Regulation of inflammation by platelets in tuberculosis

Katharine Fox

Division of Infection and Immunity
Department of Medicine
Imperial College London

Thesis submitted for Doctor of Philosophy (PhD)
March 2017
Declaration of Authenticity

I declare that this thesis was entirely written on my own. All referenced studies have been appropriately cited and given in the list of references, to the best of my knowledge. This dissertation has not been previously submitted, in part or whole, to any university of institution for any degree, diploma or other qualification.

Katharine Fox

Copyright Declaration

The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work.
Abstract

Introduction: *Mycobacterium tuberculosis* (*M.tb*) infection, causing tuberculosis (TB), kills more people than any other pathogen in the world. Innate immune cells are stimulated by *M.tb* antigens to result in an inflammatory and tissue-degrading phenotype associated with TB transmission, morbidity and mortality. Platelets are known to interact with the innate immune system and regulate matrix metalloproteinase (MMP)-driven tissue destruction but their role in the pathology of TB is not understood. My hypothesis is that platelets drive TB immunopathology through mechanisms that regulate key innate immune cell responses to *M.tb* infection.

Methods: Multiplex analysis of bronchoalveolar lavage (BALF) and immunohistochemistry of a pulmonary TB mouse model detected platelets in the *M.tb*-infected lung. I developed a novel *in vitro* TB model using freshly isolated and thrombin-activated platelets in co-culture with primary human monocytes, neutrophils and airway epithelial cells. I assessed MMP and cytokine regulation at the gene, protein and functional level, with quantitative RT-PCR, Luminex bead array, ELISA, DQ matrix degradation, western blot and bacterial colony counts.

Results: Investigation in the *M.tb*-infected lung revealed abundant platelets in a murine TB model, and activated platelet-derived factors in human BALF. Platelets upregulated specific MMPs, cytokines and chemokines in *M.tb*-infected monocytes, leading to type I collagen destruction and reduced intracellular bacterial killing. The platelet-secreted product CD40 ligand (CD40L) upregulated MMP-1 secretion, however, direct platelet-monocyte contact enhanced MMP regulation further. Platelets regulated monocytes through phosphorylated phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), extracellular signal-regulated kinase (ERK)-1/2 and p38 pathways. Interestingly, platelets inhibited early neutrophil MMP secretion in TB. Platelet-secreted factors upregulated airway epithelial cell collagenase and gelatinase activity alone and in combination with TB networks.

Conclusions: My data show that platelets are present at the site of pulmonary *M.tb* infection. Platelets regulate inflammation and tissue destruction driven by different leukocytes and respiratory epithelial cells through multiple signalling pathways.
# Table of Contents

Abstract .................................................................................................................. 2  
Table of Contents .................................................................................................... 3  
List of Figures .......................................................................................................... 7  
List of Tables ........................................................................................................... 9  
Acknowledgements ................................................................................................ 10  

Chapter 1. Introduction ........................................................................................... 11  
1. The tuberculosis epidemic .................................................................................. 11  
2. Treatment of TB ................................................................................................... 12  
3. Disease pathology ............................................................................................... 14  
4. Innate cell-mediated immunity in TB ................................................................. 16  
   4.1. Monocytes ....................................................................................................... 17  
   4.2. Neutrophils ..................................................................................................... 21  
   4.3. Airway epithelial cells .................................................................................... 24  
5. Tissue destruction in TB ..................................................................................... 26  
   5.1. The MMP family ............................................................................................ 28  
   5.2. Regulation of MMPs ..................................................................................... 30  
   5.3. MMPs in TB ................................................................................................... 33  
   5.4. The regulation of MMPs in TB: Intercellular signalling ............................ 37  
6. Platelets: the unknown player in TB ................................................................... 40  
   6.1. Platelets .......................................................................................................... 40  
   6.2. Platelets in Haemostasis ................................................................................ 44  
   6.3. Platelets in the Immune Response ................................................................. 45  
   6.4. Platelets in tissue destruction ....................................................................... 54  
   6.5. Platelets in TB ............................................................................................... 57  
7. Hypothesis and aims .......................................................................................... 59  
   7.1. Hypothesis ...................................................................................................... 59  
   7.2. Aims ................................................................................................................ 59  

Chapter 2. Materials and Methods ........................................................................ 60  
1. M.tb culture .......................................................................................................... 60  
2. Platelet preparation ............................................................................................. 61
3. Monocyte isolation .................................................................................................................. 63
4. Production of CT ..................................................................................................................... 65
5. Monocyte maturation ............................................................................................................. 66
6. Neutrophil isolation ............................................................................................................... 66
7. Cell co-culture ....................................................................................................................... 67
8. Isolation and quantification of NETs .................................................................................... 69
9. M.tb regulation of platelet-secreted factors ......................................................................... 70
10. M.tb Killing .......................................................................................................................... 70
11. Chemical inhibition and recombinant proteins ................................................................. 70
12. Sterilisation of cell culture supernatants .......................................................................... 71
13. Enzyme-linked immunosorbent assay (ELISA) ................................................................. 73
14. Luminex bead array ............................................................................................................ 74
15. Immunohistochemistry ...................................................................................................... 75
16. Western blot Analysis ........................................................................................................ 75
17. Lactate dehydrogenase (LDH) assay ................................................................................ 77
18. RNA extraction .................................................................................................................. 78
19. Quantitative Polymerase Chain Reaction (qPCR) ............................................................ 78
20. Confocal microscopy .......................................................................................................... 80
21. DQ matrix degradation assay ............................................................................................ 81
22. Accounting for platelet contributions in co-culture ............................................................ 81
23. Statistics ............................................................................................................................. 81

**Chapter 3. Platelets regulate monocyte inflammatory responses to tuberculosis** .... 83

1. Introduction .......................................................................................................................... 83
2. Results ................................................................................................................................ 85
   2.1. Platelets are present in a murine model of M.tb-infected lung. ...................................... 85
   2.2. Platelet-secreted factors are upregulated in patient lungs with active TB.................. 87
   2.3. Platelet-secreted factors correlate with MMP and IL-1β concentrations in the lung .... 89
   2.4. Optimisation of an in vitro model of platelet-regulated monocyte MMP secretion ...... 94
   2.5. Platelets regulate a specific monocyte MMP response to M.tb infection ................... 99
   2.6. TIMP concentrations do not change in the presence of platelets ............................... 102
   2.7. Platelets regulate the monocyte MMP responses at the transcriptional and post-
        transcriptional level ........................................................................................................... 104
   2.8. Platelets upregulate a functional increase in monocyte collagenase activity .......... 106
   2.9. Platelet upregulation of MMP-1 is not dependent on live M.tb infection .................. 108
Chapter 4. Platelets regulate monocytes through multi-pathway mechanisms

1. Introduction
2. Results
   2.1. *M. tb* does not directly activate isolated platelets
   2.2. Platelet regulation of monocyte gene expression is contact-dependent
   2.3. Platelet-secreted factors regulate monocyte secretion in *M. tb* infection
   2.4. Soluble CD40L significantly upregulates monocyte MMP-1 secretion
   2.5. Prolonged platelet regulation of monocyte MMP-1 is contact dependent
   2.6. HIF-1α is upregulated in *M. tb* infected monocyte-derived cells
   2.7. *M. tb* regulation of HIF-1α in MDMs
   2.8. HIF-1α is upregulated in *M. tb*-infected platelet-monocyte co-cultures
   2.9. Platelets regulate monocyte AKT and ERK-1/2
   2.10. Contact-independent platelet activation of monocyte MAPK pathways
   2.11. Platelet contact independent regulation of MMP-1 is through activation of ERK-1/2, p38 and PI3K
3. Discussion

Chapter 5. Platelets regulate TB-driven MMP activity in neutrophils and airway epithelial cells

1. Introduction
2. Results
   2.1. Platelets downregulate neutrophil MMP-8 secretion
   2.2. Platelets delay neutrophil MMP secretion in *M. tb* infection
   2.3. Platelets do not regulate neutrophil TIMP-2
   2.4. Platelets functionally downregulate neutrophil MMP activity
   2.5. Platelets regulate neutrophil cytokine responses in TB
   2.6. Platelets downregulate *M. tb*-induced NETosis
   2.7. Platelets regulate epithelial cell MMP responses in TB
   2.8. Platelets do not mediate secretion of key AEC MMP regulators
   2.9. Platelets upregulate AEC-dependent matrix destruction
   2.10. CD40L alone does not induce significant AEC MMP-1 secretion
   2.11. Platelet-secreted factors significantly regulate AEC MMP gene expression
List of Figures

Figure 1. CAT scan of TB patient lungs ................................................................. 27
Figure 2. Intracellular signalling cascades for MMP regulation .......................... 31
Figure 3. Platelets in the blood ........................................................................... 42
Figure 4. Flow cytometry analysis of purified platelet population ........................ 62
Figure 5. Thrombin-stimulated platelet activation .............................................. 63
Figure 6. Effectiveness of TB-conditioned medium .......................................... 65
Figure 7. Platelet co-culture experimental set-up ................................................ 68
Figure 8. Trans-well co-culture of platelets and monocytes ............................. 69
Figure 9. Viability of monocyte-derived cells treated with NFkB and MAPK inhibitors ...... 72
Figure 10. CD41 expression at the site of pulmonary TB pathology in murine TB model. ..... 86
Figure 11. Platelet-secreted factors are upregulated in the lungs of human pulmonary TB cases ........................................................................................................ 88
Figure 12. P-selectin correlates with MMP-1, -3, -7, -8, -9 and IL-1β in bronchoalveolar lavage fluid (BALF) ......................................................................................... 90
Figure 13. Platelet-derived growth factor correlates with MMP-1, -3, -8 and -9 in bronchoalveolar lavage fluid (BALF) ................................................................. 91
Figure 14. RANTES correlates with MMP-1, -8, -9 and IL-1β in bronchoalveolar lavage fluid (BALF). .................................................................................................. 92
Figure 15. CD40 ligand correlates with MMP-3 and -7 in bronchoalveolar lavage fluid (BALF). ........................................................................................................ 93
Figure 16. *M. tb*-driven monocyte MMP-1 and MMP-10 secretion is upregulated in platelet co-culture ........................................................................................................ 96
Figure 17. Activated platelet-secreted factors in *M. tb* infection ....................... 98
Figure 18. Platelets upregulate a specific monocyte MMP response in TB ........... 101
Figure 19. TIMP concentrations are unchanged in monocyte-platelet co-culture. ..... 103
Figure 20. Platelets upregulate monocyte MMP-1 gene and intracellular protein expression. ................................................................. 105
Figure 21. Platelets upregulate monocyte collagenase activity in TB .................. 107
Figure 22. Platelet upregulation of monocyte MMP-1 secretion is stimulated with UV-killed *M. tb* ........................................................................................................ 108
Figure 23. Platelets regulate a specific monocyte cytokine and chemokine response to TB ................................................................. 111
Figure 24. Platelet regulation of intracellular monocyte *M. tb* killing .................... 112
Figure 25. *M. tb* activation of platelets ............................................................. 126
Figure 26. Contact-dependent platelet regulation of monocyte MMP gene expression. .... 129
Figure 27. Platelet-secreted products significantly upregulate monocyte MMP-1. .... 131
Figure 28. Contact-independent platelet regulation of monocyte secretory profile. 132
Figure 29. Upregulation of monocyte MMP-1 by platelet-secreted factors .......... 134
Figure 30. Contact-independent platelet regulation of monocyte MMP-1 secretion over time.

Figure 31. HIF-1α stabilisation in *M. tb* infected monocyte-derived macrophages (MDMs).

Figure 32. *M. tb* regulation of HIF-1α stabilisation in normoxia.

Figure 33. *M. tb* regulates HIF-1α transcription through NFκB-dependent pathways.

Figure 34. Platelets enhance *M. tb*-driven monocyte HIF-1α gene expression.

Figure 35. *M. tb*-driven HIF 1α protein accumulation in platelet-monocyte co-culture.

Figure 36. Key intracellular signalling proteins are phosphorylated in *M. tb* infection of monocyte-platelet co-culture.

Figure 37. MAPK pathway activation in *M. tb* infection of monocyte-platelet co-culture over time.

Figure 38. Platelet contact-independent regulation of monocyte intracellular pathways.

Figure 39. Platelet-secreted factors target MAPKs to upregulate MMP-1.

Figure 40. Platelets decrease neutrophil MMP-8 secretion.

Figure 41. Platelet regulation of neutrophil MMP-8 and MMP-9 secretion in *M. tb* infection over time.

Figure 42. Platelets do not alter TIMP-2 concentrations in neutrophil co-culture.

Figure 43. Platelets downregulate neutrophil type I collagenase and gelatinase activity in *M. tb* infection.

Figure 44. Platelet regulation of neutrophil cytokines and chemokines.

Figure 45. Platelets downregulate NETosis.

Figure 46. Platelet stimulation of airway epithelial cell MMP-1 secretion.

Figure 47. Platelet-secreted factors upregulate airway epithelial cell MMP secretion.

Figure 48. Platelet-secreted factors do not regulate airway epithelial cell extracellular matrix metalloproteinase inducer (EMMPRIN) secretion.

Figure 49. Platelet regulation of airway epithelial cell TIMP secretion in TB.

Figure 50. Platelet-secreted products upregulate collagenase and gelatinase activity of airway epithelial cells.

Figure 51. Platelet MMP-1 regulation in TB-stimulated airway epithelial cells is not mediated by CD40 ligand (CD40L).

Figure 52. Platelet-secreted products significantly regulate airway epithelial cells MMP-1 and -10 at the gene level.
List of Tables

Table 1. Subdivisions and substrates of TB-regulated MMPs. ................................................................. 29
Table 2. Components of monocyte media conditioned with TB (CT)...................................................... 39
Table 3. Chemical inhibitors and recombinant proteins. ...................................................................... 71
Table 4. ELISA antibodies and standards. .............................................................................................. 73
Table 5. Western blot antibodies........................................................................................................... 77
Table 6. Primers and probes for quantitative PCR. ............................................................................... 79
Table 7. Requirements for patient inclusion in clinical study............................................................... 87
Table 8. Platelet markers correlate with MMPs and IL-1β in bronchoalveolar lavage fluid... 89
Table 9. Platelet-secreted MMPs and TIMPS. ........................................................................................ 94
Acknowledgements

I am very grateful for the guidance of my supervisor Professor Jon Friedland throughout this project. I also would like to thank Dr Sara Brilha for all her patience and expertise, as well as Dr Ashley Whittington, Dr Laura Nellums, Dr Danni Kirwin and Dr Cristina Loader for their suggestions, support, and encouragement both in the laboratory and outside of it. From the first day of my project through to submission the help and support of all of the members of the Friedland research group has been invaluable.

I received bronchial lavage fluid samples as a gift from Dr Shivani Singh, and murine lung samples as a kind gift from Dr Brian Robertson. I am very grateful for these donations, without which I would not have been able to carry out my investigations. I would also like to thank Dr Joanna Porter for her collaboration to perform immunohistochemistry on the murine samples.

I owe much of the last four years to continued support from the Wellcome Trust. Without their funding the project would not have been possible, and their further support to undertake an internship has made it possible for me to translate my PhD training into a new career. Furthermore, without the Wellcome Trust I would not have met my dear friends and colleagues Marie McBrien and Iris Scherwitzl, who I have been honoured to share every high and low with for the last four years and who remain the centre of my London family even as we travel onto separate paths.

My final thanks must go to my family, whose strength and resilience has been a constant source of inspiration to me. In particular, I would like to thank Amelia and her mother for bringing nothing but laughter and love to my life for the last 3 years. Also my sister, who is forever the other half of my heart, and my mother for her truly unconditional support in all that I do. Finally, I thank my father, Stephen, who taught me that to contribute to the world you must first question it, and that I might have something that was worth contributing.
Chapter 1. Introduction

1. The tuberculosis epidemic

Tuberculosis (TB) was declared a global emergency in 1993, but two decades later 2 billion people are thought to be infected and there were 10.4 million new cases in 2015 alone. TB now kills more than any other single infectious disease and remains in the top 10 causes of death globally [1].

TB is an ancient disease, caused by the bacterium *Mycobacterium tuberculosis* (*M.tb*). This is a member of the *Mycobacterium tuberculosis* complex, which is defined as the etiologic agents of TB in distinct hosts and also includes *Mycobacterium bovis* which infects cattle and is the basis for the BCG vaccine [2]. Like all mycobacteria, *M.tb* is a non-motile, non-sporulating, facultative intracellular bacterium with a waxy cell wall. This cell wall confers acid-fastness; resistance to drying, pH change and antibiotics; and distinctive immunostimulatory properties. *M.tb* is also slow growing and, unlike other mycobacteria, strictly a parasitic infection [3]. Modern strains of *M.tb* are thought to have originated 15,000-20,000 years ago, but the early progenitors may have been infecting hominids in East Africa up to 3 million years ago [4]. It is likely that *M.tb* has killed more people than any other known microbe and, in addition to this mortality, the debilitating pathology of TB has been associated with economic devastation of communities throughout that history [5, 6].

*M.tb* was one of the earliest described bacterial pathogens and there is vast literature on the bacteria. However, an ineffective vaccine and our reliance on out-of-date treatments mean TB continues to kill around 1.5 million people a year.
2. Treatment of TB

The two largest shifts in TB control were undoubtedly the introduction of the Bacillus Calmette-Guerin (BCG) vaccine in 1921 and the advent of streptomycin in 1944. The universal BCG vaccination programme was first introduced in the UK in 1956 and to date the vaccine has been given to around 3 billion people worldwide [7]. In 2005 this program was discontinued in the UK, due to its inefficiencies in TB control. The vaccine has 60-80% protective efficacy against severe forms of TB, particularly TB meningitis, in children. However, it confers limited protection for adult disease as it does not prevent initial infection or disease reactivation. Even this limited protection is highly geographically variable for reasons that remain unclear but are likely linked to contact with environmental Mycobacterium [8].

The “miracle-cure” of the TB antibiotic treatments established in the 1940s and 1950s lessened the concern around TB as a major public health concern. However, since this period there has been woeful neglect of new treatment and implementation programme development. Modern TB treatment still relies heavily on drugs developed in the mid-20th century, with the drugs isoniazid and rifampicin both first utilised in the 1950’s [9]. A six month course of these drugs form standard TB treatment, combined with pyrazinamide and ethambutol for the first 2 months. Together with streptomycin these drugs make up the first line treatment against TB, and have done so for over 50 years. The side effects of these dated treatments and the prolonged treatment time of 6-18 months fuels widespread patient non-adherence that increases the risk of transmission and mortality [10]. Non-adherence to treatment as well as flagrant antibiotic misuse has also led to the rise of a new obstacle in the control of the TB epidemic: drug resistance.

Drug-resistant TB is disease that cannot be treated by at least one of the first line TB drugs, with multi-drug resistance (MDR) defined as resistance to at least isoniazid and rifampicin. 480,000 people are estimated to have been infected with MDR-TB worldwide in 2015, resulting in 190,000 deaths per annum [1]. However, real figures may be even higher due to the severely limited point of care drug susceptibility tests available in the areas where they
are most needed [11]. Some cases of MDR-TB have also been found to have resistance to fluoroquinolones and at least one of the second-line drugs amikacin, kanamycin, or capreomycin. Cases of this extensively drug resistant TB (XDR-TB) have now been reported by 105 countries and require at least 18 months treatment, using less effective and more expensive drugs that have significantly more side effects [12].

New TB treatments are urgently required to combat drug resistance; establish shorter, simpler and less toxic treatment courses; and improve patient outcomes. Although improvements have been made to the TB drug pipeline this has so far not been reflected in significant clinical progress. The urgent need for improvement in this area has resulted in a drive to repurpose drugs that are already licenced and available for use in other diseases, for the treatment of TB. Repurposing can avoid the lengthy processes of toxicity profiling, target validation, hit-to-lead optimisation, and in vivo metabolic studies which all delay incorporation of new drugs into treatment regimens [9]. This approach has been successfully taken with moxifloxacin and linezolid, which are now used when first and second line drugs fail, and promising pre-clinical and clinical trials are underway for repurposing other drugs such as the analgesic Carprofen and the anti-psychotic Thioridazine [13]. Currently focused on drug-resistant TB, this field has the potential to offer real progress in improving treatment options for both drug-susceptible and resistant TB.

With the rise of antibiotic resistance there is substantial interest in developing treatments that target the host’s response to a pathogen using host-directed therapies [14] and this has given renewed hope to the search for anti-TB cures [15-17]. The antimicrobial drug Doxycyclin has been used to treat TB for over 60 years and recently part of this function has been attributed to its inhibition of host proteases [18]. Anti-inflammatory statins have been shown to enhance *M.tbc* killing in mice studies and human macrophages in vitro [19], and ibuprofen treatment improved survival of a TB model of highly susceptible mice, associated with better control of *M.tbc* infection [20]. The potency of a dual approach of a combined antimicrobial and host-directed treatment regimen is illustrated in the still widely-used treatment of Hepatitis C with pegylated interferon, to stimulate the host immune system, combined with ribavirin, to destroy the virus.
TB is an ideal target for host-directed therapy as it is a predominately host-driven disease, with the importance of the cell-mediated immune response as a driver of TB pathology well-established [21]. However, in TB the progress of host-directed therapy development has been slow, and is persistently restricted and hampered by our poor understanding of the pathology of TB and the role our immune response plays in it, particularly at early stages of infection [16].

3. Disease pathology

Active TB typically manifests as fever, weight loss and loss of appetite. Whilst the pathogen can infect virtually any organ in the body, the most common presentation of the disease is pulmonary, with typical symptoms including coughing and hemoptysis [22]. Behaviour such as coughing facilitates the spread of this pathogen via highly infectious aerosolised droplets which are then inhaled by others. The number of bacilli in the droplets, the virulence of the bacilli, exposure of the bacilli to ultraviolet (UV) light, degree of ventilation, and occasions for aerosolisation all influence transmission [23].

In the traditional paradigm of TB, upon inhalation of infectious droplets down into the alveoli, the bacteria are rapidly phagocytosed by alveolar resident macrophages. A highly effective infection process allows an extremely low multiplicity of infection (MOI), with less than 10 bacteria thought to be required for establishment of infection in the lung after inhalation [24]. The macrophages are thought to recognise the bacteria through pattern recognition receptors (PRR) in their membranes. These receptors belong to four main classes: opsonising receptors such as complement receptors; scavenger receptors like CD36; C-type lectin receptors such as mannose receptor; and innate immune sensors such as Toll-like receptors and nucleotide-binding-oligomerisation-domain (NOD)- like receptors [25-27]. Once phagocytosed, *M.tb* can persist in immature macrophage phagosomes and replicate, evading destruction by actively preventing phagosome maturation and acidification. In addition to the phagosome, *M.tb* is able to replicate in the cell cytoplasm and probably inhabits several intracellular niches, depending on various factors such as cell type [28].
The infected alveolar macrophages invade the epithelial layer, transporting $M. tb$ across the alveolar epithelium into the lung tissue and inducing a localised inflammatory response. This response leads to recruitment of cells from blood vessels and ultimately initiation of TB granuloma formation, with macrophages and other immune cells aggregating around the infected cells [29]. Initially, monocytes and neutrophils are recruited and accumulate to form an early granuloma. As well as being first responders, monocytes are continually recruited to the site of infection where they engulf *Mycobacterium* at a peripheral infection site and migrate deeper into tissues [30]. Antigen-specific T lymphocytes migrate to this early lesion to multiply and release pro-inflammatory cytokines such as interferon which activates macrophages to kill the intracellular $M. tb$ [31]. Further cell recruitment establishes the organised and stable structure known as a TB granuloma. Infected alveolar macrophages are at the centre of this structure and these are surrounded by a variety of cells including: neutrophils, monocyte-derived cells such as foamy macrophages and dendritic cells (DCs), and lymphocytes surrounding the outside [32]. Surrounding epithelial cells are not embedded into the internal structure of the granuloma, but do facilitate its formation [33]. The early pathology of TB may also involve a dissemination stage in the $M. tb$ life cycle as the classic hallmark of TB (creation of cavities in the lung tissue) typically occurs in the apices of the lungs or in the apical segment of the lower lobes whilst the initial deposition event occurs in the lung bases. This implies the bacilli must spread to the apices for lesion formation and progression of pathology [34]. Within these granulomas, bacterial growth stalls and enters a dormant asymptomatic (latent) state that can last for decades with the infected individuals asymptomatic and thought to be non-infectious.

Encased in a granuloma the bacteria are both contained by, and protected from, the immune system. Like many aspects of the TB host-driven pathology, the granuloma plays a complex role in bacterial clearance and immunopathology. The granuloma undoubtedly limits pathology and spread of $M. tb$ in humans with its formation essential to the control of $M. tb$ infection; protecting the host from the bacteria and its virulence. However, TB granulomas also protect the bacteria from the host immune system. Long-term the granuloma is associated with increased bacterial replication and provides a safe environment to establish bacterial latency that facilitates the bacteria surviving and reactivating decades later [29, 32, 35].
The organisation of the granuloma is particular to TB, as are the characteristic regions of necrosis within them. In around 10% of latently infected individuals the granuloma can become necrotic and the bacteria reactivates [36]. In the liquefied necrotic caseous centre of the granuloma the bacteria can multiply rapidly and ultimately result in the break-up of the granuloma [37]. This facilitates dissemination to other tissues, creation of further lesions and transmission to a new host to complete the cycle. Reactivation of *M. tb* infection results in bacterial dissemination and death in 50% of cases [3]. A wide range of both genetic and environmental immunomodulatory factors are associated with this reactivation, from HIV infection to old age. Immune suppression is a well-established risk factor for reactivation, but many individuals have no obvious defect in the immune system leaving much of this mechanism unknown [38, 39].

4. Innate cell-mediated immunity in TB

The acquired immune response, largely represented by CD4$^+$ T cells, is important to the control of *M. tb* infection in the lung but it is slow to be induced and expressed [21]. In humans, an adaptive response to *M. tb* is not generally apparent until 6 weeks after infection, as measured by a response to a tuberculin skin test (TST). This is significantly longer than for other bacterial pathogens and allows time for the bacterial population in the lungs to expand and evade immune effector mechanisms [40]. Innate immunity is thought to be central to early clearance of infection, with studies indicating that there may be bacterial clearance of *M. tb* by the innate immune system alone. For example, Verall *et al* found 50% of individuals exposed to *M. tb* did not exhibit a positive TST [41]. An alternative to early bacterial clearance is an unregulated inflammatory response, which drives presentation and persistence of disease. The imbalance between host innate cell-mediated protective and destructive action is at the centre of TB’s pathology.

There is a robust network of cells involved in the innate immune response to TB, consisting of both traditional and non-traditional immune cells. However, *M. tb* is able to subvert the functioning of this array of cells to establish a deregulated and highly inflammatory immune
response that facilitates bacterial growth and persistence and limits development of adaptive immunity [42]. A few of the key players of the innate immune cell response to TB will now be discussed in some depth.

### 4.1. Monocytes

Monocytes originate in the bone marrow from hematopoietic stem cells and comprise around 10% of leukocytes in human blood. Monocytes represent important immune effector cells, equipped with chemokine and adhesion receptors that mediate migration from blood to tissue. Extravasated monocytes survey uninfamed lung tissue for antigens to transport to draining lymph nodes while remaining largely undifferentiated [43]. In M.tb-infected tissues, monocytes have been shown to give rise to many of the macrophage and DC subsets that appear following aerosol infection with virulent M.tb, resulting in a range of monocyte-derived cells at the site of infection [44].

#### 4.1.1 Monocyte differentiation

Monocytes respond to stimuli through significant differentiation in phenotype, genotype and surface markers. Traditionally these changes have qualified monocyte-derived cells to be classified as a distinct cell type, separate from its monocyte heritage. Of the innate immune cells involved in the host response to M.tb, macrophages are considered the primordial regulators of the balance between pro- and anti-inflammatory immune responses to control bacterial replication and tissue damage. As such, macrophages have been a focus of TB research in the past [45]. Most of this study has dependent upon macrophages matured from monocytes in vitro, using stimuli such as macrophage colony stimulating factor (M-CSF). The resulting cells are classified as macrophages after 3-5 days according to morphology, cytokine responses and surface markers such as high expression of CD71 and CCR5. However, in recent years the relevance of this model for studying monocyte-derived cell function has been an increasing subject of discussion due to an improved understanding of the complexity of monocyte biology and the plasticity of monocyte-derived cells.

Morphological differences between monocyte-derived cells are unreliable discriminators due to significant changes occurring upon inflammatory stimulus. The development of
polychromatic flow cytometry has enabled the assessment of different surface markers to characterise multiple distinct DC, monocyte and macrophage subsets in humans and animal models. However, rather than clarifying the field of study this approach revealed many of the defining markers and functions that were thought to be unique were in fact shared between cell types. For example, the traditional macrophage marker CD68 does not discriminate between macrophages and dendritic cells [46] whilst CD16, previously used to discriminate macrophages to monocytes, is found on a subset of monocytes. Whilst classical monocytes in the blood are defined as CD14^+CD16^- these compose only around 80-90% of monocytes in the blood, with non-classical CD14^lowCD16^- cells as well as intermediate monocytes CD14^+CD16^- comprising the remaining 10-20% [47]. These monocytic subgroups have been accepted in their current form since 2008, and have been confirmed more recently using gene expression profiling [48]. The functional differences for these groups are not yet clear, with research hampered by the strong effect of different isolation techniques on monocyte profiles. Classical monocytes do appear to have strong phagocytic tendencies whilst the non-classical monocytes have a highly inflammatory response with properties for antigen presentation [49]. In inflamed tissue where monocyte differentiation may be triggered, the process is a gradual and plastic one. It is unclear if monocyte-derived cells such as DCs and macrophages are ontogenically distinct lineages or highly plastic cells that are able to acquire a multitude of functional modules in response to the environmental cues they receive. Indeed, accurate discrimination between these cells is so difficult that there are calls to alter nomenclature to simply be “monocyte-derived cell” followed by an indicator of functional heterogeneity [50].

This shift in our understanding of monocyte-derived cells and their development, plasticity, and function emphasises the importance of in vitro models using freshly isolated human blood monocytes stimulated in physiologically relevant ways. Such studies are urgently needed to help elucidate the mechanisms of monocyte-derived cells in the cell-mediated response to TB.

4.1.2 Monocytes in TB

Monocytes are amongst the first cells to migrate from the blood into M.tb-infected tissue. These cells bind the intracellular adhesion molecule (ICAM) through its Mac-1 binding site for extravasation into tissue such as the M.tb-infected lung [51]. Tumour Necrosis Factor
TNF-α knockout mice are known to have a dysregulated inflammatory response to TB, with an influx of neutrophils, formation of necrotic lesions, uncontrolled bacterial growth and mortality. This response is not associated with altered T cell activation but delayed monocyte recruitment into tissue. Whilst T cell responses which activate resident macrophages are sufficient for very early control of *M. tb*, without monocyte recruitment significant mortality occurs in mice models due to extensive cellular infiltration and widespread tissue necrosis [52]. Part of this immunopathology can be attributed to lack of a structured granuloma formation, as mice with altered ICAM (to prevent monocyte recruitment but allow normal T cell responses) do not form granulomas [53].

Monocyte recruitment is essential to granuloma development and its ordered structure. The basis of granuloma formation is not the aggregation of infected alveolar macrophages but increased extravasation and recruitment of uninfected monocyte/macrophages to a single infected aveolar macrophage [54]. These newly recruited monocyte/macrophages can then become readily infected through phagocytosis of infected macrophages that have undergone apoptosis [55] or by bacterial transfer between live cells [56]. 3D lung models have shown that infection of monocyte/macrophages with virulent *M. tb* is sufficient to cause cells to cluster and form an early granuloma structure [57]. In this manner, monocytes contribute to the rapid formation of granulomas after infection is established before the adaptive immune response has been mounted [58]. Furthermore, monocyte recruitment does not stop once the granuloma is established but continues to facilitate granuloma expansion and the dissemination of bacteria. Recruited uninfected monocyte/macrophage cells passage rapidly and constantly through the granuloma, in a random manner with dying cells thought to exert chemotactic signals that lead to periods of more directed motion. Infected monocyte/macrophages have increased motility compared to uninfected cells and can readily exit the primary granulomas and travel through tissue to seed distal granulomas. In one zebrafish model, 19% of newly departed monocyte/macrophages initiated granuloma formation per day and the cells were calculated to account for the majority of all granuloma expansion [55, 58]. Infected monocytes also provide a mechanism for *M. tb* dissemination in the blood. The presence of a blood-borne phase in TB is supported by the distribution of lesions throughout the lungs and other organs observed in miliary TB, and *M. tb* can occasionally be cultured from patient’s blood, although typically only those co-infected with
HIV. Zebrafish studies have also indicated that monocytes transport *M.tb* through the blood circulation as well as the tissues, to drive the early dissemination of *M.marinum* from the site of infection to the apices for lesion formation [34].

Aside from their essential role in granuloma formation and expansion, there are contradicting reports on the role of monocytes in *M.tb* killing. Monocytes can kill bacteria via phagocytosis, production of reactive oxygen species (ROS), nitric oxide (NO), myeloperoxidase, and inflammatory cytokines [59]. Pro-inflammatory cytokine stimulation of monocytes isolated from active TB patients has been shown to trigger NO production that kills *M.tb* [60]. Additionally, co-stimulation of monocytes with CD4+ T cells or natural killer (NK) cells can activate them to significantly upregulate *M.tb* killing [61]. However, *M.tb* is able to replicate inside monocytes and triggers release of pro-inflammatory cytokines such as interleukin (IL)-1β and TNF-α as well as apoptosis of the cell in a manner that does not impact bacterial survival [62, 63]. Monocyte/macrophage production of TNF-α and NO is also correlated with upregulation of T cell apoptosis, which also suggests a negative effect of monocytes on host *M.tb* killing [64]. As a whole the evidence suggests some *in vivo* killing of *M.tb* by monocytes, in a manner highly dependent on intercellular signalling.

Not all monocyte/macrophages further *M.tb* dissemination through apoptosis or exiting the granuloma. In TB, a proportion of monocytes differentiate further into epithelioid cells or foamy macrophages packed with lipid droplets, or fuse to form Langerhans-type multinucleated giant cells (MGCs). These highly differentiated cells form an essential component of the TB granuloma, thus facilitating latent *M.tb* infection [65]. Epithelioid cells are so called due to their resemblance to epithelial cells with an elongated structure and tightly interdigitated cell membranes that closely link adjacent cells. They are also highly phagocytic for *M.tb*. These cells are thought to function to create a tight granuloma structure that better contains *M.tb*, and the cells can also fuse to form multinucleated cells [66]. MGCs cannot phagocytose *M.tb* due to loss of phagocytic receptors, mannose receptor, and CD11b, but can secrete pro-inflammatory signals that facilitate tissue remodelling and further cell influx [67-69]. Monocyte-derived foamy macrophages have significantly reduced anti-microbial activity of phagocytosed *M.tb*. Instead, *M.tb* can persist in a non-replicating state with nutrients provided by cell lipid bodies that *M.tb*-containing
phagosomes migrate towards and then engulf [70]. In this manner monocytes and monocyte-derived cells are central in providing a secure reservoir of latent *M. tb in vivo*.

In addition to their role in the host immune response to *M. tb*, monocytes also have an important role in mediated responses to host co-infection. For example, in a HIV co-infection model in macaques, monocyte-produced IL-5 was attributed to a reduction in TNF-α from *M. tb*-specific CD4+ T cells [71]. The role of monocytes in HIV and TB co-infection is particularly vital in TB-associated Immune Reconstitution Inflammatory Syndrome (TB-IRIS), which is one of the most important challenges in treating HIV-TB co-infection. TB-IRIS is an exacerbated inflammatory response to TB that typically occurs in the period after combined anti-retroviral therapy that results in clinical deterioration. It is thought to complicate treatment of 25% of co-infected individuals. Monocytes have been attributed with involvement in both a person’s pre-disposition to, and development of, TB-IRIS [72]. Although the mechanism of this is still under discussion, CD14+CD16− monocytes are an independent predictor of TB-IRIS in patients and monocyte-secreted inflammatory cytokines were significantly higher in patients compared to controls [73]. This hyperactivation of monocytes may be linked to activation of the inflammasome and has been noted as a potential treatment target for TB-IRIS [74]. Through this diverse range of mechanisms and interactions, monocytes are one of the most influential players in the immunopathology of TB, with their early recruitment, infection, activity and differentiation all vital to the development of disease.

4.2. Neutrophils

Neutrophils are short-lived, phagocytic cells that are an emerging player in TB immunopathology. They are amongst the first and most numerous innate immune cell to arrive at the site of infection [75]. As well as direct killing, neutrophil products and pro-inflammatory effects influence the innate and adaptive immune system through recruitment and activation of inflammatory cells and T and B lymphocytes. Analysis of TB patient respiratory secretions have shown neutrophils to be the most commonly infected cell type [76]. They are recruited to the site of *M. tb* infection rapidly and in high numbers by chemokines and cytokines such as IL-8 and granulocyte colony stimulating factor (G-CSF) secreted by macrophages, other neutrophils and epithelial cells. This recruitment is
generally considered maximal at 24 hours (hrs) post-infection, although recruitment has been reported to continue well after this [77]. Neutrophil presence significantly contributes to the pathology of TB although the nature of their function remains a subject of debate.

Neutrophils are important mediators of TB pathology and increased bacterial growth, regulated by interferon (IFN)-γ [78]. Neutrophilia at the time of TB diagnosis is associated with delayed clearance of bacteria from sputum, and in established infection higher neutrophil counts are associated with greater bacterial growth and severity of disease [79, 80]. However, there are studies indicating early active neutrophil recruitment may be beneficial to clearing initial infection. LPS recruitment of neutrophils to the lungs of rats at the time of airborne M.tb infection, decreased downstream colony forming units (CFU) in the lung, and depleting granulocytes in mice before intratracheal infection with M.tb increased the CFU that was subsequently recovered from lungs and spleen [81, 82]. Neutrophils become activated as they are stimulated by cytokines, chemokines and M.tb products. This enhances the neutrophil respiratory burst and microbicidal function which may facilitate early clearance of M.tb through oxidative pathways and antimicrobial granule proteins [83]. Antimicrobial granules such as human neutrophil peptides (HNPs), which are members of the α-defensin family, the cathelicidin LL-37 and lipocalin 2 are released and have been particularly associated with a role in clearing M.tb from the blood [84]. In addition, apoptotic bodies of infected neutrophils can be phagocytosed by mononuclear cells to enhance restriction of M.tb growth. Increased secretion of numerous chemokines, cytokines and growth factors such as leukotrienes also drive further neutrophil recruitment, pro-inflammatory responses of other immune cells such as macrophages, and tissue destruction [85-87].

Neutrophils can readily phagocytose extracellular bacteria through opsipinisation or direct recognition via toll-like receptor (TLR)2, but may not be effective at killing intracellular M.tb [77]. Neutrophils may also alter M.tb viability through the production of neutrophil extracellular traps (NETs). NETs are formed during NETosis, a unique cell death pathway characterised by the release of decondensed chromatin and granular contents to the extracellular space. M.tb has been shown to trigger release of NETs which trap the bacteria, but, in contrast to other bacteria such as Listeria monocytogenes, are unable to kill M.tb [88]. NET formation occurs independently of M.tb-induced apoptosis but is dependent on
Chapter 1

*M.tb* phagocytosis. Interestingly, an inhibition of neutrophil elastase delays NET formation [89]. The ability of NETs to only trap and restrict growth of *M.tb* has led to the suggestion that *in vivo* their role is predominately to limit bacterial spread and enhance effective and targeted recruitment of cells. However, *in vivo* neutrophils are generally thought to contribute to local and systemic spread of infection due to significantly higher migration capacity and life span of *M.tb*-infected neutrophils [90].

Although not traditionally associated with the TB granuloma, neutrophils are often present at the site of active TB and upon activation by *M.tb* secrete chemokines. These include monocyte chemotactic protein 1 (MCP-1) and IL-8 to recruit leukocytes, and CXCR3 chemokines such as CXCL9 (also known as Monokine induced by gamma interferon (MIG)); Released upon Activation, Normal T cell Expressed and Secreted (RANTES); and MCP-1 to organise the granuloma [91, 92]. Given the requirement of a well-structured granuloma for latent *M.tb* infection this implicates neutrophils in the outcome of latent disease. In humans, higher peripheral blood neutrophil count reduces the risk of latent TB in patients with active disease. Neutrophils influence the development of T cell responses both through interactions with macrophages and DCs, and directly with secretion of cytokines such as IL-12, CC chemokine ligand (CCL)3/macrophage inflammatory protein (MIP)-1α, and MCP-1. These cytokines attract T-lymphocytes and direct their maturation. Conversely, neutrophils have also been reported to produce IL-10 during *M.tb* infection, indicating neutrophils in some circumstances might limit protective acquired immune responses [93]. DCs that have acquired *M.tb* antigen via an apoptotic neutrophil migrate more efficiently than directly infected DCs. Therefore, neutrophils mediate T cell activation with a role in DC migration to lymph nodes, and can also enhance CD4+ T cell IFN-γ release and CD8+ T cell cytotoxicity also through DC interaction [94, 95].

Together, the literature suggests that neutrophils have an initially positive role in controlling and clearing *M.tb* infection. However, after this very early stage, the highly potent inflammatory effects of neutrophils appear to exacerbate the inflammation that defines TB pathology. Some of the contradicting results from studies investigating the role of neutrophils in TB may be due to *in vitro* models which do not include intercellular communication that *in vivo* tightly control neutrophil influx and secretions. Further
investigation of intercellular regulation is needed to understand more of the role of this important yet complex player in TB pathology.

4.3. Airway epithelial cells

It is now known that cells of non-myeloid and non-lymphoid origins functionally contribute to the regulation of immune responses. These non-classical immune cells are essential to the functioning of innate and adaptive responses in TB, but less is known about their role than the classical immune cells of myeloid and lymphoid origins [96].

Epithelial cells line the airways as part of the respiratory mucosa. This mucosa consists of the airway surface liquid (a layer of innate immune factors on the luminal surface), the lamina propria (a layer of connective tissue and immune cells and the epithelium), and a layer of airway epithelial cells (AECs). These AECs are among the first cells encountered by *M. tb* and their vital role in immune defence reaches far beyond their traditional function as a simple structural barrier to infection [97].

AECs express PRR such as TLRs, C-type lectin receptors, NOD-2 receptors and mannose receptors, all of which have been implicated in the immune response to *M. tb* through recognition of *M. tb* pathogen associated microbial patterns (PAMPs) on the bacterial surface [98, 99]. These receptors signal via janus kinase/signal transducers and activators of transcription (Jak/STAT), nuclear factor kappa B (NFκB), and interferon regulatory factors (IRFs). They allow AECs to function as immune sentinels, presenting antigen to traditional immune cells and activating epithelial cell signalling pathways that lead to cytokine, chemokine and antimicrobial protein production [96]. For example, epithelial cells are an important producer of the only human cathelicidin, LL-37, which is a broad anti-microbial peptide active against a variety of bacteria including mycobacteria [100]. LL-37 also activates epithelial cells and functions as a chemoattractant for T cells, monocytes and neutrophils. Epithelial cell stimulation with BCG and *M. tb* has been shown to up-regulate cathelicidin LL-37 in an NADPH/reactive oxygen species (ROS) signalling pathway-dependent manner [101, 102]. Other AEC-secreted anti-microbial products include β-defensin 2, and hepcidin, which have both been demonstrated to play critical roles in innate immunity against mycobacteria infections. Hepcidin is an antimicrobial peptide that regulates iron homeostasis: it is
associated with the bacterial cell wall on internalised bacteria and, in vitro, inhibits *M.tb* growth and AEC expression of β-defensin 2 upon infection with *M.tb* [103, 104]. β-defensin 2 has been additionally implicated in the control of *M.tb* in a mouse model of progressive pulmonary TB, with rapid expression by AECs at an early stage correlating with control of bacilli proliferation. This was followed by a profound decrease in expression during the later progressive phase of the disease but notably, this expression was suppressed after reactivation in a latent-infection model [105]. The antimicrobial peptide β-defensin 2 is also a chemoattractant, recruiting immature DCs and memory T cells to the site of infection [106]. Through secretion of such products, AECs may play an important role in direct clearance of *M.tb* infection at an early stage.

A variety of leukocytes that are involved in TB pathology, such as monocyte-derived cells and lymphocytes, localise adjacent to AECs [107]. This facilitates a continuous interaction and intercellular communication between AECs and other cells that mediate the host response to *M.tb*. AEC secretion of cytokine and chemokines recruits, activates, and enhances survival of immune cells. *M.tb* interaction with AECs stimulates production of TNF-α; IFN-γ; G-CSF; and interleukins such as IL-1, -4, and -8. These pro-inflammatory factors recruit monocytes, neutrophils, and lymphocytes to the site of infection to facilitate bacterial clearance and are active against mycobacterial infection [99, 108]. IFN-γ induces the lung epithelium to produce the enzyme indoleamine 2,3-dioxygenase (IDO). This enzyme converts tryptophan into its kynurenine metabolites which have broad-spectrum bactericidal properties, although in TB their primary function is thought to be inhibition of IL-23, which then limits IL-17-induced cell proliferation to reduce neutrophil migration [109]. *M.tb*-infected AECs have been shown to efficiently stimulate IFN-γ from CD8+ T cells termed mucosal associated invariant T (MAIT) cells in the upper airways. A similar mechanism has been shown as important to the early response of bacterial pathogens such as *Shigella flexneri*, suggesting a role of AECs not only in supporting the early innate immune response but also assisting in priming specific adaptive immune responses through T cell interaction [96, 110]. Despite their important role in early immune defence, the role of AECs in amplification of the inflammatory response also implicates AECs in the damaging immunopathology of TB.
AECs are activated through PRRs such as TLR-3, -7, -8 and -9 in a variety of viral infections associated with airway disease, such as Influenza A virus [111] and respiratory syncytial virus (RSV)[112]. Through stimulation of these TLRs the production of cytokines such as IFNs are stimulated to promote an acute inflammatory response [113]. These cytokines are also important in chronic inflammation. For example Byers et al. used a mouse model and patients to build on research investigating chronic asthma phenotypes in post-viral murine models and show that AEC IL-33 secretion mediates disease in chronic obstructive pulmonary disease (COPD) [114]. AECs are important for the recruitment of neutrophils and in TB, a chimeric mouse model with epithelial cells unable to produce or respond to IFN-γ stimulation produced a severely hyperneutrophillic response to M.tb associated with significant mortality [115]. Conversely, inhibiting AEC recruitment of neutrophils in response to M.tb infection, through the absence of CXCL5 or its receptor CXCR2, improved pulmonary pathology in mice and enhanced survival [116]. A zebrafish model has also been utilised to investigate AEC function in TB. In this model, AECs have been shown to be essential to the development of granulomas, and as such are associated with mycobacterial growth [33]. Taken together the research clearly indicates AECs as central players of the innate immune response to TB, with far reaching and complex effects.

Cells of the innate immune response such as monocytes, neutrophils and epithelial cells are key mediators of the host response to M.tb infection. They are required for bacterial control and clearance, however together they also drive the large-scale destruction of lung tissue that is a central feature of active pulmonary disease.

5. Tissue destruction in TB

The formation of pulmonary cavities has been a noted hallmark of TB for hundreds of years (Fig 1). The cavities are the site of high mycobacterial burden and are associated with bacterial transmission, mortality and morbidity [117]. For these cavities to form, the lung parenchyma has to be destroyed. The lung extracellular matrix (ECM) is composed of macromolecules which support the complex structure of the lungs and facilitate their
function. Amongst the most abundant macromolecules are: type I collagen (a primary structural protein with a fibrillar triple helical structure), elastin, and gelatin which together confer strength, shape and elasticity [118]. The traditional paradigm of this lung destruction is that cell death in the necrotic caseous granuloma drives cavity formation [119], however, in recent years this has been refuted with publication of a substantial body of evidence indicating active degradation of the ECM by proteases. This creates lung tissue destruction that can occur on a huge scale (Fig 1). The process of this cavity formation was described in the early 1800s by the French physician René Theophile Hyacinthe Laennec who performed a series of autopsies on TB patients [120]. He noted

“Whatever the form in which the tuberculous matter develops, it begins as a grey, semi-transparent matter that little by little becomes yellow, opaque, and dense. Then it softens, and slowly acquires a liquidity like pus, and, when it is expelled through the airways, it leaves cavities, commonly called ulcers of the lung, that we will designate as tuberculous excavations” [120].

This ECM destruction is driven by a family of matrix-destroying enzymes known as matrix metalloproteinases (MMPs) [121].

Figure 1. CAT scan of TB patient lungs. Large scale breakdown of extracellular matrix (ECM) has resulted in almost complete degradation of left lung. Red arrow in the right lung indicates a smaller area of cavitation with cavity formation surrounded by a mass of waste products. This tissue degradation is driven by a family of enzymes known as matrix metalloproteinases (MMPs) and ultimately leads to organ failure and mortality in untreated TB patients.
5.1. The MMP family

5.1.1 MMP structure
MMPs are calcium-dependent zinc-containing endopeptidases that together can degrade every component of the lung ECM, as well as processing numerous bioactive molecules [122]. Since the identification of this family in 1963 there have been 28 MMPs identified, termed MMP-1 to -28. These enzymes can be either soluble or membrane bound. All MMPs have a signal peptide that targets them for secretion; a zinc ion containing catalytic domain; a flexible proline-rich hinge region; a prodomain; and a carboxy terminal hemopexin domain for substrate recognition. The membrane bound MMPs have an additional transmembrane domain and cytosolic tail.

These enzymes share structural domains but differ on substrate specificity, cellular sources, inducibility, and regulation. The MMP family is categorised by substrate specificity, as gelatinases, collagenases, stromelysins, matrilysins and others [123]. The categorisation of MMPs upregulated in TB, with a non-exhaustive list of substrates, is shown in Table 1.

5.1.2 MMP Function
Normal physiological processes such as: cell migration; cytokine and chemokine activity; embryonic development; reproduction and tissue repair; and remodelling are dependent on MMP activity [124]. For example, the collagenase MMP-1 has been shown in vivo to be required for the repair of skin wounds and this function is partially attributed to MMP break down of damaged ECM that occurs at the edge of acute skin wounds [125]. This enables new ECM components such as collagen and fibronectin to integrate correctly with intact ECM components at the wound edges. Wound cells are able to continue to produce low levels of MMPs for prolonged periods to facilitate the creation of a more normal and organised skin structure than the initial scar tissue [123]. Aside from these functions, MMPs have also been shown to control cytokine biosynthesis and direct systemic inflammation and barrier immunity through activation of signal transduction pathways. A variety of MMPs, including -1, -2, -3, -9 and -17, are involved in regulating chemokine activity through protein cleavage, for example processing pro-TNF-α to its active form [126].
Table 1. Subdivisions and substrates of TB-regulated MMPs.

Modified from Parks et al. [127], Ashworth et al. [128], Kahari et al. [129] and Ong et al. [130]. Substrates detected by in vivo or in vitro methods.

<table>
<thead>
<tr>
<th>MMP</th>
<th>Categorisation</th>
<th>Substrates</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Collagenase</td>
<td>Triple helical collagens I, II, and III</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Latent TNF</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-selectin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>α2-macroglobin</td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>Gelatinase</td>
<td>Gelatin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen IV</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proteoglycans</td>
<td></td>
</tr>
<tr>
<td>MMP-3</td>
<td>Stromelysin</td>
<td>ProMMP-1, proMMP-8 and proMMP-9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proteoglycans</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen III, IV and V</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Laminin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fibronectin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Latent TGF-β</td>
<td></td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrilysin</td>
<td>Collagen IV</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ProMMP-2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gelatin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fibronectin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Latent TNF</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elastin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pro-α defensins</td>
<td></td>
</tr>
<tr>
<td>MMP-8</td>
<td>Collagenase</td>
<td>Triple helical collagens I, II, and III</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fibronectin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>laminin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>α2-macroglobin</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>Gelatinase</td>
<td>Gelatin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen IV and V</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fibronectin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elastin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proteoglycan link protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Latent TNF</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Latent VEGF</td>
<td></td>
</tr>
<tr>
<td>MMP-10</td>
<td>Stromelysin</td>
<td>ProMMP-1, proMMP-7, proMMP-8 and pro MMP-9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proteoglycans</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen III and IV</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fibronectin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elastin</td>
<td></td>
</tr>
<tr>
<td>MMP-14</td>
<td>Membrane-type</td>
<td>Triple helical collagens I, II, and III</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gelatin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fibronectin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ProMMP-2</td>
<td></td>
</tr>
</tbody>
</table>
5.2. Regulation of MMPs

In normal physiology, the major sources of MMPs are typically wound cells such as epithelial cells and activated inflammatory cells such as neutrophils and monocyte/macrophages. As highly potent proteases, MMP activity is tightly regulated at every level.

Tight regulation of MMPs at the transcriptional level generally results in a minimal basal level of MMP production. MMP genes are late responders, not generally switched on until hours after the initial stimulus. This delay suggests MMP genes are downstream targets of the signalling pathways of early response genes which encode signalling proteins such as the mitogen activated protein kinases (MAPK), and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K). These proteins activate transcription factors such as NFkB, which in turn are able to bind to the promoters of MMP genes (Fig 2) [131]. The signalling pathways can be blocked at multiple stages through inhibitory stimuli or lack of additional activating signals for example, by sequestering transcription factors or inhibiting their phosphorylation to prevent their binding to genetic elements [132].

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor recently implicated in MMP regulation. Although constitutively expressed, the HIF-1α subunit is continually degraded by prolyl hydroxylases (PHD). When this mechanism is overcome, for example by hypoxic inhibition of PHD activity, HIF-1α protein is stable and is able to enter the nucleus to bind to HIF-1β and form HIF-1. This in turn binds to the hypoxic response elements (HREs) of a range of target gene regulatory sequences resulting in the transcription of genes involved in cellular inflammatory responses. Normoxic stabilisation of HIF-1α was first shown with lipopolysaccharide (LPS) stimulation of macrophages but a number of Gram positive infections such as Pseudomonas aeruginosa and Salmonella typhimurium have now also been shown to stabilise HIF-1α [133, 134]. This bacterial stabilisation has been shown to modulate a range of innate immune responses. For example, mice without HIF-1α in their myeloid lineage have reduced bactericidal activity and fail to restrict systemic spread of Group A Streptococcus infection [134]. In vitro this failure has been attributed to HIF-1 regulation of NO production, chemotaxis and phagocytosis [135, 136]. However, HIF-1 also targets MMPs, with MMPs such as MMP-2 and -9 having been shown to be a direct HIF-1 target, leading to clinically relevant ECM destruction [137, 138]. In pulmonary TB, lesions are
hypoxic and associated with HIF-1α, but HIF-1α can also be stabilised in *M.tb*-stimulated

In addition to the tight transcriptional control of MMP pathways, MMPs are regulated post-

transcriptionally by untranslated regions (UTR)-binding proteins. These proteins bind to

specific sequences in MMP 5'- or 3'-UTR transcripts to stabilise or destabilise the mRNAs.

**Figure 2. Intracellular signalling cascades for MMP regulation.**

Stimulation of receptors such as Toll-like Receptors (TLRs) activates preformed cytosolic proteins

by phosphorylation. Proteins central to this cascade in MMP regulation are mitogen activated

protein kinases (MAPK), which in turn activates p38 and extracellular signal-regulated kinase (ERK)

1/2, and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K). PI3K in turn activates AKT and the

mechanistic target of rapamycin (mTOR). These active proteins then activate transcription factors

such as nuclear factor kappa B (NFkB) and signal transducers and activators of transcription (STAT)

which enters the nucleus and bind to genetic sites to upregulate MMP gene expression.
For example, in immortalised keratinocytes, signalling from the \( \alpha 3\beta 1 \) integrin was required in addition to the Ras-dependent extracellular signal-regulated kinase (ERK)-1/2 signalling for stable MMP-9 mRNA that could then maintain high levels of MMP-9 protein [140]. This level of control can be manipulated using the Adenosine triphosphate (ATP) analog ATP\( \gamma \)S to upregulate IL-1\( \beta \)-induced MMP-9 expression. This enhances the binding of complexes with the mRNA stabilising factor HuR to AU-rich elements (ARE) motifs in the 3′-UTR of the MMP-9 mRNA, thus providing protection against rapid degradation [141]. Translational efficiency is thought to provide another post-transcriptional control mechanism of MMP expression. Recruitment of MMP-9 mRNA into the rough endoplasmic reticulum can be elevated through binding of proteins such as nucleolin to the 3′-UTR of human MMP-9 mRNA in response to stimulus. This enhances the efficiency of mmp-9 translation and increases protein secretion [142].

Finally, MMPs are controlled at the post-translational level. The catalytic domain contains two zinc ions, one for structural integrity and one for activity: an interaction between the zinc ion in the catalytic domain and a thiol group of a cysteine residue in the prodomain maintains the enzyme in the inactive state. Whilst membrane bound MMPs are activated intracellularly by furin like pro-protein convertases, secreted MMPs are secreted in a pro-enzyme form. To become active, these secreted MMPs must have extracellular proteolytic cleavage of their proteinase susceptible prodomain or modification of the cysteine thiol residue by tissue and plasma proteinases or opportunistic bacterial proteinases [143]. In addition to serine peptidases, stromelysins MMP-3 and -10 have been shown to be an important activating factor for MMPs. MMP-1 and MMP-8 are activated by MMP-10 through a single step cleavage of the Gly78-Phe79 peptide bond at the end of the propeptide domain resulting in increased collagen proteolysis [144, 145]. Such MMP activation by MMP-10 and -3 yields an MMP-8 enzyme with much greater specific collagenase activity than chemical MMP-8 activation. It also has a strong synergistic activation with serine proteases to produce active MMP-1 that is 10-fold more efficient that the MMP produced by serine proteases alone [146]. The role of MMP-10 in MMP-1 activation has been validated in a number of in vitro models, for example neutralisation of active MMP-10 in supernatants of stimulated keratinocytes led to decreased activation of MMP-1, and reduced keratinocyte collagen degradation [147]. Furthermore, transfection of
endothelial cells in collagen matrices, to ensure overexpression of MMP-10 increased activation of MMP-1. MMP-1 knockdown using small interfering RNA (siRNA) blocked the ability of MMP-10 overexpression to drive type 1 collagenase function [148].

The regulation of MMPs in neutrophils is profoundly different to other immune cells as the enzymes are pre-made and stored in granules. Regulation of neutrophil-dependent ECM breakdown is therefore predominately dependent on pathways that regulate the release of MMPs as opposed to their production. Similar to other cells, the neutrophil MMP response has been shown to be NFκB-dependent, and *M.tb*-infected neutrophils have upregulation of the MAPK pathway. However, inhibition of MAPK and the PI3K pathway has limited effect on reducing MMP-8 and MMP-9 secretion. Neutrophils also have upregulation of AMP-activated protein kinase (AMPK) phosphorylation, and inhibition of this factor significantly reduced neutrophil MMP-8 secretion in *M.tb* infection [149, 150].

In addition to activation at the post-translational stage, MMPs can also be inhibited. The major family of MMP inhibitors are called Tissue inhibitors of matrix metalloproteinases (TIMPs). There are four distinct TIMPs named TIMP-1, -2, -3 and -4 which control MMP activity in nucleate cells. TIMP-3 is bound to the ECM whilst TIMP-1, -2 and -4 are soluble. TIMP-2 and -4 are able to inhibit most MMPs, whilst TIMP-1 appears to have more specificity, as it is largely unable to bind to membrane-bound MMPs (MT-MMPs) but has binding to MMP-1, -3 and -7 that is stronger than the other TIMPs [151-153]. To exert their inhibitory action the TIMPs generally bind to MMPs in their active form but can also inhibit at the pro-enzyme level. For example, TIMP-2 interacts with the catalytic site of membrane bound MMP-14 and the C-terminal domain of secreted MMP-2 to form a trimolecular complex that controls the cleavage of pro-MMP-2 [154].

5.3. MMPs in TB

5.3.1 The role of MMPs in TB pathology

Upregulated collagenases, gelatinases, and stomelysins degrade the complicated meshwork of fibrillar collagen and other matrix components that make up and support normal lung structure to drive tissue destruction in TB. This ECM breakdown facilitates leukocyte influx,
tissue reconfiguration and cavity formation and is strongly associated with *M. tb* replication and human morbidity and mortality [155].

A range of MMPs and TIMPs are expressed by cells of the monocyte/macrophage lineage, neutrophils, T cells, pulmonary epithelial cells and pulmonary fibroblasts. These cells are all present at the site of infection in pulmonary TB and their reduced function in older age is associated with reduced radiological cavitation and haemoptysis in patients [156]. Specific MMPs have been found to be upregulated in TB patient’s sputum and bronchial lavage fluid (BALF) samples compared to both healthy controls and symptomatic respiratory controls, as well as those TB patients co-infected with HIV [18, 157, 158]. The upregulated MMPs in TB are generally accepted to be MMP-1, -2, -3, -7, -8, and -9. MMP-10 is also upregulated, but until recently has been overlooked due to cross-reactivation with commercial MMP-3 antibodies. These elevated MMP concentrations reduce rapidly upon therapeutic treatment of TB [159, 160]. Whilst some studies identify an increase in both TIMP-1 and TIMP-2 [113, 149] most of the evidence suggests that in active TB there is a highly concentrated subset of MMPs that are not regulated by a concurrent increase in TIMPs [158, 161].

MMP-1 concentration is correlated with extent of disease, as measured on chest radiograph and immunohistochemistry (IHC) of lung samples of patients with active TB, which showed that macrophages express MMP-1 in areas adjacent to tissue destruction [162, 163]. In TB patient sputum, the collagenases MMP-1 and MMP-8 are functionally active, causing collagen matrix destruction [150, 158]. In MMP-1, this has been shown in a positive correlation with more extensive inflammation on chest radiograph. Interestingly, when the clinically safe collagenase inhibitor Ro32-3555 was co-incubated with *M. tb*-infected human macrophages, MMP-1 activity was completely abrogated [150, 158]. Unlike *M. tb*, BCG does not cause pulmonary tissue destruction or cavitation and this has been associated with its inability to upregulate MMP-1 in macrophages at the gene or secretory level [163]. MMP-8 is also detectable in the CSF of TB patients before treatment, and in TB patients’ lung biopsy specimens in a manner that correlates with radiological and clinical disease severity. Patients with pulmonary cavities on chest radiographs have higher MMP-8 concentrations in their respiratory secretions [150, 164]. *In vivo* animal studies and *in vitro* work support these clinical associations. Mice models also indicate MMP-1 as a driver of matrix degradation in TB in the lung. Whilst mice do not produce MMP-1 and do not have caseating granulomas or
a matrix-degrading phenotype in TB, tissue degradation was established with mice manipulated to produce MMPs upon \textit{M.\textit{tb}} infection. This destruction occurred in the absence of caseous necrosis, demonstrating clearly that it is MMP-1, and not areas of granuloma necrosis, which drives tissue destruction in TB [158, 162]. MMP-8 is thought to be predominately produced by neutrophils in TB, with their rapid secretion of MMP-8 from their granules on \textit{M.\textit{tb}} infection \textit{in vitro} resulting in significant collagenase destruction. Interestingly MMP-8 is also highly associated with \textit{M.\textit{tb}}-triggered NETs which are upregulated in TB patient sputum [150].

IHC has revealed gelatinase MMP-9 secretion from monocytic cells at the heart of the granuloma in TB patient lymph nodes [165]. In addition, the concentration of MMP-9 is elevated in the cerebral spinal fluid (CSF) of TB meningitis patients compared to bacterial or viral meningitis controls. In this study, the MMP-9 per leukocyte ratio in TB meningitis was shown to be significantly higher compared with other forms of meningitis and associated with signs of tissue damage such as neurological deficit and unconsciousness [161]. Although it is classified as a gelatinase, MMP-9 also cleaves type IV collagen; an abundant collagen in the basement membrane within the lung. This activity is thought to drive MMP-9’s vital role in granuloma development. MMP-9 expression in mice is associated with significantly higher bacterial load in pulmonary \textit{M.\textit{tb}}-infection and IHC indicated this effect was due to lack of recruitment of uninfected macrophages and failure of well-organised granuloma formation [166]. A morpholino knockout of MMP-9 in \textit{Mycobacterium marinum}-infected zebrafish had reduced bacterial burden, and reduced the number of granulomas compared to wild-type (WT) zebrafish. There was also accelerated macrophage recruitment to granulomas. In this model MMP-9 expression was localised to epithelial cells surrounding the granuloma, proximal to infected macrophages [33], implicating epithelial cells as an important source of this enzyme at the site of \textit{M.\textit{tb}}-infection.

Aside from its direct role in large scale tissue destruction, MMP activity is associated with early \textit{M.\textit{tb}} dissemination, with MMP inhibitors reducing hematogenous spread of the bacterium [166]. Additionally, MMPs have been shown to modulate the activity of cytokines and chemokines which are important in the immune response to TB. For example, MMP-9 cleavage of IL-8 markedly increases its chemotactic activity whilst MMP-8 has been shown to cleave CXCL-5 to generate peptides with enhanced neutrophil-chemotactic activity [127].
These central roles of MMP’s in the immunopathology of TB have led to an interest in the enzymes as potential targets for host-directed therapy.

5.3.2 MMPs and host-directed therapy
The role MMPs play in the pathogenesis of a wide variety of communicable and non-communicable diseases such as cardiovascular disease places them as an interesting therapeutic target. Initial interest and investment in clinical MMP inhibition, fuelled predominately by cancer research, have lessened since the initial surge of clinical trials around 15 years ago, due to limited progress. The failures of this process were largely driven by a poor understanding of the complexities of MMP function and the regulation of this function, compounded by use of inappropriate animal models that did not properly reflect the disease state or MMP biology of the patients in the clinical trials. In TB, doxycycline has already been shown to inhibit collagen breakdown through inhibition of MMP-1, -3 and -8 as well as TNF-α. This was associated with inhibition of M.tb growth in guinea pig models [18]. In relevance to CNS TB, more specific MMP inhibitors have been used in brain inflammation to reduce blood brain barrier dysfunction and permeability for example to aid stroke recovery [16, 167].

Recently, there has been renewed interest in the development of synthetic inhibitors for host-directed therapies and these may have potential as adjunctive therapies in the treatment of TB. However, our limited understanding of MMP regulation continues to restrict progress in this area. In particular, our understanding of extracellular signalling in MMP responses must be expanded. As TB pathology and MMP responses are both species-specific, research in this area must often rely primarily on in vitro studies. These have been invaluable but often do not reflect that, in vivo, the cellular MMP response does not occur in isolation but as part of an intricate network of TB responses mediated and regulated by intercellular interactions. Therapeutically targeting TB granulomas through promotion of tissue healing is an exciting area of host-directed therapy, but to advance the development of host-direct therapies, MMP regulation by intercellular networks must be understood further.
5.4. The regulation of MMPs in TB: Intercellular signalling

*M.tbc* infection of cells including neutrophils, AECs and monocyte/macrophages is well established to initiate an MMP response. However, the intact granuloma’s role in containing *M.tbc* actively limits cells out-with the structure from contact with the extracellular bacterium [168]. Furthermore, a low infectious dose and growth rate means many cells in the TB immune response and granuloma formation may not be infected [169, 170]. Together this suggests that a majority of cells involved in the immune response to *M.tbc* infection and its pathology do not have direct contact with the bacterial surface. Instead, the immune response is mounted by uninfected cell responses to signals from infected cells. These intercellular communications consist both of direct cell-to-cell contact via receptors or indirect contact by secreted local growth factors, pro-inflammatory cytokines and microbial products. These communications form an intricate network of intercellular signalling between both traditional and non-traditional immune cells that is central to TB immunopathology. The network facilitates an inflammatory tissue-degrading phenotype of uninfected cells as well as mediating the response of infected cells [31, 171, 172].

The effect of these communications is perhaps most clearly established in monocytes, whose response and differentiation is determined by cell cytokines and chemokines in addition to, or instead of, *M.tbc* contact. Monocytes and their differentiated forms are essential to the host’s immune response to *M.tbc*. Monocyte maturation follows conditioning by cytokines and chemokines that are produced by cells such as other monocyte-derived cells, neutrophils and epithelial cells in response to *M.tbc* infection. For example, activation of monocytes with T Helper (Th)1 cytokines such as IFN-γ produces inducible nitric oxide synthases (iNOS) which in turn catalyses the synthesis of the antimicrobial NO [60]. Conversely, Th2 cytokines such as IL-4 and IL-13 induce monocyte-derived cell secretion of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)-β and arginase, which compete with iNOS for the use of arginine as a substrate [173-175]. This intercellular stimulation ensures that monocytes and monocyte-derived cells do not have to be directly infected with *M.tbc* to contribute to control of infection.

As well as being highly regulated by intercellular signals, monocytes are important contributors to this network. *Mycobacterial* components including the 19-kDa lipoprotein are trafficked intracellularly and released from *M.tbc*-infected monocyte/macrophages via
These exosomes can interact with naïve antigen-specific T cells to cause activation in vivo [177]. Exosome interaction with other macrophages stimulates production of pro-inflammatory mediators such as TNF-α, and promotes M.tb survival through dampening macrophage activation in response to IFN-γ [178]. Aside from microbial products, M.tb stimulation triggers monocyte secretion of an arsenal of cytokines and chemokines that have been shown to contribute significantly to an inflammatory tissue degrading phenotype. Intercellular communications from infected monocyte/macrophages have been studied in vitro through sterile filtration of supernatant of M.tb-infected monocytes. This monocyte media conditioned with TB (CT) therefore contains secreted bacterial components such as the early secreted Antigen of 6 kDa (ESAT-6) as well as the secreted chemokines, cytokines and growth factors secreted by the monocytes (Table 2). Such soluble monocyte networks have been shown to significantly upregulate a range of MMPs in a range of cells. CT alone can upregulate MMP-14 in monocytes and CT-impregnated agarose drops have been used to demonstrate clearly that monocyte networks trigger monocyte migration [179]. CT also upregulates MMP-1 secretion from fibroblasts which are an important source of this collagenase in the lung [180]. In CNS TB, CT induces MMP-9 secretion from astrocytes, whilst stimulation of microglial cells with CT down-regulates constitutive microglial MMP-2 gene expression and secretion [181, 182]. The MMP-2 downregulation was not seen with extracellular M.tb infection of the cells but mediated by pro-inflammatory mediators TNF-α, p38 MAPK and NFkB [183].

Network stimulation is a particularly important stimulus for ECM breakdown by epithelial cells. For example, direct infection of human alveolar epithelial cells has been shown to cause no CCL5 secretion whilst CT stimulates significant CCL5 secretion from primary alveolar, but not upper airway, epithelial cells [184]. IL-8 secretion from both the alveolar cell line A549’s and primary normal human bronchial epithelial cells (NHBEs) was much greater in CT-stimulated cells compared with cells infected with extracellular M.tb, at both the gene and protein level [185]. NHBE MMP-1, -3 and -9 secretion and activity is also upregulated by CT stimulation, but not direct M.tb infection [157, 171, 186]. The previously discussed role of epithelial cells in macrophage recruitment and granuloma development is thought to be driven largely by this MMP-9 secretion [33]. CT media alone is also sufficient to upregulate secretion of neutrophil MMP-8 and MMP-9 from the granules of human
isolated neutrophils. Similar to direct neutrophil infection this MMP response is NFkB dependent and PI3K independent, but has also been shown to be MAPK and AMPK dependent [150]. Where it has been investigated, CT regulation of MMP producing cells is generally dependent on its IL-1β and TNF-α concentrations, although these alone are not generally sufficient for full induction of MMP secretion [172, 181, 182]. This highlights the importance of the full complex network of cytokines, chemokines and microbial proteins secreted by cells such as monocytes. Interestingly, neutrophils do not appear to be an important contributor to the intercellular regulation of tissue destruction. Stimulation of monocytes, monocyte-derived macrophages (MDMs) and astrocytes with supernatant of infected neutrophils did not upregulate MMPs, potentially due to the limited cytokine response of neutrophils to M.tb infection [149].

Intercellular networks are essential to establishing TB immunopathology and the development of inflammatory tissue destruction. Their dynamic and cell specific effects provides context to many of the complex and opposing functions of cell-mediated immunity. A more complete understanding is needed of the extracellular regulation and triggers but his has proved difficult with our current in vitro models, which do not replicate

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Chemokines</th>
<th>Growth Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>CCL2 (MCP-1)</td>
<td>GM-CSF</td>
</tr>
<tr>
<td>IL-6</td>
<td>CCL3 (MIP-1α)</td>
<td>VEGF</td>
</tr>
</tbody>
</table>

Table 2. Components of monocyte media conditioned with TB (CT).

Media from monocytes infected with M.tb is used as a source of TB networks, and contains a variety of secreted factors that are significantly higher compared with media from uninfected controls. Modified from Sathyamoorthy, T [187].

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Chemokines</th>
<th>Growth Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>IFN-α</td>
<td>IL-8 FGF</td>
</tr>
<tr>
<td>IL-1 receptor agonist</td>
<td>CCL2 (MCP-1)</td>
<td>GM-CSF</td>
</tr>
<tr>
<td>IL-6</td>
<td>IFN-α</td>
<td>IL-8 FGF</td>
</tr>
<tr>
<td>IL-2 receptor</td>
<td>CCL3 (MIP-1α)</td>
<td>VEGF</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CCL4 (MIP-1β)</td>
<td>G-CSF</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Chemokines</th>
<th>Growth Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α</td>
<td>IL-8</td>
<td>FGF</td>
</tr>
<tr>
<td>IL-1 receptor agonist</td>
<td>CXCL9 (MIG)</td>
<td></td>
</tr>
<tr>
<td>IL-2 receptor</td>
<td>CXCL9 (MIG)</td>
<td></td>
</tr>
</tbody>
</table>
the many elements of the intercellular networks that potently influence the MMP upregulation and activity in vivo. One such component of extracellular regulation may be platelets.

6. Platelets: the unknown player in TB

In 1882 Giulio Bizzozero observed cell-like structures forming clots in disrupted tissue [188], and in 1910 these structures were termed platelets, due to their plate-like structure. Widely attributed with a simplified role in haemostasis, it was not until the last decade that the vital role of platelets in immune defence has begun to be fully appreciated. The contribution of these cells to TB responses remains almost completely unknown.

6.1. Platelets
At only 2-4 μm wide, platelets are the smallest blood particles but also one of the most numerous, only exceeded by red blood cells. A normal platelet cell count is 150-400 x10^3 per ml of blood, with platelets circulating 5-9 days before phagocytosis in the spleen and liver. A healthy adult can make 10^{11} platelets per day [189].

6.1.1 Platelet production
Platelets are produced in a process termed thrombopoiesis. Whilst the mechanisms of this process are not fully understood it is established that platelets are produced from fragments of cytoplasm derived from megakaryocytes within the bone marrow. Megakaryocytes are polyploid hematopoietic cells found only in mammals, whose major function is to produce and release platelets into circulation. Although not universally agreed, it is generally thought that megakaryocytes shed proplatelets formed by budding off the parent cell, which then go on to release platelets [190]. Although the bone marrow was historically considered the site of platelet production, more recent modelling techniques have shown that the bone marrow environment is not required for central events of thrombopoiesis. Megakaryocytes are present in the circulation and in a paradigm independent of bone marrow, the lung is the most likely site of platelet production [191]. Many studies have shown intact
megakaryocytes in the lung, the cells can be readily detected in humans lung biopsies, and
studies of animal models indicate that lung damage reduces circulating platelets [192, 193].
The most widely accepted theory is that megakaryocytes are released from the bone
marrow and, due to their size and surface adhesion molecules, are deposited in the
pulmonary microvessels - the first capillary bed to be encountered [194]. Around 90% of the
megakaryocytes are maintained in the lung microvasculature instead of transiting to reach
the arterial circulation [195]. The megakaryocytes then produce platelets, either directly or
via proplatelet production, with 98% of megakaryocytes leaving the lung devoid of
cytoplasm: a phenotype associated with platelet production [196]. The importance of these
lung-produced platelets to the total platelet population varies between studies. However, it
is generally accepted that they contribute to the majority of platelets in the lung, and are
sufficient to produce the total daily rate of platelets [191, 195, 197].

6.1.2 Platelet structure

The structure of platelets is unique (Fig 3). Although platelets have no nucleus they contain
cellular machinery such as mitochondria, which allows them to translate selectively pre-
packaged mRNA into protein and facilitates processes such as non-ATP-mediated
thrombotic signalling and platelet apoptosis [198]. In addition, the platelet cytoskeleton
(consisting predominantly of microtubules, actin microfilaments and intermediary
filaments), facilitates changes upon platelet activation. These include changes in the
spherical shape of the cell, extension of platelet pseudopodia, or platelet flattening on a
surface during spreading, depending on the type of stimulus. The cytoskeleton is also
required for the binding and positioning of signalling molecules to platelets and preventing
the platelets from being sheared in the bloodstream [199]. A tunnel system termed the
open canalicular system (OCS) connects the platelet plasma membrane to the rest of the
cell. The OCS facilitates entrance of external elements into the platelets, release of granule
contents to the exterior, and formation of filopodia during platelet activation [200, 201].
The platelet plasma membrane is composed of a phospholipid bilayer and is the site of
expression of various surface receptors and lipid rafts which help in signalling and
intracellular trafficking. These markers include CD36, P-selectin (CD62P), CD40, CD63, CD9,
G-protein-coupled receptors (GPCRs), CD41 (also known as glycoprotein (GP)IIb), GPIIb/IIIa
and Glucose transporter 3 (GLUT-3), but vary depending on platelet stimulation. They
trigger the rapid release of platelet granules which play a role in platelets’ multiple functions [202]. The membranous zone also contains membranes derived from megakaryocytic smooth endoplasmic reticulum that have been reorganised into a dense tubular system connected to the surface platelet membrane to aid thromboxane A2 synthesis and release [203]. Although the major granulocytes in humans are considered to be neutrophils, basophils and eosinophils, platelets also contain granules. These granules store over 300 proteins that can be released intracellularly or extracellularly upon stimulation. Each platelet is thought to contain around 60 granules, and with the large number of platelets present in the blood, platelets can exert extensive inflammatory effects on a scale that exceeds their individual size [204]. Platelets have three granule types: α granules, dense (δ) granules, and lysosomal (λ) granules. The platelet cytoskeleton components microtubules and microfilaments maintain platelet shape and facilitate shape changes upon activation. Platelets also contain secreted alpha (α), dense (δ) and lysosomal (λ) granules and an open canalicular system (OCS) to transport substances into and out of the cell.

Figure 3. Platelets in the blood.
A) Human Blood smear at 1000x magnitude. Wright’s stain H6455 used to demonstrate presence of neutrophils, platelets, monocytes and erythrocytes. Adapted from Hart, K Histology Photomicrographs Human Anatomy and Physiology. Accessed at http://eugraph.com/histology/cardiova/index.html. B) Platelet structure. Platelets have numerous surface receptors involved in cell adhesion and intercellular communication, including integrins such as CD41, and pathogen pattern receptors such as Toll-like receptors (TLRs) and complement receptor type 2 (CR-2). Protease-activated receptor (PAR) 1 and PAR 4 are key facilitators of platelet activation by the protease thrombin (red arrow indicates cleavage point). Platelet cytoskeleton components microtubules and microfilaments maintain platelet shape and facilitate shape changes upon activation. Platelets also contain secreted alpha (α), dense (δ) and lysosomal (λ) granules and an open canalicular system (OCS) to transport substances into and out of the cell.
granules and lysosomal (λ) granules. α granules are the most abundant of the granule types, comprising around 10% of the cell’s total volume. They contain a variety of proteins including platelet microbicidal proteins (PMP), protease inhibitors and coagulation factors which initiate the coagulation cascades. These granules fuse with surface-connected membranes of the OCS or the plasma membrane [205, 206]. δ granules contain haemostatically active molecules consisting of nucleotides such as adenosine 5′-diphosphate (ADP), bioactive amines such as histamine, and ions such as Ca^{2+}. λ granules contain factors such as proteases which modulate platelet-fibrin clot retraction for wound healing [207]. It has been reported that the distinct platelet granules are subject to synchronous or dysynchronous release, depending upon agonist specificity and potency. For example, low levels of thrombin or ADP induce δ and α degranulation, whereas λ granules are not secreted until such agonists accumulate to a much higher concentration [208]. This nuanced secretory response has been reported in infection, for example with the secretory profile of CCL5 and PDGF differing between platelet TLR-4 stimulation by Escherichia coli and a subspecies of Salmonella enterica. In this study, platelet-secreted factors induced by S. enterica stimulated a different cytokine response from peripheral blood mononuclear cells (PBMCs) compared with the platelet factors induced by E.coli infection, despite platelet surface markers CD62P and platelet factor 4 (PF4) remaining unchanged [209].

Platelets also produce microparticles. These are small lipid/protein fragments of plasma membranes that are shed from cells in the circulation and have become a focus of research interest in recent years for their role in intercellular communications [210, 211]. Microparticles engulf RNA and protein from the cytoplasm of the parent cell during their formation. This protein and RNA can then be transferred on attachment or fusion with a new cell via receptor-ligand interaction or internalisation with endocytosis[212]. Whilst a large range of cells have now been shown to produce microparticles, platelet microparticles constitute 70-90% of circulating microparticles [213, 214]. Most of these are likely to be derived directly from the platelet precursors megakaryocytes, however, platelet-derived microparticles (PDM) may also play an important role in the transport and delivery of bioactive molecules and signals throughout the body [212]. Thus, platelets have a complex and dynamic structure that is highly specialised and responsive to their multi-faceted role in the blood.
6.1.3 Platelet designation

There remains some debate on the categorisation of platelets as cells as opposed to blood particles. At the centre of this debate is the fact that platelets do not have a nucleus or supporting DNA material, apart from mitochondrial DNA. This is also the case for erythrocytes, however, these cells are afforded special dispensation as they evolved from nucleated erythroblasts, and the process of enucleation is finite. In contrast platelets are solely created from cytoplasm of the megakaryocyte, and superficially resemble cell fragments more closely than they resemble cells [215]. However, the process of platelet formation from megakaryocytes is not fully understood and may in fact involve active proplatelet signalling [190, 216]. Furthermore, the ability of platelets to respond to stimuli to alter receptor-signalling and protein secretion, as well as production of their own phospholipid vesicles and PMPs suggests a level of autonomy that could not exist in a particle defined only as a cell fragment [215]. Although a complex debate; for ease, clarity, and supported by the majority of recent literature, I will refer to platelets as cells for the remainder of this report.

6.2. Platelets in Haemostasis

Haemostasis was the first role that platelets were identified with, and it remains the primary role associated with these cells. This function is performed through adhesive and cohesive functions to form a haemostatic plug, and through activation of coagulation mechanisms by exposure of an adequate phospholipidic surface acting as a catalytic site to consolidate the hemostatic plug [217]. Activated platelet interaction with leukocytes, particularly monocytes, functions to amplify the clotting cascade in haemostasis. The first stage of this clotting function is adhesion. Platelets usually circulate in an inactive state, until various pro-aggregatory stimuli from blood vessel damage promotes the action of platelet adhesion to the subendothelial surfaces, which is the primary target site of platelet action. The platelet begins to change its shape, turn on receptors and release chemical messengers from its granules. Finally platelets aggregate to connect to each other through receptor bridges [207].
The rapid recruitment of platelets to sites of injury positions these cells to support both haemostasis and microbial recognition and control. This realisation occurred concurrently with the observation that platelet granules contain various pro-inflammatory and anti-inflammatory cytokines and chemokines that have no clear role in haemostasis [218]. Together this has sparked an increasing appreciation for the function of platelets, which extends far beyond haemostasis alone.

6.3. Platelets in the Immune Response

Platelets are now attributed with a wide range of functions in inflammation, angiogenesis, atherosclerosis, lymphatic development and tumour growth, but one of the most important platelet functions is thought to be modulating innate and adaptive immune responses [219]. Platelet activity in bacterial infection has been observed for decades, but it was not until more recent years that these observations began to be re-examined, in light of a developing understanding of platelets.

6.3.1 Direct platelet recognition and destruction of bacterial pathogens

Platelets have a direct role in protecting the host from infection. Active platelets express a range of receptors such as gC1q-R, CD62P, FcyRII, and Toll-like receptors TLR-1, -2, -4, -6, -8, and -9 [220]. The receptors on the platelet plasma membrane are dynamic and dependent on stimulation. For example, platelet TLR-1 and -6, which are expressed in vascular or atherosclerotic lesions that may involve bacterial infection, are upregulated in vitro in response to IFN-γ [221]. This is different to platelets activated by processing for patient use, which have significant upregulation of TLR-2 and -9 and decreased TLR-4 surface expression compared with inactivate platelets (as determined using P-selectin as a marker in flow cytometry) [222]. Bacteria can bind to these receptors either directly, for example with LPS recognition, or indirectly via plasma proteins bound to the bacteria such as C1q, or secreted bacterial factors such as the protease family gingipains from Porphyromonas gingivalis [223]. As well as designated immune receptors, unique receptors of the megakaryocyte cell line that are traditionally associated with a role only in haemostasis have also been shown to be involved in bacterial recognition. For example, integrins on the platelet surface such as the fibrinogen receptor GPIIb/IIIa bind to bacteria
such as *Staphylococcus epidermis* and *Streptococcus pyogenes* either indirectly via bacterial-bound fibrinogen or directly to the bacterial surface [224]. Surface proteins such as iron-regulated surface determinant (ISD) proteins in *Staphylococcus aureus* or platelet adherence protein A (PADA) in *Staphylococcus gordonii* also bind to GPIIb/IIIa [225, 226] whilst several species of the streptococcal family have proteins such as serine-rich protein A and the glycosylated streptococcal protein B that can facilitate binding to the GPIbα platelet receptor [227].

Once bound, platelets can present these bacteria to other immune cells or actively internalise them for destruction via fusion of the endosome containing the pathogen with α-granules containing PMPs. This promotes pathogen clearance from the host bloodstream and tissue [228]. These PMPs can also be secreted to kill non-internalised bacteria. Secreted PMPs include chemokines (sometimes termed kinocidins to reflect their dual role as classical chemokines with direct microbicidal activity), fibrinopeptides, and thymosin β-4. Examples include PF4, RANTES and platelet basic protein (PBP). Platelets probably release PMPs both immediately and over a prolonged period in response to relevant signals of inflammation and infection as well as microorganisms themselves [208].

Platelets secrete PMPs at sites of infection where they exert direct, rapid, and potent efficacy against many microbial pathogens and potentiate the antibacterial properties of leukocytes [229]. They have also been shown to have highly inflammatory effects, as the source of IL-1 that elicits cytokine responses from synovial fibroblasts in rheumatoid arthritis. As platelets may be the most common cell type in an inflamed arthritic joint, this mechanism could have potent clinical implications [230]. Platelets also secrete factors such as human β-defensins for anti-microbial activity [231]. In addition to anti-bacterial activity, platelets have been shown to be important for defence against parasitic infections. Platelets release PF4 upon contact with erythrocytes infected with *Plasmodium falciparum*, and this factor enters and accumulates in the infected erythrocyte via the Duffy antigen receptor for chemokines to result in cell lysis. This platelet action is required for the immune defence to *P. falciparum* [232].

Interestingly certain bacteria subvert platelet functions for their own use. For example, platelets have been shown to be essential for streptococcal biofilm formation on heart
valves that increases *Streptococcus mutans* or *S. gordonii* survival and resistance to antibiotics [233]. A similar phenomenon has been observed in other Gram positive infections, with *S. epidermidis* binding to platelet aggregates shown to induce biofilm production in platelet storage preparations [234]. Bacterial binding and activation of platelets can also further sepsis through thrombocytopenia, which is consistently associated with severe sepsis and mortality [235]. Activated platelets have a shorter timespan before phagocytic clearance, however, bacteria can shorten this further with binding of compounds such as peptidoglycan in bacterial cell walls, streptolysin O of *S. pyogenes* and pneumolysin of *Streptococcus pneumoniae*. These cause platelet apoptosis and cytotoxicity [236]. In addition bacteria such as *S. pyogenes* can use platelet binding for dissemination around the body [237] whilst *S. aureus* binds platelets to stimulate clot formation for an anchoring for colonisation of tissue, that leads to complications such as infective endocarditis [238]. This forms a complex picture of platelets in the immune response, which has not yet been fully explored.

In addition to a variety of interactions with Gram positive bacteria, platelets also have important interactions with Gram negative bacteria in a manner that is less well elucidated, but does contribute to the outcome of infection and pathology [236, 239]. For example, in a study testing very low birth weight neonates for organism-specific infection, platelet response and the frequency and duration of thrombocytopenia were significantly greater in Gram negative and fungal infections compared to Gram positive. Furthermore, persistent bacteraemia, multiorgan failure, and mortality were significantly greater in thrombocytopenic patients [240]. Bacterial factors such as the major phospholipase C from the Gram negative *Pseudomonas aeruginosa* is well established to cause platelet aggregation in a dose-dependent manner if enzymatically active [208, 241]. The cytotoxin phospholipase A2 (also from *P. aeruginosa*) is associated with a reduced leukocyte concentration, higher platelet concentration and activation, higher haematocrit, and significantly higher mortality in a pneumosepsis mouse model [242]. *Yersinia pseudotuberculosis*, but not *Yersinia entercolitica*, is known to be capable of entering platelets and inducing platelet aggregation via a mechanism involving expression of the *inv* invasin gene and the virulence plasmid pYV+ [243]. Despite the molecular interactions of
platelets with these bacteria, and the virulence versus host defence consequences of these interactions, platelet interactions with *M.tuberculosis* remains unknown.

During the process of activation, platelets express membrane proteins and release adhesive proteins, coagulation and growth factors, which can facilitate the cross-talk of platelets with cells such as leukocytes and endothelial cells. This network of intercellular communications allows platelets to have an important role in the immune system that can be independent of, and in addition to, their role in direct recognition and killing of pathogens.

### 6.3.2 Platelet interactions with the innate immune response

The acute phase response (APR) is the earliest response to infection or vascular injury. It involves the production of acute phase proteins such as C-reactive protein, serum amyloids A and P, complement proteins, and fibrinogen by the liver [244]. These destroy or inhibit the growth of microbes and exert procoagulant effects that may limit infection by trapping pathogens within local blood clots [245]. Platelets have been shown to induce this response in infections such as *P. falciparum*, where early activation of platelets triggers protective APR responses via IL-1β production. This was stark in contrast to later activation in which platelets are then associated with driving more severe forms of the disease [244].

Platelets also instigate the cell-mediated response to infection, activating innate immune cells to mount an immune response. For example, activated platelet PF4 secretion induces firm neutrophil adherence to even unstimulated endothelial cells and triggers secondary neutrophil granule exocytosis when there is an inflammatory co-stimulus such as TNF-α [246]. *In vitro*, platelet-derived TGF-β stimulated the activation of the p38 MAPK pathway in monocytes to induce cyclooxygenase (COX) 2 de novo synthesis and promote an inflammatory monocytic response [247]. Platelet-derived CD40 ligand (CD40L) is another platelet factor that has been widely reported to have vital interactions with the innate immune response. CD40L has been shown to activate DCs, monocytes and epithelial cells via the CD40 receptor to activate signalling pathways such as NFκB, and trigger secretion of a variety of inflammatory chemokines and cytokines in response to pathogen challenge [248, 249]. CD40L first exists in a trimeric form on the platelet membrane but is cleaved, most likely by MMP-9, to a soluble form [250, 251]. Whilst other cells are known to secrete this protein, over 95% of plasma soluble CD40L originates from platelets [252]. Pathogen-
activated platelets can also activate leukocytes directly. TLR-mediated signal transduction leads to increases in surface expression of P-selectin and integrin complex GPIIb/IIIa of platelets. P-selectin has been shown to be a key ligand for platelet binding of neutrophils and monocytes in a manner predominately mediated by the P-selectin glycoprotein ligand 1 (PSGL-1), although GPIIb/IIIa almost certainly plays a central role in this as well [253]. For example, Sreeramkumar et al. reported in 2014 that active platelet interaction with neutrophils enhances the neutrophil inflammatory response through interaction between active platelets and PSGL-1 clusters on the surface of neutrophil domains. These domains were extended into the lumen of injured tissue on recruitment of the neutrophil, and scanned for the presence of activated platelets. Only once neutrophils had a platelet interaction did they organise the additional receptors needed for intravascular migration and inflammation [254].

Central to a robust immune system is fast and efficient recruitment of leukocytes from the blood to tissue. Platelets are essential for rapid recruitment of immune cells to the site of infection, as has been shown in numerous in vitro and animal models of diseases [194]. For example in a murine model of acute lung injury (ALI), a disease characterised by leukocytic infiltrates into the lung parenchyma and airspaces with edema formation, mice with depleted platelets showed significantly reduced leukocyte influx into the lung [255]. Additionally, platelet-secreted chemoattractants such as P-selectin and CD40L facilitate recruitment and coordination. Platelet-secreted CD40L binds to endothelial CD40 to induce surface expression of a number of key adhesion molecules such as E-selectin, vascular cell adhesion molecule (VCAM)-1, and intercellular adhesion molecule (ICAM)-1 as well as cytokine release such as IL-8 and MCP-1. The activation of the endothelium then attracts circulating neutrophils, T cells, and monocytes to adhere to it [253].

Platelets are thought to facilitate basophil and eosinophil recruitment through PF4 stimulation of histamine secretion by basophils, which mediates adherence and ultimately extravasation of the cells [256]. Platelet-secreted enzymes such as heparanase also aid extravasation through degradation of the subendothelial matrix [257]. Platelet-surface receptors can also bind to the endothelium. This platelet binding initiates inflammatory responses and facilitates monocyte and neutrophil recruitment as, for example, platelet surface-bound P-selectin binds to leukocyte receptors to form a cell complex which
positions leukocytes for activation by inflammatory cytokines and binding to the endothelium for extravasation [253, 258]. Platelet binding to both the bacteria and host cells brings together pathogen and host immune cells, thus facilitating immune recognition of bacteria by increasing the opportunity for, and frequency of, immune cell detection of bacterial invasion [224].

Platelets enhance the host’s innate immunity by extending the life of recruited cells. Platelets achieve this though both cell-to-cell direct contact and secreted products such as PF4, which promotes monocyte survival through an anti-apoptosis signal; TGF-β, which stimulate survival of neutrophils; and CCL5, which is a survival signal to macrophages in viral lung infections [259-261]. Platelet-bound neutrophils have enhanced phagocytosis and intracellular killing of bacteria [262]. In addition TLR-4 activated platelets, activated by stimulus such as LPS, have been shown to promote formation of NETs in neutrophils recruited to sites of infection. These NETs result in bacterial clearance by trapping bacteria and concentrating antibacterial factors but are highly inflammatory, enhancing thrombosis and resulting in neutrophil death. LPS does activate neutrophils but not to trigger NETosis, unless there is co-stimulation with activated platelets [254].

Active platelets can also influence cell maturation to enhance the polarisation of the immune response. Platelets have been shown to induce morphological changes on co-incubation with CD34+ progenitor cells. The platelets shift monocyte maturation toward macrophage and foamy cell phenotypes in a mechanism thought to be dependent on internalisation of platelets, bound and activated with low-density lipoprotein, in the first 24 hours of the co-culture [263]. Macrophages and foamy cells are inflammatory markers and associated both with development and resolution of acute inflammation, as well as establishment of chronic inflammation in infectious diseases including TB. As these CD34+ progenitor cells are recruited by platelets, platelets are able to quickly and comprehensively enhance a pro-inflammatory response. The increase in the inflammatory potential of macrophages demonstrates the capability of platelets in creating and driving inflammatory responses. Interestingly, it was also found that this platelet-induced foam cell generation could be reduced to control levels using anti-inflammatory statins [264]. When binding to blood monocytes, platelets preferentially bind to CD16+ monocytes but, upon binding of classical CD16- monocytes, or through release of TGF-β, platelets upregulate CD16+ and so...
tilt the classical monocyte subset towards an intermediate or non-classical phenotype [263, 265].

Whilst most platelet responses are reported to be inflammatory there are numerous studies indicating platelets have an important function in dampening inflammation for protective effects. Many of these reports are contradictory to the platelets’ inflammatory role and these contradictions can occur even within the same model. For example, experiments in a thrombocytopenic murine model to investigate the Arthus reaction have indicated that platelets induce neutrophil infiltration into the skin and enhance edema and haemorrhage. However, in the same model platelets have also been shown to maintain vascular integrity in inflamed tissue, with inflammatory stimulus of thrombocytopenic mice able to induce massive organ bleeding [266, 267]. It is thought the source of these paradoxes is the dynamic and stimulus-dependent functionality of platelets. This is particularly well illustrated by the complex regulation of neutrophils by platelets, where studies suggest platelets have both a pro- and anti-inflammatory effect.

Platelet prevention of inflammatory bleeding is via inhibition or repair of neutrophil-driven destruction [268] and resting and thrombin-activated platelets have been shown to inhibit ROS generation and myeloperoxidase release by neutrophils [269]. In addition, platelets can inhibit the release of neutrophil elastase and sequester the serine protease already secreted [269, 270]. Aggregation of platelets and neutrophils generates lipoxin A4, which reduces recruitment through inhibition of neutrophil adhesion and extravasation [271]. In this manner platelets have been shown to reduce neutrophil-mediated tissue damage.

Platelets can also function to disrupt monocyte recruitment, with PF4 able to down-regulate the chemotactic receptors CCR1, CCR2 and CCR5 [272] and platelet-derived chondroitin sulfate A preventing firm monocyte binding to the endothelium by blocking CCL5 [273]. Secretion of CD40L induces anti-inflammatory monocyte effects through upregulation of IL-10 expression accompanied by a decrease in TNF-α and IL-6 [274]. Platelets can also sequester the heat shock protein gp96 to reduce DC activation [275]. Platelet anti-inflammatory effects have also been shown in vivo using a sepsis mouse model, where reduced platelet binding to monocytes and neutrophils led to increased concentrations of TNF-α, IL-6, CCL4, and MCP-1 [276]. Thrombocytopenic mice infected with Leishmania
parasites and encephalomyocarditis virus have similar increases of these cytokines, along with a decrease in TGF-β, where they are associated with reduced monocyte infiltration and higher mortality [277, 278]. Together, the literature on platelet interactions with the innate immune response indicates a potent but complex and dynamic role. This allows platelets to function as instigators, facilitators, modulators and enhancers of the innate immune response both through direct cell contact and granule-secreted factors.

6.3.3 Platelets coordinate adaptive immunity

Platelet effects are not limited to innate immunity, but function also to bridge the gap between innate and adaptive immunity. Platelet-secreted PF4 binds to bacteria, with a specific affinity for biphosphorylated lipid A of LPS. As PF4 is recognised by immunoglobulin (Ig)G, this complex enhances uptake of the bacteria by phagocytic cells [279]. Furthermore, through activation of DCs platelets promote maturation and presentation of antigens to T and B cells. For example, various studies have shown that complement coating of Listeria, Staphylococcus, Enterococcus and Bacillus species is recognised and bound by platelet CD42 (also known as GPIb) to enhance accumulation of the pathogen in splenic DCs and increase presentation of antigen to T cells [280]. In Listeria monocytogenes this DC presentation has also been shown to potentiate cytotoxic CD8+ T cells [281]. Platelets direct DC to sites of tissue injury and stimulate release of inflammatory chemokines and cytokines such as IL-12 (which is important in the priming and polarisation of naïve T cells), IL-14 (which stimulates proliferation of activated B lymphocytes), and IFN-γ [282]. Platelets also facilitate elimination of viral reservoirs in host cells through interaction with CD8+ T cells. Platelet depletion in a mouse model of acute viral hepatitis reduced intrahepatic accumulation of virus-specific cells in a manner that was associated with immunopathology. This function was shown to be independent of procoagulant function [283].

The secretion of cytokines and kinocidins by platelets also coordinates the polarisation of activated T cells. Binding to IgG-decorated targets causes human platelets to express CD40L as well as CCL5. These prime protective T cell-mediated immunity to bacterial pathogens and, combined with PF4, promote polarisation of Th1 cells and Th17 cells but not polarisation of Th2 cells [284, 285]. PF4 also modulates the ratio of regulatory T cells such as CD4+CD25+ T cells and non-regulatory cells such as CD4+CD25- T cells [286]. Platelet-derived CD40L, either membrane bound, secreted or delivered in PDM, forms a bridge between B
and T cell function. Platelets activate B cells and induce class-switching to produce key antibody isotypes in the immune response through CD40–CD40L interactions that facilitate antigen-specific IgG production within B cell germinal centres, in cooperation with CD4+ T cells [211, 287]. PDMs can also transfer platelet receptors to other cells. For example, PDMs have been shown to transfer CXCR4 to CXCR4-negative cells and thereby facilitate cell infection with the X4 HIV strain which depends on this receptor for cell infection. As PDMs levels are higher in HIV-infected patients than controls, receptor transfers such as this may play a role in the spread of HIV and other pathogens [288]. Thus, platelets mediate a coordinated and efficient inflammatory immune response to stimuli through intercellular communications with direct cell-cell contact; signalling molecule secretion; or direct transfer of receptors, soluble proteins and RNA in PDMs.

6.3.4 Platelets as immune barometers
Platelets have unique sensitivity to their surroundings and as such have been described as a “barometer” for infection [218, 224, 289]. For example, platelets are capable of triggering and enhancing neutrophil NETosis when activated by certain stimuli such as LPS. This can enhance bacterial clearance but is highly inflammatory and so must be carefully regulated. Platelets are significantly less sensitive to LPS than neutrophils, requiring 100 times more LPS to be activated via TLR-4 than neutrophils [290]. Consequently, they are only LPS activated in an environment with pronounced infection. In vivo, this may result in platelets only upregulating NETosis in advanced stages of infection, where there is high bacterial load and where other neutrophil responses are unlikely to be able to eliminate the bacteria [290, 291]. This phenomena is supported by the observation that platelet recognition of LPS via PF4 binding, which enhances bacterial detection by forming a new recognition site for IgG, may enhance neutrophil phagocytosis and bacterial clearance, but does not trigger NETosis. This TLR-4-independent mechanism for platelet regulation of neutrophils is much more sensitive to LPS, functioning at very low levels of bacteria [292]. Thus, platelets modulate the inflammatory response to ensure suitable reactions.

This sensitivity to disease state has led to increasing interest in platelets as markers of disease. Reliable and accessible biomarkers allow screening pre-diagnosis, determine stage of disease and treatment after diagnosis, and can be used to monitor ongoing success of treatment. Some platelet measurements are already used as biomarkers. These indices are
namely mean platelet volume (MPV) to indicate platelet production and/or destruction, the volume of blood occupied by platelets termed plateletcrit, and a marker of platelet activation termed platelet distribution width (PDW) [293]. Aside from these three indices there is significant potential for platelets to be exploited further. Platelet reactivity for different disease pathogenesis is widely dependent upon biologically active markers such as active surface receptors like CD36 and CD41, and platelet secretory products like soluble P-selectin. The expression and signalling of these markers is altered in different disease diagnosis and prognosis, potentially providing subtle differentiation that could be useful and cost effective for clinical purposes [294].

6.4. Platelets in tissue destruction
Platelet-associated MMP activity has been implicated in modulating cell migration, tissue degradation and inflammation, as well as other areas of immunopathology. This association occurs both through direct platelet production of MMPs and regulation of other cells’ MMP production.

6.4.1 Platelet-produced MMPs
Platelets themselves express and secrete certain MMPs. There have been a wide range of contradicting reports regarding which MMPs and MMP inhibitors platelets contain. At the protein level, platelets are established as sources of MMP-1, -2 and -14 as well as TIMP-1-3. MMP-3, -9, -23 and -24 are also commonly reported [295, 296]. Differing isolation techniques and leukocyte contamination may account for the discrepancies in this area. For example, the long association of platelets and MMP-9 protein and activity is likely to be due not to platelet secretion, but the binding of plasma MMP-9 to active platelets’ surface where it modulates the platelet’s activation and thrombus formation [297]. This is supported by the observation that platelets from leukocyte deficient humans have not had MMP-9 detected [298]. The extent of platelet activation and consequential MMP-9 binding is highly sensitive to blood-taking and -processing protocols, resulting in contradictory reports of secretion levels [299, 300]. Platelets also contain transcripts of MMPs and their inhibitors. They have been shown to contain mRNA of certain MMPs such MMP-1, -11, -19, and -24 as well as TIMP-1-3. However, the transcripts do not always accord with proteins
ultimately secreted, for example, platelets secrete significant MMP-2 but have no detectable transcripts [301]. The transcripts offer potential for the platelet to produce more protein after secretion of granule reserves. Platelets have been shown in vitro to respond to stimulation with TIMP-2 translation and secretion, however, it is unknown how much such translational control contributes to platelet MMP activity in vivo [301].

The pre-formed MMP protein is thought to be randomly distributed throughout the platelet cytoplasm and translocated to the extracellular space upon activation [296]. Despite the traditional assumption that platelet mRNA expression and protein patterns are a random reflection of the megakaryocyte profile, next-generation RNA sequencing has shown that megakaryocytes differentially express and sort specific MMP transcripts and protein into platelets. This occurs during the final stages of thrombopoiesis and mRNA appears to degrade little in circulating platelets [302]. The mRNA and secreted protein concentrations of platelet-derived MMPs are low, however, and given the high number of platelets present in the blood it is likely that platelet-derived MMPs are important for certain localised activity.

The major MMP produced by platelets is typically reported as MMP-1. Although active platelets have higher levels, even resting platelets can secrete MMP-1 to detectable concentrations [303]. Platelet MMP-1 is membrane bound in inactive form, but interaction with collagen is sufficient to convert the enzyme to active form. Active MMP-1 can cleave the protease-activated receptor-1 (PAR-1) at a cryptic ligand site. This leads to phosphorylation of platelet intracellular proteins, which distributes β3-integrins to areas of cellular contact and primes platelets for aggregation [304]. The predominant function for MMP-1 in this context is therefore as a driver of adherence and activation for platelets, and so platelet MMP-1 activity drives the association between high MMP-1 promoter activity haplotypes and increased risk for myocardial infarction [305]. This is supported by the fact that MMP-1 is elevated in the blocked coronary artery in comparison to peripheral blood of patients with acute myocardial infarction, although it is unknown if platelets have a positive or detrimental role in this context [306].

Platelet activation also triggers significant concentrations of MMP-2 secretion. Low levels of active MMP-2 have a pro-aggregatory effect on activated platelets by activating the PI3K
pathway that ultimately activates the GPIIb/IIIa pathway to bind fibrinogen. In addition pro-MMP-2 can bind directly to GPIIb/IIIa to be converted to active MMP-2. This process has also been suggested to enhance CD41 fibrinogen binding [295, 307, 308] and MMP-2 binding to GPIIb/IIIa has been implicated in upregulation of the inflammatory CD40L secretion by platelets [309]. Therefore low levels of MMP-2, such as the concentrations thought to be produced by platelets in vivo, may be an aggregating and inflammatory factor in platelet function. This is supported by the findings that platelet-released MMP-2 promotes arterial thrombosis in MMP-2/-/- chimeric mice [310].

6.4.2. Platelet intercellular regulation of MMP production

Whilst platelets are themselves a source of limited MMP activity they have a far reaching role in ECM degradation through regulation of the MMP activity of other cells. As previously discussed platelets play a central role in recruiting inflammatory cells such as monocytes and neutrophils to areas of infection and inflammation (see section 5.4). The potential concentration of MMPs produced by these inflammatory cells is huge and cell recruitment by platelets concentrates this proteolytic activity [296]. Aside from this general role in increasing MMP concentration through cell recruitment, platelets are able to target cells specifically to increase their MMP production and secretion. This regulation can be mediated both by direct receptor interaction and indirect secretory pathways. Most research on platelet regulation of MMPs has focused on tumour biology, where platelets have been shown to enhance tumour progression or metastasis and angiogenesis of tumour cells in a manner associated with increased gelatinase secretion [311, 312]. For example, platelets have been implicated in the upregulation of MMP-2, -7 and -9 in ovarian cancer cells by providing a primary source of lysophosphatidic acid (LPA), a simple lipid with growth factor-like signalling properties that acts on multiple G-protein coupled receptors [313]. In the lung, microvesicles derived from activated platelets have been shown to transfer the integrin CD41 from platelets to the lung cancer cell lines and stimulate the phosphorylation of MAPK p42/44 and AKT as well as the expression of MMP-14. These PDMs stimulate MMP-9 mRNA expression in the highly metastatic type II pneumocyte A549 cells [314].

Platelets have also been consistently shown to upregulate relevant inflammatory pathways in leukocytes in a variety both in vivo and of in vitro models. The importance of these interactions for MMP activity has been shown using a murine model to demonstrate that
platelets promote systemic and cardiac inflammatory responses and tissue remodelling after myocardial infarction through interaction with leukocytes for mediation of MMP-2 and MMP-9 activity [315]. In vitro platelets interact with collagen-bound monocytes to induce expression of MMP-9 through platelet P-selectin interaction with monocyte PSGL-1. This is augmented by platelet EMMPRIN expression to stimulate monocyte NFκB signalling that is known to upregulate certain MMPs [316, 317]. This has been shown to have clinical impact in rheumatoid arthritis, with significant monocyte-platelet aggregates found in patients, and monocytes from these patients producing significantly more cytokines, EMMPRIN and MMP-9 on contact with homologous platelets [318]. In neutrophils, platelets have been shown to upregulate MMP-8 and MMP-9 secretion largely through triggering of NETosis, but have also been shown to stimulate the release of neutrophil tertiary granules in mouse models of immune complex-inflamed skin independently of NET production [150, 254, 270]. Although platelet-secreted MMPs have a variety of important functions, there is no evidence that they function to directly drive the major tissue remodelling and large-scale destruction seen in inflammatory diseases such as TB. However, through intercellular regulation platelets can exert potent control of ECM breakdown. This clearly suggests a role for platelet regulation of MMPs in inflammatory tissue destruction, although to date this has not been investigated in the context of infection.

6.5. Platelets in TB

Whilst there has been no comprehensive study of the role of platelets in the host-driven pathology of TB, however, there have been several studies implicating platelet involvement. In 1998 PF4, as a marker of platelet activation, was found to be higher in pulmonary TB patients than the control group and was correlated with radiological extent of disease [319]. In 2012 Sahin et al noted that platelet count, PDW and plateletcrit were higher in pulmonary TB patients than controls. In TB patients the platelet values also correlated with other inflammatory markers, whilst they did not in controls. In addition, platelet count and activation markers were correlated with an increasing extent of disease which implies a role of platelets in the cell-based immune process of TB [320]. Most recently, a group detecting platelet activation using PMV found that this was a good marker for disease state of TB.
patients [321]. Many observational studies have shown that platelet counts increase in active pulmonary TB as well as central nervous system (CNS) TB [322-325]. Furthermore, platelets are particularly well placed for a role as early responders in pulmonary TB, which is the most common presentation of the disease, due to the intimate relationship between platelets and the lungs. Platelets are present in the lung pulmonary vessels at higher concentrations than elsewhere in the circulation, and these lung vessels may act as a platelet reservoir with release on stimulation [191]. Several studies have observed the retention of a small pool of platelets in the basal lung. Present in the lung in such high numbers, platelets are able to rapidly identify bacterial infection such as M.tb, and recruit cells to the site of infection [326]. Platelets have already been defined as being an important effector cell role in numerous inflammatory lung diseases including ALI, as described earlier, as well as cystic fibrosis, asthma and lung metastasis [327]. Platelets have also been described to change in number and function in a variety of infections involving the lung and pleurae, including influenza, bacterial sepsis and dengue fever [328, 329]. Thus, the nature of platelet synthesis, circulation and function all suggest these cells are ideally placed for a central role in TB pathogenesis.

In 2014 Feng et al published one of the only functional investigations of platelets in TB, bringing together platelet involvement in monocyte differentiation, and the role of monocyte-derived cells in TB [330]. Co-incubation of monocytes with platelets at monocyte:platelet (M:P) 1:100 ratio drove development of a foamy epitheloid phenotype that had upregulated markers of activation such as antigen presentation molecules like MHC-II, fusion markers such as CD36 and the chemoattractant CCL2. Monocytes were combined in culture with platelets and 50% of the media replaced every day for 5 days before M.tb infection. The infected cells cultured in this manner secreted significantly higher IL-10 and significantly less TNF-α than cells without platelet stimulation. In addition, the cells exhibited significantly increased BCG phagocytosis. If cultured with platelets further, the monocyte/macrophages differentiated into multinucleated foamy giant cells and this progression was exacerbated in the presence of M.tb lipomannan. Multinucleated foamy giant cells have a well-defined presence and role in TB pathogenesis, and Feng et al suggest their findings point to the presence of platelet-matured foamy cells in a human TB granuloma using IHC platelet specific protein CD42b [330]. Interestingly, analysis at gene
level of the isolated monocytes after 21 days *in vitro* co-culture with platelets identified MMP-1 as the third most upregulated gene in monocytes co-cultured with platelets, although this was not investigated further. This study indicates a role of platelets in influencing the maturation of monocyte-derived cells in the TB granuloma. However, an emerging understanding of platelet function in an array of immune cell functions suggests that platelet activity is TB is not limited to a role in monocyte maturation. Platelets potential to regulate cells such as neutrophils, monocytes and AECs at every stage of the immune response remains poorly understood and has never been explored fully in TB.

7. Hypothesis and aims

7.1. Hypothesis

Platelets drive TB immunopathology through mechanisms regulating key innate immune cell responses to *M.tb*-infection.

7.2. Aims

The aims for my project were to investigate:

1. The presence of active platelets in the tissue of pulmonary TB (Chapter 3).
2. Functional platelet regulation of monocyte cytokine and MMP activity in TB through the development and use of an *in vitro* model of TB (Chapter 3).
3. Intracellular mechanisms by which platelets regulate monocyte MMP-1 in TB (Chapter 4).
4. The role of platelets in neutrophil and stromal cell inflammatory tissue destruction in TB (Chapter 5).
Chapter 2. Materials and Methods

1. *M. tb* culture

*Mycobacterium tuberculosis* H37Rv Pasteur (*M. tb*) was cultured from stock stored at -80 °C in 30-50% glycerol. It was cultured in Middlebrook 7H9 medium supplemented with 10% OADC enrichment medium (BD Diagnostics, Oxford, UK), 0.2% glycerol, 0.02% Tween 80 and 2.5 μg/ml amphotericin with agitation at 10 rotations per minute (rpm). Culture growth was monitored with a Biowave cell density meter (WPA, Cambridge, UK) and the *M. tb* sub-cultured when the optical density (OD) reached 1.00. For infection experiments, culture at mid log growth at an OD of 0.60 was used, which corresponded to 1 x $10^8$ – 2 x $10^8$ colony forming units (CFU)/ ml. OD was correlated with CFU by performing colony counts in triplicate on Middlebrook 7H11 agar supplemented with OADC enrichment medium and 0.5% glycerol.

Whole killed bacilli with intact extracellular proteins was used in experiments outside the Category level 3 (CL3) suite. This was produced through bacterial exposure to high intensity UV light. Specifically, 1 x$10^8$ CFU were placed in a 60 mm sterile dish and double contained. 100 μl aliquot was saved as a positive control to confirm killing. The dishes were placed on a UV illuminator, covered and irradiated for 90 minutes (mins) on full power. To test killing of bacteria, 100 μl UV-killed and untreated *M. tb* were plated in triplicate on separate 7H11 agar plates and incubated at 37 °C for 6 weeks. The stock UV-killed *M. tb* was stored at -20 °C. This protocol has been optimised through previous test runs in the CL3 suite at Hammersmith hospital. Before it was removed from the secure CL3, the positive control must show excellent *M. tb* growth and the UV-treated plates must be completely devoid of any colonies. If either case was not fulfilled, samples were discarded as efficacy of killing has not been proven.
2. Platelet preparation

2.1. Platelet isolation

A platelet isolation procedure was optimised from previously published methods [299]. Blood was drawn from healthy volunteers into vacutainers with preservative-free sodium citrate (BD biosciences, San Jose, CA, USA), after an initial 1-2 mls was discarded to avoid activated platelets. The blood was gently inverted and used within 2 hrs. The blood was mixed with 50% platelet wash buffer and centrifuged for 15 mins at 200 relative centrifugal force/G-force (g) with no brake. Platelet wash buffer was made of: 10 mM HEPES; 5 mM glucose; 5 mM potassium chloride; 1 mM magnesium sulphate; 1 mM potassium phosphate; and 145 mM sodium chloride, and the pH measured to 7.4 before sterilisation with a 0.2 μm SFCA filter (Nalgene Hereford, UK). After centrifugation, the top layer was removed with transfer pipette leaving 1-1.5 ml to avoid contamination of the plasma with other cells. The platelet-rich plasma was centrifuged at 1500 g for 15 mins with 1 μM prostaglandin E (PGE) 1 (Calbiochem, San Diego, CA, USA) to avoid activation, and resuspended in platelet wash buffer. After a wash step, a count was performed diluting platelets 1 in 100 in platelet wash buffer and resting them for 15-30 mins. To activate platelets, cells were diluted to 1-5 x10^8 cells/ml and stimulated with 0.1 U/ml thrombin (Sigma Aldrich, Poole, UK) for 8 mins then centrifuged at 1200 g for 10 mins. Platelets were resuspended in media and used immediately. Cell viability was assessed with trypan blue as >99%.

2.2. Platelet purity and activation

As platelet isolation was a new technique within the laboratory flow cytometry was used to optimise isolation technique. Platelets were washed in platelet wash buffer before activation and fixed with 100 μl of cold 2% paraformaldehyde for 15 mins. They were washed at 4 °C twice in PBS and resuspended in PBS with 5% Human serum and 1% Bovine serum albumin (BSA) for 30 mins. After further washing they were resuspended in 1% BSA + 1 in 10 dilution of anti-human CD41a FITC clone HIP8 (eBioscience, Affymetrix, California, USA) and stained for 45 mins at 4 °C. A FITC mouse IgG1 isotype control (BD biosciences, San
Jose, CA, USA) was used for comparison. They were washed again and resuspended in 500 μl PBS for analysis on BD FACSCalibur flow cytometer with CellQuest (BD, Mountain View, California). Results were assessed on FlowJo software v10 (FlowJo LLC, Ashland, OR, USA). By this method, the optimised protocol was found to yield purity consistently around 95% (Fig 4).

To assess activation of platelets, flow cytometry was used to stain for P-selectin. Cells were stained with anti-human CD62P Phycoerythrin (PE) (Clone AK-4) or mouse IgG1 PE isotype control (both from eBioscience Affymetrix, California, USA). This yielded an average of 68.6 ±2.5% positive for this marker. However, cell clumping induced by thrombin activation rendered it difficult to obtain accurate readings and it is likely that this was a significant underestimate. Supernatant taken from platelets after activation was therefore used as an alternative, with secreted PF4 used as a marker of platelet activation measured by ELISA (see section 12 below for protocol). There was minimal PF4 secreted by resting platelets, whilst 10 mins thrombin incubation triggered significant PF4 secretion (p<0.0001; Fig 5), demonstrating significant activation.

**Figure 4. Flow cytometry analysis of purified platelet population.**

Purity of isolated platelet population was assessed through cell labelling with CD41-FITC or isotype control, and 10,000 events gated. Experiment was carried out in duplicate, and representative of three independent experiments. A) Histogram showing 94.6% events were CD41+ B) Distinct platelet population shown on a scatter plot of events with side scatter (SSC) and forward scatter (FSC) on a log scale.
2.3. Production of platelet supernatant

When producing platelet supernatant (PS) platelets were isolated as above, but thrombin-activated at 5x10^8 platelets/ml for 20 mins in media rather than platelet wash buffer. The cells were centrifuged at 12,000 g for 10 mins and the supernatant sterile filtered to ensure no cell contamination. The PS was then stored at -20 °C until use.

3. Monocyte isolation

Blood was taken from healthy volunteers into preservative-free heparin and diluted at least 1 in 2 in HBSS (Gibco, Life Technologies, Paisley, UK) in a T75 flask. For monocyte-derived cell experiments not involving platelet co-culture a leukocyte cone (Collingdale, NHS Blood service) was used instead of a volunteer. For this, the cells were diluted with HBSS up to a total volume 110 ml. In both sources, 35 ml of diluted blood was layered onto 15 ml Histopaque® (Sigma Aldrich, Poole, UK) the blood was then centrifuged at 480 rcf for 30 mins with the brake off. The central layer of PBMCs was pipetted off using a transfer pipette.

Figure 5. Thrombin-stimulated platelet activation.

Platelets were activated with media or 0.1 U/ml thrombin (Throm) for 10 minutes and platelet factor 4 (PF4) measured in the cell supernatant by ELISA. Bars represent mean ± SD and representative of two separate experiments. Activation protocol induces significant PF4 secretion from platelets. Statistical analysis was performed using a two-tailed unpaired T-test: ****p<0.0001.
and the cells diluted up to 50 ml with warm HBSS for a wash step at 308 rcf. This was repeated four times, with a monocyte count performed after the third wash. Monocytes were counted using adherent cells in a Neubauer counting chamber after incubation for 5 mins at 37 °C. Adherence purification was carried out by incubating cells with 0.5 mls/cm² of warm Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Life Technologies, Paisley, UK) supplemented with 10 µg/ml ampicillin and 2 mM glutamine in cell culture plates (Techno Plastic Products, Switzerland). Finally, after 1-2 hrs cells were washed with warm HBSS three times. Monocyte experiments were conducted with Dulbecco’s Modified Eagle Medium (DMEM) with 4500 mg/L glucose and L-Glutamine (Gibco, Life Technologies, Paisley, UK) and supplemented with 10% fetal bovine serum (FBS). Purity of monocyte populations with this method has been previously assessed using two-colour FACS for anti-CD3 (fluorescein isothiocyanate, FITC) and anti-CD14 (phycoerythrin, PE) and analysed on a BD FACSCalibur flow cytometer. Purity was found to be over 95%, with less than 5% cells CD3⁺.

To undertake confocal microscopy it was necessary to isolate monocytes with magnetic antibody cell sorting (MACS) and so a Pan Monocyte Isolation (Human) Kit (Miltenyi Biotec, Bisley, UK) was used. For this, blood was drawn from volunteers as above but cells were washed twice with cold HBSS at 4 °C before total PBMCs were counted, using trypan blue exclusion. After washing, the pellet was resuspended in sample buffer made with 2 mM EDTA, 0.5% BSA and PBS (pH7.2), with 30 µl buffer used for each 10⁷ PBMCs. The cells were mixed with 10 µL FcR blocking reagent and 10 µL Biotin-antibody cocktail per 10⁷ cells, and incubated for 5 mins at 4 °C. 30 µL of sample buffer per 10⁷ cells was then added with 20 µL of microbeads per 10⁷. Cells were then incubated for an additional 10 min at 4-8 °C. Finally, the volume was made up to 500 µL with sample buffer and passed through pre-prepared magnetic separation columns which were then washed with 500 µl sample buffer three times. Cells were centrifuged, resuspended in media and used immediately.
4. Production of CT

Cell culture medium from monocytes conditioned with TB (termed CT) was produced by *M. tb* infection of monocytes at multiplicity of infection (MOI) 1 for 24 hrs. Cell culture medium from unstimulated monocytes was termed CC. Supernatant was sterile filtered with 0.2 μm filter (Nalgene, Hereford, UK.). Each batch of CT and CC was tested by normal human bronchial epithelial cell (NHBE) stimulation and detection of extracellular MMP-1 concentration after 72 hrs. CT which had previously been tested before was used as a positive control and a benchmark for comparison (Fig 6). CT that elicited significant MMP-1 secretion, to concentrations that were similar to the positive control, was used for experiments.

Figure 6. Effectiveness of TB-conditioned medium. An ELISA was used to measure secreted MMP-1 concentration from monocytes stimulated with cell culture medium from monocytes conditioned with TB (CT) or controls (CC). Previously tested CT functioned as a positive control (+). Bars represent mean ± SD. An ordinary one-way ANOVA followed by Tukey’s post-test correction was used for statistical analysis: ***p<0.001.
5. Monocyte maturation

For maturation into MDMs, monocytes were cultured in RPMI 1640 media supplemented with 2 mM glutamine, 10% FBS, 10 μg/ml ampicillin and 20 ng/ml M-CSF (R&D Systems, Abingdon, UK). After 4 days the media was changed to RPMI 1640 with 10% FBS but no growth factors, and 24 hrs later the media changed again to macrophage serum free media (Gibco, Life Technologies, Paisley, UK), for the experiment to commence.

6. Neutrophil isolation

Blood from healthy volunteers were drawn in preservative-free heparin and mixed with equal volumes of 3% dextran/0.9% sodium chloride. The suspension was incubated in an upright position for 20 mins until a clearly defined interface was discerned. The leukocyte-rich plasma was pipetted and centrifuged at 250 g at 5 °C for 10 mins. The cell pellet was resuspended with 0.9% sodium chloride, layered onto Histopaque® and centrifuged at 400 x g at 20 °C for 40 mins. Thereafter, the saline layer and Histopaque® were aspirated to leave the neutrophil/erythrocyte pellet. The pellet was subjected to 30 secs of hypotonic lysis with 0.2% sodium chloride and isotonicity restored with 1.6% sodium chloride. The suspension was centrifuged at 250 x g at 5 °C for 6 mins and the cycle repeated three times. Neutrophil purity using this protocol was assessed on a BD FACSCalibur flow cytometer using PE mouse anti-CD66c (BD biosciences, San Jose, CA, USA) as over 95%. The final neutrophil pellet was resuspended in RPMI 1640 supplemented with 10% FBS for experiments. Neutrophils have been previously found to secrete more MMPs with polystyrene tissue culture plates and so 96-well flat bottom PVC plates (VWR, Leicestershire, UK) were used for all neutrophil experiments [149].
Chapter 2

7. Cell co-culture

The experimental set up for cell co-cultures is summarised in Figure 7.

7.1. Monocyte and platelet co-culture

Immediately upon preparation, autologous platelets were added to monocytes at a ratio of 100 platelets to 1 monocyte, unless otherwise stated. This created a concentration of 12.5 x10^7 platelets/ml. For controls, the same volume of media was added to the monocyte culture instead of platelets. Cells were infected immediately using \textit{M}.\textit{tb} at OD 0.6 for a MOI 1 for monocytes and 0.01 for platelets.

For trans-well experiments platelets were added to a 0.4 \(\mu\)m trans-well (Millipore, Watford, UK) at the same platelet:monocyte (P:M) of 100:1, but as more media was required the final concentration was 7.14 x10^7 platelets/ml. Trans-well culture prevented direct cell contact but allowed exchange of cell supernatant (Fig 8). For monocyte stimulation with PS, a supernatant volume equivalent to a 100:1 P:M was used. For example, 100 \(\mu\)l of PS produced from activation of 5 x10^8 platelets/ml was used to stimulate 5 x 10^5 monocytes.

For all experiments, with the exception of TIMP-1, a platelet only control was included and the value subtracted from the total co-culture concentrations to account for platelet contribution and leave only monocyte secretion.

7.2. Neutrophil and platelet co-culture

Neutrophils were infected immediately after isolation using \textit{M}.\textit{tb} resuspended in PBS as the \textit{M}.\textit{tb} culture media activates neutrophils at MOI 10 [331]. Unless otherwise stated, platelets were added to neutrophils at 100:1 platelet:neutrophil (Plt:N). This resulted in 6.25 x10^8 platelet /ml. The media used was RPMI 1604 with 10% FBS and 2 mM glutamine. For NETosis analysis phenol-red free RPMI 1640 media was used. For all experiments a platelet-only control was included and the value subtracted from the total co-culture concentrations to account for platelet contribution.
7.3. Human Bronchial Epithelial Cell and platelet stimulation

Human bronchial epithelial cells (HBECs; ScienCell, Carlsbad, USA) were cultured in Bronchial Epithelial Cell Medium (BEpiCM) supplemented with 5 ml of Bronchial epithelial cell growth supplement and 5 ml of penicillin/streptomycin solution, as per manufacturer’s instructions (ScienCell, Carlsbad, USA). All experiments were performed between passage four and five. HBECs were stimulated with CC or CT at a 1 in 5 dilution, and media or PS at a 1 in 25 dilution for 24-76 hrs as stated. Cell viability was assessed with a lactate dehydrogenase (LDH) assay, ensuring CT and PS at these dilutions did not significantly alter viability. Supernatants were collected and spun for 5 mins at 13,000 g before analysis. PS concentrations were also measured and subtracted from all experiments to account for all platelet contributions.

Figure 7. Platelet co-culture experimental set-up.

Monocytes or neutrophils were isolated from whole human blood using density purification followed by purification by hypertonic lysis for neutrophils and adhesion or magnetic activated cell sorting (MACS) for monocytes. Monocytes were infected with H37Rv *Mycobacterium tuberculosis* (*M.tb*) at a multiplicity of infection (MOI) of 1, neutrophils with MOI 10. Blood from the same donor was used to isolate platelets or produce platelet supernatant (PS). Platelets were added to monocyte or neutrophil culture before infection with *M.tb*. PS was used to stimulate monocytes from the same or a different donor, or human bronchial epithelial cells (HBECs). Solid arrow represents isolation stage; dashed arrow indicates cell stimulation.
Chapter 2

8. Isolation and quantification of NETs

For experiments involving NETs, human neutrophils were infected with *M. tb* at MOI 10 for 4 hrs in 96-well plates. A final concentration of 20 nM PMA was used as a positive control. 50 µL of micrococcal nuclease (Fermentas, ThermoFisher Scientific, Hemel Hempstead, UK), a non-processive nuclease that cuts DNA at linker sites, was added at a final concentration of 5 U/ml to each well for 10 mins at 37 °C. EDTA at a final concentration of 5 mM was used to halt the reaction. Supernatants were collected, sterile filtered and kept at 4 °C until further use. NETs were quantified using Quant-iT™ PicoGreen® (Invitrogen, Life Technology, Paisley, UK) according to manufacturer’s instructions. In brief, a standard using serial 1 in 10 dilutions of λ DNA from 2 ng/ml to 2 µg/ml diluted in 1 x TE buffer was made. 50 µL of samples and standards were loaded onto 96-well black plates, and 50 µL PicoGreen® at a 1 in 200 dilution was added in each well. The plate was then incubated at room temperature for 5 mins, and read using the fluorometer (FLUOstar Galaxy, BMG lab technologies, Germany).
9. *M.tb* regulation of platelet-secreted factors

To assess the effect of TB on active platelets, platelets were incubated with *M.tb* at a MOI of 0.01 (the equivalent of the MOI in monocyte-platelet co-culture model) for 24 hrs. Additionally, platelets were incubated with an one in three dilution of CT to assess effects of monocyte TB networks. This incubation was also for 24 hrs before the supernatant was harvested for analysis. In experiments assessing stimulation of unactivated platelets by *M.tb*, cells were incubated for 30 mins at 37 °C at the specified *M.tb* MOI or CT dilutions.

10. *M.tb* Killing

Monocyte killing of *M.tb* was assessed using CFU on Middlebrook 7H11 agar. For extracellular killing 20 µl of supernatant was diluted in water 1 in 100 before plating in duplicate. For intracellular killing, supernatant was removed and the adherent cells washed with HBSS before lysis in 500 µl water. This was diluted further in water and plated in duplicate. The plates were incubated at 37 °C for 2-4 weeks before counts were obtained.

11. Chemical inhibition and recombinant proteins

The chemical inhibitors and recombinant proteins used in this project are detailed in Table 3. Monocytes were incubated with all inhibitors for 1 hr before stimulation with *M.tb* and/or PS. Viability was assessed with a LDH assay (see section 19) and trypan blue exclusion to ensure proteins did not alter monocyte survival.

Inhibitors SB203580, PD98059, LY294002 target p38, ERK-1/2 and PI3K respectively, and are well established to inhibit MMP secretion in a variety of cells including monocytes, to levels below detection [332-334]. The MAPK inhibitors are known to have cytotoxic effects in
monocytes and so cell viability at the lowest significantly effective dose was assessed with LDH assay. Although some cytotoxicity was observed in the inhibitors it did not exceed acceptable parameters, and was not significant compared with M.tb infected controls (Fig 9). Helenalin was additionally tested for effectiveness at two doses to ensure NFkB inhibition was occurring (Fig 9B). As the drug inhibited NFkB at doses as low as 0.1 μM, 0.5 μM was used.

12. Sterilisation of cell culture supernatants

M.tb infected samples required removal of M.tb by sterile filtration before analysis outside the CL3 facility. Supernatants are centrifuged at 13,000 x g for 2 mins, and subsequently transferred to 0.2 μm PVDF centrifugal filter units (Millipore, Watford, UK) whereupon a further centrifugation at 12,000 x g for 1 min was undertaken.

Table 3. Chemical inhibitors and recombinant proteins.

<table>
<thead>
<tr>
<th>Cell stimulus</th>
<th>Company Supplier</th>
<th>Working Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant CD40 ligand</td>
<td>Thermofisher Scientific, Hemel Hempstead, UK</td>
<td>25 μg/ml</td>
</tr>
<tr>
<td>Recombinant platelet factor 4</td>
<td>Sigma Aldrich, Poole, UK</td>
<td>5 μg/ml</td>
</tr>
<tr>
<td>SB 203580</td>
<td>Enzo Life Sciences, Exeter, UK</td>
<td>1 μM</td>
</tr>
<tr>
<td>PD 98059</td>
<td>Calbiochem, Merk Millipore, Watford, UK</td>
<td>1 μM</td>
</tr>
<tr>
<td>LY 294002</td>
<td>Calbiochem, Merk Millipore, Watford, UK</td>
<td>10 μM</td>
</tr>
<tr>
<td>Helenalin</td>
<td>Enzo Life Sciences, Exeter, UK</td>
<td>5 μM</td>
</tr>
</tbody>
</table>
Chapter 2

Figure 9. Viability of monocyte-derived cells treated with NFκB and MAPK inhibitors.

(A) Monocytes were stimulated with SB 203580 (SB), PD 98059 (PD), or LY 294002 (LY), or a vehicular control for each (vSB, vPD or vLY consecutively) and M. tb infected. An LDH assay was used to assess cytotoxicity of this inhibition after 24 hours. (B-C) Monocyte-derived macrophages (MDMs) were pre-inhibited with helenalin (Hel) at 1 and 10 μM, or a vehicle control for 1 μM (V) before M. tb infection at MOI 1. (B) A lactate dehydrogenase assay (LDH) was used to assess cytotoxicity of Hel compared to a vehicle control after 8 hours infection. (C) MDM nuclear protein was isolated after 4 hours infection and western blot used to detect the p65 subunit of NFκB to assess NFκB inhibition by Hel. GAPDH was used as a reference protein. Graphs and image representative of two independent experiments. Cytotoxicity was calculated as a percentage of a positive lysed control. All inhibitors used were considered to have negligible cytotoxic effects compared with vehicular control (p>0.05). Hel significantly inhibited MDMs NFκB from 0.1 μM. Significance compared to vehicle controls was assessed using an ordinary one-way ANOVA and Tukey's multiple comparisons test: p<0.05 was taken as significant.
13. Enzyme-linked immunosorbent assay (ELISA)

MMP-1, -8, -9 and -10; TNF-α; TIMP-1 and -2; IL-8; and PF-4 concentrations in cell culture medium were measured by Duoset ELISA Development System (R&D Systems, Abingdon, UK) according to the manufacturer’s instructions. ELISA plates were coated with 100 µL of capture antibody overnight at room temperature, then washed three times with PBS/0.05% Tween. Free binding sites were blocked for 1 hr with PBS/1% BSA at room temperature. Seven standards were produced with one in two serial dilutions and samples were added to the appropriate wells and incubated for 2 hrs at room temperature. After three washes, the detection antibody was added for 2 hrs at room temperature. Three further washes were performed, and each well was incubated with 100 µl streptavidin-HRP (at 1 in 200 dilution) for 20 mins before washing was repeated. Colour change was induced by adding 100 µl substrate solution containing tetramethylbenzidine (Sigma-Aldrich, Poole, UK) per well. The reaction was stopped with 50 µL per well of 2 M sulphuric acid and the plate read at 450 nm using a microplate reader (µQuant, Biotek Instruments, UK). The ranges of the standard and the primary and secondary antibody concentrations can be found in Table 4.

<table>
<thead>
<tr>
<th>Protein</th>
<th>1st Standard (pg/ml)</th>
<th>Primary Antibody (µg/ml)</th>
<th>Secondary Antibody (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>10,000</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>MMP-8</td>
<td>4,000</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>MMP-9</td>
<td>2,000</td>
<td>1</td>
<td>200</td>
</tr>
<tr>
<td>MMP-10</td>
<td>2,000</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>2,000</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>2,000</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>TNFα</td>
<td>1,000</td>
<td>4</td>
<td>250</td>
</tr>
<tr>
<td>IL-8</td>
<td>2,000</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>PF-4</td>
<td>1,000</td>
<td>2</td>
<td>100</td>
</tr>
</tbody>
</table>
14. Luminex bead array

Luminex® corp has developed a microparticle based multiplex immunoassay for the accurate quantification of a range of analytes in very small volumes of various sample types. This was used to quantify cytokine and chemokines (Millipore, Watford, UK) and MMPs (R&D Systems, Abingdon, UK) in supernatants as well as platelet factors in bronchoalveolar lavage fluid (BALF) and supernatant samples (R&D systems, Abingdon, UK).

Specific antibodies were pre-coated onto colour-coded microparticles or beads during manufacturing. Following manufacturer’s instructions, microparticles, standards and samples were pipetted into a Luminex plate and agitated at 500 rpm for 2 hrs to allow immobilised antibodies to capture the analyte of interest. Biotinylated antibodies specific to the analyte were added and agitated for 1 hr. Next, streptavidin PE was added with agitation for 30 mins to generate a signal. Washes are performed between each step. Finally, the beads were resuspended and read using the Luminex® dual laser analyser (Bio-Rad Bio-Plex 200 System). One laser classified the bead and determined the analyte which is being detected, the second laser determined the magnitude of the PE derived signal, which is in direct proportion to the analyte bound.

MMP beads (R&D systems, Abingdon UK) were used for MMP-1, -2, -3, -7, -8, -9 and -10. The lower level of detections of the assay were 1.1, 12.6, 7.3, 6.6, 16.6, 13.7 and 3.2 pg/ml respectively. The panel of activated platelet factors consisted of PF4, P-selectin, CD40L, PDGF-BB and RANTES with a lower detection limit of 125.7, 68.2, 248.7, 3.9 and 7.5 pg/ml respectively. The panel of cytokines (Millipore, Watford, UK) consisted of IFN-γ, IL-10, macrophage derived chemokine (MDC), TNF-α, IL-12, -1β, -6, and -8 with the standard range for all of 10,000 to 3.2 pg/ml.
15. Immunohistochemistry

IHC was performed on paraffin embedded lung samples from five Balb/C M.\textit{tb}-infected mice, harvested after 28 days post infection. IHC was done in collaboration with Professor Joanna Porter at the University College of London using the Ventana Discovery XT instrument, and the Ventana DAB Map Kit (760-124). Heat induced epitope retrieval was performed in a pressure cooker using citrate buffer pH6. Anti-CD41 (Abcam, Cambridge, UK) primary antibody incubation was for 12 hrs using a 1 in 40 dilution. Rabbit anti-Rat (E0468) secondary antibody incubation was for 60 mins, using a 1 in 200 dilution. Slides were haematoxylin counterstained, dehydrated and mounted.

16. Western blot Analysis

For protein analysis of monocytes or MDMs alone, cells were stimulated and at specific time points the supernatant removed and cells washed with sterile PBS (Hyclone, GE Healthcare, Little Chalfont, UK). The cells were lysed with sodium dodecyl sulfate (SDS) sample buffer consisting of 62.5 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 50 mM dithiothreitol (DTT), and 0.01% Bromophenol blue. The lysed cells were scraped off the plate, transferred to cold microtubules and frozen at -80 °C. For protein analysis of experiments with platelet co-culture, supernatants were removed and spun down for 3 mins. The pellet was resuspended in SDS sample buffer. The same buffer was then used to lyse adherent cells as well so both adherent and non-adherent cell protein was obtained.

For isolation of the nuclear fraction of MDMs to assess NFkB activation, the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology, Rockford USA) was used. MDMs were detached from 6-well cell culture plates (Techno Plastic Products, Switzerland) and centrifuged for 5 mins at 2,200 rpm. The pellet was resuspended with ice-cold 100 µl CER I and incubated for 10 min. 5.5 µl of ice cold CER II was pipetted and incubated on ice for 1 min before pipetting again and centrifugation at 13,000 rpm. The cytoplasmic extract was transferred with to a clean pre-chilled tube and stored at -80 °C. The insoluble fraction,
which contained the nucleic protein, was resuspended in 50 µl ice-cold NER buffer. This was pipetted further and placed on ice for 10 mins. This was repeated every 10 mins for a total of 40 mins before centrifugation at maximum speed for 10 mins. The supernatant containing the nuclear extract was transferred to a clean pre-chilled tube and stored at -80 °C.

40 µL of sample was denatured at 80 °C for 10 mins prior to separation on a NuPAGE Novex 10% Bis-Tris protein gel (Novex, Life technologies, Paisley, UK) at 200 volts (V) for 45-60 mins. SDS running buffer MES and MOPS were used for small and large protein respectively, and 500 µl NuPAGE Antioxidant added to the cassette (all Novex Life technologies, Paisley, UK). ECL Rainbow molecular weight marker (Amersham, Little Chalfont, UK) was run on the gel for protein size evaluation. Subsequently, proteins were electro-transferred to a nitrocellulose membrane (Amersham Biosciences, GE Healthcare Life Sciences, Chalfont, UK) at 30 V for 90 mins and imaged using the ChemiDoc Touch Imaging System (BioRad, Laboratories, Hercules, CA, USA).

After transfer, the membrane was blocked for 1 hr with agitation in 5% milk protein (Marvel, Premier Foods Group Ltd, London, UK) and 0.1% Tween-20 (Sigma Aldrich, Poole, UK). The membrane was probed with the primary antibody incubated at 4 °C overnight. The next day the membrane was washed three times and incubated for 1 hr with HRP-linked goat anti-rabbit IgG secondary antibody or HRP-linked anti-mouse IgG secondary antibody (both Sigma Aldrich, Poole, UK). Luminescence was then detected with the ECL system (Amersham Biosciences, GE Healthcare Life Sciences, Chalfont, UK) according to manufacturer’s protocol. Membrane was exposed on a Chemidoc Touch imaging system and analysed with Image Lab version 5.2.1 (both BioRad Laboratories, Hercules, CA, USA). The membrane was stripped with Restore Plus western blot stripping buffer (ThermoFisher Scientific, Hemel Hempstead) for 15 mins for consecutive probing of phosphorylated and total forms on the same membrane. Antibody dilutions are shown in Table 5.
Table 5. Western blot antibodies.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Supplier</th>
<th>Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-human monoclonal anti-MMP-1</td>
<td>Cell Signalling Technology</td>
<td>1 in 1000</td>
</tr>
<tr>
<td></td>
<td>Leiden, Netherlands</td>
<td></td>
</tr>
<tr>
<td>Mouse anti-human monoclonal anti-β-actin</td>
<td>Sigma Aldrich, Poole, UK</td>
<td>1 in 10,000</td>
</tr>
<tr>
<td>Rabbit anti-human monoclonal anti-HIF-1α</td>
<td>Abcam, Cambridge, UK</td>
<td>1 in 2000</td>
</tr>
<tr>
<td>Rabbit polyclonal phospho- / total- ERK1/2</td>
<td>Cell Signalling Technology</td>
<td>1 in 3000</td>
</tr>
<tr>
<td></td>
<td>Leiden, Netherlands</td>
<td></td>
</tr>
<tr>
<td>Rabbit monoclonal phospho- / total- AKT</td>
<td>Cell Signalling Technology</td>
<td>1 in 2500</td>
</tr>
<tr>
<td></td>
<td>Leiden, Netherlands</td>
<td></td>
</tr>
<tr>
<td>Rabbit polyclonal phospho- / total- p38</td>
<td>Cell Signalling Technology</td>
<td>1 in 2500</td>
</tr>
<tr>
<td></td>
<td>Leiden, Netherlands</td>
<td></td>
</tr>
<tr>
<td>Mouse monoclonal anti-NFkB</td>
<td>Cell Signalling Technology</td>
<td>1 in 2000</td>
</tr>
<tr>
<td></td>
<td>Leiden, Netherlands</td>
<td></td>
</tr>
<tr>
<td>Rabbit monoclonal anti-PHD1</td>
<td>Abcam, Cambridge, UK</td>
<td>1 in 2000</td>
</tr>
<tr>
<td>Mouse monoclonal anti-PHD2</td>
<td>Abcam, Cambridge, UK</td>
<td>1 in 2000</td>
</tr>
</tbody>
</table>

17. Lactate dehydrogenase (LDH) assay

The cytotoxicity detection kit (LDH; Roche Diagnostics, Burgess Hill, UK) was used to verify toxicity in cells following manufacturer’s instructions. In brief, 50 µL of reconstituted catalysis solution was mixed with 112.5 µL of dye solution for each sample. 100 µL of this reaction mixture was mixed with 100 µL sample and incubated in the dark for 5-15 mins before the reaction was stopped with 50 µL of stop solution. The assay was read at 490 or 492 nm, with a reference wavelength over 600 nm. A positive control was gained by supernatant of cells killed with 1% triton X-100 and a low background control produced using media only. This was subtracted from all samples. Cytotoxicity was calculated as a percentage by the equation:

\[
\text{Cytotoxicity} = \frac{\text{normalised expected value}}{\text{normalised high control}} \times 100
\]
18. RNA extraction

RNA extraction was performed using the Direct-zol™ RNA extraction kit (ZymoResearch, Irvine, CA, USA). Adherent cells were washed with PBS and lysed with TRI Reagent® (Sigma Aldrich, Poole, UK). Platelet only controls were centrifuged to form a cell pellet and lysed with TRI Reagent. The sample was then mixed with an equal volume of 100% ethanol, and mRNA extracted as per the manufacturer’s instructions using the supplied RNA extraction reagents and the RNA spin columns. In brief, the mixture was transferred to a ZymoSpin in a collection tube and centrifuged. When doing a one-step PCR the DNase step was included in the RNA extraction protocol. This involved a wash step with 400 μl RNA wash buffer and incubation with 5 μl DNase I (6 U/μl) and 75 μl DNA digestion buffer in an RNase-free tube for 15 mins. The column was then washed and the purification process resumed. The column was washed twice with 400 μl RNA prewash and once with 700 μl RNA wash buffer before centrifugation for 2 mins to ensure complete removal of the wash buffer. In the final step, RNA was eluted with 30 μl RNase free water and stored at -80 °C. Total RNA was quantified by spectrophotometry (Nanodrop ND 1000, Thermo Scientific).

19. Quantitative Polymerase Chain Reaction (qPCR)

One or two-step qPCR was performed depending on starting concentrations of RNA. For both one- and two- step reactions, plasmid standards were used to calculate MMP-1 and β-actin copy numbers. These plasmid standards were kindly synthesized by Dr Catherine Ong [149]. Serial dilutions were used to generate a standard curve with a known number of gene copies. The cycle threshold for the gene of interest and reference gene was extrapolated from the standard curve to give an amount, and the gene of interest normalised to β-actin. A plasmid standard was not available for MMP-10 therefore the delta-CT method was utilised for comparative analysis. All PCR data is presented as fold changes. The fold changes were calculated by normalising each value (themselves a mean of a loading triplicate) to the
mean of the unstimulated control sample. MMP-1 and -10 primer and probes were used at 1 μM and sequences shown in Table 6.

Table 6. Primers and probes for quantitative PCR.

<table>
<thead>
<tr>
<th>Primers and probes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1 (Sigma-Aldrich, Poole, UK)</td>
<td>5’- AAGATGAAAGGTGGAACCAAAT -3’</td>
<td>5’- CCAAGAGAATGGCCGAGTT -3’</td>
<td>5’- FAM-CAGAGAGTACAATCATCGGTGTTCGGGCTC-TAMRA -3’</td>
</tr>
<tr>
<td>MMP-10 (Sigma-Aldrich, Poole, UK)</td>
<td>5’- GGACCTGGGCTTTATGGAGATATT -3’</td>
<td>5’- CCCAGGGAGTGGGCAAG -3’</td>
<td>5’- FAM- GAAGATGCATCAGGACCAATTTATC-TAMRA -3’</td>
</tr>
<tr>
<td>β-actin (Applied Biosystems, Carlsbad)</td>
<td>20x mix catalogue no. 4310881E</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

19.1. One-step quantitative reverse transcription polymerase chain reaction (qRT-PCR)

As platelets and monocytes had a small yield of RNA, qPCR was performed using the one-step RT-PCR master mix (Qiagen, Crawley, UK) according to the manufacturer’s instruction. This allows between 1 pg to 2 µg of RNA to be transcribed on a Stratagene Mx3000P platform (Agilent Technologies, La Jolla, CA, USA). The qRT-PCR cycling conditions were 50 °C for 30 mins, 95 °C for 15 mins, and 40 cycles of 94 °C for 30 secs, 60 °C for 30 secs and 72 °C for 1 min. 20x VIC-labelled β-actin control reagent (Applied Biosystems, Carlsbad, CA, USA) was used as a reference gene.

19.2. Two Step qPCR

19.2.1 cDNA synthesis

For MDM and HBEC RNA analysis a two-step quantitative PCR was performed. When using a two-step PCR reaction, cDNA was synthesised from RNA using the Quantitect® reverse transcription kit (Qiagen, Manchester, UK). The final reaction volume was 20 μl. For each sample 500 ng RNA was made up to 12 μl with RNase-free water. 2 μl of genomic DNA wipeout buffer was added and heated at 42 °C for 2 mins. A 6 μl mastermix containing 1 μl
random primers, 1 μl reverse transcriptase and 4 μl Quantiscript® RT buffer 5x was added to the RNA and heated at 42 °C for 15 mins. Primer annealing and strand extension occurred within the same reaction. The reaction was terminated by heating at 95 °C for 3 mins. The synthesised cDNA was stored at -20 °C.

19.2.2 cDNA amplification

cDNA was amplified using Brilliant II QPCR master mix (Agilent Technologies, La Jolla, CA, USA) also on a Stratagene Mx3000P platform. Each 20 μl reaction contained a 1 in 500 dilution of cDNA, primer and probes of interest, and the Brilliant II mastermix. β-actin was again used as a reference gene, chosen for the stability of its expression across different experimental conditions. The cycling conditions were: 95 °C for 10 mins and 40 cycles of 30 secs 95 °C and 1 min 60 °C.

20. Confocal microscopy

Monocytes were isolated by MACS and cultured on permanox chamber slides (Nunc Labtech, Thermo Fisher Scientific) that had been coated with 150 μg/ml of DQ collagen type 1 (ThermoFisher Scientific, Hemel Hempstead, UK) for 30 mins and washed. Thereafter, monocytes were M.tb-infected with or without platelets for 24 hrs. A platelet-only control was also included. Samples were then fixed with 4% paraformaldehyde overnight. Cells were washed twice with PBS and the chambers were removed from the slide. Fluoroshield mounting medium with DAPI (Abcam, Cambridge, UK) was added prior to application of the coverslip. Images were captured using Leica TCS SP5 confocal microscope and processed using Leica LAS AF Lite 2.6.0 (Leica Microsystems, Germany) and Image J 1.43U (NIH, USA). The florescent signal visualised in confocal images was converted for comparative numerical analysis using Image J software. Florescent reading (arbitrary units) from DQ collagen type 1 was normalised to the number of monocytes in the image visualised with DAPI stain.
21. DQ matrix degradation assay

Type I and IV collagen degradation was assessed using the EnzChek® Gelatinase/Collagenase Assay kit (Molecular Probes, Leiden, Netherlands) according to manufacturer’s instructions. In brief, 100 µL of samples and standards were added into a 96-well black fluorescence plate with 20 µL of DQ collagen type 1 or DQ gelatin at a final concentration of 250 µg/ml. The plate was incubated at 37 °C and fluorescence read at 48 hrs using a fluorometer (FLUOstar Galaxy, BMG Lab technologies, Germany) at 10% gain setting.

22. Accounting for platelet contributions in co-culture

Unless otherwise stated, platelet contributions have been accounted for in graphs of platelet co-culture. For secretion experiments, this involved subtracting concentrations from platelet-only controls performed in triplicate (or PS if used for alternative stimulation) from the relevant conditions. TIMP-1 secretion from monocyte-platelet co-culture was not subtracted, but instead included on the graph. For gene expression data, RNA from total platelet count was extracted and cycle threshold subtracted from the monocytes. In the majority of cases, analytes from platelets were undetectable, or borderline undetectable. For western blots of intracellular protein, platelet-only controls or 0 hr co-cultures are displayed on the blot where appropriate.

23. Statistics

GraphPad Prism Version 5.04 for Windows (GraphPad Software) was used for all analysis. Multiple interventions were analysed by an ordinary one-Way Anova followed by Tukey’s multiple comparison test. Multiple interventions at two time points were analysed by a two-way Anova followed by Tukey’s or Sidak’s multiple comparison. A p-value of less than 0.05 was taken as statistically significant. A p-value of less than 0.05 has been illustrated with one
star in the figures, a p-value of 0.01 with two stars, a p-value of less than 0.001 with three stars and a p-value of less than 0.0001 with four stars. For secretion analysis, experiments were all performed in triplicate on at least two occasions, while RNA analysis was performed in triplicate on a minimum of three separate experiments, unless otherwise stated. Loading triplicates were also utilised for PCRs to minimise errors. Correlation of platelet factors and MMPs were calculated with the Spearman test to generate a Spearman's rank correlation coefficient (R). An R value of equal or less than 0.4 was considered weak, whilst R of between 0.5 and 0.7 was considered to indicate a medium-strength correlation.
Chapter 3. Platelets regulate monocyte inflammatory responses to tuberculosis

1. Introduction

The immune response drives the establishment and development of pathology in TB. Active platelets have an emerging role in regulating the immune response, and extravate to the lung in inflammatory reactions such as asthma [335]. The immune response is vital to the establishment of pathology in TB. Isolated platelets have been shown to have no bactericidal activity towards *M. tb* *in vitro* [336]. However, a regulatory role of platelets in regulating TB immunopathology has never been widely investigated.

Our research group and others have previously shown an upregulation of active platelets in the circulation of TB patients compared to controls [320, 321, 337] but a presence and role of platelets in the lung of active TB has not been established. The presence of platelets can be measured using a number of markers. CD41 is expressed in the membrane of platelets (Fig 3) and associates with GPIIIa to bind several adhesion molecules such as fibrinogen, fibronectin and vitronectin. CD41 is commonly used to directly detect platelets due to its specificity to megakaryocytes and platelets [230, 338]. Additionally, platelets are major sources of numerous secreted products such as soluble CD40L and P-selectin, which can be used as markers of active platelet presence as these are only secreted upon activation [207, 252, 339].

Monocytes are an essential player in the immunopathology of TB. They are thought to have an important role in the inflammatory response to TB, with secretion of cytokines such as IL-1β associated with the development of the highly inflammatory reaction known as TB-IRIS [73]. In addition to pro-inflammatory cytokines such as TNF-α and IFN-γ, *M.tb*-infected monocytes also secrete chemoattractants such as IL-8, and immunomodulatory cytokines such as IL-12 [60, 340]. This cytokine profile is highly context specific, with for example
MDR-TB patients shown to have depressed monocyte IL-12 and TNF-α responses compared to new TB cases [60]. Monocytes and their derived cells are also important sources of a variety of MMPs which drive a tissue destroying phenotype in TB. MMP-1 is thought to be particularly important for driving early, monocyte/macrophage-derived, collagenase activity in TB [76]. The role of monocytes in the control of M.tb infection is complex and associated both with the control and facilitation of latent infection and dissemination [34, 55, 58, 62, 63]. These apparently contradictory functions may arise from the monocyte’s role as one of the most dynamic and plastic immune cells that are highly susceptible to intercellular regulation. Despite this, in TB monocytes are rarely studied in the context of intercellular networks.

Platelets are known to be important intercellular regulators of monocytes. They recruit monocytes to sites of infection [253, 258] and influence monocyte maturation to enhance a pro-inflammatory response [264]. Concurrently, platelets can have anti-inflammatory effects. For example, platelet secretion of CD40L induces anti-inflammatory monocyte effects through upregulation of IL-10 expression that is accompanied by a decrease in TNF-α and IL-6 [274]. In vivo, use of a sepsis mouse model demonstrated that reduced platelet binding to monocytes and neutrophils leads to increased concentrations of TNF-α, IL-6, MIP-1β and MCP-1, which supports a dampening effect from platelet binding [276]. 5 days in vitro stimulation of monocytes with platelets results in foamy macrophage-like cells in a process enhanced by lipoarabinomannans [330]. However, platelet regulation of TB-driven tissue destruction has never been assessed.

In this chapter I sought to fulfil my first key project aim, which was to investigate the presence of platelets in the lung of active pulmonary TB, using both a mouse model and clinical samples. I next sought to expand our understanding of the functional role of platelets in monocyte immunopathology in TB, which is currently largely unknown. Specifically, I aimed to establish an in vitro platelet-monocyte co-culture model using different ratios of monocyte to platelet cell counts, within a physiologically relevant range. I then aimed to use this model to assess in vitro platelet regulation of monocyte MMP responses, with a focus on the collagenase MMP-1. Finally, I analysed monocyte cytokines, chemokines and bactericidal activity to assess the wider immunomodulatory effects of platelet regulation of monocytes.
2. Results

2.1. Platelets are present in a murine model of *M.tb*-infected lung.

There has been limited assessment of platelet presence in the tissue of pulmonary TB. To investigate this, I first used IHC to stain lung samples from a murine model of pulmonary TB for the platelet integrin CD41 (Fig 10). Like most murine TB models, Balb/C mice do not form organised granulomas with central necrosis [341] but *M.tb* infection and dissemination through tissue does result in large areas of highly inflamed pulmonary tissue. CD41 staining indicated that there was no significant platelet presence in the TB murine lung in peripheral areas of infection with limited inflammation and cell recruitment (Fig 10A). However, there was striking CD41 visualised at the site of active infection with substantial inflammation, cell influx and tissue remodelling (Fig 10B). Platelets are anucleate cells and this is predominately reflected by the staining. Interestingly, there were significant platelets detected in the alveoli (Fig 10B). Limited CD41 association with nucleated cells can be seen on further magnification (Fig 10C), however, this is largely restricted to areas of granuloma-like structure and giant multinucleate cell formation.
Figure 10. CD41 expression at the site of pulmonary TB pathology in murine TB model. Paraffin embedded lung samples, harvested 28 days post-infection from Balb/C M.tb-infected mice, were collected for CD41 staining as a marker of platelets. (A-B) 15x magnification. (C) 40x magnification. Platelets are readily detectable in lung areas with significant TB pathology of inflammation and tissue remodelling. Images from a single mouse and representative of N=5 mice.
2.2. Platelet-secreted factors are upregulated in patient lungs with active TB

To build on my observations in a mouse TB model, and relate them to human TB pathology, I analysed TB patient BALF for the presence of factors known to be secreted by active platelets. BALF is an excellent source to analyse soluble factors in the lung with minimal invasion to the patient, and samples from a previously published study [160] offered an ideal opportunity to investigate presence of platelets in this context. The original clinical study was investigating MMP activity in pulmonary TB and inclusion criteria for patients are summarised in Table 7. Control samples were collected from individuals undergoing bronchoscopy for investigation of other pathology due to lung symptoms (typically coughing) and follow-up revealed diagnosis of: foreign body aspiration; carcinoma; lymphoma; smoking related bronchitis; small airway disease; pneumonia; and sarcoid or pulmonary vasculitis [160].

The concentrations of platelet-selected factors were compared between TB patients and symptomatic controls (Fig 11). CD40L, RANTES, P-selectin and PDGF-BB were significantly upregulated in TB cases. Compared to controls, the median of CD40L increased 2.5-fold in TB cases (p<0.001) whilst P-selectin increased 4.8-fold (p<0.01; Fig 11A-B). The median concentration of RANTES in the BALF of TB cases was 3.9-fold higher and 4.1-fold higher for PDGF-BB (Fig 11C-D; both p<0.01). No significant difference was found in PF4 concentrations between controls and TB patients (p>0.05; Fig 11E). To begin to assess a role of platelets in TB pathology, the relationship between these platelet factors and TB-upregulated MMPs was investigated by correlative analysis of the BALF samples.

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB Smear positive</td>
<td>Previous TB diagnosis</td>
</tr>
<tr>
<td><em>M. tb</em> culture positive</td>
<td>Severe Chronic Lung Disease</td>
</tr>
<tr>
<td>Over 18 years of age</td>
<td>Malignancy</td>
</tr>
<tr>
<td>Able to consent</td>
<td>HIV</td>
</tr>
<tr>
<td></td>
<td>Corticosteroids/</td>
</tr>
<tr>
<td></td>
<td>immunosuppressive drugs</td>
</tr>
</tbody>
</table>
Platelet-secreted factors are upregulated in the lungs of human pulmonary TB cases.

Bronchoalveolar lavage fluid (BALF) was collected from patients with cases of TB and symptomatic controls. The BALF was analysed for factors known to be secreted by active platelets. A magnetic bead Luminex array was used for multiplex detection of: (A) CD40 ligand (CD40L); (B) P-selectin; (C) Regulated on activation, normal T cell expressed and secreted (RANTES); (D) Platelet derived growth factor BB (PDGF-BB); and (E) Platelet factor 4 (PF4). N=17 for both symptomatic controls (Control) and TB patients (Cases). CD40L, P-selectin, RANTES and PDGF-BB were significantly upregulated in the BALF of TB cases compared with controls whilst PF4 was unchanged. The analytes are plotted on a log scale of pg/ml and represented as median ±IQR. Significance was calculated using a Mann-Whitney test with p<0.05 taken as significant: **p<0.01.
2.3. Platelet-secreted factors correlate with MMP and IL-1β concentrations in the lung

MMPs and the inflammatory cytokine IL-1β contribute to TB pathology, and have been previously measured in these clinical samples [160]. Concentrations of platelet-secreted factors were analysed to assess correlation with MMP-1, -3, -7, -8, -9 and IL-1β in the human lung (Table 8). In this data set, P-selectin correlated with all measured MMPs in a significant manner (Fig 12). MMP-3 had a weak correlation to P-selectin (R=0.4; p<0.05), whilst MMP-1, -7, -8 and -9 all had a moderate correlation (R=0.5; p<0.01; Fig 12A-E). Interestingly, IL-1β also correlated moderately with P-selectin with R=0.6 and p<0.001 (Fig 12F). PDGF-BB significantly correlated with MMP-1, MMP-3 and MMP-9 (all p<0.05) and MMP-8 (p<0.01; Fig 13). RANTES correlated with MMP-1, MMP-9 and IL-1β (all p<0.05) and MMP-8 (p<0.001; Fig 14). Lastly, CD40L was found to significantly correlate with MMP-3 (p<0.01) and MMP-7 (p<0.05; Fig 15). PF4 did not correlate with any MMPs or cytokines measured (Table 8). Together, all of the MMPs and cytokines measured correlated with at least two of the panel of analytes secreted by active platelets (Table 8). Next, a regulatory role of platelets in the immune response to TB was assessed in vitro.

Table 8. Platelet markers correlate with MMPs and IL-1β in bronchoalveolar lavage fluid.

Platelet-secreted factors: P-selectin, Platelet-derived growth factor-BB (PDGF-BB), CD40 ligand (CD40L), Regulated on activation, normal T cell expressed and secreted (RANTES) and Platelet factor 4 (PF-4) were measured as a marker of platelets. All measured MMPs correlated with at least two factors. Correlation was calculated with Spearman correlation to generate Spearman rank correlation coefficient (R Value) and a two-tailed P Value for each correlation. N=34.

<table>
<thead>
<tr>
<th></th>
<th>P-Selectin</th>
<th>PDGF-BB</th>
<th>CD40L</th>
<th>RANTES</th>
<th>PF-4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R Value</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-1</td>
<td>0.5</td>
<td>0.0009</td>
<td>0.4</td>
<td>0.02</td>
<td>0.2</td>
</tr>
<tr>
<td>MMP-3</td>
<td>0.4</td>
<td>0.01</td>
<td>0.4</td>
<td>0.03</td>
<td>0.5</td>
</tr>
<tr>
<td>MMP-7</td>
<td>0.5</td>
<td>0.005</td>
<td>0.2</td>
<td>ns</td>
<td>0.4</td>
</tr>
<tr>
<td>MMP-8</td>
<td>0.5</td>
<td>0.003</td>
<td>0.5</td>
<td>0.004</td>
<td>0.2</td>
</tr>
<tr>
<td>MMP-9</td>
<td>0.5</td>
<td>0.005</td>
<td>0.4</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.6</td>
<td>0.0002</td>
<td>0.2</td>
<td>ns</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>P Value</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-1</td>
<td></td>
<td></td>
<td>0.4</td>
<td>0.02</td>
<td>0.2</td>
</tr>
<tr>
<td>MMP-3</td>
<td></td>
<td></td>
<td>0.4</td>
<td>0.03</td>
<td>0.5</td>
</tr>
<tr>
<td>MMP-7</td>
<td></td>
<td></td>
<td>0.2</td>
<td>ns</td>
<td>0.4</td>
</tr>
<tr>
<td>MMP-8</td>
<td></td>
<td></td>
<td>0.5</td>
<td>0.004</td>
<td>0.2</td>
</tr>
<tr>
<td>MMP-9</td>
<td></td>
<td></td>
<td>0.4</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td>0.2</td>
<td>ns</td>
<td>0.4</td>
</tr>
</tbody>
</table>
P-selectin concentration in BALF of pulmonary disorders (respiratory symptomatics and TB cases) was tested for correlation with key proteases and the inflammatory cytokine IL-1β. The concentration of all analytes was detected using a Luminex Platform Correlation was calculated with Spearman correlation to generate Spearman rank correlation coefficient (R) and a two-tailed p-value is included inset for each correlation. P-selectin correlated with (A) MMP-1; (B) MMP-3; (C) MMP-7; (D) MMP-8; (E) MMP-9; and (F) IL-1β with R≥0.4 and p<0.05. Scatter plots were generated with a log scale of pg/ml for all analytes. N=34.

**Figure 12.** P-selectin correlates with MMP-1, -3, -7, -8, -9 and IL-1β in bronchoalveolar lavage fluid (BALF).
Platelet-derived growth factor correlates with MMP-1, -3, -8 and -9 in bronchoalveolar lavage fluid (BALF).

Platelet derived growth factor BB (PDGF) concentration in BALF of pulmonary disorders (respiratory symptomatics and TB cases) was tested for correlation with key proteases and the inflammatory cytokine IL-1β. Correlation was calculated with Spearman correlation to generate Spearman rank correlation coefficient (R) and a two-tailed p-value is included inset for each correlation. PDGF correlated with (A) MMP-1; (B) MMP-3; (C) MMP-8; and (D) MMP-9 in a significant manner with R≥0.4 and p<0.05. Scatter plots were generated with a log scale of pg/ml for all analytes. N=34.
Figure 14. RANTES correlates with MMP-1, -8, -9 and IL-1β in bronchoalveolar lavage fluid (BALF).

RANTES concentration in BALF of pulmonary disorders (respiratory symptomatics and TB cases) was tested for correlation with key proteases and the inflammatory cytokine IL-1β. Correlation was calculated with Spearman correlation to generate Spearman rank correlation coefficient (R) and a two-tailed p-value included inset for each correlation. RANTES correlated in a significant manner with (A) MMP-1; (B) MMP-8; (C) MMP-9; and (D) IL-1β in the BALF samples of a patient cohort with pulmonary disorders, with R≥0.4 and p<0.05. Scatter plots were generated with a log scale of pg/ml for all analytes. N=34.
Figure 15. CD40 ligand correlates with MMP-3 and -7 in bronchoalveolar lavage fluid (BALF).

CD40 ligand (CD40L) concentration in BALF of pulmonary disorders (respiratory symptomatics and TB cases) was tested for correlation with key proteases and the inflammatory cytokine IL-1β. Correlation was calculated with Spearman correlation to generate Spearman rank correlation coefficient (R) and a two-tailed p-value included inset for each correlation. CD40L correlated significantly with (A) MMP-3 and (B) MMP-7 with R≥0.4 and p<0.05. Scatter plots were generated with a log scale of pg/ml for all analytes. N=34.
2.4. Optimisation of an in vitro model of platelet-regulated monocyte MMP secretion

I next aimed to investigate a functional role of platelets in *M.tb*-driven ECM destruction using an in vitro co-culture of monocytes and platelets. As there has been no previous study of this platelet function in TB it was first necessary to optimise the in vitro culture set-up.

I first assessed platelet-only MMP responses in the context of a co-culture model. Activated platelets were incubated with *M.tb* at an MOI 0.01 for 24 hrs before secreted MMP concentrations were measured. MMP-1, MMP-2, TIMP-1 and TIMP-2 were readily detected in activated platelets (Table 9). Low concentrations of MMP-7 and MMP-9 were also detected, 101.8 ±19.0 pg/ml and 100.2 ±16.7 pg/ml respectively, whilst no significant concentration of MMP-3, -8 or -10 could be measured. As the isolated platelets secreted MMPs and TIMPs to readily detectable amounts, a platelet-only control was included in all relevant experiments to determine platelet-derived protein contribution to total concentrations.

Table 9. Platelet-secreted MMPs and TIMPS.
Concentrations (Conc) are given in pg/ml ±SD, with some analytes not detected (ND). Representative of three independent experiments.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Conc (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>439.5 ±59.0</td>
</tr>
<tr>
<td>MMP-2</td>
<td>295 ±75.6</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Trace</td>
</tr>
<tr>
<td>MMP-7</td>
<td>101.8 ±19.0</td>
</tr>
<tr>
<td>MMP-8</td>
<td>ND</td>
</tr>
<tr>
<td>MMP-9</td>
<td>100.1 ±16.7</td>
</tr>
<tr>
<td>MMP-10</td>
<td>ND</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>23161.4 ±8128.0</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>118.6 ±50.5</td>
</tr>
</tbody>
</table>
To investigate a role of platelets in monocyte MMP secretion in *M. tb* infection, monocytes were incubated with autologous activated platelets at different P:M and infected with virulent *M. tb*. MMP-1 and -10 secretion was then measured (Fig 16). *M. tb*-infected monocytes had significantly upregulated MMP-1 secretion compared to uninfected controls (p<0.01; Fig 16A). Upon co-stimulation of monocytes with both *M. tb* and platelets, MMP-1 was upregulated further in a manner dependent on platelet numbers. This upward trend is apparent at P:M 50:1, where MMP-1 increases from 5055.0 ±336.8 pg/ml to 7730.1 ±1744.5 pg/ml. At P:M 100:1 MMP-1 increased further to a significant 2.5-fold increase compared to infected monocytes alone (p<0.0001), and a 12.7-fold increase compared to uninfected controls. At higher ratios this upregulation was maintained but did not increase significantly more compared to P:M 100:1 (p>0.05; Fig 16A).

MMP-10 secretion from *M. tb*-infected monocytes was upregulated in the presence of platelets, with even the lowest platelet dose (Fig 16B). At P:M 10:1, MMP-10 increased from 1984.8 ±324.9 pg/ml to 2738.2 ±618.2 pg/ml. At 50:1 MMP-10 secretion increased further, to a concentration 2.3-fold (p<0.001) greater than *M. tb*-infected monocytes alone. Platelet upregulation of MMP-10 concentrations was maximal at a P:M 100:1 with a highly significant 2.8-fold increase compared to monocytes infected alone (Fig 16B; p<0.0001). However, higher ratios of platelets reduced MMP-10 secretion dramatically: an increase from 100:1 to 500:1 P:M was sufficient to reduce monocyte MMP-10 secretion 2.8-fold (p<0.0001), to levels equivalent to infected monocytes alone (p>0.05; Fig 16B).

As P:M 100:1 resulted in the greatest MMP upregulation in dose-response experiments, and is a ratio widely used in the relevant literature [318, 330, 342] it was chosen in the set-up of my monocyte-platelet co-culture model for MMP regulation in TB. A platelet-only control was included to account for the MMPs secreted directly from platelets, rather than monocytes, in the co-culture conditions. To confirm this is a sufficient control to quantify platelet contributions, platelet activation and secretion was investigated in the context of the monocyte co-culture environment.
Figure 16. *M.tb*-driven monocyte MMP-1 and MMP-10 secretion is upregulated in platelet co-culture.

Before infection with H37Rv *M.tb*, platelets were added to autologous monocytes with platelet:monocyte (P:M) ranging from 10:1 to 500:1. Uninfected and infected monocyte-only controls were also included. Extracellular MMP-1 and -10 concentrations were assessed by ELISA after 24 hours incubation and are expressed in pg/ml. A) MMP-1 secretion is significantly upregulated by platelets at P:M of 100:1 or greater. B) MMP-10 is increasingly upregulated by platelet co-culture until P:M 100:1. Bars represent mean ±SD and are representative of three independent experiments. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey’s multiple comparison test: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
To evaluate changes to platelets in monocyte co-culture, thrombin-activated platelets were stimulated with monocyte TB networks in the form of CT and live virulent *M. tb* at the MOI of the proposed co-culture model (MOI 0.01). Multiplex analysis then detected platelet proteins in the cellular supernatant (Fig 17). There was a small increase in P-selectin from 17735.8 ±2469.2 pg/ml to 25553.7 ±5261.0 pg/ml with CT and *M. tb* co-stimulation, but this increase did not reach significance (p>0.05; Fig 17A). No increase was seen in single stimulation with CT or *M. tb* compared with unstimulated platelets (p>0.05; Fig 17A). RANTES, PDGF-BB and CD40L (Fig 17B-D) were also measured and no change on further stimulation of activated platelets with *M. tb* or CT stimulation was detected (all p>0.05). To further confirm this, MMP-1 secretion was measured. No change in concentration was identified with *M. tb* stimulation (p>0.05; Fig 17E). As monocyte-platelet co-culture model conditions were not found to alter secretion of platelets factors after thrombin stimulation, a platelet-only control was deemed acceptable for use to assess platelet contribution to co-culture supernatant protein concentrations. This co-culture model could now be used to assess platelet regulation of monocyte MMP secretion.
Figure 17. Activated platelet-secreted factors in *M. tb* infection.

Platelets were thrombin activated *in vitro* before incubation with H37Rv *M. tb* at MOI 0.01, monocyte supernatant conditioned with TB (CT), or both, for 24 hours. The platelet secretory profile was then measured by magnetic bead Luminex array or ELISA. This involved detection of: (A) P-selectin; (B) RANTES; (C) Platelet derived growth factor BB (PDGF-BB); (D) CD40 ligand (CD40L); and (E) MMP-1 in the culture supernatant. Bars represent mean ± SD and are representative of two separate experiments. *M. tb* and CT stimulation did not alter the secretion of any of the analytes from pre-activated platelets. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey's multiple comparison test: ns p>0.05.
2.5. Platelets regulate a specific monocyte MMP response to *M. tb* infection

Having established a relevant *in vitro* co-culture model I then investigated the monocyte MMP profile that is regulated by platelets. Multiplex analysis revealed platelet regulation of a specific monocyte MMP response (Fig 18). MMP-1 secretion increased upon *M. tb*-infection of monocytes by 6.6-fold (p<0.05) and this was increased further in platelet co-culture (p<0.0001; Fig 18A). Consequently, in the presence of platelets *M. tb* infection drove a 13.1-fold upregulation in monocyte MMP-1 secretion compared to uninfected co-cultures (p<0.0001). Interestingly, platelets did not stimulate any significant MMP-1 secretion from uninfected monocytes (p>0.05; Fig 18A). MMP-7 was also induced by *M. tb* infection, increasing from undetectable levels in uninfected monocytes alone to 1231.8 ±78.8 pg/ml with infection (p<0.001; Fig 18B). However, monocytes *M. tb*-infected in the presence of platelets secreted 2.9-fold more MMP-7 than monocytes infected without platelet regulation (p<0.0001; Fig 18B). The collagenase MMP-8 was upregulated 2-fold in *M. tb*-infected monocytes alone compared to uninfected controls (p<0.001) but platelet co-incubation did not alter this response, with no significant difference in monocytes *M. tb*-infected with or without platelets (Fig 18C). Finally, MMP-10 was upregulated from 251.6 ±220.1 pg/ml in uninfected monocytes to 1972.4 ±405.4 pg/ml with *M. tb* infection. This increased further to 3423.3 ±363.2 pg/ml in the presence of platelets (p<0.001; Fig 18D) resulting in a 9.0-fold increase in *M. tb*-driven MMP-10 secretion in the presence of platelets (p<0.0001), compared to only a 6.8-fold-increase in monocytes without platelets (p<0.001; Fig 18D). Platelets alone were not sufficient to induce secretion of any of these MMPs (Fig 18A-D).

Secretion of MMP-3 and -9 was not significantly increased upon *M. tb*- infection (Fig 18E-F). There was a non-significant increase of the stromelysin MMP-3 from 9.7 ±5.2 pg/ml in control cells to 22.0 ±2.3 pg/ml (p>0.05; Fig 18E). Platelet co-incubation then significantly increased MMP-3 secretion further to 51.1 ±18.4 pg/ml (p<0.05; Fig 18E). There was high secretion of MMP-9 in uninfected monocytes of 3281.8 ±136.5 pg/ml which did not significantly increase upon infection with *M. tb*, although an upward trend was observed (p>0.05). Platelets did significantly upregulated MMP-9 in both uninfected and infected cells compared to uninfected monocytes alone (p<0.01 and p<0.05 respectively; Fig 18F). This resulted in similar MMP-9 concentrations between infected and uninfected co-cultured cells
(p>0.05; Fig 18F). It was next of interest to investigate platelet regulation of the MMP inhibitors TIMP-1 and TIMP-2.
Platelets (Plt) were incubated with monocytes at 100:1 plt:monocyte and infected with *M.tb* for 24 hours. Secreted MMPs were then measured by multiplex analysis of the cell supernatants. MMPs measured were: (A) MMP-1; (B) MMP-7; (C) MMP-8; (D) MMP-10; (E) MMP-3; and (F) MMP-9. Plt significantly upregulated monocyte MMP-9 in uninfected cells and MMP-1, -3, -7 and -10 in *M.tb* infection. Bars represent mean ±SD and are representative of three independent experiments. Statistical analysis was performed using an ordinary one-way ANOVA and Tukey’s multiple comparisons: ns p>0.05; *p<0.05; **p<0.01; ***p<0.001; ****p<0.00001.
2.6. TIMP concentrations do not change in the presence of platelets

To continue to investigate platelet regulation of monocyte MMP activity, TIMP-1 and -2 were measured. Monocytes constitutively secreted TIMP-1 to high concentrations and *M. tb* infection did not induce any changes (p>0.05; Fig 19A). Platelets alone secreted TIMP-1 to concentrations comparable with monocytes. However, co-incubation of monocytes with platelets did not result in an additive effect; TIMP-1 supernatant concentrations were unchanged by monocytes in co-culture compared with monocytes alone (p>0.05; Fig 19A). To investigate if this was due to inhibition of platelet TIMP-1 secretion by monocyte signalling pathways, active platelets were incubated for 24 hrs with *M. tb*, CT or both (Fig 19B). Accounting for TIMP-1 concentrations in CT, platelet TIMP-1 concentrations remained unchanged under all conditions (p>0.05; Fig 19B). The regulation of platelet-secreted factors on monocyte TIMP-1 secretion was assessed next, using PS to precisely account for platelet-derived TIMP-1 (Fig 19C). Platelet secreted factors, significantly reduced TIMP-1 from 13957.1 ±691.9 pg/ml in control monocytes to 10035.8 ±2012.2 pg/ml in monocytes infected with *M. tb* and stimulated with PS (p<0.05; Fig 19C).

Another important inhibitor of MMP activity in TB is TIMP-2. *M. tb*-infection of monocytes alone significantly reduced TIMP-2 secretion from 2921 ±491.4 pg/ml to 959.0 ±92.2 pg/ml (p<0.001; Fig 19D). However, TIMP-2 secretion from monocytes did not change with platelet co-culture either in uninfected or infected cells (both p>0.05; Fig 19D). I next focused on MMP-1, which is the major monocyte collagenase in TB [158], to investigate intracellular monocyte MMP-1 regulation by platelets.
Figure 19. TIMP concentrations are unchanged in monocyte-platelet co-culture.

(A) TIMP-1 concentration in a monocyte-platelet co-culture. Monocytes (Mon) were incubated with platelets (Plt) with and without M.tb infection. A Plt-only control was not subtracted and so is included to demonstrate the potential contribution to the total secreted TIMP-1 concentrations in co-culture. Despite high concentration from mon and plt alone, there was no change in total concentration of extracellular TIMP-1 in M.tb infection of mon-plt co-culture. (B) Mon regulation of plt TIMP-1 secretion in TB. Plt were incubated with monocyte media conditioned with TB (CT) and M.tb at MOI 0.01. The concentration of TIMP-1 in CT alone was subtracted to provide platelet-only concentrations. Mon TB networks do not regulate plt TIMP-1 secretion. (C) Plt regulation of mon TIMP-1 regulation. Mon were incubated with plt supernatant (PS) and M.tb, and PS TIMP-1 concentration subtracted. PS significantly reduced mon TIMP-1 secretion. (D) TIMP-2 concentration in a monocyte-platelet co-culture. Mon were incubated with plt and M.tb and secreted TIMP-2 concentrations measured after 24 hours. Plt do not regulate monocyte TIMP-2 secretion. Concentrations of secreted TIMP-1 and -2 were measured by ELISA. Bars represent mean ±SD of an experiment performed in triplicate. They are representative of two independent experiments. Statistical analysis was performed using an ordinary one-way ANOVA and Tukey’s multiple comparisons: *p<0.05; ***p<0.001.
2.7. Platelets regulate the monocyte MMP responses at the transcriptional and post-transcriptional level

MMPs are tightly regulated at every level. Having shown that platelets upregulate monocyte MMP-1 in *M. tb* infection I was interested to investigate this at the intracellular level. First, the platelet regulation of monocytes at the gene level was assessed using qRT-PCR. Monocytes infected with *M. tb* had dramatic 67.7-fold upregulation in MMP-1 mRNA compared to uninfected controls (p<0.05; Fig 20A). Platelet stimulation without *M. tb* infection was not sufficient to increase monocyte MMP-1 gene expression. However, monocyte infection in the presence of platelets resulted in a significant 4.5-fold increase in MMP-1 RNA compared to monocytes infected alone (p<0.0001; Fig 20A).

Intracellular protein was also investigated (Fig 20B). Despite platelets containing a large amount of the reference protein β-actin, the amount of platelet MMP-1 protein contributed was almost undetectable and no additional MMP-1 protein was produced from uninfected monocytes in culture with platelets compared to monocytes cultured alone. At the early MMP time point of 4 hrs, *M. tb* infection of monocytes resulted in a small upregulation of MMP-1 compared to uninfected controls. However, in monocytes infected in co-culture there was a dramatic increase in MMP-1 protein compared both to the uninfected controls and monocytes infected alone (Fig 20B). Next, the functional implications of platelet MMP-1 regulation was explored.
Figure 20. Platelets upregulate monocyte MMP-1 gene and intracellular protein expression.

(A) Platelet regulation of monocyte MMP-1 gene expression. Monocytes (Mon) were incubated with platelets (Plt) for 18 hours and the RNA harvested. Gene expression was assessed using qRT-PCR, with MMP-1 mRNA copy number normalised to β-actin mRNA copy number. Plts upregulate mon MMP-1 gene expression in *M. tb* infection. Bars represent mean ±SD of three separate experiments (N=3) with fold change calculated to uninfected mon alone. Statistical analysis was performed using an ordinary one-way ANOVA and Tukey’s multiple comparisons: *p<0.05; ****p<0.0001. (B) Plt regulation of intracellular MMP-1 production. Mon and plt were incubated for 4 hours with *M. tb*. Relative MMP-1 and β-actin protein were detected by western blot. Plt upregulate intracellular MMP-1. Image representative of two separate experiments.
2.8. Platelets upregulate a functional increase in monocyte collagenase activity

To assess platelet regulation of monocyte MMP-1 in a functional manner, confocal microscopy was utilised (Fig 21). Monocytes were cultured on DQ type I collagen (fluorescent-bound collagen which fluoresces on degradation) to visualise platelet regulation of collagenase activity. Uninfected monocytes had minimal collagenase activity (Fig 21A). This signal from uninfected monocytes was significantly upregulated upon *M.tb* infection of monocytes (Fig 21A). Co-culture with platelets did not appear to increase collagenase activity of uninfected monocytes, but dramatic collagen degradation was observed in cells co-cultured with *M.tb* infection (Fig 21A). Platelet-only controls were visualised with brightfield due to their lack of nucleus, but produced no measurable collagen degradation (Fig 21A).

Numerical analysis of the confocal images revealed *M.tb* drove a 1.8-fold increase in functional collagenase activity of monocytes (Fig 21B). Monocytes infected in the presence of platelets had 4.7-fold greater collagenase activity than uninfected co-cultured cells (p<0.001), and 2.5-fold more than monocytes infected alone (p<0.01; Fig 21B). As platelet regulation of monocyte MMP-1 requires *M.tb* stimulation of the cells, I was interested to investigate platelet regulation of monocytes with UV-killed *M.tb*. 
Figure 21. Platelets upregulate monocyte collagenase activity in TB.

Monocytes and platelets were cultured on type I collagen that fluoresces on degradation (DQ Collagen). After 24 hours, the cells were fixed and imaged with confocal microscopy. (A) Monocytes were cultured without platelets (No Plt), or with platelets (Plt) at Plt:monocyte 100:1. A platelet only control was also included (Plt only). Cells were cultured uninfected (Control) or were infected with M.tb at monocyte MOI 1 (M.tb). Cells were visualised with DAPI DNA staining and Brightfield was included in the merge image to visualise the anucleate platelets. (B) Numerical analysis of relative monocyte collagenase activity. Mean fluorescence value of DQ Collagen was normalised to monocyte cell number to generate comparative arbitrary units. Bars represent mean ±SD of four images from one experiment, representative of two separate experiments. Platelets upregulate monocyte type I collagenase activity in M.tb infection, but not in uninfected cells. Statistical analysis was performed using an ordinary one-way ANOVA and Tukey’s multiple comparisons: **p<0.01.
2.9. Platelet upregulation of MMP-1 is not dependent on live \textit{M.\textit{tb}} infection

I stimulated monocytes with UV-killed \textit{M.\textit{tb}} and compared MMP-1 secretion to that from live \textit{M.\textit{tb}} infection (Fig 22). UV-killed \textit{M.\textit{tb}} was sufficient to induce monocyte MMP-1 secretion from undetectable levels to 1087.6 ±120.8 pg/ml (p<0.0001; Fig 22). Live \textit{M.\textit{tb}} infection induced 1515.2 ±145.4 pg/ml from monocytes, which was significantly more than UV-killed \textit{M.\textit{tb}} infection (p<0.05; Fig 22). In live \textit{M.\textit{tb}} infection platelets upregulated MMP-1 secretion 4.3-fold. Interestingly, in UV-killed \textit{M.\textit{tb}} infection platelets induced a 7.5-fold increase in MMP-1 from monocytes. This resulted in similar MMP-1 concentrations between live and UV-killed \textit{M.\textit{tb}} stimulation of co-cultures, at 8176 ±197.5 pg/ml and 7804 ± 145.4 pg/ml respectively (p>0.05; Fig 22). Platelet regulation of other aspects of monocyte inflammatory TB responses was investigated.

![Figure 22. Platelet upregulation of monocyte MMP-1 secretion stimulated with UV-killed \textit{M.\textit{tb}}.](image)

Monocytes were incubated with platelets (Plt) at plt:monocyte 100:1. The cells were infected with live H37Rv \textit{M.\textit{tb}} (Live) or UV-killed H37Rv \textit{M.\textit{tb}} (UK) at a monocyte MOI of 1. Extracellular MMP-1 concentrations were assessed by ELISA after 24 hours incubation and are expressed in pg/ml. Bars represent mean ±SD of an experiment performed in triplicate and are representative of two independent experiments. Plt upregulation of monocyte MMP-1 can be induced by both live and UK-killed \textit{M.\textit{tb}}. Statistical analysis was performed using an ordinary one-way ANOVA and Tukey’s multiple comparisons: *p<0.05; ***p<0.001; ****p<0.0001.
2.10. Platelets regulate monocyte cytokine and chemokine secretion

Monocyte secretion of a variety of cytokines in the presence of platelets was assessed with multiplex analysis. TNF-α, IL-1β and IFN-γ are classic inflammatory cytokines involved in the immune response to TB [343] and were of interest in the context of a platelet co-culture. Monocytes did not constitutively express any of these cytokines, and platelet stimulation of monocytes alone was not sufficient to induce them (Fig 23A-C). IL-1β was highly upregulated upon monocyte infection with *M. tb* increasing from 1.1 ±0.3 pg/ml to 1204.8 ±115.2 pg/ml (p<0.001; Fig 23A). Strikingly, platelets increased monocyte IL-1β secretion 4.1-fold further in *M. tb* infection to 4984.2 ±225.6 pg/ml compared to infected monocytes alone (p<0.001; Fig 23A). TNF-α and IFN-γ were also significantly upregulated by *M. tb* infection (p<0.0001 and p<0.01 respectively), however, platelet co-culture did not increase these cytokines further (both p>0.05; Fig 23B-C).

*M. tb* is also known to stimulate secretion of cytokines with immunomodulatory functions in *M. tb* infection [31]. To investigate this further IL-12 was measured (Fig 23D). In this model, *M. tb* stimulated secretion of 1879.8 ±261.4 pg/ml of IL-12. However, in the presence of platelets, *M. tb* was unable to induce significant IL-12 secretion compared to uninfected controls (p>0.05; Fig 23D). There was 6.4-fold less IL-12 secretion from monocytes infected in platelet co-culture compared with monocytes infected without platelets (p<0.001; Fig 22D). Another immunomodulatory cytokine, IL-10, was upregulated in *M. tb* infection from undetectable levels to 749.6 ±187.8 pg/ml (p<0.01; Fig 23E). Converse to IL-12, IL-10 was upregulated 7.5-fold in the platelet co-culture compared with monocytes alone (p<0.0001; Fig 23E). Finally, *M. tb* infection induced dramatic upregulation of IL-6 from monocytes (p<0.0001) but the concentrations were not altered between monocytes infected alone and in co-culture (p>0.05; Fig 23F).

Finally, the chemokines IL-8 and MDC were measured (Fig 23G-H). There was a low level secretion of the potent neutrophil chemoattractant IL-8 in control monocytes, at 2132.9 ±388.0 pg/ml. In co-culture with platelets there was a small but consistent increase to 3919.0 ±674.0 pg/ml observed (p>0.05; Fig 23G). However, *M. tb* stimulated significant IL-8 secretion, reaching concentrations of 87810.5 ±17385.2 pg/ml (p<0.0001), and this was not altered with platelet co-culture (p>0.05; Fig 23G). In uninfected monocytes, platelet stimulation was sufficient to reduce MDC concentrations from 69.1 ±8.5 pg/ml to 11.3 ±8.0
pg/ml (p>0.05; Fig 23H). *M.tb* infection of monocytes alone stimulated 840.2 ±191.9 pg/ml MDC secretion (p<0.0001) but this was significantly downregulated 2.3-fold in monocyte-platelet co-culture (p<0.001; Fig 23H). Finally for this chapter, I investigated platelet regulation of monocyte bacteriocidal activity.
Figure 23. Platelets regulate a specific monocyte cytokine and chemokine response to TB.

Monocytes were incubated with platelets (Plt) for 24 hours and a panel of cytokines measured by magnetic bead Luminex array and ELISA. Extracellular cytokines measured were: (A) interleukin (IL)-1β; (B) Interferon (IFN)-γ; (C) Tumour necrosis factor (TNF)-α; (D) IL-12p70 (IL-12); (E) IL-10; (F) IL-6; (G) IL-8; and (H) Macrophage-derived chemokine (MDC). Bars represent mean ±SD of an experiment performed in triplicate and all graphs are representative of at least three independent experiments. Monocytes exposed to plt regulation and M.tb infection secrete significantly greater IL-1β and IL-10 and significantly less IL-12 and MDC. Uninfected monocyte cytokines and chemokines were not significantly regulated by plt. Statistical analysis was performed using an ordinary one-way ANOVA and Tukey’s multiple comparisons: **p<0.01; ***p<0.001; ****p<0.0001.
2.11. Platelets downregulate monocyte intracellular killing of *M. tb*

To investigate if platelet regulation has a functional effect on monocyte bacterial killing, cell supernatant and lysates were plated on media for *M. tb* growth. Minimal CFU was recovered from the supernatants, and no significant difference between monocytes infected in a co-culture and monocytes infected alone was observed (p>0.05; Fig 24). However, in cell lysates monocytes alone had 2.5-fold fewer CFU than monocytes incubated with platelets (p<0.001; Fig 24). This demonstrates significantly greater bacterial killing in monocytes alone compared with co-culture.

![Figure 24](image.png)

**Figure 24. Platelet regulation of intracellular monocyte *M. tb*. killing.**

Monocytes were infected with live virulent *M. tb* either alone or in the presence of platelets (Plt). After 24 hours the supernatant and cell lysate was diluted and plated on 7H11 agar. Colony forming units (CFU) were then counted to measure viable extracellular bacteria (Extracell) and intracellular bacteria (Intracell). Bars represent mean ±SD of three independent experiments (N=3) performed in duplicate. Whilst there was no difference between extracellular CFU, significantly more CFU were rescued from monocyte-plt co-cultures compared with monocytes cultured alone. Statistical analysis was performed using a two-way ANOVA and Sidak’s multiple comparison test: ***p<0.001.
3. Discussion

Platelets come into contact, both directly and via secreted factors, with immune cells such as monocytes in the blood capillaries surrounding the infected lung [191, 253]. Platelets have also been shown to enter and regulate cells within the lung tissue in inflammatory lung conditions such as asthma and COPD [335]. To investigate this in the context of TB I used direct staining of the platelet integrin CD41 in a mouse model of pulmonary TB. The resulting images showed platelets concentrated at the site of TB pathology in the lung. There was a clear platelet presence in some of the alveolar air pockets, which is consistent with the breakdown of the epithelial barrier resulting in the common symptom of haemoptysis. The images also indicated platelets associated with nucleate cells. This is consistent with tight platelet adherence to recruited cells as they extravate from the blood vessels, or platelet phagocytosis during monocyte maturation. Further magnification revealed most of the CD41 staining that was associated with nuclei, was associated with developing multinucleate cells. This supports previous IHC staining in human TB lung using the platelet integrin CD42b, which demonstrated that monocyte phagocytosis of platelets drives development of multinucleated giant cells [330].

After showing that platelets are present in the murine lung at the site of TB pathology, factors secreted upon platelet activation were measured in human BALF to confirm platelet presence and activity at the site of infection in humans. TB patient BALF were compared not to healthy controls but to symptomatic respiratory patients. Even compared to these patients with inflammatory lung pathologies, all of the analytes were upregulated with the exception of PF4. PF4 has been shown to be upregulated by our research group in the blood of TB patients [337]. The fact that this was not reflected in the lung may be due to the high concentration of PF4 in the symptomatic controls, with PF4 known to be vital to lung inflammation and tissue damage [344]. Additionally, PF4 may be readily reabsorbed into the lung lining leaving only a baseline residual concentration.

My analysis of platelets in BALF suggests TB highly upregulates active platelets in the lung, and together with the murine imaging demonstrates the potential for platelets to influence responses beyond cell interactions in the blood. Combined with the previously reported
activation of circulating platelets, my results present a range of contexts for platelet regulation of the cellular immune response in TB.

To begin to assess a causitive relationship between platelet activity and pulmonary tissue destruction, I sought to correlate active platelet-secreted factors with MMPs and IL-1β in the BALF samples. MMP-1, -3, -8 and -9 were significantly correlated with three of the four platelet factors upregulated in TB and MMP-7 and IL-1β correlated with two. This implicates increasing platelet analytes with increasing enzymes of tissue destruction. It would be of significant interest to assess this in the cohort of TB cases alone, however, the limited N number did not provide significant power for this. The measured MMPs and cytokine IL-1β had been previously demonstrated to be upregulated, and functionally important, in the TB lung [160]. To study this relationship in more detail I next developed an in vitro co-culture of infection.

Monocytes have a vital role in TB immunopathology in both the blood and tissue [52, 55]. I investigated platelet regulation of monocytes by establishing an in vitro model. I first optimised this model by measuring direct platelet MMP contributions. Platelets are known to secrete MMPs, however, which MMPs remains a subject of debate in the literature largely due to different platelet processing protocols. In my hands, M.tb-infected platelets that had been first activated in vitro by thrombin had readily measurable concentrations of MMP-1, 2, -7 and -9 with MMP-3 concentrations that bordered on undetectable. This accords well with the literature, where platelets are established producers of MMP-1 and MMP-2 [295, 296]. Varying concentrations of MMP-3 have also been widely reported [295, 301, 345]. MMP-9 has traditionally been reported to be secreted by platelets, but more recent investigations refute this [345]. Platelet-derived MMP-9 is now generally attributed to platelet binding of leukocyte-secreted MMP-9 in vivo which, upon in vitro activation, may be sheared off and thus appear as ‘platelet-secreted’ MMP-9 [297]. Platelets from leukocyte-deficient humans have not had MMP-9 detected [298]. Although detectable, the concentrations detected of MMP-9 in this model were low. Interestingly MMP-7 has not been previously reported in the context of platelet secretion, and so the low secretion of MMP-7 by platelets may be a new finding. Finally, detection of high concentrations of TIMP-1 and low levels of TIMP-2 concurs with previous findings [301].
Platelet interactions with monocytes have been investigated at a wide variety of concentrations. This reflects the range of ratios that are physiologically relevant due to dynamic and rapid cell recruitment, elevated platelet numbers in disease such as TB, and individual variation. P:M of 1:1-1000:1 are widely used in the literature [346-348]. To ensure a conservative model of cell-cell regulation concentrations in the mid-range of this were chosen for further investigation. Whilst monocyte MMP-1 secretion increased significantly from P:M 100:1, MMP-10 increased at even the lowest doses of platelets but decreased dramatically after 100:1. This highly dose-dependent “goldilocks effect” of platelets is well described both in vivo and in vitro and has profound clinical impact as immunotherapy targeting platelets must function within a specific range [208, 280]. For example, studies investigating the effect of platelet inhibitor aspirin on S. aureus-induced endocarditis have found aspirin to be a highly effective treatment but only when used at the dose which inhibits the platelets the correct amount: too little or too much platelet inhibition results in unchanged or even worsened clinical features. One study reported the difference to be as little as an increase from 8 mg/kg, which had significant clinical success, to 12 mg/kg, which had none [349]. The sudden decrease in MMP-10 at 500:1 P:M demonstrates sensitivity of platelet regulation, but also suggests a mechanism to restrict MMP-1 activity at high platelet densities despite the increase in MMP concentration, as MMP-10 is a key MMP-1 activator [145, 147, 148, 350]. The plateauing of MMP-1 secretion after 100:1 P:M may reflect maximal monocyte MMP-1 production, or maximal platelet regulation.

Platelets are dynamic cells potentially capable of a different secretory profile under different conditions. To assess the suitability of platelet-only controls to quantify platelet contributions in co-culture, platelet activation and secretion was investigated in the context of the monocyte co-culture environment. The results from this investigation indicated that the monocyte-platelet co-culture model conditions cannot change platelet secretion after thrombin activation. Therefore, to focus on platelet regulation of monocyte analytes, concentrations from platelet-only controls are subtracted from the total co-culture concentrations throughout this project. In this manner a co-culture of platelets and autologous monocytes at a ratio of 100:1 and using platelet-only controls was optimised to demonstrate platelet regulation of monocyte MMP responses.
In response to *M.tb* infection monocytes secreted significant concentrations of the major collagenase, MMP-1, and stromelysin, MMP-10, and these were both further upregulated by platelets. The matrylysin MMP-7 was also upregulated by platelets in *M.tb* infection. Although it is cell and stimulus specific, MMP-7 is typically regulated differentially to MMP-1. MMP-1 secretion from monocyte-derived cells is dependent on *M.tb* upregulation of p38 phosphorylation and downstream signalling to the COX-2/PGE2 pathway, whilst MMP-7 is independent of this [351]. The upregulation of both MMP-1 and MMP-7 by platelets in monocyte responses to TB therefore indicates platelet targeting of multiple intracellular pathways. Unlike MMP-1, -7 and -10, monocytes are not considered significant sources of MMP-8 [130]. Interestingly, although MMP-1 and -8 and have similar regulation and function, MMP-8 is not regulated by platelets.

Monocytes are thought to be an important source of MMP-9 *in vivo* [352]. Uniquely, platelets significantly increased MMP-9 secretion whilst *M.tb* infection did not. The lack of MMP-9 upregulation in *M.tb* infection may be due to high expression by unstimulated isolated monocytes. *In vivo* MMPs are tightly controlled and so this constitutive expression is likely a result of isolation and/or culture of monocytes. Culture of monocytes on plastic cell culture plates has been previously established to induce MMP-9 secretion which is then difficult to increase further [353]. As my model uses monocyte culture on plastic this may offer an explanation for MMP-9 pathway activation, which these data suggest platelets then upregulate further. This activation of the MMP-9 pathway in controls also explains the divergence from a previous study that found that thrombin activated platelets do not increase MMP-9 secretion from unstimulated monocytes [316]. In the future other culture protocols, such as culture on collagen, could be explored to re-examine platelet regulation of monocytes in *M.tb*. Therefore, my data as a whole indicate that platelets did not regulate secretion of any MMP in isolation but functioned in synergy with *M.tb* to enhance a specific subset of MMPs.

In comparison to monocytes, platelets secreted minimal concentrations of all proteins measured in this study, with the exception of TIMP-1. For TIMP-1 this made it difficult to distinguish the cellular contributions as there was no changes in platelet secretion of TIMP-1 induced by secreted monocyte factors. Use of supernatant from *M.tb*-infected monocytes (in the form of CT) and activated platelets (in the form of PS) yielded the suggestion that
platelet secreted products may downregulate TIMP-1 in monocytes as PS significantly reduced TIMP-1 concentrations. However, although a significant downregulation was observed it was not great enough to account for the unchanged total concentrations. The platelet regulation of monocyte TIMP-1 may be enhanced with direct contact but having confirmed this MMP inhibitor does not increase in the presence of platelets, this was not investigated further. TIMP-2 was shown to be unregulated by platelets.

Further investigation of monocyte MMP-1 regulation revealed that platelets regulate monocyte MMP-1 at the transcriptional level, and this increased gene expression was translated to increased intracellular MMP-1 protein. Normalisation of intracellular protein by western blot was difficult as platelets contained significant amounts of housekeeping proteins such as β-actin. However, inclusion of a platelet only control on the blot demonstrated that MMP-1 protein contributed by platelets was almost undetectable, below even unstimulated monocyte controls. At the functional level, confocal microscopy visualised a small amount of collagen degradation in uninfected cells, which can be attributed to monocytes attaching and detaching to the collagen-coated plate. Monocytes infected in the presence of platelets had dramatically upregulated degradation compared with all other conditions, which clearly demonstrated a role of platelets in driving ECM breakdown.

MMP-1 secretion is upregulated in virulent M.tb compared with BCG, but does not require live virulent M.tb [158, 163]. Platelet regulation of monocyte MMP-1 was also not dependent on live M.tb infection, as platelets were able to drive MMP-1 secretion with UV-killed M.tb to equivalent concentrations. This suggests that intracellular bacterial replication and bacterial secretion of products such as ESAT-6 are not required for platelet regulation of monocytes. Direct bacterial antigen stimulation of monocyte receptors is therefore sufficient to induce monocyte activation that can then be regulated by platelets to amplify MMP-1 secretion.

The increased breakdown of ECM components by monocyte MMPs has a wide range of physiological implications in vivo as monocytes are vital to the MMP-dependent establishment and maintenance of granulomas and large scale tissue destruction [158, 354]. Aside from their role in TB, monocytes have been shown to be present in abundance at
chronic inflammatory sites in a variety of diseases such as arthritis, atherosclerosis, and periodontal disease [332]. As increased monocyte MMP activity is associated with increased inflammation and cell migration it is considered a good clinical target, for example in rheumatoid arthritis [355]. The platelet MMP upregulation described in this chapter suggests for the first time that platelets may have great potential to be an important target in TB immunotherapy.

Other components of the monocytic response to *M.tb* infection were investigated next, using a panel of cytokines and chemokines. IL-1β is a classic inflammatory cytokine associated with immune control of TB. It has been shown to be essential for NO production, phagosomal acidification and maturation and adhesion molecules [31] and was therefore of interest to measure in the context of platelets. The striking increase of IL-1β secretion in monocytes infected in the presence of platelets compared to monocytes alone supports platelet upregulation of monocyte inflammatory responses. In contrast the other inflammatory cytokines measured, namely IFN-γ and TNF-α, were unchanged in the presence of platelets. The specific upregulation of IL-1β may suggest a role of platelets in the upregulation of the caspase-1-NLRP3 inflammasome. Active caspase-1 processes IL-1β. Monocytes constitutively express active caspase-1 but whilst this is required for *M.tb*-induced responses, *M.tb* stimulation has been previously shown to have little additive effect. Instead *M.tb* upregulates activation of P2X7-induced pathways by endogenously released ATP that results in increased IL-1β [356]. Platelets may contribute to this regulation, or function to drive IL-1β through further upregulation of caspase-1 activity in a previously unidentified mechanism. Whilst this has not been explored, direct monocyte contact with platelets has been shown to support monocyte caspase-3 and -9, but not caspase-8, activation to enhance cell survival [357].

The immunosuppressive cytokine IL-10 was also upregulated. IL-10 enhances mycobacterial intracellular survival and growth by suppressing the innate and adaptive immune response [358]. IL-10 knockout mice have been shown to be more resistant to *M.tb* infection and have limited bacterial growth within lung and spleen compared to wild type mice [358, 359]. Concurrently, IL-12 was significantly reduced by platelet co-culture. This cytokine is strongly associated with immune protection against *M.tb* and bacteriocidal activity [360]. As
upregulation of IL-10 has been shown to downregulate IL-12, and IL-10 inhibition increases IL-12, this feedback loop may contribute to the changes observed in my model [361, 362].

IL-8 is present in the early host response to *M. tb* infection and is known to stimulate bactericidal non-oxidative mechanisms as well as being an important neutrophil chemoattractant [92, 363]. *M. tb*-infection upregulated monocyte IL-8 secretion to significant concentrations, however, platelets did not regulate this chemokine further. Given the high concentrations measured of this chemokine, the lack of additional upregulation by platelets may be due to the maximal production of IL-8 by monocytes in *M. tb* infection. This is supported by the comparatively small, but consistent, increase seen in uninfected monocytes in platelet co-culture compared with monocytes. *M. tb* infection significantly increased MDC secretion in monocytes, but monocytes infected with *M. tb* in the presence of platelets had 50% lower secreted concentration than monocytes infected alone. MDC acts through CCR4 and has been associated with pulmonary inflammation and cell recruitment in numerous pulmonary settings. This function is driven both by direct chemoattraction of Th2 lymphocytes, monocytes, monocyte-derived dendritic cells, and natural killer cells and by driving increased secretion of other chemokines such as CXCL2 (also termed MIP-2) [364, 365].

The cytokine data in this chapter reflects findings from studies in Dengue virus which indicate platelets upregulate IL-1β and IL-10 but not TNFα from monocytes [366]. The increased IL-10 and decreased IL-12 and MDC is also a classic profile of monocytes maturing with a M2 macrophage phenotype [367, 368]. Prolonged *M. tb* infection in vitro is known to tilt monocyte derived cells towards an M2 phenotype and M2 macrophages are the major type in TB granulomas, whilst normal lung tissue contains both M1 and M2 MDMs [369]. My data indicate that platelets enhance this differentiation towards M2 pathways from the earliest time point. This observation is supported by Feng et al who used prolonged in vitro incubation with platelets of 21 days to show M2 macrophage development at the gene level [330]. M2 monocyte/macrophages are associated with significant tissue remodelling properties, including MMP secretion [370]. An M2 phenotype also has reduced pathogen killing although phagocytosis typically remains unchanged [371].
CFU analysis of cell lysates confirmed a reduction in cellular killing of *M. tb* in my model. There was significantly more viable *M. tb* recovered from cells infected in a monocyte-platelet co-culture compared to monocytes alone. Viable extracellular bacteria in the cell supernatant remained the same. This suggests that there was no difference in *M. tb* internalisation, however, it is possible that rather than directly regulating monocyte intracellular killing, platelets may bind and/or internalise *M. tb* in a manner that slows or reduces monocytyc internalisation and killing of the bacteria.

In summary, my results show for the first time that platelets enhance a tissue destructive phenotype of monocytes in TB. Active platelet secreted products were present at the site of infection in pulmonary TB, and at concentrations that correlate with IL-1β and proteases central to in TB pathology. Platelets upregulated both MMP-1 and -10 maximally at P:M 100:1, and MMP-7 and -9 were also upregulated at this ratio. A focus on MMP-1 revealed platelets upregulate transcription and translation from the earliest time post-*M. tb* infection, in a manner that was not dependent on bacterial secretions or replication. The upregulation of MMP-1, without any concurrent increase in TIMPs, resulted in a dramatic upregulation of type I collagen degradation. I also showed platelets have an immunomodulatory function, through upregulation of IL-1β and IL-10 and downregulation of IL-12 and MDC. Finally, I demonstrated increased bacterial survival in platelet co-culture of monocytes, compared with monocytes alone.
Chapter 4. Platelets regulate monocytes through multi-pathway mechanisms

1. Introduction

In the previous chapter I showed that platelets functionally regulate monocyte MMPs and cytokines in TB. I next decided to investigate the mechanism of this regulation. Platelets have been shown to be active in TB [320, 337], and bacteria and their antigens such as LPS are known to activate them [239]. *M. tb* is able to activate numerous cells of the immune system [21], and *M. tb* and platelets may come into direct contact due to a potential blood stage of *M. tb* dissemination and platelet’s presence in the *M. tb*-infected lung. Despite these connections, the mechanism driving platelet activation in TB is unknown.

Platelets have been reported to regulate monocytes through both contact-dependent mechanisms such as surface receptor interactions, and contact-independent mechanisms such as secreted factors. For example, platelet upregulation of monocyte IL-10 in Dengue virus infection was shown to be entirely dependent on direct cell contact [366] whilst monocytes incubated with platelets and collagen have increased MMP-9 secretion that is only partially dependent on direct platelet-monocyte contact [316]. Studies of platelet-monocyte interactions in rheumatoid arthritis patients have revealed this partial requirement for direct contact is due to monocyte regulation through platelet CD147 [318]. Of the many platelet-secreted proteins shown to have immunomodulatory functions, soluble CD40L and PF4 are amongst the best described in their regulation of monocyte inflammatory reactions. PF4 is a well-defined regulator of monocyte signal transduction cascades that lead to the induction of a broad spectrum of functions such as phagocytosis, respiratory burst, survival, and cytokine secretion [372]. *In vivo*, platelet-secreted PF4 drives monocyte cytokine secretion and invasion in cerebral malaria, and has been attributed with orchestrating the detrimental role of monocytes in this disease [373]. Platelets are a significant source of soluble CD40L [374, 375] and CD40L is produced at high levels in lung
and nasopharyngeal carcinoma lesions [376]. In LPS-stimulated monocytes, platelet upregulation of IL-10 and downregulation of TNF-α has been attributed to CD40L in PS [274]. CD40L significantly enhances monocyte intracellular killing through upregulation of IL-12 [377] and upregulates monocyte pro-inflammatory cytokines through interaction with CD40 [378]. This mechanism has been suggested to be relevant to the pathogenesis of autoimmune diseases such as rheumatoid arthritis [379].

MMP secretion is regulated by a complex network of intracellular signalling pathways. Recently, the transcription factor HIF-1 has been implicated in MMP regulation. HIF-1α is stabilised in *M. tb*-stimulated MDMs even in normoxia, and shown to upregulate MMP-1 secretion [139]. In normoxic environments HIF-1 formation is typically prevented with oxygen-dependent PHD degradation of HIF-1α. However, *M. tb* infection can overcome this regulation to stabilise HIF-1α, even in normoxia. Non-hypoxic activation of HIF-1α in other contexts is significantly later but more prolonged compared with hypoxia-induced stabilisation. This reflects differential regulation of HIF-1α in bacterial infection and normoxia, although the mechanism of this regulation is not fully understood. Unlike hypoxia, HIF-1α stabilisation in normoxia and bacterial infection is thought to be driven by gene expression, which in monocyte-derived cells is via NFκB [380]. HIF-1α gene expression has been shown to be central to HIF-1 activity in bacterial infection, with use of actinomycin D to abolish transcription resulting in no additional HIF-1α protein accumulation [133]. However, various post-translational mechanisms have also been implicated in normoxic stabilisation. These include changes to PHD regulation of HIF-1α through the reduction of PHD protein or activity; involvement of other mechanisms such as microRNA; or changes to hydroxylation, phosphorylation and acetylation of HIF-1α which enhance not only protein stability but also activity [381-384]. Although HIF-1α has been shown to be important in MMP regulation in TB [139], its regulation in normoxia and TB remains largely unknown with interest focused on hypoxic enviroments. Furthermore, whilst normoxic HIF-1α stabilisation is usually considered stable, the limited study of *M. tb*-stabilised HIF-1α indicates only transient and low level protein accumulation in normoxia.

Phosphorylation of key proteins in intracellular regulatory pathways is central to MMP-1 secretion (Fig 2) but is highly cell- and stimulus-dependent. For example, PGE2 in fibroblast-like synoviocytes suppresses cytokine induced MMP-1 expression, but upregulates MMP-1
in macrophages [163, 385]. Despite such variations, ERK-1/2 and mitogen-activated kinase p38 are generally central to MMP-1 regulation both in direct M.tb infection and through TB intercellular networks. They are considered to be amongst the most pivotal regulatory signalling proteins in M.tb-induced MMP-1 regulation [130]. ERK-1/2 activation has been demonstrated in vivo in inflammatory pathologies such as emphysematous lungs, and regulates MMP-1 induction by cigarette smoke in pulmonary epithelial cells [386]. In TB, MMP-1 secretion from cells including MDMs is dependent on ERK-1/2 with a significant reduction of MMP-1 observed upon ERK-1/2 inhibition. However, this regulation is again context dependent, with fibroblast MMP-1 secretion shown to be independent of ERK-1/2, indicating the importance of further study [180, 351]. In the human TB lung, IHC has revealed activated p38 in TB granulomas and M.tb-infected MDMs. p38 has been strongly implicated with a central role of MMP-1 upregulation, as well as downregulation of TIMP-1 [351]. These signalling molecules also regulate cytokines, with p38 phosphorylation required for IL-10, but not IL-8, secretion in M.tb infected monocytes [387]. Both p38 and ERK-1/2 are significantly upregulated in M.tb infection of primary human monocytes. Whilst MMP-9 regulation is predominantly regulated by ERK-1/2 in monocytes, both p38 and ERK-1/2 are vital to MMP-1 upregulation [332].

PI3K is also increasingly recognised as a key signalling cascade for MMP regulation in TB, through its phosphorylation of the downstream kinase AKT. Activation of PI3K at the AKT node is particularly relevant as this regulates MMP-1, -3 and -9 whilst other downstream components of the PI3K pathway are more targeted, such as target of rapamycin (mTOR)/p70s6 which regulates only MMP-1 and -3 [130]. M.tb rapidly phosphorylates AKT in human MDMs, and MMP secretion and gene expression is decreased in epithelial cells by blocking the PI3K pathway proximally at the AKT node [157]. AKT polymorphisms have also been associated with genetic susceptibility to pulmonary TB [388].

In this chapter I aimed to investigate the mechanism of platelet regulation of monocyte MMPs. Specifically, I aimed to assess direct M.tb activation of resting platelets as a mechanism for their activation in TB. I also aimed to determine the role of platelet-secreted products in platelet regulated monocyte responses at both the transcriptional and secretory level using PS, a trans-well model, and recombinant proteins. To elucidate intracellular mechanisms of MMP-1 regulation I first aimed to analyse M.tb regulation of HIF-1α in
monocyte-derived cells, before exploring monocyte HIF-1α gene expression and protein accumulation in the context of platelet regulation. Finally I aimed to determine the role of platelet activation of the key MAPKs PI3K, ERK1/2 and p38 in monocyte MMP-1 regulation.
2. Results

2.1. *M. tb* does not directly activate isolated platelets

First, I assessed if *M. tb* is able to activate resting platelets directly. Platelets were incubated with a vehicle control or *M. tb* at an MOI of 0.01 and 0.1. PF4 secretion was quantified as a measure of platelet activation (Fig 25A). Some PF4 was secreted by unstimulated platelets, however, *in vitro* thrombin activation induced significant platelet degranulation with PF4 concentrations increasing 2.9-fold compared to controls (p<0.01; Fig 25A). Platelets incubated with *M. tb* had no significant increase in PF4 secretion compared to the vehicle control (p>0.05) and significantly lower PF4 secretion compared with the thrombin activated positive controls (p<0.05; Fig 25A).

Platelet activation can be driven by cellular networks of communications [389]. I next investigated if platelets were activated by monocyte responses to TB by incubating resting platelets with CT (Fig 25B). Several concentrations of CT were used but there was no detectable increase in PF4 concentration compared to controls, with concentrations remaining significantly lower than the positive control of thrombin-activated platelets (p<0.01; Fig 25B). Together these data indicate that platelets are not activated by direct *M. tb* stimulation or monocyte secreted TB networks.
Figure 25. *M. tb* activation of platelets.
Platelet activation and degranulation was measured by platelet factor 4 (PF4) release and thrombin-activated platelets (+) were used as a positive control for platelet activation. (A) Resting platelets were stimulated *in vitro* with *M. tb* at an multiplicity of infection (MOI) of 0.01 and 0.1 for 30 minutes. A vehicle (V) control was also included to control for effects of the *M. tb* culture media. (B) Resting platelets were incubated in monocyte media conditioned with TB (CT) at dilution factors (DF) ranging from 100 to 2.5. Bars represent mean ±SD and are representative of two independent experiments. *M. tb* infection and CT stimulation did not activate freshly isolated platelets. Statistical analysis was performed using an ordinary one-way ANOVA and Tukey’s multiple comparisons: ns p>0.05; **p<0.01.
2.2. Platelet regulation of monocyte gene expression is contact-dependent

In Chapter 3 I showed that platelets regulate monocyte MMPs at the gene and protein level. To explore the dependency of this MMP regulation on direct cell contact between monocyte and platelets, thrombin-activated platelets were cultured in a trans-well and compared with direct platelet co-culture. The trans-well pore size prevented direct contact between monocytes and platelets but allowed exchange of secreted factors and M.tb (Fig 8). I first used this model to assess monocyte MMP gene expression (Fig 26).

At 18 hrs post-infection M.tb-infected monocytes had a 22.3-fold increase in MMP-1 gene expression compared to uninfected controls (Fig 26A). This was increased 6.5-fold further upon co-culture with platelets (p<0.0001; Fig 26A). Compared to monocytes infected alone, platelets in a trans-well set-up also had upregulated MMP-1 gene expression, with a 3.3-fold increase observed (p<0.001; Fig 26A). However, platelets in trans-well culture induced significantly less than monocytes cultured with platelets in direct contact (p<0.001; Fig 26A). MMP-10 gene expression was also investigated at this time point. MMP-10 gene expression was upregulated 52.0-fold with M.tb-infection of monocytes (p<Fig 26B). Platelets further upregulated expression by 2.6-fold (p<0.05) but this upregulation compared with monocytes infected alone was not observed with platelets in the trans-well model (p>0.05; Fig 26B). Thus, platelets regulate MMP-10 gene expression in a contact dependent manner.

To investigate the partial contact dependent MMP-1 gene expression further, a time course was undertaken. At the earliest time point of 4 hrs post-infection, there was only a small upregulation of MMP-1 gene expression observed between controls and M.tb-infected monocytes alone or in the trans-well co-culture, with a 1.6-fold and 1.2-fold upregulation respectively. However, monocytes M.tb-infected in the presence of platelets had a 2.1-fold increase compared to uninfected controls (Fig 26C). At 24 hrs, monocytes cultured with platelets in a trans-well did have an upwards trend of MMP-1 gene expression, with a 2.4-fold increase compared to M.tb infected monocytes alone (p>0.05; Fig 26C). However, with direct platelet contact gene expression increased significantly, displaying a 5.1-fold increase compared with M.tb-infected cells alone (p<0.001 Fig 26C). At 36 hrs the difference between monocytes regulated by direct and indirect platelet culture increased further, with no difference detected between monocytes M.tb infected alone and with platelets in trans-well (p>0.05; Fig 26C). In contrast, MMP-1 mRNA from monocytes with direct platelet co-
culture continued to increase and was significantly greater than the trans-well culture even at 36 hrs (p<0.001; Fig 26C). Thus, at every time point maximal monocyte gene expression is dependent upon direct platelet contact. This regulation was investigated further using assessment at the protein level.
Figure 26. Contact-dependent platelet regulation of monocyte MMP gene expression.

Monocytes were incubated with platelets (Plt) in direct culture or in a trans-well (TW) which prevented monocytes and platelets from direct contact. Cells were then infected with *M. tb* before collection of cell lysates and measurement of MMP-1 and -10 mRNA by qRT-PCR. (A-B) Monocytes were infected for 18 hours (hrs) before detection of (A) MMP-1 and (B) MMP-10 gene expression. Bars represent mean ±SD of three independent experiments (N=3). In *M. tb*-infected monocytes, platelets upregulate gene expression of MMP-1 in a partially contact-dependent manner and MMP-10 in a highly contact-dependent manner. Significance assessed with an ordinary one-way ANOVA and Tukey’s multiple comparisons test: *p<0.05; ***p<0.001.

(C) Monocytes with and without platelet co-culture were infected with *M. tb* for 6, 24 and 36 hrs before cellular RNA was collected for MMP-1 gene expression. Bars represent mean ±SD of an experiment performed in triplicate and is representative of two independent experiments. Platelet MMP-1 gene regulation increases over time compared to indirect platelet stimulation using a trans-well model. Statistical analysis was performed using a two-way ANOVA and Tukey’s multiple comparison test: **p<0.01.
2.3. Platelet-secreted factors regulate monocyte secretion in \textit{M.\textit{tb}} infection.

To explore monocyte regulation at the protein level the trans-well model was employed again, this time to detect secreted MMP-1 (Fig 27). Monocyte MMP-1 was upregulated 2.9-fold with direct platelet contact compared with monocytes infected alone (p<0.01; Fig 27A). Unexpectedly, platelets also upregulated MMP-1 without direct contact compared to infected monocytes alone (p<0.01), with MMP-1 concentrations equivalent to monocytes cultured with direct platelet contact (p>0.05; Fig 27A). To confirm this result, monocytes were stimulated with PS to emulate conditions in a solely secretory intercellular network (Fig 27B). MMP-1 concentrations induced by PS stimulation were compared to a thrombin-stimulated control (Thr) to account for any immunomodulatory effect, with thrombin added into PS during its production. Compared to this thrombin control, PS induced a significant 2.2-fold increase in MMP-1 (p<0.01; Fig 27B). As observed with trans-well culture, this upregulation was similar to levels induced by direct platelet contact (p>0.05; Fig 27B).

To expand on the platelet contact-independent regulation of monocyte secretion further I measured MMP-9 and -10 concentrations in trans-well co-culture. As I have previously shown, \textit{M.\textit{tb}-}infection in the presence of platelets induced an upward (but non-significant) trend of MMP-9 secretion from 4108.1 pg/ml to 5459.7 pg/ml (p>0.05; Fig 28A). However, this increase was not seen in monocytes infected in a trans-well platelet model (p>0.05; Fig 28A). Interestingly, MMP-10 secretion was significantly upregulated by platelet secreted networks, increasing from 530.8 ±64.1 pg/ml in \textit{M.\textit{tb}-}infected monocytes alone to 996.4 pg/ml from monocytes with platelets in trans-well culture (p<0.05; Fig 28B). However, platelets in direct contact with monocytes had 1341.3 ±119.1 pg/ml. This 26% increase on trans-well culture a non-significant but highly reproducible trend (p>0.05; Fig 28B).

I have previously demonstrated monocyte secretion of TIMP-2 and TNF-α were unchanged in direct platelet contact and these were measured in the trans-well model to assess any differences in contact-independent regulation (Fig 28C-D). As expected, TIMP-2 was significantly reduced in \textit{M.\textit{tb}} infection (p<0.0001; Fig 28C) but platelets did not alter this concentration further regardless of contact. The cytokine TNF-α also remained unchanged by platelets both in a trans-well and in direct co-culture (p>0.05; Fig 28D).
Figure 27. Platelet-secreted products significantly upregulate monocyte MMP-1.

Monocytes were infected with *M. tb* in the presence of autologous platelets (Plt) or cell-free platelet supernatant (PS) at plt:monocyte 1:100. After 24 hours, extracellular MMP-1 was analysed by ELISA. A) Plt were co-cultured with monocytes either with direct addition of plt to monocytes or through a trans-well set-up (TW) to allow exchange of cell culture supernatant and *M. tb*, but not plt or monocytes. Bars represent mean ±SD and are representative of four independent experiments. B) Monocytes were stimulated with plts or PS before *M. tb* infection at monocyte multiplicity of infection (MOI) 1. As some recombinant thrombin is present in PS from the processing stages, equivalent thrombin concentrations were added to monocytes (Thr) to control for possible effects. Bars represent mean ±SD and are representative of two independent experiments. MMP-1 was significantly upregulated by plt secreted factors, in both a trans-well and PS model of stimulation. This upregulation was equivalent to direct platelet co-culture regulation. Statistical analysis was performed using an ordinary one-way ANOVA and Tukey’s multiple comparisons test: **p<0.01.
Figure 28. Contact-independent platelet regulation of monocyte secretory profile.

Monocytes were infected with *M. tb* and co-cultured with platelets (Plt) either with indirect or direct contact. Indirect plt-monocyte contact was achieved through plt culture in a trans-well (TW) to allow exchange of cell supernatant and *M. tb* only. Cell culture supernatant was harvested at 24 hours and (A) MMP-9; (B) MMP-10; (C) TIMP-2; and (D) TNF-α measured by ELISA. Monocyte MMP, TIMP and TNF-α secretion does not alter in indirect platelet regulation with secreted factors compared to plt regulation with direct cell contact. Bars represent mean ±SD and are representative of four independent experiments. Statistical analysis was performed using an ordinary one-way ANOVA and Tukey’s multiple comparisons test: ns p>0.05; *p<0.05; ****p<0.0001.
2.4. Soluble CD40L significantly upregulates monocyte MMP-1 secretion

Platelets are known to secrete a large variety of proteins when stimulated. Amongst these, CD40L and PF4 have been shown to have potent immunomodulatory effects on monocytes [373, 378]. To examine if CD40L and PF4 mediate contact-independent platelet upregulation of monocyte MMP-1, recombinant proteins were used to stimulate monocytes (Fig 29). CD40L stimulation of monocytes increased MMP-1 secretion from 2101.1 ±76.4 pg/ml to 3277.8 ±380.6 pg/ml (p<0.01; Fig 29A). However, this was still significantly less than MMP-1 induced by PS-stimulated monocytes (p<0.01; Fig 29A). *M.tb*-infected monocyte stimulation with PF4 also caused an increase in MMP-1 secretion but this trend was not significant compared to the vehicle control (p>0.05), and PF4-stimulated monocytes had 1.8-fold less MMP-1 secretion compared to PS stimulation (p<0.05; Fig 29B). I next expanded this study of contact-independent regulation to other time points.
Figure 29. Upregulation of monocyte MMP-1 by platelet-secreted factors.

Monocytes infected with *M. tb* at a multiplicity of infection (MOI) 1, were additionally stimulated (Stim) with a vector control (V); platelet supernatant (PS); and recombinant (A) soluble CD40 ligand (CD40L) or (B) platelet factor 4 (PF4). Supernatants were harvested after 24 hours and secreted MMP-1 detected by ELISA. Graphs representative of two independent experiments and bars represent mean ±SD. CD40L, but not PF4, stimulation significantly upregulates monocyte MMP-1 secretion. However, this regulation is significantly less than the total induced by PS. Statistical analysis was performed using an ordinary one-way ANOVA and Tukey’s multiple comparisons test: ns>0.05; *p<0.05; **p<0.01.
2.5. Prolonged platelet regulation of monocyte MMP-1 is contact dependent

To assess if platelet contact regulation was time point specific, I explored the observed contact-independent MMP-1 regulation over time. Platelets upregulated monocyte MMP-1 secretion even at 18 hrs, with MMP-1 increasing from 41.5 ±19.1 pg/ml in *M.tbc*-infected cells alone to 1249.8 ±162.9 pg/ml in cells infected with platelets in a trans-well (p<0.001; Fig 30). This was an equivalent upregulation to monocytes infected with direct platelet contact (p>0.05; Fig 30). A similar trend was observed at 24 hrs, with significant upregulation of MMP secretion observed in both platelet conditions compared to *M.tbc*-infected monocytes (p<0.01; Fig 30). At 48 hrs platelet-secreted products continued to significantly upregulate MMP-1 secretion compared to *M.tbc*-infected monocytes alone (p<0.0001; Fig 30). However, direct platelet-monocyte contact resulted in 3.6-fold greater MMP-1 concentrations compared to *M.tbc*-infected monocytes alone (p<0.0001), which was significantly more than platelets co-cultured without direct monocyte contact (p<0.01; Fig 30). Having investigated platelet signalling for monocyte MMP gene expression and secretion, it was next of interest to elucidate the monocyte intracellular signalling pathways involved in platelet MMP regulation.
Figure 30. Contact-independent platelet regulation of monocyte MMP-1 secretion over time.

Monocytes were infected with *M. tb* and co-cultured with platelets (Plt) that were either added directly to culture or in a trans-well (TW) set-up. The trans-well allowed passage of cell culture media and *M. tb*, but not monocytes or plt. Supernatants were harvested after 18, 24 or 48 hours (hrs) for analysis by ELISA. Bars represent mean ±SD and are representative of three independent experiments. Plt upregulated monocyte MMP-1 secretion in a contact-independent manner at 18 and 24 hrs. However, by 48 hrs plt MMP-1 regulation was significantly enhanced with direct contact with monocytes compared to plt in trans-well. Significance was assessed by two-way Anova with Tukey’s multiple test comparison: **p<0.01.
2.6. HIF-1α is upregulated in *M.tb* infected monocyte-derived cells

I decided to investigate monocyte intracellular signalling first by examining the master regulator of inflammation, HIF-1α. I initially investigated *M.tb*-driven HIF-1α stabilisation using MDMs to confirm and expand our currently limited understanding of its accumulation in normoxia and TB. During a time course I detected stable HIF-1α in *M.tb*-infected MDMs from as early as 4 hrs after infection (Fig 31). By 8 hrs post-infection significant HIF-1α levels were detected and protein stabilisation peaked at 18 hrs post-infection. Although reduced, HIF-1α was still detected at 48 hrs (Fig 31). This indicates for the first time that *M.tb* infection results in stable accumulation of HIF-1α in monocyte-derived cells in normoxia. The regulation of this accumulation was investigated next.

![Figure 31. HIF-1α stabilisation in *M.tb* infected monocyte-derived macrophages (MDMs).](image)

Monocytes were matured to MDMs before infection with *M.tb* at multiplicity of infection (MOI) 1. Cell lysates were taken at seven time points ranging from 0.5-48 hours (hrs) and HIF-1α and β-actin protein detected by western blot. *M.tb*-infection upregulated HIF-1α stimulation from 4 hrs and peaking at around 18 hrs. Image representative of two independent experiments.
2.7. *M.tb* regulation of HIF-1α in MDMs

HIF-1α stabilisation can be regulated at several levels and this was investigated in the context of *M.tb* infection. Key inhibitors of HIF-1α stabilisation are PHD1 and PHD2. These proteins were visualised with western blot analysis to reveal a downregulation of PHD 1 in *M.tb* infection compared to controls. PHD2 remained unchanged (Fig 32A). To investigate the potential of intercellular signalling to stabilise HIF-1α in TB, MDMs were stimulated with *M.tb* or CT and HIF-1α protein compared. Interestingly, this revealed that CT-stimulated cells had greater HIF-1α stabilisation than *M.tb* (Fig 32B). Unstimulated MDMs did not show any HIF-1α stabilisation. It was next of interest to investigate *M.tb* regulation of HIF-1α at the transcriptional level.

![Western Blot Images](image)

**Figure 32. *M.tb* regulation of HIF-1α stabilisation in normoxia.**

Monocyte-derived macrophages (MDMs) were infected with *M.tb* at a multiplicity of infection (MOI) of 1. Cell lysates harvested 8 hours post-infection. A) The HIF-1α inhibitors prolyl hydroxylase (PHD) 1 and 2 were detected by western blot along with HIF-1α and β-actin. (B) MDMs were stimulated with *M.tb* or monocyte culture media conditioned with TB (CT) before cell lysates were collected for western blot analysis. *M.tb* upregulates HIF-1α in a manner associated with specific downregulation of key HIF-1α inhibitor PHD1, and enhanced by monocyte intercellular TB networks. Images representative of two independent experiments.
To investigate transcriptional regulation of HIF-1α, qRT-PCR was performed to measure HIF-1α mRNA in *M.tb*-infected MDMs (Fig 33A). There was no significant difference in HIF-1α gene expression at 2 hrs post-infection, however, by 4 hrs *M.tb*-infected MDMs had 4.0-fold more HIF-1α mRNA than uninfected monocytes (p<0.01; Fig 33A). At 8 hrs there remained a 2.6-fold increase in HIF-1α mRNA in *M.tb*-infected cells compared to uninfected cells at that time (p>0.05; Fig 33A). HIF-1α gene expression did not change over time in uninfected cells (p>0.05) but *M.tb*-infected monocytes had significantly greater HIF-1α gene expression by 4 hrs compared to 2 hrs (p<0.05; Fig 33B). After 4 hrs HIF-1α mRNA continued to increase but this was not statistically significant (p>0.05; Fig 33A).

NFkB upregulates HIF-1α gene expression in normoxia [380]. To assess the effect of HIF-1α gene transcription on protein levels I inhibited NFkB using the chemical inhibitor of p65, helenalin. HIF-1α western blot analysis revealed that NFkB inhibition resulted in significantly reduced HIF-1α in the total cell lysate (Fig 33B). This implicated NFkB-dependent gene expression as an important driver of HIF-1α. As I have now shown that platelets regulate monocytes at the gene level, and that TB intercellular monocytic networks potently upregulate HIF-1α in MDMs, it next was of interest to investigate HIF-1α in the context of platelet intercellular networks.
Figure 33. *M.tb* regulates HIF-1α transcription through NFkB-dependent pathways.

Monocytes were matured to monocyte-derived macrophages (MDMs) *in vitro* and infected with *M.tb* at a multiplicity of infection (MOI) of 1. (A) MDMs were infected with *M.tb* for 2, 4 or 8 hours (hrs) before cell lysis for RNA collection. Comparative HIF-1α gene expression was assessed by qRT-PCR with cycle threshold normalised to β-actin, and represented as a fold change of the negative control average. Bars represent mean ±SD and of three independent experiments. Significance was assessed using a two-way ANOVA and Tukey’s multiple comparison test: **p<0.05. (B) MDMs were pre-treated with the p65 subunit inhibitor Helenalin (Hel) to inhibit NFkB activation. MDMs were then *M.tb* infected. After 8 hrs HIF-1α was measured in the total cellular lysate using β-actin as a loading control. A vehicle control (V) was also included. Image is representative of two independent experiments. *M.tb*-driven HIF-1α accumulation is associated with significant upregulation of HIF-1α gene expression and is dependent on activation of the HIF-1α transcriptional regulator NFkB.
2.8. HIF-1α is upregulated in *M. tb*-infected platelet-monocyte co-cultures

I first examined monocyte HIF-1α at the transcriptional level. *M. tb* infection of monocytes significantly upregulated HIF-1α mRNA, with a 2.9-fold increase compared to uninfected cells (p<0.05; Fig 34A). Active platelets co-incubated with uninfected monocytes did not result in a significant change in HIF-1α gene expression, although there was a small 1.5-fold upward trend compared to uninfected monocytes alone (p>0.05; Fig 34A). Strikingly, infection of monocytes in the presence of platelets resulted in a 1.7-fold increase in HIF-1α mRNA compared with monocytes *M. tb* infected alone (p<0.05) and a 3.3-fold increase compared to uninfected cells in co-culture (p<0.01; Fig 34A).

To examine platelet regulation of HIF-1α further, I analysed a time course of HIF-1α transcription (Fig 34B). At 4 hrs post-infection there was a 1.5-fold increase in HIF-1α mRNA in infected monocytes alone compared to uninfected monocytes, and a similar 1.4-fold increase seen at 24 hrs post-infection (Fig 34B). Monocyte co-incubation with active platelets drove significantly more HIF-1α RNA upregulation, with an additional 2.6-fold increase in monocyte HIF-1α gene expression at 4 hrs compared to monocytes infected alone (p<0.01) and 1.5-fold increase at 24 hrs (p<0.05; Fig 34B). Interestingly, monocytes infected in the presence of platelets had only a 1.4-fold increase between time points (p<0.05) whilst *M. tb*-infected monocytes alone had a 2.3-fold increase in HIF-1α gene expression at 24 hrs compared with 4 hrs (p<0.05; Fig 34B).

Early HIF-1α accumulation in platelet-monocyte co-culture was then examined at the protein level with western blot. 0 hr controls revealed significant HIF-1α protein in co-culture compared to monocytes (Fig 35). Upon culture of platelets and monocytes together HIF-1α reduced compared to that measured at 0 hrs. After only 60 mins post-infection there was a discernable difference in HIF-1α between infected and uninfected cells and by 4 hrs there was a clear increase in *M. tb*-infected cells compared with uninfected cells (Fig 35). Due to the high HIF-1α present in platelets it was not possible to distinguish platelet and monocyte cell contributions to total HIF-1α but this protein data does indicate an increase in HIF-1α in *M. tb* infection of monocytes in the presence of platelets. I next evaluated platelet activation of key monocyte phosphorylated signalling proteins.
Monocytes were incubated with autologous platelets (Plt) at a ratio of 1:100, and infected with *M. tb* at a multiplicity of infection (MOI) of 1. RNA was collected at (A) 18 hours and (B) 4 and 24 hours (hrs) post-infection for analysis of HIF-1α gene expression by qRT-PCR. HIF-1α cycle threshold for both graphs was normalised to β-actin and represented as fold change of the uninfected control. Bars represent mean ±SD and are representative of two independent experiments. Plt upregulate HIF-1α gene expression in *M. tb*-infected, but not uninfected, monocytes. This regulation was particularly significant at early time points. Significance was assessed with an (A) ordinary one-way ANOVA and (B) a two-way ANOVA both followed by Tukey’s multiple comparison test: *p<0.05; **p<0.01.

**Figure 34. Platelets enhance *M. tb*-driven monocyte HIF-1α gene expression.**
2.9. Platelets regulate monocyte AKT and ERK-1/2

First, phosphorylation of the key regulatory protein AKT was assessed, which in the *M.tb*-infected cell is a vital downstream target of PI3K in MMP regulation [157]. Phosphorylated AKT (P-AKT) was significantly upregulated in monocytes infected with *M.tb* compared with uninfected (Fig 36A). Monocytes co-cultured with platelets had detectable levels of P-AKT but this increased dramatically upon infection. Monocytes infected in the presence of platelets had significantly more AKT phosphorylation compared to infected monocytes alone (Fig 36A). It was also of interest to investigate MAPK activation in platelet-monocyte co-culture.

Phosphorylated ERK-1/2 (P-ERK) and phosphorylated p38 (P-p38) increased in monocytes *M.tb*-infected alone compared to uninfected controls (Fig 36B). Similar to P-AKT, uninfected co-cultured cells had significant P-p38 and P-ERK, however, this increased further upon *M.tb* infection (Fig 36B). There was also significantly more total -AKT (T-AKT), -ERK-1/2 (T-ERK) and -p38 (T-p38) protein in co-cultured cells compared to monocytes alone. To further investigate this a timecourse was undertaken.

Figure 35. *M.tb*-driven HIF 1α protein accumulation in platelet-monocyte co-culture.
Monocytes were infected with *M.tb* in the presence of activated platelets (Plt), and cells lysed at 0, 1 and 4 hours (hrs) post-infection. 0 hr controls were also included for monocytes alone. Active plt contain significant HIF-1α. In plt-monocyte co-cultures there was significantly more HIF-1α protein in *M.tb*-infected cells than uninfected cells at 4 hrs. Image representative of two separate experiments.
To further investigate monocyte MAPK activation in the context of plt, a time course including a 0 hr control was undertaken. In 0 hr controls more T-AKT, -ERK and –p38 was seen in plt co-culture than for monocytes alone. At 0 hrs, co-cultured cells had high levels of P-AKT compared to monocytes alone but this reduced rapidly (Fig 37). Compared to the uninfected cells, at 1 hr post-infection there was a clear upregulation in P-AKT in the *M.tb*-infected monocyte-platelet co-culture. After 1 hr, infected cells also had significantly more P-AKT than infected cells at 0 hrs. A difference was still visible between infected and uninfected monocytes at 4 hrs post-infection but it was less marked (Fig 37).
Phosphorylation of the MAPKs p38 and ERK-1/2 was also investigated using comparison to a 0 hr control to visualise *M. tb*-driven changes (Fig 37). Platelet-derived P-p38 did not reduce as rapidly as the other proteins investigated, with similar levels visible post-infection as at 0 hrs. Between infected and uninfected monocyte-platelet co-culture a difference in P-p38 was observed at only 1 hr post infection. Uninfected monocyte-platelet co-culture induced a small increase in P-ERK over time (Fig 37). In contrast to p38, *M. tb* induced a dramatic upregulation of P-ERK, both in comparison to uninfected cells and 0 hr controls. This upregulation continued at all measured time points but appeared to peak 1 hr post-infection (Fig 37). I was next interested to assess this platelet upregulation of monocyte pathways with indirect contact.
Figure 37. MAPK pathway activation in M.tb infection of monocyte-platelet co-culture over time.

Monocytes were cultured alone or with platelets (Plt) and infected with M.tb at a multiplicity of infection (MOI) 1. Total cell lysate was harvested for western blot analysis of intracellular phosphorylated AKT (P-AKT) and total AKT protein (T-AKT); phosphorylated p38 (P-p38) and total p38 protein (T-p38); and phosphorylated ERK-1/2 (P-ERK) and total ERK-1/2 protein (T-ERK). A time course of phosphorylated and total protein was performed by harvesting monocytes alone and monocytes with Plt at 0 hours (hrs). Monocytes co-cultured with Plt were then harvested at 1 and 4 hrs post-infection. Image from one membrane, cut to remove irrelevant sample lanes. AKT, p38 and ERK-1/2 phosphorylation is upregulated in M.tb infection of Plt co-culture at 1 hr post infection. Image representative of two separate experiments.
2.10. Contact-independent platelet activation of monocyte MAPK pathways

As I have shown that platelets upregulate MMP-1 secretion in a contact independent manner it was of interest to assess MAPK pathway activation without direct contact of platelet and monocytes. First, I visualised monocyte intracellular proteins over time cultured alone or in a trans-well set-up. 1 hr post *M.tb* infection, P-AKT was upregulated in *M.tb*-infected monocytes and this slightly increased with stimulation of *M.tb*-infected monocytes with platelets in trans-wells (Fig 38). There was marked AKT phosphorylation in *M.tb*-infected monocytes at 2 hrs post-infection, which increased dramatically further with platelet stimulation. By 4 hrs post-infection AKT activation had reduced significantly despite unchanged levels of T-AKT. However, P-AKT in monocytes co-cultured with platelets in a trans-well was maintained and readily detectable (Fig 38).

An increase in P-p38 was visible in *M.tb*-infected monocytes compared to uninfected cells at 2 hrs post-infection (Fig 38). However, at this time point P-p38 was not upregulated further by platelet co-culture. By 4 hrs post-infection, there was significant P-p38 present in infected monocytes with platelet stimulation, with minimal P-p38 present in *M.tb*-infected monocytes alone. Finally, platelet upregulation of P-ERK was readily detected at the earliest time point, with a significant increase in *M.tb*-infected monocyte P-ERK with platelet regulation compared to *M.tb*-infected monocytes alone. This trend was seen at every time point but peaked at 2 hrs post-infection (Fig 38). Thus, platelets upregulate AKT, p38 and ERK-1/2 in *M.tb*-infected monocytes in a contact-independent manner. It was then of interest to assess the role of this pathway activation on monocyte MMP-1 secretion.
Figure 38. Platelet contact-independent regulation of monocyte intracellular pathways.

Monocytes were co-cultured platelets (Plt) in a trans-well to allow exchange of cellular supernatant and \( M.\text{tb} \) but prevent direct contact between plt and monocytes. Cells were infected with \( M.\text{tb} \) at a monocyte a multiplicity of infection (MOI) of 1, and total monocyte intracellular protein was harvested at 1, 2 and 4 hours (hrs) post-infection. Intracellular protein was then analysed by western blot for phosphorylated AKT (P-AKT); total AKT (T-AKT); phosphorylated ERK-1/2 (P-ERK); total ERK-1/2 (T-ERK); phosphorylated p38 (P-p38); and total p38 protein (T-p38). Plt-secreted products significantly upregulated AKT and ERK-1/2 phosphorylation at every time point, whilst p38 phosphorylation was upregulated after 4 hrs infection. Image representative of two independent experiments.
2.11 Platelet contact independent regulation of MMP-1 is through activation of ERK-1/2, p38 and PI3K

To investigate if platelet MMP-1 upregulation was driven by platelet activation of AKT, p38 and ERK-1/2 pathways, chemical inhibitors were used. As platelet activity was also likely to be affected by such inhibitors, PS was used as a replacement for platelets. First, the role of platelet AKT activation was investigated by preventing AKT phosphorylation using the PI3K inhibitor LY294002 (Fig 39A). PS significantly upregulated MMP-1 secretion from *M. tb*-infected monocytes (p<0.05) but monocyte pre-incubation with LY294002 reduced *M. tb* stimulation of MMP-1 from 3604 ±705.7 pg/ml in vehicle control cells to 947.2 ±195.1 pg/ml (p<0.05; Fig 39A). MMP-1 secretion was also significantly reduced in *M. tb*-infected monocytes stimulated with PS. Inhibiting PI3K pathway activation reduced MMP-1 concentrations in monocytes with PS stimulation 4.9-fold compared to the PS vehicle control (p<0.01), whilst *M. tb*-infected monocytes without PS stimulation was reduced 3.8-fold reduction (p<0.01; Fig 39A). Thus, platelet-stimulated monocyte MMP-1 secretion was reduced to the same concentrations as monocytes with *M. tb* alone by PI3K inhibition (p>0.05; Fig 39A).

Next, p38 phosphorylation was inhibited using SB203580 (Fig 39B). In *M. tb* infection, p38 inhibition reduced MMP-1 secretion 1.7-fold compared to vehicle monocyte-only controls (p<0.05; Fig 39B). However, there was a 2.7-fold decrease in MMP-1 secreted by *M. tb*-infected and PS stimulated monocytes compared to the co-stimulated vehicle control (p<0.0001; Fig 39B). Consequently, monocytes with reduced p38 activity had no additional MMP-1 secretion on stimulation with PS compared to monocytes with platelet stimulation (p>0.05; Fig 39B).

Finally, PD98059 was used to inhibit ERK-1/2 phosphorylation through specific targeting of mitogen-activated protein kinase kinase (MEK). There was a 2.3-fold reduction in PS-regulated MMP-1 secretion compared to the co-stimulated vehicle control (p<0.01; Fig 39C). In addition, no significant difference was observed in MMP-1 secretion between monocytes infected alone and monocytes with PS stimulation when ERK-1/2 phosphorylation was inhibited (p>0.05; Fig 39C). Therefore, inhibition of ERK-1/2, p38 and PI3K prevented upregulation of monocyte MMP-1 secretion by platelet factors.
Figure 39. Platelet-secreted factors target MAPKs to upregulate MMP-1.

Platelet regulation of monocyte intracellular pathways was investigated with chemical inhibitors. Monocytes were incubated with the (A) PI3K inhibitor LY294002 (LY29); (B) p38 inhibitor SB203580; and (C) mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 (PD). A vehicle control (V) was also included. The monocytes were then incubated with platelet supernatant (PS) and infected with *M. tb* at a multiplicity of infection (MOI) of 1. Supernatants were harvested after 24 hours for MMP-1 analysis by ELISA. Bars represent mean ±SD and are representative of two independent experiments. Inhibition of PI3K, p38 and ERK-1/2 phosphorylation significantly reduced platelet regulated MMP-1 secretion. Significance was assessed using an ordinary one-way ANOVA and Tukey’s multiple comparison test: ns p>0.05; * p<0.05; **p<0.01.
3. Discussion

In this chapter I investigated the mechanisms of platelet regulation of MMPs in *M.tb*-infected monocytes. I began by assessing if the reported platelet activation in TB is due to direct *M.tb* binding and stimulation. Bacteria can directly bind and activate platelets via a diverse range of mechanisms involving direct interactions between bacterial proteins and platelet receptors such as GPIIb-IIIa, GPIbα, FcγRIIa, and TLRs [229]. Platelet receptors are also bound and activated directly by bacterial toxins such as the *P. gingivalis* toxin gingipains which binds to PAR1 similar to thrombin, or *S. pneumoniae*’s pneumolysin which binds to platelet phospholipids [390, 391]. Platelets have also been reported to be activated by cytokines such as IL-6 and -8, such as are present in monocyte CT [392]. However, platelet exposure to neither monocyte TB networks, that include *M.tb* secreted toxins and monocyte cytokines, nor live *M.tb* induced platelet degranulation in vitro. This implicates other factors in this complex activation mechanism. There are a range of factors that have been shown in vivo to support bacterial activation of platelets such as plasma proteins like the von Willebrand factor and complement [50]. For example, plasma IgG and engagement of integrin by fibrinogen is required to support staphylococcal activation of platelets [393]. *M.tb* may therefore be able to stimulate platelets in the presence of these factors which were missing from my in vitro model.

Next, I investigated the mechanism of platelet regulation of monocyte responses using a trans-well culture model (Fig 8). This model allowed direct comparison of co-cultures with and without surface contact between monocytes and platelets. Platelet upregulation of MMP-1 gene expression was found to be partially dependent on direct platelet contact. Furthermore, a time course indicated the consistency of this trend, with differences between direct and indirect platelet regulation of MMP-1 RNA seen from the earliest time post-infection and developing further at later points. No contact-independent MMP-10 regulation was observed at the transcriptional level. Interestingly, this contact-dependent mechanism was not initially reflected at the secretory level. No significant difference was observed between direct and indirect platelet regulation of MMP-1 secretion. This was confirmed using PS stimulation and was similar to trends observed for MMP-9, TNF-α and
TIMP-2. For MMP-10 there was a small but highly consistent upward trend for monocytes stimulated directly with platelets compared with indirect co-culture which suggests a contact-dependent element for maximal MMP expression.

For MMP-1, the difference between gene and protein regulation may be due to contact independent upregulation of MMP-1 gene expression being sufficient to induce maximal translation and secretion. If cellular mRNA translation is functioning at its maximal capability there will be limited additional protein produced from the additional MMP-1 mRNA expressed with direct contact. This hypothesis is supported by the significant difference in MMP-1 secretion between monocytes with direct and indirect platelet regulation that occurred at later stages of infection, as it reflected a change in gene expression which became completely contact-dependent. Given the change observed over time for MMP-1 gene expression and secretion it is also possible that the kinetics of MMP-10 would have revealed a similar trend occurring that the single time point did not illustrate. Additionally, platelet secreted factors may enhance MMPs at the post-transcriptional level to increase mRNA stability and translation. Post-transcriptional MMP regulation has been demonstrated in a variety of mechanisms. For example, specific sequences in MMP mRNA 5’- or 3’-UTR are targeted by a variety of UTR-binding proteins to stabilise or destabilise the transcripts.

The ATP analog ATPγS upregulates MMP-9 in this manner by increasing the binding of the HuR stabilising factor to the ARE motifs present in the 3’-UTR of the MMP-9 mRNA. This protects the mRNA against rapid degradation [141]. In addition, Ras-dependent MAPK/ERK signalling maintains high levels of MMP-9 production through integrin α3β1 signalling to promote mRNA stability, and IL-10 represses HuR protein reduction of MMP-9 mRNA to improve the fibrotic processes in acute myocardial infarction [140]. Translational efficiency is also increasingly thought to play a key role in MMP regulation. For example nucleolin recruits inactive MMP-9 mRNA complexes into the rough endoplasmic reticulum to enhance the efficiency of MMP-9 translation [394]. Such post-transcriptional regulation can ensure high concentrations of secreted proteins despite a lack of additional gene expression.
My investigation into platelet intercellular MMP regulation suggests that secreted platelet products function in conjunction with direct platelet contact to regulate monocyte intracellular pathways. Platelets have been reported to secrete a host of factors (predominantly platelet granular proteins) upon activation, with one study demonstrating at least 81 proteins to be consistently present in PS of thrombin-activated platelets [395]. Therefore I next began preliminary investigations into the secreted platelet factors which drive the contact-independent monocyte MMP upregulation. To begin to assess which platelet factors are the predominate regulators of MMP-1, recombinant CD40L and PF4 were used. Many of the platelet proteins have reported immunomodulatory functions and may be involved in monocyte MMP responses, but CD40L and PF4 are amongst the best described in their regulation of monocyte inflammatory reactions. I have shown for the first time that CD40L is able to stimulate monocyte MMP-1 secretion. However, upregulation of MMP-1 by recombinant CD40L was less than that induced by PS. Recombinant PF4 stimulation did not induce significant MMP-1 secretion compared to M.tb-stimulated only monocytes. As PF4 and CD40L did not stimulate MMP-1 to concentrations achieved with PS, other proteins must also be involved in the contact independent regulation of monocyte MMP-1, and multiple proteins potentially acting on monocyte pathways in a synergistic manner.

I next sought to identify intracellular monocyte pathways targeted by platelets to regulate MMP activity. I initially focused on HIF-1α as a recently demonstrated regulator of MMP secretion in TB, although investigations of M.tb HIF-1α stabilisation have to date focused on hypoxic cavity environments [139]. HIF-1α stabilisation in normoxic M.tb infection is novel and our understanding limited and so I began investigations in a more established MDM model. Previous study using this model has shown that whilst M.tb can stabilise HIF-1α in normoxia, it is in a highly transient manner and only visible around 24 hrs post-infection of MDMs [139]. My data show for the first time that M.tb infection results in stable upregulation of HIF-1α in monocyte-derived cells in normoxia. Interestingly, some HIF-1α was also measured in unstimulated cells, suggesting a slight effect of culture or control media.

To investigate the regulation of HIF-1α stabilisation in MDMs I first measured PHD protein levels to assess changes at post translational stages. PHD1 was reduced by M.tb infection.
This finding accords with a recent investigation into HIF-1 using transcriptome analysis of TB-infected mouse lung where significant 25-30 fold reduction of PHD1 transcripts was shown compared with controls [396], but this has never been shown before at the protein level or in humans. Monocyte TB networks have been shown to upregulate HIF-1α in AECs [396], but intercellular stabilisation of HIF-1α has not been shown in monocyte-derived cells. Stimulating MDMs with CT, which contains both *M.tb* - and monocyte-secreted factors but not live *M.tb*, induced even more HIF-1α stabilisation than direct *M.tb* infection. Such a striking result clearly demonstrated that intercellular networks may be a vital regulator of HIF-1α in monocyte derived cells.

HIF-1α stabilisation in bacterial infection is thought to be primarily driven by NFkB-mediated transcriptional regulation [133, 380]. Analysis of HIF-1α gene expression revealed significant upregulation in *M.tb* infection from 4 hrs post-infection. To assess the potential role of this transcriptional upregulation on HIF-1α protein accumulation in *M.tb* infection, I targeted p65 using chemical inhibition. Inhibition of NFkB p65 subunit was associated with significant down regulation of HIF-1α protein to baseline levels at 18 hrs post-infection. This demonstrates that *M.tb* targets HIF-1α protein accumulation through NFkB-dependent gene regulation.

Having established that gene expression drives stable protein accumulation of HIF-1α in *M.tb* infection, I next investigated this in the context of my platelet co-culture. Examining trends over time supported my data from MDMs that indicated a culture effect on HIF-1α, as HIF-1α mRNA of monocytes also increased between the time points in uninfected cells. HIF-1α gene expression was also upregulated upon *M.tb* infection of freshly isolated monocytes. *M.tb* infection of monocytes in the presence of platelets upregulated HIF-1α mRNA further, in a manner that was not observed in uninfected cells. This clearly demonstrates that *M.tb* drives HIF-1α gene expression in monocytes, which is then enhanced by platelet intercellular regulation. Platelets particularly drove HIF-1α gene expression at the early stages of infection, with the largest upregulation seen at 4 hrs.

At the protein level, there was significantly more HIF-1α in *M.tb* -infected platelet-monocyte co-cultures than in uninfected cells by 4 hrs post-infection. As 0 hr co-culture revealed that platelets contain significant HIF-1α protein compared to monocytes alone, it is possible that
the trends of more protein in *M. tb* infection is a reflection of reduced HIF-1α degradation of the existing HIF-1α protein compared to uninfected controls. However, the increase in relative HIF-1α protein between 1 and 4 hrs indicates that platelets do upregulate HIF-1α protein in *M. tb*-infected monocytes, as the restricted transcriptome of platelets prevent them producing significant additional protein. In this manner platelets drove rapid HIF-1α expression at the gene and protein level in *M. tb* infection of monocytes.

Next, platelet regulation of MAPKs involved in monocyte MMP-1 regulation was investigated. I focused on the MAPKs PI3K (at the AKT node), ERK-1/2 and p38 for their role in MMP regulation in TB. These key signalling proteins drive MMP activity in part through activation of NFkB and HIF-1α in *M. tb*. For example, in LPS-stimulated monocytes activated ERK-1/2 pathways activate NFkB and initiate transcription of a range of targets including MMP-1, -3 and -9 and HIF-1α [397-400]. Measurement of AKT, p38 and ERK-1/2 phosphorylation confirmed activation of all three pathways in *M. tb*-infected monocytes was upregulated further upon co-culture. This suggests that platelets upregulate monocyte proteins, however, the increase in total protein made it difficult to accurately compare a co-culture condition with monocytes alone.

At 0 hrs, monocyte-platelet co-cultures had significantly greater phosphorylated and total protein of AKT, p38 and ERK-1/2, demonstrating that platelets contain significant amounts of these signal proteins. This is in alignment with the literature on platelets with AKT, p38 and ERK-1/2 well-established to be activated in platelets by platelet agonists such as thrombin [401, 402]. In platelets these MAPKs are activated in an integrin αIIbβ3-mediated manner by both an agonist-stimulated early activation and an outside-in integrin signal that results in more sustained activation [403]. The signalling proteins have a range of roles, for example thrombin induces platelet degranulation through the GPCR subfamily Gq which acts on PI3K pathways via AKT phosphorylation [404]. The primary role of active ERK-1/2 and p38 has also been found to degranulation but they also have roles in platelet adhesion [401, 403].

Comparison of co-culture conditions over time revealed that *M. tb* drove phosphorylation of AKT, p38 and ERK-1/2 in platelet-monocyte co-culture. AKT and ERK-1/2 phosphorylation increased over time in infected co-cultures, although this trend was not as dramatic for p38.
Whilst there was greater P-p38 in infected cells compared to uninfected cells at 1 hr post-infection, this appeared to be due not to an increase in protein compared to 0.5 hrs but reduced phosphorylated protein in uninfected cells. In addition, unlike T-AKT and T-ERK-1/2, T-p38 protein was maintained over time. The reduction in T-AKT and T-ERK-1/2 protein is likely due to processing of these proteins, with platelets are unable to replenish their protein levels due to a limited transcriptome. The stable amount of T-p38 protein detected over time maybe a result of upregulation in monocytes or prolonged maintenance of T-p38 in platelets. This potentially significant platelet contribution to T-p38 in monocyte-platelet co-culture may mask significant changes in this pathway in monocytes.

Whilst the platelet contribution at the RNA and secreted protein level could be readily accounted for, throughout my investigation of intracellular signalling at the protein level it was difficult to delineate monocyte-derived protein from platelet-derived protein. This was exacerbated with the transient nature of HIF-1α and phospho-protein as well as high levels of total protein. To directly compare the activation of these pathways in monocytes with M.tb-infection and platelets, intracellular staining of signalling proteins for analysis on flow cytometry was undertaken as monocyte specific antibodies can be used to ensure cell specific protein analysis. Unfortunately it was not possible to optimise the procedure to withstand the prolonged fixation period required for removing M.tb-infected cells from the CL3 facility. It was therefore impossible to further visualise activated monocyte signalling pathways in monocyte-platelet TB model.

Having shown that AKT, p38 and ERK-1/2 are phosphorylated in platelet-monocyte co-culture I assessed if platelets target these pathways to drive platelet contact-independent regulation of MMP-1 secretion. A trans-well set-up also ensured that no platelet protein was included in the cellular lysate, thus allowing focused visualisation of monocytic pathways only. P-AKT and P-ERK-1/2 were both highly upregulated in the presence of platelets compared to infected monocytes alone. Platelet stimulation activated ERK-1/2 faster than M.tb alone, and sustained phosphorylation of both AKT and ERK-1/2 for longer. p38 was not stimulated in the same manner, with a difference between monocytes alone and in co-culture only observed at 4 hrs. This suggests that indirect platelet contact does not drive significant activation of p38.
To build on the western blot analysis, chemical inhibitors of signalling protein were utilised. PS was used to stimulate monocytes instead of platelet themselves which would also be affected by MAPK inhibition. Analysis of cell supernatants found that the partial inhibition of these kinases was sufficient to negate any upregulation of monocyte MMP-1 secretion by platelets. This complemented the upregulation observed particularly for ERK-1/2 and AKT phosphorylation and indicates that whilst p38 is not significantly unregulated by platelets in _M.tb_ infection, its activation is a requirement for platelet mediated MMP-1 regulation. Together these data demonstrate that platelet-secreted products drive MMP-1 upregulation in TB through PI3K, p38 and ERK-1/2 MAPK pathways.

This chapter aimed to investigate the mechanism of platelet regulation of monocytes. I have shown that platelets upregulate monocyte MMP secretion in a contact-independent manner through platelet factors such as soluble CD40L. However, I also demonstrated that platelets utilise direct cell contact with monocytes for maximal transcriptional regulation and to drive prolonged MMP secretion. I showed for the first time that _M.tb_ infection drives prolonged HIF-1α expression in normoxia. This was primarily mediated through NFκB-dependent gene transcription, but may be enhanced by reduced PHD-1 degradation. Platelets enhanced monocyte HIF-1α gene expression in _M.tb_ infection in a novel mechanism that may contribute to platelet MMP regulation at the transcriptional level. Finally, I demonstrated that platelets rapidly upregulate AKT and ERK-1/2 phosphorylation and, in addition to p38, these pathways mediate contact independent upregulation of monocyte MMP-1. It was next of interest to expand my model to investigate platelet regulation of other innate immune cells that are central to TB pathology.
Chapter 5. Platelets regulate TB-driven MMP activity in neutrophils and airway epithelial cells

1. Introduction

In the previous chapters I have demonstrated a role for platelets in the monocyte response to *M.tb* infection. It was next of interest to assess if platelet MMP regulation in TB is specific to monocytes, or if platelets mediate other cells to contribute to wider TB-driven tissue destruction.

Neutrophils represent one of the first lines of defences in bacterial infections, migrating along a chemokine gradient to enter sites of infection quickly and in large numbers [86]. Neutrophils are an emerging player in TB immunopathology but are increasingly considered to be vital. They are present in the *M.tb*-infected lung at high concentrations and secrete pre-formed proteins stored in granules for a response that is unparalleled in rapidity, but with consequences that remain a subject of debate. Initial neutrophil recruitment is associated with *M.tb* infection control and bacterial clearance in a variety of pulmonary infections, including TB [81, 82, 405]. However, later neutrophil activity is thought to establish inflammatory immunopathology and uncontrolled infection [406]. Neutrophil granules are an important source of cytokines and chemokines such as IFN-γ and IL-8, which result in a range of clinically relevant effects [87, 407-409]. In humans, neutrophils secrete relatively low concentrations of cytokines such as TNF-α, but these concentrations are important *in vivo* due to the high number of neutrophils present [408]. Neutrophils are important drivers of tissue destruction in TB as they are accepted as the major source of MMP-8 in pulmonary TB, and an important source of MMP-9 and TIMP-2 [149, 150].

In *M.tb* infection, the contrasting protective and detrimental role of neutrophils in early and later stages respectively, suggests differential regulation of the cells. Platelets are well placed to contribute to early immune regulation, and direct platelet contact with
neutrophils can regulate neutrophil recruitment and degranulation in both a pro- and anti-inflammatory manner [254, 268, 410]. Platelets have been shown to be potent activators of neutrophils depending on stimulus and culture conditions. Whilst LPS-activation of platelets appears to cause platelet-driven neutrophil degranulation, whether thrombin–activated platelets are able to effectively trigger such degranulation is still a matter of debate [254, 268, 269]. These differences are likely due to the TLR-4 upregulation and stimulation driven by platelet LPS activation, which is in contrast to the TLR-3, TLR-9 and surface P-selectin expression driven by thrombin activation [290, 410-412]. Whilst some studies have shown an increase in neutrophil MMP-9 secretion with thrombin-activated platelets, others show no change [413, 414].

Platelets have also been previously reported to upregulate NETosis [415]. NETs are an important mediator of infection and inflammation and are associated with externalisation of numerous neutrophil factors including MMP-8 and -9 [416]. Platelet NET regulation was first described in 2007, when co-stimulation with LPS and platelets induced NETosis whilst neutrophil stimulation with LPS or resting platelets alone did not [290]. Platelet regulation of neutrophils has been predominately shown with direct cell contact although secreted platelet factors also have a role, for example in neutrophil chemotaxis [246, 269, 417]. Platelet regulation of neutrophils has not been investigated in the context of TB.

Whilst investigations of platelet intercellular regulation of innate immunity has focused on leukocytes, platelets are also well positioned to regulate other cell types. AECs form a continuous pseudostratified layer in the lung to create a tight barrier that protects underlying tissue from the external environment. In addition to forming this physical barrier, the active role of AECs in TB immunopathology is increasingly well-established [96, 99]. In TB AECs drive lung tissue destruction and leukocyte influx directly with MMP secretion [33, 166]. AECs also secrete MMP modulators such as TIMPs and the extracellular matrix metalloproteinase inducer (EMMPRIN, also known as CD147). AECs are important producers of secreted EMMPRIN in the human lung, where EMMPRIN potently upregulates secretion of MMPs such as MMP-1, -2, -3, -9, and -14 from cells including monocytes/macrophages, fibroblasts, and other epithelial cells [418-420].
Pulmonary TB networks drive AEC responses. For example, monocyte TB networks trigger potent AEC secretion of MMPs whilst direct *M.tb* infection does not [157, 171]. This has been validated *in vivo* using a zebrafish model where ESAT-6 induced MMP-9 in epithelial cells neighbouring *M.tb*-infected macrophages. This contributed to nascent granuloma maturation and bacterial growth [33]. In humans, AECs that are close to TB granulomas, with their large concentration of monocyte-derived cells, have also been shown to be significant producers of MMP-1 [171]. Platelets are well placed to contribute to these pulmonary networks as I have shown in Chapter 3 that active platelet-secreted factors are upregulated in the TB patient lung. Study of platelet regulation of AECs is highly limited, but CD40L, which is secreted by platelets, has been shown to activate AECs to regulate cytokines and chemokines, and sensitised CD40L-knockout mice had significantly less airway eosinophilia in both BALF and tissue in antigen challenge than controls [421, 422]. Additionally, platelet-derived TGF-β1 directs epithelial cell transition to mesenchymal-like cells in lung cancer which promotes cell motility out of the tumour microenvironment: a phenotype typically attributed to increased MMP activity [423, 424].

In this chapter I aimed to expand the role of platelets as regulators of cell-mediated tissue destruction in TB. Specifically, I aimed to assess platelet regulation of neutrophil MMP-8 and -9 and cytokine release in *M.tb* infection, and determine platelet regulation of *M.tb*-driven NET production. I then aimed to demonstrate platelet regulation of AEC tissue destruction in TB by investigating AEC secreted MMPs at the gene, protein, and functional level. Finally, I assessed indirect MMP regulation through platelet targeting of MMP modulators EMMPRIN and TIMP-1 and -2 in AECs.
2. Results

2.1. Platelets downregulate neutrophil MMP-8 secretion

To investigate platelet regulation of neutrophil MMPs in TB, neutrophils were first incubated with platelets at different ratios before M.tb infection. At 4 hrs post-infection there was a significant release of MMP-8 upon infection of neutrophils alone, with a 4.8-fold increase compared to controls (p<0.01; Fig 40). Interestingly, there was a downward trend of MMP-8 in the supernatant of neutrophils with increasing platelet co-incubation. At Plt:N of 100:1 there was a significant downregulation compared to neutrophils infected alone, decreasing from 154.8 ± 35.0 ng/ml to 106.0 ± 19.3 ng/ml (p<0.05), although this was still significantly higher than uninfected controls (p<0.01; Fig 40).

Figure 40. Platelets decrease neutrophil MMP-8 secretion.

Neutrophils were co-cultured with platelets at increasing platelet concentrations, from a platelet:neutrophil (Plt:N) of 5:1 to 100:1. Cells were infected with H37Rv M.tb at neutrophil multiplicity of infection (MOI) 10, for 4 hours before supernatants were harvested. Extracellular MMP-8 concentrations were assessed by ELISA and are expressed in ng/ml. Bars represent mean ±SD and are representative of four independent experiments. Increasing platelets result in a gradual decrease in MMP-8 concentrations. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey’s post hoc test: *p<0.05.
2.2. Platelets delay neutrophil MMP secretion in *M.tb* infection

To investigate this MMP downregulation, MMP-8 was analysed together with MMP-9 over time (Fig 41). At 2 hrs post-infection there was a 4.8-fold upregulation in MMP-8 secretion in *M.tb*-infected neutrophils compared to uninfected neutrophils (p<0.0001; Fig 41A). Platelets downregulated this MMP-8 response from 61.9 ±0.5 ng/ml in *M.tb*-infected neutrophils alone to 47.0 ±1.08 ng/ml in neutrophils infected in the presence of platelets (p<0.01; Fig 41A). The platelet downregulation observed in *M.tb*-infected neutrophils continued at 4 hrs, with a significant 1.2-fold decrease in the presence of platelets (p<0.05; Fig 41A). Interestingly, this difference was not observed by 18 hrs post-infection, where there was no significant difference in MMP-8 secretion between neutrophils alone or co-cultured (p>0.05; Fig 41A). Unstimulated controls also secreted a small amount of MMP-8, and over time this increased (p>0.05). This increase was not observed in the presence of platelets, leading to lower MMP-8 concentration in uninfected neutrophils that reached significance at 4 hrs post-infection (p<0.05; Fig 41A). Analysis between time points of *M.tb*-infected cells revealed that neutrophils infected in the presence of platelets had significantly upregulated MMP-8 secretion at 18 hrs compared to both 2 hrs (p<0.0001) and 4 hrs (p<0.001) which resulted in comparable concentrations of *M.tb*-infected neutrophils alone and in co-culture at 18 hrs.

MMP-9 supernatant concentrations were measure next. At 2 hrs post-infection MMP-9 secretion increased 5.1-fold compared to uninfected controls in neutrophils alone (p<0.0001; Fig 41B). Whilst platelets did not reduce MMP-9 concentrations in uninfected cells (p>0.05), in *M.tb*-infection there was a significant 1.4-fold decrease observed with *M.tb* infection of co-cultured neutrophils compared with neutrophils alone (p<0.01; Fig 41B). At 4 hrs this trend continued, with a significant 1.3-fold decrease (p<0.01; Fig 41B) but by 18 hrs post-infection no difference was observed between MMP-9 secretion of infected neutrophils cultured with and without platelets (p>0.05; Fig 41B). A downward trend was observed in uninfected neutrophils that were incubated with platelets compared to neutrophils alone at both 4 hrs and 18 hrs, but this did not reach significance (p>0.05; Fig 41B). Neutrophils co-cultured with platelets had a highly significant increase in MMP-9 secretion at 18 hrs compared with 4 hrs (p<0.001) whilst MMP-9 from infected neutrophils alone did not increase over this time period (p>0.05; Fig 42B).
Figure 41. Platelet regulation of neutrophil MMP-8 and MMP-9 secretion in *M. tb* infection over time.

Neutrophils were co-cultured with platelets (Plts) at plt:neutrophil 100:1 and infected with H37Rv *M. tb* at neutrophil multiplicity of infection (MOI) 10 for 2, 4 or 18 hours (hrs). Extracellular (A) MMP-8 and (B) MMP-9 concentrations were assessed by ELISA and are expressed in ng/ml. At 2 hrs post-infection there was a significant decrease in MMP-8 and -9 concentrations of neutrophils infected in the presence of plt. This was maintained at 4 hrs, but not 18 hrs. Bars show mean ±SD and are representative of three independent experiments. Statistical analysis was performed using a two-way ANOVA and Tukey’s multiple comparisons test: ns>0.05; *p<0.05; **p<0.01.
2.3. Platelets do not regulate neutrophil TIMP-2

MMPs are stored in a pro-form in the cytoplasmic granules of neutrophils, dissociating MMP-8 and -9 secretion from their activity. To investigate the functional effect of platelet MMP regulation, the MMP inhibitor TIMP-2 was measured in the neutrophil-platelet co-culture model. TIMP-2 was constitutively expressed by neutrophils but was significantly upregulated in *M. tb* infection of neutrophils, increasing 2-fold (p<0.001; Fig 42). However, neutrophil-platelet co-culture did not alter total TIMP-2 secretion (p>0.05; Fig 42). To directly assess the functional effect of platelet MMP regulation, neutrophil matrix degradation was investigated next.

**Figure 42. Platelets do not alter TIMP-2 concentrations in neutrophil co-culture.**

Neutrophils were co-cultured with platelets (Plt) at plt:neutrophil 100:1 and infected with H37Rv *M. tb* at a neutrophil multiplicity of infection (MOI) of 10, for 4 hours. Extracellular concentrations of TIMP-2 were assessed by ELISA and are expressed in pg/ml. Bars show mean ±SD and are representative of three independent experiments. TIMP-2 concentrations are unregulated by plt co-culture. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey’s post hoc test: ***p<0.001.
2.4. Platelets functionally downregulate neutrophil MMP activity

In addition to measuring inhibitor secretion, the breakdown of fluorescent-bound type I collagen and gelatin by neutrophil supernatants was measured to directly assess the functional effect of platelet MMP regulation (Fig 43). Type I collagenase activity was significantly greater for neutrophils infected in co-culture than the uninfected controls at all time points (all \( p < 0.0001 \), Fig 43A). However, compared with infected neutrophils alone there was a significant downregulation of type I collagenase activity in infected co-cultured neutrophils from the earliest time point. At 2 hrs post-infection, collagenase degradation decreased from 147.4 ±5.6 mU/ml in neutrophils alone to 107.2 ±8.9 mU/mL from neutrophils in co-culture (\( p < 0.01 \); Fig 43A). This downregulation was maximal at 4 hrs post-infection, with a significant 1.4-fold decrease (\( p < 0.001 \); Fig 43A). By 18 hrs post-infection this regulation had lessened, reducing only from 197.3 ±8.9 mU/ml in neutrophils infected alone to 159.9 ±9.8 mU/ml in neutrophils infected in co-culture (\( p < 0.01 \); Fig 43A).

The gelatinase activity of neutrophils in the presence of platelets also decreased variably over time (Fig 43B). At 2 hrs post-infection there was a significant 1.3-fold downregulation (\( p < 0.05 \)), which by 4 hrs had increased to a highly significant 1.5-fold downregulation (\( p < 0.0001 \); Fig 43B). Interestingly, at 18 hrs this increased further, with platelet co-culture reducing gelatinase activity dramatically 2.4-fold (\( p < 0.0001 \)) to values similar to the uninfected controls at this time point (\( p > 0.05 \); Fig 43B). Assessing activity over time revealed that MMP-9 activity of neutrophils infected in the presence of platelets was equivalent at 18 hrs to 2 hrs (\( p > 0.05 \), Fig 43B), indicating that platelets maintain downregulation of MMP-9 activity over time. Platelet cytokine regulation was investigated next.
Neutrophils were co-cultured with platelets (Plts) at Plt:neutrophil 100:1 and infected with H37Rv *M. tb* at a neutrophil multiplicity of infection (MOI) of 10 for 2, 4 or 18 hours (hrs). (A) Type I collagenase activity and (B) gelatinase activity of cell culture supernatants was determined with supernatant incubation on DQ matrix protein that fluoresced upon breakdown. Bars show mean ±SD and are representative of three independent experiments. There was a significant decrease in type I collagen and gelatin breakdown at all time points in neutrophils infected in plt co-culture compared with neutrophils alone. Downregulation peaked at around 4 hrs for type I collagenase whilst gelatinase downregulation peaked at 18 hrs. Statistical analysis was performed using a two-way ANOVA with Tukey’s multiple comparison test: *p<0.05; **p<0.01; ***p<0.001; ****p<0.00001.
2.5. Platelets regulate neutrophil cytokine responses in TB

Neutrophil IL-1β secretion was measured using a range of Plt:N (Fig 44A). Although concentrations of the cytokine remained low, Plt:N 50:1 was sufficient to upregulate IL-1β 1.6-fold compared to neutrophils infected alone (p<0.01; Fig 44A). This increased further at Plt:N 100:1 with a 2.1-fold increase compared to neutrophils infected alone (p<0.001; Fig 44A). Conversely, there was a slight downregulation of TNF-α observed at Plt:N 25:1 that was statistically significant (p<0.05) but not observed at higher ratios (p>0.05; Fig 44B). In contrast to these cytokines, IL-8 was secreted to high concentrations upon M.tb infection of neutrophils alone, with a 19-fold increase compared to controls (p<0.0001) but platelets did not alter the concentration of this chemoattractant further (p>0.05; Fig 44C). Although measured, no significant concentration of IL-6, -10, -12 or MDC were detected in any condition (not shown). As well as inducing neutrophil degranulation, M.tb has been previously shown to induce NETosis [88]. Therefore, I next decided to investigate this in the context of a platelet co-culture.
Figure 44. Platelet regulation of neutrophil cytokines and chemokines.

Neutrophils were incubated with platelets at platelet:neutrophil (Plt:N) of 25:1 to 100:1 and infected with *M.tb* at neutrophil multiplicity of infection (MOI) 10 for 4 hours. Cell culture supernatant was then harvest for multiplex analysis by a magnetic bead Luminex assay detecting (A) IL-1β, (B) TNF-α and (C) IL-8. Bars represent mean ±SD and are representative of two independent experiments. Neutrophils infected in the presence of platelets had significantly increased IL-1β secretion and decreased TNF-α secretion in a Plt:N dependent manner, whilst IL-8 remained unchanged. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey’s multiple comparison test: *p<0.05; **p<0.01; ****p<0.0001.
2.6. Platelets downregulate *M.tb*-induced NETosis

I assessed platelet regulation of TB-induced NETosis using PMA as a positive control for NETosis stimulation [425]. *M.tb* induced a 1.5-fold increase in NET production compared to uninfected cells (p<0.01), which was equivalent to that detected with PMA (p>0.05; Fig 45). In my co-culture model, no change in NET production was observed between neutrophils infected alone and with platelets (p>0.05; Fig 45). Furthermore, platelet culture with PMA-stimulated neutrophils led to a downregulation of NETs from 334.4 ±7.7 ng/ml in PMA-stimulated neutrophils alone to 277.2 ±40.8 ng/ml in PMA-stimulated neutrophils with platelet incubation (p<0.0001; Fig 45). The same trend was observed in *M.tb*-infected cells, where NETs decreased from 519.4 ±43.6 ng/ml in neutrophils alone to 376.6 ±41.3 ng/ml from cells in co-culture (p<0.05; Fig 45). This reduced NET production in the presence of platelets to baseline levels seen in unstimulated controls (p>0.05; Fig 45). Having shown that platelets mediate responses of neutrophils, I began investigation of platelet regulation of AECs.

![Figure 45. Platelets downregulate NETosis.](image)

Neutrophils were co-cultured with platelets (Plt) at Plt:neutrophil of 100:1 for 4 hours, with additional stimulus of *M.tb* at a neutrophil multiplicity of infection (MOI) of 10, or 20 nM phorbol myristate acetate (PMA). Cell culture supernatants were harvested and neutrophil extracellular (Extracell) DNA detected as a measure of neutrophil extracellular traps (NETs) using PicoGreen and a λ DNA standard. Bars show mean± SD. and are representative of three independent experiments. Plt did not induce significant NETosis alone, and downregulated PMA- and *M.tb*-induced NETosis. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey’s multiple comparison test: *p<0.05; **p<0.01.
2.7. Platelets regulate epithelial cell MMP responses in TB

As discussed in section 1 of this chapter, epithelial cells respond to a variety of secreted intercellular networks and function as important immune cells in TB. To examine a role of platelets in regulating AEC MMP secretion in TB, HBECs were stimulated with CT, PS, or both, and MMP-1 secretion measured (Fig 46). At 36 hrs there was already significant upregulation of MMP-1 secretion in CT-stimulated cells (p<0.05; Fig 46). However, HBEC co-stimulation with PS and CT for 36 hrs increased MMP-1 secretion from 66.5 ±7.2 ng/ml in CT-only stimulated cells to 90.3 ±0.3 ng/ml (p>0.05; Fig 46). At 72 hrs CT induced a highly significant upregulation of MMP-1 secretion, with a 8.9-fold increase compared to unstimulated cells (p<0.0001; Fig 46), but additional stimulation with PS drove MMP-1 secretion from 181.8 ± 2.2 ng/ml with CT-only stimulation to 256.8 ±15.9 pg/ml (p<0.01; Fig 46). PS-only stimulation did not drive significant MMP-1 secretion at either 36 hrs or 72 hrs incubation (both p>0.05; Fig 46).

Platelet regulation of other HBEC MMPs was also investigated at 72 hrs. First, PS upregulation of MMP-1 was confirmed using a thrombin control. PS contains residual traces of thrombin from the platelet activation stage, and this enzyme has been shown to have immunomodulatory properties on AECs [426, 427]. However, there was no significant difference between the thrombin-stimulated control and the unstimulated control in MMP secretion (Fig 47A-D). Furthermore, HBECs co-stimulated with CT and PS had 2-fold increase in MMP-1 compared to the CT-stimulated thrombin control (p<0.0001; Fig 47A). MMP-2 was also measured in PS- and CT-stimulated HBECs. There was significant constitutive secretion of MMP-2 from HBECs (Fig 47B), and this secretion did not increase upon CT stimulation. However, PS alone did increase MMP-2 secretion 2.4-fold (p<0.01; Fig 47B). Interestingly, the additional presence of monocyte TB networks in the form of CT reduced this platelet-driven response 2.5-fold (p<0.01) to concentrations equivalent to controls (p>0.05; Fig 47B).

In contrast to MMP-2, there was no detectable MMP-9 in unstimulated HBECs. Upon PS-only stimulation there was an increase in MMP-9 secretion to 54.2 ±13.0 ng/ml (p>0.05; Fig 47C) whilst CT-only stimulation induced 124.0 ±19.1 ng/ml (p<0.01; Fig 47C). However, HBEC co-stimulation with both CT- and PS- induced a 3-fold increase compared with CT-only stimulated cells (p<0.0001) with secretion of 381.1 ±48.4 ng/ml MMP-9 (Fig 47C). Finally, platelet regulation of HBEC MMP-10 was measured. PS stimulation of HBECs for 72 hrs
induced an upwards trend of MMP-10 secretion, increasing from 3.2 ±0.4 ng/ml in controls to 8.4 ±3.4 ng/ml (p>0.05; Fig 47D). CT-stimulated HBECs produced a significant 4.8-fold increase in MMP-10 compared to unstimulated controls (p<0.01) and this was increased significantly further with co-stimulation using PS and CT (p<0.0001; Fig 47D). MMP-7 was also measured but no significant concentrations were detected under any condition (not shown). To assess additional, indirect, platelet regulation of MMPs I investigated modulation of MMP regulators in the AEC model.

Figure 46. Platelet stimulation of airway epithelial cell MMP-1 secretion.

Human bronchial epithelial cells (HBECs) were stimulated with activated platelet supernatant (PS), cell culture supernatant of monocytes conditioned with TB (CT), or both PS and CT, for 36 or 72 hours (hrs). Cell supernatants were then collected and MMP-1 detected by ELISA. Bars show mean ±SD. and are representative of three independent experiments. PS upregulates CT-mediated MMP-1 secretion of AECs over time. Statistical analysis was performed using a two-way ANOVA and Tukey’s multiple comparison test: ns p>0.05; *p<0.05; **p<0.01; ****p<0.0001.
Figure 47. Platelet-secreted factors upregulate airway epithelial cell MMP secretion.

Human bronchial epithelial cells (HBECs) were stimulated with thrombin (Thr), activated platelet supernatant (PS), cell culture supernatant of monocytes conditioned with TB (CT), or both PS and CT, for 72 hours. Cell culture supernatants were then collected and MMPs detected by magnetic bead Luminex array. Bars show mean ±SD of an experiment performed in triplicate and are representative of at least three independent experiments. (A) PS upregulates MMP-1 in CT-stimulated HBECs. (B) PS upregulates HBEC MMP-2 secretion, but not upon co-stimulation with CT. (C-D) PS upregulates HBEC (C) MMP-9 and (D) MMP-10 secretion both alone and in combination with CT. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey’s multiple comparison test: ns p>0.05; *p<0.05; **p<0.01; ****p<0.00001.
2.8. Platelets do not mediate secretion of key AEC MMP regulators

In addition to secreting a range of MMPs, AECs are also important producers of soluble secreted EMMPRIN [418]. To assess if platelets could additionally regulate MMP secretion through EMMPRIN modulation, HBECs were stimulated with PS and CT and extracellular EMMPRIN detected.

There was no increase in EMMPRIN with PS-only stimulation of HBECs whilst CT induced a significant 2-fold upregulation of EMMPRIN compared with controls (p<0.01; Fig 48). However, co-stimulation of CT incubated HBECs with PS did not upregulate EMMPRIN further compared to a CT-stimulated thrombin control (p>0.05; Fig 48). TIMP-1 and -2 are also AEC secreted MMP regulators and these were investigated next.

Platelet regulation of AEC MMP inhibitors TIMP-1 and TIMP-2 was measured after 72 hrs CT

![Figure 48. Platelet-secreted factors do not regulate airway epithelial cell extracellular matrix metalloproteinase inducer (EMMPRIN) secretion.](image)

Human bronchial epithelial cells (HBECs) were stimulated with thrombin (Thr), activated platelet supernatant (PS), cell culture supernatant of monocytes conditioned with TB (CT), or both PS and CT for 72 hours. Cell culture supernatant was then collected and soluble, secreted EMMPRIN detected by magnetic bead Luminex array. Bars represent mean ±SD of an experiment performed in triplicate and are representative of three independent experiments. PS does not regulate EMMPRIN secretion in control or TB-stimulated HEBCs. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey’s multiple comparison test: ns p>0.05; **p<0.01.
and PS stimulation (Fig 49). Unstimulated HBECs secreted high concentrations of TIMP-1 (Fig 49A). However, this was significantly reduced upon stimulation with PS-only and CT-only stimulation (both p<0.0001). Co-stimulation of HBECs with both CT and PS did not reduce TIMP-1 concentrations further (p>0.05; Fig 49A). TIMP-2 concentrations did not change upon HBEC stimulation with either PS-only or CT-only but interestingly there was a significant increase in TIMP-2 secretion upon stimulation of cells with PS and CT together (Fig 49B). TIMP-2 increased from 1.7 ±0.2 ng/ml in thrombin controls to 2.9 ±0.1 ng/ml in co-stimulated HBECs (p<0.0001; Fig 49B). To assess if this platelet regulation is reflected in functional regulation of protease activity, gelatinase and type I collagen degradation was investigated.
Figure 49. Platelet regulation of airway epithelial cell TIMP secretion in TB.

Human bronchial epithelial cells (HBECs) were stimulated with thrombin (Thr), activated platelet supernatant (PS), supernatant of monocytes conditioned with TB (CT), or both PS and CT for 72 hours. Cell culture supernatant was then collected and (A) TIMP-1 and (B) TIMP-2 detected by ELISA. Bars show mean ±SD of an experiment and are representative of four independent experiments. (A) PS and CT down-regulate TIMP-1 secretion from HBECs alone but do not reduce TIMP-1 further in co-stimulation. (B) PS upregulated HBEC TIMP-2 secretion in TB, but not control, stimulated HBECs. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey’s multiple comparison test: ****p<0.00001.
2.9. Platelets upregulate AEC-dependent matrix destruction

As I have shown that platelet-secreted products regulate AEC TIMPs and MMPs such as the type I collagenase MMP-1 and the gelatinase MMP-9, I next used fluorescently bound collagen and gelatin to measure the functional effect of this regulation on HBEC protease activity (Fig 50).

Type I collagenase activity was measured first (Fig 50A). Supernatant of HBECs stimulated with PS-only had no significant increase in collagenase activity compared to unstimulated cells. HBECs stimulated with CT-only had a 2-fold increase in collagen breakdown compared with controls (Fig 50A). However, co-stimulation of HBECs with PS and CT together resulted in a 3.7-fold increase in collagenase activity compared to unstimulated controls (p<0.001; Fig 50A). This was also a significant increase compared to CT-stimulated thrombin controls (p<0.05; Fig 50A).

Gelatinase activity was measured next. Similar to type I collagen, there was no significant increase in gelatin degradation measured in PS-only stimulated HBECs compared to unstimulated cells (p>0.05; Fig 50B). Upon CT-only stimulation there was a 4.5-fold increase in gelatinase activity in a clear upwards trend that bordered on significance (p=0.055; Fig 50B). Thrombin stimulation did not alter gelatin degradation compared to CT alone (p>0.05; Fig 50B). Compared to the thrombin control, co-stimulation of HBECs with PS and CT significantly MMP upregulated gelatinase activity by 3.8-fold (p<0.001; Fig 50B). The role of CD40L in this upregulation was investigated next.
Figure 50. Platelet-secreted products upregulate collagenase and gelatinase activity of airway epithelial cells.

Human bronchial epithelial cells (HBECs) were stimulated with thrombin (Thr), activated platelet supernatant (PS), cell culture supernatant of monocytes conditioned with TB (CT), or both PS and CT for 72 hours. Cell culture supernatant was then collected and incubated on (A) type I collagen and (B) gelatin that fluoresces upon degradation. Bars show mean ±SD of an experiment performed in triplicate and are representative of three independent experiments. PS upregulates TB-mediated HBEC collagenase and gelatinase activity. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey’s multiple comparison test: ns p>0.05; * p<0.05; *** p<0.0001.
2.10. CD40L alone does not induce significant AEC MMP-1 secretion

It was of interest to investigate if MMP-1 secretion by platelet-secreted factors is mediated by platelet CD40L. HBECs were stimulated simultaneously with CT and recombinant CD40L, and MMP-1 secretion compared to HBECs co-stimulated with CT and PS. CD40L did result in an upregulation of MMP-1, from 58.1 ±12.2 ng/ml in vehicle control cells to 124.7 ±15.0 ng/ml with CD40L stimulation. However, this 2.2-fold increase was not significant in the context of the experiment as a whole, as PS co-stimulated HBECS had a highly significant 8.4-fold increase in MMP-1 secretion compared to thrombin controls (p<0.0001; Fig 51). Finally, regulation of HBEC gene expression by platelet secreted factors was investigated.

![Figure 51. Platelet MMP-1 regulation in TB-stimulated airway epithelial cells is not mediated by CD40 ligand (CD40L).](image)

Human bronchial epithelial cells (HBECs) were stimulated with thrombin (Thr), activated platelet supernatant (PS), cell culture supernatant of monocytes conditioned with TB (CT), or both for 72 hours. Cell culture supernatant was then collected and extracellular MMP-1 detected by ELISA. Bars show mean ±SD of an experiment performed in triplicate and are representative of two independent experiments. CD40L stimulation of HBECs in TB does not significantly upregulate MMP-1 secretion. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey’s multiple comparison test: ns p>0.05; ****p<0.00001.
2.11. Platelet-secreted factors significantly regulate AEC MMP gene expression

Finally, I was interested to assess if platelets target AEC MMPs at the gene expression level. First, MMP-1 gene expression was measured in HBECs stimulated with PS and CT. Although CT-only induced a significant 3.1-fold upregulation of MMP-1 mRNA (p<0.05), PS-only stimulation of HBECs did not significantly upregulate MMP-1 gene expression compared with a thrombin control (p>0.05; Fig 52A). Strikingly, co-stimulation of HBECs with both CT and PS increased MMP-1 gene expression 6.0-fold more than unstimulated controls (p<0.001), and 1.7-fold compared to the thrombin control (p<0.01; Fig 52A).

Platelet regulation of MMP-10 gene expression was also measured (Fig 52B). Interestingly, PS stimulation alone was sufficient to elevate MMP-10 mRNA 3-fold, to concentrations equivalent to CT-induced MMP-10 mRNA (p>0.05; Fig 52B). Co-stimulation with both CT and PS induced a highly significant 9.5-fold increase in MMP-10 gene expression compared to controls, and a 5.5-fold increase compared to CT-stimulated thrombin controls (p<0.0001; Fig 52B).
Figure 52. Platelet-secreted products significantly regulate airway epithelial cells MMP-1 and -10 at the gene level.

Human bronchial epithelial cells (HBECs) were stimulated with thrombin (Thr), activated platelet supernatant (PS), cell culture supernatant of monocytes conditioned with TB (CT), or both CT and PS for 24 hours. Cell RNA was then harvested and gene expression of (A) MMP-1 and (B) MMP-10 measured by qRT-PCR and normalised to β-actin gene expression. Bars show mean ±SD of fold change compared to unstimulated controls. Graphs are representative of two independent experiments. PS upregulates HBEC MMP-1 and -10 gene expression in TB. Statistical analysis was performed using an ordinary one-way ANOVA with Tukey’s post hoc test: *p<0.05; **p<0.01; ****p<0.00001.
3. Discussion

The role of neutrophils and AECs in TB has never been investigated in the context of platelets. In this chapter I sought to expand our understanding of TB immunopathology through investigation of how platelets regulate these immune cells. My data indicate that platelets delay *M. tb*-triggered degranulation of neutrophils, as *M. tb* infection of neutrophils co-incubated with platelets initially resulted in significantly reduced MMP secretion compared with *M. tb*-infected neutrophils alone, but this regulation is not maintained over time. This is a novel observation, with platelets generally described as activators of neutrophils depending on stimulus and culture conditions [290, 410-412]. Whilst the role of neutrophils in TB immunopathology is a cause of debate their involvement is typically described to be detrimental, with the exception of very early responses. This is thought to be due to the rapid degranulation of bactericidal and inflammatory factors that with early effects can limit infection but if unchecked later leads to significant pathology [77, 84, 331]. The reduction of initial, but not prolonged, neutrophil secretions in platelet co-culture therefore suggests platelets could exacerbate neutrophil responses associated with TB pathology.

MMP activity was analysed and, unlike secretion, found to be reduced at all time points. Some of the prolonged downregulation in activity can be attributed to cumulative signal formed earlier. In addition, the ELISA kits used to detect MMPs and TIMPs detect total concentrations, and therefore do not reflect any changes in TIMP-MMP complexes that would inhibit activity of secreted MMP-8 and -9. However, whilst MMP-8 regulation lessened over time, MMP-9 was maintained. This suggests other factors may be involved for MMP-9. As well as TIMP binding there are other post-translational mechanisms that could be targeted by platelets to reduce MMP activity. The presence of a proteinase susceptible “bait” region in the pro-peptide allows cleavage by proteinases, but action of the MMP intermediate or other active MMPs is often required for complete removal of the pro-peptide and activation of the enzyme. This process includes several stages which could be regulated by platelets to reduce collagenase and gelatinase activity, such as inhibiting the
action of proteinase 3 and cathepsin G which activate MMPs [428, 429]. These could contribute to the continued downregulation of gelatinase activity.

The platelet-induced delay of MMP-8 and -9 secretion may also be due to a delay in maximal *M. tb* infection of neutrophils, as MMP secretion is reduced at lower MOIs [149]. Thrombin-activated platelets form tight bonds with the surface of neutrophils to yield cell aggregates [410]. Additionally, platelet aggregation and enclosure of microorganisms in platelet–fibrin matrices can initially protect bacteria from clearance by neutrophils [208]. Both platelet binding to the neutrophil surface to form a physical barrier to *M. tb* infection and platelet binding to *M. tb* to alter internalisation of the bacterium, would be facilitated by my concentrated cell culture model and may delay *M. tb*-triggered neutrophil degranulation. However, the specific downregulation in neutrophil MMP, but not cytokine, secretion, suggested a targeted mechanism of platelet regulation.

*M. tb* infection induced TNF-α and IL-8 secretion. This is in accordance with previous literature, and confirms the role of neutrophils in the early human innate and adaptive response to TB [430]. Whilst higher concentrations of TNF-α have been reported than I detected, TNF-α secretion is partially induced in a step-wise fashion from neutrophils with IL-8 priming [430, 431] and the short time span of my experiment may not be sufficient for this to take effect. Additionally, the high concentration of neutrophils in the TB lung means that together they amount to a significant source of TNF-α.

Neutrophils are known secretors of IL-1β in humans and readily secrete this inflammatory cytokine on LPS stimulation, but they are not typically implicated as a major source of IL-1β in *M. tb* [408, 432]. Interestingly, whilst *M. tb* infection alone in my model induced only a minimally detectable concentration, additional stimulation with platelets significantly upregulated this secretion. This implicates neutrophils with the potential to be a significant source of IL-1β in TB in the context of platelet intercellular networks. I did not detect IL-6, -10, -12 or MDC from neutrophils in any condition. Again this is in accordance the majority of previous literature, although IL-6 has been reported in a previous *M. tb* study using an MOI of 1 [331]. Whilst IL-6 drives T cell responses during TB and IL-6 mRNA has been shown to be upregulated in a mouse model of TB-infected with lung [433, 434], studies suggest that
activated pulmonary epithelial cells may be the major source of IL-6 in inflammatory lung diseases rather than monocyte-derived cells or neutrophils [435, 436].

Externalisation of both MMP-8 and -9 (but not IL-8 and TNF-α) has been previously associated with NETosis, including in M.tb infection [88, 150, 416, 437]. Given the down regulation of these MMPs I decided to assess platelet regulation of TB-induced NETosis. To investigate if NETosis is altered in my co-culture set up NETs were detected 4 hrs post-infection, when MMP secretion was detected as significantly downregulated. Examination of NETosis with M.tb/PMA and activated platelet stimulation revealed a downregulation compared to stimulated neutrophils without platelets. This is a novel finding as platelet regulation of NETs produced by these mechanisms has not been investigated. Notably, it has been reported that 20–60% of isolated human neutrophils release NETs 2–4 hrs after stimulation with microbes [290, 438], and it is around this time point that maximal regulation of MMP secretion was seen by platelet co-culture. The downregulation of NETosis in this model may therefore drive the observed specific downregulation of MMP-8 and -9. In addition, the “sticky” aggregates of neutrophils and platelets that were observed to form in culture and harvesting may result in the loss of some extracellular DNA and associated proteins upon sterile filtration that is required to remove the samples from the CL3 facility. To control for this variable and confirm this novel finding different measurements of NETosis should be used, such as direct visualization of NETs by fluorescence microscopy or myeloperoxidase-deoxyribonucleic acid (MPO-DNA) ELISA.

The ability of platelet-only neutrophil stimulation to induce NETosis was also assessed. An increase in NETosis with activated platelets alone was not observed in my model. Whilst this in contrast to some previously published studies there are several variations in experimental set ups that could result in differential results, with NETosis highly dependent on time point, Plt:N, and shear stress [439]. The most important variable is likely to be the thrombin activation of platelets [292]. Thrombin stimulation primarily upregulates P-selectin which is central to neutrophil-platelet binding, but studies indicate NETosis is mediated by platelet TLR-4 in a P-selectin-independent mechanism [290, 440]. A murine study has shown P-selectin dependent, thrombin-activated, platelet upregulation of NETosis [415]. However, this study did not wash platelets after activation to remove the recombinant thrombin, and the results have been suggested to be specific to mouse and rat biology [439]. As a whole
my results support studies indicating that in humans, thrombin-stimulated platelets do not upregulate NETosis alone. I then expanded my co-culture model to investigate platelet regulation of AECs using primary HBECs.

As I have shown in Chapter 3 that platelet-secreted factors are present in the human TB lung, I used PS-stimulation to replicate these networks in vitro and investigate contact-independent platelet regulation of AECs. Thrombin can alter AEC expression of a variety of factors that influence lung and airway inflammation and remodelling such as PDGF [441], ICAM-1 [426], and ATP [427]. Most studies indicate that thrombin treatment of human epithelial cells does not induce MMP-1 or MMP-9 secretion [442] but a variety of studies in tumour biology have indicated that thrombin upregulates MMPs, such as MMP-9 in U2-OS osteosarcoma cells [443] and MMP-1 in endothelial cells [444]. In the production of PS a small concentration of thrombin was carried over and so a control with an equivalent concentration was added to account for its effects in all experiments. However, no effect on MMP or TIMP expression was induced at any level or time point from the thrombin, likely due to the very low concentrations carried over.

A PS dilution of 1 in 25 (equivalent to platelet concentration of 2 x10^7 cells/ml) did not upregulate MMP-1 or -10 secretion alone, but significantly upregulated these MMPs in combination with CT. AECs have not previously been associated with significant MMP-7 production and multiplex analysis confirmed that platelets did not induce MMP-7 secretion in HBECs. Interestingly, platelet stimulation alone was sufficient to upregulate MMP-9. Although the increase was not significant in the context of the experiment as a whole, it suggests that the presence of active platelets in the lungs drive a low level MMP response that could facilitate cell recruitment. Platelet regulation of MMP-9 was dramatically augmented in combination with monocyte TB-networks, with significant secretion of MMP-9 that could not be explained by an additive effect.

There was a high background of MMP-2 from AEC, but no increase was seen in this protease upon stimulus with CT. This is in accordance with previous studies [445]. Interestingly, PS stimulus alone drove significant increases in MMP-2. This phenomenon has not been described before but supports in vivo findings such as IHC imaging of rabbit lung in a model of pulmonary fibrosis which indicated alveolar epithelial cells as the major source of MMP-2
MMP-2 degrades basement membrane, type IV collagen, gelatin, and laminin [27] and downregulates the principal chain of type I collagen, collagen α1 production leading to significant anti-fibrotic effects [447]. Therefore through MMP-2 regulation platelets may drive additional type IV collagen breakdown, and reduce type I collagen renewal. The significant inhibition of platelet-upregulated MMP-2 by CT was unexpected but supported by work in CNS TB showing that while other MMPs were upregulated, CT concurrently downregulated MMP-2 in a microglial cell line. This was partially through TNF-α activity and dependent on NFkB and caspase-8 activation. The study suggests a mechanism for the inability to detect MMP-2 from parenchymal cells in CNS TB despite MMP-2 being upregulated in the spinal fluid [182, 448, 449]. Effects such as these demonstrate the need to contextualise in vitro M. tb infection investigations, with TB networks driving profoundly different responses to direct M. tb pathology.

I next investigated additional indirect platelet regulation of MMPs through modulation of MMP regulators EMMPRIN, TIMP-1, and TIMP-2. EMMPRIN is a potent inducer of secretion of MMPs such as MMP-9. In this capacity it has been shown to drive pathology in pulmonary fibrosis and COPD, and the increased EMMPRIN levels in smokers has been localised to bronchial epithelial cells [418, 450]. In my study PS upregulated EMMPRIN secretion in HBECs to concentrations equivalent to CT, but did not function in synergy with CT to upregulated secretion further. Although TIMP-1 was also unchanged by platelet regulation in TB, in PS-only stimulation a downregulation was observed that was equivalent to concentrations in TB stimulus. In contrast, PS-only and CT-only stimulation was not sufficient to regulate TIMP-2, but co-stimulation significantly increased TIMP-2 secretion. This differential regulation of AEC MMPs and TIMPs by PS, alone and in conjunction with CT, indicates the complexity of this network of intercellular communication.

Interestingly, TIMP-2 has been localised around AECs in vivo [446, 451] and is increased in the human TB lung compared with controls [159]. Additionally, TIMP-2 has been shown to limit tissue destruction in the lung, with neutralisation exacerbating pulmonary damage in a model of acute respiratory distress [451, 452]. Despite its role in regulating inflammatory lung tissue damage, no significant increase in TIMP-2 has been reported from in vitro TB stimulus of monocytes [158, 179], THP-1 cells [453], MDMs [163], lung fibroblasts [172] or epithelial cells [171]. Thus, the data in this chapter demonstrate upregulation of AEC TIMP-2
secretion in TB for the first time, and therefore suggest a mechanism for the AEC-derived TIMP-2 described *in vivo*. In this capacity, my data suggests that in addition to enhancing MMP-driven tissue destruction, platelets in certain contexts may also play a role in dampening effects of ECM breakdown.

To investigate the impact of platelet MMP and TIMP regulation on the AEC matrix degradation a functional assay of gelatinase and type I collagenase activity was undertaken. This assay detected significant degradation of both matrix proteins, with the increase in TIMP-2 secretion not sufficient to negate activity from the dramatic increase in MMP secretion. The functional activity of MMP-1 generally mirrored the secretory pattern, with increased collagenase activity in CT-only stimulation and an additional increase with PS co-stimulation. Gelatinase activity also reflected the major trends in secretion, however, no significant gelatin degradation was observed in PS-only stimulation despite the upregulation of gelatinases MMP-2 and -9 secretion coupled with the downregulation of TIMP-1. This may be a constraint of assay sensitivity, which has limited responsiveness to low level protease activity. In addition, the limited activity may reflect a lack of sufficient enzyme activation from the pro-form, as MMP-2 is thought to be particularly regulated by pro-peptide activation due to its high constitutive expression by many cells [132]. In this manner, platelet-only regulation increases MMP-2 secretion but requires additional signalling to translate this to functional tissue destruction. *In vivo* this could place active platelets as a primer of gelatinase activity, with secreted MMP-2 only requiring activation for ECM breakdown to begin. Together these data shows, for the first time, that platelet intercellular communications are potent contributors to TB networks that drive tissue destruction by cells of the lung epithelium. I next determined if platelet-secreted CD40L was a specific and significant driver of these communications.

Epithelial cells have been shown to express CD40 both *in vitro* and *in vivo* [421]. CD40L stimulation of CD40 on epithelial cells of various types has been shown to alter the secretion of a variety of cytokines and chemokines such as IL-1, -6, -8, -12, and TNF-α. This has significant implications in the inflammatory and fibrotic pathologies of disease such as inflammatory bowel disease and renal failures [454, 455]. I therefore decided to investigate if platelet AEC MMP regulation was mediated by CD40L secretion. I observed no significant
upregulation of MMP-1 from CD40L-stimulated HBECs. This suggests that platelet regulation of these cells is not through secreted CD40L but one or a mixture of other platelet factors.

Finally, I began investigations on the intracellular mechanism of platelet AEC MMP regulation by analysis of gene expression. I showed for the first time that platelet secreted factors target AECs at the gene level. MMP-1 gene expression was not induced by PS alone, but MMP-10 mRNA was. Platelet stimulation in combination with the TB intercellular network induced dramatic upregulation of the transcription of both genes, in manner significantly greater than an additive affect. Thus, MMP-1 and -10 are differentially regulated by platelet-secreted products at the gene level but, in the context of TB, are significantly regulated by platelets in a manner that mirrors upregulation at the secretory level.

In summary, this chapter represents a highly novel study of platelet regulation of neutrophils and AECs. My investigation into platelet regulation of neutrophil responses in TB demonstrated that platelets delay neutrophil MMP secretion without a concurrent downregulation in TIMPs. This contributes to significant downregulation of collagenase and gelatinase activity which may also be mediated by downregulation of NETosis and post-transcriptional MMP regulation. In addition, I demonstrated for the first time that platelets can substantially enhance concentrations of neutrophil-derived IL-1β, but not IL-8 or TNF-α, in TB. My investigation into platelet regulation of AECs showed that active platelet networks significantly upregulate specific AEC MMP secretion and activity in TB, and can indirectly regulate MMPs further through mediation of TIMP-1, -2 and EMMPRIN. Finally, I have shown that platelet upregulation of HBEC MMP-1 is not significantly driven by soluble CD40L, but that platelet-secreted products together with TB regulate AEC MMPs at the transcriptional level. In their capacity as a driver of the AEC MMP response, platelets may be a previously unexplored factor in granuloma formation and the inflammatory response.
Chapter 6. Discussion and Future Work

1. Discussion

In this report I first used a murine model and clinical samples to investigate the presence of active platelets at the site of infection in pulmonary TB. After this, the majority of this project used *in vitro* analysis of primary human cells to comprehensively investigate platelet regulation of the cell-mediated TB immunopathology. As described throughout this project, monocytes, neutrophils and epithelial cells are amongst the earliest and most important responders to *M.tb* infection. Each of these cells have a unique contribution to TB immunopathology, with the plasticity of monocytes facilitating a complex and dynamic range of responses to infection; neutrophil activity predominately driven by the rapid release of pre-formed proteins [254] and the increasing recognition of AECs as non-traditional immune cells central to the TB immune response [33]. To date there has been no investigation of platelet regulation of neutrophil or epithelial cells in the context of TB and only a single publication on regulation of monocyte-derived cells, which focused on foamy cell development [330]. Thus, complementing a focus on monocytes with neutrophil and AEC analysis offered an exciting opportunity to expand knowledge of platelet regulation of TB immunopathology, with a focus on MMP responses.

Despite the range of cells used in this project, and the highly cell-dependent MMP regulation, platelet regulation of the major secreted collagenase and gelatinase was observed in all three cell types. Similarities were particularly apparent in platelet regulation of monocytes and AECs, which both showed platelet upregulation of MMP-1 and -9 secretion in TB, in a contact independent manner. This unilateral targeting is interesting as *in vivo* MMP-1 and -9 are amongst the most highly upregulated MMPs in TB, and drive pulmonary tissue destruction and granuloma formation [158, 166]. Early neutrophil MMP-9 and the major neutrophil collagenase MMP-8 was also regulated, but in a downward direction. Other MMPs that have also been attributed with a role in TB pathology, such as
MMP-3, -7 and -10 [130], were shown to be platelet upregulated in a more cell specific manner. Therefore my work has demonstrated that platelets potently target cells across the spectrum of the innate immune system to specifically alter collagenase and gelatinase secretion and activity in TB.

Platelets were not able to regulate MMP-1 in uninfected cells. This trend was observed for the large majority of MMPs, cytokines and chemokines measured in all cell types. This suggests that platelets mediate the responses of monocytes, neutrophils and AECs to direct and enhance inflammation upon additional appropriate stimulus, rather than driving a particular phenotype alone. A notable exception to this trend was AEC secretion of the gelatinase MMP-2, which was significantly upregulated by platelet-only stimulus. MMP-2 and MMP-9 are key to leukocyte recruitment to the lungs and, in allergic disease, they can also establish the chemotactic gradients needed for effective clearance of inflammatory cells [456, 457]. By upregulating this process, platelets would therefore enhance effective immune responses and resolution. However, in TB this limited gelatinase response was subverted. Upon co-stimulation of AECs with PS and CT, MMP-2 secretion was dramatically downregulated compared with PS alone, whilst MMP-9 secretion was upregulated further. High concentrations of MMP-9 in TB are associated with unchecked tissue destruction and TB granuloma formation [33]. By specifically driving this gelatinase, the bacteria may therefore subvert platelet MMP regulation to drive pathology. As previously reported [458], monocytes did not secrete significant MMP-2. Platelet downregulated neutrophil MMP-8 and -9 secretion was in contrast with the increase observed in monocytes and AECs but, as discussed in Chapter 5, this may also function to drive bacterial replication and chronic tissue destruction through the delay of rapid neutrophil secretory responses.

Although there were similarities between platelet-regulated secretion profiles of different cells, I have shown that these are regulated by different mechanisms. CD40L has been shown to have significant immunomodulatory effects and use of recombinant CD40L suggested a key role of this protein in inducing MMP-1 secretion from monocytes, but not AEC. Furthermore, platelet regulation of cell gene expression differed between cell types. Direct platelet contact was required for maximal MMP-1 and -10 gene expression in monocytes, whilst PS was sufficient to readily up-regulate these MMPs at the transcriptional level of AECs. TB has been shown to upregulate neutrophil MMP transcription between 2
and 24 hrs post-infection, and this is associated with increasing MMP secretion over time [149]. Although neutrophil gene expression was not demonstrated, the absence of platelet MMP regulation after 4 hrs post-infection at the secretion level strongly suggests that platelets had no significant regulation of neutrophil gene expression. Therefore, my data indicate that platelets exert MMP regulation through direct and indirect contact of cells to drive both transcriptional and post-transcriptional control in a context dependent manner.

The complex regulatory mechanisms that I have demonstrated for platelets in cell-mediated TB responses supports the paradigm of platelets as an immune barometer; interacting with environmental stimuli to sensitively moderate responses [224, 459]. For example, I have shown that whilst MMP-1 required a P:M of 100:1, the MMP-1 activator MMP-10 increased significantly at much lower ratios. This could lead to an increase in collagen degradation with minimal platelet recruitment, which then increases with additional platelet recruitment in prolonged or increasing inflammatory stimulus. The capacity of recruited platelets to mediate ECM degradation was enhanced further by data indicating that platelet-secreted P-products were sufficient to drive MMP secretion and TIMP-1 reduction by platelets alone. However, direct platelet contact was required for prolonged monocyte MMP-1 secretion, potentially due to the lack of gene expression. In addition, at high platelet concentrations of P:M 500:1, there was a sudden and dramatic suppression of MMP-10 secretion which could result in reduced pro-MMP-1 activation. Furthermore, whilst TIMP-1 was downregulated in AECs by PS-only, it was not further downregulated in CT, ensuring that some TIMP-1 was still present in the supernatant. In contrast to TIMP-1 regulation, TIMP-2 was upregulated by PS and CT co-stimulation. Whilst these TIMP concentrations were not sufficient to counteract the increase in AEC MMP activity, in vivo they may limit run-away protease activity in this highly inflammatory environment. Thus, platelets drive inflammatory tissue destruction in TB, but interact with their environment at high levels of inflammation to modulate responses and the consequent tissue damage.

The role of platelets in TB-driven ECM destruction, and the accompanying bacterial intracellular replication described in this project suggest that platelets are an ideal target for host-directed therapies. Platelet activation of LPS stimulated MDMs, resulting in increased TNF-α, IL-6, and IL-23 secretion, can be reversed by pre-incubating platelets with dexamethasone: this glucocorticoid has therefore been suggested as a promising
therapeutic approach to treating unresolved inflammation [460]. Platelets can also be
targeted using cheap and readily-available drugs such as the platelet P2Y12 inhibitor
clopidoogrel, or acetylsalicylic acid/aspirin. Aspirin has been shown to be an effective host-
directed therapy in bacterial infections such as S. aureus where it improved patient
outcomes in a manner associated with reduced S. aureus binding to platelets and platelet–
fibrin matrices in vitro [461]. Two major clinical trials supplementing treatment for TB
meningitis with aspirin have been conducted, with one showing improved survival and a
trend towards reduced strokes with the drug, whilst the other showed no significant
difference [462, 463]. However, these results are difficult to interpret as these trials did not
randomise patients for prednisolone treatment, and steroid use significantly subverts
proper platelet function. There has never been a clinical trial assessing platelet inhibition,
for example with aspirin in pulmonary TB and based on the in vitro and in vivo work detailed
in this project, this represents a significant and exciting opportunity for drug repurposing
and treatment improvement.

2. Future work

In Chapter 3, I demonstrated that there was reduced intracellular M.tb killing in monocytes.
Moving forward, it would be of interest to investigate this further. The unaltered levels of
extracellular bacteria suggests that there was no change in phagocytosis of M.tb in platelet
co-culture. However, binding and phagocytosis as well as phagosome maturation and
intracellular replication could be assessed directly using flow cytometry or confocal
microscopy. Imaging flow cytometry using GFP-expressing M.tb allows rapid quantification
of such variables in a manner that has been shown to be highly effective for monitoring
phagocytosis, phagosomal acidification, and fusion within the same sample [464]. GFP-
expressing M.tb can also be used in confocal microscopy to visually assess bacterial binding
and phagocytosis [465], and this technique has been used effectively to show that GFP-
expressing M.tb is phagocytosed by monocytes within a three-dimensional cell culture
model of TB granuloma to drive cell aggregation [162]. The recurring issue that limits the
use of such techniques is the need for prolonged sample fixation protocols to ensure M.tb is
killed before removal from the Category 3 suite for analysis. As I demonstrated that UV-killed bacteria were sufficient to drive platelet MMP upregulation in monocytes, UV-killed *M. tb* or even BCG for some of these investigations may facilitate additional study of this regulation. However, use of such alternatives for investigations into intracellular replication of *M. tb* is not possible, and so optimised protocols or use of other facilities would ideally be utilised to allow use of appropriate virulent *M. tb* strains.

It would also be of interest to continue to elucidate the intracellular mechanism of MMP regulation which I began in Chapter 4. I have shown that secreted platelet factors used post-transcriptional regulation in monocytes to drive initial monocyte MMP-1 and -10 secretions. As discussed in Chapter 4 there are a range of processes that could be targeted for this, with platelet secreted factors shown to target a number of relevant post-transcriptional regulators. For example, P-selectin bound to platelet surfaces upregulates monocyte COX-2 at the transcriptional level. Whilst these transcripts are rapidly degraded in isolation, other platelet signalling proteins such as PDGF enhance the stability of COX-2 RNA by enhancing phosphorylation of CUG-binding protein 2 (CUGBP2), which then binds to AREs in the COX-2 3′-UTR and protects it from degradation [466] and by enhancing ARE binding of the mRNA stability factor HuR [467]. Investigations into RNA binding proteins known to be involved in MMP regulation, such as HuR and TTP [140, 468], would provide novel understanding of platelet regulatory mechanisms in general, in addition to enhancing our understanding of MMP and TB responses. Analysis of platelet-secreted factors that drive MMP secretion should also be expanded. Use of monoclonal antibodies to block receptors such as CD40 on monocytes and AEC would complement my data using recombinant protein stimulation in assessing dependency of indirect platelet regulation on analytes such as CD40L.

To complement my western blot analysis of platelet and monocyte total protein, future work could specifically investigate monocyte intracellular regulatory proteins using flow cytometry. This was attempted as described in Chapter 4, however, platelet upregulation of MMP-1 with UV-killed *M. tb* allows opportunity to revisit this methodology without the need for prolonged fixation protocols. UV-killed *M. tb* could be used in combination with western blots of H37Rv stimulus to build a comprehensive picture of platelet regulation of monocyte intracellular signalling. The siRNA knock-down of monocyte intracellular regulators would also provide more specified targeting of proteins compared to the chemical inhibitors used.
in Chapter 4. This mechanism would also allow use of platelet co-culture instead of PS, as the siRNA transfection would be limited to monocytes. SiRNA knockdown would be particularly useful for further investigations of HIF-1α, where it could reveal the HIF-1 dependency of platelet-upregulated MMP-1 secretion. NFkB knockdown could be used to confirm the results of chemical inhibition in MDMs that indicated that *M. tb* HIF-1α stabilisation is gene expression dependent, and these investigations expanded to monocyte-platelet co-culture.

In addition to driving HIF-1α gene expression, a downregulation in PHD-1 was observed in *M. tb* infection and such post-translational mechanisms should be explored further both with and without platelet culture. *M. tb* and platelets may target various post-translational mechanisms such as a reduction of PHD protein or activity, involvement of other mechanisms such as microRNA, or changes to hydroxylation, phosphorylation and acetylation of HIF-1α which enhance not only HIF-1α stability but also activity [381-384]. These have never been investigated in the context of TB. It would also be of interest to assess platelet caspase-1 regulation, particularly in monocytes. Although cytokine secretion of AEC was not carried out and remains a topic of future work in itself, IL-1β was specifically upregulated in both monocytes and neutrophils. As discussed in Chapter 3 this suggests that platelets may regulate caspase-1, as it is required for IL-1β processing. This could be investigated initially by measuring relative caspase activation in platelet co-culture compared to monocytes alone, and expanded using chemical inhibition, siRNA knock-down, and functional outputs of the caspase-1-NLRP3 inflammasome.

In addition to monocyte and AEC intracellular signalling mechanisms, neutrophil signalling would also be of interest as a topic of future work. Previous investigations have demonstrated that neutrophils are differentially regulated in direct *M. tb* infection compared to TB network stimulus, and within these stimuli MMP-8 and -9 are also differentially regulated due to their different granule storage [149]. Whilst *M. tb*-infection did not upregulate MMP-8 or -9 secretion through ERK or p38 MAP kinase phosphorylation, CT-stimulation did. It would therefore be of particular interest to investigate these proteins in the context of co-stimulation with both *M. tb* and platelet network effects. These proposed investigations into platelet targeting of intracellular signalling of immune cell MMPs would represent completely novel investigations in a still poorly understood area of TB pathology.
This project included the first investigation of platelet regulation of \textit{M.tb}- and PMA-induced NETosis. The results showed a downregulation in NETosis with co-culture of cells, but in this co-culture condition there was concern that some NETs may be lost in the sterile filtration process. This result must therefore be confirmed using immunofluorescence to visualise NETs directly, for example using DAPI DNA and histone 2B staining as well staining for associated granule proteins such as myeloperoxidase and MMP-8 [150]. The methodology used to investigate NETosis replicated secretory conditions to demonstrate a possible mechanism for the reduced MMP secretion, as discussed in Chapter 5. It indicated that activated platelets alone did not regulate NETosis which was in contrast to some previous reports that indicate that platelets upregulate the process of NETosis [469]. As discussed in Chapter 5 this lack of regulation was likely due to different platelet activation mechanisms and time points. In the future it would therefore be of interest to investigate platelet regulation of NETosis in TB more generally with NETosis data typically generated using TRAP- or LPS-activated platelets at shorter time points around 1 hr [290, 470]. To investigate this further in the context of TB, use of a lower \textit{M.tb} MOI may also yield interesting results. Recently, macrophage extracellular traps (METs) have been shown to be induced by another \textit{Mycobacterium}, \textit{Mycobacterium massiliense}, to aid interaction of the bacteria with cells and facilitate an environment for bacterial aggregation which is associated with mycobacterial survival and growth [471]. This is an exciting area of bacterial responses that in future should be explored in the context of platelets, particularly in light of the role of platelets in regulating monocyte/macrophage in mycobacterial infection described in this project.

Throughout this investigation I used primary cells and virulent \textit{M.tb} to maintain maximal possible clinical relevance. However, replication of this model with leukocytes and platelets from TB patients with and without platelet activation would provide significant further insight into MMP and cytokine responses as well as intracellular killing. Comparison of TB patient cellular responses to healthy controls may also reveal interesting trends to support \textit{in vivo} hypotheses. As platelets have already been shown to be active in TB, monocyte MMP and cytokine trends induced without \textit{in vitro} platelet activation should be investigated. Production of PS from TB patients and healthy controls in platelet co-culture may also reveal differences in platelet responses and AEC stimulation.
In addition to the clinical work, the development of an appropriate animal model would be an invaluable asset for future work on this project to develop our understanding of the clinical importance of platelets in tissue destruction. Animal investigations would allow the \textit{in vivo} assessment of the role of platelet inhibition in TB immunopathology, including regulation of tissue destruction and bacterial replication. Having already verified that platelets are present in Balb/C mouse lungs (Fig 10), a murine model of pulmonary TB could be utilised, but this model has the disadvantage of not developing mature TB granulomas and tissue destruction as seen in human disease. However, cavities have been recently reported in C3HeB/FeJ and CBA/J mice \cite{472} and this provides a new perspective on the usefulness of such models. Additionally, it has previously been shown that mice with an CS7BL6 background that express human MMP-1 under control of the scavenger receptor A promoter-enhancer have caseous necrosis in pathology much closer to that of humans \cite{162}, and such a model could be used for investigation into platelet regulatory role. A guinea-pig model of TB is also well established and although cavitation rarely develops, they do naturally have mature TB granuloma formation similar to humans \cite{473} and have been successfully used to test TB treatment doxycycline for MMP regulation \cite{18}. Thus, although the complete pathology is not replicated by any animal model, a variety of models exist that must be utilised in future work to provide a stepping stone towards translating my \textit{in vitro} findings to clinical improvements.

### 3. Final Conclusions

This project represents the most thorough investigation of platelet function in TB undertaken to date. It demonstrates for the first time that platelets functionally upregulate monocyte and AEC ECM destruction in TB. I have also shown that platelets regulate cytokine and chemokine profiles, reduce monocyte intracellular \textit{M.tbc}-killing, and delay neutrophil responses. Together the data generated from this project suggest that platelets have a detrimental impact in TB, driving wide-spread tissue destruction and inhibiting bacterial clearance. The use of primary human cell types that together represent key components of the TB immune response builds a comprehensive and highly novel image of platelets as
dynamic cells which drive TB immunopathology and have significant potential as a realistic and effective immunotherapy target.
23. Prevention, A.T.S.a.C.f.D.C.a., Diagnostic Standards and Classification of Tuberculosis in Adults and Children. This official statement of the American Thoracic Society and the Centers


Ong, C.W., Neutrophils and the regulation of matrix metalloproteinases in tuberculosis, in Medicine. 2013, Imperial College London: London, UK.


HEMATOGENOUS METASTASIS.

Integrin Mediates Interaction of Melanoma Cells with Platelets A CONNECTION TO


Dependent shedding of soluble CD40 ligand (sCD40L) from activated platelets.

Reinbo


Sawicki, G., et al., 2009.

AlphaIIbbeta3.

Choi, W.S., et al., 2008.

Coronary artery of myocardial infarction.


Myocardial infarction.

Pearce, E., et al., 2005.

PAR1 at a Cryptic Ligand Site.

Trivedi, V., et al., 2002.

Function.

Galt, S.W., et al., 2008.

Blood platelets and their inhibitors.


Room temperature activates human blood platelets.


Mastenbroek, T.G., et al., 2008.

Formation and Exert Local Collagenolytic Activity.

Mastenbroek, T.G., et al., 2008.


Araki, J., et al., 2008.

Tissue Eng Part C Methods.

Room temperature activates human blood platelets.


Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood.

Platelets, neutrophils, and neutrophil extracellular traps (NETs) in sepsis.

Ma, A.C. and P. Kubes, 2011.

Platelet TLR4 activates neutrophil extracellular traps (NETs) in sepsis.


Galt, S.W., et al., 1997.

Blood platelets


Mammalian platelets and their inhibitors.

Santos, B.S., et al., 2011.


Front Immunol, 2016. 7: p. 271.


Platelet indices: laboratory and clinical applications.


Sachais, B.S., Platelets may serve up biomarkers.


Role of metalloproteinases in platelet function.


Platelets and matrix metalloproteinases.


Mastenbroek, T.G., et al., 2007.

Platelet-Associated Matrix Metalloproteinases Regulate Thrombus Formation and Exert Local Collagenolytic Activity.


Response MMP-9 in platelets: maybe, maybe not.


Araki, J., et al., 2012.


Room temperature activates human blood platelets.

Galt, S.W., et al., Outside-in signals delivered by matrix metalloproteinase-1 regulate platelet function.


Trivedi, V., et al., Platelet Matrix Metalloprotease-1 Mediates Thrombogenesis by Activating PAR1 at a Cryptic Ligand Site.


Pearce, E., et al., Haplotypic effect of the matrix metalloproteinase-1 gene on risk of myocardial infarction.


Suzuki, H., et al., Elevation of matrix metalloproteinases and interleukin-6 in the culprit coronary artery of myocardial infarction.


Choi, W.S., et al., MMP-2 regulates human platelet activation by interacting with integrin alphallibeta3.


Reinboldt, S., et al., Preliminary evidence for a matrix metalloproteinase-2 (MMP-2)-dependent shedding of soluble CD40 ligand (sCD40L) from activated platelets.


Momi, S., et al., Loss of matrix metalloproteinase 2 in platelets reduces arterial thrombosis in vivo.


Erpenbeck, L. and M.P. Schon, Deadly allies: the fatal interplay between platelets and metastasizing cancer cells.


Lonsdorf, A.S., et al., Engagement of alpha IIb beta 3 (GPIIb/IIIa) with alpha nu beta 3 Integrin Mediates Interaction of Melanoma Cells with Platelets A CONNECTION TO HEMATOGENOUS METASTASIS.


359. Turner, J., et al., Deficiency in interleukin 10 reverses the susceptibility phenotype of CBA/J mice during infection with Mycobacterium tuberculosis. Journal of Immunology, 2012. 188.


multifarious, and multifaceted.

Gr

COPD.


Jouneau, S., et al., hemostasis and inflammation.


Dallenga, T. and U.E. Schaible, Neutrophils in tuberculosis--first line of defence or booster of disease and targets for host-directed therapy? Pathog Dis, 2016. 74(3).


Bibliography


Abbreviations

**ADP**: Adenosine 5′-diphosphate

**AEC**: Airway epithelial cell

**ALI**: Acute lung injury

**AMPK**: AMP-activated protein kinase

**APR**: Acute phase response

**ARE**: AU-rich elements

**ATP**: Adenosine triphosphate

**BALF**: Bronchoalveolar lavage

**BEpiCM**: Bronchial Epithelial Cell Medium

**BCG**: Bacillus Calmette–Guérin

**BSA**: Bovine serum albumin

**CCL**: CC chemokine ligand

**CD40L**: CD40 ligand

**CFU**: Colony forming units

**CL3**: Category level 3

**CNS**: Central nervous system

**COPD**: Chronic obstructive pulmonary disease

**COX**: Cyclooxygenase

**CR-2**: Complement receptor type 2

**CSF**: Cerebral spinal fluid

**CT**: Monocyte media conditioned with TB

**CUGBP2**: CUG-binding protein 2

**DC**: Dendritic cell

**DMEM**: Dulbecco’s Modified Eagle Medium
DTT: Dithiothreitol
ECM: Extracellular matrix
EMMPRIN: Extracellular matrix metalloproteinase inducer
ERK: Extracellular signal-regulated kinase
ESAT-6: Early secreted Ag of 6 kDa
FBS: Foetal bovine serum
FSC: Forward scatter
g: Relative centrifugal force/ G-force
G-CSF: Granulocyte colony stimulating factor
GLUT-3: Glucose transporter 3
GPCR: G-protein-coupled receptor
HBECs: Human bronchial epithelial cells
HBSS: Hanks' Balanced Salt Solution
HIF: Hypoxia-inducible factor
HIV: Human immunodeficiency virus
HNP: Human neutrophil peptide
hr: Hour
HRE: Hypoxic response element
ICAM-1: Intercellular Adhesion Molecule
IDO: Indoleamine 2, 3-dioxygenase
IFN: Interferon
Ig: Immunoglobulin
IHC: Immunohistochemistry
IL: Interleukin
iNOS: Inducible nitric oxide synthases
IRF: Interferon regulatory factor
**Abbreviations**

**ISD**: Iron-regulated surface determinant

**IQR**: Interquartile range

**Jak/STAT**: Janus kinase/signal transducers and activators of transcription

**LDH**: Lactate dehydrogenase

**LPA**: Lysophosphatidic acid

**LPS**: Lipopolysaccharide

**MACS**: Magnetic antibody cell sorting

**MAPK**: Mitogen activated protein kinases

**MCP-1**: Monocyte chemotactic protein 1

**M-CSF**: Macrophage colony stimulating factor

**MDC**: Macrophage derived chemokine

**MDM**: Monocyte-derived macrophage

**MDR**: Multidrug resistance

**MEK**: Mitogen-activated protein kinase kinase

**MGC**: Multinucleated giant cells

**MIG**: Monokine induced by gamma interferon

**MIP**: Macrophage inflammatory protein

**min**: Minute

**MMP**: Matrix metalloproteinase

**MT-MMP**: Membrane-bound MMPs

**MOI**: Multiplicity of infection

**M:P**: Monocyte:platelet

**MPV**: Mean platelet volume

**mTOR**: Mechanistic target of rapamycin

**M.tb**: *Mycobacterium tuberculosis*

**NET**: Neutrophil extracellular trap
Abbreviations

**NFkB** Nuclear factor kappa B

**NHBE**: Normal human bronchial epithelial cell

**NK**: Natural killer cells

**NO**: Nitric oxide

**NOD**: Nucleotide-binding-oligomerisation-domain

**OCS**: Open canalicular system

**OD**: Optical density

**PADA**: Platelet adherence protein A

**P-AKT**: Phosphorylated AKT

**PAMP**: Pathogen associated microbial pattern

**PAR-1**: Protease-activated receptor-1

**PBMC**: Peripheral blood mononuclear cell

**PBP**: Platelet basic protein

**PDM**: Platelet-derived microparticles

**PE**: Phycoerythrin

**P-ERK**: Phosphorylated extracellular signal-regulated kinase-1/2

**PF4**: Platelet factor 4

**PGE**: Prostaglandin E

**PHD**: Prolyl hydroxylase

**PI3K**: Phosphatidylinositol-4,5-bisphosphate 3-kinase

**PMP**: Platelet microbicidal proteins

**Plt:N**: Platelet:neutrophil

**P-p38**: Phosphorylated p38

**PRR**: Pattern recognition receptors

**PS**: Platelet supernatant

**PSGL-1**: P-selectin glycoprotein ligand 1
Abbreviations

R: Spearman’s rank correlation coefficient

RANTES: Released upon activation, normal T-cell expressed and secreted

ROS: Reactive oxygen species

rpm: Rotations per minute

RPMI: Roswell Park Memorial Institute

RSV: Respiratory syncytial virus

SD: Standard deviation

SDS: Sodium dodecyl sulfate

sec: Second

siRNA: Small interfering RNA

SSC: Side scatter

T-AKT: Total AKT protein

TB: Tuberculosis

TB-IRIS: TB-associated immune reconstitution inflammatory syndrome

T-ERK: Total extracellular signal-regulated kinase -1/2 protein

TGF: Tumour growth factor

Th: T helper

TIMP: Tissue inhibitors of matrix metalloproteinase

TLR: Toll-like receptor

TNF-α: Tumour Necrosis Factor alpha

T-p38: Total p38 protein

TST: Tuberculin skin test

UTR: Untranslated regions

UV: Ultraviolet

V: Volts

V-CAM: Vascular cell adhesion molecule
**XDR-TB**: Extensively drug resistant TB

**WT**: Wild-type