The role of antibodies in tuberculosis

by

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Dedication

For Mom, Dad, and Caron.

DECLARATION

I, *Ashley Jacobs*, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Date: 13/11/23

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Onwards.

Abstract

The role of antibodies in TB has been debated for over a century. Antibodies against *Mycobacterium tuberculosis* (*M.tb*) are detectable in persons who appear to resist *M.tb* infection, and yet humoral immune activation is a consistent biomarker of TB disease progression. Antibody responses to TB could therefore be a dual-edged sword, where antibodies promote TB pathogenesis, but some people produce antibodies that restrict the growth of *M.tb*. I therefore investigated the role of antibodies in TB in multiple clinical cohorts.

Firstly, I show the generation of fully human mAbs against *M.tb* from patients with ATB. I identify the antigen of one mAb as the secreted antigen GlnA1, and then show that this mAb promotes *in vitro* production of TNF-α, IL-6, and IL-1β in the absence of mycobacterial killing.

Secondly, I investigate antibody responses against *M.tb* in persons who live with HIV (PLWH). PLWH fail to mount a significant increase in IgG levels against *M.tb* in ATB. *M.tb*-specific IgG responses are also detectable in different cohorts of PLWH with negative interferon gamma release assay (IGRA) tests. However, greater levels of *M.tb*-specific IgG associates with IGRA conversion.

Thirdly, I show the development of flow cytometric assays to study opsonization and antibody-dependant cellular phagocytosis (ADCP) of live mycobacteria in BCG vaccinated adults. These methods show that BCG vaccination induces ADCP 28 days post-vaccination.

Fourthly, I test whether unswitched memory B cells, and specifically the marginal zone B cells (MZB) subset, are impacted by ATB. MZB respond to capsular pathogens and could thus recognize the *M.tb* cell wall. MZB are found to be depleted in ATB but are not enriched at the site of disease in PLWH with pericardial TB. The depletion of unswitched memory B cells in TB observed in this study resembles that reported in autoimmune disease.

Taken together, I provide support to the notion that at least some antibodies against *M.tb* could contribute to immunopathology in TB. The fact that PLWH are at greater risk of disseminated TB, and mount less of an antibody response could support a role for antibodies in preventing disseminated disease that remains to be studied.

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List of abbreviations

TMB	3,3',5,5'-tetramethylbenzidine
ATB	Active TB
ADC	Albumin dextrose catalase
APC	Allophycocyanin
APC-Cy	Allophycocyanin-Cyanine
Acr	Alpha-crystallin
AM	alveolar macrophages
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
Ag85A	Antigen 85A
ART	Antiretroviral therapy
BCG	Bacille Calmette-Guérin
BAFF	B-cell activating factor
BAL	Bronchoalveolar Lavage
CIC	Circulating immune complexes
CFP-10	Culture Filtrate Protein 10
CFU	Culture forming units
DMEM	Dulbecco's Modified Eagle Medium
ESAT-6	Early Secreted Antigenic Target 6 kDa
ESAT-6	Early Secretory Antigenic Target 6
ELISA	Enzyme-Linked Immunosorbent Assay
E6/C10	ESAT-6/CFP-10
FcR	Fc Receptor
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
FMO	Fluorescence Minus One
F(ab)	Fragment antigen binding
Fc	Fragment crystallisable
GlnA1	Glutamine synthetase 1
VH	Heavy chain variable region
HBHA	Heparin binding hemagglutinin
HIV	Human Immunodeficiency Virus
IgG	Immunoglobulin G

IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
IGRA	Interferon-gamma Release Assay
IL	Interleukin
LTBI	Latent tuberculosis Infection
VL	Light chain variable region
LAM	Lipoarabinomannan
MZB	Marginal Zone B Cell
MFI	Mean Fluorescence Intensity
MVA85A	Modified vaccinia Ankara-expressing Ag85A
mAb	Monoclonal antibody
MDM	Monocyte derived macrophages
M.tb	*Mycobacterium tuberculosis*
OADC	Oleic albumin dextrose catalase
OD	Optical Density
PFA	Paraformaldehyde
PFC	Pericardial fluid cells
PerCP-Cy	Peridin Chlorophyll Protein-Cyanine
PBMC	Peripheral Blood Mononuclear Cells
PLWH	Persons living with HIV
PMA	Phorbol myristate acetate
PBS	Phosphate buffered saline
Psts1	phosphate-binding transporter lipoprotein
PE	Phycoerythrin
PEG	Polyethylene glycol
PEI	Polyethylenimine
PCR	Polymerase chain reaction
QFT	Quantiferon® Gold In-Tube assay
RD	Region of difference
RT	Room temperature
scFv	Single-chain variable fragments
TB	Tuberculosis
TNF-α	Tumor necrosis factor alpha
WCL	Whole Cell Lysate

Chapter 1: The role of antibodies in tuberculosis

1.1 Introduction to TB

1.1.1 Introduction

A decade after the discovery of *Mycobacterium tuberculosis (M.tb)* by Robert Koch in 1882 the first evidence of the existence of disease neutralizing agents or 'antikörper' in serum emerged. It was inevitable that this newly identified form of immunity would be tested against tuberculosis (TB), at that time a major cause of death in Europe. In fact, much of the first characterization of antibodies was performed at the Robert Koch Institute.

The first antibody-based diagnostic tests for TB were described in 1898, and decades of curative efforts followed where people suffering from TB were transfused serum obtained from goats, sheep, rabbits, or horses inoculated with *M.tb.* Although some studies showed a beneficial effect of serum therapy, many did not, and no standardized protocol for its usage was ever designed. Staunch advocacy against serum therapy by Calmette and Guerin, the inventors of the BCG vaccine, led to the cessation of serum therapy trials in the late 1920s. Subsequent studies of antibody responses in humans showed that although antibodies against TB were detectable in most TB patients, the magnitude and specificity of serological responses varied too widely to be of diagnostic utility. In contrast, the loss of $CD4^+$ T cells in both humans and animal models was strongly implicated protection against risk of developing TB. There have therefore been decades of under-exploration into the role of antibodies in human TB that has only recently begun to reverse.

The drivers of this resurgence of interest into the role of antibodies in TB are multifactorial. A vaccine candidate designed to target cellular immunity have not shown efficacy in clinical trial. Both the human 'resister' phenotype and non-human primates with sterilizing immunity have been discovered to possess unique antibody profiles. Better tools to characterize antibodies, both at scale and at a monoclonal (mAb) level, have also revealed fresh insights into protection against TB. However, novel transcriptomic signatures for the prediction and diagnosis of TB now consistently associate humoral immunity with disease progression. Understanding what role antibodies play against *M.tb* is therefore essential to understanding both the pathogenesis and prevention of TB.

1.1.2 Epidemiology

Tuberculosis (TB) has afflicted humans for millennia and is the 10th leading cause of death worldwide. In 2007, TB exceeded human immunodeficiency virus (HIV) as the leading cause of death from a single infectious agent (1). This was unchanged until the COVID-19 pandemic, and yet even still in the year 2020 ~1.3 million people succumbed to TB. The global incidence of TB has been relatively stable for the past decade, with approximately 10 million people developing active TB per year **(**Figure 1.1**)** (1).

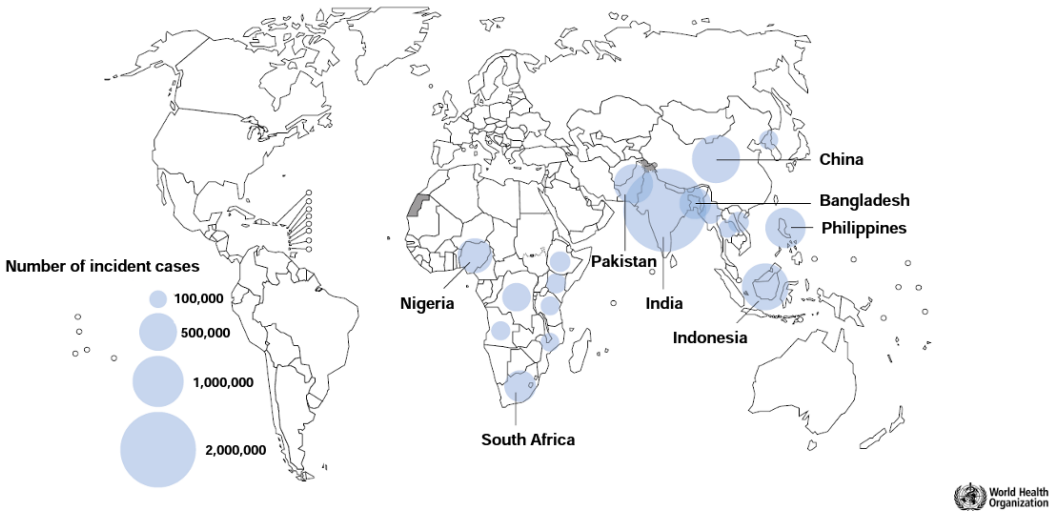

Figure 1.1 Global incidence of TB in 2020 for countries with at least 100 000 incident cases (2020) (1).

The causative agent, *Mycobacterium tuberculosis (M.tb)*, is predominantly spread by aerosol production when coughed out by individuals with respiratory TB. Approximately 1.7 billion people globally are estimated to be infected with *M.tb* in an asymptomatic and clinically quiescent state known as latent TB infection (LTBI). In this state, the WHO estimates the lifetime risk for developing TB to be between 5 and 10% (2). This estimate was derived from studies spanning several decades in which the presence of a delayed hypersensitivity reaction to a subcutaneous injection of *M.tb* antigens known as tuberculin skin testing (TST) is interpreted as indirect evidence of latent infection with *M.tb* (3).

The Bacille Calmette- Guérin vaccine (BCG) is the only licensed vaccine available for TB and has been employed globally since its first use in 1921 (4). The principal benefit of BCG vaccination is the prevention of disseminated forms of TB in infants that carry high morbidity and mortality (5). The efficacy of BCG in preventing active TB in adults is far more variable, with estimates ranging from 0% to 63% across geographical regions (6). Re-vaccination with BCG in adults could thus be seen as reasonable to boost waning immune response, but when trialled this approach did not improve the ability of BCG to protect adults from developing TB (7). The global transmission of TB has therefore not been strongly impacted by vaccination.

1.1.3 The spectrum of human TB disease

The clinical manifestations of *M.tb* disease are protean and can affect any organ system (8). Active TB's (ATB) typical presentation, however, is that of a chronic respiratory disease with cough and constitutional symptoms such as malaise, drenching night sweats, unintentional weight loss and subjective fevers (8). A diagnosis of TB is made by identifying the presence of *M.tb* in sputum or other clinical specimens by culture, nucleic acid amplification assays, microscopy, or detection of cell wall components by lateral flow device in urine (8). In the pre-antibiotic era, untreated TB carried an estimated mortality of 50% (9). Sequelae of ATB includes, but is not limited to, haemoptysis, pleural effusions, pericardial effusions, and respiratory failure (8). TB meningitis is another severe form of disease that carries significant risk of neurological impairment and death (10,11). Disseminated TB with mycobacteremia can occur in profoundly immunocompromised patients with HIV, with in-hospital mortality rates as high as ~one-third of patients (12). Despite microbiological cure, TB is also associated with long-term morbidity from both social factors and permanent impairment of lung function (13).

Yet most people (~90%) can control the replication of *M.tb* after inhaling aerosol droplets containing bacilli (1). The classical model of TB disease presumes that infection remains in a quiescent state known as LTBI unless there is a loss of immune control but can progress to ATB as post-primary TB or later reactivate due to loss of immune control (14).

A two-state paradigm of LTBI or ATB was described for many decades, despite early observation from screening programs that asymptomatic individuals could harbour culturable bacilli in sputum, or display chest x-ray pathology compatible with ATB (15). This model has been replaced by an understanding that symptomatic TB is preceded by a phase of increased bacillary replication known as incipient TB, followed by subclinical host pathology in which the presence of *M.tb* is identifiable but symptoms have not yet developed (16). Modern studies using TB GeneXpert show that individuals may be completely asymptomatic but have detectable *M.tb* DNA in induced sputum samples or radiographic changes consistent with TB (16). The gain in resolution afforded by positron emission tomography combined with computerized tomography (PET/CT) and RNA transcript signatures have allowed subclinical TB to be better defined in terms of the combined expression of inflammatory pathways as per ATB, with metabolically active pulmonary lesions (17). A study by Esmail *et al* in HIV-1 infected adults showed that individuals with subclinical TB so defined were more likely to develop symptoms and culture positive ATB within 6 months of clinical follow-up (17). Further, a transcriptomic signature study conducted in Worcester, South Africa, suggested that a 16-gene signature emerges months prior to the presentation of symptomatic TB (18).

Thus, it is likely that TB disease occurs on a spectrum **(**Figure 1.2**)**. Conversely, the notion that certain individuals could potentially be resistant to TB and clear the initial inoculum of *M.tb* is well supported

by epidemiological studies. Only 50% of close household contacts develop positive IGRA or TST tests, and negative TST tests despite exposure to *M.tb* may occur in approximately 5-10% of people (reviewed by Moller *et al*, 2018) (19).

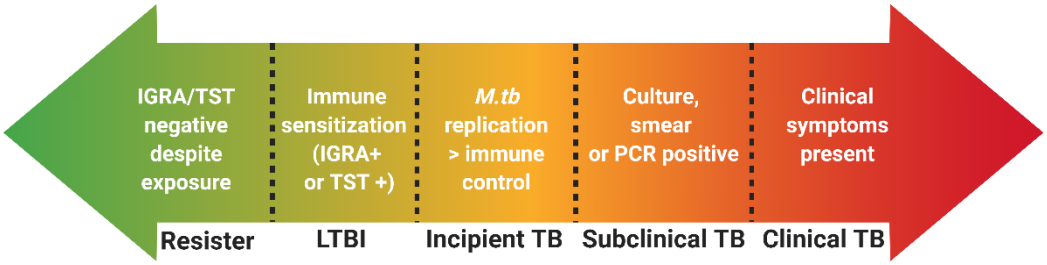

Figure 1.2 Schematic of the clinical stages of TB. Created by Biorender.com

A consensus has been sought in how best to classify patients as 'resisters' who maintain undetectable immunological assays or evidence of ATB despite definitive exposure to *M.tb* (20) Resisters must have evidence of (a) exposure to *M.tb* (b) A negative TST and IGRA test and (b) longitudinal data that this response is sustained in the absence of progression to LTBI or ATB (20). More recently, cohorts in Uganda have determined both genetic and immunological features of this phenotype, and further investigation into such individuals are likely to be critical to understanding protective immunity against *M.tb* (20,21).

Antitubercular therapy (ATT) options for drug-sensitive ATB have not improved for several decades. Combination therapy with rifampicin, isoniazid (INH), ethambutol, and pyrazinamide (RHZE) is effective at curing TB when given for a 2-month period followed by 4 months of rifampicin and isoniazid. A shorter regimen with comparable cure rates has only been described but has not been implemented (22).

1.1.4 The causative agent - *Mycobacterium tuberculosis*

As an ancient pathogen with millennia of co-evolution with humans, *M.tb* is exquisitely adapted to surviving within its host (23). Robert Koch first described the aetiology of TB in 1882, with Ziehl and Neelsen developing the characteristic acid staining of mycobacteria the following year (24,25). The slow growth (doubling in ~24hrs) of *M.tb* in culture, combined with a layer of acid-fast lipids imply a unique biology with implications for pathogenesis.

The first complete sequencing of the *M.tb* in the reference strain H37Rv identified approximately 4000 genes, with ~3906 encoding for proteins, and ~250 involved in lipid metabolism (26).

Modern studies have analysed the components of the mycobacterial cell wall as a bilayer consisting of an inner layer of mycolic acids linked to arabinogalactan and an outer layer of glycolipids and non-covalently bonded lipids **(**Figure 1.3**)** (27). This lipid layer has profound effects on how *M.tb* interacts with the host immune system, and also limits drug penetration of many antibiotics (27).

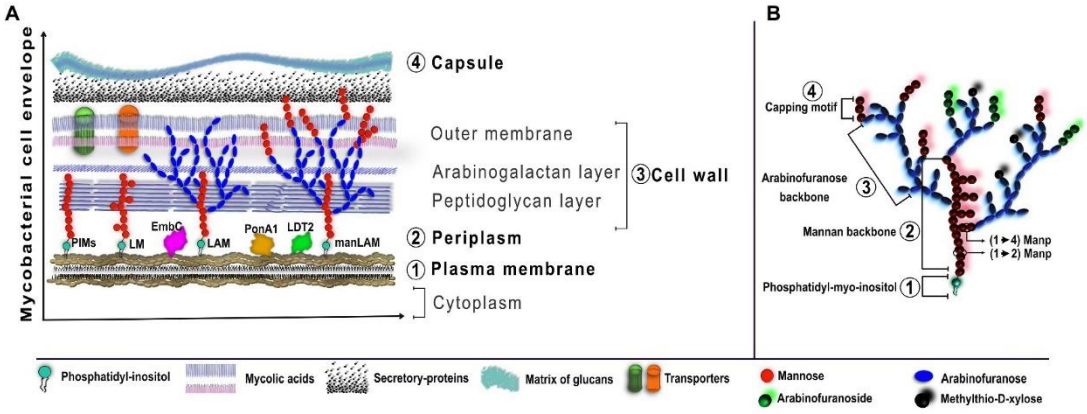

Figure 1.3 (A) The layers of the cell envelope of *M.tb*. **(B)** The structure of LAM, a major envelope glycoprotein in *M.tb* (28).

An early observation of *in vitro* cultures of *M.tb* was the accumulation of proteins secreted or shed by dead bacteria that appeared to be highly immunogenic, so-called culture filtrate proteins (CFP) (29). To engage with the extracellular environment, mycobacteria employ a unique type 7 secretion system to shuttle proteins across the hydrophobic lipid layer (30). The ESX-1 secretion system is required for full virulence of *M.tb*, facilitating the translocation of bacilli from host phagosomes into the cytosol (31). Early secretory antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) are two such proteins transported by ESX-1 that are encoded by a region of the genome deleted in *M. bovis* BCG (32). As such, detection of immune sensitization to these antigens forms the basis of IGRA testing (33,34).

As an intracellular pathogen, *M.tb* are able to persist under hypoxic and nutrient deprived conditions as might occur post macrophage internalisation (35). The metabolic adaptations of *M.tb* are extensive and involve systems to dynamically sequester of iron, carbon sources, and vitamins during infection (36).Thus, any function of antibodies against TB must contend with the unique biology of the mycobacterial cell wall, secreted antigens, and the ability for the pathogen to survive inside host immune cells.

1.1.5 Immunology of TB

1.1.5.1 Innate immunity

Natural immunity against *M.tb* must exist, as the majority of infected people do not go on to develop ATB (4). The risk for reactivation disease is highest in patients with a range of conditions resulting in impaired immunity (HIV, alcoholism, type 2 diabetes mellitus, renal failure, TNF-α inhibitors), showing that prior control of bacilli was likely mediated by host immune factors (40). Pre-antibiotic era autopsy series showed the presence of viable bacteria in granulomatous lesions in individuals who had not been ill with TB (41). Lesions containing caseous material were more likely to contain *M.tb* than healed calcified granulomas, and these bacilli were able to cause disease when inoculated into guinea pigs (41). More interestingly, most lesions macroscopically typical of TB on autopsy were sterile, whereas only 1 out of 68 contained viable bacteria (42). The non-human primate model confirms this finding, with granulomas within the same macaque varying from being sterile to multibacillary (43). It's therefore clear that the host immune system is able to contain and kill *M.tb*, but the immune factors determining the outcome of infection are incompletely understood (44).

M.tb is recognized by a range of pattern recognition receptors including toll-like receptors, Nod-like receptor, and C-type lectin receptors (45). A complex interplay exists between *M.tb* and host cells post phagocytosis or invasion, resulting in either necrosis or apoptosis (46). In the lung, alveolar epithelial cells (AECs) can become infected with *M.tb*, but alveolar macrophages (AM) are the principal cells initially involved in immunity against TB (46). However, *M.tb* can also replicate in the caseous centre of granulomas, the surface of cavities, as well as in non-phagocytic cells. The precise determinants of the fate of *M.tb* phagocytosed by AM are still being mapped, but phenotype (M1 vs M2), metabolic state and host genetics are all important (47).

1.1.5.2 T cells in TB

Cellular immunity elicited by $CD4^+$ T cells is the best understood component of adaptive immunity against *M.tb* (44). A tripoint argument for the necessity of cellular immunity in TB has been firmly established. Firstly, genetic investigation of humans with susceptibility to unusually severe or recurrent mycobacterial infection (Mendelian Susceptibility to Mycobacterial Disease), loss-of-function of interferon- γ and both its upstream (IL-12) and downstream mediators (STAT-1) have been described (45). Secondly, the global HIV epidemic has clearly demonstrated that declining numbers of $CD4^+$ T cells in humans greatly increases risk for developing active TB. Moreso, lower $CD4^+$ T cell counts also associate with risk of extrapulmonary TB and disseminated infection, including mycobacteremia (49,50). Thirdly, a series of key mechanistic experiments showed that mice with knock-out of IFN-γ, TNF-α, IL-12, STAT1, and CD4 were exquisitely sensitive to mycobacterial disease (51–53)

Despite a clear accumulation of evidence that $CD4^+$ cells are a critical component of immunity against *M.tb*, a vaccine designed to boost this response was unable to add to the protection provided by BCG in infants (54,55). The Modified vaccinia Ankara-expressing Ag85A (MVA85A) vaccine was rationally designed to stimulate polyfunctional T cells (IFN-γ, TNF-α, and IL-2 expressing) against an immunodominant antigen (Ag85A) but did not prevent active TB in infants and HIV-1 infected adults (54,55). Further, T cell epitopes may be evolutionarily conserved in *M.tb*, suggesting that recognition of these epitopes does not exert selection pressure on the organism (56). In addition, uncontrolled T cell activation has been shown to lead to atypically severe and fatal TB in individuals receiving PD-1 inhibitor immunotherapy (57). Understanding how immunity against *M.tb* is orchestrated beyond $CD4^+$ T cells is therefore of crucial importance (44). Investigation of early infection events and mucosal immunity have identified roles for innate immunity, neutrophils, and a range of lymphocyte subsets such as mucosal-associated invariant T cells (MAIT) (20).

1.1.5.3 B cells in human TB

Less is known about the role of B cells in TB, but aggregates of B cells are readily identified around granulomas, and antigen-specific B cells are detectable in the peripheral circulation of TB patients (58,59). There have been conflicting studies of how B cell knock-out mice respond to *M.tb*, with extensive debate as to what can be inferred about human TB from these experiments (57,58).

The composition of the peripheral B cell compartment is widely perturbed in ATB. Expansion of atypical and switched memory B cells, as well as reductions in circulating unswitched memory B cells have been reported (59). B cells in both LTBI and ATB express less markers of proliferation, IL-6, IL-10, and are less able to produce antibodies upon *in vitro* stimulation (56).

More recent studies have shown a favourable role for B cells in TB. When B cells were depleted in macaques using an anti-CD20 mAb, granulomas displayed poor cellular organization and trended towards higher CFU counts (60). Most recently the induction of B cells via mucosal exposure to non-tubercular mycobacteria was protective against *M.tb* challenge in mice (61). Here, B cell aggregates and germinal centre reactions was strongly associated with reduced bacillary burden.

B cells also contribute to immunoregulation and antigen presentation, functions that may influence the fate of *M.tb*-infected granulomas (62). For example, B regulatory cells isolated from patients with ATB suppressed the production of T cell IL-22 in *in vitro* co-culture (63). More recently, *ex vivo* analysis of B cells from patients with pleural TB showed that B cells at the site of disease expressed greater amounts of IFN-β than B cells isolated from peripheral blood (64). This type 1 interferon signal polarized macrophages towards an anti-inflammatory phenotype and associated with increased *M.tb* burden in a mouse model (64). Depletion of B cells via antibody in NHP, or knock-out of the B cell transcription factor BCL6 in mice hampers the accumulation of T follicular helper cells in granuloma-related

lymphoid tissue and results in impaired control of *M.tb* infection (65). These recent studies again give contradictory outcomes towards the function of B cells in TB.

Both lung immunohistochemistry of human samples and ELISA on lung tissue of NHP have shown the presence of B cells surrounding the granuloma (60,62). There are likely also spatial considerations with where human B cells interact with *M.tb* that would be challenging to recapitulate in animal models. Three-dimensional reconstruction of human lung tissue from patients with ATB showed that $CD20^{+}$ were enriched in areas of lung where necrotic debris and mycobacteria had spread into a bronchus ie. a larger airway (66). A similar cohort characterizing necrotic and non-necrotic granulomas in resected human lung tissue from patients with a poor clinical response to ATB treatment showed that non-necrotizing granulomas were enriched for B cells (66). In contrast, necrotizing granulomas associate with macrophage predominance (67).

B cells are therefore clearly a component of the human response to TB at the site of disease. These cells express a range of cytokines as described above that impact the fate of granulomas through mechanisms yet to be fully defined. However, pathogen-specific adaptive immunity by B cells is mediated by the expression of antibodies, and this function of B cells will be discussed next.

1.2 Overview of antibody biology

1.2.1 Introduction to antibodies

In 1890, Behring and Kitasato designed experiments to show that the protein fraction of blood contained a factor capable of neutralizing diphtheria and tetanus toxins (68). Rabbits were inoculated with diphtheria, and the transfer of serum from these animals to naïve animals rendered fatal doses of diphtheria toxin harmless. More so, cell-free blood from animals inoculated with tetanus was unable to protect against diphtheria, showing that this protective response was both acquired and elicited specifically against the original inoculum. These molecules were termed ‘antikörper’ or ‘antibodies’, and Behring later derived that this specific anti-toxin factor was contained in the protein fraction of serum (69). In 1939, Tiselius and Kabat discovered that adding ovalalbumin to ovalbumin-immunized rabbit serum depleted only the gammaglobulin peak on electrophoresis, thus confirming gammaglobulin proteins as antibodies or ‘immunoglobulins’ (70).

The structure of the gammaglobulin proteins was later elucidated from patients with myeloma, a malignancy of antibody-producing cells. The most abundant subtype Immunoglobulin G consisting of a dimer of heavy chain-light chain of 50kDa and 25kDa respectively joined by a disulphide bond in a hinge region **(**Figure 1.4**)** (71). The total IgG mass is therefore 150kDa. The heavy chain consists of three constant regions (CH1, CH2, CH3) and an N-terminal variable domain (VH), and light chains

have a single constant region (CL) and variable chain (VL). The light chains can be of a κ or a λ family. Pepsin treatment can cleave the disulphide bond, resulting in the creation of a $F(ab)_2$ fragment that retains its antigen binding without the ability to engage host immune cells. Papain treatment results in cleaving of both disulphide bonds in the hinge region and the creation of single heavy and light chain F(ab) fragments (72). Hypervariable regions in the Fab region create complementarity determining regions (CDRs) that dock with antigens in three-dimensional conformation. The Fc, or crystallisable fragment, consists of constant regions differing in the antibody isotype and directly engages with phagocytic, natural killer (NK) cells as well as complement.

1.2.2 Production of antibodies

Antibodies must be able to recognize an enormous array of unique pathogen molecular structures, yet B cells do not contain additional genetic information relative to other somatic cells. The Nobel prize was awarded in 1987 for solving this dilemma with the discovery of "combinatorial diversity". Transcripts from variable (V), diversity (D) and joining (J) regions of genomes are combined in early naïve B cells to form a complete heavy and light variable region**.** The full extent of recombination events and their mechanisms are reviewed by Schroeder and Cavacini (73). Between recombination of VDJ segments and pairing of heavy and light chains, the potential diversity of unique clones of antibodies is estimated at approximately 10^{12} (74)

Antibody affinity increases over time of exposure to antigen as cognate antigen binding stimulates expression of activation-induced cytidine deaminase (AID) which drives further controlled mutagenesis in a process called somatic hypermutation (75).

B cells retaining affinity to antigen thus propagate and differentiate into antibody producing plasma cells or memory B cells. T cell stimulation via a CD40/CD40L interaction stimulates a class-switching process whereby the same VDJ variable domains can be paired with differing heavy chain constant regions, resulting in antibodies with similar specificity but differing effector function (76). The importance of these processes is highlighted by deficiencies of AID in humans which results in hyper-IgM syndrome (type 2) whereby neither class switching or affinity maturation occurs, and patients present in early childhood with recurrent bacterial infections (77).

1.2.3 Antibody isotypes and subclasses

IgG: Immunoglobulin G (IgG) comprises the majority of serum antibody of which there are four subclasses in humans (IgG1, 2, 3 and 4). 10-20% of all serum proteins in humans are IgG antibodies (83). IgG subclasses differ in heavy chain constant region structure and hinge structure, thus altering binding affinity for Fc receptors (FcR) and complement **(**Figure 1.4**)**. Although the Fab region is

classically understood to confer antigen specificity, heavy chain structure has recently been shown to alters antibody flexibility and thus independently affect affinity to antigen (79). IgG1 is the most abundant IgG subclass. Both IgG1 and IgG3 are typically produced in response to protein antigens and have high affinity for FcR. Class-switching to IgG2 can occur independent of T- cell help in the presence of polysaccharide antigen, and this class of antibody has been shown to be made in response to polysaccharide-based vaccines against *Streptococcus pneumonia* and *Neisseria meningitidis* (80,81). IgG3 has the longest hinge region of all IgG subclasses, and interestingly is better able to neutralize HIV-1 than IgG1 antibodies potentially due to flexibility changes in antibody structure (82). IgG4 is associated with prolonged antigen exposure and can be increased in parasitic infections, long-term beekeepers, and individuals undergoing immunotherapy for peanut allergy (83).

IgM has a pentameric structure and is secreted by early development B cells. IgM is typically therefore low affinity polyreactive and part of the primary immune response to new pathogen. However, multimeric binding and the capacity to recruit complement effectively makes IgM effective at opsonizing pathogen. As little as one molecule of IgM can recruit enough complement to lyse a red blood cell, whereas 1000 IgG are required to do so. Natural IgM is also produced by B-1 cells in the absence of antigenic stimulation and is now implicated in many homeostatic processes (84).

IgA: Early studies highlighted the presence of an immunoglobulin in the stool of dysentery patients, the nares of influenza patients, saliva in oral typhoid vaccination, and human breastmilk (85,86). The unique structural features of IgA allow it to be secreted across mucosal barriers, and IgA is the predominant antibody in secretory fluid. Dimerized IgA associated with a joining (J chain) can be secreted and prevent pathogens from binding to epithelial cells. This appears to enhance sensitivity to bacterial proteases, and thus IgA2 is concentrated in mucosal secretion, whereas IgA1 in serum. IgA also plays an immunoregulatory role with the gut microbiome, and IgA deficient individuals display features of gut dysbiosis (87).

IgD is the least understood antibody isotype and forms a “T” shape due to heavy glycosylation and increased hinge flexibility (88). IgD is an alternative splicing arrangement of IgM, and its upregulation is associated with B cell anergy and tolerance induction (89). The majority of IgD is membrane bound, however secreted IgD has been shown to interact with pathogens infecting the upper airways, including *Haemophilus influenzae* and *Moraxella catarrhalis* (90). Circulating IgD has also been shown in human cells to modulate basophil function and trigger expression of antimicrobial peptides, IL-4, IL-13 and B-cell-activating factor of the tumour-necrosis-factor family (BAFF) (91).

IgE is present at the lowest concentration of immunoglobulin in serum and has the shortest half-life. The production of IgE is associated with hypersensitivity reactions, allergy and anti-parasitic immunity (73). The constant region of IgE binds with exquisite affinity to FcεR1 on mast cells, eosinophils, basophils, Langerhans cells to trigger degranulation. Although mainly implicated in allergy, IgE also

mediate protection against envenomation, and evidence for a tumour surveillance effect in humans is emerging (92,93).

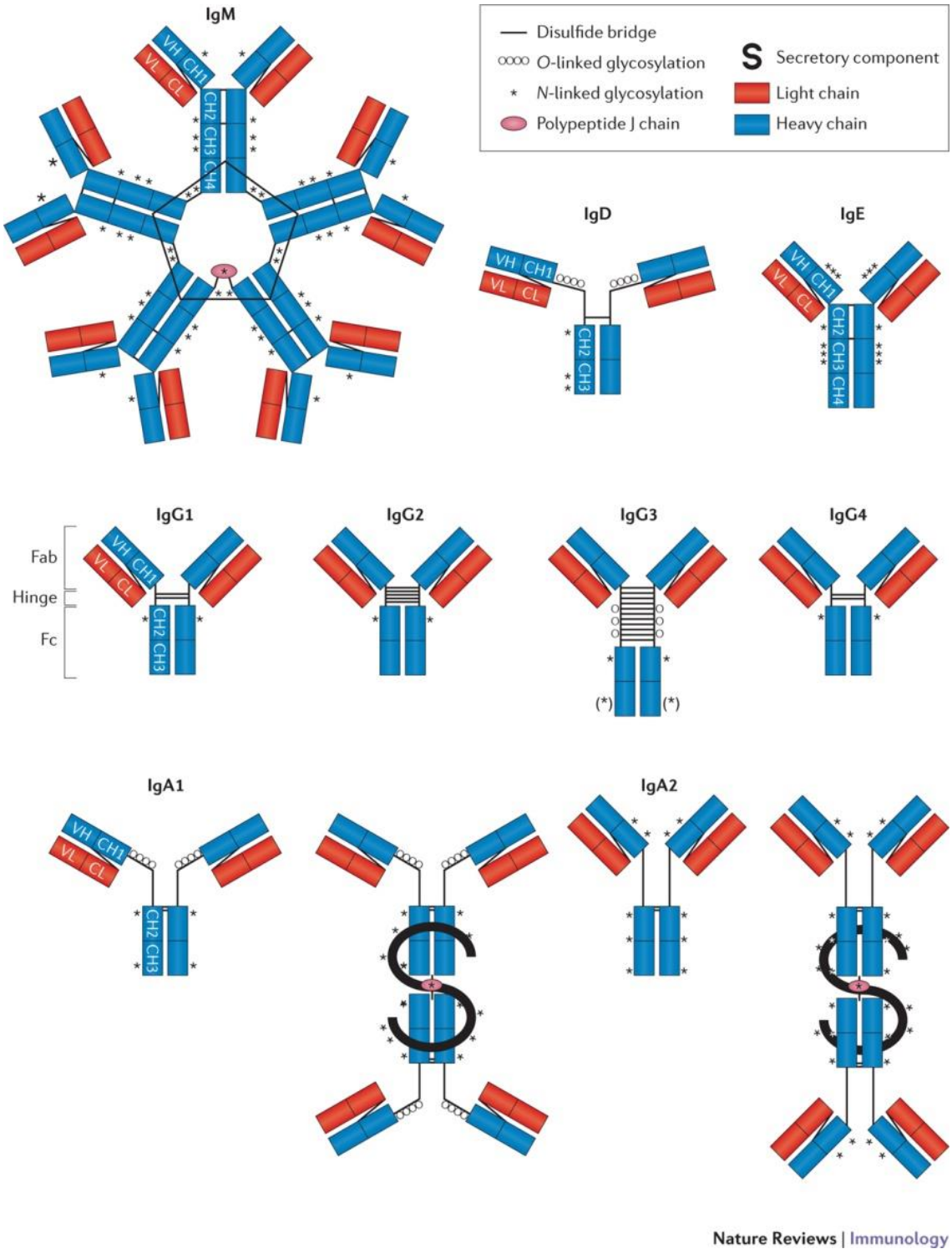

Figure 1.4 Structure of immunoglobulin isotypes and subclasses (94).

1.2.4 Functions of antibodies

Physiologically, antibodies are present in blood, mucosal surfaces, tear fluid and cerebrospinal fluid. The important of antibodies in the human immune system is shown by the evidence that most licensed vaccines work by the induction of protective antibodies (95). Antibodies exert their effect through the following mechanisms.

Direct neutralisation of toxins was the first major function of antibodies studied per Behring (68). Toxin production is a major virulence factor for numerous human pathogens, including *Bacillus anthrax*, *Staphylococcus aureus*, *Clostridium botulinum*, *Clostridium difficile* and *Escherichia coli*. Antibodies may sterically inhibit binding of toxins to host ligands or agglutinate toxin and thus render toxin inactive. MAb therapies have been approved in humans against *C. difficile* toxin B (Bezlotoxumab) and the anthrax toxin named Protective Antigen (Obiltoxaximab) (96,97). In viral infections, antibodies can directly inhibit viral entry through blockade of virus-receptor interactions. MAbs against envelope proteins gp120 that block interactions with CD4, for example, can prevent HIV replication in patients with acute HIV infection (98).

Opsonisation and complement recruitment occur when antibodies bind surface exposed antigens on a pathogen and trigger the classical complement cascade. Deposition of complement results in a pore-forming membrane attack complex (MAC) that lyses material cell walls. Antibodies vary in capacity to recruit complement, with IgM having the greatest and IgG2, IgG4 and IgA being relatively weak complement recruiters (99). Complement C1q initiates the classical complement cascade by interaction with antibody CH1 regions. Complement fixing antibodies are critical for the protection elicited by the 4CMenB vaccine against meningococcus (100).

Antibody-dependent cellular phagocytosis (ADCP) is the opsonization of pathogen via antibody that directs pathogens towards phagocytic cells expressing a range of FcR (101). The finding that antibodies aided the uptake of bacteria by host cells was first demonstrated in 1903 and reconciled the two leading theories on antibacterial immunity at the time - the humoral and phagocytic models of immunity (102). In pathogens where MAC formation may be ineffective, opsonization and complement deposition is able to trigger phagocytosis through host cell expression of Fc receptors described in more detail below.

Antibody dependent cellular cytotoxicity (ADCC) is classically understood to be crosslinking of FcγRIIIa on Natural Killer (NK) cells, stimulating the release of cytotoxic perforin and/or granzyme leading to target cell lysis. Contingent on this process happening is the presence of antigen on the target cell surface to trigger sufficient binding of IgG to cross-link the low affinity FcγRIIIa receptor. ADCC was first described in 1965, when it was observed that lymphoid cells caused ‘contact-induced’ cytotoxic death of tumour cells only when anti-serum was present (103).

NK cells are not the only cells capable of performing ADCC. Macrophages, γδ T cells and polymorphs (neutrophils, basophils, eosinophils) can perform ADCC via expression of a range of FcR (104). The

principal role for ADCC has been described in antitumour surveillance, and several oncology immunotherapy mAbs likely function via ADCC (105). However, recent roles for ADCC have been demonstrated in infectious diseases. Antibodies stimulating ADCC can set the HIV viral setpoint in elite controllers (106). Likewise, mAb114, a mAb derived from an Ebola survivor, was shown to elicit ADCC and was later demonstrated to improve case fatality rates in the 2018/2019 Democratic Republic of Congo outbreak (107). ADCC therefore plays a significant role in viral infections where viral budding from the host cell membrane provides a window of opportunity for antibody binding of exposed antigen on infected cells. The role of ADCC in other infections is still in question, but ADCC occurs in mouse models of *Chlamydia trachomatis* and schistosomiasis (108,109).

1.2.5 Fc segments and Fc receptors

Antibody effector function is mediated by the Fc portion of antibodies engaging with a range of cell types expressing FcR (Figure 1.5). Although antibodies can bind to C-type lectins, namely DC-SIGN and mannose-binding lectin, the principal mediators of immunomodulation by antibodies are the FcγR, FcμR and FcαR receptors (110). Fc receptors are a family of cell surface receptors that recognize antibodies and are linked to immunoreceptor tyrosine-based activation motif (ITAM) or immunoreceptor tyrosine-based inhibitory motif (ITIM) (110).

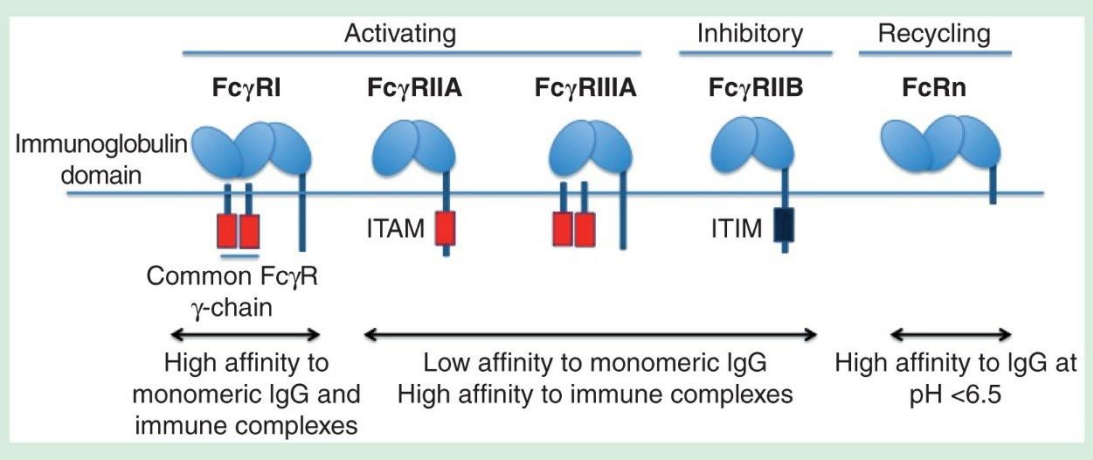

Figure 1.5 Structure and function of Fc receptors (94).

Activating FcRs bind the heavy region of the antibody (described below) and initiate downstream pathways by signalling through ITAM (111). The effects ITAM signalling is to drive internalisation of pathogen, followed by maturation of the phagosome, production of TNF-α, IL-6, IL-1β, secretion of antimicrobial peptides, metalloproteinases, antigen presentation via MHC-II expression, and respiratory burst (110). Differential effects have been observed for ADCP in different phagocytes. In neutrophils, ADCP is associated with NAPDH-dependent oxidative bursts, ROS production, degranulation, as well

as NETosis (112). In macrophages, FcR mediated phagocytosis can alter macrophage polarization between M1 and M2 phenotypes, enhance intracellular killing via phagolysosome maturation, and increase antigen presentation via MHC-II (113). Likewise for DC, ADCP results in upregulation of MHC-II and co-stimulatory molecules for T cell activation, therefore enhanced antigen presentation (113).

FcγRIIB is the only inhibitory FcR, containing ITIM, and suppresses signal transduction from antibody recognition via ITIM (113). FcγRIIB is expressed on B cells, where antibody binding modulates B cell activation for homeostasis (114). FcRn, the neonatal receptor involved in transcytosis of maternal IgG to the neonate, also regulates IgG concentration in serum by uptake and lysosomal degradation of IgG (115). Affinity of immunoglobulins for FcRN thus influences the half-life of antibodies in serum.

The Fc-FcR engagement is influenced by antibody isotype and subclass, glycosylation, and the affinity of receptors. IgG subclasses vary greatly in the number of amino acids present in the hinge region of the antibody, impacting the ability for antibodies to engage with effector receptors (116). Hinge flexibility in order goes from IgG3 > IgG1 > IgG4 > IgG2, with the elongated hinge of IgG3 conferring a higher degree of rotational freedom (117). Functionally, IgG3 may have greater ability to activate the intracellular antibody receptor TRIM21, as well as bind complement (118).

In terms of glycosylation, IgG contains a conserved glycan at position Asn297 of the CH2 heavy chains, between CH2 and CH3. Additional glycan moieties are present at the hinge region of IgG3 and IgA1 (119). Variation is generated by combinations of sialic acid, galactose, N-acetylglucosamine (GlcNAc) and fucosylation of the amino acid. Glycans at this position can alter binding of the FcR to effector cells by modulating hydrophobicity, as well as antibody half-life (120). IgG lacking glycosylation is unable to interact with FcγR. For example, ADCC activity can be increased by reducing the fucose content of mAbs (121). Direct viral neutralization of HIV by a mAb is affected by glycosylation (122). Thus, glycosylation directly impacts upon antibody function, and patterns of glycosylation are shaped by disease states and vaccination (94).

1.2.6 Advances in studying the human antibody response

Behring presciently stated in an 1895 scientific congress, “I have no fear that the thought which forms the basis of serum therapy will ever disappear out of medicine.” (123) The means to interrogate serum antibody responses has been a central scientific focus.

Quantification of antibodies is typically performed by **enzyme-linked immunosorbent assays** (ELISA), originally described in 1971 (124,125). The broad of principles of ELISA relies on covalent binding of immunoglobulins or a target antigen to plastic in laboratory plate wells, and the development of secondary antibodies conjugated to enzymes such as horse-radish peroxidase or alkaline phosphatase

that catalyse a chemiluminescent reaction when exposed to an appropriate substrate (126). Numerous clinical laboratory assays rely on this method to diagnose of a range of infectious and auto-immune conditions.

Avidity is defined as strength of binding of polyclonal antibody to an antigen and was originally measured either by the introduction of a chaotropic agent such as urea to remove weak bonds between antibody and antigen, causing low avidity antibodies to dissociate. More recently, surface plasmon resonance has largely supplanted urea assays and provides finer measurement of the strength of antibody-antigen interaction (127).

As opposed to studying serum or purified polyclonal antibody fractions, the production of **mAbs** allows the precise structural epitopes of antibody to be paired with functional assays. Immortalizing of splenic antibody producing cells through fusion with a myeloma cell to create a 'hybridoma' allowed the first isolation of mAbs (128). More recently, the ability to clone mAbs by single fluorescence activated cell sorting (FACS) and a set of primers covering the families of all potential VDJ leader sequences has revolutionized the study of human mAbs (129,130). Patient-derived antibodies are now being extensively trialled and used for the therapy of several infectious disease such as Ebola, HIV, and COVID-19 (131–133). Reverse vaccinology has also been identified as a rational means to identify novel immunogens through characterizing antibodies from patients in the convalescent phase of infections, or post vaccination where antibodies form a correlate of protection (Figure 1.6) (134). After identifying antibodies with the function that is sought (broadly neutralizing antibodies, prevention of viral entry, ADCC etc), the sequence of amino acids or crystal structure recognized by the antibody can be identified. Recombinant structural subunit immunogens can then be tested in animal models (134).

Single-chain variable fragments (scFv) can also be cloned, comprised of VH and VL regions joined by a short linker peptide (135). Multi-specific antibodies can cross-link separate antigens and both bi-specific mAbs and tri-specific scFv are being tested against HIV to overcome barriers of resistance to single mAbs (136,137).

Perhaps the biggest advances in studying humoral immunity have been the emergence of high-throughput sequencing paired to antibody cloning. Bioinformatic approaches have been used in bulk RNA sequencing to pair heavy and light chains into putative complete F(ab) sequences for *in vitro* expression (138). Characterizing antibody repertoires have been used to add resolution to understanding vaccine responses and could be useful for immunophenotyping in natural infection. The future generation of mAb production could potentially be derived from high-throughput methods of single cell sequencing to generate extensive libraries of paired heavy and light chain sequences, with bioinformatic approaches to determine putative antigen binding even prior to expression (139,140).

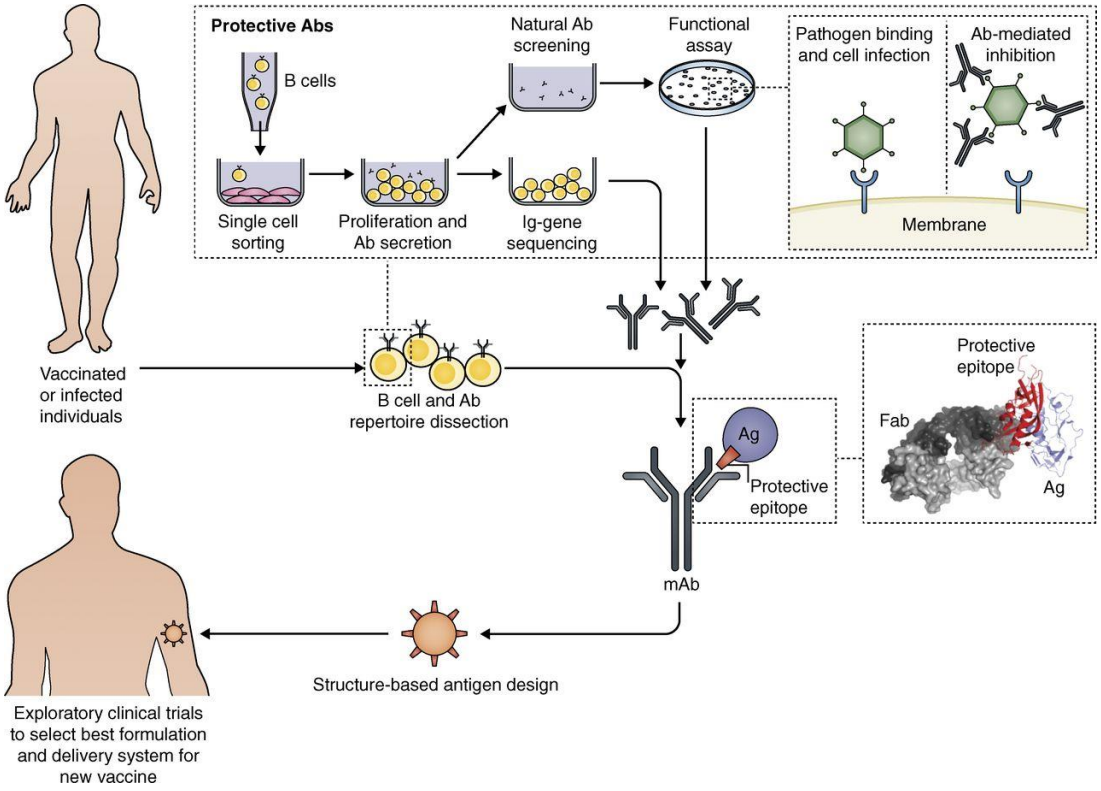

Figure 1.6 Pathway of cloning and characterizing mAbs from humans for the purpose of vaccinology (134).

1.3 The human antibody response to *M.tb* across the spectrum of clinical tuberculosis

1.3.1 Active TB

Over the last century there has been no shortage of studies examining human serum binding against a range of *M.tb* antigens. These are well reviewed by Glatman-Freedman and Casadevall in a seminal paper covering the historical literature (141).

Perhaps the defining feature of these studies is great heterogeneity in antigen recognition across individual patients, and between LTBI and ATB. For example, out of a panel of 10 culture filtrate proteins, no single antigen was recognized by all of 59 patients with ATB, and the number of antigens recognized per patient varied from one to eight. (142).

This is further compounded by the finding that approximately 10% of patients do not appear to mount a detectable titre of antibodies against mycobacterial antigens (142). To the extent that these studies using mainly ELISA based methods could determine, no single antigen or even cluster of antigens could be relied upon to distinguish ATB from LTBI. This resulted in the WHO calling a moratorium on antibody-based diagnostic kits for TB in 2012 (143).

Initial studies predominantly focussed on antigens that were noted to be highly immunogenic in western blots on *M.tb* lysates, or from technical ease-of-use in culture filtrate. ESAT-6, CFP-10, Ag85A, MPT64, the 38kDa antigen, and α-crystallin, are examples of antigens that are extensively studied as targets of the human antibody response in a range of clinical scenarios (144). Despite the above noted heterogeneity in the human antibody response, ATB generally induces a robust humoral response to *M.tb* that also correlates with higher bacterial load in sputum (144).

All subclasses and isotypes have been reported against *M.tb* antigens, but the predominant antibodies produced are IgG1 and IgG3 (145). Interestingly, ATB has also been associated with increases in IgD as well as IgG4, the role of which is not known (146).

The greatest advances in understanding the breadth of antibody responses in ATB have come more recently from high-throughput protein arrays and transcriptomics. Firstly, Kunnath-Velayudhana *et al* used a TB protein microarray representing over 1200 linearized proteins from *M.tb* H37Rv to determine the seroreactive antigen repertoire of *M.tb* (147). This array was probed with serum from individuals with ATB, and immunodominant antigens represented only 0.5% of the *M.tb* proteome (Figure 1.7). 484 proteins were recognized by serum by at least a single patient. Antigen recognition in ATB was skewed towards secreted proteins such as MPT64, Ag85A, CFP-10, and the 38kDa antigen (147) Further protein array studies in ATB have largely affirmed the already known heterogeneity of response, as well as a skewing towards recognition of secreted proteins (148–151). Most recently, large-scale Luminex assays have been used to test binding of 1680 antigens in individuals with LTBI and ATB (152). ATB was best discriminated from LTBI by antigen recognition of LAM, RV2435c, Rv3583 and Rv1528. Two other interesting findings emerged. Firstly, that ATB associates with a loss of IgM against Rv1508 and Ag85A, and secondly that IgG binding to FcR is stronger in ATB than LTBI (152).

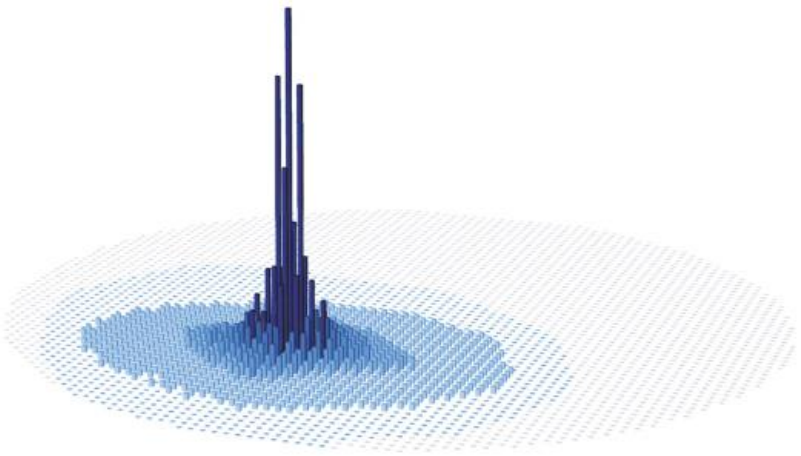

Figure 1.7 Each bar represents one of the 4,000 proteins of *M.tb*. The bar height and blue colour gradient reflects the number of reactive sera to each protein (147).

Using protein arrays validated by ELISA, out of 103 recombinant *M.tb* antigens 90% of people had antibodies against at least one antigen, but a combination of responses to six antigens (Rv0831, Rv2031, Rv2032, Rv2875, Rv0934, and Rv3874) was required to reach a sensitivity of 93% (153).

Although specific antibody has failed to generate a useful diagnostic tool in and of itself, the addition of measuring anti-Ag85B antibodies to a panel of IL-6, IL-8, IL-18 and VEGF raised the sensitivity of a rapid triage assay from 80% to 86% in adults with persistent cough (154). Even from large-scale protein arrays, it seems unlikely that antibodies could be useful biomarkers for diagnosis of TB (155).

The human repertoire of antibodies also targets non-protein antigens, but despite the biological relevance to *M.tb,* far less is known about these antibodies in TB relative to those targeting proteins. Antibodies against lipoarabinomannan, a highly immunogenic glycolipid, are the best characterized (156). A glycan array probed with sera from ATB, LTBI and controls all showed a range of seroreactivity to arabinomannan (AM) polysaccharides (157).

Follow-up work cloning antibodies from patient-derived B cells baited for AM-reactivity showed that human mAbs recognize a diverse range of epitopes across AM (158). Two high-affinity mAbs reacted to distinct polysaccharide domains, were non-competing, and were able to stain histology samples for the presence of whole *M.tb* (158). Although these two mAbs were not from individuals with ATB, it's evident that *M.tb* glycans are broadly immunogenic in humans and can contain multiple antibody epitopes.

In terms of anti-lipid antibodies, synthetic trehalose monomers and dimers are broadly reactive with patient serum (159). Other studies using antigenic preparations containing predominantly lipid, such as cord factor or membrane vesicles, show seroreactivity in ATB as well (160,161).

Diagnostic utility aside, the underlying reason for the heterogeneity of the antibody response is poorly understood. Duration of active disease prior to sampling, strain of *M.tb*, and host HLA genetic polymorphism are all plausible and under investigated explanations. There is limited data to suggest that HLA influences antigen recognition but chronological evaluation of antibody responses, or linking of strain to antibody responses has not yet been done (162,163).

Although there are unanswered questions regarding why and when certain antibody responses develop in TB, one of the hallmarks of emerging transcriptomic studies in TB has been the upregulation of FcR and complement by myeloid cells during ATB. Thus, although antibodies to specific antigens are not able to well distinguish between disease states, the overall activation of humoral immunity is consistent in ATB and even subclinical TB.

Several parsimonious TB transcriptomic signatures have now been evaluated for diagnostic utility in clinical studies. Independent of HIV-1 co-infection, FcγR1A/ FcγR1B transcription and C1q is up-regulated in persons with ATB (164–166).

Esmail *et al* validated these findings on a protein level, showing that circulating immune complexes (CIC) become more abundant in subclinical and then ATB relative to LTBI (Figure 1.8) (167). In a cohort of adolescents with LTBI in Worcester, South Africa, levels of IgG and IgA rose in those who went on to develop ATB vs those who did not prior to presentation with clinical TB (168). Elsewhere, CIC in ATB have been shown to contain a range of immunodominant humoral antigens such as Ag85A, as well as glycolipids. The antigens identified in CIC include Ag85A, 30kDa antigen, and glycolipids (LOS, DAT and PGLTb1) (169–171) Part of the challenge of detecting free LAM in the urine of PLWH is that LAM readily forms circulating immune complexes in the circulation (172).

.Smear microscopy positive patients were much earlier found to possess greater amounts of circulating immune complex than smear negative patients, suggesting CIC relates to bacterial load (173).

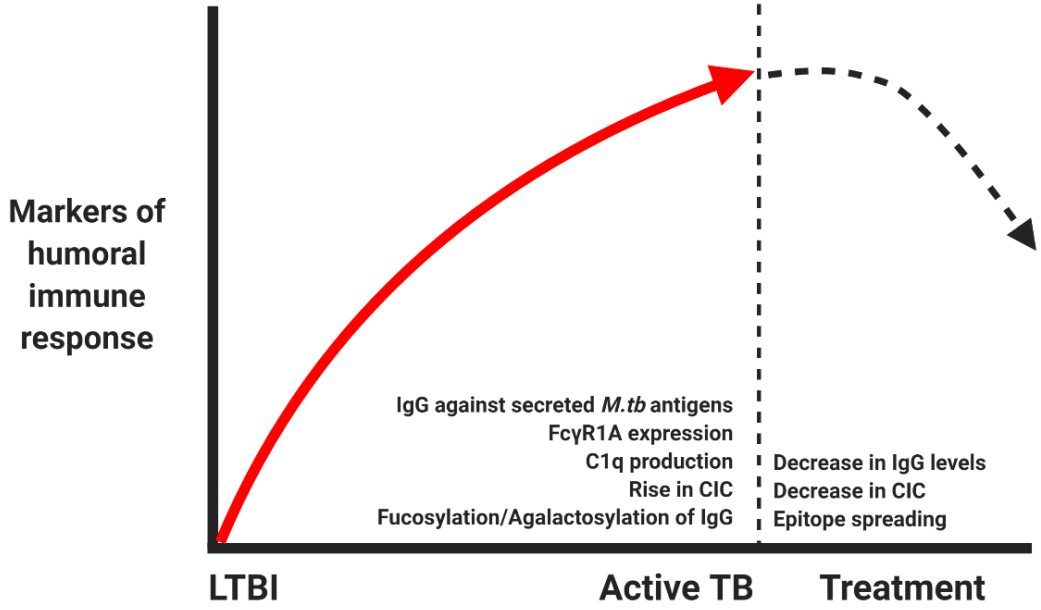

Figure 1.8 Schematic summarizing markers of humoral immunity in ATB. Created by BioRender.

Spatially, antibodies and B cells have long been known to be present in the granuloma. Human lung tissue in ATB stains intensely with rabbit anti-PPD serum. Sputum and BAL in ATB also contains *M.tb*-specific IgG and IgA, CIC and cytokines driving the expansion of antibody-producing cells (IL-4, CCL4, SOCS3) (174–176).

A substantial portion of the indepth phenotyping of cells in the granuloma comes from non-human primate data. Gideon *et al* have in a series of publications defined that the granuloma contains activated antibody-secreting B cells. Lung tissue in these animals was enriched for plasma cells and immunoglobulins that bound to CFP-10 and ESAT-6, showing that B cells in the granuloma are likely to be producing *M.tb*-specific antibodies (177,178). Further, by paired single cell sequencing and

immunophenotyping, the B cells in the granuloma were shown to be predominantly IgG or IgA expressing plasma cells.

1.3.2 Latent and subclinical TB

The *M.tb* genome contains ~50 genes co-expressed as a response to hypoxia and nutritional stress, encoded by the DosR regulon (179). Thus, a longstanding hypothesis has been that in LTBI, the antibody response would skew towards targeting antigens expressed by *M.tb* under immune pressure ie. 'latency-associated' antigens. Although many DoSR-encoded proteins are antigenic, ATB cannot be discriminated from LTBI based on their recognition by antibody (143). For example, Kimuda *et al* tested antibody responses to the DosR-encoded proteins Rv1733c, Rv0081, Rv1735c, and Rv1737c in a cohort of Ugandan individuals with either LTBI or ATB, and responses to these antigens did not differ between groups (180).

Single specific antibody biomarkers thus seem unlikely to accurately discriminate between disease states as even individual granulomas have both replicating and non-replicating populations of *M.tb*. Osada-Oka *et al* identified both Ag85A (associated with *M.tb* in growth phase) and MDP1 (associated with dormancy) expressed in the same granuloma of a patient with previous TB who underwent lung biopsy (181). Further, timing and intensity of exposure to *M.tb* also factor into the antibody response in LTBI. TST(+) persons from Ethiopia bore higher levels of antibodies to ESAT-6 and CFP-10 than TST(+) individuals from both Brazil and Denmark (182). When antibody responses in recent TST conversion was compared to individuals with a remote history of TST conversion, recent TST conversion associated with greater titres of antibody against ESAT-6, Ag85A, Acr and MDP-1 (183). In a macaque model of LTBI, antibodies against ESAT-6 occurred early (6 to 7 weeks post infection), whereas antibodies against Ag85, MPT53 and CFP-10 occurred sporadically, if at all. Interestingly, antibodies against ESAT-6 were sustainably detectable despite some animals undergoing IGRA reversion (184).

As with ATB transcriptomic signatures, subclinical TB (asymptomatic patients) shows an increase in CIC complex and FcγRI expression (167). FcγR1c expression is a component of the RISK11 score which can predict incipient TB (185). In a Gambian cohort, a single gene pair of C1q and T cell receptor-α variable gene 27 consistently predicted progression to ATB in household contacts (186). Whether this humoral response actively contributes to disease progression by a form of antibody-dependent enhancement remains to be studied in this population.

1.3.3 IGRA negative but *M.tb*-exposed

Natural outbreak events have shown the existence of individuals who, despite definitive exposure to *M.tb*, remain TST and/or IGRA negative and do not appear to be at risk of developing active disease (187). Studying such persons may therefore provide insight into immunity against *M.tb*, and thus defining the phenotype of a TB 'resister' has been a recent priority. The constituents of this phenotype are summarized below (Table 1.1)

Table 1.1 Table summarizing constituents of the resister phenotype.

No.	Criteria	Description
1	Definitive exposure to *M.tb*	• Household contacts • High transmission communities • Occupational exposure i.e. healthcare workers or gold miners
2	Immunological markers of infection	• TST • IGRA
3	Absence of features of ATB	• Symptom screening • Radiographic screening
3	Longitudinal data regarding durability of phenotype	• Absence of immunological markers of infection • Clinical signs of ATB

Phenotyping the antibody response in the resister population has been a major area of recent focus. A specific antibody response to *M.tb* in this population despite a negative TST or IGRA would be definitive that these persons were indeed exposed to *M.tb*. Questions as to the role of these antibodies in clearance of *M.tb* follow logically.

Prior to the establishment of resister cohorts, it was hypothesized that if protective antibodies in TB exist, then their utility might be in preventing the initial establishment of latent infection with *M.tb* (188). Several TST or IGRA negative cohorts have been studied without longitudinal follow-up. Anti-PPD antibodies do exist in TST negative persons and inoculating peripheral blood mononuclear cells (PBMC) with PPD and autologous serum resulted in a diverse effect on T cell activation (189). Sera where high titres of anti-PPD antibodies were present drove the proliferation of T cells.

Healthcare workers in a TB ward who remained TST negative despite occupational exposure possessed overrepresentation of an IgA gene arrangement on bulk B cell sequencing that was not present in their TST positive colleagues (190).

More recently, cohorts with longitudinal follow-up of the TST/IGRA negative phenotype have been described. A cohort of household contacts (HHC) of a pulmonary TB index case was recently described

where IGRA negative HHC were followed up for a period of seven years(21). Despite lacking sensitization to *M.tb* defined by QFT-Gold In Tube, these individuals had class-switched antibodies to ESAT-6/CFP-10, as well as higher IgM levels, antibody avidity and a unique Fc glycosylation pattern in comparison to IGRA positive HHC. Non-IFN-γ T cell responses were also described involving CD40L (CD154), CD107a, IL-2 and TNF-α. In this cohort, however, the incidence of *M.tb* was the same in both the resister and IGRA positive cohorts.

Another recent cohort of HHC in The Gambia where TST or QFT non-converters were compared to converters at baseline and at 3 months (191). The transcription profile of non-converters was enriched for B cell markers. Using a protein array, serum recognition of several antigens by IgA differed between groups. The antigens that best discriminated groups were Rv0134, Rv0629c and Rv2188c, with greater responses seen in converters. Interestingly, recognition of Rv3541c by IgG was greater in nonconverters, which may imply host benefit towards antibody recognition of this target.

Kroon *et al* described another population of resisters who were HIV-1 infected with advanced HIV. Although not definitive HHC, the cohort resided in an area in which IGRA positivity is almost universal by adolescence (192). This cohort, despite advanced HIV, did not acquire TB or both TST or IGRA positivity. Via ELISA, serum reactivity to ESAT-6/CFP-10 was frequent (~67%) and by Luminex serum reactivity to these antigens was seen in all resisters.

1.3.4 Effect of antitubercular therapy

Given the long duration of treatment for ATB, an antibody-based biomarker that could predict the successful resolution of disease is desirable. Post-ATT, the range of antigenic recognition broadens in what has been termed as epitope spreading (144). Kaplan and Chase were amongst the first to report this, showing that treatment increased the number of persons with ATT reacting to a single antigen (193). This finding of epitope spreading has subsequently been replicated further, with proposed mechanisms being either the release of previously shielded antigens from dead bacteria, or the decline of active immune evasion (144). A novel protein array probed by sera from patients pre- and post-ATT showed 14 antigens whose recognition differed significantly by treatment status (including cobL glnQ, arg, groEL) (148). Levels of IgG, IgG1, IgG2, IgG4 and IgM against ESAT-6, CFP-1 and Acr decline from time of diagnosis, between 1 and 3 months and completion of treatment. IgG3, however, did not differ during treatment (194). Further, levels of CIC also decline with ATT, but remains high in patients who do not convert to a negative sputum culture (195).

When treated with INH prophylaxis, TST positive children show a decline in IgM and IgG against alpha-crystallin (196). IgG avidity against an *M.tb* lysate preparation initially decreased in 73% of patients with treatment, but then increased towards the end of therapy. This could suggest recognition of new antigens released from bacilli killed by INH (197).

The overabundance of FcγR1A transcripts also declines with therapy, a 664-transcript signature diminished after 2 weeks, and further until the end of treatment (165,198) Cliff *et al* showed that levels of complement-related and FcγR transcripts quickly normalize post therapy, and previously depleted B cell transcripts normalize at a later time point (165).

1.3.5 Effect of HIV on the antibody response to TB

TB is the leading cause of death in people living with HIV (PLWH). Increased risk for TB occurs within the first year after HIV seroconversion and is not fully ameliorated by ART (181). At lower $CD4^+$ T cell counts, disseminated TB, TB meningitis and mycobacteremia become more frequent (200). Apart from the loss of $CD4^+$ T cells, HIV infection also results in widespread perturbation of the humoral immune system. B cell anergy results from chronic viremia that isn't fully resolved by the initiation of ART even after six months of treatment (201). The vaccine response to influenza vaccination is also blunted in PLWH, with lower titres, neutralization and avidity than HIV negative individuals (202). Thus, HIV infection could blunt the antibody response against *M.tb* or results in less effective antibodies via loss of $CD4^+$ T cell help. Given that HIV-associated TB is more frequently paucibacillary, serodiagnostics could potentially be a more useful tool in strengthening available diagnostics.

Barrera et al reported that the prevalence of detectable antibody responses against PPD declined with the progression of HIV disease (203). Among HIV-negative patients the prevalence of IgG to PPD was 50%, compared to 36% in PLWH and 5% in AIDS patients). High-throughput profiling of humoral response in HIV-associated TB has recently revealed the loss of LAM and Ag85-specific IgG responses, as well as lower titres of antibodies against ESAT6, CFP-10, and PPD (205).

There could also be a potential defect in class-switched antibody production, as IgG2 against LAM is less frequently detected in PLWH than in HIV negative individuals (204). Antibodies against AM are also significantly reduced in HIV-associated TB, with a similar deficit in IgG2 responses (206). These effects could result from loss of T follicular helper cells (Tfh) that are required for isotype switching, somatic hypermutation and proliferation of B cells (207). In keeping with this, the avidity of antibodies against Ag85A is lower in PLWH than in non-HIV infected persons (208).

The trend of declining antibody titres against TB in PLWH is not universally observed. Interestingly, it appears that antibody responses to phenolic glycolipid (PGL-Tb1) may be preserved in HIV across $CD4^+$ counts (209). Despite a mean $CD4^+$ T cell count of 98, participants maintained seroreactivity to PGL-Tb1. This finding could be accounted for by lipid presentation to B cells via CD-1 restricted T cells. IgG reactivity to MPT51 may be increased in HIV/TB coinfection relative to non-HIV TB cases (210). Whether this is because of altered bacillary replication or phenotype is unclear.

HIV-associated TB can also present as an immune reconstitution inflammatory syndrome (IRIS) after initiation of ART (211). Parsimonious host transcriptomic signatures to predict incident TB (RISK6 and Sweeney3) share some overlap with the prediction of IRIS (212). However, earlier studies have shown that antibodies against PGL-Tb1 are increased prior to ATT in individuals who develop paradoxical IRIS, whereas antibodies against ESAT-6/CFP-10 did not show the same association (213). Antibodies against LAM rise disproportionately during TB IRIS relative to controls who do not develop IRIS at the same timepoint post ATT (214). TB IRIS patients also demonstrated higher total CIC at 1- and 2-months post treatment (213). As it is thought that antigen load relates to the risk of IRIS, antibodies/CIC may track with the presence of *M.tb* and serve as a surrogate marker of overall antigen burden.

1.3.6 Humoral immunodeficiencies

If antibody responses against *M.tb* are necessary for protection against TB, it would be reasonable to assume increased risk for ATB or poor clinical outcome in persons with humoral immunodeficiency.

Although meta-analysis of the most prevalent humoral immunodeficiencies in children do not show increased risk of ATB, these studies most frequently occur in low-burden settings and where access to IVIG replacement is available (215). A range of isolated cases where atypical mycobacterial infections were observed in persons with selective IgA deficiency or hyper-IgM syndrome have been reported (216). Another unusual case of sternal and pulmonary tuberculosis in a Japanese adult male with hypogammaglobulinemia and normal T cell frequency has also been described (217).

Patients receiving mAb depletion of B cells and novel small molecule inhibitors are another population with humoral immunodeficiency. Multiple longitudinal cohorts of patients on Rituximab, an anti-CD20 mAb that depletes peripheral levels of naïve B cells, have not shown an increased risk of TB (218). However, case reports have emerged shown that severe and less common cases of disseminated TB have occurred after rituximab therapy in the absence of HIV infection (219–221). However, identifying a specific association for anti-B cell therapy with TB is difficult in such patients as concomitant use of multiple immunosuppressants is common. In patients with LTBI, rituximab was not associated with changes in QFT quantitative values, or the occurrence of TB for one year (222). This perhaps shows that patients with established LTBI do not appear to lose immune control of *M.tb* when B cells are lost, but the NHP model shows that rituximab may impact early granuloma organization (177).

Recently, belimumab, a mAb that binds to B lymphocyte stimulator was tested in a randomized, double-blind, placebo-controlled study in systemic lupus erythematosus. One death from tuberculosis meningitis was reported in the treatment arm (223).

As there are unusually severe cases of TB reported in humoral immunodeficiencies, future studies could explore whether antibody defects associate with disseminated TB.

1.4 Mechanisms of protective antibody immunity in TB

1.4.1.ADCP

In seeking a role for antibodies in TB, it must be noted that *M.tb* is opsonized by complement and IgG via the classical pathway (C1q activation) but this does not result in mycobacterial lysis (224). In fact, early studies showed that TB was able to actively replicate in a media mixed 1 in 4 with patient serum (225).

However, numerous antibody functions are contingent not only on antibody opsonization, but on engagement with host phagocytic cells (94). Antibodies are effective at opsonizing bacteria and targeting for complement-mediated lysis and antibody-dependent cellular phagocytosis (ADCP).

We have previously summarized the evidence in favour of an ADCP-driven protective antibody response in TB (Figure 1.9) (226). The crux of these studies is that opsonized *M.tb* can be more efficiently phagocytosed, killed, and processed for antigen presentation than non-opsonized bacilli. FcRs play an essential role in this process (227). This appears to hold true in animal models, although in ATB most antibodies do not directly target the mycobacterial capsule and could thus be thought to be 'non-functional' (147,228). Circumstantial evidence that this process is subverted in ATB could be (i) antigenic variability in surface antigens (ii) the loss of IgG2 against capsular antigens in advanced HIV (iii) the skewing of antibody responses to secreted antigen in ATB.

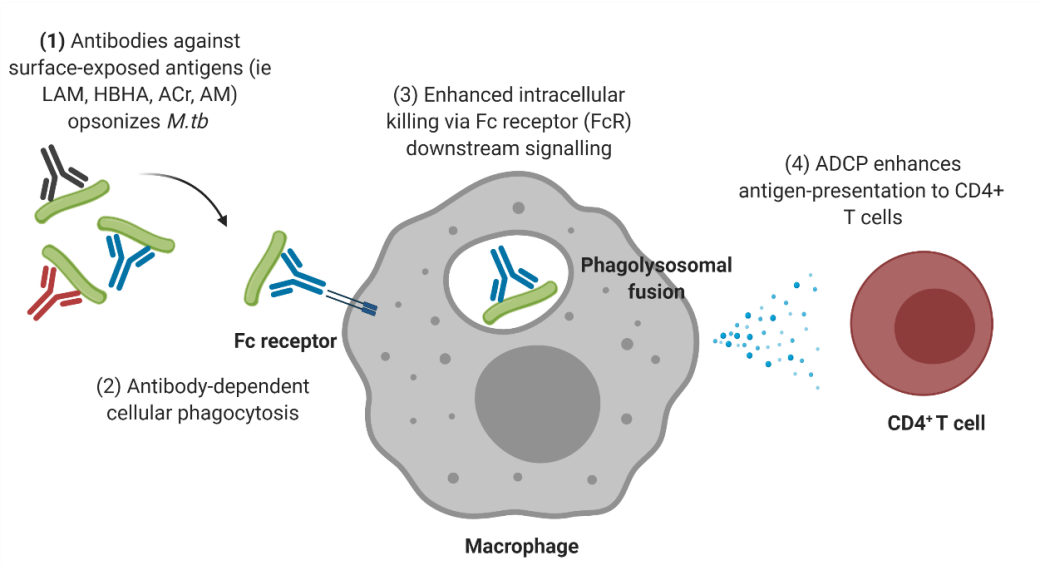

Figure 1.9 Schematic of putative mechanisms ADCP in TB. Created by BioRender.com

More recently, Lu *et al* have published two studies using systems serology to contrast the functionality of antibodies between LTBI and ATB, as well as LTBI or to the resister population (21,229). The methodology describes fluorescent beads that are conjugated to *M.tb* antigens – either PPD or ESAT-6/CFP-10 to determine phagocytosis in the human THP-1 monocyte cell line via flow cytometry. Using this assay, sera from individuals with LTBI differed markedly from those with ATB in terms of Fc region glycosylation and ADCP. Bulk sera from LTBI individuals showed greater ADCP, affinity to FcRs, phagolysosomal fusion, and inflammasome activation relative to serum from persons with ATB (229). Resisters showed very similar quantitative levels of antibodies to ESAT-6, CFP-10, LAM, and PPD, and produce antibodies that perform ADCP and ADCC.

Li *et al* showed that out of a cohort of healthcare workers from a TB treatment facility, 7/48 (14.6%) produced immunoglobulin fractions that restricted the growth of *M.tb* in mice and an *in vitro* whole blood assay (230). Depletion of opsonizing antibodies from serum removed the protective effect in whole blood.

Studying the immune response to BCG vaccination has also pointed towards a role for ADCP in protection against TB. Anti-AM antibodies in persons with both BCG vaccination and LTBI was able to perform ADCP and restrict the growth of *M.tb* in THP-1 cells (157). In adults previously not BCG exposed, BCG vaccination resulted in the induction of antibodies that drove ADCP as well as restricted the growth of BCG in a mycobacterial growth inhibition assay (231). Further, in an at-risk population of PLWH, neutrophil mediated ADCP was significantly poorer than in non-HIV infected persons (232).

Although several murine or chimeric antibodies display effects against *M.tb*, fully human mAbs against the phosphate transporter subunit (PstS1) were recently characterized (233). These mAbs were cloned from memory B cells baited for PstS1 reactivity and were able to opsonize *M.tb*. Perhaps the most salient feature of this study is that these mAbs had no inhibitory effect on PstS1's function as a transporter protein, but still were able to modestly impair the growth of *M.tb in vitro* and in mice at profoundly supraphysiological doses (500µg/ml to 1mg/ml)). This finding, and the fact that FcR blockade (CD16/CD32) abrogated protection reveals that ADCP is likely the mechanism-of-action, not direct neutralization of essential bacillary metabolic processes. Provocatively, these mAbs were cloned from a patient with ATB, further obscuring the role of antibodies in ATB and implying that either antibodies in ATB are i) 'too little, too late' ii) heterogenous in function, or iii) strongly countered by either bacterial growth or other immune defects.

1.4.2 Blockade of entry

Mucosal immunity has been a developing field of TB vaccinology. Investigation into the role of mucosal-associated invariant T cells, innate lymphoid cells and tissue-resident memory T cells in TB are ongoing (234).

In principle, secreted IgA (sIGA) could bind to inhaled bacilli and prevent invasion of host cells as is likely the case for pneumococcus and H*aemophilus influenzae* (95). SIgA crosses the alveolar epithelium, and the muco-ciliary escalatory allows pathogens to be trapped in mucous and expelled from the airways. This idea has previously been challenged in TB on the basis of to what extent sufficient concentrations of IgA could be present in the airway to capture inhaled bacilli prior to establishment of infection (235). Nevertheless, functional IgA mAbs have been cloned directly from HCW in a TB ward that blocked the entry of *M.tb* into a human alveolar epithelial cell line (236) In contrast, the same mAbs cloned into an IgG constant region drove uptake of bacilli. These findings corroborate a recent mouse study in which a humanized anti-Acr mAb protected mice against drug-resistant *M.tb* with an IgA backbone, but not when cloned with an IgG constant region (237).

The means to assess local immunity in the lung relies either on animal models, lung biopsy or BAL. A recent description of a human challenge model with pulmonary installation of PPD or BCG could be one means by which anti-*M.tb* mucosal antibodies could be studied (238). There are ethical and safety reasons why BAL or lung tissue is challenging to obtain in donors, and thus the phenotype of mucosal antibodies is not yet known in populations apart from ATB. Vaccines specifically targeting mucosal immunity via intranasal inoculation are in late-stage development for COVID-19 and could serve as proof-of-concept for future studies in TB (239).

1.4.3 ADCC

ADCC is understudied in bacterial intracellular pathogens, to the extent that no robust evidence exists as to whether it occurs in natural infection or not. There are conceptual challenges to the biological validity of this concept. Firstly, there must be antigens present in the host cell membrane of infected macrophages that are recognized by antibody. Secondly, NK cells must be able to kill both the infected host cell and the internalized bacilli.

There is, however, a reasonable epidemiological precedent for ADCC in TB. In multiple cohorts where immunophenotyping was performed on individuals prior to presentation with ATB, only the peripheral NK cell frequency associated with decreased risk of incident TB (239). Further, apart from canonical activation markers, expression of the NK cell antibody receptor FcγRIIIA (CD16), needed for ADCC, was associated with non-progression. The resister cohort from Lu *et al* also possessed antibodies with greater affinity for FcγRIIIA than those from the IGRA positive cohort (21). In the Lu *et al* study, ADCC was measured by coating ELISA plates coated with PPD, followed by serum, and then lastly incubation with NK cells (21). It is inferred that NK cell expression of CD16, IFN-γ, and degranulation markers, correlates directly to ADCC capacity. These experiments are yet to be extended to human MDM and live *M.tb*.

Although no studies in humans have shown definitively that antibodies are able to recognize *M.tb* antigens in the host cell membrane, antibodies recognizing the cell surface of infected cells does occur, and *M.tb* lipids potentially disseminate into the host cell membrane. Firstly, a mAb against a polysaccharide *Rhodococcus equi* antigen, PNAG, can bind human macrophages infected with this acid-fast intracellular pathogen (241). *R. equi* is predominantly an equine pathogen but can cause a granulomatous pneumonia in humans clinically like *M.tb*. PNAG disseminated into the host cell membrane distinct from the bacteria. Killing of *R. equi* in human macrophages was facilitated by a mAb recognizing PNAG in conjunction with complement and neutrophils, suggesting neutrophil mediated ADCC occurred (241). In mycobacteria, fluorescently labelled mycobacterial lipid phthiocerol dimycocerosate (PDIM) was shown by Cambier *et al* to embed into infected zebrafish macrophage membranes spatially distinct from visualized live bacilli (241). This process of PDIM spreading from intracellular *M. marinum* to the cell membrane of an infected cell was replicated in A549 human epithelial cells (242). Taken together, it could be hypothesized that anti-lipid antibodies are able to bind *M.tb* lipids such as PDIM that have embedded in the macrophage cell membrane, and thus target infected cells for killing by NK cells by ADCC.

1.4.4 Priming of cellular immunity

Where functional antibodies have been described in TB, it has almost always been in concert with cellular immunity. Li *et al* showed that a small fraction of HCW produced sera protective against *M.tb* challenge in a mouse model and whole blood assay (230). However, bead depletion of $CD4^+$ T cells, but not $CD8^+$ T cells, in the whole blood assay abolished the protective effect of serum (230). A human IgA mAb instilled in the nares of mice challenged with *M.tb* co-administered with IFN- γ reduced the CFU greater than IFN- γ, but not in the absence of IFN- γ (243).

The pleiotropic effect of FcR activation is well-described, and either stripping antibodies of carbohydrate, or blocking FcR, removes *in vitro* functionality of antibodies in a range of ADCP and growth assays (227,230). Antibodies could thus assist in priming or augmenting cellular immunity. In an interesting experiment, de Vallière *et al* co-incubated PBMC with DC that had phagocytosed BCG opsonized by serum from BCG revaccinated adults (244). Postvaccination serum resulted in better T $CD4^+$ proliferation and IFN- γ expression, an effect that could be abolished by pre-absorption of IgG from serum (244).

1.4.5 Limiting dissemination of disease

Hematogenous dissemination of TB was first reported in the early 1900s in which bacteremia was identified in 66.6% of patients with miliary TB, but only 6.7% of other forms of TB in the pre-treatment

era (245). Approximately 15% of TB cases occur as extrapulmonary disease, and transient mycobacteremia and/or lymphatic spread is likely the mechanism whereby distant organs are seeded with *M.tb* (246). Surprisingly, in autopsy studies of non-TB related deaths, extrapulmonary *M.tb* DNA can widely found in endothelium, kidneys, liver, and spleen (247). Mycobacteremia is also more common in advanced HIV and correlates with poor clinical outcome (12).

There therefore must be a point in time whereby extracellular *M.tb* is exposed to antibodies – either in the lymphatic system, or in blood. The fact that only a small portion of people develop extrapulmonary TB could form another role for antibodies in TB.

One of the earlier mouse mAb studies showed that prior coating of BCG with a mAb against HBHA did not reduce lung CFU, but markedly limited spleen dissemination (248). HBHA is an adhesin and facilitates entry of mycobacteria into host cells. Mice immunized with an AM-protein conjugate produced opsonized antibodies against the *M.tb* capsule (249). Passive transfer of immune serum prior to infection of naïve mice results in reduced extrapulmonary dissemination of *M.tb* to the spleen, a finding not seen in control serum from BCG vaccinated mice (249). Intravenous administration of LAM in mice results in deposition of LAM in the spleen and liver. An anti-LAM IgM mAb reduced organ deposition and resulted in clearance of LAM via biliary excretion (250).

The human data to date are circumstantial, but it appears disseminated forms of TB are associated with a particular defect in opsonizing IgG2. In PLWH, a defect in IgG2 antibodies against LAM is seen in those diagnosed with extrapulmonary TB (204). This same observation has been made in children, where IgG responses in LAM show a marked trough coincident with the age of peak incidence of disseminated TB (251). Again, children with disseminated TB had markedly reduced antibodies to LAM than those with respiratory disease despite equivalent total IgG levels (251). Antibodies of the IgG2 subclass against BCG were higher in infants who converted to QFT positivity after two years when compared to those that did not (252). In the glycan array study discussed, levels of anti-AM IgG2 were greater in patients with pulmonary disease than those with extrapulmonary TB or a miliary picture on chest x-ray (157). Notably, no such difference in anti-AM IgG1 levels were observed between pulmonary and disseminated TB (157).

The antibody response in ATB could therefore possess heterogenous functions, with some antibodies curtailing bacilli that break through granuloma control. The mAbs against PstS1 that both opsonized and restricted the growth of *M.tb* despite being derived from a patient with ATB (233). The absence of radiographic cavitation i.e. lung destruction is also associated with greater Ag85A antibody response (252).

Rituximab therapy has sporadically been linked to cases of miliary/disseminated TB, but not an increase in risk of pulmonary disease (219,220). A role for antibodies in limiting dissemination, but not reactivation TB, could be one explanation for this observation.

To test this hypothesis, the B cell and antibody compartment of patients with i) HIV-associated disseminated TB b) HIV negative persons with extrapulmonary TB could be contrasted to those whose disease is restricted to the respiratory system.

1.5 Antibodies in TB pathogenesis

There is a clear association between the increase in IgG against secreted antigens, C1q transcripts and levels, as well as FcγRIIIA/FcγRIIIB activation in the clinical progression towards incident TB. This emerging phenomenon could be interpreted that humoral immunity indirectly measures antigen burden as *M.tb* replicates, but these immune complex-receptor interactions could also be directly contributing towards disease pathogenesis.

One model (Figure 1.10) of viewing these findings is that replicating *M.tb* express secreted antigens which are then bound in CIC and taken up by myeloid cells via FcR. The overabundance of Fcγr1A/Fcγr1B would implicate monocytes, dendritic cells, and tissue-resident macrophages in the uptake of antibody, and the downstream pathways would result in expression of interferons, TNF-α and IL-1 (108).

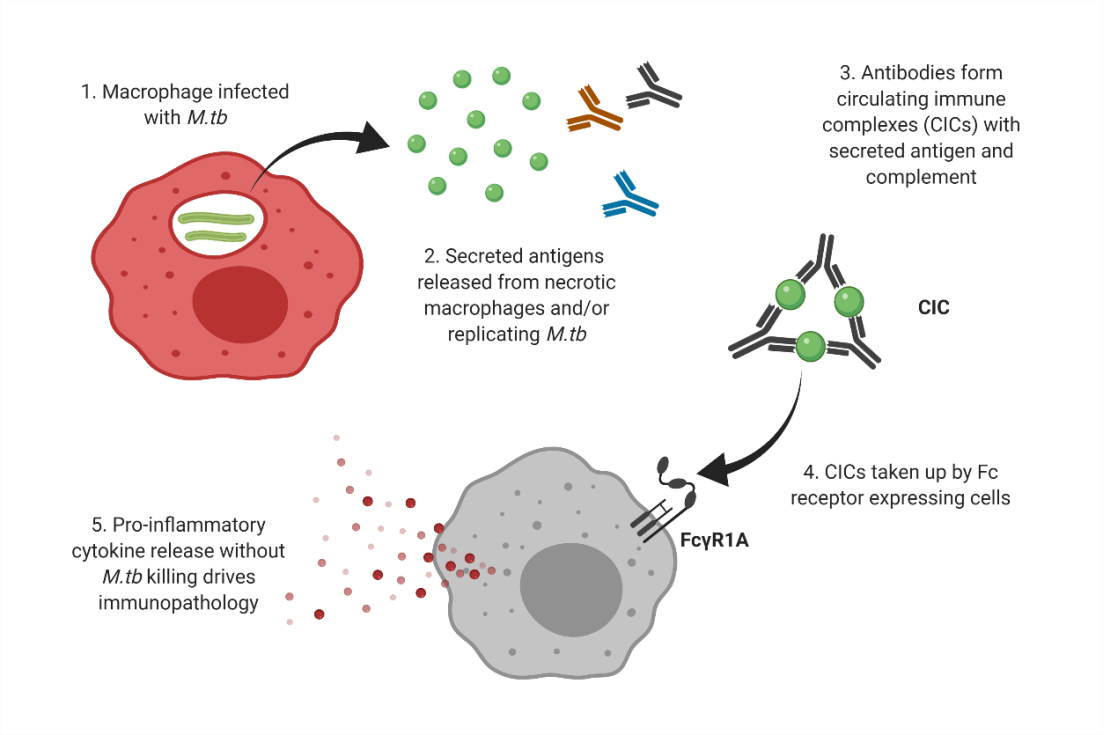

Figure 1.10 Schematic of putative mechanisms whereby antibodies could contribute to pathogenesis in TB. Created by BioRender.com

The classical model for a role of antibodies in the pathophysiology of an infectious disease is in Dengue. Antibody-dependant enhancement (ADE) of infection is central to the development of severe disease, whereby non-neutralizing antibodies traffic dengue virus to FcR- expressing cells. Pre-existing non-neutralizing antibodies from prior infection with other serotypes drives viral replication and can result in haemorrhagic fever (253). In parasitic infections, the pathogenesis of tropical splenomegaly in malaria is chronic antigen uptake distinct from parasite killing driven by anti- *Plasmodium falciparum* IgM (255,256). Leishmaniasis, another protozoal infection, is also characterized by the induction of anti-leishmanial antibodies that correlates with peak parasitemia, and the presence of CIC (257). Leishmania-induced CIC from patients with visceral leishmaniasis stimulates the production of IL-6, IL-10 and GM-CSF in PBMC (258).

The case is less strong in TB, but CIC do appear to have a proinflammatory effect. IgG1 antibodies from patients with ATB drives uptake of PPD into MDM with greater production of TNF-α, IL-10, and IL-6 relative to control serum (259). Following on from this work, Thakurdas *et al* isolated IgG1 from patient serum and showed that IgG1 specifically augments TNF-α production despite the presence of a TNF-α inhibitor (260).

Treatment of human neutrophils with CIC extracted from ATB patient sera suppressed the release of IL-4, IL-6, GM-CSF and TNF-α relative to CIC from healthy controls. CIC from ATB also modulated neutrophil respiratory burst and chemotaxis (261). TNF-α levels correlate well with disease progression in a broad range of inflammatory illnesses (262).

ATB has rarely been associated with the development of immune complex mediated glomerulonephritis, showing that these CIC can result in end-organ damage (263,264). A characterized cohort of IgA nephropathy in TB showed deposits of IgA1 and ESAT-6 on renal immunohistochemistry despite negative urine cultures for *M.tb* (265).

In post-mortem lung tissue biopsies of patients who died of TB, granulomas unintuitively rarely reveal visible *M.tb* (265). However, lung tissue in ATB stains diffusely and intensely with anti-PPD rabbit sera. The rim of cavities contains equal amounts of *M.tb* and antigens, but there is an abundance of protein antigens present in diseased tissue (266). It can therefore be envisioned that the antibody response to these antigens is unlikely to target bacilli for ADCP or ADCC, but rather form immune complexes and drive pro-inflammatory cascades by FcγR-mediated uptake in macrophages.

1.6 Antibodies and vaccination against TB

A binary viewpoint pitting humoral against cellular immunity is almost certainly an oversimplification when considering immunity required to vaccinate against TB (267). Especially since the COVID-19 era has overtly demonstrated that although neutralizing antibodies are critical for protection, T cells provide

protection against severe illness (267). Here the role of T cells is contentious, but if important, appears to be in breakthrough infection where nAbs have failed due to viral antibody escape. This could be an important lesson for the TB field where different timings and types of immunity may be required to prevent initial infection, prevent reactivation or to prevent dissemination of mycobacteria.

Despite the limited efficacy of BCG in preventing pulmonary TB, the mechanisms whereby BCG prevents dissemination in children must still be accounted for. Much work has been done to show that BCG induces a functional antibody repertoire (269) In an examination of BCG specific $CD4^+$ T cells in 5662 infants vaccinated at birth, neither IL-2, IFN-y, or TNF-α expression correlated with protection against developing TB (270). In a correlate of risk analysis of South African infants, antibodies against Ag85A were associated with decreased risk of incident TB (271).

Pre-clinically, administering BCG intravenously (IV) can induce sterilizing immunity in NHP (272). IgM responses in IV BCG vaccinated macaques associated with reduced *M.tb* burden on necropsy. Protected from susceptible macaques could be discriminated between by the presence of IgM against HspX in BAL early post-challenge, and anti-LAM IgG1 and IgM at later timepoints (272). Interestingly, a putative mechanism of protection in these animals was ADCC, with an increase in FcγR3A binding capacity and NK cell degranulation observed.

The decision to focus on protein antigens that are broadly recognized by T cells may not be a desirable strategy to induce effective antibodies. In two phase 2b trials, the candidate vaccine Modified vaccinia Ankara-expressing Ag85A (MVA85A) did not prevent incident cases of TB despite inducing polyfunctional T cells. Although Ag85A antibodies are a marker of decreased risk of TB, passive transfer of serum from mice immunized with Ag85A does not protect naïve mice (274).

Perhaps the most significant breakthrough towards defining correlates of protection against TB is the recent M72:AS01 vaccine trials (275,276). The Mtb32a and Mtb39A antigens were originally identified from serological studies in healthy TST positive individuals seeking immunodominant antigens in TB (275). M72:AS01 elicits both polyfunctional T cells and anti-M72 antibodies. This vaccine provided 49.7% protection against developing active TB in IGRA positive adults over a three-year period and presents the opportunity to determine a vaccine-induced correlate of protection against TB (276). All participants who received M72:AS01 had detectable antibody responses even 36 months post vaccination (276).

Many candidate vaccines for prevention of infection under clinical evaluation also induce antibody responses. H4:IC31 (Ag85B and TB10.4) induces antigen specific IgG1 and IgG3 70 days post vaccination but failed to prevent sustained IGRA conversion (277,278). The ID93/GLA-SE is a fusion protein-based vaccine that aims to prevent progression from LTBI to incident TB (279). ID93/GLA-SE is comprised of three immunodominant T cell antigens derived from ATB (Rv2608, Rv3619, and

Rv3620) or latency (Rv1813) (280). Despite targeting T cells, ID93/GLA-SE does induce vaccine-specific antibodies (278).

Two live-attenuated candidate vaccines to replace BCG as primary vaccination against TB, VPM1002 and MTBVAC, are in late-stage clinical testing. Although VPM1002 induces higher levels of antibodies than BCG in mice, in humans anti-PPD IgG wanes to undetectable levels within 180 days (281). This could be of concern if indeed antibodies are required for protection against *M.tb*. MTBVAC is a whole-cell vaccine of genetically modified *M.tb* with deletions in several virulence-associated genes (282). Mucosal vaccination with MTBVAC in NHP stimulates a robust IgG, IgA and IgM response in BAL fluid, and a modest increase in immunoglobulins after intradermal vaccination. Mucosal inoculation, but not intradermal vaccination, induced the production of opsonizing antibodies against *M.tb* (282). Levels of IgG and IgM in BAL fluid strongly correlated with an ADCP assay using fluorescent *M.tb* and THP-1 cells (282). Human immunogenicity studies of MTBVAC have not yet assessed antibody responses to vaccination (283). These findings also raise questions as to the impact of vaccination site and delivery on antibody production. Thomas *et al* have shown that aerosolized MVA85A vaccination raised no safety concerns in humans but did not induce anti-Ag85A antibodies in serum (283).

Whether it is necessary to induce anti-*M.tb* antibodies for protection is also controversial. A recent CMV-vectored vaccine (RhCMV/TB) containing 9 TB antigens completely protected 14 of 34 rhesus macaques from developing TB post intra-bronchial challenge (284). None of the animals in the study had vaccine-induced antibodies, but all had potent $CD4^+$ and $CD8^+$ T cell responses (284).

No scientific consensus on the optimal antigens or site of vaccination exists. If antibodies against secreted protein antigens are players in disease pathogenesis, this could be an Achilles Heel of the current direction of vaccine design. Whether antibodies may best be deployed in prevention of infection studies versus progression from LTBI in incident TB is also unclear and is contingent on where a role for antibodies is best found. Once infection is established or progressing, it may be too late for mucosal antibodies to curtail *M.tb* replication. Another concern is protecting PLWH from TB. The MVA85A vaccine was tested in PLWH and showed good immunogenicity (55). However, antibody responses are generally blunted because of HIV infection. The translational gap for an antibody-based vaccine against TB to cross to be harnessed for the next generation of TB vaccine candidates is therefore immense.

1.7 Conclusion

The role of antibodies in TB has been debated ever since the near contemporaneous discovery of *M.tb* and humoral immunity. Once understudied, seminal advances specifically with better immunophenotyping of people who appear resistant to TB, the cloning of functional human mAbs, and the antibody repertoire induced by IV BCG in NHP, shows that antibodies almost certainly have some function against *M.tb*.

How antibody epitope, isotype, glycosylation, and anatomical location, impacts this function are open questions. The nature of antigens that induce protective antibodies whether capsular glycans, lipids or membrane-associated proteins like PstS1 or ACr – is yet to be extensively catalogued. ADCP is the best studied mechanism whereby antibodies can aide killing of *M.tb* and prime cellular immunity, but ADCC or mucosal exclusion by IgA is likely underappreciated by virtue of large knowledge gaps. How these functions are impacted by HIV, and why only some individuals possess protective antibodies must be understood to better vaccinate against TB.

Paradoxically, progress in the prediction and diagnosis of ATB show that IgG against secreted proteins, CIC, and FcγR are consistent markers of disease. More research is needed to determine if this process is merely reflective of loss of immune control, or a direct contributor to the pathogenesis of TB. If the latter, this could avail new pathways for intervention by host-directed therapy.

The invention of technologies to explore human antibody responses at both scale and depth is now intersecting with the study of unique patient populations such as resisters and candidate vaccine recipients. These advances promise to shed new light on this old problem.

1.8 Aims and hypotheses

In this thesis I explore the role of antibodies in TB through an investigation of various clinical cohorts using a range of experimental methods. I have examined mAb and serological responses against *M.tb* across different clinical states, including ATB, LTBI, HIV co-infection, and BCG vaccination. I have sought to define the function of human mAbs produced by patients with ATB. Given that PLWH are at increased risk of developing disseminated forms of TB, I investigated whether the magnitude of antibody against *M.tb* is impaired in this at-risk population. In addition, whether a phenotype of TST and IGRA negative PLWH from high TB burden areas exists is underexplored. I therefore determined whether such individuals have detectable antibodies specific to *M.tb*.

Further, I showed the development of rapid flow cytometric assays to measure opsonization and ADCP using BCG vaccination as a model for mycobacterial infection. Finally, work in this thesis shows that the magnitude of antibody responses in HIV do not correlate with CD4+ T cell counts. This generated interest in testing whether T-cell independent IgM producing memory B cells (Marginal Zone B cells or MZB) are perturbed by LTBI or ATB. As MZB are activated by carbohydrate and lipid antigens, these findings are especially relevant in understanding humoral immunity against dissemination of *M.tb*.

The specific aims and hypotheses of this thesis are detailed below.

Hypothesis 1: MAbs generated from patients with ATB will differ in antigen specificity and function.

Aim 1.1: Generate a panel of fully human mAbs from patients with ATB.

Aim 1.2: Identify patient derived mAbs that recognize *M.tb* antigens.

Aim 1.3: Identify the antigen of any *M.tb*-specific mAbs.

Hypothesis 2: Mabs that bind *M.tb* will restrict the growth of *M.tb* in growth inhibition assays.

Aim 2: Test the function of anti-*M.tb* mAbs in terms of opsonization, ADCP, and bacillary killing in mycobacterial growth assays.

Hypothesis 3: A component of the immunodeficiency observed in PLWH is due to them mounting a lesser antibody response to *M.tb* across the spectrum of disease.

Aim 3: Quantify the magnitude of antibody responses across IGRA negative, LTBI, and ATB participants with and without HIV infection.

Hypothesis 4: PLWH who lack sensitization to *M.tb* from a high TB burden area will possess evidence of immune sensitization in the form of *M.tb*-specific antibodies.

Aim 4: Quantify the *M.tb*-specific antibody response in a range of cohorts with IGRA and/or TST negative PLWH.

Hypothesis 5: BCG vaccination induces an anti-mycobacterial ADCP response that can be determined with flow-cytometric assays.

Aim 5.1 Develop flow cytometric tools for the evaluation of serum opsonization capacity and ADCP of BCG.

Aim 5.2: Utilize assays developed above to quantify the effect of BCG vaccination on ADCP.

Hypothesis 6: T-cell independent IgM producing memory B cells (marginal Zone B cells or MZB) are of interest in TB as they are activated upon recognition of lipid and carbohydrate antigens. I hypothesize that the frequency and activation of these cells is impacted by ATB, resulting in their enrichment at the site of disease.

Aim 6.1: Test the frequency and phenotype of MZB in individuals with IGRA negative, LTB, and ATB.

Aim 6.2: Compare the frequency and phenotype of B cells in peripheral blood to site of disease (pericardial fluid).

Taken together, these data could provide additional insight into how antibodies may factor in both protection and pathogenesis in TB.

Chapter 2: Materials and methods

2.1 Instruments & Equipment

BD FACSAria	BD
BD LSR II	BD
Centrifuge	VWR
Electrophoresis tank and power pack	Bio RAD
BACTEC MGIT 320/960 machine	BACTEC
37°C water bath	
360° tube rotator	VWR
AxioObserver widefield microscope	Zeiss
37°C incubator with CO2	

2.2 Materials & Reagents

AgeI-HF	New England Biolabs
BD BBL MGIT tubes	Becton Dickinson
BsiWI	New England Biolabs
1.5 ml microcentrifuge tubes	Eppendorf or similar
10 x buffer	Ampliqon
12 well plates	Nunc
15 ml centrifuge tubes	Falcon
2ml screw-top microtubes	Sarstedt
3,3',5,5'-tetramethylbenzidine (TMB)	ThermoFisher
30% Acrylamide/Bis Solution, 37.5:1	Bio-Rad
48-well plates	Costar
50ml sterile polyethylene conical flasks	Sigma Aldrich

96 well plates	Nunc
AgeI-HF	New England Biolabs
Ampicillin (Amp)	Sigma-Aldrich
Anhydrous Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Anti-CD19 PE	Dako
Anti-CD20 PerCP (25µg/ml)	Becton Dickinson
Anti-CD27 FITC	Becton Dickinson
Anti-CD3 PerCP (12.5µg/ml)	Becton Dickinson
Anti-CD38 APC	Becton Dickinson
BCG Bacillus Calmette-Guerin	McShane & Wilkinson Labs
Benzonase	Sigma Aldrich
Bovine Serum Albumin (BSA)	Sigma-Aldrich
BV buffer	ThermoFisher
Carbenicillin	Sigma-Aldrich
Cell culture grade sterile water (Sigma)	Sigma Aldrich
Cell strainer	BD Falcon
Cytokine ELISA kits (Quantikine®)	RND Systems
D10 Medium	Sigma Aldrich
Dithiothreitol (DTT)	Pierce
DMEM	Sigma Aldrich
dNTP	Promega
EcoRI	Promega
ELISA plates	Nunc or equivalent
ESAT-6/CFP-10 fusion protein	Lionex.de
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Fetal Calf Serum (FCS)	ThermoFisher
Fugene 6	Promega

Glycerol	Sigma Aldrich
HEPES	Merck
Horseradish peroxidase (HRP)	ThermoFisher
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich
KpnI	Promega
L-glut/Pen-Strep	Sigma-Aldrich
L-Glutamine	Sigma Aldrich
L-Glutamine	Merck
LB media	Merck
LB plates	Merck
LIVE/DEAD Fixable Near-IR Dead Cell	ThermoFisher
Middlebrook 7H9 media	Becton Dickinson
Middlebrook OADC	Becton Dickinson
Mycobacteria stock	McShane & Wilkinson Labs
M.tb whole cell lysate	BEI Resources
M.tb antigen fractions	BEI Resources
Nitrocellulose membrane	GE Healthcare
Optimem	Gibco
PANTA/enrichment supplement	Becton Dickinson
Parafilm	Bemis NA
Paraformaldehyde	Sigma-Aldrich
PBS Phosphate-Buffered Saline	Sigma-Aldrich
PCR enzyme mix	Promega
PCR Plate	Bio Rad
PCR primers [see table x for sequence]	
PEN/STREP Penicillin/Streptomycin	ThermoFisher
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich

Plate seals	Star Lab
Polyethylenimine (PEI)	Sigma Aldrich
Qiagen HotStarTaq PCR kit	Qiagen
Qiagen miniprep kit	Qiagen
Qiagen OneStep RT-PCR kit	Qiagen
RPMI	Sigma-Aldrich
RNAse free microcentrifuge tubes	Eppendorf or similar
RNAse free tips	Gilson or similar
RNAsin (RNAse inhibitor)	Promega
RPMI-1610 Medium	Sigma Aldrich
Sall-HF	New England Biolabs
Saponin	Sigma-Aldrich
Skimmed milk	Sigma-Aldrich
Sodium Pyruvate	Gibco
Sulfuric acid	Sigma-Aldrich
Tris pH 8	Applied Biosystem
Triton -100	Sigma-Aldrich
TrypLE	ThermoFisher
Tween 80	Sigma Aldrich
Tween-200	Sigma-Aldrich
XhoI	New England Biolabs

2.3 Buffers

PBST	PBS, 0.1% Tween-20
ELISA blocking buffer	5% skim milk in PBS
FACS buffer	2% FCS in PBS
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4
R10 Medium	2 mM L-glutamine, 1 mM Sodium Pyruvate, 10% FCS
RNase-inhibiting RT-PCR catch buffer	5 ml RNase-free water, 50 µl 1 M Tris pH 8 and 125 µl RNAsin
RPMI-MGIA	RPMI-1640 Medium, 25mM HEPES , 2 mM L-glutamine
Sodium Carbonate buffer	0.05 M $NaHCO_3$, pH 9.6

2.4 Primers

Table 2.1 Forward Primer Sequences

Forward Primer	**5′ - 3′ sequence**
5′ L-VH 1	ACAGGTGCCCACTCCCAGGTGCAG
5′ L-VH 3	AAGGTGTCCAGTGTGARGTGCAG
5′ L-VH 4/6	CCCAGATGGGTCCTGTCCCAGGTGCAG
5′ L-VH 5	CAAGGAGTCTGTTCCGAGGTGCAG
5′ AgeI VH1	CTGCAACCGGTGTACATTCCCAGGTGCAGCTGGTGCAG
5′ AgeI VH1/5	CTGCAACCGGTGTACATTCCGAGGTGCAGCTGGTGCAG
5′ AgeI VH3	CTGCAACCGGTGTACATTCTGAGGTGCAGCTGGTGGAG

5′ AgeI VH3-23	CTGCAACCGGTGTACATTCTGAGGTGCAGCTGTTGGAG
5′ AgeI VH4	CTGCAACCGGTGTACATTCCCAGGTGCAGCTGCAGGAG
5′ AgeI VH 4-34	CTGCAACCGGTGTACATTCCCAGGTGCAGCTACAGCAGTG
5′ AgeI VH 1-18	CTGCAACCGGTGTACATTCCCAGGTTCAGCTGGTGCAG
5′ AgeI VH 1-24	CTGCAACCGGTGTACATTCCCAGGTCCAGCTGGTACAG
5′ AgeI VH3-33	CTGCAACCGGTGTACATTCTCAGGTGCAGCTGGTGGAG
5′ AgeI VH 3-9	CTGCAACCGGTGTACATTCTGAAGTGCAGCTGGTGGAG
5′ AgeI VH4-39	CTGCAACCGGTGTACATTCCCAGCTGCAGCTGCAGGAG
5′ AgeI VH 6-1	CTGCAACCGGTGTACATTCCCAGGTACAGCTGCAGCAG
5′ L Vκ 1/2	ATGAGGSTCCCYGCTCAGCTGCTGG
5′ L Vκ 3	CTCTTCCTCCTGCTACTCTGGCTCCCAG
5′ L Vκ 4	ATTTCTCTGTTGCTCTGGATCTCTG
5′ Pan Vκ	ATGACCCAGWCTCCABYCWCCCTG
5′ AgeI Vκ 1-5	CTGCAACCGGTGTACATTCTGACATCCAGATGACCCAGTC
5′ AgeI Vκ 1-9	TTGTGCTGCAACCGGTGTACATTCAGACATCCAGTTGACCCAGTCT
5′ AgeI Vκ 1D-43	CTGCAACCGGTGTACATTGTGCCATCCGGATGACCCAGTC
5′ AgeI Vκ 2-24	CTGCAACCGGTGTACATGGGGATATTGTGATGACCCAGAC
5′ AgeI Vκ 2-28	CTGCAACCGGTGTACATGGGGATATTGTGATGACTCAGTC

5′ AgeI Vκ 2-30	CTGCAACCGGTGTACATGGGGATGTTGTGATGACTCAGTC
5′ Age Vκ 3-11	TTGTGCTGCAACCGGTGTACATTCAGAAATTGTGTTGACACAGTC
5′ Age Vκ 3-15	CTGCAACCGGTGTACATTCAGAAATAGTGATGACGCAGTC
5′ Age Vκ 3-20	TTGTGCTGCAACCGGTGTACATTCAGAAATTGTGTTGACGCAGTCT
5′ Age Vκ 4-1	CTGCAACCGGTGTACATTCGGACATCGTGATGACCCAGTC
5′ L Vλ 1	GGTCCTGGGCCCAGTCTGTGCTG
5′ L Vλ 2	GGTCCTGGGCCCAGTCTGCCCTG
5′ L Vλ 3	GCTCTGTGACCTCCTATGAGCTG
5′ L Vλ 4/5	GGTCTCTCTCSCAGCYTGTGCTG
5′ L Vλ 6	GTTCTTGGGCCAATTTTATGCTG
5′ L Vλ 7	GGTCCAATTCYCAGGCTGTGGTG
5′ L Vλ 8	GAGTGGATTCTCAGACTGTGGTG
5′ AgeI Vλ 1	CTGCTACCGGTTCCTGGGCCCAGTCTGTGCTGACKCAG
5′ AgeI Vλ 2	CTGCTACCGGTTCCTGGGCCCAGTCTGCCCTGACTCAG
5′ AgeI Vλ 3	CTGCTACCGGTTCTGTGACCTCCTATGAGCTGACWCAG
5′ AgeI Vλ 4/5	CTGCTACCGGTTCTCTCTCSCAGCYTGTGCTGACTCA
5′ AgeI Vλ 6	CTGCTACCGGTTCTTGGGCCAATTTTATGCTGACTCAG
5′ AgeI Vλ 7/8	CTGCTACCGGTTCCAATTCYCAGRCTGTGGTGACYCAG
5′ Ab sense	GCTTCGTTAGAACGCGGCTAC

Table 2.2 Reverse Primer Sequences

Reverse Primer	5′ - 3′ sequence
3′ Cγ CH1	GGAAGGTGTGCACGCCGCTGGTC
3′ Cμ CH1	GGGAATTCTCACAGGAGACGA
3′ IgG (internal)	GTTCGGGGAAGTAGTCCTTGAC
3′ SalI JH 1/2/4/5	TGCGAAGTCGACGCTGAGGAGACGGTGACCAG
3′ SalI JH 3	TGCGAAGTCGACGCTGAAGAGACGGTGACCATTG
3′ SalI JH 6	TGCGAAGTCGACGCTGAGGAGACGGTGACCGTG
3′ Cκ 543	GTTTCTCGTAGTCTGCTTTGCTCA
3′ Cκ 494	GTGCTGTCCTTGCTGTCCTGCT
3′ BsiWI Jκ 1/4	GCCACCGTACGTTTGATYTCCACCTTGGTC
3′ BsiWI Jκ 2	GCCACCGTACGTTTGATCTCCAGCTTGGTC
3′ BsiWI Jκ 3	GCCACCGTACGTTTGATATCCACTTTGGTC
3′ BsiWI Jκ 5	GCCACCGTACGTTTAATCTCCAGTCGTGTC
3′ Cλ	CACCAGTGTGGCCTTGTTGGCTTG
3′ XhoI Cλ	CTCCTCACTCGAGGGYGGGAACAGAGTG

2.5 Software

Prism 9, GraphPad Software	GraphPad Software Inc
Image Lab Software	Bio-Rad
Geneious	Dotmatics
Fiji distribution of ImageJ	Schindelin*et al*, 2012, (285)

2.6 Cell Culture methods

2.6.1 BCG culture

Middlebrook 7H9 broth was prepared with 0.22 μm filtered ddH_2O, according to manufacturer instructions, and supplemented with 10% v/v Middlebrook, 0.2% v/v glycerol and 0.15% v/v Tween 80

(lab stock). Broth was autoclaved prior to supplementation, and 0.22 µm filtered prior to use. *Mycobacterium bovis* BCG Pasteur was cultured in prepared 7H9 broth in 50ml sterile polyethylene conical flasks, incubated at 37 °C with 150-200 rpm agitation.

2.6.2 THP-1 cell culture

THP-1 cells were cultured in RPMI-1640 + L-glut/Pen-Strep/10% FCS (R10 media). Cells were maintained at 37 °C and 5% CO2. Cells were dissociated with 5 mM EDTA in PBS for passaging and seeding for experiments.

2.6.3 HEK293T Cell Culture

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% FCS, 2 mM GlutaMAX, 50 U/ml penicillin and 50 µg/ml streptomycin. Cells were maintained at 37 °C and 5% CO_2. Cells were dissociated with 5 mM EDTA in PBS for passaging and seeding for experiments.

2.6.4 MDM differentiation and seeding

On the day required for seeding, THP-1 cell culture suspension was centrifuged at 2000 rpm for 5 minutes at a required volume per assay. Pelleted cells were resuspended in fresh R10 media and cell density was determined using a haemocytometer. 1x 10^5 cells were seeded in to wells in 96-well plates, in 200 µl R10 media with 20 nM PMA added. Cells were incubated at 37 °C with 5% CO_2, then media was replaced with fresh R10.

2.7 Molecular Biology & Immunology

2.7.1 Flow Cytometry and cell sorting

Cell samples were pelleted by centrifugation at 1500 rpm for 5 minutes. Cells were resuspended in 1ml FACS buffer and incubated for 10 min at RT before being used for cell sorting. In parallel, aliquots of 0.5 x 10^6 cells were prepared, to be used as either the unlabelled control or to be singly labelled with one antibody from the panel per aliquot, to be used as compensation controls. All aliquots were pelleted by centrifugation at 2000 rpm for 5 minutes. Cells were resuspended in 50 µl FACS buffer with anti-CD3 PerCP (4µl); anti-CD27 FITC (4µl); anti-CD38 APC (4µl); anti-CD20 PerCP (4µl); anti-CD19 PE (1µl), or each one of the antibodies separately, as appropriate for the sorting and compensation

samples. Samples were incubated for 30 min on ice before being washed with 2 ml FACS buffer and centrifuged at 2000 rpm for 5 min. Cells were resuspended in 300 – 500 µl FACS buffer and passed through a cell strainer to removed clumps of cells.

Prepared cell suspensions were sorted on a BD FACSAria with gating $CD19^{+}/CD20^{-}/CD3^{-}/CD27^{high}/CD38^{high}$. Single cells were sorted into PCR plates, with wells containing 10 µl of RT-PCR catch buffer. Row H was reserved for negative controls, where only RT-PCR catch buffer was added. Immediately after sorting each plate was sealed with a plate seal and centrifuged at 1000 rpm for 1 min. Plates were kept on dry ice during the sorting process, following by storage at -80 °C. For bulk PCR, 1000 cells were sorted as above into 1.5 ml RNase-free microcentrifuge tubes. These were immediately centrifuged at 1000 rpm for 3min and stored at -80 °C via transfer on dry ice.

2.7.2 Antibody cloning

2.7.2.1 Single cell RT-PCR for Heavy chain gamma and Lambda and Kappa chains

Following cell sorting, monoclonal antibodies were isolated from single plasmablasts as per Tiller *et al* (2008) and Wilson *et al* (2009) described briefly here (129,130). Qiagen OneStep RT-PCR kit was used for single-cell amplification of Individual IgH and IgL(k or λ) genes. Forward primers used were an equimolar mixture (100 µM) L-VH1, L-VH3, L-VH4/6, L-VH5, L-VK1/2, L-VK 3, LVK4, L-Vlambda1, L-Vlambda2, L-Vlambda3, L-Vlambda4/5, L-Vlambda6, L-Vlambda7, L-Vlambda8 (sequences listed in table 2.1). Reverse primers used were an equimolar mixture (100 µM) CgammaCH1, CK543, Clambda in ddH_2O (sequences listed in table 2.2). 15 µl RT-PCR reaction mix (0.08 µM forward primer mix, 0.08 µM reverse primer mix, 13.3 µM dNTP, 10× buffer, 0.5 µl PCR enzyme mix and ddH_2O) was added to each well, including the cell-free "negative control" wells. 1 µl stock RNA was added to one control well for a positive control. RT-PCR was run with an amplification profile of denaturation for 30 minutes at 50 °C and 15 minutes at 95 °C, followed by 50 amplification cycles of 30 seconds at 94 °C, 30 seconds at 58 °C and 1 minute at 72 °C, before a final incubation of 10 minutes at 72 °C.

2.7.2.2 Nested PCR for Heavy, Lambda or Kappa chains

IgG heavy or light (kappa or lambda) chains were amplified by nested PCR using Qiagen HotStarTaq PCR kit. Primer sequences specific to amplification of the heavy (5'AgeI VH and 3'SalI JH mixes), lambda (5'AgeI Vλ and 3'XhoI Cλ mixes), or kappa (PanVκ or 3'Cκ494) chain (sequences are listed in tables 2.1 and 2.2). 3 µl RT-PCR product was mixed with 40 µl PCR reaction mix (0.25 µM forward

primer mix, 0.25 µM reverse primer mix, 0.0125 µM dNTPs, 1x HotStarTaq enzyme, ddH_2O). PCR was run with a thermocycler profile of 15 minutes at 95 °C, 50 amplification cycles of 30 seconds at 94 °C, 30 seconds at 58 °C and 45 seconds at 72 °C, before a final incubation of 10 minutes at 72 °C.

PCR products were separated by gel electrophoresis with a 1% agarose gel. The expected heavy and light chain products were isolated from the gel using the QiAquick Gel extraction kit. PCR product sequencing was used to determine the appropriate primer set to use for PCR cloning (sequences are listed in tables 2.1 and 2.2), followed by PCR amplification as per the nested PCR reaction described above. PCR products were gel purified as above.

2.7.2.3 Expression vector cloning

Purified PCR products were restriction-enzyme digested (heavy chain gene: AgeI-HF, SalI-HF; kappa light chain: AgeI-HF, BsiWI; lambda light chain: AgeI-HF, XhoI) in the manufacturer-recommended buffers, were incubated for 6 hours at 37 °C (heavy and lambda chains genes) or 55 °C (kappa chain genes). Digestion products were gel purified and ligated into the appropriate dephosphorylated linearised expression vector – namely either human Igγ1, Igκ or Igλ expression vectors containing a murine Ig gene signal peptide sequence, a multiple cloning site upstream of the human Igγ1, Igκ or Igλ constant regions, HCMV promotor driving transcription, and an ampicillin resistance gene.

Competent DH5α *Escherichia coli* was transformed with the ligated expression vectors and screened for recombinance by ampicillin-resistant growth on selection media LB plates. Vector-positive colonies were picked and expanded by overnight liquid culture growth, followed by plasmid purification using a Qiagen miniprep kit reaction. Purified plasmid was checked for the gene insert by small-scale plasmid digestion. Heavy chain inserts were digested with EcoRI and SalI; Lambda chain inserts were digested with AgeI-HF and XhoI; Kappa chain inserts were digested with EcoRI and HindIII. 3 µl plasmid was mixed with 13 µl digestion mixture with the appropriate restriction enzymes, BSA and the manufacture recommended buffer preparation. The digestion product sizes were checked by agarose gel electrophoresis. Confirmed inserts were sequenced prior to transfection in other expression systems.

2.7.2.4 Transfection of 293T cells

293T cells were seeded at $2x10^4$ cells/ cm^2 in complete D-10 medium and incubated overnight (until 50 – 80% confluent) in standard culture conditions (37 °C and 5 % CO_2). Transfection mix was prepared by mixing 240 µl un-supplemented DMEM with 2.5 µl each of the purified heavy chain and lambda chain plasmid constructs, and 10 µl Polyethylenimine (PEI) and incubating the mix for 15 min at RT. The transfection mix was added to cells, followed by 24 hours incubation under standard culture conditions. Cell culture media was changed to UltraDOMA-PF followed by 4-5 days culture incubation

as above. Cell culture medium was collected and centrifuged at 3500 rpm for 15 minutes and stored at 4 °C until processed.

2.7.3 Enzyme-Linked Immunosorbent Assay

Fresh protein-coating solution was prepared by mixing the relevant antigen in sodium carbonate buffer, at the appropriate concentrations, as per Table 2.3. 100 µl of protein-coating buffer was added to each well in flat-bottomed 96 well microplates. The plates were then sealed and incubated overnight at 4 °C. Plates were equilibrated back to room temperature (RT), protein-coating buffer was removed, and each well was washed 3x with 100 µl PBST. Each well was then incubated with 200 µl ELISA blocking buffer and sealed for 1 hour at RT. Final volume of 500 µl of the appropriate positive and negative control antibodies were prepared in blocking buffer as per table 2.1, as well as triplicate 1 ml preparations of samples (1:100 in blocking buffer). Blocking buffer was removed from the prepared ELISA plates and plates patted dry. 100 µl of each sample or control condition were transferred to fresh wells in the ELISA plates and the plates were sealed and incubated at RT for 2 hours. Samples were removed from each plate well and wells were washed 5x with PBST and plates were patted dry. 100 µl anti-human IgG (1:500 in blocking buffer) was added to each sample and negative control well; 100 µl anti-mouse IgG (1:1000 in blocking buffer) was added to the positive control wells. Plates were sealed and incubated at RT for 1 hour. Antibody solutions were removed from each well and wells were washed 7x with PBST before the plates were patted dry. 3,3',5,5'-tetramethylbenzidine (TMB) was prepared according to suppliers' directions, and 100 µl was added to each well before the plates were incubated at RT for 25 minutes in the dark. The colour development reaction was stopped by the addition of 100 µl per well of 2M sulfuric acid and 67 µl per well of 16% paraformaldehyde. Optical density (OD) for each well, was measured at 450 nm using a microplate reader.

Table 2.3: Antigen and control preparations used for ELISA assays

Protein/ Antigen target	**Concentration in protein-coating buffer**	**Positive control antibody**	**Concentration in protein-coating buffer**	**Negative control antibody**	**Concentration in protein-coating buffer**
Recombinant Ag85A	1.6 µg/ml	Serum	1:100	Non-responder serum	1:100
M.tb whole cell lysate	2 µg/ml	Serum	1:100	Non-responder serum	1:100

ESAT-66/CFP-10 recombinant protein	4 µg/ml	Serum	1:100	Non-responder serum	1:100
M.tb extracts (cell wall, cell membrane, cytosol)	2 µg/ml	78_59 mAb	Varying	Isotype control mAb	Per 78_59 concentration

For measurement of TNF-α, IL-1β, and IL-6, RND systems Quantikine® ELISA kits were used per the manufacturers guidelines.

2.7.4 ADCP assay

THP-1 cells were differentiated to MDM in a 96 well plate two days prior to planned BCG infection, as per section 2.6.4. BCG-GFP was cultured to exponential phase as per section 2.6.1. BCG-GFP culture was pelleted by centrifugation at 3500 rpm for 10 min. The pellet was washed by reconstitution in the same volume of sterile PBS and centrifuged as above. The pellet was then resuspended in 5 ml infection media (RPMI-1640 + 10% FCS/L-glut) in a 15 ml centrifuge tube. The tube was sealed with parafilm and sonicated for 15 min. Bacterial clumps were removed by centrifugation at 2000 rpm for 2 min. The supernatant of bacterial suspension was transferred to a clean tube and OD was measured at 600 nm. The multiplicity of infection (MOI) of the suspension was calculated based on the formula MOI = OD x $1x10^8$ CFU/ml. Sample serum (1:10) was mixed with BCG-GFP (MOI 1) in infection media to a final volume of 80 µl per sample, with samples prepared in duplicate, sealed, and incubated at 37 °C for 30-60 min without shaking. R10 media was removed from differentiated THP-1 MDM, and 50 µl of BCG-GFP co-incubated with serum in infection media was added to each well. Plates were incubated for 2 hrs at 37 °C. Cells in each well were washed with 200 µl infection media. Cells were dissociated with 50 µl EDTA or TrypsinLE-PBS per well and incubated for 20-30min at RT. The cell suspensions were transferred to a clean 96-well V-bottom plate, following microscopic confirmation that cells had fully detached from the well surface. Cells were pelleted at 1500 rpm for 5 min and washed in FACS buffer, followed by fixation in 50 µl 4% PFA in PBS for 20-30 min at RT. Fixed cells were washed twice in 200 µl FACS buffer, the suspended in 50 µl FACS buffer for processing via flow cytometry.

2.7.5 Direct PBMC Mycobacterial Growth Inhibition Assay

The impact of PBMC and serum factors on the growth of mycobacteria was assessed as per Bitencourt *et al* in the McShane laboratory (231).

2.7.5.1 Preparation of bacterial growth standard curve

800 µl enriched MGIT PANTA (lyophilised PANTA reconstituted with MGIT growth supplement) was added to each of two BACTEC MGIT tubes per dilution condition to be tested. A series of seven 10-fold dilutions of freshly thawed mycobacterial stock, in PBS-Tween 80, was prepared in sterile 2 ml tubes. 500 µl of each dilution step was inoculated into two prepared MGIT tubes per condition. Each tube was tightly closed and mixed by inversion. The inoculated MGIT tubes were places into the BACTEC MGIT instrument, and the time to positivity (TTP) was recorded.

20 µl of each dilution was spotted on 7H10 or 7H11 agar plates, in triplicate, which were sealed and incubated for 10 – 28 days at 37 °C. Colonies were counted as soon as they were visible. Standard curves of TTP against equivalent input CFU were plotted and regression analysis was used to validate the fit of the data by R_2 calculation.

2.7.5.2 MGIA Assay preparation

Cryopreserved human PBMC were partially thawed in a 37 °C water bath followed by gentle agitation by pipetting and the addition of 10 ml warm R10 media without any antibiotic supplementation. Thawed cells were transferred to a fresh centrifuge tube and pelleted by centrifugation at 1500 rpm for 5 minutes. Cells were resuspended at approximately 2-3 $x10^6$ cells/ ml in R10 media without antibiotics, with 50 U/ml of benzonase. Cells were left to recover for 2 hours under standard cell culture incubation conditions. Cells were counted using a cytometer, pelleted as above, and resuspended at 10 $x10^6$ cells/ ml in RPMI-MGIA. 300 µl of cell suspension was transferred into wells in a 48-well plate, followed by 120 µl of non-heat inactivated autologous serum or plasma, matched to the donor and time-point. Mycobacteria stock was thawed at RT and diluted to 100 CFU per 180 µl in RPMI-MGIA, based on the CFU measurement assay in section 2.7.5.1. 100 CFU (180 µl) of the mycobacterial preparation was added to each sample well in the 48-well plate. The plate was incubated at 37 °C with 5% CO_2 for 96 hours. For controls, three MGIT tubes were supplemented with 800 µl enriched MGIT PANTA (as per section 2.7.5.1) to make enriched Middlebrook 7H9. 100 CFU mycobacterial preparation was added directly to two of the of supplemented tubes. The contents of the third tube were decanted, mixed with 100 CFU mycobacterial preparation to make up a final volume of 500 µl, and returned to the same MGIT tube. All three tubes were placed on to the MGIT machine.

2.7.5.3 MGIA assay processing

Prior to harvesting the cultures, 1 MGIT tube per culture well was supplemented with 800µl PANTA enrichment and label. Cultures were collected from the 48-well plate into 2ml screw-cap tubes and centrifuged at 12 000 rpm for 10 minutes. 500 µl of supernatant was removed and stored separately for downstream cytokine quantification. Wells were washed by adding 500 µl endotoxin free water and incubating at RT for 5 minutes before pipetting to detach remaining monocytes and then transferring the water to the corresponding cell and bacterial pellet. Each pellet suspension was pulse vortexed for 1 second immediately prior to transfer of 600 µl to a corresponding MGIT tube. All MGIT tubes were mixed by inversion and placed in the MGIT machine until positivity was detected.

2.7.5.4 MGIA data processing

Time-to-positivity (TTP) was recorded for all conditions and converted to CFU based on the standard curve prepared in section 2.7.5.1. Results across multiple timepoints or experiments were normalised to the direct-to-MGIT controls, by dividing the number of total CFU per sample by the number of total CFU in the direct-to-MGIT control, to give a read-out of fold-change in bacterial number, or Δlog10 CFU.

2.7.6 Flow cytometric assessment of B cells in TB

The labelling solution for the full antibody panel was made with each of the antibodies as per table 2.4 in BV buffer, to a total volume of 1200 µl. Fresh 1x FACS buffer was prepared and filtered. The near-infra red live/dead stain was dilutions 1 in 1000 in PBS, and the Fc blocker was dilution 1:40 in FACS buffer, to a final volume of 800 µl.

Table 2.4: Antibodies used to characterise B cells in TB

Antibody target	**Fluorochrome**	**Volume (µl): 1 sample**	**Volume (µl): 20 samples**
IgM	PE-Cy7	0.1	2
IgD	BB700	0.15	3
CD69	BV786	1.25	25
CD3	APC-eF780	2.5	50
CD14	BV605	2.5	50
CD27	BV711	2.5	50

CD21	PE-Cy7	2.5	50
CD23	PE	2.5	50
CD19	FITC	5	100
CD16	APC-eF780	5	100
CD1c	BV421	5	100
CD86	APC-eF780	5	100
CD45	Alexa700	5	100
Live/Dead	Near IR	--	--

Cell samples were thawed at 37 °C, diluted in a further 10 ml warm RPMI and pelleted by centrifugation at 500 x *g* for 10 minutes. Cells were resuspended in 200 µl and transferred to a 96 well plate. Cells were pelleted in the plate by centrifugation 1800 rpm for 4 min, washed with PBS, and pelleted as above. 100 µl of near-infra red live/dead stain was added to each well and incubated for 20 min at 4 °C in the dark. 100 µl PBS was added to each well, followed by centrifugation as above and washing twice in PBS as above. Cells were resuspended in 40µl freshly prepared Fc block (stock diluted 1:40 in FACS buffer) and incubated for 10 min, followed by addition of 60 µl labelling solution for the full antibody panel, freshly prepared as per table 2.4 in BV buffer. Samples were incubated for 20 minutes at RT in the dark. An equal volume of FACS buffer was added, followed by centrifugation as above and washing twice in PBS as above. Cells were resuspended in 100 µl of cytofix/cytoperm, incubated for 20 minutes and washed as above. Data was then acquired on a BD LSR II flow cytometer.

2.7.7 Screening of mAbs against live BCG

Passage 2 culture of BCG at an OD600 of 0.3-1.0 was pelleted by centrifugation at 1200 rpm for 5 minutes. 1 cm glass beads were added to the culture and the culture was shaken manually for up to 3 minutes. Bacteria were pelleted as above, and supernatant was collected and aliquoted. The bacterial pellet was washed with PBS, then resuspended in 10 µg/ml primary antibody in PBS. The labelling solution was incubated at 37 °C in a shaking incubator for 1. 5 hours, followed by washing the bacteria in PBS as above. Bacteria were fixed at in 5 % PFA in PBS for 10 minutes. Samples were washed as above in incubated with anti-Human IgG conjugated to FITC (anti-Hu IgG-FITC) for 1 hour at 4 °C with agitation. Samples were washed as above and resuspended in PBS ahead of detection by flow cytometry or by fluorescence microscopy.

2.7.8 Screening of mAbs against *M.tb* lysates

A panel of *M.tb* protein lysates (BEI Resources) was run on an automated BioRAD 12% SDS-PAGE electrophoresis gel. Binding of primary antibody (78_59) was assessed at a concentration 7.5 µg/ml, using an iBind Kit as per manufacturer's instructions.

2.7.9 Fluorescence microscopy

Antibody-bound GFP-expressing BCG were prepared as per section 2.10.7, with an anti-Human IgG conjugated to AlexaFluor 594. Bacteria were deposited in a thin layer on a 1% low-melt agarose pad on a 22 mm x 40 mm #1.5 glass coverslip. The coverslip was mounted onto a standard microscope slide. The BCG were image on a Zeiss AxioObserver inverted widefield microscope with a 100x/1.4NA Ph3 oil immersion objective, Colibri 7 LED illumination and quad-bandpass filter set 62 HE. Fluorescence and phase contrast images were collected on a photometrics P95 camera. Images were process and analyses in Fiji/image J(285).

2.7.10 Whole blood assays

Venesection was performed on donors using standard protocols. Whole blood in sodium heparin tubes was aliquoted into microcentrifuge tubes with a volume of 450 µl and incubated on a rotor at 37 °C for 24 hours with combinations of mAb and antigen as described in Chapter 3, After 24 hours samples were pelleted in a microcentrifuge at 5000 rpm for 10 minutes. The supernatant was stored for downstream ELISA assays. For *M.tb* growth assays in Biosafety Lab 3, tubes were inoculated with a standard CFU of *M.tb* H37Rv and incubated at 37 °C for 4 days as per Riou *et al* (286). Microcentrifuge tubes were then pelleted at 3000 rpm for 10 min, and the pellet inoculated into MGIT tubes for measurement of TTP.

2.8 Statistical methods

Analysis of all data was performed using GraphPad Prism (GraphPad Software Inc) with the following exceptions. For analysis of antibody clonality, Geneious (Dotmatics) was used. For flow cytometry and FACS assays, FlowJo (TreeStar) was used, and data exported into GraphPad Prism for visualization and analysis. Mann-Whitney test was used for comparing two non-parametric and unpaired groups. Kruskal-Wallis test with Dunn's correction for multiple comparisons was used for comparing more than two unpaired non-parametric groups. For two paired non-parametric groups, Wilcoxon signed-rank test was used. For more than two paired groups. For more than two paired non-parametric groups, Friedman two-way analysis of variance was used. P values are defined as significant when < 0.05.

2.9 Clinical cohorts

Samples from multiple clinical studies were used across the chapters of this thesis to address specific questions. Study name, locations in the thesis, recruitment details, human research ethics committee (HREC) approval, and reference publications with full cohort descriptions are given below (table 2.5).

Table 2.5: Clinical cohort and patient recruitment details

Study	Chapters featured	Recruitment	Ethics Approval	Reference publications
EU study	3, 4, 6	Adult participants were recruited at the uBuntu clinic, Site B, Khayelitsha. This is a peri-urban settlement of Cape Town, South Africa with a high incidence of TB cases. ATB was defined by smear positivity and/or mycobacterial culture with compatible symptoms. LTBI was defined by a positive in-house IGRA assay. Ethics was also obtained for further exploratory work relating to the role of antibodies in TB.	UCT HREC 012/007 UCT HREC 525/2014	Oni *et al* (287) Berry *et al* (288)
ASH Study	3	Adult participants were recruited from Ubuntu Clinic, Site B, Khayelitsha. ATB was defined by sputum Xpert MTB/RIF positivity. LTBI was defined as being asymptomatic with a positive IGRA.	UCT HREC 050/2015	Riou *et al* (286)
RECON study	4	HIV-1 infected adults initiating ART were recruited at the Ubuntu Clinic, Site B, Khayelitsha. Samples were collected at baseline and after 1, 3, and 6 months of ART. LTBI was determined by a positive IGRA test. Patients with symptoms suggestive of ATB, on INIH prophylaxis, or who were culture positive for *M.tb,* were excluded.	UCT HREC 245/2009 and 545/2010	Jhilmeet *et al (*289)
MVA85A (TB021) study	4	Samples were acquired during a randomized, double-blind, placebo-controlled, phase IIa vaccine study of the MVA85A vaccine. Participants were enrolled at clinical sites in Cape Town, South Africa and Dakar, Senegal. All participants were HIV-1 infected with a $CD4^+$ T cell count above 350 cells/mm^3 or more than 300 cells/mm^3 if receiving ART. ATB was defined by the GeneXpert MTB/RIF test. LTBI was defined by a positive QFT or TST.	UCT HREC 001/2010 Oxtrec 01-10	Ndiaye *et a*l (52)
HIV-1-positive persistently TB, tuberculin and IGRA negative	4	Adult PLWH in Cape Town, South Africa aged between 35 and 60 were enrolled. All participants had prolonged periods of a CD4+ cell count less than 350 cells/mm^3 , or one measurement below 200 cells/mm^3 . Participants were screened for TB symptoms, and QFT and TST were serially performed to establish the phenotype of persistent TST and IGRA negativity.	HREC of Stellenbosch University (N16/03/033 and N16/03/033A) UCT HREC 755/2016 and 702/2017	Kroon *et al* (192)
BCG infected participants (TB 038)	5	Samples were acquired during a randomised controlled study of healthy BCG naive adults from near Oxford or Birmingham in the United Kingdom. LTBI was excluded by T.spot or QFT. For the BCG arm, participants received a standard dose ($2–8 \times 10^5$ CFU) of intradermal BCG SSI.	NHS Research Ethics Service (NRES) 15/SC/0022	Wilkie *et al* (290)
Pericardial samples (IMPI-Africa study)	6	Pericardial fluid and PBMC were obtained from patients referred to Groote Schuur Hospital, Cape Town, with suspected tuberculous pericardial effusions as part of the Investigation of the Management of Pericarditis in Africa (IMPI-Africa) study.	UCT HREC REF: 102/2003, 402/2005 & 289/2007	Matthews *et al* (291)

Chapter 3: Generation and characterization of human monoclonal antibodies generated from patients with active TB

3.1 Introduction

ATB has long been known to induce hypergammaglobulinemia (141). More recently, upregulation of FcγR1A/B and C1q have also been shown to be a consistent feature of ATB. Several possibilities for the role of these antibodies can be postulated.

It is plausible that antibodies that assist in controlling *M.tb* arrive 'too little, too late' in ATB. Another possibility is that antibodies do not reach or engage with intracellular bacilli during disease and are largely bystanders in the host immune response. A third possibility is that antibodies may directly contribute to the pathogenesis of disease. Here, the rise in CIC and Fc receptor transcription that increases from asymptomatic to symptomatic TB could play a direct role in pathogenesis through induction of inflammatory cascades. Finally, all three can co-exist during ATB, and the epitope specificity, subclass, and isotype would distinguish what role an individual clone plays during infection. It is technically impossible to delineate these antibody characteristics in serum, and thus the means to clone and study mAbs individually in TB is vital.

Since the discovery of hybridoma technology, many murine mAbs have been raised against TB. MAbs against HBHA, Acr, and LAM, showing protection against *M.tb* in mouse models have been described (226). However, the technology to clone mAbs directly from humans has only emerged over the past decade. Tiller *et al* (2008), and Smith *et al* (2009) described a process of applying cell sorting to isolate antibody-producing cells and using nested PCR with degenerate primers to amplify the variable regions of the heavy (VH) and light (VL) chains of the individual B cell (292,293). The VH and VL genes can then be cloned into an IgG1 vector for expression in mammalian cells. Initial applications of this technique saw the rapid development of broadly neutralizing mAbs against a range of viral pathogens such as HIV, influenza, Dengue and Ebola (131,133,294,295). Most recently, there has been widespread adoption of human mAb generation from patients with convalescent COVID-19 infection that has resulted in clinical use of therapeutic mAbs (296). The challenge of applying this method to TB is the markedly greater number of antigens, the uncertainty as to what functions of antibodies to explore, and the question of which human population to select for in-depth antibody characterization.

Thus far, 3 publications using these methods in TB have been published, with their findings summarized in Table 3.1.

Table 3.1 Summary of studies involving the characterization of patient-derived mAbs in TB.

Author	**Year**	**Patient**	**Cells of origin**	**Antigens**	**Function**
Zimmerman *et al* (297)	2016	LTBI and ATB	Plasmablasts and memory B cells	HBHA	IgA protective IgG drives uptake of *M.tb* into alveolar epithelial cells
Watson *et al* (233)	2021	ATB	PstS1-specific B cells	PstS1	Restricts growth of *M.tb* in human macrophages and *in vivo*.
Ishida *et al* (158)	2021	LTBI	AM-specific B cells	AM	Staining of *M.tb* in mouse lung tissue

For the work in this chapter, I hypothesized that mAbs cloned from TB patients would differ in antigen recognition and thus function. Antibodies in ATB could either i) restrict the growth of *M.tb* via mechanisms discussed in chapter 1 ii) drive inflammation in the absence of killing via CIC and FcR ii) have no impact on disease models. Here, I describe the generation of fully human mAbs against *M.tb*, and the in-depth characterization of a human mAb for which the antigenic target was discovered.

3.2 Results

3.2.1 Cloning of human mAbs from patients with ATB

Nine patients enrolled in an observational study (UCT HREC 012/007 and 525/2014) with the following clinical phenotypes were selected. i) HIV negative adults, ii) Pulmonary TB with microbiologically confirmed TB iii) Pre-ATT, iv) Multi-bacillary disease as evidenced by 2^+ or 3^+ acid-fast bacilli on sputum smear microscopy. The rationale behind selecting these patients was to select those with the greatest frequency of *M.tb*-specific plasmablasts, given prior studies showing that multibacillary

patients possessed higher titres of antibodies. The method to clone mAbs from human plasmablasts was adapted from Tiller *et al* (2008) and Smith *et al* (2009), as summarized in Figure 3.1 (3,4)**.**

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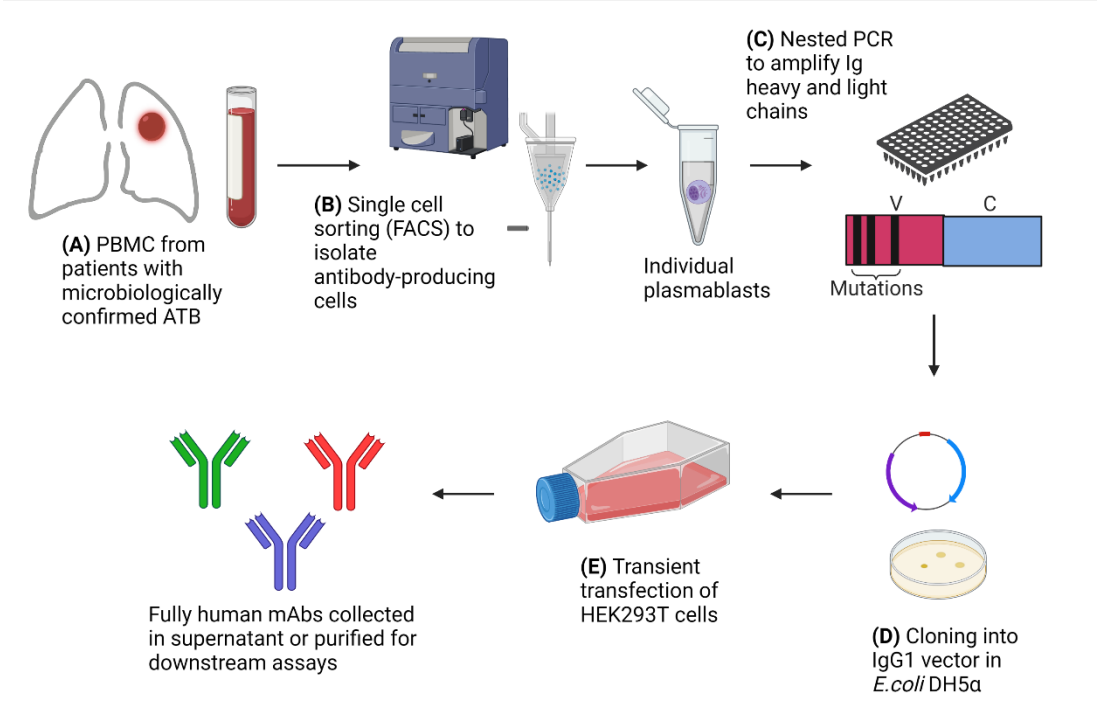


Figure 3.1 Schematic detailing methods of cloning human mAbs from patient with ATB (created in Biorender.com) **(A)** Cryopreserved PBMC from patients with microbiologically confirmed ATB was selected for cloning. **(B)** Fluorescence-activated cell sorting (FACS) was performed to isolate plasmablasts with the $CD3^{-}/CD20^{-}/CD19^{+}/CD27^{-}/CD38^{hi}$ phenotype **(C)** RT-PCR and nested PCR was performed on individual plasmablast samples to amplify VH and VL genes using degenerate primers **(D)** VH and VL genes were ligated into an IgG1 expression vector and bulked up in transformed E. coli DH5α **(E)** Intact IgG1 mAbs were expressed in HEK293T cells by transient transfection using polyethylenimine (PEI).

In brief, Fluorescence-activated cell sorting (FACS) was performed to individually isolate plasmablasts from patients with ATB. Representative gating for cell sorting is shown in Figure 3.2, with the results of frequency of plasmablasts in total PBMC.

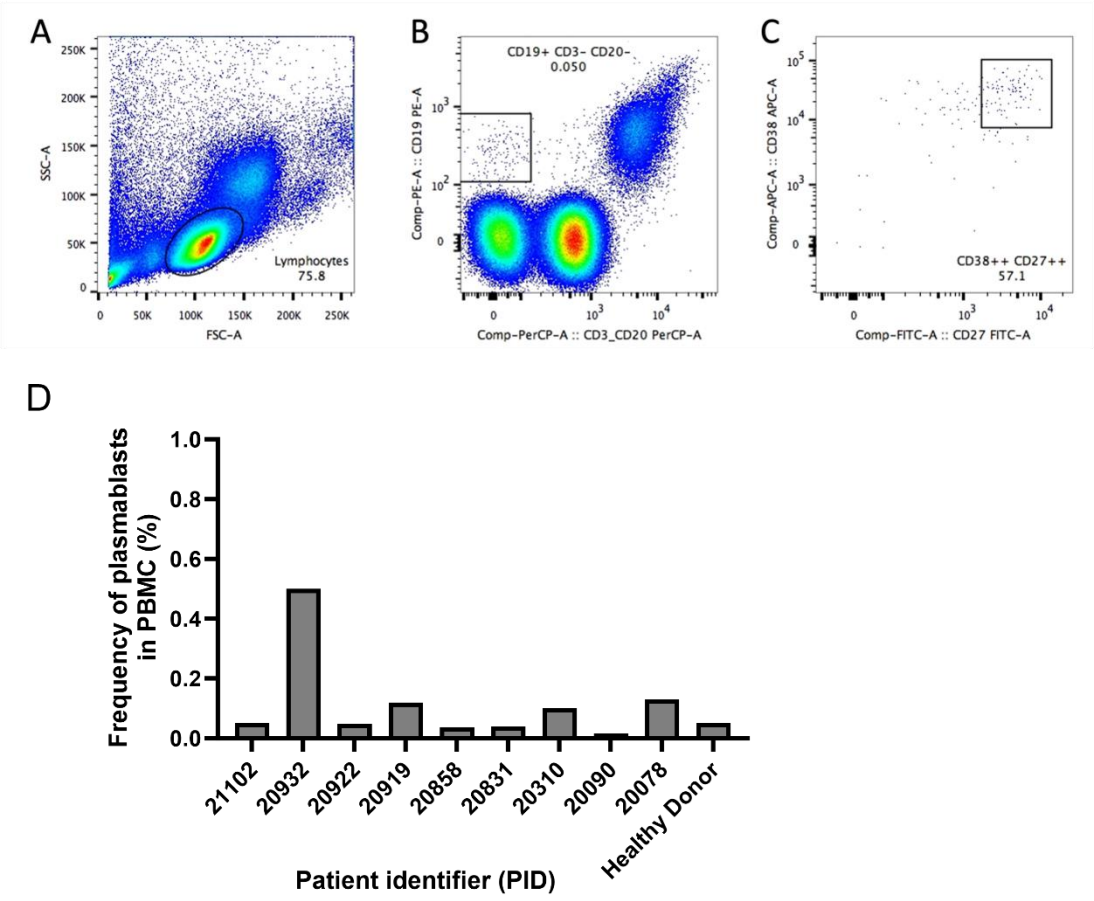


Figure 3.2 Donor representation of gating strategy for the sorting of individual plasmablasts and frequency of plasmablasts in PBMC. **(A)** After doublet exclusion, cells were size gated for the lymphocyte population. **(B)** $CD19^{+}/CD3^{-}/CD20^{-}$ cells were selected. **(C)** Next, $CD38^{hi}$ /$CD27^{+}$ were selected. **(D)** Frequency of plasmablasts in total PBMC of patients selected for mAb cloning (n=9). A healthy donor is included as a reference. PIDs = patient identifiable data.

The median plasmablast frequency amongst this group of patients with ATB (n=9) ranged from 0.016% to 0.5% (median of 0.05%). A healthy donor included in this panel possessed a plasmablast frequency of 0.05%. Due to limitations with FACS plate sorting equipment, cells were sorted into individual Eppendorf tubes containing RNAse inhibitor buffer. This limited the overall number of cells that could be sorted, with 873 individual plasmablasts isolated.

Table 3.2 Summary of yield of FACS.

PID.	Live cells (%)	PBMC acquired for FACS ($x10^{6}$)	No. of plasmablasts isolated
20078	88%	12	95
20087	82%	17.2	84
20090	81%	33	134
20310	70%	3.8	96
20831	67%	4.5	55
20919	27%	0.8	57
20858	50%	12.7	146
20922	53%	7.4	65
20932	75%	9.8	62
21102	83%	7.6	79
Totals	68	45	**873**

RT-PCR was then performed to synthesize complementary DNA, followed by nested PCR to amplify VH and VL DNA (Kappa and lambda). For Vκ, all PCR samples containing an intact VH segment and Kappa light chain were sequenced to identify the family of variable region genes. This allowed for amplification of complete Vκ segments using specific primers. A representative agarose gel is shown below in Figure 3.3. Amplified bands of the correct base pair size (~450bp for VH, ~400 for Vκ and ~400 for Vλ) were visualized with ethidium bromide, excised, purified, and then cloned into the IgG1 vector.

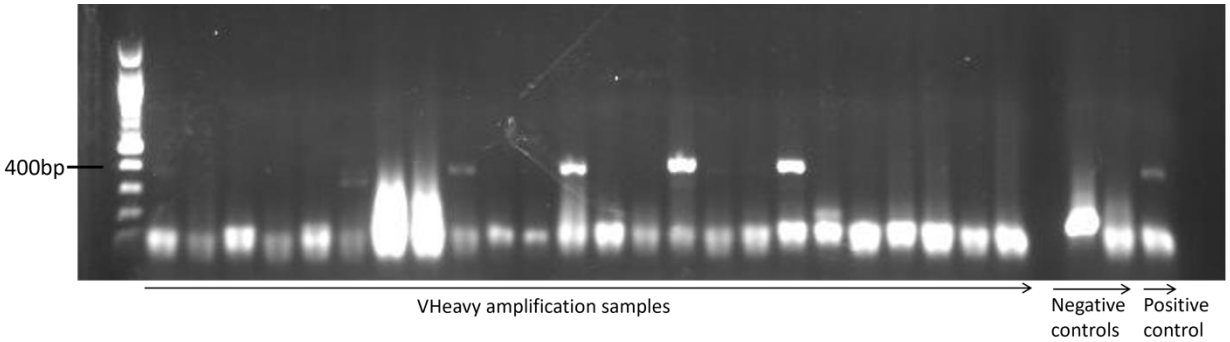


Figure 3.3 Representative agarose gel loaded with cDNA and 100bp ladder for comparison. VH genes were amplified and where successful are identified as a band in the ~450 bp region. Controls include a negative inhouse control of cDNA from non-B-cell cell culture and PBS. Positive control is of cDNA amplified from a known sample from which an anti-Dengue mAbs was cloned.

A total of 245 RT-PCR followed by nested PCR reactions were performed. The overall yield of intact sequences in this run of PCR was low, as summarised in Table 3.2**.**

Table 3.3 Summary of nested PCR on isolated plasmablasts.

	Number of intact VH + VL	**Intact paired plasmablast sequences (%)**	**MAbs expressed (n)**
20919	2 (72)	2.8	1
20932	1 (17)	5.9	0
20078	35 (120)	30	15
20310	3 (12)	25	0
20858	7 (24)	29	1
Totals	48 (245)	19.5%	**17**

Individual sorted plasmablasts underwent RT-PCR followed by separate nested PCR to amplify VH, Vκ and for Vλ. Where both VH and VL nested PCR detected bands, these are described as 'intact'. The percentage of intact cells. The total number of mAbs cloned at adequate concentrations from these VH/VL PCR products is given. Out of a total of 245 samples where RT-PCR and nested PCR reactions were performed, successful amplification of both was only found in 19.5% of samples run. From the 48 intact VH/VL sequences cloned, multiple transient transfections in HEK293T cells were performed. The concentration of mAbs produced (not shown) was ascertained by ELISA using a standard

concentration of human IgG. MAbs were taken as expressing at adequate concentrations if present in >10 µg/ml in supernatant. Thus, 17 mAbs generated from 3 patients with ATB were progressed to characterization of binding.

3.2.2 Screening of human mAbs against *M.tb* antigens

Next, the binding of 17 mAbs was tested against *M.tb* antigen preparations by ELISA. Stocks of *M.tb* were either heat-killed or sonicated, as denaturing of proteins could result in loss of 3D conformational binding sites. Binding to culture filtrate proteins was also determined, as these proteins have previously been shown to be common humoral antigens (Figure 3.4).

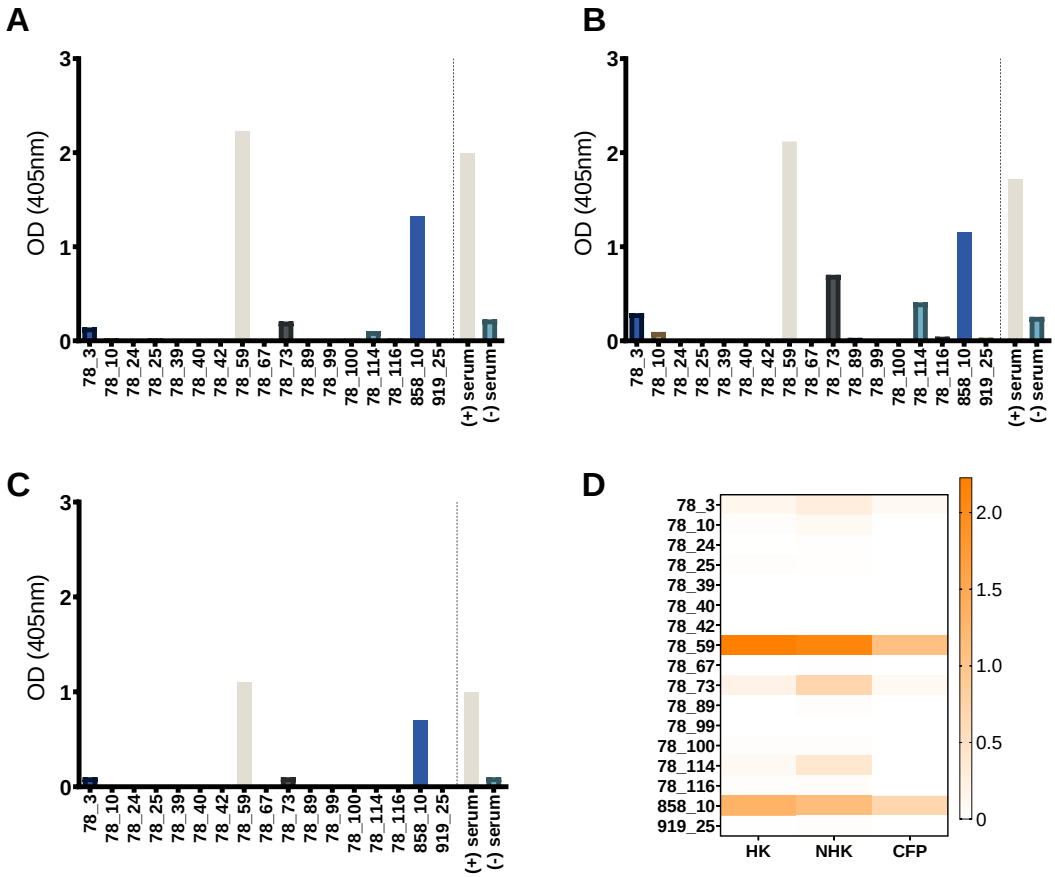


Figure 3.4 Mab (n=17) binding to *M.tb* antigens by ELISA. **(A)** Heat-killed *M.tb* H37Rv lysate. **(B)** Non-heat-killed *M.tb* H37Rv sonicate. **(C)** Culture filtrate proteins (CFP) of *M.tb* H37Rv. **(D)** Summary heatmap of 3 antigens combined (A, B, C). MAbs were diluted to a standard concentration of 10µg/ml, with antigen preparations coated at 10µg/ml. Data shown is the mean of technical duplicates. Pooled ATB serum is shown as a positive control (1:400) and donor serum from a non-BCG vaccinated person from a low-burden country is shown as a negative control (1:400).

The results of these bindings assays showed that 6 of these mAbs 78_3, 78_10, 78_59, 78_73, 78_114, and 858_10 showed binding to *M.tb* antigens. Thus, ~35% of generated mAbs from patients with ATB were *M.tb*-specific. MAbs generated from PID 20078 showed clonality (Figure 3.5).

A

MAb	VH Gene	CDR-H	Vκ gene	CDR3-κ
78_3	IGHV5-10-1*02	Unsuccessful	IGKV4-1*03 F	QQYYSTPFTF
78_59	IGHV3-7*01	ASISSEYFRPAVGSRHWVFDL	IGKV3-20*01	QQYDGSPWT
78_73	IGHV3-7*01	ARVLNLGYCNGTSCHWGLDP	IGKV3-20*01	QQYSGSPWT
78_114	IGHV3-11*05	ARETSWYFDY	IGKV3-20*01	QQYGTLPRT

B

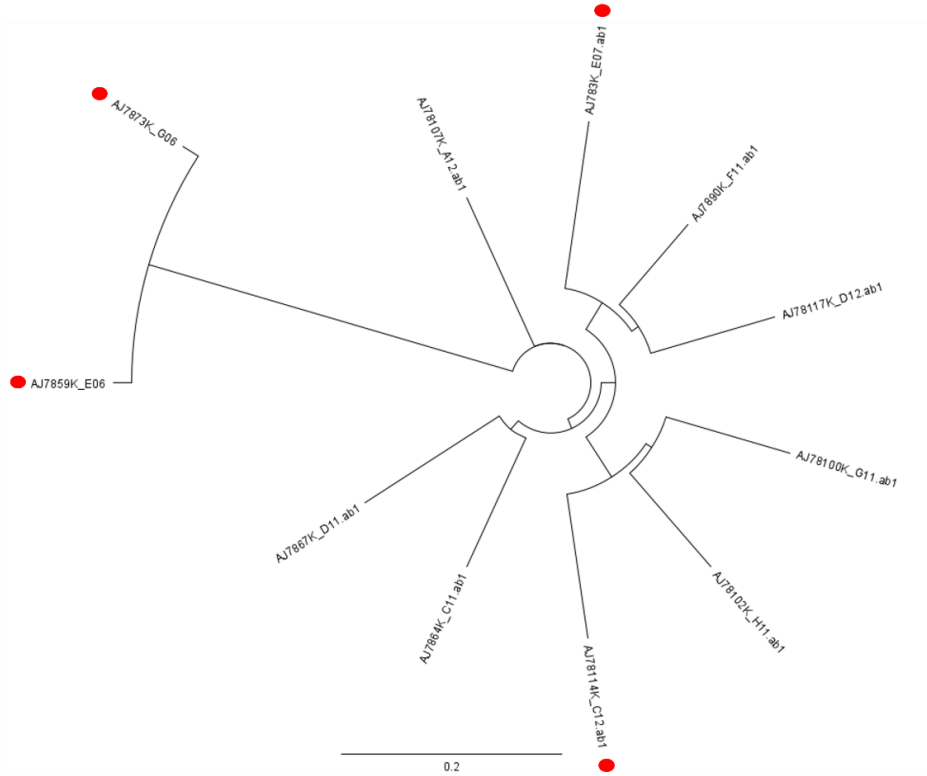


Figure 3.5 Sequencing data for *M.tb*-specific mAbs. **(A)** Table showing VH and VL families with CDR3 amino acid sequences.**(B)** Dendrogram (created using Geneious software) of Vκ gene sequences showing clonal distances between mAbs. *M.tb*-specific mAbs are highlighted with red.

All *M.tb*-specific mAbs from PID 20078 were of the Vκ family. Four of the mAbs were of the IGKV3-20*01 family. Dendrogram distance analysis for all Vκ mAbs from PID 20078 shows that 78_59 and 78_73 are closely related. Next, whether any of the generated mAbs recognized the surface of live BCG was determined (Figure 3.6). All mAbs were tested as surface-binding mAbs may not have bound via ELISA due to loss of 3D conformational epitopes or potentially lipid binding antibodies. To do so, bacterial flow cytometry with live BCG incubated with mAbs was performed. Binding was determined with addition of an anti-IgG fluorescent antibody.

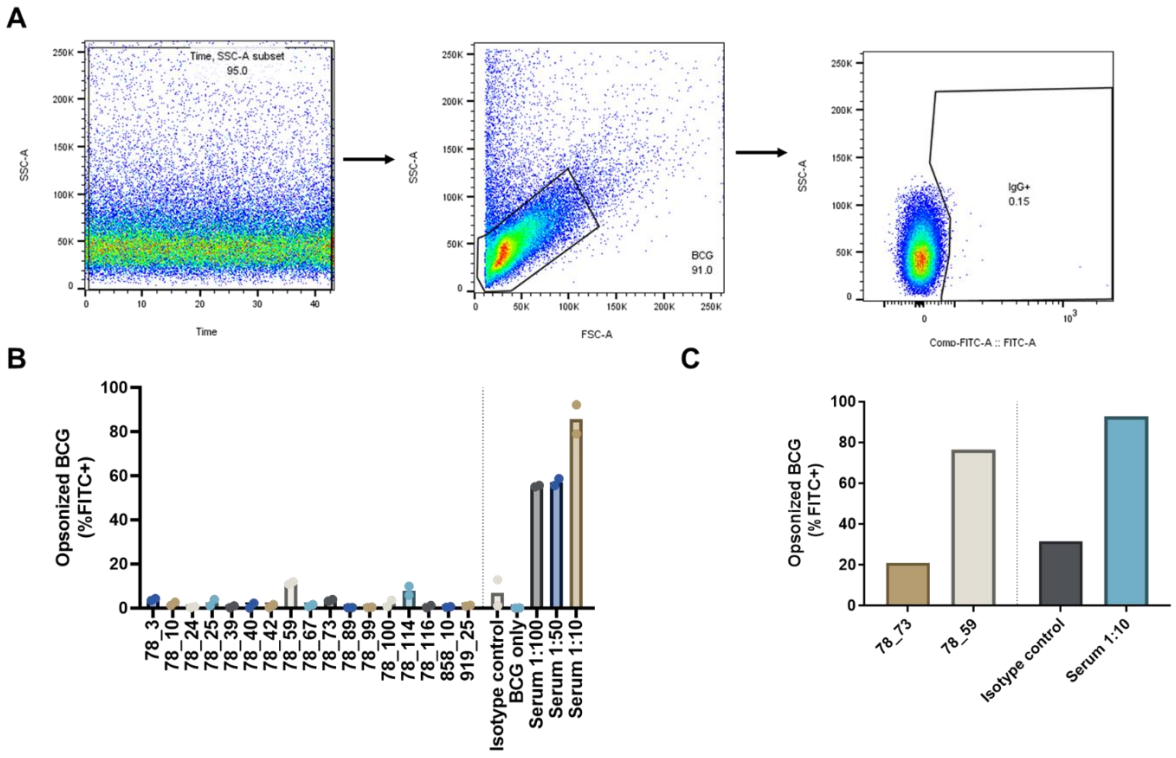


Figure 3.6 Flow cytometric assessment of BCG opsonization by mAbs. Bar graphs show the mean of replicates in which a minimum of 50,000 bacilli were acquired. **(A)** Gating strategy for BCG gated via i) time b) SSC vs FSC c) Fluorescence minus one control **(B)** Panel of mAbs (n=17) with an isotype IgG1 anti-influenza mAb, BCG only and ATB pooled serum. **(C)** Repeat experiment with protocol adjusted for greater mAb/bacteria ratio.

In this experiment, low levels of binding were seen by all mAbs. MAb 78_59 demonstrated greater binding than an isotype IgG1 control mAb. Given the low levels of binding, a negative mAb, the isotype control and 78_59 was performed again in an experiment using a higher ratio of bacilli to mAb to enhance binding. Here, it is more clearly visualized that 78_59 likely possesses ability to opsonize BCG. Taken together, only clone 78_59 recognized all antigen fractions of *M.tb* and live BCG. This clone was therefore selected for further characterization.

3.2.3 Binding characteristics of human mAb 78_59

Next, the binding of 78_59 to differing antigen fractions was performed to better characterize the nature of its antigen. (Figure 3.7).

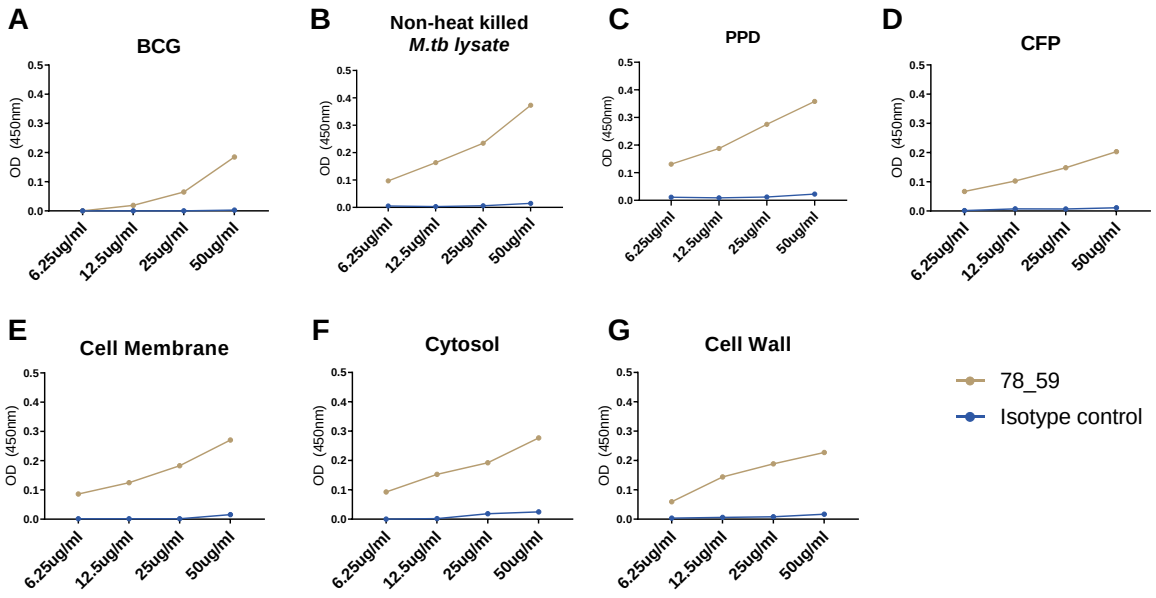


Figure 3.7 Binding curves of MAb 78.59 by ELISA **(A)** Whole cell BCG. **(B)** Non-heat-killed *M.tb* H37Rv lysate. **(C)** Purified protein derivative (PPD). **(D)** Culture filtrate proteins (CFP). **(E)** Cell membrane antigens. **(F)** Cytosolic antigens. **(G)** Cell wall antigens. Isotype control shown is an anti-malaria human IgG1 mAb.

These experiments show that 78_59 bound to both BCG and *M.tb* preparations, and thus this antibody recognizes a conserved antigenic target. The antigen is also present in the cytosol, cell membrane, cell wall, and culture filtrate. Next, western blot was performed to determine the molecular mass of the antigen. (Figure 3.8).

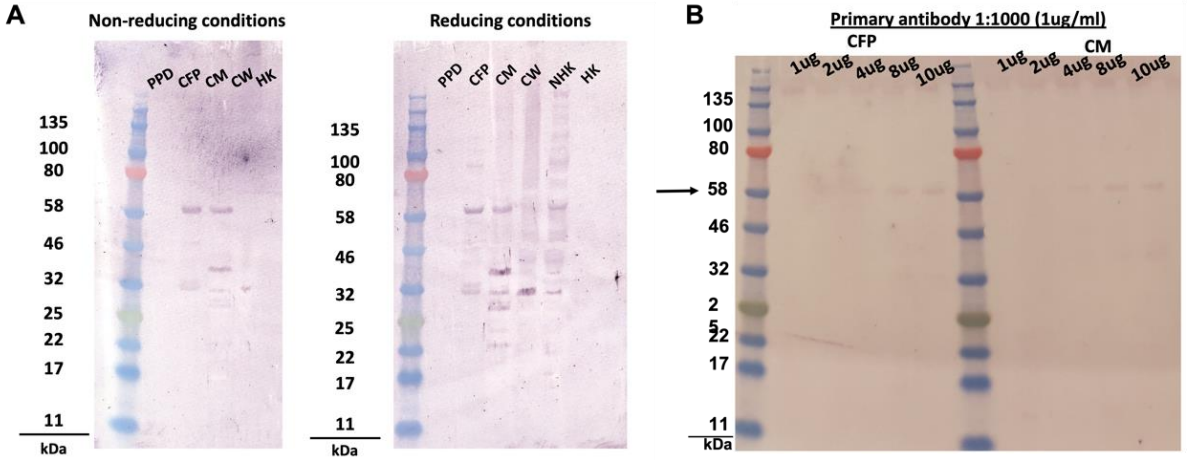
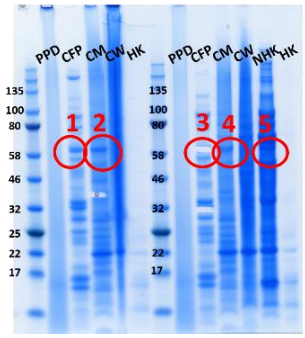


Figure 3.8 Western blot of 78_59 (1 µg/ml) against *M.tb* antigen preparations. **(A)** Non-reducing conditions using PPD, CFP, CM, CW and HK lysate. (**B)** Reducing conditions using these same antigen preparations. **(C)** 78_59 against a concentration series of CFP and CM in non-reducing conditions.

The molecular mass of the antigen recognized by 78_59 was thus ~58 kDa. Non-specific binding was also observed at the ~32 kDa mass, predominantly in reducing conditions. This could have been from excess protein, antibody, or secondary antibody. Cross-reactivity could have been created by reducing conditions, or the antigen potentially consists of conserved dimers. Western blotting against a concentration gradient of CM and CFP in non-reducing conditions was therefore repeated. Here, a concentration dependant band was seen at ~58 kDa. The 58 kDa and 32 kDa bands of Coomassie stained gel of these antigen preparations was also excised and sent for identification via mass spectrometry (Figure 3.8).

A



B

Non-reduced		Reduced		
CFP	**CM**	**CFP**	**CM**	**NHK**
GlnA1	No proteins identified	SahH	rpsB	SerA
SahH		GlnA1	GlnA1	Cpn60
			GabD2	SahH
				Syk
				GlnA1

Figure 3.8 Proteins identified in the ~58 kDa mass from *M.tb* antigen preparations. **(A)** Coomasie gel matching above western blots showing bands excised for mass spectrometry. **(B)** Summary data of proteins identified in each band via mass spectrometry.

From this experiment, only the mycobacterial protein GlnA1 was present in each excised band. It was therefore tested whether 78_59 bound recombinant GlnA1 by ELISA and western blot (Figure 3.9).

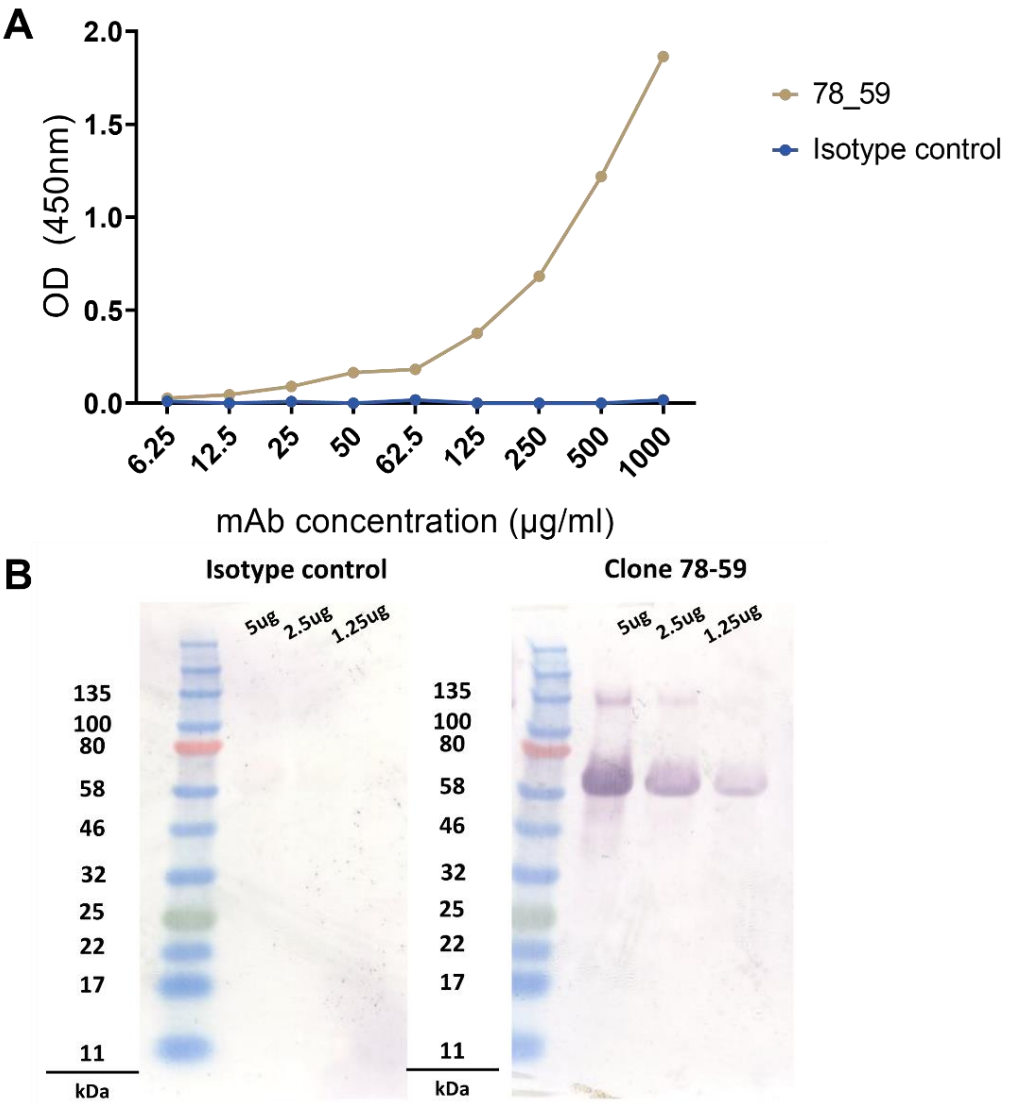


Figure 3.9 Binding of 78_59 (2 µg/ml) and isotype control (2 µg/ml) via **(A)** ELISA and **(B)** Western blot in comparison to isotype control. GlnA1 was loaded at 5 µg/ml, 2.5 µg/ml and 1.25 µg/ml in order of decreasing concentration.

78_59 showed a concentration dependent binding effect in both ELISA and western blot. No non-specific binding by the isotype control was seen. The antigen for this mAb was therefore identified as GlnA1 (Rv2220). Next, the ability of 78_59 to restrict the growth of mycobacteria in *in vitro* assays was investigated.

3.2.4 Function of human mAbs

After cloning the initial panel of anti-*M.tb* mAbs, the ability for these mAbs to directly restrict the growth of *M.tb* was tested. No prior mAbs had been shown to restrict the growth of *M.tb* by direct

inhibition, but this was examined as to not bias against a previously undescribed mechanism of function (Figure 3.10).

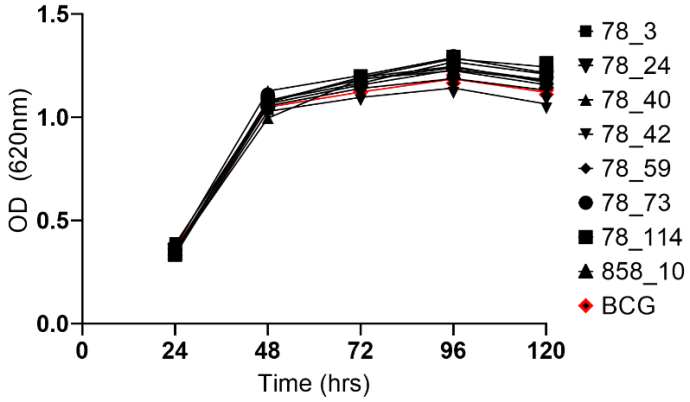


Figure 3.10 Growth curves of BCG co-incubated with human mAbs (10 µg/ml). OD of cultures in duplicates were read by spectrophotometry at each timepoint. Samples were compared at each timepoint by the Kruskall-wallis test. No effect on growth is seen. MAbs in red did not recognize *M.tb* antigens via ELISA but were included as negative controls.

From this data, none of the mAbs (including 78_59) influenced the growth of BCG in cell-free culture. ADCP capacity against BCG in human monocyte-derived macrophages (MDM) of the THP-1 cell line was tested for 78_59 (Figure 3.11).

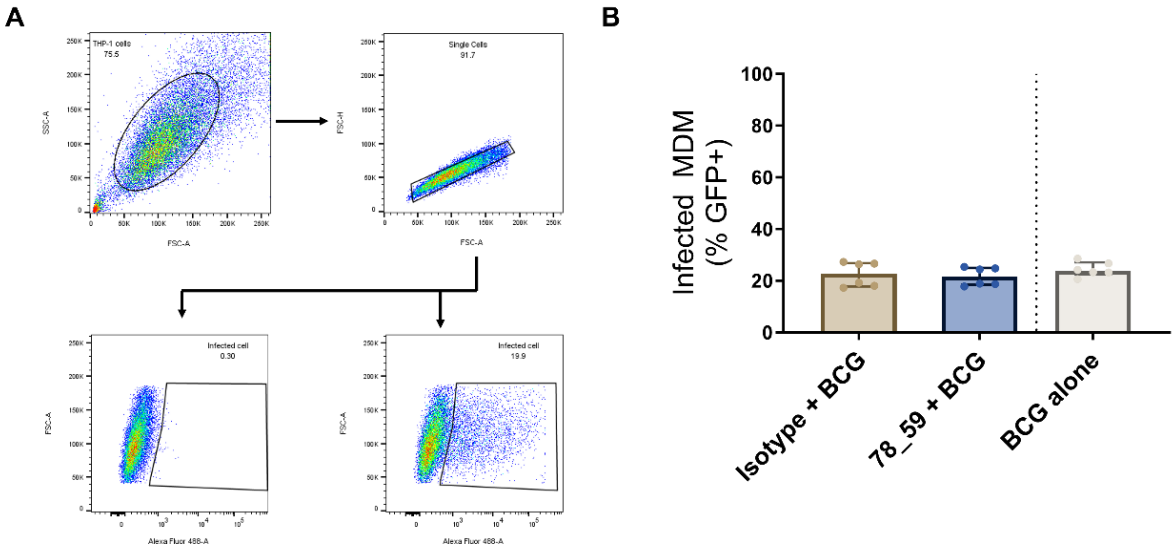


Figure 3.11 Percentage of THP-1 MDM infected by BCG-GFP as measured by flow cytometry. **(A)** Gating strategy for infected cells is as follows. Monocyte size gating (FSC vs SSC), FSC-H vs FSC-A for single cells, and SSC vs Alexa-488 (FITC/GFP). FMO control is shown in the bottom left panel, and BCG-GFP infected cells shown bottom right panel. **(B)** BCG-GFP was incubated with mAbs at (100 µg/ml) with BCG alone as a control (n=6). Data shown is from two separate experiments. Data analysed by Kruskal-Wallis with corrections for multiple comparisons.

A high concentration of mAb (100 µg/ml) was selected with intention of performing a concentration series if an effect of ADCP was seen. However, even at this concentration, 78_59 did not enhance the uptake of BCG-GP in MDM after 2 hours of incubation. As ADCP is only one hypothesized mechanism of antibody action in TB, the ability for 78_59 to restrict the growth of MTB in the human MDM was tested next (Figure 3.12).

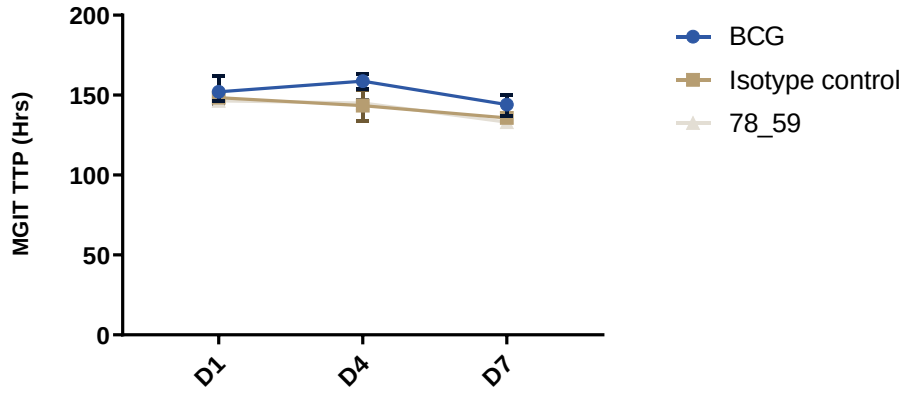


Figure 3.12 Growth of BCG in THP-1 MDM at day 1, 4, and 7 as measured by time-to-positivity (TTP) in mycobacterial growth indicator tubes (MGIT). MAbs at a concentration of 100 µg/ml or BCG alone were pre-incubated with BCG prior to infection of THP-1 MDM in triplicates for each time point. MDM were then lysed with sterile water and inoculated to MGIT tubes at D1, D4, and D7 post infection.

78_59 did not impact the growth of BCG-GFP in THP-1 MDM at any timepoint. It was reasoned that mAbs might require assistance from T cells to limit growth of mycobacteria. Thus, a mycobacterial growth inhibition assay (MGIA) previously used to measure the impact of BCG vaccination on anti-BCG serum antibody responses was adapted for use with mAbs (Figure 3.13**).** Serum, or as in this experiment, purified mAbs, are co-incubated with BCG in the presence of donor PBMC, with enumeration of CFU after 96 hours.

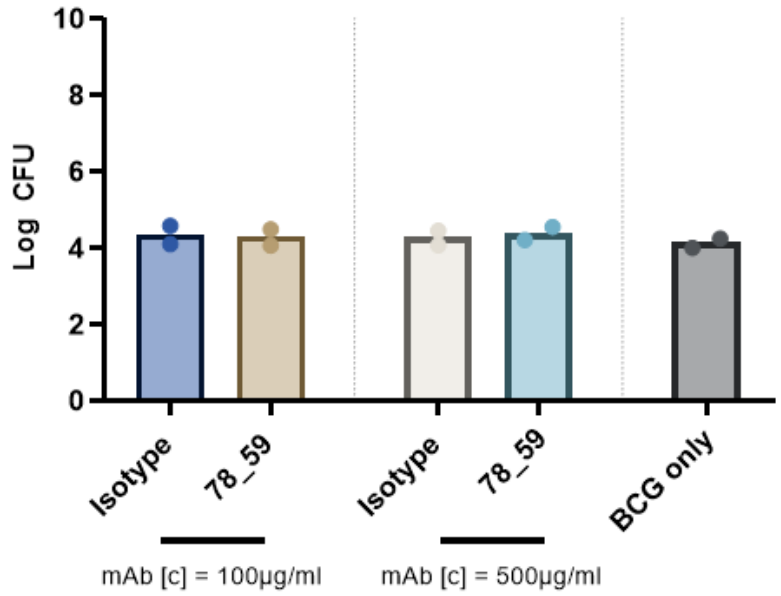


Figure 3.13 CFU of BCG in a PBMC MGIA at 100 µg/ml and 500 µg/ml of mAb (Donor n=2). Isotype antibody control compared to 78_59 with Mann-Whitney test. P values > 0.05 not shown.

The number of donor PBMC used was at this time limited by challenges in producing large amounts of mAb for use at high volume and concentration. Again, high concentrations were selected to detect small effects on growth. However, again even at this dose and in the presence of PBMC, 78_59 had no effect on the growth of BCG. Nevertheless, it was therefore hypothesized that 78_59 might still alter cytokine responses upon binding of GlnA1 in this MGIA (Figure 3.14).

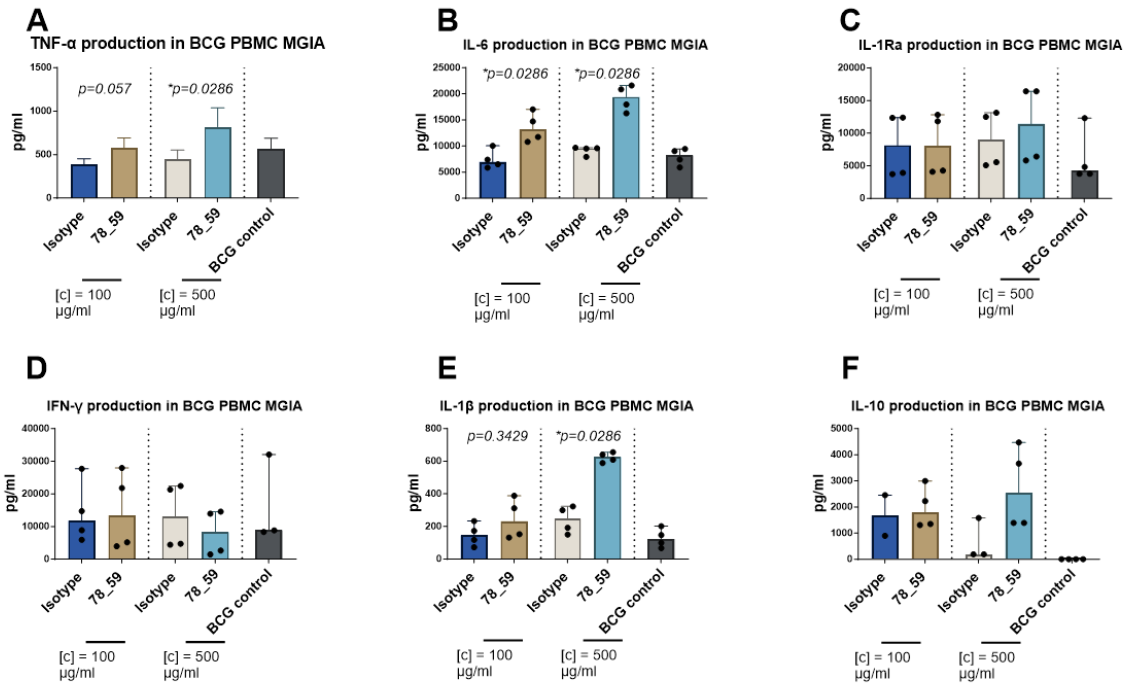


Figure 3.14 Production of cytokines measured by ELISA in BCG PBMC MGIA supernatant at day 4. **(A)** TNF-α, **(B)** IL-6, **(C)** IL-1Ra, **(D)** IFN-γ, **(E)** IL-1β, **(F)** IL-10. Isotype control, 78_59 at two concentrations (100 μg/ml and 500 μg/ml). For each cytokine and dose, isotype was compared to 78_59 by Mann-Whitney test.

In a BCG growth assay using donor PBMC, high dose 78_59 (500 μg/ml) induced greater IL-6 ($p = 0.0286$), IL-1β ($p = 0.0286$) and TNF-α ($p = 0.0286$) production relative to an isotype antibody. At a lower dose (100 μg/ml), a significant effect was detected for IL-6 ($p = 0.0286$). No effect on IL-1Ra, IFN-γ or IL-10 production was seen. Although for IL-10, the control and isotype at higher dose had technical errors which precluded statistical comparison of these groups. These findings led to postulation that antigen-antibody complexes could account for 78_59's action on cytokine production in the absence of effect on ADCP and BCG growth. These findings were therefore extended to whole blood assays (WBA) where the contribution of complement and neutrophils could amplify signal if CIC formation is important.

3.2.5 Whole blood assays with 78_59 and GlnA1

Inhouse whole blood assays (WBA) were adapted for use with mAbs. Firstly, stimulated donor whole blood samples were incubated with GlnA1 to measure induced production of IL-6, IL-1β, TNF-α. This was done to identify a) whether GlnA1 provoked this response in and of itself b) what dose of GlnA1 to progress forward to incubation with mAbs (Figure 3.15).

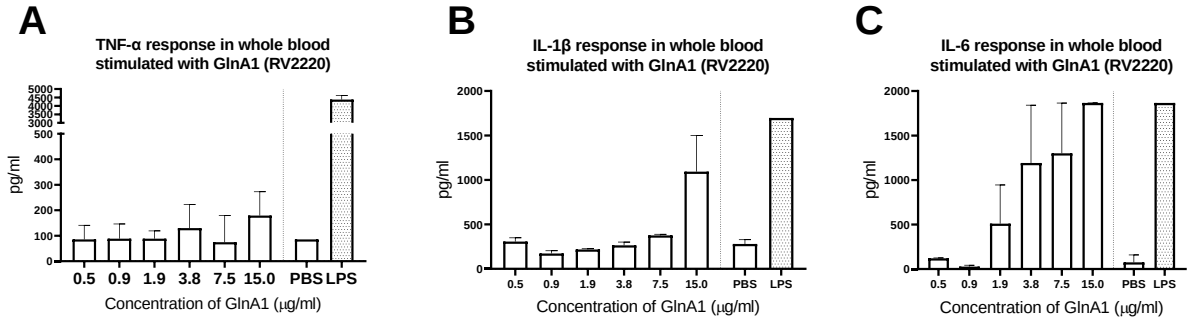


Figure 3.15 Cytokine responses in donor whole blood as measured by ELISA (n=3) **(A)** TNF-α, **(B)** IL-1β, **(C)** IL-6. Data shown is the median of donor replicates. Controls include phosphate buffered saline (PBS) as a negative control, and lipopolysaccharide (LPS). Statistical significance is tested by Kruskall-Wallis with correction for multiple comparisons.

None of the concentrations of GlnA1 induced a significant increase in multiple comparison with PBS, likely due to high inter-donor variability and small numbers. At the highest concentration of 15 μg/ml, there was a trend towards greater IL-1β expression. IL-6 at 15 μg/ml exceeded the upper limit of

detection of 1ng/ml per standard curve in this experiment. We selected the highest concentration of 15 µg/ml for downstream assays in combination with mAbs (Figure 3.16**)**.

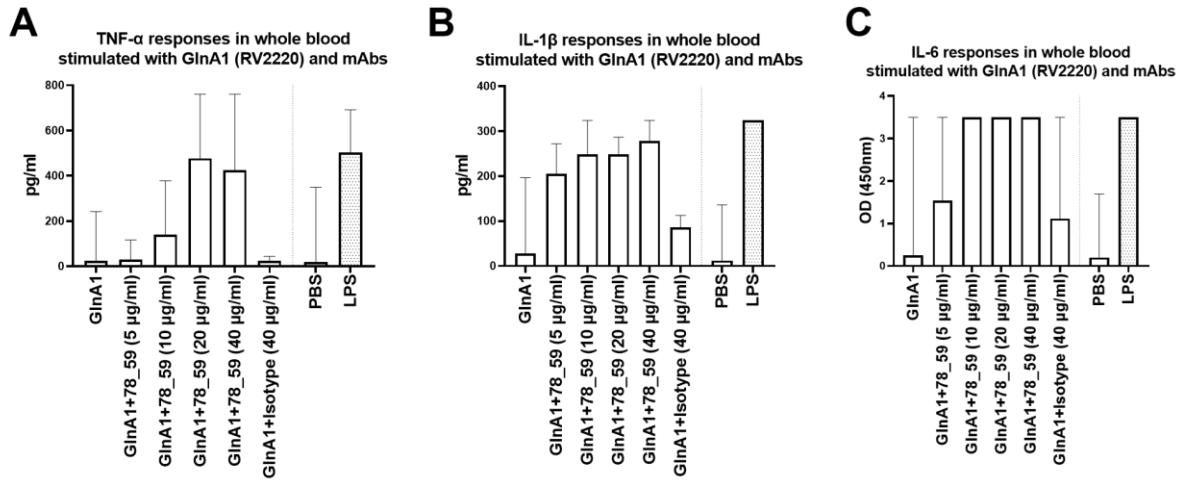


Figure 3.16 Cytokine responses in donor whole blood induced by mAbs in combination with GlnA1 as measured by ELISA (n=3) **(A)** TNF-α **(B)** IL-1β **(C)** IL-6 (shown in optical density). Data shown is the median of donor replicates. GlnA1 is added at a concentration of 15 µg/ml. Isotype control antibody is always used at the highest concentration (40 µg/ml). PBS and LPS are included as controls. Statistical significance was tested by Kruskall-Wallis with correction for multiple comparisons.

The greatest responses for TNF-α were seen in the GlnA1+ 78_59 group with concentrations of mAb above 10 µg/ml. Large variability in donor response hindered statistical significance. Results for IL-6 were again uninterpretable as they exceeded the standard curve, and raw optical density (OD) values are shown to visualize trend. For all cytokines tested, the presence of 78_59 increased the median response. These experiments were extended to 8 donors (Figure 3.17).

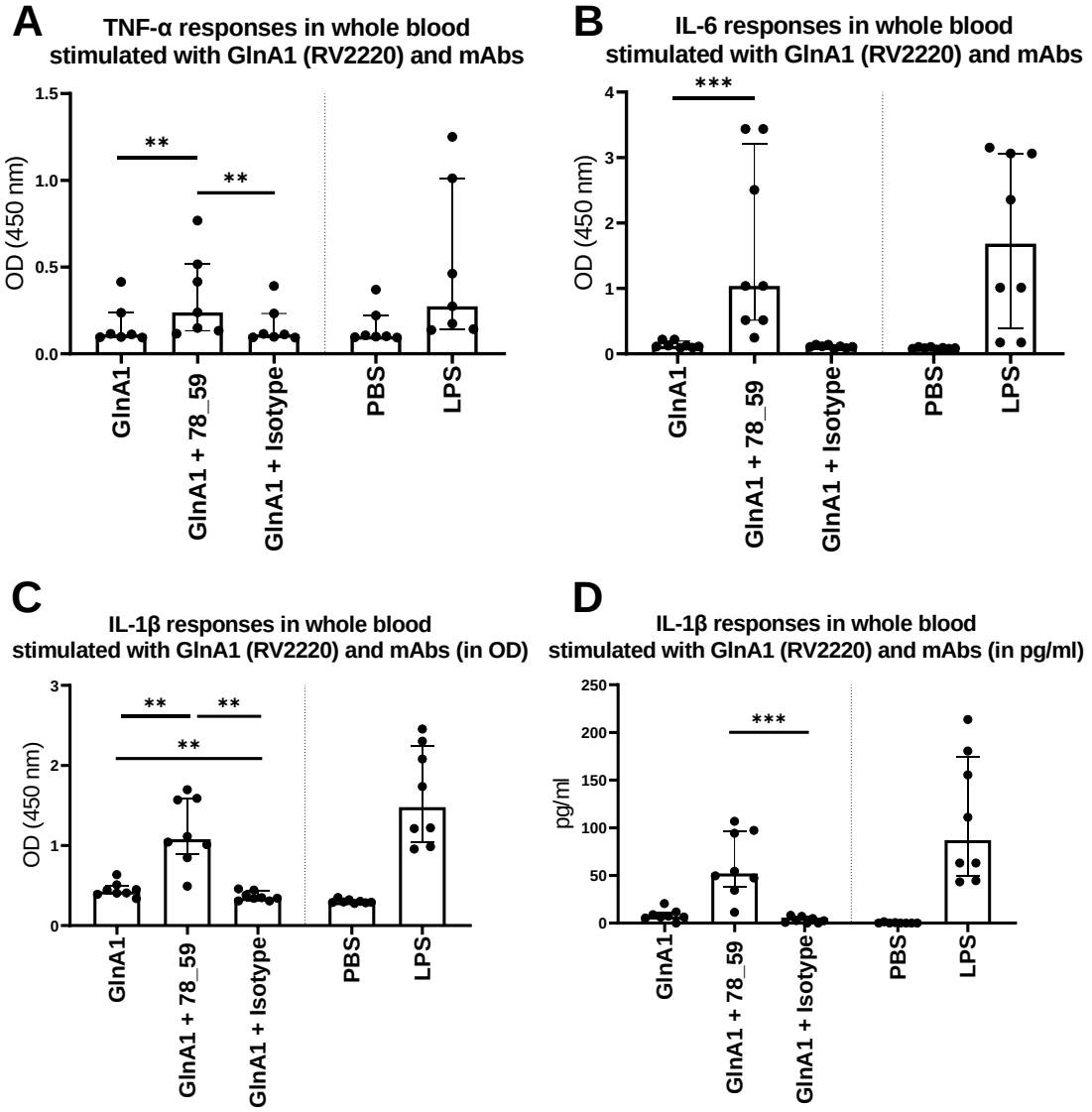


Figure 3.17 Cytokine responses in donor whole blood inoculated with GlnA1 in the presence of mAbs, as measured by ELISA (n=8) **(A)** TNF-α (OD) **(B)** IL-6 (OD) **(C))** IL-1β (OD) **(D)** IL-1β (calculated values). Data shown is the median of donor replicates with interquartile range. GlnA1 is added at a concentration of 15 µg/ml. Isotype control antibody is always used at the highest concentration (40 µg/ml). PBS and LPS are included as controls. Statistical significance is tested by Kruskall-Wallis with correction for multiple comparisons. P values > 0.05 are not shown.

Absolute calculated values for several measurements exceeded the upper limit of detection for the ELISA assay standard curve of 1ng/ml for TNF- α, 250pg/ml for IL-1β, and 300pg/ml for IL-6. When combined with GlnA1, 78_59 induced a greater amount of IL-1β than isotype antibody. For cytokines in raw optical density data, 78_59 with GlnA1 resulted in greater TNF-α expression than GlnA1 alone or GlnA1 in combination with an isotype control antibody. A greater IL-6 response was also seen between GlnA1 alone and GlnA1 combined with 78_59. Next, donor whole blood was inoculated with

M.tb H37Rv to test whether additional immune factors of the WBA would impact mycobacterial growth when combined with mAbs (Figure 3.18).

In brief, a known stock of *M.tb* H37Rv was inoculated into 600 µl of donor blood in duplicates. 20µg/ml of 78_59, isotype control, or equivalent volume of phosphate buffered saline (PBS) was added to donor blood prior to incubation. Samples were incubated at 37 degrees in constant rotation prior to cell pelleting, lysis and seeding of the pellet into MGIT tubes for time-to-positivity assessment in a BD MGIT machine.

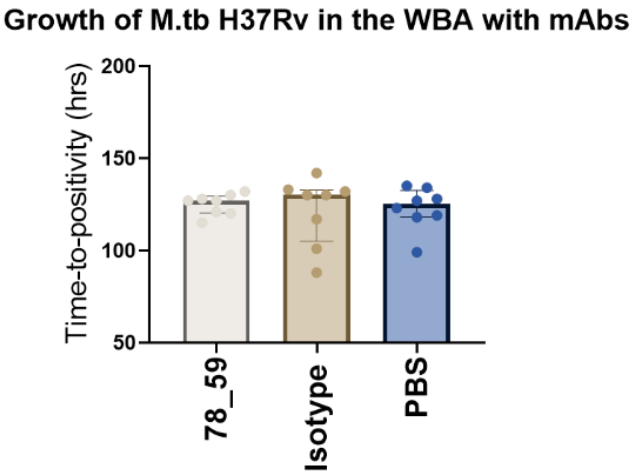


Figure 3.18 Effect of mAbs on growth of *M.tb* in whole blood in donors (n=8). 78_59 was compared to isotype and PBS controls. Each datapoint represents a single donor. PBS, phosphate buffered saline. Statistical significance is determined by Mann-Whitney test between isotype control and 78_59.

78_59 again showed no impact on mycobacterial growth relative, to isotype control, after 96hrs of incubation. The next step would be to test the production of IL-6, IL-1β, and TNF-α in the supernatant of this experiment. It would also be required to repeat prior cytokine quantification assays with a dilution series for samples where concentration exceeded the standard curve.

3.2.6 Recognition of BCG by 78_59 is impacted by bacterial metabolism

In addition to ELISA, fluorescent microscopy was performed to further validate the binding of 78_59 to live mycobacteria. GlnA1 expression is controlled by nitrogen availability, and it could also be reasoned that bacterial metabolism *in vitro* impacts binding of 78_59 in the previously described assays. It was therefore tested whether culture media containing high or low nitrogen concentrations altered the binding patterns of 78_59 to live BCG (Figure 3.19).

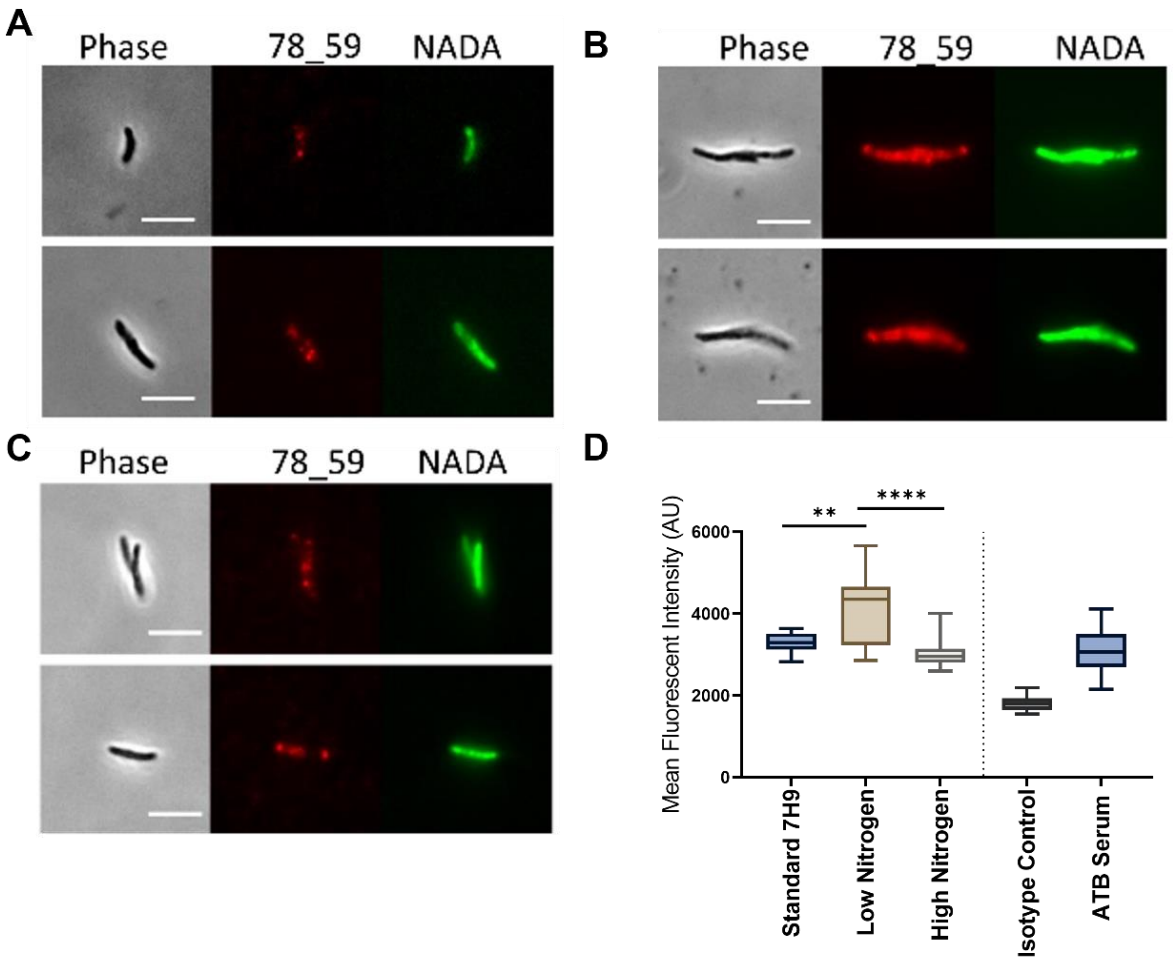


Figure 3.19 Impact of bacterial metabolism on mAb surface binding determined by wide-field fluorescence microscopy. Live bacteria are detected by phase contrast imaging and NADA staining (green), while surface-binding of mAb is detected with Alexafluor 647-conjugated anti-human IgG. Representative images are shown in; **(A)** BCG grown in standard Middlebrook 7H9 media co-incubated with 78_59 (100 µg/ml). **(B)** BCG grown in low nitrogen media co-incubated with 78_59 (100 µg/ml). **(C)** BCG grown in high nitrogen media co-incubated with 78_59 (100 µg/ml). **(D)** Quantification of surface-bound fluorescence signal (Mean Fluorescent Intensity [MFI] in AU) of 78_59 on BCG grown in different media. Data represented is acquired from at least 100 individual bacilli, with median MFI and interquartile range shown. Scale bars = 5 µm.

In standard media, 78_59 binds to live BCG in discrete punctate clusters as seen with widefield microscopy. These results confirm prior ELISA and flow cytometric assays. The MFI of IgG staining is also significantly lower in standard Middlebrook 7H9 media than in low nitrogen conditions. The availability of GlnA1 at the cell surface is thus likely increased by nitrogen-depleted culture conditions, presenting more surface-exposed antigen for antibody binding.

3.2.7 Serum recognition of GlnA1 across the clinical spectrum of TB

Although antibodies targeting GlnA1 did not restrict the growth of *M.tb*, it was of interest to understand how frequently such antibodies arise in humans. Thus, the prevalence of antibody responses to GlnA1 across the clinical spectrum of TB was determined. It was hypothesized that if antibodies targeting GlnA1 are generated by bacillary replication, then a greater percentage of individuals with ATB would possess such antibodies than those with LTBI. HIV infection might also impact bacterial load, and thus the magnitude or prevalence of antiG-GlnA1 antibodies (Figure 3.20).

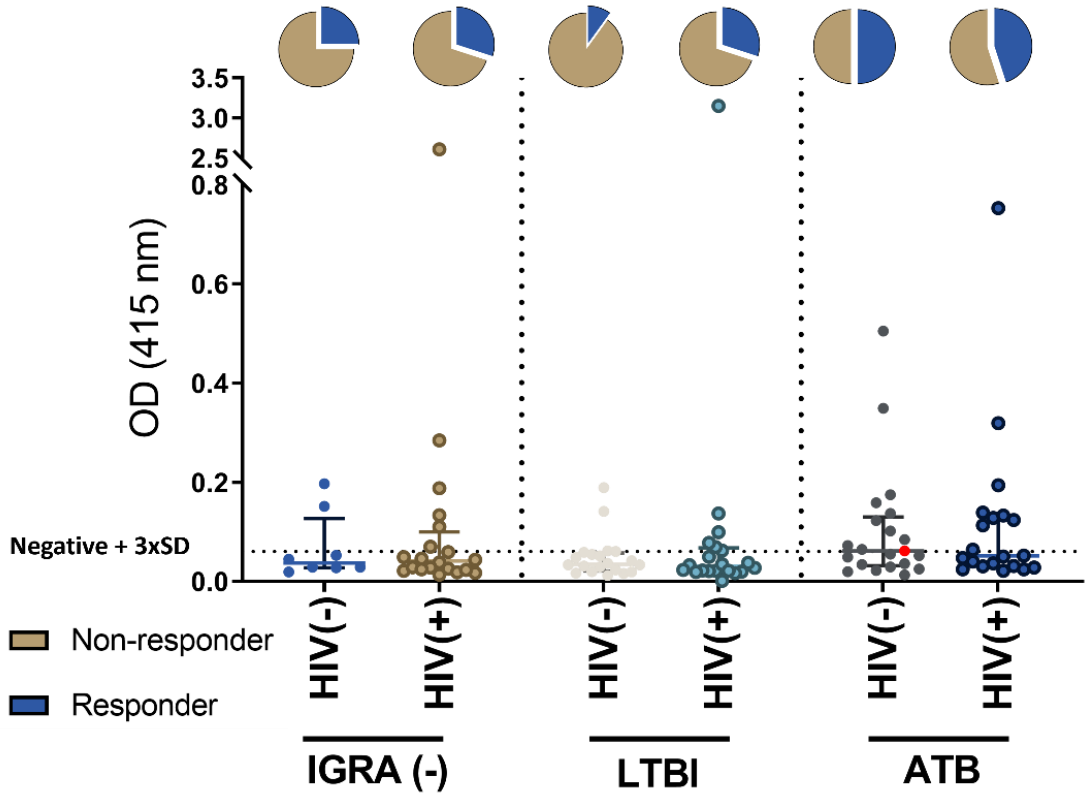


Figure 3.20 Seroreactivity to GlnA1 in humans across the clinical spectrum of TB. IGRA negative HIV- (n=8), HIV+ (n=20), LTBI HIV- (n=20), LTBI (n=20) and ATB HIV- (n=20), HIV+ (n=20). Negative control + 3xSD was used to define a cut-off of positivity. Red dot in the HIV-/ATB = PID 20078 (Donor from whom mAb 78_59 was derived). Pie charts represent proportion of responders versus non-responders. Different in percentage of responders to non-responders was measured by Chi-square test. Magnitude of antibodies was compared by Kruskall-Wallis test, with correction for multiple comparisons.

Antibody responses against GlnA1 were detectable in between 20-45% of all populations. In the IGRA negative population, these antibodies could have arisen either from cleared *M.tb* infection or from BCG

vaccination. It is also interesting to note that PID 20078 did not possess a high magnitude of antibody response against GlnA1. The proportion of anti-GlnA1 serological responders in each group were as follows: IGRA-/HIV- 25%, IGRA-/HIV+ 30%, LTBI/HIV- 10%, LTBI/HIV+ 30% , ATB/HIV- 50%, and ATB/HIV+ 45%. However, the magnitude of responses did not differ across clinical groups or across HIV infection status when compared by Chi-square test.

3.3 Discussion

In this chapter I sought to characterize mAbs generated from patients with ATB. Patients with ATB were selected for unbiased plasmablast IgG cloning, as the likelihood of obtaining pathogen-specific mAbs is greatest in individuals with an active infection (293).

Given that the initial yield of mAbs was lower than anticipated, several aspects of the patient selection and cloning could have been improved. Firstly, as was performed in the CAPRISA study to generate mAbs against HIV, donors could have been pre-selected by prior serological studies identifying the participants with the most potent *in vitro* antibody function (13). MAbs derived from a resister as defined in chapter 1, or individuals with LTBI would have the strongest biological credibility of contributing to the clinical phenotype if an *in vitro* function was detected. A future study could select persons for mAb cloning by first discovering persons with LTBI or a resister phenotype with unusually potent antibodies.

Secondly, methods to enrich for cloning of antigen-specific antibodies have been developed since this work. Ishida *et al* (2021), and Watson *et al* (2021) have shown B cell baiting by fluorescently labelling *M.tb* antigens for cell sorting (158, 233). An antigen-agnostic approach for the purpose of novel target discovery was specifically selected for this study, which would reduce the proportion of mAbs generated that are antigen specific. However, strategies to boost the yield of antigen-agnostic mAbs could include sorting of pools of memory B cells prior to stimulation with antigens and a B cell stimulation cocktail (CD40L, IL-21 and BAFF (14). Pools containing anti-*M.tb* IgG by ELISA would then be selected for sequencing and cloning of all possible pairs of VH and VL. Another novel tool is magnetic B cell sorting, which can also be combined with antigen stimulation and identification of antigen-specific B cells by a fluorescent labelling (15). These methods could improve the ratio of mAbs cloned to antigen-specific mAbs in the future.

Several aspects of the cloning protocol could have been better optimized to increase the yield of mAbs produced. We generated 17 mAbs over multiple transfection experiments that generated concentrations greater than 10 µg/ml in HEK293T cells. The initial nested PCR yield showed that most single cell tubes (~80%) did not contain intact VH and VL genes (Table 3.2). Here, a panel of tubes could have

been selected for amplification of a housekeeping gene to determine if cell sorting had failed to sort cells at all, or if cells sorted were not of the B cell lineage. If cell sorting had failed, this step could have been repeated prior to running more PCR reactions. Next, out of the 48 mAbs cloned, less than half expressed well. At this point, transfection in parallel with alternative transfection agents such as lipofectamine could have been tested to increase mAb expression. MAbs could also have been concentrated using filter columns or protein A/G bead purification and then binding tested by ELISA as described above.

I described the cloning of 5 mAbs from ATB patients that bound *M.tb* antigen preparations. From International ImMunoGeneTics (IMGT) and Geneious Prime analysis, 3 of the mAbs from PID 20078 appear to be clonally related. In order to better understand the emergence of clonal responses in *M.tb,* bulk sequencing of VH/VL would be necessary.

As the strength of evidence for antibody functionality in TB focussed on opsonizing capacity, I specifically advanced 78_59 for characterization. Initially overlooked, clone 78_114 also displayed a low level of surface binding and could have been tested in conjunction with 78_59. Interestingly, this mAb bound *M.tb* lysate but not culture filtrate protein. To expand upon the hypothesis that antibodies against secreted antigens are pro-inflammatory but do not enhance cellular killing of *M.tb*, all such mAbs must be characterized and especially non-opsonic antibodies.

The number of human mAbs where antigens have been identified in *M.tb* remains small. The only characterized human mAb antigens are against LAM, HBHA, and PstS1 (10–12). I used western blot with extraction and digestion of the 58 kDa band from a range of subfractions of *M.tb* to identify a putative pool of antigens for 78_59. The success of this process relied upon there being a limited number of antigens at this molecular mass, and binding being unhindered by the linearization process of western blotting. In this way, I show that this mAb's antigenic target was GlnA1 (Rv2220). This strategy may be useful to define the antigens of further mAbs, and where unsuccessful, protein or glycan arrays as well as co-immunoprecipitation can be explored.

It is interesting to note that despite showing binding to the surface of BCG by ELISA and flow cytometry, 78_59 did not exhibit ADCP or have effect on the growth of BCG/*M.tb*. We consider multiple potential explanations for why this is the case. GlnA1 is a dodecameric enzyme produced in the cytosol of virulent mycobacteria and is involved in the production of the mycobacterial poly-L-glutamine layer (16). GlnA1 is present in culture filtrate and the mycobacterial cell wall. GlnA1 could therefore dissociate from the cell wall during infection, either by being shed or actively secreted by bacilli. Thus, mAbs may only bind temporarily to surface exposed GlnA1 before the antigen is dissociated, limiting the opportunity to traffic *M.tb* towards FcR expressing cells.

The expression of GlnA1 is also strongly regulated by nitrogen availability (17). As seen by microscopy, in standard culture medium, binding of 78_59 appeared to be limited to distinct foci. GlnA1 may

therefore be too sparsely present at the mycobacterial surface to trigger cross-linking and activation of Fc receptors by antibody. A future experiment could test the effect of 78_59 on ADCP or WBA with *M.tb* cultured in low versus high nitrogen availability conditions.

GlnA1 is also essential for intracellular survival of *M.tb,* and so binding with 78_59 likely doesn't inhibit its enzymatic function to an extent that results in mycobacterial growth inhibition. This is similar to findings that anti-PstS1 mAbs require FcR (CD16/CD32) to restrict growth of *M.tb,* suggesting an antibody effector functions as the mechanism of action. If mAbs impacted metabolic function, this could be determined by comparing transcription profiles of opsonized vs non-opsonized mycobacteria.

Another avenue of exploration as to why 78_59 possessed no *in vitro* bacterial killing potential is the IgG1 backbone. Zimmerman *et al* (2016) showed that the isotype of antibody contributes to function, and here cloning 78_59 into an IgA or IgM backbone could alter the outcomes of our assays (297). Recently, an IgM mAb against LAM has been shown to restrict the growth of *M.tb* relative to an isotype control in a WBA (18).

Lastly, the binding strength of 78_59 to GlnA1 could simply be too weak to create a stable antibody-antigen bond. Surface plasmon resonance could determine if this is the case. The overall avidity of serum against the mycobacterial surface declines during ATB for unknown reasons (19). Affinity of mAbs against *M.tb* antigens is largely undetermined, and how affinity maturation evolves in ATB is difficult to study relative to viral infections given how patients present to healthcare with insidious onset of symptoms. One model could be to test whether anti-*M.tb* mAbs against the same antigens possess greater affinity after ATT. In summary, we used three assays to test the impact of 78_59 on mycobacterial growth. This mAb did not affect the growth of BCG or *M.tb* in THP-1 macrophages, PBMC, nor in WBA.

When interpreting the WBA data, further characterization of donor i) HIV status, ii) LTBI status by QFT, and iii) pre-existing antibodies against GlnA1 will be needed. Additional technical controls of the same experiments repeated only with 78_59, and endotoxin contamination must also be performed. Experiments in the PBMC MGIA and the WBA were from different batches of mAb cloning and purification, yet results were consistent across both experiments. Purified preparations for 78_59 should also be tested for microbial contamination by culture and plating. These controls would confirm that the elevated levels consistently seen in the 78_59 condition was not due to technical factors such as LPS or microbial contamination, nor that clone 78_59 drove this cytokine response in and of itself.

It is also possible that the PBMC and WGA were tested at timepoints too short to determine an effect on growth by mAbs. Initially optimized for T cell stimulation, longer incubation periods up to 168 hours need be tested to confirm that the outcome doesn't change over time. However, the 96-hour timepoint is adequate for effects of post-vaccination serum on BCG growth to be seen in the PBMC MGIA (18). The initial PBMC and WBA are also statistically limited by numbers of biological replicates. Further

validation of these results must therefore test the effect of mAbs in whole blood derived from well-characterized donors at adequately powered sample sizes. However, the WBA remains a viable means whereby to test the function of human mAbs against *M.tb* in a mechanistically unbiased manner.

One of our consistent findings was the stimulation of TNF-α, IL-1β, IL-6 production by 78_59 incubated with BCG or *M.tb* in PBMC or whole blood respectively. This pattern of cytokine production relates to bacterial antigens being taken up via FcγRIIA (20). Serum from patients with ATB has been shown to augment the production of TNF-α and IL-6 in MDM upon stimulation with PPD (21). Further, GlnA alone stimulates mouse-derived DC cells to produce IL-1β, IL-10, and IL-12p70 (22) It is plausible then that 78_59 functions similarly to these studies, by driving uptake of GlnA1 via CIC formation and FcR engagement to stimulate a pro-inflammatory cascade. Here, I also provide additional context that this process does not assist in host immune control of *M.tb*.

This finding is also biologically plausible, given emerging data to show that GlnA1 peptide is the most abundantly detected peptide in serum extracellular vesicles (82%) in LTBI (23). Thus, soluble mycobacterial GlnA1 is present in the circulatory system of humans. Although TNF-α is necessary for the control of TB, a double-edged sword exists where elevated levels of TNF-α also associate with severity of disease (24,25). As the FcγR/CIq signatures emerge during the progression from subclinical to ATB, antibodies against secreted antigens could therefore directly contribute to inflammation and symptom onset.

To extend this work, mAbs from ATB must be characterized at scale. Here, it is plausible that mAbs against secreted antigens in ATB all share the same function as a group. This hypothesis is relevant given that TB vaccine design is focussed on immunodominant T cell proteins of this class. Ag85A, as in the MVA85A vaccine, falls into this category, and does not elicit protective antibodies in mice (26). GlnA1 has also been evaluated in pre-clinical vaccine models with impact on CFU post *M.tb* challenge (27). Whether synergy between the antibody and cellular response is necessary to prevent TB by vaccination is unknown.

If CIC and antibodies play an active role progression of TB disease, this pathway could be targeted by inhibition of caspase 1 or Fc receptor signalling. Host-directed therapy aims to shorten the duration of chemotherapeutic agents and/or mitigate tissue damage that may result in long-term clinical sequelae. One potential agent is VX-765, which attenuates IL-1β production in a range of infection and inflammation-mediated diseases (28). Decoy antibodies that block FcγRIIA for the treatment of CIC-mediated autoimmune disease have also been developed (29). Next generation host-directed therapies could explore blocking these pathways in ATB and potentially TB IRIS.

Taken together, this chapter describes the adaptation of human mAb generation to patients with ATB. I demonstrate the indepth characterization of a single clone which bound to a range of mycobacterial antigen preparations by flow cytometry, ELISA, and fluorescent imaging. I also describe the process

whereby the antigenic target of this mAb was discovered as GlnA1 (Rv2220), a putative TB vaccine candidate. No prior mAbs against GlnA1 have been generated or studied. This mAb did not enhance ADCP or mycobacterial killing in a range of assays. However, relative to an isotype antibody control, the anti-GlnA1 mAb increased IL-1β, IL-6 and TNF-α production in both PBMC and whole blood. These findings support a potential link between antibody responses in the form of CIC formation with FcR uptake and disease pathophysiology in ATB. As GlnA1 is homologous across BCG and *M.tb*, it is notable that such antibodies occur in approximately 1 in 5 persons across the spectrum of human TB infection. Antibodies against GlnA1 were also readily identified in PLWH. In chapter 4, I expand upon this work by investigating the human antibody response against *M.tb* in PLWH.

Chapter 4: Antibodies in HIV-associated TB

4.1 Introduction

In 2020, approximately a third of total deaths in persons living with HIV (PLWH) were due to TB (313). Multiple factors contribute to the increased severity of TB in PLWH. Advanced HIV disease predisposes patients to disseminated forms of disease TB such as miliary TB, *M.tb* blood stream infection (BSI) and TB meningitis (314). The diagnosis of TB is also more challenging in PLWH owing to a lesser likelihood of detecting *M.tb* in sputum in patients with TB (315). Further, treatment of HIV-associated TB carries the risk of additional morbidity from immune reconstitution inflammatory syndrome (IRIS), and drug interactions with anti-retroviral therapy (ART) (314,316).

The predominant immunological mechanism whereby PLWH are more susceptible to TB is thought to be via the loss of $CD4^+$ T cells (317). HIV infection results in declining numbers of $CD4^+$ T cells through a number of host and viral-mediated mechanisms (318). The risk of developing TB, dissemination of disease, and mortality all strongly associate with declining $CD4^+$ T cell counts (314).

However, immune defects against *M.tb* other than the loss of $CD4^+$ T cells in PLWH exist. Even on ART, or with $CD4^+$ T cells counts above 700 cells/mm^3, PLWH still carry an additional 4.4 times increased risk of TB (7). Further, susceptibility to TB in PLWH begins during the first year of diagnosis with HIV infection (8). HIV infection also broadly impairs B cell function and blunts the antibody responses to multiple vaccinations (9–11).

The role of antibodies in protection against opportunistic infections (OI) in PLWH is understudied relative to studies focussing on cellular immunity. Recently, an inverse relationship between antibodies targeting a cryptococcal capsular polysaccharide and mortality in PLWH with cryptococcal antigenemia has been shown (12). A role for antibodies in protection against *Pneumocystis jirovecii,* the first described HIV-associated opportunistic infection, has also been proposed (13)

HIV infection alters both epitope recognition and subclass antibody production against *M.tb*. Most PLWH without ATB (95%) possess antimycobacterial antibodies, however this rate of declined to 1 out of 20 (5%) in advanced HIV in one early study (203). IgG2 responses against LAM were absent in PLWH diagnosed with extrapulmonary TB (15). Certain antigens, such as MPT51, may be more frequently recognized by PLWH with ATB than HIV uninfected persons (16).

More recently, van Woudenbergh *et al* (2020) showed that HIV infection broadly altered antibody profiles against *M.tb* (17). IgG levels against LAM, Ag85, ESAT/CFP-10 and purified protein

derivative (PPD) were decreased in PLWH with ATB (17). In LTBI, PLWH had lower IgM responses to *M.tb* than HIV uninfected persons independent of $CD4^+$ T cell counts (17).

Little is known about how the impact of HIV on antimycobacterial antibody production relates to risk of developing TB. In this chapter, we sought to test the hypothesis that PLWH fail to mount as significant an antibody response to *M.tb* as HIV negative persons across the spectrum of human TB disease. The aim of characterizing multiple cohorts was to glean context from comparison to HIV uninfected persons, as well as in PLWH where outcomes relating to acquiring TB were known.

We firstly tested whether differences existed in the magnitude of antibody responses to *M.tb* at the point of ATB diagnosis when compared to PLWH with LTBI or HIV negative persons. Next, we measured IgG response against *M.tb* in cohorts with longitudinal data. Next, we tested how antibody responses are altered by initiation of ART in PLWH with LTBI. We then characterized the phenotype of antibody responses in 3 cohorts of IGRA negative PLWH.

Similar IGRA negative cohorts of resisters have been studied with the goal of finding genetic or adaptive immune responses that protect against TB (18). The mechanisms whereby highly exposed but persistently IGRA negative individuals do not appear to acquire infection have predominantly been studied in HIV negative people, who are at less risk of TB than PLWH. HIV negative resisters have been shown to possess IgM specific to *M.tb*, class-switched antibodies against a range of mycobacterial antigens, and non-canonical T cell responses (19). We therefore determined how IgG responses against *M.tb* specific antigens relates to TST induration. Next, we assessed the IgG response in two cohorts of PLWH with longitudinal data. We investigated whether IGRA negative PLWH from a high burden area who had developed TB despite profound immunocompromise, as well as PLWH who underwent IGRA conversion or developed ATB, possessed IgG responses specific to *M.tb*.

4.2 Results

4.2.1 The impact of HIV infection on anti-*M.tb* IgG levels

IgG responses to *M.tb* H37Rv whole cell lysate (BEI resources) were measured by enzyme linked immunosorbent assay (ELISA), described in detail in the methods section. *M.tb* whole cell lysate (WCL) was selected as an antigen pool containing the broadest range of *M.tb* antigens. Firstly, IgG levels were measured in cryopreserved serum samples of HIV infected and uninfected individuals with ATB or LTBI (Table 4.1)**.** Two cohorts were characterized (UCT HREC 012/007 and 050/2015), with the major difference being how LTBI was determined described below.

Table 4.1 Definitions of LTBI, ATB, and median $CD4^+$ T cell counts (cells/mm^3) in cohort 1 and 2 below.

	Definition of LTBI	Definition of ATB	LTBI N=	LTBI Median (IQR) $CD4^+$ T cell counts	ATB N=	ATB Median (IQR) $CD4^+$ T cell counts
Cohort 1	Inhouse IGRA. Intracellular staining of CD4+ T cells post stimulation with WCL	Sputum culture	35	323 (250-463)	35	211 (105-484)
Cohort 2	QuantiFERON-TB Gold-In-Tube	Sputum culture or GeneXpert	27	551 (362-663)	34	161 (72-312)

Median antibody responses were compared across LTBI and ATB for HIV+ and HIV- groups (Figure 4.1). Further, the association between IgG levels against *M.tb* WCL and $CD4^+$ T cell counts was tested.

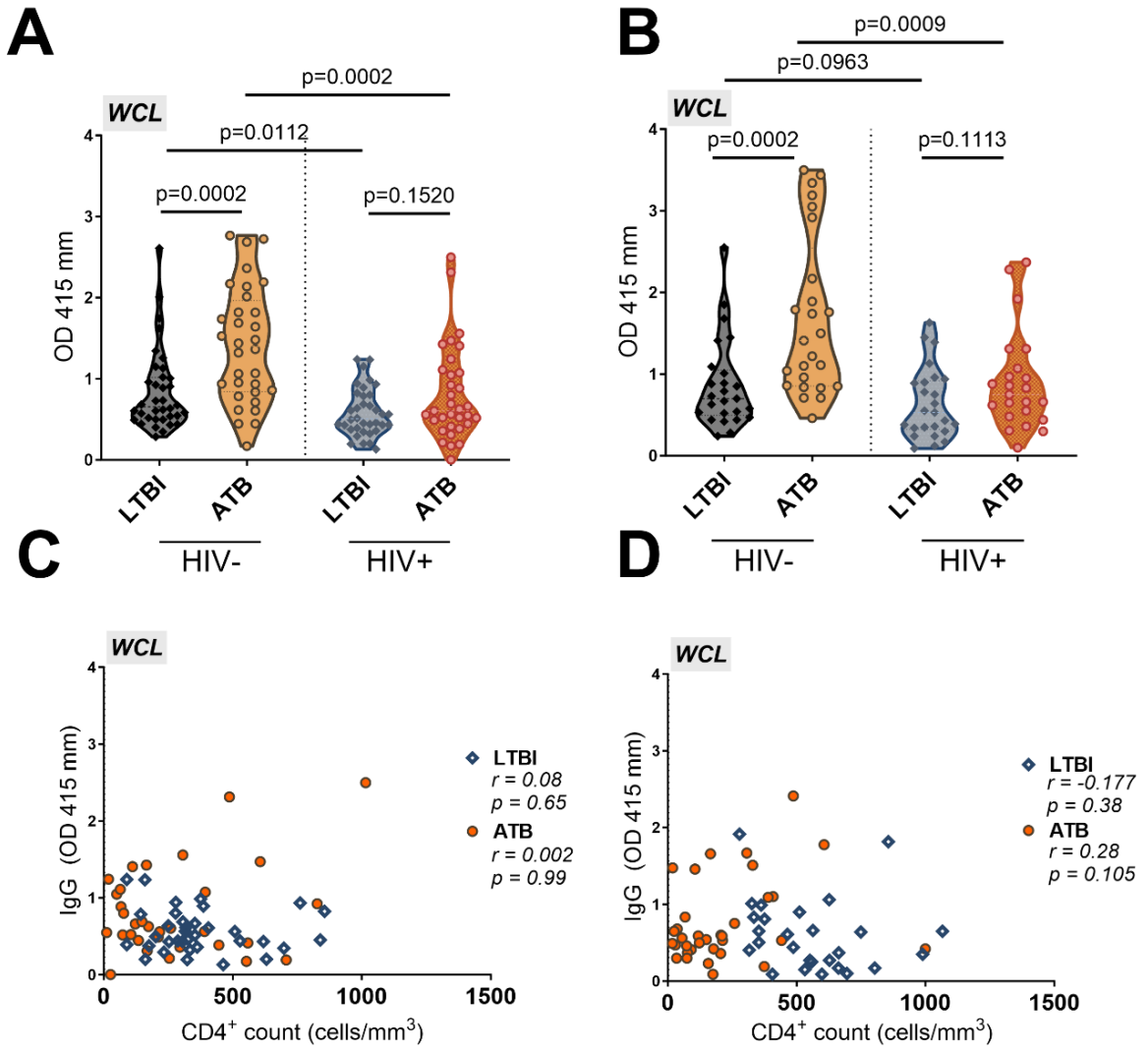


Figure 4.1 IgG levels against *M.tb* WCL in HIV infected and uninfected persons with LTBI or ATB. Results shown are in optical density (OD) and represent the mean value of technical triplicates normalized to a control serum per plate. **(A)** Cohort 1. HIV-/LTBI n=34. HIV-/ATB n=32. HIV+/LTBI n=36. HIV+/ATB n=36. **(B)**

Cohort 2. HIV-/LTBI n=25. HIV-/ATB n=25. HIV+/LTBI n=25 HIV+/ATB n=23. Violin plots with median responses and interquartile range are shown. Significance was determined by Mann-Whitney test. **(C)** Scatterplot demonstrating the relationship between anti-*M.tb* whole cell lysate IgG responses (OD 415mm) and $CD4^+$ T cell counts (cells/mm^3) for PLWH with both LTBI and ATB in Cohort 1. **(D)** Scatterplot demonstrating the relationship between anti-*M.tb* whole cell lysate IgG responses (OD 415mm) and $CD4^+$ T cell counts (cells/mm^3) for PLWH with both LTBI and ATB in Cohort 2. Significance was determined by Spearman rank co-efficient test.

In both cohorts, the magnitude of anti-*M.tb* WCL IgG responses were significantly greater in HIV-uninfected patients with ATB when compared to those with LTBI (HIV-/LTBI 0.66 [0.50-1] vs HIV-/ATB 1.4 [0.84-1.96], p = 0.0002) and (HIV-/LTBI 0.7 [IQR 0.5-1.05] vs HIV-/ATB 1.41 [0.86-2.55], p = 0.0002) respectively. However, PLWH failed to mount a similar significant increase in IgG levels between ATB and LTBI (HIV+/LTBI 0.51 [0.38-0.8] vs HIV+/ATB 0.61 [0.43-1.1], p = 0.15) and (HIV+/LTBI 0.53 [0.32-0.92] vs HIV+/ATB 0.75 [0.48-1.1], p = 0.11) respectively. In the first cohort where IGRA status was defined by inhouse flow cytometry, PLWH with both LTBI and ATB had lower anti-*M.tb* WCL IgG than HIV uninfected persons. In the second cohort, defined by QFT positivity, there was no difference across HIV status in LTBI, but PLWH with ATB had lower antibody responses when compared to HIV uninfected persons.

There was no correlation between $CD4^+$ T cell count and the magnitude of anti-*M.tb* IgG response in the HIV+/LTBI or HIV+/ATB groups. Given the blunted total IgG responses to *M.tb* in PLWH, it was next determined whether particular IgG subclasses were differentially impacted by HIV infection (Figure 4.2).

Both HIV- (HIV-/LTBI 0.039 [0-12] vs HIV-/ATB 0.21 [0.06-0.79], p = 0.001) and HIV+ groups (HIV+/LTBI 0.02 [0-0.08] vs HIV+/ATB 0.12 [0.03-0.2], p = 0.02) with ATB had greater magnitudes of IgG1 than those with LTBI. Only the HIV- group showed a significant IgG2 response during ATB (HIV-/LTBI 0.3 [0.14 – 0.45] vs HIV-/ATB 0.67 [0.18-0.59], p = 0.035). There were no differences seen between LTBI and ATB for either group when testing IgG3 and IgG4 responses against *M.tb* WCL. Neither were any differences between HIV- and HIV+ LTBI or ATB groups for any of the IgG subclasses. Levels of anti-*M.tb* IgG4 were not well detected in this assay likely due to a combination of low abundance and assay technical parameters such as over-dilution of serum.

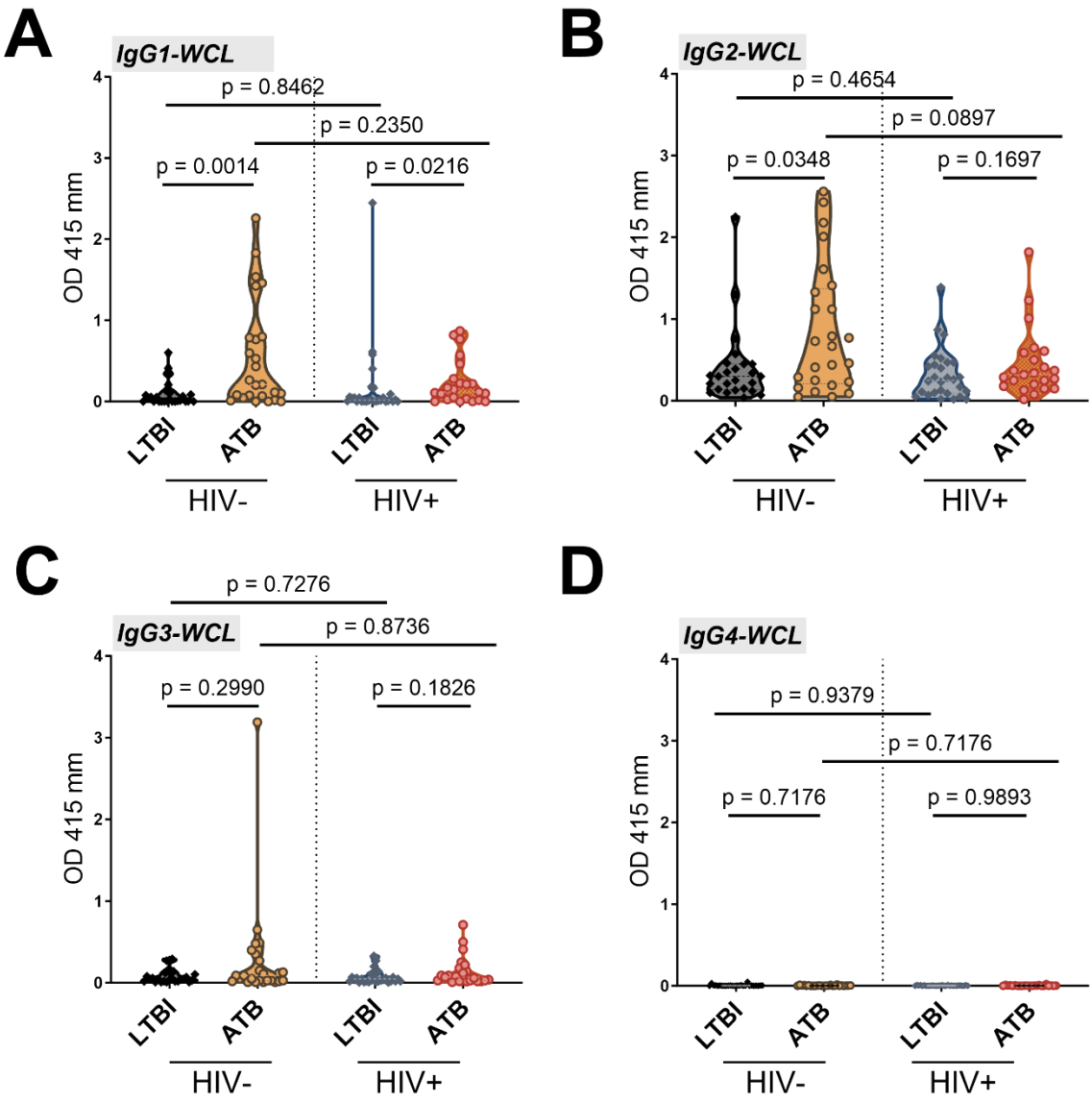


Figure 4.2: IgG subclass class detection against *M.tb* WCL in cohort 2. HIV-/LTBI n=25. HIV-/ATB n=25. HIV+/LTBI n=25 HIV+/ATB n=23 **(A)** IgG1, **(B)** IgG2, **(C)** IgG3, **(D)** IgG4. Violin plots with median responses and interquartile range are shown. Significance was determined by Mann-Whitney test.

4.2.2 Anti-*M.tb* IgG responses post initiation of antiretroviral therapy (ART).

Next, the effect of ART on IgG levels against *M.tb* in PLWH with LTBI was determined (UCT HREC 245/2009 and 545/2010). Given that ART reduces risk of developing ATB, it was postulated that ART could alter levels of IgG against *M.tb* during immune reconstitution. In this cohort, LTBI was again defined as IGRA positive per an inhouse IGRA assay, and HIV viral suppression was noted in all participants at the Week 48 timepoint (Figure 4.3). The median CD4+ counts at Week 0 (baseline) was

95 cells/mm3 (IQR 33-121), and all participants had documented virological suppression during the course of follow-up.

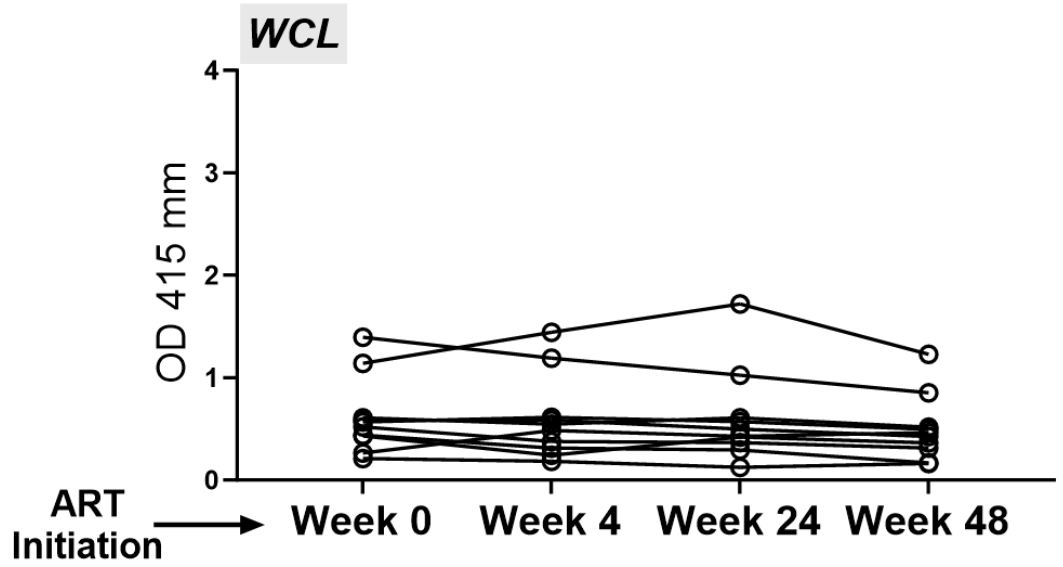


Figure 4.3: IgG levels against *M.tb* WCL in PLWH with LTBI after initiation with ART (n=10). Each dot represents the mean OD from triplicate wells for an individual participant at that timepoint. Groups were compared using Friedman's test with Dunn's correction for multiple comparisons.

Initiation of ART in PLWH with LTBI did not significantly influence IgG levels against *M.tb* WCL in a 48-week period. Next, levels of antimycobacterial antibodies in IGRA and/or TST negative PLWH were determined.

4.2.3 Antibody responses to *M.tb* in IGRA negative PLWH

IgG responses to a commercial ESAT-6/CFP-10 (E6/C10) fusion protein were measured by ELISA. This antigen, containing two RD-1 proteins, was used to determine *M.tb* specific antibody responses, whereas antibodies against WCL were taken to more broadly represent antimycobacterial antibody responses.

The relationship between IgG responses and the extent of TST skin induration as a continuous measurement in millimetres was also tested. It was hypothesized that pre-existing antibodies against *M.tb* may be lower in PLWH than in HIV uninfected persons (Figure 4.4)

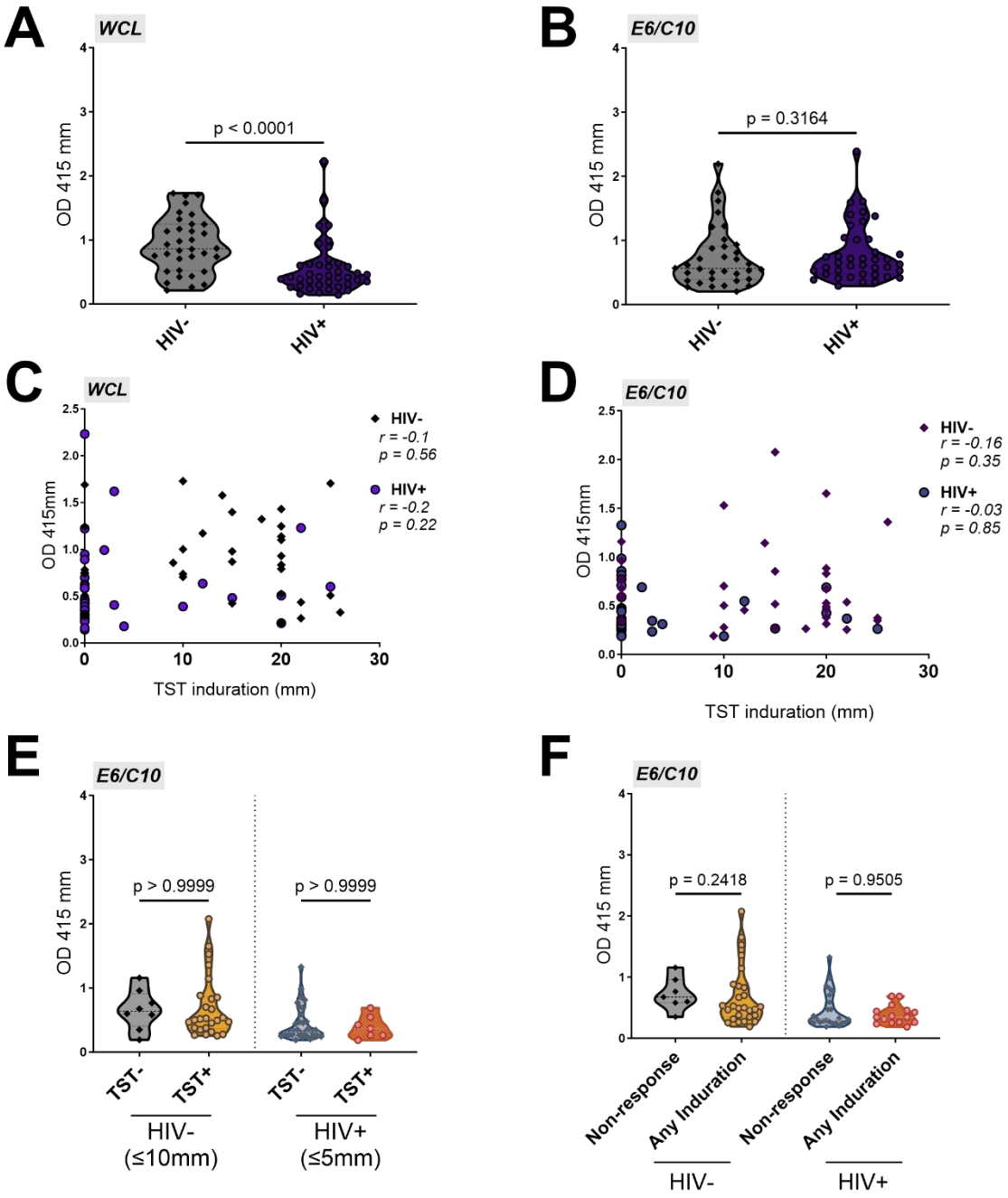


Figure 4.4: IgG responses against *M.tb* antigens HIV-/IGRA- (n = 34) and HIV+/IGRA- (n = 40) persons. **(A)** WCL **(B)** E6/C10 **(C)** Scatterplot of TST induration (mm) vs Anti-*M.tb* WCL IgG levels (OD 415mm). **(D)** Scatterplot of TST induration (mm) vs Anti-ESAT-6/CFP-10 IgG levels (OD 415mm). **(E)** Groups stratified according to WHO standardized cut-offs for TST positivity. HIV-/TST- n=8, HIV-/TST+ n=26, HIV+/TST- n=33, HIV+/TST+ n=7. **(F)** Groups stratified by TST anergic (0mm) or any induration (>0mm). HIV-/TST=0mm n=7, HIV-/TST>0mm n=27, HIV+/TST=0mm n=25, HIV+/TST>0mm n=15 Violin plots with median responses and interquartile range are shown. significance was determined by Mann-Whitney test. For scatterplots, significance was determined using Spearman rank co-efficient test.

IgG responses to *M.tb* WCL were greater in IGRA negative HIV negative individuals than in PLWH ($p < 0.0001$). However, this difference was not seen for IgG against E6/C10. There was no association between IgG levels against E6/C10 and TST induration. Stratifying patients into categories of TST result according to World Health Organization accepted cut-offs, or according to TST non-response versus responsiveness, did not reveal any difference in anti-ESAT-6/CFP-10 IgG levels. PLWH who tested negative via IGRA and TST thus possessed antibodies generated by exposure to *M.tb*. Next, the relationship between IgG against *M.tb* and risk of developing TB was determined in longitudinal cohorts.

4.2.4 Antibody responses to *M.tb* in longitudinal studies of IGRA negative PLWH

We assessed IgG responses against *M.tb* WCL and E6/C10 in sera obtained by the MVA85A vaccine trial (UCT HREC 001/2010 and OxTREC 01-10) where PLWH were followed for two years to monitor the development of LTBI or ATB (316). This enabled us to compare the baseline immune characteristics of PLWH who later developed LTBI or ATB with those who did not. In this cohort, patients included were HIV infected with $CD4^+$ T cell counts above 350 cells/mm^3, or above 300 cells/mm^3 if on ART. All patients had QuantiFERON-TB Gold (QFT) values below the threshold for positivity on enrolment. The antibody response to *M.tb* was determined on enrolment to the study, prior to intervention, and thus are not reflective of a vaccine response. Neither did MVA85A prevent incident TB in this trial (315. IgG levels were compared in QFT negative PLWH who underwent QFT conversion (converters) during longitudinal follow-up to those who remained QFT negative (non-converters) (Figure 4.5).

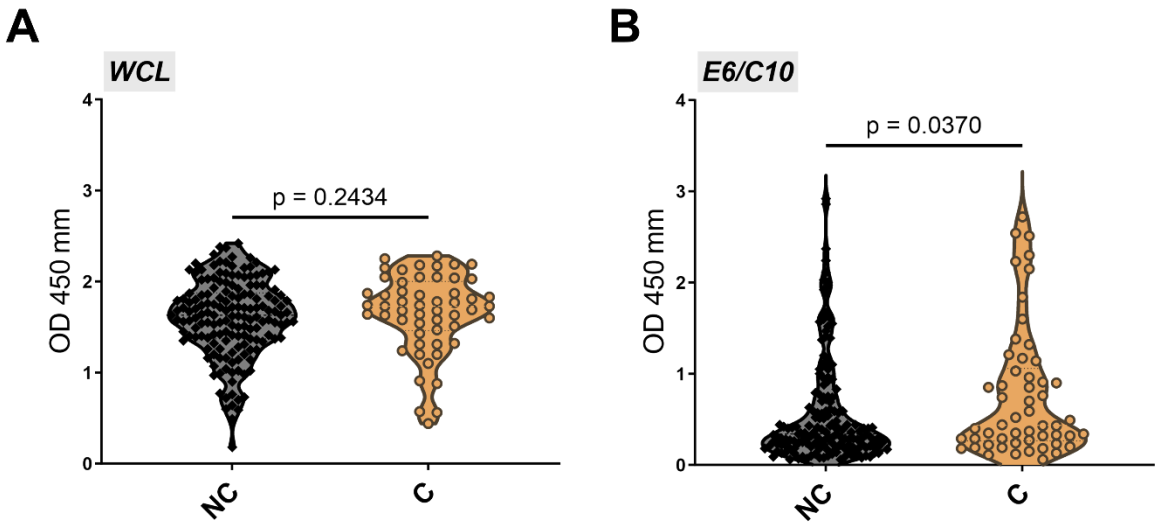


Figure 4.5: IgG responses to *M.tb* lysate in QFT non-converters (NC) (n=162) compared to QFT converters (C) (n=54)**. (A)** *M.tb* WCL **(B)** ESAT-6/CFP-10. **(C)** Violin plots with median responses and interquartile range are shown. Significance was determined by Mann-Whitney test.

IgG levels against *M.tb* WCL did not differ between QFT converters (C) and non-converters (NC). However, QFT converters possessed greater levels of IgG against E6/C10 at baseline than non-converters (NC 0.33 [0.06 - 0.59] vs C 0.44 [0.06 – 1], p = 0.037). Next, independent of QFT result at baseline, IgG responses in PLWH who developed microbiologically confirmed ATB were compared to those who did not (Figure 4.6). Cases were matched to controls (M Lesosky) based on age, gender, study site, time on study, and availability of samples. IgG levels against the vaccine antigen Ag85A, an immunodominant protein, were also tested based on previous data from infants where IgG levels against Ag85A were associated with reduced risk of developing TB (265).

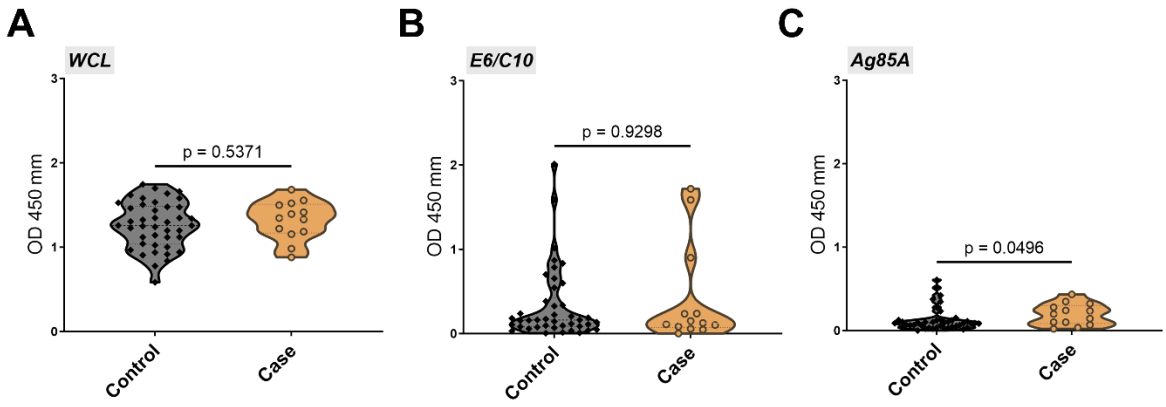


Figure 4.6: IgG responses to *M.tb* lysate in cases (n=13) compared to controls (n=42)**. (A)** *M.tb* WCL **(B)** E6/C10. **(C)** Ag85A. Violin plots with median responses and interquartile range are shown. Significance was determined by Mann-Whitney test.

No differences between PLWH who developed ATB vs controls were seen for WCL or E6/C10. However, IgG responses against Ag85A were greater in PLWH who went on to develop ATB than those who did not *(*p = 0.0496*)*. Given that here antibodies against *M.tb* associated with risk of QFT conversion and ATB, the antibody profiles of PLWH in a high burden area who did not develop TB despite low $CD4^+$ T cell counts were tested.

4.2.5 Antibody responses to *M.tb* in IGRA negative PLWH vaccine trial or resister

Antibodies against *M.tb* WCL and E6/C10 were determined in a cohort of persistently IGRA negative PLWH from a high TB burden area with no history of having TB (HREC Stellenbosch University N16/03/033A, UCT HREC 755/2016, 702/2017) (21). Here, participants had either a single $CD4^+$ T cell count < 200 cells/mm^3, or two $CD4^+$ T cell counts < 350 cells/mm^3 at least 6 months apart prior to ART initiation. Immune reconstitution was defined as a last $CD4^+$ T cell count > 200 cells/mm^3. These patients then underwent serial IGRA testing, and were defined as HIV negative, IGRA and TST non-reactive if having 3 negative IGRA tests and a TST of 0 mm induration. This phenotype was denoted

as HITTIN - HIV-1-positive persistently TB, TST and IGRA negative (HITTIN). IgG responses specific to *M.tb* in this population were determined (Figure 4.7).

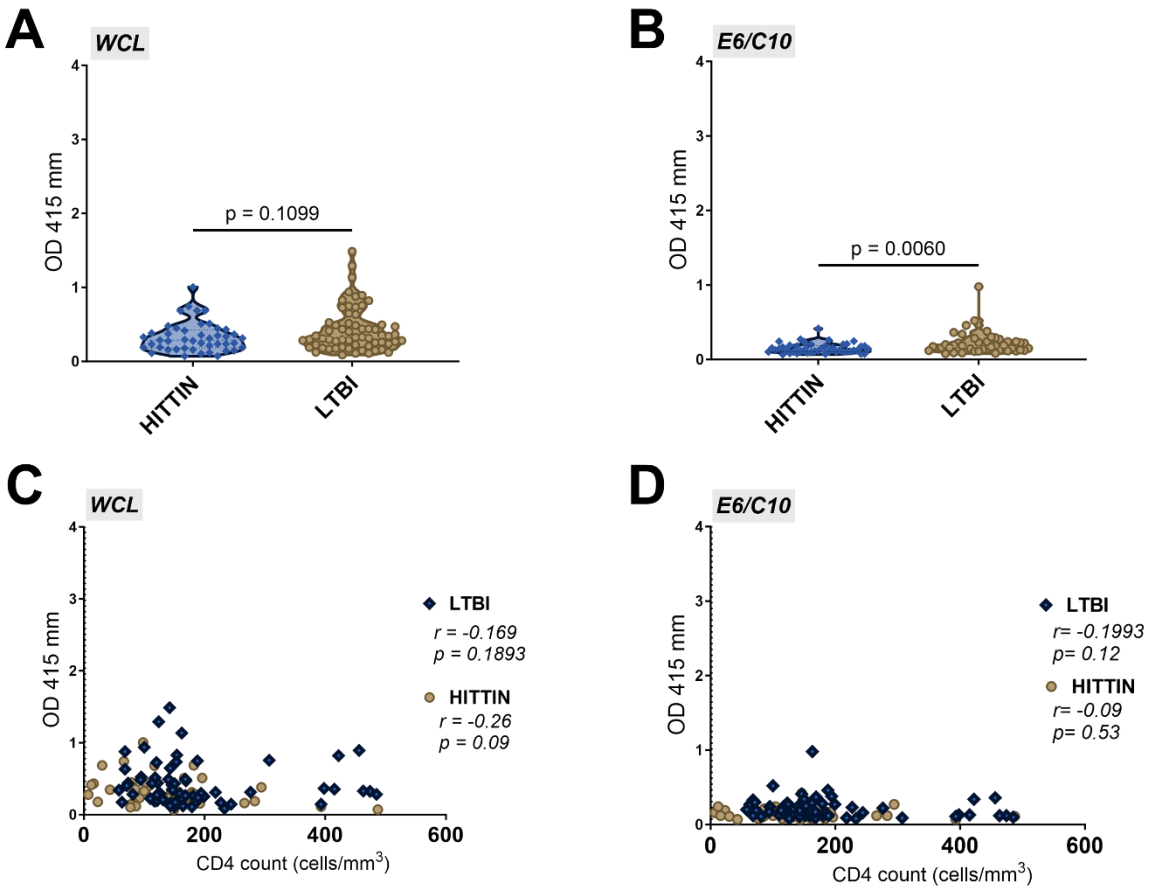


Figure 4.7: IgG responses to *M.tb* in HITTIN (n=67) compared to PLWH with LTBI (n=62)**. (A)** WCL **(B)** E6/C10. **(C)** Scatterplot demonstrating the relationship between anti-*M.tb* WCL IgG responses (OD 415mm) and $CD4^+$ T cell counts (cells/mm^3) for HITTIN and LTBI groups. **(D)** Scatterplot demonstrating the relationship between anti-*M.tb* E6/C10 IgG responses (OD 415mm) and $CD4^+$ T cell counts (cells/mm^3) for HITTIN and LTBI groups. Violin plots with median responses and interquartile range are shown. Significance was determined by Mann-Whitney test. For scatterplots, significance was determined using Spearman rank co-efficient test.

HITTIN possessed lower levels of IgG against E6/C10 than IGRA+ controls (p = 0.006). However, levels of antimycobacterial antibodies (WCL) did not differ. Again, IgG levels against WCL and E6/C10 had no correlation with $CD4^+$ T cell counts.

Taken together, IGRA negative PLWH from multiple cohorts show IgG responses to E6/C10. Given the implications of E6/C10 specific antibodies in HITTIN, titration of serum with uncoated well background was performed to eliminate non-specific plate binding as a cause for these responses.

Levels of non-specific binding could otherwise be interpreted as a signal of positive serological recognition of E6/C10. A subset of 15 HITTIN serum samples were used selected from high, middle, and low responders in the above data (Figure 4.8).

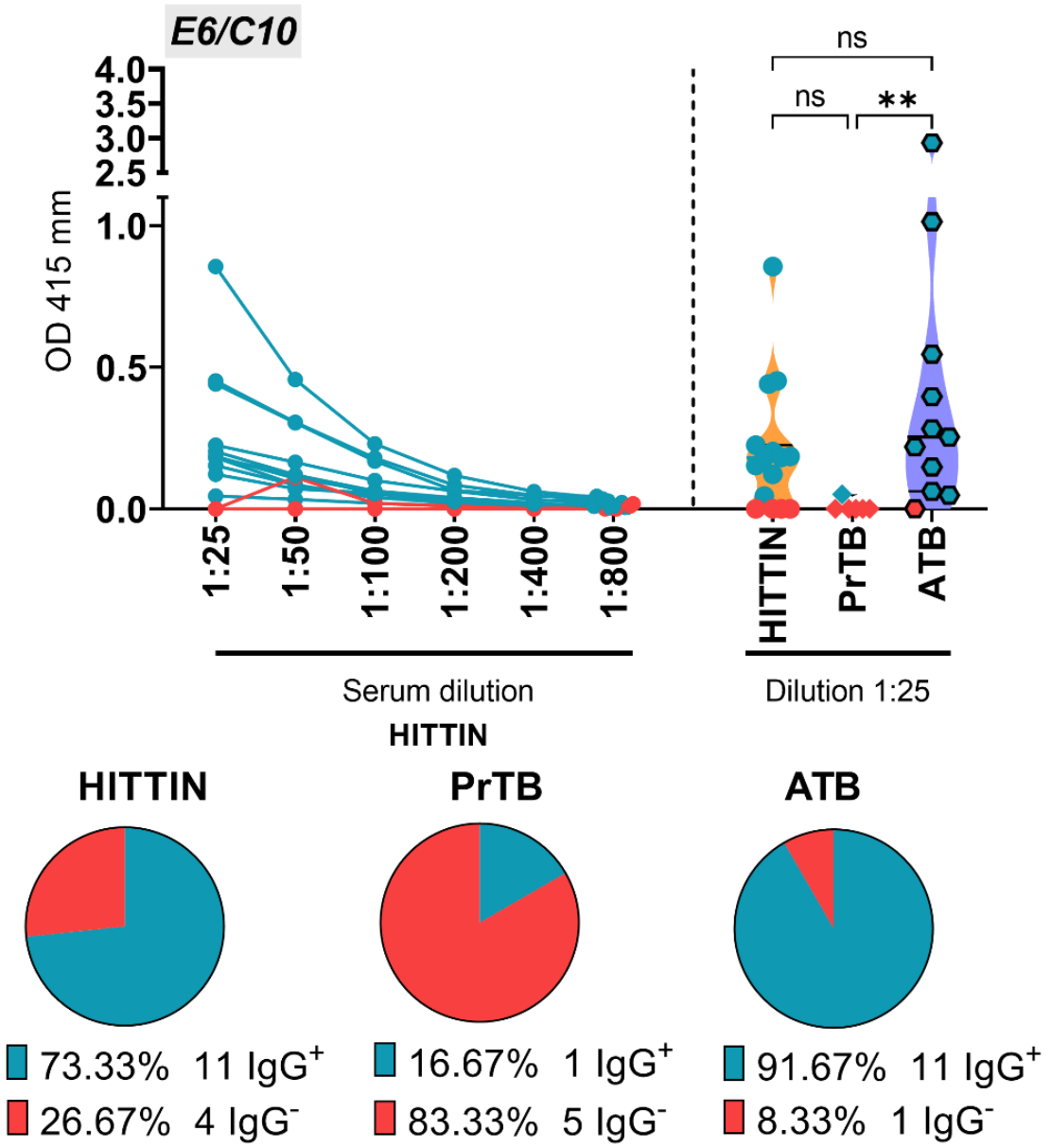


Figure 4.8: Titration curves of serum antibody responses against E6/C10 in HITTIN (n=19). Optical density (OD 415mm) values from antigen uncoated wells at each dilution were subtracted from serum OD values at each dilution. Serum dilutions range from 1:25 to 1:800. Absent binding curves are shown in red. Controls include 6 individuals with previous TB (PrTB) (n=6) and PLWTH with ATB (n=12). Here, violin plots with median and interquartile ranges are shown. Groups are compared using Kruskal-Wallis test with Dunn's post-test correction for multiple comparisons. * = $P \leq 0.05$. ** = $P \leq 0.01$. *** = $P \leq 0.001$. ****= $P \leq 0.0001$.

IgG titres with background subtraction reveals that the majority of HITTIN included in this assay (>2/3) did possess antibodies specific to *M.tb* (E6/C10). Control groups of PLWH with previous TB or ATB were included for comparison to groups with a definite history of TB. There was no statistically significant difference between IgG levels at a 1:25 dilution against E6/C10 in HITTIN compared to the previous TB or ATB groups. However, PLWH with previous TB had lower IgG levels than those with ATB ($p = 0.005$).

4.3 Discussion

In this chapter we show that IgG responses against *M.tb* are impacted by HIV infection. In two cohorts, we validated that ATB is characterizable by an increase in antimycobacterial IgG (22). In PLWH with ATB however, the IgG response to *M.tb* WCL was blunted. PLWH also had impaired IgG2 responses, whereas ATB was associated with greater antigen specific IgG2 levels in HIV uninfected people. A lack of IgG2 response to LAM has previously been shown to associate with extrapulmonary TB (15). A potential explanation for this is the dysregulation of IFN-γ production due to HIV, with IFN- γ being a key inducer of IgG2 responses (328).

The clinical context for these findings is that TB patients with HIV-1 coinfection are less likely to have cavitatory lung disease, and more likely to present with disseminated TB (2). As discussed in chapter 1, a 'too little, too late' model for the emergence of protective antibodies during TB is possible. A mAb against HBHA limited the dissemination of *M.tb* in mouse models, and an anti-PstS1 mAb cloned from a patient with ATB modestly restricted the growth of *M.tb* (23,24). Therefore, PLWH could be predisposed towards disseminated disease, in part, by an insufficient antibody response to restrict hematogenous spread of *M.tb*.

In this work, the initiation of ART in PLWH with LTBI did not impact IgG levels against *M.tb* WCL. Hypothetically, IgG responses could have been altered in response to antigen availability if immune reconstitution led to the killing of *M.tb* over the time period. Unmasking IRIS responses could potentially associate with increased IgG levels (25). However, no change in anti-*M.tb* WCL IgG levels were seen over the course of 48 weeks in these participants.

However, IGRA positivity doesn't necessarily imply ongoing *M.tb* infection, given that most IGRA positive persons do not develop TB, and chemotherapeutic treatment of LTBI only alters the host transcriptomic response in approximately one third of people (26). Thus, these patients may no longer be infected with viable *M.tb*, and thus initiation of ART would not result in killing of mycobacteria with stimulation of a humoral immune response. It is also possible that the timepoint of 48 weeks was too short a timepoint to see restoration of circulating IgG levels, given that B cells are still dysfunctional a

year after initiation of ART (11). It is also possible that isotype switching, or avidity changes occur post ART initiation due to restored T cell help.

Next, we investigated antibody responses across three cohorts of IGRA negative PLWH. Although interest in this phenotype has grown, studies have not yet included PLWH. In our data, IGRA negative PLWH had lower levels of IgG against WCL than HIV uninfected persons. However, this was not the case for IgG against the secreted antigens E6/C10, where no difference was seen. One explanation is that antibodies against E6/C10 and other protein antigens are relatively preserved, however HIV may impair responses to antigens such as glycolipids and carbohydrates found in WCL.

ESAT-6 and CFP-10 are secreted antigens encoded in the RD-1 segment of the *M.tb* genome that are not expressed by BCG; thus, these antibodies are thought to arise from prior infection with *M.tb* (27). Antibodies against ESAT-6 could potentially arise from NTM such as *M. kansasii*, but the burden of such infections is substantially lower than that of *M.tb* (28). Here, IgG to E6/C10 did not relate to TST or IGRA status. Different mechanisms could account for the presence of IgG against E6/C10 in the absence of IGRA or TST positivity. Firstly, antigen-specific T cell responses could wane due to HIV mediated loss of $CD4^+$ T cells. Secondly, clearance of *M.tb* infection could also result in declining T cell responses with IgG levels sustained by long-lived plasma cells. Thirdly, non-IFN-γ T cell responses, such as CD40L/CD154 expression seen in Ugandan resisters, could predominate in these patients (19). Lastly, antigen specific T cells could compartmentalize to the lung over time and no longer be peripherally detectable.

Next, we showed that IgG levels against E6/C10 are higher in individuals who undergo IGRA conversion relative to those who do not. This data accords with the notion that IgG levels against secreted antigens relates to bacterial load. It is unlikely that anti- E6/C10 antibodies emerge prior to cognate T cell response, thus the limit of detection for QFT (ESAT-6, CFP-10, TB7.7) could underestimate true sensitization to *M.tb* antigens.

When examining IgG levels in PLWH who went on to develop ATB, E6/C10 levels were not different between PLWH who developed ATB during follow-up and controls. However, IgG responses against Ag85A levels were significantly greater in those who developed ATB. For the analysis of cases of ATB, both controls and cases were grouped independent of IGRA result at baseline, owing to limited numbers of patients who developed ATB during the 2-year follow-up period. This work was predicated on the finding that antibody responses against Ag85A in BCG vaccinated infants were greater in those that did not go on to acquire ATB (29). However, this trend was not seen in HIV infected adults. As to why Ag85A was significantly increased, but E6/C10 was not, could be accounted for by Ag85A being the most frequently identified antigen in circulating immune complexes during ATB (30,31). These results lend further weight to the argument discussed that activation of humoral immunity (FcR, C1q, IgG) is upregulated during the progression from subclinical and ATB (32).

In the third IGRA negative cohort, extensive clinical characterization was performed to ensure that IGRA negative PLWH had undergone periods of advanced immunosuppression rendering them susceptible to TB. The key question was whether such individuals remained IGRA negative despite exposure to *M.tb*, or simply because they had never been exposed to *M.tb* in the first place. Here we show that it is likely that the majority of these PLWH who were persistently IGRA and TST negative were indeed infected with *M.tb* at some point in time yet did not develop disease. Mthembu *et al* have recently described the existence of anti-E6/C10 antibodies in PLWH, with increasing discordance at lower $CD4^+$ T cell counts (334).

There were also several technical aspects to these experiments that could be improved upon. Firstly, the 'hook' or prozone has been described where the presence of very high amounts of antibody in serum sterically hinders the binding of antigen-specific immunoglobulin to the coated protein, resulting in falsely low or even negative signal from the ELISA experiment (33).

Although these assays were performed with serum at a 1:100 dilution, ideally, the HIV/ATB groups should undergo titration of serum to determine whether a hook effect did occur. Next, subtraction of chemiluminescent signal (OD) from non-coated background wells was not always performed owing to clinical sample availability and the number of samples required to run in large clinical studies (34). Here, background of secondary was subtracted, and samples were normalized to control serum on every plate. To determine whether responses reflect non-specific binding of serum to the ELISA plate, or true antigen responses, background subtraction is required as was shown in Figure 4.8.

In Figures 4.6 and 4.7, owing to performing this assay in a different laboratory, a different secondary antibody was used. Here it is seen that the WCL ELISA background responses are high above background. To correct this, titration of the secondary antibody should have been performed and the time to stopping the substrate reaction could be shortened.

IgG4 was also poorly detected in this study. IgG4 is the least abundant subclass, representing 4% of total IgG in normal serum (5). This could account for why the assay was unable to detect differences in IgG4 production. To optimize the signal from this ELISA, greater concentrations of serum should be used with a positive control or purified IgG4 to determine the optimal dilution of serum.

This work could also be expanded in several directions. Recently, IgM responses have emerged as a correlate of protection in non-human primates with sterilizing immunity against *M.tb* induced by intravenous BCG vaccination (272). Further, a population of resisters studied in Uganda were characterized as having IgM responses against a broad range of *M.tb* antigens, including ESAT-6/CFP-10 (19). Thus, whether HIV impacts IgM levels against *M.tb*, especially in the HITTIN cohort, is of interest.

Early work on extrapulmonary TB in HIV suggested that individuals with TB lymphadenitis lacked IgG2 against LAM (15). $CD4^+$ expressing T follicular helper cells, lost in HIV infection, are necessary

for B cell class-switching and maturation (37). Thus, assays could be extended to determine the impact of HIV on IgG subclass recognition of other glycolipids.

Beyond quantitative antibody responses, antibody function against *M.tb* in PLWH is largely understudied. Neutrophil-mediated opsonophagocytosis of mycobacteria via CD16 and CD35 is impaired in PLWH, but the effect of HIV on other functions of antibody is unknown (339). One approach here could be to compare the effect of purified serum immunoglobulins from both PLWH and HIV uninfected persons in *M.tb* whole blood growth assays, controlling for concentration of anti-*M.tb* antibodies. The hypothesis being that PLWH produce poorer affinity antibodies, have a restricted breadth of target recognition, or impaired antibody effector function. It is plausible that IFN-γ is required to mediate the effect of mAbs, and thus here mAbs should also be tested in WBA samples derived from otherwise healthy PLWH where this signalling pathway may be deficient. I did not test whether a differential effect for 78_59 exists between HIV infected and uninfected WBA, but this could form the substrate of future investigation.

Defects in the antibody compartment in PLWH could also be studied directly from patients with advanced HIV and mycobacteremia (340). Recently, *ex vivo* fluorescent imaging and flow cytometry of *M.tb* BSI have visualized mycobacteria isolated directly from patient blood samples (341). Tagging IgG on *M.tb* in whole blood with a fluorescent secondary antibody against human immunoglobulin could be an approach to rapidly quantify opsonizing antibody directly from clinical samples. It could also address a fundamental question as to whether antibodies bind *M.tb* during hematogenous spread..

As a whole, PLWH have blunted IgG response to *M.tb* across IGRA negative, LTBI, and ATB. We also show that detection of antibodies arising from prior exposure to *M.tb* are detectable in different cohorts of IGRA negative PLWH. In cohorts with longitudinal follow-up, greater antibody responses against E6/C10 or Ag85A associate with disease progression, either to LTBI or ATB. This finding fits in with transcriptomic signatures showing the upregulation of Fc receptor and C1q expression during sub-clinical TB (167,342).

Yet, the majority of PLWH with low $CD4^+$ T cells who did not develop ATB despite living in a high transmission area, possessed antibodies specific to *M.tb.* The presence of these antibodies was valuable to confirm that these PLWH had an adaptive immune response specific to *M.tb* and therefore must have had been exposed at some point in time. Why they had no history or developing TB despite profound immunosuppression is an area of ongoing study. Whether these antibodies are part of the apparent protection against disease or help prevent dissemination of disease in PLWH could be explored in future studies.

Chapter 5: Towards serum antibody functional assays in BCG vaccination

5.1 Introduction

BCG is a live-attenuated vaccine to prevent TB that was developed by serial passage of *Mycobacterium bovis* (342). BCG has an excellent safety profile and is recommended by the World Health Organization in countries with a high burden of TB and/or leprosy (344). BCG vaccination's effectiveness in preventing against adult pulmonary TB is inconsistent, ranging from 0 to 73%, and the principal rationale behind its continued use is to reduce the incidence of extrapulmonary TB in children (345,346).

The mechanisms whereby BCG protects against TB in these circumstances are incompletely understood. The frequency and cytokine profiles of BCG-specific $CD4^+$ T cells were previously shown to not associate with protection against IGRA conversion in children, as was rationally anticipated (346). However, more recently BCG-specific T cells that secrete IFN-γ did associate with reduced risk of TB in infants (270). In the same study, infants who did not go on to develop ATB had greater levels of anti-Ag85A IgG at baseline (270). Tanner *et al* have reviewed the consistency of findings that BCG vaccination leads to an antibody response (348) Beyond measuring the magnitude of responses induced by BCG vaccination, the function and role of such antibodies are yet to be comprehensively characterized. This has implications for improving upon the immune response induced by BCG, as many candidate vaccines are designed as a prime-boost strategy post BCG vaccination.

Even though BCG vaccination has partial clinical efficacy, its use in humans provides a model to investigate the human antibody response against mycobacteria. BCG retains close genetic homology to *M.tb*, does not require containment level 3 biosafety protocols, and is safe to administer across species (329).

The ongoing use of BCG vaccination as a fruitful discovery tool is highlighted by studies using intravenous (IV) BCG vaccine to elicit sterilizing immunity in non-human primates, and proposed phase 1 trials to evaluate mucosal immunity induced by aerosolized BCG (272,348).

Antibody-dependant cellular phagocytosis (ADCP) is postulated to be an important mechanism whereby antibodies may control the growth of *M.tb* (226). Recognition of the surface of live mycobacteria is a contingent condition for antibodies to perform ADCP. Although ELISA on live *M. bovis* BCG have been demonstrated, these methods are time-consuming and have high background

signal (302). Opsonization assays are therefore necessary. In this chapter, I sought to develop proof-of-concept for assays that could be used to evaluate antibody functionality in clinical samples.

The aims of this study therefore were therefore to first develop a flow cytometry-based assay to test serum ADCP capacity stimulated by BCG vaccination. Secondly, I aimed to determine the feasibility of a method to rapidly assess bacterial opsonization by serum antibodies using flow cytometry.

5.2 Results

5.2.1 Towards measuring ADCP in BCG vaccination

A flow cytometry assay was developed to determine ADCP for serum, with gating strategy identical to that described in chapter 3 for mAbs (Figure 5.1A). In brief, THP-1 were cultured in 96-well plates and then infected with BCG-GFP in the presence of serum. After a washing step, GFP^+ macrophages were enumerated by flow cytometry. We sought to determine conditions for testing the effect of BCG vaccination on serum ADCP capacity. The variables to optimize this assay included multiplicity-of-infection (MOI), time post infection, concentration of serum incubated with BCG-GFP (Figure 5.1B-D).

From this data, an MOI of 10, a timepoint of two hours as a midpoint between 1h30m and 2h30m, and a serum concentration of 1:10 were selected for downstream experiments. This serum concentration condition was selected as the higher serum concentration did not produce markedly greater ADCP responses, and a concentration of 1:10s better conserved serum stocks for future experiments. Discrimination between Donors and BCG control was observed at an MOI of 1 (Donor 2 vs BCG alone, $p = 0.013$) and 10 (Donor 2 vs BCG alone, $p = 0.03$). From this data it appears that the increase in ADCP is mostly driven by vaccinated donor 3 and would require testing with a bigger sample size to discriminate between donors. For the time course assay, no timepoints were different statistically. There is also inter-assay variability between the MOI and time course experiments, which could be explained by variable expression of GFP.

Thus, ADCP was next tested in a clinical cohort of United Kingdom citizens receiving BCG vaccination as adults (NHS Research Ethics Service (NRES) Committee South Central—Oxford B (REC reference 15/SC/0022) as described in publication by Wilkie *et al* (2022) (349). Healthy BCG-naïve adults residing in the United Kingdom were enrolled. LTBI was excluded by IGRA testing. Volunteers received a standard clinical dose of $2–8 \times 10^5$ CFU BCG SSI intradermally. For the below (Figure 5.2), pilot assays 6 randomly selected samples from the group receiving BCG were selected, and samples were tested at D0, D14, D28, and D84 post-vaccination.

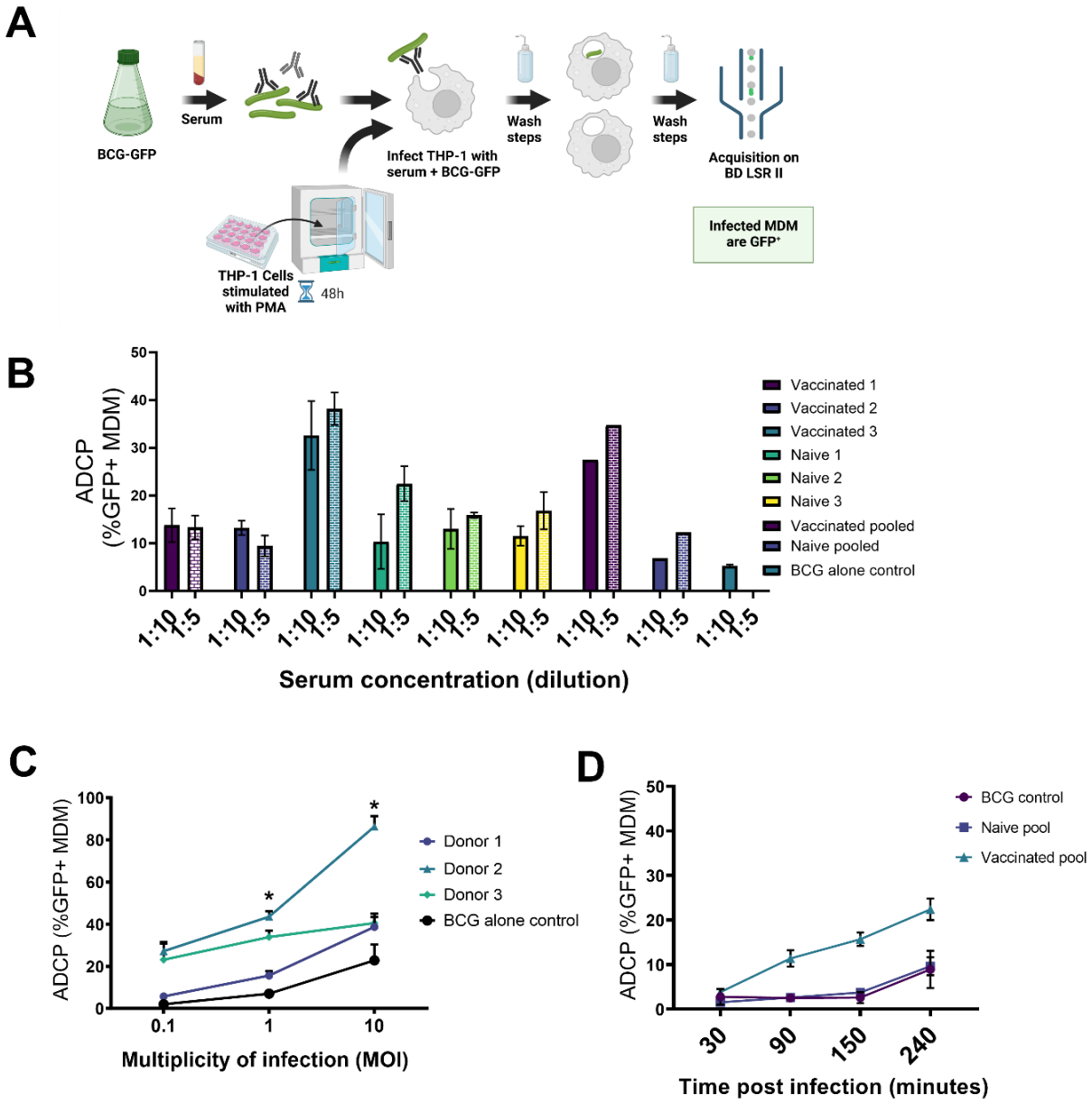


Figure 5.1 Development of flow cytometric assay to measure ADCP in THP-1 MDM **(A)** Schematic outlining assay protocol. **(B)** Testing of serum concentrations 1:10 vs 1:5 for a range of serum samples. Mean of duplicate samples is shown for each sample, apart from BCG alone without serum control samples which are single values and hence no error bars are shown. **(C)** MOI curve of three donors included in the BCG vaccinated pool. Data shown is mean with error. **(D)** Time course of ADCP using an MOI of 1. Data shown is the mean of technical duplicates. For **(C)** and **(D)**, Samples were compared using Kruskal-Wallis test with Dunn's correction for multiple comparisons. * = $p<0.05$.

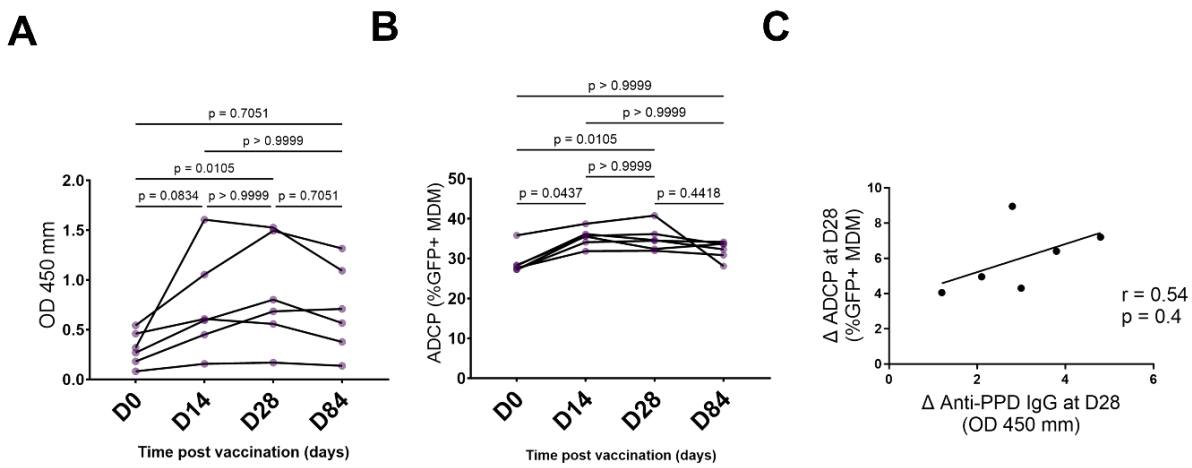


Figure 5.2 Effect of BCG vaccination in adults on IgG responses to PPD, and on ADCP (n=6). **(A)** IgG levels against PPD at D0, D14, D28, and D84 post-vaccination. Data points shown are paired samples with each point representing the median of technical triplicates. **(B)** ADCP in THP-1 cells as ascertained by flow cytometry. Values shown are the mean of duplicates for each sample. Significance was determined by comparing the median at each timepoint using Friedman's Test for paired non-parametric data with Dunn's correction for multiple correction. **(C)** Scatterplot of the fold change between anti-PPD IgG at D0 and 28 vs the fold change between ADCP (%GFP+ THP-1) at D0 and D28. Samples were compared using Spearman's rank coefficient test.

BCG vaccination induces an antibody response to mycobacterial proteins (PPD) which trended towards significance at D14 (p = 0.0834) and was statistically significant at 28 days post vaccination (p = 0.0105). In Figure 5.2B, BCG vaccination is shown to induce a significant ADCP response at both D14 (p = 0.0437) and D28 (p = 0.0105) post-vaccination. The increase in ADCP in the absence of a significant increase in IgG at D14 could be due to statistical powering, or perhaps the unmeasured effect of IgM complement recruitment in this assay. Despite the lack of correlation between the fold change increase of anti-PPD IgG and ADCP levels from D0 to D28, samples from these timepoints were selected per conventional timepoints in the evaluation of vaccine induced antibody responses.

Next, to the contribution of complement and FcR in ADCP were determined. The role of Fc receptor mediated uptake via FcγRIIIA (CD16) and FcγRIIA (CD32) were tested via cross-linking by commercial anti-CD16 and CD-32 mAbs at concentrations recommended by the manufacturer for flow cytometry (1:100) (Figure 5.3). The effect of heat-inactivation of serum at 56 degrees Celsius was also tested to assess the influence of complement in this assay. Complement is degraded at this temperature whereas serum immunoglobulins remain intact.

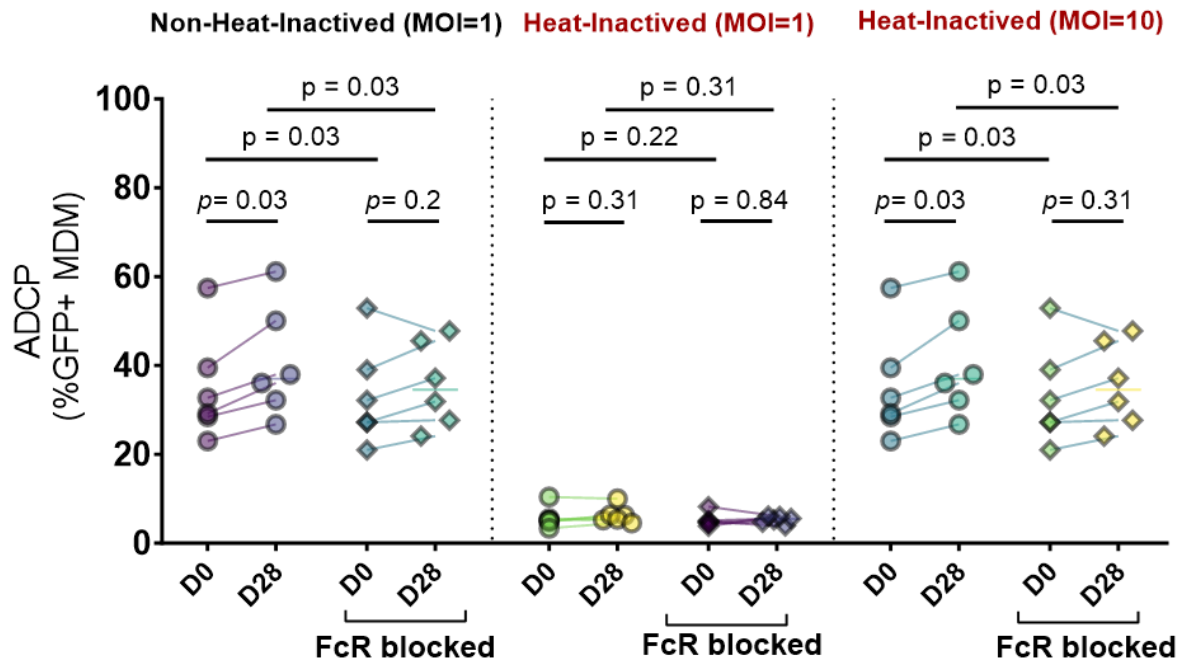


Figure 5.3 Effect of heat inactivation, FcR blockade and MOI on ADCP in BCG vaccination (n=6). Each datapoint represents the mean of technical duplicates. Samples at D0 and D28 are compared with or without mAbs crosslinking CD16/CD32. Two heat-inactivation conditions are shown, firstly with an MOI of 1, and next with an MOI of 10. Samples were compared by multiple Wilcoxon matched-pairs signed rank tests.

In non-heat inactive conditions without FcR blockade, BCG vaccination again enhanced serum ADCP at D28 post vaccination (p = 0.03). This enhancement is reduced by the presence of mAbs cross-linking Fc receptors and preventing activation via serum immunoglobulins. Heat-inactivation of serum results in a large drop in ADCP capacity, with less uptake of mycobacteria and no differences between D0 and D28, as well as no effect of FcR blockade. Complement and immune complex uptake therefore appears to contribute towards phagocytosis of mycobacteria. As the uptake of BCG-GFP was lowered by heat-inactivation, the MOI was then increased to an MOI of 10 better test if an effect for Fc-receptor blockade was still present. At an MOI of 10, there was a significant difference at D0 compared to D28 serum. This effect was again reduced in the presence of Fc receptor blockade (p = 0.03). BCG vaccination therefore induces an ADCP response, with the contributors thereto including IgG, complement and FcR.

5.2.2 Towards a bacterial flow cytometry assay to opsonization of BCG

Next, the feasibility of a same-day flow cytometry assay to measure opsonization of live *M. bovis* BCG was tested. Stocks of BCG-GFP were cultured as per for ADCP assays above. To measure opsonization, live BCG-GFP was co-incubated with serum for two hours, and then washed and fixed with paraformaldehyde (Figure 5.4A). A secondary fluorescently conjugated antibody binding the constant

region of IgG was used at commercially recommended concentration (1:100) to determine antibody remaining bound to BCG-GFP. Data was acquired by flow cytometry as below (Figure 5.4B-E).

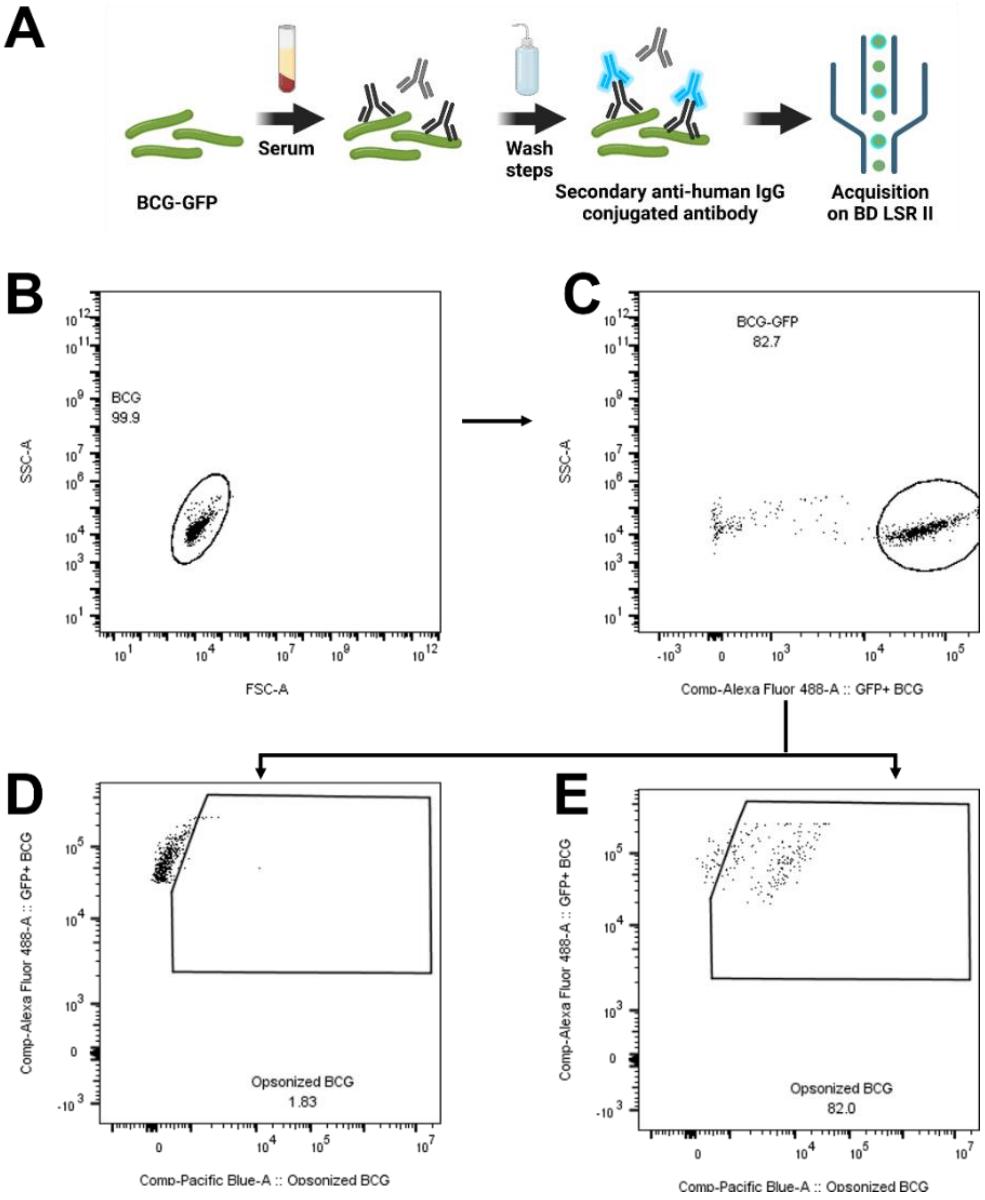


Figure 5.4 Exploratory studies to rapidly measuring BCG opsonization via bacterial flow cytometry. **(A)** Schematic of assay design; **(B)** SSC-A vs FSC-A, **(C)** SSC-A vs Alexa-488 (GFP^+), **(D)** FMO gating on BCG with secondary anti-IgG V450 (Pacific Blue) conjugated antibody, **(E)** Demonstration of positive sample on gating derived from FMO control.

This was then used in a pilot experiment to test whether bacterial flow cytometry could discriminate serum opsonizing capacity between BCG vaccinated and BCG naïve persons (Figure 5.5).

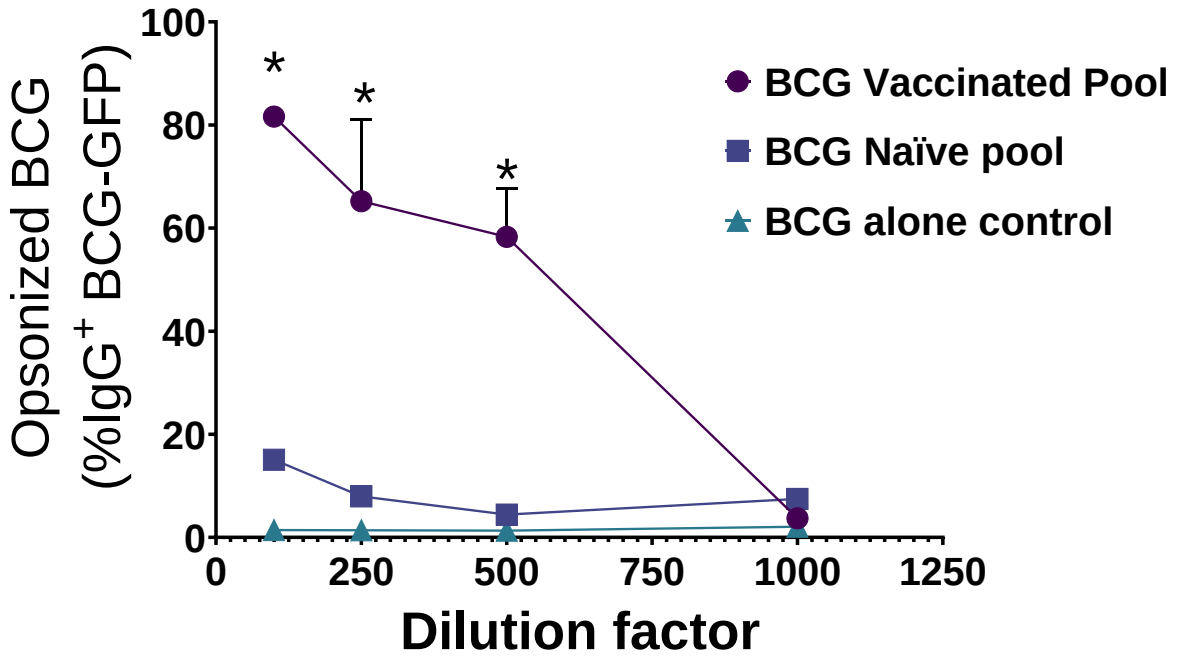


Figure 5.5. Opsonization of control pool serum in BCG vaccination with concentration series of serum. Pooled donor serum of BCG vaccinated individuals (n=3) were compared to BCG naïve individuals (n=3) and BCG with secondary antibody only. Data points pictured represent the mean of triplicates with error shown. Data points were compared using Kruskal-Wallis with Dunn's correction for multiple comparisons. * = $p<0.05$.

Bacterial flow cytometry discriminated between BCG vaccinated pool serum and control at dilutions of 1:500 ($p = 0.0219$), 1:250 ($p = 0.0417$), and 1:100 ($p = 0.0219$). However, no significant differences were seen when compared for BCG naïve serum. A concentration-based effect for serum dilution is also noted. Low background signal in the control suggests non-specific binding of antibody to BCG is not a major factor in this assay. This assay may therefore be a viable means to measure opsonization capacity. in serum samples.

5.3 Discussion

In this chapter, I undertook the development and application of assays to measure the function of antibodies induced by BCG vaccination. Understanding the antibody response to BCG vaccination is likely similar to that of the response induced by *M.tb* infection, these results would still need to be validated using virulent strains of *M.tb* with primary human MDM. Future studies could use live/dead reporter strains of *M.tb* that are now available to develop similar flow cytometry-based antibody

functional assays. or imaging experiments (350). A necessary next step would also be to determine the fate of phagocytosed mycobacteria by measuring CFU post-infection in the same assays.

A key limitation to the experiments in this chapter was that the number of samples run was not powered optimally for statistical analysis. The strength of findings in preliminary assay design could have been bolstered by technical replicates and repeating experiments. When applied to clinical samples, power calculations should be included in experimental design to better predict required sample size (350).

Although differences in immunogenicity across different strains of BCG have been shown, studies have not extended specifically to humoral immunogenicity, and particularly, antibody function (351). Although it is possible that differing strains of BCG vary in ability to evade or suppress the function of antibodies, this was beyond the ability to test in this thesis.

The data that is described above shows proof-of-concept for antibody functional assays that later were refined by the McShane laboratory and used describe the induction of BCG-specific opsonizing and ADCP performing antibody responses (231). In this study by Bitencourt *et al*, BCG vaccination was shown via B cell enzyme-linked immunosorbent spot assay (ELISpot) to induce a BCG-specific population of plasmablasts. Purified IgG showed best opsonization capacity at D84 post vaccination, with opsonization capacity correlating to ADCP performance (231). Chen *et al* have also described a relationship between ADCP and antibodies raised against surface-exposed arabinnomannan by BCG vaccination (352).

The role for Fc receptors in TB is now well described (226). Blockade of CD16 and CD32 in a whole blood assay abrogated reduction of *M.tb* CFU mediated by purified immunoglobulins isolated from healthcare workers (230). Bitencourt *et al* developed CD16/CD32 blockade on ADCP assays further to show that Fc receptor mediated ADCP is boosted by BCG vaccination (231). In Figure 5.3, an isotype mAb control to ensure non-specific binding of mAb did not non-specifically block engagement of FcR should have been included.

The bacterial flow challenge requires further optimization regarding a ratio of bacilli to serum, given the low number of bacilli captured in each run. Such an assay has advantages over ELISA in terms of testing binding to live bacilli, same-day sample preparation and rapid acquisition. Bitencourt *et al* in the same laboratory refined this method using purified IgG from BCG vaccinated adults to show that opsonization was greatest at D84 post vaccination, and that opsonization correlated strongly with ADCP (231). For exploratory purposes, it would also be interesting to know if serum contains IgA or IgM that opsonizes mycobacteria. However, secondary antibodies targeting these subclasses was not used in this experiment. In future, this technique could be adapted to an automated plate acquisition flow cytometer for high-throughput screening of serum opsonizing capacity.

It is thought that protective antibodies against *M.tb* would opsonize bacilli and facilitate FcR-mediated phagocytosis and killing. However, whether antibodies also recognize the surface of infected cells to

facilitate ADCC is unknown. Future functional assays in TB should also address ADCC as a potential mechanism of antibody-mediated protection. ADCC has previously been tested in TB by measuring NK cell activation after contact with the Fc portion of anti-PPD antibodies, which may not recapitulate natural cellular infection events with live mycobacteria (229). Using similar methods to the above, MDM infected with BCG-GFP could be stained by serum antibodies and bound cells could be labelled by secondary antibody for enumeration via flow cytometry.

As complement has long been understood to play a role in phagocytosis of *M.tb*, it will be of interest to better understand the role of IgM in TB, as IgM is a far more potent activator of complement immune complex formation than IgG (84,353) Future experiments could investigate the contribution of IgM to ADCP in TB via blockade of FcμR or depletion of IgM from serum. In the next chapter, we tested the hypothesis that IgM-producing B cells are activated in TB infection and disease.

Chapter 6: Marginal Zone B cells in TB

6.1 Introduction

Prior work in this thesis has predominantly focussed on IgG responses, but several lines of inquiry generated interest in T cell independent antibody responses and IgM. Firstly, in chapter 4 we identified that levels of IgG against *M.tb* did not relate to $CD4^+$ T cell counts in PLWH. This was unexpected, as serological memory against pathogens such pneumococcus and measles declines with the loss of $CD4^+$ T cells (354). One explanation was that B cell activation was uncoupled from cognate T-B in interaction. In the previous chapter, ADCP induced by BCG vaccination was found to be impacted by heat-inactivation of complement in serum. Enhanced ADCP also occurred at Day 14 post-vaccination, a timepoint where vaccine-induced IgG levels had not yet peaked. IgM is known to recruit complement ~1000x more effectively than IgG and may therefore have played an unmeasured role in recruiting complement and opsonophagocytosis (355).

Lastly, collaborators in the Gengenbacher laboratory showed that a subset of IgM-expressing memory B cells known as marginal zone B cells (MZB) were enriched in the lungs of mice infected with *M.tb* (Unpublished data). The role of these cells in TB remains poorly characterized and is of interest given that MZB are able to respond to circulating lipid antigens, which are targets in TB disease, independent of germinal centre reactions (356).

One of the key roles of MZB in infection is to produce IgM against bloodborne pathogens encapsulated by lipid and/or carbohydrates (357). There may be opportunity for these cells to be activated in TB, as haematogenous spread of *M.tb* and likely TB antigen does occur, especially as a serious complication of TB in advanced HIV. Infection with *M.tb* induces a specific IgM response, as serum IgM against ESAT-6/CFP-10 is detectable in both resisters and individuals with LTBI (21). Such IgM responses may also play a functional role in TB, as IgM titres against PPD, LAM, PstS1 and Apa are the immune markers that best associate with reduced bacillary load in the IV BCG model in NHP (272). A humanized IgM mAb against LAM restricted the growth of *M.tb* in a whole blood assay, whereas its class-switched IgG clone failed to do likewise (272).

B cell differentiation is canonically determined by CD27 and IgD expression into naïve ($CD27^-IgD^+$), unswitched memory ($CD27^+/IgD^+$), switched memory (CD27+/IgD^-), and double-negative ($CD27^-/IgD^-$) B cells (359). IgM is produced by B-1 like B cells, follicular B cells, and unswitched memory B cells (8). Although contentious, there is growing evidence that transitional B cells that undergo somatic hypermutation but remain IgM-expressing in humans align phenotypically with MZB that are readily

identified in the spleens of mice (356). Evidence for the existence of these cells comes from examining cells extracted post-splenectomy in humans and biopsy of mucosal lymphoid associated tissue (360–362). Here, additional markers such as CD1c and CD21 further argue that these cells are not just found in the marginal zone but correlate functionally to circulating MZB in humans (362). Follicular B cells make up the majority of circulating B cells and generate antibodies by germinal centre licensing (356). In comparison, MZB contribute around 15% of circulating B cells in humans and produce somatically mutated IgM against lipids and polysaccharides independent of T cell stimulation as part of the early response to circulating pathogen (356).

Epidemiological data may support the hypothesis that MZB are important in human TB. Splenectomy reduces circulating memory IgM^+ B cells without impacting pre-existing antigen-specific IgG levels (363). Splenectomised patients without other comorbidities are 1.9x more likely to be diagnosed with pulmonary TB than matched controls (364). Although anecdotal, case studies of TB lymphadenitis and TB meningitis post-splenectomy have also been reported (365,366).

In humans, *Du Plessis et al* identified that MZB frequencies, defined by the markers ($CD19^+/IgM^+/CD23^-/CD27^+$), are reduced in frequency at time of TB diagnosis relative to a control group diagnosed with lung disease other than TB, and that their frequency in PBMC increased after 6 months of antitubercular therapy (17). In multidrug resistant TB, patients had lower frequencies of unswitched memory B cells ($CD27^+/IgD^+$) than healthy controls (18).

We therefore sought to validate whether the unswitched B cell compartment and thus MZB are perturbed across the spectrum of TB, using markers that are likely to align closer with robust definitions of MZB (362). We further postulated that the MZB would be increasingly activated across the spectrum of human TB disease, as defined by expression of CD69 and CD86. These markers were identified in the Gengenbacher lab as expressed in activated MZB (unpublished data) and are also well described as activation (CD69) and co-stimulatory molecules (CD86) upregulated upon B cell exposure to antigen (367). We also sought to define whether MZB migrate to the site of disease in a cohort of PLWH with TB pericardial effusions.

6.2 Results

6.2.1 Optimization of B cell flow cytometry panel

In collaboration with the Gengenbacher laboratory, a panel for the identification of B cell subsets and MZB was adapted for use in humans. To do so, PBMC samples from 2 healthy donors were used to adapt the panel to the host laboratory. First, fluorescence minus one (FMO) controls were performed. Adequate separation on the other markers was determined (Figure 6.1).

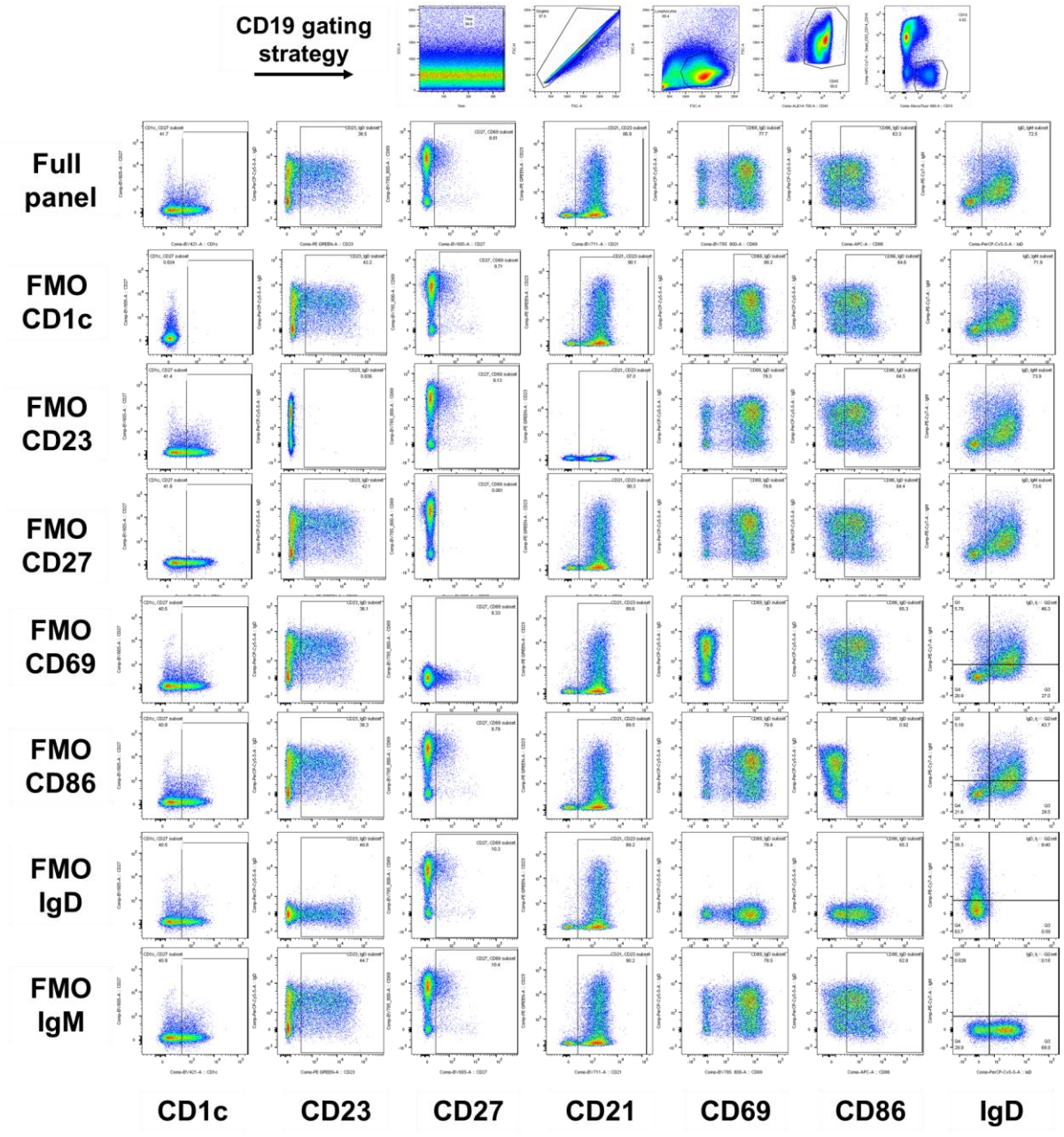


Figure 6.1 FMO gating of markers for characterization of B cells in TB.

Next, given that IgM and IgD markers in pilot runs were initially partially off the scale of detection, the concentration of the IgD and IgM antibodies were titrated to determine an optimal concentration (Figure 6.2).

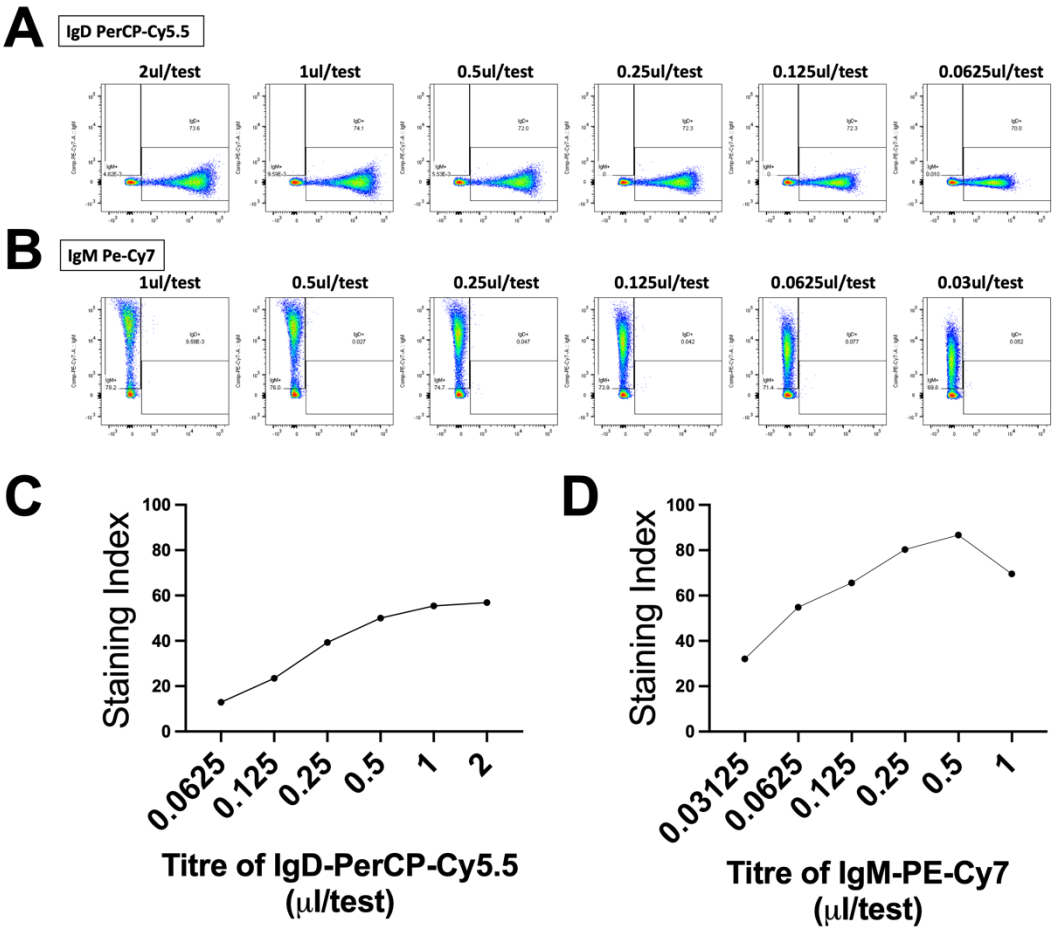


Figure 6.2 Concentration series of IgD and IgM antibody titration. (A) Gating of IgD and, (B) IgM with calculated staining index. (C) IgD staining index curve, (D) IgM staining index curve.

Based on this data, optimal staining index for IgD was selected at a titre of 1, and for IgM a titre of 0.5 for downstream experiments. Representative gating strategy was thus performed and taken forward for initial experiments on donor samples (Figure 6.3).

B cells were defined as single $CD45^+$ and $CD19^+$ lymphocytes. IgD was then gated against CD27 to determine differentiation phenotype. Boolean gating was then used to define CD21, CD1c. CD23 and IgM expression. Further gating for CD69 and CD86 was performed in the MZB population ($CD45^+/CD19^+/IgD^+/CD27^+/IgM^+/CD21^+/CD1c^+/CD23^-$) to ascertain activation status. This panel was tested on donor samples under stimulation conditions to determine whether MZB as well as activation markers could successfully be detected in human samples (Figure 6.4). Here, MZB were stimulated using *M.tb* WCL (10µg/ml), PPD (10µg/ml), and gamma-irradiated whole cell *M.tb* H37Rv (10µg/ml) and assessed by flow cytometry at 2 hours, 8 hours, and 16 hours post-stimulation.

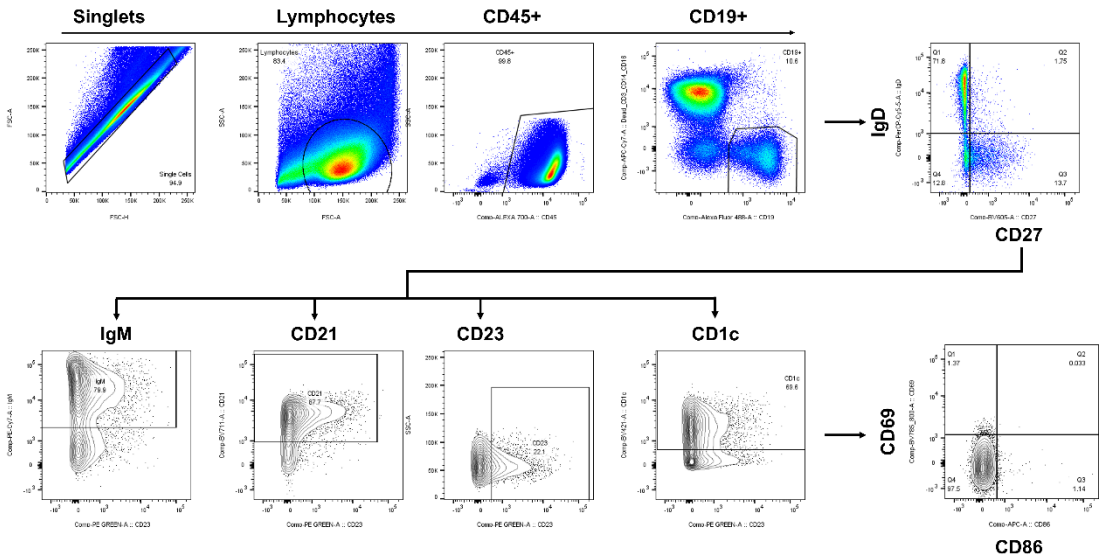


Figure 6.3 Representative gating strategy to define markers.

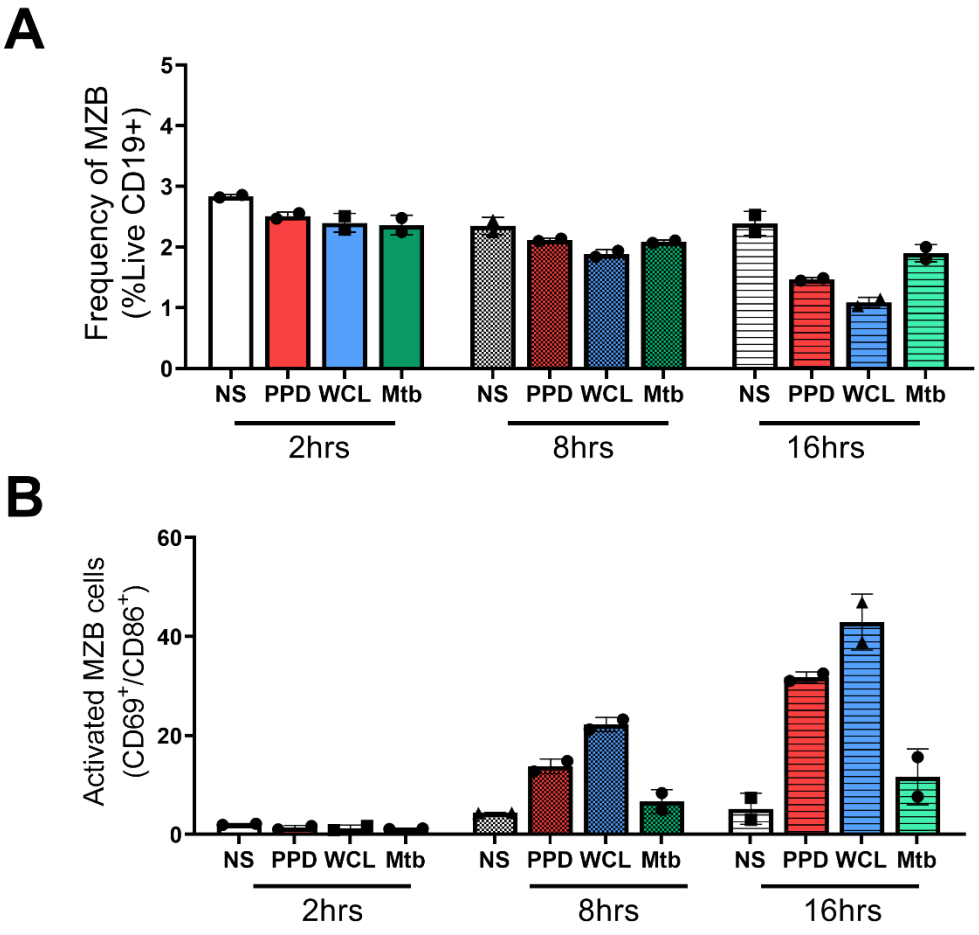


Figure 6.4 Preliminary determination of MZB frequency and activation in human donor samples. MZB are defined as $CD19^{+}/CD27^{+}/IgD^{+}/IgM^{+}/CD21^{+}/\ CD23^{-}/CD1c^{+}$. NS = non-stimulated. PPD = purified protein derivative. WCL = whole cell lysate. Mtb = gamma irradiated *M.tb* **(A)** Frequency of MZB in live CD19+ cells across three different timepoints. **(B)** Dual expression of CD69 and CD86 in MZB post-stimulation. Data shown is the mean with standard deviation of 2 samples. Samples were compared using ANOVA with Bonferroni's correction for multiple comparisons.

MZB were readily detectable using this panel in human samples. A mean MZB frequency of 2.84% was seen in unstimulated conditions. Although not statistically significant in this preliminary experiment, the frequency of MZB drops with stimulation and at both 8 hour and 16-hour timepoints. Dual expression of CD69/CD86 is induced by all stimulation conditions, but importantly MZB in the non-stimulated condition also express CD69/CD86 at the 8 hour and 16-hour timepoint. Taken together, it was noted that MZB are able to be enumerated with this panel in donor samples, and that they respond to *M.tb* antigens. However, because of decreased frequency over time as well as in stimulation conditions, the non-stimulated condition was taken forward for clinical samples.

6.2.2 B cells in patients with TB

The frequency and activation of B cells was determined in a biobanked cohort of HIV uninfected individuals who were either IGRA negative, IGRA positive (designated LTBI), or were diagnosed with active TB (Table 6.1) (UCT HREC 012/2007). ATB in this cohort was defined as smear and/or culture positivity and/or assigned to the ATB by a study clinician based on symptoms and radiographic disease. Limited clinical data exists for IGRA negative participants.

Table 6.1 Clinical characteristics for samples.

	IGRA-	LTBI	ATB	P value
Number	10	10	10	
Sex (% female)		3 (30%)	2 (20%)	>0.99
Age (years±SD)		21.87±2.33	38.98±24.11	*0.0386
Weight (kg)		65.8±9.52	61.375±14.93	0.4551
Height (cm)		170.7±7.06	165.75±7.96	0.1812
BMI (kg/m^2)		22.64 ± 3.69	22.14±3.63	0.7769
Mantoux (mm)	9.90 (0-20)	15.2 (12.25-20)		0.2747

BMI = body-mass index (kg)/height(m)^2. P values for sex were determined using Fischer's exact test. P values for age, weight, height, and BMI were defined by student's t-test. Mantoux induration (mm) was compared using Mann-Whitney test.

Significant differences were seen in age, with patients with ATB being on average older than those participants with LTBI. No significant differences were seen with respects to sex, weight, height, or BMI. IGRA negative participants did not have significantly lower Mantoux induration. Next, the phenotype of B cells was compared across these clinical phenotypes to assess whether TB had any impact on the distribution of B cell subsets (Figure 6.5).

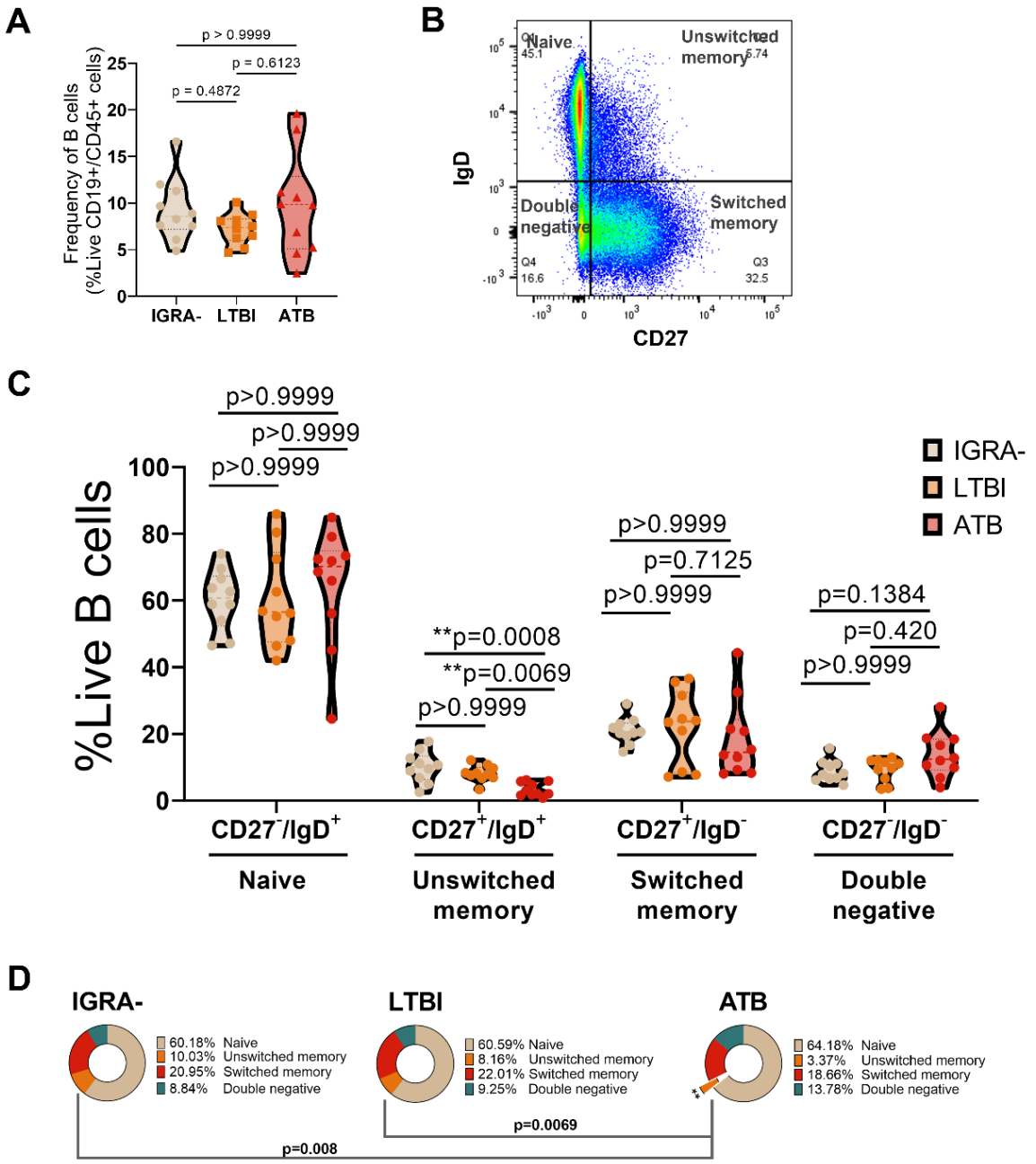


Figure 6.5 B cell frequencies in IGRA- (n=10), LTBI (n=10) and ATB (n=10) participants. **(A)** Frequency of total B cells in live lymphocytes. **(B)** Representative plot of IgD vs CD27 to determine B cell phenotype. **(C)** Frequency of naive B cells ($CD27^-/IgD^+$), unswitched memory B cells ($CD27^+/IgD^+$), switched memory B cells ($CD27^+/IgD^-$), and double negative B cells ($CD27^-/IgD^-$). Distributions are shown by violin plot. **(D)** Pie charts demonstrating proportions of B cells across clinical groups. Samples were compared by Kruskal-Wallis test with Dunn's correction for multiple comparisons. * $p<0.05$, ** $p<0.001$, ***$p<0.0001$.

The frequency of live B cells did not differ across clinical groups. Canonical states of B cell differentiation were determined by gating IgD against CD27, and here only the frequency of unswitched

memory B cells differed across clinical groups. Significantly lower unswitched memory B cells were seen in ATB when compared to both IGRA- (p = 0.008) and LTBI participants (p = 0.0069). The unswitched memory B cell compartment encompasses IgM producing B cells and would thus also contain MZB. Therefore, the expression of IgM and other markers of marginal zone B cells (CD1c, CD21) and markers of follicular B cells (CD23) were characterized (Figure 6.6).

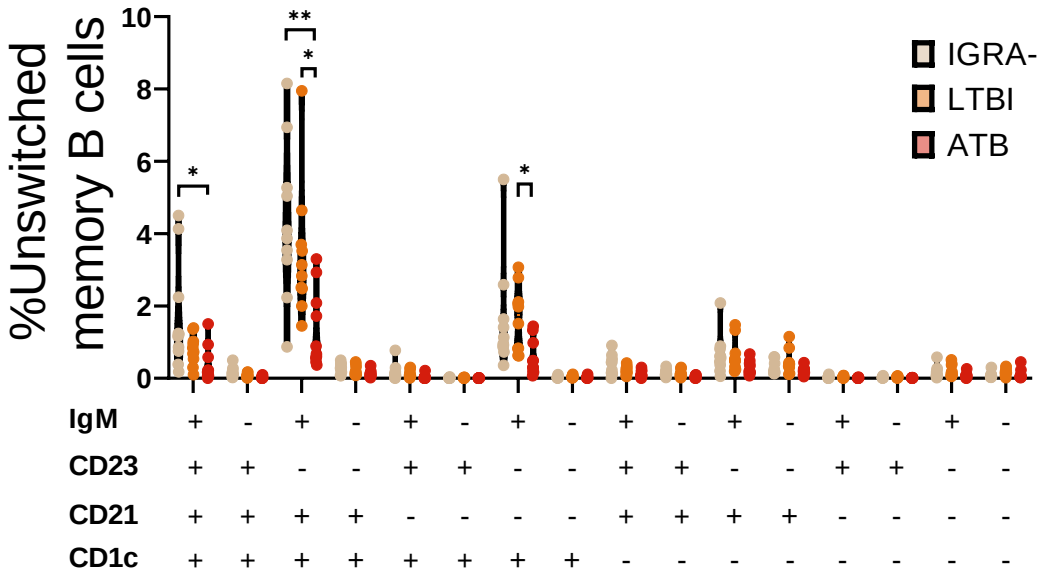


Figure 6.6 Phenotyping unswitched memory B cells in IGRA- (n=10), LTBI (n=10) and ATB (n=10) participants. Samples were compared by Kruskal-Wallis test with Dunn's correction for multiple comparisons. * p<0.05, ** p<0.001, ***p<0.0001.

The most abundant cell phenotype across these markers is of the $IgM^+/CD23^-/CD21^+/CD1c^+$ phenotype. Cells expressing these markers have been identified as circulating MZB in humans. Other phenotypes of IgM-expressing cells were also present at lesser frequencies in ATB than IGRA- participants. Interestingly, low frequencies of non-IgM expressing cells were also seen in the unswitched memory B cell compartment. Given the abundance of $IgM^+/CD23^-/CD21^+/CD1c^+$, the stepwise decrease in frequency across clinical groups, and the surrounding literature to support this phenotype as MZB, this phenotype was taken forward for further characterization. Next, the expression of the activation markers CD69, CD86 in MZB was assessed (Figure 6.7).

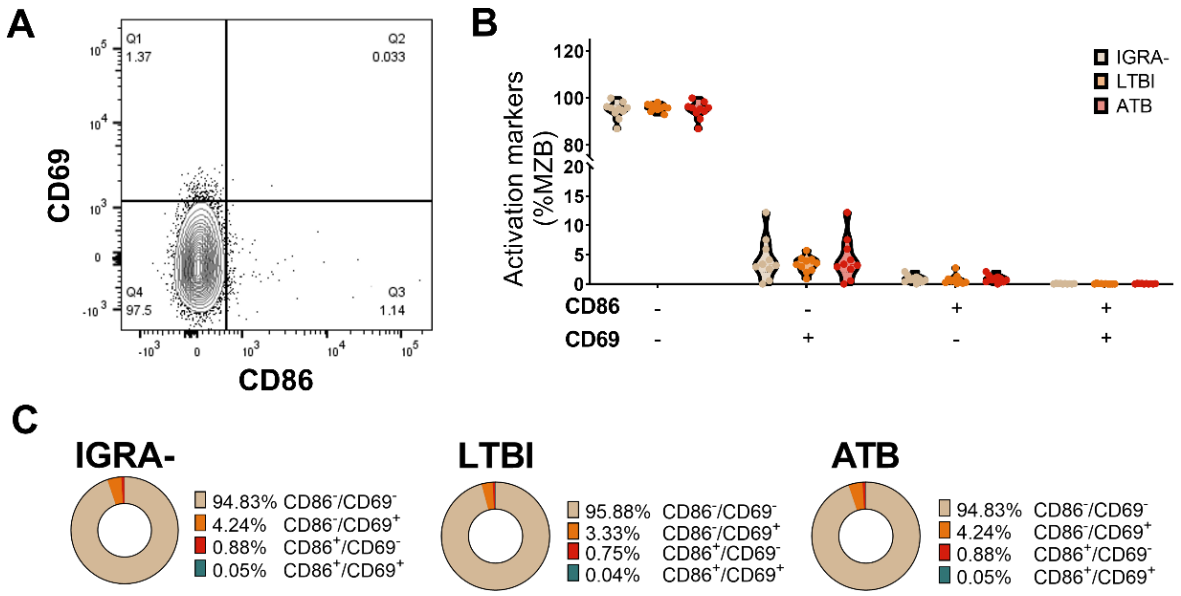


Figure 6.7 B cell activation in IGRA- (n=10), LTBI (n=10) and ATB (n=10) participants. **(A)** Representative plot of CD69 vs CD86 to determine B cell activation. **(B)** Frequency of CD86 and/or CD69 expression in MZB. Distributions are shown by violin plot. **(C)** Pie charts demonstrating proportions of B cell activation marker expression across clinical groups. Samples were compared by Kruskal-Wallis test with Dunn's correction for multiple comparisons.

Although the expression of CD69 and CD86 was induced by *M.tb* antigen stimulation in healthy donors, expression of these markers did not differ across clinical groups. *Ex vivo* phenotyping of MZB shows that most of these cells were $CD69^-/CD86^-$, even in patients with ATB. As IgD and IgM are splicing variants and surface expression of either marker has been linked to either cellular activation or anergy, the mean fluorescent intensity (MFI) of IgD and IgM was also compared across clinical states (Figure 6.8)

Surface IgM expression in MZB did not differ across clinical groups. However, IgD expression was significantly downregulated in ATB relative to both IGRA- ($p = 0.0049$) and LTBI ($p = 0.0248$) participants. One possibility for why frequencies of MZB are lower in ATB could be cellular migration to the site of disease.

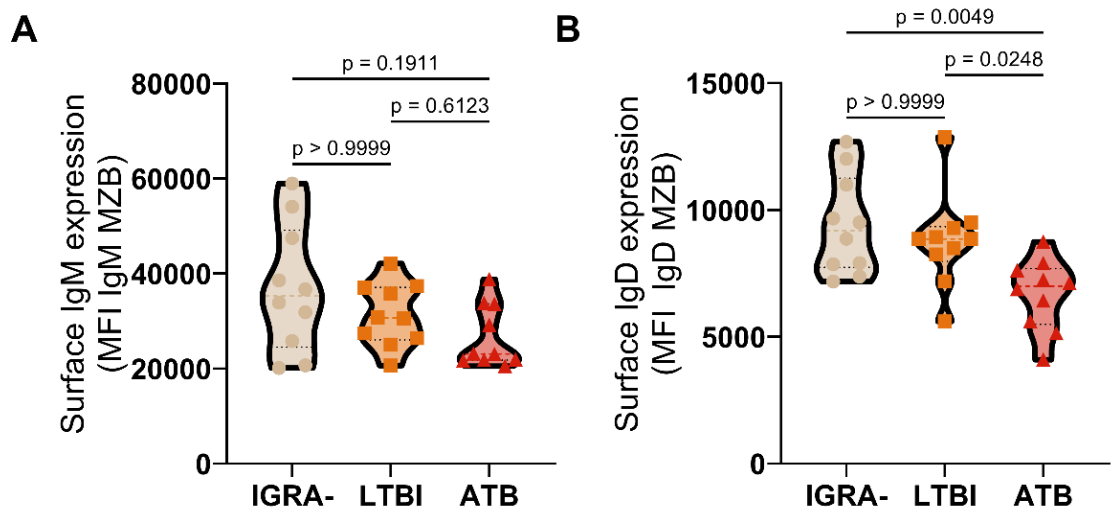


Figure 6.8 MZB expression of IgM and IgD in IGRA- (n=10), LTBI (n=1) and ATB (n=10) participants. **(A)** MFI of IgM in MZB across clinical groups, **(B)** MFI of IgD in MZB across clinical groups. Samples were compared by Kruskal-Wallis test with Dunn's correction for multiple comparisons.

6.2.3 B cells in patient with pericardial TB

To test whether B cells compartmentalize to the site of disease, a pilot study was conducted comparing peripheral PBMC to pericardial fluid in patients with HIV-associated TB (102/2003, 402/2005, and 289/2007). The frequency and activation of MZB in pericardial fluid was compared to PBMC in cryopreserved samples from PLWH with pericardial TB, described below (Table 6.2).

Table 6.2 Clinical details for PLWH with pericardial TB (n=6).

Patient ID	PBMC	PC	Age	Sex	HIV status	Fluid ADA	TB Culture	CD4 count
1	+	+	20	F	+	87	-	65
2	+	-	32	M	+	85	+	157
3	+	+	20	F	+	97	N/A	N/A
4	+	+	68	M	+	N/A	N/A	N/A
5	-	+	30	F	+	23.5	N/A	559
6	-	+	21	F	+	59	N/A	300
	N= 4 (66%)	N=5 (83%)	32±18.6	F = 3 (50%)	100%	85 (IQR=50.8)	1 (16.6%)	270±215.4

Fluid ADA = adenosine deaminase, a marker of lymphocyte activation that aids in the diagnosis of pericardial TB. For age, mean with standard deviation is shown. For ADA and CD4 T cell count (cells/mm^3), median with interquartile range is shown.

The diagnosis of TB pericardial effusions was frequently not microbiologically confirmed, and thus limited culture positivity confirmation exists. Next, the frequency and phenotype of B cells was compared between PBMC and PFC (Figure 6.9).

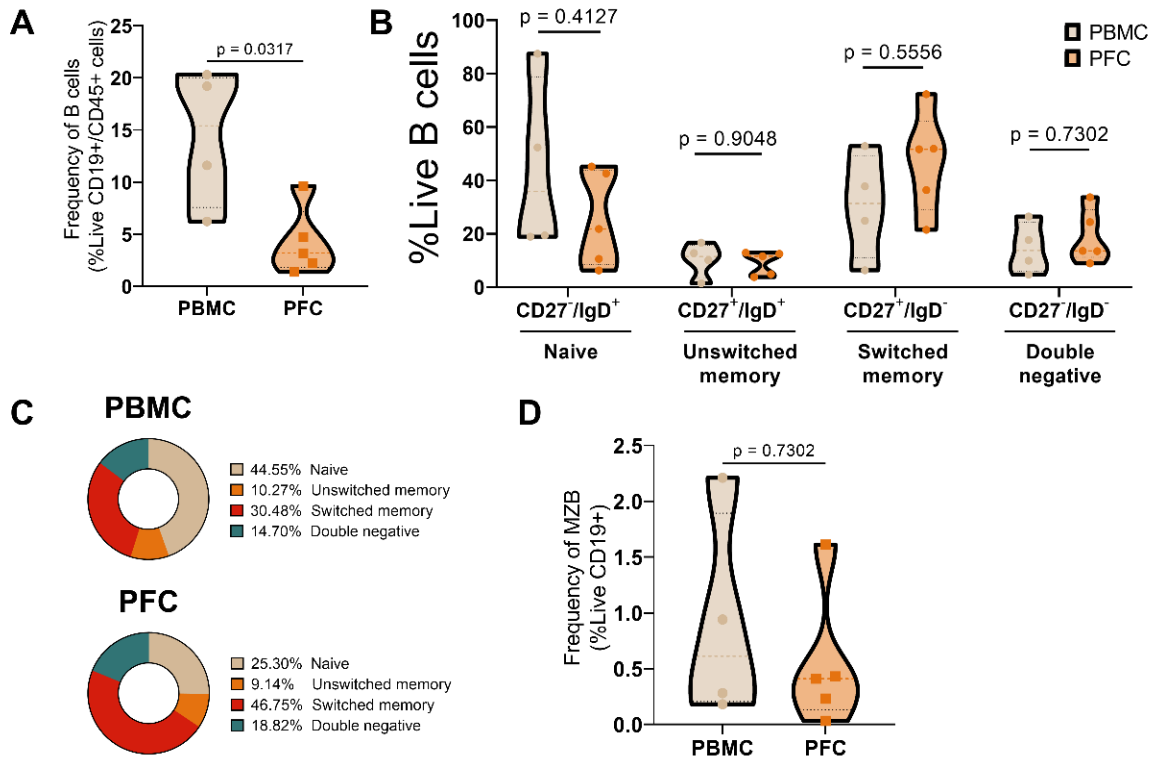


Figure 6.9 Frequency and phenotype of B cells in PLWH. PBMC (n=4) and PFC (n=5). **(A)** Total B cell frequency, **(B)** B cell phenotyping across clinical groups. Sample distribution is shown by violin part. **(C)** Pie charts showing distribution of B cell phenotypes. Samples were compared were compared by Mann-Whitney test.

In PLWH with TB pericardial effusions, the overall frequency of B cells ($CD19^+$ cells) was lower in PFC than in PBMC ($p = 0.0303$). B cells were therefore not enriched in the site of disease compartment in pericardial TB. When maturation phenotype was assessed, no subset of B cells differed significantly between PBMC and PFC. The frequency of MZB in total B cells also did not differ between PBMC and PFC. Next, whether B cells at site of disease were more activated was tested (Figure 6.10).

From these data, the activation of MZB as defined by CD69 and CD86 expression, did not differ between PBMC and PFC. The baseline activation of MZB in this population appears higher than that in HIV uninfected persons seen previously. Next, the MFI of IgM and IgD were compared between PBMC and PFC.

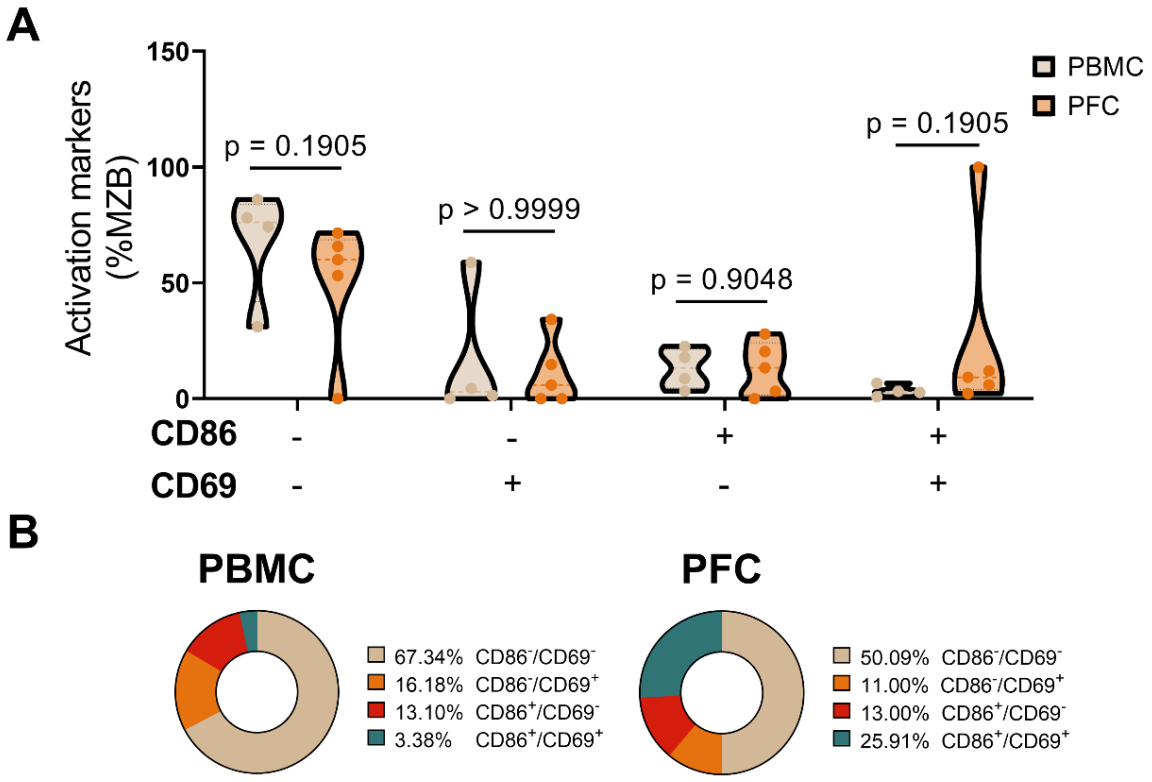


Figure 6.10 MZB activation in PBMC (n=4) vs PFC (n=5). **(A)** Frequency of CD86 and/or CD69 expression in MZB. **(B)** Pie charts demonstrating proportions of B cell activation marker expression across clinical groups. Sample distribution shown by violin plot. Samples were compared by Mann-Whitney test.

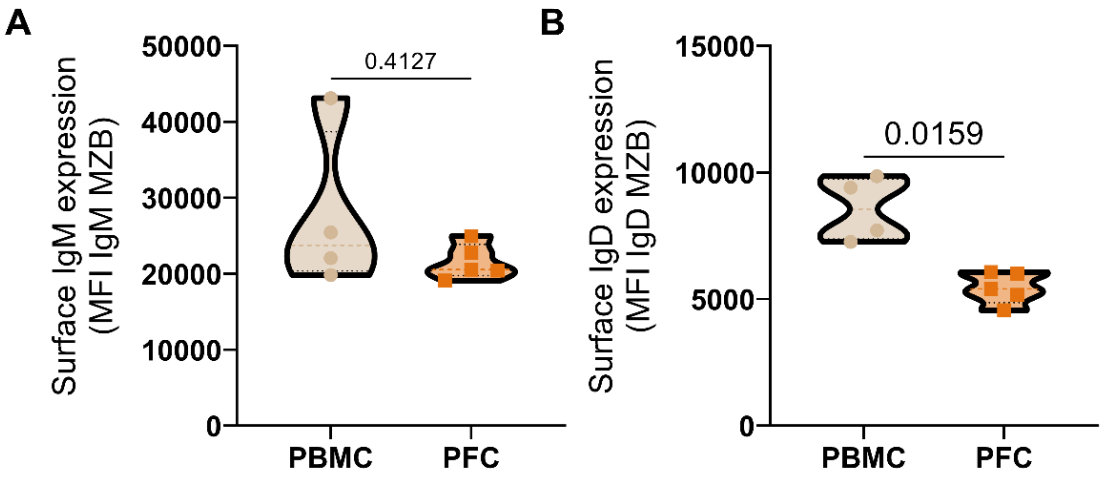


Figure 6.11 MZB MFI of IgM and IgD in PBMC (n=4) versus PFC (n=5). **(A)** MFI of IgM in MZB across clinical groups, **(B)** MFI of IgD in MZB across clinical groups. Samples were compared by Kruskal-Wallis test with Dunn's correction for multiple comparisons.

The MFI of IgM did not differ between PBMC and PFC. However, similar to prior findings of IgD expression being downregulated in ATB when compared to IGRA- and LTBI participants, the MFI of IgD was also lower in PFC than in PBMC ($p = 0.0159$). The possible interpretations of this finding are discussed below.

6.3 Discussion

In this chapter, I show that the unswitched memory B cell compartment is decreased in patients with ATB disease when compared to IGRA- and LTBI participants. With the cell surface markers assessed in this study, the major cell phenotype in this compartment was identified as $IgM^+/CD23^-/CD21^+/CD1c^+$. These cells fit current rigorous definitions for human MZB that may help distinguish MZB from other IgM expressing B cells (362). Although defects in the unswitched B cell compartment have been described in TB, the phenotyping to include IgM expression as well as confirm CD21 and CD1c expression has not previously been reported (368). Putative MZB ($CD27^+/IgM^+/CD23^-$) are decreased in frequency at the time of TB diagnosis but increase after treatment (59).

This alteration in circulating B cell populations is not unique to this dataset and may have implications for understanding the pathogenesis of TB. Several autoimmune diseases such as systemic sclerosis and multiple sclerosis display a depletion in circulating unswitched memory B cells as a feature of disease activity (369,370). MZB production of self-binding IgM plays a major role in the pathogenesis of systemic lupus erythematous (SLE), a severe autoimmune disease hallmarked by autoantibodies against intranuclear antigens. A common pathogenic denominator may be the presence of large amounts of antigen that the antibody response is unable to clear. As previously discussed, the antibody response to *M.tb* in ATB is predominantly targeted to secreted antigen which is unlikely to opsonize and facilitate bacterial killing. To support this hypothesis, sorted B cells from patients with ATB proliferate poorly and produce less antibody and IL-6 upon stimulation (56).

When further defined, the unswitched memory B cell compartment was predominantly composed of $IgM^+/CD23^-/CD21^+/CD1c^+$ cells. These cells fit the predefined markers for MZB and were present at significantly lower frequencies in ATB than in IGRA- and LTBI participants. Other non-IgM expressing cells were present in this compartment, and their phenotype and function remains to be characterized. Three possibilities were proposed for the fate of MZB in ATB. Firstly, MZB could be lost via cell death. The mechanisms whereby MZB proliferation and apoptosis are regulated have been recently explored and appear to differ from those required for follicular B cells (371). Homeostatic apoptosis occurs in FOB, but MZB, which frequently respond to self-antigen, must also have mechanisms whereby to limit activation that could occur in ATB (371).

The next possibility is that MZB either differentiate from their original phenotype, or their precursors unable to mature into detectable MZB. There is more evidence for the latter, with the developmental lineage of MZB being impaired in systemic lupus erythematous, another condition in which decreased frequencies of MZB are seen (372).

A further hypothesis which we tested was that MZB migrate to the site of disease. This was originally proposed from mouse data in which MZB but not FOB selectively populated the lungs of mice infected with *M.tb* (Gengenbacher laboratory, unpublished data). To test this, MZB were characterized in the pericardial fluid of patients with HIV-associated pericardial TB. Although the sample size was not optimal to distinguish the differences in B cell populations in PBMC compared to site of disease (PCF), there were lower frequencies of total B cells in the pericardial fluid than peripheral circulation in these patients. Although it is possible that pericardial fluid as a known paucibacillary site of disease does not sufficiently promote migration of B cells, in a similar cohort of patients the PCF was shown to be enriched for $CD4^+$ T cells (373). Thus, it appears that MZB are not lost from the peripheral circulation to site of disease.

Although CD69 and CD86 were detectable in donor samples stimulated with a range of *M.tb* antigens, the expression of these markers by MZB did not differ between clinical groups or site of disease. Regarding the mechanism of these responses, MZB are well described to express toll-like receptors and also respond to lipopolysaccharide (374). It could be that other markers of B cell activation and antibody production such as CD38 or CD138 may be more relevant to distinguish between clinical conditions. Alternatively, the number of MZB encountering circulating antigen in HIV uninfected persons or in pericardial fluid could be too low to detect. However, the key phenotypic change noted in MZB was a decrease in the surface expression of IgD across clinical groups as well as between PBMC and PCF in PLWH with pericardial TB.

IgD and IgM are splicing variants of the same heavy chain mRNA for surface-expressed immunoglobulin receptor isotypes, whose differential expression serves to modulate the axis between anergy and activation (375). Peripheral B cells expressing high levels of IgD and low levels of IgM B cell receptor (BCR) are functionally anergic (376). This is proposed as a homeostatic mechanism whereby B cells can modulate activation in the presence of self-antigen, and autoregulate to limit activation (377). Although controversial, the IgM and IgD BCR may signal differently upon response to the same antigen (378). There is little data to speculate upon regarding a function for our finding that MZB IgD expression is reduced both in ATB relative to less severe disease states, as well as between peripheral blood and site of disease. Putatively, a decrease in IgD expression could signal downregulation of tolerance mechanisms as in the alleviation of a braking mechanism on the activating IgM receptor. However, further functional studies for MZB are needed to address this question.

Whether MZB contribute to protection against TB by production of anti-*M.tb* IgM also requires testing. Future work could be to quantify *M.tb*-specific IgM in paired serum from these participants, and test whether frequency and activation of MZB correlates with these values. To functionally determine whether IgM produced by MZB have any role against *M.tb*, sorting and stimulation of MZB could be performed as was performed for autoantibodies in lupus (379). Purified IgM from human MZB could be used to evaluate ADCP and *M.tb* killing in whole blood assays as previously described in this thesis.

Whether pericardial fluid contains antibodies against antigens elsewhere tested in this thesis ie. GlnA1, ESAT-6/CFP-10, and whole cell lysate, remains to be studied. Comparing purified antibody fraction from pericardial fluid to serum would help define the function of antibodies that are enriched at the site of disease. However, these studies would not be powered adequately for diagnostic utility, and the challenges in antibody diagnostic studies are discussed in chapter 1.

To approach this question from a clinical perspective, it could also be of interest to compare the function and activation of MZB in PLWH between those with contained pulmonary disease and those with disseminated TB. The rationale being that urinary LAM and mycobacteremia is more readily detectable in patients with advanced HIV (12). MZB are thought to respond to capsulated organisms and lipid in the bloodstream, and there could therefore be a role for these cells in preventing disseminated disease.

In conclusion, I identified that the unswitched B cell compartment, and specifically MZB, are decreased in frequency in ATB. The hypothesis that these cells migrated to the site of disease could not be confirmed in this data. I also identified a currently unexplained role for decreased IgD expression in MZB across the spectrum of clinical TB, as well as between peripheral circulation and site of disease. The overall pattern of B cell perturbation here is also seen in autoimmune disease and may be suggestive of immunopathology in the B cell compartment during ATB worthy of further exploration.

Conclusions

The role of antibodies has long been understudied in TB, but this has recently changed. Several human mAbs targeting a range of antigens are now known to restrict the growth of *M.tb* in pre-clinical models. Serological responses against *M.tb* are also known to exist in people who appear to resist TB. Now, the challenge in harnessing antibodies for better vaccines and therapies will be to understand why some antibody responses are beneficial, and others are not.

In Chapter 3, I generated human mAbs by cell sorting plasmablasts from patients with ATB. Out of an initial panel of 17 mAbs cloned from 3 patients, I found 5 that bound *M.tb* antigen pools. I showed that one clone (78_59) targeted GlnA1 (Rv2220) a secreted antigen of *M.tb*. Despite opsonizing live BCG, 78_59, did not enhance phagocytosis or reduce the growth of mycobacteria. Across PBMC and whole blood assays with GlnA1 alone, BCG or *M.tb* H37Rv, 78_59 appeared to increase in TNF-α, IL-6, and IL-1β production relative to an isotype mAb. Antibody recognition of GlnA1 was not only observed in patients with ATB, but also in both IGRA negative and latently infected persons with and without HIV infection.

This leads me to speculate that the activation of humoral immunity in the transition of subclinical to ATB is not merely a marker of disease but actively contributes to its progression. In this model, replicating bacilli secrete antigen are bound by antibodies in the lung and/or blood, form immune complexes with circulating complement, and are then taken up by FcR-expressing cells. This process wouldn't necessarily help infected macrophages kill *M.tb*, but would drive the inflammatory cascade that results in the characteristic fever and weight loss of TB.

In chapter 4, I showed that the PLWH do not mount as great an antibody response to *M.tb* as HIV uninfected person between the states of LTBI and ATB. Perhaps in advanced HIV, this defect in immunity contributes to why bacilli can survive in the bloodstream and disseminate further.

The characterization of the resister phenotype has yielded much insight into immunity against *M.tb*. but PLWH who are IGRA negative have not been well studied. I show the detection of antibodies against E6/C10 in three cohorts of IGRA negative PLWH. TST induration is used as a test for exposure to *M.tb*, yet had no correlation with anti-E6/C10 IgG responses.

I detected antibodies against E6/C10 in the majority of IGRA negative PLWH who had not developed TB despite documented periods of advanced immunodeficiency. Yet, IGRA negative PLWH who undergo QFT conversion during follow-up conversion appear to have greater anti-E6/C10 IgG levels than those who do not. Antibody responses may therefore be a better marker of prior exposure to *M.tb* than TST and may emerge prior to QFT is able to detect the cognate T cell response. The mechanisms whereby these individuals do not acquire TB with high risk remains to be fully explained.

In chapter 5, I showed the development of functional assays against mycobacteria using flow cytometry. These assays could detect an effect of BCG vaccination on ADCP and opsonization of mycobacteria. They were also useful in designing methods to test the function of human mAbs. Future work could build on this toolkit to rapidly evaluate antibody function against live *M.tb* in clinical studies.

Finally, in chapter 6 I characterized the phenotype of IgM-producing B cells in TB. I showed that a reduction in the unswitched memory B cell compartment occurs in LTBI and ATB. Most of these cells were of the MZB phenotype. I next tested whether this finding could be explained by MZB honing to the site of disease. MZB were not enriched in the pericardial fluid of PLWH with extrapulmonary TB. An unexpected result from these experiments was the downregulation of B cell IgD across clinical groups and when comparing PBMC to PFC. The B cell phenotype of downregulated IgD and depleted unswitched memory B cells are reminiscent of literature on autoimmune diseases where pathological antibodies are the very hallmark of pathology.

In conclusion, several findings from this thesis lead to me propose that the role of antibodies and B cells in the pathophysiology of TB must be explored further. Yet other studies show that functional antibodies against TB do exist, and in PLWH an ineffective antibody response may be another gap in the armour. Are antibodies therefore a double-edged sword in TB, contributing to both the pathology and prevention of TB? The solving of this paradox has great implications for designing new tools for the eventual eradication of TB.

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