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By

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Committee in charge:

Professor Lee W. Riley, Chair
Professor Sangwei Lu
Professor Karsten Gronert

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Abstract

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Professor: Lee W. Riley, Chair

Tuberculosis (TB) faces difficult challenges for its treatment and control. Both the diagnosis of TB and *Mycobacterium tuberculosis (M. tuberculosis)* drug susceptibility testing take weeks and clinicians often do not know if the patient is taking an appropriate set of drugs until complications or even death occur. Consequently, early determination of a successful drug therapy response in individuals infected with *M. tuberculosis* is urgently needed. Since the *M. tuberculosis* cell wall is comprised of a diverse repertoire of lipids, we examined the possible role of these lipids as antigens for serologic response during *M. tuberculosis* infection. This dissertation is focused on the examination of lipid-antibody response as a potential biomarker used to monitor treatment response in *M. tuberculosis* infected hosts. Briefly, Chapter 1 describes the current pitfalls of monitoring tuberculosis treatment with current methods, including acid-fast bacilli (AFB) smear and culture conversion. Chapter 1 also covers the definition of biomarkers and the rationale to use *M. tuberculosis* cell wall lipids to develop an anti-lipid-antibody based test. Evidence from a similar biomarker-based test for syphilis is presented. The chapter also discusses the biological basis which guided the lipid-antibody biomarker search and discovery.

Chapter 2 describes the use of a serum bank from patients with pulmonary TB provided by the World Health Organization–Tropical Diseases Research consortium (WHO-TDR) to identify *M. tuberculosis* lipid candidates as targets of antibody response. These samples were used to look for an antibody response to multiple mycobacterial lipids resolved by thin-layer chromatography immunoblot (TLC-I). This approach allowed us to identify *M. tuberculosis* cardiolipin by mass spectrometry and we determined that the IgM antibody response to cardiolipin can be used as a biomarker of infection.

In Chapter 3, we investigate the biological evidence behind the production of anti-phospholipid IgM antibody during TB infection and anti-TB treatment. For this purpose, we used the Cornell mouse model of infection to monitor the change in IgM antibody response against four phospholipids including cardiolipin (CL), phosphatidyl choline (PTC), phosphatidyl ethanolamine (PE) and phosphatidyl inositol (PI) over the course of *M. tuberculosis* infection and treatment. We separated BALB/c mice into three groups

including acute infection (AI), chronic infection (CI), and healthy control (HC). Both AI and CI groups were infected via the aerosol route with *M. tuberculosis* strain H37Rv at day 0. The AI group was treated from 4 to 12 weeks post-infection, while the CI group was treated from 20 to 28 weeks post-infection. We also measured the levels of pro-inflammatory cytokines, IL-5 and MCP-1. We observed that in treated AI mice, anti-phospholipid IgM antibody levels decreased compared to those of healthy mice at all time points. Anti-PTC IgM antibodies remained significantly higher in CI mice than in AI mice at all time points post-infection. The anti-PTC IgM antibody levels in CI mice decreased to levels similar to those of AI and HC mice at 32 weeks post-infection. The anti-phospholipid IgM antibody levels correlated with the bacterial load in the lungs, with treated mice showing fewer *M. tuberculosis* colony-forming units (CFU) after eight weeks of treatment. Furthermore, IL-5 was mainly produced by the site of infection in the lung and decreased with anti-tuberculosis treatment within the CI mice group.

Finally, in Chapter 4, we examine the use of anti-phospholipid IgM antibody changes as a biomarker for treatment response in patients with smear positive pulmonary TB. Serum samples were obtained from pulmonary TB patients at the start and end of the intensive phase of treatment (40 doses of anti-TB combination therapy) enrolled from Kampala, Uganda in a CDC-TB Trials Consortium randomized clinical trial. The samples were screened for IgM antibody levels against five commercially available phospholipids by an in-house ELISA assay. The lipid antigens included CL, PI, PE, PTC, and sphingolipid (SL). IgM antibody levels to CL, PE, PI, PTC and SL significantly decreased following anti-TB drug treatment in patients without lung cavities on their baseline chest radiograph. In contrast, patients with cavitary TB showed an overall increase in the anti-phospholipid IgM antibody response following anti-TB drug treatment, notably with a significant increase in anti-PE antibody levels. Thus, anti-lipid IgM response appears to be a useful biomarker for treatment response, especially in those with non-cavitary disease.

Chapter 5 summarizes the conclusions in support of using the anti-phospholipid IgM antibody response as a useful biomarker for monitoring TB treatment response. This novel biomarker test would greatly facilitate TB management in resource-poor settings. The development of a point of care (POC) test based on anti-phospholipid IgM antibody will be an affordable and highly sensitive alternative to microscopy or culture testing for monitoring treatment response in individuals with TB. Chapter 5 also gives examples of three platforms that might be used for the development of such a POC test. However, we note that it is necessary to explore the specificity of this assay further by testing patients with HIV infection, latent TB infection, and non-TB pulmonary diseases.

Dedication

I am very pleased to dedicate this dissertation thesis to my parents, Amador and Zoraida Goodridge. They have both provided me with a solid education since I was born. This has been key in achieving a PhD degree at one of the top universities in the world.

Also, I dedicate this dissertation to my sister, Mayanin Goodridge, as motivation for her own future career. I am sure that this thesis represents for her proof that academic success is reachable with constant effort and dedication.

Finally, I dedicate this thesis to my girlfriend, Colleen E. Lynch. She has been my key support during the most difficult year of my entire life. With her, I was and still am able to find lots of love and energy that has made me stronger to continue my life's journey.

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Chapter 1

Introduction to tuberculosis biomarker-based tests

I. Definition: Tuberculosis (TB) is an infectious disease caused by members of the *Mycobacterium tuberculosis* complex, which includes *Mycobacterium tuberculosis* (*M. tuberculosis*), *M. caprae, M. microti, M. pinnipedii*, *M. africanum,* and *M. canettii* (the latter two are responsible for a small number of cases in Africa). These three species mainly affect humans while *M. bovis,* which is also part of the *M. tuberculosis* complex group, is involved primarily with cattle infection. Mycobacteria other than tuberculosis (MOTT) can cause pulmonary TB in humans. These are also known as non-tuberculous mycobacteria (NTM) and affect mainly immunocompromised patients as well as the elderly (Taiwo and Glassroth 2010).

Initial exposure to TB occurs by inhalation of aerosolized *M .tuberculosis* bacilli and inhalation is the main route of transmission of pulmonary TB. Initial infection usually goes unnoticed and may be detected by the tuberculin skin test (TST) within 2 to 10 weeks after infection. Early lung lesions commonly heal, leaving no residual changes except for occasional pulmonary lymph node calcification. However, about 10% of those initially infected will eventually develop active disease, half of them during the first 2 years following the infection. The remaining 90% of infected individuals will harbor the bacteria in a latent stage and never develop active disease (Heymann 2004).

Additionally, recurrence of TB can result from a relapse of disease from the same strain that caused the original episode or from infection by another strain, also called "exogenous re-infection" (Cole, Davis et al. 2005). TB recurrence is defined as a second episode of disease after a patient has completed treatment and has been deemed cured of tuberculosis (WHO 2008).

II. Epidemiology of Tuberculosis: TB occurrence is worldwide. The annual incidence varies by world region with the highest burden of the disease found in African and Asian countries. According to the World Health Organization (WHO), nearly 2 billion people—one-third of the world's population—have been infected with the *M. tuberculosis* bacilli. Annually, 8 million people become ill with TB, and nearly 2 million people die from the disease worldwide. In 2004, an estimated 14.6 million people had active disease with 9 million new cases (WHO 2007). In the United States, the incidence is 4.4 per 100,000 inhabitants per year. In the state of California an incidence of 7.5 per 100,000 inhabitants was reported in 2007, including 2,726 new cases, more than any other state in the country. Specifically, the City of San Francisco reported 142 new TB cases during the same period (CDC 2008). Another study in the same city showed a relapse rate of about 1.0% in TB patients and 9.3% in TB-HIV co-infected patients (Nahid, Gonzalez et al. 2007). These low incidence rates are a stark contrast to those from other world regions. For example, Latin America countries, such as Panama, have reported an incidence of 45 per 100,000 inhabitants (WHO 2010) However, the major burden of TB worldwide is in Africa and South Asia, which reach average incidence rates of 340 and 180 per 100,000 inhabitants respectively (WHO 2007).

III. Immune response to *M. tuberculosis* infection: Lung granuloma has been described as the hallmark of pulmonary tuberculosis (Ulrichs and Kaufmann 2006). Granuloma is defined as

a complex, organized immunological structure comprised of differentiated, interdigitated macrophages (also called epithelioid cells) which are surrounded by other immune cells, such as T and B lymphocytes and natural killer (NK) cells (**Figure 1.1**). These cell types are organized in a fashion that forms a cellular wall, which prevents the pathogen from disseminating throughout the host and focuses the immune response to the site of mycobacterial infection. Development of proper granuloma structure is controlled by chemokines and cytokines produced by local tissue cells and infiltrating leukocytes (Ulrichs and Kaufmann 2006). In addition, coordinated activation of cells is crucial for productive granuloma development and thus for long term containment of the pathogen to the site of infection (Ulrichs and Kaufmann 2006).

For years it was believed that tuberculous granulomas were stable cell structures serving to encircle barriers to "wall off material that cannot be destroyed" (Mariano 1995). Recently, increasingly more evidence suggests that granulomas are highly dynamic structures (Cosma, Humbert et al. 2004; Volkman, Clay et al. 2004; Egen, Rothfuchs et al. 2008). Once granulomas are established, these structures maintain themselves by cell turnover, which involves local proliferation of macrophages and continuous immigration of monocytes. Work done by Dannenberg and colleagues in rabbits has shown that the constituent granuloma macrophages have a relatively short life span of several days, and that the maintenance of the granuloma structure depends largely on monocyte migration within the lung (Ando, Dannenberg et al. 1972). Epithelial cells have been shown to live 3 to 4 weeks whereas giant cells survive for only a few days (Mariano, Nikitin et al. 1977) (Papadimitriou, Memmos et al. 1979). These cell turnovers exert pressure not only on intracellular *M. tuberculosis* but also on the host immune system to maintain proper containment of bacteria. As the cells harboring intracellular bacteria die, bacteria are released. The release of bacteria stimulates recruitment of new macrophages and other host immune cells to the site of primary infection in order to reinforce and maintain the granuloma structure. It has been observed that the granuloma structure is in constant motion at the innate and adaptive immunity stages of infection (Volkman, Clay et al. 2004; Clay, Volkman et al. 2008; Egen, Rothfuchs et al. 2008). In order for proper granuloma to be maintained there must be constant crosstalk between the bacterium and the host immune system at the primary site of infection. Successful maintenance of the granuloma leads to containment of the pathogen to the site of primary lesion.

Cavity is a hallmark of advanced stages of TB. In those individuals with advanced TB, the granuloma develops a caseous necrosis center. These caseous necrotic sites enlarge and establish communication with the bronchial tree, thus forming a cavity. The cavities are able to access oxygen, allowing the *M. tuberculosis* bacilli to multiply in high numbers. Quantification of the liquefied material within a single cavity has been shown to have 10^7–10^9 bacilli; this indicates that patients with cavitary TB can readily transmit the disease to others (Dannenberg 2009). Cavitary TB is identified using chest ray, which reveals cavitary lesions. Further cavity classification is made by measuring the cavitiy's diameter and determining whether cavities are present in both lungs. Cavitary TB is classified according to large (>4cm) or small (<4 cm) cavity lesions. Bilateral cavitary TB indicates the presence of cavities in both lungs.

IV. Treatment of Tuberculosis: The identification of acid fast bacilli (AFB) in stained smears from sputum or other bodily fluids together with clinical and epidemiological findings consistent with TB are elements for presumptive diagnosis of active disease and usually justify initiation of anti-TB treatment. For most cases of drug susceptible disease, a 6-month regimen is recommended including isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB) for the first 2 months followed by INH and RIF for 4 months. Currently, most regions administer this regimen following the guidelines under the Direct Observed Treatment Strategy, or DOTS (WHO 2007). The application of this strategy has been shown to be highly effective and reliable in the treatment of most infected patients (Singla, Sarin et al. 2009).

A. Multi-drug resistant tuberculosis: Multi-drug resistant tuberculosis (MDR-TB), defined as TB caused by organisms that are resistant to at least INH and RIF, threatens the progress made in controlling the disease. The emergence of extensively drug-resistant TB (XDR-TB), defined as MDR-TB that is also resistant to all fluoroquinolones and to at least one of three injectable second-line drugs (i.e., amikacin, capreomycin or kanamycin), intensifies this threat (WHO 2008). In general, the drug resistant tuberculosis data are limited. However, since 1994 the WHO and the International Union against Tuberculosis and Lung Disease (The Union) have conducted global drug resistance surveillance in selected sites. In the 2008 WHO/Union Report, the proportion of new cases and/or incidence of resistance to any drug increased in Peru, the Republic of Korea, Lithuania, and Russia (WHO/IUATLD 2008). The highest drug resistance proportions and/or rates occurred in areas where TB control practices have not been rigorously monitored, suggesting that poor TB control practices may be the basis for the high degree of drug resistance observed.

Although treatment for patients with MDR-TB has been available in high-income countries for many years, this is not the case for resource-limited countries. The management of MDR-TB is complex, time-consuming and demanding to both the patient and provider. Thus, it is often recommended that this management be implemented only in hospitals equipped with appropriate isolation units, stringent infection control measures, multidisciplinary medical teams, and access to high quality reference laboratories. In developed countries such as United States, such treatment costs up to $180,000 per case (Moore-Gillon 2001). The management of MDR-TB in resource-limited countries is even more challenging due to limited infrastructure, the need for more trained personal, and lack of proper medications. Currently, international efforts are being directed at the introduction of MDR-TB management programs in low-income countries. The WHO and several partners launched the DOTS-plus treatment model in the late 1990's. In this model, the use of the regular DOTS strategy is complemented with second-line drugs provided at concession prices by the pharmaceutical industry. These drugs are carefully introduced and monitored in the health care system and within the framework of local TB control programs. This new effort falls under the umbrella of the Green Light Committee (GLC). The GLC acts as an expert review and WHO advisory body, which ensures that participating countries are using the standardized regimens

along with appropriate health care infrastructure and the technical and financial resources of the countries involved.

The development and implementation of new tools to monitor treatment response would strengthen the recently introduced DOTS-plus strategy (Grandjean and Moore 2008). Adequately altering treatment in a timely manner will help to limit the spread of MDR-TB and even XDR-TB strains to susceptible populations and therefore decrease their incidence.

V. Current state of TB diagnosis: The most common methods for TB diagnosis are the observation of AFB smears in sputum and *M. tuberculosis* growth in cultures from sputum. Developed nations, including the US, also use culture to diagnose TB (2000). It is widely accepted that *M. tuberculosis* culture has a higher sensitivity and detection rate of pulmonary TB (80%) than smear microscopy (Levy, Feldman et al. 1989). The American Thoracic Society has also indicated that the growth of the organisms is necessary for precise species identification; drug susceptibility testing requires culture of the organisms; and that genotyping of cultured organisms may be useful to identify epidemiological links between patients or to detect laboratory cross-contamination (2000). However, sputum culture and AFB smear have certain disadvantages. Sputum smear has poor sensitivity and specificity. One-half of all new cases of pulmonary tuberculosis are smear negative at initial diagnosis and other pathogens can appear as AFB. During anti-tuberculosis therapy, the clearance of AFB from sputum is often protracted, and staining cannot distinguish viable from nonviable bacilli. Diagnosis by culture is also problematic as the slow growth rate of *M. tuberculosis* requires up to eight weeks in order to detect a culture positive result. (Desjardin, Perkins et al. 1999). During this prolonged period, the culture test can easily be contaminated, further extending the time to report a positive diagnosis.

While the AFB smear is the method recommended by the WHO for low-income settings, in more affluent nations, the tuberculin skin test (TST) is widely used for TB screening (Farhat, Greenaway et al. 2006). The TST is used to diagnose latent TB infection, but has a relatively low specificity because it cross-reacts with Bacillus Calmette-Guérin (BCG) and Mycobacteria other than tuberculosis (MOTT). The BCG strain is used for vaccination in developing countries and MOTT are frequently found in environmental sources such as tap water. This renders the interpretation of a positive TST difficult, especially in regions with high MOTT infection rates and BCG vaccinated populations (Farhat, Greenaway et al. 2006). Additionally, the test requires at least three days for results and its sensitivity is low in certain groups, such as patients with Acquired Immune Deficiency Syndrome (AIDS). Novel assays which measure IFNγ release assays (IGRAs) e.g., QuantiFERON TB Gold (Cellestis, Carnegie, Australia), and T-SPOT TB (Oxford Immunotech, Oxon, UK) are faster (results can be obtained overnight) and are more specific since these IGRAs use *M. tuberculosis* specific antigens (Lalvani 2007). Nevertheless, there are still unanswered questions regarding the usefulness of IGRAs in TB-endemic countries and in high-risk individuals, such as HIV co-infected individuals (Andersen, Doherty et al. 2007).

Other diagnostic approaches suffer from additional drawbacks. Methods such as flow cytometry, ELISA, and ELISpot assays are restricted to the antibodies that are available and the markers chosen are generally selected on the basis of single marker studies. Measuring single molecules is insufficient when studying complex diseases such as TB.

Nucleic acid amplification tests (NAATs) have also been developed for TB diagnosis; and several gene targets within the *M. tuberculosis* genome have been used. The accuracy of commercial and *in-house* NAATs have been analyzed extensively (Pai, Flores et al. 2003; Pai, Flores et al. 2004; Flores, Pai et al. 2005; Ling, Flores et al. 2008). Overall, both commercial and *in-house* NAATs have estimates of accuracy that are highly heterogeneous. In the meta-analysis of 84 studies conducted by Flores *et al.*, results indicated that NAAT sensitivity ranged from 9.4% to 100%, and specificity varied from 5.6% to 100%. A similar meta-analysis by Ling *et al* found similar results to Flores *et al.* suggesting that commercial NAATs alone cannot be recommended to replace conventional tests for diagnosing pulmonary TB. In addition, NAATs positive results only reveal whether or not there is *M. tuberculosis* bacilli in a given sputum sample and do not indicate additional details about the physiopathology status of the infected host. Of course, NAATs are very helpful in diagnosing AFB negative TB patients and extrapulmonary TB. Overall, the use and application of NAATs has a limited and very specific benefit for TB diagnosis and control.

A recently developed *ex vivo M. tuberculosis* gene amplification test (GeneXpert MTB/RIF) can also be used to diagnose drug-resistant TB. GeneXpert MTB/RIF is based on automated sample processing amplification of the *M. tuberculosis rpoB* gene. Clinical studies have shown both 100% sensitivity and specificity using GeneXpert MTB/RIF with AFB smear positive patients (Helb, Jones et al. 2010). The results are available within two hours, which is slightly longer than AFB smear tests, but significantly shorter than culture testing. However, GeneXpert MTB/RIF has some disadvantages. First, it is only intended to identify active pulmonary TB and does not detect latent disease (Walzl, Ronacher et al. 2011). Second, GeneXpert MTB/RIF is an expensive test. The estimated price per test is 60 USD, whereas an AFB smear can cost as little as 2 USD (Scherer, Sperhacke et al. 2009). Diagnosis using solid and liquid sputum culture together costs no more than 20 USD in low-resources settings (Chihota, Grant et al. 2010). In spite of this higher cost, WHO has endorsed the use of GeneXpert MTB/RIF for immediate implementation in high burden settings where highly sensitive and specific tests are lacking.

New diagnostics for tuberculosis are urgently needed to replace or facilitate the use of AFB smear and culture tests in the clinical management of patients with pulmonary TB (Foulds and O'Brien 1998). The global control of TB requires the use of more sensitive tools for rapid detection of active disease, monitoring treatment, and differentiating latent TB infection (LTBI) from active forms of the disease. Improved technologies such as GeneXpert MTB/RIF might be useful for reference laboratories and general hospitals, but may not be practically applied in low-resources settings where funds for TB control programs and technical capacity are limited (Grandjean and Moore 2008).

A. Pitfalls in monitoring TB treatment response: Immediate treatment of patients with pulmonary TB is effective in controlling the spread of disease. Once the TB patient initiates the drug therapy, he or she becomes less contagious to other close contacts. The TB transmission is then blocked and no new individuals are infected. However, the TB treatment is 6 months long and several factors interfere with achieving therapy success. Lack of treatment compliance by the patient and intermittent availability of anti-TB drugs in low resource settings contribute to this difficulty with treatment success. In order to avoid treatment failure, it is strongly recommended to monitor the therapy of patients with pulmonary TB. .

The monitoring of treatment response is based on the evaluation of symptoms, monthly sputum smear microscopy and culture conversion. AFB sputum smear evaluation usually occurs at 1, 2, 5 and 6 months after treatment initiation. The clearance of AFB from sputum has low sensitivity, is often protracted, and staining cannot distinguish viable from nonviable bacilli. The conversion of a culture from positive to negative 1 to 2 months after the start of treatment correlates with the sterilizing activity of the drugs administered and is considered the best predictor of treatment success. However, because of the slow growth rate of *M. tuberculosis,* this measure can be determined only after weeks or months of treatment (Desjardin, Perkins et al. 1999). Treatment monitoring by serial chest radiographs is also not recommended. Radiological abnormalities may persist for months after a good treatment response, often due to permanent scarring. However, performing an end-of-treatment (EOT) chest X-ray in patients with pulmonary TB is recommended in order to establish a new baseline of the patient's anatomy to serve as a reference in case of relapse or re-infection.

The use of AFB smears with low sensitivity and delayed bacterial culture conversions for monitoring treatment have hampered the effort for TB control worldwide. These circumstances make treatment monitoring a challenging task and as a result, health care professionals have to rely mostly on clinical expertise and available epidemiological data to determine treatment effectiveness (Heymann 2004). This situation calls for the development of tests that will allow for efficient clinical management of patients undergoing treatment for pulmonary TB. This dissertation presents the discovery, research and validation of a new biomarker-based test to monitor anti-TB treatment response.

VI. Rationale for using a biomarker-based test: Most TB diagnostic and prognostic tests are based on detection of pathogen signals. In most cases, these types of tests are specific and sensitive. However, the technologies designed to enhance detection of pathogen signals become more expensive and often require ever-more complicated technology. This situation poses a huge challenge when trying to implement these tests in low resource settings. Indeed, the major burden of TB is located in areas where economical resources and science and technology capabilities are sparse. Thus, many of the TB control programs are not able to purchase expensive cutting-edge technology to run their diagnosis

and prognosis activities. In addition, it is likely that the laboratory personnel in those areas do not have training and are not in touch with the current pipeline test.

The alternative to pathogen signal detection is biomarker detection. These tests are often simpler, cheaper and affordable for immediate implementation in high burden settings located in remote areas. In addition, biomarker-based tests are easily transformed into point of care devices, completely portable and with minimal technological requirements. This facilitates the use and application not only for TB control program activities but also for vaccine development, clinical trials and monitoring the treatment response. Indeed, Walzl *et al* recently highlighted the need for host biomarkers to diagnose TB and provide correlates of risk of TB (Walzl, Ronacher et al. 2008; Walzl, Ronacher et al. 2011). We believe that shifting the global effort against TB to the development, implementation and use of biomarker-based tests will have a major impact in TB control worldwide.

A. Biomarker definition: Biological indicators or biomarkers generally include biochemical, molecular, genetic, immunological or physiological signals of events in biological systems (NATIONAL-RESEARCH-COUNCIL 2006). These events are depicted as a continuum between an exposure to a microbial infection and the resulting clinical effect. Biomarkers traditionally have been classified as indicators of exposure or susceptibility to a disease or condition. For the purposes of the present study, we are interested in biomarkers of effect, e.g., biomarkers that measure biochemical, physiologic, behavioral, or other alterations in the host that, depending on magnitude, can be recognized as associated with an established or possible health impairment or disease. Moreover, we are interested in using the antibody response to specific *M. tuberculosis* lipids (effect) as a biomarker of the infection stage in patients undergoing treatment. In this case, the antibodies are a characteristic that can be objectively measured and evaluated as indicators of active disease and therefore meet the current biomarker definition (GROUP 2001; Jacobsen, Mattow et al. 2008).

B. Use and application of biomarkers in TB. As mentioned above, the identification of biomarkers has been the major focus of global efforts against TB in recent years. This search has lead to the identification of a wide variety of biomarkers that can potentially be used in TB diagnosis and treatment monitoring. Most biomarkers are based on the detection of *M. tuberculosis* or its products, as well as the detection of products from the host's immunological response during active disease or latent infection. The exact application will depend on the nature of the biomarker and subsequent validation in clinical studies. To date, few biomarkers have proven effective for assessing all stages of TB and in most cases, biomarkers have a single or dual application. Recently, Walzl *et al* revised the immunological biomarkers available to study TB (Walzl, Ronacher et al. 2011). They emphasized that the identification of biomarkers is highly dependent on our understanding of tuberculosis pathogenesis. Indeed, the marked heterogeneity of the host immune response and bacterial metabolism within the infected host will influence the validation of

biomarkers to study different TB stages. With this in mind, the discovery and identification of a biomarker-based test can be separated into four methods of use: 1) diagnosis of active disease; 2) differentiation of active disease from LTBI; 3) prediction of progression from LTBI to active disease and 4) monitoring treatment response. The correct application of a TB biomarker for any of these uses will require a stepwise identification process as well as strict clinical validation studies.

Biomarker-based tests using a combination of molecular profiles will likely have higher power than a single molecule (Immunodiagnosis Conference 2008; Wazl et al 2008 review). Such increased power is necessary to characterize the disease status of an individual receiving TB treatment. Sufficient characterization of a patient's disease status would allow for correct classification of individual therapy stages. Thus, monitoring biomarkers will properly indicate recovery as well as therapy responsiveness within different patients (Jacobsen, Mattow et al. 2008) facilitating appropriate follow-up for TB patients during drug therapy.

The specific uses of biomarker-based tests will vary depending on the stage of TB infection. The diagnosis of active disease requires biomarkers to rapidly identify contagious individuals. This is a key element that contributes to the efforts for stopping TB transmission. On the other hand, differentiation between active and LTBI assumes that biomarkers are able to reflect the undergoing physiopathology of infection within the lung (Walzl, Ronacher et al. 2011). Because of this, cytokine release assays (CRA) have provided a more sensitive and specific biomarker-based test than the TST. However, the role of CRA in high incidence areas has not been determined yet. Detecting progression from LTBI to active disease requires biomarkers with higher discriminatory power. For example, the widely used interferon gamma release assay has a large intermediate range, where results cannot be determined as positive or negative. These ambiguous results create uncertainty for the clinician and increase the cost of TB control programs by increasing the number of test repeats in order to observe a positive result.

Finally, the response to anti-tuberculosis treatment requires a novel biomarker-based test with three potential applications. First, pretreatment biomarkers with predictive ability could identify the requirements of individual patients for specific treatment regimens. Second, early treatment response biomarkers could confirm the effectiveness of anti-tuberculosis therapy for both clinical management and within clinical trials for new drugs. Third, an end of treatment biomarker for relapse could facilitate the evaluation of shortened drug regimens in clinical trials. These potential applications demonstrate the need for discovery and validation of novel biomarkers to increase the repertoire of tests available for improved TB clinical management.

C. Lipid-antibody biomarker: It has been previously described that a large proportion of the *M. tuberculosis* genome is dedicated to the synthesis and catabolism of lipids and a large proportion of the cell wall consists of complex lipids (Brennan and Nikaido 1995; Senaratne, Sidders et al. 2008; Dunphy, Senaratne et al. 2010). The plasma membrane of *Mycobacterium* contains phospholipids found in other bacteria as

well as numerous lipids unique to *Mycobacterium*, notably, 2-alkyl-3-hydroxy fatty acids made up of up to 90 carbons. Other lipid-containing molecules unique to the *M. tuberculosis* complex are phenolic glycolipids, polyacyltrehalose and lipooligosaccaharides (Brennan and Nikaido 1995). These different mycobacterial lipids are preferentially distinguished by the immune system at several levels. For instance, lipoarabinomanan lipids represent an antigen recognized by Toll-like receptors of the innate immune system. Other lipids are presented by the CD1 molecules to T lymphocytes such as the mycolic acids and glucose monomycolates (Lang and Glatman-Freedman 2006). Because of their unique presence in mycobacteria, these lipids provide a valuable biosignature useful to identify host biomarkers. (Jacobsen, Mattow et al. 2008)

Recently it has been shown that lipid molecules can activate T lymphocytes through their presentation by CD1 molecules (De Libero and Mori 2005; Mendelson, Walters et al. 2005; Lang and Glatman-Freedman 2006; De Libero and Mori 2008). These lipid molecules act as antigens capable of inducing IgM, IgA, and IgG antibody humoral responses. As opposed to peptide antibodies, which are generated by B-2 B cells, the lipid antibodies are mainly produced by B-1 B cells, which represent about 5% of the entire B cell population (**Figure 1.2**). In general, the B-1 B cells are very different from B-2 B cells since the former have very little or no memory and they are mainly located in pleural and peritoneal cavities. B-1 B cells express high levels of IgM and do not require T cell help as they self-renew from pre-existing B1 cells. Interestingly, these cells lack somatic hypermutation and have restricted diversity in the V region (Kindt, Goldsby et al. 2007). B-1 B cells can differentiate into two distinctive lineages including B1a and B1b B cells each with a distinctive phenotype (Tung, Mrazek et al. 2006). The characteristics of these B-1 B cell suggest that the antibodies produced by B-1 B cells contribute to the innate immunity of the host that is stimulated by the active infection status in the host.

We propose that B-1 B cell-related humoral responses might be used as biomarkers of infection because the level of antibody response to *M. tuberculosis* lipids may correlate with bacterial burden during infection. Specifically, we hypothesize that the antibody response to lipids may decrease with successful treatment as the bacterial load decreases in the infected host. Such change, therefore, could serve as a marker for a positive response to treatment. In fact, this method closely emulates the technique for monitoring response to syphilis treatment. Syphilis is a disease for which the etiologic agent cannot be artificially grown in the laboratory, and will be discussed further in the rationale below.

D. Biomarker focus of this dissertation: The use of antibody responses to lipids as a biomarker for monitoring treatment response is an established practice. As mentioned above, the classic example of this approach is syphilis treatment, in which *"natural"* antibodies to lipids are measured to determine the effectiveness of the treatment. The traditional approach to syphilis serodiagnosis has two steps. The first step involves screening with a non-treponemal test such as the rapid plasma regain test (RPR). This

is followed by a confirmatory testing of the reactive specimens using a treponemal test such as the *Treponema palladium* particle agglutination assay (TPPA) (Peeling and Hook 2006). Interestingly, the non-treponemal test -RPR- usually reverts to negative after treatment with penicillin in contrast to the treponemal tests. For that reason, the RPR is extensively used to monitor the effectiveness of the syphilis treatment given the fact that the specific tests (protein-based), such as the TPPA, remain positive indefinitely after the early or late stage of the disease (Lee and Kinghorn 2008). More specifically, the non-treponemal test (RPR) measures the IgM and IgG antibodies to lipoidal material released by damaged host cells, lipoprotein-like material, and possibly cardiolipin released from the treponemes. These antibody titers were shown to decline four-fold by the third month, and continued to decline to an undetectable level after the first year of treatment (Larsen, Steiner et al. 1995).

VII. Closing remarks

Lipid-antibody biomarker-based tests are reasonable alternatives to monitor anti-TB therapy. Until now, the antibody response has only been used for diagnosing TB in humans. Recent evidence shows there is a wide repertoire of antibody responses during active disease. However, the utility of the lipid-antibody response to monitor TB therapy still remains unknown. The dissertation research results presented here delineate the discovery, biological understanding and validation of a lipid-antibody biomarker useful for monitoring treatment response in mammalian hosts. The results given provide the foundation for a new type of test that could contribute to TB control worldwide. The evidence presented here also contributes to our understanding of the lipid-antibody biology, which could allow others to identify new areas where they can be applied.

VIII. Figures

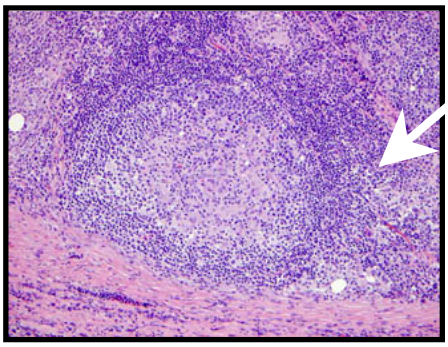


Figure 1.1. A cellular tuberculosis granuloma. An inner core of epithelioid macrophages (larger light pink cells in the center) is surrounded by an outer ring of lymphocytes and neutrophils (smaller dark purple cells). The arrow indicate the area of accumulation of T cells. Hematoxilin and Eosin stain at 20X objective.

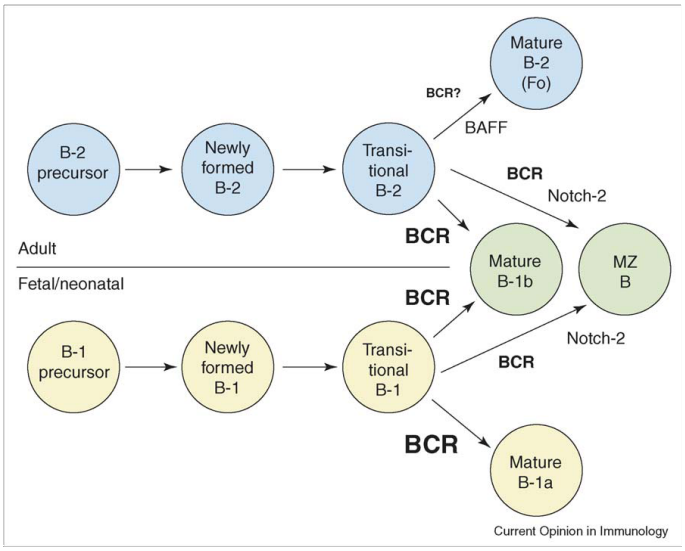


Figure 1.2. Developmental precursors of B-1 and B-2 cell. Note that depending on the signal from B Cell Receptor (BCR), Notch-2 receptor and B cell Activation receptor (BAFF), the precursor can be differentiated into B-1a, B-1b, MZ or B-2 B cells.

CHAPTER 2

Lipid-antibody screening and identification

I. Introduction

The identification of a lipid-antibody biomarker suitable for monitoring TB treatment response involves two key elements from both the pathogen and the host: 1) the screening of lipid candidates from *M. tuberculosis* and 2) the array of humoral responses in the infected host. Both elements are complex and identifying the useful components requires a high throughput screening strategy. For instance, *M. tuberculosis* harbors a wide variety of lipid molecules that can be used as an antigen. These lipids can be found in the cell wall or cytoplasm and may differ according to the growth stage of the bacteria. The host will respond differently to *M. tuberculosis* infection depending on his/her immunological status, nutritional levels, previous exposure, and even ethnic background. Thus, the search for a lipid antibody candidate appropriate for both the pathogen and host immune system can consume valuable time and financial resources. For these reasons, we screened crude *M. tuberculosis* lipids by a new method of thin layer chromatography-immunoblot (TLC-I). This method allowed us to determine which mycobacterial lipids could be used as a basis for a biomarker test to monitor treatment response. This chapter shows the preliminary data obtained using this approach.

Our findings suggest that the antibody response against *M. tuberculosis* lipids varies during different stages of TB infection. First, we used an *in vivo* mouse model based on the Cornell animal model of TB infection, and second, we compared the presence of antibodies in serum samples drawn from humans that were AFB smear positive TB patients, non-TB patients or healthy controls. The non-TB group included patients with pneumonia caused by pathogens other than *M. tuberculosis.* In this way, we were able to identify the lipid antigens to which antibodies can be used as a biomarker of anti-tuberculosis treatment response.

II. Methods

A. ***TLC-I lipid screening in Cornell mouse model****:* BALB/c mice were infected via inhalation of aerosolized *M. tuberculosis* H37Rv reference strain. At 4 weeks post-infection, half of the mice were treated for 8 weeks with antibiotics (INH and PZA) *ad libitum.* Treatment led to a state of infection in the mice where bacilli could not be recovered from any of the organs by culture on 7H9 agar plates. Blood was collected from the mice at day 1 and at week 12 post-infection *(p.i.)* to study the antibody response to multiple *M. tuberculosis* lipids resolved by TLC. All experiments with animals were approved by the ACUC Animal Use Protocol # R228-1211B.

In brief, *M. tuberculosis* H37Rv (ATCC #27294) reference strain was cultured in 7H9 liquid media for 14 days at 37°C without CO_2. Total lipids from packed bacterial cells were extracted for two hours with chloroform:methanol (2:1, v/v). Bacterial debris were separated by centrifugation and the supernatant containing the lipids was stored at

-80°C until use or evaporated under nitrogen gas and dissolved in 300µl chloroform:methanol (C:M) (2:1, v/v) for application in TLC-I.

A 20µl volume of extracted lipids was spotted onto a silica gel 60 plate and separated in a chromatography chamber with solvent systems including C:M:($CaCl_2$)=65:35:7. A set of the separated lipids was revealed by phosphomolybdic acid. Another set of separated lipids was fixed with 0.5% polyisobutylmethacrylate. The subsequent immunoblot was performed by first blocking the TLC plate with 3% bovine serum albumin in phosphate buffer saline (BSA-PBS) and then incubating the plate with a 1:100 dilution of mouse serum in BSA-PBS 3% overnight at 4ºC (Fujita, Doi et al. 2006). The TLC plate was then washed with PBS six times for 10 minutes each time. Immediately after washing, the TLC plate was incubated with a 1:50,000 dilution of rabbit anti-mouse IgG-HRP for 2 hours at room temperature (RT) and washed with PBS six times for 10 minutes each time. Finally the TLC-I was developed with enhanced chemiluminescence (ECL) autoradiography with a substrate from Pierce Thermo Fisher Cat No. 34087 (Rockford, IL, USA).

B. ***TLC-I screening in patients with pulmonary TB and pneumonia*****:** A set of 20 serum samples was obtained from the World Health Organization-Tropical Diseases Research (WHO-TDR) serum bank (Nathanson 2008). This set of samples included 10 patients with pulmonary symptoms suggestive of pneumonia other than TB (non-TB patients) and 10 patients with pulmonary TB diagnosed by AFB smear, culture and chest X-ray (TB patients). A third group included 20 samples from healthy individuals was obtained commercially from Equitech-Bio Inc (Kerrville, TX, USA). These individuals did not have any pulmonary symptoms, cough or fever, and were negative for HIV, hepatitis B and C, syphilis, Chagas disease, and West Nile, per FDA regulations. Additional information about the epidemiological data and risk factors of the patients are included with the data set. All serum samples were received frozen, thawed once on ice, split into small aliquots, and frozen again for further use in the TLC-I. Samples did not experience thaw-freeze cycles as they were maintained in cold chain to ensure sample quality before arrival at the UC Berkeley laboratory (Nathanson 2008) IRB approval was obtained from the committee for protection of human subjects (CPHS) at UC Berkeley 2010-02-791.

C. ***Thin layer chromatography immunoblot (TLC-I):*** Mycobacterial lipids were prepared as described above for the animal model. Extracted lipids were spotted in a silica gel 60 plate and separated using the same conditions described above. TLC plates were then incubated with a 1:1000 dilution of patient serum (*vs.* 1:50 dilution used for mice) in 3% low fatty acid bovine serum albumin (LFA-BSA) overnight at 4º C. The TLC was then washed with PBS six times for 10 minutes each time. The TLC plate was immediately incubated with a 1:50,000 dilution of goat anti-human conjugated IgM-HRP for 2 hours at RT and washed with PBS six times for 10 minutes each time. The TLC-I was developed using enhanced chemiluminescence autoradiography with a substrate from Pierce Thermo Fisher Cat No. 34087 (Rockford, IL, USA).

D. ***Lipid identification by mass spectrometry:*** The lipid antigen within the TLC-I profile of interest was identified by a stepwise wet chemistry procedure. Briefly,

mycobacterial lipid extracts were fractionated by silica column chromatography. Lipid fraction F6 containing lipids that migrated to the same location as our lipid band of interest in the TLC-I (R_f=0.6) were selected for further identification. TLC-I was performed to confirm the IgM antibody response to fraction F6 (**Figure 2.4**). The lipid fraction was then dried under nitrogen gas flow and reconstituted in chloroform:methanol (1:1). Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) measurements were performed by quadruple time-of-flight (Q-tof Premier, Waters, Milford, MA) and LTQ Orbitrap XL (Thermo, Waltham, MA) instruments. Mass spectra were recorded with electrospray ionization in the negative ion mode.

E. ***Lipid-protein overlay assay (LPOA):*** Commercial nylon strips with cell membrane lipids were obtained from Echelon Biosciences Inc Cat. No. P-6002 (Salt Lake City, UT, USA). Each strip membrane had 15 spots with the following synthetic cell membrane lipids: trigliyceride (TG), diacilglycerol (DAG), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL), phosphatidylinositol (PI), phosphatidylinositol phosphate (PI-4-P), phosphatidylinositol diphosphate (PI-4,5-P), phosphatidylinositol triphosphate (PI-3,4,5-P), cholesterol (CHOL), sphingomyelin (SM), 3-sulfogalactosylceramide (SG), and one blank solvent spot **(Figure 2.6).** WHO-TDR and healthy control serum sample sets were analyzed using modifications of the manufacturer's protocol. Briefly, strips were blocked with 3% low fatty acid bovine serum albumin (LFA-BSA) for one hour. Lipid strips were then washed once with phosphate buffer saline (PBS) at RT. The strips were then incubated with a 1:1000 dilution of patient serum in 3% LFA-BSA overnight at 4ºC. Next, the strips were washed with PBS twelve times for five minutes each time at RT and immediately incubated with a 1:20,000 dilution of goat anti-human conjugated IgM-HRP for 2 hours at RT. The nylon strips were then washed with PBS 18 times for 5 minutes. The strip was developed with enhanced chemiluminescence (ECL) autoradiography with a substrate from Pierce Thermo Fisher Cat No. 34087 (Rockford, IL, USA).

E. Statistical Analysis: The presence of IgM in the TLC-I and LPOA assays in TB, non-TB and HC groups were compared by Fisher Exact test using a two way table analysis. Data are presented in percentages. Comparisons were considered significant with $p < 0.05$.

III. Results

A. ***TLC-I lipid screening in Cornell mouse model****:* Differences were observed in antibody response between treated and untreated mice during the course of infection. Antibodies against some mycobacterial lipids were not detectable at day 0 of the infection (**Figure 2.1**). After the eighth week of treatment (12 weeks post-infection) there were differences in the TLC-I patterns shown in treated and untreated mice (**Figure 2.2**). At this time point, the untreated mice showed

signals of a strong antibody response to some of the mycobacterial lipids, whereas treated mice showed decreases in antibody response to the same mycobacterial lipids. These differential results also correlated with the bacterial load in the lungs of infected mice, with the treated mice showing lower numbers of *M. tuberculosis* colony-forming units (CFU) than untreated mice at the same time point.

In order to determine if the differential response observed by the TLC-I method in the mouse experiment is appropriate for monitoring the response to treatment in a human host, we used human serum to examine the lipid antibody response in 1) those with active TB disease before treatment, 2) those with pulmonary disease other than TB, and 3) healthy individuals.

B. ***TLC-I screening in patients with pulmonary TB and pneumonia*:** Our preliminary results showed differences in antibody response to polar lipid fractions between TB patients and non-TB patients. IgM antibodies against some mycobacterial lipids were not detectable in non-TB patients. In contrast, the TLC-I showed a different banding pattern in the serum of TB patients, with a strong IgM antibody response to several mycobacterial polar lipids. The banding pattern indicated that an IgM antibody response to polar lipids occurred significantly more frequently in the TB patient serum (100%) versus the non-TB patient serum (60%) (p=0.043 Fisher Exact test) (**Figure 2.3**). These results suggest that the detection of anti-mycobacterial polar lipid IgM antibodies by TLC-I may serve as a potentially useful biomarker test for monitoring TB treatment response. The application of these IgM antibodies for diagnosis purposes might be beneficial.

C. ***Lipid identification by mass spectrometry:*** The mass spectra exhibited a doubly charged negative ion at m/z = 701 and a singly charged negative ion at m/z = 1404 (**Figure 2.5**). The accurate mass measurements of these ions, in combination with MS/MS measurements, identified this lipid as cardiolipin ($C_{77}H_{146}P_2O_{17}$). These results suggest that this lipid was the main target of IgM antibody response in the studied fraction.

D. ***Lipid-protein overlay assay (LPOA) screening:*** Since cardiolipin is a member of the phospholipid family, we hypothesized that other phospholipids would elicit a similar IgM antibody response in TB patients. We looked for commercial sources of phospholipids that are known to be present in the cell wall of *M. tuberculosis* and used them to test for the presence of these antibodies during active disease.

TB patients have higher levels of IgM antibodies against bovine cardiolipin. Among the cell membrane lipids in the LPOA strip, IgM antibodies against cardiolipin were found in 89% of TB patients from the WHO-TDR sample set (**Table 2.1, Figure 2.7).** This was significantly higher than the percentage of

healthy controls with the IgM antibodies (17%), but not significantly different from non-TB pneumonia patients (78%). IgM antibodies against cholesterol and sphingomyelin were also found in significantly higher proportions of TB patients compared to healthy individuals. The 12 remaining synthetic cell membrane lipids showed no significant differences when comparing all three groups. There were non-significant differences between the AFB positive patients and the non-TB pneumonia group for the cell wall lipids including TG, DAG, PA, PS, PE, PC, PG, PI, PI-4-P, PI-4,5-P and SG. No additional trend was observed within these non-significant lipids for the AFB group *vs.* the non-TB group.

IV. Discussion

The above experiments show differential antibody response against *M. tuberculosis* lipids in serum from infected and uninfected hosts. Both murine and human experiments showed that there is an increased lipid-antibody response in these hosts during TB infection. Specifically, mice that were infected with *M. tuberculosis* showed higher antibody levels compared to mice that received anti-tuberculosis treatment. Similarly, human hosts infected with TB also showed higher antibody signals in the TLC-I screening test compared to hosts with non-TB pneumonia, indicating IgM type antibodies as a potential biomarker for untreated TB infection. TLC-I analysis showed no consistent pattern within the IgG and total Ig antibodies from the human serum samples.

Unlike humans, mice have only one (CD1d) of the 5 known subtypes of CD1 molecules (CD1a-e). Therefore, mice may not be as efficient as humans in presenting lipid antigens. Thus, the above results are only a starting point from which to identify an appropriate lipid-antibody biomarker useful for monitoring treatment in humans.

The IgG antibody response for lipid antigens has been described as very diverse, which could relate to the poor utility of IgG as a marker for diagnosing TB (Steingart, Dendukuri et al. 2008). Perhaps IgG antibody produced during TB is secreted not only by B-2 B cells, but also by marginal zone (MZ) cells, which have both B-1 and B-2 B cells properties. This situation would alter any antibody response associated with TB lipids. In addition, the portion of IgG detected by the TLC-I may be produced by memory B cells and not related to the infection status in the lung of TB patients. Unlike IgG antibodies, IgM antibodies, especially those generated by B-1 B cells, showed a consistent pattern with the TLC-I screening. This allowed us to pursue and identify which of the lipids associated with these patterns could be tested as a biomarker to monitor the TB treatment response.

Cardiolipin (CL) from *M. tuberculosis* was identified as the major lipid antigen from our antibody biomarker test using mass spectrometry from a TLC eluate and a column chromatography fraction. Our experiments showed that the *M. tuberculosis* CL antigen is capable of distinguishing patients with TB from those with non-TB pneumonia. These findings allowed us to rapidly assess commercial sources of this and other lipids with similar structures for further evaluation as a biomarker test to monitor TB treatment response. The next chapter will describe the biological basis of this anti-lipid response, focusing on what is known about the role of B-1 B cell activation and secretion of IgM antibodies during TB and anti-TB therapy in animal models.

Analysis of the human serum samples using the LPOA showed that TB patients have significantly higher IgM antibody levels to cell membrane lipids, including cardiolipin, cholesterol, and sphingomyelin, than healthy individuals (Table 2.1). High levels of anti-cardiolipin IgM antibody were also present in 78% of non-TB pneumonia patients. However, there were no significant differences in these antibody levels between non-TB patients and the TB patients within the WHO-TDR sample set. The different phospholipid antigens in the nylon strip membrane might explain these results. While the TLC-I uses total lipids extracted from *M. tuberculosis*, the LPOA uses a set of synthetic lipids produced from theoretical structures, making the commercial LPOA incomparable with our TLC-I screening tool. However, the former test provides evidence that support our initial TLC-I findings in the human and animal studies. Indeed, the LPOA results correlate with the previous identification of CL in eluate of TLC-I by mass spectrometry, indicating that TB patients have higher levels of IgM antibody against CL originating from the *M. tuberculosis* cell membrane.

The CL structure from bacteria and bovine are similar. Previous studies have shown that CL has four fatty acid chains and two polar heads with the phosphate group linked by an acyl chain (Jensen, Tomer et al. 1987; Gutberlet, Dietrich et al. 2000). The only difference between bacterial and bovine CL is the number of double bonds within the fatty acid chain. CL from *E. coli* has only two double bonds, whereas bovine CL has two double bonds per fatty acid chain, for a total of eight double bonds. The structure of *M. tuberculosis* CL has not been described yet. Our study describes the mass spectrum suggestive of CL molecules found in the eluate of the TLC of *M. tuberculosis* H37Rv lipids. The exact structure and double bond locations still remain unknown and require additional characterization by nuclear magnetic resonance (NMR) strategies. However, it is not understood if variations in the number of double bonds accounts for the antibody affinity difference. Reis *et al* demonstrated that fatty acid chains with double bonds are highly susceptible to oxidation (Reis, Domingues et al. 2004). Other groups have shown that natural antibodies have a higher affinity to oxidized cardiolipin and oxidized lipid transporter molecules such as LDL (Tuominen, Miller et al. 2006; Gounopoulos, Merki et al. 2007; Chou, Hartvigsen et al. 2008; Chou, Fogelstrand et al. 2009; Binder 2010). Taken together, this evidence suggests that bovine CL is a good surrogate lipid to use as an antigen to detect IgM antibodies in TB infected humans. If the IgM antibody levels show changes over the course of infection, this lipid antibody biomarker will be a suitable tool to monitor the treatment response during anti-TB drug therapy. *M. tuberculosis* CL is not commercially available and difficult to purify. Thus, we proposed to use bovine CL and other similar phospholipids to determine the IgM antibody levels during TB treatment in human and animal hosts. In Chapter 3 we will discuss anti-phospholipid IgM antibody biomarkers and the biology behind the secretion of these anti-phospholipid IgM antibodies in infected mice.

Together these results indicate that cardiolipin, either from *M. tuberculosis* or from commercially available sources, are suitable biomarker targets for TB and can potentially be used to monitor TB treatment response. Since the techniques used to identify CL as a biomarker are based on the intensity of chemioluminiscence, we were not able to quantify the exact amount of antibodies involved in host response. A quantitative method such as ELISA should be used to determine relative quantitative differences in the level of antibodies present

in these patients during treatment. Chapter 3 and 4 will describe findings for the quantification of anti-phospholipid IgM antibody during treatment of acutely and chronically infected mice, as well as during the intensive phase of treatment in humans.

V. Figures

Fig. 2.1a

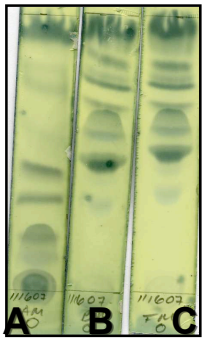


Fig. 2.2a

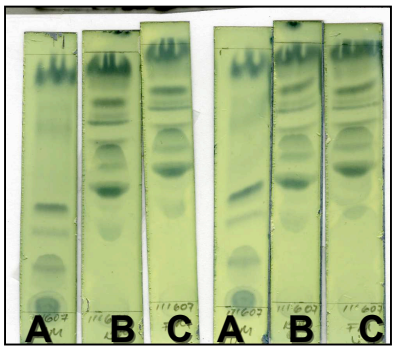


Fig. 2.1b

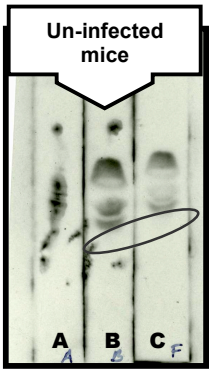


Fig. 2.2b

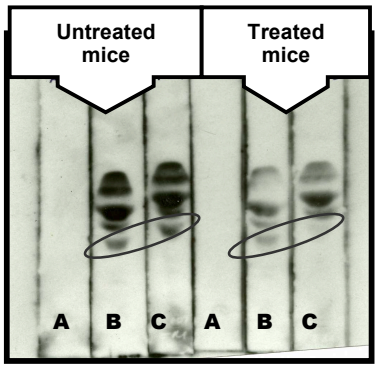


Figure 2.1a: TLC stained with phosphomolybdic acid before the TLC-I. **Figure 2.1b:** TLC-I of mouse serum at day 0 of infection (uninfected mice) using different solvent systems: **A=** C:M:W =100:14:0.8, **B=** C:M:W = 60:30:6, **C=** C:M:$CaCl_2$ = 65:35:7.

Figure 2.2a: TLC stained with phosphomolybdic acid before the TLC-I. **Figure 2.2b:** TLC-I of mouse serum at 12 week after infection from treated (**T**) and untreated (**UT**) animals using different solvent system: **A**= C:M:W =100:14:0.8, **B**= C:M:W = 60:30:6, **C**= C:M:$CaCl_2$ = 65:35:7.

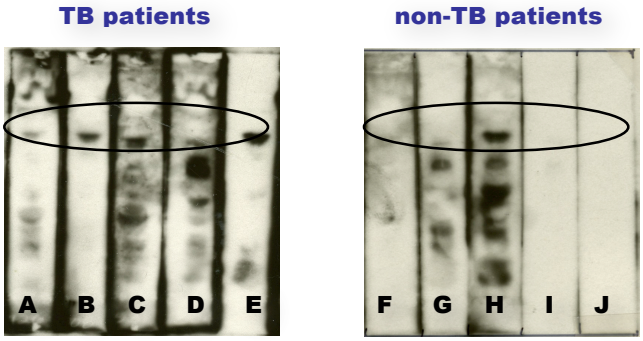


Figure 2.3: TLC-I with human samples: Mycobacterial lipids were spotted onto silica gel plates and separated by C:M:$CaCl_2$ = 65:35:7 as a solvent system. The TLC plate was incubated with serum samples from **TB-patients** (**left panel**) and non-TB patients (**right panel**), washed, and followed by a second incubation with goat anti-human IgM-HRP. Lanes A through E each show an individual TB patient and lanes F through G each show an individual non-TB patient. These reactions were developed by chemiluminescence autoradiography.

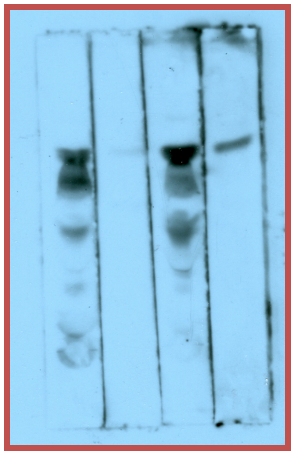


Figure 2.4: Confirmatory TLC-I: Whole lipid extract of *Mycobacterium tuberculosis* (**lanes 1 and 3**) and lipid fraction F6 (**lanes 2 and 4**) and were spotted and onto silica gel plates and separated by C: M:$CaCl_2$ = 65:35:7 as a solvent system. The TLC plates was incubated with serum samples from healthy individuals (**lanes 1 and 2**) and TB-patients (**lanes 3 and 4**), washed, and followed by a second incubation with goat anti-human IgM-HRP. The reactions were developed by chemiluminescence autoradiography.

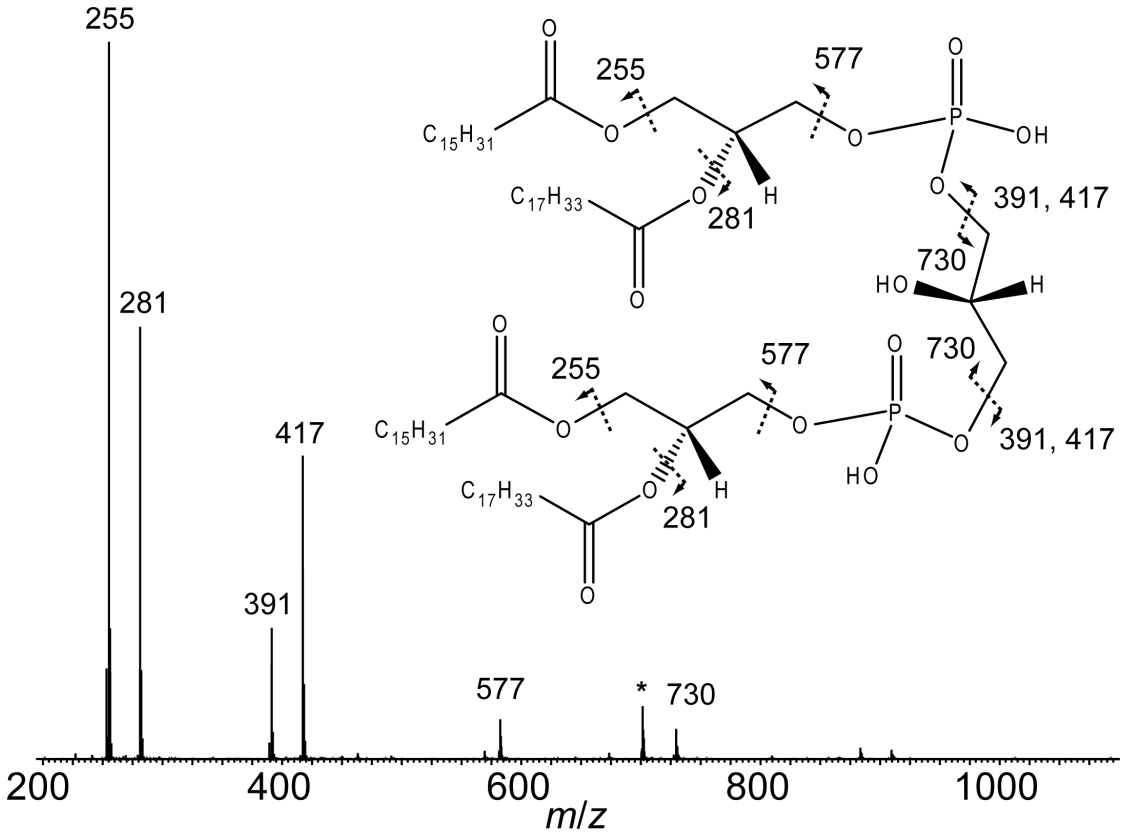


Figure 2.5: Tandem mass spectrum of lipid biomarker candidate in fraction F6: *M. tuberculosis* lipid fraction F6 was dried and resuspended in C:M (1:1) and analyzed by tandem mass spectrometry in negative ion mode. Graph shows tandem mass spectrum resulting from collision-induced dissociation of a doubly charged negative ion at mass-to-charge ratio (*m*/*z*) 701.5. The identified CL has two fully saturated C16 fatty acid chains, and two C18 fatty acid chains each containing one unit of unsaturation. The singly charged fragment ions at *m*/*z* 391 and 417 are secondary fragmentation products, namely, resulting from cleavage at phosphate and loss of C16 and C18 fatty acid chains, respectively. Residual precursor ion (*m*/*z* 701.5) is denoted by the asterisk.

Table 2.1: Presence of IgM antibodies against cell wall lipids in TB, non-TB and HC.

Self Lipid	AFB(+) N=9 %	Non-TB N=9 %	HC N=18 %	AFB(+) *vs* non-TB *P value*	AFB(+) *vs* HC *P value*
TG	0	0	6	*n.s.*	*n.s.*
DAG	33	0	0	*n.s.*	*n.s.*
PA	22	33	67	*n.s.*	*n.s.*
PS	56	56	100	*n.s.*	*n.s.*
PE	11	44	0	*n.s.*	*n.s.*
PC	11	11	0	*n.s.*	*n.s.*
PG	100	100	89	*n.s.*	*n.s.*
CL	89	78	17	*n.s.*	***0.0001***
PI	100	89	83	*n.s.*	*n.s.*
PIP	89	89	61	*n.s.*	*n.s.*
PIP2	0	11	0	*n.s.*	*n.s.*
PIP3	11	22	0	*n.s.*	*n.s.*
CHOL	44	22	0	*n.s.*	***0.007***
SM	33	22	0	*n.s.*	***0.028***
SGC	67	56	28	*n.s.*	*n.s.*

***n.s.** = not significant; **AFB(+)** = patient with pulmonary TB diagnosed by a positive AFB smear; **non-TB** = patients with pulmonary disease other than TB (pneumonia); **HC:** healthy controls; cell wall lipid abbreviations described in Methods section.*

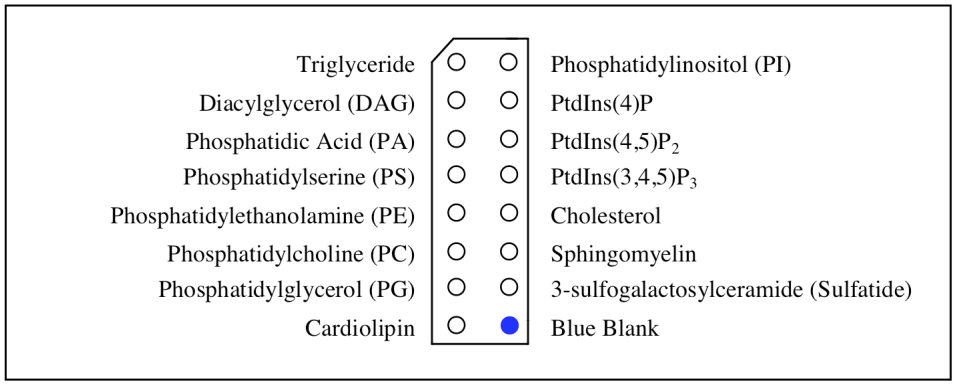
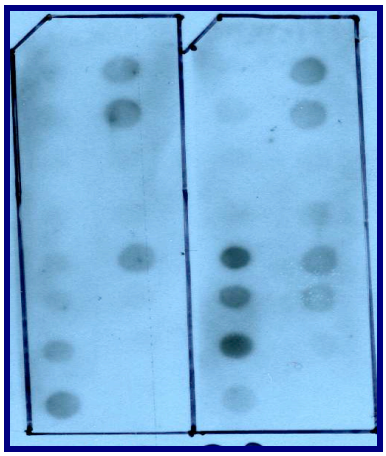


Figure 2.6: Lipid locations in the lipid-protein overlay assay (LPOA) strip: Image shows the position of 15 different biologically active lipids prespotted on the hydrophobic strip membrane. Every spot contained 100 pmol of the annotated cell membrane lipid. The LOPA strips were stored at 2 to 8 °C and used once. The LPOA strips have a diagonal cut on their top left corner and are spotted with Xylene Cyanol FF (blue) at the bottom right blank corner to assist with orientation of the strip. Ponceau S staining (pink) was added to the lipid spots.



A **B**

Figure 2.7: Lipid-protein overlay assay: Image shows the results of autoradiography of IgM antibodies against cell wall lipids in TB and non-TB using the LPOA strip. Spotted lipids are shown in Figure 2.6. Panel **A** shows a non-TB patient; panel **B** shows a TB patient. Circle spots represent chemilumiscence signals for the presence of IgM antibody after a 1 min exposure of the film.

CHAPTER 3

Biological basis for anti-phospholipid IgM antibody production during TB infection and treatment in mice

I. Introduction

Anti-phospholipid IgM antibodies have a non-traditional B cell origin. It is widely accepted that two types of B cells contribute to the production of antibodies against lipid antigens: B-1 and B-2 B cells. Both are able to make antibodies against lipids, however they each do this in a different way. B-2 B cells require migration into the secondary lymph organ (SLO) and T helper cells for activation; whereas B-1 B cells are ready and able to secrete antibodies within the infection site (Hardy 2006; Montecino-Rodriguez and Dorshkind 2006). B-1 cells can be subdivided further into two lineages known as B-1a and B-1b (Hardy 2006). As discussed in Chapter 1, B-1 cells have very little or no memory and they are mainly located in pleural and peritoneal cavities, whereas B-2 cells primarily circulate in peripheral blood (**Figure 1.1**, Chapter 1). B-1 cells express high levels of IgM antibodies and do not require T cell helpers, since they self-renew from pre-existing B-1 cells (Deenen and Kroese 1993). Additionally, B-1 cells have a shorter half-life than B-2 cells with B-1a cells having a 50% renewal time of 38 days, while the B-2 cell renewal time is 45 days (Deenen and Kroese 1993). B-1 cells also lack somatic hypermutation and thus have restricted diversity in the variable region of antibody heavy and light chains (Hardy 2006). These characteristics suggest that natural antibodies produced by B-1 cells contribute to the innate immunity of the host (Baumgarth, Tung et al. 2005; Racine, Chatterjee et al. 2008). These natural antibodies are mainly of the IgM type and are polyreactive with an estimated half-life of about 8 hours (Sigounas, Kolaitis et al. 1994). Despite this knowledge, the exact pathways of B-1 B cell activation and development during TB infection are not well understood and are still debated.

B-1 cells respond rapidly and strongly to host-derived innate signals, such as interleukin 5 (IL-5), and to pathogen signals, which mainly consist of lipids, such as lipopolysacharides and phosphorylcholine (Takatsu, Kouro et al. 2009). IL-5 is produced from the T cells that are recruited during TB infection and granuloma formation. It is not clear if the lung alveolar epithelial cells also produce and sustain levels of IL-5. However, the high ratio of epithelial cells to lymphocytes in the lung supports the idea that epithelial cells are the primary IL-5 producing cells during infection. The secretion of IL-5 may be favorable for *M. tuberculosis* because it skews the host response towards Th2-type antibody mediated immunity, which is not known to have a protective role during TB infection. Interestingly, in cells collected from the air spaces within the lungs of TB patients by a bronchoalveolar lavage (BAL) procedure, there was a higher frequency of purified protein derivative (PPD)-specific IL-5 secreting cells than in their peripheral blood monocyte cells (PBMC) (Morosini, Meloni et al. 2005). It is still unclear whether epithelial cells or lymphocytes in the BAL are the main cell repertoire responsible for the secretion of IL-5. In addition, the levels of IL-5 expressed *in situ* gradually increased with the progression of TB compared to the levels of IL-5 from circulating cells. Thus, disease severity may influence the levels of IL-5 in the lung (Somoskovi, Zissel et al. 1999). IL-5 is not detectable in pleural fluids surrounding the lungs of TB patients, possibly because pleural fluid cells and circulating blood take up this cytokine. This evidence supports the conceptual model that during active disease, IL-5 is released in the lung and, together with lipids derived from *M. tuberculosis*, provides the signals required to stimulate and sustain the proliferation and survival of secreting B-1 cells and therefore, the innate B cell response at the site of infection (Barnes, Lu et al. 1993; Takatsu, Kouro et al. 2009).

B-1 B cells migrate to the lung of the infected TB host. Recent evidence from Russo *et al* in 2010 showed that in mice infected with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), B-1 cells are present within the pulmonary lesions (Russo and Mariano 2010). They also showed that B-1 B cells migrate from the peritoneal cavity to the infected lung, modulate the histological pattern of the inflammation, influence the influx of other cells to the infected lung, and favor resistance to the mycobacteria (Russo and Mariano 2010). In addition, Barberio showed that B-1 B cells *in vitro* secrete IL-10 in response to lipopolysaccharide (LPS) (Barbeiro, Barbeiro et al. 2011). In support of this evidence, studies by Chan *et al* have provided histological sections of lungs from infected mice that reveal the presence of B cells and their progressive increase in number during the course of TB infection in mice (Tsai, Chakravarty et al. 2006; Maglione, Xu et al. 2007; Maglione and Chan 2009). We have yet to determine the specific role that B-1 B cells have in the formation of granuloma and lymphoid follicles within the lungs of the infected host. Nevertheless, when considered together, these observations imply that during active disease, the activation of B-1 B cells occurs inside the lung and depends on IL-5 levels and pathogen associated molecular patterns (PAMPs), most likely lipopolysaccharide (LPS) and phospholipids. Others have described the accumulation of tuberculin-like lipid molecules that occurs inside the cavity and caseous granulomas (Shima, Dannenberg et al. 1972; Converse, Dannenberg et al. 1996; Cosma, Humbert et al. 2004; Helke, Mankowski et al. 2006; Swaim, Connolly et al. 2006). It is possible that phospholipids originate not only from replicating bacilli but from dead host cells within the granulomatous caseous lesions.

B cells regulate the recruitment of macrophages to the *M. tuberculosis* infection site within the lung. As discussed in Chapter 1, the granuloma formation by macrophages and lymphocytes is the hallmark immune response for infection control and containment within the lung. A recent report showed that levels of IL-10 are higher in co-cultures of B cells with macrophages *in vitro.* Chan *et al* has also demonstrated that the total B cell response within the lungs of infected mice might inhibit cell mediated immunity and favor disease progression (Maglione, Xu et al. 2007; Maglione and Chan 2009). Previous results from our laboratory using a novel adjunctive therapeutic vaccine to treat TB in mice, showed a significantly lower macrophage:lymphocyte ratio in vaccinated versus unvaccinated mice (Miyata 2011). Thus, we hypothesize that TB healing or successful treatment will be reflected by a decrease in anti-phospholipid IgM in the serum of treated mice.

We propose that the decrease in the bacterial burden in the lung during TB treatment will be reflected by a reduction in lipid antigens, and therefore a reduction in the responsive signals derived from the stimulation and development of B-1 cells. The natural IgM antibody levels should decrease in proportion to the decrease in pathogen lipids as well as the degree of lesion healing upon successful treatment (Romanowski, Sutherland et al. 1991). We anticipate a distinct antibody level change according to acute or chronic infection status in our mouse infection model.

In Chapter 2, we showed biological evidence supporting the hypothesis that antibody responses due to B-1 cells may be used as biomarkers of infection, since the level of antibody response to *M. tuberculosis* lipids may correlate with bacterial burden during active disease. Therefore, measurable changes in the level of antibody response could serve as a marker for

successful response to treatment. To test this hypothesis, we studied changes in levels of anti-phospholipid IgM antibody during TB infection and treatment in mice during acute and chronic infection. We also investigated the levels of IL-5 and other pro- and anti-inflammatory cytokines in the infection site and in the secondary lymphoid organ.

II. Methods

A. ***Cornell model of mouse infection:*** Eight-week-old female BALB/c mice (Jackson Laboratories) were maintained and studied at UC Berkeley's Biosafety Level 3 (BSL3) Northwest animal facility. We infected the mice with *M. tuberculosis* H37Rv (American Type Culture Collection strain 25618) via aerosol route with the inhalation exposure system (IES, Glas-co, Terre Haute, Ind.) using a protocol previously adapted in our laboratory (Cheigh, Senaratne et al. 2010; Marjanovic, Miyata et al. 2010). Two batches of 30 mice each were placed in the IES and exposed to a single *M. tuberculosis* bacilli solution for 60 min intervals. The right lung was obtained from mice grouped in the day 1 post-infection time-point to assess the initial bacterial inoculum. Then, additional right lung samples were obtained at 4, 12, 20, and 28 weeks post infection. To assess the bacterial burden, we homogenized right lungs in PBS-Tween (0.05%), and plated the homogenate onto Mildebrook 7H11 agar plates containing oleic acid-albumin-dextrose-catalase supplement (OADC) (Becton Dickinson), 0.5% glycerol, and cyclohexamide (l00 ug/ml Sigma Aldrich). Plates were read and colony forming units (CFU's) per lung were enumerated 21 days later.

B. ***Antibiotic treatment in mice:*** At 4 weeks after infection, mice were given isoniazid (100 μg/mL) and pyrazinamide (15 mg/mL) in drinking water *ad libitum* for 8 weeks. These mice were considered to be acutely infected (AI). Mice designated as chronically infected (CI) received the same treatment, but this was started at week 20 post-infection (*p.i.*). Mice were followed in order to evaluate clinical symptoms, lung CFU counts, and organ cytokine levels. Healthy control (HC) mice were not infected and did not receive any treatment. Three mice from each group were used for organ collection at each time-point.

C. ***Determination of cytokines and anti-phospholipid IgM antibodies*****:** Whole blood, left lung and spleen were collected at the time of sacrifice. Blood was allowed to coagulate for 30 min and was centrifuged at 13,000 rpm for 15 min. Serum was separated and sterilized by filtration by a 0.2 μm disc filter (Difco, CA, USA) and stored at -80°C. Left lung and spleen were homogenized in PBS-Tween (0.05%) and centrifuged at 13,000 rpm for 15 min. The supernatant was filter sterilized as described above and then stored at -80°C. Standard procedures for cleaning, disinfection and transport of samples to the BSL2 laboratory were followed. For comparison and to assess specificity, we used a *Salmonella* mouse infection model to compare the anti-cardiolipin IgM levels. Briefly, BALB/c mice were infected with 1 x 10^E CFU of *Salmonella* per mouse intragastrically. Infected mice were anaesthetized and blood was collected via cardiac puncture at day 2, 5, 7, 14 and 28

p.i. Collected blood was mixed with heparin to 50 units/ml and centrifuged at 8,000 rpm for 5 minutes. As healthy controls, we used 70-day old uninfected BALB/c mice. Serum was collected and stored at -70 C until use.

In the BSL2 laboratory, mouse serum from TB and *Samonella* infected mice was used to assay for anti-phospholipid IgM antibody by an in-house ELISA, which will be described in greater detail in Chapter 4. IL-5, MCP-1, IL-10, TNFα, and IFNγ concentrations from serum, lung, and spleen supernatants were determined with commercial kits from eBioscience (Cat No. 88-7054-22, 88-7054-22, 88-7104, 88-7324-22, 7314, San Diego, CA, USA). The limits of detection (LOD) for these kits were 4, 15, 30, 8 and 15 pg/ml for IL-5, MCP-1, IL-10, TNFα, and IFNγ, respectively. Samples were analyzed according to the manufacturer's protocol. Standard curves were prepared following the manufacturer's instructions and cytokine concentrations are given in pg/ml.

D. Statistical Analysis: The mean CFU counts of *M. tuberculosis* recovered from the organs of each mouse group as well the anti-phospholipid antibody levels and cytokine levels were compared with the Student's *t*-test. Data are presented as line and bar graphs that indicate the mean and standard deviation for each group of 3 mice. All assays were performed in triplicate. Differences between the mean CFU counts, antibody and cytokine levels obtained from each group were considered significant at $p < 0.05$.

III. Results

A. ***Bacterial load in CI and AI mice decreases with treatment:*** The *M. tuberculosis*-exposed mice in our study followed two different timelines of infection and anti-TB treatment. The initial inoculum enumeration at 24 hours *p.i.* for both sets of aerosol infected mice reached an average of 73 and 101 CFU per lung, respectively. Statistical analysis of these two inoculum levels revealed no differences (p= 0.6460, data not shown) between the two groups. Thus, the average initial inoculum for our study sample was estimated at 87 CFUs. We randomized all of the infected mice into two groups across our study; AI and CI. At week 4, both AI and CI groups reached a total average of 10^5 to 10^6 CFUs per lung. This is consistent with the number of bacteria previously found by our laboratory using this model of mouse infection (Cheigh, Senaratne et al. 2010; Marjanovic, Miyata et al. 2010).

At week 12 *p.i.,* after eight weeks of treatment with INH and PZA, the AI mice group showed no detectable *M. tuberculosis* bacilli by culture. This group continued to have no detectable bacilli at weeks 20 and 28 *p.i.* In contrast, CI mice had 10^5 CFU per lung at week 20 *p.i.* The CFUs decreased to non-detectable levels after eight weeks of treatment at week *28 p.i.* **(Figure 3.1).**

B. ***IL-5 levels are higher in lung than serum and spleen:*** There were higher levels of IL-5 in the lung than in the spleen of CI and AI mice at week 20 *p.i.* The lungs of CI

and AI mice achieved average IL-5 levels of 1242 and 873 pg/ml, whereas the spleen of these mice reached an average of 136 and 328 pg/ml, respectively. No detectable levels of IL-5 were found in the serum of AI, CI or HC mice groups. At week 12 *p.i.,* there was no change in levels of IL-5 in the lungs of the AI and CI mice. At week 28 *p.i.,* the levels of IL-5 in CI mice after anti-TB treatment showed a decrease to levels similar to those of the HC and AI groups (**Figure 3.2**).

C. ***IFNγ and IL-10 are lower in spleen of AI mice after treatment*:** Spleen homogenate supernatant showed significantly lower levels of IFNγ at week 12 *p.i.* (week eight of treatment) in AI mice compared to the CI mice which had not received treatment at that time (p=0.002). The spleen IFNγ levels in AI mice were similar to healthy controls. In contrast, the spleen of the AI treated group did not show significantly lower levels of IL-10 than CI group. These lower levels were also comparable to those of the healthy control mice. TNFα was almost undetectable in AI and HC mice, whereas CI mice showed higher levels of this cytokine **(Figure 3.3).** There were no significant differences in the levels of TNFα, IFNγ and IL-10 in the lungs of AI and CI mouse groups at week 12 *p.i.* **(Figure 3.4).**

D. ***Levels of anti-phospholipid IgM antibody in AI mice decrease after treatment.*** Levels of anti-cardiolipin IgM antibodies showed a significant decrease at week 12 after the treatment of AI groups. There were no significant differences in the levels of anti-phospholipid antibodies detected at day 1 and week 4 post-infection in the AI and CI groups. AI mice showed anti-phospholipid IgM antibody levels similar to those of healthy mice at all time points. The IgM antibody response against CL, PTC, PI and SL was significantly higher in CI mice at 12 weeks *p.i.* than in AI mice. There was no significant difference in anti-PE IgM antibody levels (p=0.0635) between CI and AI mice after treatment **(Figure 3.5a to 3.5e).**

Anti-PTC IgM antibodies remained significantly higher in CI mice than in AI mice at 12, 20 and 28 weeks *p.i.* The anti-PTC IgM antibody levels in CI mice decreased to levels similar to those of AI and HC mice at 32 weeks *p.i.* (data not shown). This indicates that the sensitivity of the IgM response to PTC is greater, since the anti-PTC levels changed to normal levels in response to successful anti-TB treatment in chronically infected mice. The IgM levels correlated with the bacterial load in the mouse lung, with treated mice showing no *M. tuberculosis* CFU after eight weeks of treatment.

a. In the *Salmonella* mouse model of infection, the levels of anti-cardiolipin IgM at days 2, 5, 7, 14 and 21 days *p.i.* were lower than the levels of anti-cardiolipin IgM antibody in AI and CI mice at week 4 *p.i.* in the Cornell mouse model (**Figure 3.7**). The *Salmonella* infected mice at day 28 *p.i.* show levels of anti-cardiolipin IgM antibody similar to those of AI and CI mice at week 4 *p.i.* The 10 week-old healthy control mice from the *Salmonella* mouse model (non-infected mice) showed lower levels of anti-cardiolipin IgM antibodies than the 12 week-old healthy control mice from the Cornell mouse

model (see **Figure 3.5a** and **Figure 3.7**)

E. ***MCP-1 decreases in lungs of AI mice after treatment.*** At week 12 *p.i.* lung levels of MCP-1 were significantly lower in AI mice after treatment when compared with CI mice (1028 *vs* 1567 pg/ml, p=0.011). Spleen MCP-1 levels of AI, CI, and HC mice were significantly lower than the levels found in the lung. There were no detectable levels of MCP-1 in serum from all three groups **(Figure 3.6).**

III. Discussion

This study aimed to produce preliminary data and to provide initial insight into the biology of B-1 B cell activation and the production of anti-phospholipid IgM antibodies using the Cornell model of animal infection. These findings need to be complemented with further immunohistological studies looking at B-1 B cell surface markers to understand their distribution within the lung and their role in granuloma formation and resolution. Our laboratory is currently establishing and adapting protocols to accomplish this. In addition, *ex-vivo* experiments using primary culture of B-1 B cells isolated from the lungs of TB infected mice will provide detailed information about cellular interactions *in-vivo.*

In this dissertation research, BALB/c mice were infected with *M. tuberculosis* by aerosol route and then treated with anti-tuberculosis drugs. In contrast to the murine experiments presented in Chapter 2, we treated mice during acute infection (week 4 *p.i.*) and chronic infection (week 20 *p.i.*) stages to determine changes in anti-phospholipid IgM antibody response. Additionally, we investigated the contribution of IL-5 to the activation of B-1 B cells and measured two pro-inflammatory cytokines, IFNγ and TNFα, and one anti-inflammatory cytokine, IL-10. We also evaluated the role of MCP-1 as an associated biomarker cytokine related to macrophage activation during TB infection and inflammation processes.

Our study showed that anti-phospholipid IgM antibody levels against CL significantly decreased at week 12 *p.i.* after eight weeks of treatment. In contrast, the anti-PTC, PI, and SL IgM did not decrease, but showed significantly lower levels after eight weeks of treatment in acutely infected mice at the same time point. These lower IgM levels corresponded to a reduction in the bacterial load. Levels of anti-PTC IgM remained significantly low in AI mice at up to 28 weeks *p.i.* In contrast, there were no significant difference in anti-PE IgM antibody levels between chronically infected mice and acutely infected mice after treatment. When we compare these results with the *Salmonella* infection model, the levels of anti-cardiolipin IgM antibody were found to be similar to those in the acute infection in mice infected with *M. tuberculosis*. The *Salmonella* infected mice died at one month *p.i.* and did not achieve higher IgM antibody levels similar to those of CI mice during *M. tuberculosis* infection. These findings suggest that IgM response to infection does not occur in all types of infection, since *Salmonella* infected mice did not show elevated anti-cardiolipin IgM at the time of death, when infection reached its maximum phase.

We also found that IL-5 levels are higher in the lungs of CI mice versus HC mice before anti-TB treatment. The spleen had significantly lower levels of IL-5 and serum had no detectable levels as measured by our in-house ELISA platform. These results support the

hypothesis that most of the IL-5 dependent B-1 B cell activation takes place within the lung. Further investigation has been initiated in our laboratory to evaluate histological lung sections from infected mice, which will help to answer this question. We plan to search for cell surface markers in lung collections of BALB/c and B6 mice infected with *M. tuberculosis* wild type, Erdman and BCG strains as well as other strains with lipid metabolism mutations, including *mce1A*$^{-/-}$, *mce1R*$^{-/-}$ and *fad5*$^{-/-}$. Observations by other researchers allow us to hypothesize that T lymphocytes recruited to the lung upon TB infection might contribute to IL-5 secretion and subsequent activation of B-1 B cells (Takatsu, Kouro et al. 2009). While many groups have shown that the major cellular sources of IL-5 are Th2 cells, Tc2 cells, mast cells, eosinophils and γδT cells, there is also evidence that NK cells, NK T cells, or nonhematopoietic cells, including epithelial cells, have the ability to secrete IL-5 (Tominaga, Matsumoto et al. 1988; Desreumaux, Janin et al. 1992; Warren, Kinnear et al. 1995; Muramatsu, Sankaranand et al. 1999; Montecino-Rodriguez, Leathers et al. 2006; Sakuishi, Oki et al. 2007). However, our work has not yet determined which cell repertoires are responsible for the secretion of IL-5 in the lungs of the TB infected hosts.

The location of B-1 B cells within *M. tuberculosis* infected lung is not clear. While others have demonstrated that B cells are located within the lung by detecting plasma cell surface markers in lung tissue, the exact location of B-1 B cells during TB infection has not been investigated. Interestingly, polyclonal activation of B cells upon infection has recently been described in another mycobacterial infection-leprosy. Studies of serum from leprosy patients have identified a wide spectrum of auto-antibodies including anti-cardiolipin (aCL), a rheumatoid factor and anti-phospholipid antibody (Saraux, Allain et al. 1996; de Larranaga, Forastiero et al. 2000). We expect to gain a complete understanding from future projects of how B-1 B cells migrate into the lung during infection; as well as the series of changes that these cells undergo during anti-TB treatment.

The evidence presented above supports our hypothesis that anti-phospholipid IgM antibodies might serve as a biomarker-based test to monitor anti-TB treatment. Our results show an increased level of anti-phospholipid IgM antibodies during acute and chronic infection. We also observed that healthy control mice have a continuous increase in these anti-phospholipid IgM antibodies with age. These findings in healthy mice are consistent with previous observations by Montecino *et al* where B-1 B populations in the peritoneal cavity also increased with the age of mice (Hardy 2006; Montecino-Rodriguez and Dorshkind 2006). Thus, it is expected that older healthy mice will have higher basal levels of anti-phopholipid IgM antibody. Our study also showed this increase. We also demonstrated that higher levels of IL-5 are found in the lung versus the serum and spleen. It is unclear if IgM antibody levels may contribute to host defense or immunopathology. The correlation of IgM antibodies with the progressive infection in mice suggests that these antibodies play no role in protection. In contrast, these antibodies are a direct reflection of the inflammation status of TB infection within the lung, and a decrease in these antibodies reflects a reduction of the bacterial burden in the lung. Thus, measures of this anti-phospholipid IgM antibody in serum might be a suitable biomarker for monitoring treatment response. Chapter 4 will provide the results of a validation study in humans we conducted to test this hypothesis.

IV. Figures

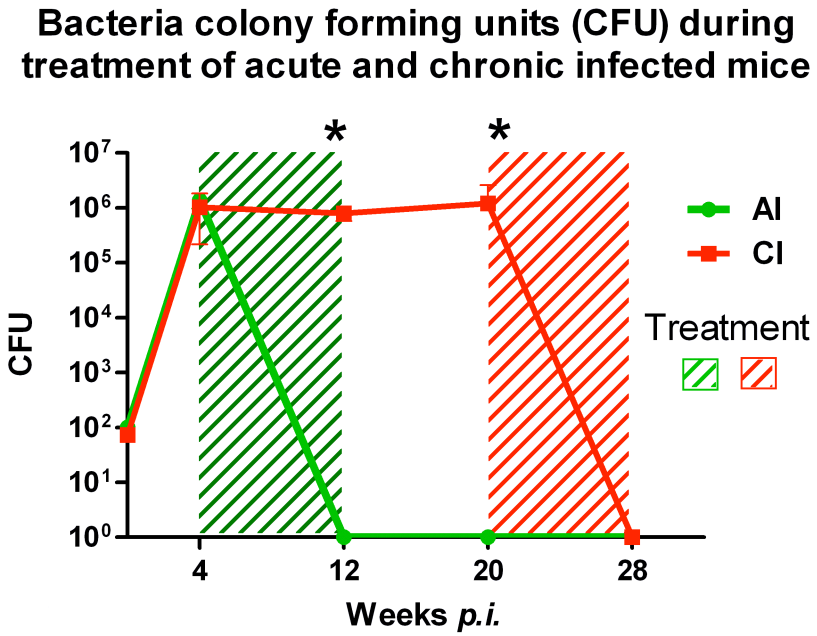


Figure 3.1: Bacterial burden in infected mice

Colony forming unit enumeration per lung collected from infected mice at the specified time points. The green line represents acutely infected (AI) mice that recived treatment starting at week 4 p.i.. The red line represents chronically infected (CI) mice that received treament starting at week 20 p.i.. Green and red shaded areas indicate the eight week treatment periods of AI and CI groups respectively. Significant differnces between both groups are designated by an asterisk ($p < 0.05$).

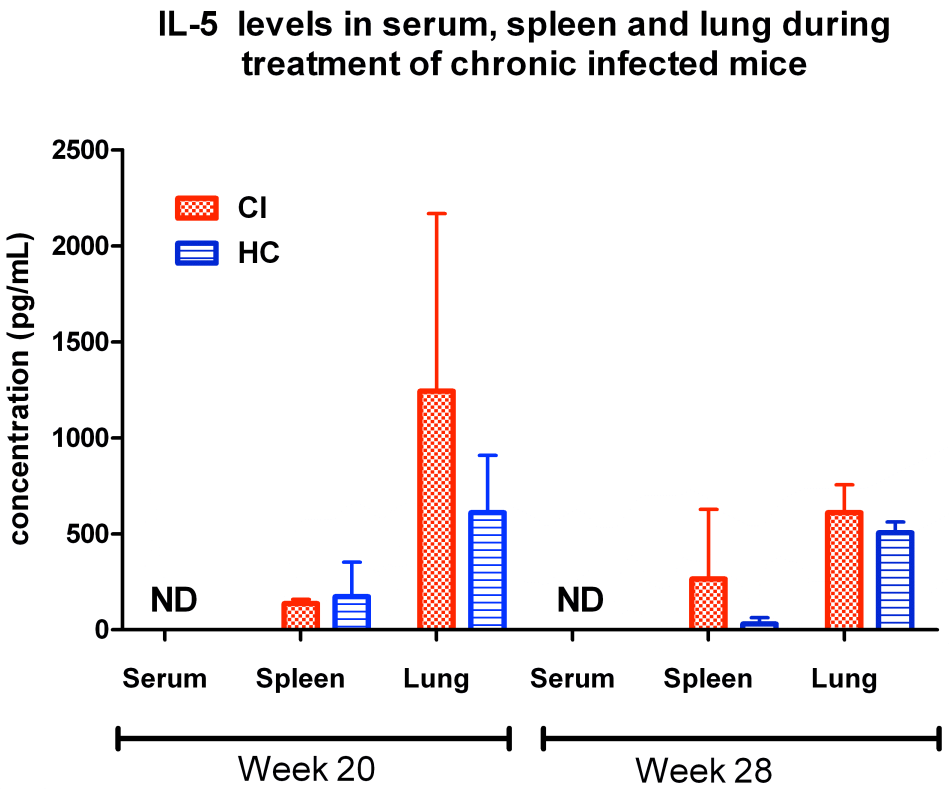
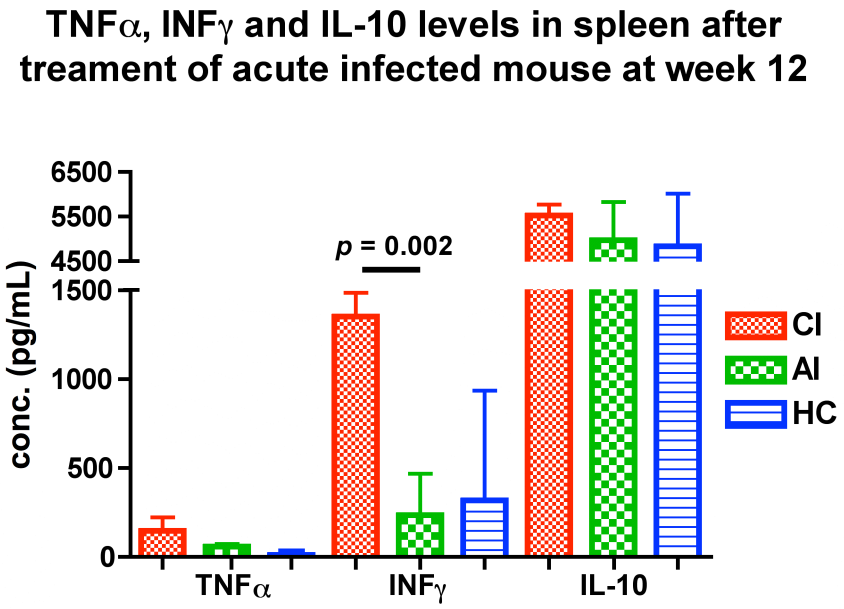


Figure 3.2: Levels of IL-5 during the treatment of CI mice group. Levels of IL-5 are shown in pg/ml. Each measure was obtained by assays using samples from three mice per assay. Red bars correspond to chronic infection (CI) and blue bars to healthy control mice (HC).



<u>Figure 3.3:</u> Pro- and anti-inflammatory cytokine levels in the spleen of mice at week 12 ***p.i.*** Levels of TNFα, IFNγ and IL-10 are shown in pg/ml. Significant differences are indicated with a *p* value and a black bar. Each measure was obtained by assays using samples from three mice per assay. Red bars correspond to chronic infection (CI), green bars correspond to acute infection (AI) and blue to healthy control mice (HC)**.**

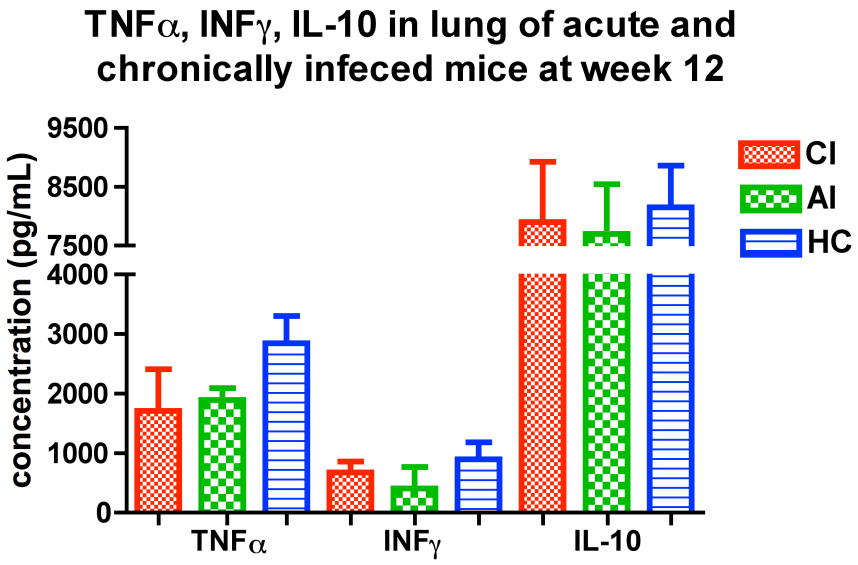
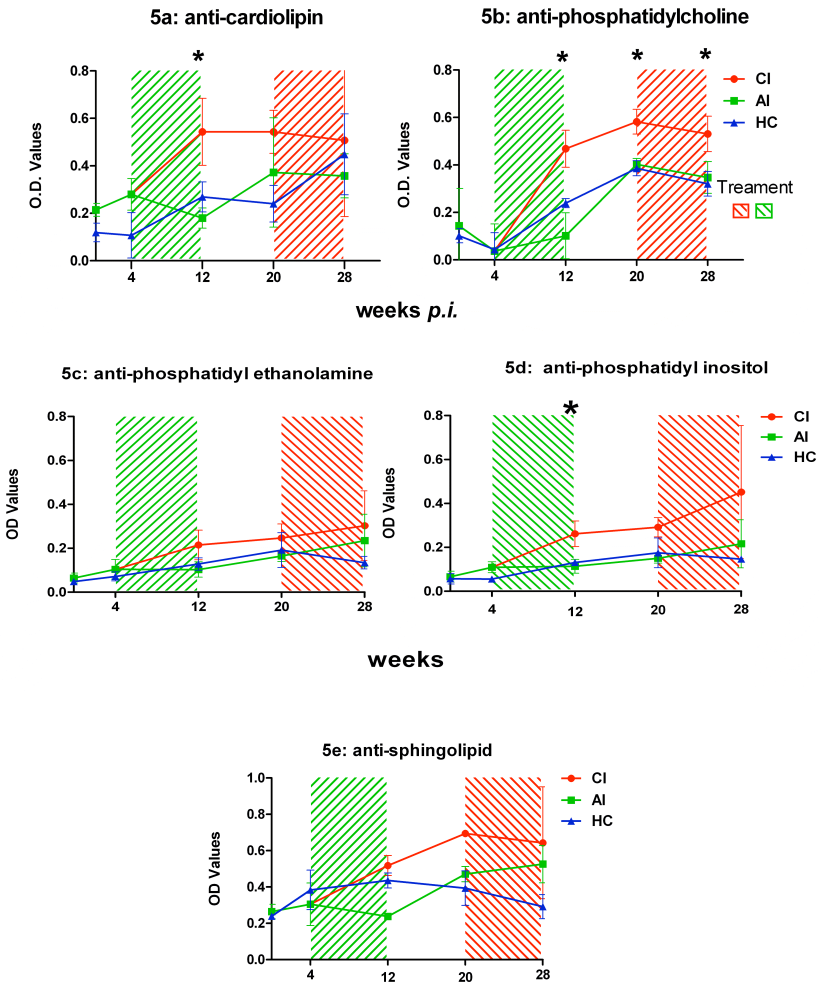


Figure 3.4: Pro- and anti-inflammatory cytokine levels in the lungs of infected mice at week 12 *p.i.* Levels of TNFα, IFNγ and IL-10 are shown in pg/ml. Each measure was obtained by assays using samples from three mice per assay. Red bars correspond to chronic infection (CI), green bars correspond to acute infection (AI) and blue bars to healthy control mice (HC).



<u>Fig 3.5a-3.5e:</u> Levels of anti-phospholipid IgM antibodies in the Cornell model of mouse infections. Levels of IgM antibodies in serum are shown in O.D. Significant differences between the groups at a given timepoint are indicated by an asterisk in the X axis ($p < 0.05$). Each measure was obtained by assay of serum from three mice per assay. Red lines correspond to chronic infection (CI), green lines correspond to acute infection (AI) and blue lines to healthy control mice (HC). Green and red shaded areas indicate the eight week treatment periods of AI and CI groups respectively.

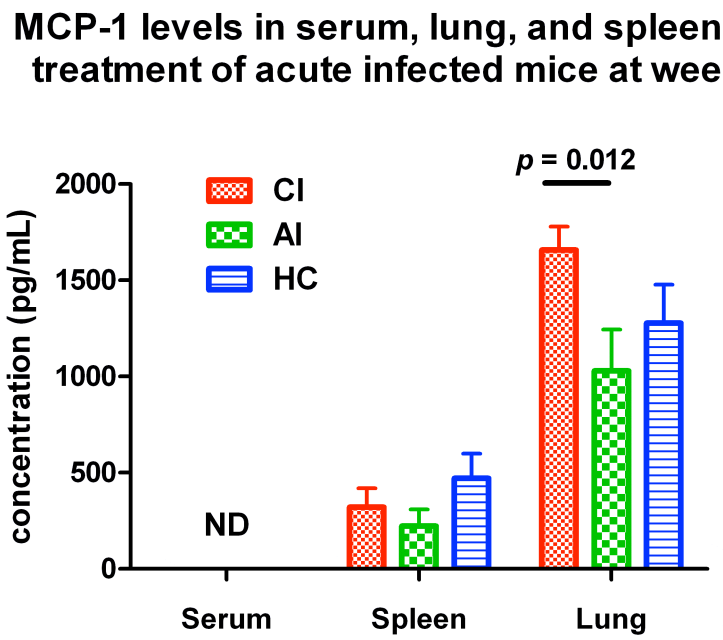


Figure 3.6: MCP-1 levels in serum, lung, and spleen after treatment of acute infected mice at week 12 *p.i.* Levels of cytokines are shown in pg/ml. Significant differences are indicated with a *p* value and black bar. Each measure was obtained by assays using samples from three mice per assay. Red bars correspond to chronic infection (CI), green bars correspond to acute infection (AI) and blue bars to healthy control mice (HC).

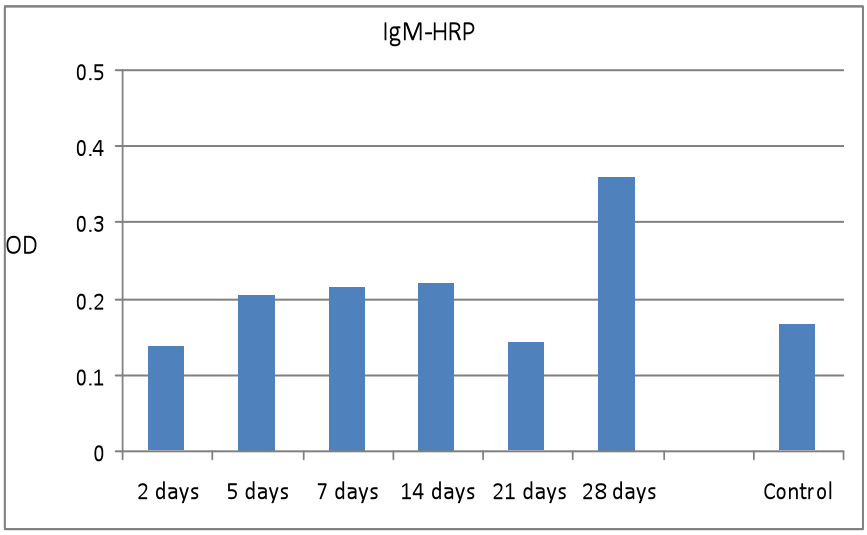


Figure 3.7: anti-cardiolipin IgM antibody levels during Salmonella model of mice infection. Levels of IgM antibodies in serum are shown in O.D. Groups of 2-5 mice were used to collect serum at each time point following oral infection by *Salmonella*. For the uninfected mice (control), mice were sacrificed at approximately 70 days of age.

CHAPTER 4

Validation of anti-phospholipid IgM antibodies as biomarker for monitoring TB treatment response

I. Introduction

The anti-lipid antibody response in mice is different than in human hosts. Mice have an incomplete set of CD1 molecules whereas humans have all isoforms of CD1 including CD1a through CD1e; mice only have CD1d and CD1e (Lang and Glatman-Freedman 2006). Differences in CD1 molecules might contribute to a different pattern of anti-phospholipid IgM antibody response during TB infection and treatment. In addition, our findings reported in Chapter 3 are limited by the mice strain used. In general, BALB/c mice are more susceptible to TB infection and the cell mediated immune response is weaker. In contrast, B6 mice have been shown to be more resistant to artificial models of TB infection. In addition, BALB/c mice do not generate lung cavities and these animals do not cough (Yamamura, Maeda et al. 1986; Dannenberg 2009). Another limitation with our animal model is that current bioethical regulations at UC Berkeley did not allow blood extraction from the same mouse at consecutives time-point. This situation prevented us from evaluating the anti-phospholipid IgM antibody level changes within an individual animal host. For these reasons the findings in Chapter 3 are not necessarily applicable in a human host. Thus, a human validation study is required to determine the utility of a biomarker-based test using anti-phospholipid IgM antibody to evaluate TB treatment response.

The anti-lipid antibody response is highly variable in humans. As mentioned in Chapter 1, Steingart *et al* described the lack of sensitivity of lipid-antibody response for diagnosis tests (Steingart, Dendukuri et al. 2008). Little is known about how much the anti-lipid antibody response changes in the same patient during TB infection and treatment. It is widely accepted that anti-phospholipid IgM antibodies produced by B-1 B cells are self-reactive and they have been associated with autoimmune diseases such as systemic lupus erythematosus and anti-phospholipid syndrome (Ravirajan, Harmer et al. 1995; Tebo, Jaskowski et al. 2008). The self and poly-reactive properties of anti-phospholipid IgM antibodies facilitate their rapid clearance from the blood stream. IgM will bind to self-phospholipid motifs in host cells and lipid transporter structures, such as oxidized low-density lipoprotein (oxLDL) (Binder and Silverman 2005), and these self-binding properties increase their clearance rate compared to antibodies with a highly specific protein antigen target. The rapid clearance rate may permit the observation of changes in anti-phospholipid IgM antibody levels during a course of treatment for diseases such as tuberculosis.

We propose that the B-1 B cell-produced IgM antibody response may serve as a biomarker for monitoring TB treatment in humans. That is, IgM antibody response against phospholipids may decrease as the bacterial load decreases with successful treatment. Such a response could serve as a marker for effective treatment response and an end-point assessment in clinical trials of new anti-TB drug regimens.

II. Methods

XII. ***Patient specimens*****:** Serum samples were obtained at baseline (before initiation of drug therapy) and at the end of the intensive phase of therapy from 40 HIV-negative patients with acid-fast bacilli (AFB) smear and culture-confirmed pulmonary tuberculosis (PTB). These patients were enrolled in the Center for Disease Control and

Prevention–Tuberculosis Trials Consortium (CDC-TBTC) randomized clinical trial conducted in Kampala, Uganda. The patient cohort was composed of two groups: 20 culture-positive patients (slow responders) and 20 culture-negative patients (fast responders) defined by culture status at the end of 40 doses of anti-TB combination treatment, which corresponds to eight weeks of treatment (5 doses per week). Patients were further categorized according to disease severity based on the findings of pre-treatment chest radiographs (limited, moderate, and extensive based on a validated grading scheme) and whether or not cavitary lesions were present (cavity and no cavity) (Falk A 1969). Serum samples were screened for levels of IgM antibodies against five phospholipids extracted from bovine sources available commercially (Avanti Polar Lipids, Alabama, USA).

XIII. ***Enzyme-linked immunosorbent assay (ELISA)****:* The phospholipid antigens included cardiolipin (CL), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PTC), and sphingolipid (SL). Lipids were diluted to 10 mg/ml in ethanol and 50µl of the solutions were dried overnight in flat bottom well polystyrene ELISA plates (Fisher Scientific, USA). ELISA plates were blocked with 100µl of 3% low fatty acid bovine serum albumin (BSA) (USBiologicals, USA) and washed with phosphate buffered saline (PBS) pH 7.4. Frozen serum samples were thawed twice and diluted 1:100 in 3% low fatty acid BSA. The diluted samples were added to the plate and incubated for one hour at room temperature (RT), followed by three washes with PBS. Then, 100µl of 1:5,000 goat-derived anti-human IgM was labeled with horseradish peroxidase (HRP) (Thermo Scientific, Ill), diluted in PBS pH 7.4, and incubated at RT for 1hr and washed again with PBS. Finally, 100 µl of tetramethylbenzidine substrate (TMB) (Thermo Scientific Pierce, Illinois, USA) was added and the reaction was stopped immediately with 50µl of sulfuric acid 1M.

Reactions were read within ten minutes at 450nm in a spectrophotometer (Cambridge Technologies, Massachusetts). The results were read out as the average of optical densities (O.D.) of triplicate assays. Standard curves for our in-house ELISA were prepared with polyclonal human IgM (Thermo Scientific Pierce, Illinois, USA), which yielded a correlation coefficient R^2 of 0.99. The concentration of IgM in serum samples (in µg/ml) was calculated based on this standard curve. High, medium, and low IgM level control samples were included in each assay. Assays with control sample results outside two standard deviations were discarded and repeated.

XIV. ***Statistical analysis*****:** All statistical analyses were conducted with STATA (Version 11, STATA Corp., Texas). The two-sample Wilcoxon rank-sum test was used to compare lipid-specific IgM concentration differences between fast and slow responders (at baseline and at the end of intensive phase of treatment). We performed a step-wise procedure to test for the following variables suspected to be biologically relevant to IgM antibody response during treatment: age, gender, body mass index (BMI), bilateral abnormalities, AFB smear status at baseline, cavitary disease, and disease severity. Variables were added to the basic model in a stepwise fashion. Variables were eliminated from the full model if they failed to achieve significance at the

5% level in order to obtain the best fit and simplest model. The resulting model included cavity classification and disease severity as significant variables. The Wilcoxon signed-rank nonparametric test was used to compare lipid-specific antibody differences between groups for cavity and disease severity variables. The Committee for Protection of Human Subjects at UC Berkeley (UCB#2010-06-1752) approved this sub-study. The protocol for the parent clinical trial (conducted by the TB Trials Consortium sponsored by the U.S. Centers for Disease Control and Prevention; ClinicalTrials.gov number, NCT00694629) was approved by the Center for Disease Control and Prevention IRB and the local IRBs at all participating sites. All patients gave informed consent for participation.

III. Results:

The CDC-TBTC sample set was composed of 40 HIV-negative patients (29 males and 11 females) with smear and culture-confirmed pulmonary TB. The baseline characteristics of the study subjects are shown in Table 1. The average age was 29 years (range 19 to 53 years of age). Seventy-seven percent of these patients were non-smokers, one patient had a past history of excessive alcohol use, and one patient had used non-injectable drugs within the last year. **(Table 4.1).** Patients with negative culture after 2 months of treatment showed a higher pretreatment BMI. This is similar to previous findings where BMI was the strongest predictor of culture status (Visser, Grewal et al. 2011). Three patients had limited radiographic disease, 21 had moderate disease, and 16 had extensive disease. Fifty-eight percent had cavitary lesions.

The anti-phospholipid IgM antibody response was highly variable among these patients. A global analysis of our sample set showed heterogeneous results for all five anti-phospholipid IgM levels, regardless of culture conversion status (data not shown). A comparison of the change in IgM antibody levels between TB patients who remained culture positive and those who culture converted after 40 doses of intense therapy, revealed no significant differences using a two-sample *t*- test (data not shown).

The multi-variable model included age, gender, body mass index, bilateral abnormalities, smear status at baseline, cavity extension and disease extension. This model revealed no contribution of the variables to changes in antibody levels upon treatment completion. Thus, we opted to use a simpler model using "cavity classification" as a proxy for disease severity and adjusted for "age". The "cavity classification" variable showed a uniform distribution across three categories, including no cavity (n= 17), small cavity (n= 9), and large cavity (n= 14). This simpler model allowed us to study the anti-phospholipid IgM antibodies as a biomarker for TB treatment response, despite the variability of the antibody levels within our sample set.

Analysis of this simpler model consistently showed a decrease in IgM anti-phospholipids antibodies among TB patients with no lung cavities. The mean IgM concentration decreased significantly in four of the five phospholipids, including PE, PI, PTC and SL (paired *t*-student p= 0.008, 0.016, 0.020, and 0.043 respectively). The distribution of delta change among all patients was scattered due to the presence of outliers in the cavity classification categories (data not shown). The median values of antibody change were analyzed with a two-sample Wilcoxon rank-sum test that found a significant decrease for all five lipids, CL, PI, PE, PTC, and SL in non-cavitary TB patients (Wilcoxon rank-sum test p= 0.036, 0.006, 0.001, 0.007, 0.040 respectively, see **Figure 4.1**). The antibody level reduction was different for each lipid as we

observed a 22.7%, 11.1%, 15.4%, 24.3% and 18.0% concentration reduction of IgM anti-CL, PE, PI, PTC and SL, respectively (data not shown).

In contrast, patients with cavitary TB showed an overall increase in the anti-phospholipid IgM antibody response following anti-TB drug treatment. A significant increase was observed in anti-PE antibody levels (p= 0.025, 95% C.I.-2.287, -0.113).

We evaluated our biomarker test's ability to identify successful treatment response in non-cavitary TB patients. We defined a positive biomarker test as a decrease in anti-phospholipid IgM antibodies and a negative test as an increase or no change in anti-phospholipid IgM antibodies. The outcome was successful treatment response. The sensitivity values were 88.2%, 70.6%, 76.5%, 88.2% and 76.5% for each anti-CL, PE, PI, PTC and SL IgM, respectively, with an overall sensitivity of 80% (Table 4.2). The sensitivity was 94% in non-cavitary disease patients when the decrease in IgM response was assessed against 2 or more of the phospholipids (CL and PTC, or CL, PTC, and PI).

IV. Discussion

Changes in serum anti-phospholipid IgM antibodies appear to be a useful biomarker test to monitor treatment response in TB patients, especially in patients with non-cavitary disease. The decrease in patients without cavitary disease may be related to the decrease in bacterial burden and healing of lung lesions in these patients. In contrast, patients with cavitary disease showed a significant increase in antibody levels after 40 doses of treatment. This increase may reflect liquefied caseum containing high levels of *M. tuberculosis* bacilli and their lipid debris, as well as host cells' phospholipids released during the inflammatory process (Converse, Dannenberg et al. 1996; Dannenberg 2009). This increase might be transient during treatment, but additional studies assessing antibody levels at later time points are needed to support this suggestion.

The pathophysiology of cavity formation and healing during TB is not completely understood (Gadkowski and Stout 2008). Evidence from human cavities suggests that Th2 cytokines, such as IL-4 and TNFα, may play a role in cavity formation (Somoskovi, Zissel et al. 1999). These Th2 cytokines may antagonize host defenses and cause tissue necrosis in TB patients' lungs (van Crevel, Karyadi et al. 2000). Early work by Yamamura *et al.* showed that a lipid-protein mixture from heat-killed bacilli sensitized and elicited cavities in white male rabbits (Yamamura, Maeda et al. 1986). Interestingly, this study demonstrated that mycobacterial proteins alone were able to produce granulomas, but not cavities. More recently, it has been shown that mycobacterial lipids act as adjuvants as mycobacterial proteins combined with synthetic adjuvants were able to elicit cavity formation (Helke, Mankowski et al. 2006). Once the cavity breaks open and communication with the bronchial tree is established, more oxygen enters allowing the bacilli to multiply further inside the cavity (Palaci, Dietze et al. 2007). Routine cultures have measured 10^7- 10^9 bacilli within the liquefied material of a single cavity.

We hypothesize that IgM secreting B-1 B cells are activated upon treatment by the transiently enhanced inflammation induced by dying *M. tuberculosis* in cavitary lesions. Necrotic cells accumulate lipids in the caseum and this may explain the increase in the anti-lipid IgM antibody levels we observed in treated patients with cavitary disease in this study. Both lipids from dead host cells and from *M. tuberculosis* in the released caseous material contain

sufficient levels of antigens to activate the B-1 B cells and induce anti-phospholipid IgM antibody production. It has also been observed that TB patients have higher levels of PPD-specific IL-5 secreting cells in their bronchoalveolar lavage (BAL) than peripheral blood mononuclear cells (PBMC) (Morosini, Meloni et al. 2005). More recently, it was shown that increased levels of neutrophils and granulocytes occur during TB pathogenesis in guinea pigs and humans (Ordway, Palanisamy et al. 2007; Eum, Kong et al. 2010; Russo and Mariano 2010). These studies indicate that $CD4^+$ lymphocytes are initially the predominant inflammatory cells within the lungs. After 30 days, increased B cells and granulocytes have been associated with worsening lung pathology. Furthermore, lungs of BCG infected BALB/c mice have shown a significant increase in B-1 B cells at 40 days post infection (Russo and Mariano 2010). Thus, self-lipids, mycobacterial lipids, and IL-5 secreted by granulocytes generate the signaling required for full activation of B-1 B cells. The increase in anti-phospholipid IgM antibodies observed in cavitary TB patients might be a reflection of T cell-independent B-1 B cell activation upon cavity liquefaction during TB treatment.

The percentage of TB patients with cavitary disease at initial diagnosis varies by geographical region. Countries with low and mid TB incidence such as Saudi Arabia, Denmark, and Brazil report cavities on the initial chest X-ray in 14%, 42%, and 36% of TB patients, respectively (Nyman, Brismar et al. 1996; Wilcke, Askgaard et al. 1998; Gomes, Saad Junior et al. 2003). In contrast, Nigeria, a country with a high prevalence of TB, has reported the presence of cavities in up to 46% of cases (Andreu, Caceres et al. 2004). Our biomarker anti-phospholipid IgM antibody test will be useful to monitor the treatment of 64-86% of the patients suffering from TB, depending on the cavitary TB prevalence in the region.

The change in anti-phospholipid IgM antibody levels may also serve as a valuable test to monitor TB treatment in HIV-positive patients. As mentioned previously, B-1 B cells do not require T cells for activation. Therefore $CD4^+$ and $CD8^+$ cell counts will not affect the anti-phospholipid IgM antibody levels produced in patients infected with HIV. In addition, HIV-positive patients generally do not develop cavitary TB because of a weakened cell-mediated immune response. The anti-phospholipid IgM antibodies in TB-HIV infected patients could be useful in monitoring disease severity.

The proportions of non-cavitary TB patients that showed a decrease in IgM response were 88.2%, 70.6%, 76.5%, 88.2% and 76.5% for IgM anti-CL, PE, PI, PTC and SL, respectively (Table 2). Since all of the patients responded to the treatment, these values also represent the sensitivity of our IgM biomarker ELISA test. At eight weeks of treatment, these values were all higher than the reported 60-80% sensitivity of the 2-month sputum culture conversion method currently in use to monitor treatment response (Liu, Shilkret et al. 1999; Salihu, Aliyu et al. 2003; Alguire and Medicine 2008; Uzundag Iseri, Dulkar et al. 2010). Since a large proportion of sputum cultures from newly diagnosed patients are negative before treatment, the sensitivity of the traditional monitoring test is actually even lower. The anti-phospholipid IgM ELISA does not require sputum culture results.

This study is limited by the fact that it included patients who all responded to the drug regimens given. Our test still needs to be studied with a larger sample size that also includes patients who do not respond to treatment. If our biomarker-based test can be shown to also have high specificity for those that respond to treatment, it can serve as a low-cost method to monitor response to treatment. The bovine phospholipids used in this study were all readily commercially available and can be used in low concentrations. In most countries, chest x-rays

are routinely performed as part of TB diagnostic work ups. Thus, the combination of chest x-ray and anti-lipid IgM response results could serve an inexpensive and sensitive approach to monitor response to TB treatment.

V. Future directions

The findings of this dissertation research open a new set of questions. The discovery of *M. tuberculosis* cardiolipin as lipid biomarker, together with the anti-phospholipid IgM antibody response in animal models and its the validation in the human host anti-TB therapy does not answer all the questions. It still remains unclear whether or not this biomarker-based test is useful in HIV co-infected patients. Additionally, it is necessary to determine if this biomarker-based test is useful for monitoring the treatment of other infectious diseases of the lung, like pneumonia. We must also determine if our biomarker test's performance will differ between fast and slow responders, both with initial non-cavitary TB. In addition, we believe it is necessary to understand the biology behind the increase in anti-phospholipid antibodies during cavitary TB. Our current mouse model of infection does not produce cavitary TB, but rabbits and guinea pigs hosts can be used in order to address this issue. It is essential to determine the exact location of B-1 B cell activation within the infected lung. Immunohistochemistry using surface cell markers for IgM, CD5 and CD11b will be a key in this research. Finally, the clearance dynamics of B-1 B cells during lung healing upon anti-TB treatment needs to be clarified. In summary, the results reported here represent one small portion of a huge area of basic and applied research. Our laboratory has recently started analyzing surface markers to study the cell repertoire within the lung of infected mice. We expect to soon generate new information to facilitate the study of these remaining unanswered questions.

VI. Tables and figures

Table 4.1. Demographic and clinical characteristics of TB patients who responded early (sputum culture negative at 8 weeks of treatment) and late (sputum culture positive at 8 weeks).

	Culture negative at 8 weeks (n = 20)	Culture positive at 8 weeks (n = 20)
Demographic characteristics		
Age; median (IQR)	25.0 (6.0)	30.0 (4.0)
Male gender	13 (65%)	16 (85%)
Social characteristics		
Smoking history	4 (20%)	5 (25%)
Excessive alcohol use in the past	0 (0%)	1 (5%)
Past drug use last year (injection)	0 (0%)	0 (0%)
Past drug use last year (non-injection)	0 (0%)	1 (5%)
Clinical characteristics		
BMI; median (IQR)	19.8 (2.8)	18.6 (2.5)
HIV infected	0 (0%)	0 (0%)
Any co-morbid condition	1 (5%)	0 (0%)
TB diagnosis		
Any cavity at baseline chest radiograph	12 (60%)	11 (55%)
Bilateral cavitation	1 (5%)	2 (10%)
Cavity classification		
Cavity absent	8 (40%)	9 (45%)
Cavity present with < 4cm diameter	5 (25%)	4 (20%)
Cavity present with ≥ 4cm diameter	7 (35%)	7 (35%)
Extent of Chest x-ray involvement		
Limited	0 (0%)	3 (15%)
Moderate	14 (70%)	7 (35%)
Extensive	6 (30%)	10 (50%)
Any bilateral abnormalities	8 (40%)	12 (60%)
AFB smear		
2+	4 (20%)	0 (0%)
3+	6 (30%)	7 (35%)
4+	10 (50%)	13 (65%)
Days to detection; median (IQR)	5.84 (2.54)	5.83 (2.38)
TB treatment		
Received pretreatment	4 (20%)	1 (5%)
Days of pretreatment	0 (0%)	0 (0%)

Table 4.2: Biomarker test evaluation for monitoring treatment response in non-cavitary TB[a].

	Non-Cavitary TB decreased	Sensitivity %
CL	15/17	88.2
PE	12/17	70.6
PI	13/17	76.5
PTC	15/17	88.2
SL	13/17	76.5

[a]Number of non-cavitary TB patients that showed a decrease in anti-phospholipid IgM antibody levels. Values for sensitivity were determined for each lipid using contingency table analysis.

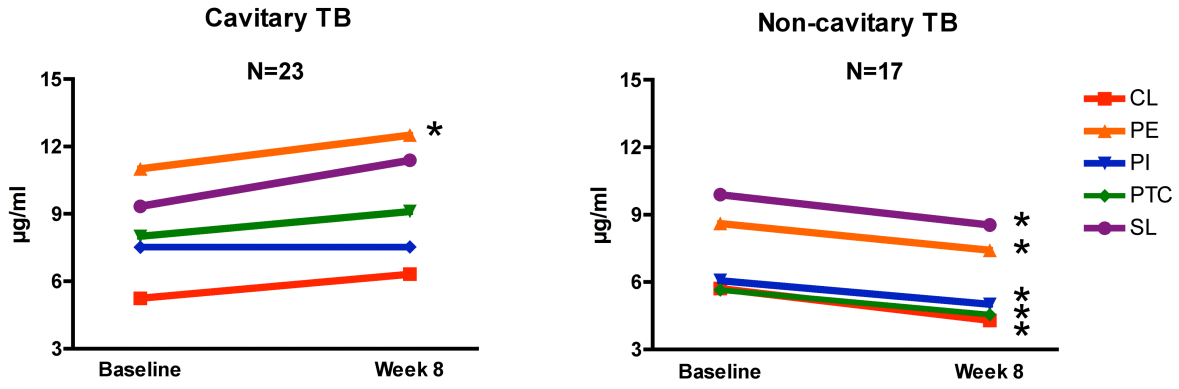


Figure 4.1: Plots of the median change of IgM anti-phospholipids antibody levels after 40 doses of intensive phase anti-TB drug therapy. Antibody levels were determined by in-house ELISA assay; and results are shown in concentration (µg/ml). The panels compare the antibody decrease in cavitary TB patients vs non-cavitary TB patients for each of the five phospholipids including Cardiolipin (CL); phosphatidyl ethanolamine (PE); Phosphatidyl Inositol (PI); Phosphatidyl Choline (PTC); Sphingolipid (SL). Asterisks indicate a significant increase or decrease ($p<0.05$) in the corresponding anti-phospholipid IgM antibody.

CHAPTER 5

CONCLUSIONS

Tuberculosis (TB) is a major threat to public health worldwide. The global effort for TB control urgently requires a major shift to improve the tests that are currently used for TB diagnosis and prognosis of treatment outcome. Until now, pathogen-signal based tests have been implemented without a dramatic effect in reducing the TB burden. The pathogen signal-based tests are more expensive and technologically complicated. In contrast, biomarker-based tests are cheaper and simpler alternatives for rapid implementation in low resources settings where TB is highly prevalent. This dissertation thesis proposes to measure anti-phospholipid IgM antibodies as a biomarker-based test for monitoring TB treatment response.

Chapter 1 discussed TB as a major threat to public health worldwide and described the current challenges in diagnosis and treatment as well the rationale and need for new biomarker-based tests. This chapter also illustrated the wide repertoire of lipids located within *M. tuberculosis* cell walls that can be used as potential targets of novel lipid-antibody-based biomarkers. Specifically, antibodies generated by B-1 B cells have the characteristics of an innate immunity response and therefore might reflect infection status in a host. This chapter also discussed the biological understanding which allowed us to hypothesize that anti-lipid IgM antibodies secreted by B-1 B cells might reflect the burden of *M. tuberculosis* bacilli inside the lungs and that these antibodies decrease with anti-tuberculosis treatment. A similar antibody monitoring approach has been demonstrated to monitor the treatment of syphilis. This dissertation thesis proposes that in patients with pulmonary TB, the levels of anti-lipid IgM antibodies might also decrease with treatment.

The lipid screening and discovery approach described in Chapter 2 resulted in the identification of *M. tuberculosis* cardiolipin (CL) as the major lipid antigen during active disease. The TLC-I technique developed in our laboratory guided us in screening most of the lipid antigens found in *M. tuberculosis* cell wall. We identified a band in the TLC-I containing a lipid antigen to which 100% of TB patients showed an IgM antibody response. Wet chemistry including flash chromatography, two dimensional TLC and mass spectrometry allowed us to identify cardiolipin in this band fraction. This finding allowed us to hypothesize that CL, as well as other phospholipids, might be useful antigens for a lipid-antibody biomarker-based method to monitor the treatment response.

Chapter 3 attempted to understand the biology of anti-phospholipid IgM antibody production and decrease during TB infection and treatment of mice. Our findings indicate that indeed, anti-phospholipid IgM levels against different phospholipids, including CL, PTC, PI, and SL, decreased significantly after eight weeks of treatment in acute infected mice (AI). Levels of anti-PTC IgM antibodies remained significantly low in AI mice up to 28 weeks *p.i*. We found no significant difference in anti-PE IgM antibody levels between AI mice and CI mice. Interestingly, higher levels of IL-5 were found in the lungs of infected mice. This finding raises new questions about which cells within the lung secrete IL-5 during TB infection and how this relates to B-1 B cell activation. We also observed that the levels of the chemokine MCP-1 secreted by macrophages decrease after treatment of acute infected mice. Taking these findings together, we hypothesize that anti-phospholipid IgM antibodies produced by B-1 B cells may be a useful biomarker to monitor treatment response in infected mice.

Finally, Chapter 4 presented the results of a study testing the use of anti-phospholipid IgM antibody as a biomarker for monitoring the treatment response in human hosts with pulmonary TB. Here we studied IgM antibodies during the first intensive phase of anti-TB drug therapy using an *in-house* ELISA platform. Our findings showed that patients with non-cavitary TB significantly decrease in anti-CL, -PTC, -PI, -PE and -SL IgM antibodies during the first 8 weeks of treatment. In contrast, during cavitary TB there is an increase, with a significant increase only in anti-PE IgM antibodies. No significant differences in IgM levels for any lipids were found between fast and slow responders. The sensitivity of this biomarker-based test in non-cavitary TB patients ranged from 71% to 88%. We anticipate that additional research is needed to understand the significance of using this as a biomarker of cavitation or severity of disease.

All these observations together allowed us to propose the use of anti-phospholipid IgM antibodies as the basis for a biomarker-based test to monitor TB treatment response. Currently other members in our laboratory are continuing with the development of a point-of-care (POC) test for use in limited resource settings. There are three platforms under evaluation, including lateral flow assay (LFA), mini-ELISA (*m*ELISA), and silica embedded polyacrylamide (SEP). Prototype devices will be tested in collaborator sites in Brazil and Panama as we plan to collaborate with TB control programs in high burden cities within these countries. In addition, our laboratory has started to investigate the cellular dynamics within the lung of infected mice during treatment and vaccination. This approach will initially include the characterization of cell repertoires of histological sections of mice lung using surface cell markers and con-focal florescent microscopy. We anticipate that both investigations will provide new insights that will facilitate a better understanding of the use and biology of the anti-phospholipid antibodies. We strongly believe that this new information, together with the biomarker-based test proposed here, will have a large positive impact on the management of patients with pulmonary TB and the efforts to control the disease worldwide.

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