Variation in beta-defensin expression in the control of *Mycobacterium tuberculosis*

Mr Joshua Rosenheim

Division of Infection and Immunity

University College London

Supervisor: Professor Mahdad Noursadeghi

Thesis for Doctor of Philosophy

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Abstract

Tuberculosis is a bacterial infection caused by *Mycobacterium tuberculosis* (Mtb) and remains a leading cause of death by an infectious agent worldwide. The intracellular survival and replication of Mtb within macrophages is a determinate of Mtb persistence and pathogenesis. Our understanding of the physiological mechanisms behind human macrophage restriction of Mtb remains limited. Beta-defensins are antimicrobial peptides proposed to mediate antimicrobial restriction of Mtb. I investigated their induction and cellular source in the tuberculin skin test (TST) as an in vivo human experimental challenge model. This model revealed striking inter-individual variation in expression of beta-defensins independent of interferon-gamma (IFNγ). To elucidate the causes of expression variation in TST RNA-sequencing data, genome-wide single nucleotide polymorphism array profiling, gene modules analysis, and typing of genetic copy number were used. These data attributed inter-individual variation in expression to both differences in genetic copy number and variation in cytokine signalling upstream of beta-defensins. Beta-defensins are expressed by epithelial and myeloid cells, but it was not known which cell types were producing beta-defensins in vivo during anti-Mtb immune responses. To address this knowledge gap, single cell RNA-sequencing (scRNA-seq) and spatial gene expression with RNAscope fluorescence in situ hybridisation (FISH) were employed, revealing inducible expression exclusively limited to epithelial cells. Publicly available scRNA-seq and assay for transposase-accessible chromatin using sequencing (ATAC-seq) data were interrogated to support conclusions. Whether beta-defensins represent a physiologically important mechanism of antimicrobial defence against Mtb required further study. Using a fluorescent Mtb-infection model, I quantified intracellular and extracellular Mtb growth in human monocyte-derived macrophage (MDM) culture by flow cytometry. I demonstrated variable production of beta-defensins by airway epithelial cells in vitro and found no effect of secreted beta-defensins on macrophage control of Mtb. Taken together these data suggest beta-defensins do not contribute towards antimicrobial restriction of Mtb early during infection in humans.

Impact statement

Our understanding of protective immunity against Mtb infection remains limited. Insights from human observational data and animal models highlight the importance of cell-mediated immunity, namely macrophage responses augmented by Mtb-specific Th1 cells. Whilst such responses are often necessary for protection, they are not sufficient, and many who develop active TB do not have inferior cell-mediated immunity. Additionally, there exists evidence that individuals can achieve protection independent of IFNγ. TB is a complex disease and up to 95% of exposed individuals never progress to active TB. Elucidating the mechanisms by which the immune system achieves control of infection are crucial in our efforts to develop efficacious vaccines and stratify patients most at risk of disease.

An in vivo human experimental Mtb challenge model revealed highly variable beta-defensin expression among healthy, Mtb-sensitised participants. The expression of beta-defensins are one of the earliest immune responses to respiratory infection with the potential to impact the establishment of Mtb within a host. For the first time, I reveal a complex regulatory network in vivo in which genetically encoded copy number variation and inter-individual differences in cytokine responses influence their expression in TB. I also present evidence supporting beta-defensins as part of an IL-17-mediated immune response.

Numerous studies describe beta-defensins as directly antimycobacterial peptides but do not address the physiological significance of this Mtb-killing activity. I demonstrate that physiological concentrations of pneumocyte-derived beta-defensins are insufficient at restricting Mtb growth in macrophages. This is an important step towards elucidating the role of beta-defensins in vivo and suggests they do not function to directly kill Mtb. This discovery necessitates a change in focus for beta-defensins in TB. Future studies should aim to address a role for beta-defensins in augmenting IL-17 immunity in vivo**.**

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Abbreviations

ABS – Pooled type AB human serum
AEC – Airway epithelial cell
AMP – Antimicrobial peptide
ANOVA – Analysis of variance
ATAC-seq – Assay for transposase-accessible chromatin using sequencing
ATII – Alveolar type II pneumocytes
AUROC – Area under the receiver operating characteristic curve
BAL – Bronchoalveolar lavage
BCG – Bacillus Calmette-Guerin
BCL – Binary Base Call
BDRU – Beta-defensin repeat unit
BSL-3 – Biosafety level 3
CC – Cytokine cocktail
CCA – Canonical correlation analysis
CCL – Chemokine ligand
CCR – Chemokine receptor
CD – Cluster of differentiation
CDNA – Complementary deoxyribonucleic acid
CEBPB – CCAAT enhancer-binding protein beta
CFP-10 – 10 kDa culture filtrate protein
CFU – Colony forming unit
CHIM – Controlled human infection model
CITE-seq – Cellular indexing of transcriptomes and epitopes using sequencing
CNV – Copy number variation
DC – Dendritic cell
DMEM – Dulbecco's Modified Eagle Medium
DNA – Deoxyribonucleic acid
E2C – E2-crimson
ECACC – European Collection of Authenticated Cell Cultures
ELISA – Enzyme-linked immunosorbent assay
EQTL – Expression quantitative trait loci
ESAT-6 – Early secretory antigenic target 6 kDa
FC – Fold change
FCS – Foetal calf serum
FISH – Fluorescence in situ hybridisation
FFPE – Formalin-fixed, paraffin-embedded
FSC – Forward scatter
GDA – Global Diversity Array-8
GFP – Green fluorescent protein
GM-CSF – Granulocyte-macrophage colony-stimulating factor

GWAS – Genome-wide association studies
HaAEC – Human primary alveolar pneumocytes
HBD – Human beta-defensin
HiDi – Highly deionized
HIRV-TB – Human immune response variation in tuberculosis
HNP – Human neutrophil peptide
HRC – Human random control
IFN – Interferon
IGRA – Interferon gamma release assay
IL – Interleukin
INH – Isoniazid
ISH – In situ hybridisation
Kb – Kilobase
KC – Keratinocyte
KDa – Kilodalton
LOF – Loss-of-function
LPS – Lipopolysaccharide
LTBI – Latent tuberculosis infection
Lys – Lysine
MAIT – Mucosal Associated Invariant T
ManLAM – Mannose-capped lipoarabinomannan
Mb – Megabase
M-CSF – Macrophage-colony stimulating factor
MDM – Monocyte-derived macrophages
MHC – Major histocompatibility complex
MIC – Minimum inhibitory concentration
MMP – Matrix metalloproteinases
MOI – Multiplicity of infection
MR1 – MHC class I-related protein 1
MRNA – Messenger ribonucleic acid
Mtb – *Mycobacterium tuberculosis*
NF-κB – Nuclear factor-κB
NK – Natural killer
NKT – Natural killer T
NLRP3 – NLR family pyrin domain containing 3
NHP – Non-human primate
NRAMP1 – Natural resistance-associated macrophage protein 1
OD – Optical density
PAMP – Pathogen-associated molecular pattern
PBS – Phosphate-buffered saline
PCA – Principal component analysis
PCR – Polymerase chain reaction
PDIM – Phthiocerol dimycocerosate

PFA – Paraformaldehyde
PRR – Pattern recognition receptor
PRT – Paralogue ratio test
QC – Quality control
QPCR – Quantitative polymerase chain reaction
RD1 – Region of difference 1
RhBD – Recombinant human beta-defensin
RNA – Ribonucleic acid
RNA-seq – Ribonucleic acid sequencing
RNS – Reactive nitrogen species
ROS – Reactive oxygen species
ROX400 – MapMarker® 400 X-Rhodamine
RPMI – Roswell Park Memorial Institute Medium 1640
RT – Reverse transcription
ScRNA-seq – Single cell ribonucleic acid sequencing
ScSHC – Single-cell significance of hierarchical clustering
SNP – Single nucleotide polymorphism
TB – Tuberculosis
TBE – Tracheobronchial epithelial
TCR – T cell receptor
Th – T helper
TLR – Toll-like receptor
TNF – Tumour necrosis factor
TPM – Transcripts per million
TST – Tuberculin skin test
Ub – E3-Ubiquitin
UMAP – Uniform manifold approximation and projection
UMI – Unique Molecular Identifier

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1. Introduction

1.1. Tuberculosis

Tuberculosis (TB) is a disease predominantly resulting from infection with *Mycobacterium tuberculosis* (Mtb) and a leading cause of death from an infectious agent worldwide (Bagcchi, 2023). It is estimated that in 2021 there were 10.6 million cases of TB disease globally resulting in 1.6 million deaths, an unfortunate increase from 2020, reversing many years of gradual decline and reflecting the widespread healthcare disruptions due to the Covid-19 pandemic (Bagcchi, 2023). Prevalent immunological memory for Mtb, reflected in positive interferon gamma release assays (IGRA) or tuberculin skin tests (TST), is thought to represent a far greater number who experience subclinical, asymptomatic latent TB infection (LTBI) (World Health Organization, 2018). Modelling predicts 5-10% of those infected develop active disease (Houben and Dodd, 2016) in which the uncontrolled growth of Mtb drives lung inflammation and pathology. Most cases of active disease develop within 2 years of the initial infection (Behr, Edelstein and Ramakrishnan, 2018) but can occur decades later (Lillebaek *et al.*, 2002). Current TB treatment relies on antibiotics administered over the course of several months (Furin, Cox and Pai, 2019). Despite treatment combining several drugs, the long duration of antibiotic courses increases the risk of multidrug-resistant TB (MDR-TB) developing (Dheda *et al.*, 2014). In 2021 an estimated 450,000 cases of disease were multidrug-resistant or rifampicin-resistant TB (MDR/RR-TB). Although just 3.6% of new cases were MDR/RR-TB, a concerning 18% of cases occurred among those who were previously treated. MDR/RR-TB treatment requires personalisation and substantially longer treatment durations (World Health Organisation, 2022).

Recent advances in the field include improved diagnostics (Boehme *et al.*, 2010), new antibiotics for the treatment of drug-resistant TB (Nyang'wa *et al.*, 2022), and a promising vaccine eliciting three years of protective immunity (Tait *et al.*, 2019). Additionally, shorter treatment regimens for drug-susceptible TB have been proposed with the added advantage of reduced patient loss to follow-up (Borisov *et al.*, 2018; Menzies *et al.*, 2018; Swindells *et*

al., 2019; Paton *et al.*, 2023), but there remain concerns over the higher prevalence of relapse when compared to standard regimens. The development of tools that inform clinical decision-making around when to end treatment will be complimentary to this approach, such as the use of sensitive host transcriptional biomarkers for short-term TB risk (Gupta *et al.*, 2020).

Blood transcriptional biomarkers can accurately discriminate active TB from latent TB, both in high burden settings and independent of HIV status (Roe *et al.*, 2016; Gupta *et al.*, 2020; Turner *et al.*, 2020). Many of these biomarkers score transcriptomic changes in circulating immune cells, indicative of incipient TB but not causal, that predate the development of clinically presenting active TB (Zak *et al.*, 2016; Gupta *et al.*, 2020). This offers the ability to screen individuals most at risk of developing TB in the short-term and therefore in need of preventative therapy, before the onset of symptoms and immunopathology. However, such biomarkers reflect Mtb-agnostic canonical immune responses to many infections and cannot be considered specific for TB, thus limiting their sensitivity (Mulenga *et al.*, 2021). Phenotypic T cell signatures assessed by flow cytometry also discriminate stages of TB but with additional technical complexity over blood tests (Halliday *et al.*, 2022).

Despite such advances, our basic understanding of what constitutes protective immunity remains limited. There exists extensive disease heterogeneity and evidence of concurrent disease progression and resolution within a single individual (Barry *et al.*, 2009; Lin *et al.*, 2014). Complex immunological responses within distinct granuloma may dictate the outcome of granulomatous inflammation (Gideon *et al.*, 2022), thus we can no longer assume uniformity in the immune response within an infected organ. Instead, each foci of disease should instead be viewed as a distinct host-pathogen interaction. The exact immune processes governing such outcomes remain to be elucidated and are a significant limitation in the progression of the field.

1.2. Mycobacterium tuberculosis

Mtb is a member of the *Mycobacteriaceae* family of aerobic and non-motile bacilli that are characteristically resistant to conventional acidic decolourisation (Forbes *et al.*, 2018). The

family is divided into slow and fast-growing bacteria (Tsukamura, 1967). Mtb is slow growing making laboratory research time-consuming. Whilst only a handful of family members cause serious disease in humans, immunocompromised individuals are susceptible to infection from numerous non-tuberculous mycobacteria (Henkle and Winthrop, 2015).

The mycobacterial cell envelope is a complex structure with up to 8-fold more lipid content than other gram-positive bacteria (Daffé, 2015). The envelope comprises an inner plasma membrane similar to most prokaryotes (Jackson, 2014). Surrounding the membrane is a polysaccharide-rich cell wall covalently bound to mycolic acids and interspersed with numerous other lipids (Jackson, 2014; Jankute *et al.*, 2015). The synthesis of mycolic acid is targeted by key anti-TB drugs (Winder and Collins, 1970; Winder, Collins and Whelan, 1971).

As a facultative intracellular pathogen, Mtb dedicates a significant proportion of its genome to encoding immune evasion proteins, being well adapted for survival within macrophages (McKinney *et al.*, 2000; Noss *et al.*, 2001; Raynaud *et al.*, 2002; Rengarajan, Bloom and Rubin, 2005). Mtb encodes approximately 4,000 genes with 5 times the number of enzymes dedicated to lipid metabolism than *E. coli* (Cole *et al.*, 1998). Many of these capsule lipids modulate the human immune response to benefit Mtb (Chan *et al.*, 1991; Axelrod *et al.*, 2008; Murry *et al.*, 2009; Day *et al.*, 2014; Cambier *et al.*, 2017; Quigley *et al.*, 2017). Nearly 10% of the genome comprises members of PE and PPE protein families (Cole *et al.*, 1998), several of which are secreted by type VII secretion systems (Abdallah *et al.*, 2009; Bottai *et al.*, 2012; Sayes *et al.*, 2012) and appear to be important virulence factors (Iantomasi *et al.*, 2012; Saini *et al.*, 2016). Early secretory antigenic target 6 kDa (ESAT-6) and 10 kDa culture filtrate protein (CFP-10) proteins are major immunodominant antigens in TB (Skjøt *et al.*, 2000). The genes for both ESAT-6 and CFP-10 reside in the region of difference 1 (RD1) genomic locus, the loss of which is central to the attenuation of *M. bovis* in the live-attenuated vaccine Bacillus Calmette-Guerin (BCG) (Mahairas *et al.*, 1996; Pym *et al.*, 2002; Hsu *et al.*, 2003; Lewis *et al.*, 2003).

Mtb does produce a toxin, demonstrated to trigger eukaryotic cell death by necrosis (Danilchanka *et al.*, 2014; Pajuelo *et al.*, 2021), but toxins are not generally thought to drive disease. Consensus is that pathology in active TB arises from a dysregulated immune response, driving the destruction of lung tissue and failure of the organ. Thus, it is essential that we understand the components of host immunity that prevent progression to active TB.

1.3. Pathogenesis of human Mycobacterium tuberculosis infection

Inhaled aerosolised droplets containing the bacillus first contact lung-resident alveolar macrophages and alveolar epithelial cells. Key host pattern recognition receptors (PRRs), including Toll-Like Receptors (TLRs), C-type lectin receptors, and scavenger receptors on these cells recognise mycobacterial pathogen-associated molecular patterns (PAMPs) and respond by secreting pro-inflammatory cytokines and chemokines to recruit leukocytes to the lung (Ernst, 1998; Whitsett and Alenghat, 2015).

TB is a granulomatous inflammatory disease in which macrophages control the slow-growing bacteria by engulfing extracellular Mtb (Pai *et al.*, 2016). However, early during infection, alveolar macrophages represent an intracellular niche for Mtb (Cohen *et al.*, 2018). Soon after, blood monocytes migrate into the lung in a CCL2-dependent manner (Lu *et al.*, 1998; Cambier *et al.*, 2017). However, these also fail to control Mtb growth (Aston *et al.*, 1998, p. 199; Cambier *et al.*, 2017) and may serve as an intracellular niche in the absence of T cell-mediated macrophage activation (Leemans *et al.*, 2005). In contrast, total abrogation of macrophages in zebrafish let to substantially higher *M. marinum* burden (Clay *et al.*, 2007, p. 200), indicating that they do control infection some degree in zebrafish TB models.

Granuloma centres that undergo necrosis are a hallmark of TB pathology (Dheda, Barry and Maartens, 2016), but the mechanism of infected cell death at earlier stages of disease has also been implicated in pathogenesis. Apoptotic cell death is generally viewed to be protective as it reduces viable bacteria and augments antigen presentation (Oddo *et al.*, 1998; Keane, Remold and Kornfeld, 2000; Schaible *et al.*, 2003; Hinchey *et al.*, 2007; Martin *et al.*, 2012). Conversely, necrosis facilitates escape of large numbers of viable bacteria into the

extracellular space (Gan *et al.*, 2008; Lerner *et al.*, 2017). Virulent Mtb strains appear to induce more necrosis and less apoptosis of infected cells (Keane, Remold and Kornfeld, 2000; Danelishvili *et al.*, 2003).

Failure of macrophages to clear Mtb precipitate formation of a granuloma, a large multinucleated cellular aggregate with surrounding immune cells (Ramakrishnan, 2012). The molecular mechanisms driving macrophage fusion remain poorly understood (Pereira *et al.*, 2018). The formation of granulomas was thought to require Mtb-specific adaptive immunity (Saunders and Cooper, 2000), but studies in zebrafish have shown that granulomas form in the absence of an adaptive immune system (Davis *et al.*, 2002). Granulomas can be established by a single bacterium (Lin *et al.*, 2014), highlighting its effectiveness at evading macrophage killing mechanisms. There exists vast heterogeneity in granuloma states between and within individuals. Infected individuals can present with several granulomas of different trajectories (Barry *et al.*, 2009; Lin *et al.*, 2014), influenced by bacterial burden and the tissue microenvironment (Gideon *et al.*, 2022). Typically, bacterial burden in granulomas decreases following maturation of the adaptive immune response (Wolf *et al.*, 2008; Lin *et al.*, 2014).

However, early formation of organised granulomas favours Mtb survival and dissemination (Davis and Ramakrishnan, 2009; Cronan *et al.*, 2016; Gautam *et al.*, 2018). The appearance of macrophage aggregates coincided with a doubling of viable Mtb (Volkman *et al.*, 2004), dependent on RD1. Disrupting the organisation of the granuloma improved survival (Cronan *et al.*, 2016), possibly by increased neutrophil and T cell access (Cronan *et al.*, 2016; Gautam *et al.*, 2018). Permissive monocyte recruitment is augmented by mycobacterial glycolipids (Cambier *et al.*, 2017) and blocking recruitment led to smaller granulomas (Lu *et al.*, 1998). In contrast, there were no long-term differences in bacterial burden implying equivalent control of infection by adaptive immunity (Lu *et al.*, 1998).

Containment within granulomas drives Mtb into dormancy, suppressing metabolic activity and replication as an adaptation to the hostile environment (e.g. hypoxia) (Chao and Rubin,

2010). A group of around 50 genes known collectively as the DosR regulon control this dormancy phenotype (Boon and Dick, 2002; Voskuil *et al.*, 2003; Galagan *et al.*, 2013). Dormancy may contribute to Mtb persistence during chronic infection.

Reactivation of Mtb can occur following host immunosuppression that affects cell-mediated immunity, such as the impaired CD4+ T cell function in HIV infection or with anti-tumour necrosis factor (TNF) therapy (Havlir, 1999; Keane *et al.*, 2001; Botha and Ryffel, 2003; Corbett *et al.*, 2006; Jambo *et al.*, 2014). More recently, PD-1/PD-L1 cancer immunotherapy has been shown to reactivate Mtb (Morelli *et al.*, 2022), highlighting a pathogenic role for uncontrolled T cell responses. Non-communicable diseases, social conditions, as well as lifestyle choices can lead to immunosuppression and increase TB risk (Marais *et al.*, 2013). However, reactivation also occurs in otherwise healthy individuals, the causes of which are yet to be determined.

Mtb is an obligate airborne pathogen with transmission inextricably linked to virulence (Brites and Gagneux, 2012). Granuloma centres caseate and undergo necrosis, eventually liquefying (Dheda, Barry and Maartens, 2016), containing large numbers of viable bacteria (Capuano *et al.*, 2003). Mtb-containing fluid that leaks into lung cavities is coughed up, aerosolising the bacteria and facilitating onward transmission (Chen *et al.*, 2021). The production of matrix metalloproteinases (MMP) drives lung destruction and cavitation via the degradation of collagen (Elkington *et al.*, 2011; Ong *et al.*, 2015). Monocytes, neutrophils, fibroblasts, and epithelial cells have all been demonstrated to secrete MMPs in response to Mtb (O'Kane, Elkington and Friedland, 2008; Volkman *et al.*, 2010, p. 200; Elkington *et al.*, 2011; Ong *et al.*, 2015). Immunosuppressive host-directed therapies may be beneficial to long-term lung function by reducing cavitation (Miow *et al.*, 2021; Wallis *et al.*, 2021). In targeting different aspects of TB, immunosuppression in conjunction with antibiotics may well complement one another.

1.4. Genetically encoded risk for tuberculosis

There is strong evidence that genetically encoded variances do contribute risk for developing TB disease. There is a substantially elevated TB risk among monozygotic twins versus dizygotic twins (Comstock, 1978). TB is a complex disease requiring a coordinated and appropriate response of multiple immune cell subsets for protection (Flynn and Chan, 2001). Furthermore, there exist several disease states between acute infection and a chronically infected, overburdened TB lung (Pai *et al.*, 2016). Variation can occur at any point along this pathway of proper host defence, including phagocytosis, pattern recognition, cytokine production, immune cell recruitment and induction of effector mechanisms, and may contribute to susceptibility independently of one another.

Rare genetic disease, termed Mendelian susceptibility to mycobacterial disease (MSMD), comprise those with genetic mutations affecting interactions between phagocytes and T helper 1 (Th1) cells via IFNγ (Bustamante *et al.*, 2014). Deleterious mutations in IFNGR1, IFNGR2, STAT1, JAK1, IRF8, SPPL2A, IL12B, IL12RB1, IL12RB2, IL23R, ISG15, TYK2, RORC, CYBB and NEMO all lead to MSMD (Bustamante *et al.*, 2014). These inherited deficiencies in IFNγ-signalling highlight the importance of IFNγ-immunity in mycobacterial control and led to the belief that IFNγ is necessary for protection. However, observations of TST and IGRA negative, Mtb seropositive individuals who did not develop TB have challenged this dogma (Kroon *et al.*, 2020). In most cases of active disease patients have intact IFNγ-responses, suggesting IFNγ is not sufficient for protection (Elias, Akuffo and Britton, 2005; Mittrücker *et al.*, 2007). Recently, reports of a loss-of-function (LOF) mutation in TNF was found to selectively impair reactive oxygen species (ROS) production in macrophages and increase TB susceptibility (Arias *et al.*, 2024). It is interesting to note that LOF mutations in RORC, essential for Th17 cell development (Ivanov *et al.*, 2006), elevate mycobacterial disease risk (Okada *et al.*, 2015), although these patients also had diminished IFNγ-responses to BCG.

There have been myriad candidate gene and genome-wide association studies (GWAS) focusing on TB susceptibility with limited success (Abel *et al.*, 2018). These two approaches

differ in whether they are a priori hypothesis-driven or not (Jorgensen *et al.*, 2009), but often reveal weak effect sizes indicating complex regulatory gene networks required for protection (Schadt, 2009). Human leukocyte antigen class II alleles have long been associated with risk of TB (Bothamley *et al.*, 1989; Goldfeld, 1998, p. 199; Ravikumar *et al.*, 1999; Sveinbjornsson *et al.*, 2016), although a recent large-scale meta-analysis failed to replicate most associations (Schurz *et al.*, 2022). It is presumed that such alleles confer risk due to their central role in antigen presentation to CD4+ T cells (Caruso *et al.*, 1999). Several genetic variants influencing macrophage antimicrobial effector mechanisms have also been associated with disease risk (Bellamy *et al.*, 1998; Greenwood *et al.*, 2000; Malik *et al.*, 2005; Möller *et al.*, 2009; Velez *et al.*, 2009). Given the extent to which population genetics influences inheritance and therefore GWAS associations, candidate genes require validation both in populations that differ in genetic diversity from the discovery cohort, as well as with further mechanistic experimental validation.

1.5. Innate immunity in tuberculosis

1.5.1. Macrophage control of Mtb infection

Control of mycobacterial infection requires activated macrophages (Walker and Lowrie, 1981). The intracellular survival and replication of Mtb within macrophages is a determinate of Mtb persistence and pathogenesis. Mtb survival in macrophages depends on the both cell-intrinsic microbicidal activity as well as bacterial virulence (Flynn and Chan, 2001). Whilst myeloid cells may provide an intracellular niche for Mtb that shields it from the immune system, activated macrophages have several effector mechanisms that control infection (Figure 1.1). This is a critical step in preventing cascade towards TB disease. However, our understanding of the physiological mechanisms behind human macrophage restriction of Mtb growth remains limited.

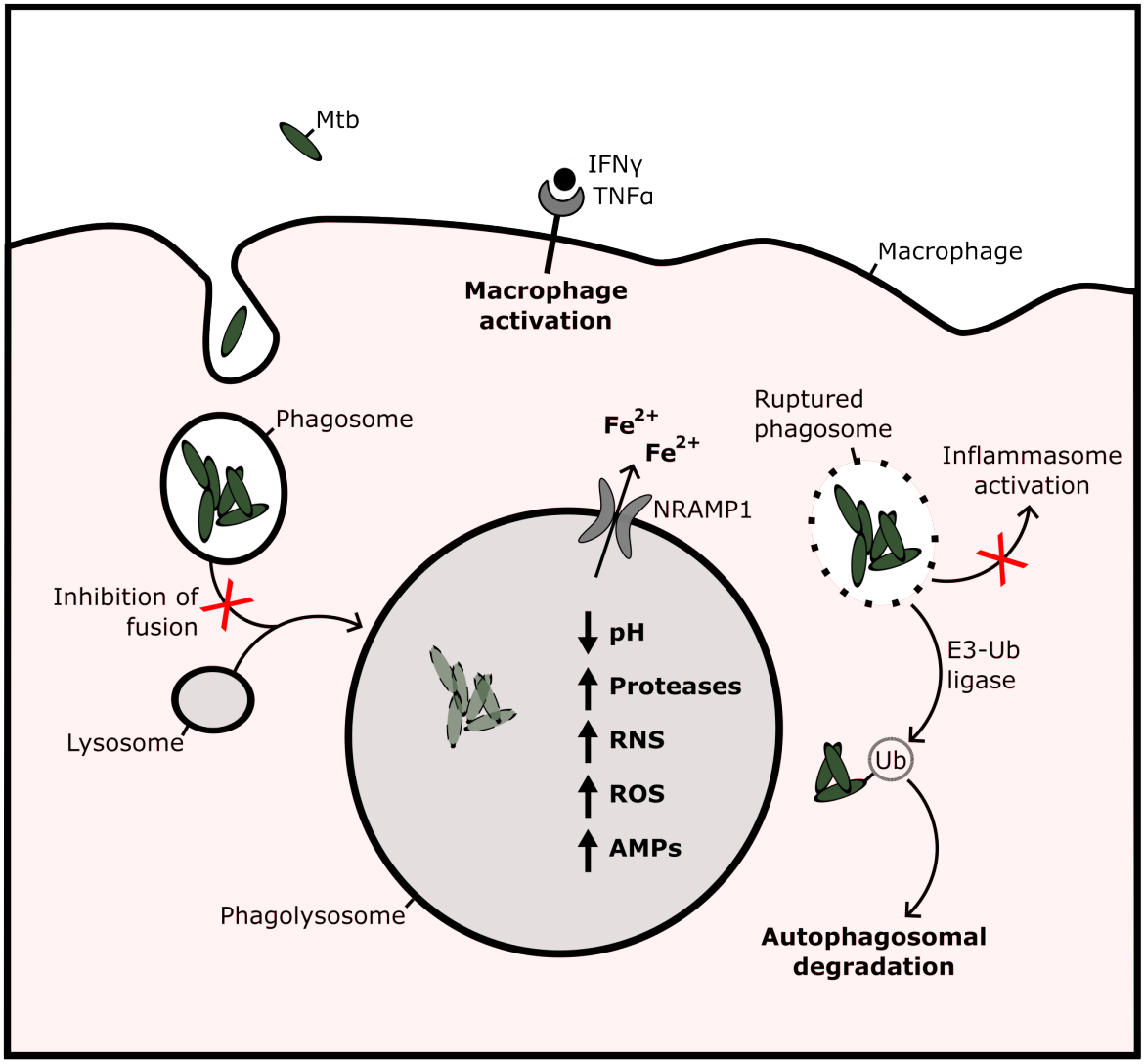


Figure 1.1. **Antimycobacterial mechanisms of macrophages.** Activated macrophages direct phagocytosed Mtb towards phagolysosomes for proteasomal degradation. The phagosome can be arrested by Mtb, facilitating intracellular replication. Mtb can also rupture the phagosome to escape into the cytosol. Cytosolic sensing of Mtb will trigger inflammasome activation and pyroptosis but this pathway is antagonised by Mtb. Additionally, cytosolic Mtb and arrested phagosomes may be directed towards autophagosomal degradation. Activated macrophages can also produce reactive nitrogen species (RNS), reactive oxygen species (ROS), antimicrobial peptides (AMPs), and restrict the intracellular availability of iron, all of which may limit the survival of Mtb.

IFNγ and TNFα synergise to activate macrophages and enhance their killing capacity (Flynn and Chan, 2001). TLR2 signalling through MyD88 is important for macrophage self-priming via the production of TNFα (Underhill *et al.*, 1999; O'Neill and Bowie, 2007) and antimicrobial nitric oxide generation in mouse macrophages (Brightbill *et al.*, 1999; Shi *et al.*, 2003). Synergy between TLR2 and vitamin D receptor signalling also induced an antimicrobial peptide (AMP), LL37, in THP-1 cells that augmented Mtb control (Liu *et al.*, 2007). Mice lacking MyD88 were very susceptible to Mtb (Fremond *et al.*, 2004; Scanga *et al.*, 2004). As is usually the case with Mtb, exposed surface glycolipids on Mtb have been shown to inhibit TLR2 signalling (Noss *et al.*, 2001; Blanc *et al.*, 2017).

The route of Mtb entry into macrophages is diverse (Aderem and Underhill, 1999) and can influence Mtb survival. For example, the engagement of macrophage mannose receptor by mycobacterial mannose-capped lipoarabinomannan (ManLAM) results in Mtb-containing phagosomes with decreased lysosomal fusion, thus facilitating the intracellular survival of Mtb (Kang *et al.*, 2005). Virulent Mtb strains were found to engage the macrophage mannose receptor to a greater extent than attenuated strains (Schlesinger, 1993). The presence of several host factors also differentially affect uptake. Notably, surfactant proteins secreted by alveolar type II pneumocytes (ATII) can either increase (Downing *et al.*, 1995; Gaynor *et al.*, 1995) or decrease (Ferguson *et al.*, 1999) macrophage phagocytosis of Mtb.

Phagocytosis of extracellular Mtb culminating in phagolysosome fusion and proteasomal degradation will eliminate the bacteria (Savina and Amigorena, 2007). However, Mtb employs several mechanisms to delay and block this process (Lugo-Villarino and Neyrolles, 2014). Mtb interferes with the recruitment of Rab proteins that coordinate phagosome trafficking (Seto, Tsujimura and Koide, 2011). Surface lipids delay acidification of the late phagosome (Axelrod *et al.*, 2008) and interfere with phagolysosome maturation (Fratti *et al.*, 2003; Vergne, Chua and Deretic, 2003). Secreted enzymes degrade PI3P (Vergne *et al.*, 2005), essential for phagosome maturation (Fratti *et al.*, 2001), and inhibit recruitment of a critical membrane proton pump to the Mtb-containing phagosome (Sturgill-Koszycki, 1994; Wong *et al.*, 2011).

Deletion of several other genes attenuates Mtb in macrophages via increased phagolysosome fusion (Walburger *et al.*, 2004; Bach *et al.*, 2008).

Mtb appears to not only arrest the phagosome but initiate escape into the cytosol, possibly to induce host cell death. ESAT-6 secretion system-1 (ESX1) is a large multi-protein secretion apparatus and major mycobacterial virulence factor. EAST-6, exported by ESX1, mediates phagosome rupture and escape (van der Wel *et al.*, 2007; Smith *et al.*, 2008) in synergy with the cell wall lipid phthiocerol dimycocerosate (PDIM) (Quigley *et al.*, 2017). Deletion of ESX1 limited the ability of Mtb to infect neighbouring macrophages and is central to BCG attenuation (Hsu *et al.*, 2003; Guinn *et al.*, 2004). The presence of Mtb and bacterial components in the cytosol can lead to innate sensing and NLR family pyrin domain containing 3 (NLRP3) inflammasome activation, resulting in inflammatory cell death (Houben *et al.*, 2012; Simeone *et al.*, 2012). However, caspase-1-dependent inflammasome activation appears to be antagonised by Mtb to benefit its survival in vivo (Master *et al.*, 2008).

Macrophages can also direct Mtb towards autophagosomal degradation (Gutierrez *et al.*, 2004; Watson, Manzanillo and Cox, 2012). Parkin and Smurf1 are the key E3-Ubiquitin (Ub) ligases which catalyse degradation of Mtb. Polyubiquitination, in which substrate is tagged with long Lysine (Lys)48- or Lys63-residue-linked chains of Ub, marks the intracellular target for proteasomal degradation via p62 or NBR1 recruitment (Manzanillo *et al.*, 2013; Franco *et al.*, 2017). IFNγ has been shown to upregulate autophagic machinery in macrophages (Gutierrez *et al.*, 2004). Sensing of cytosolic Mtb DNA via the cGAS-STING pathway is an important trigger of autophagy in macrophages, requiring ESX-1 (Watson, Manzanillo and Cox, 2012; Watson *et al.*, 2015; Bernard *et al.*, 2020). The importance of autophagy in host defence against Mtb has been questioned (Behar and Baehrecke, 2015), primarily due to a study of mice deficient in numerous essential autophagy factors (Kimmey *et al.*, 2015). Authors found no differences in TB pathogenesis across mice with defective autophagic machinery compared to wild type. Since then, the deletion of several of these autophagy factors in human macrophages demonstrated their importance in autophagy-mediated

restriction of intracellular Mtb (Aylan *et al.*, 2023), but it is unclear whether these findings translate in vivo.

Macrophages produce RNS, ROS, and AMPs to directly kill Mtb (MacMicking *et al.*, 1997; Liu *et al.*, 2007; Idh *et al.*, 2017). ROS appear to be an essential anti-Mtb effector mechanism in human alveolar macrophages in vivo (Arias *et al.*, 2024). RNS play a pivotal role in mouse macrophage killing of Mtb. Deletion of nitric oxide synthase in mice rendered them extremely susceptible to Mtb infection (MacMicking *et al.*, 1997). Macrophages isolated using bronchoalveolar lavage (BAL) washes of human TB lung were found to express NOS2, the gene for inducible nitric oxide synthase (Nicholson *et al.*, 1996), but human alveolar macrophages inhibit Mtb growth ex vivo independently of NOS2 (Aston *et al.*, 1998). Head-to-head comparisons have revealed substantially lower NOS2 expression and nitric oxide production in human macrophages compared to murine macrophages (Thoma-Uszynski *et al.*, 2001; Gross *et al.*, 2014). A recent study has highlighted epigenetic silencing of the NOS2 gene in human macrophages (Gross *et al.*, 2014).

Mouse and human macrophages also differ in their ability to produce AMPs. Mouse macrophages upregulate mouse beta-defensin 4 (orthologous to human beta-defensin (hBD) 2) in response to Mtb-infection (Peng *et al.*, 2024) and CD44 signalling (Singh *et al.*, 2022). Overexpression of hBD2, encoded by DEFB4, in human monocyte-derived macrophages (MDM) augmented Mtb control (Kisich *et al.*, 2001), but direct evidence of human macrophage expression of DEFB4 is limited (Duits *et al.*, 2002; Rivas-Santiago *et al.*, 2005; Rodriguez-Carlos *et al.*, 2020; Díaz *et al.*, 2023). LL-37, the only cathelicidin family member expressed in humans (Dürr, Sudheendra and Ramamoorthy, 2006), is upregulated by MDMs in response to Mtb (Rivas-Santiago *et al.*, 2008), can restrict Mtb growth (Liu *et al.*, 2007), and may be antagonised by the efflux pump Rv1258c (Lin *et al.*, 2016).

Lastly, macrophages restrict the intracellular availability of key nutrients (Appelberg, 2006). Macrophages use natural resistance-associated macrophage protein 1 (NRAMP1) to efflux divalent transition metals out of phagosomes (Hood and Skaar, 2012). In turn, Mtb utilises

mycobactin to capture host iron and shuttle it back towards the Mtb-containing phagosome (Luo, Fadeev and Groves, 2005; Boradia *et al.*, 2014). The importance of iron deprivation in macrophage-restriction of Mtb is highlighted by instances of iron excess in humans and animal models (Gangaidzo *et al.*, 2001; Lounis *et al.*, 2001; Schaible *et al.*, 2002; McDermid *et al.*, 2013). Additionally, there is a metabolic switch in Mtb enabling cholesterol metabolism as a primary carbon source (Griffin *et al.*, 2012) that is essential for persistence (Pandey and Sassetti, 2008). Mtb also appears to require several phosphate transport genes for survival in murine macrophages (Rengarajan, Bloom and Rubin, 2005) implying intracellular phosphate restriction.

There is considerable evidence supporting each macrophage killing mechanism in restricting the growth of Mtb in vitro and in animal models. However, despite decades of research, the relative contribution of each of these mechanisms towards control of Mtb infection in humans is still unknown.

1.5.2. Alveolar pneumocytes

Cells comprising the lung epithelium play a functionally important role in defence against respiratory pathogens (Hernández-Santos *et al.*, 2018; Davis and Wypych, 2021). ATIIs are an important immunomodulatory cell within the pulmonary alveolus (Brandt and Mandiga, 2024). ATIIs respond to Mtb infection by secreting cytokines and chemokines (Lin, Zhang and Barnes, 1998; Barclay *et al.*, 2023) to orchestrate the immune response. Crosstalk with infected macrophages augmented ATII response to Mtb (Reuschl *et al.*, 2017). As mentioned previously, ATII-derived surfactant proteins influence the phagocytosis of Mtb (Downing *et al.*, 1995; Gaynor *et al.*, 1995; Ferguson *et al.*, 1999) and may even supress macrophage function in TB (Pasula *et al.*, 1999).

Several ATII-derived antimicrobial effector molecules have been proposed to contribute towards anti-Mtb immunity. When treated with vitamin A, Mtb-infected bronchial epithelial cells upregulate hBD2 and hBD3 mRNA that coincides with a decrease in bacterial burden (Jacobo-Delgado *et al.*, 2021). The overexpression of hBD1 in ATIIs effectively limited

intracellular Mtb growth within these cells in vitro, whilst deletion of the hBD1 gene augmented Mtb growth (Chen *et al.*, 2024). ATIIs respond to Mtb-infected macrophages by upregulating hBD2 mRNA in an interleukin (IL)-1β-dependent manner (Reuschl *et al.*, 2017). HBD2 restricted macrophage-free Mtb growth in vitro (Reuschl *et al.*, 2017). However, the physiological significance of this finding in human infection remains unclear.

Mtb can invade ATIIs (McDonough and Kress, 1995; Bermudez and Goodman, 1996) but they are less permissive to infection than macrophages (Reuschl *et al.*, 2017). Mtb induced necrotic cell death of ATIIs in vitro (Dobos *et al.*, 2000) by modulating the expression of several apoptosis-related genes (Danelishvili *et al.*, 2003). Necrosis benefits Mtb and is associated with virulence (Dobos *et al.*, 2000; Gan *et al.*, 2008). Infected ATIIs can present antigen on major histocompatibility complex (MHC)-II to CD4+ T cells to stimulate IFNγ release (Debbabi *et al.*, 2005). It is not known whether ATIIs represent a significant reservoir for Mtb during infection in vivo.

1.5.3. Other innate immune responses

The innate immune system relies on the recognition of conserved pathogen products (ie. PAMPs) by pre-existing, germline encoded receptors that do not vary (Janeway and Medzhitov, 2002). There exists myriad germline encoded innate receptors distributed throughout cellular compartments (Ishii *et al.*, 2008). Together, they enable the innate immune system to recognise the diverse microbial world. Innate immune responses to infection are rapid (Janeway and Medzhitov, 2002). There is evidence that innate immunity contributes to anti-Mtb immunity in a variety of cell types in addition to macrophages.

Activated neutrophils can kill Mtb in vitro (Brown, Holzer and Andersen, 1987). Rapid, CXCL8-dependent neutrophil recruitment occurs (Cheng *et al.*, 2017) and can efficiently prevent Mtb infection (Pedrosa *et al.*, 2000; Sugawara, Udagawa and Yamada, 2004). However, neutrophils constitute the greatest proportion of Mtb-infected cells within the BAL and sputum of active TB patients (Eum *et al.*, 2010) and several studies have linked neutrophil-responses with the pathogenesis of TB (Eruslanov *et al.*, 2005; Berry *et al.*, 2010; Nouailles *et al.*, 2014; Ong *et al.*,

2015; Yeremeev *et al.*, 2015; Moreira-Teixeira *et al.*, 2020). ATII-derived chemokines may play a role in exacerbating neutrophil infiltration and disease (Nouailles *et al.*, 2014).

Comparatively little is known about the role of mast cells in TB. Mast cells are found distributed throughout lung tissue at rest (Metcalfe, Baram and Mekori, 1997) and are sufficient to restore TLR2-dependent innate control of infection in mice (Carlos *et al.*, 2009). They are important orchestrators of inflammation, releasing several key cytokines and chemokines. In addition to secreting TNFα and CXCL8 (Marshall, 2004), mast cells are a dominant source of IL-17 in several inflammatory diseases (Lin *et al.*, 2011; Kenna and Brown, 2013) and are found to express IL-17 in TB granulomas (Garcia-Rodriguez *et al.*, 2021). A study of chronic TB infection in non-human primates (NHP) implicated mast cells with worse disease (Gideon *et al.*, 2022), whilst in human TB lungs post-mortem there was increased mast cell infiltration in fibrotic tissue (Garcia-Rodriguez *et al.*, 2021).

Mucosal-associated invariant T (MAIT) cells are a subset of T cells that express T cell receptors (TCRs) with restricted diversity (Tilloy *et al.*, 1999; Reantragoon *et al.*, 2013). They constitute approximately 4% of CD3+ T cells in the lung and have polyfunctional responses to infection with aspects of innate, type 1, and type 17 immunity (Provine and Klenerman, 2020). MAIT cell TCRs are not restricted by classical MHC-I or MHC-II molecules but rather by MHC class I-related protein 1 (MR1) (Treiner *et al.*, 2003; Reantragoon *et al.*, 2013). They secrete IFNγ following the recognition of Mtb-antigens presentation on MR1 (Gold *et al.*, 2010), though TCR-signalling is not a pre-requisite for activation (Provine and Klenerman, 2020).

Natural killer (NK) cells are cytotoxic lymphocytes that secrete cytokines (Strowig, Brilot and Münz, 2008) and have certain immune memory characteristics (O'Leary *et al.*, 2006; Cooper *et al.*, 2009; Sun, Beilke and Lanier, 2009). Unlike T and B cells, they cannot rearrange their germline encoded receptors to generate antigen-specificity (Raulet, Vance and McMahon, 2001) and thus form part of the innate immune system. NK cells can secrete IFNγ in response to Mtb (Vankayalapati *et al.*, 2004). IL-22, also produced by NK cells, increased macrophage growth restriction of Mtb (Dhiman *et al.*, 2009).

NK cells can directly kill Mtb-infected alveolar macrophages and monocytes (Vankayalapati *et al.*, 2005) and their frequency amongst peripheral blood mononuclear cells (PBMC) was associated with latency in human TB infection (Roy Chowdhury *et al.*, 2018). Additionally, NK cells can augment the ability of cytotoxic CD8+ T cells to lyse infected monocytes (Vankayalapati *et al.*, 2004) as well as modulate other T cell subsets (Zhang, 2006; Roy *et al.*, 2008), including lysing expanded regulatory T cells (Treg).

Dendritic cells (DCs) are better at antigen presentation than macrophages and other immune cells (Merad *et al.*, 2013) making them central to the priming of naïve T cells. DCs phagocytose Mtb and migrate to lung draining lymph nodes (Wolf *et al.*, 2007, 2008) where they initiate protective T cell responses (Tian *et al.*, 2005; Olmos, Stukes and Ernst, 2010). DCs also secrete Mtb antigens into the interstitial space within lymph nodes which can then picked up by uninfected DCs and presented to T cells (Srivastava and Ernst, 2014), thus augmenting the response.

However, infected DCs disseminate Mtb to the lung draining mediastinal lymph nodes (Humphreys *et al.*, 2006). Interestingly, they have even been shown to migrate out of chronic granulomatous TB lesions to distal tissues (Schreiber *et al.*, 2011), though in this case it was not apparent whether this disseminated viable bacteria. Mtb should not benefit from disseminated infection to distal tissues as it transmitted to new hosts via the lungs (Chen *et al.*, 2021).

1.5.4. Initiating adaptive immunity

The adaptive immune system comprises two lymphocyte lineages, T and B cells (Boehm and Swann, 2014). The process of receptor gene rearrangement differentiates it from innate immunity and is essential for generating antigen specific responses (Janeway and Medzhitov, 2002). Effectors cells can be long-lived in vivo, enabling the adaptive immune system to generate rapid antigen-specific response upon reinfection (Lam, Lee and Farber, 2024). These processes are the basis of durable immunological memory and vaccine-elicited protection.

Optimal host defence against Mtb requires coordination between innate and adaptive immune systems (Flynn and Chan, 2001).

The initiation of the adaptive immune system requires antigen presentation alongside pro-inflammatory cytokines and co-stimulation (Janeway, 2001). In this regard, the innate immune system activates and educates adaptive immunity. MHC class II presentation is necessary to elicit protective CD4+ T cells (Caruso *et al.*, 1999), but is directly antagonised by Mtb (Hmama *et al.*, 1998; Noss *et al.*, 2001). Mtb inhibits pro-inflammatory cytokine production by infected macrophages (Reed *et al.*, 2004; Blanc *et al.*, 2017) and reduces surface CD80 expression (Saha *et al.*, 1994), an important co-stimulatory molecule (Mir, 2015). In doing so, Mtb may modulate antigen presentation to T cells and thus shape the T cell receptor repertoire (Musvosvi *et al.*, 2023).

When compared with other respiratory pathogens the initiation of adaptive immunity appears delayed (Janeway, 2001; Reiley *et al.*, 2008; Wolf *et al.*, 2008; Lin *et al.*, 2014). Whilst animal models revealed a plateau in Mtb growth that coincided with the onset of T cell-mediated immunity (Wolf *et al.*, 2008; Lin *et al.*, 2014), this took several weeks to develop. Adaptive immunity may be delayed due to the slow growth of Mtb (Tsukamura, 1967) as well as the direct antagonism of inflammation and antigen presentation (Hmama *et al.*, 1998; Noss *et al.*, 2001; Divangahi *et al.*, 2010; Blanc *et al.*, 2017).

T cell memory induced by Mtb challenge conferred some protection to secondary infection with decreased bacterial burden after several weeks (Kamath and Behar, 2005). Curiously, this immune memory offered no long-term survival benefit in these mice. This highlights the effectiveness of Mtb in establishing itself within the host and the challenges faced by vaccine development, which rely on the induction of antigen-specific memory.

1.6. Adaptive immunity in tuberculosis

Mtb-specific cell-mediated immunity protects from disease, highlighted by MSMD (Bustamante *et al.*, 2014), T cell depletion with HIV infection (Havlir, 1999; Corbett *et al.*,

2006; Jambo *et al.*, 2014), and experimental T cell depletion in NHP models of TB (Lin *et al.*, 2012; Winchell *et al.*, 2023). However, T cell activation has also been implicated in mediating pathogenesis. PD-1/PD-L1 checkpoint inhibition to treat cancer can reactivate latent tuberculosis (Barber *et al.*, 2019; Anand *et al.*, 2020; Crawley *et al.*, 2020; Kato *et al.*, 2020). Moreover, Mtb has been shown to specifically sense pro-inflammatory IFNγ to drive bacterial metabolism and growth in vitro (Ahmed *et al.*, 2022). Mtb can therefore be considered to actively respond to the host immune response, though how this might mediate pathogenesis in vivo is yet to be determined.

1.6.1. T helper 1 and 17 responses

CD4+ Th1 cells are essential for control, emphasised by MHCII and CD4+ T cell-deficient animals (Caruso *et al.*, 1999; Mogues *et al.*, 2001; Lin *et al.*, 2012), and a rapid influx of Mtb-specific CD4+ T cells to lung could protect against experimental Mtb challenge (Sakai *et al.*, 2014). However, this protection may not be entirely mediated by IFNγ-producing Th1 cells (Gallegos *et al.*, 2011).

An Mtb-specific Th17 cell phenotype was enriched in a highly exposed, IFN-response-negative cohort, deemed to have resisted infection (Sun *et al.*, 2024), whilst Th17 cells correlated with vaccine induced protection in NHPs (Dijkman *et al.*, 2019). Mouse models also illustrate the importance of the IL-17 response to virulent Mtb (Okamoto Yoshida *et al.*, 2010; Wozniak *et al.*, 2010; Freches *et al.*, 2013; Gopal *et al.*, 2014). Furthermore, single cell RNA-sequencing (scRNA-seq) of heterogenous NHP TB granulomas associated a mixed Th1-Th17 T cell population and cytotoxic CD8+ T cells with reduced bacterial burden (Gideon *et al.*, 2022). These low burden granulomas enriched for such T cell subsets appeared later on in infection, raising the question as to whether they might necessarily be protective throughout the entire course of tuberculosis. Although none of the functional T cell subsets identified in the study have been experimentally validated, exogenous IL-17 decreased Mtb growth in an in vitro human granuloma model system (Ogongo *et al.*, 2021). Precisely how IL-17 mediated this

effect in this model is unclear, though authors noted an increase in nitric oxide production that may have directly killed Mtb.

Contrasting this body of evidence for protective Th17 responses, Th17 cells may also contribute to pathogenesis later in disease. IL-17 cytokine activity is enriched in active TB disease and demonstrated to drive lung pathology when in excess (Cruz *et al.*, 2010; Pollara *et al.*, 2021). In mice with a defective autophagic pathway, the concentration of IL-17 in the lung mirrored bacterial burden (Franco *et al.*, 2017). Therefore, IL-17 responses could be exaggerated in late stages of disease where there is uncontrolled bacterial replication. Interestingly, an IL-17 immune axis revealed in a distal skin challenge model decreases following treatment (Pollara *et al.*, 2021), suggesting active TB primes the immune system (Budzik *et al.*, 2020; DiNardo *et al.*, 2020). In contrast, the frequency of Mtb-specific Th17 cells in human TB lung tissue inversely correlated with plasma IL-1β (Ogongo *et al.*, 2021) indicating Th17 cells may reduce pathogenic inflammation. It is worth noting that certain genetic variants which increase IL-1β production augment macrophage control of Mtb in vitro (Eklund *et al.*, 2014). Thus, the timing and magnitude of the Th17 response may be crucial.

Curiously, T cell epitopes are unusually conserved among members of the M. tuberculosis complex of mycobacteria (Comas *et al.*, 2010; Copin *et al.*, 2014) suggesting an evolutionary advantage in driving T cell recognition. This might be related to the role of Mtb-specific T cell responses in preventing dissemination of the bacteria beyond the lung (Jiang *et al.*, 2024), seen as a dead-end in the life cycle of Mtb (Chen *et al.*, 2021). Supporting this, CD4+ T cell-depleted HIV-infected TB patients were less likely to transmit Mtb to close contacts than HIV-uninfected TB patients (Espinal *et al.*, 2000).

1.6.2. Other T cell subsets

Th2 cells could not inhibit growth of Mtb in vivo (Gallegos *et al.*, 2011) and are thought to antagonise protective Th1 responses (Tan *et al.*, 2012; Ashenafi *et al.*, 2014). Similarly, Tregs limit the control of Mtb infection by suppressing Mtb-specific effector T cells (Chen *et al.*,

2007; Scott-Browne *et al.*, 2007). Nonetheless, at some point in the disease pro-inflammatory signalling becomes detrimental to the host and therefore Tregs may be protective later on.

CD8+ lymphocytes are critical for early control of tuberculosis in macaques (Chen *et al.*, 2009; Winchell *et al.*, 2023) and contribute by killing intracellular Mtb (Stenger *et al.*, 1998; Cho *et al.*, 2000; Ernst *et al.*, 2000). Gamma-delta T cells and CD1a, b, and c-restricted T cells can theoretically recognise and respond to Mtb (Panchamoorthy *et al.*, 1991; Moody *et al.*, 2000) but their importance in vivo is poorly understood. Gamma-delta T cells are a source of IL-17 (Lockhart, Green and Flynn, 2006) whilst CD1a, b, and c-restricted T cells can secrete IFNγ (Montamat-Sicotte *et al.*, 2011).

The protective role of T cells in TB is both complex and perhaps temporal in nature. Granulomas that formed later during infection in the context of adaptive immune responses were more likely to have lower bacterial burden (Gideon *et al.*, 2022), suggesting the exact timing of Mtb-specific immunity determines outcome.

1.6.3. Humoral immunity

Given the role of cell-mediated immune responses in restricting Mtb growth in vivo, humoral immunity is often overlooked. B cells contribute via the production of both Mtb-specific antibodies and cytokines (Maglione and Chan, 2009) and are present in the human TB granuloma (Ulrichs *et al.*, 2004). There are documented cases of protective humoral immunity in TB both in vivo and in vitro, but efficacy varies considerably between studies and there exist several instances where Mtb-specific antibody titres correlate with human TB pathology (Glatman-Freedman and Casadevall, 1998).

Results from NHP TB models are conflicting (Phuah *et al.*, 2016). B cell depletion typically reduced granuloma sizes and increased the relative bacterial burden per granuloma, though the exact outcome was very individualised. Interestingly, NHP deficient in B cells have exacerbated Th17 immunity (Phuah *et al.*, 2016), mirroring that seen in mice (Kozakiewicz *et al.*, 2013). These mice also had an augmented IL-17-mediated neutrophil influx, suggesting B

cells may be protective against pathogenic neutrophil responses (Kozakiewicz *et al.*, 2013). Whilst the absence of B cells in NHPs had no impact on acute disease pathogenesis (Phuah *et al.*, 2016), longer duration studies may yet reveal a key role for B cells in chronic Mtb infection.

1.7. Balancing immune responses in tuberculosis

The development of TB is a multistep process in which the immune system first attempts to clear and then to contain Mtb. Whether active disease arises from acute infection, a secondary re-infection, or re-activated dormant bacilli, the immune system must respond appropriately in order to prevent growth and eliminate Mtb. Throughout infection human immunity is finely poised between protective and pathogenic responses. It is well documented that immunodeficiencies elevate risk of disease (Bustamante *et al.*, 2014; Okada *et al.*, 2015), but evidence is now accumulating that excessive inflammation can be just as detrimental (Barber *et al.*, 2019; Anand *et al.*, 2020; Crawley *et al.*, 2020; Kato *et al.*, 2020), highlighted by less cavitary disease in immunocompromised patients (Kwan and Ernst, 2011). Interestingly, T cell-mediated responses to Mtb antigen challenge correlated with TB incidence (Comstock, Livesay and Woolpert, 1974), though this may reflect social settings, including the relative exposure of individuals to patients with active TB, rather than underlying immunology.

TNF is a pleiotropic cytokine (Van Loo and Bertrand, 2023) with a dichotomous role in TB. The importance of TNF in TB is displayed in individuals undergoing anti-TNF therapy, who have a substantially elevated disease risk (Keane *et al.*, 2001), rare LOF mutations in the human TNF gene (Arias *et al.*, 2024), and in animal models depleted of TNF (Flynn *et al.*, 1995; Lin *et al.*, 2010). However, it also exacerbates inflammation and is sufficient to drive virulence (Tsenova *et al.*, 1999). TNF has been shown to induce pathogenic necrotic cell death in THP-1 cells and zebrafish macrophages infected with mycobacteria (Roca *et al.*, 2019).

Eicosanoids are lipid mediators of inflammation and are modulated by Mtb to promote necrosis and survival in vivo (Divangahi *et al.*, 2009, 2010). In zebrafish, a balance between pro-inflammatory leukotriene B4 and anti-inflammatory lipoxin A4 eicosanoids was necessary

for optimal TNF responses to mycobacterial infection (Tobin *et al.*, 2010). Human genetic polymorphisms modulating eicosanoid responses have been associated with TB meningitis mortality (Tobin *et al.*, 2010, 2012). Heterozygotes were most protected, supporting a necessary balance in TNF responses to Mtb infection. Pro-and anti-inflammatory eicosanoids have also been shown to be spatially distributed in TB granulomas (Marakalala *et al.*, 2016), with anti-inflammatory eicosanoid mediators enriched in the granuloma periphery. This balance of inflammation around the granuloma may serve to protect lung tissue.

Type I IFN responses have been widely established as pathogenic in TB. IFN response genes were upregulated in the peripheral blood of patients with active TB (Berry *et al.*, 2010). Although this finding did not discriminate between type I and type II IFN, there are several lines of evidence implicating type I IFNs in pathology. Mtb virulence has been linked with an induction of type I IFNs in mice (Manca *et al.*, 2001, 2005), perhaps due to increased phagosome escape of Mtb triggering NOD2-dependent type I IFN release (Pandey *et al.*, 2009). There has been a documented case of IFNα treatment for chronic hepatitis D infection exacerbating TB disease (Telesca *et al.*, 2007), whilst in mouse models, poly-IC-induced type I IFN was extremely detrimental (Antonelli *et al.*, 2010). Type I IFNs also promote human macrophage death in vitro (Lee and Nathan, 2024). Additionally, they may inhibit protective type II IFN (IFNγ) responses and induce anti-inflammatory IL-10 (McNab *et al.*, 2014). However, a transcriptomic signature for type I IFN activity in TSTs inversely correlated with severity of TB disease in humans (Szydlo-Shein *et al.*, 2024) and STAT2 signalling may be protective during the acute stages of Mtb infection (Szydlo-Shein *et al.*, 2024).

As expected, anti-inflammatory cytokines and those that inhibit IFNγ, TNFα, and Th1 responses all worsen disease. Consequently, IL-10-deficient mice have enhanced Th1 responses and are less susceptible to infection (Redford *et al.*, 2010). IL-10 is also a marker of T cell unresponsiveness in TB (Boussiotis *et al.*, 2000). Nevertheless, pro-inflammatory signalling can also be detrimental. IL-10 was a correlate of protective immunity in NHPs (Dijkman *et al.*, 2019), perhaps indicating a necessary balance of inflammation for optimal response to Mtb infection.

ScRNA-seq of heterogenous NHP TB granulomas implicated mast cells and plasma cells as part of a general type 2 immune environment with worse disease (Gideon *et al.*, 2022). IL-4, a classical Th2 response cytokine, enhances macrophage permissivity to Mtb (Khan *et al.*, 2022) and is associated with chronic disease and reactivation in mice (Hernandez-Pando *et al.*, 1996; Howard and Zwilling, 2001). Furthermore, IL-4 drives E-cadherin expression in macrophages (Van Den Bossche *et al.*, 2009) that limits neutrophil access to granuloma centres in vivo (Cronan *et al.*, 2021) and thus may facilitate Mtb persistence. Although IL-4 and IL-10 deficient mice faired no differently during experimental Mtb infection (North, 2001), it may well be necessary to assess IL-4/IL-10 blockade in more relevant TB models.

Taken together, the timing and magnitude of inflammation following Mtb infection is central to outcome. Weak or delayed inflammation can facilitate bacterial growth and lengthen the time before mature adaptive immunity develops. Conversely, excessive inflammation is for all intents and purposes a necessity for transmission, leading to extensive tissue damage and fibrotic scarring. When studying pathology in human TB, it is important to consider the fact that those presenting to the clinic represent failed immunity. Whilst such patients can highlight pathogenic immune responses, most infected individuals who do not progress to disease, and are thus assumed to have appropriate immunity, are missed.

1.8. Models of tuberculosis

The complexity and chronic nature of tuberculosis infection necessitates important consideration for the type of models used. Animal models offer sufficient intricacy to model features of disease as time progresses, from the initial phase of Mtb challenge right through to chronic stages and beyond, but the use of non-human hosts to model human infection assumes this host-pathogen interaction is conserved. Conversely, in vitro models can use primary human immune cells but need strategic design if discoveries are to translate in vivo.

1.8.1. In vivo models

Despite the many advantages of mouse models, including ease of genetic manipulation and the many well-established inbred strains, the mouse is not a natural host for Mtb and organised granulomas are typically absent (Young, 2009). Mice lack functional lung MMP1 expression, perhaps explaining the absence of cavitating TB disease in non-humanised mouse models (Elkington *et al.*, 2011; Calderon *et al.*, 2013). Additionally, there are known differences in the antimicrobial capacity of human and murine macrophages (Thoma-Uszynski *et al.*, 2001; Gross *et al.*, 2014; Peng *et al.*, 2024). Crossbreeding of inbred mouse strains, known as diversity outbred mice, has faithfully generated populations with heterogeneous outcomes following Mtb infection (Smith *et al.*, 2022; Kurtz *et al.*, 2024). These mice have been used for genetic TB susceptibility experiments, but insights have so far been limited (Smith *et al.*, 2022; Kurtz *et al.*, 2024). Guinea pigs and rabbits both make comparatively better models as chronic disease mirrors humans more closely, but have higher costs associated with their laboratory care alongside limited genetically inbred strain availability (Young, 2009).

Models leveraging natural host-pathogen interactions exist. Zebrafish larvae are transparent genetically tractable hosts that establish granulomas when infected with *M. marinum* (Ramakrishnan, 2013). Zebrafish models have shown that granulomatous inflammation facilitates Mtb persistence (Cronan *et al.*, 2021), detailed the mechanism of optimal TNF responses governed by eicosanoid production (Tobin *et al.*, 2010), and elucidated STAT2-dependent protective type I IFN immunity (Szydlo-Shein *et al.*, 2024). However, zebrafish models of TB are not without limitations. During their larval stage zebrafish lack an adaptive immune system, developing as they age to adulthood, at which point zebrafish are no longer transparent (Ramakrishnan, 2013).

NHPs serve as excellent models of TB, recapitulating the granulomatous inflammation and heterogeneity of disease seen in humans (Lin *et al.*, 2014; Mothé *et al.*, 2015; Gideon *et al.*, 2022), though inter-species differences exist (Maiello *et al.*, 2018). There also exists the ability

to investigate simian immunodeficiency virus co-infection and immunosuppressive therapies in these animals (Diedrich *et al.*, 2010; Lin *et al.*, 2010; Mehra *et al.*, 2011; Foreman *et al.*, 2016). Generally, the biggest limitation to NHP research comes from the costs associated with curating and maintaining the animals (Scanga and Flynn, 2014).

1.8.2. In vitro models

With in vitro models, one can infect primary human cells with Mtb alongside controlling and modulating variables to test hypotheses. Infecting primary human MDMs are preferable to cell lines (e.g. THP-1) given differences in Mtb replication dynamics and cytokine responses between the two (Stokes and Doxsee, 1999; Hoppenbrouwers *et al.*, 2022). Consideration must also be given to the conditions in which they are differentiated in as this can lead to functional differences in macrophage responses (Khan *et al.*, 2022). Primary blood monocytes are straightforward to obtain from human donors but are not the first phagocyte involved in Mtb infection (Pai *et al.*, 2016). It is possible to use primary human alveolar macrophages recovered from BAL (Reuschl *et al.*, 2017). These models can interrogate acute interactions between host and pathogen, with additional cells in added in bilayers (Birkness *et al.*, 1999; Reuschl *et al.*, 2017) to mimic the alveolus, but do not recapitulate the granulomatous inflammation and chronic disease state.

There have been several approaches to modelling granulomatous inflammation in vitro. Three-dimensional granuloma models can incorporate myeloid and lymphoid cells present in PBMC (Guirado *et al.*, 2015). In addition, the presence of extracellular matrix enables the study of pathogenic, tissue-remodelling responses (Tezera *et al.*, 2017). These can run for several weeks to monitor long-term responses to intervention (Tezera *et al.*, 2017). However, once established, cells cannot be added, thus one cannot model cell recruitment to a granuloma over time (Elkington *et al.*, 2022). In addition, if research questions can be addressed with simpler models then adding complexity can be counterproductive, increasing noise without a tangible benefit. With a disease as complex as TB, every model has trade-offs that need to be addressed.

1.8.3. Tuberculin skin tests

The TST is a well-tolerated diagnostic test for LTBI (Huebner, Schein and Bass, 1993), in which purified protein derivative (PPD) of Mtb is injected intradermally and a localised, delayed type IV hypersensitivity reaction occurs at the injection site. Here, memory CD4+ T cells recognising Mtb antigens augment inflammation in a largely IFNγ-dependent manner (Tsicopoulos *et al.*, 1992; Markowitz, 1993; Tomlinson *et al.*, 2011; Bell *et al.*, 2016). The Noursadeghi group has pioneered genome-wide transcriptional profiling of biopsies at the site of TSTs to dissect the molecular components of anti-Mtb immunity (Tomlinson *et al.*, 2011; Bell *et al.*, 2016; Pollara *et al.*, 2021). Biopsies taken 48 hours after challenge in individuals with a positive skin test reaction capture a comprehensive IFNγ-mediated response to Mtb antigens in vivo (Chu *et al.*, 1992; Tomlinson *et al.*, 2011; Bell *et al.*, 2016).

There are several key advantages of this model. Because antigen challenge is performed in vivo, TSTs circumvent sampling of the immune system, a limitation of in vitro PBMC models. Any circulating leukocyte can theoretically be recruited to the site of inflammation. However, relevant cell types may still be missed. Alveolar macrophages and certain lung-resident memory T cell populations (Takamura *et al.*, 2016) will not circulate in blood and thus cannot be expected to contribute to TST responses.

Importantly, the TST reflects anti-Mtb immunity from the site of disease (Bell *et al.*, 2016). A TST transcriptional signature was identified, comprising significantly enriched transcripts in TSTs versus saline skin challenge. Mean expression of this TST transcriptional signature correlated precisely with the genome-wide molecular perturbations found in diseased human TB lung (Bell *et al.*, 2016). The cellular response to PPD injection constitutes immune cells responding to both tissue injury and antigen, and it is worth noting that TST response genes do not account for antigen specificity. At 48 hours post-challenge, responses can be considered to reflect acute rather than chronic infection, akin to new infections or what might occur when Mtb disseminates. However, heterogeneity at the site of chronic TB disease

(Barry *et al.*, 2009; Lin *et al.*, 2014; Gideon *et al.*, 2022) is unlikely to be recapitulated in a distal skin challenge.

Finally, responses to a standardised stimulus enables the head-to-head comparison of anti-Mtb immunity between individuals. Biopsies can be easily stored long-term before downstream RNA isolation, thus removing time-sensitive laboratory processing that may affect sample integrity. Therefore, TSTs serve as a human antigen-challenge model for anti-Mtb immunity to interrogate transcriptional responses that may determine the outcome of infection.

1.8.4. Human TST response variation

Transcriptional profiling was performed on TSTs from 167 IGRA-positive LTBI individuals recruited to the human immune response variation in TB (HIRV-TB) study. TST transcriptomes from LTBI participants were compared against transcriptomes from control TSTs using a saline injection. 3478 TST response genes were identified by outlier analysis. Given the contribution underlying genetics makes towards TB disease risk (Comstock, 1978), it was hypothesised that the variation in immune responses to infection contributes to the variation in outcome. Focus was on variable responses that correlated weakly with IFNγ. Whilst IFNγ is necessary for protection against mycobacterial disease (Bustamante *et al.*, 2014), it is not sufficient for protection and IFNγ responses are a poor correlate of outcome (Elias, Akuffo and Britton, 2005; Mittrücker *et al.*, 2007).

In my preliminary analysis of TST immune response variation, the transcript variance for TST response genes were plotted against transcript correlation with IFNγ (Figure 1.2). This revealed considerable inter-individual variance in immune responses, much of which did not correlate with IFNγ. A bimodal distribution of transcript variation was apparent, reflecting genes for which certain individuals had little to no expression of. Amongst these highly variable transcripts were numerous genes for TCR diversity alongside DEFB4A and DEFB103B.

These encode hBD2 and hBD3, respectively, members of a large AMP family that has been implicated in macrophage control of Mtb.

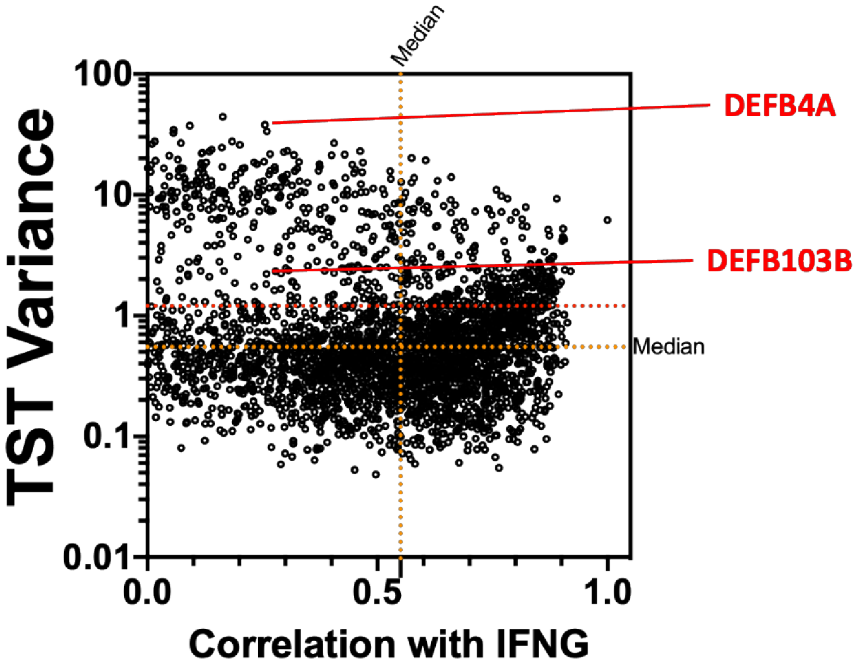


Figure 1.2. **Inter-individual variation of the anti-Mtb response in TSTs on day 2.** In my preliminary analysis of day 2 TST transcriptomic data from 167 LTBI participants, variance of each TST response gene (y-axis) is plotted against the gene's correlation with IFNγ (x-axis). Each point represents a single gene. Dotted orange lines show median values whilst the dotted red line shows the mean TST variance.

1.9. Antimicrobial peptides

Short cationic AMPs are found throughout evolution (Pazgier *et al.*, 2006; Lazzaro, Zasloff and Rolff, 2020). AMPs form an integral part of the innate immune system in humans and have long been viewed as broad-spectrum antimicrobials due to their ability to directly lyse a wide range of pathogens, including gram-positive and gram-negative bacteria, enveloped viruses, and fungi (Zhang and Gallo, 2016) In addition to this lytic function, AMPs have an ever-growing list of non-lytic, immunomodulatory functions being attributed to them (Zhang and Gallo, 2016) giving weight to a perhaps underappreciated role in vivo. There exist two well studied AMP families in humans, cathelicidins and defensins (Ganz, 2003; Dürr, Sudheendra and Ramamoorthy, 2006).

There is considerable diversity in AMP structure, length, and net-positive charge, yet all share a common attribute in their ability to interact with lipid membranes (Brogden, 2005). This is driven by electrostatic interactions between the positively charge peptides and negatively charged phospholipids on target membranes. Eukaryotic and prokaryotic membranes differ in their constituent lipids, with prokaryotic outer membranes predominantly composed of negatively charged phospholipids (Matsuzaki, 1999). Consequently, AMPs show preferential selectivity towards bacterial membranes. Membranes of plant and mammalian cells sequester negatively charged phospholipids on their inner membrane, unlike bacterial cells, which may contribute to overall protection from host AMPs (Matsuzaki, 2009). Cholesterol found in mammalian cell membranes may further stabilise membranes and reduce the cytopathic effect of host AMPs (Matsuzaki, 1999). Crucially, lytic activity can be inhibited by increasing the media ionic strength or salt concentration in vitro (Bals *et al.*, 1998; Morrison *et al.*, 1998; Vylkova *et al.*, 2007), supporting this functional electrostatic interaction.

Another important feature of AMPs is their amphipathic nature (Brogden, 2005), with a partitioning of hydrophobic and hydrophilic amino acids in their tertiary structure possibly facilitating membrane interactions and multimerisation. It is typical of AMPs to require enzymatic processing in order to function, with most transcribed as much larger precursor

proteins and stored in this inactive conformation (Liu and Ganz, 1995; Sørensen *et al.*, 2001). For certain AMPs, the pro-protein sequences mask the cationic charge of the mature peptide and may have arisen as a means of preventing cytotoxicity in the producing cell (Valore *et al.*, 1996; Satchell *et al.*, 2003). However, some beta-defensins appear to retain their lytic activity even in this pro-form (Valore *et al.*, 1996).

1.9.1. Defensins

Defensins are a large family of multifaceted AMPs (Ganz, 2003) that have been proposed to mediate antimicrobial restriction of Mtb (Dong *et al.*, 2016). The functional cationic peptides are small, approximately 30 amino acids in length, and are characterised by a cysteine-bridge-stabilised tertiary structure (Taylor, Barran and Dorin, 2008). Like many AMPs, defensins are thought to form pores in target membranes (Kagan *et al.*, 1990). They are synthesised as larger precursor proteins until processing, the exact timing of which is sub-family specific (Yount *et al.*, 1995; Ganz, 2003). Within animals the defensin family can be divided into three sub-families – alpha-defensin, beta-defensin, and theta-defensin - based on the location of their cysteine framework (Ganz, 2003). Both alpha and beta-defensins constitute a key component of the human innate immune system, whilst the theta-defensin family, found in some Old World primates, is not expressed in humans due to premature stop codons (Nguyen, Cole and Lehrer, 2003). Alpha and beta-defensins have a triple-stranded beta-sheet structure (Ganz, 2003) and members of the family are found as both monomers and dimers in solution (Hill *et al.*, 1991; Sawai *et al.*, 2001; Schibli *et al.*, 2002). Beta-defensins have been shown to kill gram-negative bacteria more efficiently than gram-positive (Wei *et al.*, 2009). The inverse is true for alpha-defensins (Wei *et al.*, 2009).

1.9.2. Genetics of human beta-defensins

Human alpha and beta-defensin genes cluster on chromosome 8p23.1 on which lies a large beta-defensin repeat unit (BDRU) of approximately 300 kilobase (kb) (Hollox, Barber, *et al.*, 2008; Machado and Ottolini, 2015). In silico approaches have identified other transcribed

beta-defensin genes residing on chromosomes 6 and 20 that have remained uncharacterised (Schutte2002).

The genes encoding hBD2 and hBD3, DEFB4 and DEFB103, respectively, both reside within the BDRU (Figure 1.3). The BDRU is subject to extensive copy number variation (CNV) with anywhere from one to twelve copies found per diploid genome (Hollox, Armour and Barber, 2003). CNV of this locus is not unique to humans having been observed in rhesus macaques (Ottolini *et al.*, 2014). Most commonly, individuals are found to have between two and seven copies (Hollox, Barber, *et al.*, 2008). In exceptionally rare cases, individuals may have just a single BDRU copy (Hollox, Barber, *et al.*, 2008). This suggests there may be an evolutionary disadvantage to encoding too few copies, such as an increased susceptibility to infection, though this has not been documented. DEFB1, encoding hBD1, resides outside of the BDRU and is not copy number variable (Hollox, Armour and Barber, 2003).

A duplication event of the BDRU has resulted in identical gene paralogs in a head-to-head orientation (Giglio *et al.*, 2001). Within the human reference genome, paralogs are denoted with a suffix *A* for the centromeric copy, or *B* for the telomeric copy. Inversion of hBD paralogs was thought to be a common event but was later shown to occur in just 27% of individuals (Sugawara *et al.*, 2003). However, this cohort comprised just 50 individuals with limited genetic diversity.

Genetic copy number has been associated with protein expression in cervical samples (James *et al.*, 2018), however hBD2 protein production has also been demonstrated to correlate better with local inflammation than it does with genetic copy number (Aldhous, Noble and Satsangi, 2009). Two large cohorts of individuals with psoriasis were enriched for increased BDRU copy number compared to control populations (Hollox, Huffmeier, *et al.*, 2008). Similar observations were made for Crohn's disease that have since been disputed (Fellermann *et al.*, 2006; Aldhous *et al.*, 2010; Bentley *et al.*, 2010)**.** This association of beta-defensin copy number with autoimmune disease suggests a central role in augmenting inflammation.

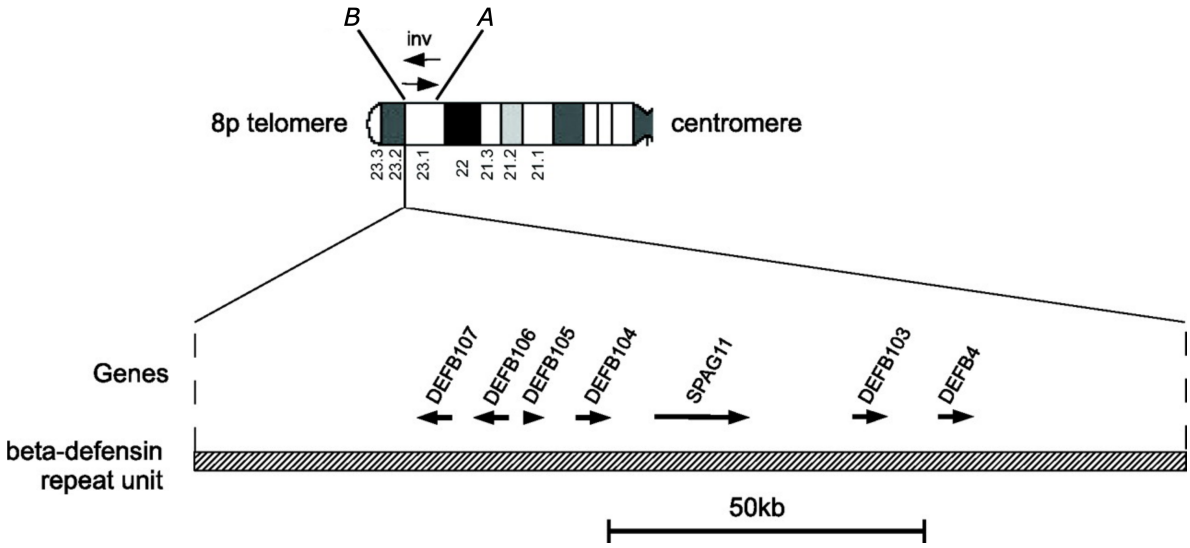


Figure 1.3. **8p23.1 genomic locus showing a single beta-defensin repeat unit.** The BDRU is a large genomic locus of approximately 300 kb located on chromosome 8p23.1. There are six beta-defensin genes encoded within the BDRU including both DEFB4 and DEFB103. BDRU paralogs lie in a head-to-head orientation, denoted with a suffix *A* for the centromeric copy and *B* for the telomeric copy. (Figure adapted with permission from Bakar, Hollox and Armour, 2009).

1.9.3. Expression and regulation of human beta-defensins

Beta-defensins are broadly distributed throughout the human body, expressed predominantly by epithelial cells in skin and at mucosal surfaces (Harder *et al.*, 1997, 2001; Bals *et al.*, 1998; McNamara *et al.*, 1999; Kaiser and Diamond, 2000). HBD2 was first isolated from psoriatic skin lesions (Harder *et al.*, 1997), being one of the most abundant peptides found in these lesions. Not long after, hBD3 was also isolated from psoriatic lesions (Harder *et al.*, 2001).

DEFB1, encoding hBD1, is constitutively expressed (Goldman *et al.*, 1997; Valore *et al.*, 1998). In contrast, both DEFB4 and DEFB103 are induced by inflammation. Several bacterial PAMPs, TNFα, IL-1β, IL-17A, and IL-22 have all been shown to upregulate their transcription (Harder *et al.*, 2000; García *et al.*, 2001; Tsutsumi-Ishii and Nagaoka, 2003; Kao *et al.*, 2004; Wolk *et al.*, 2004; Joly *et al.*, 2005; Pivarcsi *et al.*, 2005; Liang *et al.*, 2006; Reuschl *et al.*, 2017). Mycobacterial lipids can induce hBD2 expression in airway epithelial cells (Rivas-Santiago *et al.*, 2005).

Intracellular signalling pathways that culminate in nuclear factor-κB (NF-κB) and CCAAT enhancer-binding protein beta (CEBPB) translocation into the nucleus are central to the induction of hBD2 and hBD3 (Nagy *et al.*, 2005; Huang *et al.*, 2007; Jang *et al.*, 2007; Kao *et al.*, 2008). The 5' region upstream of both genes contains consensus NF-κB and CEBPB binding sites (Diamond *et al.*, 2000; Tsutsumi-Ishii and Nagaoka, 2003; Kao *et al.*, 2008). CEBPB is important for IL-17 signalling (Gaffen *et al.*, 2014), regulating *Defb4* expression (murine homologue of DEFB4) in murine oral cells (Simpson-Abelson *et al.*, 2015). In one study, a direct comparison between cytokines highlighted IL-17 as uniquely potent at inducing hBD2 expression in primary human tracheobronchial epithelial (TBE) cells (Kao *et al.*, 2004). In contrast to mouse data, there was no hBD3 induction in TBE cells with IL-17 (Kao *et al.*, 2004).

There is limited data supporting the expression of DEFB4 or DEFB103 in primary human monocytes, monocyte-derived macrophages, and alveolar macrophages (Duits *et al.*, 2002; Tsutsumi-Ishii and Nagaoka, 2003; Rivas-Santiago *et al.*, 2005; Nickel *et al.*, 2012; Rodriguez-

Carlos *et al.*, 2020; Díaz *et al.*, 2023). The strongest upregulation of both beta-defensins is in PMA-differentiated THP-1 cells infected with Mtb (Rodriguez-Carlos *et al.*, 2020; Díaz *et al.*, 2023), but these cells are known to differ from primary human macrophages in their transcriptional responses (Hoppenbrouwers *et al.*, 2022). Three studies have demonstrated DEFB4 expression in primary human phagocytes (Duits *et al.*, 2002; Rivas-Santiago *et al.*, 2005; Nickel *et al.*, 2012). It is debatable whether one is physiologically relevant, with an exceptionally modest increase following Mtb infection at a multiplicity of infection (MOI) of 350:1 (Rivas-Santiago *et al.*, 2005). A combination of LPS and IFNγ could induce DEFB4 expression in alveolar macrophages (Duits *et al.*, 2002). In contrast, DEFB4 was consistently expressed by blood monocytes and MDMs both with and without this LPS/IFNγ stimulus (Duits *et al.*, 2002). These findings have not been replicated. In another study, LPS upregulated DEFB4 in monocytes from just two of five donors (Rivas-Santiago *et al.*, 2005), suggesting there might be inter-individual variation in expression. Authors demonstrated no DEFB4 expression in monocytes or MDMs infected with Mtb (Rivas-Santiago *et al.*, 2005). However, Mtb-infected human MDMs have been observed to upregulate hBD2 mRNA under hypoxic conditions (Nickel *et al.*, 2012), designed to mimic the hypoxic environment of the granuloma (Tsai *et al.*, 2006).

1.9.4. Antimicrobial activity of beta-defensins

Beta-defensins are broad-spectrum antimicrobials that function to disrupt target membrane integrity (Ganz, 2003). Bacterial species with a greater negative surface charge were more susceptible to defensin-mediated killing (Cabak *et al.*, 2020), with hBD3 preferentially interacting with negatively charged lipid monolayers (Böhling *et al.*, 2006). Assessment of hBD3 binding affinity shows strong interactions with various phosphatidylinositols and phosphatidic acid (Phan *et al.*, 2016). Conversely, it had no affinity for cholesterol and phosphatidylcholine, both of which are major lipid constituents in the outer membranes of eukaryotic cells. Defensin interaction with anionic phospholipids is believed to initiate pore-like structure formation (Poon *et al.*, 2014). Whilst some defensins are cytotoxic to mammalian cells at high concentrations, the presence of serum in culture media inhibited this

effect (Lichtenstein, 1991). Notably, the concentrations required for eukaryotic cell lysis were substantially higher than the reported minimum inhibitory concentration (MIC) for numerous pathogens (Harder *et al.*, 2001; Sahly *et al.*, 2006; Lee and Baek, 2012; Zharkova *et al.*, 2019).

Mature hBD1, 2, and 3 have net charges of +4, +6, and +11, respectively (Pazgier *et al.*, 2006). Antimicrobial activity of hBD3 is typically greater than for hBD2 (Lee and Baek, 2012), perhaps reflecting this difference in net charge. MICs vary depending on strain and experimental conditions, but the lowest reported in literature are approximately 1.5 μg/mL for hBD2 and 0.3 μg/mL for hBD3 (Sahly *et al.*, 2006). HBD3 has been shown to kill bacteria at and above physiological salt concentrations (Harder *et al.*, 2001). Dimerisation of hBD3 may contribute towards its salt-insensitive antimicrobial activity (Schibli *et al.*, 2002).

1.9.5. Proposed mechanisms of AMP-mediated cell lysis

Four major models of membrane lysis by AMPs have been proposed: barrel-stave, toroidal, disordered toroidal, and the carpet model (Figure 1.4) (Brogden, 2005). In the barrel-stave model, peptides form transmembrane pores by directly inserting into the lipid core of the target membrane before aggregating into a pore-like structure (Vogel and Jähnig, 1986; Oren and Shai, 1998). This membrane pore facilitates the leakage of cytosolic contents, leading to cell death. For decades, AMPs have been assumed to follow this model based on ion conductance experiments that implied step changes in conductance, indicative of monomers leaving or joining the pore (Yang *et al.*, 2001). However, one of the best described examples of this barrel-stave model has been called into question (Yang *et al.*, 2001). Unlike barrel-stave, the toroidal model does not require oligomerisation into a tertiary structure (Matsuzaki *et al.*, 1996). Instead, when AMPs insert into the membrane they remain in contact with the polar lipid heads, thus causing a bend in the membrane and a central pore (Yang *et al.*, 2001). A disordered toroidal model has been proposed following molecular dynamic simulations in which only one AMP was found to reside within the terminal pore (Leontiadou, Mark and Marrink, 2006). Although there is no experimental data to support this model, the disordered nature would make it a more thermodynamically stable pore than either of the previous two

models. Lastly, there exists the carpet model, where AMPs behave in a detergent-like manner, interacting with and covering the membrane's surface (Pouny *et al.*, 1992). In this model, there is no peptide insertion but the disaggregation of membrane lipids via micelle formation.

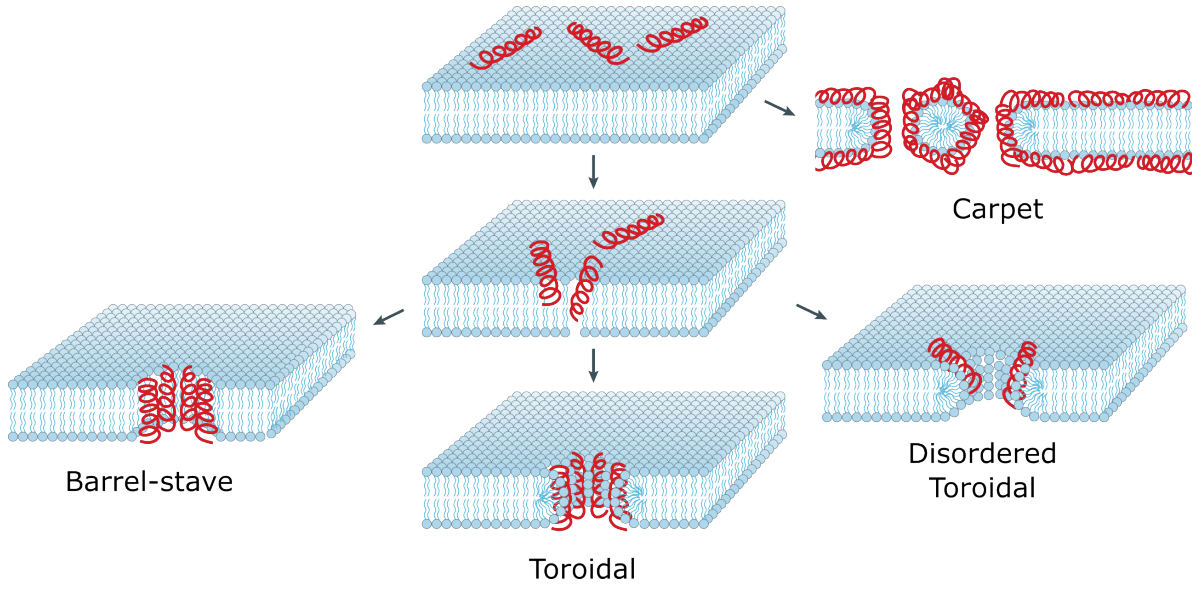


Figure 1.4. **Proposed mechanisms of antimicrobial peptide lipid membrane disruption.** AMPs localise to target membranes driven by electrostatic interactions with the negatively charged phospholipid heads. In both the barrel-stave and toroidal models, AMPs directly insert themselves into the lipid bilayer, causing a central pore in the membrane. The disordered toroidal model follows the toroidal model but requires just one AMP for pore formation. In the carpet model, AMPs behave like a detergent to disaggregate membrane lipids. (Figure adapted with permission from Brogden, 2005).

Each model of AMP membrane lysis has supporting evidence. Most often, the studied AMP differs and thus does not discount the validity of other models. Therefore, the exact mechanism may well be dependent on the peptide, its concentration, and the constituent lipids in the target membrane. Furthermore, the precise experimental conditions, such as the pH, temperature, and salt concentration, may further impact the mechanism of lysis.

Cytoplasmic AMP accumulation has been observed (Rivas-Santiago *et al.*, 2008; Semple and Dorin, 2012; Zins *et al.*, 2014). Sub-lethal concentrations of hBD3 upregulated the expression of several ABC membrane transporters in *S. aureus* (Sass *et al.*, 2008), perhaps as a means of countering cytosolic accumulation of hBD3. There are several lines of evidence that AMPs interact with cytosolic or membrane-bound targets (Brogden, 2005). Both human alpha-defensin-1 (hNP1) and a cathelicidin isolated from porcine neutrophils (Shi *et al.*, 1994) were found to rapidly block protein and nucleic acid synthesis in *E. coli* (Lehrer *et al.*, 1989; Boman, Agerberth and Boman, 1993). HNP1 interacts with a cell wall precursor lipid to kill gram-positive bacteria (De Leeuw *et al.*, 2010). Similarly, hBD3 interferes with cell wall synthesis (Sass *et al.*, 2010). AMP interactions with the peptidoglycan synthesis pathway mirror that seen for several classes of antibiotics (Breukink and De Kruijff, 2006). It is possible that AMPs gain access to intracellular targets via the permeation of the lipid membrane in a two-step mechanism of killing.

1.9.6. Bacterial resistance to antimicrobial peptides

Given the ubiquity of AMPs throughout evolution it comes as no surprise that bacteria have evolved mechanisms to resist their activity. The alteration of net surface charge can limit AMP cytotoxicity. Genes of the *dlt* operon incorporate D-alanine into the cell wall of gram-positive bacteria (Perego *et al.*, 1995). Mutations that reduced D-alanine transfer onto teichoic acids in the cell wall increased the net negative charge and therefore susceptibility to human alpha-defensins (Peschel *et al.*, 1999). AMP-resistant gram-negative bacteria were found to have modified membrane lipopolysaccharide (LPS) by removing acidic phosphate groups (Vaara *et al.*, 1981; Tran *et al.*, 2006). This was attributed to the PhoP-PhoQ system (Gunn *et al.*, 1998).

Mutations in this LPS-modifying pathway decreased virulence in mice (Gunn *et al.*, 2000). Gram-negative bacteria may even use this system to sense AMPs and promote the expression of essential virulence genes in vivo (Miller, Kukral and Mekalanos, 1989; Bader *et al.*, 2005). Importantly, Mtb encodes an enzyme that mediates resistance to AMPs by altering membrane potential (Maloney *et al.*, 2009), indicating this is an important mechanism of Mtb-growth restriction in vivo.

Efflux pumps can actively remove cytosolic AMPs from the cell (Blair *et al.*, 2022). Gram-positive bacteria were shown to respond to culture with various AMPs by upregulating the expression of membrane transporters (Sass *et al.*, 2008; Majchrzykiewicz, Kuipers and Bijlsma, 2010). Additionally, drug-resistant clinical isolates have been found to overexpress efflux pumps conferring drug-resistance (Yoon, Courvalin and Grillot-Courvalin, 2013; Prickett *et al.*, 2017).

In addition to adapting to the presence of AMPs, bacterial proteases, whether they are intracellular, membrane-bound, or secreted, can actively degrade AMPs (Schmidtchen *et al.*, 2002; Koziel *et al.*, 2010; Strempel *et al.*, 2013; Kany *et al.*, 2018). The production of phospholipid-rich outer membrane vesicles can also protect bacterial cells (Kulkarni, Swamy and Jagannadham, 2014). By functioning as a cell-free target for AMPs, they can sequester cationic peptides from the bacterial membrane (Urashima *et al.*, 2017; Balhuizen *et al.*, 2021).

There are valid concerns around the development of bacterial resistance to AMPs. Pro-longed, sub-lethal exposure to the human cathelicidin LL-37 in vitro increased tolerance (Sass *et al.*, 2008; Kubicek-Sutherland *et al.*, 2017; Urashima *et al.*, 2017). However, short, cationic AMPs that target pathogen membranes are ubiquitous in nature (Lazzaro, Zasloff and Rolff, 2020). Given this apparent conserved function, adaptation of the prokaryotic membrane to resist AMPs must come at some cost to bacterial fitness.

1.9.7. Non-lytic functions of human beta-defensins

HBDs have a growing list of non-lytic functions being attributed to them. Both hBD2 and hBD3 exhibit chemotactic activity for CCR6 and CCR2-expressing cells (Yang *et al.*, 1999; Niyonsaba, Ogawa and Nagaoka, 2004; Röhrl *et al.*, 2010a; Kim, Yang and Jang, 2019). Both hBD2 and hBD3 chemoattract cells at 2-100 ng/mL, substantially lower concentrations than is required for bacterial killing (Wu *et al.*, 2003; Sahly *et al.*, 2006). It is interesting consider that many chemokines share the same characteristic tertiary structure, charge, and antimicrobial activity as beta-defensins (Yang *et al.*, 2003).

Additionally, beta-defensins can prime immune cells (Funderburg *et al.*, 2007; Barabas *et al.*, 2013; Wanke *et al.*, 2016) and induce pro-inflammatory cytokine production (Boniotto *et al.*, 2006; Jin *et al.*, 2010; Niyonsaba *et al.*, 2010; Judge *et al.*, 2015). HBD3 induces IFNγ secretion from NK cells (Judge *et al.*, 2015). It also increases co-stimulatory molecule surface expression on monocyte-derived DCs (Funderburg *et al.*, 2007), in turn augmenting this NK cell IFNγ response (Judge *et al.*, 2015).

Priming was shown to be independent of CCR6/2 receptor recognition (Barabas *et al.*, 2013) with studies supporting TLR1 and TLR2 as a beta-defensin receptor (Funderburg *et al.*, 2007, 2011). Whilst hBD2 concentrations between 2–10 μg/mL induce pro-inflammatory cytokine production (Boniotto *et al.*, 2006; Jin *et al.*, 2010), further increases in concentration also led to anti-inflammatory IL-10 production (Boniotto *et al.*, 2006). Interestingly, suppression of LPS-induced inflammation by hBD3 has been described (Semple *et al.*, 2010), attributed to interactions with TLR receptors as opposed to its ability to bind cell-free LPS (Semple *et al.*, 2011). In addition to leukocytes, hBD2-4 have all been shown to induce IL-18, MCP-1, MIP3a, RANTES, IL-6, IL-10, and IP-10 expression in keratinocytes (Niyonsaba *et al.*, 2005) as well as induce their migration (Niyonsaba *et al.*, 2007).

In summary, beta-defensins appear to be multifaceted peptides. They display broad-spectrum antimicrobial activity in addition to numerous immunomodulatory effects across several cell types important for anti-Mtb immune responses. Induced during acute infection,

they form an essential component of germline encoded immunity, directly restricting pathogen growth and potentially orchestrating the early immune response. Therefore, beta-defensins may have an underappreciated role in vivo.

1.10. Beta-defensins in tuberculosis

The TST transcriptome revealed a cluster of highly variable genes with poor correlation with IFNγ (Figure 1.2) which were of interest as candidate genes for protective immunity. Given the central role of macrophages in TB, I focused on candidate genes with literature supporting involvement in macrophage control of Mtb, DEFB4A and DEFB103B.

Both genes encode AMPs that are directly bactericidal against mycobacteria in vitro (Corrales-Garcia *et al.*, 2013; Reuschl *et al.*, 2017; Su *et al.*, 2018) and can localise with intracellular Mtb (Rivas-Santiago *et al.*, 2005). Studies using infected human MDMs transfected with DEFB4 mRNA (Kisich *et al.*, 2001), and mouse models of TB (Peng *et al.*, 2024), imply a protective role for hBD2 in reducing bacterial load and lung inflammation. Data from this mouse model suggested Mtb inhibits NF-kB signalling and thus *Defb4* induction (Peng *et al.*, 2024), though loss of this inhibitory activity also induced several other cytokines and chemokines that augment anti-Mtb immunity. In vitro hypoxic conditions upregulated hBD2 mRNA in human MDMs infected with Mtb (Nickel *et al.*, 2012). Therefore, it is plausible that hBD2 may restrict Mtb growth within the hypoxic environment of the granuloma (Tsai *et al.*, 2006).

Plasma concentrations of hBD3 correlate with pulmonary TB severity (Bongiovanni *et al.*, 2020), whilst cutaneous TB augmented expression of DEFB4 and DEFB103 compared to normal skin (Zhao *et al.*, 2016). The induction of these beta-defensins perhaps directly reflects changes in bacterial burden. However, DEFB4A and DEFB103B are enriched in TSTs from active TB patients, with TST expression subsequently decreasing following the completion of anti-TB therapy (Pollara *et al.*, 2021). This suggests some aspect of disease drives their expression, such as changes in their epigenetic regulation or an increase in cytokine responses that induce beta-defensins. Whether such changes are in consequence of increased bacterial burden, precede increases in bacterial burden, or even precipitate such events, remains to be

elucidated. Most studies of beta-defensins in the context of Mtb infection focus on their potential as a therapeutic agent (Dong *et al.*, 2016), but it is not known whether the hBD response reflects a physiologically relevant component of anti-Mtb immunity.

1.11. Hypothesis

Inter-individual variation in DEFB4 and DEFB103 expression in the TST represents a potential determinant for differential outcomes of Mtb infection. I hypothesised that the variation in the expression of DEFB4 and DEFB103 in response to Mtb infection is due to genetic copy number variation, and contributes to Mtb growth restriction by macrophages.

1.12. Research objectives

To investigate this hypothesis, I addressed the following research objectives:

1. What is variation in beta-defensin expression due to?
2. Do macrophages upregulate beta-defensin expression in the in vivo immune response to Mtb?
3. Does augmented beta-defensin expression lead to greater Mtb restriction in macrophages?

2. Methods

2.1. Study participants

Individuals with LTBI, confirmed by positive peripheral blood IGRAs using QuantiFERON Gold Plus Test (Qiagen) with absence of clinical or radiological evidence of TB disease, were recruited onto the HIRV-TB study (Principal investigator: M Noursadeghi), approved by the NHS Research Ethics Committee (IRAS: 242062, REC: 18/LO/0680). At baseline, all participants donated peripheral blood for genomic DNA isolation and were subjected to TSTs in their forearm; an intradermal injection of 0.1 mL 2U tuberculin (Serum Statens Institute). LTBI participants recruited to the HIRV-TB study were segregated into two cohorts. For cohort 1, puncture biopsies were taken 48 hours post-TST at the site of the TST. Biopsies were stored in either: RNA*later* Stabilization Solution (Invitrogen) and transferred to -80°C to stabilise total RNA for downstream tissue RNA isolation and sequencing; or 10% neutral buffered formalin solution to stabilise the tissue for image analysis. An additional TST with puncture biopsy was performed 7 days after the administration of tuberculin, for use in the expression quantitative trait loci (eQTL) analysis. For cohort 2, blister suction cups were placed across the site of the TST for 1-3 hours until a satisfactory blister had formed over the area of inflammation. The blister was punctured and the fluid aspirated for scRNA-seq.

Individuals with a microbiologically confirmed Mtb infection and accompanying clinical symptoms, resulting in an active TB diagnosis, were recruited onto the MONITOR study before starting treatment (Principal investigator: M Noursadeghi), approved by the NHS Research Ethics Committee (IRAS: 280133, REC: 20/LO/1217), or the Tuning TB study (Principal investigator: G Tomlinson), approved by the NHS Research Ethics Committee (IRAS: 197718, REC: 16/LO/0776). MONITOR TB participants were subjected to TSTs as previously described. 48 hours post-TST, puncture biopsies were taken at the site of the TST and stored in 10% neutral buffered formalin solution to stabilise the tissue for image analysis. Tuning TB participants were subjected to TSTs as previously described or had an intradermal injection of saline solution in their forearm. 48 hours post-injection, puncture biopsies were taken from

the site of injection. Biopsies were stored in RNA*later* Stabilization Solution (Invitrogen) and transferred to -80°C to stabilise total RNA for downstream tissue RNA isolation and sequencing.

2.2. Tissue processing

For processing for RNA sequencing, TST biopsies were equilibrated to room temperature for 30 minutes before transferring to CK14 tissue lysis tubes (Bertin instruments) containing 350 µL of Buffer RLT (Qiagen) supplemented with 1% 2-Mercaptoethanol (Sigma). Tissue was homogenised using a Precellys Evolution Homogeniser (Bertin Instruments). Tubes were pulsed for 6 x 23 second cycles at 6300 rpm, resting for 2 minutes between cycles on ice. RNA from homogenised tissue was isolated using RNeasy Mini Kit (Qiagen) obtain high- quality total RNA before using TURBO DNA-free Kits (Thermo Fisher Scientific) to remove residual genomic DNA. RNA concentrations were quantified using Qubit 2.0 Fluorometer (ThermoFisher Scientific). RNA integrity scores were determined using the 4200 Tape Station (Agilent). All kits were used according to the manufacturer's instructions.

For TST biopsies taken for image analysis, these were immediately stored in 10% neutral buffered formalin solution for 24 hours at room temperature to fix the tissue. After 24 hours, these were transferred to phosphate-buffered saline (PBS) (Gibco) prior to embedding in paraffin wax, in accordance with the Advanced Cell Diagnostic's RNAScope Multiplex Fluorescent v2 Assay protocol. Formalin-fixed, paraffin-embedded (FFPE) TST biopsies were cut into 3 µm sections and mounted onto SuperFrost Plus adhesion slides for downstream RNAscope applications.

4 mL blood samples from HIRV-TB participants were taken at baseline in EDTA BD Vacutainer blood collection tubes (VWR) before being aliquoted and stored at -80°C until processing. For genomic DNA isolation, blood samples were thawed at room temperature before processing with the QIAamp DNA Blood Maxi kit (Qiagen). DNA concentrations were quantified using Qubit 2.0 Fluorometer (ThermoFisher Scientific). DNA integrity scores were determined using

the 4200 Tape Station (Agilent). All kits were used according to the manufacturer's instructions.

2.3. RNA-sequencing

For genome-wide mRNA-sequencing, samples were shipped to the Pathogen Genomics Unit (UCL). Complementary DNA (cDNA) libraries were generated using Kappa HyperPrep Kits (Roche) and sequenced on the Illumina NextSeq 550 system using NextSeq 500/550 High Output 75 Cycle Kits (Illumina), resulting in a median of 25 million 41 bp paired-end reads per sample. All kits were used according to the manufacturer's instructions. The Pathogen Genomics Unit performed all sequencing quality control steps and the conversion of Binary Base Call (BCL) files to FASTQ format.

RNAseq data was mapped to Ensembl Human GRCh38 release 111 using Kallisto (v0.46.1), or HISAT2 (v2.2.1). Raw read counts were normalised to transcripts per million (TPM) values, summed at gene level, and annotated with Ensembl gene ID, gene name, and gene biotype using the Bioconductor packages tximport (v1.20.0) and biomaRt (v.2.48.0) in R (v4.1.0).

2.4. Single cell RNA-sequencing

For single cell mRNA-sequencing, TST blister fluid was aspirated and erythrocytes lysed with RBC Lysis Buffer (Invitrogen). The cell suspension was centrifuged to pellet the cells before Fc receptor blockage with Human TruStain FcX (BioLegend). Staining for cell surface antigen capture was performed using TotalSeq-C Human Universal Cocktail (BioLegend) for 30 minutes at 4°C, according to the CITE-Seq protocol (Version: 2019-02-13, New York Genome Center Technology Innovation Lab). For single cell capture and barcoding, stained single cells were loaded onto the 10x Chromium Controller (10x Genomics) at a maximum of 20,000 cells per lane, using the 5' Single Cell Immune Profiling with Feature Barcoding kit (10x Genomics). Barcoded cDNA was processed for single cell sequencing library preparation according to the manufacturer's instructions. Library concentration and quality were evaluated using the Qubit 2 Fluorometer (Invitrogen) and High Sensitivity D5000 kit with the 4200 TapeStation

System (Agilent). Prepared 5' gene expression libraries were outsourced to Novogene for sequencing on the Illumina NovaSeq 6000 system targeting 20,000 150 bp paired-end reads per cell. Novogene performed all sequencing quality control steps and the conversion of BCL files to FASTQ format.

The final approach for processing and clustering of TST blister scRNA-seq data was decided upon with Carolin Turner (University College London). This occurred throughout the continuation of laboratory work required to generate this TST blister dataset and was mostly in the format of weekly meetings to discuss progress. The aim was to develop a robust single cell analysis pipeline that would be deemed suitable for subsequent datasets with minimal modification. FASTQ data was processed using the Cell Ranger (v7.1.0) (10x Genomics) 'multi' pipeline, including read alignment to the Ensembl Human GRCh38 reference (gene expression reference version 2020-A and VDJ-T reference version 7.1), filtering, barcode counting, and unique molecular identifier (UMI) counting. Data were processed using Bioconductor packages in R (version 4.1.1). To remove low-quality cells (those with few UMIs, few expressed genes, or a high number of mitochondrial reads), it was decided that any cell residing beyond three absolute median deviations below the median for a given quality metric would be discarded. This approach enables consistently robust exclusion of low-quality cells from any given dataset. Furthermore, this approach is not tied to any specific analysis pipelines and can easily be adopted to past and future datasets. Doublets were identified by scDblFinder and removed, and a gene sparsity filter was used to remove genes detected in <0.1% of all cells. This sparsity filter was subsequently modified in my analysis of single cell expression of beta-defensin genes, to enable the retention of selected beta-defensin genes. Dataset dimensionality was reduced using highly variable genes (modelGeneVar and getTopHVGs functions from the scran package) before samples from 31 participants were integrated (mutual nearest neighbours algorithm with k=50) using the top 50 principal components. Cell clustering was performed with the Louvain algorithm. The optimal clustering resolution was set using single-cell significance of hierarchical clustering (scSHC) as a post-hoc test, which evaluates whether clusters could have arisen from a single larger

cluster using a bootstrapped null distribution of the Ward linkage. The approach worked as follows; stepwise increases in the cluster resolution were made, starting at 0.2 and increasing by 0.2 each time, to find the resolution that gave the maximum number of statistically confirmed Louvain clusters using scSHC. Upon deciding on this maximum resolution, subsequent rounds of clustering were performed on each cluster with a comparable approach. This continued until no further clusters could be statistically validated, or the clusters contained fewer than 500 cells. Clusters were then manually annotated for cell ontogeny using a priori gene markers based on the expected cell types in TSTs. Finally, the clusters were merged to depict the 13 distinct cell lineages presented in my analysis. Uniform manifold approximation and projection (UMAP) visualisation was performed with the runUMAP function in scater (v1.18.6) using the default top 50 principal components. The percentage of cells expressing each marker, and the average expression of each marker, were visualised with the plotDots function in scater (v1.18.6).

Processing and clustering of human TB granuloma scRNA-seq data was performed with Kieran Killington (UCL). FASTQ data was processed using Cell Ranger (v7.1.0) (10x Genomics) with read alignment to the Ensembl Human GRCh38 reference (gene expression reference version 2020-A). All filtering, barcode counting, and UMI counting parameters were kept as detailed in the original manuscript; cells with <200 genes or with >35% mitochondrial reads were discarded. Principal component analysis (PCA) dimensionality reduction, data integration (canonical correlation analysis (CCA) method), clustering were performed using Seurat (v3). There was no integration with the healthy lung tissue dataset (Habermann *et al.*, 2020). Clustering resolution was set at 0.2 to obtain broad cell clusters. Uniform manifold approximation and projection (UMAP) visualisation was performed with the RunUMAP function in Seurat (v3). Cluster annotation used a priori gene markers based on the expected cell types in human TB granulomas. The percentage of cells expressing each marker, and the average expression of each marker, were visualised with the DotPlot function in Seurat (v3).

2.5. Analysis of ATAC-seq data

Publicly available ATAC-seq datasets derived from unstimulated human lung epithelial cells (GEO accession: GSE183873, European Nucleotide Archive accession: PRJNA762259) (Kadur Lakshminarasimha Murthy *et al.*, 2022), resting human MDM at the end of differentiation in vitro (GEO accession: GSE147306, European Nucleotide Archive accession: PRJNA613719) (Song *et al.*, 2021), and human monocytes/MDM throughout an LPS stimulation time-course experiment (GEO accession: GSE147307, European Nucleotide Archive accession: PRJNA613718) (Song *et al.*, 2021) were visualised using Integrative Genomics Viewer (v2.16.0) (Robinson *et al.*, 2011). ATAC-seq data from alveolar macrophages was not made publicly available (Staitieh *et al.*, 2023), but insight into the chromatin accessibility surrounding beta-defensin gene paralogs was obtained from correspondence with the senior author.

2.6. Cell type and cytokine activity module analysis

Derivation of cell type specific and cytokine activity modules are described in detail previously (Bell *et al.*, 2016; Byng-Maddick *et al.*, 2017; Pollara *et al.*, 2021; Turner *et al.*, 2024). Genes comprising specific peripheral blood immune cell modules were identified in published dataset (ArrayExpress accession number E-GEOD-22886) as follows; significantly upregulated genes were identified by a t-test with Welch's approximation ($p<0.05$) and a four-fold change threshold in comparison to the unstimulated cell type. Where a four-fold change threshold identified too few genes for the module, a two-fold change filter was used. Module specificities were then validated in a published genome-wide dataset of purified cell types (ArrayExpress accession number E-GEOD-28490). Module sensitivities were validated in further published genome-wide datasets describing histologically confirmed changes in cell abundance (ArrayExpress accession numbers E-GEOD-22886, E-GEOD-28490, E-MTAB-2547, and E-GEOD-16844). Cytokine response modules were derived from datasets in which primary human keratinocytes were stimulated with a selection of recombinant human cytokines (GEO accession numbers GSE12109 and GSE36287) using the same approach as the

peripheral blood immune cell modules. Module specificities were validated in an independent dataset (GEO accession number GSE36287).

TST-specific cell type modules were identified in the TST blister scRNA-seq dataset (Turner *et al.*, 2024) using pseudobulked expression by cluster. Cell type-specific differential gene expression by two-sided Wilcoxon tests were performed by comparing the given cell type against all other cells in the dataset. The top 50 significantly upregulated genes, ranked by decreasing log2 fold difference with an adjusted p-value threshold of <0.05, were chosen. Signatures were validated in an independent COVID inflammatory lung disease scRNA-seq dataset (Mehta *et al.*, 2024).

2.7. Single nucleotide polymorphism genotyping

Isolated genomic DNA from HIRV-TB study participants were hybridised to the Global Diversity Array-8 (GDA) BeadChip (Illumina) according to the manufacturer's instructions. The GDA, a high-density single nucleotide polymorphism (SNP) microarray, is optimised for cross-population SNP imputation and was selected due to the diverse ethnic groups featured in the HIRV-TB and Tuning TB studies.

2.8. Expression quantitative trait loci analysis

EQTL analysis including genotype imputation was kindly performed by Ping Zhang (Oxford University). A threshold of 1 megabase (Mb) extending up and downstream from the transcriptional start site (TSS) of any given gene was used to identify cis-acting SNPs. The present eQTL analysis incorporates TST transcriptomic data from day 2 (N=216) and day 7 (N=158) TSTs in LTBI participants (HIRV-TB study) and additional day 2 TSTs from active TB participants (Tuning TB study) (N=41). For multiple testing correction, the dataset was permutated 1000 times with scrambled genotypes and p-values ranked. An adjusted p-value was set for each gene based on ranked p-values.

2.9. Quantitative polymerase chain reaction

Reverse transcription (RT) to generate cDNA from TST RNA samples was performed with 20 ng of cDNA using TaqMan RT reagents (Applied Biosystems) and random hexamers. Quantitative polymerase chain reaction (qPCR) for DEFB4 and DEFB103 mRNA expression was performed using TaqMan inventoried assays (Applied Biosystems) according to the manufacturer's instructions. GAPDH mRNA expression was used as an internal reference gene for each qPCR. Cycle threshold data were analysed using Microsoft Excel.

2.10. Triplex paralogue ratio test

The triplex paralogue ratio test (PRT) has been described in detail (Hollox, 2017). Triplex PRT comprises of two PRT assays (PRT107A and HSPD21) and a third multiallelic ratio test (indel rs5889219) to give three independent measures of beta defensin repeat unit copy number. Each primer pair amplifies multiple regions of the genome, resulting in several polymerase chain reaction (PCR) products per assay. PRT107A and HSPD21 assays amplify a copy number variable amplicon residing within the BDRU, and a copy number stable reference amplicon from elsewhere in the genome. Each assay then utilises the ratio between amplicons as a measure of copy number. The indel rs5889219 assay amplifies a multiallelic variant that differs in length between repeats. The assay does not result in an integer value for copy number but rather a ratio of one to three amplicons, the total sum of which being divisible by the BDRU copy number. Each amplicon differs in length by a few base pairs, enough to enable separation by DNA electrophoresis without adversely affecting PCR amplification kinetics. Each test is performed in duplicate with differing fluorescently-tagged primers facilitating quantitation.

PRT107A PRT amplifies a region encoded within the beta defensin repeat unit and a copy number stable paralog, encoded on chromosome 11. PRT107A forward primer (AGCCTCATTTAACTTTGGTGC) and reverse primers (GGCTATGAAGCAATGGCCTA) amplify two amplicons of 154 bp and 156 bp in size. PRT107A forward primers incorporated either FAM or HEX fluorescent tags. HSPD21 PRT also amplifies a region encoded within the beta defensin

repeat unit and a copy number stable paralog, encoded on chromosome 21. HSPD21 forward primer (GAGGTCACTGTGATCAAAGAT) and reverse primers (AACCTTCAGCACAGCTACTC) amplify two amplicons of 172 bp and 180 bp in size. HSP21 reverse primers incorporated either FAM or NED fluorescent tags. Indel rs5889219 forward primer (AAACCAATACCCTTTCCAAG) and reverse primers (TTCTCTTTTGTTTCAGATTCAGATG) amplify a potential of three amplicons of 125 bp, 128 bp, and 130 bp in size. Indel rs5889219 forward primers incorporated either HEX or NED fluorescent tags. HSPD21 and PRT107A tests were performed in duplex using 10 ng of genomic DNA. PCRs begun with denaturation at 95°C for 5 min followed by 22 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 70°C for 1 minute, followed by 70°C for 40 minutes. The indel rs5889219 assay was performed separately, requiring 25 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 70°C for 1 minute, followed by 70°C for 20 minutes.

Amplicon separation was performed by Source Bioscience. PRT amplicons from the same sample were pooled into one run with MapMarker® 400 X-Rhodamine (ROX400) marker and highly deionized (HiDi) formamide before separation using the 3730XL DNA capillary electrophoresis instrument (Applied Biosystems). Areas under amplicon peaks were identified using Peak Scanner software (v1.0) (Thermo Fisher). Raw ratios between area under amplicon peak from either PRT assays were normalised by linear regression using 6 human random control (HRC) DNA samples from the ECACC collection (Sigma) (HRC ID: c0088, c0207, c0849, c0913, c0940, c0969). The six independent copy number estimates are combined using a maximum- likelihood approach to calculate the absolute probability of the normalised data corresponding to an integer value of 1 to 9. Data is assumed to follow a Gaussian normal distribution and equal probability is assumed for each copy number. For PRT107A and HSPD21 assays, 9 probabilities are generated, whereas the indel rs5889219 assay has 25 estimates owing to different amplicon ratios corresponding to the same integer value. The highest probabilities are combined to estimate copy number for each individual. Every copy number call is associated with a p-value reflecting confidence in said call, with p-values of greater than 0.01 resulting in sample exclusion.

2.11. HEK293T transfection

HEK293T cells (provided by David Stirling, University College London) were seeded into T-75 flasks (Corning) at 25% confluency in Dulbecco's Modified Essential Medium, high glucose (DMEM) (Sigma) supplemented with 10% foetal calf serum (FCS) (Sigma). After 24 hours, culture media was aspirated and fresh DMEM with 10% FCS was added, supplemented with 250 µL Opti-MEM (Gibco), 30 µL FuGENE 6 (Promega), and 2 µg of lentiviral plasmid vector (SFXUG v2) per T-75 flask. SFXUG plasmid constructs encoded DEFB4 or DEFB103 under a spleen focus-forming virus (SSFV) promoter, and a reporter green fluorescence protein (GFP) gene under the Human Ubiquitin C promoter, for the constitutive expression of both genes. Efficient transfection was confirmed visually by the presence of GFP positive cells 48 hours post-transfection.

At 48 hours post-transfection, cells were detached using Trypsin-EDTA (0.25%) (Gibco) and pelleted by centrifugation, 400 xg for 5 minutes. Cells were fixed using 4% paraformaldehyde (PFA) for 24 hours with constant gentle agitation before washing with PBS (Gibco) and subsequent paraffin embedding, as per ACDBio's RNAscope assay protocols. Fixed, paraffin embedded HEK293T cell pellets were cut into 3 µm sections and mounted onto SuperFrost Plus adhesion slides for downstream RNAscope applications.

2.12. RNAscope staining and image acquisition

Mounted FFPE TST biopsy sections were prepared and stained with mRNA probes specific for DEFB4, DEFB103, ANPEP (encoding CD13), CD14, CD68, and CD3D, using the RNAscope Multiplex Fluorescent Reagent Kit v2 Assay (ACDBio) according to the manufacturer's instructions. DEFB4 and DEFB103 mRNA probes were bioinformatically designed by ACDBio upon request. 3-plex RNAscope™ Positive Control Probes and 3-plex RNAscope™ Negative Control Probes (ACDBio) were also used. Positive Control Probes target human POLR2A, PPIB, and UBC mRNA and were used to assess sample RNA integrity. Negative Control Probes target bacterial dapB mRNA in all three channels and thus assess non-specific staining. Whole-slide multispectral imaging of TST sections was performed using PhenoImager HT (Akoya

Biosciences). Phenochart and inForm Tissue Analysis software (Akoya Biosciences) were used to process whole slide scans covering the entire tissue into tiled 40X images for downstream spectral unmixing and analysis. In addition to exporting separate Tag Image File Format (TIFF) images for each fluorescence channel, Phenochart utilises known emission spectra for Opal™ fluorescent dyes (Akoya Biosciences) to detect tissue autofluorescence and export as a separate TIFF image.

2.13. Image analysis

Image analysis was performed using QuPath software (v0.5.0) (Bankhead *et al.*, 2017). First, processed 40X images were stitched together using QuPath-Merge unmixed files to pyramid.groovy, which converts multiple TIFF fields of view to a single image. QuPath was then used to spectrally unmix Opal™ fluorescent dyes (Akoya Biosciences) and remove tissue autofluorescence for the entire tissue section. Cell segmentation was performed with the StarDist extension in QuPath (Bankhead *et al.*, 2017; Schmidt *et al.*, 2018) using a 5-pixel boundary expansion of the edge of the DAPI nuclear stain to approximate single cells. The small boundary maximises the likelihood that signal within a segmented object originated from a single cell. Within single cells, the mean fluorescence intensity for each Opal™ fluorescent dye (Akoya Biosciences) was determined.

The RNAscope assay is highly sensitive to low levels of gene expression. However, it was observed that TST tissue sections stained with the 3-plex RNAscope™ Negative Control Probes (ACDBio) had evidence of non-specific staining. Therefore, every TST section assessed for target mRNA expression was paired with a section simultaneously stained using the Negative Control Probes. Negative control probe scans were used to obtain a mean and standard deviation of signal intensities for each channel representing this non-specific staining. These values were then used to convert channel intensities for the mRNA probes of interest into z-scores, and any signal >2 standard deviations (z-score=2) away from the corresponding channel in the negative control probe scan was retained. A binary approach based on this z-score threshold was used to define whether a cell expressed the target mRNA or not.

2.14. Cell culture

All steps of cell culture were performed at room temperature unless stated otherwise. Human primary MDM were prepared fresh from individual healthy human donors for each experiment. Briefly, blood was drawn and PBMC isolated by density centrifugation using Ficoll-Paque Plus (Cytiva) at 800 xg for 20 minutes. After washing three times with PBS (Gibco), PBMC were resuspended in Roswell Park Memorial Institute Medium 1640 with L-glutamine (RPMI) (Gibco) supplemented with 5% heat-inactivated (56°C for 30 minutes) pooled type AB human serum (ABS) (Sigma). $4x10^6$ PBMC were seeded per well of 24-well tissue culture plates (TPP) and left to adhere for 1 hour at 37°C, 5% CO_2, at which point non-adherent cells were removed by washing again. Adherent cells were incubated for three days at 37°C, 5% CO_2 in RPMI supplemented with 10% heat-inactivated (56°C for 30 minutes) autologous serum and 20 ng/mL macrophage colony-stimulating factor (M-CSF) (R&D Systems). On day 3, cells were washed again and culture media replaced with fresh RPMI supplemented with 5% ABS, before adherent cells were left for a further three days at 37°C, 5% CO_2 to fully differentiate. On day 6, MDM were transferred to biosafety level 3 (BSL-3) laboratories for infection experiments.

Airway epithelial cell (AEC) lines (A549, BEAS-2B, Calu-3, Detroit 562) (a kind gift from Ann-Kathrin Reuschl, University College London) were grown in T-75 flasks (Corning) at 37°C, 10% CO_2 in DMEM (Sigma) supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma). Culture media was refreshed every 2-3 days. For passage, AECs were detached from tissue culture flasks using Trypsin-EDTA (0.25%) (Gibco) and pelleted by centrifugation, 400 xg for 5 minutes. Fresh culture media was added to the pellet and AECs then split at an appropriate ratio for passage, maintaining confluency between 20 – 80%. When seeding for experiments, AECs were detached as previous and seeded into the appropriate size tissue culture flask (Corning) or plate (TPP).

Human primary alveolar pneumocytes (haAEC) (EP71AL, Epithelix) from three healthy human donors (ATII0835, ATII0889.02, 05AB0937.02) were grown in T-75 flasks (Corning) at 37°C 5%

CO_2 in Pneumocyte Culture Medium (EP11AL, Epithelix). Every 3 days, culture medium was aspirated and replaced with fresh Pneumocyte Culture Medium. HaAEC were not passaged. HaAEC were detached from tissue culture plates using Trypsin-EDTA (0.25%) (Gibco) and seeded into the appropriate size tissue culture flask (Corning) or plate (TPP).

2.15. Mtb culture

Mtb (H37Rv strain) constitutively expressing E2-Crimson (E2C) fluorescent protein (E2C H37Rv) (a kind gift from Maximiliano Gutierrez, The Francis Crick Institute, London, UK) was grown in Middlebrook 7H9 medium (BD Biosciences) supplemented with 10% Middlebrook ADC Growth Supplement (BD Biosciences), 0.2% glycerol (Glycerol), 0.05% Tween™ 80 (Sigma), and 40 μg/mL hygromycin (Invitrogen) at 37°C with agitation (90 rpm). Growth was monitored bi-weekly by measuring optical density at 600 nm wavelength (OD_{600}) and Mtb cultures passaged when approaching end of log-phase ($OD_{600} \geq 1$).

2.16. Mtb-infection experiments

On the day of infection, the OD_{600} of Mtb cultures were measured and those in mid-log-phase growth (OD_{600} = 0.6 – 1.0) chosen for disaggregation. To disaggregate, 2 mL of Mtb culture was pelleted by centrifugation at 2000 xg for 5 minutes. 1 mL of supernatant was removed and approximately five sterile 2 mm glass beads added to the pellet. Cultures were vigorously agitated by hand for 1 minute before leaving for 5 minutes to allow aerosols to settle. Bacteria were gently resuspended with 1 mL of PBS (Gibco). Residual clumps were left to settle for several minutes and the OD_{600} measured from the top half of the liquid. The concentration of Mtb in disaggregated cultures was discerned using previously determined Mtb concentrations for given OD_{600} measurements.

An appropriate volume of disaggregated Mtb was added to RPMI (Gibco) supplemented with 5% ABS (Sigma) to result in a MOI of 1:1. Rested, fully differentiated MDMs on day 6 of culture were washed once with PBS (Gibco) before infection with 200 μL of the prepared Mtb in RPMI

supplemented with 5% ABS. Infected MDMs were returned to the incubator at 37°C for 4 hours to allow for internalisation of Mtb.

After 4 hours MDMs were vigorously washed three times with PBS to remove extracellular bacteria. For supernatant transfer experiments, 1 mL of the appropriate AEC culture supernatant was added to the well. In the case of experiments using recombinant human beta-defensin (rhBD) 2 and 3 (Peprotech), 1 mL of RPMI supplemented with 5% ABS and the appropriate concentration of rhBD was added to the well. In addition, one well received 1 mL RPMI supplemented with 5% ABS and 50 ng/mL of isoniazid (INH) (Cambridge Bioscience). Plates were then returned to the 37°C incubator and left for 120 hours.

2.17. Harvest and fixation of infection experiments

For each infection experiment, the Mtb from a single well of infected MDM cultures were harvested at 4 hours post-infection (after the removal of extracellular bacteria) to obtain a baseline for Mtb growth. At the relevant subsequent time point, supernatant was mixed using a pipette and the extracellular Mtb in supernatant collected. Cells were then vigorously washed with PBS (Gibco) before lysis for 6 minutes using distilled water with 0.05% Tween™ 80 (Sigma). Lysate was mixed using a pipette and the bottom of the well scraped to ensure the collection of all intracellular Mtb. All samples were then fixed with PFA (4% final concentration) for 15 minutes before removal from the BSL-3 laboratory.

2.18. Flow cytometry

CountBright™ Absolute Counting Beads (Invitrogen) were mixed with samples before running on a BD LSRFortessa™ Cell Analyzer (BD Biosciences). Gating and analysis was performed using FlowJo™ Software (v10.6.2) (BD Biosciences).

To account for clumping of bacteria, gated E2C H37Rv events are multiplied by the mean fluorescence intensity of gated events in the PE-Cy5 channel to give the total Mtb load for the sample. The PE-Cy5 mean fluorescence intensity and FCS value of events increase linearly

with live bacteria (Mehta, 2020). Total bacterial load per sample was calculated using the equation below:

(E2C H37Rv count x mean fluorescence) x (Beads count x # beads added)

2.19. Cytokine stimulation experiments

AECs and haAEC were seeded into the appropriate tissue culture dish for the given experiment. For the evaluation of hBD2 and hBD3 production, $1x10^5$ AECs and haAEC were seeded in DMEM 10% FCS (AECs) or Pneumocyte Culture Medium (haAEC) per well of 12-well tissue culture plates (TPP), and left to adhere for 24 hours at 37°C (10% CO_2 for AECs, 5% CO_2 for haAEC). Alternatively, when generating supernatant for supernatant transfer experiments $2.1x10^6$ AECs were seeded into T-75 flasks (Corning). After adhering, cells were washed once with PBS (Gibco) and fresh culture media added supplemented with 10 ng/mL each of recombinant human IL-17A, IL-1β, and TNFα (Peprotech), or fresh culture media alone. Supernatants were harvested at the relevant time-point and stored at -80°C for enzyme-linked immunosorbent assay (ELISA) measurement of beta-defensin concentration and supernatant transfer experiments.

2.20. Enzyme-linked immunosorbent assay

The concentration of hBD2 and hBD3 in samples were assessed using Human BD-2 and BD-3 Standard ABTS ELISA Development Kits (Peprotech). OD_{405} measured using a Multiskan™ FC Microplate Photometer (Thermo Scientific) with wavelength correction set at OD_{650}. All kits were used according to the manufacturer's instructions.

2.21. Statistical analyses

Linear regression models, multivariate correlation analyses, and regression analyses were performed in R. Linear regression and correlation analyses utilise calculation of the Pearson correlation coefficients. Hierarchical clustering for correlation matrices used the complete-linkage clustering method. Permutation analysis for eQTL multiple testing correction was

performed by Ping Zhang. Post-hoc significance analysis for statistical validation of Louvain clusters in the scRNA-seq TST blister dataset was performed by Carolin Turner. Odds ratios were performed in Microsoft Excel. One-way analysis of variance (ANOVA) tests were calculated in Graphpad Prism 10. For repeated measures ANOVA, individual variances were computed for each comparison.

3. Inter-individual variation in beta-defensin expression

3.1. Objectives

The objective of this chapter was to elucidate the mechanisms of inter-individual variation in DEFB4 and DEFB103 expression in the TST. Transcriptional responses to antigen can vary for several reasons. Given biological heterogeneity in tissue, the abundance of transcripts within RNA-seq datasets reflects both changes in the magnitude of expression and the abundance of transcribing cell types (Kukurba and Montgomery, 2015). Thus, the increased recruitment of immune cells expressing beta-defensin genes to the TST would be reflected with an increased number of beta-defensin transcripts. In this case, the magnitude of DEFB4 and DEFB103 expression per cell may not vary between individuals; rather, the absolute number of cells differs.

Underlying genetic variation can also lead to differences in expression. SNPs may affect regulatory regions around coding genes (Fairfax and Knight, 2014), for example, by influencing the strength of transcription factor binding to the DNA. In addition to SNPs, genetic variation can take place in the form of CNV (Stranger *et al.*, 2007). Here, the total amount of DNA in the genome varies due to duplication or deletion events across larger genomic loci. This is an important consideration as many beta-defensin genes have extensive CNV in the human genome (Hollox, Armour and Barber, 2003).

DEFB4 and DEFB103 expression was independent of IFNγ. Therefore, variation in an IFNγ-independent immune response may inhibit or augment beta-defensin expression. Within the TST, there may be a contribution from each of these described mechanisms. To inform the future studies on the physiological role of beta-defensins in TB, it is important to elucidate the mechanisms influencing the expression of DEFB4 and DEFB103 in vivo. This chapter of work aimed to address their contribution to inter-individual variation in beta-defensin expression in the TST.

3.2. Discrepancies in beta-defensin gene paralog expression

Since the first assessment of transcript variance in the TST had been made, in which DEFB4A and DEFB103B were highlighted as highly variable (Figure 1.2), the LTBI cohort had been expanded to 216 individuals. As before, the variance of TST response genes was compared to their correlation with IFNγ. Both genes remained highly variable between participants from the complete cohort (Figure 3.1A). For DEFB103B, the variance had increased and was now comparable with DEFB4A, though the latter did show a modest decrease in variance. The exact correlation with IFNγ was also marginally different but remained well below the median for TST response genes. Thus, DEFB4A and DEFB103B expression are highly variable, IFNγ-independent features of the TST response.

These two genes were not the only variable transcripts that were poorly correlated with IFNγ. However, I focused on the variance in beta-defensin expression as they are reported to be directly antimycobacterial (Corrales-Garcia *et al.*, 2013; Reuschl *et al.*, 2017; Su *et al.*, 2018) and may be expressed by human macrophages in TB (Rivas-Santiago *et al.*, 2005; Nickel *et al.*, 2012). In general, most genes in the upper left quadrant had high variance due to the absence of expression in certain individuals (Figure 3.1A), including numerous VDJ gene segments. To illustrate this, the variance of several genes expressed in the TST on day 2 was examined (Figure 3.1B); DEFB4A and DEFB103B and their gene paralogs, DEFB4B and DEFB103A, in addition to IFNγ and TNFα as key TST-response cytokines (Bell *et al.*, 2016), and the top two most variable VDJ gene segments. A pseudocount of 0.001 is added during log transformation of TPM expression values. Therefore, a Log2 TPM of -9.97 reflects no gene expression.

IFNγ and TNFα typify high and low TST variance, respectively, whilst highly variable DEFB4 gene paralogs spanned the entire dynamic range of TST gene expression. It was not immediately apparent why DEFB4A was more variable than DEFB4B but appeared to be due to an elevated number of individuals who had no expression of the former. In contrast, DEFB103 paralogs appeared to bisect the dynamic range of DEFB103 expression with very little overlap. Overall, DEFB4A and DEFB103B had the highest proportion of individuals with

no expression of the given gene paralog. In addition to this, their expression was more variable than IFNγ and TNFα.

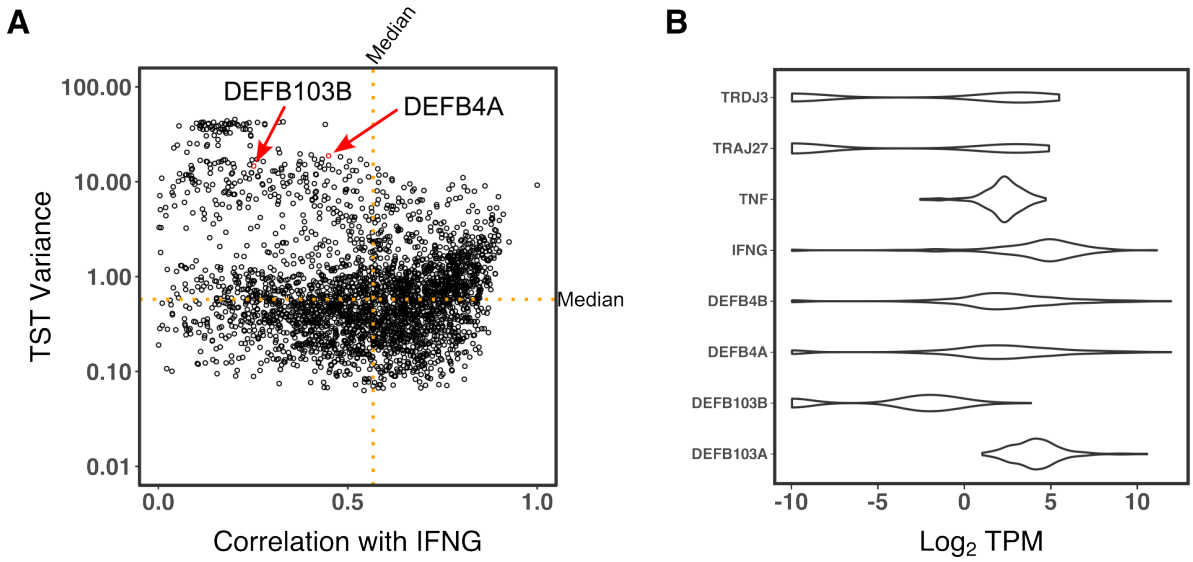


Figure 3.1. **Variation in beta-defensin expression in TSTs on day 2. (A)** Inter-individual variation (y-axis) and correlation of the anti-Mtb response with IFNγ (x-axis) in the TST transcriptome on day 2 from 216 LTBI participants. Each point represents a single gene. **(B)** Violin plots showing the distribution of log2-transformed TPM TST gene expression across individuals. The distribution of expression for highly variable VDJ gene segments, beta-defensin gene paralogs, and two key cytokines for anti-Mtb immunity, IFNγ and TNFa, are shown (N=216).

These discrepancies in paralog expression were nonetheless unexpected. Beta-defensin gene paralogs are presumed to be of identical sequence, both encoded within separate BDRUs differentiated only by their distance from the centromere (Giglio *et al.*, 2001). It was unclear why the expression of one paralog would differ from the other. For the most part, the expression of beta-defensin paralogs were well correlated with one another (Figure 3.2A,B). However, correlation was generally weaker at the lower end of expression, particularly between DEFB103 paralogs (Figure 3.2B). With instances of low expression, it was sometimes evident that the individual had expression for only one of the paralogs. This was the case for both DEFB4 and DEFB103. As seen before, DEFB4A and DEFB103B had the most instances of no expression, reinforcing why they were first highlighted as highly variable TST response genes.

It was unclear whether this reflected genuine, sole expression of a single paralog or resulted from the read alignment process. Kallisto is a tool for the mapping of sequencing data that utilises pseudoalignment (Bray *et al.*, 2016). Instead of aligning entire sequencing reads to a reference genome, Kallisto quantifies expression by dividing reads into shorter chunks (k-mers) before identifying transcripts that the shorter sequence is compatible with. Kallisto then uses a probabilistic method to collapse all the coding regions of the genome for which the k-mer aligns to into the transcript it most likely originated from. Despite utilising pseudoalignment, Kallisto has been demonstrated to perform accurate and computationally efficient alignment (Bray *et al.*, 2016). It was plausible that pseudoalignment may not be optimal for sequencing read alignment at the BDRU locus given the identical nucleotide sequences. I hypothesised that pseudoalignment was unable to accurately align reads to the correct loci, resulting in the binning of all reads into a given paralog when expression was towards the lower end.

To address this, I remapped a subset of the TST RNA-seq dataset using HISAT2, a true read alignment program (Kim *et al.*, 2019). HISAT2 alignment again revealed discrepancies between beta-defensin paralogs. However, in contrast to Kallisto, there was instead an increased discordance between DEFB4 paralogs (Figure 3.2C) whilst DEFB103 paralog

expression was more correlated (Figure 3.2D). In general, results from both alignment tools were comparable (Figure 3.2E-H). However, HISAT2 did not perform better than Kallisto as an alignment tool. This head-to-head comparison revealed that the two approaches to read alignment found different individuals with no expression of a given paralog. As HISAT2 offered no benefit to Kallisto mapping, I opted to continue using pseudoaligned RNA-seq data for downstream analyses.

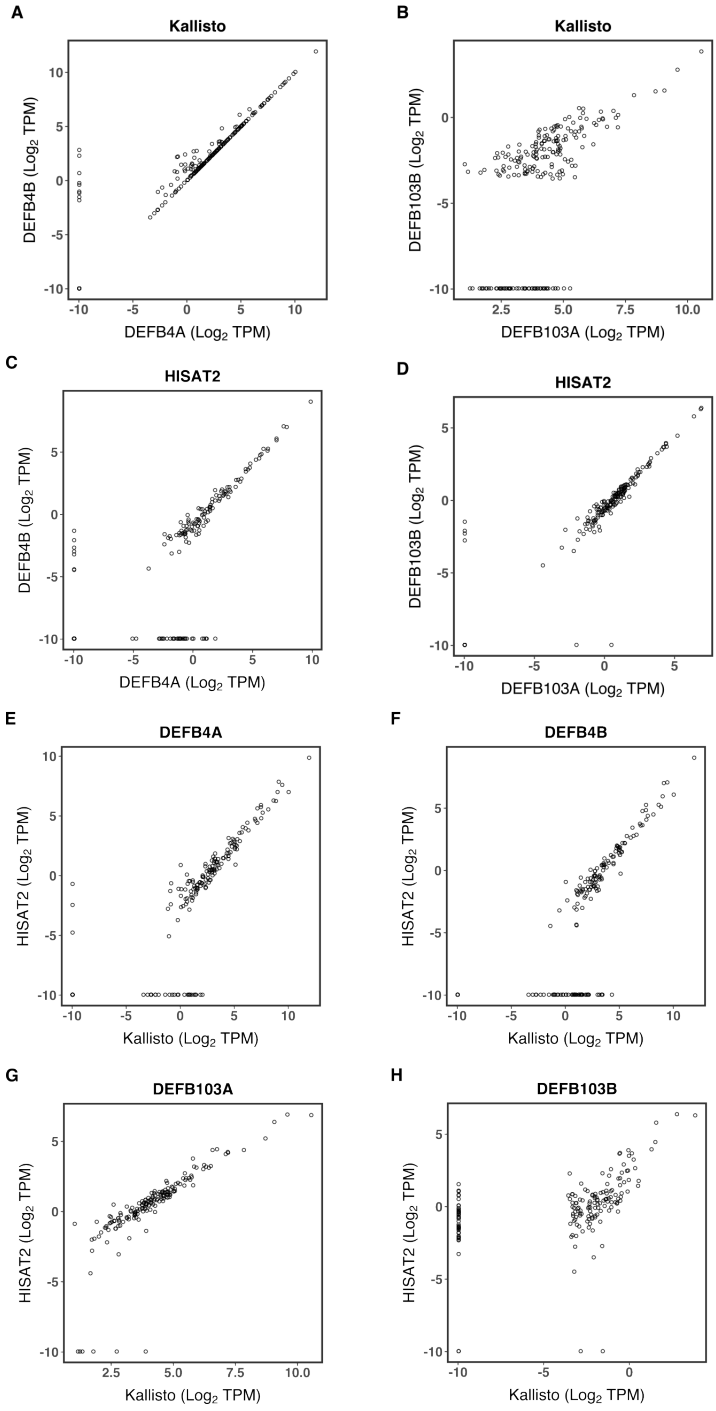


Figure 3.2. **Comparison of RNA-seq read alignment tools on beta-defensin paralog gene expression.** Log2-transformed TPM expression in TSTs on day 2 for DEFB4 and DEFB103 gene paralogs **(A+B)** pseudoaligned with Kallisto (N=216) or **(C+D)** using HISAT2 alignment (N=179). Comparison of log2-transformed TPM expression aligned by Kallisto and HISAT2 for **(E)** DEFB4A, **(F)** DEFB4B, **(G)** DEFB103A, and **(H)** DEFB103B in TSTs on day 2 (N=179).

3.3. Summing beta-defensin gene paralog expression

To accurately associate features with expression variation, the discordance between beta-defensin paralogs needed to be addressed. I used qPCR to alternatively measure beta-defensin expression in a subset of the LTBI cohort. Unlike RNA-seq, qPCR uses specific primers to amplify cDNA (from mRNA) of a gene target. These primers do not distinguish between beta-defensin paralogs and therefore quantify the total amount of beta-defensin gene expression in the sample. In general, qPCR expression values correlated well with RNA-seq data (Figure 3.3). Weaker correlation coefficients for DEFB4A and DEFB103B were evident, both of which had samples with no expression assessed by RNA-seq (Figure 3.3A,E).

The discrepancies between paralog gene expression could be resolved by summing RNAseq expression of both paralogs (Figure 3.3C,F). In doing so, instances of zero paralog expression were somewhat accounted for by virtue of there being expression of the other paralog. Summing paralog expression did result in a modest decrease in correlation with qPCR expression for one of the paralogs (DEFB4B and DEFB103A) (Figure 3.3B,D), but offered a substantial improvement in correlation for the other paralog (Figure 3.3A,E).

The identical coding sequence of beta-defensin paralogs means that following expression, the peptide made during mRNA translation will also be identical. Thus, summing the expression of both paralogs is a more accurate reflection of the total beta-defensin mRNA transcribed by the cell. Summation is preferable to taking the mean expression across the two paralogs as it is less affected by zero expression values. Paralog-summed gene expression enabled a single measurement of the beta-defensin expression for downstream analyses.

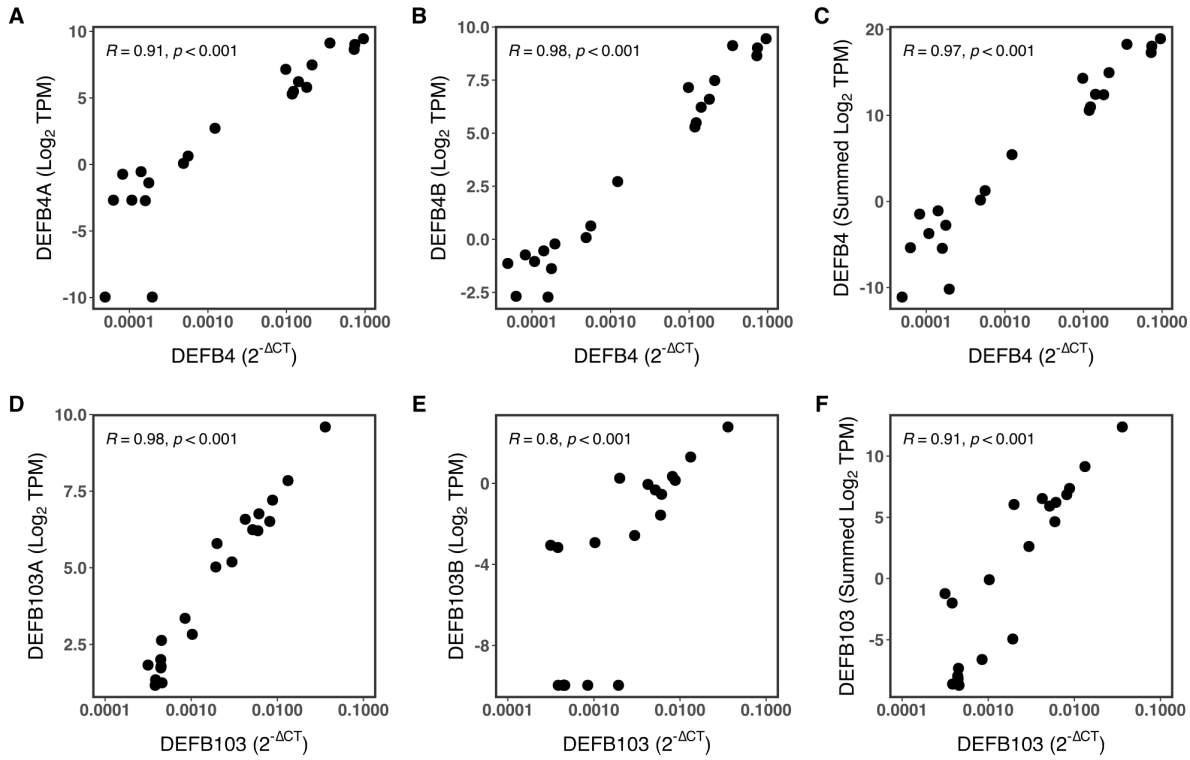


Figure 3.3. **Paralog-summed beta-defensin gene expression.** Comparison of **(A-B)** DEFB4 and **(D-E)** DEFB103 paralog gene expression in RNA-seq data (Kallisto, log2-transformed TPM) versus qPCR data ($2^{-\Delta CT}$) from TSTs on day 2 (N=21). **(C, F)** Comparison of beta-defensin expression following the summation of beta-defensin gene paralogs in RNA-seq data (Kallisto, log2-transformed TPM) and qPCR data ($2^{-\Delta CT}$) (*R* = Pearson correlation coefficient) (N=21).

3.4. Immune cell recruitment to the TST on day 2

With a solution to paralog expression, I next addressed whether differences in the abundance of blood-derived immune cells could account for the variation in beta-defensin expression in the TST. During the type IV hypersensitivity reaction that occurs in TSTs, neutrophils and monocytes are some of the first immune cells recruited from the periphery (Platt *et al.*, 1983). Monocytes differentiate at sites of inflammations into MDMs with both having been reported to express DEFB4 (Rivas-Santiago *et al.*, 2005; Nickel *et al.*, 2012; Cambier *et al.*, 2017). Therefore, differential recruitment of monocytes may contribute to the variation in beta-defensin expression between individuals. It is not possible to deconvolute bulk RNA-seq data to gain insight into the cell type abundance comprising the sample, but validated transcriptional signatures that discriminate between cell types can serve as surrogate measurements of abundance (Bell *et al.*, 2016; Pollara *et al.*, 2017).

I evaluated the relationship between the abundance of monocytes and neutrophils and beta-defensin expression. These two modules were validated in peripheral blood samples and represent key innate immune cells during the immune response against Mtb (Bell *et al.*, 2016; Pollara *et al.*, 2017). There was no obvious relationship between monocyte abundance and beta-defensin expression (Figure 3.4A,B). or between neutrophils and DEFB103 (Figure 3.4E). However, neutrophil abundance did correlate moderately well with DEFB4 expression (Figure 3.4D). Neutrophils are not reported to express DEFB4. Thus, this relationship between neutrophil abundance and DEFB4 expression likely reflects a shared immune axis. Supporting this, hBD2 has been shown to chemoattract neutrophils via CCR6 (Niyonsaba, Ogawa and Nagaoka, 2004).

Gene expression across individual TST transcriptomes were then regressed against the cell type module score to predict the residual variance not explained by cell abundance. After regression, the residual variance for all TST response genes were calculated and their correlation with IFNγ evaluated, as before. Both DEFB4A and DEFB103B were retained as two of the most variable TST response genes independent of IFNγ (Figure 3.4C,F). Therefore, the

beta-defensin expression could not be predicted based on estimates of monocyte and neutrophil abundance.

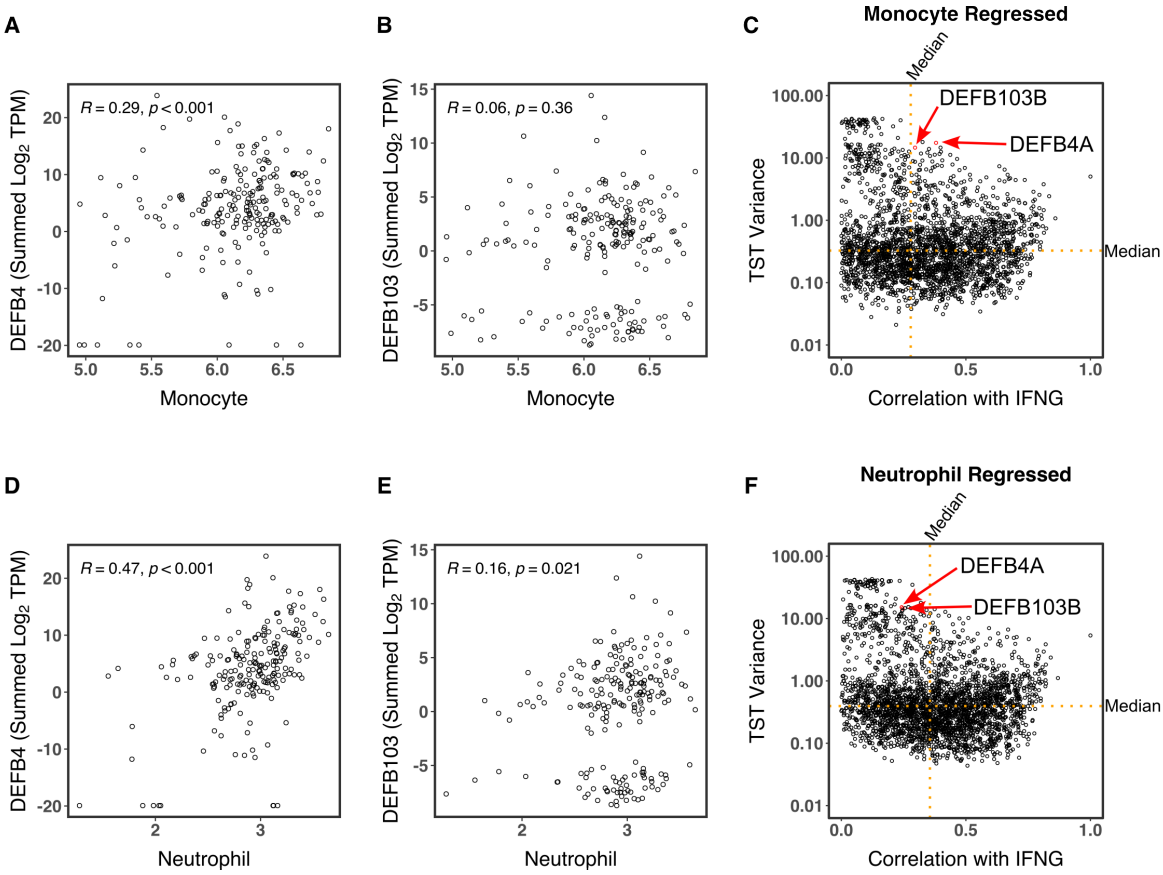


Figure 3.4. **Regression analysis of myeloid cell abundance with beta-defensin expression in TSTs.** Comparison of **(A-B)** blood monocyte and **(D-E)** neutrophil transcriptional signature scores with paralog-summed, log2-transformed DEFB4 or DEFB103 TPM gene expression in TSTs on day 2 (N=216). **(C, F)** Inter-individual variation (y-axis) and correlation of the anti-Mtb response with IFNγ (x-axis) in TST transcriptomes regressed against blood monocyte and neutrophil transcriptional signature scores (N=216). Dotted orange lines show median values.

Validated immune cell transcriptional signatures have also been identified in a scRNA-seq dataset of day 2 TST responses (Turner *et al.*, 2024). Unlike the previously used peripheral blood-derived signatures, these modules reflect cell types specifically identified in TSTs on day 2. Most cells in this dataset were lymphocytes, with validated cell type modules available for CD4+ T cells, CD8+ T cells, γδ T cells, and NK cells. In addition, a more general transcriptional signature specific for myeloid lineage cells had been validated. This module reflected the abundance of macrophages, several DC subsets, and Langerhans cells (Turner *et al.*, 2024).

A correlation analysis between calculated module scores and paralog-summed beta-defensin expression in TSTs on day 2 highlighted a high degree of co-correlation across all cell type modules (Figure 3.5). Consequently, most of cell type module scores correlated with beta-defensin expression to about the same degree as each other, with the exception of NK cells. Additionally, module scores consistently correlated more so with DEFB4 expression than DEFB103. Complete-linkage hierarchical clustering of the correlation matrix further illustrated the co-correlation between cell abundance, making it challenging to discern any causal effect regarding immune cell recruitment to the TST. However, given most of the cell types shown here are not reported to express DEFB4 and DEFB103, there was little to suggest that cells expressing beta-defensin had increased abundance in TSTs leading to transcript variance.

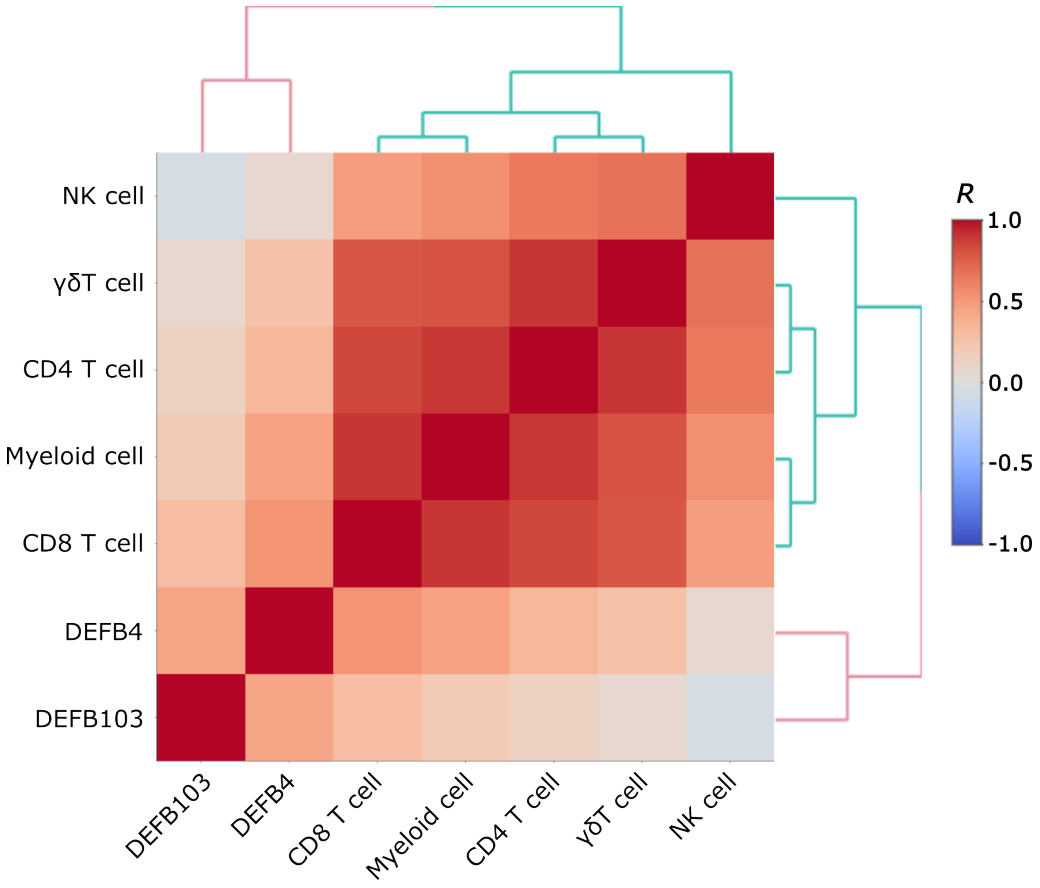


Figure 3.5. **Correlation analysis of TST blisters cell type signatures with beta-defensin expression in TSTs.** Transcriptional signature scores for TST blister cell types were calculated in RNA-seq data from TSTs on day 2 in 216 LTBI participants. A Pearson correlation matrix of cell type signature scores and beta-defensin expression is shown (*R* = Pearson correlation coefficient). The dendrograms show the hierarchical clustering (complete-linkage method) with colours reflecting the two clades. (γδ=Gamma-delta, DEFB4=Paralog-summed DEFB4 expression, DEFB103=Paralog-summed DEFB103 expression).

3.5. Single nucleotide polymorphisms proximal to beta-defensin genes

Genetic variation can give rise to differences in gene expression between individuals (Wright *et al.*, 2014). SNPs are the most frequent form of genetic variation in the human genome in which a single nucleotide in the genetic sequence varies. Most SNPs do not reside in coding regions but exert their effect by regulating expression of nearby or distant genes (Rockman and Kruglyak, 2006). EQTL analysis evaluates the association of a particular SNP with changes in the expression of a given gene (Fairfax and Knight, 2014). When the SNP and gene are within a certain threshold from one another in the genome, these can be said to be cis-acting. Cis-acting SNPs may occur within transcription factor binding sites, promoter, enhancer, and repressor regions adjacent to a gene, thereby influencing expression. Due to linkage disequilibrium it can be challenging to determine the precise causal SNP found associated with a trait. Trans-acting SNPs can reside anywhere in the genome greatly increasing the number of potential associations. Therefore, trans associations come with a significant statistical penalty for multiple testing.

The eQTL analysis was performed by collaborators Ping Zhang and Julian Knight at the University of Oxford. All participants who underwent transcriptional profiling of their TSTs were also genotyped with additional in silico genotype imputation (Burdick *et al.*, 2010). The analysis was restricted to cis-acting SNPs by limiting the distance between SNP and gene to 1 megabase (Mb) up and downstream of the TSS. 158 of the 216 LTBI participants also had a TST biopsied on day 7 RNA-sequenced, which were included in the eQTL analysis to increase statistical power. In addition, 41 individuals with active TB also underwent transcriptional profiling of their TSTs on day 2. These were similarly genotyped and included in the final analysis. This almost doubled the sample size to 415. Genotype permutation was used to establish the frequency of random associations, from which an adjusted p-value threshold could be calculated for detecting statistically significant associations.

None of the cis-acting SNP associations with DEFB4A, DEFB4B, and DEFB103B passed the threshold for statistical significance (Figure 3.6). Additionally, no SNPs were associated with

DEFB103A expression. The eQTL analysis was repeated using only TSTs on day 2 from 216 LTBI participants, and by with looking for associations with paralog-summed beta-defensin expression. In both cases, no cis-acting SNPs were significantly associated with changes in DEFB4 or DEFB103 expression (data not shown).

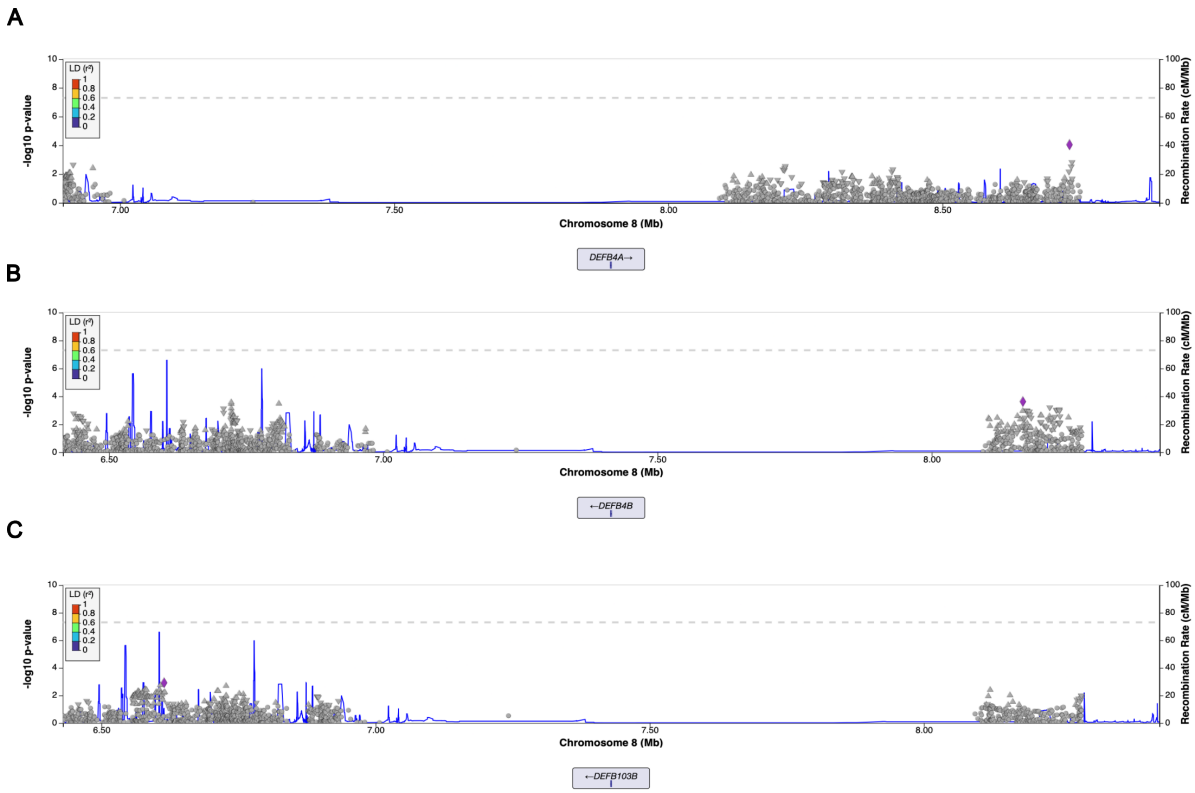


Figure 3.6. **EQTL analysis of cis-acting SNPs.** Regional association plot of the 8p23.1 genomic locus, showing associated SNPs within 1 Mb of **(A)** DEFB4B, **(B)** DEFB4B, and **(C)** DEFB103B (N=415). Purple diamonds reflect the most significant SNP association. The dotted line reflects the adjusted p-value for significance. Data from a combined cis-eQTL analysis in TSTs on day 2 (N=216) and day 7 (N=158) from LTBI participants (HIRV-TB study) and TSTs on day 2 from active TB participants (Tuning study) (N=41).

3.6. Beta-defensin copy number variation

Genetic CNV is a common occurrence in the human genome and contributes to variation in gene expression (Stranger *et al.*, 2007). Structural polymorphism leading to duplication of coding DNA can mediate gene dosage effects, whilst deletion events can reveal detrimental recessive mutations (Hollox, Barber, *et al.*, 2008). Both DEFB4 and DEFB103 are subject to extensive CNV, associated with rates of psoriasis (Hollox, Huffmeier, *et al.*, 2008) and hBD2 protein concentration in the cervix (James *et al.*, 2018). Due to the considerable range in BDRU copy numbers, conventional methods of typing copy number lack the sensitivity to discern integer values for copy number (Fode *et al.*, 2011).

The paralog ratio test is a PCR-based assay for typing copy number first developed specifically for assessing beta-defensin CNV (Aldhous *et al.*, 2010). Using a single primer pair, two regions of the genome are amplified. One of these regions resides within the BDRU and is therefore subject to the same copy number variation as the beta-defensin genes. The other region lies elsewhere in the genome, in a copy number stable location, and therefore serves as a control. The ratio of amplicons following PCR reflects copy number. By using a single primer pair and ensuring amplicons are of similar length and GC content, PCR kinetics vary minimally (Hollox, 2017).

To increase accuracy, triplex PRT evaluates three genomic loci (Figure 3.7) (Bakar, Hollox and Armour, 2009). PRT107A and HSPD21 assays utilise the ratio between amplicons as a direct measurement of copy number. The indel rs5889219 assay amplifies a multiallelic variant that differs in length between repeats. The ratio of amplicons from multiallelic variants sum to an integer divisible by the BDRU copy number. Each test is performed in duplicate with differing fluorescently-tagged primers facilitating quantitation. Compared to qPCR and PRT-based copy number assays, triplex PRT was found to be more accurate and precise whilst avoiding the systematic biases of qPCR (Fode *et al.*, 2011). Triplex PRT types BDRU copy number and thus, as both are encoded within the BDRU, DEFB4 and DEFB103 share the same absolute value.

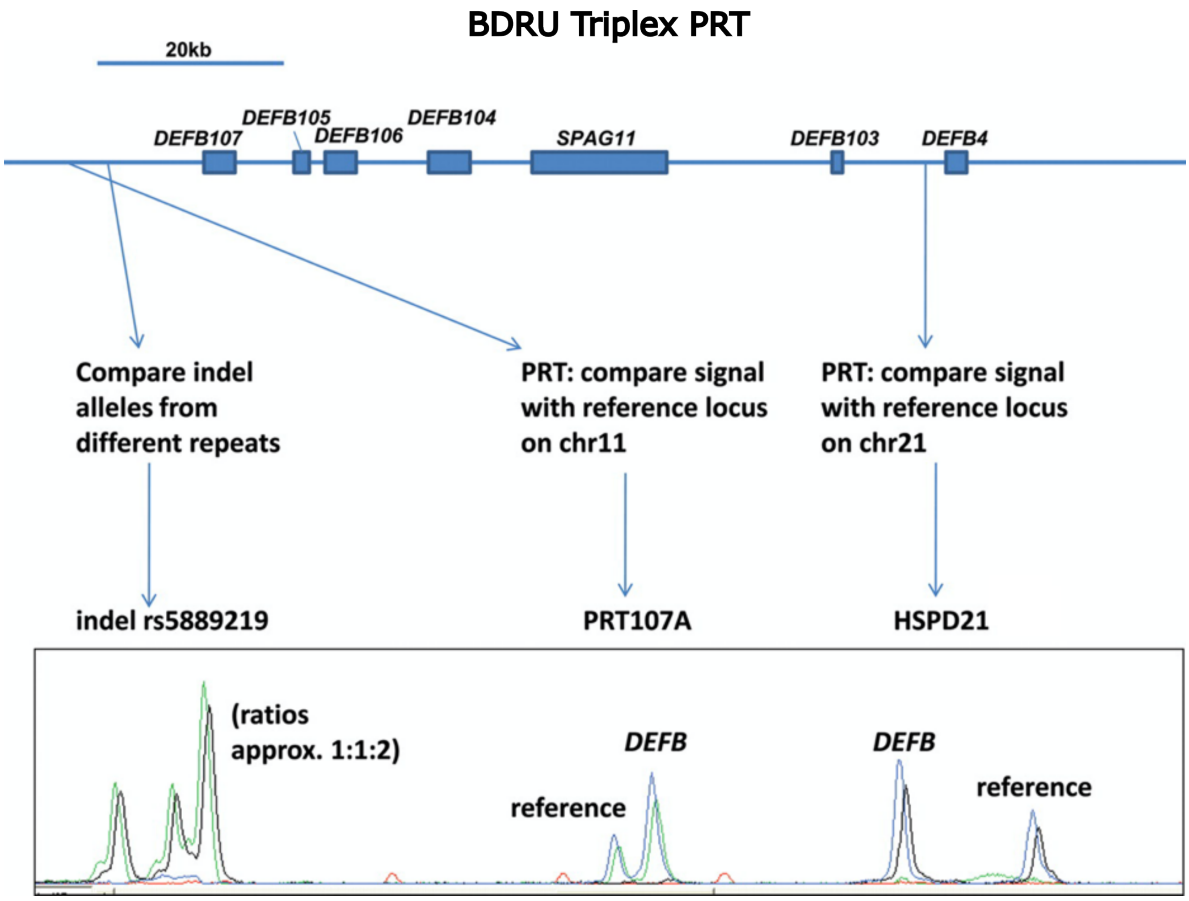


Figure 3.7. **Triplex PRT to assess beta-defensin copy number.** The copy number variable BDRU showing the approximate genomic locations of the PRT-based triplex test. An example of results from the test is shown below. Each test amplifies a region within the BDRU. PRT107A and HSPD21 assays also amplify a copy number stable reference locus elsewhere in the genome. The ratio of PRT107A and HSPD21 assay amplicons reflects BDRU copy number. The indel assay rs5889219 assesses a triallelic polymorphism of three varying sizes, the ratios of which sum to an integer value divisible by the BDRU copy number. All amplicons are of differing sizes (within a few base pairs) thus allowing separation by DNA capillary electrophoresis. Primer pairs incorporate a fluorophore to facilitate accurate quantitation of amplicons. Each assay is performed in duplicate with alternate fluorophores, resulting in 6 independent tests for BDRU copy number. (Figure adapted with permission from Aldhous *et al.*, 2010).

I used 6 HRC DNA samples from the ECACC collection as reference standards for each triplex PRT, which was included in each PCR plate. These samples have all been previously typed for beta-defensin copy number (Fode *et al.*, 2011). Based on these known values of integer copy number, raw amplicon ratios from the triplex PRT can be adjusted to increase accuracy (Hollox, 2017). Amplicon ratios for unknown samples are then adjusted using the linear regression equation generated with the reference DNA (Figure 3.8A-D). Adjusted ratios are referred to as "normalised". Normalised PRTs revealed clustering around integer values (Figure 3.8E-G), with the HSPD21 assay having notably better precision than PRT107A.

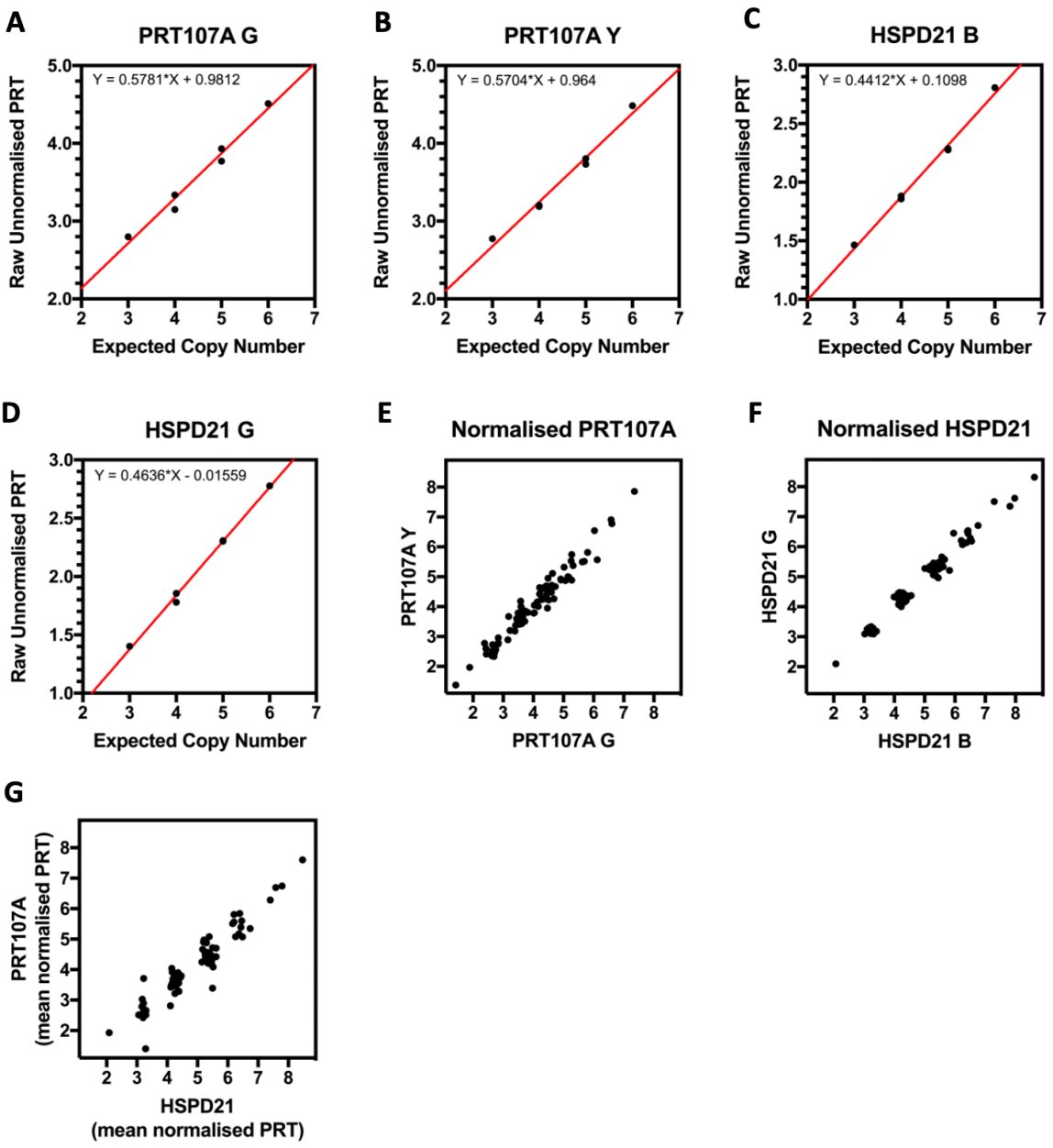


Figure 3.8. **Validation of triplex PRT genotyping of BDRU copy number. (A-D)** Raw PRT estimates of beta-defensin copy number in 6 HRC DNA from the ECACC collection (N=6). **(E-F)** Normalised PRT estimates of BDRU copy number from each assay in LTBI participants (N=81). Each assay is suffixed with a letter denoting the fluorophore colour (G=Green, Y=Yellow, B=Blue). **(G)** Comparison of normalised PRT107A and HSPD21 assay BDRU copy number estimates in LTBI participants (N=81). Representative data from one 96-well plate PCR.

Final calling of integer copy number was done with a maximum likelihood approach following a gaussian distribution for each integer value. This resulted in an associated p-value for each test. Only tests with p-values smaller than 0.01 were retained. Using the triplex PRT, all 216 LTBI participants were typed for BDRU copy number. Accurate copy numbers could be called for 212 of the 216 participants. Copy numbers were normally distributed around a median of 4 copies per diploid genome (Figure 3.9A), and fell within the range of 2 to 8 copies most often observed in populations (Bakar, Hollox and Armour, 2009). Approximately 35% of individuals had 4 copies, 30% had 5, 20% had 3, and 10% had 6.

The relationship between BDRU copy number and paralog-summed DEFB4 and DEFB103 expression was evaluated. It appeared that in both cases the expression of beta-defensins trended upwards with increasing copy number. A significant positive correlation between BDRU copy number and expression was evident for DEFB103 (Figure 3.9C). This relationship was weaker for DEFB4, with a smaller effect size and no statistical significance (Figure 3.9B). Thus, the data supported a modest contribution of genetic copy number towards increased DEFB103 expression in the TST.

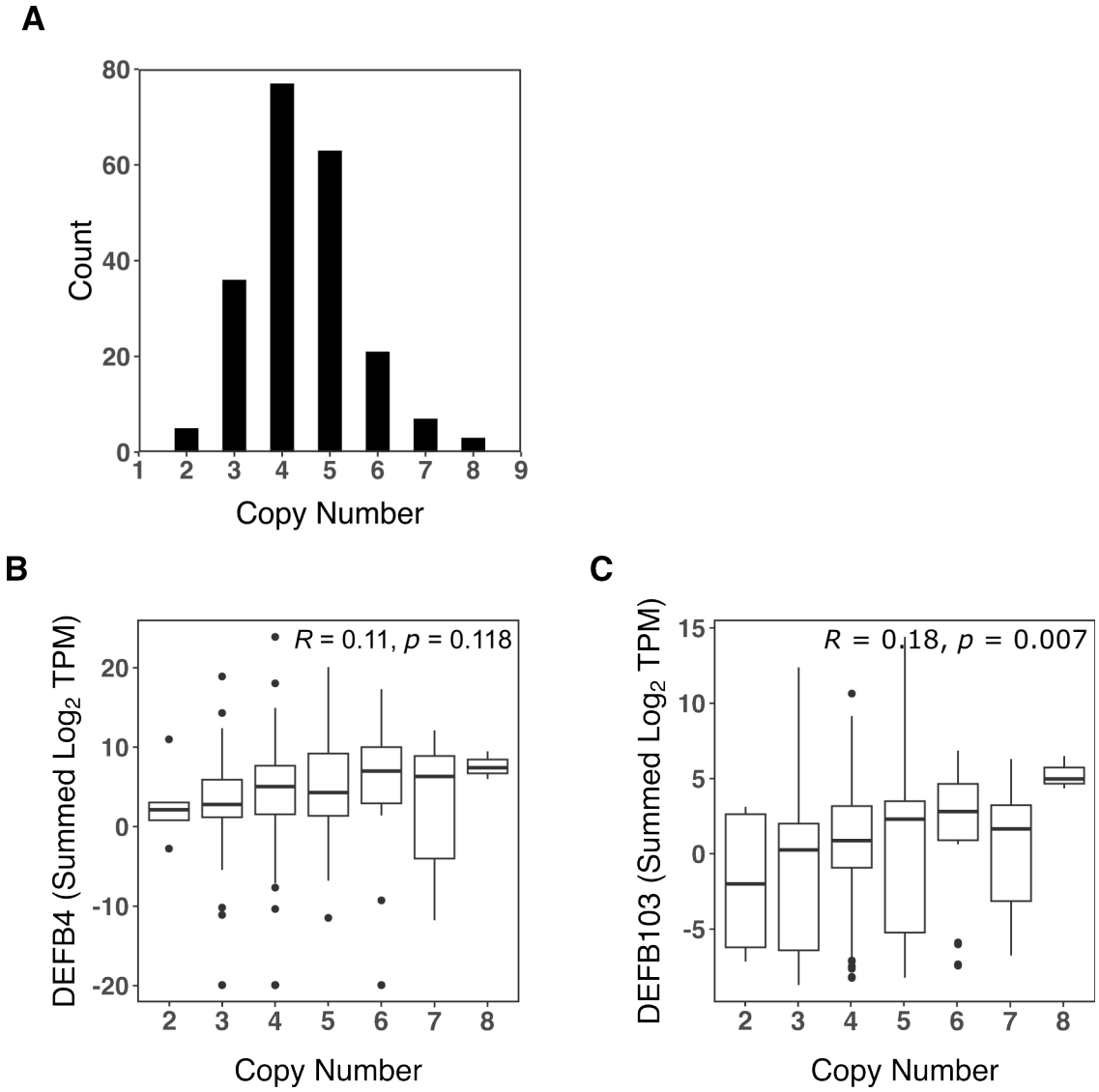


Figure 3.9. **Beta-defensin copy number estimates and comparisons with gene expression in TSTs. (A)** Distribution of BDRU copy number assessed by triplex PRT among 212 LTBI participants who underwent transcriptional profiling of their TSTs on day 2. Comparison of copy number variation with paralog-summed **(B)** DEFB4 or **(C)** DEFB103 expression in TSTs on day 2 (*R* = Pearson correlation coefficient) (N=212).

3.7. Inter-individual variation in PPD-induced cytokine activity

DEFB4 and DEFB103 are both inducible AMPs with expression upregulated by pro-inflammatory cytokines (Kao *et al.*, 2004, 2008; Nagy *et al.*, 2005; Huang *et al.*, 2007; Jang *et al.*, 2007). Given the TST model utilised a standardised antigen dose, I reasoned that inter-individual variation in cytokine signalling might contribute to the differences in DEFB4 and DEFB103 expression.

Paired TSTs from a cohort of active TB participants were available. Here, each participant had a concomitant and independent intradermal injection of a saline solution when undergoing a TST. As expected, the expression of both DEFB4 and DEFB103 were strongly induced following PPD challenge (Figure 3.10). This demonstrated that the induction of beta-defensin expression is specific to antigen-mediated pro-inflammatory responses in skin and not simply in response to skin tissue damage caused when administering tuberculin.

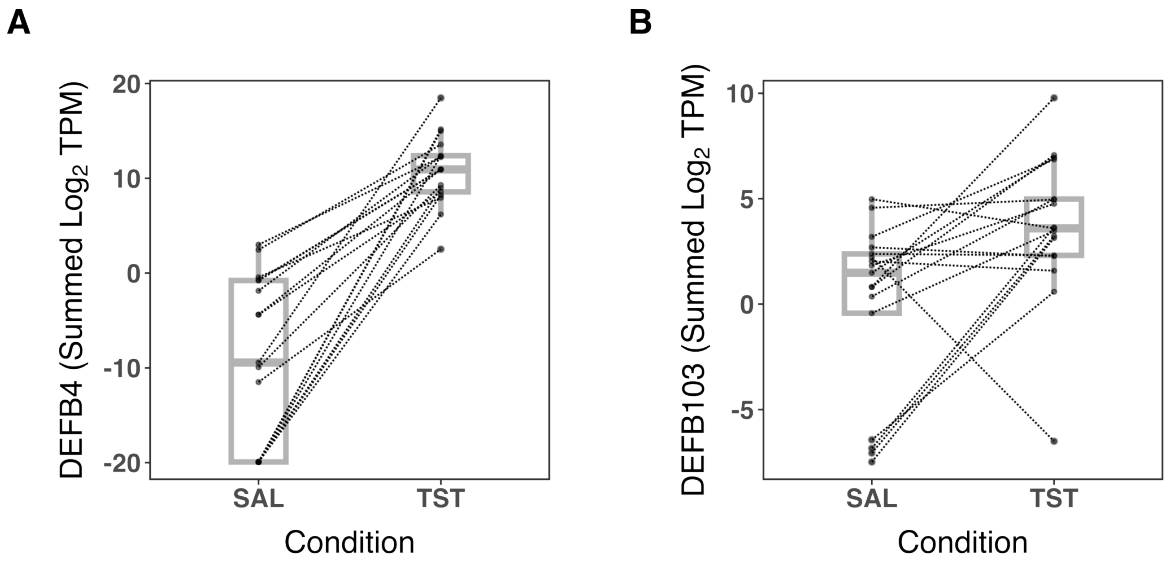


Figure 3.10. **DEFB4 and DEFB103 expression is induced in TSTs.** Participants with active TB recruited to the Tuning TB study underwent simultaneous transcriptional profiling of TSTs and saline skin challenges on day 2. Paralog-summed expression of **(A)** DEFB4 and **(B)** DEFB103 was compared between the two skin tests (SAL=saline skin challenges). Dotted lines specify paired samples (N=17).

Next, I explored how cytokine activity related to the inter-individual variation in DEFB4 and DEFB103 expression. Direct measurements of cytokines, whether at the mRNA or protein level, in complex models can miss their peak quantitation and fail to separate their biological presence from noise (Bell *et al.*, 2021). Transcriptional modules of cytokine activity comprise genes predicted downstream of cytokine signalling and reflect the biological activity of a given cytokine. Transcriptional signatures for the activity of various cytokines have been previously identified and validated in independent datasets (Pollara *et al.*, 2021). These transcriptional response modules reflect cytokine-specific gene upregulation in keratinocytes following cytokine stimulation. Keratinocytes will generate a strong transcriptional signal in TSTs.

There was a strong degree of co-correlation for nearly all cytokine activity modules (Figure 3.11). Whilst no causal relationships can be assessed with this approach, there is a considerable body of evidence that several of these cytokines, notably TNFα, IL-1β, IL-17A, and IL-22 can induce beta-defensin expression (Harder *et al.*, 2000; Tsutsumi-Ishii and Nagaoka, 2003; Kao *et al.*, 2004; Wolk *et al.*, 2004; Joly *et al.*, 2005; Pivarcsi *et al.*, 2005; Liang *et al.*, 2006). Transcriptional responses from two cytokines, IL-22 and IL-17, were evidently less co-correlated with the activity of other cytokines.

Despite upregulating beta-defensin expression in human primary keratinocytes (Wolk *et al.*, 2004), IL-22 activity correlated the least of all the cytokines with DEFB4, and not at all with DEBF103 expression. In contrast, complete-linkage hierarchical clustering of the correlation matrix grouped IL-17 activity with DEFB4 expression. IL-17 was the only cytokine uniquely clustered with beta-defensin expression, evident at whichever level the dendrogram was cut. Compared to the cytokine activity modules, the correlation between BDRU copy number and expression was much weaker, indicating a greater effect size for cytokine activity than genetic variation.

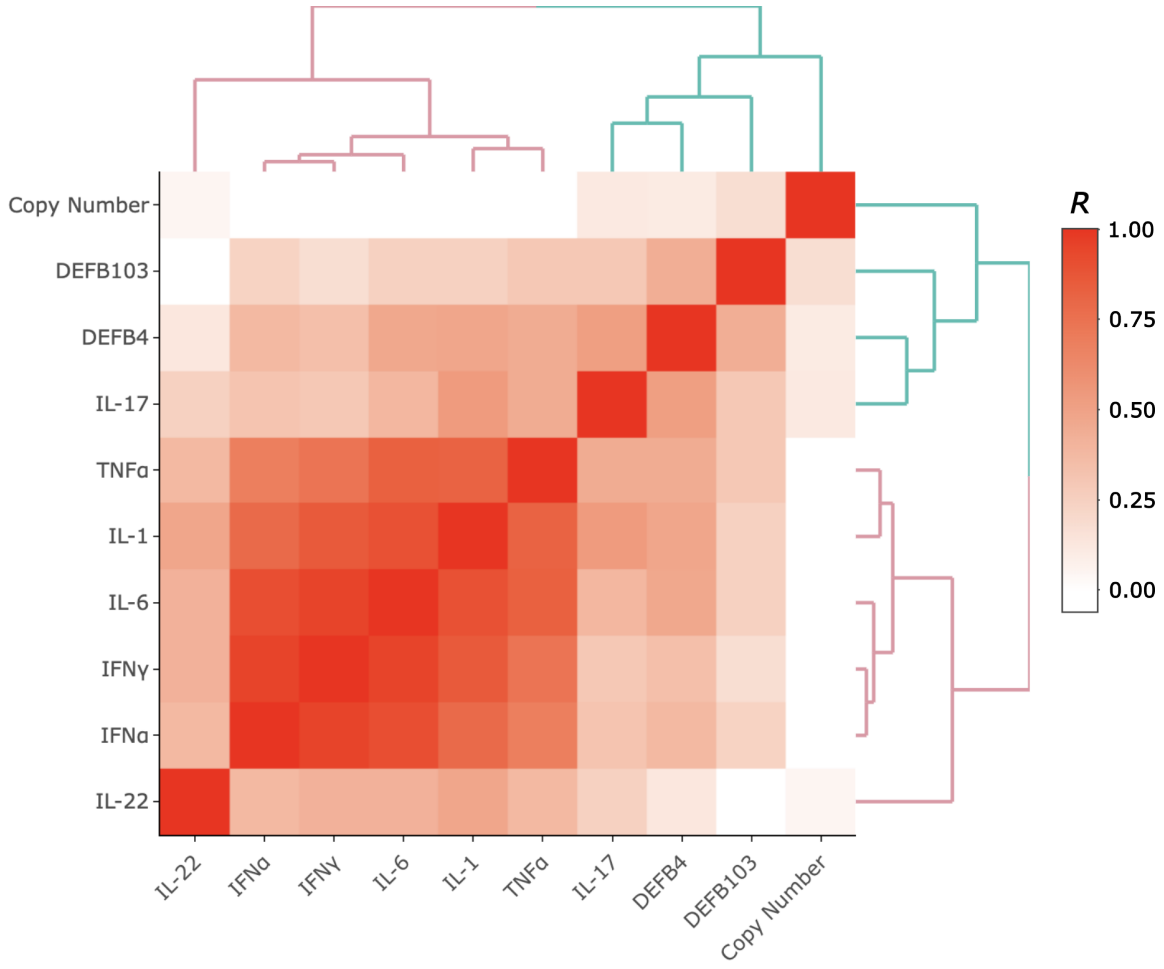


Figure 3.11. **Correlation analysis of cytokine activity signatures with beta-defensin expression in TSTs.** Transcriptional signature scores for cytokine activity were calculated in RNA-seq data from TSTs on day 2 in 212 LTBI participants. A Pearson correlation matrix of cytokine activity signature scores, beta-defensin expression, and BDRU copy number is shown (*R* = Pearson correlation coefficient). The dendrograms show the hierarchical clustering (complete-linkage method) with colours reflecting the two clades. (DEFB4=Paralog-summed DEFB4 expression, DEFB103=Paralog-summed DEFB103 expression, Copy Number=BDRU copy number).

To further illustrate this relationship with IL-17 activity, the previous figure showing BDRU copy number compared with beta-defensin expression (Figure 3.9B-C) was stratified by the IL-17 module score (Figure 3.12). This demonstrated that most individuals with the highest expression of beta-defensin in the TST also had the highest transcriptional score for biological IL-17 activity. This was most apparent with DEFB4, but still evident with DEFB103. Additionally, when comparing beta-defensin expression among individuals with the same genetic copy number, there was a clear relationship between IL-17 activity and DEFB4 expression (Figure 3.12A).

In general, for any given copy number, individuals with more IL-17 activity had more expression of beta-defensins. Whilst this cannot imply a causal relationship, IL-17A potently upregulates DEFB4 mRNA in vitro (Kao *et al.*, 2004). Other cytokines assessed in this analysis may also induce beta-defensin expression and contribute to inter-individual variation in TSTs.

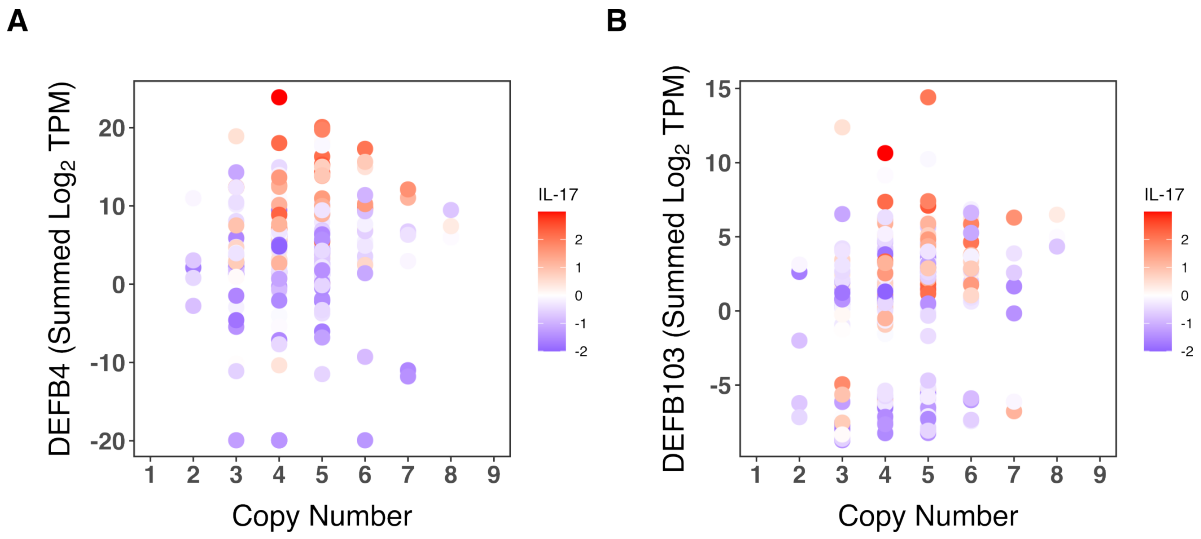


Figure 3.12. **IL-17 cytokine activity with beta-defensin expression in TSTs on day 2**. Comparisons of BDRU copy number variation with paralog-summed **(A)** DEFB4 or **(B)** DEFB103 expression in TSTs on day 2 from 212 LTBI participants were stratified by the transcriptional signature score for IL-17 cytokine activity.

3.8. Discussion

In this chapter, I evaluated the potential biological mechanisms that may have contributed towards inter-individual variation in beta-defensin expression in TSTs. I revealed inducible DEFB4 and DEFB103 expression that was associated with increases in pro-inflammatory cytokine signalling, myeloid and T cell abundance, and with BDRU copy number.

In general, most of the cell type and cytokine activity transcriptional modules were highly co-correlated. Thus, it was difficult to determine which of these, if any, were causative in driving inter-individual variation in beta-defensin expression. Human monocytes and macrophages may express DEFB4 and DEFB103 (Duits *et al.*, 2002; Tsutsumi-Ishii and Nagaoka, 2003; Rivas-Santiago *et al.*, 2005; Nickel *et al.*, 2012; Rodriguez-Carlos *et al.*, 2020; Díaz *et al.*, 2023) and therefore differential recruitment of these cells might have led to inter-individual variation in expression. However, the abundance of blood-derived monocytes alone did not correlate with DEFB4 and DEFB103. Furthermore, whilst myeloid cell abundance correlated with expression, so too did the various T cell transcriptional signatures, and a similar relationship was seen for neutrophil abundance and DEFB4 expression. None of these latter cell types express beta-defensin genes. The observed relationships between neutrophils and T cells may therefore reflect a shared immune axis. Importantly, beta-defensins are known to have chemokine-like properties. Both hBD2 and hBD3 chemoattract cells via interactions with CCR6 and CCR2 chemokine receptors (Yang *et al.*, 1999; Niyonsaba, Ogawa and Nagaoka, 2004; Röhrl *et al.*, 2010a; Kim, Yang and Jang, 2019). Thus, beta-defensins can mediate the recruitment of T cells, neutrophils, DCs, and monocytes to the TST, and perhaps explains why the respective transcriptional signatures correlated with DEFB4 and DEFB103 expression in the TST. I cannot exclude the possibility that macrophage abundance in the TST varies between individuals and drives DEFB4 and DEFB103 expression variation.

As was seen with immune cell types, most of the cytokine activity modules correlated with one another. Of those tested, TNFα, IL-1β, IL-17A, and IL-22 have all been reported to induce the expression of DEFB4 and DEFB103 (Harder *et al.*, 2000; Tsutsumi-Ishii and Nagaoka, 2003;

Kao *et al.*, 2004; Wolk *et al.*, 2004; Joly *et al.*, 2005; Liang *et al.*, 2006; Reuschl *et al.*, 2017). Consequently, the biological activity of any of these cytokines may have contributed to the inter-individual variation in beta-defensin expression in TSTs. Confounding this, IL-6 and type I IFN were also correlated with DEFB4 and DEFB103 to a similar extent. Neither of these induce beta-defensin expression in vitro (Harder *et al.*, 2000; Kao *et al.*, 2004; Reuschl *et al.*, 2017). Using complete linkage clustering, the IL-17 cytokine activity module was found to be more closely related to DEFB4 expression than with any of the other cytokine module scores. This suggested that IL-17 may play an important role in regulating beta-defensin responses in the TST.

IL-17 is a potent inducer of DEFB4 expression in human primary respiratory epithelial cells (Kao *et al.*, 2004). Additionally, in the original IL-17 transcriptional signature, DEFB4 was one of the predicted downstream biomarkers for IL-17 activity in vivo (Pollara *et al.*, 2021). I modified this IL-17 activity module to remove DEFB4 prior to the correlation analysis, but together with previously reported in vitro data, reinforces IL-17 as a key cytokine for initiating and augmenting DEFB4 expression during in vivo immune responses. It is worth noting that authors found no DEFB103 expression with IL-17 treatment (Kao *et al.*, 2004), though there may exist differences between transcriptional responses in respiratory epithelium and that in skin. DEFB4 encodes hBD2, which can mediate the recruitment of T cells via CCR6 (Yang *et al.*, 1999). CCR6 is an important mediator of Th17 cell migration (Yamazaki *et al.*, 2008) and the expression CCR6 among CD4+ Th cells was associated with increased IL-17 production (Lyu *et al.*, 2019). These indicate that the observed relationship between beta-defensins, T cells, and IL-17 activity might be biologically plausible.

In collaboration with Ping Zhang, I demonstrated that SNPs neighbouring beta-defensin genes did not influence their expression to a significant degree. At the given sample size in this study, I remained underpowered to detect any associations around the beta-defensin locus. TSTs represent a complex heterogenous population of responding non-immune and immune cells, each with their own expression pattern. Thus, analysis of the entire sample may miss eQTLs present in only a subset of the total cell population (Fairfax and Knight, 2014). In future,

the bioinformatic deconvolution of the TST transcriptome may facilitate cell-specific eQTLs. Furthermore, eQTLs may be present in one tissue (i.e. skin) but not in another (i.e. lung). Whilst this is unrelated to elucidating the genetic influence on expression in TSTs, it is an important consideration when translating findings from the TST model of TB to the disease itself.

All potential SNPs linked with beta-defensin expression in this analysis were distal from the BDRU, and the extensive structural polymorphism of this locus is likely to limit sensitivity across this region (Bakar, Hollox and Armour, 2009). Only one study has reported a SNP associated with changes in DEFB4 or DEFB103 expression, having used an a priori hypothesis-driven approach involving Sanger sequencing of the specific locus (Kurt-Bayrakdar *et al.*, 2020). Had any SNPs been strongly linked to expression, fine-mapping by sequencing would have taken place to find causal associations, but this may have been a more suitable approach in the first instance. In contrast to SNPs, structural variation of the BDRU was significantly associated with DEFB103 expression. The effect size found in this analysis was comparable to other reports of BDRU CNV (James *et al.*, 2018).

CNV is typically thought to mediate a gene dosage effect, whereby the presence of more coding DNA leads to more gene product upon expression (Hollox, Barber, *et al.*, 2008). However, most occurrences of CNV in the human genome involves a doubling or halving of the total number of genes. In contrast, extensive CNV of the BDRU can add additional complexity by increasing distances between regulatory non-coding regions and the coding region itself (Hollox, Barber, *et al.*, 2008). Thus, one cannot assume a linear relationship between copy number and beta-defensin expression. The impact of the inversion polymorphism of this locus on expression is unknown and has not been considered here (Sugawara *et al.*, 2003; Bakar, Hollox and Armour, 2009).

The absence of gene expression in certain individuals was not unique to beta-defensins and led to high variability for those most variable TST response genes. There was a modest contribution to inter-individual variation by the nomenclature of beta-defensin gene paralogs

in the reference genome, but much of the variance in beta-defensin expression was unrelated to discrepancies between paralogs. The dynamic range of DEFB4 and DEFB103 expression observed in the TST was among the greatest for any gene and far beyond that explained by copy number distribution. Assuming the gene dosage effect, a doubling of copy number (i.e. from 4 copies to 8) can be expected to lead to a doubling in the total gene expression (Hollox, Barber, *et al.*, 2008). For DEFB4 and DEFB103, the observed expression spanned a considerably greater range than accounted for by this effect. Given the complexity of in vivo immune responses, the small effect size for CNV is perhaps unsurprising. Previous work has demonstrated a stronger correlation between the beta-defensin response and localised measurements of inflammation than with genetic copy number (Aldhous, Noble and Satsangi, 2009). Several steps must occur to induce beta-defensin expression, including PRR recognition of PAMPs, cytokine upregulation, secretion, and receptor binding in turn. Variation along this pathway can confound associations between copy number and gene expression. It is worth noting that in general, modules correlated better with DEFB4 expression than with DEFB103. These relationships might have masked the effect size of copy number variation on DEFB4 expression. Supporting this, the effect size for CNV on DEFB103 expression was greater that for DEFB4, and the only one to have reached statistical significance.

Many of the relationships described in this chapter are confounded by their high degree of co-correlation, and thus cannot imply causality. However, I have been able to infer directionality for several of these relationships using supporting data from published literature. It is worth noting that there has been no discovery component to this, and there may remain some other contributing factor towards the variation in beta-defensin expression that has been missed. Nonetheless, together these data imply that the inter-individual variation in DEFB4 and DEFB103 expression is driven by biological differences in PPD-induced cytokine production, most likely as part of a IL-17-mediated response, with a modest contribution by genetic copy number of the BDRU locus.

4. Beta-defensin expression is limited to epithelial cells in TB

4.1. Objectives

The focus of this chapter was to identify which cell types expressed DEFB4 and DEFB103 during anti-Mtb immune responses. These beta-defensins are widely reported to be expressed by epithelial cells, particularly those at mucosal surfaces and in skin (Harder *et al.*, 1997, 2001). However, innate immune cells also express antimicrobial peptides. Alpha-defensins are major effector proteins in neutrophils (Ganz, 2003) and constitute up to 50% of the total protein content in neutrophil granules (Rice *et al.*, 1987). Additionally, the only human cathelicidin, LL-37, is expressed by Mtb-infected human macrophages (Dürr, Sudheendra and Ramamoorthy, 2006; Rivas-Santiago *et al.*, 2008), and can restrict Mtb growth (Liu *et al.*, 2007). Macrophage production of hBD2 and hBD3, however, is less well documented. There is evidence that in certain contexts, hypoxia or with high MOI Mtb-infection, primary human macrophages upregulate DEFB4 mRNA (Rivas-Santiago *et al.*, 2005; Nickel *et al.*, 2012). Evidence for macrophage DEFB103 expression in TB comes only from the immortalised THP-1 cell line (Rodriguez-Carlos *et al.*, 2020; Díaz *et al.*, 2023). It is unclear whether these responses happen in vivo.

It is not possible to accurately deconvolute the TST transcriptome to address whether human macrophages express DEFB4 and DEFB103. Instead, I took two approaches using cutting-edge single cell technologies. The first was to explore beta-defensin expression in a scRNA-seq dataset of TSTs suction blisters on day 2. This dataset mirrored the bulk TST transcriptome evaluated in chapter 3 at the single cell level. I generated this dataset, leading all sample processing and cDNA library construction steps. To complement this, I evaluated spatial, single cell expression across TSTs using RNAscope, a tissue RNA-FISH assay.

4.2. Single cell sequencing of TST suction blisters

TSTs were performed in a new cohort of Mtb-sensitised LTBI participants recruited to the HIRV-TB study. Transcriptional responses were captured on day 2. However, instead of taking

a puncture biopsy at this timepoint, a suction blister was induced at the site of the TST. This resulted in the extravasation of responding leukocytes into the blister fluid, which could then be pierced and the immune cells directly harvested from the fluid (Theocharidis *et al.*, 2022). I led the laboratory side of this work whilst the bioinformatic analysis was conducted together with Carolin Turner, University College London, to decide on the optimal analysis approach detailed in chapter 2.4. The figures and analysis of beta-defensin expression contained herein was conducted separately from other publications using this dataset (Turner *et al.*, 2024). TST suction blisters were performed in 31 individuals resulting in an integrated dataset of 63,881 cells (Figure 4.1A).

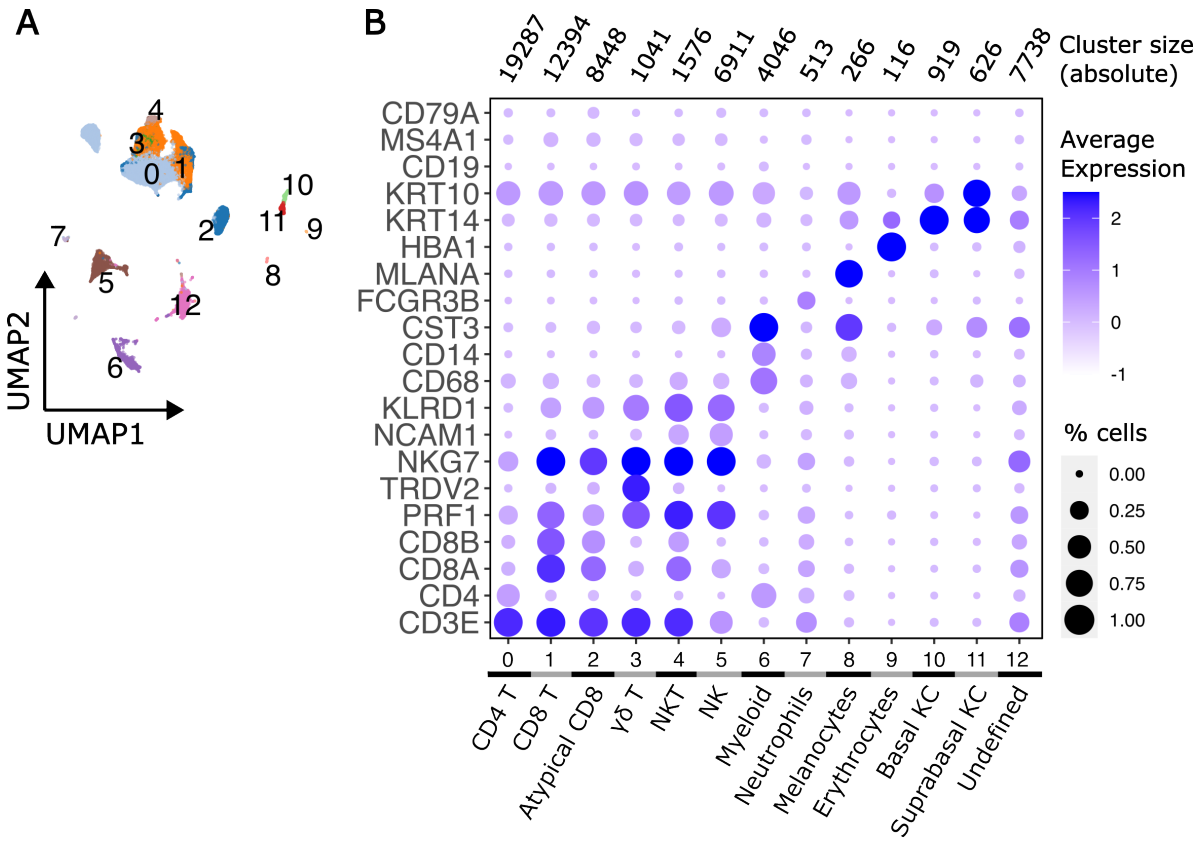


Figure 4.1. **ScRNA-seq of suction blisters at the site of TSTs on day 2. (A)** Clustering of integrated scRNA-seq data from 63,881 cells in TST suction blisters on day 2 (N=31). The inferred cell type for clusters 0 to 12 can be seen along the x-axis of panel B. **(B)** Canonical marker gene expression by cell cluster with inferred cell type and absolute cluster size (γδ=Gamma-delta, KC=Keratinocytes). Dot size represents the percentage of cells expressing each marker and colour shows the average log normalised expression of the marker for each cluster. The average expression colour scale is capped at -1 and 2.5.

To cluster cells, sequential rounds of clustering were performed until clusters could either no longer be separated with statistically assured validity or had reached an arbitrary limit of 500 cells per cluster (Turner *et al.*, 2024). This enabled greater separation of cell populations that had remained grouped together after just a single round of clustering. After sequential rounds of clustering there were 101 defined clusters representing a mix of cell lineages. Many of these clusters represented the same cell lineage in different transcriptional states. Consequently, clusters were annotated and merged to reflect the 13 distinct cell lineages identified based on canonical marker gene expression (Figure 4.1B) (Turner *et al.*, 2024).

Clusters 0 to 4, identified as different T cell subsets, constituted nearly two-thirds of all captured cells. Approximately half of these were annotated as CD4 T helper cells. There were also two large CD8 T cell populations, separated by differential CD8A and CD8B gene expression. Smaller clusters of γδ T cells and NKT cells could be identified as a result of the sequential clustering approach, having been initially grouped with CD8 T cell and NK cell clusters, respectively. No B cells were present in TST suction blisters. Although B cells have been reported in delayed type IV hypersensitivity reactions in skin (Platt *et al.*, 1983) their presence was infrequent (<2% of all cells). Nonetheless, suction blisters may be biased towards capturing cells with a greater propensity to extravasate into the blister fluid.

There were also several populations of innate immune cells. The largest were clusters 5, annotated NK cells, and clusters 6, annotated myeloid cells, reflecting a heterogenous population of macrophages, DCs, and Langerhans cells. Finer separation of this myeloid cell cluster required three full rounds of clustering with most cells segregating based on function (i.e. antigen-presenting or antimicrobial) prior to ontogeny (Turner *et al.*, 2024). Very few neutrophils were captured and could only be annotated based on slightly elevated FCGR3B (CD16b) expression. 10x scRNA-seq platforms have well-documented limitations surrounding neutrophils including the difficulties with distinguishing them from empty droplets, though these can be overcome with alternative data processing strategies (Wigerblad *et al.*, 2022). It is also possible that few neutrophils remain present in the skin 2 days post-challenge (Platt *et al.*, 1983).

In addition to leukocytes there were small populations of non-immune cells. Clusters 8 to 11 could be annotated as melanocytes, erythrocytes, and keratinocytes, respectively. Suprabasal keratinocytes were identified as a distinct population from basal keratinocytes based on elevated KRT10 expression (He *et al.*, 2020). The capture of keratinocytes and melanocytes in TST suction blisters suggests that during blister formation these cells are dissociated from the skin, likely due to the tissue injury that occurs upon blister formation. There remained a large cell cluster (cluster 12) for which no ontological label could be delineated. The cells in this cluster exhibited low numbers of total unique molecular identifiers (UMI) (i.e. total reads) and detected genes, characteristic of low-quality cells that had not been removed during quality control (QC) steps (Turner *et al.*, 2024).

Using the cluster annotations based on the expression of established marker genes, I evaluated the expression of DEFB4 and DEFB103 in cells captured from suction blister blisters at the site of TSTs on day 2. At first, it was noted that no cells in the filtered dataset post QC expressed DEFB4 or DEFB103. This was due to the use of a gene sparsity filter, which removed genes that were expressed in <0.1% of all cells in the total dataset. Gene sparsity is a challenging component to scRNA-seq data analysis as it can either represent missing genes due to technical reasons (i.e. a failure to capture the transcript; loss during reverse-transcription), or simply the absence of gene expression (Laehnemann *et al.*, 2019). Typically, sparsity filters are used to reduce dimensionality prior to clustering. To circumvent this absence of beta-defensin expression, the sparsity filter was retained but DEFB4 and DEFB103 genes were allowed to pass.

Of 63,881 cells, only 37 cells expressed either beta-defensin gene (DEFB4A = 31, DEFB4B = 1, DEFB103A = 3, DEFB103B = 2) (Figure 4.2). Most expressed DEFB4A with just a few captured cells expressing DEFB103 gene paralogs. The majority were from the suprabasal keratinocyte cluster (35 cells). Additionally, a single cell from the NK cell cluster, and one undefined cell, expressed DEFB4A. Inter-individual variation in beta-defensin expression was also evident in this dataset. Although not every participant had cells expressing DEFB4 and DEFB103, the cells in question originated from several different individuals (data not shown).

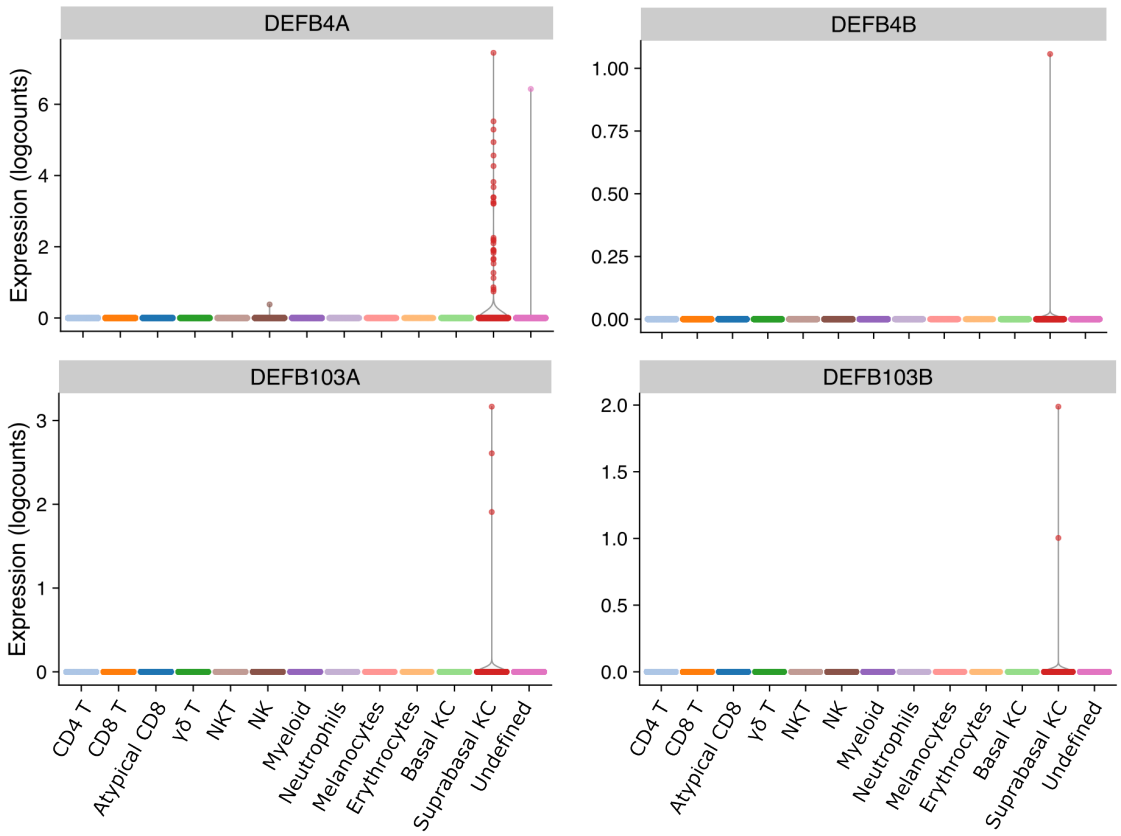


Figure 4.2. **Beta-defensin expression in single cells captured in day 2 TST suction blisters.** Log normalised expression of DEFB4 and DEFB103 paralogs in 63,881 cells across 13 cell clusters identified in TST suction blisters on day 2. Cluster annotations are based on canonical marker gene expression (γδ=Gamma-delta, KC=Keratinocytes). Dots represent single cells from an integrated scRNA-seq dataset of TST suction blisters from 31 LTBI participants.

Single cell analysis of TST responses supported PPD-induced beta-defensin expression among suprabasal keratinocytes of the skin epithelium. However, this conclusion comes from a very limited sample size of just 37 cells. As seen in bulk RNA-seq of TSTs, individuals often had very low expression of DEFB4 and DEFB103 genes (Figure 3.1B, Figure 3.2). This may have exacerbated occurrences of gene dropout in these individuals. Genes with low expression in a given cell are often missed due to the technical challenges surrounding the sequencing of picogram RNA quantities per cell (Lun, McCarthy and Marioni, 2016). It also remained possible that a subset of macrophages expressing beta-defensin had restricted motility and limited extravasation into the blister fluid, as has been observed with other myeloid cells (Rojahn *et al.*, 2020). These limitations were addressed by using RNAscope, a tissue RNA-FISH assay, to perform spatial gene expression in puncture biopsies of TSTs.

4.3. Spatial expression of beta-defensins in TSTs

RNAscope measures spatial, single cell expression in formalin-fixed, paraffin-embedded (FFPE) tissue sections. Using probes specific for target mRNA, gene expression across the entire tissue is assessed in substantially more cells per TST than is economical with scRNA-seq. RNAscope amplification steps fluorescently label single RNA molecules making it sensitive to low levels of expression (Atout, Shurrab and Loveridge, 2022). Additionally, evaluating expression across the entire TST tissue section removed potential bias surrounding cell extravasation into blister fluid (Rojahn *et al.*, 2020).

Many human coding genes have pre-made RNAscope probes available for purchase. However, DEFB4 and DEFB103 were both off-catalogue requests made to the supplier. As with qPCR probes, the probes do not differentiate between beta-defensin gene paralogs. After bioinformatically designing probes based on the complementary base sequence, I evaluated the specificity of either probe. I cloned DEFB4 and DEFB103 DNA into respective lentiviral plasmid vectors and used these to overexpress each beta-defensin in transfected HEK293T cells. This resulted in monolayers constitutively expressing DEFB4 (293TΔDEFB4)

and DEFB103 (293TΔDEFB103), respectively, which were then formalin-fixed and paraffin-embedded for the RNAscope assay.

The DEFB4 probe only detected RNA in 293TΔDEFB4 monolayers whilst the DEFB103 probe only detected RNA in the 293TΔDEFB103 monolayers (Figure 4.3). This convincingly demonstrated sensitive and specific detection of beta-defensin expression. There was variable signal intensity between cells in both monolayers, reflecting different transfection efficiencies. At rest, untransfected HEK293Ts did not express DEFB4 or DEFB103.

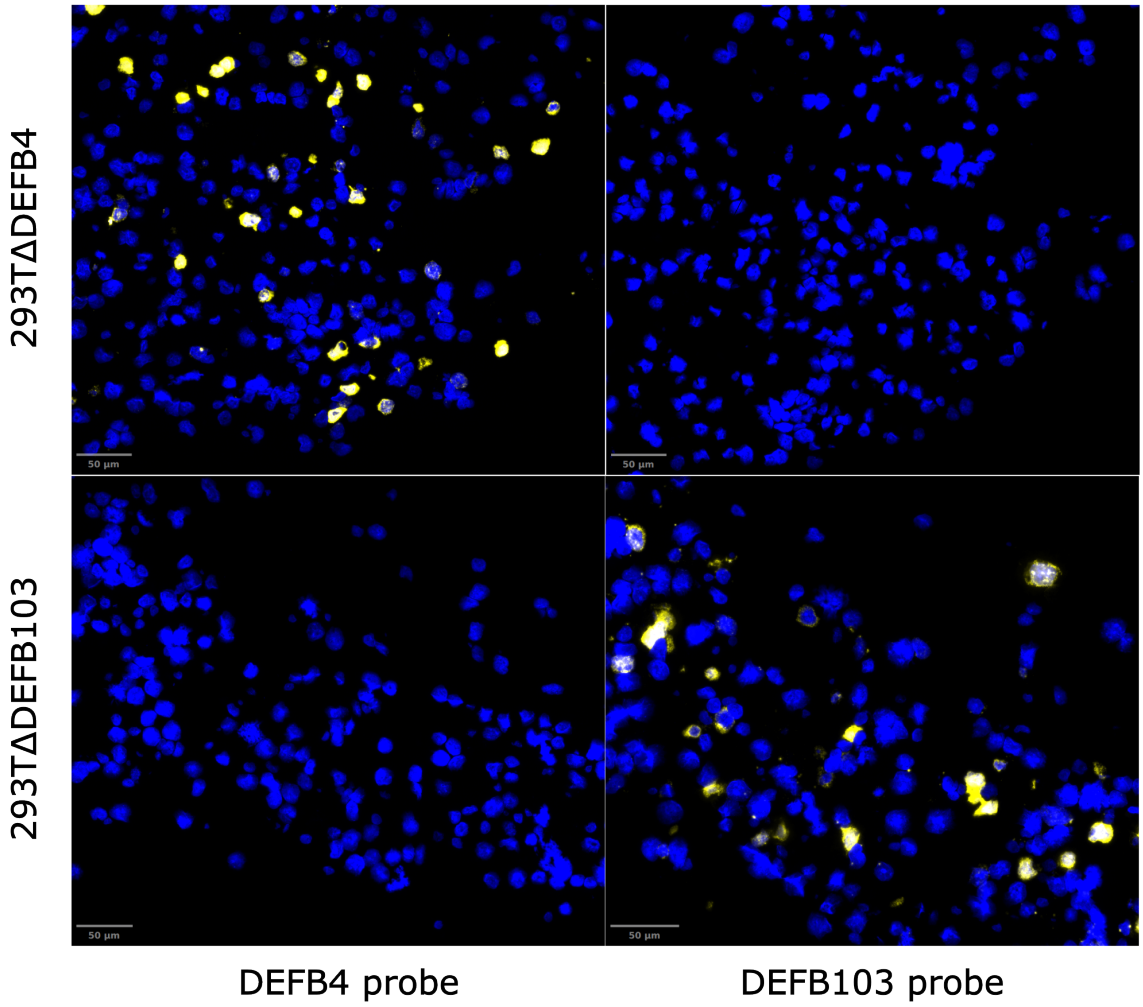


Figure 4.3. **Validation of DEFB4 and DEFB103 RNAscope probe specificity.** HEK293T cell monolayers transfected with plasmids encoding DEFB4 or DEFB103 mRNA and a GFP reporter gene. DEFB4 and DEFB103 expression was evaluated in FFPE transfected monolayers using custom probes designed for the RNAscope assay. Representative images from each monolayer are shown (293TΔDEFB4=HEK293T cells overexpressing DEFB4, 293TΔDEFB103=HEK293T cells overexpressing DEFB103).

As scRNA-seq of TST blisters revealed beta-defensin expression in keratinocytes, I addressed whether there was evidence of macrophage expression of these genes in the TST. Using RNAscope, I identified macrophages based on the expression of established marker genes. We had RNAscope probes for macrophage marker genes already available in house, CD14 and CD68, in addition to a more general myeloid marker in CD13. The RNAscope V2 assay used here is limited to assessing expression across four channels in addition to a nuclear stain, though there are variations to the assay that enable up to 12 markers (Dikshit *et al.*, 2022). Consequently, I wanted to evaluate which macrophage marker would be the best single marker gene. This would then allow for the identification of macrophages expressing beta-defensin. Alongside this, the decision was made to include a probe for CD3D, ensuring that there would be a control cell marker to inform confidence in the specificity of signal co-localisation.

To inform marker gene selection, I assessed the cell type specific expression of CD13, CD14, and CD68 in cells from the TST blister dataset (Figure 4.4A). Unsurprisingly, all three markers were enriched in cluster 6, annotated as myeloid lineage cells. CD13 was the most specific myeloid cluster marker of the three, followed by CD14, whilst CD68 had low to moderate expression across most cell clusters. CD68 was the only marker also expressed in the cluster of suprabasal keratinocytes. Generally, expression of CD14 was greater amongst cells annotated as myeloid than in any other population. In contrast, CD68 expression was much lower in the myeloid cell population. Whilst CD68 expression was clearly enriched among myeloid cells, cells from lymphocyte clusters (T, NKT, and NK cells) that expressed CD68 had comparably moderate expression.

TSTs on day 2 from one LTBI and one active TB participant were stained for CD13, CD14, and CD68 expression by RNAscope (Figure 4.4B,C), to evaluate marker gene expression overlap in cells across the TST. There was considerable overlap in cells expressing more than one marker and no single marker captured the entire combined population of myeloid cells. Of the three markers, CD13 captured the smallest population of cells, whilst CD14 and CD68 were more comparable in the absolute number of cells identified. Given the overlap of both CD14 and

CD68 expression, both appeared to classify the same cell population. CD14 was selected as the optimal macrophage marker due to it being generally more specific for myeloid cells in TST blisters (Figure 4.4A).

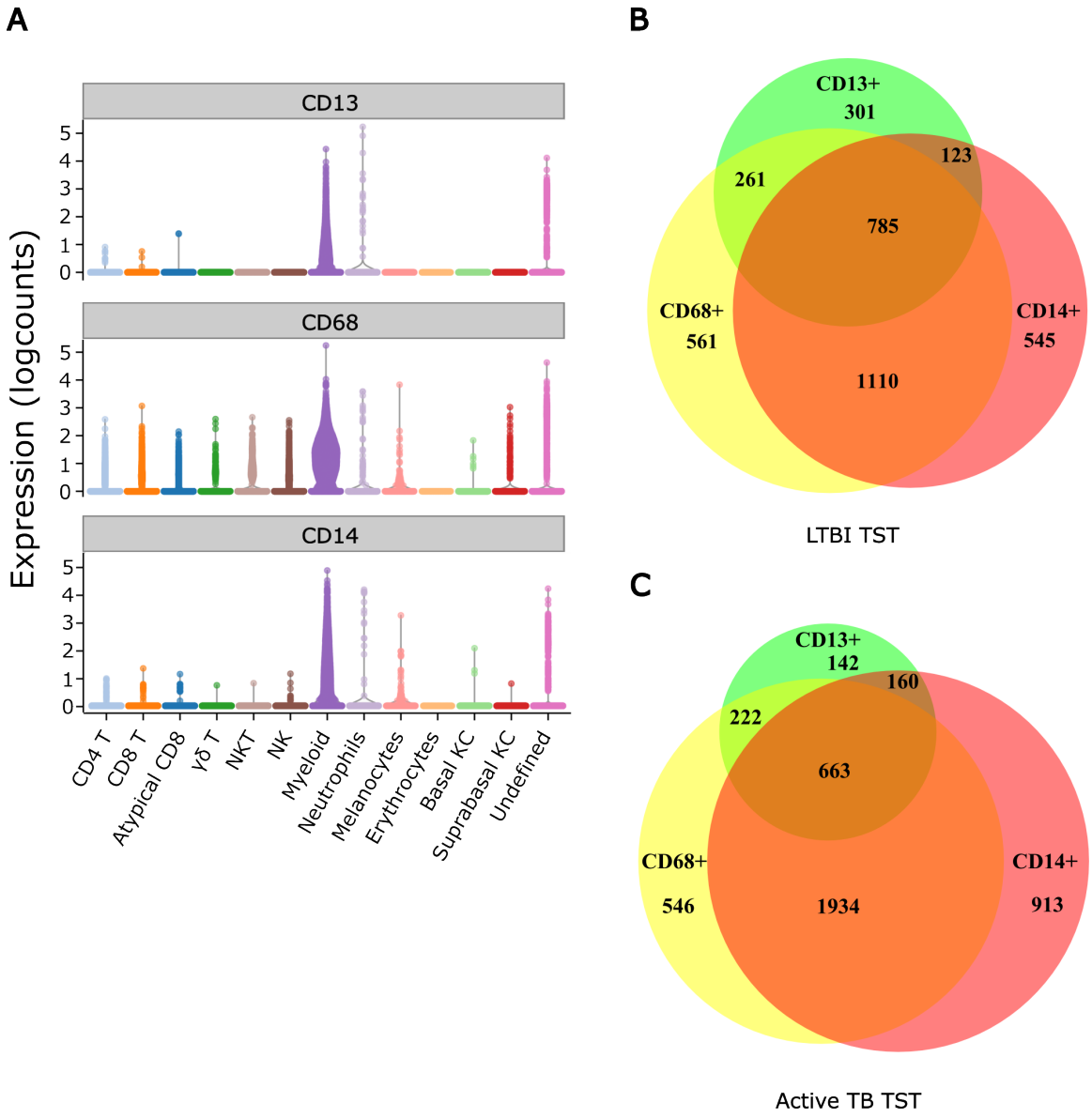
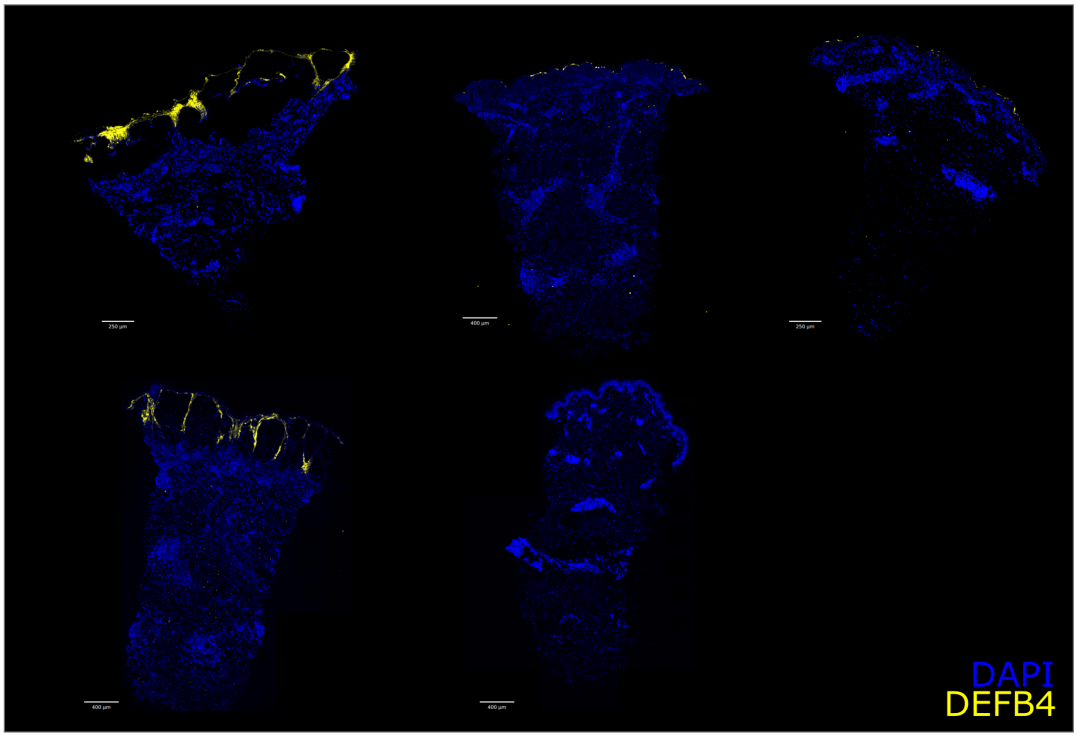


Figure 4.4. **Single cell expression of myeloid cell marker genes. (A)** Log normalised expression of canonical myeloid lineage marker genes in 63,881 cells across 13 cell clusters identified in TST suction blisters on day 2. Cluster annotations are based on canonical marker gene expression (γδ=Gamma-delta, KC=Keratinocytes). Dots represent single cells from an integrated scRNA-seq dataset of TST suction blisters from 31 LTBI participants. **(B-C)** The overlap of cells expressing canonical myeloid lineage marker genes by RNAscope. Marker gene co-expression was evaluated across the entire tissue section from one LTBI and one active TB participant TST on day 2.

Next, tissue sections of TSTs on day 2 from LTBI and active TB participants were stained for DEFB4 and DEFB103 expression in addition to single gene macrophage and T cell markers. This revealed inter-individual variation in the expression of both beta-defensins (Figure 4.5), as had been first observed in the bulk RNA-seq dataset of TSTs on day 2 (Figure 3.1). Previously, DEFB4A expression has been shown to be enriched in TSTs from those with active TB (Pollara *et al.*, 2021). The RNAscope dataset here found no enrichment of DEFB4 among active TB participants (Figure 4.5), likely reflecting the comparatively limited sample size (48 active TB participants (Pollara *et al.*, 2021) versus 5 in this study). The expression of DEFB4 and DEFB103 was generally limited to the epidermal layer of TSTs, corroborating evidence of their expression among keratinocytes in TST suction blisters.

A



B

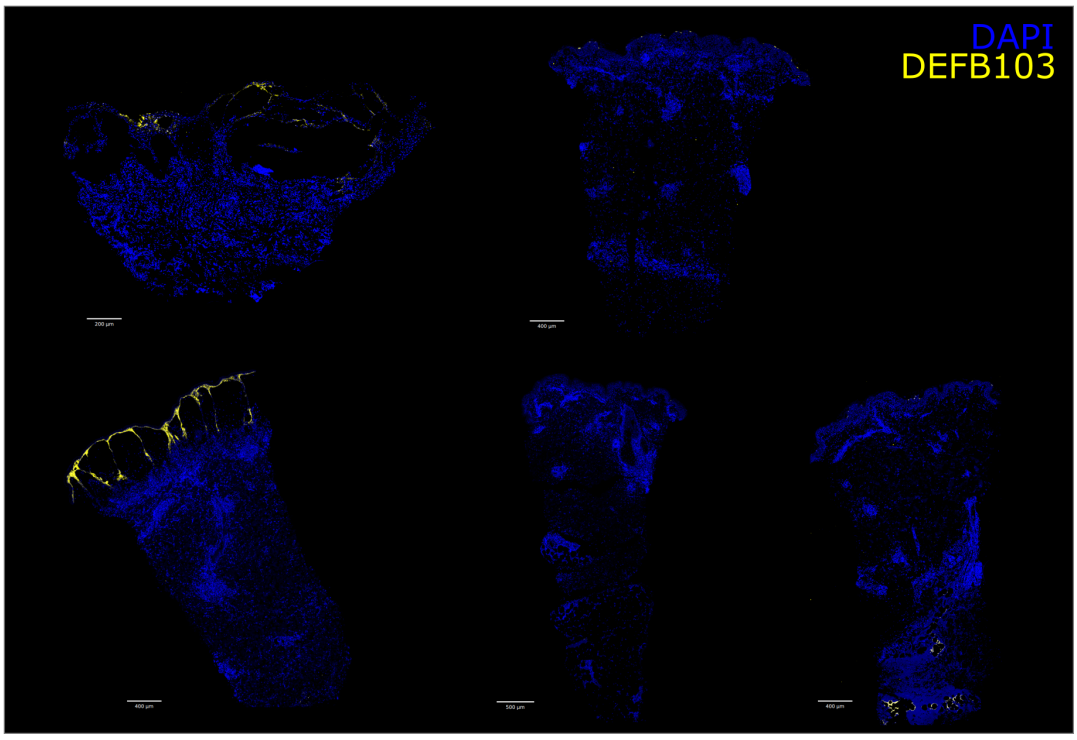


Figure 4.5. **Distribution of DEFB4 and DEFB103 RNA in tuberculin skin tests on day 2.** The spatial distribution of **(A)** DEFB4 and **(B)** DEFB103 expression across TSTs on day 2 was evaluated by RNAscope. Beta-defensin mRNA is shown in yellow, DAPI nuclear stain in blue. For each panel; top row = active TB participant TSTs on day 2 from the MONITOR study, bottom row = LTBI participant TSTs on day 2 from the HIRV-TB study.

CD14 and CD3D expression identified numerous immune cells adjacent to one another, often in close proximity to beta-defensin mRNA (Figure 4.6A). RNAscope enables quantitative assessment of spatial gene expression. I used the DAPI nuclear stain to segment individual cells within the TST (Figure 4.6B), with paired scans using a negative RNAscope probe mix to set thresholds for signal positivity. Expression of CD14 and CD3D served as the ontological basis for cell annotation, and the degree of beta-defensin co-expression with either marker evaluated. The number of CD14+ macrophages and CD3D+ T cells across individual TSTs varied substantially, by around one order of magnitude (Figure 4.6C). On average, TSTs had approximately 2,500 macrophages and T cells, respectively, identified by single marker gene expression.

The number of double-positive cells, expressing either beta-defensin and one of the cell lineage markers, also varied considerably between TSTs, but the absolute numbers for both cell types were close to identical (Figure 4.6C). Compared with the total number of macrophages and T cells identified across individual TSTs, there were generally very few cells that expressed beta-defensin. At most, approximately 100 double-positive cells were seen in each cell population. There were two TSTs which were stained with only CD14 and DEFB4 probes, and so did not have a population of CD3D+ cells (Figure 4.6C,D). The expression of DEFB4 and DEFB103 was always assessed independently of one another, but there did not appear to be substantial differences between the two.

There is no evidence that CD3D+ T cells express beta-defensin genes. Therefore, these served as a control population with those T cells identified as expressing beta-defensin representing stochastic signal co-localisation. Most TSTs had paired populations of macrophages and T cells, enabling a direct comparison of the percentages of beta-defensin expressing immune cells (Figure 4.6D,E). The co-localisation of beta-defensin expression in macrophage and T cell populations occurred at a similar frequency across the TST. For several TSTs, beta-defensin positive T cells as a fraction of all T cells was greater than that for macrophages. Less than 10% of identified macrophages were double positives in all but one of the TSTs. For one individual, approximately 20% of macrophages were DEFB4+, whilst a staggering 70% of

DEFB4+ T cells were found in this TST. Thus, despite several hundred instances of beta-defensin positive macrophages, it was evident that there was no enrichment of signal colocalisation in macrophages versus T cells. This suggested that none of these cells represented genuine observations of beta-defensin-expressing macrophages.

Odds ratios were calculated at 0.79 and 0.24 for DEFB4 and DEFB103 expression, respectively, in CD14+ versus CD3D+ cells. This statistically confirmed a greater likelihood of observing beta-defensin expression among T cells than macrophages in the TST. It is difficult to establish ground-truths in cell segmentation for quantitative image analysis (Caicedo *et al.*, 2017) particularly given the heterogeneity in macrophage morphology (Hourani *et al.*, 2023). The close proximity of cells throughout the TST may have contributed to inaccuracies in cell segmentation that resulted in infrequent beta-defensin expression among macrophages and T cells. Taken together, single cell analyses of TSTs on day 2 attributed beta-defensin expression exclusively to keratinocytes.

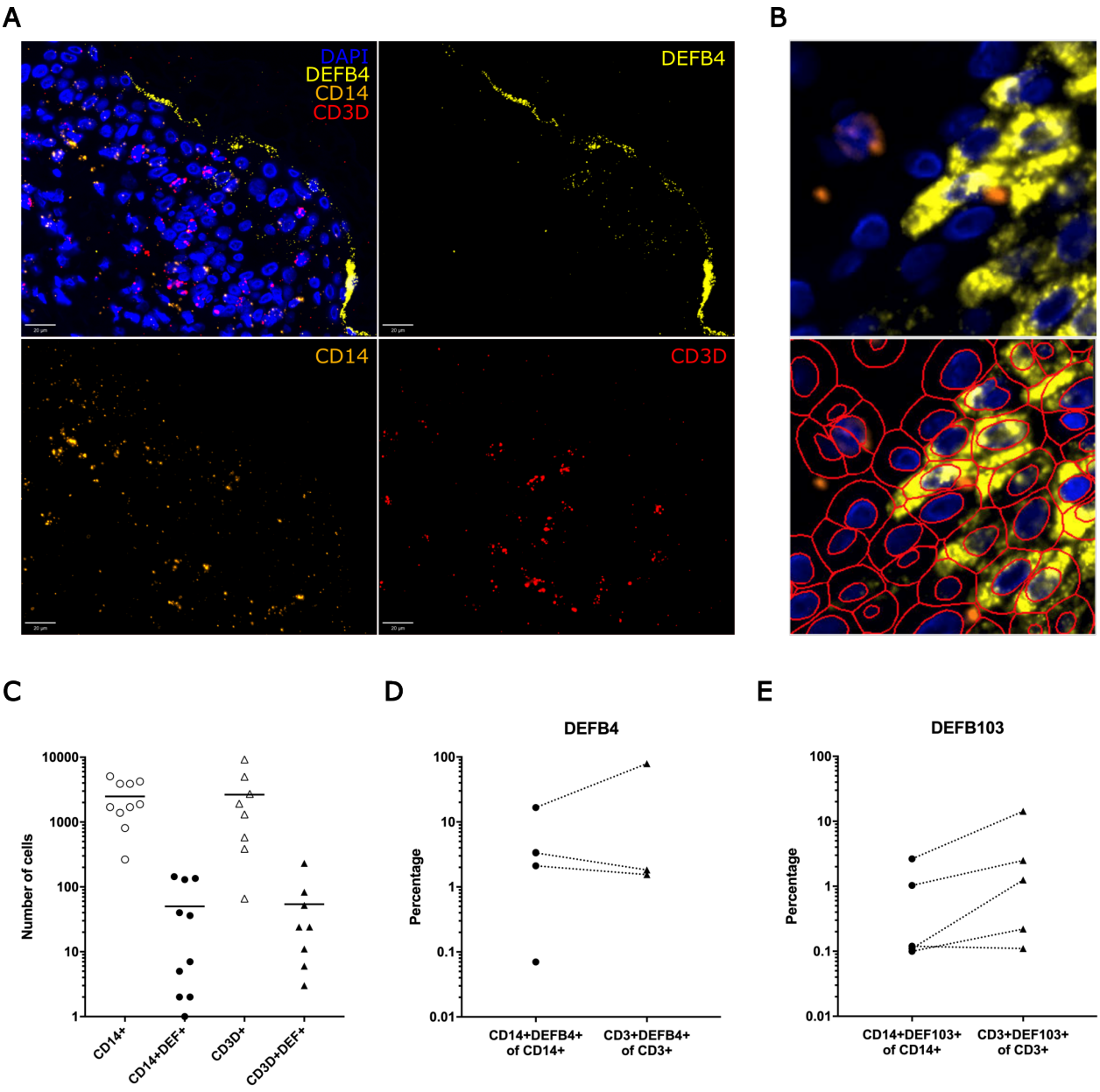


Figure 4.6. **Co-localisation between beta-defensin RNA expression and single gene immune cell markers. (A)** Representative images from a TST on day 2 (active TB participant) showing the distribution of beta-defensin and marker gene expression in the epidermis. DEFB4 (yellow), CD14 (orange), and CD3D (red) mRNA is shown. DAPI nuclear stain in blue. **(B)** Representative images of cell segmentation using DAPI. Red lines mark the nuclear boundary and extended perimeter, within which expression is quantified. **(C)** The absolute numbers of CD14+ macrophages (open circles), CD3D+ T cells (open triangles), and beta-defensin positive macrophages (closed circle) or T cells (closed triangles) (DEF=DEFB4 or DEFB103 expression) (N=10). Symbols represent an individual TST whilst lines are the mean. **(D-E)** The percentage of DEFB4-positive or DEFB103-positive macrophages and T cells as a total of the respective cell populations (N=5). Lines represent paired populations from one TST. Two TSTs stained for DEFB4 expression had no contemporaneous CD3D stain.

4.4. Beta-defensin expression in human TB lung granulomas

Beta-defensin expression in keratinocytes, the major cell type in human epidermis (Barker *et al.*, 1991), is known (Harder *et al.*, 1997, 2001). These specialised skin epithelial cells are not present in the lung. However, respiratory epithelial cells have been shown to upregulate DEFB4 expression in response to Mtb infection in vitro (Rivas-Santiago *et al.*, 2005; Reuschl *et al.*, 2017). Furthermore, alveolar macrophages are the major tissue-resident macrophage in the lung (Davies *et al.*, 2013) and do not participate in TST responses. One study has demonstrated DEFB4 expression in alveolar macrophages stimulated with LPS and IFNγ (Duits *et al.*, 2002), but evidence of DEFB4 upregulation in response to Mtb occurred with very high MOI (350:1) that may not be physiologically relevant (Rivas-Santiago *et al.*, 2005). Nonetheless, evaluation of anti-Mtb immune responses in skin was insufficient at addressing beta-defensin expression in these cell types.

A recent study performed scRNA-seq of human TB lung granulomas (Wang *et al.*, 2023). In this study, 6 active TB patients underwent resection of highly inflamed regions of the lung. In collaboration with Kieran Killington, University College London – who processed the raw data and performed all QC, dimensionality reduction, and clustering steps – I evaluated cell type specific DEFB4 and DEFB103 expression. All QC parameters were kept the same as in the original manuscript but with the omission of a separate healthy lung tissue scRNA-seq dataset (Habermann *et al.*, 2020). This resulted in an integrated dataset of 79,486 cells from 6 active TB patients (Figure 4.7A). A lower clustering resolution was set at 0.2 to reduce the number of cell communities, and only a single round of clustering was performed.

Cells from human TB lung granulomas were clustered into 12 distinct populations, separated into 8 cell lineages labelled based on established marker gene expression (Figure 4.7B). As with the TST blister dataset (Figure 4.1), most cells were annotated as T cells. However, there existed a substantially larger population of myeloid lineage cells than were evident in the TST. This may reflect a limitation of suction blisters in requiring cells to extravasate into the fluid to be captured (Rojahn *et al.*, 2020), potentially favouring the capture of lymphocytes.

Alternatively, it may be that there are simply more cells of myeloid lineage present in granulomas than are in the TST.

There were very few cells expressing DEFB4 (Figure 4.7C), as was also seen in the TST blister dataset. None of the captured cells from human TB lung granulomas expressed DEFB103. DEFB4A was the only gene of interest for which there was expression, with a limited number of cells (59 out of 79,486 cells) with captured DEFB4A mRNA transcripts. 43 of these cells were annotated as pneumocytes (Figure 4.7B). These were most likely ATII cells given their primary role in pulmonary surfactant secretion (Han and Mallampalli, 2015) and the enrichment of surfactant gene expression in this cluster. Across the various leukocyte populations there was limited, infrequent expression of DEFB4A. Only a few cells from several of these clusters expressed DEFB4A; 0.02% of all myeloid cell (3/14828) and 0.01% of all T cells (5/49419) expressed DEFB4, compared with 1.9% of alveolar pneumocytes (43/2258).

Five cells from the undefined cell cluster had high expression of DEFB4A. It is worth noting that for this cluster, though no cell type annotation could be given, around 25% of composite cells expressed surfactant genes. This was in addition to approximately 25% of the cluster expressing CD68 and CD3D, respectively. Thus, it is likely that the undefined cell cluster represented a mix of different cell types. These may have been grouped together due to poor cell quality, as was seen in the TST blister dataset (Figure 4.1), though this was not explored here. Similarly, whilst two cells from cluster 3 (annotated as myeloid cells) expressed DEFB4, this cluster had evidence of surfactant gene expression perhaps indicating a mixed cell population.

Alveolar pneumocytes are not considered part of the TB granuloma (Cadena, Fortune and Flynn, 2017). Their presence in this dataset may reflect resection of a larger tissue area during surgery, capturing cells adjacent to the granuloma in addition to those comprising it. Therefore, this dataset supports the expression of DEFB4 by alveolar pneumocytes neighbouring human TB lung granulomas. There was no evidence to suggest that cells within the TB granuloma express DEFB4 or DEFB103.

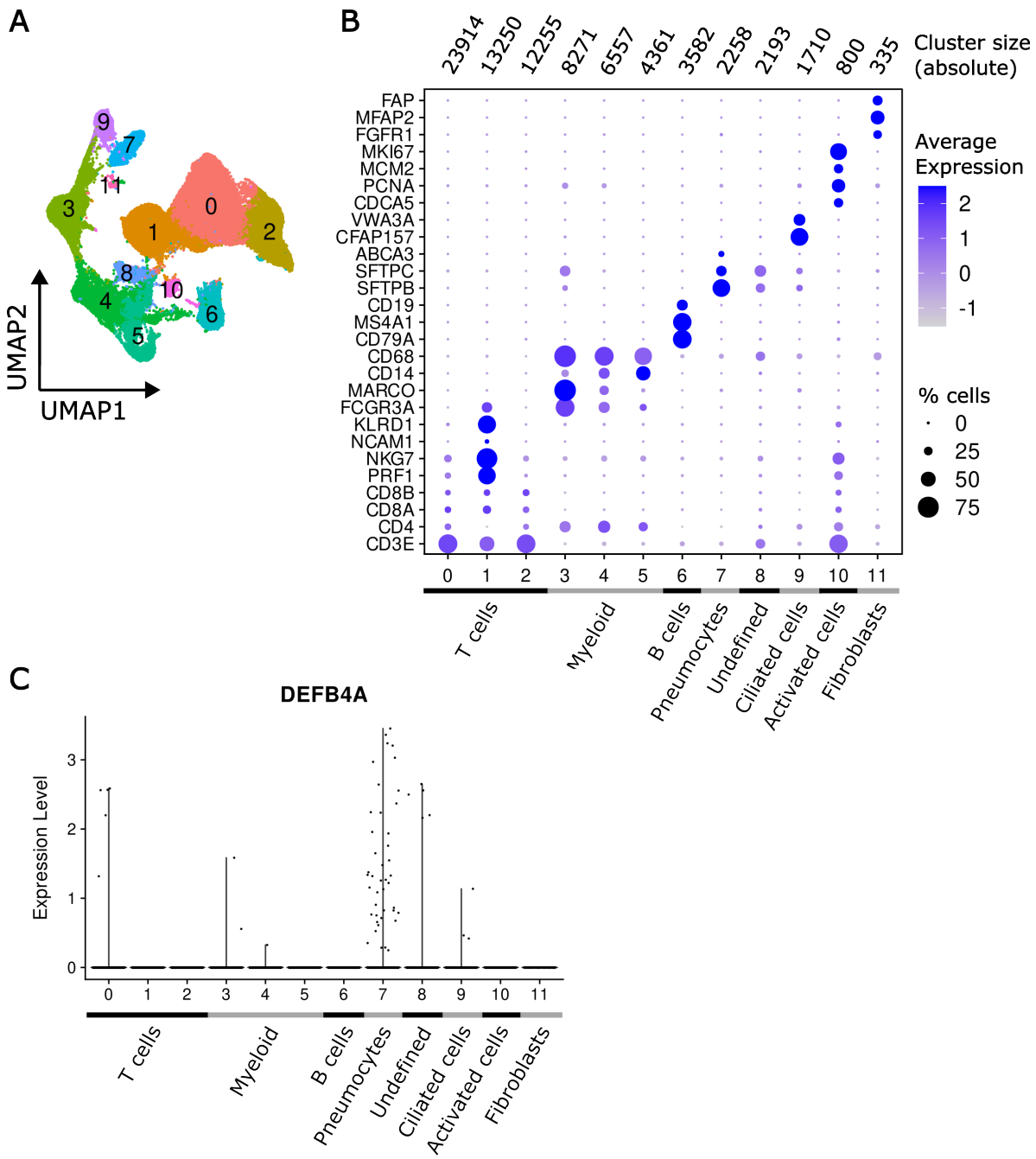


Figure 4.7. **Single cell RNA-seq of human TB lung granulomas. (A)** Clustering of integrated scRNA-seq data from 79,486 cells in human TB lung granulomas (N=6). **(B)** Canonical marker gene expression by cell cluster with inferred cell type and absolute cluster size (Activated cells=Proliferating lymphocytes). Dot size represents the percentage of cells expressing each marker and colour shows the average log normalised expression of the marker for each cluster. The average expression colour scale is capped at -1.5 and 2.5. **(C)** Log normalised DEFB4A expression in 79,486 cells across 12 cell clusters from human TB lung granulomas (N=6). Cluster annotations are based on canonical marker gene expression.

4.5. Chromatin accessibility of beta-defensin genes

The epigenetic regulation of gene expression can underpin cell type specific expression of certain genes (Jaenisch and Bird, 2003). ATAC-seq evaluates chromatin accessibility (Grandi *et al.*, 2022), where open chromatin around a gene enables access for transcription factors and RNA polymerase to rapidly initiate transcription. Where chromatin is closed, no transcription is thought to occur. I explored the chromatin accessibility around DEFB4 and DEFB103 gene paralogs in published ATAC-seq datasets from human primary lung epithelium, monocytes, and macrophages.

Chromatin proximal to the TSS of DEFB4A and DEFB4B was open in resting lung epithelial cells (Figure 4.8). Conversely, the chromatin surrounding DEFB103 gene paralogs was closed. The finding is concordant with the absence of DEFB103 expression in human TB lung granulomas (Figure 4.7). In resting human primary MDM there was little evidence of open chromatin around any of the beta-defensin gene paralogs in question. Additionally, LPS stimulation of human primary monocytes and MDMs seemingly had no effect on chromatin accessibility and remained closed throughout the time course. Data presented here does not reflect monocyte and macrophage responses to Mtb, thus it remains unclear whether such findings translate to TB. However, previous studies showed that LPS stimulation upregulated DEFB4 expression in human primary monocytes and MDMs in vitro (Duits *et al.*, 2002; Rivas-Santiago *et al.*, 2005). Such findings were not supported by the data presented here.

I also explored the chromatin accessibility in human primary alveolar macrophages (Staitieh *et al.*, 2023). The raw data has not yet been made publicly available, but processed ATAC-seq data had no sequenced reads aligning to regions adjacent to DEFB4 and DEFB103 genes (data not shown). In ATAC-seq data, only regions of open chromatin generate sequencing reads (Grandi *et al.*, 2022), thus an absence of reads across a genomic locus indicates closed, inaccessible DNA.

Together these data provide a mechanistic insight into the expression of beta-defensins in lung epithelium. Furthermore, they suggest an inability of human monocytes and

macrophages to upregulate DEFB4 and DEFB103 expression during conventional antibacterial responses that may be due to epigenetic regulation.

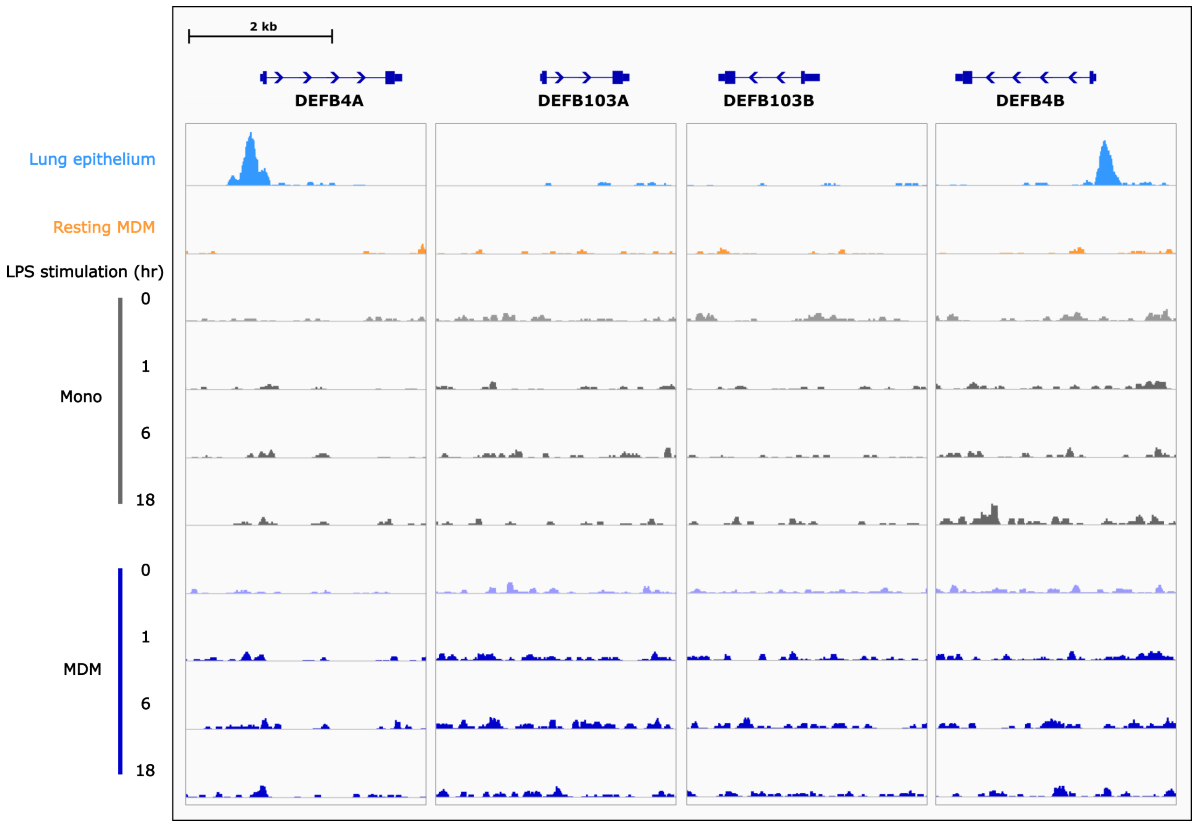


Figure 4.8. **Epigenetic regulation of human beta-defensin genes in epithelial and monocyte lineages.** Sequenced reads aligning to accessible regions of chromatin neighbouring DEFB4 and DEFB103 gene paralogs is shown. Colours represent the different cell types and/or conditions (Mono=Monocytes). The y-axis scaling is the same across all samples. Publicly available ATAC-seq data is from resting human primary lung epithelium (GEO accession: GSE183873, European Nucleotide Archive accession: PRJNA762259), resting human primary MDM at the end of differentiation in vitro (GEO accession: GSE147306, European Nucleotide Archive accession: PRJNA613719), and human primary monocytes/MDM throughout an LPS stimulation time-course experiment (GEO accession: GSE147307, European Nucleotide Archive accession: PRJNA613718).

4.6. Discussion

In this chapter, I have presented evidence supporting beta-defensin expression in epithelial cells during anti-Mtb immune responses in vivo. Single cell analysis of TSTs by scRNA-seq and RNAscope demonstrated DEFB4 and DEFB103 expression exclusively among keratinocytes. Corroborating this, scRNA-seq data from human TB lung granulomas attributed DEFB4 expression to alveolar pneumocytes.

In contrast to previous findings in respiratory epithelial cells (Harder *et al.*, 2001), there was no DEFB103 expression in alveolar pneumocytes. This may be for several reasons. The authors of this publication evaluated DEFB103 expression in primary tracheal epithelial cells. There may exist differences in DEFB103 responses between specialised respiratory epithelial cell types. Authors also noted a significant upregulation of DEFB103 in response to *P. aeruginosa*, but very minimal upregulation with TNFα and did not evaluate DEFB103 expression in the presence of mycobacterial antigens. Thus, it may be that DEFB103 is not induced in pneumocytes during anti-Mtb responses. Additionally, I found evidence of closed chromatin surrounding DEFB103 genes in resting human primary lung epithelial cells (Figure 4.8), indicative of epigenetic silencing. However, the absence of DEFB103 expression the lung may also relate to the challenges surrounding mRNA capture of lowly expressed genes (Lun, McCarthy and Marioni, 2016). Among keratinocytes from TST suction blisters, the absolute expression of DEFB103 was notably lower than that for DEFB4 (Figure 4.2), suggesting epithelial cells in general may express lower amounts of DEFB103.

I found no evidence that human macrophages express DEFB4 or DEFB103 in response to Mtb. The absence of macrophage expression among TST blister cells could have been due to the aforementioned limitations of scRNA-seq approaches regarding low levels of gene expression (Lun, McCarthy and Marioni, 2016). However, RNAscope had no such limitation. Furthermore, RNAscope identified approximately 25,000 CD14+ macrophages across all the TSTs, substantially more than the 4046 cells comprising the myeloid cluster in TST blisters. Nonetheless, beta-defensin expression in CD14+ cells was not enriched versus that in CD3D+

cells. This suggested that the colocalisation of CD14 and beta-defensin expression resulted from the close proximity of two single-positive cells, rather than reflecting genuine double-positive cells.

The use of dual marker staining was not performed for two reasons. Considering the overlap between CD14+ and CD68+ cells in the TST, it was reasoned that the use of dual macrophage markers would be of limited benefit versus the increased reagent costs. Furthermore, the decision was made to use only three of the available four channels for the RNAscope assay due to high levels of tissue autofluorescence across TSTs. Tissue-endogenous fluorescence occurs with the natural emission of light from the molecular components of tissue (Brodie, 2020). This can result in a low to moderate degree of light emission, typically towards the shorter wavelength end of the emission spectrum (Baschong, Suetterlin and Laeng, 2001), that lowers the signal to noise ratio. This limits many fluorescence-based analyses in tissue, such as the colocalisation of signals (Whittington and Wray, 2017).

There is no autofluorescence quenching approach to RNAscope. Instead, the assay utilises patented bioinformatic spectral unmixing to separate signals, including tissue autofluorescence. The extracellular matrix, particularly that in normal human skin, has a high degree of autofluorescence across many wavelengths that can be enhanced by the FFPE process (Brodie, 2020). Common workarounds for RNAscope include placing the target with highest expression in the green channel (Secci *et al.*, 2023) to account for this increased background signal. Unfortunately, due to the high degree of tissue autofluorescence, I found the green channel unusable across most TSTs and decided to avoid it altogether. Attempts to quench this tissue autofluorescence were unsuccessful. This left me with just three available channels, in addition to the DAPI nuclear stain. It was for these reasons that I settled on using only CD14 expression as a marker for macrophages in the TST.

There are limitations in using the expression of a single gene to identify a given cell type. Of note, cell-surface expression of CD14 is often used to identify classical monocytes, and CD68 for macrophages (Ahmed *et al.*, 2024). However, monocytes differentiate into macrophages

at sites of inflammation and retain CD14 expression (Sharygin *et al.*, 2023; Ahmed *et al.*, 2024). Supporting this, there was considerable overlap between CD14+ and CD68+ cells in the TST (Figure 4.4B,C), suggesting that the majority of cells identified are differentiated macrophages. Despite there being minimal expression of CD14 among keratinocytes captured in the scRNA-seq TST blister dataset, human primary keratinocytes can express CD14 (Song *et al.*, 2002). It is possible that a fraction of CD14+ cells were in fact beta-defensin expressing keratinocytes. If this were the case, this reduces the likelihood that CD14+ cells observed to express DEFB4 and DEFB103 were genuine macrophages. This does not however explain why there was a similar frequency of beta-defensin expressing CD3D+ cells. There are alternative macrophage markers that have not been explored here. CD163 may be a more specific marker for monocytes and macrophages (Lau, Chu and Weiss, 2004), and would be worth considering for future characterisation of macrophages in the TST.

The scRNA-seq dataset from human TB lung granulomas had very limited DEFB4 expression across all cells, captured in just 59 out of 79,486 cells (approximately 0.07%). It was evident that expression was enriched in the cluster of pneumocytes. Alveolar pneumocytes and respiratory epithelial cells have robust data supporting DEFB4 expression (Harder *et al.*, 2000; Kao *et al.*, 2004; Reuschl *et al.*, 2017), and although the absolute sample size was limited, 73% of all DEFB4 expressing cells were from the pneumocyte cluster.

Conversely, there was only sporadic DEFB4 expression across several other clusters. T cell and myeloid cell clusters both had a small number of cells expressing DEFB4 within them, constituting 0.01% and 0.02% of all cells in the cluster respectively. Similar numbers of both T cells and myeloid cells expressed DEFB4, perhaps indicating a failure to remove all doublets or background RNAs. It is difficult to ascertain whether expression in three myeloid cells represents evidence of DEFB4 expression in macrophages. It is not general practice to draw conclusions around cell type gene expression based on so few cells due to the considerable heterogeneity between any single cell in scRNA-seq datasets (Zhang *et al.*, 2023). The clustering of cells for scRNA-seq data analysis is essential due to the relatively low number of genes captured in any one cell (Lun, McCarthy and Marioni, 2016) and for this reason, gene

expression is typically evaluated at the level of clusters. Furthermore, clustering is highly sensitive to data pre-processing steps (Zhang *et al.*, 2023). Considering this, a limitation of my analysis was the absence of any metric to determine the statistical robustness of clusters (Kanter, Dalerba and Kalisky, 2019; Grabski, Street and Irizarry, 2023). Nonetheless, there was no enrichment of DEFB4 expression in myeloid cell clusters.

Alveolar macrophages are present in this dataset. In the original manuscript, high expression of MARCO characterised alveolar macrophage clusters (Wang *et al.*, 2023). High FCGR3A expression has also been associated alveolar macrophages (Frankenberger *et al.*, 2012). I found cluster 3 to be enriched for MARCO and FCGR3A expression and thus likely represents this alveolar macrophage population. However, this cluster may constitute a mixed myeloid lineage population owing to the low clustering resolution used here. Were I to use sequential rounds of clustering (Turner *et al.*, 2024), I may have been able to parse alveolar macrophages from other myeloid cell types. Despite this, alveolar macrophages did not appear to contribute towards the beta-defensin response in vivo.

This dataset is limited to resected TB lung granulomas from just 6 males (Wang *et al.*, 2023), all of which had highly inflamed lung tissue as a result of their TB. The immune responses captured in this dataset reflect dysregulated immunity, and their clinical status in addition to prolonged failure of anti-TB treatment indicated poor immune control of infection (Wang *et al.*, 2023). It may be that in those whose macrophages do express beta-defensins, they exhibit better control of Mtb growth and thus the individual does not progress to active TB.

I cannot rule out the possibility of beta-defensin expression in human macrophages prior to granuloma formation, and therefore having a role in achieving sterilising immunity. However, the best evidence supporting DEFB4 expression in Mtb-infected human primary macrophages is during experimentally induced hypoxia (Nickel *et al.*, 2012), expected to occur in granuloma centers (Tsai *et al.*, 2006). Therefore, one might expect this dataset to be the best-case scenario for finding evidence of macrophage DEFB4 expression in vivo. I did explore another published TB granuloma scRNA-seq dataset from an Mtb-infected NHP model (Gideon *et al.*,

2022), which comprised of both early and late-stage granulomas. However, there was no beta-defensin expression in any of the cells from this dataset (data not shown). Limitations of single cell sequencing technologies might necessitate a hypothesis-driven approach to studying macrophage beta-defensin expression in TB, such as the enrichment of macrophages from human lung tissue samples prior to more sensitive gene expression assays. Evidence that human alveolar macrophages express beta-defensins is sparse and addressing this should be an important focus of future work.

5. Epithelial cell-derived beta-defensins do not contribute to macrophage restriction of Mtb growth

5.1. Objectives

HBD2 and hBD3 have both been demonstrated to kill mycobacteria (Corrales-Garcia *et al.*, 2013; Reuschl *et al.*, 2017; Su *et al.*, 2018). Data from in vitro and in vivo TB models also support a role within macrophages for the restriction of Mtb by beta-defensin 2 (Kisich *et al.*, 2001; Peng *et al.*, 2024). Contrary to several previous studies (Duits *et al.*, 2002; Tsutsumi-Ishii and Nagaoka, 2003; Rivas-Santiago *et al.*, 2005; Nickel *et al.*, 2012; Rodriguez-Carlos *et al.*, 2020; Díaz *et al.*, 2023), I have found no evidence of DEFB4 and DEFB103 expression by human macrophages in TB. Instead, the evidence suggests their expression in vivo is restricted to epithelial cells. IL-1β-mediated cross talk between Mtb-infected alveolar macrophages and alveolar pneumocytes has been shown to upregulate DEFB4 expression in the latter (Reuschl *et al.*, 2017). Despite this, it was not clear whether the paracrine production of hBD2 by pneumocytes could restrict the growth of Mtb in human macrophages.

Numerous studies have demonstrated that pro-inflammatory cytokines induce DEFB4 and DEFB103 expression in airway epithelia and pneumocytes (Harder *et al.*, 2000; García *et al.*, 2001; Tsutsumi-Ishii and Nagaoka, 2003; Kao *et al.*, 2004; Reuschl *et al.*, 2017). However, none of these studies evaluated the beta-defensin response at the protein level, instead measuring mRNA expression. Not only is mRNA expression evident before secreted protein, but the correlation between the two in human datasets is known to be relatively poor (Vogel and Marcotte, 2012). I investigated whether the inducible beta-defensin response in pneumocytes at the protein level was sufficient to restrict Mtb growth.

5.2. Cytokine-induced variation in beta-defensin production

I stimulated alveolar pneumocytes with a cocktail of pro-inflammatory cytokines, IL-17A, IL-1β, and TNFα, all at 10 ng/mL. In addition to upregulating DEFB4 expression in airway

epithelial cells (Kao *et al.*, 2004), each respective cytokine activity module correlated well with beta-defensin expression in TSTs (Figure 3.11), though it is worth reiterating that this in no way implied causality. For example, the IL-6 activity module correlated with beta-defensin expression to about the same degree as the other cytokines, despite IL-6 having been shown not to induce expression in respiratory epithelia (Harder *et al.*, 2000).

I evaluated the production of hBD2 and hBD3 in four commonly used airway epithelial cell (AEC) lines, A549, BEAS-2B, Calu-3, and Detroit 562 (Figure 5.1A). This revealed variable hBD2 secretion between the AEC lines. In contrast, there appeared to be no production of hBD3 in response to cytokine treatment. In hBD2-producing cells, the concentration in the supernatant rose steadily over three days to a maximum concentration of approximately 4 ng/mL in Detroit 562 cultures. After three days, rapidly growing AEC lines reached confluency and started to die. This maximal concentration was notably well below the minimum reported to kill Mtb in liquid culture (Reuschl *et al.*, 2017). However, in one study the amount of hBD2 secreted by immortalised cell lines was approximately 10-fold less than from human primary cells (Kao *et al.*, 2004). Therefore, I repeated this cytokine stimulation experiment with three human primary alveolar pneumocytes (haAEC), isolated from the lungs of three healthy individuals.

The haAEC contain a mix of type I and type II alveolar pneumocytes and divide slowly over several days, but cannot be cultured long-term. To mirror the experiments with AECs, haAEC were stimulated with 10 ng/mL of IL-17A, IL-1β, and TNFα concomitantly (Figure 5.1B). The results were comparable to AECs, with variable production of hBD2 between donors, no measurable hBD3 response, and a steady increase in hBD2 concentration to approximately 5 ng/mL in supernatant. This absence of hBD3 production may been related to the epigenetic silencing around the DEFB103 gene in lung epithelium (Figure 4.8). Alternatively, it may be that some other combination of cytokines is necessary to induce hBD3 secretion. The variation in hBD2 secretion suggested some intrinsic ability of the different pneumocytes to respond to the cytokines.

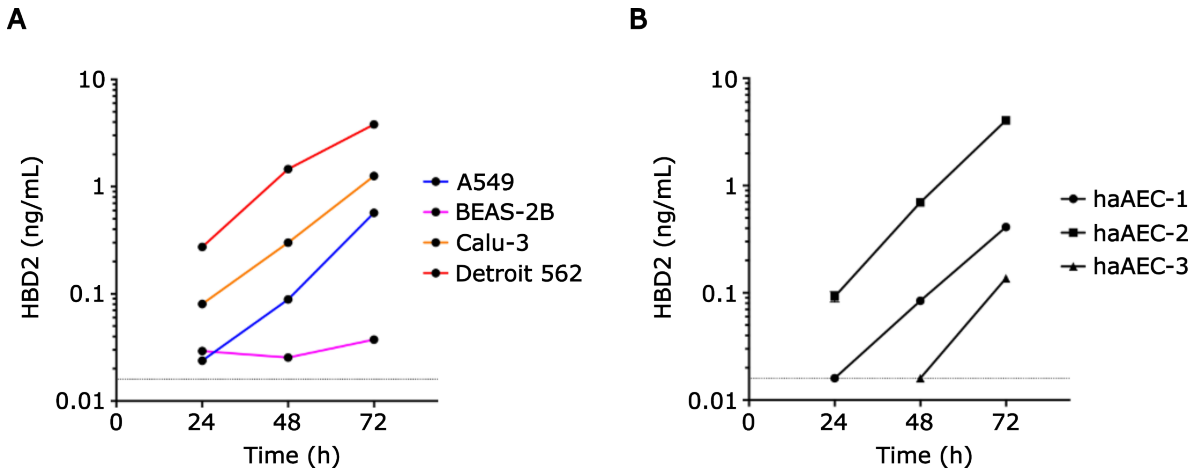


Figure 5.1. **Cytokine-induced beta-defensin production by airway epithelial cells. (A)** Four human airway epithelial cell lines were stimulated with a cocktail of pro-inflammatory cytokines (IL-17A, IL-1β, and TNFα all at 10 ng/mL) for three days. The concentration of hBD2 and hBD3 was measured in the supernatant at each time point by ELISA (N=2). No hBD3 was measured hence data is not presented here. **(B)** The cytokine stimulation experiment was repeated with human primary alveolar pneumocytes (haAEC-1=ATII0835, haAEC-2=ATII0889.02, haAEC-3=05AB0937.02) from three healthy human donors. The concentration of hBD2 and hBD3 was measured in the supernatant at each time point by ELISA. No hBD3 was measured hence data is not presented here. For each donor, three independent time course experiments were performed using the same stock aliquot of human primary pneumocytes. Error bars reflect the standard deviation around the mean. The dotted line reflects the limit of detection (15.6 pg/mL) of the ELISA.

5.3. High throughput flow cytometric assay for Mtb quantitation

I established that hBD2 concentration in supernatant of cytokine-stimulated pneumocyte cultures was approximately three orders of magnitude below what had been required to restrict Mtb growth (Reuschl *et al.*, 2017). However, this study evaluated Mtb killing in the absence of human macrophages. It was also not known what concentration of hBD2 would restrict Mtb growth in the presence of macrophages. The only study to demonstrate growth restriction of Mtb by hBD2 in human primary macrophages made no measurements of the hBD2 concentration (Kisich *et al.*, 2001).

Beta-defensins can get inside macrophages (Semple and Dorin, 2012) where they may end up more concentrated than they were extracellularly. They may also enhance macrophage killing mechanisms, having been reported to prime other innate immune cells (Funderburg *et al.*, 2007; Barabas *et al.*, 2013; Wanke *et al.*, 2016) and augment pro-inflammatory cytokine secretion (Boniotto *et al.*, 2006; Jin *et al.*, 2010; Niyonsaba *et al.*, 2010; Judge *et al.*, 2015). Therefore, it remained a possibility that in the presence of human macrophages the concentrations necessary for Mtb growth restriction are lowered substantially. Alternatively, certain subcellular compartments may harbour bacteria whilst limiting beta-defensin accumulation, such that they never reach concentrations adequate for killing.

To assess the growth of Mtb in human primary MDMs, I used a high throughput flow cytometric assay that quantifies mycobacterial load in the extracellular and intracellular compartments of Mtb-infected macrophages in vitro (Figure 5.2). MDMs are differentiated from peripheral blood monocytes of healthy human donors using M-CSF and infected with a constitutively fluorescent Mtb strain H37Rv. At the experimental endpoint, cultures are harvested and fixed, and a known quantity of fluorescent counting beads are added to each sample. Individual bacteria and counting beads are measured using flow cytometry to accurately quantify bacterial load (Figure 5.2A). Mtb growth is evaluated by comparing bacterial load after 5 days to the number of internalised bacteria 4 hours post infection

(Figure 5.2B). Additionally, the number of bacteria are multiplied by the fluorescence to account for clumping of Mtb as it grows.

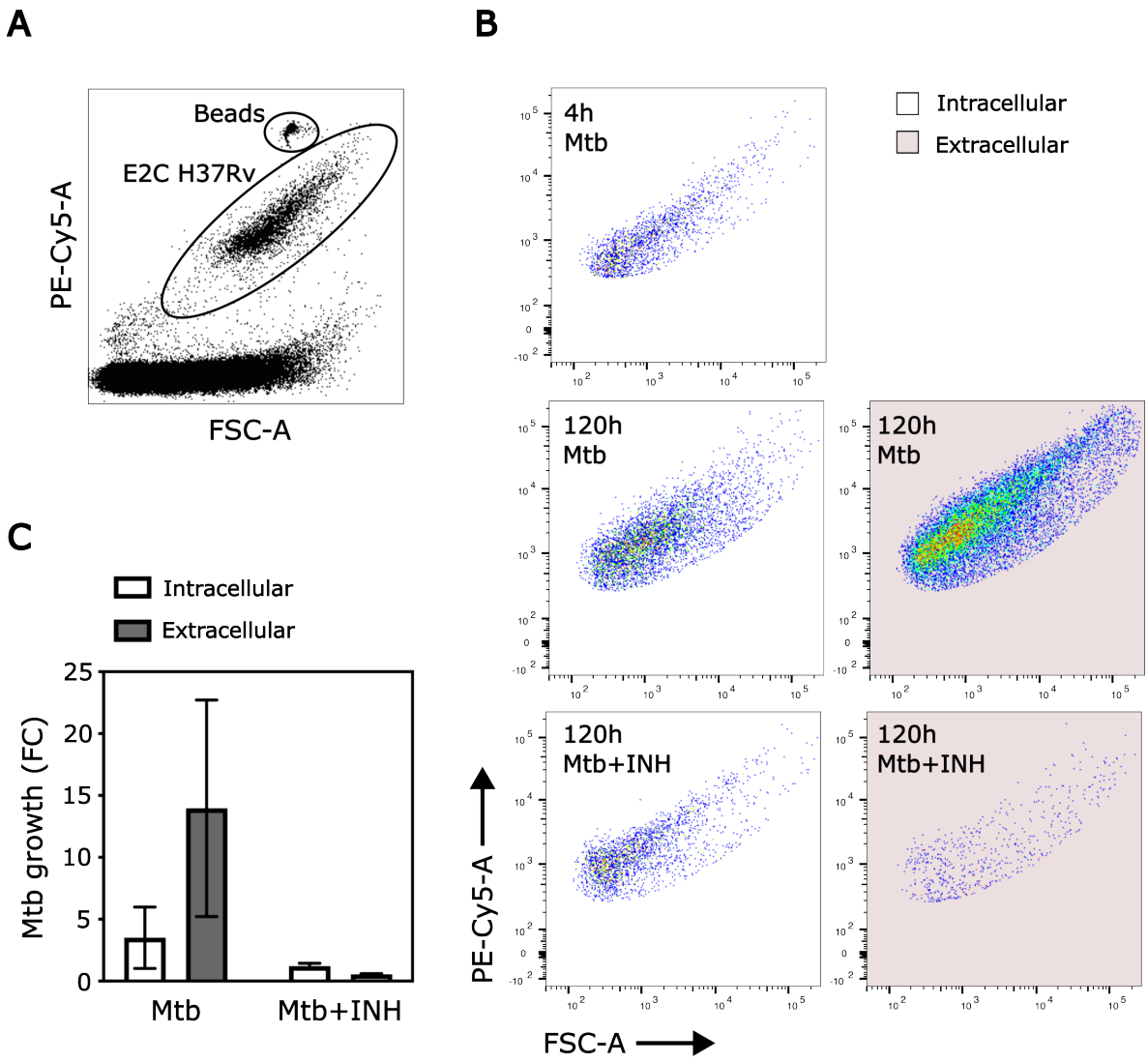


Figure 5.2. **High throughput flow cytometric assay for Mtb quantitation. (A)** Representative gating strategy to distinguish fluorescent Mtb (E2C H37Rv) and counting beads from debris. **(B)** Representative flow cytometry plots of E2C H37Rv populations from one experiment, showing the 4 hour baseline sample and those at 120 hours post infection with and without 50 ng/mL of isoniazid. **(C)** Mtb load in each compartment at 120 hours, as a fold change from internalised Mtb 4 hours post infection, in infected MDMs with and without 50 ng/mL of isoniazid. MDMs were infected at a MOI of 1:1. (INH=Isoniazid). Mean ± SD of four separate experiments.

Flow cytometry has been used to quantify intracellular mycobacterial load (Barclay *et al.*, 2023). Measurements by flow cytometry correlate with bacterial number assessed by traditional colony forming unit (CFU) counting (Barclay *et al.*, 2023). However, my data showed that over the course of 5 days, Mtb grows both intracellularly and extracellularly (Figure 5.2B,C). Most growth in fact occurs extracellularly, highlighting that Mtb is not an obligate intracellular pathogen, and studies should equally consider extracellular growth as they do intracellular. Overall, human primary MDMs differentiated with M-CSF were unable to restrict Mtb growth (Figure 5.2C). Isoniazid, which is bactericidal against growing Mtb (Winder and Collins, 1970), limited the growth in both compartments. For reasons that are as yet unclear, isoniazid had comparatively limited efficacy against intracellular Mtb.

5.4. Paracrine beta-defensin 2 does not augment macrophage control of Mtb

Using high throughput flow cytometric quantitation of Mtb growth, I addressed whether hBD2 secreted by pneumocytes could augment Mtb growth restriction by human primary MDM. AECs were stimulated with cytokines as before. After 3 days, the hBD2-enriched supernatant was collected, along with supernatant from unstimulated AEC monolayers. Mtb-infected macrophages were then cultured in the presence of these supernatants for 5 days. Baseline restriction of Mtb growth varied considerably between the four donors (Figure 5.2C). To enable a direct comparison across donors, the total Mtb growth in the presence AEC culture supernatants was normalised to the growth in infected MDM cultures only.

Total Mtb growth was comparable across all the conditions and remained unchanged regardless of whether AECs were stimulated with cytokines or not (Figure 5.3). The distribution of Mtb intra- or extracellularly varied between donors. Some had a 2–3-fold increase in intracellular Mtb after 5 days of culture (Figure 5.3B). However, these donors had a concomitant decrease in the extracellular compartment (Figure 5.3C), such that the total amount of Mtb in the entire well was relatively unchanged (Figure 5.3A). Whether this shift in Mtb distribution reflects biological differences between donors, or technical variation in harvesting between experiments, is undetermined. Of note, macrophage viability was not

measured but has been shown by others in the group to be generally unaffected by Mtb infection over 5 days.

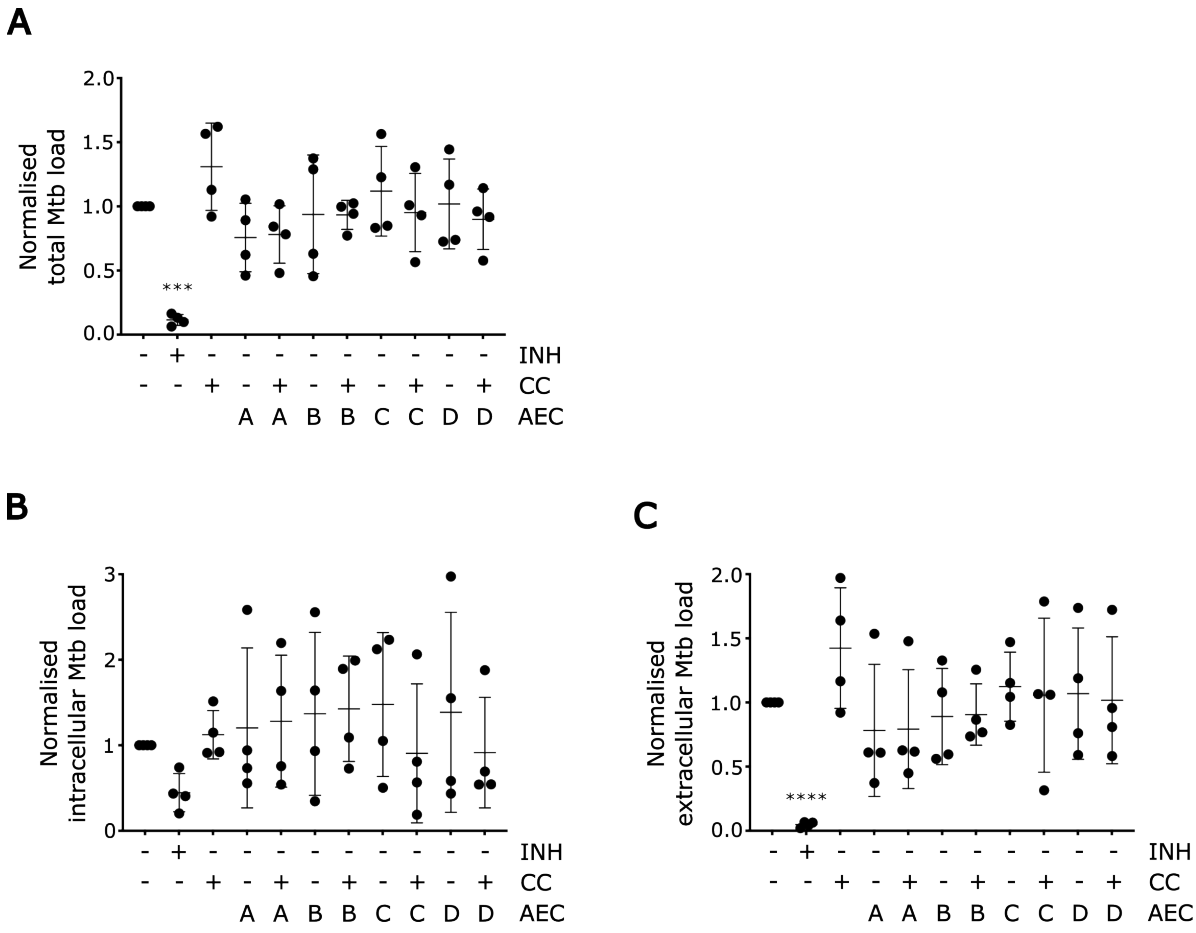


Figure 5.3. **Effects of stimulated airway epithelial cells on Mtb growth in human primary MDM.** The **(A)** total, **(B)** intracellular, and **(C)** extracellular Mtb load in human primary MDM 120 hours post infection was quantified by flow cytometry. Load has been normalised to the Mtb-only condition in the respective compartment for each experiment. MDMs were infected at a MOI of 1:1. 4 hours post infection, supernatant was transferred from resting and cytokine-stimulated A549 (A), BEAS-2B (B), Calu-3 (C), and Detroit 562 (D) cultures. (INH=50 ng/mL of isoniazid, CC=Cytokine cocktail containing 10 ng/mL of IL-17A, IL-1β, and TNFα, AEC=Airway epithelial cell supernatant). Mean ± SD of four separate experiments. Repeated measures one-way ANOVA (Geisser-Greenhouse correction) with Dunnett's test was used to compare load with Mtb-only control. P value 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

I measured the concentration of hBD2 in each of the transferred supernatants by ELISA. This allowed me to compare the hBD2 concentration with the total load of Mtb in the well after 5 days (Figure 5.4). Mtb load was normalised to the 'Mtb + CC' condition after 5 days. Here, Mtb-infected MDMs received culture media containing 10 ng/mL of IL-17A, IL-1β, and TNFα. This media was identical to that used to stimulate AECs. Consequently, the only difference between this condition, and those receiving supernatant from cytokine-stimulated AECs, would be factors secreted by pneumocytes in response to stimulation. Interestingly, in 2 of 4 donors, the addition of cytokines resulted in a 50% increase of the total Mtb load (Figure 5.3A). This could be largely attributed to increases in extracellular Mtb (Figure 5.3C). It is for this reason that the normalised Mtb loads presented in Figure 5.4 tend to be below 1, especially for extracellular Mtb counts (Figure 5.4C).

There was no relationship between hBD2 concentration and total Mtb growth (Figure 5.4A). All data points using cytokine-stimulated AEC supernatants were included. The varying hBD2 concentration reflects the different hBD2 response between the four AEC lines. Linear regression best-fit lines highlight this absence of a relationship. Even with intracellular Mtb, where there appears to be a slight downward trend with increasing hBD2 concentration (Figure 5.4B), there are broad 95% confidence intervals and a lack of statistical significance. As with the total Mtb count, extracellular Mtb growth remained unchanged with increasing hBD2 concentration (Figure 5.4C).

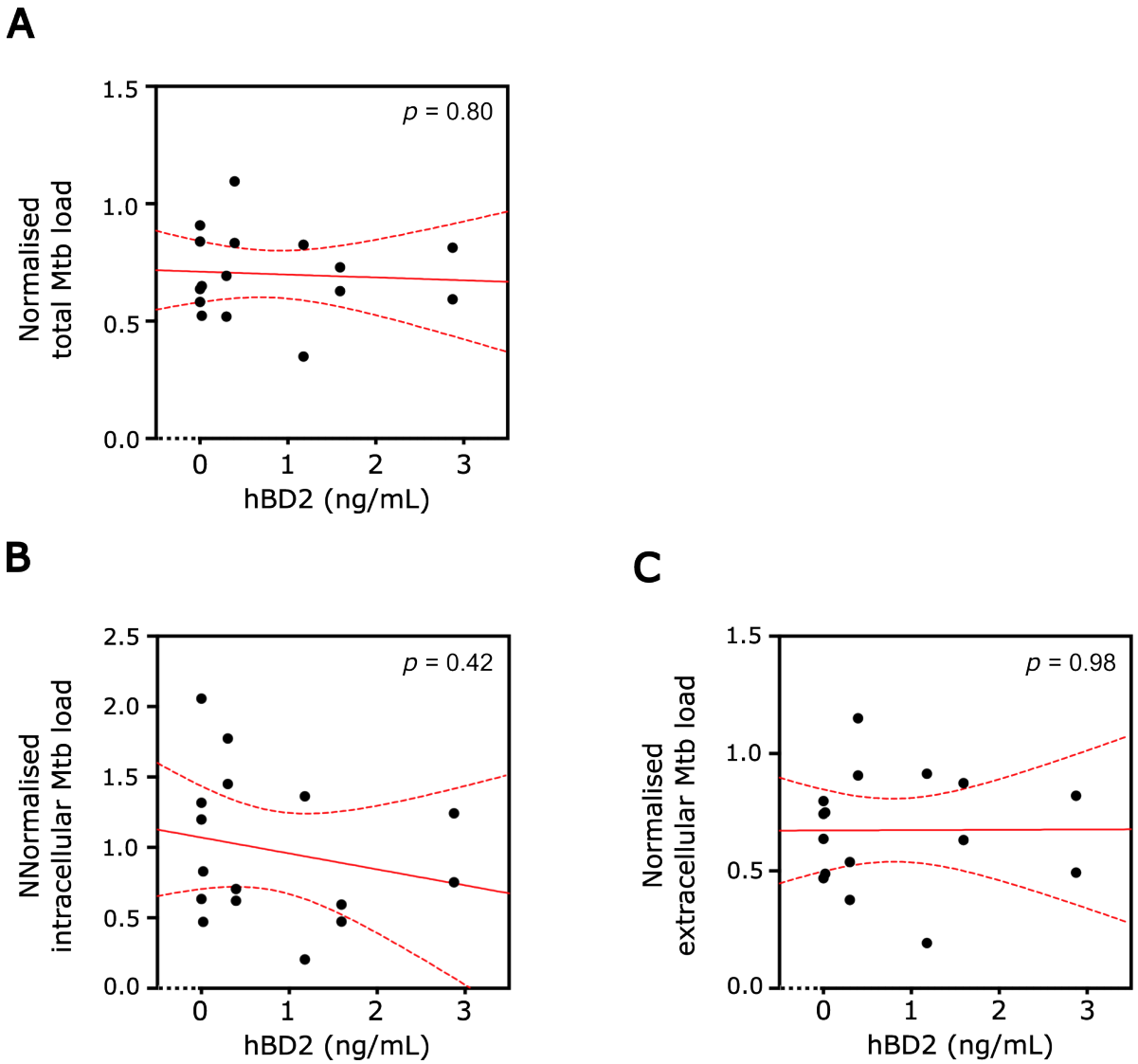


Figure 5.4. **HBD2 concentration does not correlate with Mtb growth restriction.** The concentration of beta-defensin, measured by ELISA, in transferred AEC culture supernatants was compared with the Mtb load in the respective well 120 hours post infection. The normalised Mtb load and hBD2 concentrations are from the four separate experiments shown in Figure 5.3. At the 120 hour infection end-point, Mtb load in each compartment was normalised to the Mtb-infected MDMs cultured in the presence of the cytokine cocktail. Data shown are the AEC+CC conditions (N=16). Linear regression best-fit line with 95% confidence bands.

5.5. Beta-defensins restrict macrophage-free Mtb growth

HBD2 secretion by pneumocytes is not the only secretory response to cytokine stimulation (Reuschl *et al.*, 2017). Whilst secreted hBD2 was not observed to impact macrophage control of Mtb, there may have been other factors that counteracted the protective effects of hBD2. Incidentally, the cytokine cocktail alone did appear to increase Mtb growth (Figure 5.3A), suggesting that pro-inflammatory factors can limit mycobacterial control.

Furthermore, I had been unable to induce hBD3 secretion by pneumocytes. Even though I found no evidence of DEFB103 expression in human TB lung granulomas (Figure 4.7), and data suggesting DEFB103 may be epigenetically silenced in lung epithelia (Figure 4.8), the lack of hBD3 secretion may have been related to the chosen cytokine stimulus (García *et al.*, 2001; Kao *et al.*, 2004).

To address both points, I used both recombinant hBD2 and hBD3. Recombinant hBD2 and hBD3 have been shown to restrict mycobacterial growth measured by CFU counts (Reuschl *et al.*, 2017; Su *et al.*, 2018). To demonstrate that the high throughput flow cytometric assay employed here was sensitive to mycobacterial restriction by beta-defensin, I first titrated rhBD2 and rhBD3 onto macrophage-free Mtb cultures. Beta-defensin 2 could restrict Mtb growth at 2500 ng/mL (Figure 5.5). Below this, concentrations were insufficient to kill Mtb, with this finding in line with previous reports that hBD2 restricts Mtb growth at 2.5 µg/mL (Reuschl *et al.*, 2017). Importantly, the maximum concentration of hBD2 in stimulated pneumocyte cultures was approximately 1000-times below this concentration (Figure 5.1). A similar trend was observed for hBD3 that did not reach statistical significance (Figure 5.5).

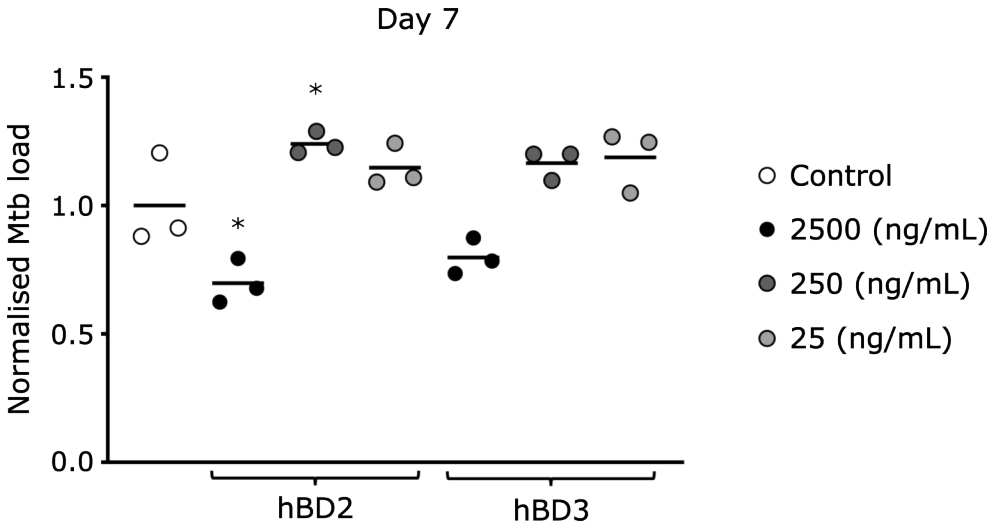


Figure 5.5. **Recombinant beta-defensin restriction of macrophage-free Mtb growth.** Recombinant human beta-defensin (hBD2 or hBD3) was added to growing Mtb liquid culture at the indicated concentration. At day 7, Mtb load was quantified by flow cytometry. Mtb load was normalised to the mean of the three replicates of Mtb only cultures (control). Lines represent the mean of three separate experiments. One-way ANOVA with Dunnett's test was used to compare load with control. P value 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

Next, I supplemented Mtb-infected human primary MDMs with recombinant beta-defensin. Mtb growth in the absence of any recombinant beta-defensin was elevated (Figure 5.6A) when compared with the growth that had been observed in supernatant transfer experiments (Figure 5.2C). It was unclear whether this reflected differences in the ability of donor macrophages to control Mtb growth. As seen previously, isoniazid restricted Mtb growth (Figure 5.6B) but with limited efficacy against intracellular Mtb (Figure 5.6C).

I titrated rhBD down to a concentration comparable to the maximum observed in stimulated pneumocyte cultures (Figure 5.1). This revealed no effect of beta-defensin on macrophage control of Mtb growth (Figure 5.6B-D). There were differences in the normalised intracellular and extracellular load between donors (Figure 5.6C-D) that had little effect on total Mtb growth. For one condition (Mtb-infected MDMs with 10 ng/mL of rhBD2) there was no intracellular Mtb (Figure 5.6C). This did not reflect augmented Mtb growth restriction. Instead, a shift of Mtb into the extracellular space meant that the total bacterial load in this condition was comparable to the control (Figure 5.6B). Technical variation in sample harvesting, donor differences, or macrophage death in this condition may explain this. Taken together, these data reveal that hBD2 and hBD3 cannot restrict Mtb growth below a concentration of 2.5 μg/mL.

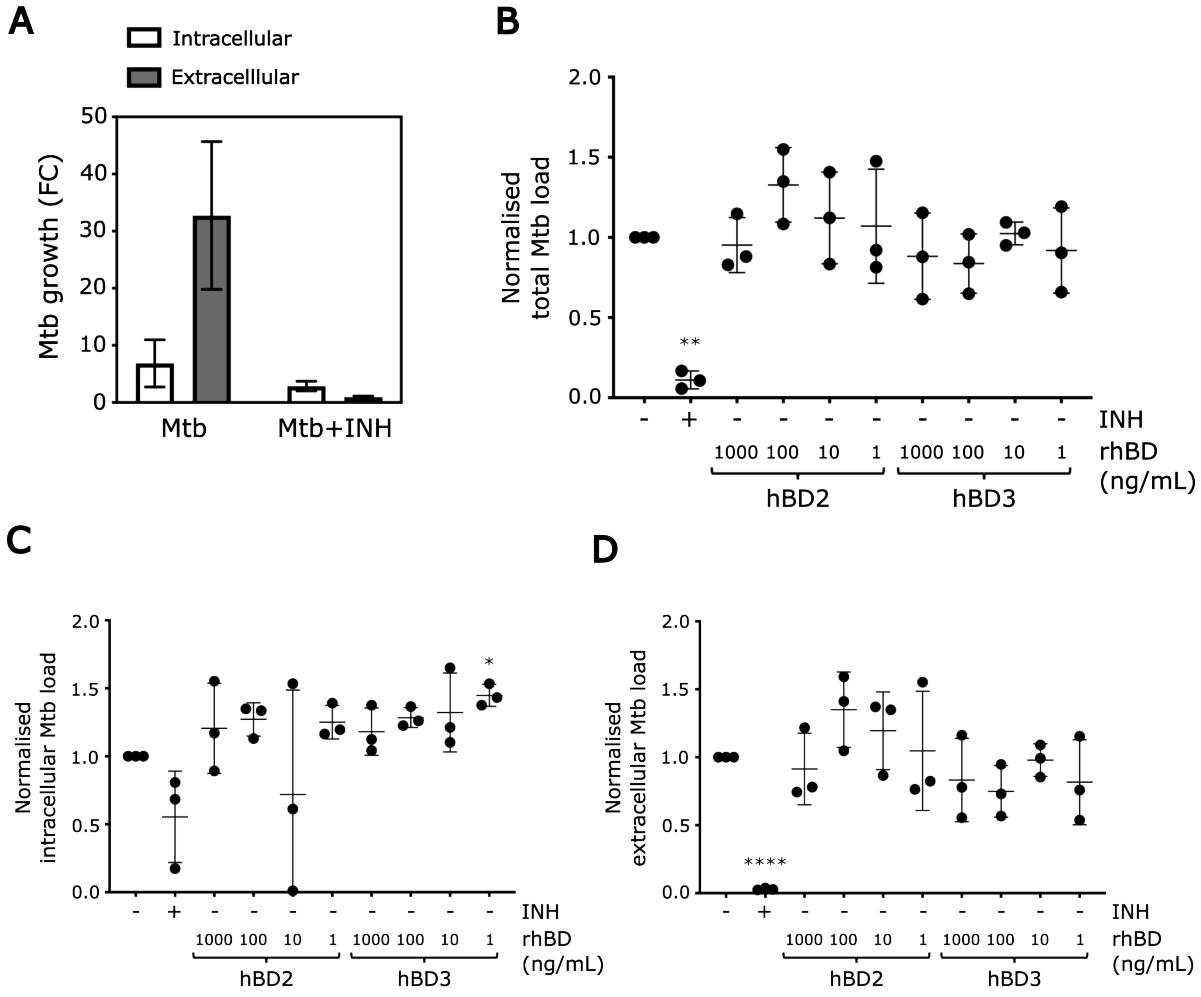


Figure 5.6. **Effects of beta-defensin on Mtb growth in human primary MDM. (A)** Mtb load in each compartment at 120 hours, as a fold change from internalised Mtb 4 hours post infection, in infected MDMs with and without 50 ng/mL of isoniazid. The **(B)** total, **(C)** intracellular, and **(D)** extracellular Mtb load in human primary MDM 120 hours post infection was quantified by flow cytometry. Load has been normalised to the Mtb-only condition in the respective compartment for each experiment. MDMs were infected at a MOI of 1:1. 4 hours post infection, recombinant beta-defensin (hBD2 or hBD3) was added at the indicated concentration. (INH=50 ng/mL of isoniazid). Mean ± SD of three separate experiments. Repeated measures one-way ANOVA (Geisser-Greenhouse correction) with Dunnett's test was used to compare load with Mtb-only control. P value 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

5.6. Discussion

In this chapter, I have demonstrated that the amount of hBD2 secreted by alveolar pneumocytes is insufficient to restrict Mtb growth. Furthermore, paracrine beta-defensin did not appear to augment macrophage control of Mtb. This is an important advancement of our understanding of beta-defensins in TB. Previous work has shown that beta-defensins are expressed by pneumocytes (Harder *et al.*, 2000; García *et al.*, 2001; Reuschl *et al.*, 2017) and are directly antimycobacterial at high concentrations (Corrales-Garcia *et al.*, 2013; Reuschl *et al.*, 2017; Su *et al.*, 2018), but the work here is the first to address whether this inducible beta-defensin response in alveolar pneumocytes can augment macrophage control of Mtb growth.

I used a cytokine cocktail consisting of IL-17A, IL-1β, and TNFα because this resulted in variable production of hBD2 in alveolar pneumocytes. However, these cytokines did not induce hBD3 secretion. In keratinocytes, IL-1β and TNFα have been shown to induce DEFB103 expression (Wolk *et al.*, 2004; Joly *et al.*, 2005), whilst TNFα did induce DEFB103 expression in respiratory epithelial cells (Harder *et al.*, 2001). In contrast, IL-17 treatment strongly induced DEFB4 expression in human primary TBE cells but not DEFB103 (Kao *et al.*, 2004). Differences here may reflect a delay in hBD3 secretion compared to gene upregulation. This could have been addressed by measuring concomitant gene expression. Although DEFB103 was highlighted as IFNγ-independent in the TST, in vitro experimentation demonstrates a substantial induction of DEFB103 expression with IFNγ that is not evident for DEFB4 (García *et al.*, 2001; Wolk *et al.*, 2004; Joly *et al.*, 2005). Therefore, DEFB103 may well form part of IFNγ responses in vivo, and the addition of IFNγ to the cytokine cocktail might have been sufficient to mediate hBD3 secretion from pneumocytes. Overall, the concentrations of hBD2 that I observed in cytokine-stimulated pneumocyte cultures was within that ranges reported in other studies. In vitro, AEC and THP-1 cell cultures 48 hours after Mtb infection produced in the range of 10–20 ng/mL of several beta-defensins (Rodriguez-Carlos *et al.*, 2020), whilst another comparable study reported just 100-1000 pg/mL of hBD2 and hBD3 (Marin-Luevano *et al.*, 2021). At most, human primary respiratory epithelial cells secreted approximately 50 ng/mL of hBD2 over 24 hours in response to 100 ng/mL of IL-17 (Kao *et al.*, 2004).

I did not find any evidence of hBD-mediated Mtb growth restriction in the presence of human macrophages. Limited stock availability of recombinant beta-defensin in conjunction with the time remaining for my laboratory work meant that the maximum concentration of rhBD2 I could use for macrophage-infection experiments was 1 μg/mL. At these maximal concentrations I was unable to show any degree of Mtb-growth restriction. Whilst this finding is supported in the case of hBD2 (Reuschl *et al.*, 2017), I have not shown proof-in-principle that hBD-mediated restriction can occur in this model. However, the absence of restriction in this model supports the suggestion that beta-defensins 2 and 3 are unlikely to directly kill Mtb in vivo.

In the absence of macrophages, 2.5 μg/mL of rhBD2 was sufficient to decrease Mtb growth. This finding should translate to the restriction of extracellular Mtb growth even in the presence of macrophages. However, I cannot extrapolate this to internalised Mtb, the killing of which may require even higher concentrations. Curiously, isoniazid did not appear to be as effective against intracellular Mtb as it did against extracellular bacilli. This might indicate the presence of an intracellular macrophage compartment that isoniazid cannot access, within which Mtb is able to survive. The same may be true for the paracrine production of beta-defensins and further limit their antimycobacterial efficacy in vivo.

I convincingly demonstrated that 1 μg/mL of rhBD3 does not restrict Mtb growth in human macrophages, with data indicating a minimum of 2.5 μg/mL is necessary to see modest extracellular reduction in Mtb growth. Previous work found hBD3 had a MIC for Mtb of 24 μg/mL (Corrales-Garcia *et al.*, 2013). Reduced concentrations have been shown to kill other mycobacteria, namely *M. bovis* (Su *et al.*, 2018), but still required a minimum of 10 μg/mL. My data is limited by a lack of statistical significance for hBD3 killing, but suggests that hBD2 is the more lytic AMP regarding Mtb. Here, I have shown the opposite to several studies which found hBD3 to be more lytic than hBD2 (Sahly *et al.*, 2006; Lee and Baek, 2012).

The high throughput flow cytometric assay used here has two main advantages. Firstly, it is less time consuming than counting CFUs. Secondly, it is more accurate, accounting for

clumping of bacteria during growth (Mehta, 2020). It has some limitations; dead bacteria will continue to fluoresce for a given period of time and it is not clear what time duration is needed for fluorescence to be lost. Dead bacteria may contribute to the bacterial load in certain cases, reducing assay sensitivity. Isoniazid demonstrated a clear reduction in load, suggesting the assay is sensitive enough to differentiate substantial growth restriction between conditions. However, there may have been a modest reduction in Mtb growth in the presence of beta-defensin that was missed as a result of fluorescent, dead bacteria. Despite this, the flow cytometric quantitation of Mtb growth in liquid culture was able to differentiate a statistically significant reduction in bacterial growth in the presence of 2.5 µg/mL of rhBD2, a concentration in line with what has been reported elsewhere (Reuschl *et al.*, 2017). My data indicates there is insufficient growth restriction below this concentration.

The four immortalised airway epithelial cell lines had differential rates of cell division, varying from approximately 24 hours for A549 and BEAS-2B lines, to several days for Calu-3s and Detroit 562 lines. I did set up co-culture experiments, with the aforementioned cell lines seeded into transwell inserts, but found that due to the differential growth rates extended culture durations were not possible. Transwell inserts have limited surface area and the AEC lines soon outgrew them. I maintained AEC co-culture for just 24 hours, relying on macrophage-derived cytokines to induce DEFB4 and DEFB103 expression, but found no growth restriction or measurable beta-defensin in supernatants (data not shown). Thus, I chose a more reductionist approach to modelling the impact of beta-defensins on Mtb growth by instead transferring supernatants across from pneumocyte cultures. This removed any impact of differential growth rates between AEC lines, enabled pre-stimulation of AECs with a cocktail of cytokines, and allowed for accurate measurements of the hBD2 concentration in the supernatant without disturbing the Mtb-infected MDM culture.

I might have considered using granulocyte-macrophage colony-stimulating factor (GM-CSF) to differentiate blood monocytes. GM-CSF is essential for alveolar macrophage function in vivo (Shibata *et al.*, 2001) and in vitro (Nakata *et al.*, 1991). However, GM-CSF and M-CSF differentiated macrophages controlled Mtb growth to a similar degree (Khan *et al.*, 2022).

Alternatively, the isolation of human primary alveolar macrophages is feasible (Reuschl *et al.*, 2017) and might be more relevant to modelling the initial stages of Mtb infection.

There are other in vitro models that incorporate both macrophages and polarised epithelial cells, with physiological characteristics such as mucus production (Parasa *et al.*, 2013). In contrast to this model, alveolar pneumocytes were only ever cultured as monolayers without polarisation of the epithelial cell layer. Whilst this presents itself as a limitation, polarised respiratory epithelial cells do not secrete substantially more hBD2 (Kao *et al.*, 2004). It may be that other aspects of the model (i.e. mucus production) concentrates hBD2 in certain areas, enough to restrict Mtb growth. It is unclear whether these characteristics would have any impact on macrophage control of internalised Mtb.

Adding complexity to in vitro models requires careful consideration as it can increase noise and limit effect sizes. In using a reductionist approach, I could address whether beta-defensins directly restrict Mtb growth in the presence of macrophages without additional noise from hBD2 potentially modulating other components in the system. This has demonstrated that the inducible beta-defensin response in alveolar pneumocytes is not capable of augmenting human macrophage control of Mtb.

6. Conclusion and future direction

This thesis focused on addressing a variable, inducible beta-defensin response as a component of IFNγ-independent immunity in TB. I examined what features of the host immune response and underlying genetics contributed towards the variation in expression in the TST, and in which cell types were they expressed. My data supports that during TB, the induction of DEFB4 expression in alveolar pneumocytes is part of an IL-17 driven response. There was a modest contribution of host genetics towards beta-defensin expression, in the form of CNV of the BDRU locus, in line with what has been reported previous (Aldhous, Noble and Satsangi, 2009; James *et al.*, 2018).

I found no direct effect of secreted beta-defensins on macrophage control of Mtb. However, several lines of evidence suggest that they may be involved in modulating protective Th17 immunity (Dijkman *et al.*, 2019; Gideon *et al.*, 2022; Sun *et al.*, 2024). HBD2 is a CCR6 ligand (Yang *et al.*, 1999; Röhrl *et al.*, 2010b) and can mediate the migration of T cells with Th17 potential to sites of inflammation (Yang *et al.*, 1999; Yamazaki *et al.*, 2008; Lyu *et al.*, 2019). HBD2 selectively induced cell migration from as little as 2 ng/mL (Yang *et al.*, 1999), comparable to the concentrations observed in cytokine-stimulated pneumocyte cultures in vitro. Supporting a protective role, *defb4* KO in mice led to worse disease (Peng *et al.*, 2024), with increased Mtb burden, suggesting DEFB4 augments protective immune responses in vivo. However, DEFB4 expression also correlated with the transcriptional abundance of neutrophils in the TST. As a potent neutrophil chemoattractant (Niyonsaba, Ogawa and Nagaoka, 2004), hBD2 may also function to drive pathogenic immunity in TB. Both excessive IL-17 activity (Pollara *et al.*, 2021) and pneumocyte-mediated neutrophil recruitment (Nouailles *et al.*, 2014) have been implicated in TB pathogenesis.

Evidence of a functional immune axis involving IL-17, neutrophils, and beta-defensins exists in vivo. In mice, impairment of IL-17 decreased Defb3 and Defb4 (DEFB103 and DEFB4 orthologs) expression with *C. rodentium* infection (Ishigame *et al.*, 2009), whilst IL-17 overexpression increased neutrophil infiltration (Hurst *et al.*, 2002). IL-17 is also major driver

of psoriasis pathogenesis in humans (Zenobia and Hajishengallis, 2015). Interestingly, BDRU copy number is elevated in psoriatic patients versus healthy volunteers (Hollox, Huffmeier, *et al.*, 2008), supporting a role for beta-defensins in augmenting pathogenic inflammation. Whilst there are several copy number variable genes encoded within the BDRU locus, hBD2 is one of the most abundant proteins found in psoriatic scales (Harder *et al*., 1997). These observations do not necessarily translate to TB. It may be that hBD2 responses have little effect on immune control of Mtb and serve only as a biomarker for disease severity and pathogenic immunity in chronic infection (Bongiovanni *et al.*, 2020; Pollara *et al.*, 2021).

Annotated as AMPs, HBD2 and hBD3 have a wealth of in vitro studies supporting pathogen killing (Ganz, 2003). Yet antimicrobial activity in vitro may not necessarily translate in vivo. Mice lacking *defb4* had increased bacterial burden 30 days after Mtb challenge (Peng *et al.*, 2024), but authors never explored whether this related to direct effects on Mtb or by some other mechanism. Nor is antimicrobial activity a feature unique to AMPs with several chemokines possessing lytic activity (Yang *et al.*, 2003). The concentrations required to restrict Mtb growth (Corrales-Garcia *et al.*, 2013; Reuschl *et al.*, 2017), or indeed the growth of several other bacterial pathogens (Sahly *et al.*, 2006; Lee and Baek, 2012), are considerably greater than the concentrations at which they chemoattract several immune cell types (Yang *et al.*, 1999; Niyonsaba, Ogawa and Nagaoka, 2004; Röhrl *et al.*, 2010a; Kim, Yang and Jang, 2019). The MIC of beta-defensins in vitro is also influenced by factors such as the starting concentration of the pathogen. Lower AMP concentrations may be sufficient to restrict a small number of Mtb in vivo thereby protecting the host against new infection, but such experiments are challenging due to the small numbers of bacteria required. It is interesting to consider how hBD2 might restrict Mtb growth given the known complexity of the mycobacterial cell envelope and wall (Jackson, 2014; Jankute *et al.*, 2015). There is evidence that hBD2 accumulates on the Mtb cell wall but it is unclear whether it forms pores to mediate killing (Rivas-Santiago *et al.*, 2005). Human alpha-defensin 1, which has a comparable MIC for Mtb, has been shown to interact with and disrupt Mtb DNA (Gera and Lichtenstein, 1991; Sharma and Khuller, 2001).

When evaluating the antimycobacterial activity of hBD2 and hBD3 in vitro, a key question remains; what is a physiologically relevant concentration of beta-defensin in the lung? Several studies have measured beta-defensin protein concentrations in BAL fluid. In human mycobacterial infections, including pediatric TB, the concentration of hBD2 in BAL fluid reached just 7 ng/mL (Ashitani *et al.*, 2001; Cakir *et al.*, 2014), well below that required for antimicrobial activity. Substantially higher concentrations of alpha-defensin have been found in the BAL of adults with active TB, at approximately 1.5 µg/mL, but it is important to stress that this concentration reflects the sum of human alpha-defensins 1, 2, and 3 (Ashitani *et al.*, 2002). Across other lung diseases individual beta-defensin peptides do not appear to reach concentrations above a few ng/mL (Mukae *et al.*, 2007; Yanagi *et al.*, 2007; Harimurti *et al.*, 2011). The highest concentration of hBD2 in BAL is found in patients with cystic fibrosis (Chen *et al.*, 2004). Here, hBD2 was found at a maximum of approximately 6.2 µg/mL, but it worth noting that this may relate to the impaired mucus clearance typical of cystic fibrosis (Morrison, Markovetz and Ehre, 2019).

For beta-defensins to be lytic in vivo they may require concentration within specific physiological compartments, such as an enrichment in mucus or the phagosome (Rivas-Santiago2008). There is evidence that this occurs with the human alpha-defensins in neutrophil granules (Ganz *et al.*, 1985). Additionally, localised secretions of alpha-defensins in the gut are estimated to reach 100 mg/mL (Ayabe *et al.*, 2000). HBD2 is found in psoriatic lesions at a concentration sufficient to mediate antimicrobial killing (Ong *et al.*, 2002). The excessive IL-17 activity in psoriasis (Ghoreschi *et al.*, 2021) presumably drives hBD2 secretion from keratinocytes resulting in such high concentrations. However, whether these localised enrichments occur during human Mtb infection in the lung remains unanswered.

Beta-defensins have been considered to function in vivo as alarmins (Oppenheim and Yang, 2005), both augmenting innate immune responses and actively driving cell recruitment to sites of inflammation. Future studies will need to address the potential immunomodulatory effector functions of beta-defensins and their implication in TB pathogenesis. Animal models of TB will be more suited to dissecting this in vivo role due to the complexity of orchestrating

responses of several immune cell types in tissue, including cell recruitment difficult to model in vitro.

To conclude, hBD2 and hBD3 are epithelial cell-derived AMPs, induced during anti-Mtb immune responses in vivo. Both are key beta-defensins in the skin (Dong *et al*., 2022) and hence contribute to immune responses captured in the TST. In the lung, hBD2 but not hBD3 is secreted by alveolar pneumocytes and appears unlikely to contribute directly towards early Mtb growth restriction by macrophages due to insufficient localised concentrations. However, given their chemotactic activity at minimal concentrations, future studies of beta-defensins should focus on their participation in IL-17-driven immunity and their role in mediating cellular recruitment to the site of disease.

7. References

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8. Appendix

My work addressing the role of variable beta-defensin expression during anti-Mtb immune responses was disrupted by the Covid-19 pandemic and led to several years during which substantial research time was dedicated to Covid-19-related projects. These focused on addressing transcriptional biomarkers of acute viral infection, in an observational cohort of highly exposed healthcare workers from the first week of lockdown in the UK, and in a controlled human infection model (CHIM) of SARS-CoV-2 infection. This additional work directly resulted in the form of three publications (Gupta *et al.*, 2021; Chandran *et al.*, 2022; Rosenheim *et al.*, 2023), two of which are first author publications. Further details on my specific work in these projects is summarised below.

From the first week of the UK national lockdown in March 2020, I had access to peripheral blood RNA samples from a cohort of healthcare professionals working in London based hospitals. Blood RNA had been sampled weekly, where possible, alongside PCR-based laboratory testing for contemporaneous SARS-CoV-2 infection. I processed 169 blood RNA samples in the laboratory to prepare them for RNA-seq. These comprised of available RNA samples within three weeks of the first positive PCR test from those with PCR-confirmed infection, a sample 24 weeks post infection (convalescent), and baseline blood RNA samples from a control group with no PCR-confirmed infection. Following the generation of this dataset, I worked closely with Rishi Gupta (University College London) to learn how to perform bioinformatic analysis that evaluates the sensitivity and specificity of blood transcriptional biomarkers. Following a systematic literature search performed by Rishi Gupta and Lucy Bell (University College London), 20 distinct transcriptional signatures for viral infection were identified. Despite moderate to strong Spearman rank correlation between the expression of all signatures, indicative of shared type I IFN regulation of constituent genes in each, a single gene, IFI27, was found to have superior accuracy for discriminating contemporaneous nasopharynx geal PCR-positive SARS-CoV-2 infection from test-negative controls (Table 1). Crucially, this biomarker performed well irrespective of

symptoms. These findings supported further evaluation of blood *IFI27* transcripts as a biomarker for early phase SARS-CoV-2 infection, and its use for screening individuals at high risk of infection, such as contacts of index cases, to facilitate early case isolation and early use of antiviral treatments as they emerge. I wrote this manuscript together with Rishi Gupta and Lucy Bell which was published in The Lancet Microbe in 2021 (Gupta *et al.*, 2021), with shared first authorship.

Table 1. Validation metrics of whole-blood RNA signatures for discrimination of participants with PCR-confirmed SARS-CoV-2 infection at first week of PCR positivity

	AUROC	**Sensitivity**	**Specificity**	**Adjusted p value**
IFI27	0·95 (0·91–0·99)	0·84 (0·70–0·93)	0·95 (0·85–0·98)	..
Sweeney7	0·95 (0·91–0·99)	0·82 (0·67–0·91)	0·95 (0·85–0·98)	0·85
Zaas48	0·93 (0·88–0·98)	0·61 (0·45–0·74)	0·95 (0·85–0·98)	0·088
Pennisi2	0·91 (0·86–0·96)	0·58 (0·42–0·72)	0·95 (0·85–0·98)	0·088
IFI44L	0·90 (0·84–0·96)	0·55 (0·40–0·70)	0·95 (0·85–0·98)	0·039
AndresTerre11	0·89 (0·83–0·95)	0·55 (0·40–0·70)	0·95 (0·85–0·98)	0·021
Henrickson16	0·89 (0·82–0·96)	0·55 (0·40–0·70)	0·93 (0·83–0·97)	0·0093
TrouilletAssant6	0·87 (0·80–0·94)	0·53 (0·37–0·68)	0·93 (0·83–0·97)	0·008
Lydon15	0·86 (0·79–0·94)	0·58 (0·42–0·72)	0·95 (0·85–0·98)	0·0046
Herberg2	0·84 (0·76–0·92)	0·5 (0·35–0·65)	0·93 (0·83–0·97)	0·0034
Sampson4	0·84 (0·76–0·92)	0·5 (0·35–0·65)	0·93 (0·83–0·97)	0·0027
Sampson10	0·83 (0·74–0·92)	0·5 (0·35–0·65)	0·95 (0·85–0·98)	0·0021
RSAD2	0·83 (0·74–0·91)	0·47 (0·32–0·63)	0·93 (0·83–0·97)	0·0021
MX1	0·82 (0·74–0·91)	0·45 (0·30–0·60)	0·95 (0·85–0·98)	0·0017
Tsalik33	0·79 (0·70–0·89)	0·39 (0·26–0·55)	0·98 (0·9–1·0)	0·0011
Lopez7	0·79 (0·69–0·88)	0·37 (0·23–0·53)	0·98 (0·9–1·0)	0·00080

IFIT3	0·75 (0·64–0·86)	0·45 (0·30–0·60)	0·93 (0·83–0·97)	0·00027
OLFM4	0·62 (0·51–0·74)	0·03 (0·0–0·13)	0·98 (0·9–1·0)	<0·0001
Sweeney11	0·60 (0·48–0·73)	0·16 (0·07–0·30)	0·96 (0·88–0·99)	<0·0001
Yu3	0·59 (0·47–0·71)	0·05 (0·01–0·17)	1 (0·93–1·0)	<0·0001

Data are point estimates (95% CIs). Includes 38 contemporaneous SARS-CoV-2-positive samples and 55 SARS-CoV-2-negative samples. Discrimination is shown as area under the receiver operating characteristic curve (AUROC). Sensitivity and specificity are shown using predefined thresholds of 2 SDs above the mean of the uninfected control population (Z2). p values show pairwise comparisons to best performing signature with Benjamini-Hochberg adjustment (false discovery rate 0·05). Equivalent data for discrimination between test-negative controls and participants with SARS-CoV-2 infection 1 week before positive PCR test are in appendix 1 (p 7).

In addition, the laboratory work I had done on this project led to another related publication in Cell Reports Medicine (Chandran *et al.*, 2022). This publication described the temporal kinetics and relationships between the earliest immune responses to infection with an unbiased systems-level approach using the same dataset I had generated from healthcare workers sampled weekly before, during, and after incident SARS-CoV-2 infections during the first epidemic wave in London. Differentially expressed transcripts were identified by comparison of profiles from the time of first positive viral PCR to those of uninfected controls, and using upstream regulator enrichment analysis to identify pathways responsible for this differential gene expression, found rapid type I IFN and virus-specific T cell responses to infection that precede the appearance of virus-specific antibodies.

I subsequently became involved in a study of CHIM for acute SARS-CoV-2 infection. This provided the perfect opportunity to expand upon the previously identified blood transcriptional biomarkers for acute respiratory viral infection (Gupta *et al.*, 2021). Diagnostic tests for SARS-CoV-2 virus are widely available and formed an essential part of the response strategy to the COVID-19 pandemic, such as PCR for viral nucleic acids. However, such tests remain limited in the early and late stages of an infection. One of the major limitations of our previous publication on viral biomarkers (Gupta *et al.*, 2021) had been the uncertainty around the timepoint of incident infection, which limited interpretations of the temporal dynamics for the identified transcriptional biomarkers. As before, I processed several hundred blood RNA samples in the laboratory to prepare them for RNA-seq. I then processed the RNA-seq data and analysed this dataset to assess the sensitivity and specificity of blood transcriptional biomarkers. Full details of the methods involved in generating the dataset in this studied are detailed here (Rosenheim *et al.*, 2023). In brief, blood RNA for sequencing was isolated from whole blood samples collected whilst RNA was extracted from SARS-CoV-2 challenge nasopharyngeal swabs and curettage samples. I led the laboratory processing and RNA-sequencing of the 374 blood samples and 96 nose swabs used in this analysis.

I classified replicative infection as occurring in individuals with evidence of increasing viral load and consecutive positive PCR-tests. Among the 36 participants, 18 developed a

replicative infection. 2 participants who did not develop a replicative infection became seropositive for SARS-CoV-2-specific antibodies between the time of recruitment and the initiation of the study. These two participants were excluded from the analysis.

As had been previously revealed (Gupta *et al.*, 2021), all of the blood transcriptional signatures could discriminate replicative from non-replicative SARS-CoV-2 infection, measured by AUROC. I found that most signatures provided perfect discrimination of replicative infection from days 4 to 10 (Figure 8.1), with variation between signatures occurring either side of this time period. I noticed that the temporal kinetics of AUROCs appeared a dichotomise signatures; those that discriminate well early on following inoculation perform less well in the latter phase of infection, and vice versa. I identified two single gene biomarkers that discriminated this early and late phase type I IFN response to acute SARS-CoV-2 infection (Figure 8.2). Whilst both genes are recognised as IFN-stimulated genes, IFI27, but not MX1, appeared to be subject to differential transcriptional regulation exclusively in myeloid cells of peripheral blood. The delayed and sustained upregulation of IFI27 extended well beyond the peak of viral titres in the nose. In contrast, MX1 expression tracked nose viral load and virus culture positivity more tightly (Rosenheim *et al.*, 2023).

We tested the generalisability of our findings to see whether this early and late type 1 IFN-induced immunity was a feature of the immune response to SARS-CoV-2 infection. We explored MX1 and IFI27 expression in publicly available longitudinal blood transcriptomic data from other human challenge studies involving influenza, human rhinovirus, and respiratory syncytial virus. Despite these experiments being limited to only 6 days of sampling, it was apparent that across all respiratory viral infections the expression of MX1 peaked early and preceded IFI27 upregulation (Rosenheim *et al.*, 2023). This suggested that two distinct waves of type 1 IFN-inducible immunity are key features of the immune response to acute respiratory viral infection. Combined use of both biomarkers offer the ability to differentiate stages of acute viral infection in contacts of an index case or among triage patients in hospitals. I wrote this manuscript, in revision with Nature Communications, whilst a preprint of this work is available on medRxiv (Rosenheim *et al.*, 2023).

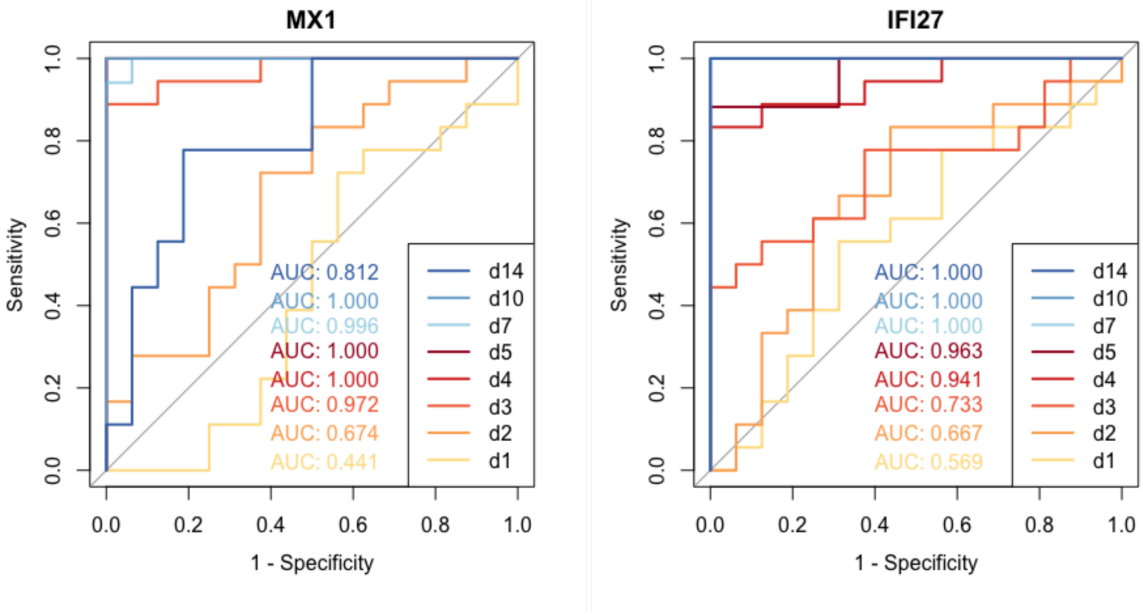

Figure 8.1. **Receiver operating characteristic curve illustrating the top performing single gene biomarkers.** Receiver operating characteristic curve (AUROC) for MX1 and IFI27 genes when discriminating replicative (N=18) from non-replicative infection (N=16), stratified by time after virus challenge (d = study day).

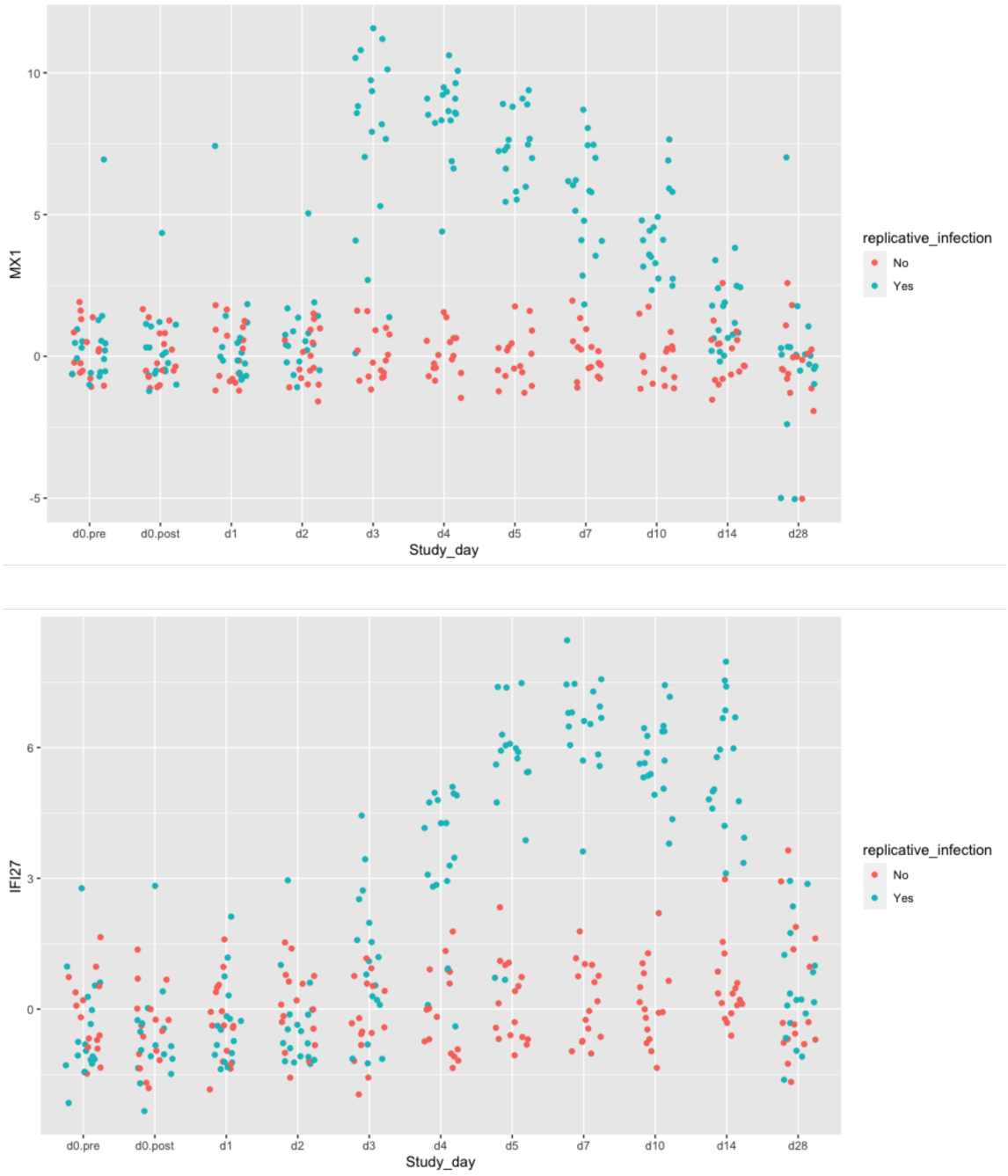

Figure 8.2. **Signature scores over time for the top performing single gene biomarkers.** Individual signature scores (y-axis) for MX1 and IFI27 from each participant over time (x=axis). Participants are classified into two groups, replicative (N=18) and non-replicative (N=16) infection. The time course begins on the same day as inoculation, with the first sample (d0.pre) taken hours prior to inoculation, and the second sample on that day (d0.post) taken hours after inoculation (d = study day).