberculosis are often asymptomatic but may develop a symptomatic pneumonia. Individuals with primary progressive disease may present with, cough, haemoptysis and weight loss and is most commonly seen in children and young adults. Patients with post-primary disease most commonly experience a chronic productive cough and marked weight loss and often experience low-grade pyrexia, dyspnoea and fatigue. Symptoms are often insidious and persist from weeks to months [25].

1.3.3 HIV co-infection

HIV co-infection poses special challenges to the clinical management of patients with active tuberculosis. The risk of active tuberculosis increases soon after infection with HIV and the manifestations of pulmonary tuberculosis are similar to those of HIV-negative individuals. At CD4 counts of less than 200, the presentation of TB may be atypical, with subtle infiltrates, pleural effusions, hilar lymphadenopathy, and other forms of extrapulmonary TB in up to 50% of patients. At CD4 counts of less than 75, pulmonary findings may be entirely absent, manifested as a non-specific chronic febrile illness with high early mortality [26].

1.4 Tuberculosis diagnosis

1.4.1 Latent infection

Screening and treatment for latent *M.tb* infection is indicated in the groups in which the prevalence of latent infection is high [27]. These include:

- Foreign born persons from regions in which tuberculosis is endemic
- Those in whom the risk of reactivation is high e.g. patients with HIV infection or diabetes and persons receiving immunosuppression
- Recent and close contacts of individuals of patients with tuberculosis [28-30]

Latent TB infection can be diagnosed with either a tuberculin skin test (TST) or an interferon-gamma release assay (IGRA). The TST was first described by Koch in 1890 and developed by Mantoux in 1907. The test relies on the principle of delayed hypersensitivity to recruit memory T cells to the site of intradermal injection of purified protein derivative (PPD) of *Mycobacterium bovis*. It is less expensive than an IGRA and in patients with culture confirmed TB, has similar levels of sensitivity (75-90%). TSTs have several disadvantages including cross-reactivity of PPD with non-tuberculous mycobacteria and the BCG vaccine. Other limitations include false-negative results in immunocompromised patients, young persons and older persons [31].

IGRA testing is based on the assessment of the release of interferon (IFN)- γ by CD4 cells. Currently, two standardised IGRAs have been developed to measure IFN- γ responses to infection in blood samples; QuantiFERON-TB GOLD test (Cellestis) and T-SPOT TB (Oxford Immunote) assay. QuantiFERON uses ELISA to measure the amount of IFN- γ released in response to in vitro stimulation of whole blood with *M.tb* antigens and T-SPOT uses ELISA to

count the number of IFN- γ producing cells on precoated plates. Both assays use highly *M.tb* specific antigens such as early secreted antigen 6 (ESAT 6) and culture filtrate protein (CFP10) which are not present in BCG or most environmental mycobacteria thus providing specificity available with TST. The sensitivity and specificity of IGRAs vary according to the population being tested but a recent comprehensive review calculated the pooled sensitivity of QFT-GOLD and TB-SPOT to be 83% and 90% respectively with a specificity of 99% and 88% respectively [32]

1.4.2 Active infection

Worldwide, acid-fast bacilli (AFB) sputum smear microscopy is the most widely used method for identifying tuberculosis cases [33, 34]. The main advantages of microscopy are that the test is inexpensive and rapid. Major disadvantages include low sensitivity and specificity, particularly in HIV-positive individuals and the absence of drug susceptibility information.

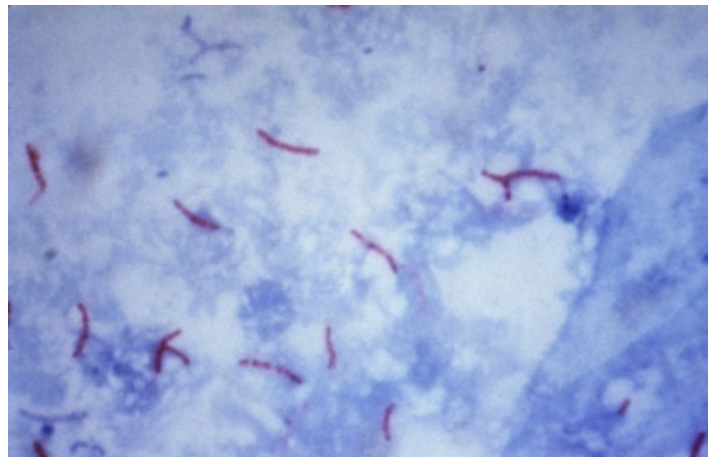


Figure 2: Ziehl-Neelsen stained sputum slide showing acid-fast bacilli.

Adapted from <http://biomedfrontiers.org/diabetes-2014-1-4/>

M.tb culture therefore remains the recognised gold standard for confirming diagnosis of tuberculosis. In recent times, liquid-based culture technologies have resulted in faster and more sensitive. Compared to solid-culture on Lowenstein-Jenson medium which requires approximately 4-6 weeks for detectable growth and a further 2-3 weeks for species specific identification, these newer methods allow recovery of mycobacterial species from 2 weeks from clinical specimens. Culture-based methods suffer from a number of disadvantages including the need for biosafety category 3 facilities and extensive training of personnel, particularly when liquid culture technologies are used.

The development of molecular methods, typically involving PCR, has allowed the rapid identification of *M.tb* with a number of commercially developed tests available. The pooled sensitivity of these test has been calculated as approximately 95% for smear-positive samples and approximately 75% for smear negative samples, while their performance for extrapulmonary specimens is usually much lower [35, 36]

The Xpert MTB/RIF assay is a PCR-based test which has been developed as a point-of-care TB diagnostic test [37]. Xpert MTB/RIF has a sensitivity of 98-100% in smear-positive respiratory specimens and 57-83% in smear-negative specimens.

1.4.3 Drug-resistant tuberculosis

Whilst conventional drug-sensitivity testing (DST) on Lowenstein-Jensen medium requires 2-4 weeks from primary culture, broth-based fully automated culture systems report results within 5-8 days from a primary culture. Such systems are now routinely used for first- and second-line anti-TB DST of *M.tb* in developed countries [38].

The emergence of MDR-TB has led to the development of novel methods to facilitate the rapid diagnosis of drug-resistant TB. In resource-poor settings, a number of inexpensive, non-commercial phenotypic methods have been developed to assess DST with high levels of agreement with conventional DST. The microscopic observation drug susceptibility (MODS) test in which sputum is directly inoculated into Middlebrook 7H9 broth containing first-line anti-TB drugs, and the cording growth of *M. tuberculosis* is detected by an inverted microscope, exhibits $\geq 95\%$ agreement with conventional DST for INH, RIF and MDR-TB [39]. The nitrate reductase (NR) assay is able to detect $\geq 95\%$ of MDR-TB and XDR-TB samples by detection of pink-red colour due to the reduction of nitrate to nitrite by viable *M.tb* [39]. Molecular methods detect resistance-associated mutations in the target genes of *M.tb* and can yield results within 1-2 days. Identification of the 81-bp RRDR of the *rpoB* gene present on 90-95% of RIF-resistant strains allows the identification of most MDR-TB strains as 85-90% of RIF-resistant *M.tb* isolates are also resistant to INH [40]. The ability of Xpert MTB/RIF assay to rapidly diagnose *M.tb* as well as identify drug resistance has resulted in endorsement by the WHO and represents a potential key tool in tackling drug-resistant disease globally [1].

1.5 The matrix metalloproteinase (MMPs) family

Metalloproteinases are endopeptidases that contain an active site Zn^{2+} and are divided into subfamilies based on evolutionary relationships and structure of the catalytic domain. The metzincin subfamily of metalloproteinases are characterised by 3-histidine zinc-binding motif and a conserved methionine turn following the active site. The members of the

metzincin family are the reprotlysins or ADAMS (A Disintegrin And Metalloproteinase), serralytins, astacins and matrixins (MMPs).

Initially described in the involuting tail of tadpoles by their ability to degrade collagen, a total of 25 vertebrate MMPs have been described of which 24 are found in humans [41]. MMPs are translated as an inactive enzyme which required cleavage to its active form. All MMPs contain a conserved pro and catalytic domain, with the exception of MMP-23. The prodomain is approximately 80 amino acids and contains the consensus sequence PRCXXPD. The catalytic domain contains three conserved histidines in the sequence HEXXHXXGXXH, which ligate the active Zn^{2+} site. With the exception of MMP-7, -23 and -26, MMPs have a flexible proline-rich hinge region and a hemopexin-like C-terminal domain, which functions in substrate recognition. Other domains are restricted to subgroups of enzymes. MMPs share a strikingly similar 3-D structure as well as gene arrangement suggesting that they originated from a common ancestor gene. At least eight of the known human MMP genes (MMP-1, -3, -7, -8, -10, -12, -13, and 20) are clustered on chromosome 11 at 11q21-23. Other MMP genes are scattered among chromosomes 1, 8, 12, 14, 16, 20, and 22 [42]. MMP gene expression and secretion can be triggered by a variety of factors including growth factors, cytokines, physical stress, oncogenic cellular transformation and components of infectious pathogens among others [43]. Various factors can activate MMPs via the conformational change that disrupts the Cys- Zn^{2+} binding (cysteine switch) and leads to intermediate activation followed by autocatalytic cleavage of the propeptide from the core protein that renders the enzyme fully active [44].

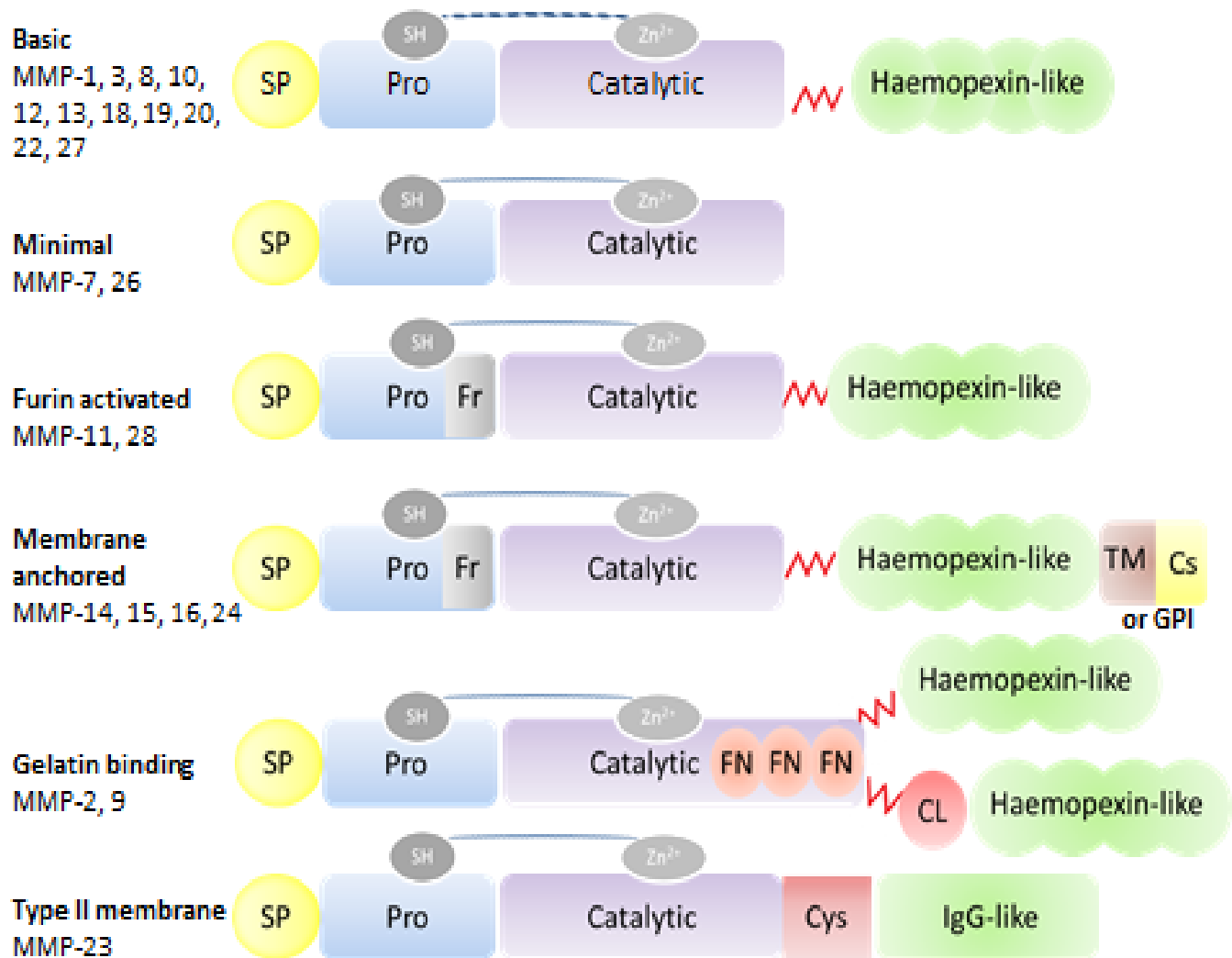


Figure 3: Schematic structure of major MMP groups. Adapted from Parks *et al.* Matrix metalloproteinases as modulators of inflammation and innate immunity, *Nature Reviews Immunology* 4, 617-629 (August 2004)

MMPs can be broadly defined by their substrate specificity into the following groups

Name	Alternative name	ECM Substrate	Other substrates
Collagenases			
MMP-1	Collagenase-1	Type I, II, III collagen, aggrecan	
MMP-8	Collagenase-2	Type I, II, III collagen, aggrecan	
MMP-13	Collagenase-3	Type I, II, III collagen, aggrecan	
Gelatinases			
MMP-2	Gelatinase-A	BM, Type IV, V and XI collagen, gelatin, aggrecan, laminin	CCL-7, CXCL12, β -amyloid
MMP-9	Gelatinase-B	BM, Type IV, V and XI collagen, gelatin, fibronectin, laminin	Latent TGF β & VEGF, α 1-proteinase inhibitor, CXCL8, CXCL1, 4 & 11, β -amyloid
Stromelysins			
MMP-3	Stromelysin-1	Fibronectin, laminin, Type IV collagen, aggrecan	Latent TGF β , E-cadherin, IL-1 β , β -amyloid
MMP-10	Stromelysin-2	Laminin	ProMMP-1, 7, 8 & 9
MMP-11	Stromelysin-3	Type IV collagen, aggrecan, laminin	Apoptosis
Elastases			
MMP-7	Matrilysin	Elastin, gelatin, fibronectin, Type IV collagen, aggrecan, laminin	E-cadherin, syndecan-1, pro=defensins, FasL, latent TNF α , CXCL1
MMP-12	Metalloelastase	Type V collagen, laminin, elastin, fibronectin	Latent TNF α , CXCL11, α 1-proteinase inhibitor, CXCL1, 3, 5, 6 & 8, CCL2, 7, 8 & 13
Membrane-type			
MMP-14	MT1-MMP	Type I, II, III collagen, laminin, aggrecan	ProMMP-2, syndecan-1, SLPI, DR-6, CCL7
MMP-15	MT2-MMP	Fibronectin, aggrecan, laminin, perlecan	ProMMP-2, antiapoptotic
MMP-16	MT3-MMP	Type III collagen, fibronectin, gelatin, laminin, vitronectin	ProMMP-2
MMP-24	MT5-MMP	Proteoglycans	ProMMP-2, cadherins
GPI anchored			
MMP-17	MT4-MMP	Gelatin	ProMMP-2, ADAMTS4
MMP-25	MT6-MMP	Type IV collagen, gelatin, fibronectin, fibrin, laminin	α 1-proteinase inhibitor, ProMMP-2
Others			
MMP-19	RAS1	Type IV collagen, aggrecan, laminin, fibronectin, gelatin	T-cell development, IGFB-3
MMP-20	Enamelysin	Type V collagen, aggrecan, amelogen, ameloblastin	
MMP-21		Gelatin	
MMP-28	Epilysin	Casein	

Table 1: Summary of major MMP groups and published substrates

1.5.1 MMPs in health and disease

MMPs have been implicated in a number of disease processes which involve aberrant ECM turnover such as arthritis, tumour invasion and malignancy. However, as well as their role in ECM remodelling, MMPs perform multiple roles in the normal immune response such as facilitating leukocyte recruitment, cytokine and chemokine processing and defensin action[45]. Typically, MMPs are not expressed in normal healthy tissue but can often be detected in repair or remodelling processes, as well as in diseased or inflamed tissues. They are released from a broad range of cells at sites of infection, including leucocytes (monocytes, macrophages, lymphocytes and neutrophils), fibroblasts and epithelial cells, and play a number of physiological roles in the normal immune response to infection including remodelling of the extracellular matrix, facilitating cell migration, processing cytokines, and activating defensins [45]. However, excess MMP activity may lead to tissue destruction and immunopathology that favours pathogen dissemination or persistence by breaking down barriers to spread or by creating an immunoprivileged site that is poorly accessed by host immune cells [46]. To prevent this, MMP production is tightly regulated. In addition to control on the transcriptional level, MMPs are compartmentalized and secreted as pro-enzymes that require proteolytic cleavage. What is more, upon activation, they are controlled by tissue inhibitors of metalloproteinases (TIMPs) which bind MMPs in a 1:1 manner to prevent enzymatic activity MMP activity is controlled at the transcriptional level as well as by compartmentalisation and secretion as pro-enzymes requiring proteolytic cleavage [47]. The balance of MMPs to TIMPs therefore determines matrix turnover, where either an excess of MMPs or a deficit of TIMPs may result in ECM degradation.

1.5.2 Regulation of MMP activity

Under physiological conditions the activity of MMPs is tightly regulated to prevent excessive proteolytic activity and tissue destruction. As with other proteinases, the action of MMPs can be regulated at the level of gene transcription, translation and secretion of latent enzyme, proenzyme activation, and inactivation by endogenous inhibitors.

Proenzyme activation

In addition to the “cysteine switch” mechanism, several MMPs contain a RXXR or RRKR sequence between the pro- and catalytic domains. These function as a target sequence for pro-protein convertases or furins; this is known as a furin cleavage site. Not all MMPs are activated in this manner, for example, proMMP-2 is known to be activated by active MMP-14 through its cell surface interaction [48]. Currently the mechanism of activation in some MMPs is not fully described and remains the subject of ongoing investigation both within our group and by others.

Compartmentalisation

Compartmentalisation is an important regulatory control mechanism which prevents the indiscriminate release of proteinases. Typically, proteinases such as MMPs are anchored to the cell membrane, thereby maintaining a high enzyme concentration locally and targeting their catalytic activity to specific substrates in the pericellular space

Endogenous inhibitors of MMPs

MMPs activities are inhibited by two major types of endogenous inhibitors: α_2 -macroglobulin and Tissue Inhibitors of Metalloproteinases (TIMPs).

α_2 -macroglobulin

Human α_2 -macroglobulin is a plasma glycoprotein of 725 kDa comprising of four identical subunits of 180kDa. It acts by trapping the proteinase whilst in their fluid phase within the macroglobulin. The complex is then rapidly cleared by endocytosis via the receptor low density lipoprotein related protein-1.

TIMPS

TIMPs are selective inhibitors of MMPs consisting of 184-194 amino acids. They comprise 4 main classes TIMP-1, TIMP-2, TIMP-3 and TIMP-4 which can be subdivided into an N-terminal and C-terminal subdomain. Each domain contains three conserved disulphide bonds and the N-terminal domain folds as an independent unit with MMP inhibitory activity. TIMPs inhibit all known MMPs although TIMP-1 has only limited inhibitory activity against MT1-MMP, MT3-MMP, MT-MMP and MMP-19. TIMP-1 null mice and TIMP-2 null mice do not exhibit obvious abnormalities. TIMP-3 null mice develop emphysema-like alveolar damage [49] and faster apoptosis of mammary epithelial cells after weaning [50] indicating that TIMP-3 is a major regulator of MMP activity in vivo.

1.5.3 MMPs and cytokines

Several studies have indicated that MMPs can directly or indirectly affect the activity of various cytokines that function in inflammation and repair processes, including interferon- β , vascular endothelial growth factor (VEGF), epidermal growth factors, fibroblast growth factors and transforming growth factor- β [51]. Similarly, IL-1 β , a potent pro-inflammatory cytokine, requires proteolytic processing by IL-1 β -converting enzyme (ICE) for activation has been shown to be modulated by MMPs. At least three members of the MMP family, MMP-

2, -3 and -9 can cleave and activate the IL-1 β precursor. Furthermore, after activating IL-1 β , MMP-3 degrades the biologically active cytokine [52], which can also be inactivated in vitro by MMP-1, -2 and -9 [52]. These data indicate a dual role for MMPs in biphasic modulation of inflammatory-mediator activity whereby MMPs are involved in both activation and inactivation

1.6 MMPs and their role in TB pathogenicity

In order to exert its pathogenic effects *M.tb* must drive destruction of the extracellular matrix and destroy its fibrils to allow transmission. Increasing evidence is implicating MMPs activity in the TB-related tissue destruction with MMPs being the only enzymes capable of destroying all components of lung fibrillar collagen at neutral pH. Initial studies of *M.tb* pathogenicity and MMPs demonstrated that mycobacterial lipoarabinomannan (LAM), a major antigenic wall component of *M.tb* increases MMP-1 and MMP-9 in the human THP-1 cell line [53]. Virulent *M.tb* up-regulates MMP-1 more potently than the weakened vaccine strain *M.bovis* BCG in primary human macrophages [54] whilst examination of lung specimens from Tb infected patients indicate that MMP-1 is expressed and secreted in macrophages around the granuloma. Additionally, both MMP-1 and MMP-9 were found to be up-regulated in primary human lung cells stimulated with filtered, conditioned media from *M.tb*-infected monocytes (CoMTb). These results were not seen with direct infection indicating that MMPs are up-regulated via a monocyte-dependent network that involves both host and pathogen derived soluble factors [55-57]. Much of the investigation of MMPs and Tb pathogenicity has focused on clinical disease and more recently on novel animal models in which to study the disease. Elevated MMP-9 levels have been found in the

pleural fluid and serum of Tb infected patients [58] whilst elevated levels of MMP-1 and MMP-3 have been found in the sputum and bronchoalveolar lavage (BAL) samples of Tb infected patients, irrespective of HIV status [59]. In terms of extra-pulmonary disease, elevated levels of MMP-9 have been demonstrated in the cerebrospinal fluid and brain tissue of patients with tuberculous meningitis [60]. In addition, it has been shown that CNS tuberculous disease causes a down-regulation of glial MMP-2 secretion via TNF- α , NF-KB, p38 and caspase 8 dependent network [61].

Until recently, the study of MMPs and TB pathogenicity in animals has been somewhat limited due to the lack of good models in which to study the disease. The mouse for example, does not express MMP-1 nor does it form cavitary disease. This has been overcome by the recent development of MMP-1 transgenic mice. Within these mice it has been demonstrated that, *M.tb* infection increased MMP-1 expression, resulting in alveolar destruction in lung granulomas and significantly increased collagen breakdown [59]. It has also been shown that MMPs may play a major role in the induction of tuberculous granuloma formation [17]. In these studies which utilise *Mycobacterium marinum* (Mm) to infect transparent zebrafish larvae, the widely accepted model of the granuloma as an immunoprotective mechanism to curtail and encase mycobacteria has been challenged [62]. Recently it has been shown that ESAT 6 from TB drives epithelial cell MMP-9 secretion which then regulates a cytokine gradient for monocyte recruitment to the granuloma. [17]. In this study the early granuloma was shown to expand bacterial numbers with MMP-9 promoting granuloma formation and virulence as well as accelerating macrophage recruitment to the granuloma.

There is mounting evidence for the role of MMPs in TB pathogenicity and increasingly, a better understanding of MMP activity is starting to challenge some of the accepted models of granuloma formation, tissue destruction and disease transmission.

1.7 Hypoxia and its role in innate immunity

In a given environment, oxygen availability is usually defined as anaerobic or anoxic (no available oxygen), hypoxic (reduction in available oxygen compared to atmospheric pressure) or normoxic (atmospheric pressures of usually 21% O₂ at sea level or an O₂ [pO₂] partial pressure of 159mmHg). The oxygen percentage in healthy tissue ranges from 2.5-9% O₂ but markedly decreases in tumours, wounds and sites of infection to levels of ≤1% O₂ [63-65]. In the healthy human lung, alveolar pO₂ is approximately 100-110mmHg (~14% O₂). Local hypoxia found in inflamed and infected sites relates to a number of factors; decreased perfusion secondary to microvascular injury and thrombosis associated with the metabolic activities of the infectious pathogen and the recruited inflammatory cells.

The principle cells of the innate response to inflammation are cells of the myeloid lineage, monocytes/macrophages and neutrophils. These cells phagocytose invading microorganisms and release numerous antimicrobial molecules and pro-inflammatory mediators. Phagocytic cells must therefore be adapted to generate energy and function effectively in oxygen-deprived areas. In recent times, these adaptive responses have been demonstrated to be orchestrated by the transcription factor hypoxia-inducible factor or HIF

1.8 Hypoxia-inducible factor (HIF)

HIF is a heterodimeric helix-loop-helix transcription factor, the expression of which is controlled by both oxygen and iron [66]. HIF-1 was discovered by the identification of a hypoxia response element (HRE; 5'-RCGTG-3') in the 3' enhancer gene for erythropoietin (EOP), a hormone that stimulates erythrocyte proliferation and undergoes hypoxia-induced transcription [67, 68]. HIF is comprised of a constitutively expressed β -subunit and an oxygen-dependant α -subunit. HIF-1 β is also known as the aryl hydrocarbon nuclear translocator (ARNT), which was originally identified as a binding partner of the aryl hydrocarbon receptor [69]. These proteins belong to the basic helix-loop-helix-Per-ARNT-Sim (bHLH-PAS) protein family. The bHLH and PAS motifs are required for heterodimer formation between the HIF-1 α and HIF-1 β subunits, and the downstream basic region is responsible for specific binding to the HRE DNA sequence. Three forms of HIF have been identified, HIF-1 α , HIF-2 α and HIF-3 α and all are known to respond to changes in available cellular oxygen [70].

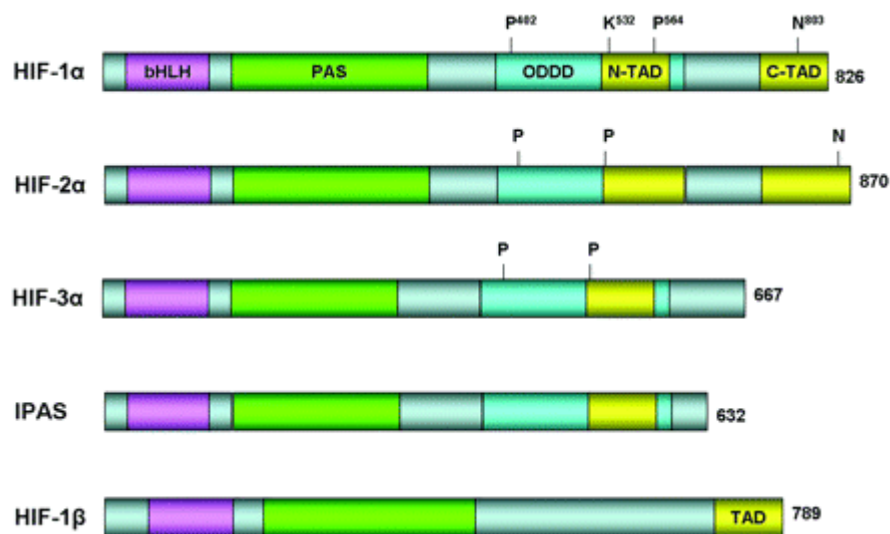


Figure 4: Domain structure of human HIF- α and HIF-1 β . Adapted from Ke, Q. and Costa, M. Hypoxia Inducible Factor-1. *Mol Pharmacol*. 2006 Nov; 70(5):1469-80

In comparison the HIF-1 α which is universally expressed, HIF-2 α is predominately expressed in the lung, endothelium and carotid body [71]. HIF-3 α was discovered later and is also expressed in a wide variety of tissues [72]. In addition, a splice variant of HIF-3 α , inhibitory PAS (IPAS) which is found predominantly in the Purkinje cells of the cerebellum and corneal epithelium, was subsequently discovered [73]. HIF- α (HIF-1 α , HIF-2 α , HIF-3 α and IPAS) contains an oxygen-dependent degradation domain (ODDD) that mediates oxygen-regulated stability through the hydroxylation of two proline (P) residues and the acetylation of a lysine (K) [74]. HIF-1 α and HIF-2 α contain two transactivation domains; N-TAD and C-TAD. The C-TAD in particular has been shown to interact with co-factors such as CBP/p300 to activate gene transcription [75]

1.8.1 Regulation of HIF

The transcription and synthesis of both HIF-1 α and HIF-1 β are constitutive but HIF-1 α protein has a short half-life ($t=2-5$ mins) and is highly oxygen sensitive. In normoxia, HIF-1 α protein is rapidly degraded resulting in little or no detectable HIF-1 α protein. Thus the stability and transactivational function of HIF is controlled principally at the post-transcriptional level by hydroxylation, ubiquitination, acetylation and phosphorylation [76].

In normoxia, the HIF α -subunits are unstable as cells continually synthesised and degraded. This process is primarily regulated by a family of prolyl hydroxylases called PHD1, PHD2 and PHD3 [77, 78]. Under normoxic conditions, PHDs catalyse the Fe(II)-dependent hydroxylation of specific prolyl residues within the oxygen-dependent domain of HIF- α . Once hydroxylated, the von-Hippel-Lindau (pVHL) tumour suppressor protein binds to α -subunits and identifies them for ubiquitination and proteosomal degradation [79]. A further level of control is provided by asparaginyl hydroxylase factor inhibiting HIF (FIH). FIH

inhibits p300-CREB-binding protein (CBP)-mediated transcriptional co-activation of the HIF genes.

Under low oxygen levels (hypoxia), PHD activity decreases. This results in stabilisation of the α -subunits which translocate to the nucleus and heterodimerises with the β -subunit along with co-factors p300/CBP. The resultant HIF complex binds to core pentanucleotide sequences known as hypoxic-response elements (HREs) in the promoter regions of target genes.

Besides hypoxia, HIF has also been shown to be regulated by oxygen-independent mechanisms. Cytokines, growth factors, environmental stimuli and other signalling molecules have been implicated in regulating HIF in normoxic conditions [80-83].

HIF signalling pathway is considered to be the master regulator of both immune cell function and survival [79]. HIF is present in all mammalian cells and is known to regulate the expression of several hundred genes which function in a variety of host cellular and systemic responses to low oxygen levels. It has been shown to influence metabolism, angiogenesis, vascular tone, cell differentiation, and apoptosis as well being involved in cancer and many other physiological conditions such as inflammation and infection. Recently, HIF has been shown to act as the master regulator of macrophage and neutrophil inflammatory and innate immune function [84].

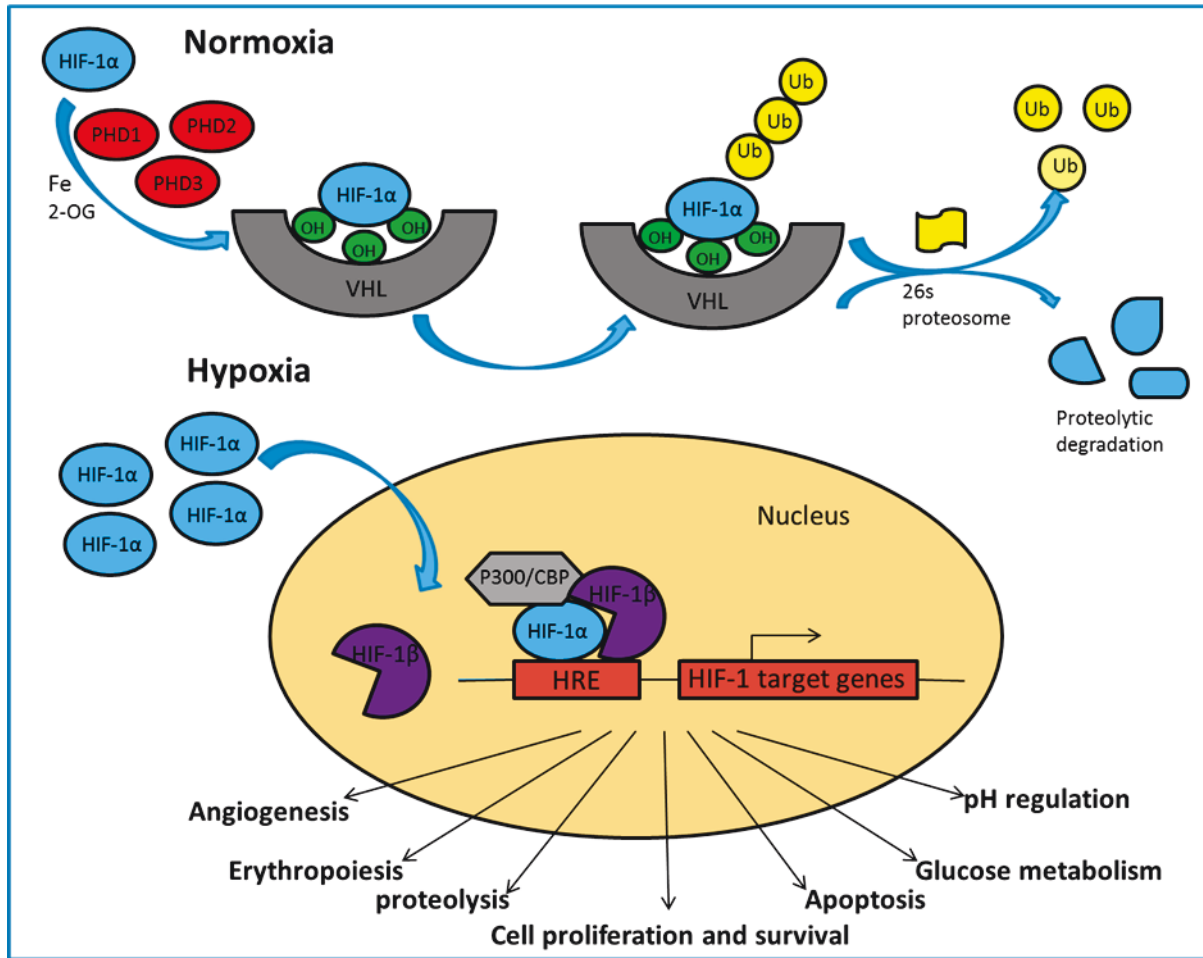


Figure 5: Schematic representation of HIF-1 regulation in normoxia and hypoxia. Adapted from http://journals.cambridge.org/fulltext_content/ERM/ERM7_06/S1462399405009117sup007.htm

1.8.2 HIF and innate immunity

It has been shown that hypoxia increases phagocytic rates in a HIF-dependent manner [85, 86] and that macrophages from mice deficient in HIF-1α show a diminished capacity to kill Gram-negative and Gram positive bacteria when compared to macrophages from wild-type littermates [86]. Conversely, activation of the HIF pathway through deletion of the VHL-tumour suppressor protein or pharmacological inducers supported myeloid cell production of defence factors and improved bactericidal capacity. HIF-1 has been shown to induce leukocyte β2 integrin expression thus promoting neutrophils binding to epithelium [87] as

well as inhibiting neutrophil apoptosis via NF- κ B activity [88], thereby prolonging the effective window for these cells to participate in phagocytic killing. A striking and perhaps unexpected finding is that bacterial exposure is a stronger stimulus for HIF-1 α activation than hypoxia itself [86]. Several clinically important bacterial species such as Group A Streptococci (GAS), methacillin-resistant *S.aureus* and *Salmonella* species can induce HIF-1 α with HIF-1 α stabilisation demonstrated even at normoxia. These findings indicate the complex nature of HIF signalling and the need for further analysis of its pathways as well as its role in innate immunity.

In addition to oxygen-dependent activation pathways, HIF can also be induced by inflammatory cytokines, growth factors and bacterial products at normoxic conditions, although the underlying molecular pathways are not fully elucidated. Pro-inflammatory cytokines TNF α and IL-1 β have been shown to increase accumulation and transcriptional activity of HIF-1 α . The fact that HIF can be activated in response to inflammatory cytokines indicates HIF may play an important role in inflammation.

In addition to cytokines, bacteria and bacterial products such as LPS also stimulate HIF activity under normal oxygen levels. Several pathways have been reported to be involved in this process including NF- κ B [79, 84, 89], ROS [90], and PHDs [91].

The finding that bacteria can be stronger activators of the HIF pathway rather than hypoxia in addition to the demonstration of bacteria-induced HIF protein stabilisation in normoxia has been the subject of much investigation. Recently, the mechanistic explanation for these phenomena has been found to reflect a close, synergistic relationship between HIF and the central regulator of innate immunity, NF- κ B.

1.8.3 NF- κ B in innate immunity

NF- κ B is a dimeric transcription factor composed of subunits from two subfamilies: the NF- κ B subfamily comprised of p50 and p52 and the Rel subfamily comprised of Rel A, also known as p65, Rel B and c-Rel. All NF- κ B subunits have Rel homology domains that facilitate DNA-binding and dimerisation but only the Rel proteins have C-terminal transactivation domains [92]. As a result, all NF- κ B subunits with the exception of Rel B can form homo- or heterodimers resulting in a high degree of combinatorial diversity but only dimers containing Rel proteins can act as transactivators. Currently, the p50/p65 heterodimer represents the most abundant of the Rel dimers [92].

NF- κ B influences gene expression events that impact cell survival, differentiation and proliferation in addition to its pivotal role in innate immunity. For this reason, the activity of NF- κ B is tightly regulated at multiple levels. The principal mechanism for regulating NF- κ B is through the inhibitory I κ B proteins (inhibitor of NF- κ B), and the kinase that phosphorylates I κ Bs, namely, the I κ B kinase (IKK) complex [93]. The IKK complex consists of two catalytic subunits (IKK α and IKK β) and one regulatory subunit (NEMO). This signalling pathway through the heterodimeric IKK complex is known as the canonical NF- κ B signalling pathway [94]. An alternative noncanonical pathway also exists which is mediated through activation of IKK α homodimers [95]. A number of post-translational modifications also modulate the activity of the I κ B and IKK proteins as well as NF- κ B molecules themselves [92].

1.8.4 Regulation of NF- κ B by hypoxia

Hypoxic activation of NF- κ B has been identified in a wide array of cells types and has been the subject of intense investigation. Most notable, key inflammatory genes including TNF α , IL-6 and MIP-2 have been shown to be directly activated by hypoxia in an NF- κ B-dependent manner. Although the exact mechanisms involved have not been fully elucidated, the hypoxia sensitive component appears to be at the level of (or upstream) of the IKK complex, as evidenced by demonstration of IKK β activation in cells exposed to hypoxia [84, 91]. A possible explanation linking HIF and NF- κ B regulation is through the oxygen-sensing prolyl hydroxylase (PHDs) enzymes which also regulate HIF. Evidence from Cummins *et al.* demonstrates that inhibition of the PHDs leads to increased NF- κ B signalling by inhibition of IKK β [91].

1.8.5 HIF and NF- κ B interdependence

A number of studies have indicated that significant cross-talk occurs between HIF and NF- κ B. TNF α -induced HIF-1 α stimulation requires NF κ B at the level of HIF-1 α protein stabilisation without affecting its mRNA level [96, 97]. Similarly, IL-1 β acts on HIF-1 α protein stability by triggering NF κ B activity and inhibiting VHL-mediated protein degradation [96]. More recently, an in vivo study by Ruis *et al* demonstrated that NF- κ B upregulates HIF-1 α through transcriptional upregulation and furthermore showed that basal NF- κ B is a prerequisite for constitutive HIF-1 α expression [84]. A further level of support for HIF and NF- κ B cross-talk is the finding that HIF can alter NF- κ B signalling by upregulating p65 and IKK α in neutrophils [88].

1.9 Hypoxia and tuberculosis

It is evident from a variety of human, animal and *in vitro* studies, that oxygen tension is intimately associated with the outcome of *M.tb* infection. A growing body of research demonstrate that *M.tb* growth, metabolic and transcriptional profile are acutely sensitive to varying levels of oxygen *in vivo* and *in vitro* [18, 98]. Tuberculous granulomas have long been considered the hosts defence mechanism against the bacteria. By encasing the bacilli, the granuloma restricts access to oxygen and exposes the organism to an acidic environment as well as other immune effectors, such as nitric oxide. This hypoxic environment does not necessarily lead to eradication of the bacillus. Several studies indicate that hypoxia causes Tb to enter a state of bacteriostasis or slow growing replication which can last the lifetime of the host [99]. In this model of latency, these mycobacteria are viewed as the seeds of reactivation which under certain permissive conditions, for example HIV infection, will then progress to cause active disease.

1.9.1 Oxygen status and reactivation

Several indirect observations have supported the view that Tb infections are preferentially associated with the most oxygen-rich sites in the body [100]. In humans, reactivation from latency most commonly occurs within the upper lobes of the lungs, an area shown to be the single most oxygenated region in the body. Similarly, quadrupeds such as rabbits and cows develop Tb infection in the dorsal portion of the lungs, a region corresponding to the most oxygen-rich site of the animal [101]. Strikingly, if *M.tb* infected rabbits are maintained in an upright position for 11 hrs a day, the Tb lesions are found to be predominantly in the apical lung zones [102]. It has long been postulated that reduced oxygen tension is associated with inhibition of *M.tb* growth. In the pre-antibiotic era, one of the most effective treatments for

pulmonary tuberculosis involved the use of artificial pneumothorax, a process in which the affected lung is temporarily collapsed thereby restricting the supply of both blood and oxygen. Maintaining infected animals at reduced oxygen tension slows disease progression and results in reduced pathology [103]. Similarly, human epidemiological studies indicate that rates of Tb transmission and/or disease decrease at higher altitudes where oxygen tensions are lower [104]. Despite the mainly observational evidence from human studies, it is interesting that no study of tissue oxygen tension has been undertaken in humans. Currently, our understanding of tissue oxygenation and hypoxia is based on animal models of TB infection, many of which indicate that TB granulomas are in fact profoundly hypoxic.

1.9.2 Investigation of hypoxia in current animal models of TB infection

Several animal models are currently used to study TB infection. These include mice, zebrafish, guinea pig, rabbit and non-human primates. Whilst a number of studies have been undertaken to examine the presence of hypoxia within these models, it is not until recently that specific animal models have been developed to assess the impact of hypoxia on the immunopathology of TB infection.

Hypoxia and the mouse model of TB infection

Mice, most notably BALB/c mice, are the most commonly used animal model to study *M.tb* infection. The advantages of this model include low cost and space requirements, availability of inbred strains and immunological reagents. Unfortunately, caseation and cavitation, two hallmarks of human TB associated with dramatic changes in oxygen tension are almost never seen in infected mice [59]. Granulomas in mice are generally small and

poorly differentiated and studies by Aly *et al.* and Via *et al.* indicate that TB lesions in mouse lungs are not hypoxic [105, 106]. More recently, several groups have sought to utilise C3HeB/FeJ (Kramick) mice to more closely represent human phenotypic changes. In this setting, *M.tb* infection results in the formation of caseous necrosis within granulomas with surrounding regions of hypoxia, as demonstrated by positive staining for the hypoxia tracer pimonidazole (Pimo) [107, 108]. Interestingly, C3HeB/FeJ mice demonstrate reduced responsiveness to pyrazinamide and moxifloxacin when compared with BALB/c mice and different. There is also evidence that hypoxia within granulomas of C57BL/6 mice might alter host immune responses resulting in the upregulation of the serine proteases cathepsin G, neutrophil elastase and proteinase 3 [109]. Interestingly, when C57BL/6 mice are infected with *M.tb* by dermal inoculation, they appear to develop a phase of latent disease, a phenomenon not seen in BALB/c mice. As such, this mouse model has the potential to offer a novel mechanism by which to assess new vaccines and anti-tuberculosis chemotherapy.

Hypoxia and the zebrafish model of TB infection

Zebrafish are sensitive to infection with *Mycobacterium marinum* (Mm) whose embryo stage is transparent allowing for the live imaging of early TB infection and granuloma formation[17]. Currently the limited immunological reagents means oxygen tension in zebrafish lesions has not been reported. There has however been recent investigation of HIF signalling in Mm infection in zebrafish which demonstrates that HIF-1 α signalling occurs in early Mm infection and that activation of this pathway aids host clearance of Mm infection [110]. Additionally, the antimicrobial effect of HIF-1 α stabilization, and HIF-2 α

reduction, were demonstrated to be dependent on inducible nitric oxide synthase (iNOS) signaling at early stages of infection.

Hypoxia and the guinea pig model of TB infection

Guinea pigs are exquisitely sensitive to *M.tb* infection and as such do not undergo a latent phase. Mature lesions develop caseous necrosis which are positive for the hypoxia marker pimonidazole hydrochloride (PIMO) [106].

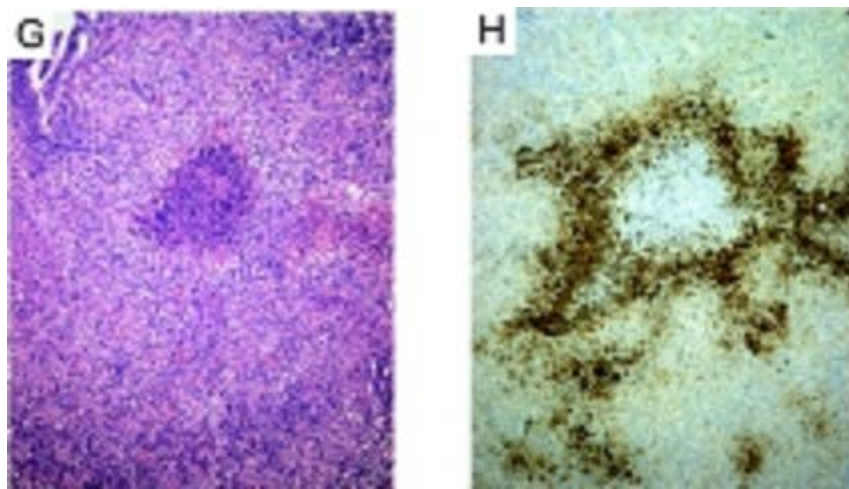


Figure 6: H &E stain of caseous necrosis (G) and PIMO stain (H) in a guinea pig. Via *et al.* Infect Immun. 2008 June; 76(6): 2333–2340.

Guinea pigs have been widely used to model TB infection and represent an attractive model in which to study tissue hypoxia. Unfortunately, their use is limited by the space and moderate cost requirements as well as the relative lack of immunological reagents.

Hypoxia and the rabbit model of TB infection

Like the guinea pig, rabbits develop hypoxic lesions around areas of caseous necrosis. Rabbits however are resistant to infection with *Mycobacterium tuberculosis* and are instead infected with *Mycobacterium bovis*, to which they are highly sensitive. Necrotic granulomas

are positive for PIMO whilst rabbits kept at 95% O₂; 5% CO₂ i.e. hyper-oxygenated, demonstrate decreased PIMO uptake compared to rabbits housed at 21% O₂; 5%CO₂ [106]. In addition, direct measurement of granulomas using a PO₂ probe placed into visible granulomas revealed mean ($\pm$ SE) pO₂ measurements of 2.66 ± 0.70 mm Hg compared to 59.46 ± 5.03 mm Hg in grossly normal lung tissue ($P < 0.001$) [106].

Non-human primate

Primates develop TB disease in remarkably similar way to humans with evidence of a latent phase as well as caseous necrosis and hypoxia within pulmonary lesions [111]. Unlike the rabbit and guinea pig, immunological reagents are plentiful but the high costs involved as restricted the use of this model for studying TB. In addition to demonstrating evidence of hypoxia in the rabbit and guinea pig, Via *et al.* demonstrate evidence of PIMO activation in 100% of necrotic granulomas in *M. tuberculosis*-infected cynomolgous macaques [106].

Given this body of evidence, it is surprising that the nature of oxygen tension and its relevance to disease activation has never been adequately investigated in humans. This is likely to be due in part to the invasive nature of many of the tools used to assess hypoxia. Polarography using a pO₂ probe requires an accessible site and the endogenous hypoxia tracer pimonidazole needs to be administered intravenously prior to an invasive biopsy. Investigators therefore have sought to utilise *in vitro* systems, transcriptional analysis of human TB granulomas, and *M.tb* genome sequencing to better understand pathogen and host responses to hypoxia.

1.10 *In vitro* systems of hypoxia

1.10.1 The Wayne model

The most widely accepted *in vitro* system of hypoxia is the Wayne model which, despite improvement, is still troubled by issues of reproducibility [112]. In this model, a sealed standing culture of *M.tb* with a defined volume of air above it is allowed to incubate over a period of days. By defining and restricting the volume of air available, a self-generated oxygen gradient is produced which slowly depletes the bacteria of oxygen. The concomitant shift in *M.tb* physiology produces two distinct states of non-replicating persistence (stages 1 and 2) [99]. These stages reflect discrete metabolic and drug-susceptibility states compared with log phase growth. As might be expected, the synthesis of proteins is markedly reduced in this system [113]. This is however accompanied by the increased expression of certain genes namely the alternative sigma factors sigB, sigE and sigH. Alternative sigma factors drive broad changes in transcription and may help drive the adaptation to hypoxia [114, 115].

1.10.2 The defined hypoxia model

This model allows researchers to determine the atmospheric conditions and maintain them throughout the course of an experiment, usually within an incubator or dedicated hypoxia workstation. Use of this approach has determined that bacterial replication is halted at oxygen tensions of 1% or less but the bacteria remain viable supporting the theory of non-replicating persistence [116, 117].

1.10.3 Transcriptional analysis

Analysis of the initial transcriptional response to hypoxia indicates approximately 100 genes with altered expression and nearly all the up-regulated genes were found to be under the control of the two-component transcriptional regulation system DosR/DosS, or DosR regulon [18]. Of the characterised genes which are down-regulated, many are involved in protein synthesis, lipid synthesis and aerobic metabolism, indicating a shift to reduced metabolic activity. Interestingly, the expression of DosR regulon genes is not synonymous with the “dormancy response” of *M.tb*. Evidence from several groups shows that the DosR regulon is induced during stages of active growth [118] and that loss of DosR results in only mild phenotypic changes in many models of latency [119]. The DosR regulon may therefore participate in a more focussed manner to promote survival during periods of extended respiratory, nitrosative and redox stress.

Evidence from transcriptional profiling of human TB granulomas again supports the finding of altered transcriptional responses which favour bacterial survival. Using RNA-RNA in-situ hybridisation, Fenhalls et al. demonstrate upregulation of *icl*, *narX*, and Rv2557 and Rv2558, within the lymphocyte “cuff” directly surrounding the necrotic center [120]. These four genes have all been implicated in bacterial persistence and concur with data from *in vitro* studies using the Wayne model.

In a recent systems analysis of *M.tb* regulatory and metabolic networks using ChIP-Seq, key transcription factors induced by hypoxia and subsequent re-aeration were further investigated. In addition to the key role of DosR, the gene Rv0081 was found to act as the central mediator of the initial response to hypoxia whereas a larger stimulon termed the enduring hypoxic response (HER) is induced in later hypoxia [121]. In addition the negative

transcriptional regulator KstR which control a large regulon mediating cholesterol degradation and lipid and energy metabolism was found to significantly impact on this network [122]. Collectively, the data reveals an interconnected subnetwork linking hypoxic adaptation, lipid and cholesterol degradation, and lipid biosynthesis in the pathogen response to hypoxia but do not address hypoxia in the host.

1.11 Hypoxia and MMPs

Hypoxia is present in several disease processes which involve pathophysiological mechanisms related to hypoxia-induced extracellular remodelling. These include chronic obstructive pulmonary disease (COPD), atherosclerosis and arthritis [123, 124]. In relation to vascular remodelling, overexpression and activation of MMPs strongly correlates with clinical manifestations of atherosclerosis [125]. In patient samples, levels of active intraplaque MMP-9 were found to be higher in unstable coronary plaques and were found to be strongly associated with unstable angina compared with stable coronary plaques [126].

Hypoxia and HIF-1 α have been implicated in a wide range of malignant disease processes. HIF-1 α overexpression is found in a number of cancers including ovarian, breast, lung, head-and-neck and gastric cancers amongst others [127-129]. HIF is correlated with p53 accumulation and cell proliferation suggesting a role for HIF-1 α in cancer progression. MMPs contribute to invasion and metastasis of tumour cells by degrading the basement membrane and ECM, which then enables migration and spread of cancer cells. Several studies indicate increased HIF-1 α mediated MMP activity in malignancy. In human breast

cancer, hypoxia and HIF-1 α upregulate MMP-9 expression and promotes tumour invasion [130]. Similarly, hypoxia increases MMP-2 expression in oesophageal cancers via a HIF-1 α mediated pathway and contributes to hypoxia-induced invasion and metastasis [131].

Recent studies investigating the role of hypoxia in inflammation indicate that changes in oxygen levels affect MMP activity and a number of key regulators of MMP expression including the transcription factors NF- κ B and AP-1, have been linked to HIF [84]. It has been shown that the hypoxic environment of the rheumatoid synovium promotes MMP-1 and MMP-3 gene expression and secretion thereby contributing to direct tissue destruction observed in the disorder [132]. In the context of Tb infection, little is known of the impact of oxygen levels on MMP expression and activity. Moreover the role of hypoxia during active Tb infection has not been elicited with most research focusing on latent disease. In fact, whilst animal studies indicate that Tb granulomas are hypoxic, there has been no research performed to determine whether human lesions are hypoxic.

1.12 The radiological manifestations of pulmonary TB

Historically, pulmonary tuberculosis has been divided into primary and post-primary infection. It can be difficult to distinguish between the two radiologically (as well as clinically) as there tends to be a large amount of overlap between the two. The main imaging modality used globally, if this is available, is a plain chest radiograph. In the developed world computerised tomography (CT) is increasingly being used. Nuclear medicine techniques are occasionally used but are mostly employed when the diagnosis is debated.

1.12.1 Primary tuberculosis

At radiology, primary TB manifests itself as four main entities: parenchymal disease, lymphadenopathy, miliary disease and pleural effusion.

Parenchymal disease

Typically, parenchymal disease manifests as dense, homogenous parenchymal consolidation in any lobe. Its appearance is often indistinguishable from that of bacterial pneumonia, however it can be differentiated from bacterial pneumonia on the basis of radiographic evidence of lymphadenopathy and lack of response to conventional antibiotics [133].

Lymphadenopathy

Radiographic evidence of lymphadenopathy is seen in up to 96% of children and 43% of adults [133]. Lymphadenopathy is typically unilateral and right sided, involving the hilum and right paratracheal region, although it is bilateral in about one third of cases. Nodes greater than 2cm in diameter with low attenuation at its centre, secondary to necrosis at CT are highly suggestive of active disease [134].

Miliary disease

Miliary disease affects 1-7% of patients with TB. The classic radiographic findings are of evenly distributed small 2-3mm nodules. These are sometimes interspersed with focal or diffuse consolidation where the nodules have coalesced.

Pleural effusion

A pleural effusion is seen in approximately one quarter of patients with a proven diagnosis of TB [135]. It is often the only manifestation of TB and is usually unilateral. Ultrasound scanning often reveals a complex septated effusion

1.12.2 Post primary TB

As already discussed, significant radiological overlap occurs between the features of primary and secondary TB. Post primary disease can manifest as parenchymal disease, airways involvement and pleural extension.

Parenchymal disease

Typically, parenchymal disease occurs as patchy, poorly defined consolidation, particularly in the apical and posterior segments of the upper lobes. Additionally, cavitation, the hallmark of postprimary TB is seen in approximately 50% of patients [136]. Cavities are usually multiple and occur within areas of consolidation.

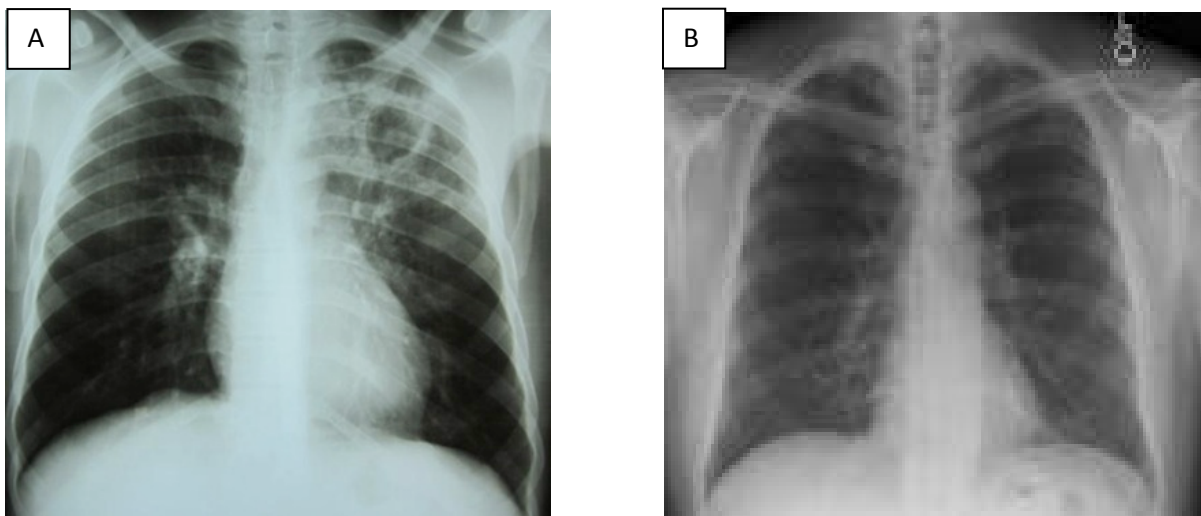


Figure7: A.) Plain chest x-ray demonstrating left upper lobe cavity B.) Normal chest x-ray.
Adapted from http://www.sharinginhealth.ca/pathogens/bacteria/mycobacterium_tuberculosis.html

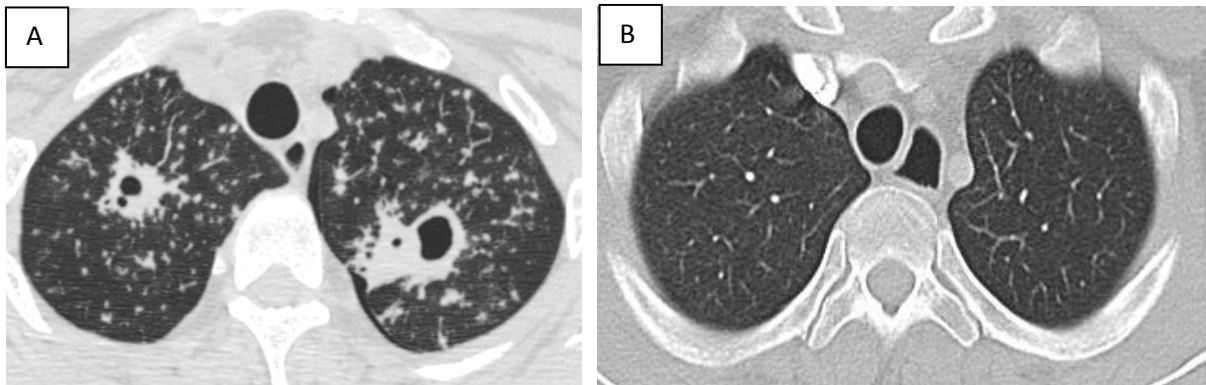


Figure 8: A. High resolution CT scan showing apical consolidation and cavitation of pulmonary tuberculosis. B. Normal CT chest for comparison.

http://main.rudn.ru/_new/russian/win/tub1/tubeng2012/02_diag/02_09_rentgen/02_09_07_caverna/02_09_08_4%20CT-scan/CT-scan.htm

Airways disease

Airways disease may be seen on CT as tree-in-bud opacification[136]. This finding represents endobronchial spread of infection and indicates active disease. Other airways changes include bronchial stenosis leading to lobar collapse and obstructive pneumonia.

Pleural extension

Pleural effusions are seen in about 18% of individuals with postprimary TB. The pleura may become thickened and may progress to a tuberculous empyema and ultimately a bronchopleural fistula. Residual pleural thickening and calcification may occur.

1.13 Nuclear medicine imaging of TB

Nuclear medicine techniques are frequently employed in the investigation of pyrexia of unknown cause, of which tuberculosis often forms part of the differential diagnosis. Several techniques are available including Gallium-67 citrate, Indium 111-labelled autologous leucocyte scintigraphy and fluorodeoxyglucose positron emission tomography (FDG-PET). Both Gallium-67 and Indium 111 have a sensitivity of approximately 76% in detecting extrapulmonary tuberculosis but neither are specific for detecting TB compared with other infective causes [137, 138]. Similarly, FDG-PET has a high sensitivity for TB lesions but its uptake is increased in malignant disease as well as other granulomatous lesions. One study indicates that it might be possible to distinguish between TB and malignant lesions by using carbon-11 choline PET. In this study, the standardised uptake value (SUV) of C¹¹-choline was found to be high in malignant lesions and low in TB lesions whereas all lesions were found to have high uptake with FDG-PET [139].

1.14 Assessment of tissue oxygenation

The measurement and assessment of tissue oxygenation has been primarily influenced by the field of tumour biology. Tumour hypoxia has long been recognised to confer poorer prognosis and poorer response to therapy[140]. Many therefore have sought to elucidate the role of oxygen in solid tumours in order to improve cancer therapy. Various methods to assess the level of oxygenation have been developed including invasive measurement using pO₂ electrodes and fibre-optic probes, immunohistochemical detection of exogenously administered drugs that label hypoxic tumour cells and of proteins overexpressed in

response to hypoxia as endogenous markers. More recently, non-invasive imaging tissue techniques have been established to allow assessment of tissue hypoxia, disease progression and treatment responsiveness.

1.14.1 pO₂-polarography

Several groups have attempted to measure tissue oxygenation using a directly inserted pO₂ probe. In particular, head and neck and cervical cancers have been studied due to their relatively accessible site. This method uses a very fine 300 µm cathode needle, which is inserted into the site of interest and a current generated by ionisation of oxygen is measured to provide an estimate of oxygen concentration at the site of insertion [141]. This method has been validated and is considered the gold standard of hypoxia measurement by the American Cancer Institute [142].

1.14.2 Exogenous hypoxia markers

Currently, pimonidazole (Pimo) is considered to be the “standard” exogenous hypoxia marker [143]. The binding of Pimo to thiol containing proteins has been shown to dramatically increase below an oxygen concentration of 10 mmHg [144]. At present, Pimo is injected intravenously prior to an invasive biopsy and immunohistochemical staining being carried out. The small patient numbers within the published data means the prognostic role of Pimo is unknown [145]. Furthermore the need for invasive biopsies has limited the clinical application of Pimo. Its utility therefore remains mainly primarily in laboratory research as a marker of severe tissue hypoxia.

1.14.3 Non-invasive imaging probes for hypoxia

Several non-invasive methods to detect tissue hypoxia have been developed including functional imaging modalities such as positron emission tomography-computed tomography (PET-CT), single-photon emission computed tomography (SPECT) and magnetic resonance imaging/magnetic resonance spectroscopy (MRI/MRS) and surrogate markers of hypoxia, for example carbonic anhydrase IX (CA-IX).

1.14.4 PET-CT and SPECT

PET-CT and SPECT permit the non-invasive *in vivo* assessment of tissue hypoxia by measuring the spatiotemporal distribution of hypoxia specific tracers.

1.14.5 [¹⁸F] fluoromisonidazole FMISO

Nitroimidazoles have long represented an attractive therapeutic agent in tumour biology because of their unique behaviour in hypoxic environments arising from their high electron affinity. The past 40 years has seen the development and therapeutic trials of this group as chemical modifiers that increase the sensitivity of hypoxic tissues to radiation or chemotherapeutic agents, and as bioreductive prodrugs, or hypoxia selective cytokines. These agents have since been adapted for the labelling procedure of hypoxic cells *in vivo*.

[¹⁸F]fluoromisonidazole (FMISO) is a lipophilic compound that enters the cell by diffusion. Once inside the cell, the nitro group is reduced by a series of nitroreductase enzymes which include cytochrome p450 reductase, xanthine oxidase and oxdioreductase. In the presence of high intracellular oxygen, the resulting radical anion is converted back to the intact nitro group and the compound will leave the cell via diffusion [146] and return into the circulation. Under hypoxic conditions a sequence of single electron reductions takes place,

leading to irreversible covalent binding to intracellular macromolecules[147]. FMISO is then irreversibly trapped within the hypoxic tissue. Whilst several radionucleotide hypoxic tracers exist, [^{18}F]FMISO represents the most widely investigated and validated of this group. *In vivo* investigation of rat rhabdomyosarcoma found significant correlation between the hypoxic volumes identified by [^{18}F]FMISO PET and the volumes detected by PimO.^{[148].} In studies of the correlation between concentrations of titrated misonidazole ([^3H]MISO) and oxygen pressures using EMT6/Ro multicell spheroids, accumulation of MISO near the central necrosis was observed where oxygen tensions lower than 10mmHg were measured[149]. Interestingly, no [^3H]MISO was detected in the area of central necrosis but since MISO requires intact nitroreductase enzyme activity, this is perhaps not a surprising finding. There have been numerous human studies conducted to evaluate the relationship between tissue pO_2 and FMISO uptake[150]. These have confirmed a strong correlation between tissue pO_2 and [^{18}F]FMISO uptake irrespective of the method used to assess tissue pO_2 [151]. Thus far no study has been able to demonstrate a direct linear relationship between pO_2 measurement and amount of FMISO taken up by tissues. It is therefore not possible at present to measure the pO_2 of tissues using this non-invasive method.

1.14.6 Other 2'-nitroimidazole based hypoxia tracers

Since the advent of [^{18}F]FMISO, several other fluorinated and iodinated agents which utilise the nitroimidazole basic structure as its base have been developed. These include ^{18}F -fluoroazomycinarabinofuranoside (^{18}F -FAZA) and its iodinated counterpart $^{123}\text{I}/^{124}\text{I}$ -IAZA. In preclinical trials, ^{18}F -FAZA is more rapidly cleared from the vascular compartment than [^{18}F]FMISO resulting in a higher tumour to blood ratio. This increased ratio enables the investigator to more clearly delineate areas of hypoxia from non-hypoxic tissue. ^{124}I -IAZA

exhibited a slightly lower tumour-to-blood ratio compared to [^{18}F]FMISO at three hours but the longer physical half-life of the tracer allowed for later time-point imaging [152]. Both agents have since been validated in human studies of lymphoma, head and neck tumours and lungs tumours but the advantage of these tracers over [^{18}F]FMISO has not been fully proven.

1.14.5 Dithiosemicarbazone compounds

Dithiosemicarbazone compounds provide investigators with immense flexibility since they can be rapidly labeled with a variety of copper isotopes of varying half-lives, including ^{60}Cu ($t_{1/2}=23.7$ min), ^{61}Cu ($t_{1/2}=9.74$ min), and ^{64}Cu ($t_{1/2}=12.7$ hrs). Unfortunately, some of these tracers have shown quite variable absolute uptakes among different cell lines at the same pO_2 [153]. These results indicate that whilst tracer uptake in tumours is high in most clinical studies, this uptake may not be solely as a consequence of tumour hypoxia.

1.14.6 Magnetic resonance (MR) techniques

Magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) are tools used in a wide variety of clinical settings. Specialised MRI techniques such as blood oxygen level dependent (BOLD)-MRI and Overhauser MRI (OMRI) have been shown to have potential to demonstrate tissue hypoxia. BOLD-MRI uses endogenous signals like deoxyhaemoglobin and OMRI uses exogenous paramagnetic contrast agents such as triphenyl-methyl radicals. The most widely used MR method to detect tissue hypoxia has been fluorine MRS/MRI to assess tumour hypoxia. It is a sensitive technique and gives a measure of interstitial oxygen tension [154]. One of the main disadvantages of this method is the false high value of oxygen tension when compared with Eppendorf electrodes [155],

something which is difficult to overcome without administering levels of tracer likely to cause hepatic toxicity. BOLD-MRI therefore represents a far more attractive option to assess tissue hypoxia as no exogenous marker is required. Since deoxyhaemoglobin is highly sensitive to blood flow, the technique has encountered several obstacles leading to both false positive and negative results. As such a new technique called FLOOD (flow and oxygen dependent contrast) has been developed and techniques are constantly being designed to overcome the issue of in-flow [156].

HYPOTHESIS AND AIMS

With the background knowledge that hypoxia is present in several animal models of TB infections and that hypoxia drives MMP-mediated tissue destruction in other inflammatory conditions, my hypothesis is that:

- Tissue hypoxia increases MMP gene expression and secretion in tuberculosis (TB) contributing to inflammation and direct tissue destruction.

The specific aims of my project are to investigate:

- The extent of hypoxia in human subjects using PET-CT
- The effect of hypoxia on MMP/TIMP gene expression and secretion
- The effect of hypoxia on the transcriptional regulation of MMPs
- The activation of transcription factors and relevant MMPs in tissue biopsies from TB cases

CHAPTER 2: METHODS

[¹⁸F]FMISO PET-CT scans

Ethical approval for the study was obtained from East London and City Research Ethics Committee and Imperial College Healthcare Trust Joint Research Office (Study no. 10/H0703/43), with further approval by the UK Administration of Radioactive Substances Advisory Committee (ARSAC). Patients were recruited by Dr M Belton from Imperial College Healthcare NHS Trust with a confirmed microbiological diagnosis of TB, either on acid-fast smear and or *M.tb* culture. All patients had abnormal plain chest radiographs and had received less than 2 weeks of anti-TB therapy.

PET-CT scans were performed on a Siemens mCT (Siemens Medical, Erlangen, Germany) at the Department of Nuclear Medicine Charing Cross Hospital, Imperial College NHS Trust, London. [¹⁸F]FMISO was synthesized in the Wolfson Brain Imaging Centre, Cambridge University. Patients received 406 ± 6 MBq [¹⁸F]FMISO intravenously followed by a 90 minute uptake period to promote contrast between normoxic and hypoxic tissue on tissue-to-background (TBR) images, and also to allow the free [¹⁸F]FMISO concentration in tissue to reach equilibrium with that in blood, a pre-requisite for Patlak plot determination of influx rate (K_i) [61]. Immediately following a low dose CT scan for attenuation correction, patients underwent a 45 minute single bed position PET scan acquired in list mode. As Patlak analysis requires knowledge of input function to the tissue, three venous blood samples (90, 135 and 145 minutes post-injection) were taken in order to scale to a population-based [¹⁸F]FMISO arterial plasma function. The latter was produced from 6 controls scanned at

The Wolfson Brain Imaging Centre, University of Cambridge as part of a study on stroke (PI: Prof J-C Baron), which included late venous sampling as part of the study protocol.

[¹⁸F]FMISO PET-CT data analysis

List mode PET data were reconstructed into 9x5min frames using OSEM [62] (8 subsets, 4 iterations, 5mm FWHM 3D Gaussian post-smoothing filter) into a 168x168x74 array with voxel dimensions 4.07 x 4.07 x 3.00mm. Corrections were applied for randoms, dead time, normalization, scatter, attenuation, sensitivity and decay. To reduce the impact of patient motion during PET acquisition, the last 8 frames of the dynamic PET image series were non-rigidly registered to the first frame using symmetric normalization (SyN) with mutual information as the similarity metric [63]. All PET frames were then registered to CT using affine transformation. The last three of the registered frames (120-135mins post injection) were converted to a TBR image through normalization with the radioactivity concentration in a bilateral lateral muscle region assumed to represent normoxic tissue. All 9 frames were used to produce a K_i map using Patlak plot analysis. Regions of interest (ROI) were defined on the TBR images and ROI values were determined by placing the ROIs on the TBR and K_i maps.

Cell culture

A549 cells

Lung adenocarcinoma A549 cells (ECACC 86012804) were cultured in RPMI 1640 (Invitrogen, UK) with 10% fetal calf serum (FCS), 2 mM glutamine, and 10 mg/ml ampicillin. Subculture

was performed by washing the cell monolayer twice with 10ml of warmed HBSS, adding 5ml of trypsin for 2 mins and then neutralising with 5ml of RPMI 1640. Cells were then seeded at 2-4,000cells/cm² for further passaging or 35-50,000 cells/cm² depending on the type of experiment being undertaken. Experiments were performed on cells between passage 8-16 and all experiments were performed in RPMI 1640 with 2 mM glutamine, and 10 mg/ml ampicillin (no FCS added).

Normal Human Bronchial Epithelial cells (NHBE)

NHBE cells were purchased from Lonza as cryopreserved cells and were regenerated and maintained according to supplier's instructions. In brief, NHBEs were cultured in T75 tissue culture flasks in 15ml basal cell epithelial growth medium (BEBM) supplemented with 52 µg/ml bovine pituitary extract, 0.5µg/ml hydrocortisone, 0.5ng/ml human recombinant epidermal growth factor, 0.5µg/ml epinephrine, 10µg/ml transferrin, 5µg/ml insulin, 0.1ng/ml retinoic acid, 6.5 ng/ml triiodothyronine and 10µg/ml ampicillin. Medium was changed on day 1 and day 3. Once the cells had reached 80-100% confluence (generally day 6), to subculture, the cell monolayer was washed twice with 10ml warmed HBSS, trypsin was added for 2 minutes and then neutralized with 10ml of trypsin neutralising solution. Cells were seeded at a density of 60,000cells/cm² for experiments.

Monocyte purification and maturation into macrophages

Monocytes were isolated from healthy blood donors (UK Blood Transfusion Service) using Ficoll-Paque (GE Healthcare, Little Chalfont, UK) density centrifugation and a standard 1 hour adherence protocol. In brief, the buffy coat from a single blood donor was diluted 50:50 with HBSS, 35ml was layered onto 15ml of Ficoll-Paque and centrifuged at 1500 rpm

for 30 mins. The resulting monolayer was then washed with HBSS and spun for 5 mins a total of 5 times. Total monocytes were calculated by counting the number of adherent cells in a Neubauer counting chamber after incubation for 5 minutes at 37°C. Cells were subsequently plated on to tissue culture plates or 60mm dishes at a density of 250,000 cells/cm² resuspended in RPMI 1640 (Invitrogen, UK) containing 2mM glutamine and 10µg/ml ampicillin. After 1 hour at 37°C in a humidified, 5% CO₂ incubator, cells were washed a total of 3 times; once with the existing RPMI supernatant and then twice with 1 ml of warmed HBSS. Monocytes were then ready for infection with *M.Tb*.

Differentiation into macrophages was carried out over a period of 4 days in medium containing 10% FCS and 100ng/ml M-CSF (R & D, Abingdon, UK). After 4 days in the growth factor containing media, the media was changed to RPMI with 10% FCS but no growth factors, and 24 hours later the experiment was commenced

TB culture

The virulent *M.tb* strain H37Rv Pasteur was cultured in Middlebrook 7H9 broth (BD Biosciences) with 10% ADC enrichment medium (BD Biosciences), 0.2% glycerol, 0.02% tween 80 and 2.5µg/ml amphotericin for infection of cells. An optical densitometer (Biowave Cell Density Meter: WPA) was used to assess the growth of *M.Tb*. On a weekly basis, *M.tb* was subcultured by transferring 100-400µl of ultra-sonicated broth into 2ml of fresh 7H9, giving a variety of optical densities (OD) for experiments of the next week. Experiments were performed with cultures having an OD of 0.5-0.7, corresponding to 10⁸ colony forming units/ml (CFU/ml) and the mid-log growth phase. Optical density was

correlated with CFU by performing colony counts in triplicate on Middlebrook 7H11 agar supplemented with OADC enrichment medium and 0.5% glycerol.

GFP-Tb

To construct the GFP-expressing *M.tb* strain, *M.tb* was transformed with the dual reporter plasmid pSMT3LxEGFP (Fig. 9). This plasmid is based on the pSMT3 *Escherichia coli*/mycobacteria shuttle vector and contains the human codon-optimised and fluorescence-enhanced EGFP gene from pEGFP (Clontech, Nottingham, UK) cloned under the control of mycobacterial 19 kDa promoter, and the *luxAB* genes from *Vibrio harveyi* under the control of *hsp60* promoter. Positive clones were selected by growing on plates and media supplemented with 50 μ g/ml hygromycin (Roche Diagnostics, Mannheim, Germany).



Figure 9: Diagram of GFP-expressing *M.tb* strain

Conditioned media preparation

Conditioned media from *M.tb* infected monocytes (CoMTb) was produced in batches by infecting monocytes seeded in 3ml RPMI on 60mm dishes (see 4.1.3 above) at a multiplicity of infection (MOI) of 1 (which equates to ~70µl of TB broth with an OD of 0.6). A control conditioned media from uninfected cells (CoMCont) was also produced in the same way but without the infection step. After 24 hours, the cell medium culture was harvested, centrifuged at 13,000rpm for 5 minutes and filtered to remove infectious material using a 0.2µm anapore filter (Whatman, UK). All CoMTb batches were tested by MMP-1 ELISA on either A549 or NHBE cells and only those producing a good MMP-1 response were subsequently used in experiments. CoMTb batches were aliquoted and stored at -20°C.

Direct infection protocol

After 5 days maturation, the supernatant from 24 well plates of MDMs was removed and replaced with 400µl of macrophage serum free medium (MSFM, Invitrogen, UK) in each well prior to infection at a MOI of 1 (~5ml *M.tb* broth). Cells were incubated at 37°C for 2 hours after which non-phagocytosed *M.tb* was removed by suctioning the supernatant and replacing with 400µl per well of fresh medium. Cell supernatants for analysis were harvested at 72 hours and spun at 13,000 rpm for 5 minutes using a 0.2µm anapore filter (Whatman, UK). Samples were then stored at -20°C. Direct infection of samples for HIF-1α Western Blot analysis was performed in the same way but without replacing the supernatant at 2 hours.

CoMTb experiment protocol

Experiments involving A549 cells were performed using RPMI 1640 and those involving NHBE cells used BEBM supplemented as per the manufacturers' instructions (see above). Experiments were performed 24 hours after the seeding of tissue culture plates at the appropriate density. These were 70% for transfection experiments and 80-100% for all other experiments. Apart from dose-response experiments, all studies were performed using CoMTb at a 1:5 dilution. Previous kinetic experiments of the JSF laboratory have shown that the 72 hour time point is the appropriate time to harvest supernatants for MMP secretion quantification.

LDH cytotoxicity assay

The cytotoxic impact of hypoxia on cultured cells was assessed by measurement of lactate dehydrogenase (LDH) in cell culture supernatants. LDH levels are measured using Cytotoxicity Detection Kit (Roche Applied Sciences) according to the manufactures instructions. Briefly, cell culture supernatants were harvested at 72 hours along with supernatants from unstimulated cells (low control) and those having undergone complete chemical lysis (high control) with 5µl 1% triton X per well. A reaction mixture of 250µl of reconstituted bottle 1 (catlaysis) and 11.25ml of bottle B (dye solution) was prepared and 100µl added per well to a 96 well tissue culture plate. 100µl each of samples and controls were then added to each well followed by 50µl of Stop solution. The plate was then shaken at a speed of 500rpm for 10 seconds and the samples analysed immediately on Bio-Orbit

1253 luminometer (Labtech International) at 490nm, with a reference wavelength over 600nm.

LDH cytotoxicity of experimental samples were calculated using the following equation

$$\text{Cytotoxicity} = \frac{\text{exp. Value} - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

Colony counting

Following 72 hours of infection with *M.Tb*, MDMs were lysed with 100µl of sterile 0.1% Triton X and 2µl of the lysate diluted with 198µ of 7H9 Middlebrook 7H9 broth (BD Biosciences) with 10% ADC enrichment medium (BD Biosciences), 0.2% glycerol and 0.02% tween 80. 5µl of serial 1 in 10 dilutions were then plated (in triplicate) onto 7H11 agar plates Middlebrook 7H11 agar (BD Biosciences) with 10% OADC enrichment medium (BD Biosciences) and 0.005% glycerol and colony counting performed between 2 and 3 weeks after initial plating based on the assumption that one colony represented a single Tb bacilli.

Generation of hypoxia

All cells were incubated at 37° in a humidified atmosphere containing either 5% CO₂ (normoxia) and the required oxygen level. Initial experiments were either performed using a dedicated hypoxic chamber (kindly loaned by Patrick Maxwell, UCL) adapted from an anaerobic jar (Oxoid) or a hypoxic incubator (Galaxy CO14S, New Brunswick).



Figure 10: Oxoid hypoxic chamber. Prior to each incubation, the chamber was secured in a horizontal position. Plates were loaded along with a T-25 flask containing sterile water to ensure sufficient humidification. After sealing, the chamber was evacuated (-0.6 Bar) and filled with a hypoxic gas mixture (1% O₂, 5% CO₂) (repeated 3x as per manufacturer's instructions) and placed in a standard incubator set to 37°C.

Subsequently, a custom designed hypoxia workstation was built and approved for use with Containment Level 3 pathogens (Coy Laboratories). Prior to commissioning the workstation, a mock up to the exact proposed dimensions was made out of cardboard. This was then placed within the Class I cabinet and airflow testing performed to ensure that the workstation did not interfere with airflow. The cabinet was made from acrylic which could withstand formaldehyde fumigation in the event of a large spill of *M.tb* however small spills were managed as others in the CL3 with alcohol decontamination. The design of the workstation incorporated an airlock facility as well as a temperature controlled humidified incubator allowing the handling and manipulation of cells without any exposure to ambient air. Stable O₂ and CO₂ levels were maintained through sensors which continuously monitored and adjusted gas levels. The workstation was commissioned by a Coy approved engineer who ran temperature and humidity testing and compared these to a control CO₂ incubator in the CL3 to ensure that comparable environments were present in both. In

addition, CO₂ and O₂ levels were measured using gas sampling meters. Sensors were calibrated once per month according to the manufacturers' instructions.

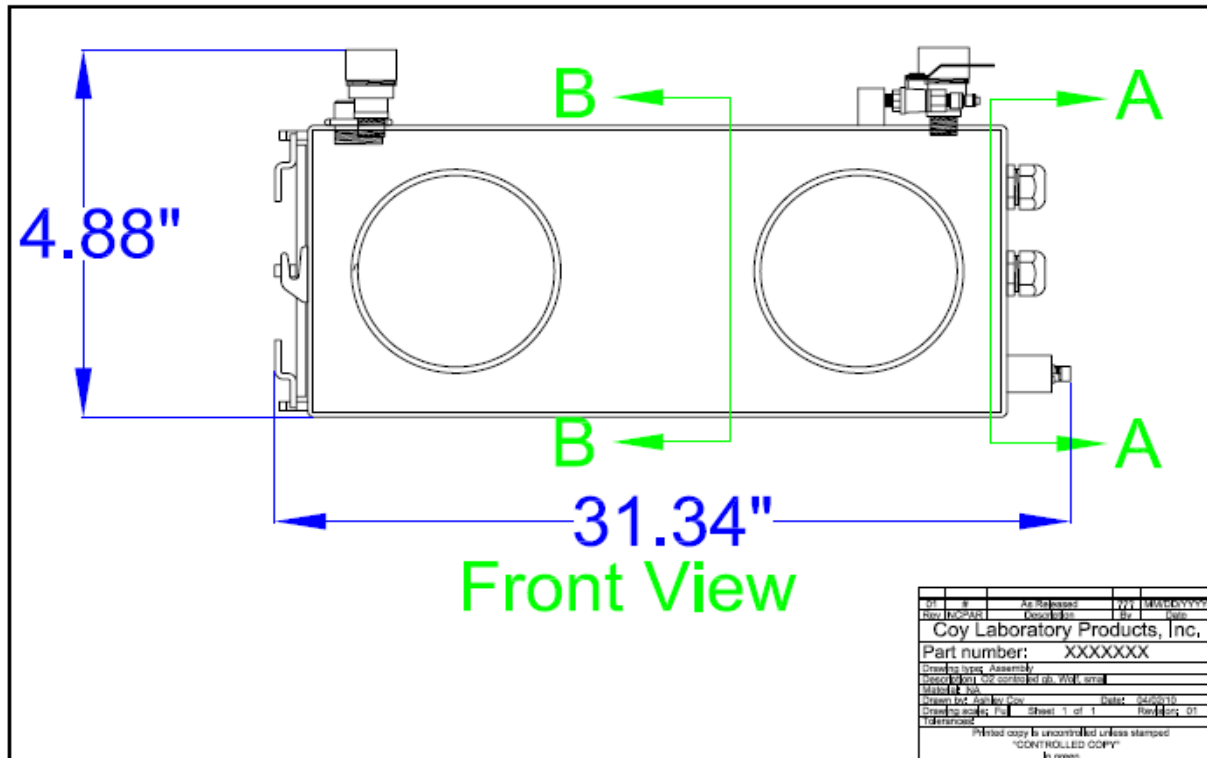


Figure 11: Technical drawings of specially commissioned hypoxia workstation designed to fit within Class I cabinet in the CL3 facility of the JSF laboratory

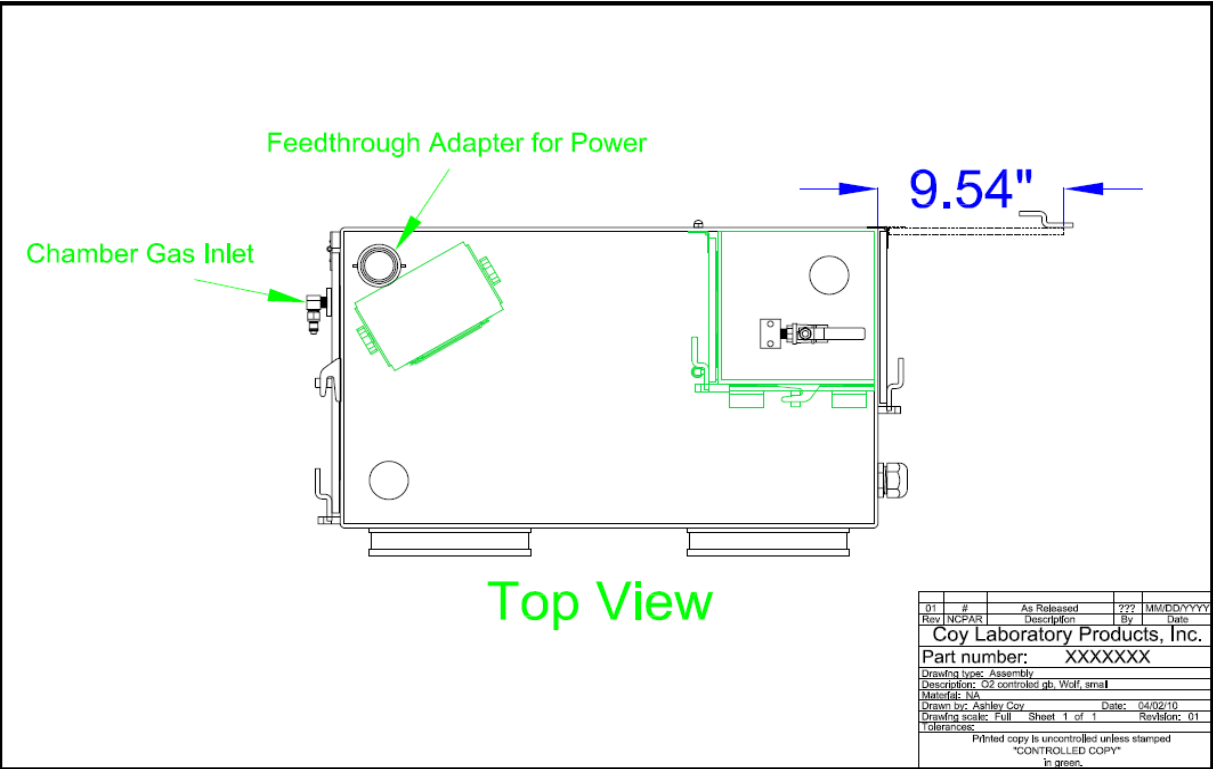
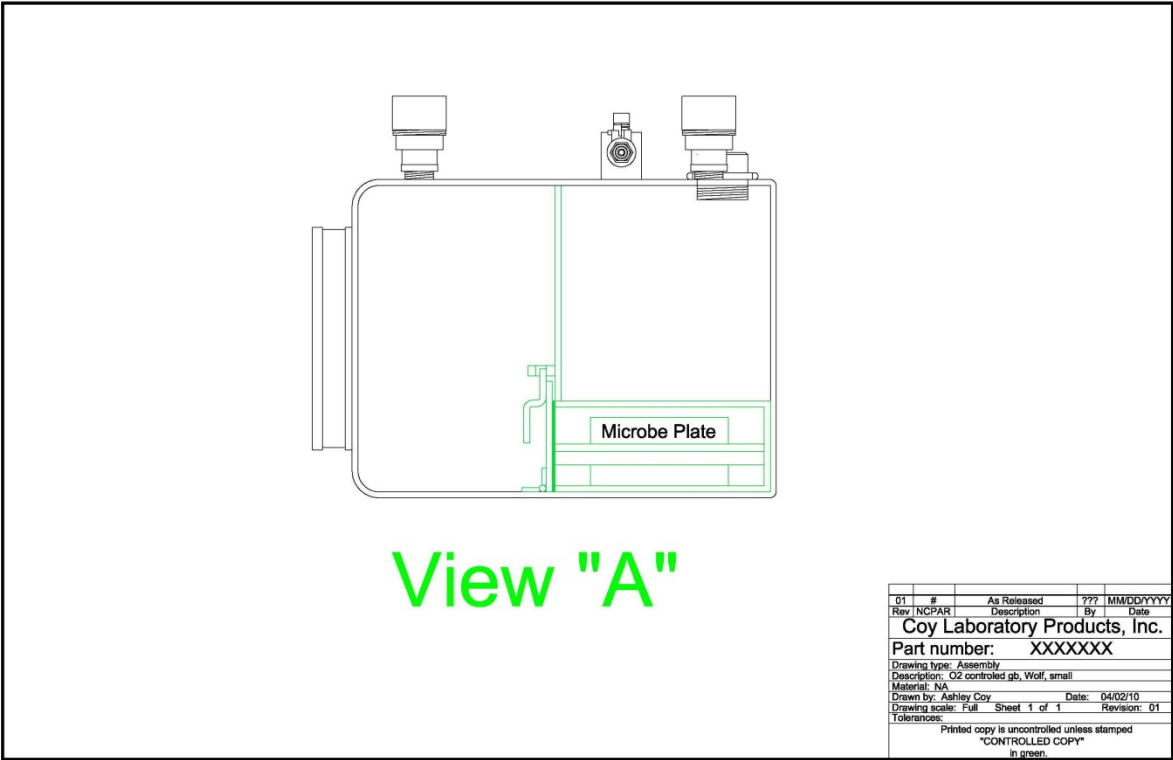


Figure 11 continued

HIF-1 α stabilisation

The pan hydroxylase inhibitor 2-oxoglutarate dimethyloxallylglycine (DMOG) was used to inhibit HIF prolyl and asparaginyl hydroxylases (PHD1/2/3 and FIH respectively). By blocking the activity of these hydroxylases, DMOG stabilises HIF-1 α and activates the HIF system. Cell viability at the completion of experiments (72 hrs) was assessed by trypan blue exclusion assay

Enzyme Linked Immunosorbent Assay (ELISA)

MMP and TIMP levels in cell culture medium were measured by ELISA (R&D Systems, UK) according to the manufacturer's instructions. Briefly, a 96-well plate was coated with the appropriate capture antibody and incubated at room temperature (RT) overnight. Wells were then blocked for 1h using 0.5% BSA in PBS. Standard curve (loaded in duplicate) and supernatants from 72h-conditioned cell culture were added at the appropriate dilution to each well and incubated for 2h hours at RT. Biotinylated detection antibody was then added and incubated for 1 hour at RT followed by HRP-conjugated streptavidin, incubated for 20min. The substrate tetramethylbenzidine (TMB) was added to the wells, and the reaction terminated using 1M H₂SO₄. MMP-1, MMP-3, MMP-9 and TIMP-1 levels were quantified by measuring optical density at 450nm, with a 540nm reference wavelength.

Fluorokine[®] MAP Assay (Luminex xMAP[®] analysis)

Luminex analysis (R&D systems, UK) is a microparticle based multiplex immunoassay for the accurate quantification of human MMPs 1, 2, 3, 8, 9, 12 and 13 in various sample types (serum, plasma and cell culture supernatants). This assay provides simultaneous quantification of these MMPs in a single small volume. MMPs 1, 3 and 7 were measured according to the manufacturers' instructions. In brief, MMP specific antibodies are purchased pre-coated onto colour-coded microparticles or beads. Microparticles, standards and samples are pipette into 96 well luminex plates and shaken at a speed of 500rpm for 2 hours to allow the immobilised antibodies (on the suspended beads) to capture the MMP of interest. After washing away any unbound substances, biotinylated antibodies specific to the MMPs of interest are added and shaken for a further 2 hour at 500rpm. Finally, streptavidin PE (phycoerythrin) were added to generate a signal and the beads resuspended and read using a Luminex[®] dual laser analyser. The first laser classifies the bead and determines which MMP is being detected whilst the second determines the magnitude of the PE derived signal, which is directly proportionate to the amount of MMP bound. In addition to MMP analysis, 30-plex analysis of a broad spectrum of cytokine, chemokines and growth factors was performed using the Luminex platform in collaboration with other members of the JSF group.

Zymography

Zymography is a substrate-based SDS-PAGE electrophoresis, using 11% polyacrylamide gels and is used to detect MMPs by the degradation of their preferential substrate as well as by

their molecular weight. Proteins are separated in a denaturing in an SDS-PAGE containing a specific substrate that is co-polymerised with the acrylamide. Following electrophoresis, the gel is washed with Triton X-100 which restores enzyme structure and proteolytic activity. The renatured MMPs in the gel digest the substrate when incubated in appropriate activation buffer i.e. collagenase buffer. This enzyme activity is then seen as white bands on Comassie stained gels. Densitometric image analysis is performed by digital image acquisition (UVP) followed by band quantification with NIH Image version 1.61 (Scion Image).

Casein zymography

Casein zymography to analyse MMP-1 concentrations was performed as previously described (Leber *et al*, 1997). Briefly, 20µl aliquots of cell culture supernatant with 5x loading buffer (0.25 M Tris pH 6.8, 50% glycerol, 5% SDS, bromophenol blue) were run on 11% acrylamide gels impregnated with casein (Invitrogen) at 125V for 2 hours (buffer 25 mM Tris, 190 mM glycine, 0.1% SDS). After incubation in 2.5% Triton X-100 (Amresco 0694-1L) for 1 hour with agitation and two brief washes in collagenase buffer (55 mM Tris base, 200 mM sodium chloride, 5 mM calcium chloride, 0.02% Brij, pH 7.6), gels were incubated for 40 hours in fresh collagenase buffer at 37°C. Caseinolytic activity was detected by staining the gels with 0.1% Coomassie blue (Pharmacia Biotech, Sweden) in 1:4 acetic acid: water for 1 hour with agitation and then destaining for 15 min with 1:3:6 acetic acid: methanol: water. All experimental samples were run in parallel with 10 ng recombinant MMP-1 (Merck) to standardize between gels.

Gelatin zymography

Gelatin zymography was performed for the detection of MMP-9. 20µl aliquots of cell culture supernatant mixed with 5x loading buffer (0.25M Tris pH 6.8, 50% glycerol, 5% SDS, bromophenol blue) were run on 11% acrylamide gel impregnated with 0.1% gelatine (SIGMA G2500) at 180V for 3-4 hours (buffer 25mM Tris, 190 mM glycine, 0.1% SDS). After incubation in 2.5% Triton X-100 (Amresco 0694-1L) for 1 hour with agitation (to remove the SDS), the gel was rinsed twice in 1x low salt collagenase buffer (55mM Tris base, 200mM NaCl, 5mM CaCl₂, 0.02% Brij-35, pH 7.6) prior to incubation at 37⁰C for 16 hours in fresh collagenase buffer. The following day, gelatolytic activity was detected by a single step stain/de-stain method using 0.02% Comassie blue (Pharmacia Biotech, Sweden) in 1:3:6 acetic acid: methanol: water.

Anti-HIF-1α Western blotting

Cells were initially washed with 1ml sterile PBS and then lysed in SDS sample buffer (62.5mM Tris, 2% SDS, 10% glycerol, 50mM DDT, 0.01% bromophenol blue). For MDMs directly infected with M.Tb, samples were spun at 13,000 rpm for 5 mins then filtered through a 0.2µm anapore filter (Whatman) to ensure the removal of any infectious material. Sample could then be safely removed from the CL3 facility. 20µl samples were heat-denatured at 70°C for 10min and separated on a NuPAGE Bis-Tris 4-12% gel (Invitrogen) at 200V (Running buffer: 50mM MOPS, 50mM Tris, 0.1% SDS, 1mM EDTA) for 1h. Gels were electro-transferred to a nitrocellulose membrane (Amersham, UK) and blocked for 1h with 5% milk protein, 0.1% Tween-20. The membrane was probed with anti-HIF1α Ab (1:1,000

dilution) (BD Biosciences, UK) overnight, washed, and incubated with goat anti-mouse IgG horseradish peroxidase (HRP)-conjugate secondary Ab (1:1,000 dilution) (Jackson ImmunoResearch) for 1h. Loading control was performed using the rabbit-anti p38 Ab (1:1,000 dilution) (Cell Signalling Technology) and goat anti-rabbit IgG HRP conjugate (1:1,000 dilution) (Cell Signalling Technology) Luminescence was detected with the ECL system (Amersham) according to manufacturer's protocol.

Transformation of competent *E.Coli* with MMP-1 promoter constructs

10 µl of plasmid DNA was added to chilled sterile polypropylene tubes containing 100 µl of competent *E.coli* cells (Invitrogen). Cells were gently mixed using a pipette tip and placed on ice for 30 mins. In order to assess transformation efficiency, 5 µl of control DNA was added to an additional chilled polypropylene tube containing competent cells. Cells were then heat shocked in a 42°C water bath for 45 secs and immediately placed on ice for 2 mins. 0.9ml of SOC medium (Invitrogen) was added to each tube and cells were then incubated in a shaking incubator (at 225rpm) at 37 °C to allow expression of antibiotic resistance. After 1 h the contents of each tube was diluted 1:10 and 1:100 with SOC medium and plated on 15% LB agar plates containing 0.1mg/ml ampicillin. Plates were incubated overnight at 37°C until colonies developed. A single colony from each plate was selected and used to inoculate a starter culture containing 5ml of sterile LB media (Sigma) and 0.1 mg/ml ampicillin. The starter culture was placed on a shaking incubator 150rpm at 37°C for 8 hours to allow the *E.coli* cells to reach late logarithmic phase. 400µl of each started culture was then added to 200ml LB media and placed in a shaking incubator for 16

h at 37°C. *E.coli* cells were harvested by centrifugation in a 50ml falcon at 6000g for 15 mins at 4°C following which supernatants were drained and discarded. Cell pellets were frozen and stored at -20 °C if not proceeding immediately to DNA plasmid purification.

Alkaline Lysis and plasmid DNA purification

Alkaline lysis and plasmid DNA purification was performed using the Qiagen Maxi Kit® according to the manufacturers' protocol. In short, bacterial pellets were resuspended in 8ml buffer P1 containing 100 µg/ml RNase A solution and mixed by pipetting until no clumps remained. 8ml of buffer P2 was then added and the cells suspension gently mixed by inversion of the falcon 4-6 times. The suspension was then allowed to incubate at room temperature for 5 mins after which 8ml of chilled buffer P3 was added. Following gentle mixing, the lysate was placed on ice for 20 mins. The lysate was then centrifuged at 13000rpm for 30mins at 4°C after which the supernatant containing the plasmid DNA was promptly removed and placed in a 50ml falcon and centrifuged at 13,000rpm for a further 15 mins at 4°C. Supernatants were immediately placed in the Qiagen tips-500 equilibrated with 8ml buffer QBT and the column allowed to empty by gravity. The tip was washed x2 with 25ml buffer QC and the plasmid DNA then eluted with 12ml buffer QF into a clean 50ml centrifuge tube. 8.5ml of isopropanol was then added to the eluted DNA in order to precipitate the DNA. The DNA was then centrifuged immediately at 12,000rpm for 30 mins at 4°C and the supernatant carefully decanted. The DNA remaining pellet was washed with 4ml of 70% ethanol and centrifuged at 12,000rpm for 10 mins after which the supernatant was removed and the pellet allowed to air dry. The dried pellet was resuspended in 500 µl TE buffer (pH 8.0) and DNA OD 260/280 measured for quantitation. 50 µl aliquots were then stored at -80°C for subsequent use.

Promoter-reporter assay

A549 cells were seeded in 12-well plates at 160,000 cells/well in RPMI 1640 containing 2 mM glutamine, 10% FCS, and 10 µg/ml ampicillin. Cells were transfected 24 h later in serum-free medium with 0.8 µg of MMP-1 promoter DNA inserted into the firefly luciferase expression plasmid pGL2-basic (gift from Dr. I. Clarke, University of East Anglia, East Anglia, U.K.) and 0.08 µg of control reporter plasmid, pRL-TK, constitutively expressing low level *Renilla* luciferase activity, suspended in 2.4 µl of FuGene 6 (Roche). After 12h, cells were stimulated with CoMTb at a 1:5 dilution in the presence or absence of 0.25mM DMOG (4h pre-incubation period). After another 24h, cells were washed with 100µl of sterile PBS, lysed in 100µl of Passive Lysis Buffer (Promega) and frozen at -80°C. Luciferase activity of extracts was measured using the Dual-Luciferase Reporter Assay System (Promega) analyzed with the Bio-Orbit 1253 luminometer (Labtech International). *Renilla* luciferase activity was used to normalize firefly activity to control for transfection efficiency. The following construct were used: -4372 (WT), -3830, -2942, -2001, -1551, -1194, -517 (Figure 12).

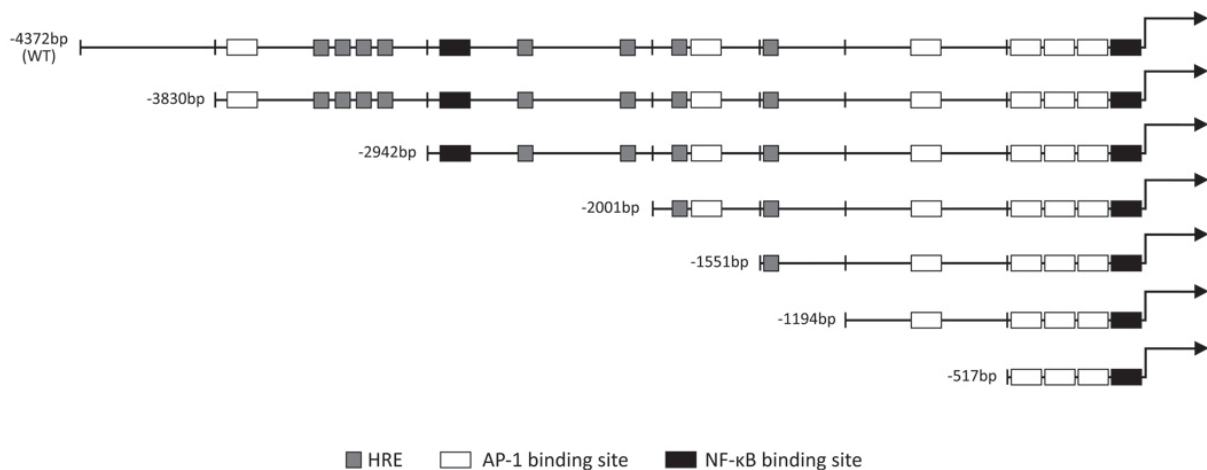


Figure 12: MMP-1 promoter truncation constructs. HIF-1, AP-1 and NF-κB binding sites are indicated

RNA extraction (early experiments)

RNA was extracted from cultured cells using Qiagen RNeasy® Mini Kit and according to the manufacturers' protocol. Cell-culture medium was completely aspirated and discarded after which 175µl of precooled (4⁰ C) Buffer RLN was added. Cells were gently scrapped, transferred to a microcentrifuge tube then incubated on ice for 5 mins. The lysate was then centrifuged at 4⁰ C for 2 mins at 300 x g. The supernatant from this tube was then transferred to a new centrifuge tube and the centrifuge heated to 20-25⁰ C. 600µl of Buffer RTL was added to the supernatant and then briefly vortex to ensure complete mixing. 430µl of ethanol (96-100%) was added to the homogenised lysate and mixed by pipetting. Up to 700µl of the sample was transferred to an RNeasy spin column placed in a 2ml collection tube and centrifuged for 15 s at ≥10,000rpm. The flow-through was then discarded and this step repeated with the remaining sample. 700µl Buffer RW1 was then added to the RNeasy spin column and centrifuged 15 s at ≥10,000rpm to wash the spin column and the flow-through discarded. Next, 500µl Buffer RPE was added to the RNeasy spin column and centrifuged 15 s ≥10,000rpm and the flow-through discarded. This step was then repeated a second time. Finally, the RNeasy spin column was placed in a 1.5ml collection tube and 30-50µl RNase free water was added directly to the spin column membrane. The sample was centrifuged for 1 min at ≥10,000rpm to elute the RNA. Samples were stored at -80⁰ C if not proceeding immediately to cDNA synthesis.

cDNA synthesis (early experiments)

cDNA synthesis by reverse transcription, using the extracted RNA as a template, was performed as a prerequisite for Quantitative Real Time PCR. For each sample a volume of

RNA equivalent to 250ng, 500ng, 1µg was diluted up to 11µl with RNase-free water. 1 µl of random primers (Invitrogen) was added to each sample. The samples were heated at 65°C for 10 minutes. A mastermix containing 5x 1st strand buffer, 10mM dNTPS, DTT, RNase OUT and Superscript III (Invitrogen) in the correct proportions was made. The samples were placed on ice for 1 minute. 8µl of the mastermix was added to each sample. And they were incubated at 50°C for 1 hr, then 75°C for 15 mins. If not proceeding to RT-PCR straight away the cDNA samples were stored at -20°C.

RNA extraction (2011 onwards)

RNA was extracted from cultured cells using PureLink RNA[®] Mini Kit (Invitrogen) and according to the manufacturers' protocol. Cell-culture medium was completely aspirated and discarded after which cells were washed with 1ml of sterile PBS and 1ml of trizol was added. Cells were gently scraped and transferred to microcentrifuge tubes and 200µl of chloroform added per sample. Samples were shaken vigorously by hand and incubated at room temperature for 2 mins. Samples were then centrifuged at 12,000g for 15 mins at 4°C and the colourless upper phase (350µl) transferred to a sterile RNase free microcentrifuge tube. 350µl of 70% ethanol was added to the samples which were then vortexed and inverted until all visible precipitate had dispersed. Samples were then transferred to a spin cartridge placed in a 2ml collection tube and centrifuged at 12,000g for 15 secs at room temp following which the flow through was discarded. 700µl of buffer I was added to the spin cartridge and centrifuged at 12,000g for 15 secs and the flow through discarded. 500µl of buffer II was then added to the cartridge and centrifuged for 15 sec and the flow through discarded. This step was then repeated with a further 500µl of buffer II. The spin cartridge

was then centrifuged at 12,000g for 1 min to dry the membrane after which the RNA bound to the cartridge membrane was eluted with 30µl of RNase free water. The spin cartridge was centrifuged for 1 min at 12,000g at room temperature and this step was then repeated with the same 30µl of RNase free water to increase the RNA yield. RNA concentrations were then determined using the nanodrop and stored at -80°C.

cDNA synthesis (2011 onwards)

cDNA synthesis by reverse transcription, using the extracted RNA as a template, was performed as a prerequisite for Quantitative Real Time PCR. cDNA synthesis was performed using QuantiTect Reverse Transcriptase Kit (Qiagen) according to the manufacturers' protocol. RNA samples were diluted to an RNA equivalent value to the quantity required up to 12µl RNase free water and incubated on ice. 2µl of gDNA wipeout buffer was added to each sample to remove genomic DNA. Samples were heated to 42°C for 2 min and immediately placed on ice. A reverse transcriptase mastermix was made from Quantiscript Reverse Transcriptase, Quantiscript RT buffer 5x and RT primer mix in the correct proportions, mixed and incubated on ice. 6µl of reverse transcriptase mastermix was added to each sample and the tubes gently flicked and briefly spun to mix and the mastermix placed on ice. The samples were then heated to 42°C for 15 min and then immediately heated to 95°C for 3 min. If not proceeding immediately to RT-PCR, cDNA was stored at -20°C

RT-PCR

cDNA was prepared at a 1:50 dilution in RNase free water. Five standards were prepared using the sample with the highest expected level of the gene of interest and serial 1:5 dilutions. The gene of interest was normalised to 18s however since this time the JSF group have moved to β -actin as its primary reference gene. A mastermix was prepared with specific probes (0.5 μ l FAM for MMP-1 from Sigma; 0.125 μ l for 18S from Applied Biosystems) and primers (0.25 μ l for MMP-1 and 0.125 μ l for 18S) , Brilliant II 12.5 μ l and nuclease free water up to a volume of 15 μ l. 15 μ l mastermix was mixed with 10 μ l of standard or sample then 15 μ l added to the wells of Framestar 96-well plates which was then centrifuged at 300rcf for 5 mins at 4 degrees. The plate was centrifuged at 300 rcf for 5 minutes at 4°C. RT PCR was performed on a Stratagene Mx3000Pro machine with an initial 10 minute denaturation step at 95°C followed by 50 cycles at 95°C for 30 seconds then 60°C for 1 minute. MMP-1 gene expression was normalised to 18s. Data was expressed as fold change compared to control.

siRNA

Monocytes were isolated and differentiated into MDMs. MDMs were transfected using DharmaFECT 3 transfection reagent with either non-targeting control siRNA or HIF-1 α siRNA smartpool (Dharmacon, FisherScientific, Loughborough, UK), according to the supplier's instructions. Transfection medium was replaced with RPMI at 8hrs and cells allowed to rest for 16hrs, at which time the MDMs were infected with *M.tb* H37Rv (MOI= 1). Cell culture supernatant and cell lysates were harvested for HIF-1 α and MMP-1 mRNA and protein

secretion analyses. To access transfection efficiency, MDMs were also transfected with 25nM FAM conjugated non-targeting control siRNA, detached from the plates with cell dissociation buffer (Gibco, Invitrogen) and analysed by flow cytometry (FACSCalibur, BD Biosciences). Transfection efficiency was 80.4%.

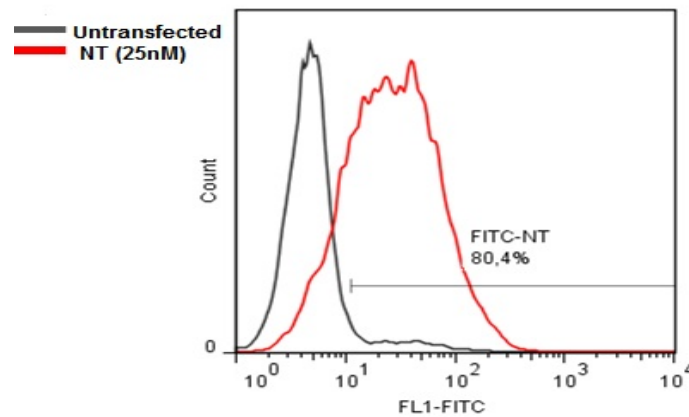


Figure 13: FACS analysis of siRNA transfection efficiency of primary human MDMs with FAM NT siRNA

Confocal microscopy

Primary human monocytes were matured to macrophages in 4-well Permanox plastic chamber slides (Thermo Fisher Scientific Inc. UK). Cells were fixed for 3 days following infection with 4% paraformaldehyde in PBS for 30 minutes. Cells were permeabilized with 0.5% saponin and blocked with 1% BSA for 1 hr. Cells were incubated with anti-MMP-1 primary Ab (1:200, Abcam, UK) overnight, washed and incubated with goat anti-mouse secondary Ab (1:100, Abcam, UK) and labelled with FITC for 2 hrs. Nuclei were visualized using DAPI. The images were scanned under an x60 oil immersion objective and to avoid bleed-through effects, each dye was scanned independently by a Leica TCS SP5 equipped with an argon-krypton mixed gas laser. Image analysis was with Leica confocal software (Leica Microsystems, Germany) and Image J v1.48a.

Immunohistochemistry

HIF-1 α immunohistochemistry was performed on formalin-fixed, paraffin-embedded lung biopsies from six patients with culture-proved *M.tb* infection and six non-infected control samples. Immunohistochemistry staining was performed using Bond III fully automated staining system with the Bond Polymer Refine Detection system and associated reagents (Leica Microsystems, Newcastle-Upon-Tyne, UK). Antigen retrieval was carried out using Epitope retrieval pH 6 (ER1) at 100°C for 30mins. The antibody HIF1- α (Abcam AB1) was applied for 30mins at a dilution of 1/500. The detection system uses DAB as chromogen and section were counterstained with haematoxylin.

Statistical analysis

Statistical analysis of the data was carried out using Prism GraphPad software. The main statistical test used was a one-way ANOVA, for comparison of multiple groups, with Tukey correction for multiple comparisons. The student t-test was used for direct comparison of two groups, where relevant. Unless stated otherwise, all experiments were performed in triplicate and performed at least twice. A p value ≤ 0.05 was considered significant.

CHAPTER 3: INVESTIGATION OF HYPOXIA IN HUMAN PULMONARY TB USING PET-CT

Introduction

Radiological imaging of pulmonary TB has been performed in several modalities including plain x-rays, CT scanning and nuclear medicine techniques such as FDG-PET. In the clinical setting, plain CXR is the most commonly used radiological technique used to monitor both disease progression and response to treatment. CT is frequently employed in the developed world to aid with diagnosis and monitor treatment, particularly in cases of drug-resistant disease. The role of FDG-PET in the diagnosis of *M.tb* infection has been limited by its inability to reliably distinguish between *M.tb* infection and lung malignancy [157, 158].

The imaging of hypoxia in patients with TB, pulmonary or extra-pulmonary, has not previously been performed. Similarly, no studies to confirm the presence of hypoxia, by any modality, in TB patients have been undertaken. Of the methods available to measure tissue oxygenation, imaging with the hypoxia tracer ^{18}F -FMISO using PET-CT was considered to be the most appropriate for my study. The use of pO₂ polarography requires an accessible site to insert the probe and pimonidazole requires an invasive excision biopsy, neither of which is appropriate in pulmonary disease. The decision to use the tracer ^{18}F -FMISO was based on two fundamental principles, 1.) ^{18}F -FMISO is the most extensively studied hypoxia tracer with no known harmful side-effects in humans, 2.) The half-life ($t_{1/2}$) of ^{18}F -FMISO is 110 mins meaning that that most of the tracers will have dispersed by the end of the study. This means that at the end of the study, an individual can continue with their daily activities with minimal disruption. It also meant the period of isolation from children and/or pregnant

women was only 24 hrs from the start of the study, which is equivalent to a standard FDG PET-CT study. The $t_{1/2}$ of ^{18}F -FMISO was also an important consideration as the tracer was produced by the Wolfson Brain Imaging Institute, University of Cambridge and then transported in a specially designed vehicle to Charing Cross Hospital. A tracer with a shorter $t_{1/2}$ would have decayed en-route and not been suitable for use.

The protocol for the study and methods of data analysis are contained within the Methods section of this thesis. In order to cause the least disruption to the patient, our protocol involved late PET-CT imaging (90 minutes after administration of ^{18}F FMISO). This allowed for patients to be scanned at the earliest time that the tracer is known to have reached equilibrium in the blood. In addition, our patients had x3 venous samples from a single cannula performed as opposed to the arterial blood samples of other ^{18}F FMISO studies, which can be significantly more uncomfortable for patients. Samples were then scaled according to a population-based ^{18}F FMISO arterial plasma input function scale produced from n=6 controls scanned at Wolfson Brain Imaging Centre, University of Cambridge as part of a study on stroke (PI: Prof J-C Baron); which included late venous sampling as part of the study protocol.

The study was approved by East London and City Research Ethics Committee and Imperial College Healthcare NHS Trust Joint Research Office. The study was also approved by UK Administration of Radioactive Substances Advisory Committee (ARSAC). Patients were recruited from Imperial College Healthcare NHS Trust during the period Jan 2011-Jan 2013. PET-CT scan were conducted within the department of Nuclear Medicine at Charing Cross Hospital. Patients were recruited according to the following inclusion and exclusion criteria and asked to sign a consent form prior to their participation in the study.

Inclusion criteria	Exclusion criteria
Confirmed diagnosis of pulmonary TB (either smear or culture)	Prisoners, patients unable to give consent, children under the age of 18 years
Evidence of one or more radiographic abnormalities associated with TB e.g. cavities, nodules, consolidation, fibrosis or calcific changes	Patients with pre-existing lung disease of any aetiology
Treatment naïve or within 4 weeks of having commenced anti-TB medication	Individuals with a history of previously treated TB
HIV negative	Individuals with a past history of claustrophobic anxiety disorder

Table 2: Patient criteria for recruitment to clinical study

Patients with HIV infection were excluded as TB infection in HIV co-infected individuals shows different clinical features to HIV negative patients. In particular, these patients do not develop cavitary lung disease. Pregnant women were excluded because of the potential teratogenic effects of radiation to a developing fetus. Finally, the initial exclusion excluded smokers from the study and this might act as a hypoxia confounder. As the recruitment process progressed it became evident that smokers formed a significant proportion of the TB population and such an exclusion was not reflective of the general TB population. Smokers and ex-smokers were therefore included in the study along with non-smokers.

Results

In total, six patients were recruited to the study however, PET-CT scans were only performed in five individuals. This was because of failure of production of [^{18}F]FMISO on one occasion meaning there was insufficient yield available to perform the scan. Despite the inclusion criteria detailing that patients should not have received four or more weeks of

anti-TB medication, all patients underwent their scan within 14 days of commencing treatment with 4 of the patients being scanned within 8 days.

Clinical and laboratory data collected at the start of the study are recorded in Table 3 and Table 4. Antibiotic sensitivity results were verified by the UK National Reference laboratory (Health Protection Agency) and were added later (usually 6-8 weeks after commencing treatment).

Data from the scans along with gamma counts of the three blood samples were transferred to the Wolfson Brain Imaging Institute, University of Cambridge where they were analysed. The amount of uptake of [^{18}F]FMISO in a specific region of interest (ROI) was measured at the end of the scan i.e. 145 minutes after injection of the tracer and recorded as the standardised uptake value (SUV). The same frames then underwent Patlak analysis to perform K_i mapping where K_i represents the rate of influx of tracer and is a more specific marker of hypoxia. It was possible to undertake Patlak analysis since the study protocol was designed in such a way so as to allow a total of nine five minute dynamic acquisition windows to be performed. During the dynamic acquisition windows, the influx and efflux of tracer were measured and the background blood levels of [^{18}F]FMISO digitally subtracted. This data was then used to produce K_i images which demonstrate the trapping of tracer within regions of severe hypoxia ($\text{pO}_2 < 10\text{mmHg}$).

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Sex (M/F)	M	F	F	F	F
Age (years)	49	22	31	37	62
Country of origin	Romania	Mongolia	Nepal	Philippines	Morocco
Height (cm)	178	160	153	157	156
Weight (kg)	64	53.2	44.9	45.3	48
Duration of symptoms	6 months	3 months	1 month	1 month	1 month
Symptoms					
Cough	+	+	+	+	+
Weight loss	+	-	+	+	-
Fever	+	+	+	+	+
Haemoptysis	+	-	-	-	-
GI symptoms	-	-	+	-	-
Lethargy	-	-	-	-	+
Smoking status	Current 30 pack years	Never	Never	Never	Never
CXR					
Consolidation	+	+	+	+	+
Cavitation	+	+	-	+	+
Fibrosis	-	-	+	-	-
Sputum smear	AFB +++	AFB +++	AFB +	AFB +	AFB +++
Days to <i>M.tb</i> culture positivity	3 days	7 days	26 days	7 days	5 days
Antibiotic sensitivity	Fully sensitive	Resistant isoniazid + streptomycin	Fully sensitive	Resistant rifampicin, isoniazid + prothionomide	Fully sensitive

Table 3: Patient demographics, chest radiograph and microbiology results

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Haematology					
Hb (g/dl)					
WCC ($\times 10^9$ /l)	10.8	10.2	10.1	12.7	10.4
Neutrophil ($\times 10^9$ /l)	7.5	7.7	10.4	16.4	10.7
	6.0	4.4	7.3	12.8	8.7
Biochemistry					
Na (mmol/l)					
K ⁺ (mmol/l)	129	141	134	132	132
Urea (mmol/l)	4.0	4.1	4.8	4.0	4.0
Cr (μ mol/l)	3.5	3.5	2.4	3.1	8.1
	52	53	55	65	97
CRP (mg/l)	189	10.6	150	69	312
Liver function					
Bilirubin (μ mol/l)					
ALT (IU/l)	10	n/a	6	11	12
ALP (IU/l)	39	n/a	13	29	<6
Total protein (g/l)	135	n/a	95	114	113
Albumin (g/l)	62	n/a	70	72	75
	39	n/a	27	24	49
HIV status					
	Negative	Negative	Negative	Negative	Negative
Vitamin D (nmol/l)					
	26.0	28.6	24.3	25.9	14.9

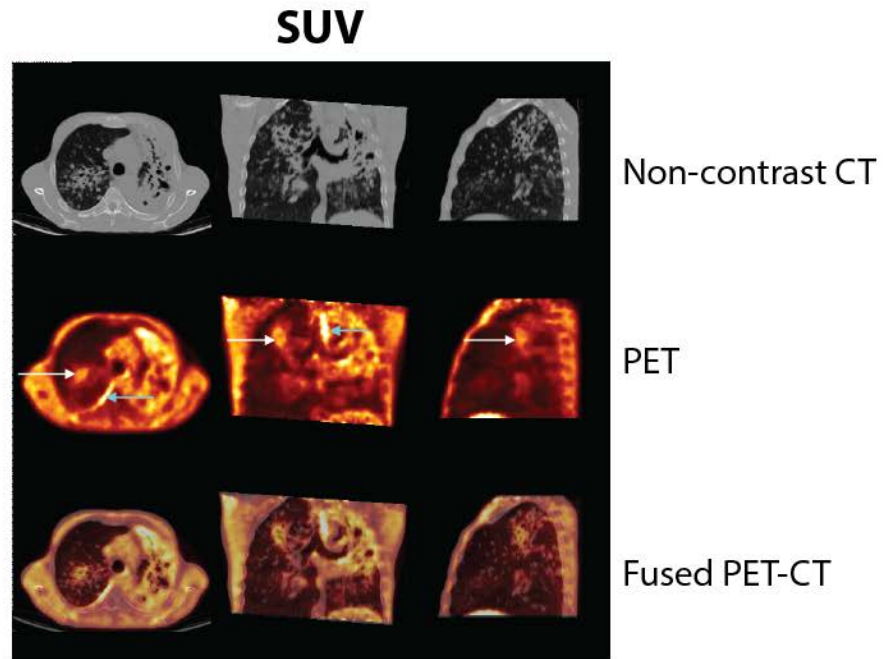
Table 4: Blood analysis at time of presentation

n/a, not available

Patient 1

Results from Patient 1 revealed evidence of high uptake of [^{18}F]FMISO throughout both lung fields at 145 minutes (SUV). In particular, uptake in the left upper lobe and right upper lobe were extremely high (Figures 14 and 15). Once Patlak analysis was performed, high K_i areas were seen in the right upper lobe and sporadically within the left upper lobe. Interestingly, when the changes present in the left lung were analysed most of the regions of high SUV did not correlate with high K_i mapping (Figure 15). This discordance can be explained by analysing the Time Activity Curves (TAC) along with SUV and K_i mapping (Figure 16). It is expected that blood [^{18}F]FMISO levels should decrease over time as the tracer decays. As such, the TAC in a region of interest will have the same shape of curve if the tracer is decaying and not accumulating in that area. The TACs for Patient 1 show that the curves for the left upper and left lower lobes follow the same curve for blood [^{18}F]FMISO levels. It is likely therefore that the changes seen in most of the left lung are due to hyperperfused, normoxic lung tissue. In direct contrast, the changes in the right upper lobe demonstrate high SUV, high K_i and increasing TAC of [^{18}F]FMISO indicating accumulation of [^{18}F]FMISO and hence severe hypoxia. The co-registered PET-CT images did not show any significant emphysema in this patient therefore the smoking status of this patient was not a confounder.

A.



B.

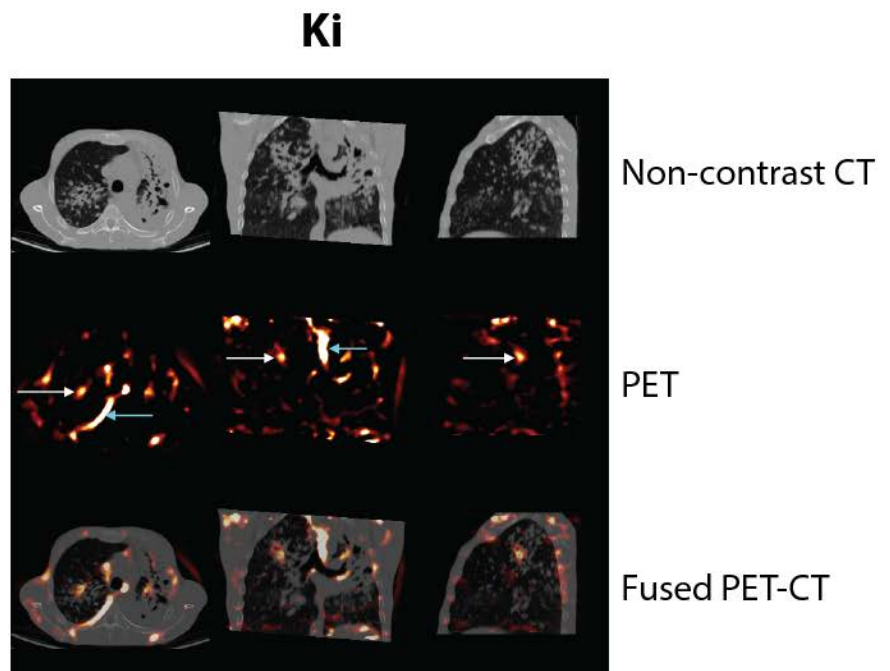
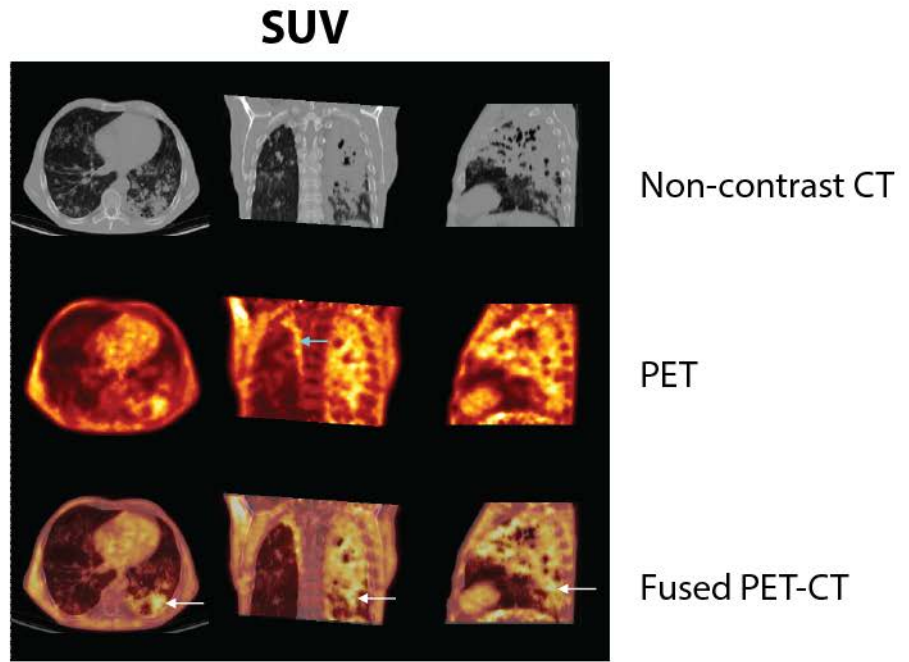


Figure 14: ^{18}F -FMISO PET-CT images of right upper lobe lesion in Patient 1. Standardised uptake values (SUV) of ^{18}F -FMISO were assessed for the region of interested (ROI) 145 mins post-injection of tracer **(A)**. CT and PET images were fused to allow spatiotemporal assessment of tracer uptake. The same ROI and frame was analysed using Ki mapping **(B)** to identify evidence of trapping of ^{18}F -FMISO and thus severe hypoxia. (White arrows=regions ^{18}F -FMISO trapping; blue arrows=movement artefact)

A.



B.

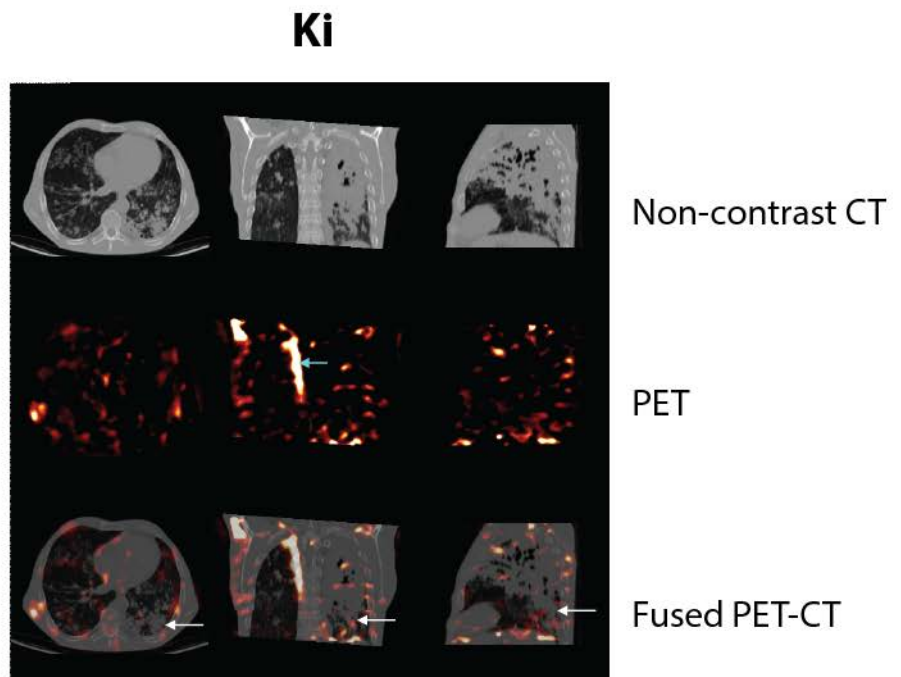
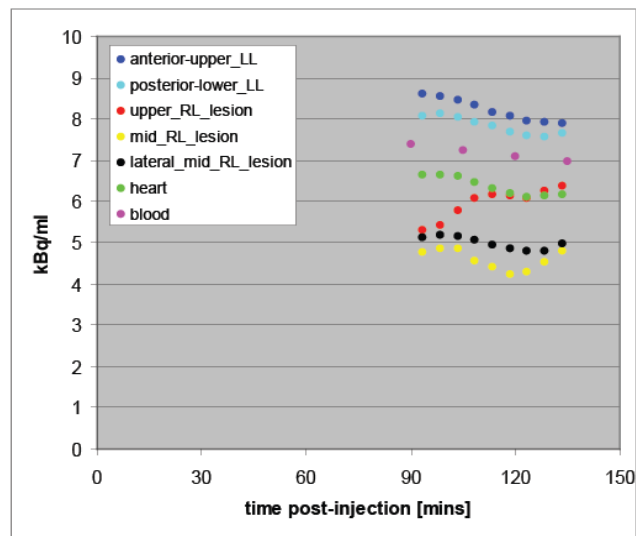


Figure 15: ^{18}F -FMISO PET-CT images of posterior aspect of left lower lobe lesion in Patient 1. (A) Standardised uptake value of the left lower lobe lesion demonstrated high uptake of ^{18}F -FMISO at 145 mins post injection. **(B)** Ki modelling demonstrates no significant trapping of tracer, indicating pO_2 levels of $>10\text{mmHg}$.

White arrows=regions of ^{18}F -FMISO uptake; blue arrows=movement artefact

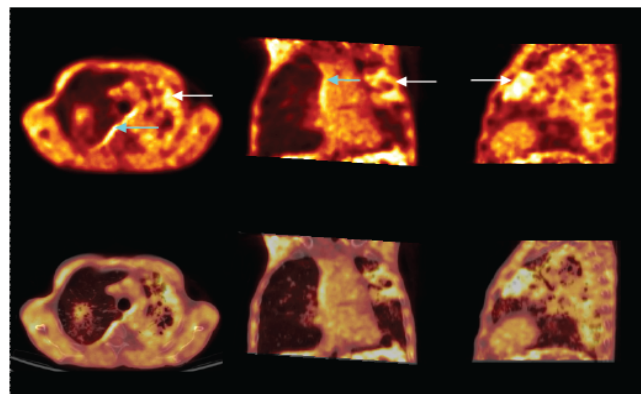
A.

Time Activity Curve (TAC)



B.

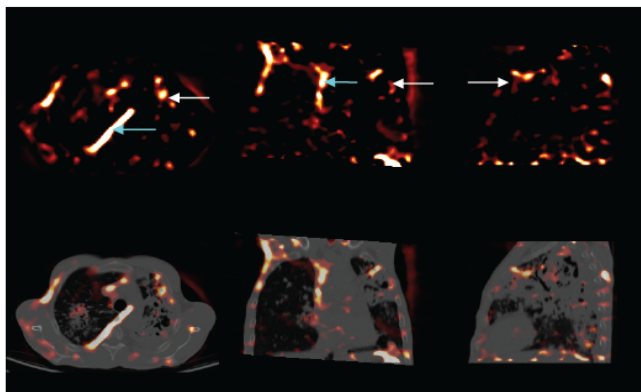
SUV



PET

Fused PET-CT

Ki



PET

Fused PET-CT

Figure 16: Time Activity Curves and PET-CT images for Patient 1. (A) TACs for the left upper lobe, left lower lobe and heart have the same shape as blood levels of FMISO indicating equilibrium has been reached. In direct contrast, the right upper lobe region shows TAC increasing with time demonstrating accumulation of trapped tracer over time. (B) The high SUV in the anterior left upper lobe is not matched by Ki except for 3 small areas indicating most of tracer is not being trapped.

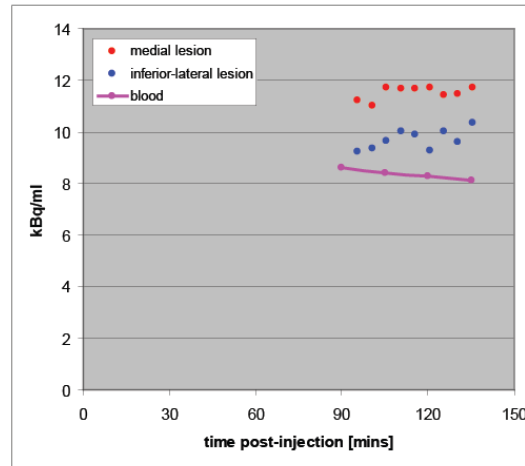
White arrows=tracer uptake; blue arrow=movement artefact

Patient 2

The results from Patient 2 demonstrated regions of severe hypoxia in the area surrounding the right upper lobe cavity (Figure 17B). This lesion demonstrated variable uptake within the surrounding area and hence the ROIs were divided into medial and inferolateral (Figure 17A). Both ROI demonstrated increasing TACs whereas the TAC for blood, as would be expected, declined. There was a marked uptake of [^{18}F]FMISO as indicated by SUV at 145 mins post-injection of tracer. K_i modelling demonstrated evidence of severe hypoxia in the two regions of interest. Despite a lower concentration of [^{18}F]FMISO, the TAC produced a steeper slope in the inferolateral region meaning the K_i was in fact higher. It is not currently possible to correlate K_i with pO_2 however it is possible to extrapolate that the degree of hypoxia in this region is greater than the medial region. There was no uptake of tracer within the cavity itself and this is an expected finding since [^{18}F]FMISO is trapped through a series of reductive steps which only occur in living (non-necrotic) cells. On this occasion, data was analysed on a “non-rigid registration” protocol and this enabled almost complete elimination of the movement artefact seen with patient 1.

A.

Time Activity Curve



B.

SUV

Ki

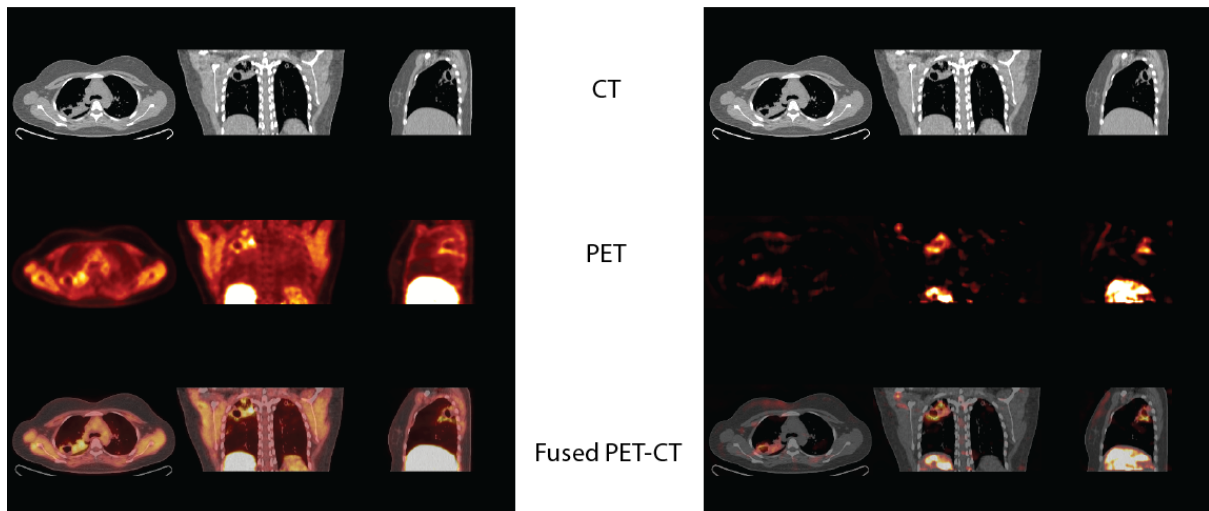
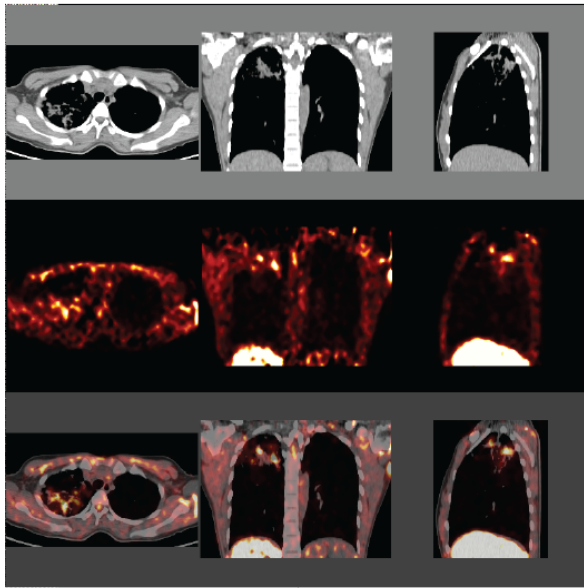


Figure 17: Time Activity curves and ^{18}F -FMISO PET-CT imaging in Patient 2. Data from Patient 2 demonstrate the presence of severe hypoxia. **(A)** TAC values of ^{18}F -FMISO in the right upper lobe lesion increase over time indicating accumulation and trapping of tracer. This is in direct contrast to blood levels of ^{18}F -FMISO which, as expected decline over time. **(B)** SUV values at 145 mins and Ki mapping demonstrate high uptake of tracer and trapping of tracer within the region of consolidation surrounding the right upper lobe cavity (high uptake is also seen in the liver, the site of metabolism of ^{18}F -FMISO).

Patient 3

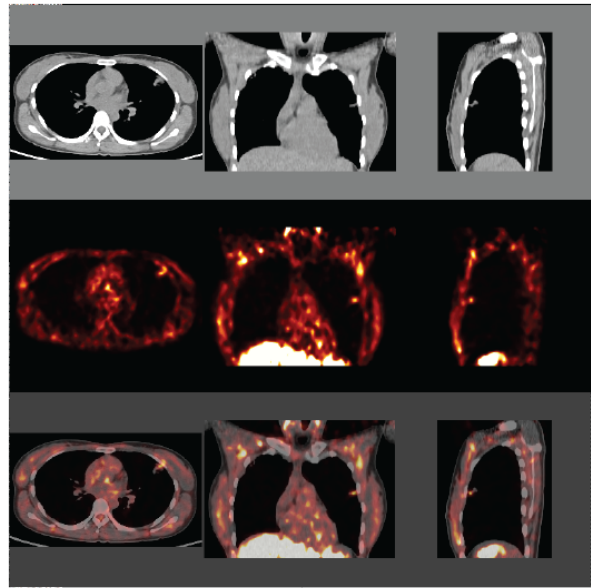
Analysis of the data from Patient 3 demonstrated evidence of severe hypoxia within the right upper lobe and left lower lobe (Figure 18A and B). The chest x-ray of this patient demonstrated heterogeneous changes which were confirmed on CT scan with evidence of consolidation, cavities and fibrosis. Several different ROIs were analysed and concordance between SUV and K_i was seen throughout all lesions (Figure 18C). On this occasion high K_i values were seen in small isolated areas of consolidation (Figure 18B) as well as around areas of destructive pathology (Figure 18A). The TAC showed a steady state or increase in all ROIs demonstrating accumulation of tracer in direct contrast to the blood values which decreased over time (Figure 18C).

A.



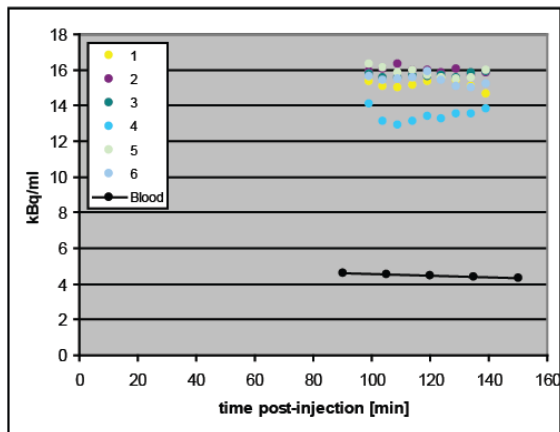
Ki Right upper lobe

B.

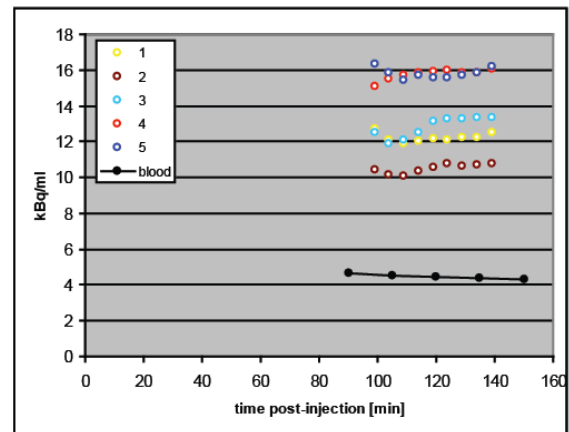


Ki Left lower lobe

C.



high SUV lesions



high Ki lesions

Figure 18: Ki mapping and TACs for Patient 3. Results from Patient 3 demonstrate regions of severe hypoxia in TB lesions distributed throughout both lungs. **(A)** Ki mapping of lesions within the right upper lobe and left lower lobe **(B)** demonstrate trapping of tracer within regions of consolidation and in the area surrounding a pulmonary cavity (upper panel-non-contrasted CT; middle panel-PET; lower panel-fused PET-CT image). **(C)** TAC for patient 3 show static or increasing levels of [18F]FMISO in regions of interest. All regions with high Ki values corresponded to high SUV values

Standardisation of results

Following the scans of Patients 4 and 5, attention focussed on how to analyse the data from all 5 patients collectively and how to best normalise the findings across the patient group. In particular, it was necessary to determine how best to normalise the data to remove the high background seen in the tissues in some of the scans. In order to achieve this, the last three of the registered frames (120-135mins post injection) were converted to tissue-to background (TBR) images through normalisation with the radioactivity in a bilateral lateral muscle region (assumed to represent normoxic tissue). Initially, SUV values were compared to TBR values to evaluate if the two measurements were concordant. Mean TBR values were demonstrated to correlate with mean SUV across all regions of interest (ROI) within individual lesions across all 5 patients (Figure 19) with TBR values overall measuring slightly lower than SUV.

Average TBR levels ($\pm$ SD) in the lateral muscle regions were then used to calculate background normoxic K_i values for the group. The upper K_i limit for normoxic tissue was calculated as 0.0005 min^{-1} and subsequent K_i values were considered positive i.e. hypoxic if they were greater than this threshold. K_i mapping of the same ROIs evaluated revealed all patients had K_i values greater than background in at least one region of interest except for patient 4 whose values were on the limit (Figure 20). Heterogeneity was seen both within individual lesions and across the patient group with the exception of patient 2 who demonstrated evidence of hypoxia in all ROIs. In total, 5 individual lesions (x1 per patient) with 21 ROIs were evaluated. Of these, 11 ROIs had K_i values greater than background and were therefore considered hypoxic.

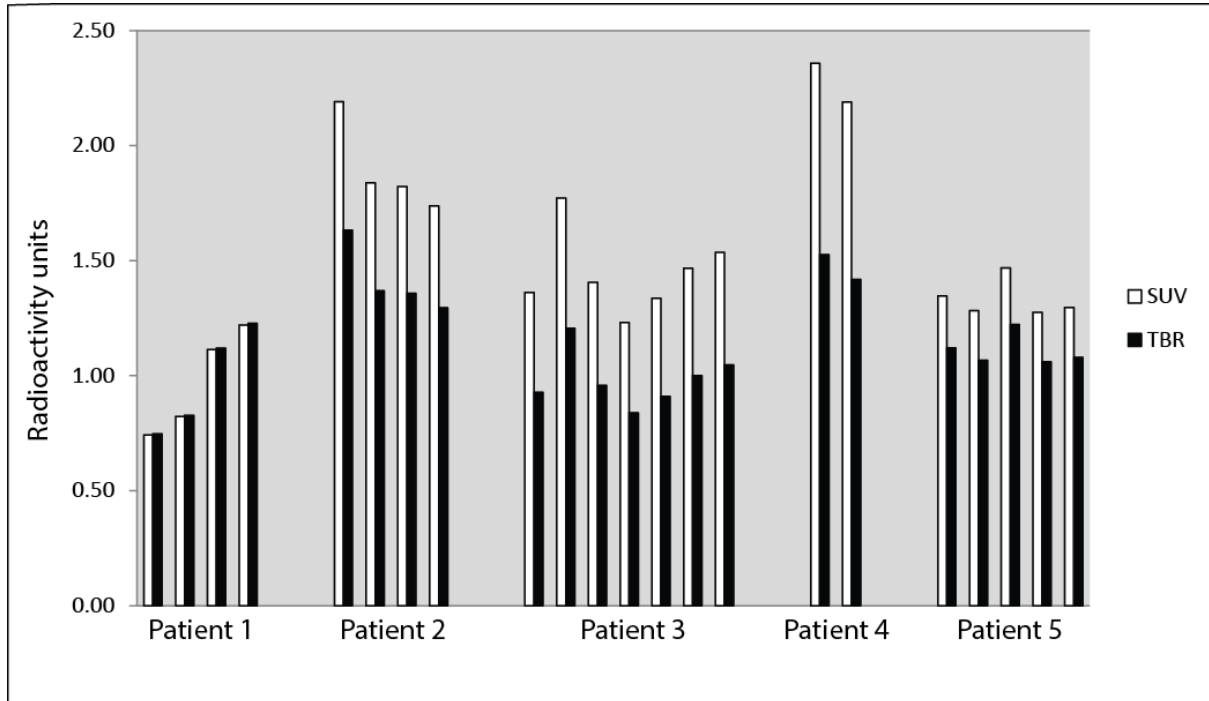
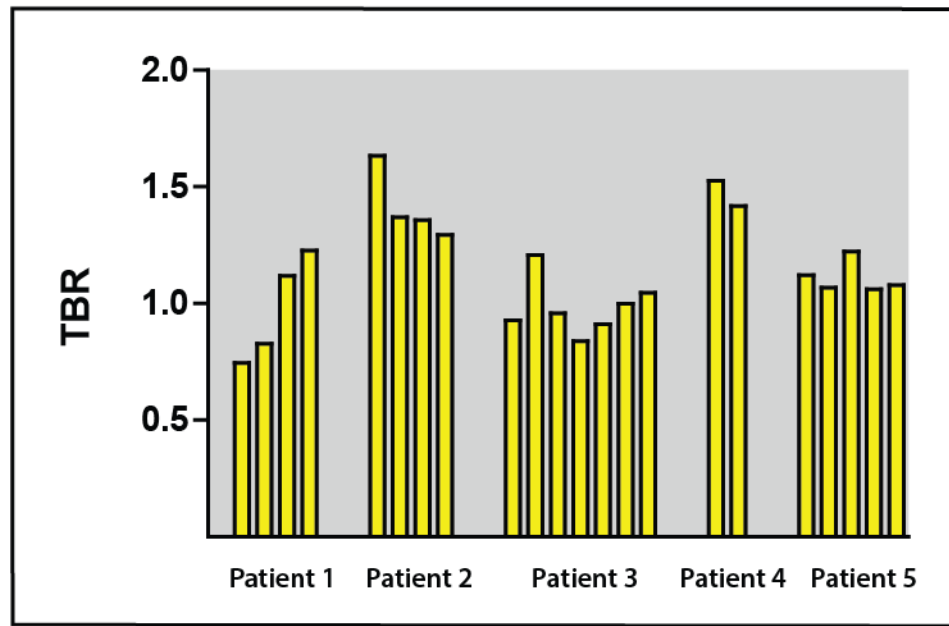


Figure 19: Standardised uptake value (SUV) compared to tissue-to background ratios (TBR) across 21 regions of interest. TBR values correlated to SUV across all ROIs in all 5 patients. TBR values were mainly lower than SUV.

A.



B.

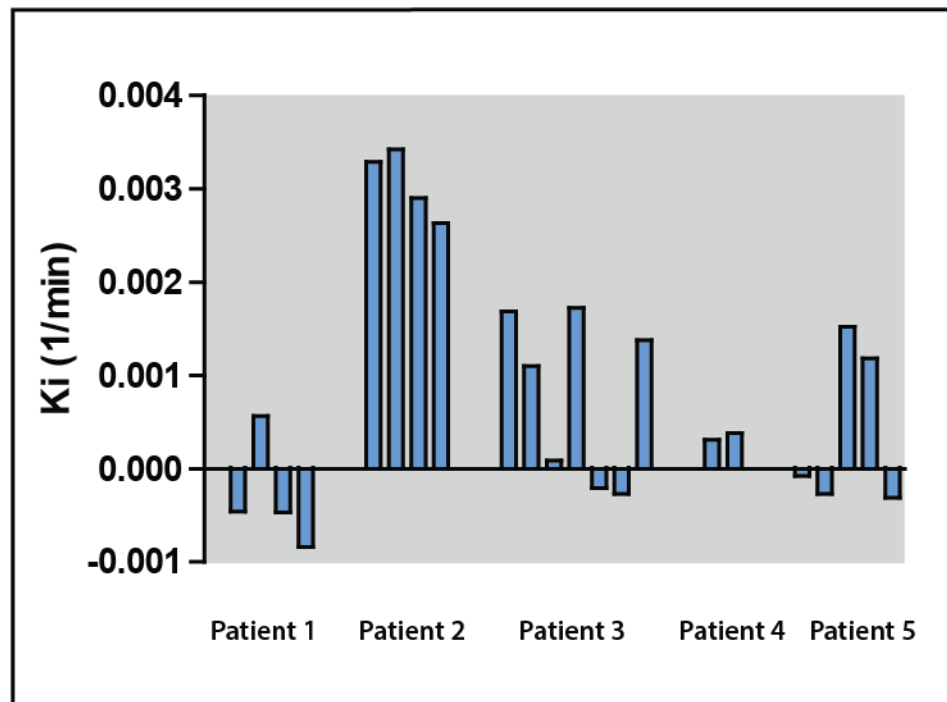


Figure 20: Standardisation of K_i mapping across all 5 patients (A) TBR values greater than unity are found for at least one lesion in all patients (each individual bar represents one region of interest, ROI). (B) Regional K_i values demonstrate heterogenous trapping of $[^{18}\text{F}]\text{FMISO}$ within individual lesions and between patients (upper K_i limit for normoxic tissue = 0.0005 min^{-1}).

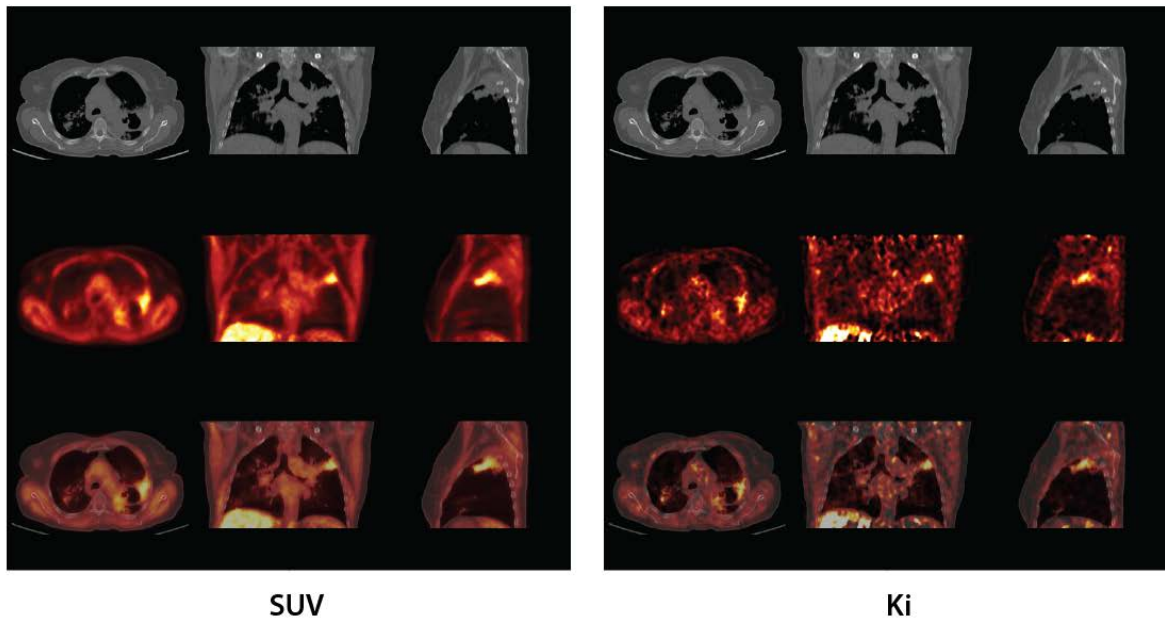
Patient 4

Analysis of data from patient 4 demonstrated the importance of accurate documentation of the steps involved in our specific protocol. Initial analysis demonstrated that the radiologically abnormal regions on the CT scan contained regions of severe hypoxia (Figure 21A). Subsequent review of the time of administration of the tracer and time of blood samples revealed that a discrepancy had occurred. The outcome of this discrepancy was that the peripheral blood values were calculated incorrectly. Final analysis demonstrated high tracer uptake but low K_i values (Figure 21B).

Patient 5

Following the standardisation of results across the five patients, the results from Patient 5 were evaluated using TBR and subsequently using TBR to calculate K_i . TBR demonstrated evidence of high tracer uptake in radiologically abnormal areas of lung as well as within background tissue. K_i mapping revealed heterogenous changes with hypoxia present in the right lower lobe lesion and not in the left sided lesion (Figure 22). The results from patient 5 therefore demonstrated the presence of severe hypoxia with heterogenous changes seen.

A.



B.

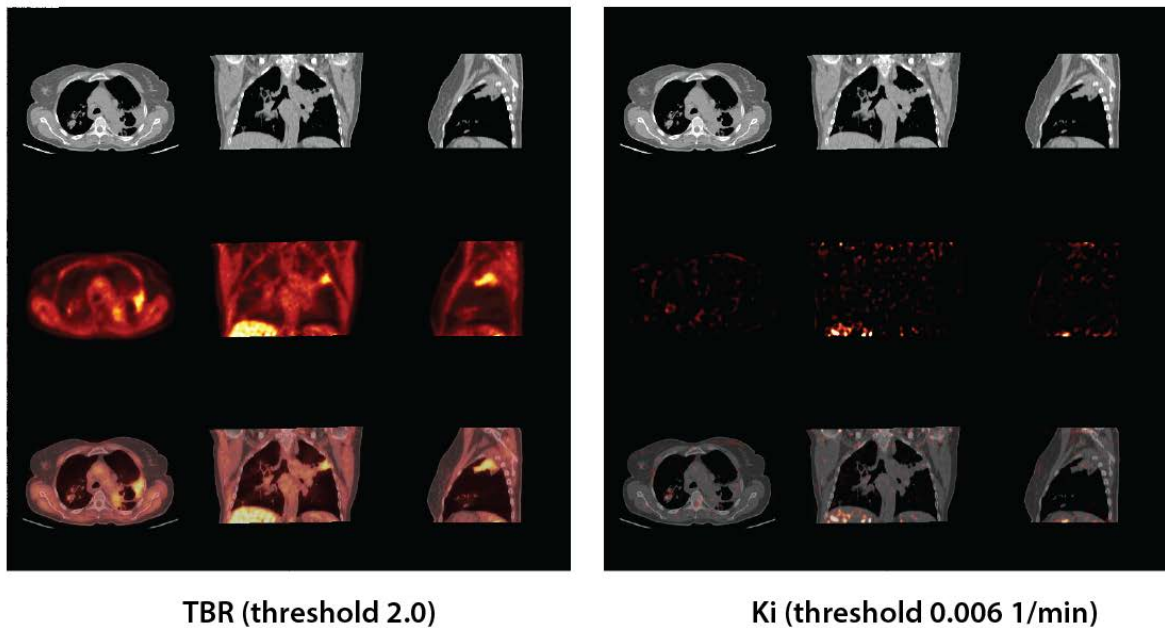


Figure 21: PET-CT Results from Patient 4. (A) Initial analysis of data from patient 4 demonstrates significant tracer trapping and hypoxia (Ki) in the left upper lobe. (B) Repeat analysis using adjusted blood gamma counts demonstrates high TBR and tracer uptake in this lesion. Standardisation across the 5 patients with a threshold of 0.006 l/min demonstrates no evidence of severe hypoxia on PET-CT imaging.

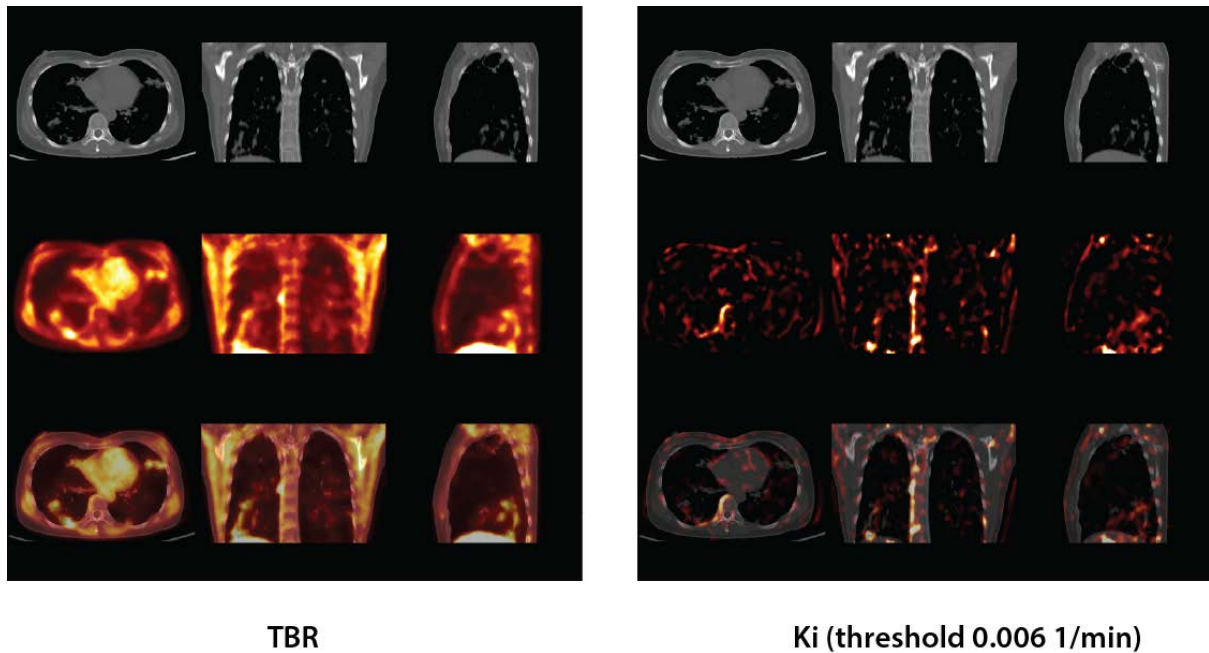
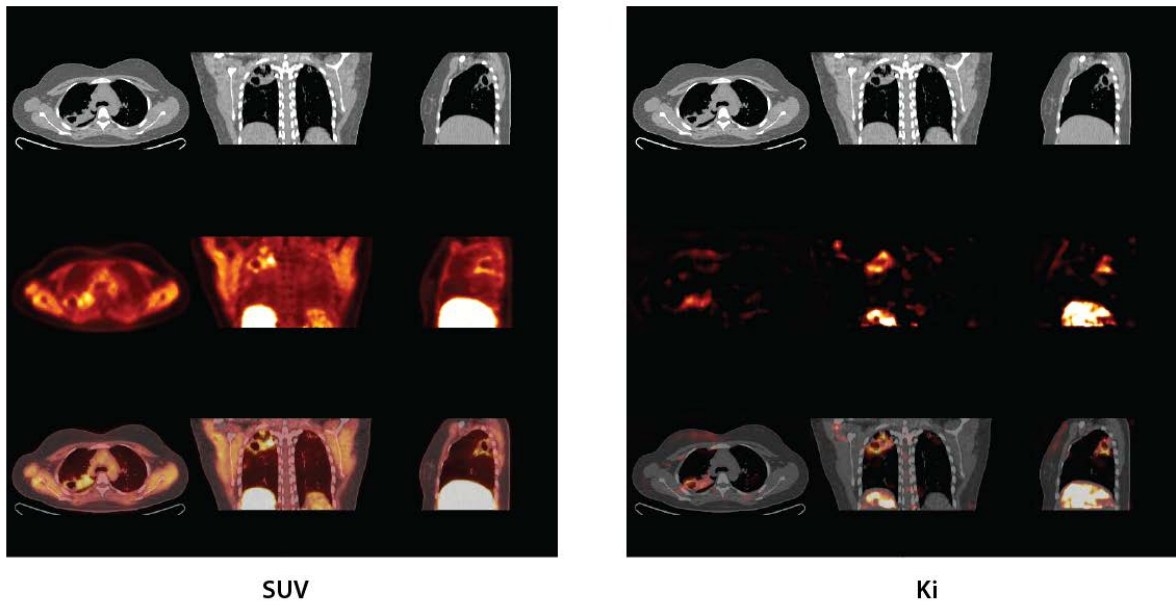


Figure 22: PET-CT images from Patient 5. TBR images show high uptake of tracer in right lower lobe and left lower lobe lesion. Ki mapping demonstrates that severe hypoxia is only present in the right sided lesion.

A.



B.

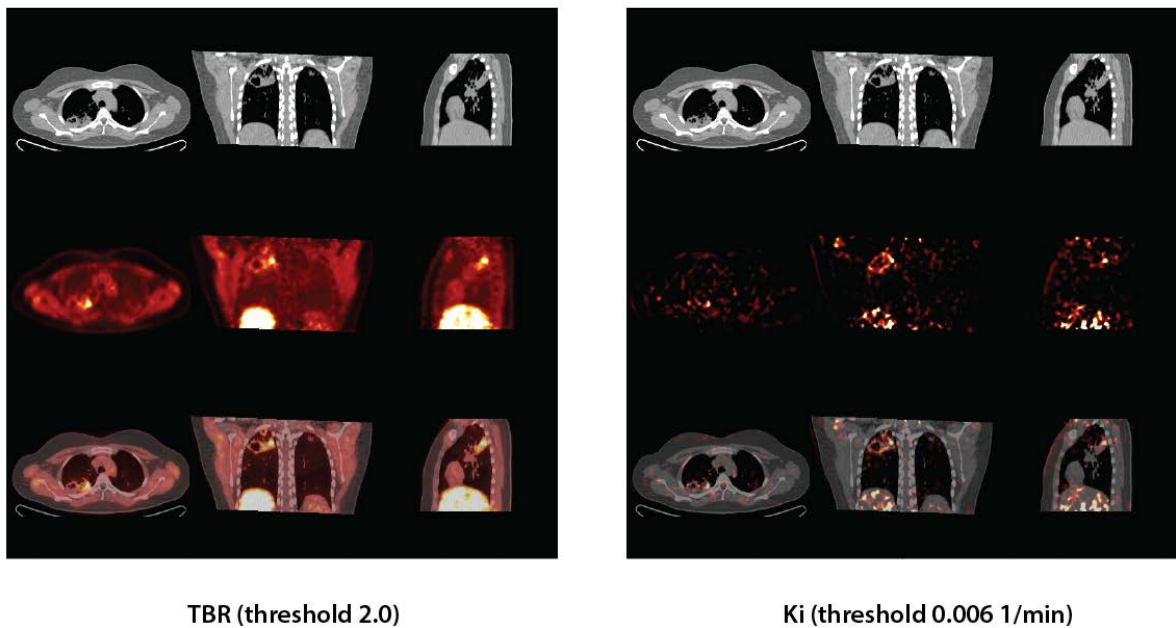


Figure 23: Comparison Ki images from Patient 2. Both SUV and TBR images demonstrate significant uptake and trapping of tracer. (A) Ki calculated using SUV shows high tracer trapping. (B) When analysis is performed using TBR to calculate Ki and background threshold of 0.006 l/min is used, severe hypoxia is still present in the right upper lobe lesion.

Discussion

My clinical study is the first to demonstrate the presence of severe hypoxia within pulmonary lesions during active tuberculosis infection. Hypoxia has been demonstrated in certain animal models of *M.tb* infection but the methods used are not suitable for human subjects, most notably invasive pO_2 probes, excision biopsy or necropsy.

The results of this clinical study were performed in collaboration with the Department of Nuclear Medicine Imperial College and the Wolfson Brain Imaging Centre (WBIC), University of Cambridge. Although studies have been previously performed at WBIC using ^{18}F -FMISO to image hypoxia in both animal and human studies, no similar studies have been performed in the context of human infection. Before commencing with the study, a protocol was designed taking in to account patient factors, transportation issues and data collection and analysis requirements.

Patient factors included the need to transport the patient in a timely fashion to Charing Cross Hospital, minimising the duration of the scan and performing venous blood sampling compared the arterial blood sampling that was being conducted in studies at WBIC. ^{18}F -FMISO kinetics is such that the earliest point that the tracer reaches equilibrium in the blood compared to tissues is 45 minutes. This time-point was therefore selected for my study. By closely examining ^{18}F -FMISO kinetics we were able to limit the tracer uptake duration to 90 minutes and scan time to 45 minutes (compared to 120 mins and 90 mins respectively in a current research study of unconscious stroke patients). It is possible that an increased uptake time might have allowed greater uptake of in hypoxic tissues. For example the time-activity curves for patients 2 and 3 suggest that tracer uptake in some lesions were increasing with time. Additionally, arterial blood sampling might have improved peripheral

^{18}F -FMISO measurements since a greater number of historical controls are available but these factors needed to be balanced with the comfort of the patient.

Transportation issues were overcome by producing sufficiently large batches of ^{18}F -FMISO which allowed for decay of tracer during the 2.5 hour transfer between Cambridge and London. Unfortunately on one occasion the yield of ^{18}F -FMISO was not sufficient to take account of this limitation. Were the scan being performed closer to the site of production, it would have been possible to produce a further batch in time. On this occasion, the scan was cancelled and could not be rescheduled.

Finally, data collection issues were addressed by collecting data in List Mode (a numerical format which can be reconstructed into images) which allowed the transfer of large volumes of anonymised data. Venous blood samples were analysed immediately at Charing Cross Hospital using standardised gamma counting methods and data transferred to WBIC the following day. On one occasion (patient 4), the time points for collection of blood samples was not recorded properly meaning that the analysis was incorrect. This was subsequently rectified but might have been avoided if data collection and analysis were being performed on the same site.

Once a patient had been recruited to the study, the main factors which determined when the scan would be performed was the fixed day and time slot within the Department of Nuclear Medicine at Charing Cross Hospital and the notice required for production of tracer at the WBIC. This limiting factor resulted in a minimum of 6 days delay in performing the scans. My study limited the duration of treatment prior to scanning to less than 1 month however every effort was made to perform the scans as early as possible to reduce the impact of anti-TB therapy. I was able to perform scans in 4/5 patients within 8 days of

having commenced anti-tuberculosis treatment. Current national guidelines recommend a 2 week isolation period for patients starting anti-TB therapy however many studies demonstrate that patients often remain sputum smear positive for longer than this time [159, 160]. Several studies demonstrate that sputum conversion rates both on smear and culture or often longer than this period [161, 162]. Prolonged conversion times are associated with high bacillary counts at diagnosis, lung cavitation and a prolonged period of symptoms [161]. Of the 5 patients recruited to my study, 3 were strongly smear positive (3+) and median time to culture positivity was 9.6 days (range 3-27 days). This indicates a high burden of disease and likely longer sputum conversion times although it is only possible to postulate since this data was not recorded. The largest human study to examine changes in *M.tb* lesions in the early phase of anti-TB therapy assessed changes in SUV_{max} by FDG-PET in 21 individuals [163]. Scans were performed prior to commencing treatment and after 1 month. The median SUV_{max} at T=0 was 8.6 (range 3.1-15.5) and 1 month median SUV_{max} was 5.3 (range 1-11.9). All but 2 patients demonstrated a decrease in SUV_{max} at 1 month with a median decrease of 31%. Interestingly, of the two patient whose SUV_{max} did not decrease, 1 was shown to have smear positive *M.tb* at 3 months despite appropriate therapy whilst the other was subsequently demonstrated to not to have TB but in fact, malignant disease. This study demonstrates early metabolic changes occurring response to anti-TB therapy and given the recent findings linking hypoxia and HIF signalling to metabolic responses in inflammation [164], might have been relevant to my study. Interestingly, a prospective pilot study of HIV positive patients examining SUV_{max} at start of therapy and after 4 months found using FDG-PET found no difference between responders and non-responders [165]. It is possible that earlier anti-TB treatment might have affected my

results however the impact of anti-TB therapy on the pulmonary lesions in the patients in my study will remain undetermined.

One possible explanation for the presence of hypoxia in my study might be that hypoxia occurs as a consequence of the severe inflammation and consolidation seen in several pneumonic processes. It was not possible to compare the hypoxia in TB patients compared to other forms of respiratory infection or bacterial pneumonia since no study of this type has yet been performed. ^{18}F -FMISO PET-CT has only been utilised to investigate hypoxia in the context of infection in one study. In this study of 26 patients with nasopharyngeal carcinoma, ^{18}F -FMISO PET-CT scans were performed to assess and clinically correlate hypoxia in periodontal disease following radiotherapy [166]. The authors of this study report high rates of sensitivity and specificity in detecting odontogenic infections but interestingly bacterial pathogens were only identified in less than 50% of patients. Furthermore, the analysis did not include Patlak K_i evaluation and therefore did not assess for possible excess vascular delivery to the region of interest. Since high tracer uptake might represent high tracer delivery secondary to high blood flow, my study used Patlak K_i mapping to identify ^{18}F FMISO trapping and thus specifically regions of hypoxia. The maximum TB lesion K_i value (0.005 min^{-1}) is equal to the highest K_i value in an ^{18}F FMISO study of head and neck cancer by Wang et al [167] which puts the degree of hypoxia in this study into context. As discussed previously, it is not currently possible to provide a direct correlation between ^{18}F FMISO uptake and pO_2 but the results confirm evidence of severe hypoxia. Since my study is the first to image hypoxia in the presence of confirmed *M.tb* infection, I was keen to ensure that all possible steps were taken to assess the results using both internal and external controls. My protocol included the measurement of ^{18}F FMISO

levels in peripheral blood thus providing an internal control for any possible movement artefact. In addition the finding of stable or increasing K_i values in lung lesions compared to blood values, which declined over time, further supports the finding of tracer trapping demonstrated by K_i measurements. Comparison of abnormal lung lesions compared to structurally normal lung demonstrated no uptake of tracer in normal lung tissues in any of the five patients, providing a further internal control. Finally, peripheral blood measurements were scaled to a historical control population of 12 patients in order to compare results to external controls.

My data revealed that heterogeneity existed both within individual lesions and between lesions, consistent with the emerging concept of multiple TB microenvironments existing within a single patient [168]. Similarly studies conducted in rabbits infected with *M.tb* HN878 and non-human primates infected with *M.tb* Erdman strain reveals heterogenous disease within pulmonary lesions. Interestingly, when lesions are visualised over time using either CT or FDG-PET, temporal-spatial changes are seen with new lesions often appearing whilst principal lesions resolve [169, 170]. My results demonstrated that not all lesions are hypoxic however, since [^{18}F]FMISO is only taken up in regions of severe hypoxia, it is possible that regions of hypoxia existed that were not detected using this probe i.e. greater than 1-2% pO_2 but less than the 9-10% pO_2 present in normal healthy lung tissue. My study did not assess how hypoxic lesions changed during the course of therapy and might be considered for future studies assessing TB therapeutics.

Standardisation of results across the five patients using TBR involved examining at a single lung lesion with high SUV and comparing data both within an individual lesion and across patients. Whilst this is a useful method to examine lesions across patients, this method

however did not examine multiple lesions within the same patient and might therefore underestimate the number of lesions containing hypoxic regions. It would certainly be of interest to determine the extent of hypoxia within different lesions within the same patient but this analysis was not undertaken due to time limitations (each patient registration took nine days to perform and needed to be performed without compromise to patient diagnostic services). Since Ki mapping performed using SUV revealed multiple lesions within a single patient with high Ki values, it is likely that hypoxia exists in several lesions.

Until now, the study of hypoxia in the context of *M.tb* infection has focussed on pathogen responses to hypoxia which indicate transition to a state of latent disease. My study is the first to demonstrate that hypoxia exists in humans during active *M.tb* infection. Research demonstrating hypoxia in the context of active infection is emerging but until now has only been conducted in animal models. Of note, the presence of hypoxia in these models has been shown to affect antimicrobial killing [107, 171]. My clinical research therefore has implications not only for furthering our understanding of the disease in humans but also has implications for how we study the disease within the laboratory.

In conclusion, [¹⁸F]FMISO PET-CT demonstrates for the first time the presence of severe hypoxia within active human pulmonary TB infection. This novel finding serves not only to improve our understanding of the environment in which the infection occurs in humans but, provides insights which might aid the development of future new therapies.

CHAPTER 4: HYPOXIA AND MMPs IN RESPIRATORY EPITHELIAL CELLS DURING TB INFECTION

Introduction

The pulmonary epithelium is the first barrier to *M.tb* and respiratory epithelial cells are the most abundant cells in the lungs covering an alveolar surface area of approximately 70m². Although macrophages are the first immune cells to encounter *M.tb* in the alveoli and secrete a series of inflammatory mediators, signalling also occurs between stromal cells and inflammatory leucocytes, often accentuating the host immune response.

Previous studies from the Friedland group have shown that MMP secretion from normal human bronchial epithelial cells (NHBEs) is up-regulated when stimulated with filtered, conditioned media from *M.tb*-infected monocytes (CoMTb)[46]. These results were not seen with direct infection with *M.tb* indicating that MMPs are up-regulated via a monocyte dependent network that involves both host and pathogen derived soluble factors[172]

The *in vitro* TB network used in my experiments is an established model in the Friedland laboratory (description in Methods). Each individual batch of CoMTb that was generated was done so with a batch of conditioned media from uninfected monocytes (CoMCont) from the same donor as a control. Batches were then tested in A549 respiratory epithelial cells where cells were stimulated with CoMTb or CoMCont and the supernatants harvested at 72 hours. The Friedland group have confirmed that CoMCont does not stimulate MMP production from epithelial cells. A comparative analysis of cytokines and chemokines in CoMTb and CoMCont has been performed several times in our department using the Luminex multiplex platform. Table 5 summarises grouped analyses from the Friedland

group of batches of CoMTb and CoMCont. The analysis has also been used to confirm that donor-related variability in the cytokine and chemokine profiles of CoMTb is within statistically acceptable limits.

Cytokine/chemokine	CoMCont		CoMTb		p value
	Mean (pg/ml)	SEM	Mean (pg/ml)	SEM	
GM-CSF	3	0	559.8	374	<0.0001
G-CSF	3	0	1178	446.2	<0.0001
IL-1 β	131.8	44.5	26274	8815	<0.0001
IL-6	1	0	41992	14227	<0.0001
TNF α	1	0	26910	17403	<0.0001
IFN γ	undetectable	N/A	Undetectable	N/A	N/A
IL-12	2	0	1592	1072	<0.0001
IL-1RA	505.5	171.1	7970	2032	0.0020
CXCL-8	27.75	8.9	94865	49375	<0.0001
MIP-1 α	4	0	198693	94784	<0.0001
MCP-1	7.25	1.25	1426	642.4	<0.0001
MIG	47	22	147.3	48.2	ns
IL-17	undetectable	N/A	undetectable	N/A	N/A

Table 5: Summary of principle cytokines and chemokines measured in CoMTb and CoMCont. Primary human monocytes were infected with *M.tb* MOI=1 and supernatants harvested at 24hrs. Cytokines and chemokines were analysed by Luminex multiplex platform

This chapter explores the effect of HIF and hypoxia on MMP activity. It aims to identify the key MMPs affected by hypoxia and the mechanisms which may drive this activity.

Results

DMOG increases MMP-1 expression in A549 cells stimulated with CoMTb

In order to investigate the effect of HIF-1 α on MMP expression in respiratory epithelial cells stimulated with CoMTb, I initially used the 2-oxyglutarate analogue dimethyloxalyl glycine (DMOG) to inhibit the HIF prolyl and asparaginyl hydroxylases and therefore activate the HIF system (Fig. 24). HIF-1 α stabilisation by DMOG was confirmed in A549 cells and is discussed in Chapter 6 (Fig 25 p117)

Experiments were initially conducted in A549 cells, a commonly used respiratory epithelial cell line which is a widely accepted cellular model in respiratory disease. HIF-1 α stabilisation in cells pre-incubated with DMOG caused an increase in MMP-1 secretion in a dose-response manner (Figure 25A). Doses above 0.5mM caused significant cell death on trypan blue exclusion assay and these results were not included in future experiments. In CoMTb stimulated cells, DMOG 0.25mM also caused a 95-fold in MMP-1 gene expression at 6 hrs and 40-fold increase at 24 hrs (Figure 25B). By comparison, CoMTb stimulation alone causes a 5-fold increase in MMP-1 gene expression at 6 hrs and 10-fold increase at 24 hrs. Analysis of the other main MMPs known to be secreted by A549 cells demonstrated that DMOG significantly increased MMP-9 secretion in A549 cells (Figure 25D) and that MMP-3 secretion was unaffected by DMOG (Figure 25C).

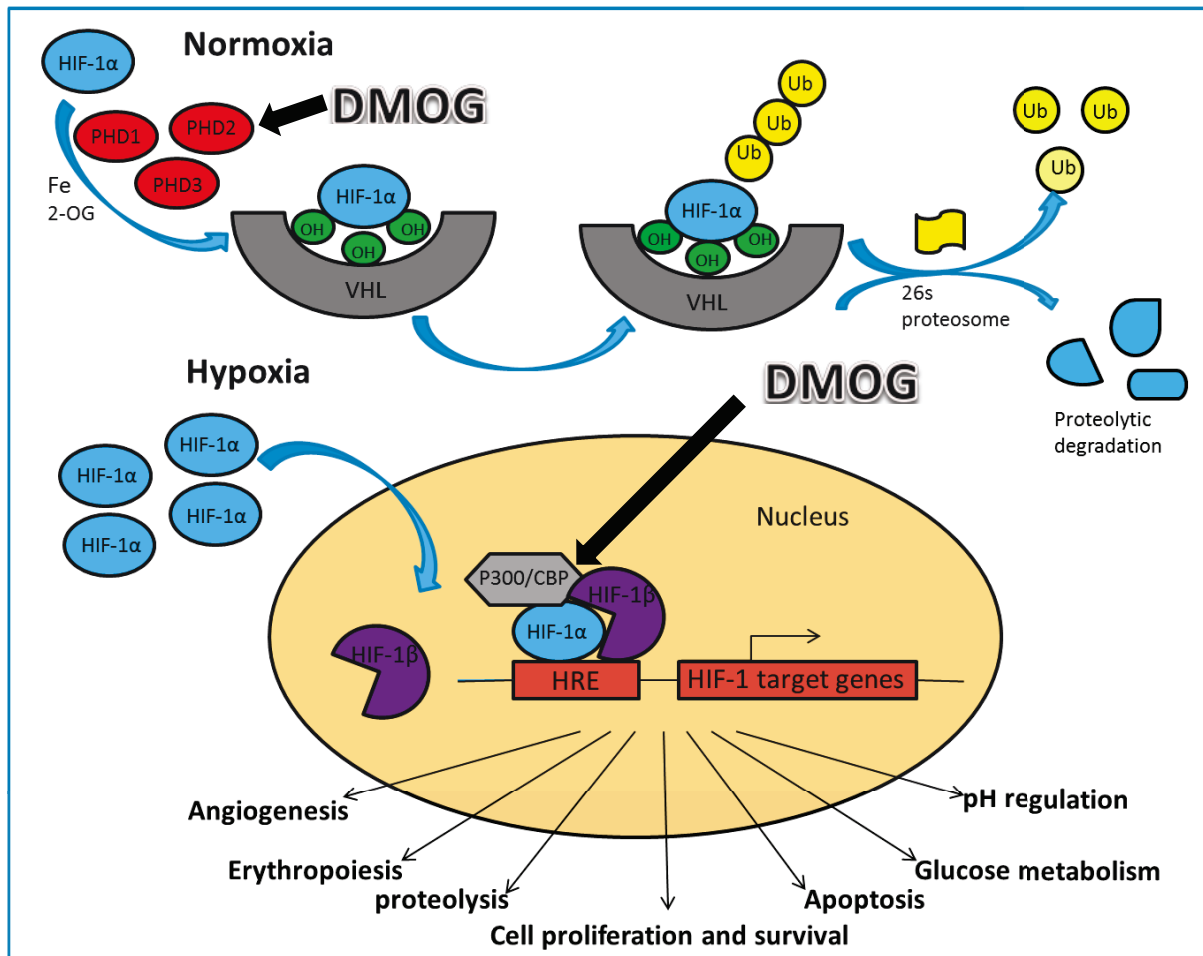


Figure 24: Schematic demonstrating blockade of HIF by prolyl hydroxylases (PHD1, PHD2 and PHD3) and factor inhibiting HIF (FIH) which binds p300/CBP thereby promoting stabilisation of HIF-1 and activation of the HIF pathway.

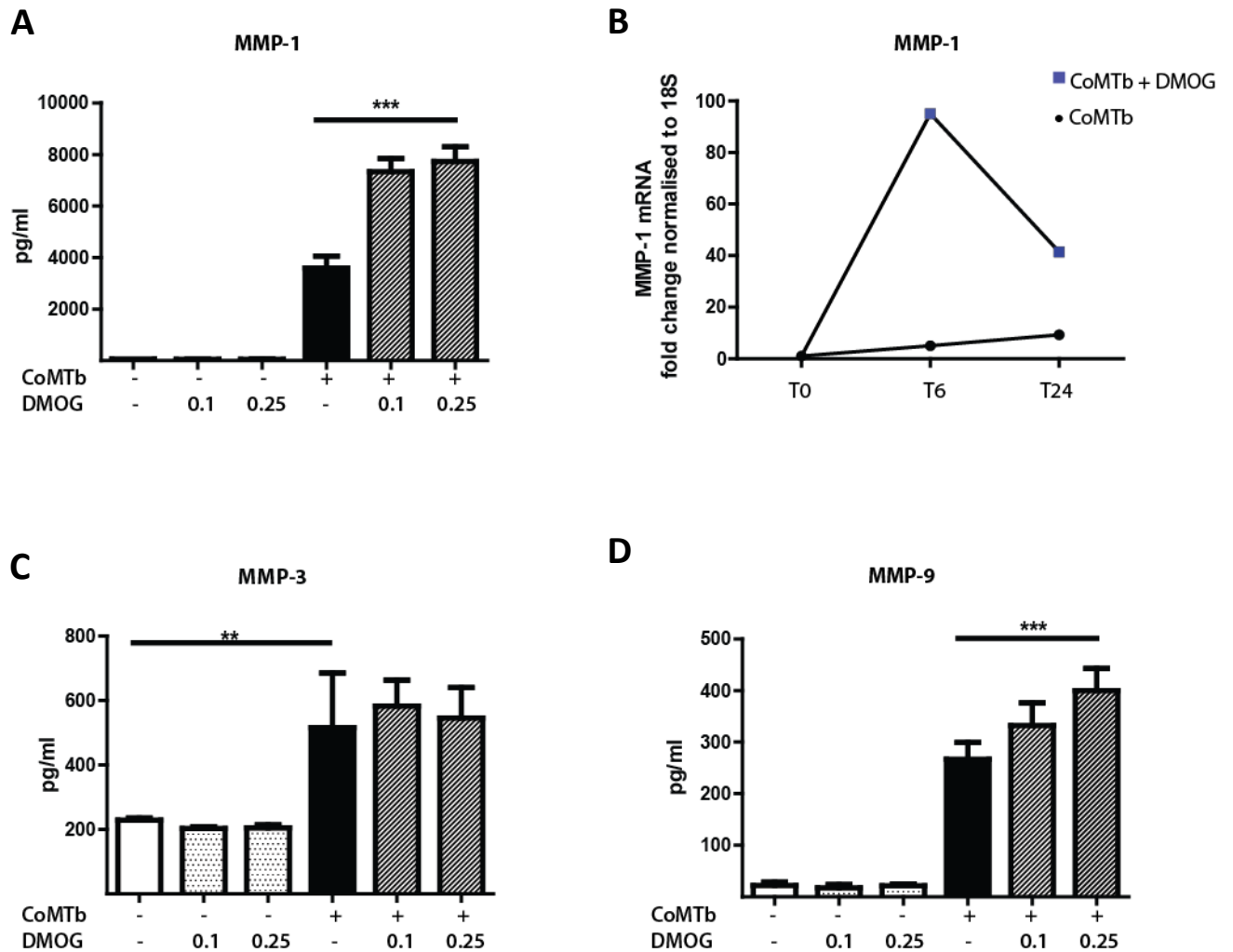


Figure 25: Effect of HIF-1 α stabilisation on MMP secretion from CoMTb stimulated A549 cells. A549 cells were pre-incubated with DMOG for 2hrs and then stimulated with CoMTb. Supernatants were harvested at 72 hrs and MMPs measured by Luminex. **(A)** DMOG increases MMP-1 secretion in CoMTb stimulated cells. **(B)** DMOG increases MMP-1 gene expression at 6 and 24hrs compared to CoMTb stimulated cells. **(C)** DMOG does not affect MMP-3 secretion in either control or CoMTb infected cells. **(D)** DMOG (0.25mM) causes increased MMP-9 secretion in CoMTb stimulated cells. Bars represent mean (+/- SD) of conditions in triplicate and are representative of at least 2 independent experiments.

DMOG decreases TIMP-1 secretion from CoMTb stimulated A549 cells

One of the ways in which MMP activity is regulated is by the specific tissue inhibitors of MMPs (TIMPS) which bind MMPs in a 1:1 manner. Previous data from the JSF group define a matrix destructive phenotype whereby CoMTb stimulation of respiratory epithelial cells results in increased MMP activity which is not associated with a corresponding rise in TIMP activity [46]. It was important therefore to determine whether DMOG affected TIMP activity. I first performed CoMTb dose-response experiments to confirm the previously reported findings by our group. Compared to control cells, stimulation with CoMTb did not affect TIMP-1 secretion (Figure 26A). These findings remained consistent irrespective of the concentration of CoMTb, in particular, TIMP-1 secretion did not increase with increasing concentration of CoMTb. I then assessed the effect of DMOG (0.25mM) on TIMP-1 secretion in cells stimulated with CoMTb (1:5dilution). Pre-incubation with DMOG decreased MMP-1 secretion in control cells ($p<0.01$) and also caused a significant decrease in MMP-1 secretion in CoMTb stimulated cells (Figure 26B; $p<0.001$). Together, these results demonstrated that inhibition of the PHDs and FIH with DMOG causes a significant increase in MMP-1 and MMP-9 secretion which is not associated with a compensatory increase in TIMP-1 secretion.

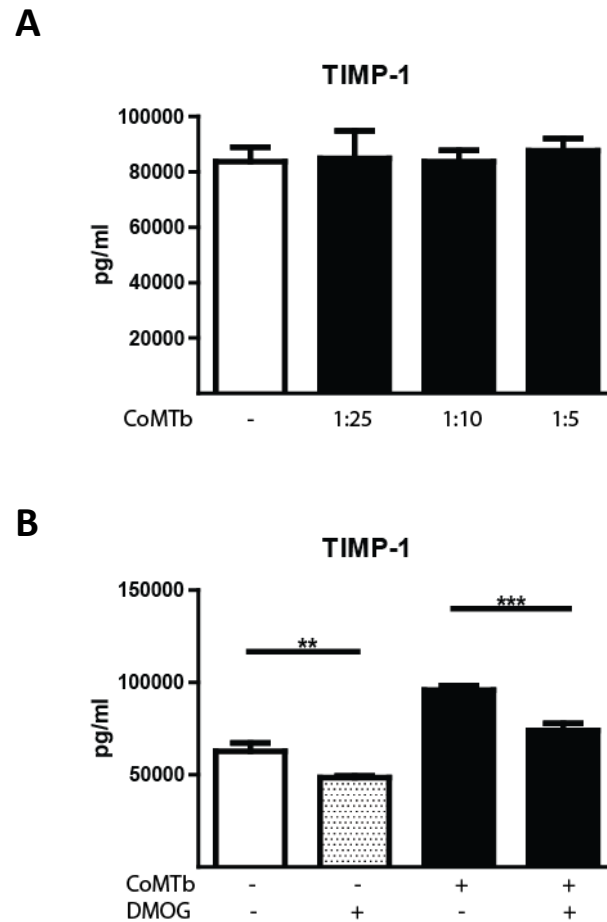


Figure 26: Effect of DMOG on TIMP-1 secretion in respiratory epithelial cells. A549 cells were stimulated with CoMTb and TIMP-1 levels measured after 72hrs. **(A)** Stimulation of A549 cells with CoMTb at increasing concentrations causes no change in TIMP-1 compared to control cells. **(B)** Pre-incubation with DMOG (0.25mM) for 2 hrs decreases TIMP-1 levels in unstimulated cells ($p < 0.01$) and in cells stimulated with CoMTb (1:5) ($p < 0.001$). Bars represent mean ($\pm$ SD) of conditions in triplicate and are representative of at least 2 independent experiments.

Hypoxia at the level seen in humans increases MMP-1 secretion in A549 cells

Having established the effect of HIF-1 α stabilisation through PHD inhibition, I then sought to determine the effect of hypoxia on MMP activity. In order to do this, I used the fixed model of hypoxia where cells were either placed in an adapted Oxoid anaerobic jar filled with a calibrated gas mixture of 1% oxygen and 5% carbon dioxide or a hypoxic incubator where oxygen levels could be set between 1 and 19% oxygen and CO₂ maintained at 5% (see Methods). In these experiments, no pre-incubation in hypoxia took place and cells were placed immediately into their respective incubators following stimulation with CoMTb. Based on my clinical studies, I initially examined the effect of 1% oxygen on MMP-1 activity in A549 cells and then went on to investigate the effect of different oxygen levels. Incubating CoMTb stimulated cells (1:5 dilution) cells in 1% oxygen caused a highly significant increase in MMP-1 and MMP-9 secretion (Figure 27A and C) compared with cells incubated in 21% oxygen. At lower concentration of CoMTb (1:25 and 1:10), the increase in MMP-1 secretion caused by 1% oxygen did not reach statistical significance. Interestingly, when casein zymography was performed, there was evidence of proteolytic activity from all samples incubated in hypoxia, even at the lower CoMTb concentrations (Figure 27B). Casein zymography is a semi-functional assay of proteolytic activity and cells incubated in hypoxia all showed evidence of retained MMP-1 proteolytic activity. In comparison, no MMP-1 proteolytic activity was seen in cells incubated in normoxia.

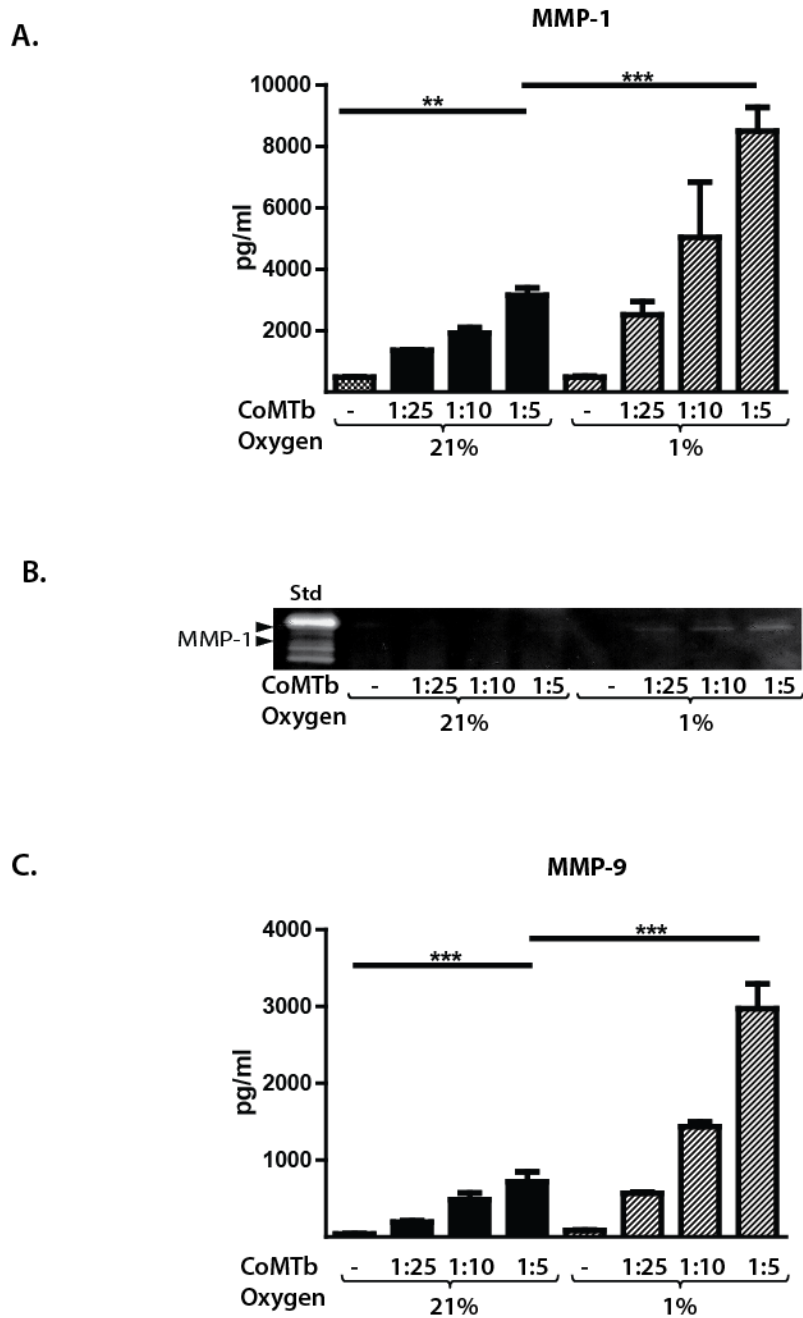


Figure 27: Hypoxia (1% O₂) and effect on MMP-1 and MMP-9 protein secretion from CoMTb stimulated A549 cells. A549 cells were stimulated with CoMTb (1:5) and then incubated at either 21% O₂ or 1% O₂. Cell culture supernatants were harvested at 72 hrs. **(A)** 1% O₂ causes a highly significant increase in MMP-1 secretion compared to 21% O₂ (p<0.001). **(B)** Casein zymography demonstrates that secreted MMP-1 protein from CoMTb stimulated cells incubated in hypoxia is proteolytically active. **(C)** Hypoxia causes a highly significant increase in MMP-9 protein secretion (p<0.001). Bars represent mean (+/- SD) of conditions in triplicate.

MMP-1 confocal microscopy

Thus far experiments in A549 cells demonstrated increased MMP-1 secretion from CoMTb cells at 72 hours and a significant increase in MMP-1 secretion in the presence of 1% O₂. I attempted to visualise MMP-1 from A549 cells using confocal microscopy. Cells were grown in Permax slides and then stimulated with CoMTb (1:5) and cells fixed at 72 hrs. No MMP-1 staining was seen in these cells at T=0hrs (data not shown). A small amount of MMP-1 staining was seen in control cells (normoxia and hypoxia) at 72 hrs with increased staining seen in CoMTb stimulated cells. Of note, some but not all cells incubated in hypoxia demonstrated increased MMP-1 staining within the cytoplasm of the cell (Figure 28).

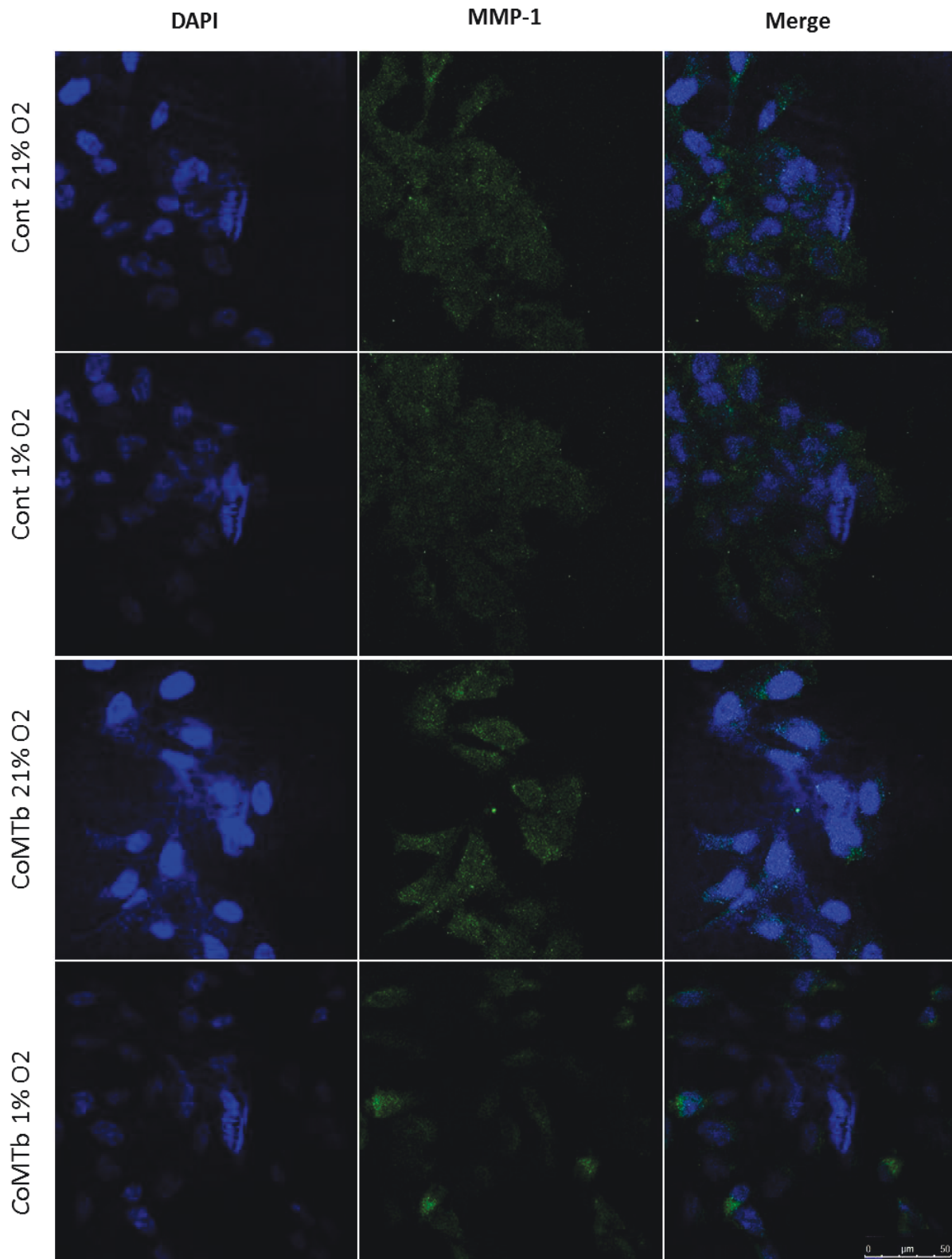
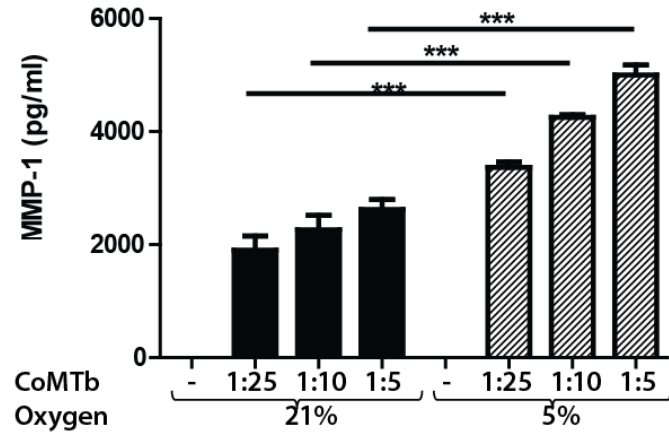


Figure 28: MMP-1 confocal microscopy of CoMTb-stimulated A549 cells in normoxia and hypoxia. A549 cells were stimulated with CoMTb and incubated in either 21% or 1% O₂. Cells were fixed 72 hrs post stimulation. Nuclear staining was shown using DAPI. Increased MMP-1 staining is seen in CoMTb-stimulated cells compared with control cells. More pronounced staining seen in some CoMTb-stimulated cells incubated in hypoxia and was localised to the cell edge in some instances.

Varying degrees of hypoxia differentially affects MMP-1 secretion in A549 cells stimulated with CoMTb

I then proceeded to examine the effect of different levels of oxygen on MMP-1 secretion to investigate whether there was a level of oxygen which triggered an increase in MMP-1 secretion. Experiments performed at 5% oxygen revealed a pattern of increased MMP-1 secretion which closely resembled MMP-1 secretion at 1% oxygen. A highly significant increase in MMP-1 secretion in 5% oxygen was seen at all CoMTb concentration in a CoMTb dose-response manner (Figure 29A; $p < 0.001$). The results of CoMTb dose-response experiments at 10% oxygen compared to 21% oxygen were unexpected and were not predicted by my previous DMOG experiments. Incubating CoMTb stimulated cells at 10% oxygen caused a significant decrease in MMP-1 secretion compared to cells incubated in 21% oxygen (Figure 29B). My initial concern was that I might have harvested the supernatants incorrectly, however multiple repeated experiments at 10% oxygen produced the same results of a decreased in MMP-1 secretion.

A.



B

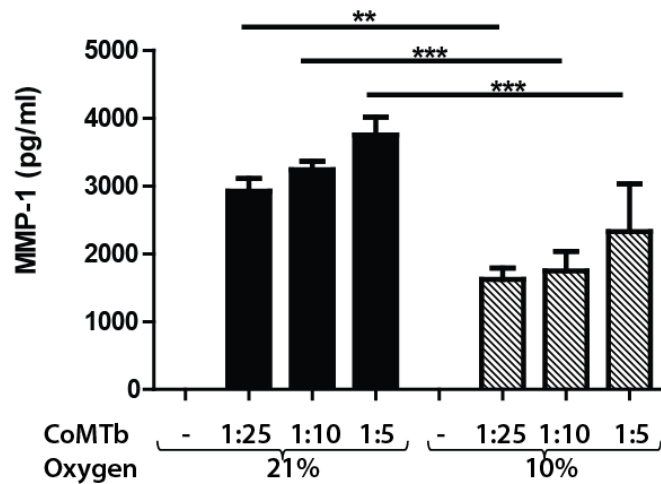


Figure 29: Effect of varying degrees of hypoxia (5% and 10% O₂) on MMP-1 secretion from A549 cells. A549 cells were stimulated with increasing concentrations of CoMTb and incubated at either in hypoxia or normoxia. Supernatants were harvested at 72hrs. (A) Incubation at 5% O₂ resulted in a highly significant increase in MMP-1 protein secretion ($p < 0.001$) at all concentrations of CoMTb. (B) Incubation at 10% O₂ results in a significant decrease in MMP-1 secretion compared to cells incubated at 21% O₂. Bars represent mean ($\pm$ SD) of conditions in triplicate and are representative of at least 2 independent experiments.

DMOG increases MMP-1 and MMP-9 secretion in primary respiratory epithelial cells

Following on from my work in A549 cells I then progressed to investigating hypoxia in primary human bronchial epithelial cells (NHBEs). Initially I performed a series of DMOG dose-response experiments in NHBE cells stimulated with CoMTb (diluted 1:5 as per the standard protocol in the JSF laboratory). Pre-incubation of samples with DMOG prior to stimulation with CoMTb resulted in a dose-dependent increase in MMP-1 secretion (Figure 30A). Increasing the length of pre-incubation with DMOG had no effect on the increase in MMP-1 secretion (Figure 30A). Next, I sought to establish the effect of DMOG (0.25mM) on key MMPs known to be up-regulated by the TB networking effect of CoMTb. Luminex analysis demonstrated that HIF-1 α stabilisation significantly increased MMP-1 ($p<0.001$) and MMP-9 ($p<0.001$) secretion in NHBE cells stimulated with CoMTb (Figure 30B).

Trypan blue exclusion assay performed at 72 hours demonstrated significant cell death at 0.5 and 1.0mmol concentrations of DMOG and is the most likely explanation for the decrease in MMP-9 secretion seen above 0.25mM (Figure 31A). Subsequent dose-response experiments using lower concentrations of DMOG once again confirmed the significant increase in MMP-9 in cells pre-incubated with DMOG and stimulated with CoMTb (Figure 31B; $p<0.05$). Gelatin zymography (Figure 31C) demonstrated the proteolytic activity to be increased in both the DMOG samples and CoMTb stimulated cells. The addition of DMOG to CoMTb stimulated NHBE cells resulted in a large increase in MMP-9 proteolytic activity. When the zymogram was analysed by densitometry, this increased proteolytic activity was found to be greater than an additive effects of either DMOG or CoMTb alone (Figure 31D).

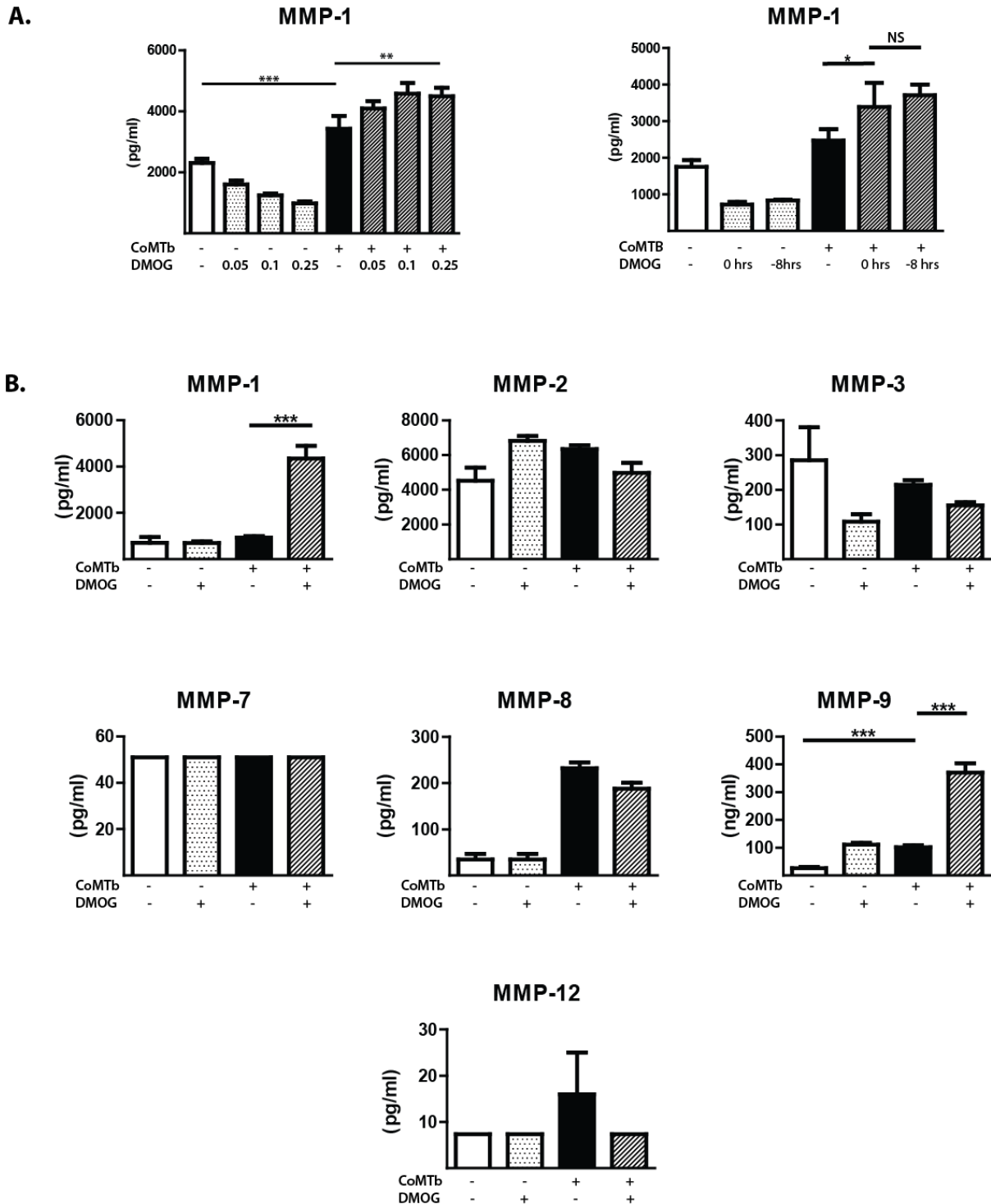


Figure 30: Effect of DMOG on TB networking effects in NHBE cells stimulated with CoMTb. NHBE cells were pre-incubated with DMOG and then stimulated with CoMTb (1:5). Supernatants were harvested at 72hrs. **(A)** DMOG causes a dose-dependent increase in MMP-1 secretion. **(B)** Length of pre-incubation with DMOG (0.25mM) has no effect on the amount of MMP-1 protein secretion. **(C)** DMOG (0.25mM) causes a highly significant increase in MMP-1 and MMP-9 ($p < 0.001$) protein secretion in NHBE cells stimulated with CoMTb compared to CoMTb stimulation alone. Bars represent mean ($\pm$ SD) of conditions in triplicate and are representative of at least 2 independent experiments.

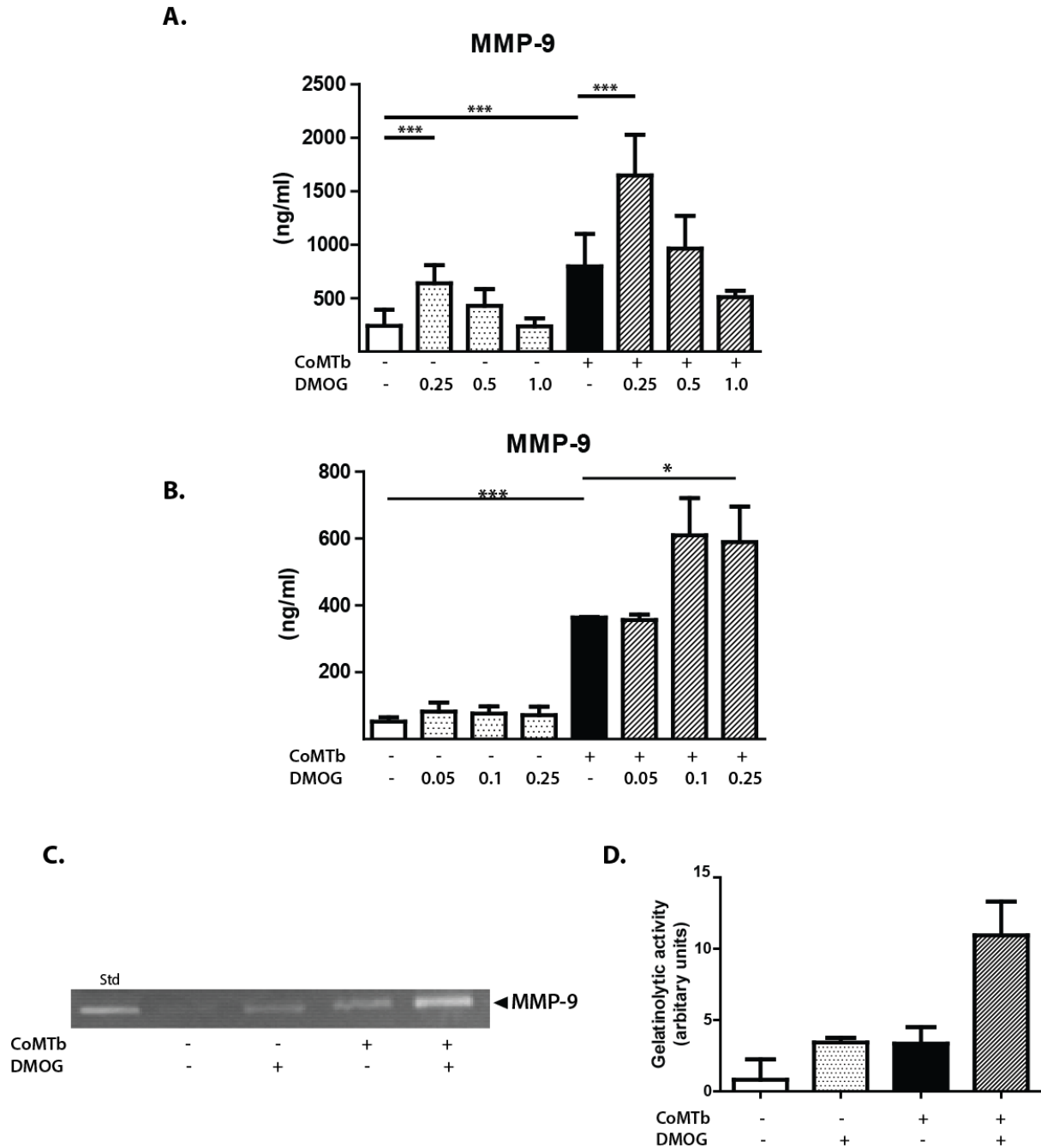


Figure 31: Effect of DMOG on MMP-9 protein secretion and proteolytic activity in NHBE cells. NHBE cells were pre-incubated with DMOG for 2hrs and then stimulated with CoMTb (1:5). Supernatants were collected at 72hrs. **(A)** DMOG (0.25mM) causes increased MMP-9 secretion in control unstimulated cells and cells stimulated with CoMTb. Significant cell death occurs at concentrations of 0.5mM and above associated with decreased MMP-9. **(B)** DMOG (0.1mM and 0.25mM) cause increased MMP-9 protein secretion. **(C)** Gelatin zymography demonstrates increased proteolytic activity in cells incubated with DMOG. **(D)** Densitometry of gelatin zymogram demonstrates a cumulative effect of CoMTb stimulation and DMOG. Bars represent mean (+/- SD) of conditions in triplicate and are representative of at least 2 independent experiments.

Hypoxia (1% pO₂) increases MMP-1 secretion in NHBE cells stimulated with CoMTb

I then proceeded to examine the effect of hypoxia on MMP-1 activity in NHBE cells. Experiments performed using the fixed model of hypoxia demonstrated that 1% O₂ caused a significant increase in MMP-1 secretion in CoMTb stimulated cells (Figure 32A; $p < 0.001$). In contrast to the previous A549 experiments, no difference was seen in MMP-1 secretion when cells were incubated at 5% O₂ (Figure 32B). Incubation at 10% O₂ caused a decrease in MMP-1 secretion compared to cells incubated at 21% oxygen but this difference did not reach statistical significance (Figure 32C). In order to assess the dose-response effect of oxygen on MMP-1 secretion, I performed parallel experiments where NHBE cells were incubated in 1%, 5% or 21% oxygen in the adapted Oxoid jar, hypoxic incubator and standard CO₂ incubator respectively. Once again, I was able to confirm that MMP-1 secretion is only up-regulated in severe hypoxia i.e. 1% pO₂ (Figure 32D; $p < 0.01$). LDH cytotoxicity assay confirmed that the increased MMP-1 was not a consequence of hypoxia induced cytotoxicity (Figure 32E).

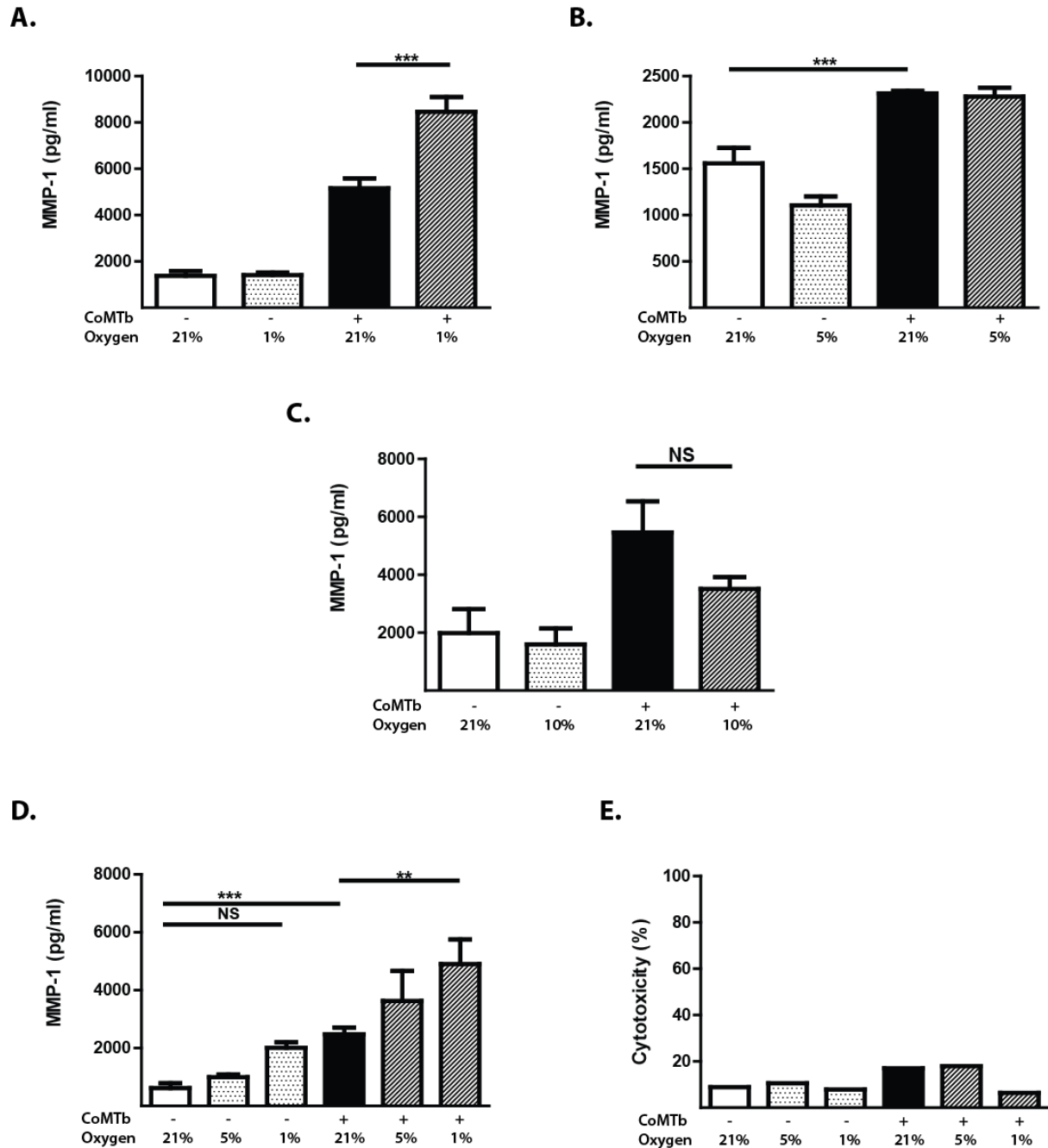


Figure 32: Effect of different oxygen levels on MMP-1 secretion in NHBE cells stimulated with CoMTb. NHBE cells were stimulated with CoMTb and incubated at different oxygen levels. MMP-1 levels were measured by ELISA at 72hrs. **(A)** 1% O₂ causes a highly significant increase in MMP-1 protein secretion from cells stimulated with CoMTb compared to cells incubated at 21% O₂. **(B)** 5% O₂ does not affect MMP-1 protein levels in CoMTb stimulated cells compared to 21% O₂. **(C)** MMP-1 protein levels are unchanged in cells incubated at 10% O₂ compared to 21% O₂. **(D)** Only severe hypoxia (1% O₂) causes increased MMP-1 secretion from CoMTb stimulated cells. **(E)** Hypoxia (1% and 5% O₂) does not increase cells cytotoxicity as measured by LDH release assay. Bars represent mean (+/- SD) of conditions in triplicate and are representative of at least 2 independent experiments.

DMOG and hypoxia cause increased MMP-1 promoter activity in respiratory epithelial cells stimulated with CoMTb

Having investigated the effect of hypoxia and DMOG on MMP activity in respiratory epithelial cells, I then proceed to investigate the mechanisms by which this might occur. I first sought to investigate the effect of hypoxia on MMP-1 promoter activity. The investigation of MMP-1 promoter activity required successful transient transfection of the WT-MMP-1 promoter into primary NHBE cells. Transfection of NHBE cells has been successfully performed within the JSF group however all these experiments have been performed in normoxia only. I attempted multiple transfection experiments using NHBEs in hypoxia. On all occasions the combination of transfection reagents and hypoxia (1% O₂) resulted in >50% cell death (assessed by trypan blue exclusion assay). Consequently, transfection experiments were performed using A549 respiratory epithelial cells

Compared to control cells, CoMTb stimulation caused a significant increase in WT-MMP-1 activity (Figure 33A; $p < 0.001$). DMOG (0.25mM) alone did not affect MMP-1 promoter activity. Pre-incubation of cells with DMOG resulted in a significant increase in WT-MMP-1 promoter activity in CoMTb stimulated cells (Figure 33A; $p < 0.01$). When DMOG (0.25mM) and 1% O₂ were compared, 1% O₂ was a stronger stimulus for MMP-1 promoter activity than PHD inhibition (Figure 33B).

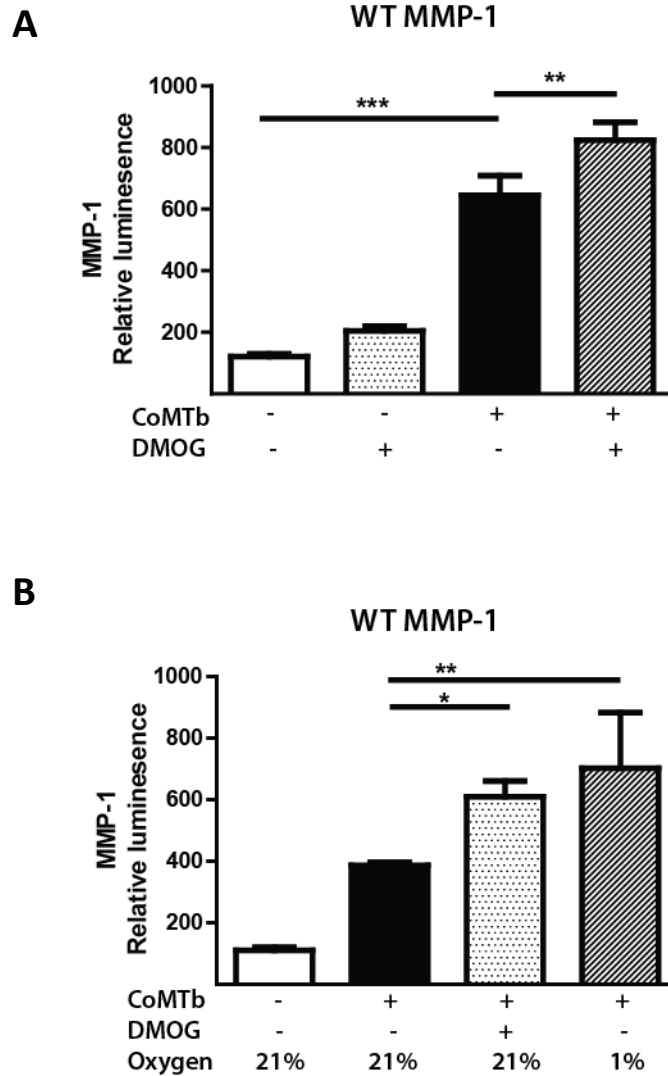
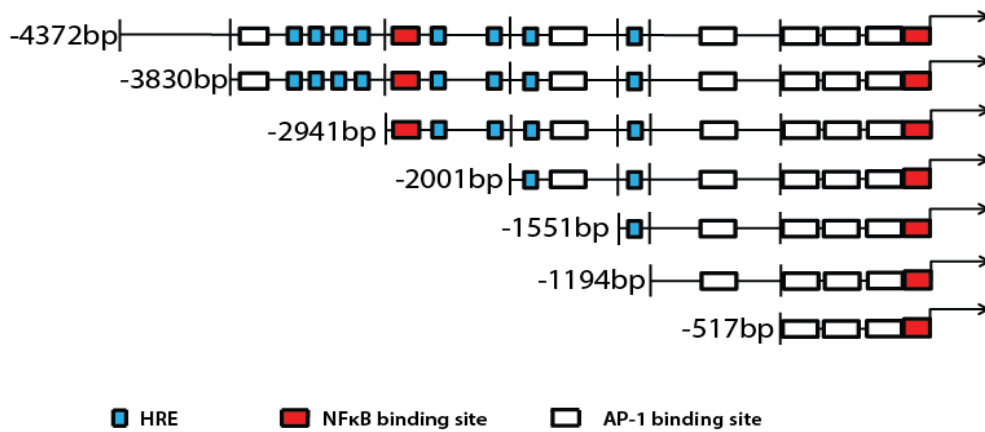


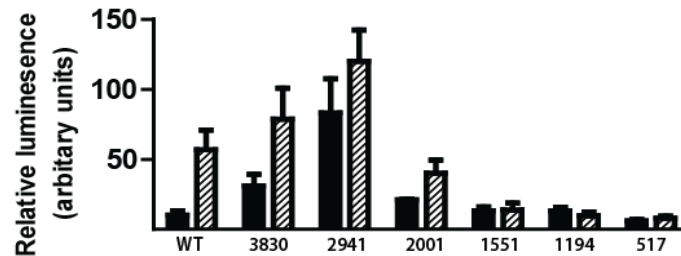
Figure 33: Effect of DMOG and hypoxia on MMP-1 promoter activity in A549 cells. A549 cells were transfected with WT-MMP-1 promoter and then incubated with DMOG or at 1% O₂. Relative luminescence was measured at 24hrs by dual luciferase assay. **(A)** DMOG (0.25mM) causes a significant increase in MMP-1 promoter activity in CoMTb stimulated cells ($p < 0.01$). **(B)** DMOG and hypoxia (1% O₂) both increase MMP-1 promoter activity in CoMTb stimulated cells. Hypoxia is a stronger stimulus for MMP-1 promoter activity than DMOG. Bars represent mean ($\pm$ SD) of conditions in triplicate and are representative of at least 2 independent experiments.

I next proceeded to further dissect the effect of hypoxia (1% O₂) on specific elements within the MMP-1 promoter. *In silico* analysis of the MMP-1 promoter sequence revealed a number of putative hypoxia response elements (HREs) which act as HIF binding sites (Figure 34A). Analysis also revealed consensus binding sites of NFκB and AP-1 transcription factors which are known to regulate MMP activity and have recently been linked to HIF. A549 respiratory epithelial cells were transiently transfected with either WT-MMP-1 promoter or MMP-1 promoter truncation constructs and then stimulated with CoMTb in the presence of normoxia or hypoxia. In normoxia, deletions upstream of -2942 resulted in increased MMP-1 promoter activity indicating the presence of inhibitory elements (Figure 34B). In contrast, truncations downstream of -2942 had a negative effect on MMP-1 promoter activity. The presence of hypoxia resulted in increased MMP-1 promoter activity, compared to normoxia, in deletion constructs upstream of -2001. Since NFκB is intimately involved in the regulation of HIF activity and a consensus NFκB binding site was identified in this promoter region, I sought to manipulate this site to dissect its effect on MMP-1 activity. Site-directed mutagenesis of the NFκB binding site between -2942 and -2001 resulted in a decrease in MMP-1 promoter activity in normoxia (Figure 34C). Strikingly, mutagenesis of the NFκB binding site reduced the highly significant increase in MMP-1 promoter activity seen in hypoxia to baseline levels. Site-specific mutagenesis of the AP-1 site between -2000 and -1551 did not affect WT-MMP-1 promoter activity in normoxia or hypoxia (34C).

A.



B.



C.

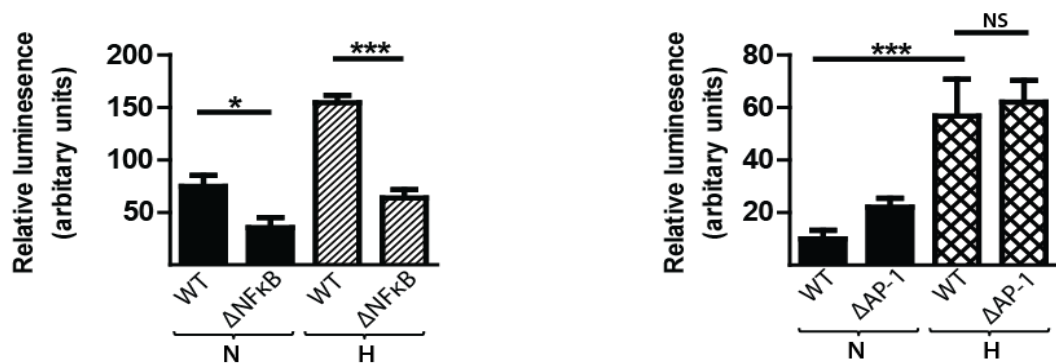
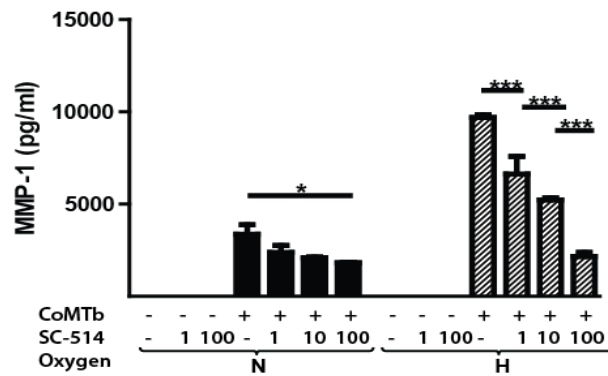


Figure 34: Effect of hypoxia on transcription factors within the MMP-1 promoter. (A) In silico analysis of the MMP-1 promoter reveals putative hypoxic response element (HRE) binding sites as well as consensus NFκB and AP-1 binding sites. (B) Relative luminescence data following transfection of A549 respiratory epithelial cells with either WT-MMP-1 promoter or a series of MMP-1 promoter deletion constructs in the presence (shaded bars) or absence (solid bars) of hypoxia. The effect of hypoxia is absent in constructs from -1551. (C) Site-directed mutagenesis of the NFκB binding site decreases MMP-1 promoter activity in response to CoMTb in normoxia (solid bars) or hypoxia (shaded bars). Site-directed mutagenesis of AP-1 does not affect MMP-1 promoter activity in hypoxia.

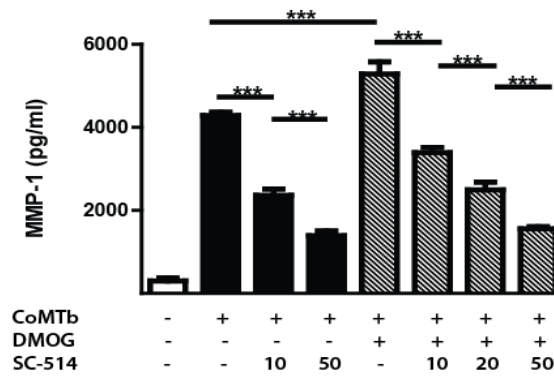
Chemical inhibition of NFκB leads to decreased MMP-1 secretion in CoMTb stimulated cells in hypoxia

To further investigate the role of NFκB during hypoxia in CoMTb-driven MMP-1 expression, I inhibited NFκB activity at two different levels. Initially, I used SC-514 to inhibit IKK-β activity and then used helenalin to inhibit the p65 subunit of NFκB [173]. Inhibition of IKK-β resulted in a significant decrease MMP-1 secretion in normoxia in A549 cells stimulated with CoMTb. (Figure 35A; $p < 0.05$). 1% O₂ causes a 2.9-fold increase in MMP-1 secretion in CoMTb cells compared to 21% and this increase was significantly reduced by SC-514, in a dose-dependent manner (Figure 35A; $p < 0.001$). When cells were treated with DMOG and SC-514, CoMTb-driven MMP-1 secretion decreased in a dose-dependent manner (Figure 35B). A maximum dose of 50μM was used in this experiment as DMOG (0.25mM) and SC-514 (100μM) combined caused significant cytotoxicity and cell death. Inhibition of the p65 subunit of NFκB (RelA) using helenalin resulted in a significant decrease in MMP-1 secretion in A549 respiratory epithelial cells (Figure 35C). Surprisingly, helenalin demonstrated greater inhibitory effects on MMP-1 secretion at lower doses with no inhibition of MMP-1 secretion seen at 10μM. Collectively, the results of chemical inhibitor studies of NFκB indicate that both IKK-β and RelA are required for hypoxic up-regulation of *M.tb*-driven MMP-1 secretion.

A.



B.



C.

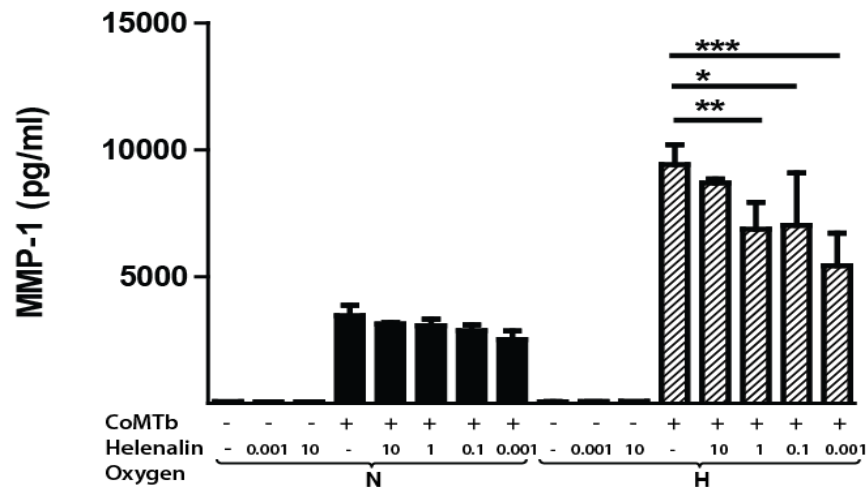


Figure 35: Effect of NF κ B chemical inhibition on CoMTb-driven MMP-1 secretion. (A) Inhibition of the IKK β subunit of NF κ B with SC-514 causes a dose-dependent decrease in MMP-1 secretion in normoxia and hypoxia (1% O₂). The greatest effect is seen in hypoxia. **(B)** SC-514 decreases MMP-1 secretion in A549 cells treated with DMOG (0.25mM). **(C)** Helenalin causes decreased MMP-1 secretion in cells incubated in hypoxia (1% O₂). Bars represent mean (+/- SD) of conditions in triplicate and are representative of at least 2 independent experiments.

Discussion

Pulmonary cavitation is fundamental to the global success of *M.tb*, as it facilitates mycobacterial transmission to new hosts. The biochemistry of the lung matrix predicts the involvement of MMPs in tissue destruction. Previous studies have shown that a number of these enzymes, including MMP-1 and MMP-9, are implicated in *M.tb* pathogenesis and that their expression is up-regulated in lung epithelial cells via a monocyte-dependent network that involves both host- and pathogen-derived soluble factors [46, 59]. I have demonstrated for the first time that hypoxia and HIF activation (by DMOG), augments this effects and cause increased expression of MMP-1 and MMP-9 in respiratory epithelial cells. This increase is not accompanied by an increase in the specific tissue inhibitor of MMP-1 (TIMP-1). The results of my cellular experiments demonstrate therefore that hypoxia further deviates the secretory profile towards matrix degradation and tissue destruction.

I found that the increase in MMP-1 and MMP-9 protein seen in hypoxia resulted in increased proteolytic activity. Both casein and gelatin zymography demonstrated evidence of increased substrate breakdown in the presence of hypoxia or DMOG. Interestingly, DMOG increased MMP-9 proteolytic activity even in unstimulated cells. Since zymography is a semi-functional assay, other methods to study collagen degradation might have included DQ collagen and assessment by confocal microscopy.

My investigation of MMP-1 by confocal microscopy demonstrated evidence of increased MMP-1 staining in some A549 cells stimulated with CoMTb incubated in hypoxia. I chose to visualise MMP-1 at 72 hrs post-stimulation as this is the time-point known to show the maximal changes in MMP-1 secretion. It is possible that performing confocal microscopy

earlier, for example at 48hrs might have improved my ability to detect cellular MMP-1, prior to secretion of the protein into the cell supernatant. I attempted to perform dual staining for MMP-1 and HIF-1 α but found no evidence of HIF-1 α staining at 72 hrs. Again, it is possible that the time-point selected was not appropriate and further kinetic experiments would need to be performed. Overall, the results from confocal microscopy compliment my experiments examining MMP-1 gene expression and protein secretion but the technique does not provide quantitative results beyond direct visualisation of the protein.

My experiments performed at different levels of oxygen in parallel might be confounded by differences between the ambient conditions in each incubator. I attempted to control for these factors by placing temperature and humidity sensors within incubators. In addition, I performed experiments on cells cultured at the same time with sensors set at the same level of oxygen e.g. 1% O₂ to ensure parity between conditions. Humidity levels in the Brunswick Hypoxic incubator were consistently lower than the Oxoid jar or Hypoxia Workstation (87% +/- 2%) compared to >95%. In addition, temperature and humidity levels were highly sensitive to opening and closing of the incubator door, therefore experiments were designed in such a way as to minimise these factors. Nonetheless, these differences and varying the level of oxygen did not result in an increase in cell cytotoxicity. Differences in ambient conditions therefore are unlikely to have affected my experiments but, an improvement to the experimental conditions would be the purchase of a larger incubator which would better accommodate repeated opening of the incubator door.

The decrease in MMP-1 secretion at 10% oxygen compared to 21% oxygen is not accounted for in any current literature and is a surprise finding. It is possible that 10% oxygen is more representative of physiological oxygen levels in the lung and adaptive changes have

occurred to lessen the tissue destructive effects of the MMPs. My work focused principally on the factors driving MMP expression but did not investigate the features associated with 10% O₂ which lead to a *decrease* in MMP-1 activity. Investigation of changes in MMP-1 gene expression and MMP-1 secretion kinetics would have been useful but also assessment of the chemokine and cytokine profile might have identified potential key regulators. It would have also been useful to look at downstream regulatory pathways such as P38 MAPK to assess if these were affected by different oxygen levels. Such work might provide insights into potential therapeutic interventions which result in decreased MMP-1 expression.

My findings of hypoxia-driven MMP expression are consistent with findings from the hypoxic rheumatoid synovium where hypoxia and HIF have been demonstrated to drive MMP-mediated tissue destruction [174]. In addition, HIF-driven MMP expression has been described in a number of malignant conditions [130, 175]. HIF-1 α stabilisation increases MMP-9 activity in breast cancer cell lines [130] and HIF and MMP-9 expression in breast cancer is associated with increased metastatic potential and invasiveness [176].

Since MMP-1 performs the rate-limiting step in the degradation of type I collagen, and that it was the most consistently up-regulated MMP in my experiments, I focused on MMP-1 as the primary host factor involved in lung tissue damage observed in TB. I have shown that DMOG significantly increases MMP-1 mRNA accumulation in A549 cells stimulated with CoMTb. Additionally, both DMOG and hypoxia up-regulate MMP-1 promoter activity in cells transfected with the full-length promoter.

Having demonstrated that hypoxia potently up-regulates MMP-1, I sought to investigate how manipulation of key transcription factors, known to regulate MMPs, might modulate MMP-1 secretion in hypoxia. NF- κ B has been shown to be a key regulator of HIF as well a

key transcription factor involved in the regulation MMP-1 [61, 177, 178]. By blocking the I κ B subunit of NF- κ B with SC-514, I demonstrated a significant down-regulation of MMP-1 secretion. This decrease occurred in normoxia and with DMOG pre-incubation or in hypoxia. Interestingly p65 blockade with helenalin, had no effect in normoxia but caused a significant decrease in MMP-1 secretion in cells incubated in hypoxia. This finding indicates that NF κ B regulation of MMP-1 activity might involve differing regulatory mechanisms in hypoxia compared to normoxia.

In silico analysis of the MMP-1 promoter sequence revealed a number of putative HREs indicating potential involvement of HIF-1 in MMP-1 transcriptional control. My work investigating the role of HIF pathways and MMP activity are described in Chapter 6. Site-directed mutagenesis of the HRE's between -3850 and -1551 however, would have indicated if the HIF complex regulates transcriptional activity of MMP-1. In addition, I would have like to have manipulated elements within the MMP-9 promoter to investigate if NF- κ B, HIF and AP-1 are involved in the transcriptional regulation of MMP-9 in hypoxia.

My finding of hypoxia-driven MMP expression during TB infection were mimicked by DMOG indicating that this pathway is potentially open to therapeutic manipulation. Previous trials of MMP inhibitors have proved unsuccessful due to intolerable side-effects [179]; a more targeted approach might therefore improve these therapies. More recently, the use of DMOG to manipulate the HIF pathway has been investigated in animal models of infective and inflammatory conditions. DMOG pre-treatment has been shown to be protective in a murine model of colitis [180] and protective against LPS-induced sepsis [181]. DMOG therapy is currently being investigated in the context of inflammatory bowel disease [182].

My cellular model cannot fully predict MMP responses *in vivo*, but my finding that DMOG potently up-regulates MMP activity might mean that alternative approaches are required. One approach might be to overexpress the PHDs and ascertain if this leads to a decrease in MMP secretion. The development of MMP-1 and MMP9 transgenic mice by the JSF group means that these studies are now possible and represent potential future avenues by which the interplay of hypoxia, the PHDs and MMPs might be explored.

In summary, my investigation of hypoxia and MMPs in respiratory epithelial cells has demonstrated that MMP-1 and MMP-9 are potently up-regulated under hypoxia conditions and in response to prolyl hydroxylase inhibition. My results reveal that hypoxia augments the previously described mechanism by which *M.tb* may drive pulmonary cavitation in the lung and thus create a niche within the host for its survival and transmission.

CHAPTER 5: HYPOXIA AND MMPs IN HUMAN MONOCYTE-DERIVED MACROPHAGES

Introduction

Macrophages are one of the first host immune cells to encounter *M.tb*. Initially macrophages phagocytose the bacilli and then proceed to recruit peripheral macrophages and monocytes to the site of infection to encase the bacilli. In data previously published by the JSF group, direct infection of monocyte-derived macrophages by *M.tb*, but not BCG, causes potent up-regulation of MMP-1 and MMP-7 [183]. Further systematic profiling of all known MMPs and the related A disintegrin and metalloproteinases (ADAMs) in primary human macrophages infected with *M.tb* shows up-regulation of MMP-3 and MMP-10 [183]. Finally, analysis of MMP-1 and MMP-7 from lung biopsy specimens of TB patients demonstrates expression within epithelioid macrophages and Langerhans giant cells within granulomas. Hypoxia is known to influence macrophage function and survival [184, 185] but the effect of hypoxia on *M.tb*-infected macrophages has not been evaluated.

In this chapter, I have examined the effect of hypoxia and HIF stabilisation on MMP activity in MDMs directly infected with *M.tb*. This chapter examines the key MMPs affected by hypoxia and the mechanisms by which this occurs. Additionally it explores whether the effect of hypoxia on MMP activity is influenced by *M.tb* phagocytosis and intracellular replication. Finally it also investigates the role of NFκB in hypoxia-driven MMP activity during direct infection.

Results

HIF-1 α stabilisation with DMOG increases MMP-1 and MMP-3 protein secretion in *M.tb* infected MDMs

To assess the effect of HIF-1 α stabilisation on MMP activity, I initially examined the effect of DMOG on MMP-1/-3 and -7, the main MMPs secreted in response to *M.tb* infection. The investigation of MMP-10 was being conducted by a colleague within the JSF group and therefore was not covered in my study. HIF-1 α stabilisation with DMOG caused a highly significant increase in MMP-1 secretion in *M.tb* infected MDMs occurring in a dose-response manner (Figure 36A; $p < 0.001$). Concentrations of greater than 0.5mmol resulted in marked cell death, as assessed by trypan blue exclusion assay. Casein zymography revealed that secreted MMP-1 was proteolytically active (Figure 36B). This increased proteolytic activity corresponded with increasing concentrations of DMOG.

DMOG caused a significant increase in MMP-3 secretion from *M.tb* infected MDMs although no dose-response relationship was identified (Figure 36C). DMOG did not impact significantly on MMP-7 secretion in *M.tb* infected MDMs except at a concentration of 0.05mmol where a reduction in MMP-7 secretion was observed (Figure 36D).

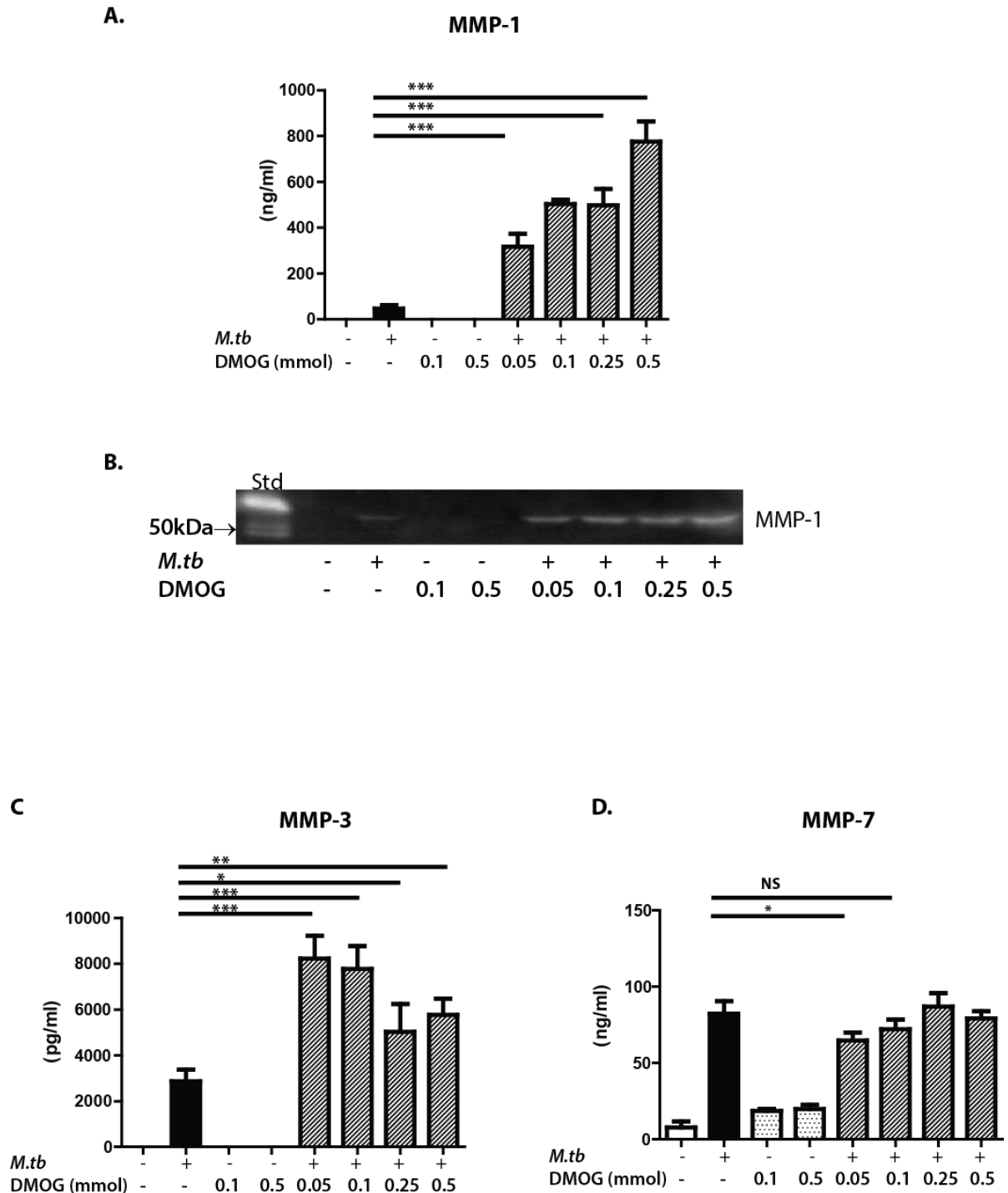


Figure 36: Effect of DMOG on MMP secretion from MDMs directly infected with *M.tb*. MDMs were pre-incubated with DMOG (0.05mM, 0.25mM and 0.5mM) for 2 hrs and then infected with *M.tb* (MOI=1). Supernatants were harvested at 72 hours and MMP-1 levels measured by Luminex. **(A)** DMOG significantly increases MMP-1 secretion in a dose-response manner ($p < 0.001$). **(B)** Casein zymography demonstrates that secreted MMP-1 protein is proteolytically active. **(C)** DMOG causes a highly significant increase in MMP-3 secretion. **(D)** DMOG has no effect on MMP-7 secretion except at a dose of 0.05mM where it causes a decrease in MMP-7 protein levels ($p < 0.05$). Bars represent mean ($\pm$ SD) of conditions in triplicate and are representative of at least 2 independent experiments.

Hypoxia at the level seen in human disease increases MMP-1 and MMP-7 secretion in *M.tb* infected MDMs

I next proceeded to examine the effect of hypoxia (1% O₂) on MMP secretion in *M.tb* infected MDMs. Cells were infected with *M.tb* H37Rv and placed in the same incubator at 21% O₂ and 5% CO₂. After 2hrs cell culture supernatants were removed and replaced with new medium. MDMs were then incubated at either 21% O₂ or 1% O₂. These steps were taken with all experiments to ensure try and control for any differences in phagocytosis that might occur in hypoxia. Compared to cells incubated at 21% O₂, hypoxia (1% O₂) caused a significant increase in MMP-1 and MMP-7 secretion in MDMs infected with *M.tb* (Figure 37). Hypoxia did increase secretion of MMP-1 and MMP-7 in uninfected cells but this result did not reach statistical significance. In contrast to cells pre-incubated with DMOG, MMP-3 secretion was not affected by hypoxia (Figure 37).

All cells were inspected by light microscopy at 72 hrs and although cells incubated in hypoxia appeared more activated i.e. elongated with stellate appearance, not difference in cell death was seen (as assessed by trypan blue exclusion assay).

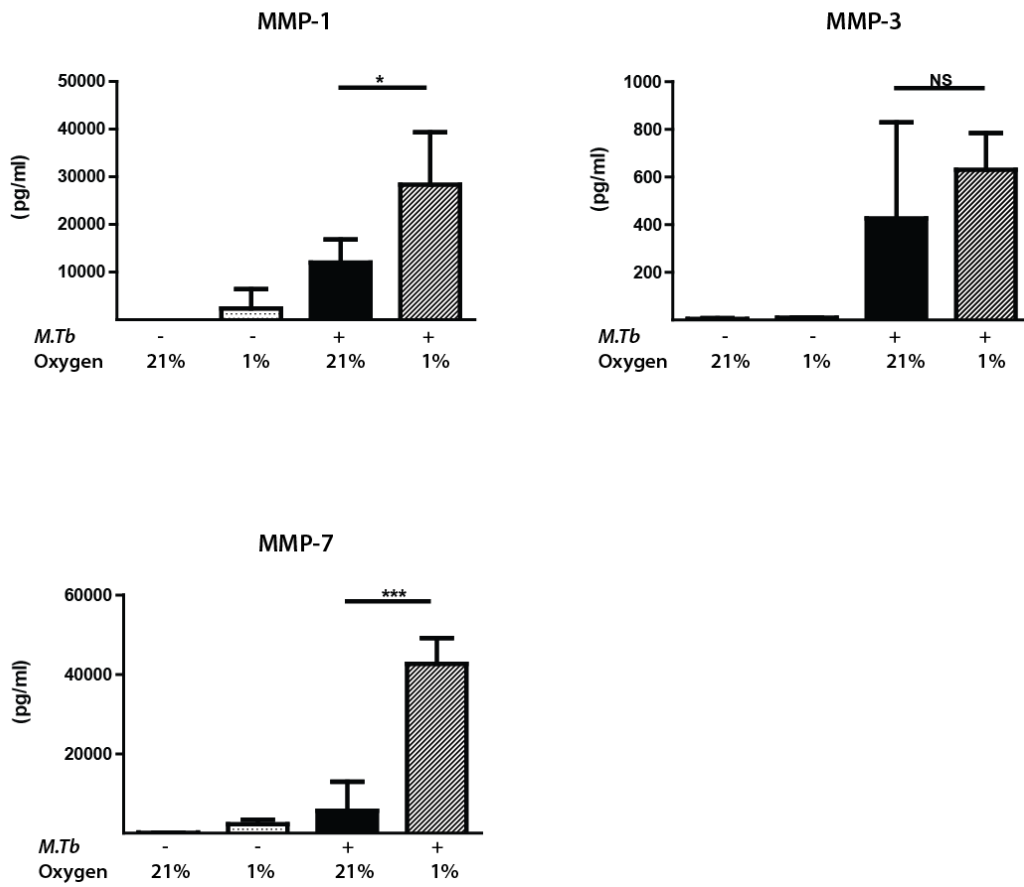


Figure 37: Effect of 1% oxygen on MMP secretion from MDMs directly infected with *M.tb*. MDMs were directly infected with *M.tb* (MOI=1) and incubated with either normoxia (21% O₂) or hypoxia (1% O₂). Supernatants were harvested at 72 hrs and MMPs measured by Luminex. 1% O₂ causes a significant increase in MMP-1 (p<0.05) and MMP-7 protein (p<0.001) secretion compared to cells incubated at 21 %O₂. Hypoxia (1% O₂) has no effect on MMP-3 protein secretion from *M.tb* infected MDMs. Bars represent mean (+/- SD) of conditions in triplicate and are representative of at least 2 independent experiments.

Increased MMP-1 secretion in MDMs infected with *M.tb* is not accompanied by a change in TIMP-1 levels

The concordance between the effect of DMOG *and* hypoxia on MMP-1 secretion determined that subsequent investigations focussed on MMP-1 activity. In the first instance, I looked to compare the effect of hypoxia on *M.tb* infected MDMs with a control stimulus. LPS is known to stimulate MMP-1 secretion in MDMs [186]. Furthermore, LPS activity has been shown to be activated by hypoxia [187]. Compared to control cells incubated in normoxia, LPS stimulation (100ng/ml) caused a 2.3 fold increase in MMP-1 secretion, a result which did not reach statistical significance (Figure 38A; $p>0.05$). *M.tb* infection (MOI=1) caused a 20-fold increase in MMP-1 secretion compared to LPS-stimulated cells. When cells were incubated in hypoxia, *M.tb* infection caused a 28-fold increase in MMP-1 secretion compared to LPS ($p<0.001$) (Figure 38A).

Next, I proceeded to examine the effect of hypoxia on TIMP-1 the specific tissue inhibitor of MMP-1. In uninfected MDMs, hypoxia (1% O₂) caused a significant increase in TIMP-1 secretion (Figure 38B). 1% O₂ had no effect on TIMP-1 secretion in *M.tb* infected MDMs demonstrating that increased MMP-1 secretion caused by hypoxia was not accompanied by a rise in its specific inhibitor. Casein zymography demonstrated increased caseinolytic activity in *M.tb*-infected MDMs in hypoxia (Figure 38C).

The results of two independent experiments (single samples run in triplicate) demonstrated that the greatest difference in MMP-1 mRNA was seen at 24hrs (Figure 38D). Hypoxia caused a 2800-fold increase in MMP-1 mRNA in *M.tb* infected MDMs compared to cells incubated in normoxia (Figure 38D; $p<0.001$).

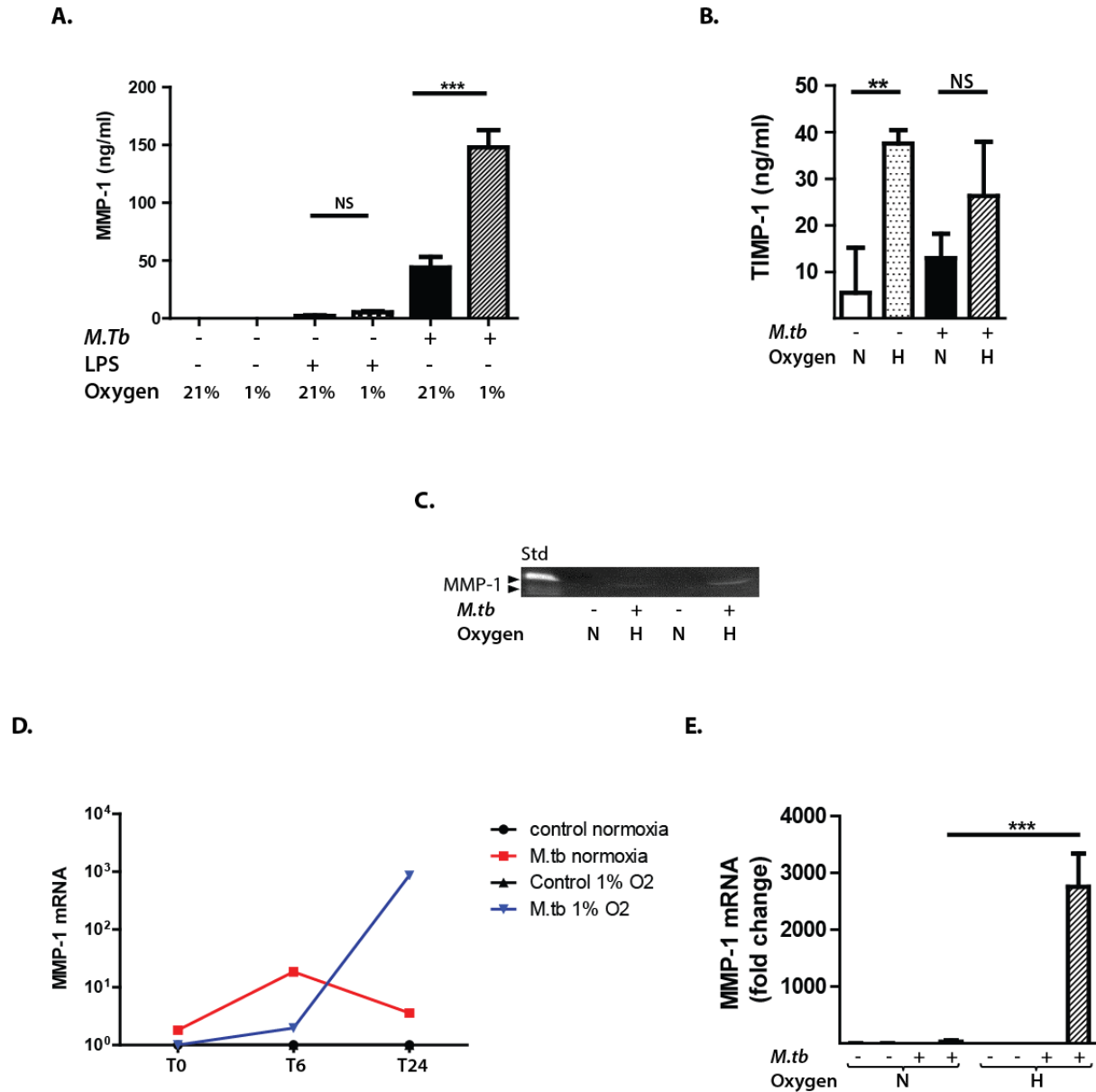


Figure 38: Effect of 1% O₂ on MMP-1 and TIMP-1 activity in MDMs. MDMs were either stimulated with LPS (100ng/ml) or directly infected with *M.tb* (MOI=1). Supernatants were harvested for MMP-1 and TIMP-1 protein secretion at 72 hrs. Cells were lysed at the various time points and RNA extraction performed to assess MMP-1 gene expression. **(A)** 1% O₂ causes a highly significant increase in MMP-1 protein secretion from *M.tb* infected cells compared to LPS and control cells (p<0.001). **(B)** 1% O₂ causes increased TIMP-1 secretion from control cells and has no effect on *M.tb* infected MDMs (p>0.05). **(C)** Casein zymography demonstrates that increased secreted MMP-1 protein in hypoxia is proteolytically active. **(D)** MMP-1 kinetic experiments show that 1% O₂ causes increased MMP-1 gene expression, maximal at 24 hrs. **(E)** 1% O₂ causes a 2,800-fold increase in MMP-1 gene expression (p<0.001) at 24 hrs from MDMs infected with *M.tb* compared to normoxia (N=21% O₂; H=1% O₂). Bars represent mean (+/- SD) of conditions in triplicate and are representative of at least 2 independent experiments.

Confocal microscopy demonstrates increased MMP-1 staining in *M.tb*-infected MDMs incubated in hypoxia

I proceeded to assess MMP-1 activity by confocal microscopy. Macrophages were matured on Permanox chamber well slides as my attempts to mature the cells on glass slides resulted in poor cell adherence and fewer cells than seen on standard tissue culture plates. MMP-1 staining and confocal imaging has not previously been performed within our group and there is no published work in this area so a series of optimisation experiments were required to determine antibody concentrations. MMP-1 staining at t=0 demonstrated no MMP-1 staining, either in control or *M.tb* infected cells (data not shown). I performed MMP-1 staining at 72hrs since this is the time of maximal MMP-1 secretion from MDMs. The results of two independent experiments demonstrated increased MMP-1 staining in the cytoplasm of MDMs infected with *M.tb* (Figure 39). Hypoxia caused increased MMP-1 staining compared to normoxia in *M.tb* infected cells. Interestingly, MMP-1 staining was seen in control cells at 72hrs (normoxia and hypoxia) despite no measurable MMP-1 in the cell supernatant. In these experiments the merge images contained the bright field view in order to better visualise the morphological changes that occurred during the course of the experiment.

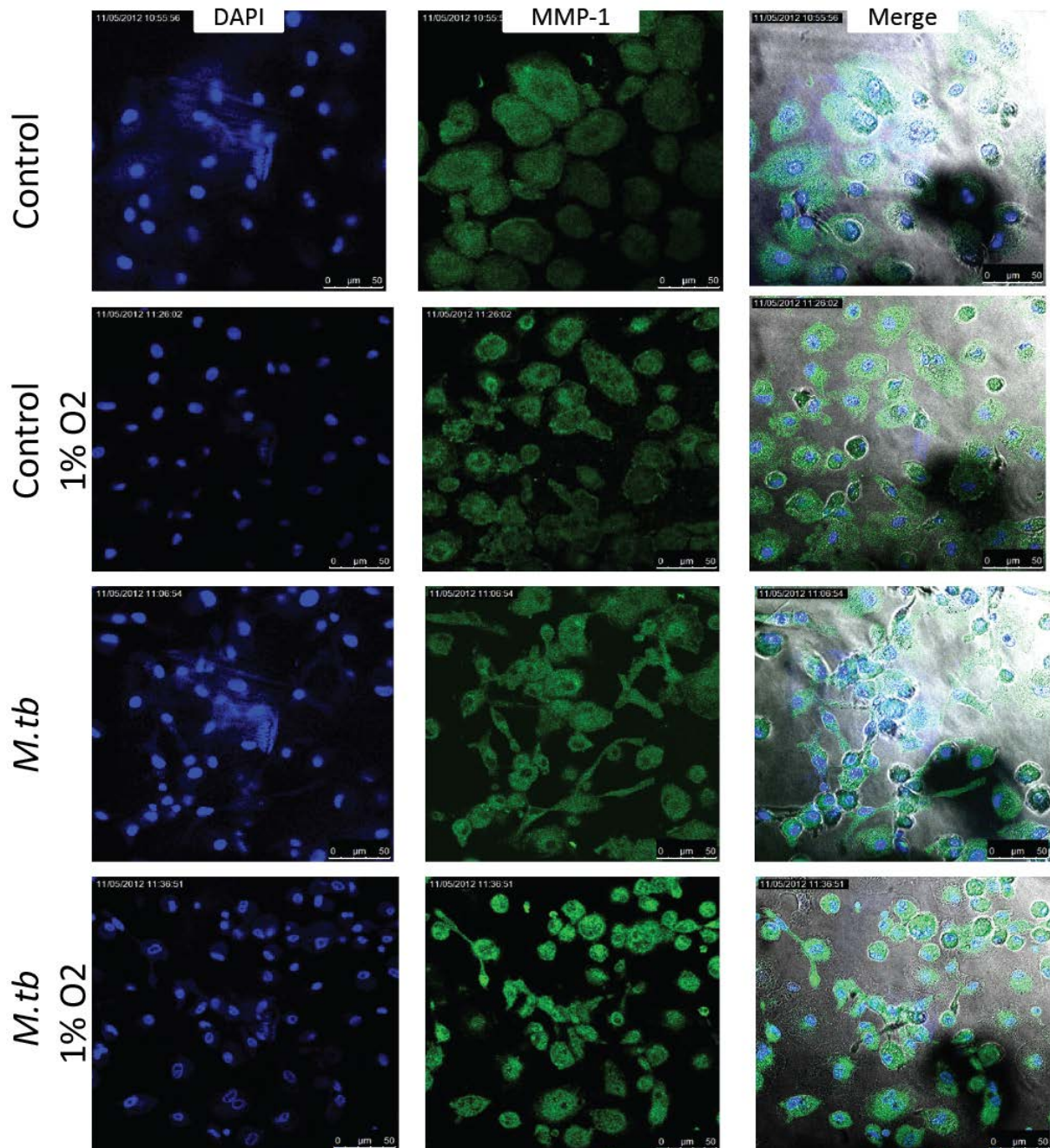


Figure 39: MMP-1 confocal microscopy of *M.tb* infected MDMs in normoxia and hypoxia. MDMS were infected with *M.tb* and incubated at either 21% O₂ or 1% O₂. Cells were fixed and *M.tb* killed with 4% paraformaldehyde prior to permeabilisation and staining for MMP-1. Nuclei were stained with DAPI. MMP-1 staining is seen in all cells at 72 hrs compared to t=0 (data not shown). Increased MMP-1 staining is seen in *M.tb*-infected MDMs incubated at 1% O₂ compared to control cells and *M.tb* infected cells incubated in normoxia.

Incubating *M.tb*-infected MDMs at varying levels of hypoxia has differential effects on MMP-1 secretion

I next proceeded to investigate the effect of different oxygen levels on MMP-1 secretion. I investigated the effect of 1%, 5% and 10% oxygen in three independent experiments. In keeping with my findings in respiratory epithelial cells, 5% oxygen caused a significant increase in MMP-1 secretion (Figure 40B; $p < 0.05$) whilst 10% oxygen caused a decrease in MMP-1 secretion compared to MDMs infected with *M.tb* and incubated at 21% oxygen (Figure 40C; $p < 0.001$). I performed parallel experiments comparing 1%, 10% and 21% oxygen and although 10% oxygen did cause a decrease in MMP-1 secretion, this result did not reach statistical significance (Figure 41A). Sub-group analysis demonstrated that the decrease in MMP-1 secretion at 10% was statistically significant when compared with normoxia alone (Figure 41B; $p < 0.01$).

Next I sought to determine if there were any factors which might be associated with the changes in MMP-1 secretion at different levels of oxygen. Light microscopy demonstrated little evidence of gross morphological change in the appearance of the cells between 21% and 10% oxygen (Figure 41C). The appearances seen at 1% oxygen of highly activated cells were consistent with those seen throughout the course of my cellular experiments. Interestingly, I was at this point assisting a colleague in the group and one of the purposes of this particular experiment was to investigate the changes in pH of the cell supernatant 72hrs post *M.tb* infection. At 21% and 10% oxygen, the cell culture supernatant of control cells became more alkaline over time (in keeping with the buffering effect of 5% CO₂) whereas control cells incubated at 1% oxygen remained at pH 7.2. Strikingly, when cells were infected with *M.tb*, cells incubated at 21% and 1% O₂ became markedly acidotic at 6.48 and

5.7 respectively (Figure 41D). *M.tb* infected MDMs incubated at 10% O₂ experienced no change in pH during the course of the experiment.

The mechanisms by which these pH changes occur at different oxygen levels and the effect of pH on *M.tb*-driven immunopathology are now the focus of a clinical research training fellowship being conducted within the Friedland group.

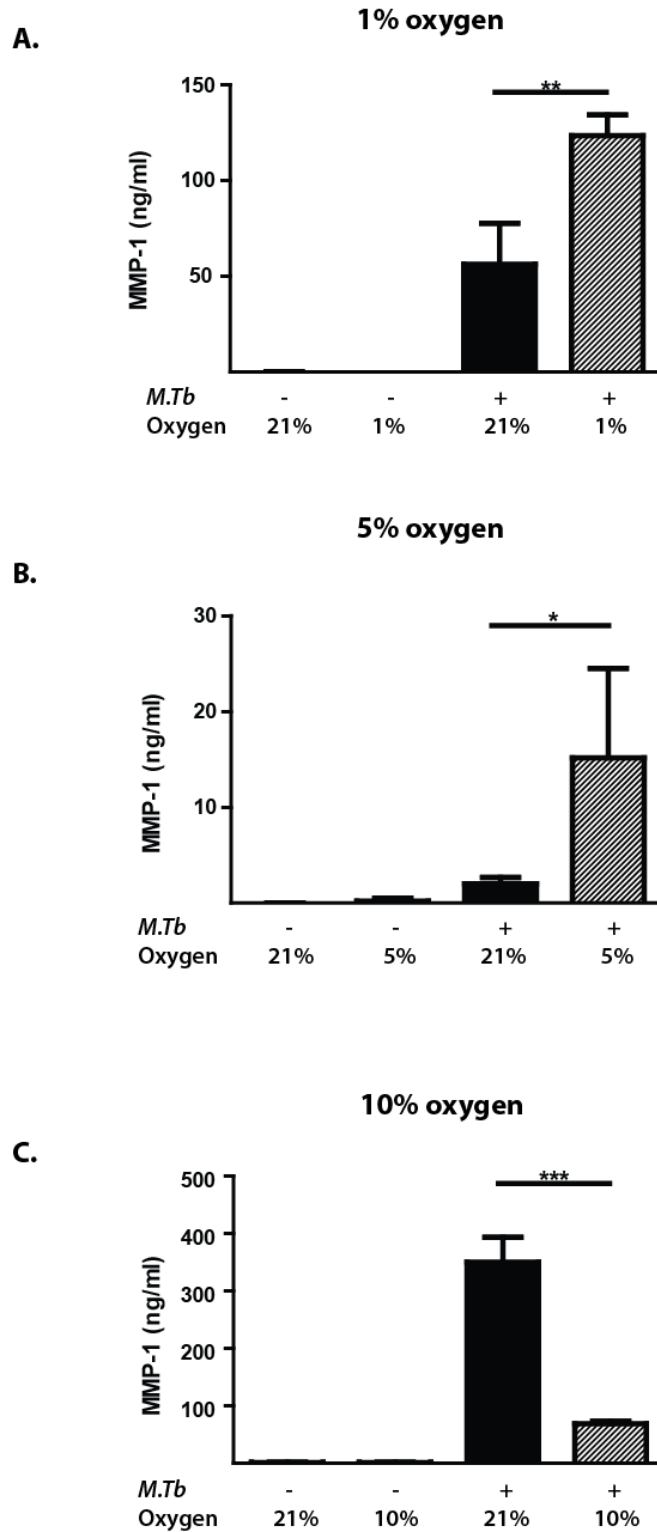


Figure 40: Effect of 1%, 5% and 10% oxygen on MMP-1 protein secretion from *M.tb* infected MDMs. MDMs were infected with *M.tb* (MOI=1) and incubated with either 21% O₂ or hypoxia (1%, 5% or 10% O₂). Supernatants were harvested at 72 hrs and MMP-1 measured by ELISA. **(A & B)** 1% and 5% O₂ causes a significant increase in MMP-1 protein secretion compared to 21% O₂. **(C)** 10% O₂ causes a reduction in MMP-1 secretion compared to *M.tb*-infected cells incubated at 21% O₂. Bars represent mean (+/- SD) of conditions in triplicate and are representative of at least 2 independent experiments.

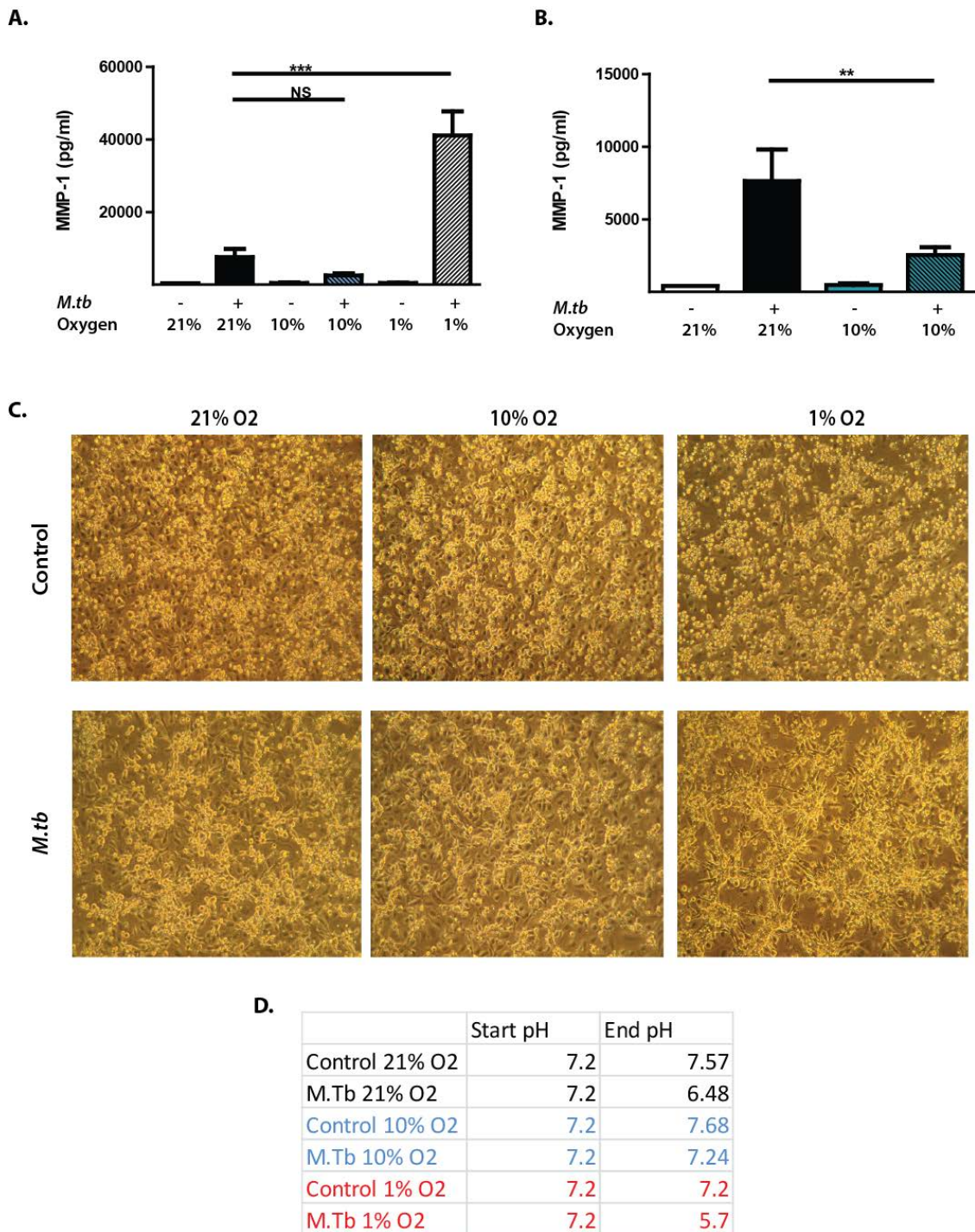


Figure 41: Investigation of the effect of 10% oxygen compared to 1% and 21% O₂ on MMP-1 secretion, cell morphology and cellular pH. MDMs were directly infected with *M.tb* (MOI=1) and incubated at 1%, 10% or 21% O₂. MMP-1 protein secretion by ELISA, cell morphology and cell culture pH was assessed at 72 hrs. **(A)** Compared to 21% and 1% O₂, 10% O₂ causes a decrease in MMP-1 protein secretion but the levels do not reach statistical significance. **(B)** 10% O₂ causes less MMP-1 secretion from *M.tb*-infected MDMs when compared to 1% O₂ only (p<0.01). **(C)** Light microscopy reveals no significant morphological changes to account for differences in MMP secretion. **(D)** *M.tb* infection in normoxia and severe hypoxia (1% O₂) cause acidification of cell culture supernatants at 72 hrs. pH is unaltered after 72 hrs in MDMs incubated at 10% O₂.

Investigation of hypoxia on *M.tb* phagocytosis using GFP-TB

An emerging body of evidence indicates that myeloid cells adapt to in response to hypoxia and that hypoxia increases the phagocytic ability of macrophages. I therefore proceeded to explore if hypoxia might be impacting on the amount of bacilli being phagocytosed following direct infection and whether therefore the changes in MMPs might be accounted for by an increased percentage of infected macrophages.

I initially investigated *M.tb* phagocytosis in MDMs using GFP-TB by confocal microscopy. Each experimental condition was performed in duplicate. Cells were fixed at 2hrs post *M.tb* infection and 10 random fields were selected for each experimental conditional. The percentage of MDMs per field containing GFP-TB were counted independently by myself and a colleague and the median value between the two assessments was taken was the overall percentage. In this initial experiment, hypoxia resulted in a significant increase in phagocytosis of *M.tb* at 2hrs (Figure 42B). I then attempted to refine this experiment by more accurately counting the number of macrophages using to DAPI to identify the number of nuclei and additionally to look at later time points. My results in MDMs infected with GFP-TB and incubated at 21% O₂ at 8 hours revealed that not only were individual macrophages infected with more than one bacilli but also that distinguishing individually infected cells was challenging (Figure 43). At 24 hours, the GFP signal had marked changed and in contrast to the elongated bacilli seen at 8 hrs, the signal had become more diffuse and less apparent (Figure 44). The results in hypoxia could not be analysed since no GFP signal was seen either at 8 hrs or 24 hrs.

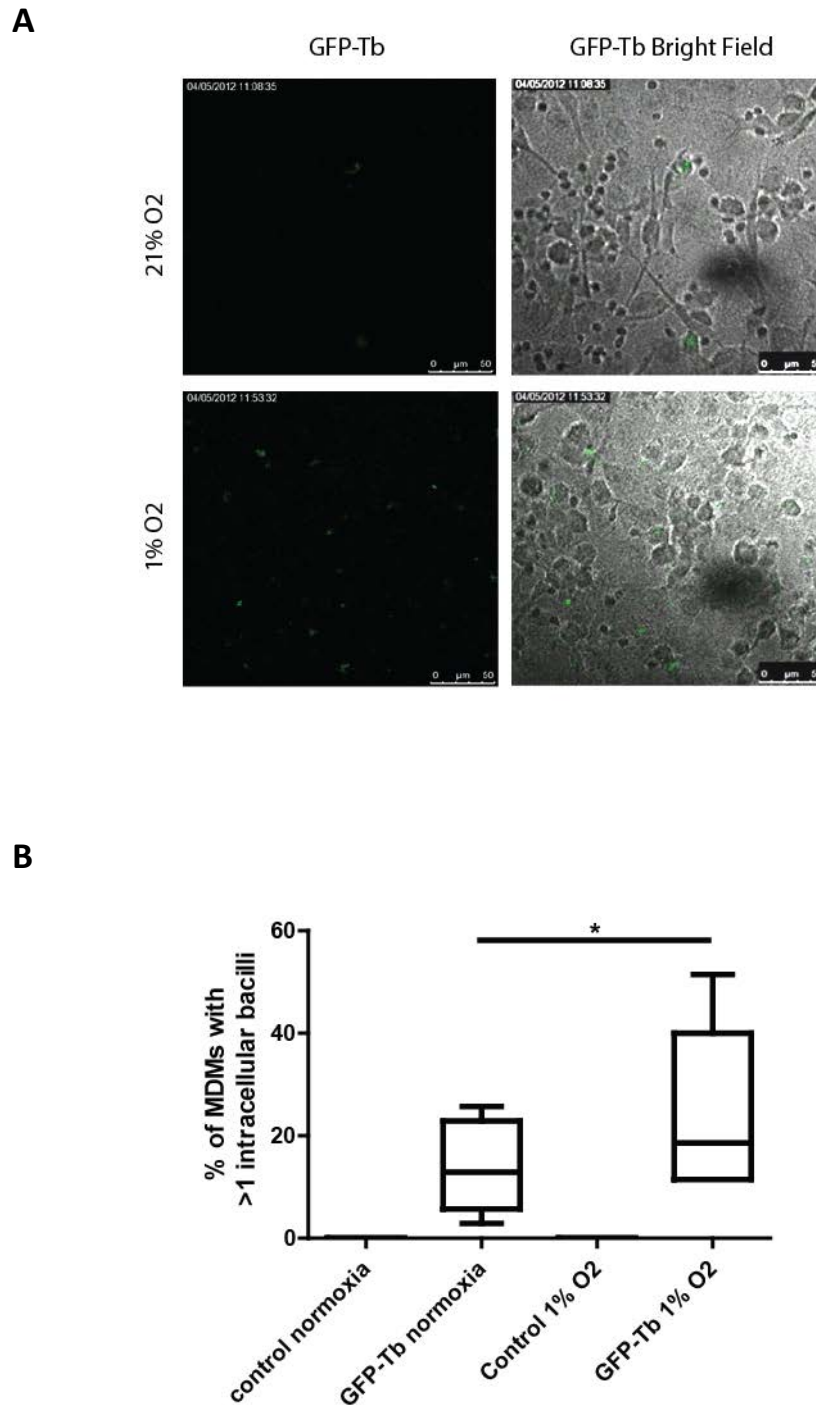


Figure 42: Effect of hypoxia (1% O₂) on phagocytosis of GFP-Tb at 2 hrs. MDMs were directly infected with GFP-Tb (MOI=1). Cells were fixed and GFP-Tb killed with 4% paraformaldehyde after 2hrs. x10 random fields were assessed for each condition by two independent investigators and average percentages of intracellular bacilli per field were recorded. **(A)** Representative images for permanox wells incubated in normoxia (21% O₂) and hypoxia (1% O₂). **(B)** Hypoxia increases the percentage of intracellular GFP-Tb bacilli 2 hrs post-infection (p<0.05). Data representative from 2 independent experiments.

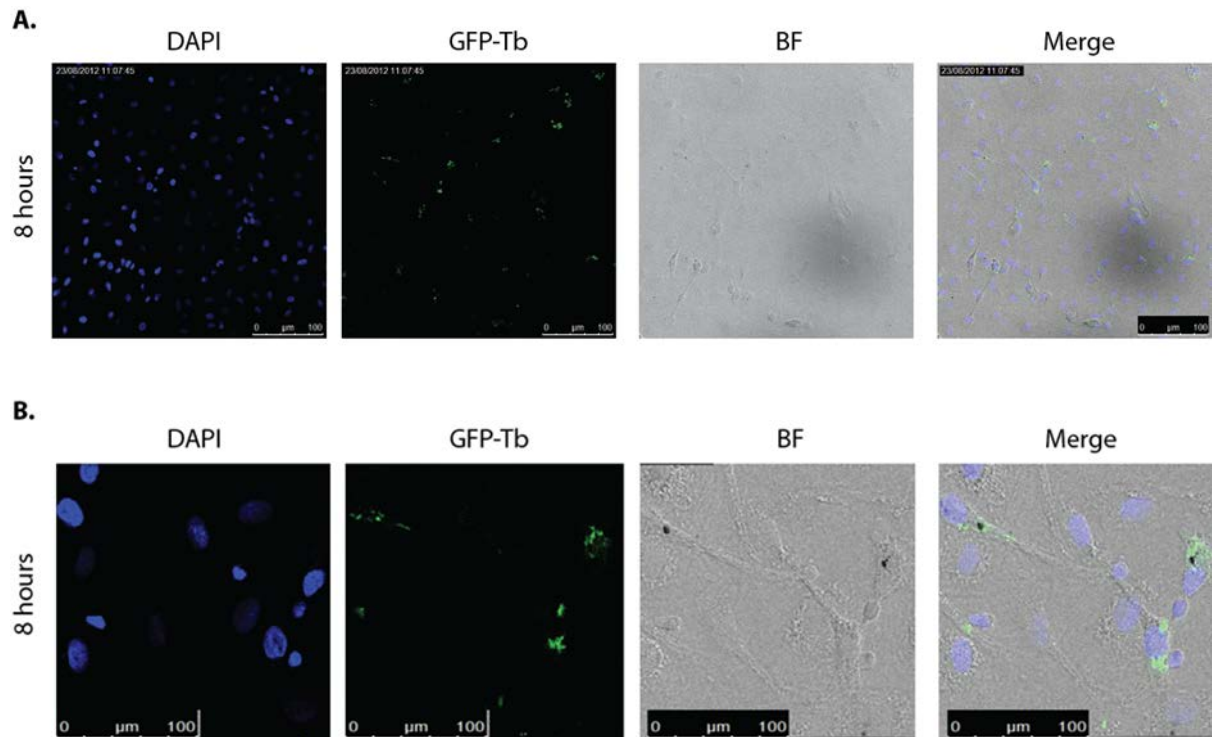
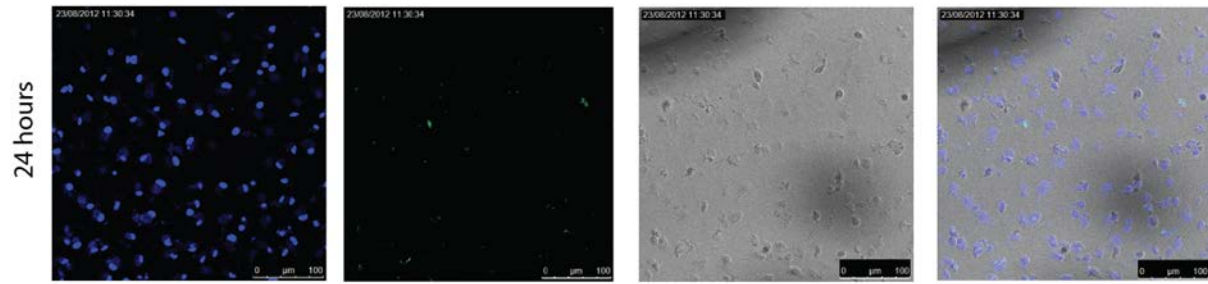


Figure 43: Phagocytosis of GFP-Tb at 8hrs in normoxia. Representative imaged of intracellular bacilli 8 hrs post-infection. **(A)** Nuclei are stained with DAPI (far left), GFP-Tb (middle left), brightfield (middle right) and merged images (far right). **(B)** Higher powered images reveal that individual MDMs are frequently infected with more than one bacilli.

A



B

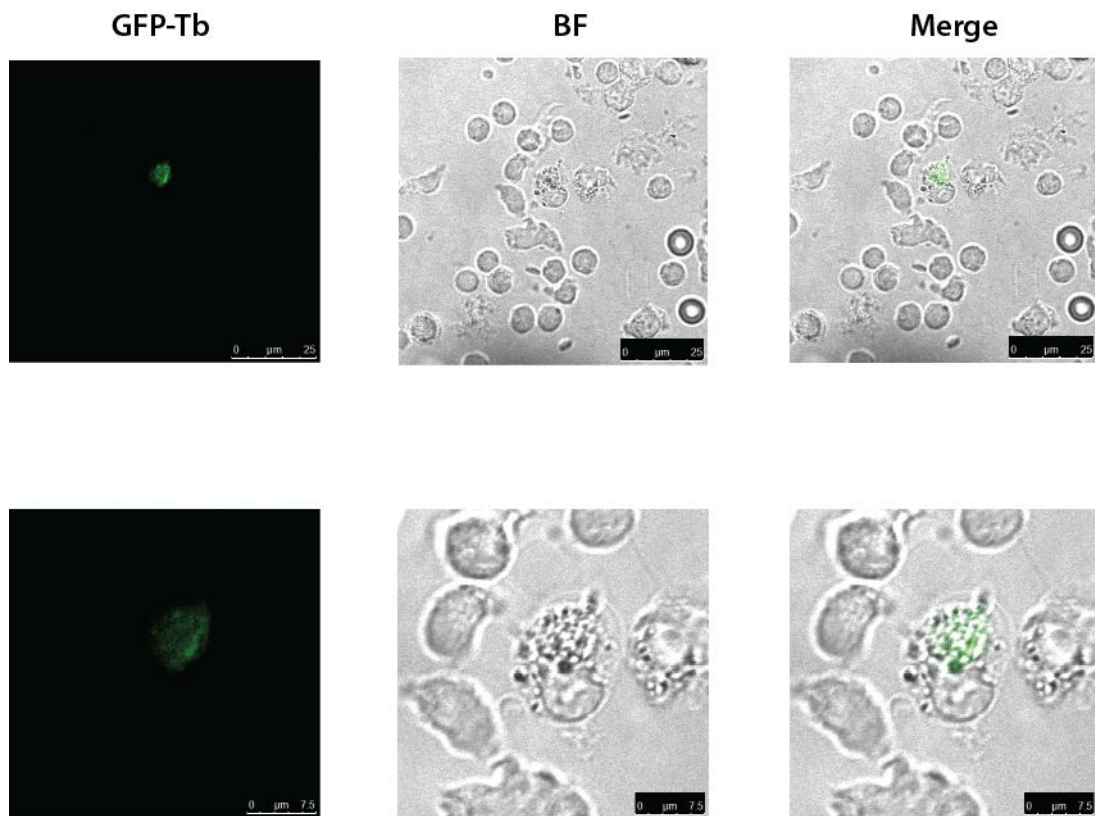


Figure 44: Phagocytosis of GFP-Tb at 24hrs in normoxia. Representative imaged of intracellular bacilli 24 hrs post-infection. **(A)** Nuclei are stained with DAPI (far left), GFP-Tb (middle left), brightfield (middle right) and merged images (far right). **(B)** Higher powered images reveal that GFP signal is significantly altered 24 hrs post-infection, and becomes more diffuse.

Further review of the scientific literature revealed that this result might have been predicted. Green fluorescent protein is a highly fluorescent protein of the bioluminescent jellyfish *Aequorea victoria*. This 238 amino-acid is present as a monomer in solution and emits green light upon excitation with ultraviolet or blue light [188]. This step fundamentally requires molecular oxygen to dehydrogenate the α,β bond of residue 66 and is the slowest step in GFP chromophore formation with a time constant of 2 to 4 hrs *in vivo* for wild-type GFP [189]. It is likely therefore that I was able to visualise GFP-TB at my 2 hr timepoint and not at 8 and 24 hrs because of this reason.

An alternative to GFP-TB I considered was auramine-rhodamine staining but given the difficulty in quantifying intracellular TB in macrophages, I opted to investigate intracellular infection by other methods.

Increased MMP-1 secretion during *M.tb* infection in hypoxia does not require bacterial replication

My first step was to establish whether hypoxia affected the intracellular replication of *M.tb* within MDMs. In order to do this, I infected MDMs with *M.tb* at MOI=1. In keeping with all my previous experiments, cell culture supernatants were replaced at 2 hrs post-infection with fresh medium. This step is standard to many laboratory infection protocols involving bacteria. It has also been tested many times within the JSF laboratory and shown to produce reproducible rates of *M.tb* infection. Cells were lysed at 72hrs following incubation in either 21% or 1% oxygen. Colony counting was then performed after 3 weeks. Hypoxia did not affect the number of viable bacilli indicating that hypoxia had no significant effect on the intracellular replication of *M.tb* (Figure 45A). Since hypoxia had no effect on

intracellular replication, I then investigated whether the increase in MMP-1 secretion seen in *M.tb* infected MDMs required the presence of actively replicating mycobacteria. Previously published data from the JSF group demonstrates that increased MMP-1 secretion is seen when MDMs are stimulated with either uv-killed *M.tb* and *M.tb* supernatant, both of which contain no live bacteria [59]. Hypoxia (1% O₂) increased MMP-1 protein secretion in MDMs stimulated with either uv-killed *M.tb* or *M.tb* supernatant (Figure 45B; $p < 0.001$). Collectively, these results demonstrate that hypoxia upregulates MMP-1 expression in *M.tb* stimulated cells and this process does not require bacterial replication.

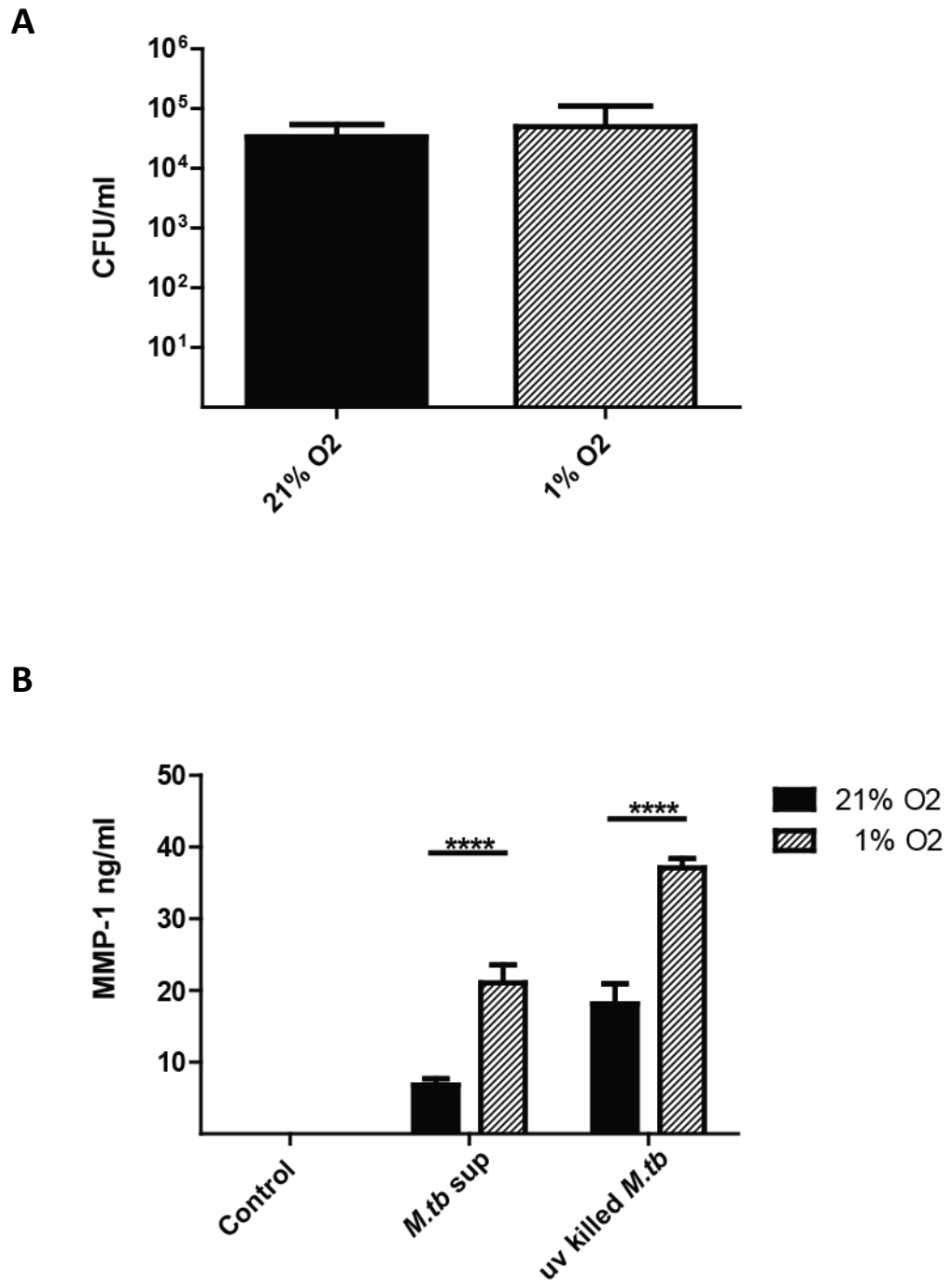


Figure 45: Effect of hypoxia (1% O₂) on intracellular replication of *M.tb* and MMP-1 secretion following “non-live” *M.tb* stimulation of MDMs. MDMs were infected with *M.tb* (MOI=1). Cell lysates were collected at 72 hrs and plates on 7H9 agar plates. Colony counting was performed at 3 weeks. **(A)** Hypoxia had no effect on *M.tb* CFU’s. MDMs were stimulated with either *M.tb* supernatant or uv-killed *M.tb*. Supernatants were harvested at 72 hrs and MMP-1 protein measured by ELISA. **(B)** Hypoxia augments MMP-1 secretion ($p < 0.001$) from MDMs stimulated with “non-live” *M.tb*. Bars represent mean ($\pm$ SD) of conditions in triplicate and are representative of at least 2 independent experiments.

Inhibition of NFκB causes decreased MMP-1 secretion in *M.tb*-infected MDMs incubated in hypoxia or with DMOG

My results from respiratory epithelial cells indicated a pivotal role of NFκB during *M.tb* infection in hypoxia. NFκB is known to critically regulate MMP activity in CNS TB infection [61] but there has been no investigation of its role in the presence of hypoxia and *M.tb* infection. I therefore proceeded to explore if NFκB signalling was important in regulating MMP-1 secretion in human MDMs directly infected with *M.tb*. In order to this I used the chemical inhibitor helenalin, shown to inhibit NFκB in previous work by our group[190]. HIF signalling was activated using both DMOG and hypoxia at 1% O₂.

Cells were pre-incubated with DMOG (0.25mM) and helenalin (1.0μM) for 2 hrs and then infected with *M.tb* (MOI=1). Helenalin caused a decrease in MMP-1 secretion in cells treated with DMOG (Figure 46A; p<0.001.) Dose-response experiments demonstrated this finding even at higher doses of helenalin in the presence of DMOG (Figure 46B). Helenalin caused a decrease in MMP-1 secretion from *M.tb*-infected cells but this decrease was not statistically significant (Figure 46B).

When *M.tb* infected cells were incubated in hypoxia (1% pO₂), helenalin caused a highly significant decrease in MMP-1 secretion compared to cells incubated in normoxia (Figure 46C; p<0.001). Collectively these results indicate that NFκB signalling might form a key role in hypoxia-driven MMP-1 secretion during *M.tb* infection.

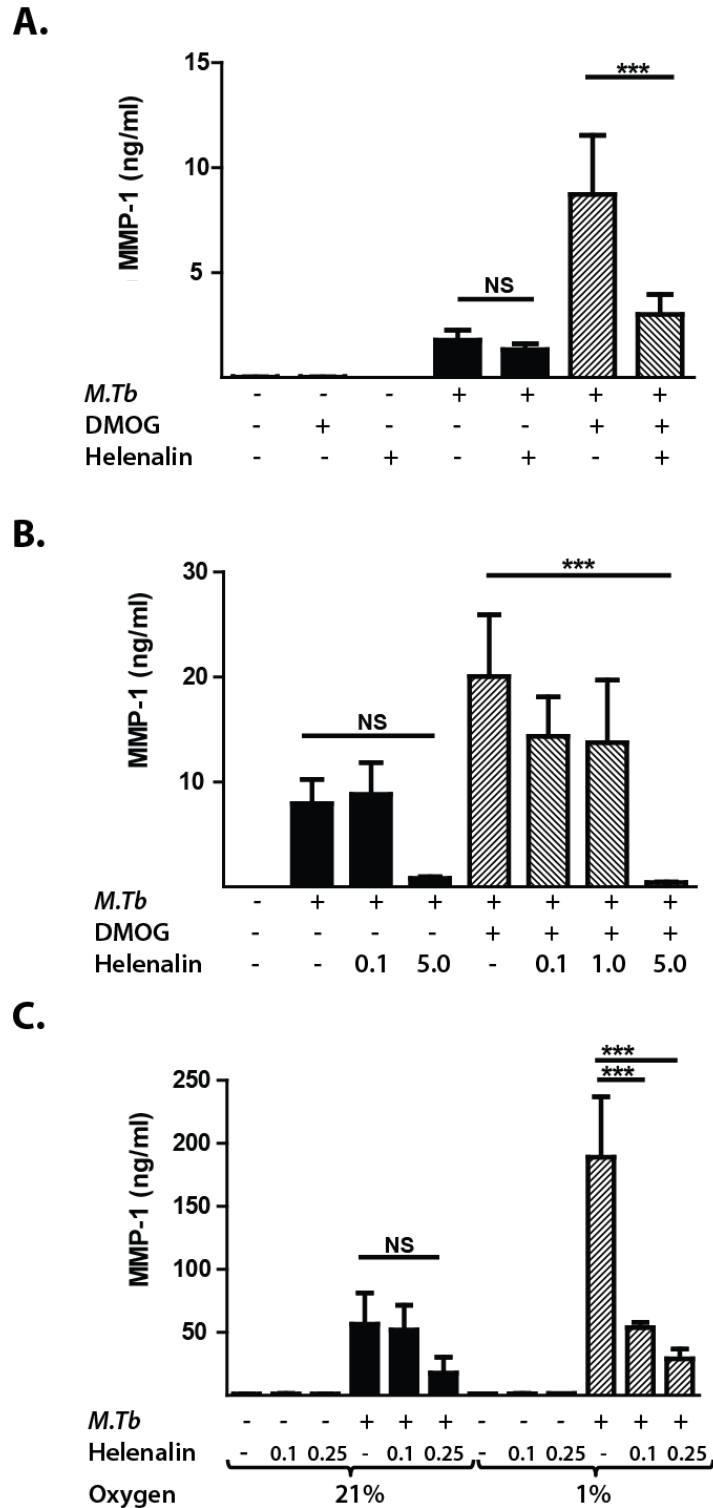


Figure 46: Effect of helenalin on MMP-1 secretion in *M.tb*-infected MDMs treated with DMOG or incubated in hypoxia (1% O₂). (A) Helenalin (1.0μM) decreases MMP-1 secretion from *M.tb*-infected MDMs pre-incubated with DMOG (0.25mM). (B) p65 subunit inhibition causes decreased MMP-1 secretion in *M.tb*-infected MDMs, but this effect does not reach statistical significance compared to DMOG treated cells. (C) Helenalin causes a highly significant decrease in *M.tb*-infected MDMs incubated in hypoxia. Bars represent mean (+/- SD) of conditions in triplicate and are representative of at least 2 independent experiments.

Discussion

The results of my investigation in MDMs showed that MMP-1 gene expression and secretion were significantly increased and was functionally active in *M.tb*-infected human macrophages in hypoxia compared to normoxia and, there was no compensatory increase in the inhibitor TIMP-1. Consistent with my findings in respiratory epithelial cells, NF- κ B signalling pathways were found to regulate MMP-1 secretion in hypoxia. Additionally, 10% O₂ causes decreased secretion of MMP-1.

Diverse lines of investigation are implicating MMP-1 as a key protease in TB pathology. MMP-1 causes collagen destruction in *M.tb*-infected transgenic mice [59] and *M.tb* infection upregulates MMP-1 more potently than *M.bovis* BCG in human macrophages [183]. In human TB granulomas, MMP-1 expression is up-regulated 606-fold compared to uninfected lung [191]. Similarly, MMP-1 was the most potently upregulated gene in macrophages from patients who developed TB compared with those with latent disease [23].

My results revealed increased proteolytic activity in MDMs directly infected with *M.tb* which was markedly increased by both hypoxia and DMOG. Zymography is a semi-function assay of caseinolytic activity and gives an indication of total proteolytic activity. Subsequent to these experiments, other techniques have been employed within our group to demonstrate collagen breakdown for example using DQ collagen. Increased collagen degradation is seen in neutrophils and monocytes directly infected with *M.tb* (data unpublished). Further experiments might have included the assessment of collagen degradation using this technique both in normoxia and hypoxia and whether this corresponded to infected cells

(using auramine-rhodamine to stain *M.tb*). Since my experiment involved maturing monocytes to macrophages over 5 days, a series of further experiments would be needed to assess if maturation on collagen affected this process and additionally, the effect on MMP-1 secretion. An alternative approach would be to assess MMP-1 activity in normoxia and hypoxia from monocytes. Since MMP activity is normally measured at 24hrs or less in *M.tb* infection, the effect of seeding on collagen is likely to be reduced. Hypoxia has been shown to prolong monocyte survival and thus might act as a confounder in this experiment [192]

I found divergent MMP-1 and MMP-7 responses in response to DMOG and hypoxia (1% O₂). Although I did not examine the potential mechanisms by which this process occurs, there is evidence that MMP-1 and MMP-7 secretion from macrophages occurs by differing processes. For example, *M.Tb*, but not important proinflammatory cytokines involved in orchestrating the host immune response, increase MMP-1 secretion from macrophages whereas MMP-7 expression and secretion can be stimulated by TNF- α alone [183]. In addition, divergent MMP-1 and MMP-7 response occur in response to TNF- α stimulation and steroid treatment suggests that the upstream regulation of these MMPs differs [59].

Although my confocal experiments using GFP-Tb were less successful, owing to the oxygen responsive elements of green florescent protein, I demonstrated that not all MDMs became infected with *M.tb*. It is interesting therefore that the increased MMP-1 staining seen in *M.tb* infection and hypoxia was almost universal. This demonstrates that direct *M.tb* infection is unlikely to be the only mechanism driving MMP-1 production and that TB-networks might be exerting an effect. I showed that increased MMP-1 secretion in hypoxia from *M.tb*-infected MDMs was not dependent on actively replicating bacilli and my findings

are supported by recently published data investigating the interaction between macrophages and hypoxia. Nickel *et al.* demonstrate that hypoxia (1% O₂) at increasing MOIs does not affect *M.tb* colony forming units after 4 days of infection.

I attempted to perform HIF-1 α staining using confocal microscopy but this was not successful. My initially experiments were performed using antibodies against HIF-1 α in addition to MMP-1 antibodies. Care was taken to ensure that there was no cross-reactivity between the antibodies and that the colour channels were sufficiently distinct, but I was unable to detect HIF-1 α staining by confocal microscopy. One possible explanation is the time-point selected. MMP-1 staining was performed at 72hrs which is the maximal time point for MMP-1 protein secretion in MDMs however, since HIF-1 α stabilisation frequently occurs within hours of exposure to pro-inflammatory stimuli, this time point might have been too late. Recently published data reveals early upregulation of HIF targets genes in response to *M.Marinum* infection in a zebrafish model [110] suggesting I may have selected the incorrect time point. The experiment might therefore have been improved by performing HIF-1 α kinetic experiment using confocal microscopy as well as assessing HIF-1 α staining independently of MMP-1 staining.

The role of NF κ B was investigated using chemical inhibitors and in consistent with my findings in respiratory epithelial cells, NF- κ B inhibition decreases MMP-1 secretion in M.tb-infected cells in hypoxia (or pre-incubated with DMOG). I only used helenalin to inhibit the p65 subunit of NF- κ B but would like to have inhibited other subunits. An alternative approach would be to transfect MDMs with NF- κ B subunits based on the results of the chemical inhibitor experiments. Transfection of MDMs had not been performed

successfully within our laboratory but following the results using HIF-1 α siRNA (see Chapter 6) it would certainly now be possible to undertake these experiments.

I showed that incubating *M.tb*-infected MDMs at 10% O₂ decreased MMP-1 secretion compared to incubation at 21% O₂. The finding of neutralisation of pH was extremely interesting as *M.tb* is known to evade host immune cells by effecting mechanisms which neutralise pH within phagocytic cells [193]. Research is ongoing in this area within the JSF group and might provide insights into the mechanisms by which 10% O₂ decreases MMP activity,

In conclusion, hypoxia is a positive stimulus for MMP expression in MDMs, potentially allowing *M.tb*, an aerobic bacterium, to escape the hypoxic environment of the granuloma and spread to new hosts.

CHAPTER 6: HYPOXIA AND THE ROLE OF HIF DURING *M.tb* INFECTION

Introduction

Activation of HIF during bacterial infection has been described in several studies with increased levels of HIF-1 α demonstrated in response to *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* [89]. In addition, HIF stabilisation has been described in a number of viral and parasitic infections [64].

Pathogen	Effect on HIF stabilization	Effect of altered HIF on disease pathogenesis
Bacteria		
<i>Streptococcus pyogenes</i>	Increases	Activation of phagocyte killing
<i>Streptococcus agalactiae</i>	Increases	Activation of phagocyte killing
<i>Pseudomonas aeruginosa</i>	Increases	Activation of phagocyte killing
<i>Salmonella typhimurium</i>	Increases	Activation of phagocyte killing
<i>Bartonella henselae</i>	Increases	Angiogenesis and vascular lesions
<i>Chlamydia pneumoniae</i>	Decreases	Promotes intracellular survival of bacterium
Bacterial components		
Lipopolysaccharide	Increases	Phagocyte activation, but with high systemic doses, cytokine storm and sepsis syndrome occur
Siderophores	Increases	Activation of mucosal defences
Viruses		
Respiratory syncytial virus	Increases	VEGF release and airway oedema
Vesicular stomatitis virus	Increases	Type I interferon production and cellular resistance to virus
Hepatitis B virus	Increases	Neovascularization and oncogenesis
Hepatitis C virus	Increases	Neovascularization and oncogenesis
Human papilloma virus	Increases	Neovascularization and oncogenesis
Human herpesvirus 8	Increases	Reactivation of virus from latency
Parasites		
<i>Toxoplasma gondii</i>	Increases	Preserves viability of infected host cell
<i>Leishmania amazonensis</i>	Increases	Development of skin ulcers

HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

Table 6: Infectious organisms and effect on HIF. Adapted Nizet V. *Nature* Sept 2009: Vol 9. No.9

Interestingly, some studies have shown that bacterial exposure can be a more potent stimulus for HIF-1 α than hypoxia itself, and bacteria-induced HIF-1 α stabilisation can occur readily under conditions of normoxia [86].

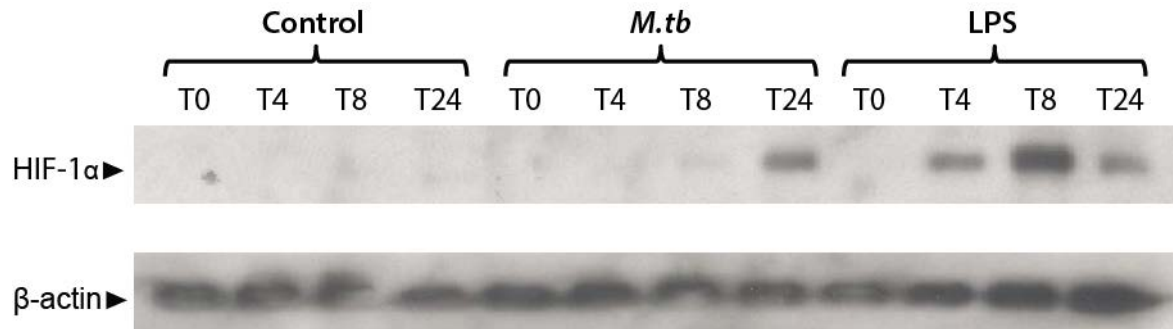
Although investigation of HIF activity has been conducted in several infective processes, the presence or role of HIF during TB infection has not been previously investigated. Following my finding of severe hypoxia within TB lesions in patients, I investigated the role of HIF-1 α during Tb infection *in vitro* and in patients with a confirmed diagnosis of pulmonary *M.tb* infection.

Results

***M.tb* infection drives HIF-1 α stabilisation in MDMs**

My initial investigation of HIF-1 α activity during *M.tb* infection involved determining whether HIF-1 α stabilisation occurred during direct infection of MDMs. LPS (100ng/ml) was used as a positive control since it is known to stabilise HIF-1 α maximally at 8hrs in primary MDMs [187]. Direct infection with *M.tb* (MOI=1) caused in HIF-1 α stabilisation at 24hrs compared to 4hrs with LPS (Figure 47A). This is the first time that *M.tb*-driven HIF-1 α stabilisation has been described. I then proceeded to determine the kinetics of HIF-1 α stabilisation in *M.tb* infected MDS. HIF-1 α stabilisation occurred maximally at 24hrs and declined thereafter (Figure 47B). By comparison, some HIF-1 α accumulation was seen in control cells from 24hrs which persisted to 72hrs.

A.



B

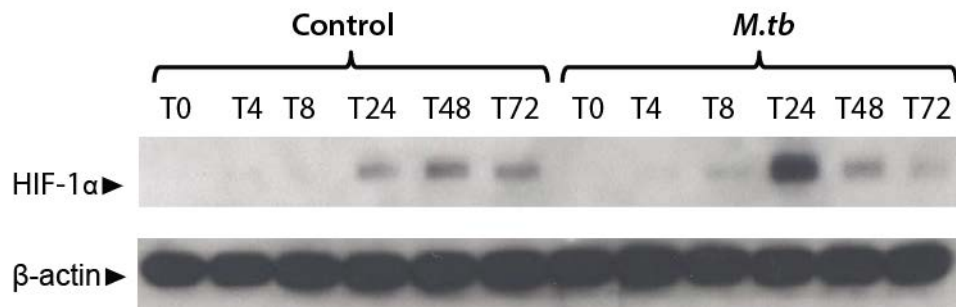


Figure 47: HIF-1α western blotting of MDMs directly infected with *M.tb*. MDMs were infected with *M.tb* (MOI=1). Cells were incubated at 21% O₂ and 5% O₂ and lysed at serial time points to determine HIF-1α kinetics. **(A)** *M.tb* infection drives accumulation from 8 hrs post-infection. Maximal accumulation occurs at 24 hrs. LPS (100ng/ml), used as a positive control, drives HIF-1α accumulation from 4 hrs and is more sustained. **(B)** Maximal HIF-1α stabilisation occurs at 24 hrs post-infection. Blots are representative of at least 2 independent experiments

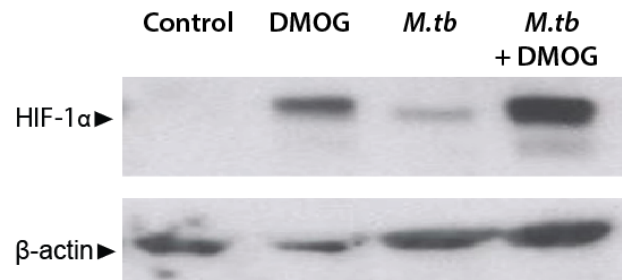
DMOG and hypoxia (1% pO₂) drive stabilisation of HIF-1 α

I then proceeded to investigate the effect of DMOG and hypoxia (1% O₂) on *M.tb*-driven HIF-1 α accumulation. Pre-incubation of MDMs with DMOG (0.25mM) for 2 hours prior to *M.tb* infection resulted in an increase in HIF-1 α protein compared to DMOG alone (Figure 48A). Next I examined the effect of hypoxia (1% pO₂) on *M.tb*-driven HIF-1 stabilization. Since hypoxia driven HIF-1 α protein is known to degrade within two minutes of exposure to ambient air [194], it was necessary to conduct these experiments within a custom designed hypoxia workstation (Coy Laboratories, Michigan, USA) The presence of hypoxia (1% O₂) caused prolonged and increased HIF-1 α accumulation in MDMs infected with *M.tb* compared to cells incubated in normoxia (Figure 48B). HIF-1 α stabilisation occurred as early as 4 hours in cell incubated in hypoxia compared to 24hrs in normoxia. Collectively, these results demonstrate that direct *M.tb* infection results in HIF-1 α accumulation in both normoxia and hypoxia.

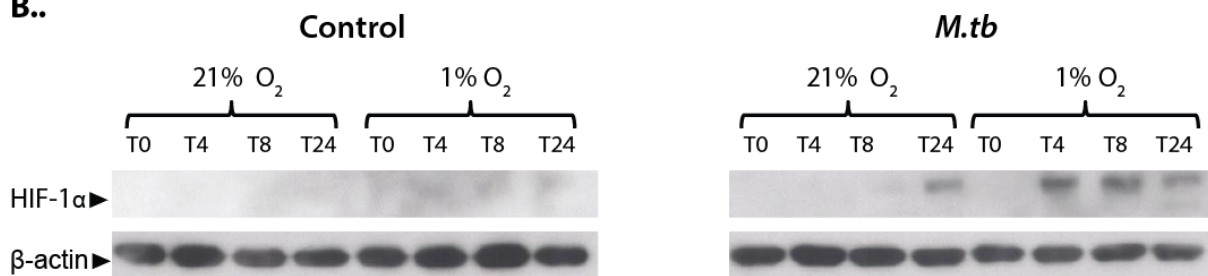
HIF-1 α stabilisation is not specific to *M.tb* infection

As part of the investigation of HIF-1 α activity in TB infection, I was keen to determine if there were any TB specific components driving this stabilisation. In particular, if these might be related to virulence factors ESAT-6 and CFP-10, which are present in *M.tb* but absent in BCG. MDMs from the same donor were plated and cells infected at MOI=1 with either *M.tb* or BCG. HIF-1 α western blotting demonstrated a faint band corresponding to HIF-1 α at 8hrs in and 24hrs in *M.tb* infected cell in normoxia. By comparison, cells incubated in hypoxia demonstrated evidence of HIF-1 α stabilisation at 8hrs in both *M.tb* infected cells and those infected with BCG (Figure 48C).

A.



B..



C.

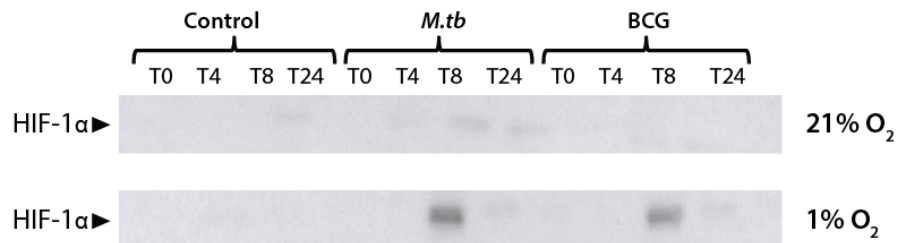


Figure 48: Effect of DMOG and hypoxia (1% O₂) on HIF-1α stabilisation in MDMs infected with *M.tb* and BCG. (A) MDMs were pre-incubated with DMOG (0.25mM) for 2 hrs and then infected with *M.tb* (MOI=1). Cells were incubated in normoxia (21% O₂). Lysates were harvested 8 hrs post-infection. DMOG and *M.tb* co-infection causes a marked increase in HIF-1α accumulation compared to either DMOG or *M.tb* infection alone. (B) MDMs were incubated at either 21% O₂ or 1% O₂ within a hypoxic workstation. 1% O₂ causes earlier and more prolonged HIF-1α stabilisation in *M.tb* infected MDMs compared to cells incubated at 21% O₂. (C) Both *M.tb* and BCG infection (MOI=1) causes stabilisation of HIF-1α in hypoxia (1% O₂) 8 hrs post-infection. Blots are representative of at least 2 independent experiments.

CoMTb and DMOG cause HIF-1 α stabilisation in A549 cells but not NHBEs

I then proceeded to investigate whether HIF-1 α stabilisation occurred as a consequence of direct infection alone or also occurred via TB-networking effects. In A549 respiratory epithelial cells stimulated with CoMTb, HIF-1 α induction was present in cells from 6 hours even in the absence of hypoxia or DMOG exposure (Figure 49A). As would be predicted, DMOG stabilized HIF-1 α , but this effect was markedly increased by concurrent stimulation with CoMTb. Investigation in primary cells (NHBEs) produced much less clear results. NHBEs exhibited high basal levels of HIF-1 α and pre-incubation with DMOG and/or stimulation with CoMTb stimulation resulted in reduced HIF-1 α levels (Figure 49B). It is possible that the high number of growth factors present supplemented in the NHBE growth medium might be driving HIF-1 α accumulation therefore the effect of DMOG and/or CoMTb are minimal.

These results confirm that during *M.tb* infection, HIF-1 α stabilization occurs both by direct infection in MDMs and via an *M.tb* networking effect (using CoMTb) in A549 respiratory epithelial cells.

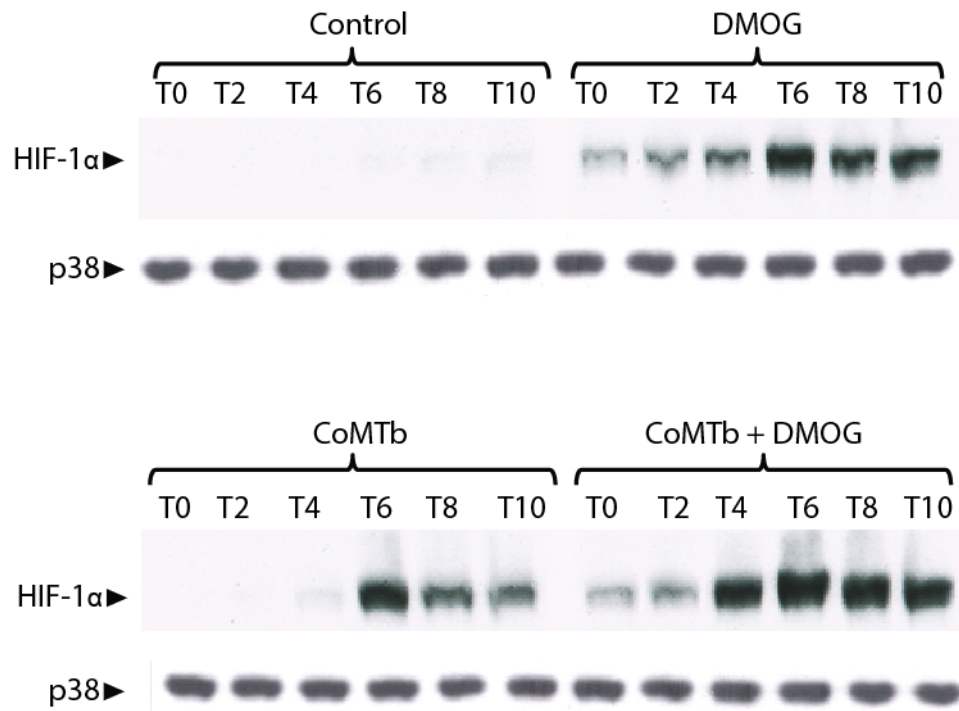
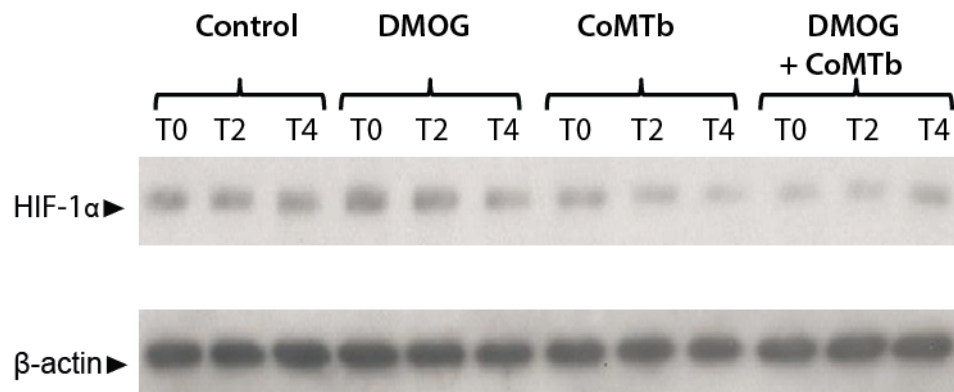
A.**B.**

Figure 49: Effect of DMOG on HIF-1α accumulation in respiratory epithelial cells stimulated with CoMTb. Respiratory epithelial cells (A549 and NHBEs) were pre-incubated with DMOG (0.25mM) for 2 hrs and then stimulated with CoMTb (1:5). Cell lysates were harvested at various time points to determine HIF-1α kinetics. **(A)** Both DMOG and CoMTb stimulation cause stabilisation of HIF-1α in A549 cells. Maximal accumulation occurs at 6hrs. Co-stimulation causes a cumulative increase in HIF-1α stabilisation. **(B)** NHBE cells display high resting levels of HIF-1α. CoMTb and DMOG (0.25mM) cause decreased HIF-1α accumulation at 4 hrs post-stimulation. Blots representative of at least 2 independent experiments.

Protocol re-optimisation

At this point during my investigations of HIF-1 α , the ECL reagent supplied by GE Healthcare was discontinued and replaced with ECL-Prime, a significantly more sensitive reagent. The consequences of this were that although the developing process became more sensitive, the assay was frequently too sensitive and detected significant amounts of background protein. Initially, I attempted to shorten exposure time but a reduction from 4 minutes to 2 seconds still resulted in uninterpretable blots. Our group tested a number of alternative commercially available developing reagents but all proved too insensitive. After a number of re-optimisation experiments involving reducing the amount of protein loaded, diluting both the primary and secondary antibodies and lengthening times of protein blocking steps I was able to perform western blot analysis of interpretable quality. Another key factor that arose from these optimisation experiments was that HIF-1 α is highly sensitive to freeze-thaw cycles with almost no HIF-1 α present following x1 freeze-thaw cycle. The same was not true of subsequent investigation of the PHDs which tolerated up to x4 freeze-thaw episodes.

Role of HIF-2 α during *M.tb* infection is indeterminate

Having investigated HIF-1 α activity in both direct and indirect infection I then proceeded to investigate if *M.tb* infection caused stabilisation of HIF-2 α . I performed multiple experiments and western blots to examine HIF-2 α activity in MDMs directly infected with *M.tb*. I was able to detect evidence of HIF-2 α activity on one occasion where I detected a faint band in hypoxia (Figure 50). Unfortunately, I was not able to reproduce these results on any further experiments despite multiple modifications to my Western blot protocol e.g. loading more protein and increasing antibody concentrations.

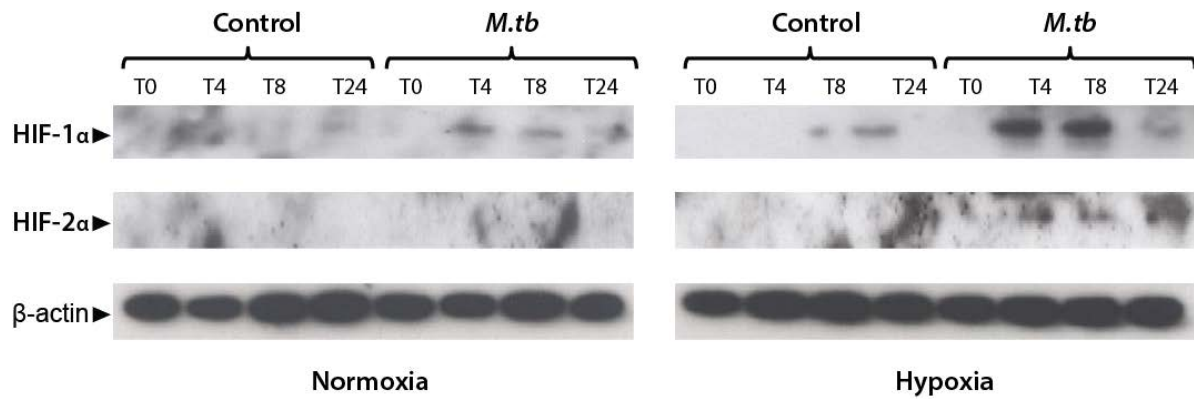


Figure 50: Effect of hypoxia (1%) on HIF-2α stabilisation in MDMs infected with *M.tb*. MDMs were directly infected with *M.tb* (MOI=1) and incubated in either normoxia (21% O₂) or hypoxia (1% O₂ hypoxia workstation). Cell lysates were harvested at various time points to determine HIF-1α and HIF-2α kinetics. Hypoxia causes increased and earlier accumulation of HIF-1α in MDMs infected with *M.tb* compared to cells incubated at 21% O₂. Hypoxia causes a faint band on western blot corresponding to HIF-2α from 4 hrs post-infection until 24 hrs post-infection. Blots from one experiment.

***M.tb* infection increases prolyl hydroxylase activity in normoxia and PHD1 and PHD2 only in hypoxia**

Next I sought to investigate the activity of the prolyl hydroxylases in response to *M.tb* infection both in normoxia and hypoxia. My aims were to ascertain if the PHD response to *M.tb* were altered compared to control uninfected cells and if so, was this response different if the cells were incubated in hypoxic conditions.

Investigation comparing control cells to those infected with *M.tb* in normoxia demonstrated that cells infected with *M.tb* demonstrated greater induction of PHD1, PHD2, PHD3 and FIH (Figure 51). Membranes were stripped and re-probed for the PHDs to try and control for differences in protein loading. FIH was run of a separate western blot and may have overheated given the bands seen. These differences were seen even at T0 which is interesting since the cells were seeded and retrieved from the same tissue culture plate. These changes suggest that the PHD response to *M.tb* infection occurs very rapidly, possibly within minutes.

When responses in normoxia were compared to responses in hypoxia, there was marked differences both between control and *M.tb* infected cells and between each condition in normoxia and hypoxia. Initially comparing the control cells in normoxia to control cells in hypoxia revealed that PHD1 activity was relatively unaltered (taking in to account the high background interference on the blot). The activity of PHD2 was markedly increased in hypoxia particularly at 24hrs. By comparison, the activity of PHD3 and FIH were relatively unaltered by hypoxic conditions.

When HIF-1 α levels from *M.tb* infected cells incubated in normoxia were compared to cells incubated in hypoxia there was once again differential expression of PHDs. PHD1 and PHD2 levels were greater at 24 hrs in *M.tb* infected cells incubated in hypoxia than cells incubated at normoxia. Levels of PHD3 in *M.tb* infected MDMs incubated in hypoxia were either unchanged or slightly decreased at 24hrs whilst FIH was decreased in hypoxia. Repeat experiments demonstrated the same changes occurring with PHD responses whilst no significant difference has noted in FIH activity.

Collectively, these results demonstrate that in addition to driving HIF-1 α stabilisation, *M.tb* infection causes changes in expression of PHD1 and PHD2, with the most significant increases occurring in hypoxic conditions.

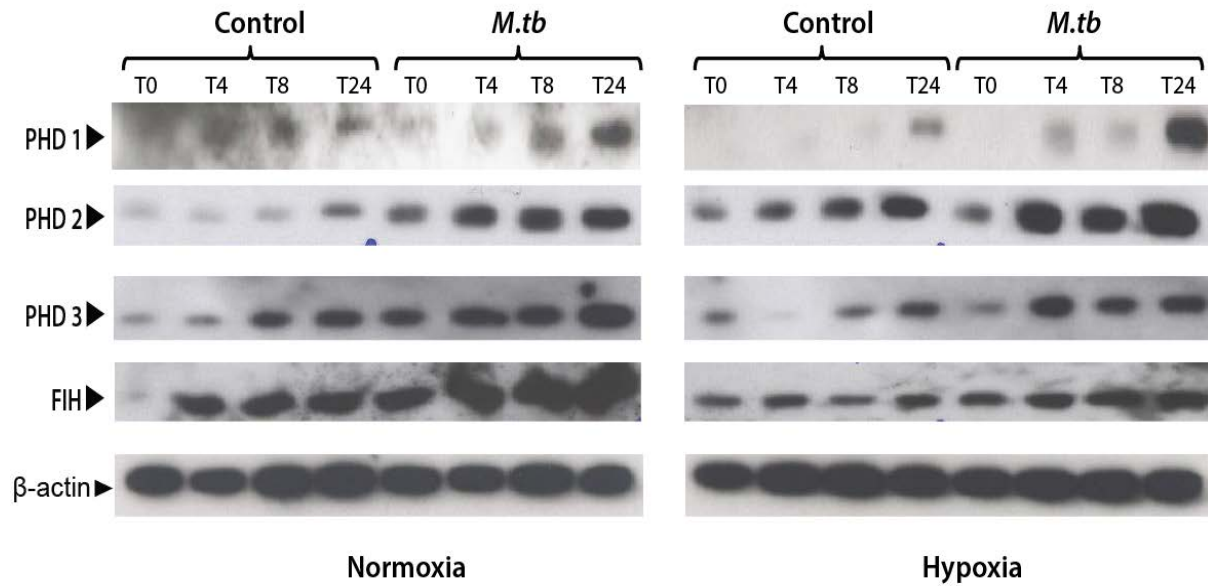


Figure 51: Investigation of effect of hypoxia on prolyl hydroxylase activity in response to *M.tb* infection. MDMs were directly infected with *M.tb* (MOI=1) and incubated in either normoxia (21% O₂) or hypoxia (1% O₂, hypoxia workstation). Cell lysates were harvested at 0, 4, 8 and 24 hrs. *M.tb* infection causes increased levels of PHD1, PHD2 and PHD3 protein levels on western blot compared to control cells. Hypoxia causes increased protein levels of PHD2 in control cells compared to cells incubated in normoxia. 1% O₂ causes increased levels of PHD1 and PHD2 in *M.tb*-infected MDMs compared to cells incubated at 21% O₂. Representative blots taken from one experiment performed independently on three occasions.

HIF-1 α inhibition decreases MMP-1 secretion in *M.tb* infected MDMs incubated in hypoxia

Having demonstrated that *M.tb* infection drives HIF-1 α , I then sought to determine whether inhibiting HIF-1 α impacted on MMP-1 activity during TB infection. Initially, I used LW6 chemical inhibitor (Merck Millipore, UK) to inhibit hypoxia-induced accumulation of cellular HIF-1 α protein in MDMs directly infected with *M.tb*. Although LW6 caused a decrease in *M.tb* driven MMP-1 secretion in human MDMs in normoxia, this result did not reach statistical significance (Figure 52; $p>0.05$). In contrast, inhibition of HIF-1 α activity in hypoxia caused a highly significant decrease in MMP-1 expression in a dose-dependent manner. Cell toxicity was assessed by trypan blue exclusion assay and since significant cell death was seen at doses $>100\mu\text{mol}$, these results were excluded from the final analysis.

Since chemical inhibitors can be associated with non-specific off-target effects, I then proceeded to use HIF1 α siRNA to inhibit HIF-1 α activity. I was assisted in these experiments by my colleague Sara Brilha. In order to assess transfection efficiency, MDMs were transfected with FAM conjugated NT siRNA and analysed by flow cytometry. Transfection efficiency was confirmed as 80.4% (Figure 13). Knockdown of HIF-1 α mRNA was confirmed to be 73% in hypoxia compared to *M.tb* infected cells. Interestingly, there was little upregulation of HIF mRNA by *M.tb* infection compared to control either in normoxia or hypoxia. This finding suggests that most of the increased HIF-1 α activity associated with *M.tb* infection might be occurring at the post-transcriptional level.

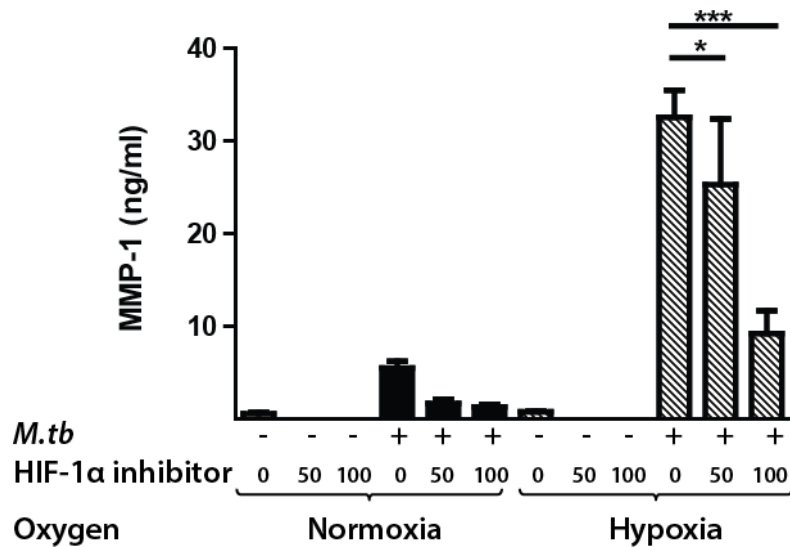


Figure 52: Effect of HIF-1 α chemical inhibition on MMP-1 secretion in *M.tb*-infected MDMs. MDMs were pre-incubated with LW6 (50 and 100 μ M) for 2 hrs and then directly infected with *M.tb* (MOI=1). Cells were incubated in normoxia (21% O₂) or hypoxia (1% O₂). Supernatants were harvested at 72 hrs post-infection. Inhibition of HIF-1 α decreases MMP-1 secretion in *M.tb*-infected MDMs in normoxia but this result does not reach statistical significance. HIF-1 α chemical inhibition causes a dose-dependent decrease in MMP-1 protein secretion in *M.tb*-infected MDMs incubated in hypoxic conditions. Bars represent mean (+/- SD) of conditions in triplicate and are representative of at least 2 independent experiments. **p<0.01, *p<0.05

HIF-1 α western blot demonstrated almost complete knockdown of HIF-1 α protein in *M.tb* infected MDMs in normoxia and significant, although not complete knockdown in hypoxia (Figure 53B). Consistent with the gene expression data, transfection of MDMs with non-targeting siRNA had no impact on HIF-1 α levels.

Next, I assessed whether inhibiting HIF-1 α using HIF-1 α siRNA affected MMP-1 activity. HIF-1 α siRNA caused a significant decrease in MMP-1 expression at the level of both gene expression and protein secretion. In normoxia, although a decline in MMP-1 mRNA levels was seen, this result did not reach statistical significance (Figure 54A). There was however a significant decrease in MMP-1 protein seen in normoxia in cells treated with HIF-1 α siRNA (Figure 54B; $p<0.01$). In hypoxia, HIF-1 α inhibition with HIF-1 α siRNA caused a highly significant decrease in MMP-1 activity at the level of gene expression and protein secretion ($p<0.001$).

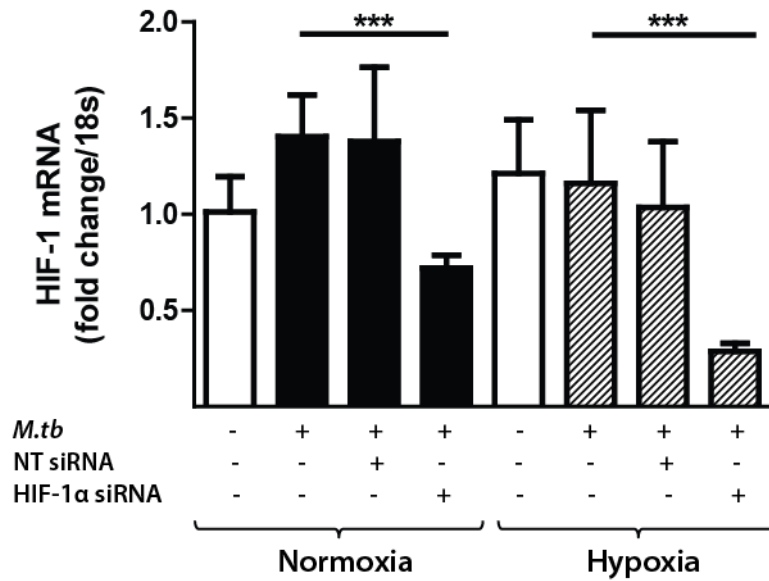
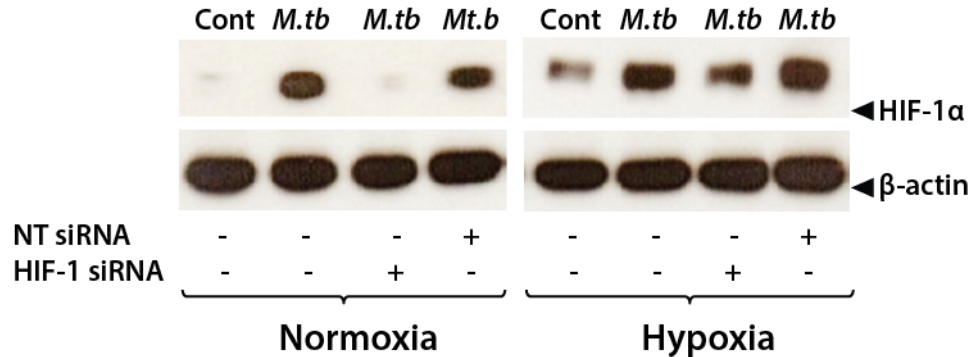
A**B**

Figure 53: HIF-1α knockdown and effect on HIF-1α in *M.tb*-infected MDMs incubated in normoxia and hypoxia. MDMs were transiently transfected with either NT or HIF-1α siRNA (25nM). Cells were infected with *M.tb* (MOI=1) and incubated in either normoxia (21% O₂, hypoxia workstation). **(A)** HIF-1α siRNA but not NT siRNA causes a significant decrease in HIF-1α gene expression in *M.tb*-infected MDMs in normoxia and hypoxia at 4 hrs post-infection ($p < 0.001$). **(B)** HIF-1α western blot at 8 hrs post-infection shows HIF-1α siRNA causes complete knockdown of HIF-1α protein in *M.tb*-infected MDMs in normoxia. A marked decrease occurs in hypoxia. No change in HIF-1α protein is seen with NT siRNA. Bars represent mean ($\pm$ SD) of conditions in triplicate and are representative of at least 2 independent experiments. Representative western blot from 2 independent experiments

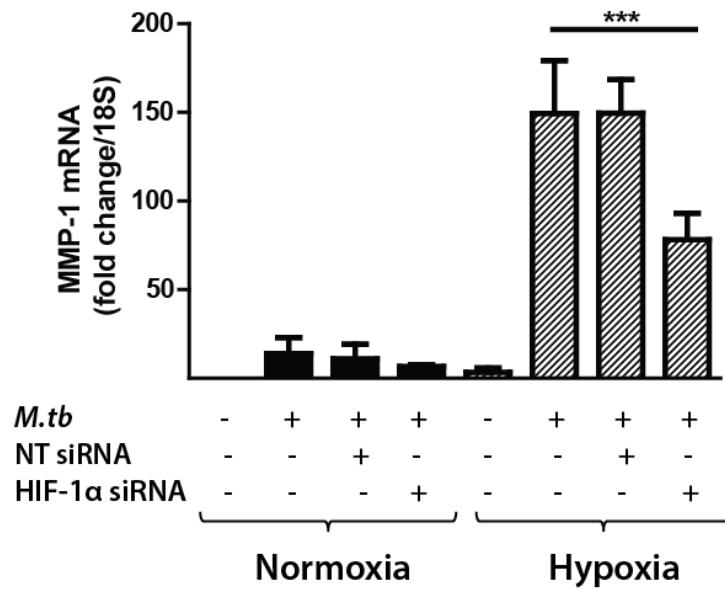
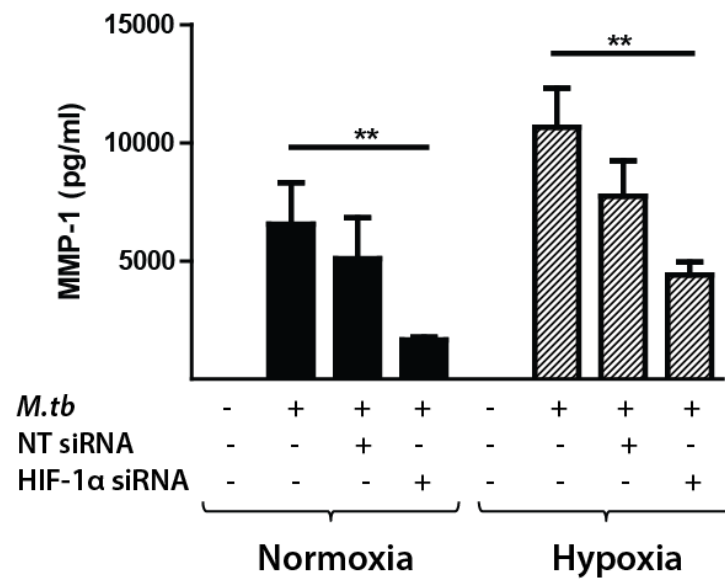
A.**B.**

Figure 54: Effect of HIF-1α siRNA on MMP-1 gene expression and protein secretion. MDMs were transfected with either HIF-1α siRNA or NT siRNA (25nM). Cells were infected with *M.tb* (MOI=1) and incubated in normoxia (21% O₂) or hypoxia (1% O₂). MMP-1 gene expression was analysed 24 hrs post-infection and MMP-1 protein secretion at 48 hrs by Luminex. **(A)** HIF-1α siRNA causes a significant decrease in MMP-1 gene expression from *M.tb*-infected MDMs in hypoxia ($p < 0.001$). **(B)** HIF-1α knockdown significantly decreases MMP-1 protein secretion in *M.tb*-infected cells in both normoxia and hypoxia ($p < 0.01$). No change in MMP-1 gene expression or protein secretion is seen with NT siRNA. Bars represent mean ($\pm$ SD) of conditions in triplicate and are representative of at least 2 independent experiments.

HIF-1 α siRNA decreases MMP-1 secretion in A549 stimulated with CoMTb in hypoxia

I was interested to investigate whether HIF-1 α siRNA might influence MMP-1 activity in respiratory epithelial cells given the findings described in chapter 5 and the results of HIF-1 western blotting. Previous work done by our group has demonstrated successful transfection of A549 and NHBE cells using Fugene 6 and Dharmacon smartpool siRNA. I therefore transfected A549 cells with HIF-1 α using our previously validated protocol and subsequently stimulated them with CoMTb (1:5 concentration). HIF-1 α siRNA caused a 44% decrease in MMP-1 protein secretion compared to control, non-transfected cells or NT siRNA (Figure 55). It would have been important to demonstrate evidence of HIF-1 α knockdown, as I had done with my experiments with MDMs, but this was not possible to due to time constraints. These experiments would have also been improved by assessed the effect of HIF-1 α siRNA on MMP-1 gene expression.

Collectively the results of the chemical inhibitor and RNA interference experiments demonstrate evidence of HIF-1 α signalling pathways driving MMP-1 activity during TB infection.

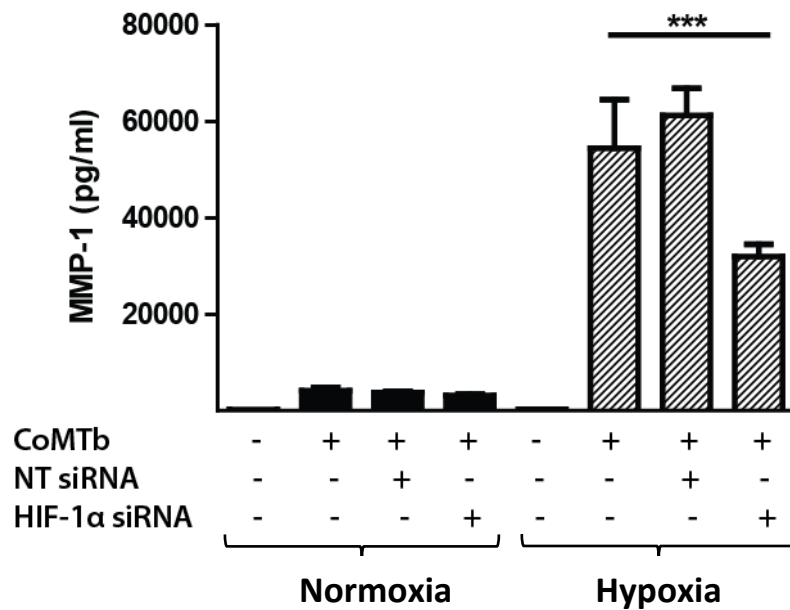
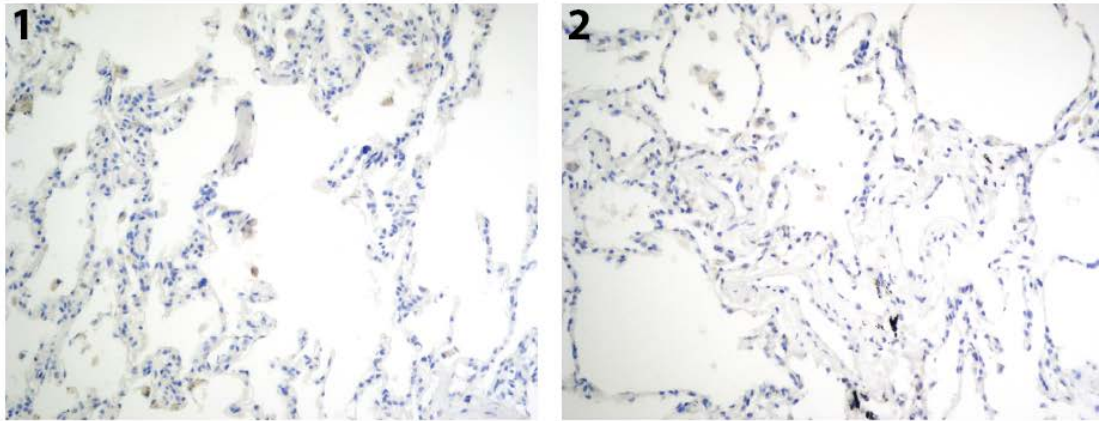


Figure 55: Effect of HIF-1α siRNA on MMP-1 secretion in A549 cells. A549 cells were transfected with either HIF-1α siRNA or NT siRNA. Cells were stimulated with CoMTb (1:5) and incubated in either normoxia (21% O₂) or hypoxia (1% O₂). Supernatants were harvested at 48hrs post CoMTb stimulation and measured by ELISA. HIF-1α siRNA induces a 44% decrease in MMP-1 protein secretion ($p < 0.001$) compared to untransfected cells in cells incubated in hypoxia. HIF-1α siRNA has no effect on cells incubated at 21% O₂. Bars represent mean (+/- SD) of conditions in triplicate and are representative of at least 2 independent experiments.

Lung biopsies from patients with TB demonstrate increased HIF-1 α immunoreactivity compared to controls

The finding of *M.tb* infection promoting HIF-1 α stabilisation *in vitro* then led me to investigate whether this finding was also present *in vivo*. Analysis was performed by Professor Francesco Mauri and Dr Tyler Lloyd within the Department of Histopathology, Imperial College NHS Trust. Initial optimisation of the technique was first performed on a positive control sample from a patient with ovarian cancer and then adapted for lung tissue. Immunohistochemistry from six control samples demonstrated no evidence of HIF-1 α staining (Figure 56A). By contrast, samples from all six patients with a confirmed diagnosis of pulmonary *M.tb* demonstrated positive staining for HIF-1 α within and around TB granulomas (Figure 56B). Of note, HIF-1 α staining was seen within macrophages and multinucleated giant cells (Figure 57).

A.



B.

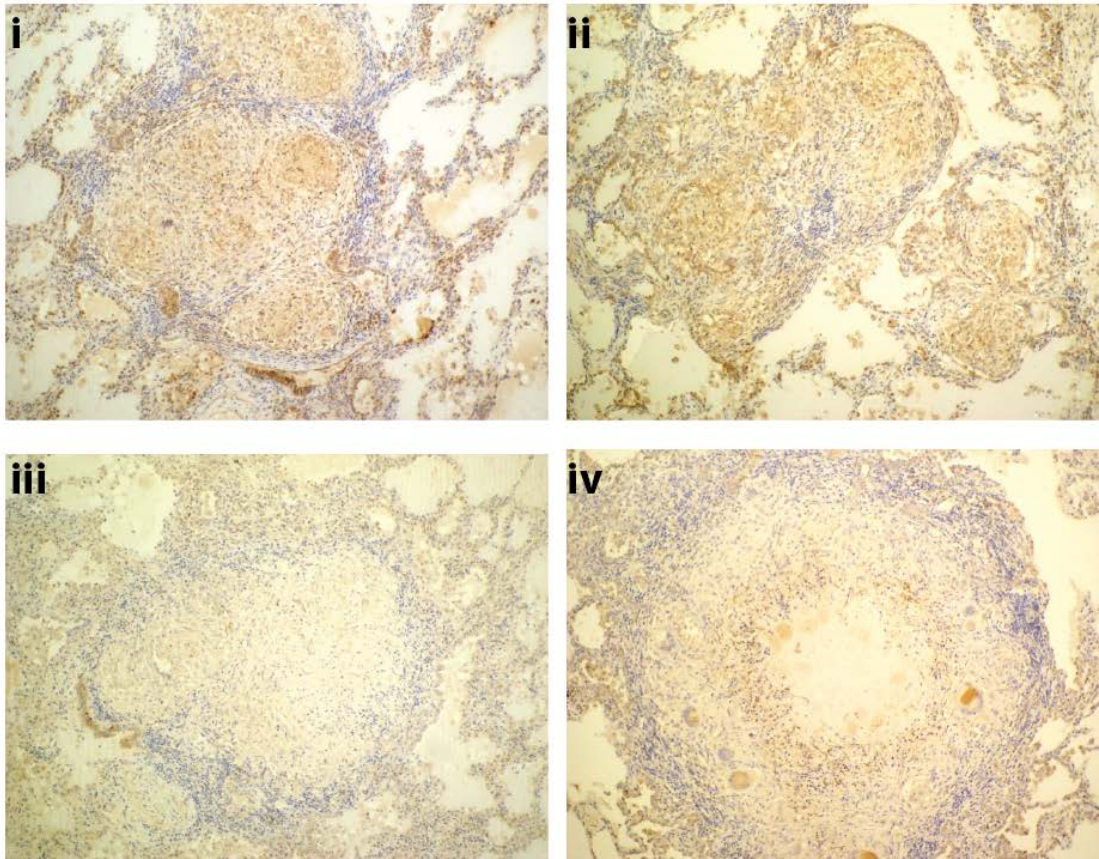
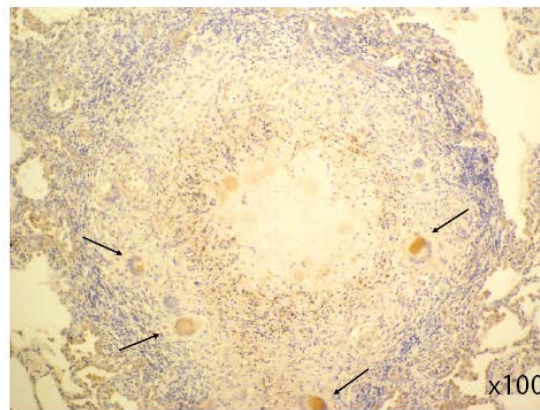


Figure 56: HIF-1 α immunohistochemistry. Immunohistochemistry was performed on lung biopsy samples from 6 controls and 6 patients with a confirmed microbiological diagnosis of TB. **(A)** Samples from control patients, 2 representative images are shown, do not demonstrate immunoreactivity for HIF-1 α . **(B)** TB patients demonstrate positive immunohistochemical staining for HIF-1 α within TB granulomas (brown). No HIF-1 α staining is seen in regions of caseous necrosis. Granulomas from 4 individual patients are shown (i-iv). Magnification x100

A.



B.

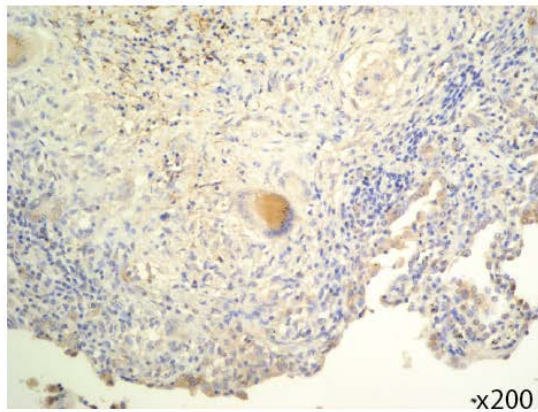
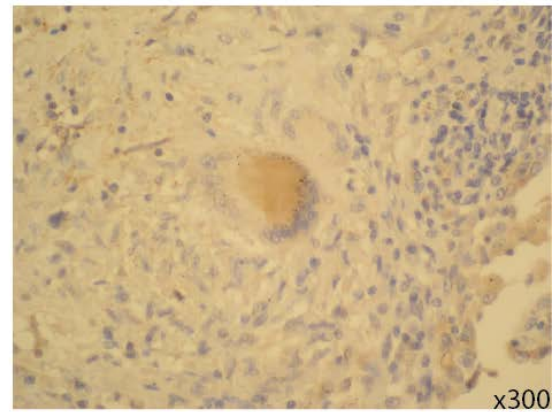


Figure 57: HIF-1 α localisation in human TB granulomas. (A) Positive HIF-1 α staining is prominent within macrophages and multinucleated giant cells (arrows). **(B)** High powered images of multinucleated giant cell from image A.

Discussion

My results demonstrate a pivotal role of HIF-1 α -dependent signalling pathways in driving MMP-1 expression during *M.tb* infection. I have shown for the first time that *M.tb* infection causes HIF-1 α stabilisation even in normoxia. In addition, my results demonstrate that HIF-1 α inhibition decreases MMP-1 gene expression and protein secretion. Finally, immunohistochemistry reveals evidence of HIF-1 α in patient samples.

HIF stabilisation due to infective organisms has been described in several studies [195, 196] but this is the first time that *M.tb*-driven HIF-1 α stabilisation has been described. I found that HIF-1 α stabilisation occurred following infection with both *M.tb* and BCG indicating that HIF activation might be a generalised feature of mycobacterial diseases. It would have been interesting to investigate HIF stabilisation during other mycobacterial infections to assess this further. Additionally, it would be interesting to assess if HIF levels differed in clinical strains compared to laboratory strains. I performed this experiment twice and although subtle, on each occasion the amount of HIF-1 α appeared slightly less in BCG compared to *M.tb* infection. Cells were infected with equivalent optical densities of TB cultures but it would have been useful to perform colony counting to ensure that CFU's were equal between the two conditions. Additionally, measurement of HIF-1 α protein levels by ELISA (R&D) might have been useful. I had intended to stimulate the cells with both ESAT-6 and CFP-10 but the marginal difference in HIF-1 α between *M.tb* and BCG and subsequent bacterial contamination of ESAT-6 and CFP-10 cultures meant I did not pursue this line of investigation.

My investigation of chemokines, cytokines and growth factors responses during *M.tb* infection and in response to DMOG pre-incubation reveal factors that might be driving HIF accumulation (Appendix 2). Of note, the cytokines IL-1 β and TNF α have all been shown to be key drivers of HIF-1 α stabilisation in other infections and inflammatory conditions [80]. Interestingly, although TNF- α increases in response to *M.tb* infection, no significant change occurs with DMOG indicating that TNF α is not responsible for the increased HIF-1 α stabilisation (Figure 49A). The results of the Luminex 30-plex examined the effect of DMOG on key cytokines, chemokines and growth factors in *M.tb*-infected MDMs but this analysis requires repetition using cells incubated in hypoxia. Additionally, analysis of respiratory epithelial cells should be performed to assess *M.tb* networking effects. The different responses in MMP-3 and MMP-7 secretion in *M.tb*-infected MDMs (Figure 36 and 37) in response to DMOG and 1% O₂ for example, might be driven by altered cytokine and chemokine profiles.

I have shown that hypoxia and HIF-1 α -dependent pathways regulate MMP-1 gene expression and protein secretion. My results are consistent with data from the hypoxic rheumatoid synovium where hypoxia and HIF-1 α have been shown to regulate MMP-driven tissue destruction in hypoxic fibroblasts [174, 197]. The advantage of assessment of the rheumatoid synovium is the ability to directly measure pO₂ levels to confirm the presence of hypoxia from cells taken from this region. It would be useful to evaluate if HIF-1 α responses in alveolar macrophages, which might be considered comparatively hypoxic, differed to peripheral blood MDMs in response to *M.tb* infection.

I found no significant evidence of HIF-2 α activity during my investigations. This contrasts with studies in osteoarthritic cartilage destruction where HIF-2 regulated MMP activity [198]. My experiments did not include a positive control for HIF-2 α which might have improved the experiment but overall, I did not find any significant evidence of HIF-2 α activity during *M.tb* infection in either normoxia or hypoxia. One possible explanation for this is the level of hypoxia used. Fang *et al.* for example, examined HIF-1 and HIF-2 activity in primary MDMs using O₂ levels of 0.1% [199]. Improvements to my assessment of HIF-2 α activity would have included using alternative HIF-2 α antibodies and measurement of HIF-2 α mRNA levels in response to *M.tb* infection in normoxia and hypoxia.

My investigation of regulation of HIF-1 α during *M.tb* infection focussed principally on the prolyl hydroxylases. The PHDs have been shown to be key regulators of HIF-1 α -mediated neutrophil survival in response to hypoxia [88]. Of note, PHD3 is strongly induced in response to infective and inflammatory stimuli and is essential for hypoxic regulation of neutrophilic regulation of inflammation in humans and mice [200]. My investigation of PHD activity was performed in human monocyte-derived macrophages and demonstrated changes in PHD activity in response to both *M.tb* infection and to hypoxia compared to control cells. These findings demonstrate changes in the PHD's protein levels but do not specifically address which features of *M.tb* infection might be driving HIF-1 α accumulation. Maximal induction of the PHDs occurs 24 hrs post-infection and indicates that the PHD response might occur in response to *M.tb*-induced HIF-1 α stabilisation. Since HIF-1 α degradation is principally controlled by the PHDs, inhibition of the individual PHDs and examining the effect on HIF-1 α stabilisation might provide further insights into the mechanism by which this occurs.

My study did not investigate the effect of HIF-1 α inhibition on NF κ B and given the inter-dependent role of these two transcription factors, it is likely that HIF-1 α siRNA would have affected NF κ B activity. Future experiments would include measuring NF κ B activity in *M.tb* infected MDMs treated with HIF-1 α siRNA focussing on the individual NF κ B subunits.

Examination of patient lung specimens showed evidence of HIF-1 α expression in samples from TB patients. HIF-1 α localised to macrophages and multinucleated giant cells, immune cells also known to be key sources of MMPs [46, 201]. My finding contrasts with HIF immunoreactivity in other infections where HIF-1 α is found predominantly in neutrophils [202].

Collectively, my data demonstrates evidence of HIF-1 α activation *in vitro* and *in vivo* during *M.tb* infection. My work is complimented by two recent studies which demonstrate evidence of HIF-1 activation in response to TB infection. Whole-blood gene expression profiling shows that HIF-1 is up-regulated in TB patients compared to controls but not compared to patients with sarcoidosis[203]. In the zebrafish model of TB infection, expression of the HIF-1 target gene *PHD3*, is seen in response to early infection in leucocytes. Furthermore, stabilisation of HIF-1 α signalling with DMOG causes decreased *Mm* burden [110]. Manipulation of the HIF pathway therefore represents a potential target for future therapeutics against TB.

In conclusion, I have shown that *M.tb*-infection causes HIF-1 α stabilisation even in the absence of hypoxia and HIF-1 α signalling pathways regulate MMP-1 activity in TB infection.

CHAPTER 7: CONCLUSIONS

In this first detailed study of hypoxia in human tuberculosis, I have demonstrated the presence of severe hypoxia within areas of lung consolidation and around pulmonary cavities. Hypoxia increases gene expression and secretion of MMP-1, a key collagenase that causes tissue destruction and immunopathology in pulmonary TB. In addition, *M.tb* infection directly drives HIF-1 α accumulation, which is further increased in hypoxia to synergistically activate immunopathogenic signalling pathways.

My clinical study demonstrated hypoxia in human TB using [^{18}F]FMISO PET-CT scanning for the first time in patients with an infectious disease. Hypoxia has not previously been found in man although reported in diverse animal models of TB, all of which have somewhat different pathology to human disease [106-108]. Until now, the investigation of hypoxia and TB has focussed primarily on pathogen responses to oxygen restriction, controlled by the two-component regulation system dosR/dosS (or dosR regulon) [204-206]. Direct assessment of hypoxia *in vivo* typically involves the utilization of polarographic oxygen electrodes which can provide absolute measurements of tissue oxygenation at the sampling location. However, this procedure is technically demanding and suffers from a number of limitations including invasiveness, high susceptibility to sampling errors and the fact that only easily accessible locations can be interrogated [207]. [^{18}F]FMISO PET-CT scans define regions of hypoxia without the need for invasive probes, as previously shown in tumour biology [151, 208] and cerebral ischaemia [209, 210]. Since high tracer uptake, as quantified by standardized uptake value (SUV) or TBR, may represent high tracer delivery to a region of interest rather than tracer trapping under hypoxic conditions, I determined the influx rate of

[^{18}F]FMISO into the trapped tissue compartment (K_i) so as to more specifically identify tissue hypoxia. The maximum TB lesion K_i value (0.005 min^{-1}) is equal to the highest K_i value in an [^{18}F]FMISO study of head and neck cancer by Wang et al [167], which puts the degree of hypoxia in this study into context. My protocol included the measurement of [^{18}F]FMISO levels in peripheral blood, thus providing an internal control for any possible movement artefact. In addition, the finding of stable or increasing K_i values in lung lesions compared to blood values, which declined over time, further supports the finding of tracer trapping demonstrated by K_i measurements. My data reveals that heterogeneity exists both within individual TB lesions and between lesions, consistent with the emerging concept of multiple TB microenvironments existing within a single patient [168].

The finding of hypoxia within lung lesions directed my cellular experiments. MMP-1 gene expression and secretion were significantly increased and was functionally active in *M.tb*-infected human macrophages in hypoxia compared to normoxia and there was no compensatory increase in the inhibitor TIMP-1. Thus, hypoxia is a further driver to the pro-inflammatory tissue destructive phenotype characteristic of TB, where disease is more due to host response than the direct effects of mycobacteria. Consistent with this, hypoxia had no effect on mycobacterial growth in my experiments.

Hypoxia-dependent, monocyte driven up-regulation of MMP-1 gene expression in human respiratory epithelial cells required the transcription factor NF- κ B. NF- κ B and HIF-1 α interact to regulate innate immunity and inflammation. NF- κ B critically regulates MMP activity in TB infection of the central nervous system [211] but has not been investigated in the context of hypoxia-driven MMP expression in TB infection. NF- κ B is both directly affected by hypoxia

and modulates HIF-1 α expression as well as being regulated by prolyl hydroxylases. The precise nature of the interaction between HIF-1 α , prolyl hydroxylases and NF- κ B in TB requires further interrogation and is currently being investigated by the JSF group.

M.tb caused significant accumulation of HIF-1 α in direct infection and via a networking effect even in the absence of hypoxia, with a synergistic increase in HIF-1 α expression in hypoxia or following treatment with DMOG. To my knowledge, this is the first time that hypoxia-independent HIF-1 α stabilization has been observed in any mycobacterial infection, although such an effect has been reported in other specific infections [195, 196, 212].

I have now shown expression of HIF-1 α within TB granulomas localizing the activity to the host inflammatory response and in particular to epithelioid macrophages and multi-nucleate giant cells, which we have previously shown are key in secretion of a number of MMPs [183, 201]. I have demonstrated using both the chemical inhibitor LW6 and targeted siRNA that HIF-1 α regulates MMP-1 expression in TB. Consistent with my data in macrophages and respiratory epithelial cells in TB, hypoxia and HIF-1 α have been reported to regulate MMP-driven tissue destruction in hypoxic fibroblasts within rheumatoid synovium [174].

In summary, I show for the first time that human TB lesions are severely hypoxic. Hypoxia increases MMP-1 gene expression and secretion during *M.tb* infection, and *M.tb* infection independently increases HIF-1 α activity even under conditions of normoxia. Taken together, my data suggests that *M.tb* drives a HIF-1 α -dependent proinflammatory tissue destructive cascade that may lead to cavitation and promote disease transmission, representing a potential future therapeutic target in the era of increasing drug resistant TB.

CHAPTER 8: FUTURE WORK

My study was able to confirm the presence of severe hypoxia during *M.tb* infection but since only a single scan was performed, was not able to assess if the extent of hypoxia changed over time or in response to treatment. Future studies might include follow-up scans after commencing treatment e.g. 8 weeks to see if this impacted on the extent of hypoxia. Such a study ideally would involve scanning patients who were treatment naïve with repeat scans being correlated to clinical and microbiological response.

[¹⁸F]FMISO PET-CT scans demonstrated severe hypoxia within active tuberculosis infection but the technique currently does not allow the quantification of level of oxygen within these lesions. It is quite possible that hypoxia exists within TB lesions that cannot be detected by [¹⁸F]FMISO, i.e. greater than 2% O₂ but less than the 9-14% found in normal healthy lung tissue. Findings from some of patients demonstrated uptake of tracer within axillary lymph nodes (patients 2, 3 and 5) indicating that severe hypoxia might exist within lymph nodes. At present, it is not possible to confirm this finding using [18F]FMISO PET-CT scan as the method has not been validated for lymph node disease. One possible way to overcome this would be to directly insert a pO₂ Eppendorf into accessible lymph nodes to measure tissue pO₂. The advantage of this method is that the probe might also be able to assess areas of relative hypoxia i.e. 2-10% pO₂.

My results from both respiratory epithelial cells and macrophages revealed an unexpected effect of 10% oxygen on MMP-1 secretion. Although the results did not reach statistical significance in NHBE cells, incubating cells at 10% oxygen consistently results in lower levels

of MMP-1 protein compared to cells incubated at 21% oxygen. It is arguable that 10% oxygen more closely resembles physiological oxygen levels within the lungs but this would only be true of healthy lungs because until now, oxygen levels in *M.tb* infected lungs has not been explored. It is possible to postulate that cellular changes occur at 10% O₂, such as the stabilising of pH seen in my macrophage experiments, which act to reduce the tissue destructive effect of MMPs. Possible future work in this area might include analysis of secreted chemokines and cytokines in response to varying oxygen concentrations as well assessing the effects on HIF and the PHDs. It would also be interesting to measure TIMP levels to see if these are altered by the degree of hypoxia. These findings might provide insights into how the pro-inflammatory profile of *M.tb* infection might be manipulated to lessen the tissue destructive effects of *M.tb* infection.

The results of my study demonstrate that both HIF and NF-κB signalling pathways are involved in *M.tb*-driven MMP secretion. Since the activity of HIF and NF-κB are intimately related future work should involve more in depth exploration of how their activity is regulated during *M.tb* infection. I demonstrated that *M.tb* infection drives HIF-1α stabilisation but did not explore the effect of the *M.tb* infection on NF-κB activity in either normoxia or hypoxia. Such investigation could be performed using Trans-Am kits to initially assess the effect of *M.tb* infection on NF-κB subunits in normoxia and hypoxia. The effect of hypoxia on NF-κB could be further evaluated by examining the effect on NFκB gene expression. Further investigation would include assessing the effect of HIF-1α inhibition on NF-κB expression using siRNA and conversely exploring if NF-κB inhibition affects HIF-1α activity during *M.tb* infection.

My investigation of the activity of the prolyl hydroxylases during *M.tb* infection and in response to hypoxia (1% O₂) revealed a number of interesting findings. I found differences between control MDMs and *M.tb*-infected cells in normoxia and augmented changes in hypoxia. In particular, PHD 2 protein increased significantly. My findings contrast with the effect of hypoxia in neutrophils where PHD 3 activity has been shown to critically regulate neutrophil function [200]. Furthermore, my results contrast with changes in PHD expression in pro-inflammatory macrophages where PHD3-encoding *EGLN3* gene is specifically expressed by *in vitro*-generated proinflammatory M1(GM-CSF) human macrophages at the mRNA and protein level [213]. In order to explore these responses further, PHD mRNA kinetics should be measured to examine the effect of *M.tb* infection and hypoxia on PHD gene expression. This could then be investigated further by inhibition of the PHDs using both chemical inhibitors and siRNA to assess the effect of specific PHD inhibition on MMP activity.

The finding of severe hypoxia during active TB infection and augmentation of the host proinflammatory response to *M.tb* infection has implications for the other host immune cells. Work by other members of the JSF group has already commenced examining the effect of hypoxia on neutrophils and apoptosis during *M.tb* infection. Preliminary data I was involved with also indicates that MMP-10, a stromolysin implicated in cell migration is upregulated in hypoxia in *M.tb*-infected monocytes. This preliminary data is included in a Fellowship application by the JSF group.

Ultimately, the effect of hypoxia and HIF represents a potential future avenue for the development of new therapies and treatment strategies aimed at reducing host inflammatory tissue destruction.

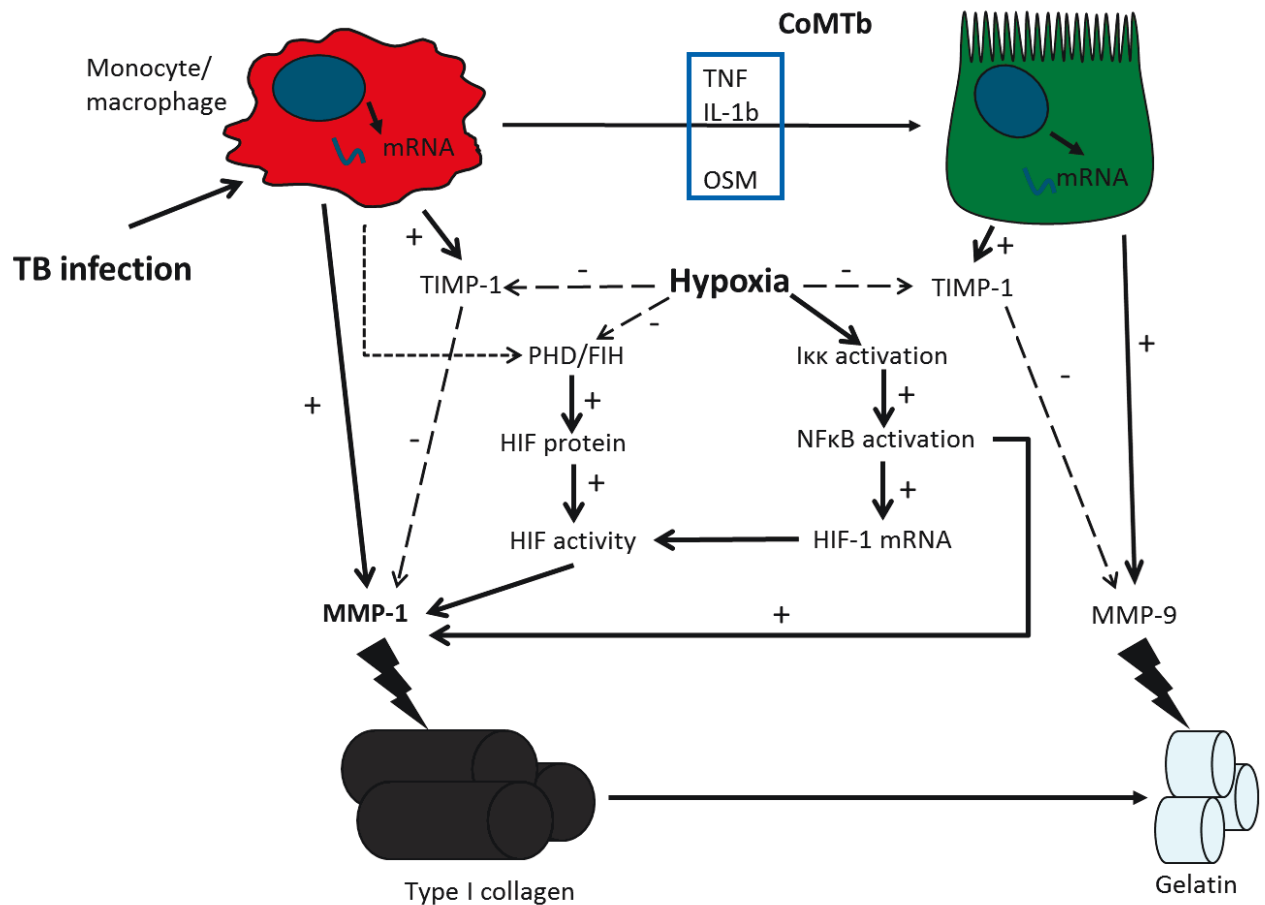


Figure 58: Schematic diagram of postulated mechanism by which hypoxia modulates MMP activity during TB infection.

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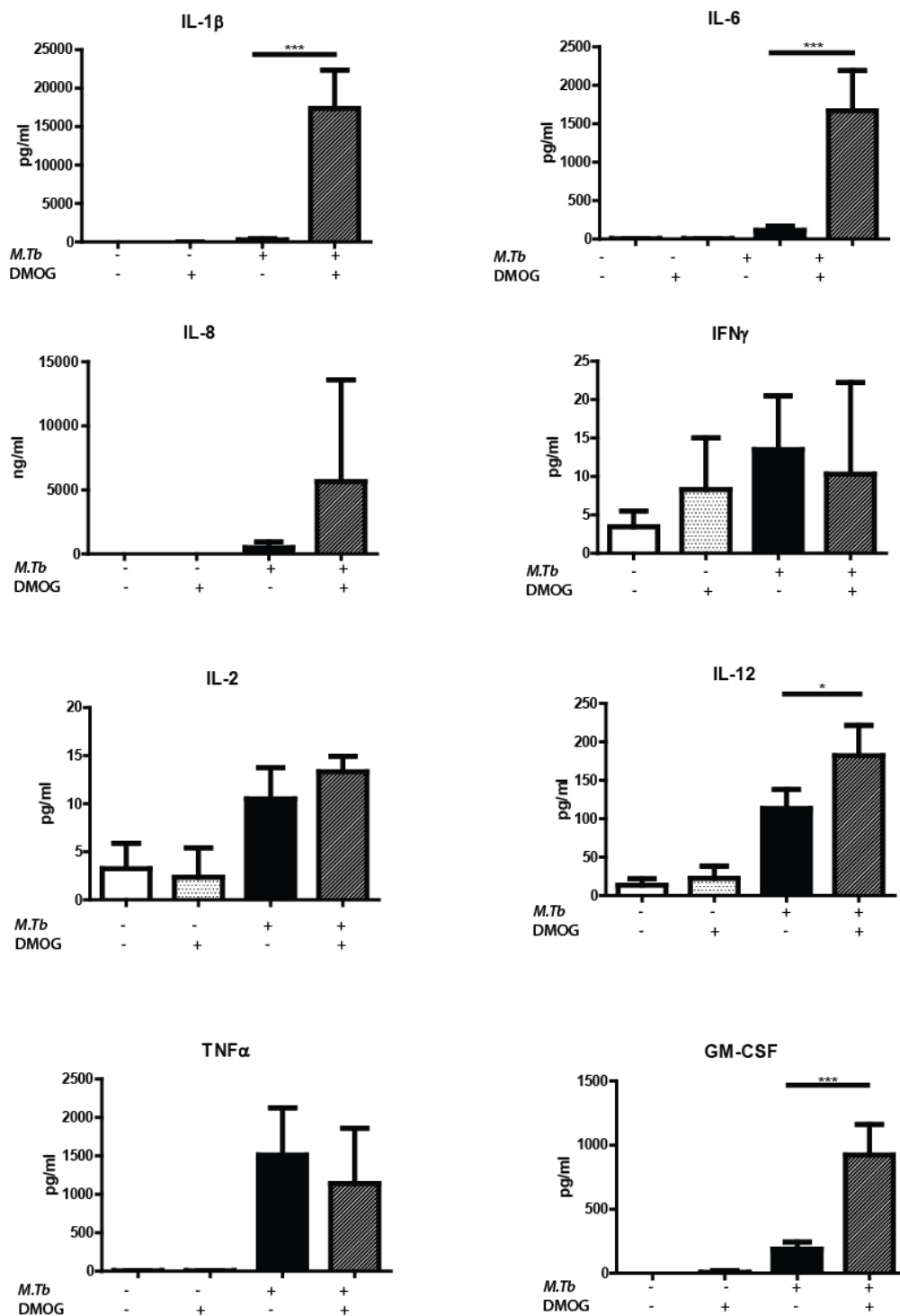
APPENDIX 1: Abbreviations

A549	adenocarcinoma cell line
Ab	antibody
BCG	Bacille Calmette-Guèrin (vaccine)
BSA	bovine serum albumin
CoMTb	conditioned media from monocytes stimulated with <i>M.tb</i>
DMOG	dimethyloxalylglycine
ECM	extracellular matrix
FCS	fetal calf serum
GAS	group A streptococcus
HBSS	Hank's balanced salt solution
HIF	hypoxia inducible factor
HIV	Human Immunodeficiency Virus
HRE	hypoxia responsive element
HRP	horseradish peroxidase
<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
M-CSF	monocyte colony stimulating factor
MDM	monocyte-derived macrophage
MDR	multi-drug resistant
MMP	matrix metalloproteinase
MOI	multiplicity of infection
M.tb	<i>Mycobacterium tuberculosis</i>
NHBE	normal human bronchial epithelial cell
OD	optical density
PBS	phosphate-buffered saline
PHD	prolyl hydroxylase
RNA	ribonucleic acid

RT	room temperature
RT-PCR	real-time polymerase chain reaction
SDS	sodium dodecylsulphate
siRNA	small interfering ribonucleic acid
TF	transcription factor
TB	tuberculosis
TB supp	tuberculosis supernatant
TIMP	tissue inhibitor of metalloproteinase
TMB	tetramethylbenzidine
uv-killed	ultraviolet killed
VHL	von Hippel Lindau protein
XDR	extensively drug resistant

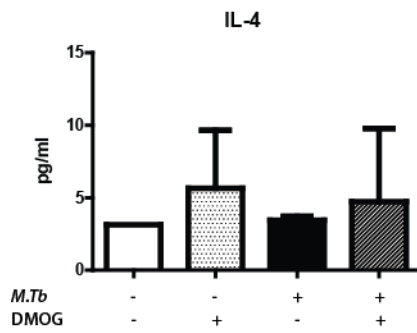
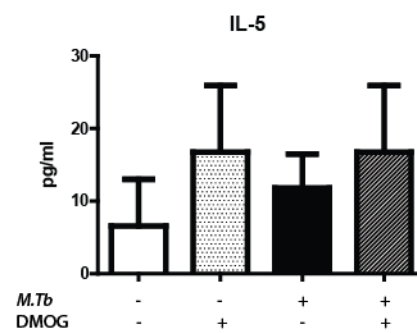
APPENDIX 2: Results of 30-plex Luminex analysis

Th1 response

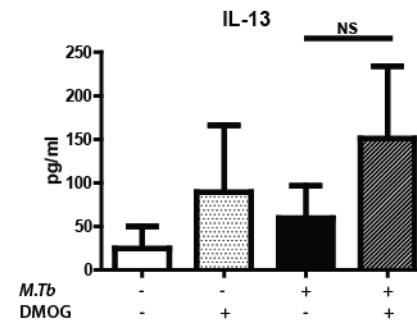


MDMs pre-incubated with DMOG (0.25mM) and infected with *M.tb* (MOI=1). Supernatants were harvested 72 hrs post-infection and analysed by Luminex. Representative data from 2 independent experiments performed in triplicate. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

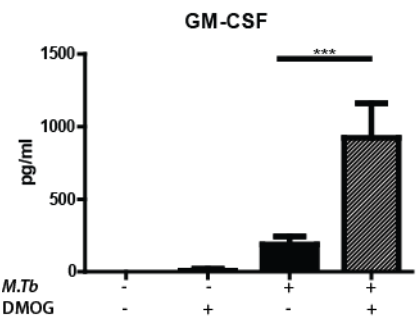
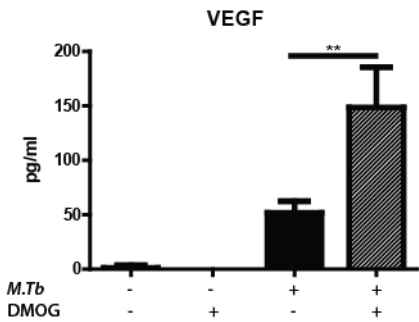
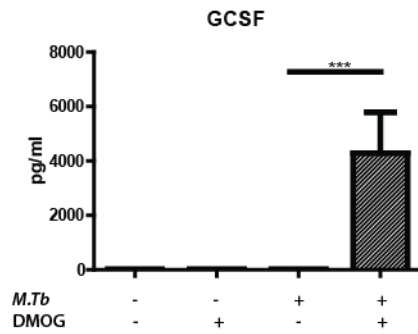
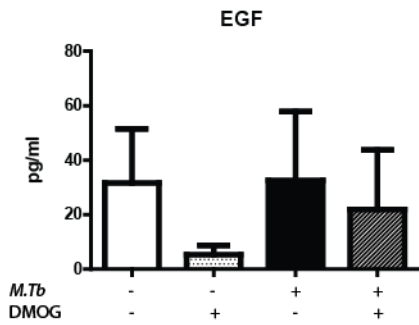
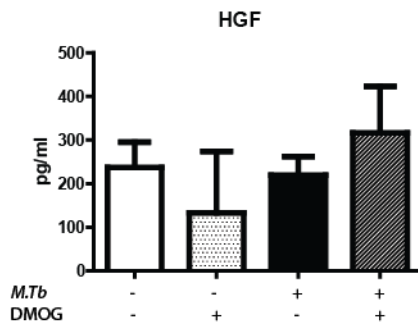
TH2 response



IL-10 inconclusive results between experiments

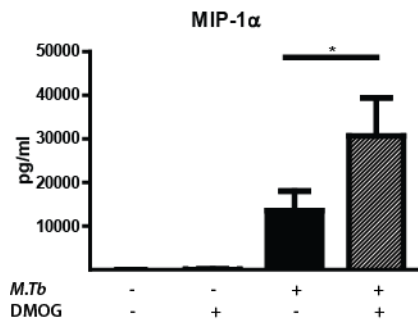
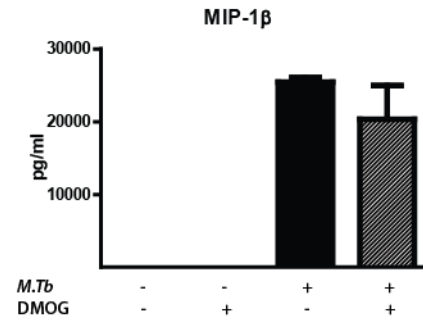
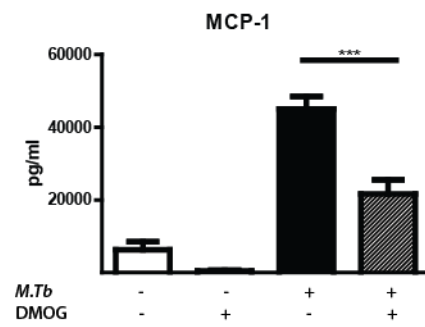
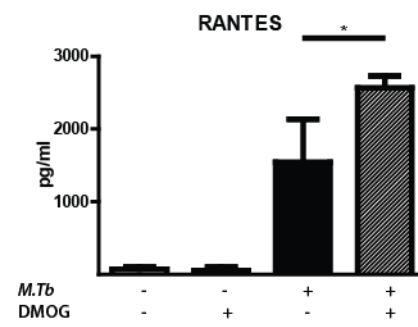
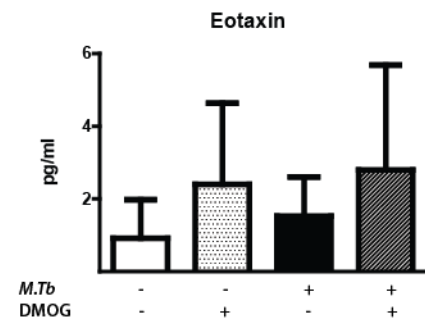
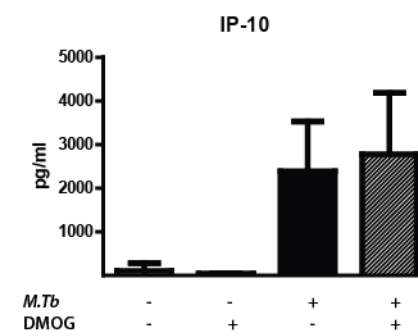
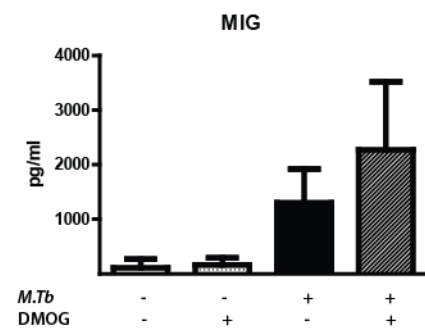


Growth factors

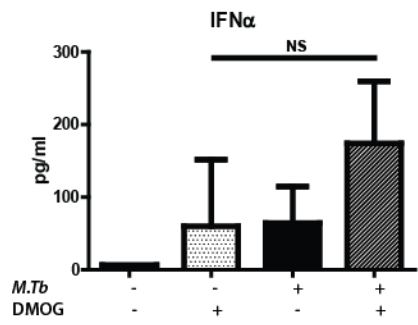
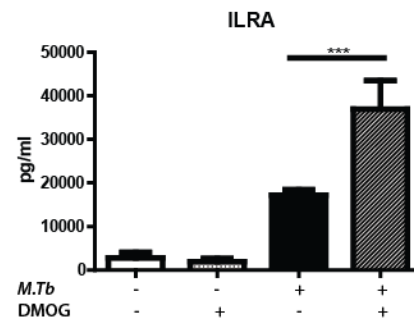
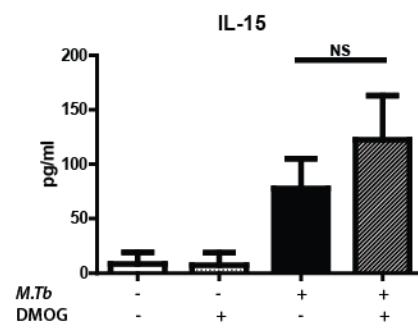


FGF inconclusive results b/n experiments

Chemokines



Others



APPENDIX 3: PUBLICATIONS AND PRESENTATIONS

Presentations and awards

Severe hypoxia exists within pulmonary tuberculosis lesions and augments matrix metalloproteinase-mediated immunopathology

Moerida Belton*, Sara Brilha, Tim Fryer, Kuldip Nijran, Paul T. Elkington, Jon S. Friedland

British Thoracic Society Winter Meeting 2013. *Oral presentation*

Winner of BTS/BLF/BALR Early Career Investigator Prize

Hypoxia Increases Matrix Metalloproteinase-Driven Immunopathology During Tuberculosis Infection: Evidence From In Vitro Studies And Patients With Active Pulmonary Disease

M.Belton*, P. Elkington, K. Nijran, N. Patel, L. Tezera, T. Fryer, A. Al-Nahhas, J.S. Friedland

American Thoracic Society 2013 International Conference, Philadelphia. *Oral presentation*

American Thoracic Society Abstract Scholarship

British Lung Foundation Travel Award

Cellular oxygen levels modulate tuberculosis immunopathology

Moerida Belton*, Marcin Dembek, Paul Elkington, Jon S. Friedland

British Infection Association 15th Annual Meeting 2012, *Oral presentation*

Winner British Infection Association Best Original Paper

Hypoxia modulates tuberculosis immunopathology

Moerida Belton*, Paul Elkington, Marcin Dembek, Jon S. Friedland

Annual Academic Trainee Event 2012, Imperial College London

1st Prize for poster

Tissue oxygen levels modulate tuberculosis immunopathology

Moerida Belton*, Marcin Dembek, Paul Elkington Jon. S. Friedland

22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 2012.
Oral presentation

Hypoxia modulates tuberculosis immunopathology

Moerida Belton*, Paul Elkington, Marcin Dembek, Jon S. Friedland

Keystone Symposia 2012 Alberta Canada, Advances in Hypoxic Signalling: From Bench to Bedside. *Poster presentation*

Papers

Hypoxia and HIF-1 α -dependent MMP-1 secretion in human pulmonary tuberculosis

Moerida Belton*, Sara Brilha, Roido Manavaki , Francesco Mauri, Kuldip Nijran, Young T. Hong, Neva Patel, Marcin Dembek, Liku Tezera, Justin Green, Rachel Moores, Franklin Aigbirhio, Adil Al-Nahhas, Tim Fryer, Paul T. Elkington, Jon S. Friedland
Submitted