Assessing the Potential of Monoclonal Antibodies Against Mycobacterium Tuberculosis

Assessing the Potential of Monoclonal Antibodies Against *Mycobacterium tuberculosis*

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Abstract

Tuberculosis infects approximately one-quarter of the world's population and is the leading cause of death by a single infectious disease, responsible for a reported 1.3 million deaths in 2017. Though *Mycobacterium tuberculosis*, the causative bacteria, is treatable with antibiotic therapy, the increased prevalence of drug resistant strains as well as the variable efficacy of the only widely approved vaccine have highlighted the need for novel approaches to therapeutic and vaccine development. Historically, it has been believed that the protective immune response against *M. tuberculosis* is attributed almost exclusively by cell-mediated immunity with humoral immunity being largely dismissed. Despite the skepticism that has surrounded the role of antibodies against this intracellular pathogen, there has been an increase in evidence suggesting that they can be involved in a variety of protective capacities. In particular, extensive study of the variable (Fab) and constant (Fc) domains has become possible in recent years, and there is a strong possibility that antigen-specificity, innate immune signaling, glycosylation patterns and other related characteristics impact a monoclonal antibody's protective abilities. Utilizing a specially optimized version of the Golden Gate cloning system, production of mAbs with chosen Fab domains and immunoglobulin subclasses is possible, allowing for the assessment of the influence of each component. By confirming antigen-specific binding, ability to induce innate cell effector functions, and protection conferred against live bacterial challenge, results indicate that antibodies can potentially be important mediators of defense against this global pandemic.

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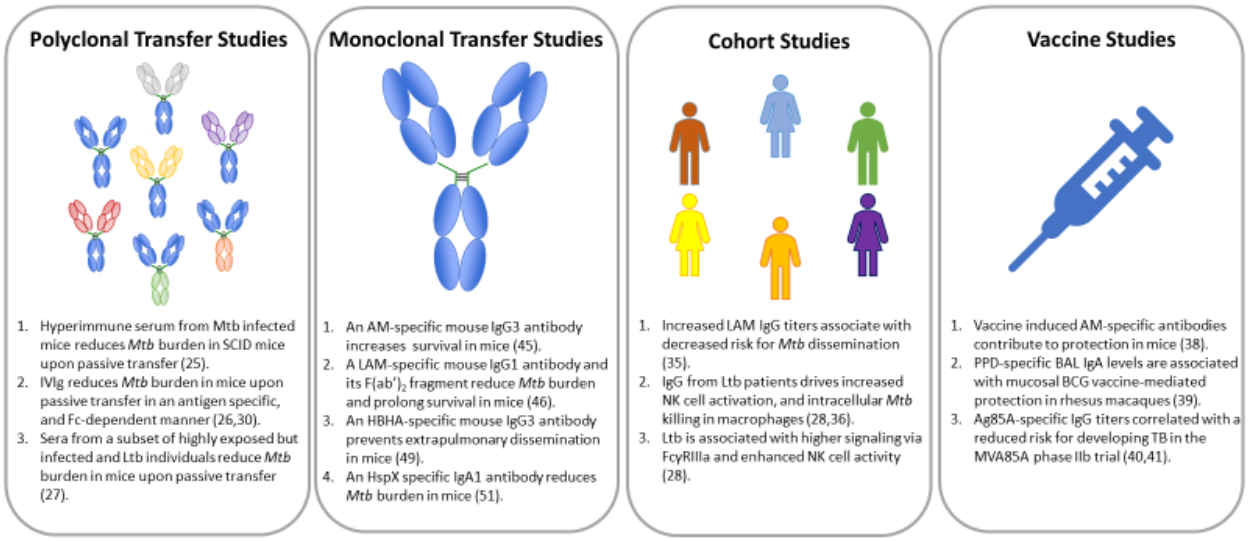

Table 1. Summary of key study findings supportive of roles for antibodies against tuberculosis.

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1. Background

1.1: Antibodies and Tuberculosis

Mycobacterium tuberculosis (*Mtb*), the causative bacteria of tuberculosis (TB), infects approximately one-quarter of the world's population and is the leading cause of death by single infectious disease, responsible for a reported 1.3 million deaths in 2017[1]. *Mtb* infection does not always lead to TB disease, presenting in a spectrum of states ranging from a largely asymptomatic latent infection (Ltb) to the transmittable active disease (Atb). While treatable via antibiotic regimens, the rise of drug-resistant strains has emphasized the need for developing novel methods of antibacterial therapies. Bacillus Calmette–Guérin (BCG) remains the only widely approved TB vaccine. Developed nearly a century ago using a live attenuated strain of *Mycobacterium bovis*, its protective efficacy is highly inconsistent, varying significantly by age group and geographic location among other factors[2,3]. Historically, the protective response against TB has been attributed almost exclusively cell-mediated immunity (CMI) with the role of humoral immunity remaining unexamined until more recent years. Given the lack of clarity surrounding the role of antibodies in this context, the vast majority of vaccine development efforts have focused on inducing T cell responses, leaving the function of the humoral immune system understudied[4,5].

1.1.1: Historical Focus on CMI

The critical importance of cell-mediated immunity against TB has been well-documented in decades worth of studies and CD4+ T cells in particular are viewed as the primary immunological axis mediating host defense[6]. A lack of these cells has been shown to lead to increased manifestation of active disease pathologies in both animal models and humans. Depletion of CD4+ T cells in mice and cynomolgus macaques resulted in increased lung CFUs,

enhanced bacterial dissemination (increased splenic CFUs), and decreased rates of survival[7–9]. Humans with low CD4 counts, often caused by human immunodeficiency virus (HIV) infection, were also less capable of controlling disease, resulting in a significant increase in the rates of active disease[10,11].

In contrast, the importance of the humoral immune system remains far less completely defined. Though antibodies typically represent the correlate of immunity in most clinically approved vaccines[12], they have been largely discounted in the context of *Mtb* due to their supposed irrelevance in controlling intracellular bacteria[4,6,13]. The idea that humoral immunity is of little importance in *Mtb* infection is partly related to the perceived dichotomy between it and cellular immunity. The paradigm maintaining that the Th1 response protects against intracellular pathogens while CMI defends exclusively against extracellular agents has created the long held belief in the apparent division of labor between these two systems[14]. As a result, the absence of widely accepted evidence supporting a protective role for antibodies has led to the assumption that they are unnecessary for *Mtb* defense. Though there has been an influx of potentially supportive data produced by more modern studies citing roles in innate immune cell recruitment and antibacterial effector functions, the historically negative perception of antibodies against *Mtb* has caused them to remain a contentious subject in the field.

Recent evidence from passive transfer and cohort studies, vaccine trials, and monoclonal antibody (mAb) therapeutics make a strong case that antibodies can positively impact the host response to tuberculosis and that such roles warrant further investigation (Table 1).

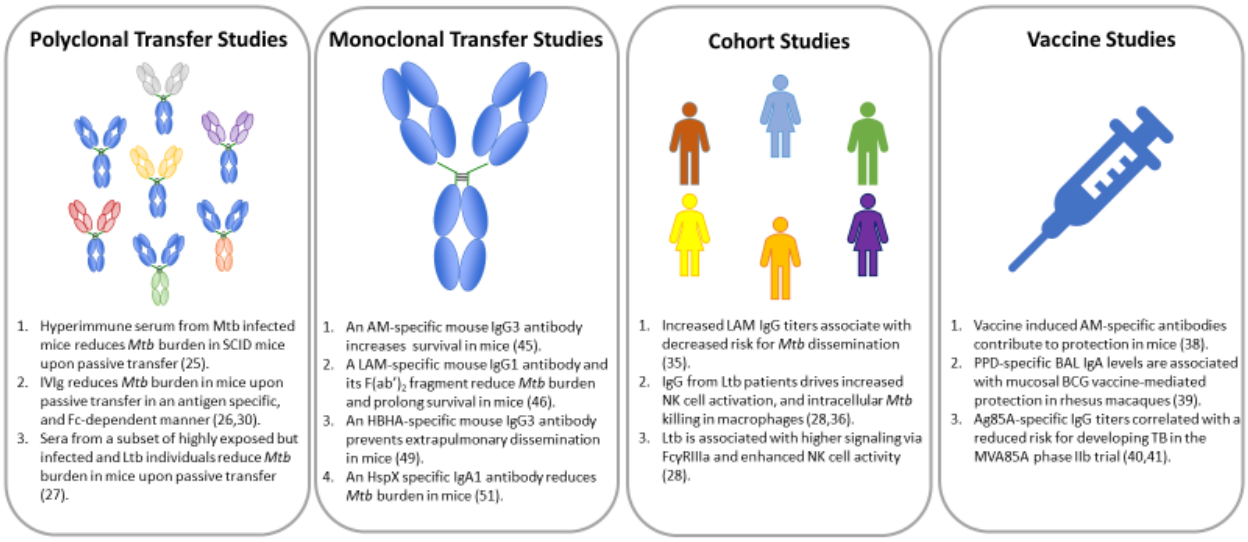

Table 1. Summary of key study findings supportive of roles for antibodies against tuberculosis.

1.1.2: Passive Transfer Studies

Among the earliest studies on combating tuberculosis were serum transfer experiments conducted in the late 1800s. Attempting to build on the positive results of such trials against a variety of infectious diseases, a range of animal and human protocols were conducted yielding inconsistent results. The Henry Phipps Institute immunized cows with heat-killed concentrate of *Mtb* bacilli and then derived serum for administration[15]. Despite promising results attained using this methodology against other diseases, transfer of cow serum provided no benefit to tuberculosis patients[16,17]. Work by Viquerat and De Schweinitz similarly attempted to use other animal models (horse, donkey) to generate protective serum, but again these efforts were largely unsuccesful[16–18]. More recent studies have also struggled to show success via passive transfer.

Serum derived from New Zealand rabbits intravenously infected with *Mtb* was administered to mice challenged with BG. This immunization not only failed to confer protection, but instead enhanced disease, suggesting that the presence of *Mtb*-specific antibodies might be detrimental in certain cases[19].

Despite these results, there are also several studies finding that antibody transfer can be conducive to antibacterial function both *in vivo* and *in vitro*. An early study deriving serum from immunized guinea pigs reported bactericidal activity against *Mtb in vitro*[16]. Other studies from this time concluded that passive transfer of immune bovine serum to 412 TB-infected patients induced complete resolution of disease in 16% of those treated with reduced clinical symptoms in 40% and bacterial clearance from the sputum in 43% of patients[20]. Another study using immunized donkey serum reported that 83% of recipients were cured of disease[18]. Similarly to this, separate studies using horse serum showed significant disease benefit in over 80% of treated individuals, though attempts to replicate these results were less successful, making it difficult to cross-compare these findings[17,21,22]. Overall, the small sample sizes, differences in disease severity, inconsistent clinical endpoints, lack of sufficient controls, and non-replicable results have weakened the impact of these studies[17,18,21,22]. While the results together indicate that different people have different antibody responses, the aforementioned inconsistencies made it difficult to use these studies as a strong foundation for further antibody/serum trials. Additionally, it was around this time that antibiotics became more prevalent, showing more consistent antimicrobial properties and somewhat undermining the efforts to develop effective antibody-based therapies, which were more complex and incocnsistent[23,24].

Despite the lack of clarity surrounding these findings, there are a number of studies conducted within the past two decades that seem to offer more reliable support for protection

conferred by passive transfer. Guirado et al created a liposome packaged preparation of *Mtb* concentrate and administered it to mice in combination with rifampicin and isoniazid[25]. Immune serum was then drawn from these mice and applied to *Mtb* infected SCID mice lacking functional T, B, and NK cells. Through the 10 week post-infection timepoint, antibody-recipient mice experienced reduced lung CFUs compared to littermate controls[25]. These results are especially notable because they suggest the possibility that certain antibodies may have the ability to mediate host defense at least somewhat independently of cellular immunity. Similar results were seen in a study using human intravenous immunoglobulin (IVIg) to *Mtb*-infected mice. IVIg is gathered and pooled from large sample populations which frequently include those previously exposed to *Mtb*, BCG vaccination, or undiagnosed latent infection. As a result, IVIg preparations often contain *Mtb*-specific immunoglobulins. When IVIg known to include these antibodies were passively administered to mice, significant decreases in bacterial burden were recorded for both early and late infection states[26]. A 2017 study by Li et al reported that the efficacy of serum derived from humans (applied to mice) appeared to vary according to infection state of the donor. Sera derived from Beijing healthcare workers who were latently infected or highly exposed, but maintained TB-negative clinical diagnoses offered protection against mice infected via aerosol[27]. Specifically, sera from approximately half a dozen workers fitting these descriptions caused a 2-3-fold reduction in lung CFUs compared to sera from individuals with Atb disease, which offered no protection. Interestingly, these positive effects were abrogated in the absence of CD4+ T cells, indicating that certain antibodies are able to function independently of CMI while others are more dependent. Additionally, these findings also suggest antibodies and T cells could work synergistically in mediating anti-bacterial defense. A potential explanation for this mechanism is that antibodies can mediate protection via direct or indirect Fc-

receptor signaling interactions. For example, there is growing evidence that constant domain glycosylation patterns alter an antibody's ability to induce certain immune cell effector functions[28]. Olivares et al investigated the impact of Fc-deglycosylated IVIg (composed of *Mtb*-specific antibodies), finding that EndoS-mediated removal of Fc-glycans significantly reduced the protective capabilities of antibodies[29]. Prior work by the same group had discovered that depleting IVIg preparations of these TB-specific immunoglobulins eliminated their protective properties[30]. Taken together, these findings suggest that antibody binding and subsequent Fc-receptor interactions were responsible for conferring protection[30]. Taking all these studies into account, there appears to be a clear rationale for more thorough examination of the mechanisms by which antibody specificity and Fc interactions with various immune cell types govern the ability to protect against tuberculosis.

1.1.3: Cohort Studies

One of the main arguments against the importance of TB-antibodies that remains today is the mixed findings of many cohort studies. A study from China following patients with X-linked agammaglobulinemia (XLA) reported that these individuals did not appear to be at increased risk for active disease despite lacking mature B cells and normal antibody titers[31]. Similarly, patients with defective Bruton's tyrosine kinase genes, manifesting in compromised humoral immunity, also show no indication that humoral immune deficiencies predispose individuals to increased risk of tuberculosis disease[12,32]. It is important to note that many of these patients had received IVIg (high likelihood of containing *Mtb*-specific antibodies) for other unrelated infections[12,31,32]. Given the efficacy of IVIg discussed in the previous section[26,29,30], these studies should likely not be taken as definitive evidence against a role for antibodies in this context. It should also be noted that certain individuals with XLA are still able to produce some IgM or IgG, meaning that

antibody mediated immunity should not be dismissed in these instances. Another argument against TB-antibodies has been that B cell deficiency does not appear to increase risk of contracting Atb. A study and analysis of patients receiving the B cell depleting monoclonal antibody, rituximab, did not report a significantly increased risk of disease[33,34]. A caveat to these findings is that rituximab has highly limited depleting effects on antibody secreting plasma cells within the bone marrow that do not express CD20. Additionally, the monoclonal does not decrease pre-existing antibody titers, which remain relatively stable over time[33,34].

Despite this, there are also numerous historical and recent cohort studies with evidence that antibodies are beneficial against TB. A 1990s study of children from the UK and southeast Asia found that titers of IgG specific for the bacterial cell wall glycolipid, lipoarabinomannan (LAM), correlated with reduced risk of disseminated disease in a manner independent of age and geographic location[35]. Troughs in LAM antibody levels coincided with peak bacterial dissemination. Serum from those with disseminated disease had significantly reduced ($p<0.05$) titers of LAM-specific antibodies as determined by binding ELISA. Recent studies have also observed variations in functional antibody profiles that are associated with latent or active disease states[28]. This 2016 study by Lu et al highlighted differences in ability to drive innate immune effector functions that were largely attributable to distinct IgG Fc-glycosylation patterns. For example, Ltb patient IgG showed enhanced binding to FcγRIIIa (NK cell receptor), causing this group to show increased antibody-dependent cellular cytotoxicity (ADCC) and NK cell degranulation. In addition, Ltb patient antibodies also had enhanced ability to drive intracellular bacterial killing in macrophages, offering a potential explanation for this degree of bacterial control. Consistent with this, a multi-cohort study intended to find immune factors associated with Ltb, observing that increased FcγRIIIa signaling and upregulated NK cell

activation correlated with this controlled state of disease[36]. These positive findings strongly suggest that antibodies can play protective roles against *Mtb* infection despite much older beliefs to the contrary. In order to further asses this, it is important to better comprehend the influence that an antibody's target and Fc-domain properties have on governing its protective potential.

1.1.4: Vaccine Trials

Though a vast majority of all successful vaccines currently used confer protection by inducing an antibody response, design of tuberculosis vaccines has focused almost exclusively on stimulating CMI due to the aforementioned assumptions about the role of humoral immunity[37]. To date, these efforts have generally not yielded superior results to BCG, which was first introduced in the early 1920s. In spite of these obstacles, there have been a number of vaccine trials conducted in mice, macaques, and humans that have suggested that antibodies can influence vaccine efficacy. In mice, Prados-Rosales et al engineered a TB vaccine aimed at selectively inducing an *Mtb*-specific antibody response[38]. Using the capsular polysaccharide arabinomannan (AM) conjugated to either antigen 85B (Ag85B) or *Bacillus anthracis* protective antigen, this study reported significant reduction in lung bacterial burden in *Mtb* infected mice. Similar efficacy was observed when immune serum derived from vaccinated mice was administered to naïve mice prior to *Mtb* infection. In rhesus macaques, lung-specific antibody responses were linked to protection. Subjects were immunized with BCG either intradermally (standard) or via endobronchial instillation (mucosal). Upon repeated low-dose challenges with *Mtb*, the mucosal vaccination group experienced reduced bacterial burden and disease pathology in the lungs compared to the standard immunization group[39]. It was also observed that the mucosal group had robust PPD-specific IgA antibody responses in the bronchoalveolar lavage fluid. In humans, a number of recent clinical trials show evidence of antibodies as correlates of

protection against tuberculosis. A 2009 phase 2b trial used a recombinant Vaccinia Ankara virus engineered to express Ag85A (MVA85A) to boost cellular immune responses in BCG vaccinated individuals[40]. Despite not showing significantly improved efficacy compared to BCG vaccination, the vaccine was shown to be safe and tolerable. A 2016 follow up analysis to this trial found that the presence of Ag85A-specific IgG titers correlated with reduced risk of TB disease[41]. Another novel vaccine developed in the past few years is the M72/AS01$_E$ adjuvant fusion design[42]. Combining *Mtb* antigens known to induce robust T cell responses (*Mtb*32A and *Mtb*39A) with AS01 adjuvant, this phase 2b trial showed 54% protection against progression to active disease in *Mtb*-infected adults. While a significant portion of this is rightly attributed to CMI, it is interesting to note that immunization also stimulated a significant anti-M72 protein-specific IgG response that remained 26-fold higher than pre-vaccination levels through 12 months post-administration time point[43]. Accounting for the role of these proteins towards bacterial virulence, it is possible that both canonical virulence blocking antibodies and non-canonical antibody effector might contribute to a vaccine's effectiveness.

Together, these vaccine studies in animals and humans suggest that antibodies can indeed have potential roles in mycobacterial defense. Though it is important to note that the robust CMI responses induced in each study are likely the major mediators of the protective effects observed, there is a wealth of evidence supportive of beneficial roles for humoral responses, both independently as well as in tandem with T cell immunity. Specifically, defining the exact importance of antibody target specificity and better understanding the contextual factors (including disease severity, route of therapeutic delivery, timing of immunization) associated with vaccination will allow for advancement of therapeutic design strategies.

1.1.5: Monoclonal Antibodies

One method of addressing these gaps in our knowledge is through the generation and testing of monoclonal antibodies. Due to their singularly defined antigen specificities and modifiable constant domains, mAbs facilitate our ability to assess the impact each of these features has on antibacterial protection. A study in the late 1990's used splenocytes from BALB/c mice immunized with heat-inactivated *Mtb* to generate LAM (5c11) or mycolyl-arabinogalactan-peptidoglycan complex (4f11)-specific IgMs as well as the AM-specific IgG3, 9d8[44]. While 5c11 and 4f11 were essentially ineffective, 9d8 recipient mice infected with *Mtb* had increased survival times[45]. Lung tissue stains revealed that protected mice had enhanced bacterial containment within granulomas that are described as better organized than those observed in controls. iNOS staining for nitric oxide (antimycobacterial product of activated macrophages) revealed that 9d8 mouse macrophages were more prevalent surrounding bacterial clusters hinting at their role in hindering further dissemination[45]. While these results indicate that 9d8 mediated protection at least partly by enhancing immune cell responses, they can also be interpreted as evidence that target specificity and immunoglobulin isotype are potential determinants of an antibody's protective capabilities. Subsequent monoclonal experiments have also shown various correlates of protection against a range of *Mtb*-associated antigens through a variety of expected and unexpected mechanisms. Hamasur et al reported that a LAM-specific IgG1 clone, SMITB14, was able to significantly decrease lung and spleen CFUs in *Mtb* challenged mice, resulting in overall improvements in survival rates[46]. To further examine the mechanism of protection, the $F(ab')_2$ domain of SMITB14 was isolated, removing the constant domain that is required for recruiting and signaling innate immune cells. Interestingly, administering these truncated antibodies to mice by the same procedure, increases in survival similar to the complete mAbs was observed, indicating that binding activity alone was sufficient

to drive a significant degree of protection. Taken together with previously mentioned IVIg data, it can be surmised that antibodies are able to effect function not only through stimulating antimicrobial effector functions, but also via binding blockade, mirroring the neutralization of viruses[47]. Additionally, studies involving mAbs specific for other *Mtb*-associated antigens including heparin-binding hemagglutinin adhesin (HBHA) and heat shock protein X (HspX) were also able to show examples of protection *in vivo*. HBHA represents a target of interest due to its role in facilitating extrapulmonary bacterial dissemination[48]. Accordingly, application of an HBHA-specific mAb to *Mtb*-infected mice caused reduction in spleen, but not lung CFUs[49]. HspX, a stress-induced intracellular and cell wall-associated protein, is another target of interest based on its protein folding function[50]. When given an HspX-specific IgA1, infected mice had decreased bacterial burden in the lungs[51]. These findings are of notable interest because IgA interacts with the Fc-receptor CD89 (FcαR1), which is found in humans, but not in normal mice. The study by Balu et al found that transgenic mice expressing human CD89 were protected by mAb injection, but their CD89-negative littermate controls were not, indicating that Fc-mediated functions drive these effects. Overall, these monoclonal experiments have demonstrated that antibodies can protect against tuberculosis *in vivo*, though the mechanism by which they do so remain somewhat unclear. Expanded study of immunoglobulin target specificities and Fc-dependent and independent routes of protection will contribute to the continued development of antibodies as potential TB therapeutics.

1.1.6: Potential Applications

In order to strengthen the case supportive of TB antibodies, additional work can be done to expand the repertoire of relevant antigen targets as well as to better understand the mechanisms by which immune effector functions are mediated. Generating monoclonals against

a diverse array of *Mtb* antigens will further define the protective potential of unexplored targets and by pairing this with continued study of Fc-mediated actions, our ability to assess the value of inducing specific functions (NK cell cytotoxicity, macrophage phagocytosis, etc) will be greatly enhanced (Figure 1). Understanding each of these antibody components in tandem will be critical for developing the next generation of therapeutics and vaccines to combat tuberculosis.

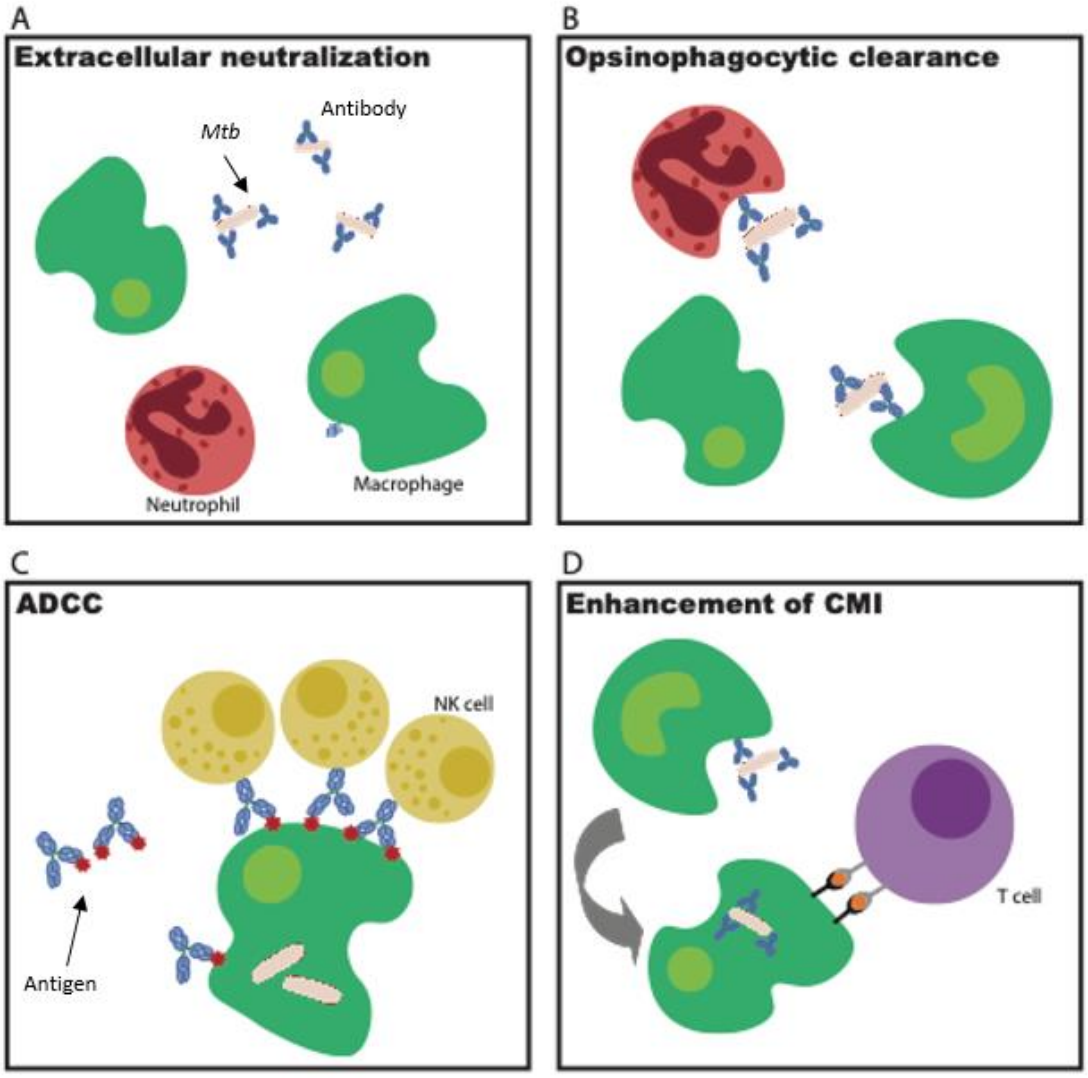

Figure 1. Antibody functions of interest. A: Extracellular neutralization of bacteria, preventing cellular invasion and/or mediating bacterial clearance by immune cells. B: Fc-mediated uptake of antibody-bound bacteria by phagocytic cell types. C: Antibody dependent cellular cytotoxicity

where by antibodies induce NK cell activation in response to cellular infection. D: Antibody-supported enhancement of T cell anti-bacterial functions.

1.1.7: Antigen Selection Criteria

In this project, alanine and proline rich antigenic glycoprotein (Apa) and phage growth factor *E. coli* large (GroEL) were chosen as antigen targets of interest. Apa is a 45kDa secreted protein that is known to elicit a Th1 response in PPD positive individuals. It is a fibronectin attachment protein that is known to remain attached to the cell surface long enough to associate with pulmonary surfactant protein A (PSP-A) on macrophages, facilitating bacterial entry into the cell[52]. It has also been shown that infected guinea pigs vaccinated with *apa* DNA and a poxvirus recombinant boost experienced decreased splenic CFUs[53]. For these reasons, it is believed that Apa represents a viable target for monoclonal antibodies.

GroEL is a 60kDa chaperonin that associates with growth factor *E. coli* small (GroES) in order to enable correct protein folding. It is found to be upregulated by *Mtb* during macrophage infection and there is evidence that TB positive individuals have titers of specific antibodies against it[54]. Together with GroES, HBHA, and HspX (heat shock proteins), it makes up roughly 60% of the components of PPD, making it a relatively abundant target in this context. There is also some evidence that GroEL has also been found on the cell surface, suggesting that it is potentially a physiologically relevant target for antibodies as well[55].

2. Materials & Methods and Results

2.1: Introduction

To understand the contributions of antigen specificity and constant domain to an antibody's ability to protect against TB, Fab regions of varying specificities were expressed on selected Fc-variants and then tested in functional and bacterial growth restriction assays. Variable heavy and light chain sequences were obtained from a collaboration with Aeras and the Kepler Laboratory of Computational Immunology at Boston University. Mice were immunized with either Apa or GroEL antigen and sequences were derived from plasmablasts. Pairs were down-selected based on sequence conservation across multiple animals. These pairs were modified for compatibility with the Golden Gate Cloning system and used to transform Stellar Competent bacteria. DNA extracted from these cells was combined with sequences for Fc-variants and used to transfect 293F cells, producing monoclonal antibodies of chosen specificity and constant domain. These antibodies were tested for binding in human IgG ELISAs before being analyzed for effector function. Monoclonals able to induce these innate immune cell functions were then identified by their ability to restrict *Mtb* growth in whole human blood.

2.2: Materials and Methods

In order to assess the contributions of antigen specificity and Fc-domain to an antibody's immune capabilities, we utilized a modified version of the Golden Gate cloning system to generate monoclonals with known Fab targets on a human IgG1 backbone (Figure 2).

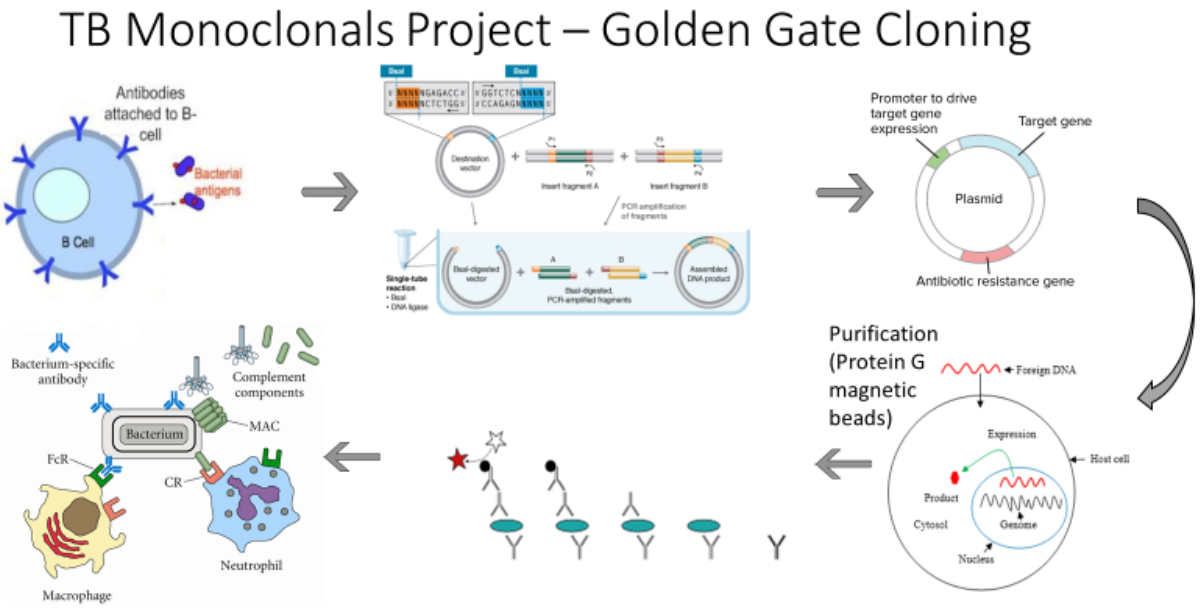

Figure 2. Overview of the production and testing pipeline. Variable heavy and light chain sequences are derived from antibodies responsive to B cell baiting. The Golden Gate cloning platform is used to insert these constructs into vectors that can be used to transform Stellar Competent cells. Protein-coding DNA is extracted from transformed cells and used to transfect modified Human embryonic kidney 293 cells. Produced antibodies are purified from the supernatant and then assessed for binding capability and functional activity.

2.2.1: Generation of Variable Heavy and Light Chain Sequences

Nine mice were immunized with Apa and MPL/Alhydrogel adjuvant and six were immunized with GroEL and QS-21 adjuvant. From these mice, 692 Apa V_H/V_L pairs and 734 GroEL pairs were derived, of which 16 Apa and 17 GroEL matches were selected based on their conservation across multiple subject lineages. Collaborators at Aeras provided us with matched variable heavy (V_H) and light (V_L) segments that had been isolated using Atreca's Immune Repertoire Capture technology.

2.2.2: Antibody Cloning

These pairs and IgG1 Fc domain were cloned into pUC plasmids in a manner allowing for the annealing of any variable segment pair to the IgG1 backbone. The ligation reaction utilized the pKL352 plasmid (kanamycin resistance), variable and constant segments, furin-2a, bovine serum albumin (BSA), BsaI, T4 DNA ligase, and ATP (Figure 3).

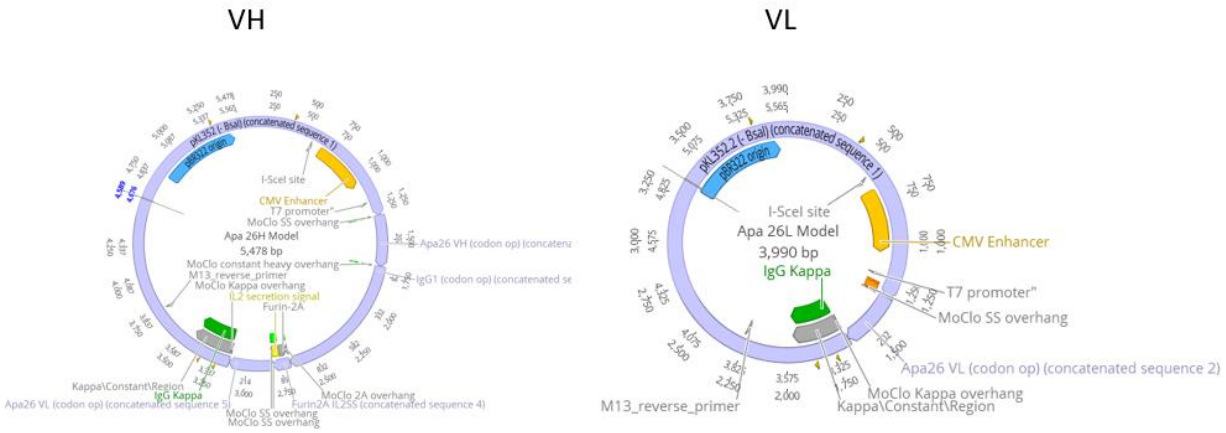

Figure 3. Variable heavy and variable light chain plasmid design. Apa clone 26 is depicted here.

Following molecular cloning assembly, the reaction mixture was used to transform Stellar Competent cells. Transformed bacteria were incubated with SOC outgrowth media before plating on kanamycin-coated plates. Following overnight incubation, colonies were selected based on their expression of kanamycin resistance genes. Colony PCR was performed to determine the presence of the plasmid DNA inserts. DNA was isolated from these bacteria using the Qiagen QIAprep Spin Miniprep Kit. DNA was checked by SYBR Safe DNA Gel Stain and sequence accuracy was confirmed by Sanger Sequencing.

2.2.3: Antibody Expression

Plasmid DNA was combined with opti-MEM reduced serum media and filtered using a 0.22μm syringe apparatus, after which polyethylenimine was added. Following a 30 minute

incubation, the transfection mixture was added to 293F at $0.4x10^6$ cells/mL in 293 Freestyle Expression Medium. After incubation at 37°C for 5 day, supernatant was collected, and Protein G magnetic beads were added and incubated overnight at 4°C. Antibodies were purified and tested for production confirmation and quantity by ELISA. Bolt™ 4-12% Bis-Tris Plus LDS reducing gels were used to denature antibodies and confirm correct protein fragment sizes.

2.2.4: ELISA for Antigen Binding

To assess the binding capabilities of these monoclonal antibodies, PPD and antigen-specific ELISAs were performed. ELISA plates were coated with either PPD or specific antigens (Apa or GroEL) at a concentration of 2ug/mL. Antibodies were serially diluted 5-fold, starting at an initial concentration of 3.5ug/mL. The eBioscience human IgG ELISA protocol was used.

2.2.5: Antibody Dependent Cellular Phagocytosis (ADCP)

The functional properties of the generated antibodies were determined by functional assays including ADCP. PPD was biotinylated using Sulfo-NHS-LC-biotin before filtering, washing, and brought to a working concentration of 1mg/mL. Biotinylated PPD was then coupled with Neutravidin beads (FITC) and incubated overnight. Following incubation, the coupled protein beads were pelleted and resuspended in 5% BSA in PBS to block non-specific binding interactions. Antibodies were diluted in PBS at concentrations of 1ug, 0.5ug, 0.1ug, and 0.05ug per well. The prepared PPD-neutravidin beads were pelleted and resuspended in PBS and added to 10uL of each antibody dilution in a 96-well round-bottom plate. Assay plate were then incubated for 2 hours at 37°, 5% CO_2. During incubation, THP-1 human monocytes from culture were adjusted to a concentration of $0.125x10^6$ cells/mL. After incubation, the assay plate(s) were washed with PBS and then pelleted at 2000 x g for 10 minutes. Pellets were resuspended with the re-concentrated THP-1 cell suspension and then incubated overnight at 37°C, 5% CO_2. After

overnight incubation, the plates were removed from the incubator and resuspended in 4% paraformaldehyde. Data acquisition was performed using a BD LSRII flow cytometer. Analysis was performed using FlowJo software to calculate a phagocytic score, defined as (%bead+ cells x FITC MFI)/100.

2.2.6: Antibody Dependent Neutrophil Phagocytosis (ADNP)

Ability to induce innate cell function was also assessed in terms of neutrophil phagocytosis. The same antibody dilution series and PPD-coated NeutrAvidin beads used in the ADCP assay described previously are used here. All elements of that protocol used here remain the same through the antibody + PPD bead 2 hour incubation. HIV negative blood was collected from donors in the Ragon Clinical Research and treated with acid citrate dextrose (ACD) anticoagulant. Blood was mixed with 3% dextran and then incubated. Following incubation, supernatant was removed and pelleted, after which ammonium-chloride-potassium (ACK) lysing buffer was introduced. Polymorphonuclear cells (PMNs) were isolated and resuspended at a concentration of $5x10^5$ cells/mL in R10 media. The isolated PMNs were then added to the antibody-PPD bead complexes and stained with Biolegend anti-human CD66b Pacific Blue™ for identification of neutrophils. After incubation, assay plates were washed with PBS and resuspended in 4% PFA for fixation before acquisition on a BD LSRII flow cytometer. Analysis was performed on FlowJo to determine the phagocytic score: (%bead+ CD66b+ cells x MFI)/100 to represent the degree of antibody-induced neutrophil phagocytosis of PPD-coated beads.

2.2.7: *Mtb-lux* Growth Restriction in Human Whole Blood

The ability of the mAbs to restrict *Mtb* growth in human blood was assessed by whole blood assay (WBA). A luciferase reporter expressing H37Rv *Mtb* strain (*Mtb-lux*)[56] was passaged in 7H9 media using Zeocin to eliminate mutant development. Whole human blood was

collected from HIV negative patients on the mornings of assay set up days. Donor blood was infected with *Mtb-lux* at an MOI of $1x10^6$ *Mtb*/mL blood and added to 96-well plates containing antibodies pre-diluted in modified R10 media without phenol red. Plates were read for luminescence by Tecan Spark every 24 hours from 0-120 hours post-infection. Bacterial growth curves were generated from these readings, allowing for the assessment of the antibodies' ability to control both extracellular and intracellular infection over time.

2.3: Results

2.3.1: Antigen and PPD Binding ELISA Results

Figures 4 and 5 show results for binding of Apa and GroEL clones to their specific antigens as well as to PPD in human IgG ELISAs. Optical density is measured at 450nm, with antibody clones selected for higher OD (binding) to both Apa/GroEL and PPD. Apa clones 26, 94, and 104 were chosen especially for their ability to bind to PPD relatively better than the other monoclonals. The same rationale was applied to select GroEL clones 27, 40, and 66 as candidates of interest for further assessment in the functional assays.

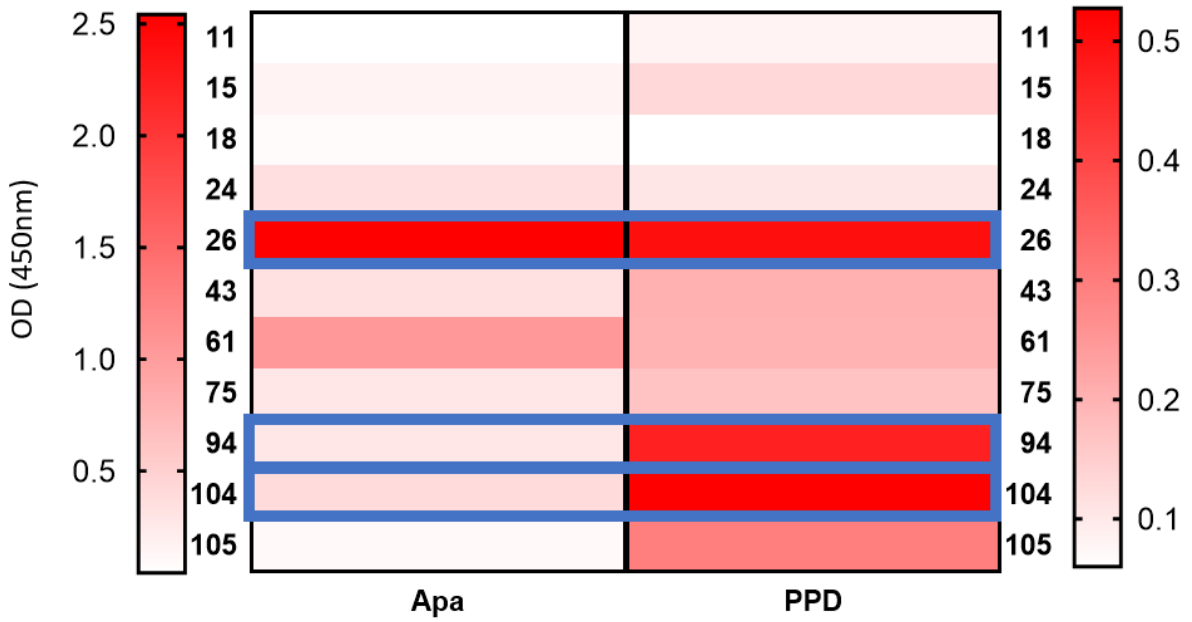

Figure 4. Human IgG ELISA of Apa antibody clones against Apa and PPD.

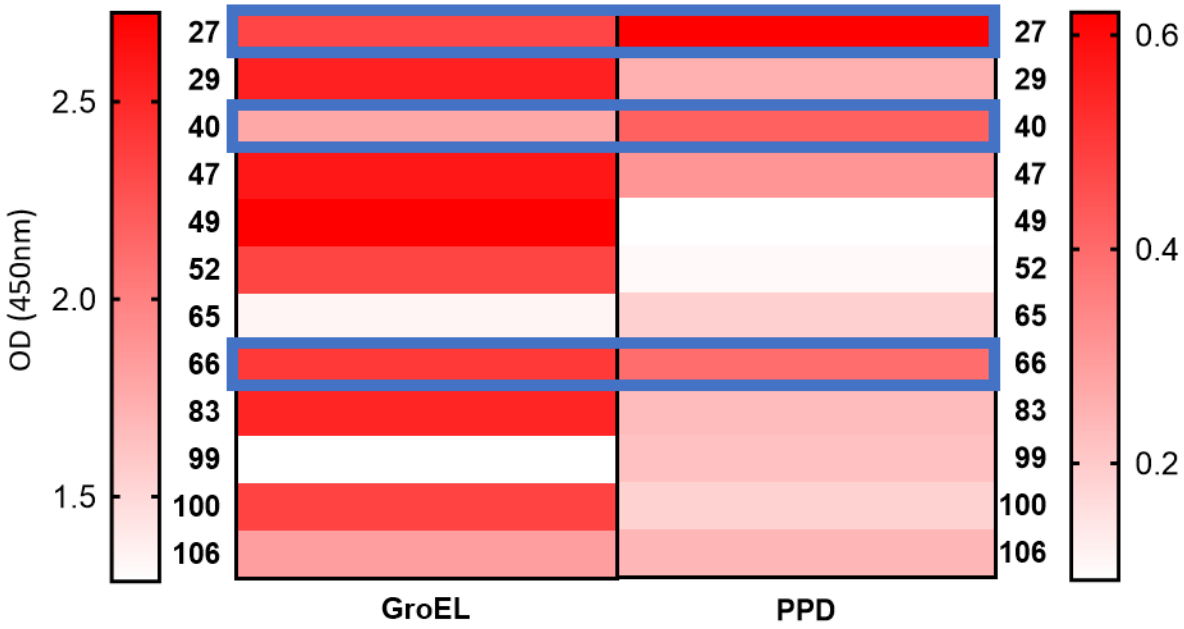

Figure 5. Human IgG ELISA of GroEL antibody clones against GroEL and PPD.

2.3.2: ADCP Results

Figure 6 shows phagocytic scores from the antibody dependent cellular phagocytosis assays using titrations of the six total Apa and GroEL clones selected for their ability to bind to specific antigen and PPD in the human IgG ELISA. A higher phagocytic score indicates that an antibody is able to induce a greater degree PPD-coated bead uptake by THP-1 monocytes. The "hook" effect seen in the cases of Apa 104 and GroEL 27 is not uncommon. It is possible that higher concentrations of antibody can be oversaturating, causing binding antibodies to overcrowd each other, leading to decreased phagocytosis. As a result, lower concentrations of antibodies can initially show increased phagocytic scores before then titrating out as expected.

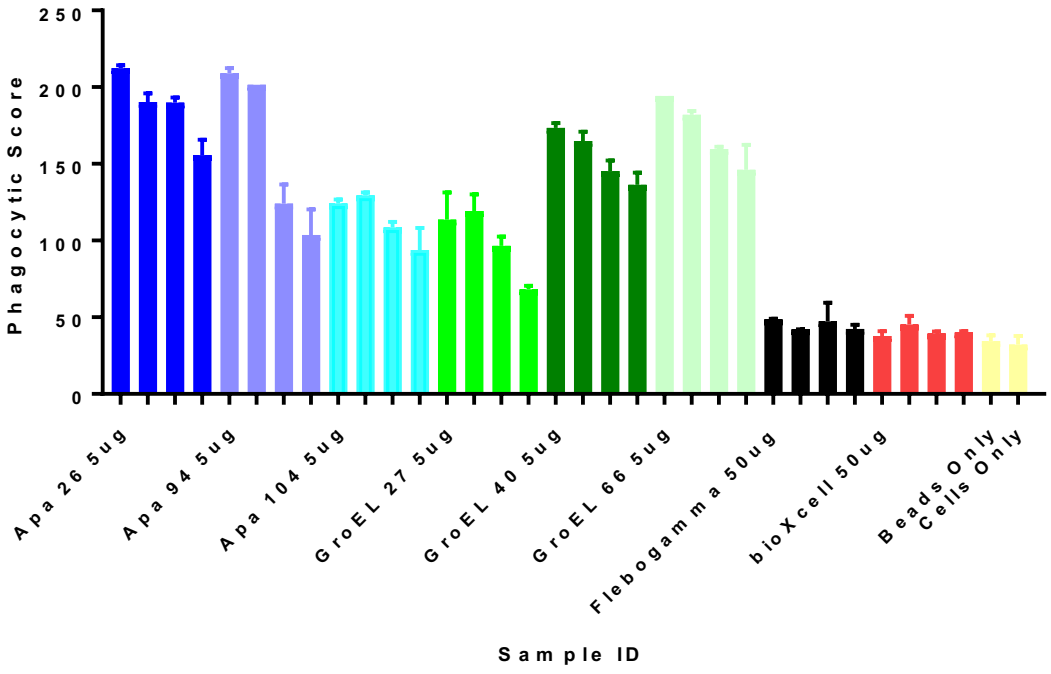

Figure 6. Antibody dependent cellular phagocytosis of the Apa and GroEL clones selected in Figures 4 & 5 for binding capability.

2.3.3: ADNP Results

Figure 7 shows phagocytic scores for the selected Apa and GroEL clones from the antibody dependent neutrophil phagocytosis assay. The score represents an antibody's ability to induce uptake of PPD-coated beads by neutrophils isolated from human whole blood. Similarly to the ADCP assay, the antibody concentration hook effect is not unexpected, though it is not seen here.

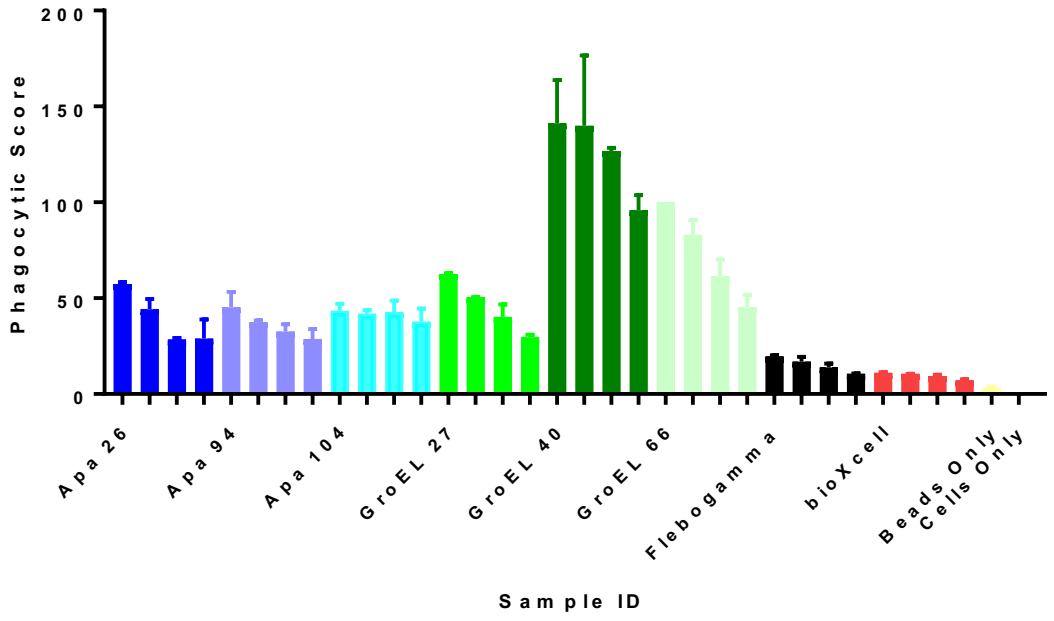

Figure 7. Antibody dependent neutrophil phagocytosis of the selected Apa and GroEL clones. For each clone, there are four serial dilutions (3 fold) starting at 2ug/well.

2.3.4: Human Whole Blood Assay Results

Figure 8 shows data from human whole blood infected with *Mtb-lux*. Bacterial luminescence is used to measure of bacterial growth, shown below as relative luminescence units

(RLU) vs time post infection. An antibody's ability maintain lower RLUs represents its ability to restrict bacterial growth.

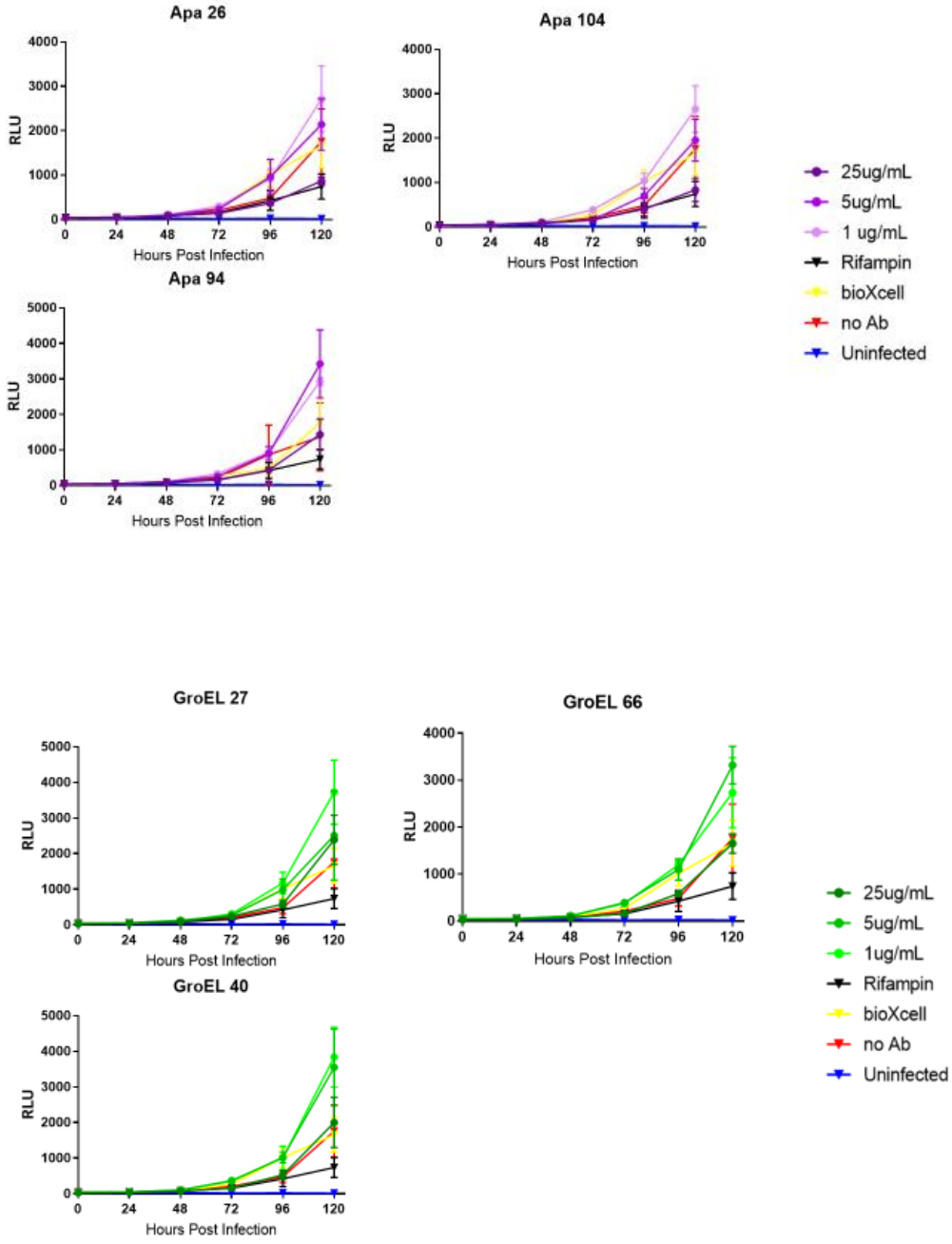

Figure 8. Human whole blood assay. The top graphs show data from Apa 26, 94, and 104. Bottom graphs show data from GroEL clones 27, 40, and 66.

3. Discussion

As seen in the previous section, the selected Apa and GroEL antibody clones varied in their abilities to bind to PPD or live *Mtb* bacteria, as well as in their capacities to induce cell-mediated uptake. The ADCP assay (Figure 6) shows that all 6 clones are able to induce monocyte phagocytosis of PPD-coated beads at a significantly higher level than the non-specific IgG control (bioXcell). Figure 7 shows that these clones are also able to induce neutrophil uptake of PPD beads, though the Apa clones appear to mediate less of this function relative to the GroEL monoclonals. These observations are consistent with the biological profile of these antigens as well as their connection to both phagocytosis assays. Separate phagocytosis assays are necessary because monocytes and neutrophils express different receptors. Monocytes are known to constitutively express FcγR1 (CD64), a high affinity IgG receptor. Accordingly, all clones induce a significant degree of function in the ADCP assay. While neutrophils are also known to have CD64, they do not have the same expression pattern as monocytes, instead expressing it primarily in response to inflammatory stimuli such as interferon gamma[57]. It is therefore not surprising to see lower neutrophil activity in the ADNP assay. It is important to note that these statements are based on the clones' phagocytic scores compared to the scores of the controls only within that specific assay. While both ADCP and ADNP utilize the same method of scoring, direct score-to-score comparisons cannot be made between assays.

It is possible that the relative abundance of each antigen influences the results of these assays. GroEL, together with related heat shock proteins GroES, HspX, and DnaK dominates the

composition of PPD, combining to account for nearly 60% of its total make up. In comparison, Apa is estimated to account for 2% of PPD's contents[58]. This difference can potentially explain the functional differences between Apa and GroEL clones seen in Figure 7. The Apa antibodies are able to bind to PPD (consistent with the binding ELISAs), but not to the same degree as the GroEL antibodies. As a result, they still cause some degree of neutrophil phagocytosis, but noticeably less than the GroEL clones do.

Difference in antigen biological profile is also the likely factor influencing the results of the whole blood assays. GroEL is a chaperonin and only expected to be found intracellularly in live *Mtb* bacteria. Due to this, it is unlikely that the GroEL antibodies were able to consistently encounter and bind to it in the context of the WBA, rendering them largely unable to restrict bacterial growth (Figure 8). In contrast, Apa has been found to remain associated with the cell wall long enough to interact with other proteins during infection. Accordingly, the Apa clones perform better in the WBA. Surface bound Apa is located and bound by the antibodies, allowing them to then mediate restriction of microbial growth (ability shown in ADCP and ADNP especially), resulting in the lower RLUs seen in Figure 8.

It appears that the results of the two phagocytosis assays and the whole blood assay are consistent with each other as well as to the biological characteristics of the antigens being explored. Given that GroEL is far more common in PPD than Apa is, it is not surprising to see that the GroEL clones are able to consistently induce phagocytosis in both cell types, with notable differences (vs Apa) apparent in the neutrophil experiment. Despite this, GroEL is not known to be available on the cell surface of live bacteria, preventing consistent antibody binding and Fc-mediated restriction of bacterial growth. Apa may not be as common in the context of

PPD, but is shown to be expressed on the cell surface, allowing for those clones to be more effective than the GroEL specificities against live bacteria.

Though completely defined mechanisms for antibodies in tuberculosis remains unclear, the data presented here offers support to the notion that humoral immunity can induce beneficial effector functions against *Mtb* infection.

3.1. Limitations

There are a number of caveats and limitations that must be considered. First, Fab sequences were derived from antigen-baited mice. Though humanized mouse models of tuberculosis are available, there is no guarantee that the selected clones are necessarily the same as those that would be derived from infected humans. As result, antibodies using variable regions of murine origin may be unexpectedly immunogenic when applied in the context of human samples. This would be especially relevant in the human whole blood assay where, however unlikely it may be, antibodies using variable regions of murine origin could have off-target binding, potentially skewing the reported results. There are also limitations that apply to both the binding ELISAs as well as the phagocytosis assays regarding PPD. Purified protein derivative is made from sterilized, denatured TB bacteria. The resulting inoculant contains nearly 5,000 unique antigens, many of which will never be expressed in the same locale as antibodies during in the context of live bacterial challenge. As a result, data showing protection against PPD antigen must be considered carefully, as antibodies may be responding to antigens that they will never actually encounter on live mycobacteria. A caveat to the whole blood assay is that *Mtb* is a respiratory pathogen, and so does not typically reside in the blood[59]. While it should be noted that some cell types in human whole blood are the same as those that would encounter *Mtb* in the lungs, it is a reach to take the results of these assays as truly representative of a respiratory

infection. Despite these limitations, all of these systems provide a useful means of assessing *in vitro* protective effects of antibodies based on their antigen-binding specificities and capacity to enact Fc-mediated cellular immunity.

3.2. Ongoing and Future Research

Further studies are currently being done on antibodies of different specificities, especially those specific for non-protein antigens of interest such as LAM. Not only will these mAbs broaden the range of specificities that have been characterized, but provide much more in depth evidence about whether or not binding affinity alters Fc function or magnitude of response. Cloning of the previously mentioned specificities will be done on a panel of 20 Fc-variants. This panel of antibodies will be characterized according to the same series of assays used above. Glycan engineering is also of interest. It has previously been shown the antibodies derived from ATB and LTB individuals have unique, identifiable glycosylation patterns[28]. Lu et al show that these patterns also influence an antibody's ability to induce Fc-mediated effector functions, including ADCP. In keeping with these breakthrough findings, it would be of high interest to glycan engineer high affinity mAb clones in order to enhance their functional capabilities. In addition, clones that perform well in all of the previously described assays will be adapted for application in humanized mouse models. These experiments will address some of the caveats previously mentioned, especially those regarding the limitations of *in vitro* testing models for *Mtb*. Provided success here, murine infection experiments would begin the process of optimizing these to eventually be tested in non-human primate models and clinical trials further in the future.

Together, these findings are supportive of beneficial roles for antibodies against tuberculosis. There is evidence that the preliminary binding and functional activity of specific

protein-targeting antibodies translates to observable bacterial restriction in *in vitro* testing of human blood samples, suggesting that continuing this project and incorporating an increased variety of antigen specificities and Fc domains will potentially lead to the generation of even more effective monoclonals. In conjunction with cell-mediated immunity, the humoral immune response may one day provide a much needed solution to the global tuberculosis pandemic.

4. Bibliography

Bibliography

1. *Global Tuberculosis Report 2018*. (2018).
2. Brandt, L. *et al.* Failure of the Mycobacterium bovis BCG vaccine: Some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infect. Immun.* **70**, 672–678 (2002).
3. Fine, P. E. M. Variation in protection by BCG: implications of and for heterologous immunity. [Published erratum appears in Lancet 1996;347:340]. *Lancet* **346**, 1339–1345 (1995).
4. Achkar, J. M. & Casadevall, A. Antibody-Mediated Immunity against Tuberculosis: Implications for Vaccine Development. *Cell Host Microbe* **13**, 250–262 (2013).
5. Casadevall, A. Antibody-based vaccine strategies against intracellular pathogens. (2018). doi:10.1016/j.coi.2018.04.011
6. Casadevall, A. Antibody-mediated protection against intracellular pathogens. *Trends Microbiol.* **6**, 102–107 (1998).
7. Caruso, A. M. *et al.* Mice Deficient in CD4 T Cells Have Only Transiently Diminished Levels of IFN-□, Yet Succumb to Tuberculosis 1. *J Immunol J. Immunol. by guest March* **162**, 5407–5416 (1999).
8. Lin, P. L. *et al.* CD4 T Cell Depletion Exacerbates Acute Mycobacterium tuberculosis While Reactivation of Latent Infection Is Dependent on Severity of Tissue Depletion in Cynomolgus Macaques. *AIDS Res. Hum. Retroviruses* **28**, 1693–1702 (2012).
9. Yao, S. *et al.* CD4+ T Cells Contain Early Extrapulmonary Tuberculosis (TB) Dissemination and Rapid TB Progression and Sustain Multieffector Functions of CD8+ T and CD3- Lymphocytes: Mechanisms of CD4+ T Cell Immunity. *J. Immunol.* **192**, 2120–2132 (2014).
10. Lawn, S. D., Butera, S. T. & Shinnick, T. M. Tuberculosis unleashed: The impact of human immunodeficiency virus infection on the host granulomatous response to Mycobacterium tuberculosis. *Microbes Infect.* **4**, 635–646 (2002).
11. Geldmacher, C. *et al.* Preferential infection and depletion of Mycobacterium tuberculosis –specific CD4 T cells after HIV-1 infection . *J. Exp. Med.* **207**, 2869–2881 (2010).
12. Jacobs, A. J., McShane, H., Mongkolsapaya, J., Screaton, G. R. & Wilkinson, R. J. Antibodies and tuberculosis. *Tuberculosis* **101**, 102–113 (2016).
13. Mackanes, G. B. *Resistance to Intracellular Infection*. *Source: The Journal of Infectious Diseases* **123**, (1971).
14. Abebe, F. & Bjune, G. The protective role of antibody responses during Mycobacterium tuberculosis infection. *Clin. Exp. Immunol.* **157**, 235–243 (2009).
15. Flick, L. *Serum treatment in tuberculosis*. (1905).
16. Maragliano, C. Le sérum antituberculeux et son antitoxine. *Rev Tuberc* 131–138 (1896).
17. Glatman-Freedman, A. & Casadevall, A. Serum therapy for tuberculosis revisited: Reappraisal of the role of antibody-mediated immunity against Mycobacterium tuberculosis. *Clin. Microbiol. Rev.* **11**, 514–532 (1998).
18. Gamgee, A. Dr. Viquerat's Treatment of Tuberculosis. *Lancet* 811–813 (1894).
19. Forget, A., Benoit, J. C., Turcotte, R. & Gusew Chartrand, N. Enhancement activity of anti mycobacterial sera in experimental Mycobacterium bovis (BCG) infection in mice. *Infect. Immun.* **13**, 1301–1306 (1976).

20. Alexander Marmorek, B. D. *Antituberculous serum and 'vaccine.'* (1903).
21. Paquin, P. The treatment of tuberculosis by injections of immunized blood serum. *JAMA J. Am. Med. Assoc.* **XXIV**, 842 (1895).
22. Lemen, J. R. Three years of serum therapy in tuberculosis - Harvard University. (1898). Available at: https://hollis.harvard.edu/primo-explore/fulldisplay?docid=01HVD_ALMA21201435238000394l&context=L&vid=HVD2&lang=en_US&search_scope=everything&adaptor=Local Search Engine&tab=everything&query=any,contains,Three years of serum therapy in tub. (Accessed: 4th March 2019)
23. Feldman, W. H., Karlson, A. G. & Hinshaw, H. C. Streptomycin in Experimental Tuberculosis. The Effects in Guinea Pigs following Infection by Intravenous Inoculation. *Am. Rev. Tuberc. Pulm. Dis.* **56**, 346–59 (1947).
24. Murray, J. F., Schraufnagel, D. E. & Hopewell, P. C. Treatment of tuberculosis: A historical perspective. *Ann. Am. Thorac. Soc.* **12**, 1749–1759 (2015).
25. Guirado, E. *et al.* Passive serum therapy with polyclonal antibodies against Mycobacterium tuberculosis protects against post-chemotherapy relapse of tuberculosis infection in SCID mice. *Microbes Infect.* **8**, 1252–1259 (2006).
26. Roy, E. *et al.* Therapeutic efficacy of high-dose intravenous immunoglobulin in Mycobacterium tuberculosis infection in mice. *Infect. Immun.* **73**, 6101–9 (2005).
27. Li, H. *et al.* Latently and uninfected healthcare workers exposed to TB make protective antibodies against Mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 5023–5028 (2017).
28. Lu, L. L. *et al.* A Functional Role for Antibodies in Tuberculosis. *Cell* **167**, 433–443.e14 (2016).
29. Olivares, N. *et al.* The protective effect of immunoglobulin in murine tuberculosis is dependent on IgG glycosylation. *Pathog. Dis.* **69**, 176–183 (2013).
30. Olivares, N. *et al.* Prophylactic effect of administration of human gamma globulins in a mouse model of tuberculosis. *Tuberculosis* **89**, 218–220 (2009).
31. Chen, X.-F. *et al.* Clinical characteristics and genetic profiles of 174 patients with X-linked agammaglobulinemia. *Medicine (Baltimore).* **95**, e4544 (2016).
32. Doğru, D., Kiper, N., Ozçelik, U., Yalçin, E. & Tezcan, I. Tuberculosis in children with congenital immunodeficiency syndromes. *Tuberk. Toraks* **58**, 59–63 (2010).
33. Alkadi, A., Alduaiji, N. & Alrehaily, A. Risk of tuberculosis reactivation with rituximab therapy. *Int. J. Health Sci. (Qassim).* **11**, 41–44 (2017).
34. Kimby, E. Tolerability and safety of rituximab (MabThera®). *Cancer Treat. Rev.* **31**, 456–473 (2005).
35. Costello, A. M. *et al.* Does antibody to mycobacterial antigens, including lipoarabinomannan, limit dissemination in childhood tuberculosis? *Trans. R. Soc. Trop. Med. Hyg.* **86**, 686–92
36. Roy Chowdhury, R. *et al.* A multi-cohort study of the immune factors associated with M. tuberculosis infection outcomes. *Nature* **560**, 644–648 (2018).
37. Glatman-Freedman, A. The role of antibody-mediated immunity in defense against Mycobacterium tuberculosis: Advances toward a novel vaccine strategy. *Tuberculosis* **86**, 191–197 (2006).
38. Prados-Rosales, R. *et al.* Enhanced control of Mycobacterium tuberculosis extrapulmonary dissemination in mice by an arabinomannan-protein conjugate vaccine.

PLOS Pathog. **13**, e1006250 (2017).
39. Dijkman, K. *et al.* Prevention of tuberculosis infection and disease by local BCG in repeatedly exposed rhesus macaques. *Nat. Med.* **25**, 255–262 (2019).
40. Tameris, M. D. *et al.* Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: A randomised, placebo-controlled phase 2b trial. *Lancet* **381**, 1021–1028 (2013).
41. Fletcher, H. A. *et al.* T-cell activation is an immune correlate of risk in BCG vaccinated infants. *Nat. Commun.* **7**, (2016).
42. Van Der Meeren, O. *et al.* Phase 2b Controlled Trial of M72/AS01E Vaccine to Prevent Tuberculosis. *N. Engl. J. Med.* **379**, 1621–1634 (2018).
43. Penn-Nicholson, A. *et al.* Safety and immunogenicity of candidate vaccine M72/AS01E in adolescents in a TB endemic setting. *Vaccine* **33**, 4025–4034 (2015).
44. Glatman-Freedman, A., Martin, J. M., Riska, P. F., Bloom, B. R. & Casadevall, A. Monoclonal antibodies to surface antigens of Mycobacterium tuberculosis and their use in a modified enzyme-linked immunosorbent spot assay for detection of mycobacteria. *J. Clin. Microbiol.* **34**, 2795–2802 (1996).
45. Teitelbaum, R. *et al.* A mAb recognizing a surface antigen of Mycobacterium tuberculosis enhances host survival. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 15688–15693 (1998).
46. Hamasur, B. *et al.* A mycobacterial lipoarabinomannan specific monoclonal antibody and its F(ab′)2 fragment prolong survival of mice infected with Mycobacterium tuberculosis. *Clin. Exp. Immunol.* **138**, 30–38 (2004).
47. Hangartner, L., Zinkernagel, R. M. & Hengartner, H. Antiviral antibody responses: The two extremes of a wide spectrum. *Nat. Rev. Immunol.* **6**, 231–243 (2006).
48. Menozzi, F. D. *et al.* Identification of a heparin-binding hemagglutinin present in mycobacteria. *J. Exp. Med.* **184**, 993–1001 (1996).
49. Pethe, K. *et al.* The heparin-binding haemagglutinin of M. tuberculosis is required for extrapulmonary dissemination. *Nature* **412**, 190–194 (2002).
50. Yuan, Y. *et al.* The 16-kDa alpha-crystallin (Acr) protein of Mycobacterium tuberculosis is required for growth in macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9578–83 (1998).
51. Balu, S. *et al.* A novel human IgA monoclonal antibody protects against tuberculosis. *J. Immunol.* **186**, 3113–9 (2011).
52. Ragas, A., Roussel, L., Puzo, G. & Rivière, M. The *Mycobacterium tuberculosis* Cell-surface Glycoprotein Apa as a Potential Adhesin to Colonize Target Cells via the Innate Immune System Pulmonary C-type Lectin Surfactant Protein A. *J. Biol. Chem.* **282**, 5133–5142 (2007).
53. Kumar, P., Amara, R. R., Challu, V. K., Chadda, V. K. & Satchidanandam, V. The Apa protein of Mycobacterium tuberculosis stimulates gamma interferon-secreting CD4+ and CD8+ T cells from purified protein derivative-positive individuals and affords protection in a guinea pig model. *Infect. Immun.* **71**, 1929–37 (2003).
54. Monahan, I. M., Butcher, P. D., Betts, J. & Banerjee, D. K. Differential expression of mycobacterial proteins following phagocytosis by macrophages. *Microbiology* **147**, 459–471 (2001).
55. Hickey, T. B. M., Thorson, L. M., Speert, D. P., Daffé, M. & Stokes, R. W. Mycobacterium tuberculosis Cpn60.2 and DnaK are located on the bacterial surface, where Cpn60.2 facilitates efficient bacterial association with macrophages. *Infect. Immun.* **77**, 3389–401 (2009).

56. Andreu, N. *et al.* Optimisation of Bioluminescent Reporters for Use with Mycobacteria. *PLoS One* **5**, e10777 (2010).
57. Dale, D. C., Boxer, L. & Liles, W. C. The phagocytes: neutrophils and monocytes. *Blood* **112**, 935–945 (2008).
58. Cho, Y. S. *et al.* Deciphering the proteome of the in vivo diagnostic reagent "purified protein derivative" from Mycobacterium tuberculosis. *Proteomics* **12**, 979–91 (2012).
59. Ernst, J. D. The immunological life cycle of tuberculosis. *Nat. Rev. Immunol.* **12**, 581–591 (2012).