THESIS

EVALUATION OF CLINICAL PARAMETERS FOR DETECTION OF EARLY ENDPOINT

CRITERIA IN GUINEA PIGS EXPERIMENTALLY INFECTED WITH

MYCOBACTERIUM TUBERCULOSIS

Submitted by

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ABSTRACT

EVALUATION OF CLINICAL PARAMETERS FOR DETECTION OF EARLY ENDPOINT CRITERIA IN GUINEA PIGS EXPERIMENTALLY INFECTED WITH MYCOBACTERIUM TUBERCULOSIS

Tuberculosis (TB) is a global epidemic caused by the bacteria *Mycobacterium tuberculosis.* Despite extensive research and funding to improve diagnostic and treatment strategies, the emergence of multi drug resistant and extensively drug resistant strains of *M. tuberculosis* are on the rise. Vaccines present a solution to the failing diagnostic and treatment strategies by preventing incidence of disease. Guinea pigs are a common animal model to test TB vaccine candidates, but are difficult to evaluate in terms of overall health status. We hypothesized that using parameters within the complete blood count, serum biochemistry, and urinalysis assays we would identify biomarkers that could be used as early endpoint criteria for guinea pigs experimentally infected with *M. tuberculosis.* Using groups of BCG-vaccinated or saline-treated guinea pigs we measured biomarkers over the life of guinea pigs both pre-challenge and after challenge with laboratory strain *M. tuberculosis* H37Rv. Our study showed significant differences between groups of guinea pigs, as well as compared to pre-challenge values. Specifically, blood urea nitrogen, urine specific gravity, serum proteins, alanine transferase, hematocrit, leukocytes, and body weight should be monitored when establishing early endpoint criteria in guinea pigs experimentally infected with *M. tuberculosis.*

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Chapter 1: Introduction

1.1 Epidemiology

Tuberculosis (TB) is a global epidemic, affecting approximately one-third of the world's population. As a disease that has many subclinical carriers and is spread via aerosol, transmission often occurs between individuals unbeknownst to them. Additionally, high population density and minimal access to health care contribute to the staggering numbers of people infected, with diagnosis and treatment completely unavailable to many (World Health Organization, 2013). Although progress is being made in the diagnosis and treatment of TB worldwide, the Human Immunodeficiency Virus 1 (HIV-1) epidemic, increasing drug resistance, and other epidemiological factors complicate the efforts for global TB eradication (World Health Organization, 2013).

The World Health Organization (WHO) reported in 2012 there were 8.6 million new cases of TB and 1.3 million TB deaths. Of those deaths, 0.3 million were associated with HIV co-infection. The number of TB deaths is staggering, and many would be prevented with access to facilities for diagnosis and proper treatment. Approximately 90% of cases can be effectively cured with drugs that have been available for decades. TB is still a disease of poverty, which is complicated by overcrowding and poor nutrition. Unfortunately, many people infected with the deadly disease do not have access to health care, nor are they able to comply with the 9 months of treatment necessary to cure TB even with access to health care (World Health Organization, 2013).

Additionally, the increase of the multi-drug resistant TB epidemic in Eastern Europe among drug-users and prisoners is the underlying reason for the increase in TB cases in this region. The most potent risk factor for TB is among HIV-positive patients. People infected with HIV are 20 times more likely to develop TB than those that are HIV-negative (World Health Organization, 2013; Dannenberg and Collins, 2007). Other risk factors include heavy alcohol consumption and smoking, with smoking doubling the risk for TB infection. Diabetes mellitus increases the risk of TB about three times, compared to those

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without diabetes. Just as with any infectious disease, the use of immunosuppressive drugs increases the risk for contracting TB as well. And, lastly, there are genetic variants known to effect susceptibility in macrophages, vitamin-D metabolism, nitric oxide synthase, and interferon-gamma pathways (Lawn and Zumla, 2011).

To further compound this health crisis, incidence of Multi-Drug Resistant TB (MDR-TB) and Extensively Drug Resistant TB (XDR-TB) are on the rise, with newly identified strains of completely drug resistant TB. MDR-TB is classified as *M. tuberculosis* that is resistant to two first-line drugs--isoniazid and rifampicin. XDR-TB is classified as *M. tuberculosis* that is also resistant to all fluoroquinolone antibiotics, and three of the injectable aminoglycosides (capreomycin, kanamycin, and amikacin) (Lawn and Zumla, 2011). According to the WHO, globally, an estimated 3.6% of new cases and 20.2% of previously treated cases have MDR-TB, with the highest levels in Europe and central Asia. In 2012, there were an estimated 450,000 new cases and 170,000 deaths from MDR-TB globally. XDR-TB is also on the rise, with an estimated 9.6% of MDR cases having XDR-TB. With only 34 of the 107 countries reported to have MDR-TB reaching the treatment success target of >75%, there is an imperative need for improved treatment outcomes.

Despite the inability of many people to seek treatment, the global TB rate is on the decline. The WHO reports that since 1990, the TB mortality rate has fallen by 45% and the incidence of new TB cases is declining in most countries as well. Much of this success has been due to the international effort to control TB by the WHO. The directly observed treatment, short-course (DOTS) strategy and its successor, the Stop TB Strategy, has helped successfully treat 56 million people between 1995 and 2012.

1.2 Pathogenesis

Tuberculosis is an infectious disease caused by the bacteria *Mycobacterium tuberculosis*. *M. tuberculosis* is a facultative intracellular bacteria that requires an aerobic environment, has an acid-fast staining profile, is non-motile, non-encapsulated, and non-spore forming (Kassim and Sherris 2004). Although humans are the primary host, *M. tuberculosis* can also infect other animal species. Tissue tropism is primarily the lungs (pulmonary TB) but the organism can spread to other locations, commonly

including the liver and the spleen (extrapulmonary TB). The disease is spread from person to person through aerosolization of the bacteria when an infected individual expels bacteria, typically through coughing. Although *M. tuberculosis* is highly infectious, a very small proportion of people exposed to it will actually develop disease. It is estimated that the lifetime risk of developing active clinical TB after inhalation of an aerosol from an infectious patient is approximately 10% (Watt, Hosseini, et al. 2009). Those persons that do not develop active clinical disease either clear the bacteria altogether, or the bacteria remain within the host, which then achieves a delicate balance between latency/dormancy, and active infection. Latency is defined by the ability of the host immune system to control over-replication of the bacterium, so that the patient remains free from extensive tissue damage and symptoms (Lawn and Zumla, 2011). This state can remain for many years, with reactivation occurring at a much later date, or not at all. Although an estimated 2 billion people carry *M. tuberculosis* in a latent state, the mechanism of latency is largely unknown (Lawn and Zumla 2011).

Once the bacteria is inhaled into the lungs, it is ingested by an alveolar macrophage. The predominant view of pathogenesis is that *M. tuberculosis,* once within a macrophage, exists within the phagosome and inhibits its fusion with the lysosome, which was first shown by electron microscopy in 1971, and has been corroborated by additional studies since (Armstrong and Hart, 1971, 1975). How the bacillus achieves this function has yet to be understood. Contrary to this "classic" paradigm is a shift toward accepting the idea that *M. tuberculosis* has the ability to escape the phagosome and exist within the cytosol of infected host cells. This is predicted to occur within days of initial infection, and can increase to up to 40% of bacilli surviving in the cytosol, which is visible on EM (Houben, Demangel et al. 2012). There is also some evidence that point to phagosomal damage via specialized secretion system at later time points in the course of infection (van der Wel, Hava et al. 2007; Houben, Demangle, et al. 2012). Although the classic view of TB pathogenesis, and this newer idea of phagosomal escape, seem to be opposing, it is entirely possible that both of these pathogeneses can occur in synchrony.

It is within the macrophage that the bacillus leaves the capillary bed of the alveolus and travels to the interstitium of the lung via airspace surface or lymphatic capillaries (Chakerian, Alt et al. 2002; Wolf,

Desvignes et al. 2008; Urdahl, Shafiani et al. 2011). Once in the lung parenchyma, the bacillus undergoes a battle against resident alveolar macrophages for survival. It becomes a delicate balance of the host adaptive immune system controlling infection with a primary T-cell response, while also controlling inflammation in the host. At some point the bacillus overpowers the host immune system and infection becomes an active disease state, and it remains controversial whether that response is host-driven or bacteria-driven (Orme, 2014). Interstitial inflammation becomes evident as fluid accumulates from leaky vessels, which allows for further accumulation and subsequent infection of neutrophils, monocytes, and macrophages to the site of infection. It is through these infected cells that *M. tuberculosis* can spread to other organs via the blood stream and lymphatic system. Within the lung parenchyma, these cells form a granuloma—which is defined as focal, compact collections of inflammatory cells in which mononuclear cells dominate—and is the hallmark of *M. tuberculosis* infection. The central core is made up of neutrophils that have undergone various stages of degranulation and necrosis- both due to nutrient deprivation (due to low oxygen tension in the core of a granuloma) as well as direct killing of bacilli (Pedrosa, Saunders et al. 2000). This process contributes to the central core of necrosis within the granuloma; as neutrophils degranulate they release enzymes that are damaging to the lung parenchyma and vascular endothelium. The bacterial cell wall of the bacillus is good at scavenging these radicals, but the vascular endothelium of the host is not. The result is severe local microvascular oxidative damage, as well as compression by a growing granuloma. This is an example where one of the primary anti-microbial defenses of neutrophils—production of toxic oxygen radicals and other chemicals—also contributes to the pathology of the disease (Chan, Fujiwara et al. 1989; Palanisamy, Kirk et al. 2011) Additionally, low oxygen tension in the core of the granuloma creates a challenge for host T cells and macrophages to adequately infiltrate the site of infection.

In addition to surviving, the bacillus must also adapt to the changing environment in the host. Bacteria that survive in these conditions enable latency genes (DosR) that allow them to survive undetected by the host immune system for some time (Boon and Dick, 2011). Meanwhile, the host immune system continues to respond with necrosis, which creates a large, cavitated granuloma; allowing

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for escape and transmission of the organism. Although the granuloma is typically viewed as an immune response that is advantageous to the host by isolating an infectious pathogen, *M. tuberculosis* is somewhat unique in that it also benefits from the granuloma by escaping the host immune system if it is able to survive long enough within the central necrotic core. Although granuloma formation is associated with local control of the infection, it is also responsible for transmission in humans and some animals—namely non-human primates. As the core becomes more necrotic it becomes liquid and caseous, and invades into surrounding alveoli and larger airspaces. The thick sputum is then coughed up and produces aerosols of infective droplets. Additionally, by residing in a somewhat protected environment, *M. tuberculosis* is shielded from penetration by antibacterial agents as well. It is common medical knowledge that antibiotics do not penetrate granulomas very well (Ekins, 2014). At this stage of the *M. tuberculosis* lifecycle, the long held belief is that the bacillus will go into a dormant or latent stage within the granuloma (Lawn and Zumla, 2011). One, emerging concept hypothesizes that the bacillus actually lives as an extracellular organism within the central core of necrosis. In order to accomplish this new "lifestyle" the organism scavenges iron and copper, and flourishes on the carbon source provided by cholesterol released from dead and dying neutrophil cell walls until granuloma cavitation and escape (Pandey and Sasseti 2008, Orme 2014). This emerging theory of tuberculosis pathogenesis is gaining merit by Orme and colleagues via the guinea pig model, which emphasizes the potential of the bacillus to be spending greater than 90% of its life in an extracellular phase (Obregon-Henao, Shanley et al. 2012). Whereas the current, widely accepted model emphasizes the latent bacteria living in macrophages, from which they become reactivated and disease manifests itself in the host. An additional challenge to the former model is suggestion that the bacillus may also reside within neutrophils, and that neutrophils do not simply act as scavenging cells (Eum, Kong et al. 2010).

The role of neutrophils is being redefined in the pathogenesis of TB. In a similar fashion, the role of NK cells is gaining more attention in recent years. Several studies have elaborated on Lurie's initial studies in genetically resistant and genetically susceptible rabbits. More recently, there is evidence that shows genetically TB-susceptible rabbits have 20 times more bacilli 7 days after infection when

compared to genetically TB-resistant rabbits. This evidence points to a strong innate response early in infection, because T-cells do not respond within 7 days (Lurie, Zappasodi et al. 1955; Allison, Zappasodi et al. 1962; Dannenberg and Rook 1994). Additionally, NK cells have been implicated in both an innate response and adaptive immune response, and may have an ability to maintain a balance of inflammation in response to *M. tuberculosis* infection (Barnes, Samten et al. 2009). Studies show that *M. tuberculosis*-activated NK cells respond by producing IFN-gamma, which is a proinflammatory cytokine that starts a cascade of signals to induce CD8+ T cells to produce more IFN-gamma and more inflammation (Vankayalapati, Klucar et al. 2004). The same study shows that when NK cells are activated by *M. tuberculosis* stimulated monocytes, they actually lyse adaptive T-regulatory cells, which prevents suppression of the immune response. (T-regulatory cells are the regulatory arm of the adaptive immune response, typically suppressing proinflammatory cytokine cascades.)

Whether or not a person that has been exposed to *M. tuberculosis* develops disease comes down to the cellular level as well. In human populations, the likelihood is that a person will inhale a single bacillus, or few bacilli in a respiratory droplet. If the bacilli survive the trip into the lungs without first being expelled by normal exhalation, they will find their way down to the terminal airway, or alveolus. In the alveolus lives resident macrophages (alveolar macrophages) whose primary role is to phagocytize inhaled particles. Since the number of bacilli a person is exposed to is extremely low in most cases, the macrophage has the advantage. Disease will likely never manifest in these patients due to the low exposure dose and ability of the alveolar macrophages to destroy the bacteria before it can elicit a T-cell immune response. In other cases, perhaps there is a clump of bacteria in the respiratory droplet, rather than one or a few bacilli. Now the odds are in favor of the bacteria surviving, due to sheer numbers. In the event they do survive, it will take several weeks for the immune system to respond, as the doubling time is very slow in *M. tuberculosis*. This latter hypothesis is the current standard for modeling TB in animals. By exposing animals to an increased number of bacilli (~15-20 in guinea pigs, ~25 to macaques, and ~100 to mice), we increase the odds that they will in fact, develop fulminant TB; therefore enabling researchers to study it (Orme, 2014).

1.3 Clinical Presentation

Although TB is predominantly a respiratory disease, it can cause disease in any organ, especially in immunocompromised people; with often-vague clinical signs. Primary TB develops in the lungs after exposure to *M. tuberculosis*, and develops the classic pathology in humans, guinea pigs, and nonhuman primates of caseating granulomas. As the immune system continues responding, the granulomas heal, although rarely ever becoming sterile. Post-primary, adult-type, or secondary TB (all synonymous) occurs in people that have developed immunity to TB (Hunter, Olsen, et al 2011). It is different from primary TB in many ways including clinical presentation. Secondary TB lesions are restricted to upper lung lobes and do not spread to local lymph nodes nor disseminate to other organs, in contrast to primary TB. The majority of patients with secondary TB recover spontaneously without therapy. Interestingly, those that do not recover actually account for the majority of clinical cases and subsequent transmission (Hunter, Olsen et al. 2011).

Another, more rare, form of human TB is the miliary form. It once affected the young most frequently, but with vaccination, and chemotherapeutics as a common treatment modality for TB, the miliary form now affects the middle aged and elderly most frequently. Miliary TB has an interesting pattern of tiny, disseminated, caseous granulomas in the lungs- resembling millet seeds. It is very difficult to diagnose as the onset is insidious and clinical signs are nonspecific. Similar to primary TB, miliary TB can present with an onslaught of non-specific clinical signs including fever, night sweats, anorexia, weight loss, cough, weakness, and several others (Kim, Langston et al. 1990; Mert, Bilir et al. 2001). Kim and colleagues found a miliary pattern is not always detected with chest radiographs, lab data can be inconclusive, and bronchoscopy and biopsy haven't proven to be reliable tools in the diagnosis of miliary TB; and histologic evaluation of tissue biopsy sections is usually necessary to make a rapid diagnosis.

In a study by Kim and colleagues, clinical findings in miliary TB did not differ in symptomology from other forms of active TB. Laboratory data showed mild anemia, leukocytosis was not prominent, but neutrophilia was frequently present (66% of cases). Hypoxemia was often found- with 12/30 patients having a PO2 <60mmHg. Hypocapnia was common as well and the mean Alveolar-arterial gradient was

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45mmHg. Serum sodium was often low and liver function tests were minimally abnormal. Diffuse, polyclonal hypergammaglobulinemia was often seen. Sterile pyuria was found in 32% of cases but did not seem to correlate with the isolation of *M. tuberculosis* from urine. Culture results showed organisms in the sputum of 12/33 patients. Urine and bronchoalveolar lavage fluid infrequently yielded samples positive for acid-fast bacilli. Sputum (76%), gastric aspirates (75%), and urine (59%) were frequently culture positive while cerebrospinal fluid was never positive. A biopsy result showed granulomatous inflammation, but seldom revealed acid-fast organisms and seldom was culture-positive. Bone marrow was histologically abnormal in 41% of cases, with the majority of abnormalities showing noncaseating granulomas. Liver biopsy samples were abnormal in 92% of cases (despite apparently normal liver function tests), showing granulomatous inflammation, but were rarely culture positive. Transbronchial biopsy samples were abnormal in ⅝ cases, three of which were culture positive. Open lung biopsy consistently yielded tissue that was both abnormal histologically and culture-positive.

1.4 Diagnosis

A patient presenting with fever of unknown origin, night sweats, and weight loss must be considered for TB, similar to a patient presenting for persistent and productive cough. Unfortunately, there is no gold standard test with high enough specificity and sensitivity for TB to quickly rule in or out the disease. Further confounding the difficulties of diagnosis are those patients with latent TB or those with subclinical disease. In HIV-positive patients, there must always be a high index of suspicion because the risk of TB is high in this cohort. One study found that conventional screening for cough lasting 2-3 weeks typically has less than 50% sensitivity for active TB, and about 20% of patients positive for TB had no symptoms at all (Getahun, Kittikraisak et al. 2011).

Currently, the diagnostic method of choice for identification of active TB infection is cytology of sputum. In conjunction with sputum smear tests is chest radiography, which is not a sensitive tool alone for detecting pulmonary TB, but is highly suggestive in combination with appropriate history and clinical signs of TB. Sputum smear tests and chest radiography together is the mainstay of diagnostic tests, especially in areas where TB is at the highest prevalence: low-income and middle income-countries

(Lawn and Zumla 2011). Because the detection rate is unacceptably low in areas where TB is the most prevalent, the lack of a sensitive and specific diagnostic test that is economically feasible remains a huge hurdle in the global battle against TB. There is a great need for an accurate point-of-care test that can be used at all levels of the healthcare system and socioeconomic status. Although many tests are commercially available, including serology, line probe assays, automated liquid culture systems, nitrate assays, immune-based assays, and many others—each is not without its shortcomings and lack of practicality (Pai, Zwerling et al. 2008; Lawn and Zumla 2011).

Another diagnostic challenge is identification of latent TB infection (LTBI). The tuberculin skin test (TST) is currently the most commonly used, and oldest, diagnostic test for LTBI; although it is not a perfect method. TST is based on the detection of delayed-type hypersensitivity reactions to purified protein derivative (PPD) injected into the skin. (PPD is made of up a mixture of antigens shared by several mycobacterial species). The site of injection is evaluated 48-72 hours after injection. In a negative patient, the site of injection appears no different than surrounding skin. In a positive patient, the site of injection will have a red, raised appearance of variable character and size resulting from delayed-type hypersensitivity response to the antigen. TST is a very simple test with low cost and can be performed by a trained individual without the need for a specialist or laboratory; which makes it especially attractive in high burden, low income countries where medical resources are limited. Epidemiologic studies have shown that treatment of LTBI based off of a positive TST reduces the risk of active TB by 90% (Ferebee 1969, American Thoracic Society 2009). Although there are some clear reasons for the attractiveness of TST as a diagnostic tool of choice for LTBI, TST does not have good specificity—commonly giving rise to false positive or false negative results for a variety of reasons, and specificity is limited by the cross reaction of PPD with the Bacille Calmette-Guerin (BCG) vaccine (Richeldi 2006). False test results are not benign either; false positive results can cause a person to undergo unnecessary chemoprophylaxis, which has a laundry list of potential toxic side effects. False negative results can delay treatment of TB resulting in worse disease and potentially higher transmission rates. Both false-positive and false-negative

results can increase subsequent morbidity and potential mortality that is avoidable with a more sensitive and specific testing method.

Other diagnostic methods are vast, but none have proven to have high enough specificity, sensitivity, and practicality for use as a widespread diagnostic tool. These tests include the widely touted Interferon gamma assays (IGRAs).The advantages of IGRA testing over TST include requiring only a single visit to a health-care professional, automated testing (which will reduce reader bias), and the ability to screen people repeatedly exposed to TB (Richeldi 2006, Kunst 2006). Some drawbacks to IGRAs include the need to perform a blood test, blood must be processed within 12h of collection, and some laboratory technical expertise is also necessary. Care should be taken when interpreting results of the IGRA test, since a positive IGRA does not necessarily indicate active disease, whereas a negative IGRA does not conclusively rule out active TB. Lastly, and most importantly, these tests can be cost-prohibitive in some regions and are therefore not in widespread use regardless of the potential for early diagnosis of TB.

In 2010, Boeme and colleagues made great strides in advancing the use of nucleic acids for diagnostic purposes. The automated TB assay (Xpert MTB/RIF) utilizes real time PCR to test for the presence of *Mycobacterium tuberculosis* (MTB) and the resistance to rifampin (RIF) - a staple drug used in TB therapy. Although the test is slightly less sensitive than controlled lab studies, the time to receive test results is so much faster than traditional diagnostic methods that it results in faster time to initiate drug treatment. Boeme cited reducing time to start treatment from 56 days to 5 days, and dropout rates from 39% to 15% in patients with smear-negative, culture-positive TB. If more patients can be correctly identified in this manner, despite lower sensitivity than other tests, there is potential to not only cure more patients of disease, but also decrease transmission rates and further spread of disease (Vickerman, Watts et al. 2003).

Researchers are looking to identify biomarkers in patients with active TB not only for diagnostic purposes, but for monitoring and treatment as well. Lawn and Zumla define a biomarker as, "...a characteristic that is objectively measured and assessed as an indicator of normal biological processes,

pathogenic process, or pharmacological responses to therapeutic intervention." Therefore, biomarkers are able to provide information about health status in addition to identifying a disease process, and may be able to help predict reactivation risk, cure, eradication, vaccine efficacy, and endpoints for clinical trials. Tuberculosis-specific biomarkers are needed for many reasons including providing surrogate endpoints, identifying candidate selection for drug discovery, shortening time for licensing of new drugs, development, and assessment of new vaccines, and many other reasons. Researchers are using multiplexed assays to compare gene expression between patients with active TB, patients with latent TB, and healthy people with no exposure to TB (Berry, Graham et al. 2010; Jacobsen, Repsilber et al. 2007; Mistry, Cliff et al. 2007). Additionally, there is evidence that several biomarkers used in proteomics, transcriptomics, and metabolomics may be even more specific when combined with genomics assays (Jacobsen, Repsilber et al. 2007).

1.5 Treatment

First line treatment for TB includes several antibiotics for an extensively long period of time. The mainstay of treatment is rifampin, which works by interrupting bacterial replication through the inhibition of RNA polymerase. This specific medication along with several others including isoniazid, pyrazinamide, ethambutol, and streptomycin make up the anti-TB drug cocktail that is used as the primary line of anti-TB medication in the United States—which are also the targets of some of the most important genes involved in drug resistant strains of *M. tuberculosis* (Centis, Cirillo et al. 2007). One of the significant drawbacks is that this cocktail must be taken daily for many months without interruption. Compliance is difficult, especially in those patients that are drug users and live in impoverished areas, which, not coincidentally, comprise the highest number of TB cases worldwide. Additionally, lack of compliance contributes to increased transmission and drug resistant forms of TB, which are on the rise. Currently there are multi-drug resistant, extensively drug-resistant, and completely drug resistant forms of TB. Treatment of MDR-TB is possible with well-designed treatment regimens and compliance; however it can be difficult to obtain second-line anti-TB drugs that are also considerably less potent, more expensive, and have more severe side effects. Subsequently, the increase and spread of XDR-TB is

correlated with inappropriate treatment of MDR-TB that results in further drug resistance. The appropriate diagnosis and treatment of LTBIs would prevent MDR-TB as well as XDR-TB, and allow the medical community to make significant advances in curing TB globally. As simple as that sounds, employing that strategy is very difficult. Diagnosis of MDR-TB is especially cumbersome due to inadequacies of field sample collection and diagnosis, as well as compliance to treatment regimens. Improvement in diagnostic capabilities paired with new drug development is crucial to gain control over the spread of MDR-TB. New drugs are needed that are less toxic and more powerful against *M. tuberculosis*-- thus reducing duration of treatment and increasing patient compliance. The general principles for choosing an effective treatment regimen against MDR-TB include 1) use at least four drugs certain or highly likely to be effective, 2) do not use drugs for which resistance crosses over, 3) eliminate drugs that are not safe in the patient, 4) include drugs in a hierarchical order based on potency, and 5) be prepared to prevent, monitor, and manage adverse effects for each of the drugs selected (Centis, Cirillo et al. 2007).

Research involving the discovery of new drugs is difficult and time consuming. For example, the two largest trials of isoniazid preventive therapy involved more than 50,000 participants and a 10-year follow up period (Barry, Boshoff et al. 2009). This caliber of patient recruitment and duration of study prevents new advances from coming down the pipeline quickly, despite the desperate need for new treatments. (Interestingly, more research is being conducted to better diagnose latency and to discover biomarkers that identify reactivation of TB rather than new treatments for TB). Some scientists believe that the estimated 2.5 billion people at risk for developing active TB are not actually at risk; that the number is actually much smaller than that (Barry, Boshoff et al. 2009). The trouble lies in detecting those that are truly at risk for active disease, and thus who would benefit from medical intervention. The theory here, is that instead of treating one-third of the world's population with chemotherapeutics, we could target the smaller subset that is actually at risk for spreading the epidemic, and treat only those individuals. Thereby, scientists and medical professionals could more easily customize the course of

chemotherapy to a fraction of people that could have their disease specifically targeted by novel anti-TB chemotherapeutics (Barry, Boshoff et al. 2009).

1.6 Vaccination

In light of the overall inadequacy of global diagnostic and treatment strategies for TB, it becomes increasingly important to prevent the spread of TB in order to eradicate the disease. *Mycobacterium bovis* Bacille Calmette-Guerin (BCG) is the only available vaccine against TB. Since its inception in the 1920's, BCG has been administered to more than 3 billion people, which makes it the most widely used vaccine globally (Anderson and Doherty 2005). Initial studies quickly established BCG's effectiveness in children, and it is widely touted for ending the TB epidemic in Europe. Currently, the WHO recommends vaccinating all infants with a single intradermal dose of BCG in countries with a high risk of TB infection (Anderson and Doherty 2005).

Although BCG is a very well established vaccine, it is not globally accepted as a disease prevention strategy. Early in the history of the BCG vaccine, distribution of the initial strain to many larger producers resulted in genetic drift; thus not all BCG strains are equally effective, and some have shown to have serious negative consequences (Behr 2001). Extensive field trials have shown the efficacy in adults to be anywhere between 0-80%, with the least efficacious being in the regions with the highest TB rate (Fine 1995). It is estimated that globally, the BCG vaccine only prevents about 5% of all possible vaccine-preventable deaths due to TB (World Health Organization 2009). Although the BCG vaccine has evolved over the years, the most common explanation for its lack of efficacy in adults is due to exposure to environmental mycobacteria. Environmental mycobacteria can cross react and mask or inhibit the protective effects of BCG, and each global region has its own environmental strains.

Another downside to vaccination with BCG is that it interferes with one of the main diagnostic tests for TB- the tuberculin skin test (TST). The effects of BCG vaccination can last up to 15 years, and will result in potential false-positive TST, which requires additional resources to differentiate vaccinated versus infected individuals. This is a tremendous hurdle to overcome, especially in the developing world where TB incidence is the highest and the problem is often compounded by co-infection with HIV.

Because it is standard protocol to vaccinate infants in endemic areas, it requires more investment to diagnose a true *M. tubeculosis* infection in adolescents and adults, especially when booster vaccines have been given. Where early treatment would be more advantageous, time is instead spent diagnosing a true positive (or negative) infection.

Although BCG is the most widely used vaccine worldwide, it's mechanism of protection is largely unknown. In mouse models, BCG vaccination reduces bacterial burden and dissemination, but does not protect against infection (Soysal, Millington et al. 2005). In humans, neonatal BCG vaccination can prevent against meningeal dissemination and miliary TB, but does not appear to protect against infection in all cases. BCG may be more protective against developing infection in children that are exposed to *M. tuberculosis*, especially in cases where exposure comes from a live-in family member with active disease (Soysal, Millington et al. 2005). Despite evidence that BCG can be effective in certain subpopulations, the lack of knowledge regarding its mechanism of action makes it difficult to determine which populations would benefit most from prophylactic vaccination.

In spite of improving diagnostics for TB, the need for a novel vaccine to prevent disease in the first place is still paramount. The majority of TB researchers are in agreement that a potent vaccine will be necessary to eradicate the disease, despite intermittent advances in diagnosis and treatment. Vaccine researchers generally focus on one of two methods to create a groundbreaking TB vaccine- either modifying and improving the existing BCG vaccine, or replacing the BCG vaccine altogether. Several methods are being looked into including subunit vaccines, recombinant BCG vaccines, auxotrophic vaccines, atypical mycobacterial strains, DNA vaccines, and heterologous prime boost approach (Tyagi, Nangpal et al. 2011). Leading edge research is making great strides using live mycobacterial vaccines as well as subunit vaccines. Work in animal models have shown that adding deleted genes (due to genetic drift) back into BCG may restore its protective abilities (Behr 2011). Other researchers are using mutants expressing the Listeriolysin O gene from *Listeria monocytogenes* with hopes of damaging the phagosome membrane, which would increase the amount of antigen necessary to mount a strong immune response (Conradt, Hess et al. 1999). With the sequencing of the *M. tuberculosis* genome, researchers working with

subunit vectors have been able to make great strides. Live vector vaccines, including recombinant

vaccinia and adenovirus, have shown promise in animal models and have moved on to human clinical

trials (Scriba, Tameris et al. 2010). Many TB researchers believe that the only way to mount a strong

enough immune response to completely protect against *M. tuberculosis* infection is actually with

modified-live vaccines. And, finally, some researchers are looking into a booster vaccine for BCG.

Recently scientists found that boosting BCG-vaccinated individuals either within a few months of

primary vaccination, or years later, mounts a similar protective response (Scriba, Tameris et al. 2010).

This gives scientists hope that it will be possible to boost the millions of people worldwide that have

already been vaccinated with BCG, protecting adults or elderly in which BCG has waning protection.

1.7 Animal Models

The success in finding a new vaccine for TB relies heavily on animal models to prove concept,

safety and efficacy. Although no animal model is a perfect replica of the immune response that occurs in

a human infected with *M. tuberculosis*, there are several models to choose from that are utilized for

different phases of study, and are imperative for scientific advancement.

The mouse model is the most widely used animal model used in research globally, and the same

is true for TB research. The costs of animals as well as the ease of housing mice in large numbers are

significant reasons why mice are commonly used. More importantly, scientists know a lot about the

mouse genome and there are many inbred strains to choose from. The genome of a mouse can be easily

manipulated to add or delete certain sequences; thus creating a mouse lacking a component of the immune

system, or even lacking a functional immune system altogether. In addition, gene disruption can allow

scientists to evaluate the importance of a single molecule or set of molecules by studying how the host

immune system responds compared to an immunocompetent individual (Orme 2003). Many reagents are

available for use in mice as well, including antibodies, fluorescent stains, and many others for molecular

assays including ELISA, flow cytometry, and immunohistochemistry. Specific to TB, mice are

extensively used to model various aspects of the disease, and are particularly useful in understanding the

basic immunological response to TB. Common studies are those of mutant strains of *M. tuberculosis,*

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immune response, drug safety and efficacy, as well as screening for vaccine candidates. Mice can also be infected by various routes- via aerosol is the most accurate way to mimic transmission in humans. Intravenous, intraperitoneal, subcutaneous, intranasal, and several other routes can also be used to inoculate mice. Following infection, there is dissemination of bacteria to local lymph nodes, which is detectable within 2 weeks of infection (Flynn 2006). Similar to humans, dissemination to the spleen and liver is common in mice. The pathologic response to *M. tuberculosis* is slow in mice, as it is with humans, and will eventually lead to death of the mouse if allowed to progress to advanced stages. In more recent years, scientists have shown that mice have a quiescent or latent form of TB as well. While in a chronic stage, bacteria replicate at a reduced rate. And, similar to humans, if they become immunosuppressed or the immune response is interrupted experimentally, mice will have a robust reactivation of infection and succumb to fulminant TB (Flynn 2006).

Although there are clear advantages to using mice in TB studies, there are also some significant drawbacks. The size of the mouse poses a real challenge when studying pulmonary disease. The mouse bronchial tree is not only very small, but less complex as well. It lacks accessory vasculature and has limited alveolar lymphatic drainage. Additionally, a true latent form of TB is difficult to replicate in mice. Although they do show a chronic stage of infection where the mycobacterial organism replicates at a much slower state, mice are not in a truly latent stage where replication has ceased completely. In humans in a latent stage, bacterial numbers are undetectable. In mice, infection is well controlled, but bacterial numbers remain relatively high. Because there is constant bacterial load, the disease is always progressing in mice, albeit slowly (Flynn, Capuana et al. 2003; Flynn 2006).

Another significant drawback to using mice in TB studies is the difference in pathology between mice and humans. Humans develop distinct pulmonary granulomas in response to *M. tuberculosis* infection. The mouse response to *M. tuberculosis* infection is similar in that it includes granulomatous inflammation, but there is not a true tubercle or granuloma. The human granuloma is well circumscribed with a central core of neutrophils and lymphocytes, surrounded by macrophages (Pedrosa, Saunders et al. 2000). In contrast, the mouse develops a more diffuse granulomatous inflammation that lacks the

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structure of a true granuloma. While it still contains lymphocytes and macrophages, it is not organized into a well-circumscribed lesion. Additionally, granulomas in humans can become caseous and necrotic, fibrotic, calcified, or even cavitary. And any one of these granuloma types can coexist in a human lung infected with *M. tuberculosis* at any given time. The mouse does not have different stages of granulomatous inflammation. Instead, there is little heterogeneity in the granulomatous response, and it does not typically become necrotic or cavitated (Orme 2003). Because the various stages of granulomas in the human creates varied microenvironments for the bacillus at the same time, it is important to be able to model this type of bacterial pathogenesis and effects due to said microenvironment. Because the mouse does not display this type of pathology, they become a less than ideal model for pathogenesis studies.

Rabbits are a significant historical animal model used for TB research. Lurie and colleagues were able to identify genetically susceptible and resistant rabbit strains, but those strains do not exist today. Rabbits are relatively resistant to infection by *M. tuberculosis* naturally, although certain clinical strains TB may confer infection in some rabbits (Flynn 2006). For most TB studies in rabbits, *M. bovis* is used for infection and/or challenge, and response to infection with *M. bovis* is similar to the human response to *M. tuberculosis*. Rabbits develop caseating necrotic pulmonary granulomas that may develop cavitation, as well as tuberculous meningitis as seen in children (Flynn 2006).

Downsides to studying rabbits in this fashion include the difficulty of housing in groups per animal welfare regulations, as well as the expense. Additionally, there are limitations regarding available reagents used for rabbits-- since they are not commonly used, there is not a pressing demand to develop more reagents for immunoassays and other assays.

Non-human primates have been used historically for TB research, both for vaccines and novel drug testing. Monkeys are natural hosts of the disease, which makes them excellent for studying transmission as well as pathogenesis, as it is very similar to the human immunological response to *M. tuberculosis* infection. Historical data shows that macaques were highly susceptible to infection, but recent data shows that, similar to humans, the results of exposure can vary widely (Capuano, Croix et al. 2003; Flynn, Capuano et al. 2003; Gormus, Blanchard et al. 2004; Flynn 2006). Additionally, human

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criteria for latent *M. tuberculosis* infection can be applied to monkeys. Humans with latent TB are defined as infected by positive, delayed-type hypersensitivity reaction to mycobacterial antigens (or have a positive PPD as discussed earlier). Monkeys with a positive PPD and negative clinical signs are also classified as latently infected. Additionally, a monkey with latent TB infection can spontaneously reactivate or be induced to reactivate through immunosuppression. This feature is also identical to human TB pathogenesis. More importantly, the pathology observed in monkeys is nearly identical to the pathology seen in humans-- granuloma lesions can become solid and have a necrotic, caseous center with variable amounts of fibrosis and cavitation; and a spectrum of granuloma progression can exist in the same monkey concurrently (Capuano, Croix et al. 2003).

The fact that non-human primates are the only true animal models of latent tuberculosis, and the disease pathogenesis is nearly identical to human pathogenesis, makes them quite the attractive model. Unfortunately, there are some very significant drawbacks to using non-human primates as a model for TB that have made their use in TB studies very limited. The expense and difficulty of housing these species are large hurdles to overcome. Primate facilities need to be specifically designed and constructed to maintain safety to the animals as well as the humans involved, but also to prevent escape. Primate facilities also require a very large staff of animal caretakers, veterinarians, veterinary technicians, and animal behaviorists dedicated solely to primate health and enrichment. Because primates transmit TB infection via aerosol (unlike other animal models), their use necessitates housing at an ABSL-3 level of biocontainment. On top of housing difficulties, many facilities are reluctant to conduct research that could be the potential nidus of a large-scale TB outbreak (to either humans or primates) if a break in security were to occur.

Guinea pigs are extensively used in TB research, partly because of their sensitivity to fulminant infection. Once exposed via aerosol with a low dose, guinea pigs develop progressive and fatal disease usually within a year (Basaraba et al. 2006). Their lung pathology more similarly reflects that of human infection, contrary to other rodent counterparts. The granuloma developed in guinea pigs is well circumscribed with a central core of heterophils (the guinea pig equivalent to human neutrophils) with a

surrounding population of lymphocytes and macrophages. The granuloma can also become centrally necrotic and cavitated with varying degrees of fibrosis and calcification. The granulomas are not as protective to the guinea pig as in humans; as they do not adequately contain the bacteria. Because of their pattern of susceptibility to low-dose infection and subsequent development of fulminant TB, guinea pigs are often thought to be the most sensitive animal model to tuberculosis infection. Due to their high susceptibility and inadequacy in controlling infection, guinea pigs are thought to be an ideal animal model for vaccine studies with the rationale that the best vaccine needs to protect against the most susceptible host (Flynn 2006). Guinea pigs have also demonstrated their use as a model of dissemination, as late stage TB is often found not only in the lungs of guinea pigs, but also commonly in the liver, spleen, and lymph nodes, and less commonly in the eye, adrenal gland, mammary chain, eye, and brain (Palanisamy, Smith, et al. 2008)

Guinea pigs are relatively easy to house and care for. They are naturally a very docile and social species that are inexpensive to obtain and easily housed in ABSL-3 conditions (which are recommended but not required). They pose very minimal health risks to their human caretakers aside from the development of allergies to dander, as they are not aggressive and rarely bite, and do not naturally harbor zoonotic diseases. Guinea pigs are large rodents but small animals which make them easy to handle as well.

The downsides to using guinea pigs as a model of TB infection are minimal but mainly include the lack of useful reagents, similar to the problem in rabbits. In contrast to the murine model, which has an abundance of reagents, immunological reagents for guinea pigs are not as readily available. Although development of new reagents for guinea pigs is ongoing, the availability of materials for immunoassays including flow cytometry and immunohistochemistry still remain limited.

1.8 Hypothesis and Specific Aims

Guinea pigs are used commonly as an animal model used for tuberculosis vaccine candidates. One caveat to using guinea pigs is their strong behavioral tendency as a prey species to mask disease until it is very severe. When evaluating animals that have been infected with *M. tuberculosis*, it is important to

be able to critically evaluate their health status-- not only for efficacy data, but also to provide the most humane care to animals used in research. Although we know guinea pigs have been experimentally infected, the progression of disease is often unknown and often quite variable. Vaccine candidates can range from highly efficacious to minimally protective, and health monitoring is one of the parameters that is used to evaluate each test vaccine. Currently, guinea pigs may or may not lose weight in response to infection, and weight loss does not always correlate to severity of disease. Additionally, many guinea pigs do not have evidence of premonitory signs, including decreased activity, cyanosis, dyspnea, or tachypnea.

In both human and animal medicine, the first step to recognizing deficiencies in organ function is to take blood and urine samples and evaluate them for a complete blood count, serum biochemistry, and urinalysis. Parameters within these assays are often used to diagnose systemic disturbances such as renal disease or hepatocellular disease. However, these biomarkers also have the potential to be interpreted as criteria for humane endpoints in guinea pigs experimentally infected with *M. tuberculosis.* It therefore became our focus to utilize these simple, minimally invasive tests not to diagnose systemic organ dysfunction, but to determine an objective set of criteria using testable biomarkers that could be used to establish humane endpoints, as well as to examine correlation with severity of disease.

Central Hypothesis: If we evaluate urine and blood parameters in conjunction with temperature and body weight over the life of the guinea pig, then we will be able to identify biomarkers that can be used to establish early endpoint criteria in the guinea pig model of tuberculosis.

Specific Aims:

I- We will collect urine and blood for serum biochemistry, complete blood count, and urinalysis every thirty days over the life of guinea pig—either vaccinated with BCG or saline-treated, and challenged with *M. tuberculosis.*

II- We will use gross necropsy, organ CFU counts, and histopathology to characterize severity and dissemination of TB-related disease in BCG-vaccinated or saline-treated guinea pigs.

III- We will collect blood under various anesthetic regimens to assess the effects of each anesthetic on a normal, naïve guinea pig

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Chapter 2. Methods

2.1 Animals

Female, out-bred Hartley guinea pigs weighing 450-500 grams were purchased from Charles River Laboratories (Wilmington, MA). Guinea pigs were maintained under animal biosafety level 3 barrier conditions in isolator cages (Thoren, Hazleton, PA) for the entire period of the experiment. They had access to standard guinea pig chow (Harlan, Madison, WI), water ad libitum, and daily hay cubes (Rocky Mountain Lab Supply, Centennial, CO).Guinea pigs were housed on ⅛" corn cob bedding (Harlan, Madison, WI) with red huts (Bio Serve, French Town, NJ) for enrichment. All animals were pair housed until inoculated, then singly housed until the end of the experiment. All experimental procedures were approved by the Colorado State University Institutional Animal Care and Use Committee.

2.2 Mycobacterium species

Mycobacterium bovis BCG Pasteur (TMCC 1011) strain was grown in Proskaur and Beck (P&B) medium with 0.1% Tween 80 (Sigma, St. Louis, MO) to mid-log phase (Appendix A, Izzo Lab SOP). Aliquots were stored at -80C and thawed before use. *M. tuberculosis* H37Rv (TMCC 102) was initially grown for three passages as a pellicle on P&B medium to produce seed stocks. Working stocks with a maximum of six passages were expanded from the seed stocks in P&B medium with 0.1% Tween 80. Working stocks were prepared at the mid-log phase, and aliquots were stored at -80C (Appendix A, Izzo Lab SOP).

2.3 Vaccinations and Infection

Ten guinea pigs were vaccinated with 10^3 CFU of BCG Pasteur and an additional 10 guinea pigs were treated with saline via the intradermal route on the ventrum, using a tuberculin syringe attached to a 26-gauge, ½" needle. Two weeks after vaccination, guinea pigs had blood collected for baseline values. Guinea pigs were then allowed to rest for 8 weeks before they were exposed to 10-20 CFU of *Mycobacterium tuberculosis* H37Rv via the respiratory route using a Madison aerosol chamber (Madison,

WI). Working stocks of *M. Tuberculosis* H37Rv were diluted to 10^6 CFU/ml in sterile distilled water and placed in the nebulizer jar. Animals were exposed to the aerosol for 5 minutes. Animals were then returned to their home cages (Appendix A, Izzo Lab SOP).

2.4 Experimental Design

Guinea pigs were monitored daily for the duration of study. Body temperature was measured daily to track progression of disease using an RFID microchip (IPT-300 Bio Medic Cada Systems [BMDS], Inc, Seaford, DE) implanted subcutaneously over the dorsum (Appendix A, Izzo Lab SOP). Body temperature of individual animals were assessed each day at approximately the same time of day using a DAS-6006/7 scanner transponder (BMDS). Body weight was measured weekly. Prior to inoculation and every 30 days after, guinea pigs were lightly anesthetized with isoflurane gas at 4-5%, with an O2 flow rate of 0.75-1.0L/minute, and placed in dorsal recumbency. Urine was collected in a sterile polystyrene tube as it was voided upon induction of anesthesia. 2.5-3.0ml blood was collected from the cranial vena cava under isoflurane anesthesia using a 26 ⅝ gauge, 0.5" needle attached to 3ml Luer lock syringe (BD Biosciences, Mountain View, CA). Blood was immediately transferred into appropriate tubes containing no additive, heparin, or potassium EDTA (BD Biosciences, Mountain View, CA). Blood for hematology was submitted in an EDTA tube to the Colorado State University Diagnostic Lab and run on an ADVIA 120 Hematology System (Siemens, Loveland, Colorado). Blood for serum chemistry was run on an Abaxis VS2 (Abaxis, Union City, CA). Blood was collected from the EDTA tube for blood gas analysis, and was run cage-side upon completion of collection on an Abaxis iStat (Abaxis, Union City, CA). Urine was analyzed using a dipstick (Fisher Scientific, Pittsburgh, PA). The color change was noted using the chart on the dipstick bottle. If leukocytes were positive, the urine was to be plated and characterized for gram positive or negative cell wall. No urine samples had positive growth, so the culture procedure was not performed.

An additional 45 naïve guinea pigs were used as anesthetic controls. Each guinea pig had blood collected for complete blood count, serum chemistry, and blood gas analysis under the following conditions: 1) alert, 2) isoflurane anesthesia, and 3) ketamine and xylazine anesthesia. For alert blood

draws, guinea pigs were placed in dorsal recumbency with a towel wrapped around the caudal half of the body to prevent excessive movement. The handler held the forepaws between the thumb and forefinger, and placed them caudal to the shoulders in a flexed position in line with the body. The blood collector restrained the head with the index finger behind the occipital crest and the thumb gently across the rostral mandible to stabilize the head. Once the alert guinea pig was adequately restrained, blood was collected from the cranial vena cava in the same manner as previously described. Blood collection under isoflurane anesthesia was performed in the same manner as previously described. Guinea pigs were allowed at least one week rest between blood collection under Isoflurane anesthesia and ketamine and xylazine anesthesia to allow for adequate washout. Ketamine (MWI, Boise, Idaho) and xylazine (Akorn, Inc., Decature, IL) were administered using 26 ⅝ gauge, 0.5" needle attached to 1ml Luer lock syringe (BD Biosciences, Mountain View, CA) into the muscle of the proximal hind limb. Guinea pigs were manually restrained for this procedure and immediately placed back into their home cage until the anesthetic took effect. Once guinea pigs were adequately anesthetized, as determined by lack of a response to toe pinch, blood was collected and analyzed for complete blood count, serum chemistry, and blood gas analysis as previously described.

2.5 Necropsy and Histology

Guinea pigs were allowed to survive until they met an established set of endpoint criteria implemented by the animal care and use committee. Criteria included being moribund or exceeding 15% weight loss, and /or showing evidence of respiratory compromise (increased respiratory effort and/or rate). Guinea pigs were sedated with ketamine (MWI, Boise, Idaho) and xylazine (Akorn, Inc., Decature, IL) administered intramuscularly into the proximal hind limb. Once at a surgical plane of anesthesia (assessed by lack of response to toe pinch), guinea pigs were placed in dorsal recumbency. The ventral thorax was saturated with 70% isopropyl alcohol (Sigma-Aldrich, St. Louis, MO) and blood was collected from the heart using a 3ml Luer lock syringe with 20 gauge, 1" needle attached. Blood was then transferred to appropriate tubes for complete blood count, serum chemistry, and blood gas analysis as previously described. Guinea pigs were then euthanized with intracardiac administration of sodium

pentobarbital (MWI, Boise, ID) (Appendix A, Izzo Lab SOP). Organs were harvested for both culture

and histology. The right cranial lung lobe, half of the spleen, one liver lobe, one kidney, half the brain,

and one eyeball were evaluated for CFU numbers. Bacterial load was determined by plating organ

homogenates onto nutrient 7H11 agar supplemented with oleic acid-albumin-dextrose-catalase (Sigma,

St. Louis, MO). Colonies were enumerated after 21 days of incubation at 37C. The remaining lungs,

spleen, liver, kidney, brain and eyeball were evaluated for histology, as well as the stomach, small

intestine, colon, cecum, and mediastinal and mesenteric lymph nodes. Organs were placed into formalin

for 2 weeks, then trimmed for histology. Tissues were embedded in paraffin and sections cut and stained

with hematoxylin and eosin (H&E; IHC Tech, Aurora, CO). Photomicrographs were taken using an

Olympus BX41 microscope attached to a Dell Precision computer with DP2-BSW software for image

capture.

2.6 Statistics

Statistical analysis was done using SAS 9.3. A separate repeated measures analysis was

performed for each response variable. Response variables included blood urea nitrogen (BUN),

creatinine, albumin, globulin, total protein, alkaline phosphatase (ALP), alanine aminotransferase (ALT),

total leukocytes, hematocrit, heterophils, lymphocytes, and monocytes. The within-subjects factor is time

(0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, and 420 days) and the between-subjects

factor is treatment (BCG or Sham). A time*treatment interaction term was also included in the model and

a random effect for animal (nested within treatment) was used to account for repeated measures. Contrasts

were used to estimate and test comparisons of interest. A Tukey adjustment was used to control for

multiple testing (separately for each response variable).

In addition, a repeated measures analysis based on differences (versus pre-challenge) was also

conducted. Using only the first and last (scheduled) measurement for each animal, a paired t-test was

performed for each response variable. (This analysis is looking for overall changes between first and last

scheduled values.) In addition, a two-sample t-test was run for each response variable to look for

differences between the two treatment groups. The difference was calculated as Last-First. (This analysis

is looking for differences between the two groups.) Using Pearson (standard) correlation, the correlation between lung CFUs and survival time, and spleen CFUs and survival time were assessed.

Blood parameters were also analyzed in naïve guinea pigs to determine the effects of anesthetic protocol on serum biochemistry, complete blood count, and blood gas parameters. Statistical analysis was done using SAS 9.4. A separate repeated measures analysis was performed for each response variable. The within-subject factor is treatment. Pairwise comparisons were used to estimate and test differences between the treatments. A Tukey adjustment was used to control for multiple testing—separately for each response variable.

Guinea pigs were divided into two groups, ten of which were in the BCG-vaccinated group and ten were inoculated with saline. All guinea pigs were micro-chipped and vaccinated 10 weeks prior to challenge, then bled for baseline blood values 8 weeks prior to challenge. All guinea pigs were challenged with approximately 10-20 colony forming units (CFU) of *Mycobacterium tuberculosis* H37Rv. Every thirty days after challenge, all guinea pigs were bled for complete blood count (CBC), serum biochemistry, and venous blood gas, and urine was collected and evaluated for proteinuria, pH, urine specific gravity, ketonuria, white blood cells, and red blood cells. Guinea pigs were euthanized based on established criteria for humane endpoints, which included, but was not limited to, weight loss >15% and respiratory distress. Based on these criteria, guinea pigs were euthanized at various times from 67 days to 441 days post challenge. Four guinea pigs, all in the BCG-vaccinated group, lived to 441 days without clinical signs. At this time these 4 guinea pigs were considered survivors and the study was terminated.

3.1 Serum Biochemistry and Urinalysis

Blood Urea Nitrogen (BUN) and Creatinine

We found that guinea pigs had significant changes in BUN between groups only at the day 90 and 150 time points (Fig 1a). They were similar at the pre-challenge time point, and the changes did not correspond to any significant events clinically. Within the saline-treated group there were no significant differences between time points compared to pre-challenge values. The BCG group did, however, show some changes. There was a significant decrease in the BCG group at days 150, 180, 240, and 270 compared to pre-challenge values (Fig 1a). Infection resulted in an initial decrease in BUN in both groups, suggesting a similar metabolic response, independent of the immune status at the time of infection. From days 60 to 90, BCG-vaccinated guinea pigs recovered their BUN levels to pre-infection levels, while the saline-treated group did not. Therefore, early serum BUN may become an early marker

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of a difference between vaccinated and non-vaccinated guinea pigs, but more guinea pigs will need to be tested. In addition, there was a significant effect of treatment over time in the BCG group (Fig 1b).
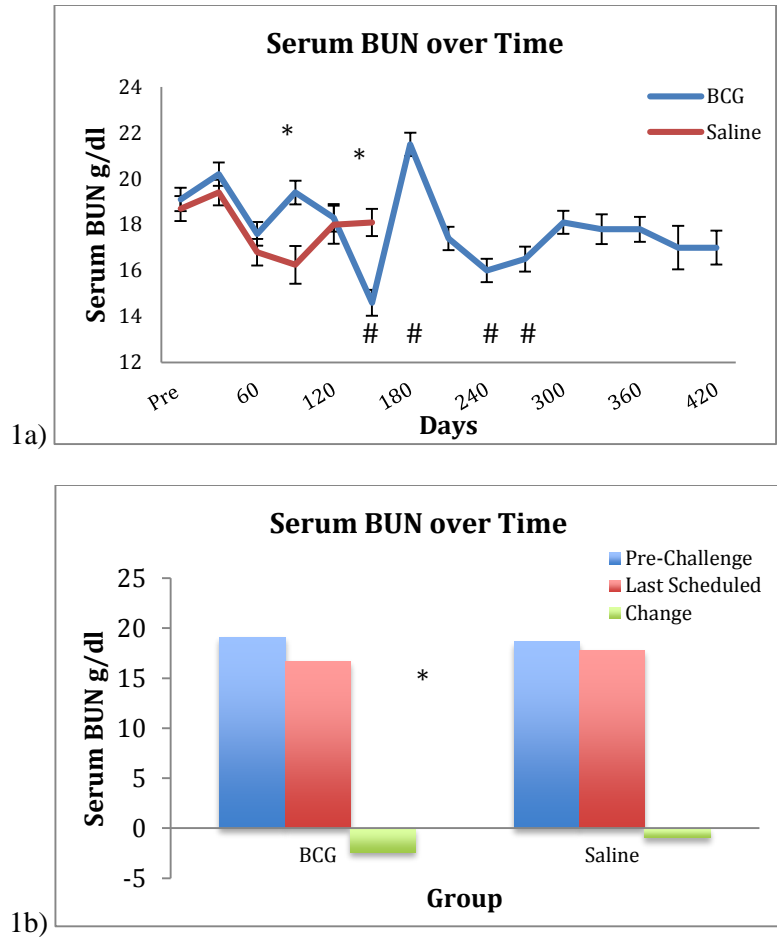

1a)

1b)

Fig 1 a) Comparison of serum BUN in BCG-vaccinated and saline-treated animals from pre-challenge through the last scheduled blood draw. Significant differences (*) occur comparing between groups at day 90 (p = 0.02) and day 150 (p = 0.04). There are no differences within the saline-treated group comparing pre-challenge BUN to each time point thereafter. There are significant differences (#) within the BCG-vaccinated group comparing pre-challenge BUN to the following time points at 150d (p <0.01), 180 (p = 0.02), 240 (p <0.01), and 270 (p = 0.015). **(b)** Comparison of serum BUN between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change calculated as (Last Scheduled – Pre-Challenge). There was a significant effect (*) of treatment over time between groups (p = 0.01)

Serum creatinine was measured with little statistical significance between groups, for which the only time point was day 150 (Fig 2a). The saline-treated group showed a gradual increase in serum creatinine while the BCG-vaccinated group remained fairly consistent throughout the experiment (Fig 2a). Additionally, there was a significant effect of treatment over time, indicating the increase between the last-scheduled and pre-challenge serum creatinine value was bigger in the saline-treated group (Fig 2b).
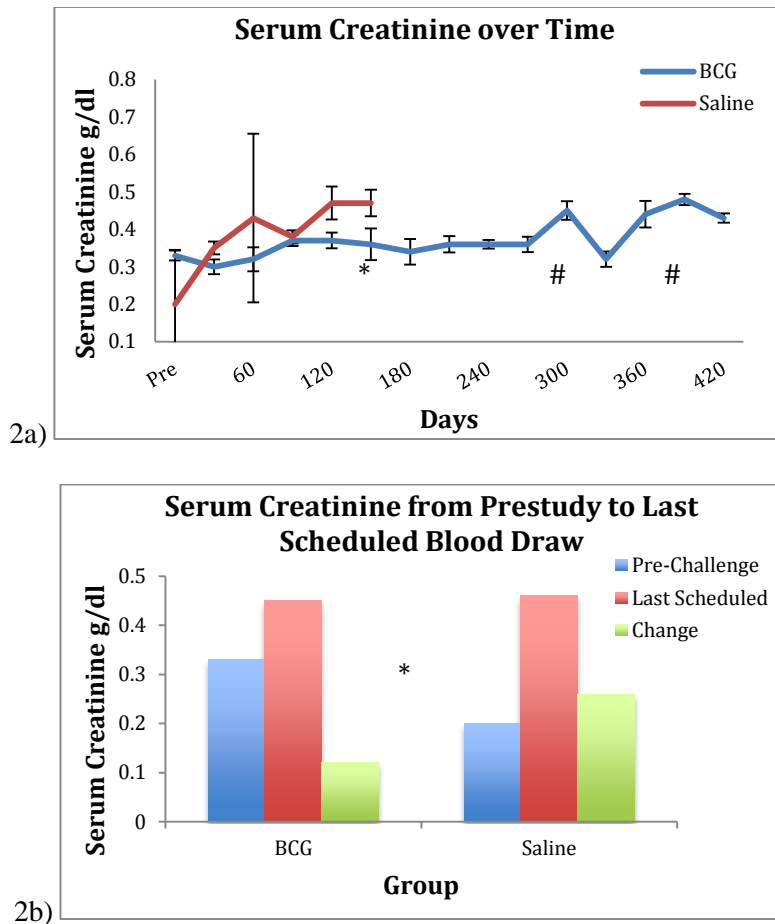
Serum Creatinine over Time

2a)

Serum Creatinine from Prestudy to Last Scheduled Blood Draw
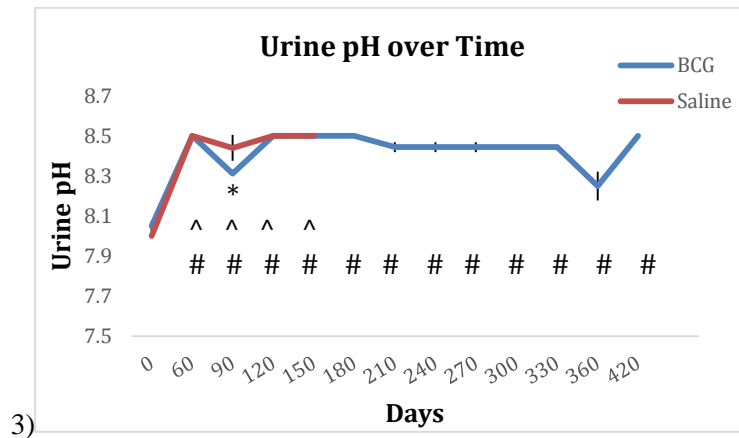
2b)

Fig 2 a) Comparison of serum creatinine in BCG-vaccinated and saline-treated animals from pre-challenge through the last scheduled time point. Significant differences (*) occur comparing between groups at day 150 (p <0.01). There are significant differences (^) within saline-treated group compared to pre-challenge creatinine to day 150 (p <0.01). There are significant differences (#) within the BCG-vaccinated group comparing pre-challenge to days 300 (p = 0.05) and 390 (p = 0.04). **b)** Comparison of serum creatinine between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change calculated as (Last Scheduled – Pre-Challenge). There was a significant effect (*) of treatment over time between groups (p <0.01).

Urine

Urine was collected as it was voided pre-challenge and every 30 days post infection. It was then evaluated using a urine dipstick for parameters including urine pH, urine specific gravity, white blood cells, red blood cells, protein, ketones, and bilirubin. Consistently throughout the study, regardless of time point or vaccination status, guinea pigs had a urine pH of 8.0 or 8.5 and urine specific gravity of 1.008 to 1.035. Protein was found randomly and in variable amounts from mild to excessive, while white blood cells, red blood cells, ketones, and bilirubin were never present; therefore statistics were completed for urine pH, urine specific gravity, and urine protein. Urine collection was attempted on every animal at every time point, but some animals failed to void enough urine to analyze at various times. In addition,

there was no urine analyzed at the 30 day time point due to inability to acquire urine dipsticks in time for collection.

The only observed outcomes for urine pH were 8 or 8.5 due to the nature of analysis using urine dipsticks. However, there was statistical significance comparing groups at the day 90 (p = 0.03) due to a higher average urine pH in the saline-treated group (Fig 3) . Within the both groups there was significance compared to pre-challenge values at all following time points (saline-treated p < 0.01, BCG-vaccinated p< 0.02). Statistically, this is a reflection of the fact that all guinea pigs had a pre-challenge urine pH of 8.0, and a urine pH of 8.5 at most of the following time points.

3)

Fig 3 Comparison of urine pH in BCG-vaccinated and saline-treated animals from pre-challenge through the last scheduled blood draw. Significant differences (*) occur comparing between groups at day 90 (p= 0.03). There are significant differences (^) within saline-treated group compared to pre-challenge urine pH at all the following time points (p <0.01). There are significant differences (#) within the BCG-vaccinated group comparing pre-challenge at all the following time points (p < 0.01).

There were a few of very influential outliers with very high protein values that are driving the urine protein results, but did not appear to be associated with a better or worse outcome (Fig 4a and 4b). However, the very high urine protein values appeared at the same time point that the urine specific gravity decreased in the saline group (at 60 days, Fig 5a), but normalized afterward. These events could be related and require further investigation. Comparing groups, there were significant differences at days 60 and 120 (p < 0.01), with the saline group showing an increase at both time points. Within the saline-treated group, there were significant differences as days 60 and 120 (p < 0.01) compared to pre-challenge values. Within the BCG-vaccinated group, there were significant differences at days 210 and 300 (p < 0.05) compared to

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pre-challenge values. Lastly, there was a significant effect of treatment over time in the saline-treated group (Fig 4b).
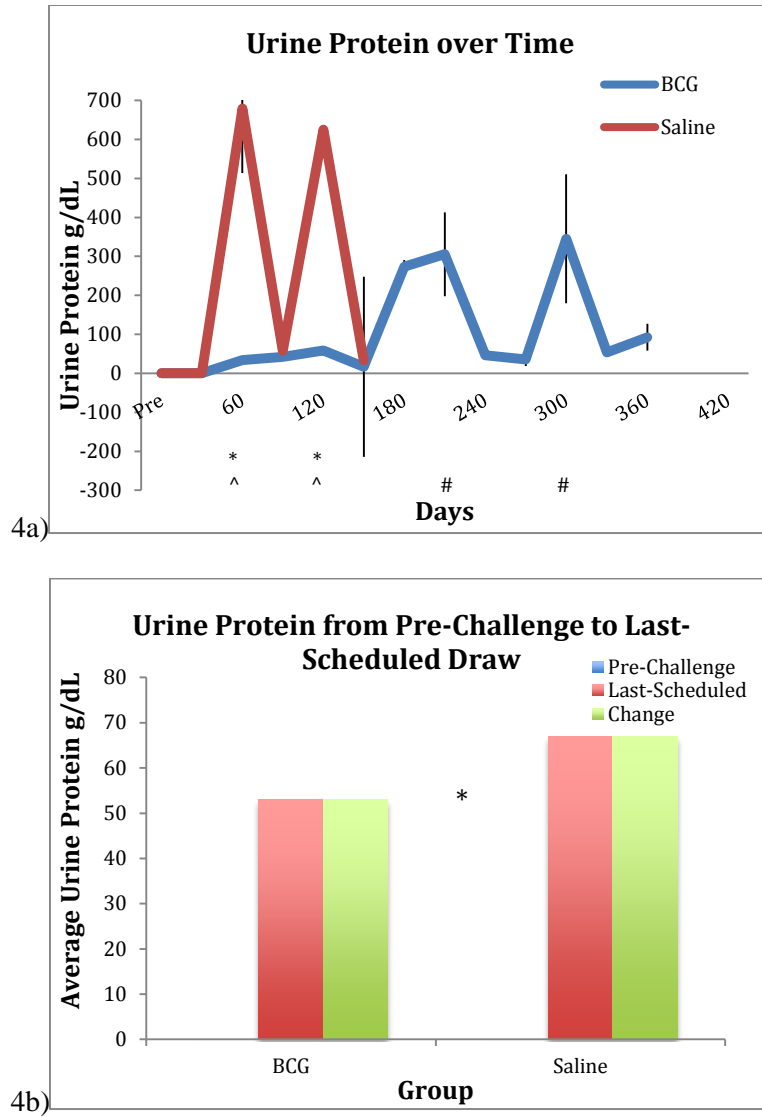

4a)

4b)

Fig 4 a) Comparison of urine protein in BCG-vaccinated and saline-treated animals from pre-challenge through the last scheduled time point. Significant differences (*) occur comparing between groups at days 60 (p <0.01) and 120 (p = 0.01). There are significant differences (^) within saline-treated group compared to pre-challenge creatinine to days 60 and 120 (p <0.01). There are significant differences (#) within the BCG-vaccinated group comparing pre-challenge to days 210 (p = 0.05) and 300 (p = 0.04). **b)** Comparison of serum creatinine between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change calculated as (Last Scheduled – Pre-Challenge). There was a significant effect (*) of treatment over time between groups (p = 0.02).

There were more influential changes when evaluating the urine specific gravity both between groups and within groups. Between groups there was a significant difference at day 60. Within both groups, there is a decrease comparing the pre-challenge value to all the following time points (Fig 5a).

Additionally, there was a significant effect of treatment over time in the saline-treated group (Fig 5b). This indicates there was a decline in the USG post infection, although this decline was greater in the saline-treated group at day 60. The USG also declined at a slower rate in the BCG-vaccinated group.
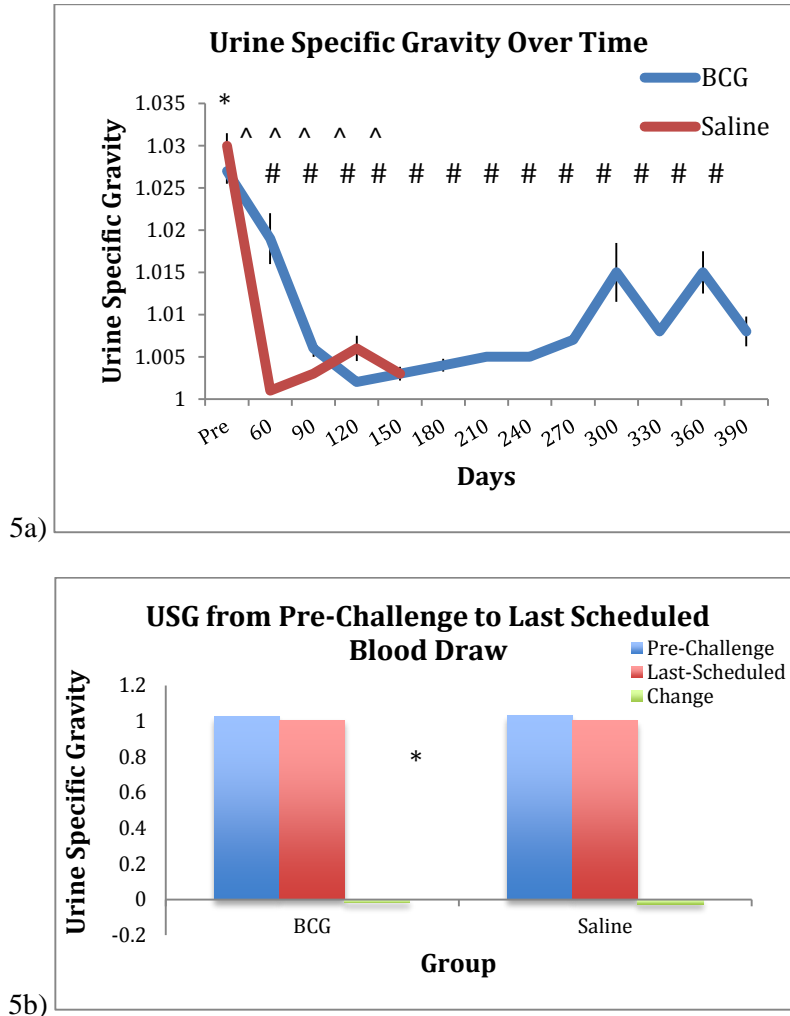

5a)

5b)

Fig 5 a)- Comparison of urine specific gravity (USG) in BCG-vaccinated and saline-treated animals from pre-challenge through the last scheduled time point. Significant differences (*) occur comparing between groups at day 60 (p <0.01). There are significant differences (^) within saline-treated group comparing pre-challenge USG to all the following time points (p <0.01). There are significant differences (#) within the BCG-vaccinated group comparing pre-challenge to all of the following time points except day 60 (p < 0.01). Note SE bars are graphed, but SE were so small compared to the scale on the chart that most of them are not visible. **b)** Comparison of urine specific gravity between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change calculated as (Last Scheduled – Pre-Challenge). There was a significant effect (*) of treatment over time between groups (p <0.01).

Albumin

There is a significant difference in albumin levels between groups of guinea pigs at the pre-challenge time point, due to a lower concentration serum albumin in the saline-treated group (Fig 6a).

This difference prior to infection may be an indication of effect of prior vaccination with BCG on serum albumin levels. Within the BCG-vaccinated group, the animals showed significant differences at all time-points when compared to pre-challenge (Fig 6a). This is, in part, due to a fluctuating change in albumin, which starts as a decrease, then a slight overall increase after day 30. Although the trend is increasing after 30 days in the BCG-vaccinated group, the overall change is still a decrease due to the sharp decrease within the first 30 days. When evaluating the saline-treated group, there is also a significant decrease within the first 30 days of infection, but then a steady increase, which is larger in proportion to the BCG-vaccinated group (Fig 6a). Overall, the trend is the same in both groups- with a decrease within 30 days, then a steady increase, but the degree of increase of albumin in the saline-treated group is larger compared to the BCG-vaccinated group (Fig 6b).

The paired results show that, when comparing pre-challenge to the last scheduled blood draw, there is a significant difference in all animals, indicating they do change in over time when comparing pre-challenge serum albumin concentrations (Fig 6b). Additionally, there is a difference when comparing the same values between BCG-vaccinated and saline-treated groups. This corroborates the notion that the BCG-vaccinated group is somewhat protected from developing severe disease, and shows less of a change than the saline-treated group.
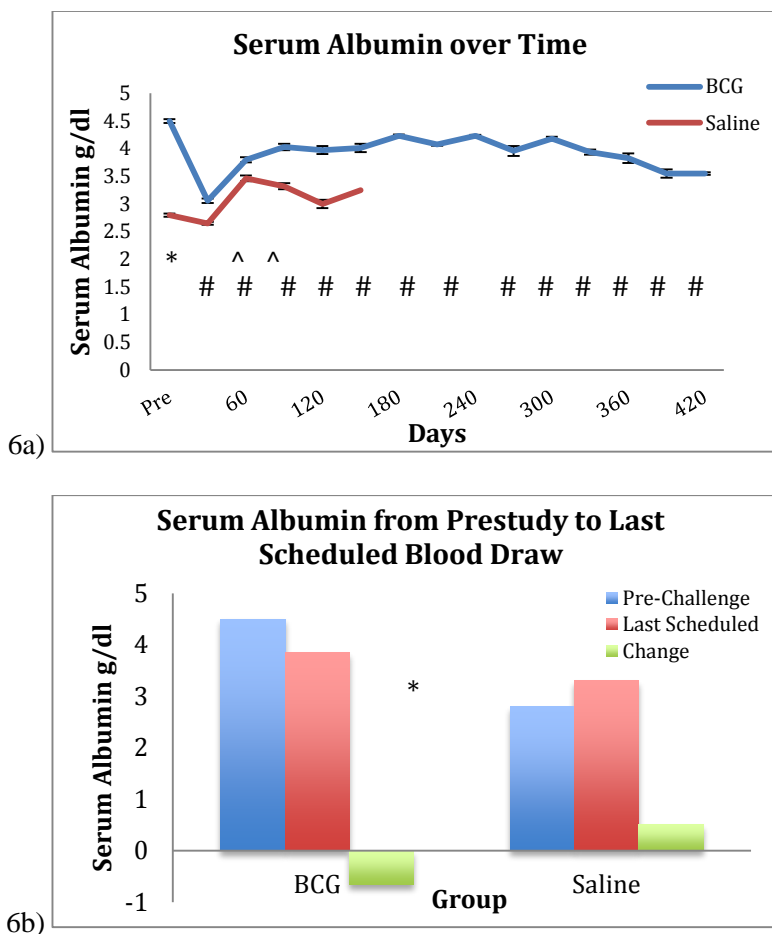
6a)

6b)

Fig 6 a) Comparison of serum albumin in BCG-vaccinated and saline-treated animals from pre-challenge through the last scheduled blood draw. Significant differences (*) occur comparing between groups only at the pre-challenge time point ($p < 0.01$). Significant differences (^) occur within the saline-treated group comparing pre-challenge values to days 60 and 90 ($p < 0.01$). Significant differences (#) occur within the BCG-vaccinated group comparing pre-challenge values to all the following time points, except day 240 ($p < 0.01$, at day 180 $p = 0.03$) **b)** Comparison of serum albumin between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change calculated as (Last Scheduled – Pre-Challenge). There was a significant effect of treatment over time between groups ($p < 0.01$).

Globulin

Serum globulin levels are significantly different between groups prior to inoculation with *M. tuberculosis*. On the contrary, the saline-treated group had a lower level of pre-challenge serum globulins, which is likely indicative of a more physiologic level in guinea pigs. There were also significant differences between groups at times 90, 120, and 150 days post inoculation (Fig 7a).

Within the saline-treated group, there are significant changes from pre-challenge compared to all following time-points, indicating that serum globulin levels change dramatically within 30 days of infection and continue to rise thereafter (Fig 7a). Significant changes occur within the BCG-vaccinated

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group within 30 days as well, first decreasing from pre-challenge values. However, after 180 days, the serum globulins increase resulting in no statistically significant difference later in the course of disease compared to pre-challenge values, except at day 390 (Fig 7a). Graphically this is show as the BCG-vaccinated group having a decrease in serum globulins during the first 90 days of infection, which then recovers to almost pre-challenge values. While in the saline group, the serum globulin levels just rise.

Additionally, when comparing animals from pre-challenge to last scheduled blood draw, there is a significant difference in the degree of change between BCG-vaccinated and saline-treated animals. The increase in serum globulins in the saline-treated group is greater than the decrease in serum globulins in the BCG-vaccinated group (Fig 7b).
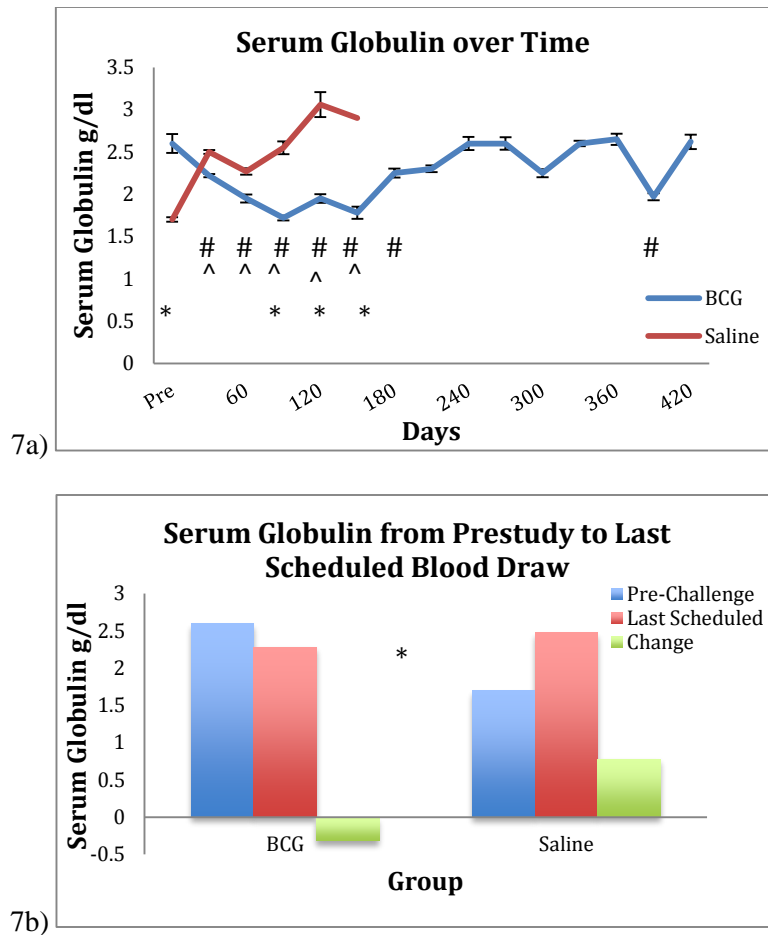
7a)

7b)

Fig 7 a) Comparison of serum globulin in BCG-vaccinated and saline-treated animals from pre-challenge through the last scheduled blood draw. Significant differences (*) occur between groups at pre-challenge and days 90, 120 and 150 ($p < 0.01$). Significant differences (^) occur within the saline-treated group comparing pre-challenge values to all the following time points ($p < 0.01$, at day 150 $p = 0.02$). Significant differences (#) occur within the BCG-vaccinated group comparing pre-challenge at days 60, 90, 120, 150, 180, and 390 ($p < 0.01$, at day 120 p=0.02, day 180 $p = 0.03$). (**b**) Comparison of serum globulin between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change calculated as (Last Scheduled – Pre-Challenge). There was a significant effect of treatment over time ($p < 0.01$).

Total Protein

Total protein is a composite value reflecting both albumin and globulin levels in the serum. In our study, total protein is only significantly different between groups at the pre-challenge blood draw, which is due to both albumin and globulin differences between groups (Fig 8a). Interestingly, although there are significant changes between groups at later time-points comparing both serum albumin and serum globulin, there is not a difference in total protein except at the pre-challenge value.

Within the BCG-vaccinated group, there is a difference at several time-points compared to the pre-challenge draw with clustering both in the early and late stages of disease, with the middle stage

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having no significant differences from pre-challenge values (Fig 6a, 7a, 8a). However, the saline-treated group shows a steady increase from pre-challenge values. Although the saline-treated group does show a steady increase, the maximum serum protein concentration was actually lower than that achieved by the BCG-vaccinated group. This observation is largely due to the lower overall serum albumin concentration in the saline-treated group, despite the overall higher globulin level.

When comparing the serum total protein level from baseline to the last scheduled blood draw, there is a significant difference between groups (Fig 8b). This is likely due to the opposing significant changes of both albumin and globulin having a normalizing effect when protein is looked at in totality. The BCG-vaccinated group had an overall decrease in total protein while the saline-treated group showed an overall increase when comparing pre-challenge values to the last scheduled blood draw, which is statistically represented as a significant effect of treatment over time (Fig 8b).
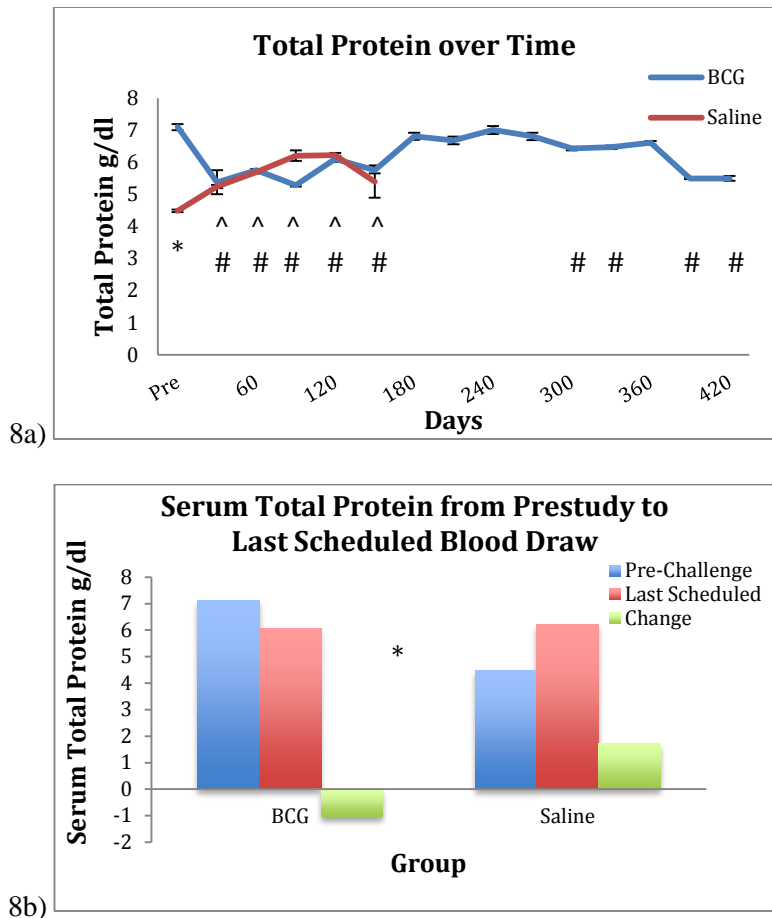
8a)

8b)

Fig 8 a) Comparison of serum total protein in BCG-vaccinated and saline-treated animals from pre-challenge through the last scheduled blood draw. Significant differences occur (*) between groups at the pre-challenge time point ($p < 0.01$). Significant differences (^) occur within the saline-treated group comparing pre-challenge values to all the following time points ($p < 0.01$). Significant differences (#) occur within the BCG-vaccinated group comparing pre-challenge values to days 30, 60, 90, 120, 150, 300, 330, 390, and 420 ($p < 0.01$, day 330 $p = 0.01$). **b)** Comparison in serum total protein between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change which was calculated as (Last Scheduled – Pre-Challenge). There was a significant effect of treatment over time between groups ($p < 0.01$).

Liver Enzymes

Alkaline phosphatase (ALP) is an inducible enzyme that can be elevated due to elevated

isoenzymes from the liver, bone, and gastrointestinal tract. Because of its propensity to change in

response to several organ systems, it is not reliably a liver-specific enzyme. However, ALP can be used in

conjunction with other parameters in a serum biochemistry assay to indicate changes in liver function.

Alanine aminotransferase (ALT) is a more specific measure of liver function than ALP in guinea pigs, but

it is not as specific to the liver as in other animals. Since ALT is not induced by other organ systems to

the same degree as ALP, and can be elevated due to hepatocellular damage, it can still be used as a measure to assess liver damage (Harkness J, Turner P, et al 2010).

When evaluating serum ALP in our study population, there is a significant difference between groups only at the pre-challenge time point, as the values are essentially the same between groups after inoculation (Fig 9a). Since both groups of animals were age and weight matched, there should be no difference in serum ALP between groups due to growth and influence by other organ systems such as the bone. This also implies that there were no changes in ALP to indicate that the BCG-vaccinated group was any more protected from disease that the saline-treated group, although it is well established that BCG is protective in guinea pigs. Due to the large differences from pre-challenge values to post-inoculation values, there was a statistically significant effect of treatment over time (Fig 9b).
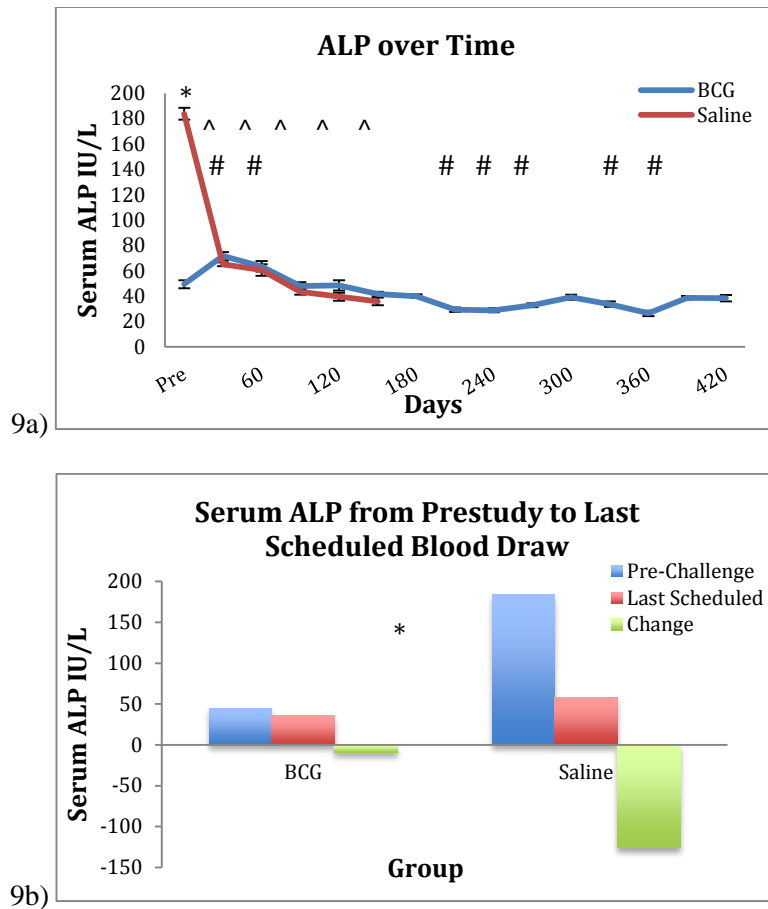
Fig 9 a) Comparison of serum ALP in BCG-vaccinated and saline-vaccinated animals from pre-challenge through the last scheduled blood draw. Significant differences (*) occur between groups at the pre-challenge time point (p <0.01). Significant differences (^) occur within the saline-treated group comparing to pre-challenge at all of the following time points (p <0.01). Significant differences occur within the BCG-vaccinated group comparing to pre-challenge to days 30, 60, 210, 240, 270, 330, and 360 (p < 0.01, day 60 p = 0.03, day 270 p = 0.04). **b)** Comparison of serum ALP between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change which was calculated as (Last Scheduled – Pre-Challenge). There was a significant (*) effect of treatment over time (p <0.01).

Serum alanine aminotransferase (ALT) values were different between groups prior to inoculation, but not to the degree that we saw with ALP (Fig 10a). In contrast to the ALP values, groups of guinea pigs were different also at day 150, due in part to a greater increase in serum ALT in the saline-treated group. The BCG-vaccinated group also had an increase in serum ALT over time, but peaked at a lower value that the saline-treated group, and began to decline about midway through disease progression (Fig 10a). Additionally, the BCG-vaccinated group showed an overall decrease in serum ALT while the saline-vaccinated group showed an increase when comparing pre-challenge values to the last-scheduled blood draw, which is represented statistically as a significant effect of treatment over time (Fig 10b).
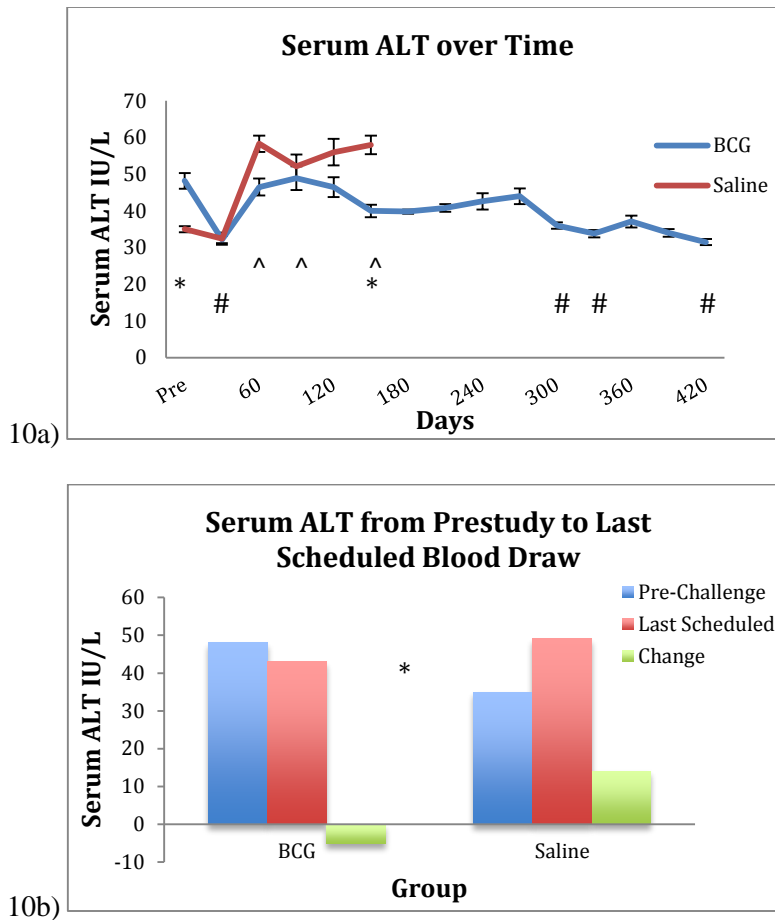
39

Serum ALT over Time

10a)

Serum ALT from Prestudy to Last Scheduled Blood Draw

10b)

Fig 10 a) Comparison of serum ALT in BCG-vaccinated and saline-treated animals from pre-challenge through the last scheduled blood draw. Significant differences (*) occur comparing between groups at pre-challenge and day 150 (p =0.03). Significant differences (^) occur within the saline-treated group comparing pre-challenge to days 60, 90, and 150 (p <0.01). Significant differences (#) occur within the BCG-vaccinated group comparing pre-challenge to days 30, 300, 330, and 420 (p <0.01, days 300 and 420 p= 0.02). **b)** Comparison of serum ALT between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change which was calculated as (Last Scheduled – Pre-Challenge). There was a significant (*) effect of treatment over time (p <0.01).

Summary

When evaluating the serum chemistry parameters in totality, there are clear trends and many

significant differences when comparing within groups, between groups, and at varied time points

throughout the study. In a comparison of averages, it is easy to see visually that there are distinct

differences is some parameters while others were more similar between groups (Fig 11a and 11b).

However, as a group, none of these parameters alone proved to be good predictors of severity of disease

or death. Serum proteins and liver enzymes did show the most significant changes among all the

parameters evaluated with the serum biochemistry panel, both within groups and between groups of BCG-vaccinated and saline-treated animals.
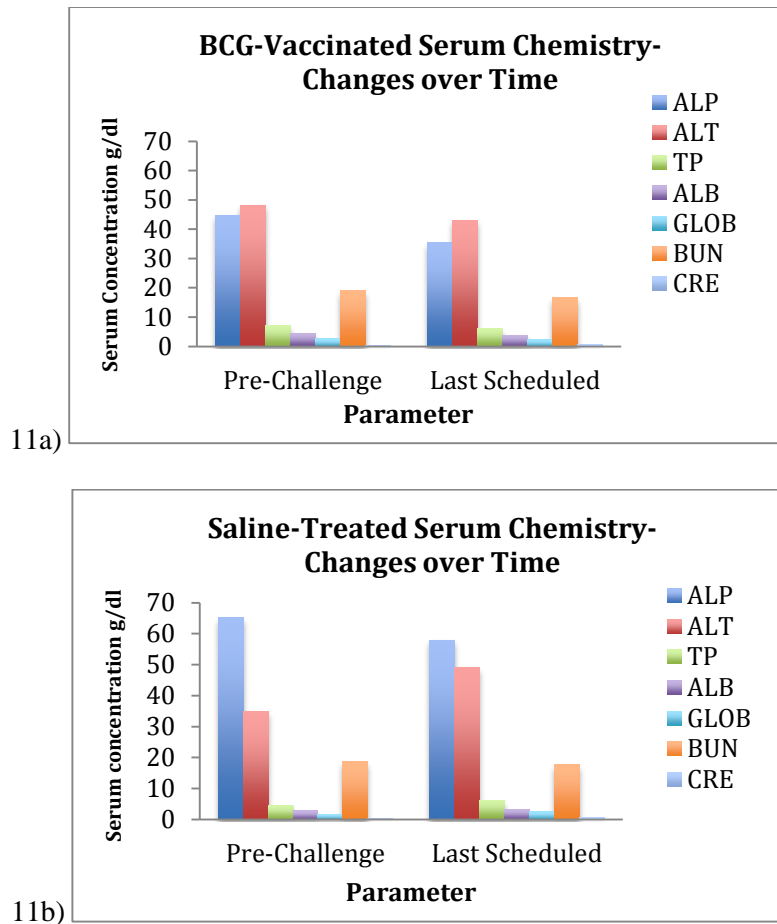

11a)

11b)

Fig 11 Summary of changes in serum chemistry parameters within **(a)** BCG-vaccinated animals and **(b)** saline-treated animals. We compared time points from pre-challenge to the last scheduled blood draw. Creatinine is not visible on the graph as the value is considerably small when plotted on the y-axis scale.

3.2 Complete Blood Count
Hematocrit

Hematocrit is a measure of the percentage of red blood cells in the circulating blood volume. In our guinea pigs, hematocrit was only different between groups of BCG-vaccinated and saline-treated animals at day 30, due to a more dramatic increase from pre-challenge values in the saline-treated group compared to the BCG group (Fig 12a). Within both the groups, the hematocrit continued to increase throughout the course of disease, resulting in a significant increase from pre-challenge values at all following time points except days 390 and 420 in the BCG-vaccinated group (Fig 12a). Although both

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groups showed a dramatic increase from pre-challenge hematocrit values, they changed so similarly that there was no effect of treatment over time (Fig 12b).
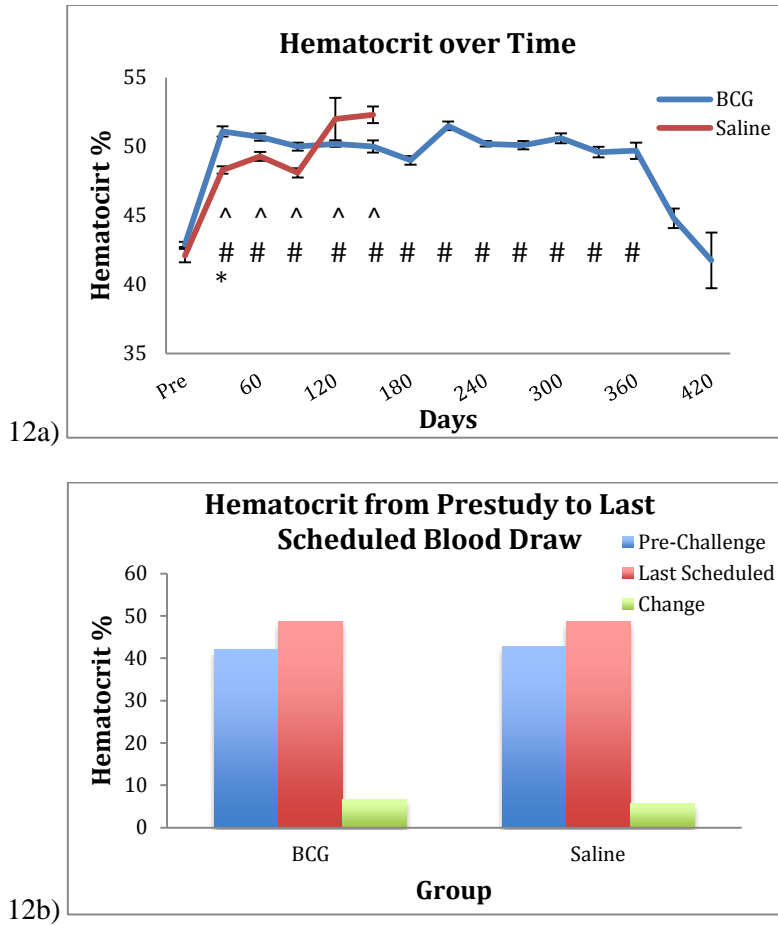

12a)

12b)

Fig 12 a) Comparison of hematocrit in BCG-vaccinated and saline-treated animals from pre-challenge through the last scheduled blood draw. Significant differences (*) occur between groups at day 30 (p <0.01). Significant differences (^) occur within the saline-treated group comparing pre-challenge to all the following time points (p <0.01). Significant differences (#) occur within the BCG-vaccinated group comparing pre-challenge to all the following time points through day 360 (p <0.01). **b)** Comparison of hematocrit between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change which was calculated as (Last Scheduled – Pre-Challenge). There is no significant effect of treatment over time.

Leukocytes

When evaluating leukocytes (white blood cells), we look at the total leukocyte count as well as the individual cell types that comprise the leukocyte population, which included heterophils, lymphocytes, and monocytes. Between groups of guinea pigs, the total leukocyte count was different at days 30, 90, and 120, with an overall more dramatic increase in the saline-treated group (Fig 13a). Within the BCG-vaccinated group, the only significant differences from pre-challenge values were at days 390

and 420, representing an increase and then a decrease, respectively (Fig 13a). We attributed these changes to be due to ability of the BCG-vaccinated group to have a more efficient immune response capable of controlling infection, which results in fewer immune cells in the peripheral blood.

The saline-treated group showed changes at all time-points when compared to pre-challenge values, except at day 30. This reflects the saline group having an appropriately slow response to acute infection, and a more robust response overall (Fig 13a). Although the saline-treated group showed an overall increase and more robust response to infection, there was not enough of a difference between groups to have a significant effect of treatment over time (Fig 13b).
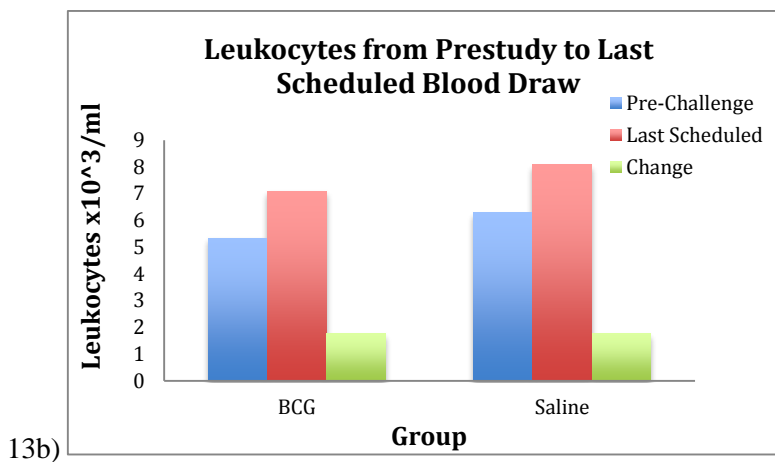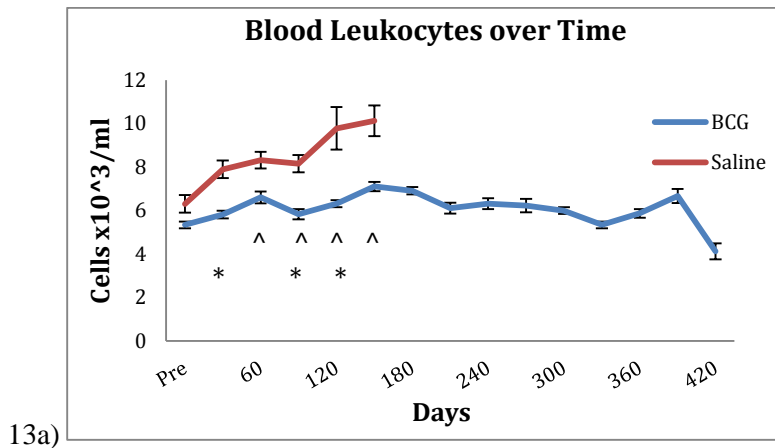

13a)

13b)

Fig 13 a) Comparison of peripheral blood leukocytes in BCG-vaccinated and saline-treated animals from pre-challenge through the last scheduled blood draw. Significant differences (*) occur between groups at days 30, 90, and 120 (p =0.02). Significant differences (^) occur within the saline-treated group comparing pre-challenge to days 60, 90, 120, and 150 (p <0.01). Significant differences (#) occur within the BCG-vaccinated group comparing pre-challenge to days 60, 120, 150, 180, 210, 20, 270, 390, and 420 (p <0.01, day 120, 240, and 420 p = 0.02, day 210 p = 0.04, day 270 p = 0.03). **b)** Comparison in peripheral blood leukocytes between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change which was calculated as (Last Scheduled – Pre-Challenge). There is no significant effect of treatment over time.

Heterophils

Heterophils are the guinea-pig counterpart to neutrophils as described in other mammals. They are the primary cell type in acute inflammatory responses with toxic eosinophilic granules that are responsible for microbial destruction and development of pathologic lesions. When comparing heterophils between saline-treated and BCG-vaccinated groups, there are only significant differences at days 30 and 90 (Fig 14a). When visually evaluating the data, it appears as though the BCG group having a more delayed response and the saline-treated group having a greater magnitude of response (Fig 14a).

Despite an obvious increasing trend in the saline-treated group, there was no significant increase from pre-challenge heterophil values at any later time point. The BCG-vaccinated group had significant differences from pre-challenge values at days 150 and 390 (Fig 14a). Additionally, there was no significant effect of treatment over time between groups, despite the increasing trend in the saline-treated group (Fig 14b).
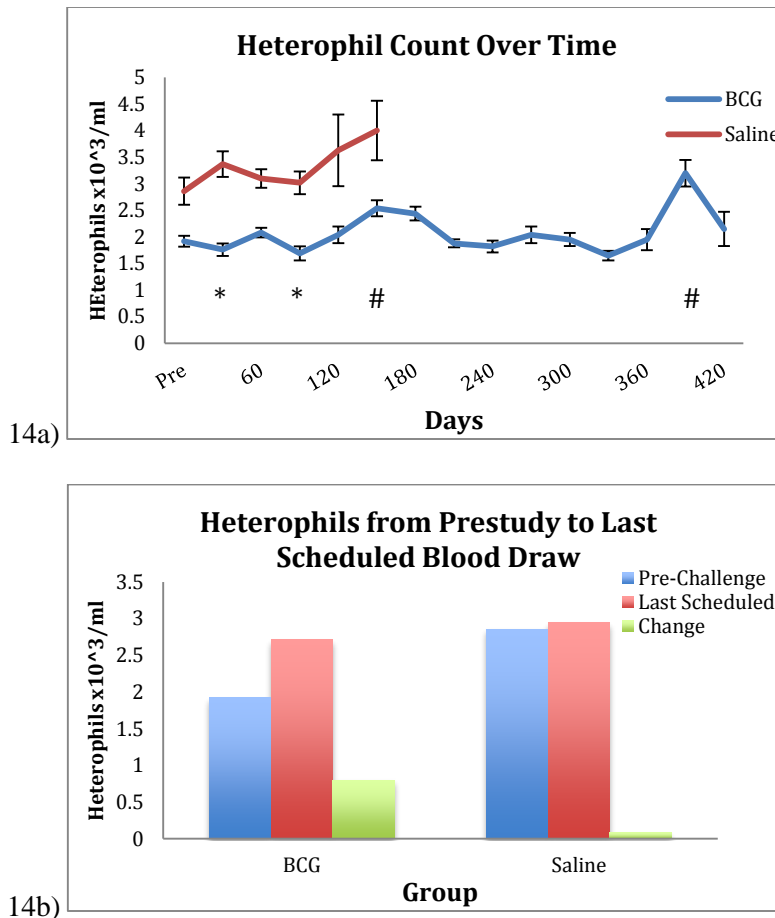
14a)

14b)

Fig 14 a)- Comparison of peripheral blood heterophils in BCG-vaccinated and saline-treated animals from pre-challenge through the last scheduled blood draw. Significant differences (*) occur between groups at days 30 and 90 (p <0.01, p= 0.02 respectively). There are no significant differences within the saline-treated group comparing pre-challenge to all the following time points. There are significant differences (#) within the BCG-vaccinated group comparing pre-challenge to days 150 (p = 0.03) and day 390 (p < 0.01). **b)-**Comparison in peripheral blood heterophils between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change which was calculated as (Last Scheduled – Pre-Challenge). There is no significant effect of treatment over time.

Lymphocytes

There was no significant difference between groups at any time point, nor were there any differences within the BCG-vaccinated group at any time point. However, the saline-treated group showed a significant difference when comparing pre-challenge to all the following time points. Graphically, this is depicted as the lymphocyte count changing significantly within the first 30 days of infection in both groups of animals, and continuing to increase in the saline-treated group throughout the course of infection (Fig 15a). Although the BCG-vaccinated group had a slight initial increase, the lymphocyte counts remained fairly stable throughout the infection, with a significant decrease at day 420.

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When comparing groups over time, the lymphocyte count changed similarly resulting in no significant effect of treatment over time (Fig 15b).
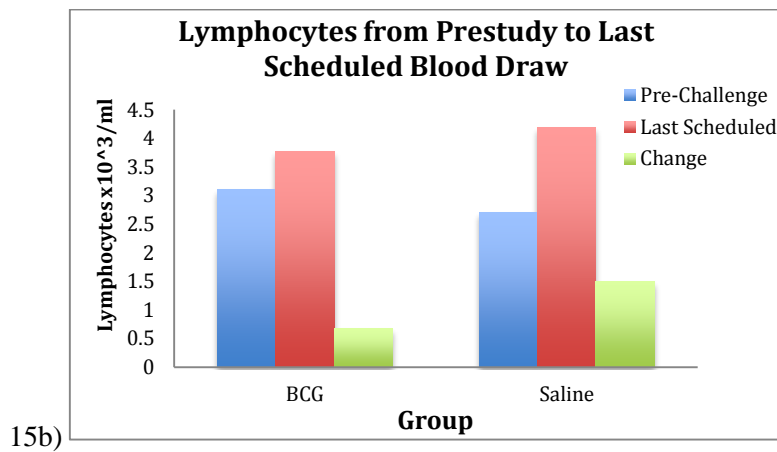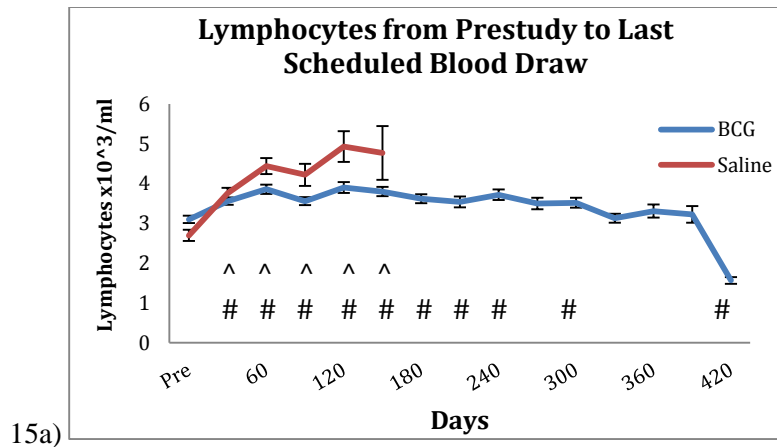

15a)

15b)

Fig 15 a) Comparison of peripheral blood lymphocytes in BCG-vaccinated and saline-treated animals from pre-challenge through the last scheduled blood draw.. There were no significant differences when comparing time points between groups. Significant differences (^) occur within the saline-treated group comparing pre-challenge to all the following time points ($p < 0.01$). There were significant differences (#) within the BCG-vaccinated group comparing pre-challenge to all of the following time points except day 270, 330, 360, and 390 ($p < 0.01$, at day 300 p = 0.01). **b)** Comparison of peripheral blood lymphocytes between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change which was calculated as (Last Scheduled – Pre-Challenge). There is no significant effect of treatment over time.

Monocytes

Monocytes are different between groups at days 90 and 120, but at no other time (Fig 16a). There were no significant changes within the BCG-vaccinated group at any time point. The saline-treated group showed changes from pre-challenge at all time points except day 150. Graphically, this is reflected as a sharp increase in the saline-treated group within the first 30 days of infection, with less drastic increases

at the following time points (Fig 16a). There is no data for the BCG-vaccinated group at day 180 due to data transfer complications.
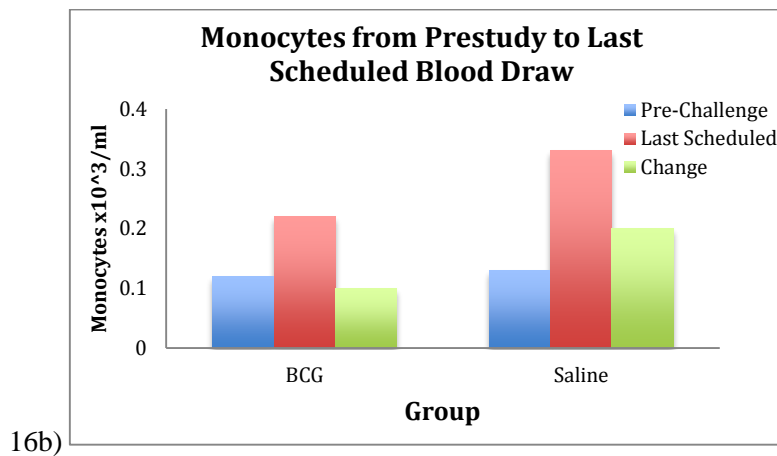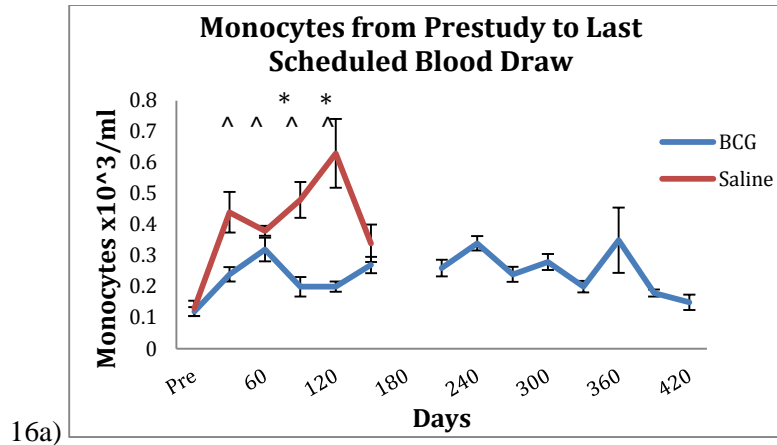

16a)

16b)

Fig 16 a) Comparison of peripheral blood monocytes in BCG-vaccinated and saline-vaccinated animals from pre-challenge through the last scheduled blood draw. Significant differences (*) occur between groups at days 90 and 120 ($p = 0.01$). Significant differences (^) occur within the saline-treated group comparing pre-challenge to days 30, 60, 90, and 120 ($p < 0.01$). There are significant differences (#) within the BCG-vaccinated group comparing pre-challenge to days 60, 240, 300, and 360 ($p < 0.01$, day 60 $p = 0.01$, day 300 $p = 0.05$). **b)** Comparison in peripheral blood monocytes between BCG-vaccinated and saline-treated animals from pre-challenge to the last scheduled blood draw, including the change which was calculated as (Last Scheduled – Pre-Challenge). There was no significant effect of treatment over time.

Summary

When evaluating the inflammatory cells in totality, the BCG-vaccinated and saline-treated groups of guinea pigs responded to disease in a similar fashion, but at different times post inoculation. There are several significant differences and trends both between groups and within groups of animals. The most compelling are the increase in heterophils and monocytes within the saline-treated group compared to the

BCG-vaccinated group. Although graphically the groups look very different, statistically none of the individual leukocytes showed a significant effect of treatment over time.
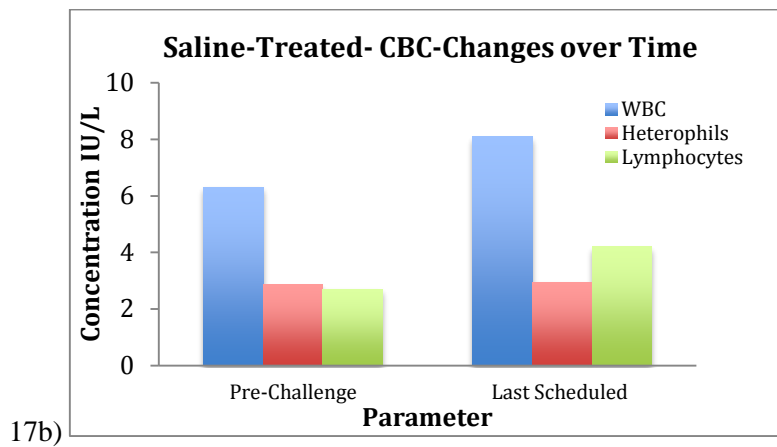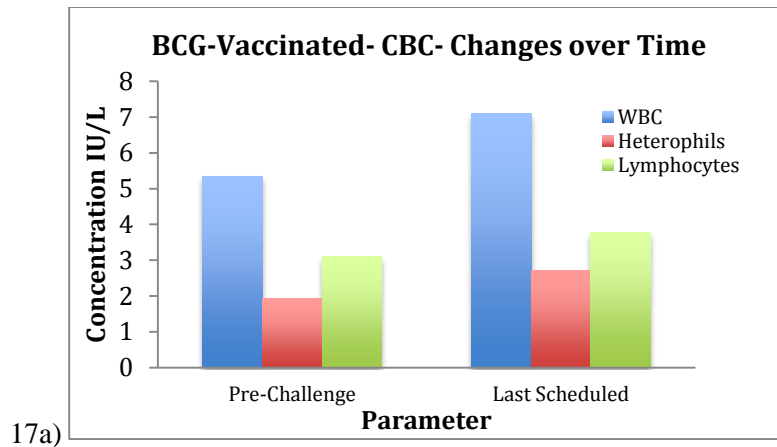

17a)

17b)

Fig 17- Summary of changes in complete blood count parameters within **(a)** BCG-vaccinated animals and **(b)** saline-treated animals. We compared time points from pre-challenge to the last scheduled blood draw in both groups.

3.3 Blood Gasses

Blood gasses were evaluated at the same time points as serum biochemistry and complete blood counts. At all times, except the pre-challenge time point, blood was collected improperly into a potassium EDTA tube (rather than a lithium heparin tube). The additive in this tube is highly acidic had deleterious effects on all of the measured blood gas parameters including pH, pCO2, p02, HCO3-, and lactate. We are therefore unable to use this data when identifying parameters for early endpoint criteria in guinea pigs infected with *Mycobacterium tuberculosis.*

3.4 Body Temperature

All guinea pigs were given a subcutaneous microchip prior to vaccination and challenge, and temperatures were monitored daily. The average temperature for the BCG-vaccinated group prior to inoculation was 39.39°C, with a minimum of 38.3° C and a maximum of 39.9° C. The average temperature for the saline-treated group prior to inoculation was 39.33 °C, with a minimum of 39.0° C and a maximum of 39.8° C (Fig 18). Both groups of guinea pigs had very similar body temperatures throughout the experiment, within their thermoneutral zone and consistent with homeostasis (Terril and Clemons, 1998).

At the last temperature recorded for each guinea pig (just prior to death), the BCG-vaccinated group had an average of 38.31°C, with a minimum of 37.8° C and a maximum of 39.3° C. At the same time, the saline-treated group had an average of 39.15°C, with a minimum of 38.3° C and a maximum of 40° C (Fig 18). The trend for both groups was decreasing over time, and neither group showed evidence of pyrexia or hypothermia associated with disease. One animal in the saline-treated group did show an increase above 40°C, to 40.6°C, but then returned back to 40°C prior to euthanasia within 4 days of that increase. No other animals exhibited temperatures above 40° C at any time in either group.
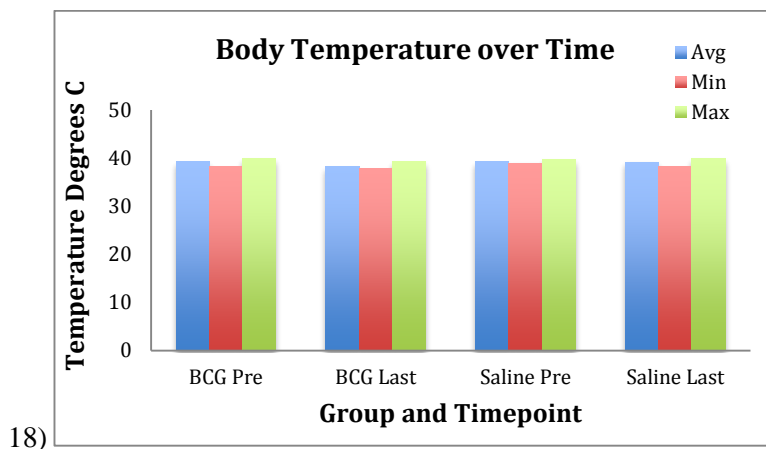
18)

Fig 18- Comparison of body temperatures from pre-challenge (pre) and the last scheduled blood draw (last) in both BCG-vaccinated and saline-treated animals. Average, minimum, and maximum temperatures were recorded and graphed.

3.5 Body Weight

All animals were weighed weekly beginning prior to vaccination and inoculation. Pre-challenge body weights were lower than the average adult weight because guinea pigs were young when the study started, and continued to gain weight for most of the experiment. BCG-vaccinated guinea pigs had an average pre-challenge body weight of 597.4 grams and saline-treated guinea pigs had an average pre-challenge body weight of 619.4 grams. The average body weight at the measurement just prior to death was typical of average for an adult female guinea pig in both groups. The BCG-vaccinated group had an average terminal body weight of 915.8 grams and the saline-treated guinea pigs had an average terminal body weight of 742.6 grams (Fig 18). Overall, saline-treated guinea pigs lived a shorter life and gained less weight than the BCG-vaccinated guinea pigs.

We also evaluated the maximum weight in guinea pigs and the time from max weight to terminal weight, which was an overall decrease in both groups. In the BCG-vaccinated group, the average maximum weight was 984 grams and an average terminal body weight of 915.8 grams, with the time to terminal body weight taking 8.1 weeks. The saline-treated group had an average maximum body weight of 762.8 grams and an average terminal body weight of 742.6, with the time to terminal body weight taking 5.1 weeks. This shows that although the saline-treated guinea pigs were not as large and lived shorter lives, they also progressed slightly faster in their weight loss. Few guinea pigs reached 10% total body weight loss, and only 1 guinea pig in the BCG-vaccinated group lost greater than that with 21% total body weight loss over 10 weeks' time. However, several guinea pigs in both groups were at their maximum weight at the time of the terminal measurement, specifically one animal in the BCG-vaccinated group and four animals in the saline-treated group.
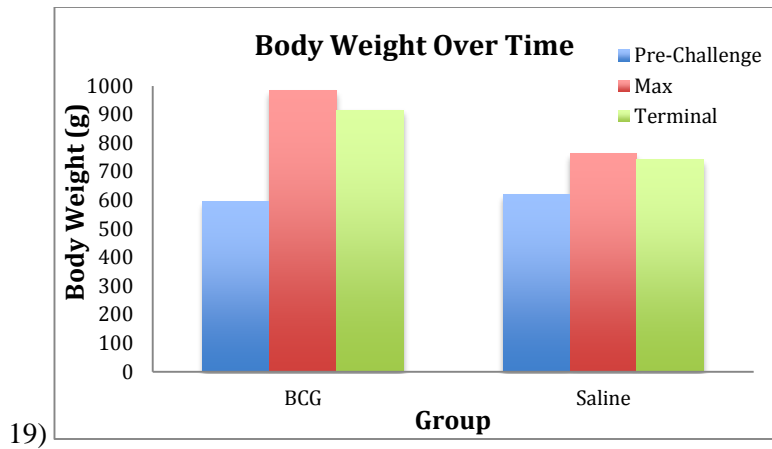
Fig 19- Comparison of body weight among BCG-vaccinated and saline-treated animals at various time points. Recordings graphed are pre-challenge, maximum weight achieved throughout the study (max), and terminal body weight (terminal).

3.6 Survival and Colony Forming Units

All animals were euthanized based on criteria for humane endpoints, rather than predetermined time points. Criteria included decreased activity, increased respiratory effort, weight loss greater than 15%, cyanosis of the extremities and ears, and palpable organomegaly. Survival days were plotted for both saline-treated and BCG-vaccinated animals (Fig 20). The saline-treated group had the earliest mortality and shortest survival period, with the first death at 67 days post challenge and the last at 180 days post challenge. The BCG-vaccinated group had a much longer survival period, with the first death at 117 days, and the last four animals at 441 days. The last four animals to be euthanized in the BCG-vaccinated group did not show clinical signs of disease at any time during the experiment. These four animals were considered survivors and the study was terminated.
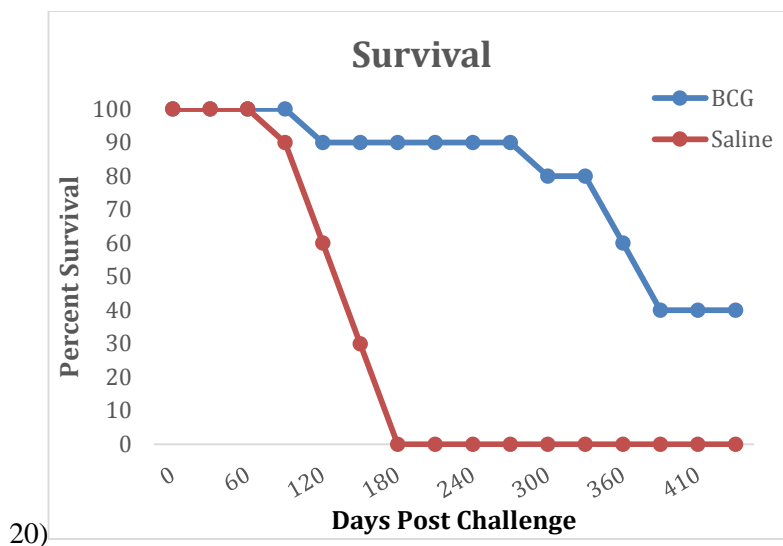
Fig 20 Graph of percent survival plotted over time in days, of both BCG-vaccinated and saline-treated animals.

20)

Colony forming units (CFUs) were used to objectify severity of infection by extrapolating quantity of viable microbes in a given organ homogenate. The higher the CFU count, the higher the bacterial burden in the organ being evaluated. We calculated CFUs by plating organ homogenates (lung, spleen, liver, brain, eye ball) and incubating for 21 days. Lung and spleen CFUs were inversely correlated with survival days (-0.768) and (-0.694), respectively (Fig 21a and 21b). The lowest CFUs were in animals that lived the longest (four survivors). The BCG-vaccinated group had 7 animals with no detectable CFUs in the lungs (of those, 4 were survivors), while the saline-treated group only had 2 animals with no detectable lung CFUs. The animal with the shortest survival time of 67 days had 4.53 CFUs in the lungs. The highest level of CFUs was 8.33 in an animal that lived 188 days. Both these animals were saline-treated as well.

In addition to typical extrapulmonary lung lesions—liver and spleen—some individual animals had detectable CFUs in other organs. One animal in the BCG-vaccinated group had CFUs in the brain, while 2 animals in the saline-treated group had CFUs in the brain. Also, one animal in the saline-treated group had CFUs in the eyeball (Fig 21a and 21b).
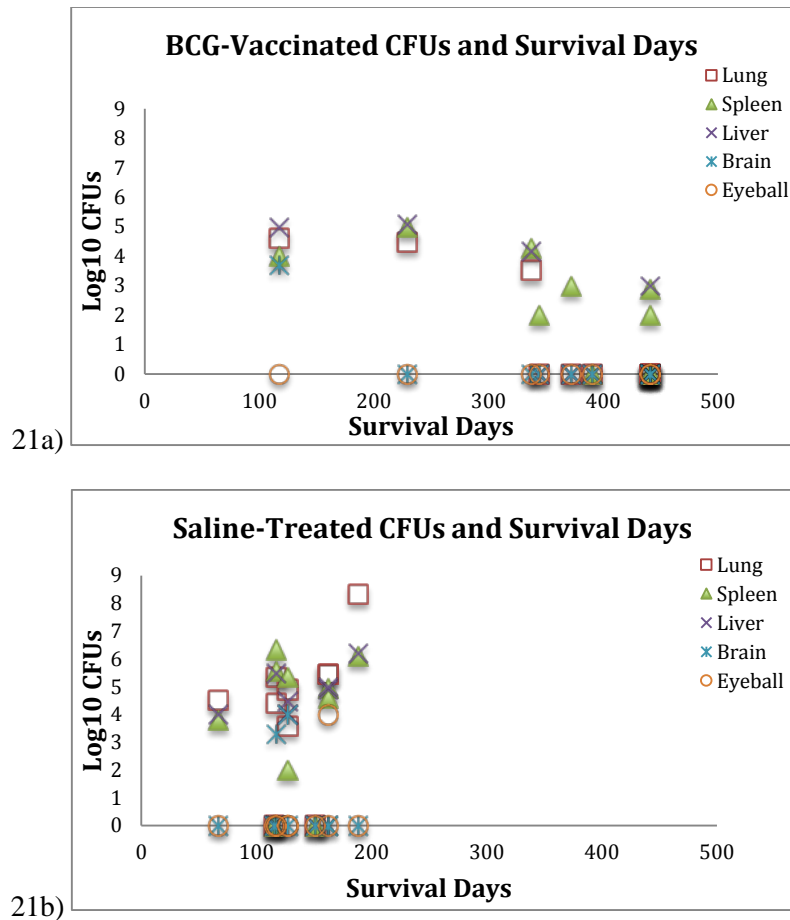
Fig 21 Comparison of survival days and various organ CFUs within the **(a)** BCG-vaccinated animals and **(b)** saline-treated animals. Organs evaluated were lung (square), spleen (triangle), liver (grey X), brain (blue X), and eyeball (orange circle).

3.7 Histopathology

Histopathology involves evaluating serial sections of organs for microscopically detectable changes. Organs harvested for histopathology included heart, lung, spleen, liver, kidney, brain, eyeball, lymph nodes, and sections of the gastrointestinal tract. Tissues were formalin fixed and embedded in paraffin, and sections were cut and stained with hematoxylin and eosin. Slides were then evaluated by a board certified pathologist on a scale of 0-15, with zero having no appreciable lesions and 15 having very severe and complicated lesions. Lungs were scored based on total organ involvement, primary lesions, secondary lesions, necrosis, mineralization, and fibrosis. Spleens and livers were scored based on lesion severity and extent. Accessory organs were scored based on presence or absence of lesions and severity of lesions (Table 1, Fig 22).

Lung tissues had predictably the highest overall scores, with the saline-treated group having the highest scores among them. The same trend continued with the other organs—BCG-vaccinated animals had lower scores than saline-treated animals. Some animals in both groups had lesions in the liver, lungs, and spleen. All four survivors had zero as a score for the lungs, while one had lesions in the spleen (total score = 2) and one had lesions in the liver (total score = 4). The animal with the worst lung lesions (total score = 15) was saline-treated and lived the shortest time of 67 days.
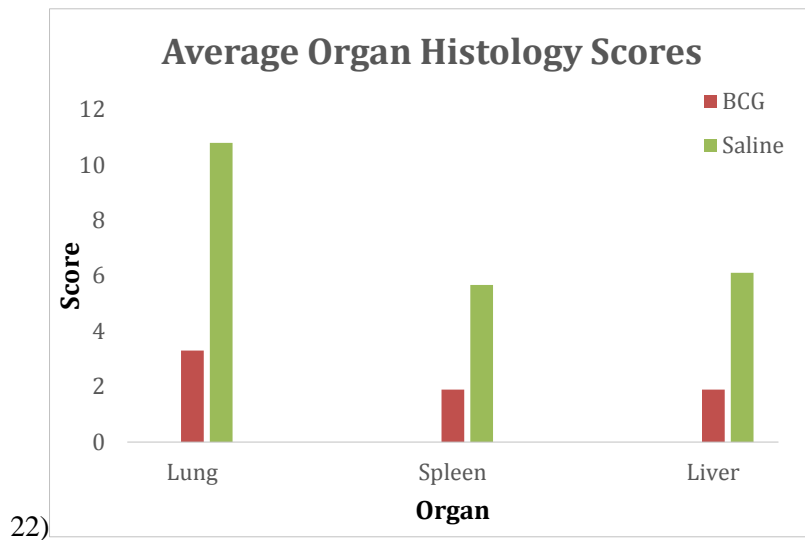
22)

Fig 22- Average organ histology scores of pulmonary and common extrapulmonary lesions in both BCG-vaccinated and saline-treated animals.

Table 1 - Chart of lung, spleen, and liver lesions. Lesions were scored by a veterinary pathologist. Scale is from 0 to 15, with 0 being no lesions and 15 having the most severe lesions. Lungs were scored by: total lung involvement (involve), primary lesions (primary), secondary lesions (sec), necrosis (nec), mineralization (min), and fibrosis (fib). Spleen and liver lesions were scored by severity and extent. Animals 1-10 are BCG-vaccinated and animals 11-20 are saline-treated. There is no data for animal #16 as it was not included in the pathologist's report.

Animal	Lung						Total	Spleen		Total	Liver		Total
	Involve	Prima	Sec	Nec	Min	Fib	Score	Severity	Extent	Score	Severity	Extent	Score
1	0	0	0	0	0	0	0	1	1	2	0	0	0
2	2	0	2	2	0	2	8	3	4	7	3	2	5
3	0	0	0	0	0	0	0	0	0	0	0	0	0
4	1	0	1	0	0	0	2	0	0	0	3	1	4
5	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0
8	3	2	3	2	0	2	12	3	3	6	3	2	5
9	2	1	2	2	0	2	9	2	2	4	3	2	5
10	1	0	1	0	0	0	2	0	0	0	0	0	0
AVG	0.9	0.3	0.9	0.6	0	1	3.3	0.9	1	1.9	1.2	0.7	1.9
11	3	2	3	2	2	2	14	3	4	7	3	3	6
12	4	2	4	2	1	2	15	3	1	4	3	2	5
13	3	2	3	2	2	2	14	3	4	7	3	4	7
14	3	3	2	2	0	2	12	4	4	8	3	4	7
15	0	0	0	0	0	0	0	0	0	0	3	2	5
17	1	2	1	1	2	2	9	3	3	6	4	2	6
18	3	2	3	2	0	2	12	3	4	7	4	2	6
19	3	3	3	0	2	2	13	1	3	4	3	2	5
20	3	0	3	0	0	2	8	4	4	8	4	4	8
AVG	2.56	1.78	2.4	1.2	1	2	10.8	2.6667	3	5.67	3.3333	2.78	6.11

Other Extrapulmonary Lesions

Several organs (in addition to the liver and spleen) in both groups were affected by either granulomatous inflammation or actual granuloma formation. These included the heart, lymph nodes, gastrointestinal tract, and brain (Table 2). Most of the animals had sections of lymph nodes submitted for histopathology, all of which showed significant lymphadenopathy. Lymph nodes submitted included the mesenteric and mediastinal nodes. Lesions ranged from granulomatous inflammation with a score of 2-3, to >90% effacement of the node due to severe granulomatous inflammation and fibrosis, with extension

into surrounding tissues. Additionally, several animals had focal granulomatous inflammation in the myocardium, as well as granulomas within the epicardium and adventitial fat. Only one animal had brain lesions, and this was a BCG-vaccinated animal that died early in the experiment.

Table 2- Summary chart of unexpected extrapulmonary TB lesions. An "x" denotes presence of lesions. Animals 1-10 were BCG-vaccinated and animals 11-20 were saline-treated. There is no data for animal #16 as it was not included in the pathologist's report.

Animal #	Lymph Nodes	GIT	Heart	Brain
1				
2	x	x		
3			x	
4	x			
5				
6			x	
7				
8	x		x	x
9			x	
10				
11	x		x	
12	x			
13	x		x	
14	x		x	
15				
17	x			
18	x		x	
19				
20	x			
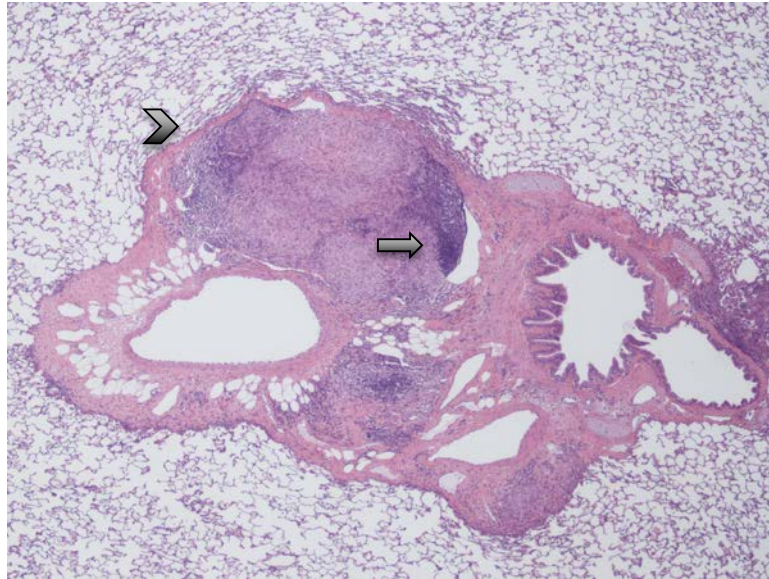
Fig 23- Lung lesion from saline-treated animal #12 which had the most severe score of 15. Presence of discrete granuloma with central necrosis, fibrosis (arrow head), and mineralization (arrow). 40X magnification
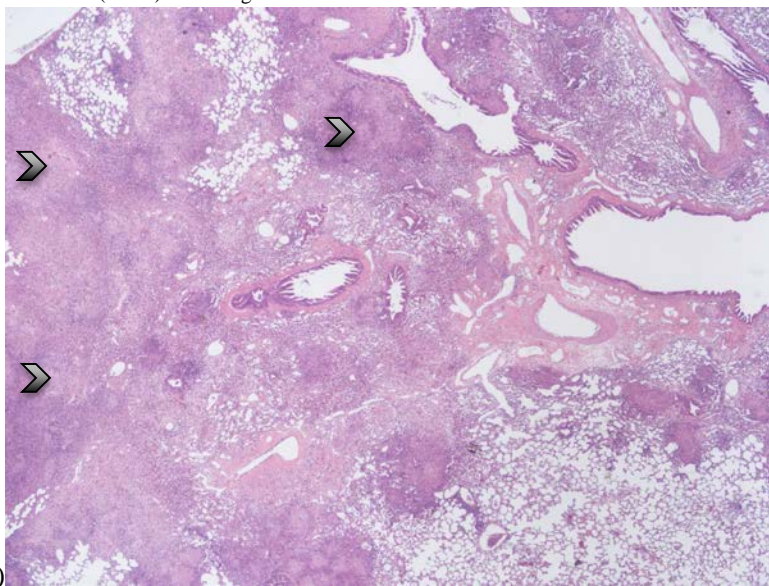
Fig 24- Lung lesion from saline-treated animal #12 with a sever score of 14. Presence of multifocal granulomas with necrosis (arrow head), fibrosis, and mineralization. 20X magnification.
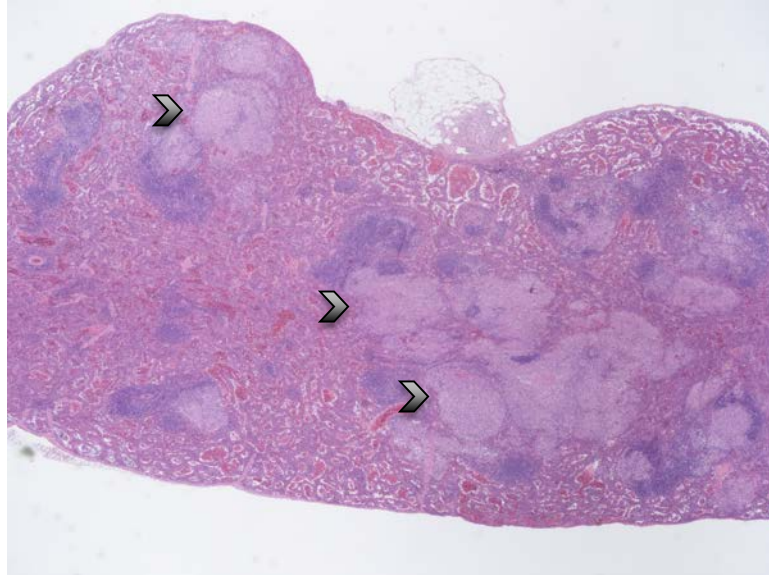
Fig 25- Highest scored spleen lesion from BCG-vaccinated animal #2 with a score of 7. Multifocal granulomas are present with evidence of necrosis (arrow head) and fibrosis. 20X magnification
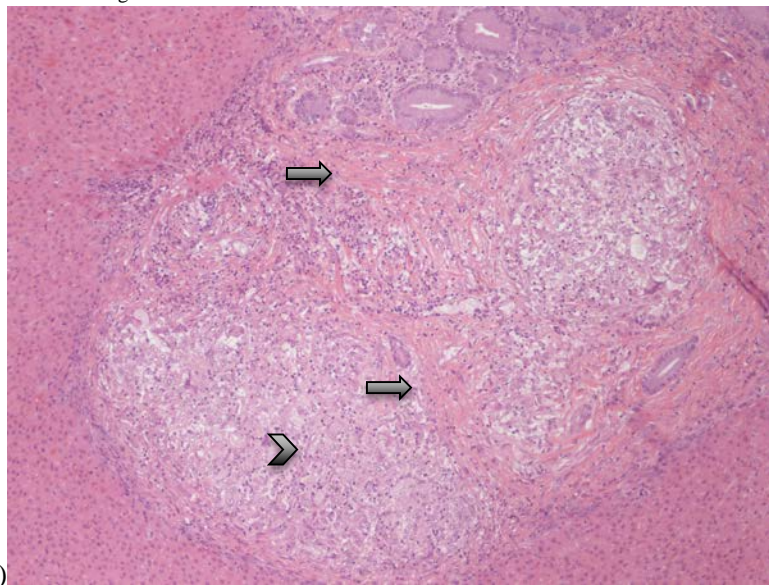
Fig 26- Liver granuloma from saline-treated animal #17, with a total score of 6. Evidence of necrosis (arrow head) and fibrosis (arrow). 100X magnification
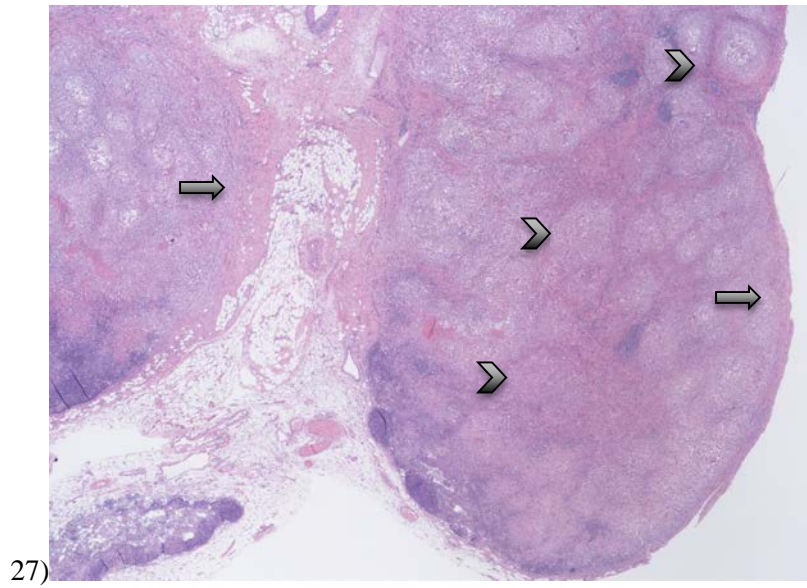
27)

Fig 27- Severe mediastinal lymph node lesion from saline-treated animal #11. Approximately 75% of the lymph node is effaced by coalescing granulomas (arrow head), which appear variable. There is severe fibrosis of the capsule (arrow). 20X magnification.
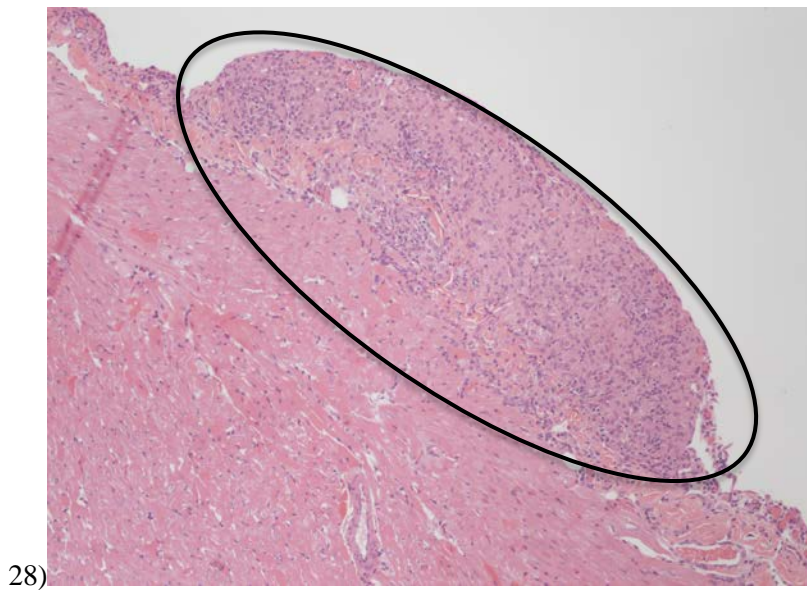
28)

Fig 28- Heart lesion from saline-treated animal #13. There is multifocal granulomatous inflammation (circled) in the epicardium of the left ventricle. 100X magnification.

29)
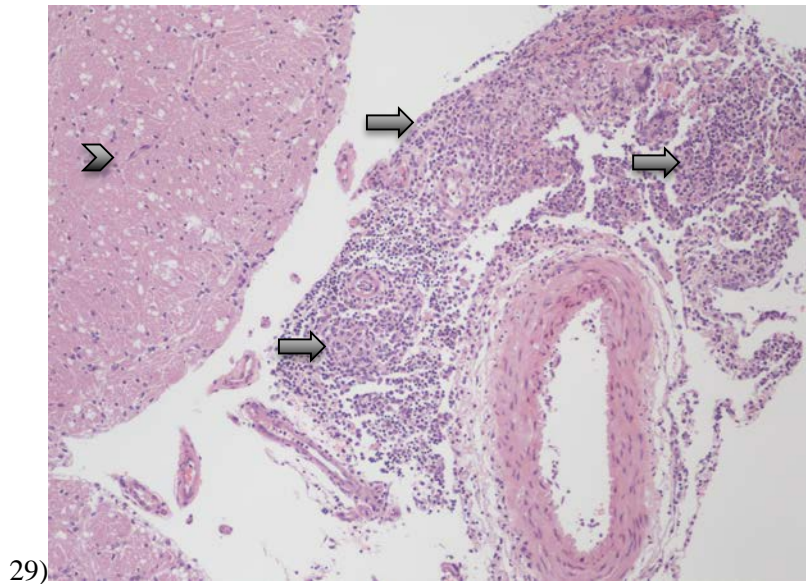
Fig 29- Brain from BCG-vaccinated animal #8. There is focally extensive and severe granulomatous meningitis (arrows) ventrally along the brainstem. The neuropil (arrow head) appeared relatively unaffected. 100X magnification.

3.8 Anesthetic Controls

For Specific Aim III, we evaluated the serum biochemistry and complete blood count parameters, as previously described, in 45 uninfected guinea pigs to assess the effects of the inhaled anesthetic isoflurane and the injectable combination of ketamine and xylazine anesthesia when compared to alert guinea pigs. Our results overwhelmingly point to the combination of ketamine and xylazine having the most profound effect on blood parameters, which are summarized in the following graphs (30a-d). Statistical significance is depicted when comparing alert to isoflurane (*), alert to ketamine and xylazine (^), and isoflurane to ketamine and xylazine (+).

30a)

30b)

30c)

30d)
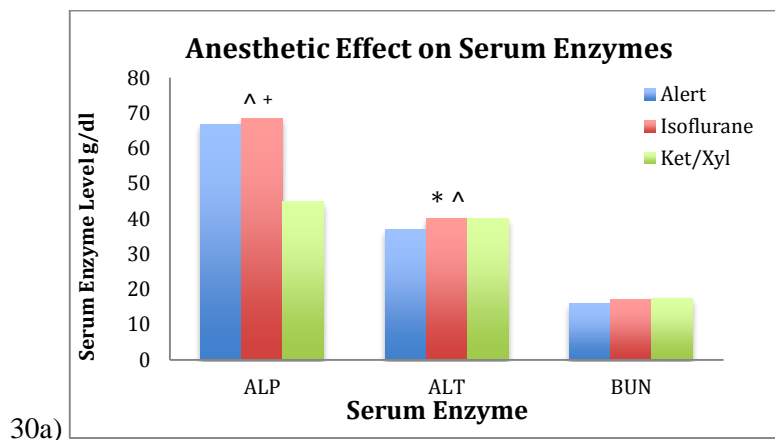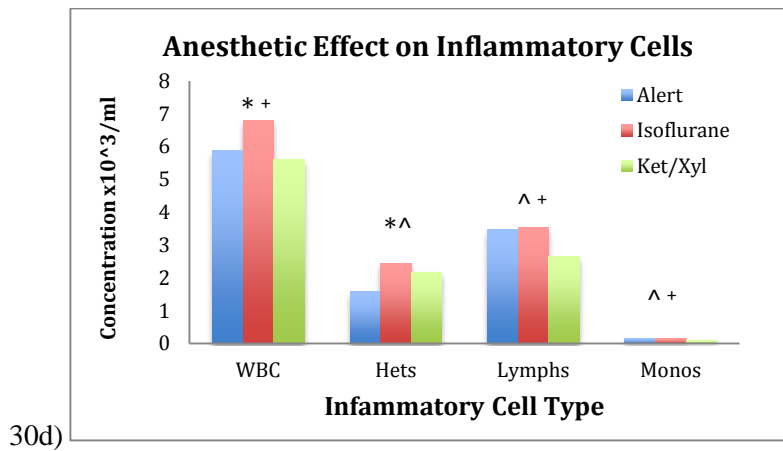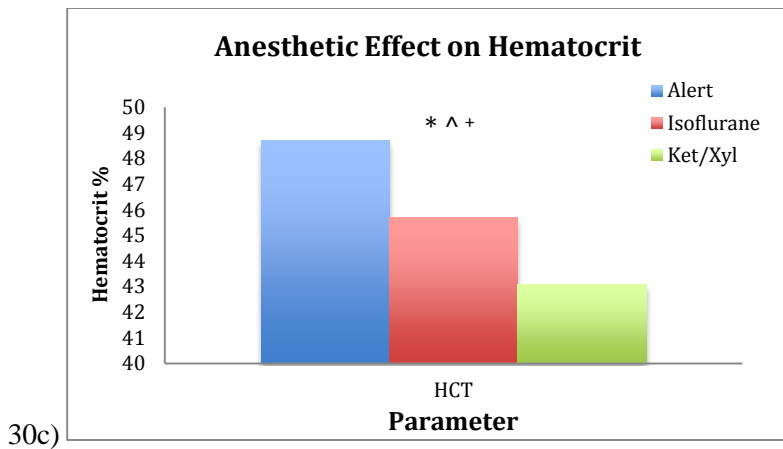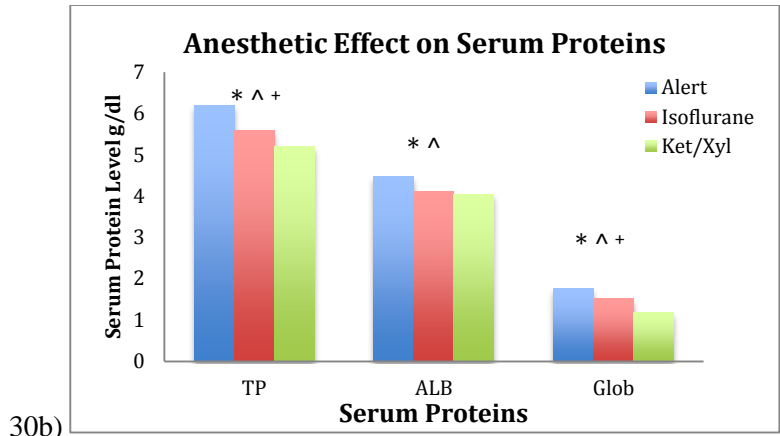
Fig 30 Summary of changes due to anesthetic effect in **(a)** serum enzymes ALP, ALT, and BUN; **(b)** serum total protein, albumin and globulin; **(c)** hematocrit; and **(d)** white blood cells, heterophils, lymphocytes, and monocytes. Anesthetics were: no anesthetic (alert), isoflurane, or combination of ketamine and xylazine (Ket/Xyl). Significant differences occurred when comparing parameters in alert and isoflurane anesthetized animals (*), alert and ketamine and xylazine anesthetized animals (^), and isoflurane and ketamine and xylazine anesthetized animals (+).

Serum Biochemistry

Evaluation of organ specific enzymes for changes due to anesthetic protocol showed mixed results. Renal values of BUN and creatinine were not changed at all due to either anesthetic when compared to each other and to alert controls. Serum proteins, globulin, and serum ALP were all significantly decreased in response to both anesthetics, with ketamine and xylazine having a more profound effect on all values than Isoflurane (Fig 30a and 30b). Both anesthetics significantly increased serum ALT compared to alert values (Fig 30a). These results are not a surprise, as isoflurane is completely metabolized by the respiratory system with minimal residual organ effects, while ketamine and xylazine are both largely metabolized by the liver, with minimal renal excretion.

Complete Blood Count

Evaluation of hematocrit showed significant effects by both isoflurane and ketamine and xylazine anesthesia compared to alert values (Fig 30c). With Isoflurane having only a mild (but significant) effect, and ketamine and xylazine having a more profound effect on decreasing hematocrit levels. Although the decrease in hematocrit in both anesthetized groups was significant, this did not correspond clinical signs of anemia in our guinea pigs.

The leukogram also showed similar effects of isoflurane and ketamine and xylazine anesthesia. Specifically, the total leukocyte count significantly increased with isoflurane, but decreased with ketamine and xylazine administration compared to alert leukocyte counts (Fig 30d). Additionally, both isoflurane and ketamine and xylazine anesthesia increased peripheral heterophils count to a similar degree from alert levels. Both the lymphocyte and monocyte counts were more affected by ketamine and xylazine administration than isoflurane administration (Fig 30d).

Chapter 4. Discussion

In the interest of looking at research guinea pigs as both veterinary patients and as a vaccine model for TB, we set out to evaluate clinical signs and measured values in guinea pigs experimentally infected with *Mycobacterium tuberculosis*. This posed a challenging situation, as guinea pigs are excellent at masking disease. Like other prey species, guinea pigs display normal behaviors—eating and drinking, urinating and defecating, social interactions, etc. in the face of severe disease. It is only when the guinea pig is very near death that it becomes evident there is a pathologic process occurring. Specifically in guinea pigs infected with *M. tuberculosis*, we don't typically see clinical indicators of disease until it has become very severe and progressive. Observed signs can include respiratory distress and cyanosis, and more unpredictably weight loss and decreased activity. However, these signs tend to occur suddenly and without prior indication, and sometimes guinea pigs die without any premonitory signs. Therefore, it became our focus to identify other parameters detectable in the blood or urine that may be used to establish earlier endpoint criteria, although the guinea pig may appear outwardly normal.

Our prediction when starting this project was that there would be a parameter or set of parameters that could help identify criteria for early endpoints in guinea pigs experimentally infected with *M. tuberculosis.* After extensive data collection in two groups of guinea pigs exposed to *M. tuberculosis*— one group vaccinated with BCG, and one group treated with only saline—we found that identifying disease in guinea pigs is much more difficult than anticipated. We expected that guinea pigs would mask their disease exceptionally well in regard to evaluating behavior and external parameters, which they did. However, we also expected to find biomarkers that would indicate otherwise, as evaluated by serum biochemistry, complete blood count, and urine assays. It was our goal to utilize two easy, minimally invasive, whole body blood tests that are typically used to identify systematic abnormalities (renal disease, for instance), in a different manner. We aimed to identify biomarkers that were specifically due to changing metabolic demands secondary to infection with *Mycobacterium tuberculosis*, with or without

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systemic dysfunction. Although our search was not exhaustive, we did find that many commonly evaluated parameters in the blood did change in a way that have potential to establish criteria for earlier intervention. Specifically, the blood urea nitrogen (BUN), urine specific gravity (USG), serum proteins, alanine aminotransferase (ALT), total leukocyte count, and heterophil count have significant implications for utility as criteria for early endpoints.

Additionally, our data corroborate earlier studies that indicate TB pathogenesis in guinea pigs is very similar to that in humans. As previously mentioned, Kim and colleagues found that human TB patients show a slowly progressive, mild, non-regenerative anemia without prominent leukocytosis, but often with neutrophilia. Although our guinea pigs were never classified as anemic based on established reference ranges, there was a decrease in hematocrit in the animals we called survivors. It is possible, that if given enough time and protection from fatal infection, guinea pigs may predictably and reproducibly show a form of anemia of chronic disease. Also, Kim and colleagues found that human TB patients have relatively normal liver function tests, and there is an increase in serum immunoglobulins. Our animal data parallels these findings with minimally changed serum liver enzyme values and an elevation in serum globulins. Interestingly, despite seemingly normal bloodwork, bone marrow and liver biopsies often show abnormalities including granulomatous inflammation without the presence of organisms in humans, which we also found to be true with regard to the liver histopathology in guinea pigs.

In humans, genitourinary forms of extrapulmonary TB are one of the more common forms (Iseman, 2000), but this has not historically been true for the guinea pigs model. An average of 22 years between time of primary *M. tuberculosis* infection to symptoms of genitourinary pulmonary TB was found in one study in humans (Iseman, 2000), which may explain why we do not see a correlate in guinea pigs; they simply do not live long enough to develop this form of extrapulmonary TB. In our guinea pigs, there are early signs of genitourinary change as early as 60 days post inoculation within the saline-treated group, and starting at 90 days post-inoculation within the BCG-vaccinated group when evaluating urine specific gravity and serum BUN. Additionally, some guinea pigs did show a decrease in hematocrit with chronicity, which may correlate to signs of anemia of chronic disease and decreased erythropoietin

production from the kidneys. In particular, one surviving animal had a terminal hematocrit of 30, but lived throughout the experiment without clinical signs. However, no guinea pigs showed sterile pyuria or histopathologic renal changes after euthanasia. In order to accurately evaluate the genitourinary system in guinea pigs infected with *M. tuberculosis,* experiments would need to control for food and fluid intake as well as excretion in conjunction with serum biochemistry, complete blood count, and urine parameters.

The liver is an organ responsible for producing many proteins and enzymes that can represent both the liver's function as well as immune function. Measured serum proteins include albumin and globulins. Albumin is made solely by the liver, is a negative acute-phase protein, and contributes considerably to vascular oncotic pressure. Serum globulins are a measure of several proteins that are both produced by the liver and the immune system. Immune globulins are commonly referred to as antibodies. Although serum globulins can sometimes be interpreted as an indication of liver function, they are more likely an indication of immune response in our study. However, we cannot say that is certain since antibodies were not directly measured.

Although it has been established that *M. tuberculosis* disseminates to the liver of guinea pigs, the degree is variable and somewhat dependent on immune status. The BCG vaccine has not been shown to be protective against development of disease in guinea pigs, similar to humans, but it does provide some protection against acquiring very severe and disseminated forms of disease. Measures to evaluate the liver are vast, and typically include evaluation of enzymes detectable in the blood. Alkaline phosphatase (ALP), alanine transferase (ALT), glucose, albumin, and globulin were measured parameters in the serum biochemistry assay used for this study. Briefly, ALP is an inducible enzyme where elevations can be due to changes in isoenzymes of the liver, bone or gastrointestinal tract. ALT is more indicative of hepatocellular damage, but is not organ specific in guinea pigs. Glucose is highly variable, and can be increased or decreased due to many factors including cirrhosis, stress, post-prandial sampling, sepsis, chronic renal insufficiency, artifact of sample storage, and many others. Because changes in glucose can be affected by many variables other than the liver specifically, we did not utilize glucose as a liver-function enzyme. Albumin and globulin are both serum proteins that are made by the liver. Albumin

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decreases as both a negative acute-phase protein and directly related to decreased production by the liver. Globulin, however, is not nearly as specific. There are globulins made by the liver, and globulins produced by the immune system, which are commonly known as antibodies- however, both are measured in the serum as globulins without specificity of the source.

Although the saline-treated group had more severe liver lesions than the BCG-vaccinated group on gross necropsy and histopathology, neither group showed severe changes in serum levels of liver enzymes. While ALP was significantly higher in the saline-treated group at the pre-challenge time point compared to the BCG-vaccinated group, the groups were essentially the same after inoculation. This could indicate either an elevation in serum ALP from physiologic values in the saline group, an effect of vaccination in the BCG group, or a combination of both. Although ALP can be elevated in response to bone growth and gastrointestinal tract (GIT) pressures as well as in response to liver pathology, our guinea pigs were age and weight matched, therefore controlling for bone growth and GIT variables. Guinea pigs were also different in their measured ALT values prior to inoculation, with the BCG-vaccinated group higher than the saline-treated group. We speculate that the differing pre-challenge ALT may have something to do with immune status. While the BCG-vaccinated group recovers the serum ALT to pre-challenge values fairly quickly, there is a subsequent steady decrease throughout the remainder of the study. Conversely, saline-treated guinea pigs showed a significant and steady increase in ALT over time. This is likely due to hepatocellular damage in the liver from hepatic granulomas in response to infection. Histopathologically, guinea pigs in the saline-treated group had much more severe and disseminated lesions affecting the hepatic parenchyma; which correlates well with an increase in serum ALT over time as a direct result of hepatocellular damage. This data also corresponds well to what Kim and colleagues found in humans—relatively normal liver function tests with abnormal liver biopsies in 92% of patients. While ALP, ALT, albumin, and globulin are not used as evaluation of liver function specifically, perturbations in these parameters can all be due to hepatocellular leakage and damage to the liver.

When evaluating serum proteins, total protein reflects the additive value of both serum albumin and globulin. Total protein is directly measured in the serum, as is albumin, while globulin is calculated by taking the difference of total protein and albumin. Albumin is a large plasma protein that contributes significantly to oncotic pressure and can be falsely elevated in times of dehydration. Albumin can be decreased in times of compromised liver function as well as in response to inflammation, as a negative acute phase protein (Ritchie RF, Palomaki GE, et al 1999). Serum globulins are both made by the liver and the immune system. An increase in serum globulin levels can be indicative of the liver producing more protein in response to inflammation, as well as the immune system producing antibodies in response to infection. A decrease in globulin can also be reflective of liver function; however, it is an unreliable means to evaluate liver function and is not usually interpreted in that fashion. More specifically, immunoglobulins are evaluated as a measure of inflammation, and are not produced by the liver. They are a large component of calculated serum globulins however, which makes interpreting serum globulin somewhat difficult. It is not possible to differentiate liver-produced globulins from immunoglobulins with serum chemistry analysis, but it is generally assumed that an increase in this value is due to an inflammatory response and is likely due to an increase in the immunoglobulin component.

In our guinea pigs, the BCG-vaccinated group had a significantly higher total protein at pre-challenge measurements, which was attributable to a higher globulin level reflecting an immune response to vaccination. After infection, the BCG-vaccinated group had an initial decrease followed by a steady increase in total protein, while the saline-treated group had a steady increase immediately following inoculation. The initial decrease in the BCG-vaccinated group is likely a return to normal levels following immunization, with a later increase as a response to infection. Both the BCG-vaccinated and saline-treated groups had a sharp decrease in albumin within the first 30 days of infection, with a subsequent steady increase afterward. The initial decrease reflects the role of albumin as an acute phase protein. The subsequent increase is slightly puzzling, as there are not many differentials for increased albumin. One theory is that our guinea pigs could have been slightly dehydrated, which results in

hemoconcentration and a falsely elevated albumin as a result. Further studies that measure food and fluid intake would need to be conducted to characterize this theory more definitively.

Similar to serum albumin, the serum globulin was significantly different between groups at the pre-challenge time point, which is due to prior vaccination and an increase in immune activity in the BCG-vaccinated group. The saline-treated group shows a sharp increase in serum globulins within the first 30 days following inoculation, followed by a more subtle increase throughout the remainder of the experiment. Although we are unable to distinguish the specific globulin proteins with a serum biochemistry assay, we speculate that this finding more accurately represents the immune response to infection in the form of increased antibody production. There was a significant effect of treatment over time in the saline-treated group, which was graphically depicted as globulin continuing to increase with time, as TB related disease was more severe in the saline-treated group.

Other parameters that are used to evaluate liver-specific pathology in humans and other animals include gamma-glutamyl transferase (GGT), aspartate transaminase (AST), sorbitol dehydrogenase (SDH), lactate dehydrogenase (LDH) and total bilirubin. Total bilirubin was a component of our serum chemistry profile, but results were consistent and unremarkable. It may be worth evaluating serum AST, GGT, and SDH in addition to ALP, ALT, and serum proteins, to identify additional hepatic enzymes that could be used as biomarkers for early endpoint criteria. Because hepatic forms of extrapulmonary TB are predictable and consistent in both vaccinated and non-vaccinated guinea pigs, identifying a hepatic biomarker for early endpoint criteria would beneficial and applicable to all guinea pigs enrolled in the study.

The complete blood count evaluates red blood cell count (hematocrit), total white blood cell count (leukocytes), as well as the individual white blood cells detectable in the peripheral circulation. Leukocytes are comprised of heterophils, lymphocytes, and monocytes primarily, with lesser influence by basophils and eosinophils. In guinea pigs, peripheral blood lymphocyte concentration stays fairly constant as the animal grows and volume increases, which is an important point in our guinea pigs as they continued to grow throughout the study (Harkness J, Manning P, 1976)

In both groups, there is a significant increase in hematocrit within the first 30-60 days after infection, which reflects an acute response to hypoxia. As the hematocrit continues to increase in the saline-treated group for the remainder of the experiment, hematocrit slowly decreases in the BCG-vaccinated group, with a final steep decrease between days 360 and 420. While the increasing hematocrit in the saline-treated group likely shows a continued response to hypoxia by producing more red blood cells, the BCG group does not show the same response. It is possible that as the disease becomes chronic in, hematocrit levels become stable. At 360 days the remaining four animals have a distinct decrease in hematocrit, which continues to drop until the end of the study. This could be a reflection of a shift to anemia of chronic disease in the survivors, although more research would need to be done to confirm that speculation.

In our guinea pigs, the saline-treated group had a larger degree of leukocytosis than the BCG-vaccinated group, and the saline group developed more severe inflammation over time. Our data show that although guinea pigs vaccinated with BCG do develop classic TB lesions, the severity and dissemination is much less than saline-treated animals. This may be due to a synergistic effect between decreased bacterial burden and an appropriately decreased immune response; the opposite of which is seen in non-vaccinated animals. This correlates with longer lifespan, decreased severity of pathology, and lower bacterial burden in the BCG group. The inflammatory response is significantly dampened in BCG-vaccinated guinea pigs, which is an advantageous attribute rather than detrimental. At the same time, it is very possible that the immune response in saline-treated guinea pigs is overzealous relative to bacterial burden, resulting in the severe pathology that we have seen in several of our study animals. However, this still leaves us wondering from what the BCG-vaccinated animals die, since it doesn't appear to be from severe and disseminated TB.

In acute inflammation, heterophils are expected to increase dramatically and soon after infection. They are the inflammatory cell responsible for phagocytosis and destruction of microorganisms, and can be found in the tissues or peripheral circulation. Lymphocytes typically increase in chronic cases of inflammation and neoplasia. In both groups of guinea pigs in our experiment, there was an increase in

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total leukocyte count attributable to heterophils, lymphocytes, and monocytes; although the total leukocyte count most closely paralleled changes in heterophils in both groups. This increase occurred within the first 30 days in the saline-treated group, due to all subsets of leukocytes. The BCG-vaccinated group responded similarly, although at a lower level. This reflects well the vaccination status and relatively mild inflammatory response of the BCG group, since it is known that the vaccine protects against very severe forms of disease. Additionally, this reflects the immune response in the saline group to more organisms present in the lungs, which ultimately results in more immune cells responding to infection.

Both BCG-vaccinated and saline-treated animals show similar heterophil counts in the peripheral blood both at the pre-challenge time point. However, the saline-treated group does have significantly higher heterophil count at several time points during the progression of disease, and has a more pronounced increase over time. This indicates that although the saline-treated group experiences greater inflammation than the BCG-vaccinated group, as well as more severe TB-associated lesions histopathologically, the difference does not present itself as consistent in peripheral blood. This could indicate several things. First, heterophils in the saline-treated group may be more aggressive and degranulate at a higher rate than heterophils in the BCG-vaccinated group, which causes more tissue destruction and pathology, and may be cytokine driven. The second possibility is that our sampling interval was too large to see indication of severe heterophilia in peripheral blood. We do know that heterophils are called to the pulmonary parenchyma and a common route from the bone marrow to the tissue is through the vasculature. However, it may be true that heterophils are only in the vasculature for a short period before extravasating into tissue, further confirming that TB is a disease of tissues rather than blood. Third, it is possible that heterophils travel more in the lymph fluid than in the vasculature (similar to lymphocytes), and once they're in the tissues they stay there until they die. Therefore, severe peripheral blood heterophilia may never be an actual component of the inflammatory response in our study, regardless of widespread tissue inflammation.

When comparing guinea pig lymphocytes to human lymphocytes and the response to inflammation, guinea pigs are unique in that daily lymphocyte production is much higher than in humans, and the thymus is a major contributor (Yoffey, 1956). Also, lymphatic vessels are a main route of transfer of thymocytes to the general circulation in the guinea pig, largely due to an extensive efferent lymphatic network around the thymic artery. This is also in contrast to nearly every other mammalian species, including humans (Kotani et al 1966). We may be seeing this in our guinea pigs in the response of lymphocytes over time between the two groups of guinea pigs, as the BCG-vaccinated group does not show an exaggerated response. Even in the saline-treated group, although there is a distinguishable lymphocytosis, it is not to the magnitude described in humans.

With regard to peripheral blood lymphocytes there were no significant differences between the groups of saline-treated and BCG-vaccinated guinea pigs at any time point. There was a significant elevation within both groups at 30 days, which indicates an initial increase in lymphocytes in the peripheral blood that remains constant throughout the disease process, independent of immune status. Again—we may be seeing that there is not a detectable peripheral inflammation in guinea pigs, but rather a more severe tissue inflammatory component and lymphocyte travel through lymph fluid.

Monocytes constitute a very small proportion of inflammatory cells in the peripheral blood, but are still important cells to consider. They are a phagocytic leukocyte that have a role in chronic inflammation, and differentiate into macrophages. At days 90 and 120, the saline-treated group had significantly higher monocytes than the BCG-vaccinated group. This demonstrates chronicity of disease in the saline-treated group, as monocytes migrate to the tissues and differentiate into macrophages. There was no difference between pre-challenge and last-scheduled blood draw, which indicates that although the saline-treated group had higher monocyte count at some time points, the overall change is similar between groups. Both groups have an increase in peripheral monocytes over time, which reflects that TB becomes chronic in guinea pigs independent of immune status.

When evaluating the overall inflammatory response in peripheral blood of guinea pigs infected with *M. tuberculosis*, there is minimal significant difference between groups of saline-treated and BCG-

vaccinated guinea pigs. However, this does not correlate to the histopathology and organ CFU count, which is more severe in the saline-treated group. This apparent dichotomy may be due to the methods for detecting inflammation. It is also possible that we are seeing what both Kotani and Yoffey described decades ago: 1) guinea pigs do not have high levels of circulating inflammatory cells, 2) inflammatory cells may only circulate for a short period, and 3) these cells may also travel through lymph fluid as well as blood (Kotani et al. 1966). Additionally, there may be other parameters of greater interest to evaluate for inflammation, such as fibrinogen. Fibrinogen is commonly used in horses and cattle to detect inflammation, as they predictably have low levels of circulating leukocytes and the complete blood count can often appear unremarkable in the face of severe inflammation. Although fibrinogen is most often evaluated in human medicine as a factor in clotting, it is evaluated as an acute phase protein in some veterinary species, and may be worth looking at in our guinea pig patients as well.

Blood gasses are often used to evaluate gas exchange. They are comprised of partial pressures of oxygen and carbon dioxide, blood pH, bicarbonate anion, and lactate levels in the peripheral blood. The most ideal sample is an arterial sample, as it has been freshly oxygenated and is the best indicator for the efficacy of gas exchange across the lungs. However, a venous sample can be used as long as it is evaluated appropriately for the type of sample.

The pre-challenge evaluation of blood gasses revealed normal and healthy guinea pigs in both groups, with appropriate blood pH and gas exchange. However, within 30 days we found that our guinea pigs became severely acidemic with the blood pH averaging about 6.8 in all animals independent of immune status. This finding was both very exciting and puzzling, because blood pH is physiologically very tightly regulated, and all animals with a blood pH of 6.8 would be moribund, and our guinea pigs appeared healthy. In addition, all guinea pigs maintained a low blood pH throughout the experiment.

After extensive research in textbooks and literature of pulmonary physiology, as well as consultation with experts in the field of clinical pathology and emergency veterinary medicine, we came to the conclusion that a prolonged, sustained, severely acidemic pH was not compatible with life, and must be the result of some sort of sampling error. A quick comparison of blood gasses analyzed in guinea

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pigs infected with *M. tuberculosis*, using both blood from EDTA tubes and lithium heparinized tubes in the same animal, we found that the potassium EDTA used for all the blood gasses in our study had severe consequences on measured values of blood pH. In a side-by-side comparison of blood from the same guinea pig, all of the EDTA samples showed low blood pH, while all of the heparinized samples showed a more acceptable blood pH. Hence, the additive (potassium EDTA) was falsely decreasing the blood pH (and possibly other parameters) when analyzing the blood gasses.
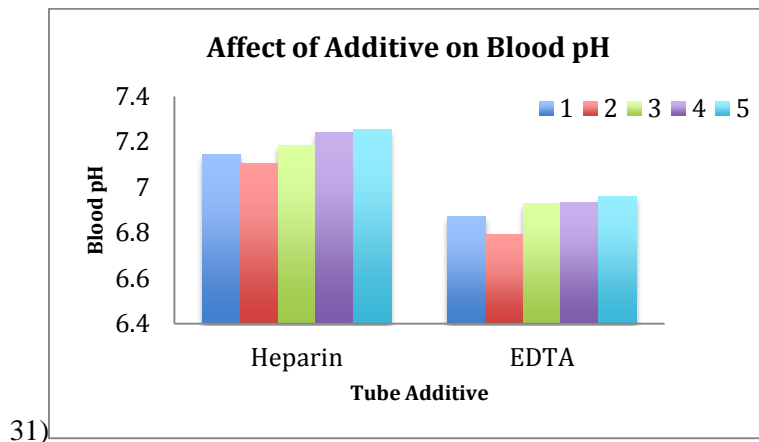
31)

Fig 31- Chart comparing the effect of additive to blood tubes on blood pH. Heparinized tubes are evaluated on the left, and Potassium EDTA tubes evaluated on the right. We used 5 animals, numbered 1-5.

In light of these results, we have eliminated the blood gas data from our significant findings, since we are unable to adequately interpret these results as a component of infection with *M. tuberculosis* due to the sampling error. However, we do feel as though there is great potential for evaluating blood gasses to both determine disease state and identify early endpoint criteria in guinea pigs infected with *Mycobacterium tuberculosis*.

Due to curious observed effects when collecting data throughout the study as well as at the terminal time points, we conducted a comparison of anesthetic effect on blood parameters in healthy, uninfected guinea pigs, in order to control for potential effects of disease. It was our observation that some values, specifically those related to the liver, collected while under ketamine and xylazine anesthesia seemed quite a bit different than those collected under isoflurane anesthesia. To confirm our suspicions, we collected blood from 45 naïve guinea pigs under 3 conditions- 1) unanesthetized, 2)

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isoflurane anesthesia, and 3) ketamine and xylazine anesthesia. We then ran a complete blood count, serum chemistry, and blood gas analyses to assess the same parameters for effects of anesthetic, and not disease.

Complete Blood Count

Evaluation of hematocrit levels showed that both anesthetics significantly decrease this value. While none of our guinea pigs were classified as anemic due to this decrease, the decrease could affect clinical and research evaluations. When evaluating guinea pigs on an individual basis, an animal that is on the low end of the normal hematocrit range could conceivably be falsely classified as anemic if the sample was collected under anesthesia, which could have both clinical and research implications. For research purposes, the anesthetic effect could produce results that may be falsely interpreted as a consequence of disease or experimental manipulations.

The leukogram was also influenced by anesthetic choice—the most notable changes were an increase in total leukocyte count and decrease in lymphocytes due to ketamine and xylazine, and both anesthetics increased heterophils. Thus, when evaluating the leukogram for inflammation, these anesthetic-induced changes could alter interpretation of the leukogram. As with changes in the hematocrit, a classification of an inflammatory leukogram versus a normal leukogram could have an impact on treatment and euthanasia decisions.

Serum Biochemistry

The changes on serum biochemistry values were mixed. Renal enzymes BUN and creatinine were not changed by either anesthetic, which is due to the primary respiratory or hepatic metabolism of both anesthetics, with little (if any) metabolites reaching the kidneys for urinary elimination.

On the contrary, both serum proteins and liver enzymes were significantly affected due to anesthetics. Total protein was decreased in response to both isoflurane and ketamine and xylazine. Albumin was mildly decreased while globulin was more significantly decreased by ketamine and xylazine than isoflurane. Ketamine and xylazine caused a decrease in ALP and increase in ALT. A decrease in

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serum protein and changes in liver enzymes suggest hepatic metabolism of injectable anesthetics. Isoflurane, which is metabolized and eliminated n the lungs, had no change.

Core body temperature, as measured by a subcutaneously implanted RFID chip, remained very consistent throughout the study. Temperatures stayed between 38 and 40°C throughout the study in all animals, with no apparent trends associated with disease.

Body weights increased in both groups of animals throughout the study. This is a result of the younger guinea pigs maturing during the study. At 20 weeks post infection, the BCG group had a higher average body weight than the saline group. This may be due to chronic illness in the saline-treated group of animals.

Colony Forming Units (CFUs) were calculated for several organs including the heart, lung, liver, spleen, brain, kidney, and eyeball. CFUs and pathology scores were the highest in the lungs of saline-treated guinea pigs. However, some saline-treated animals had below detectable levels of CFUs in the lungs, despite having histopathology consistent with TB. In these cases, it is possible the organisms responsible for inciting tissue inflammation may have been destroyed while the immune system continues to respond, possibly due to persistent antigenic stimulation; ultimately resulting in vast tissue destruction in the absence of the organism . This demonstrates how an overzealous immune system can lead to pathology. The BCG-vaccinated individuals experience far less severe pathology.

In addition to liver and spleen lesions, we identified two animals in the saline-treated group and one animal in the BCG-vaccinated group showed positive CFUs in the brain, with one having corresponding histopathological changes. One animal in the saline-treated group showed CFUs in the eyeball, in the absence of histopathologic changes. The liver and spleen are established secondary sites of infection in guinea pigs, while the kidneys are expected and common site of extrapulmonary TB in human patients due to their fine vascular stroma, which can be prone to thromboembolism, but has yet to be seen in the guinea pig model. Meningeal forms of TB are somewhat common in children with TB, from which the BCG vaccine protects, and was exemplified in our guinea pigs as well.

There were four animals in the BCG-vaccinated group that were survivors. These four individuals did not show signs of disease associated with *M. tuberculosis* infection both in their behavior and in their measured values. Body weights and temperatures were stable, and activity levels remained normal throughout the study. We terminated these animals at 441 days post infection. Upon evaluation of organ CFUs and histopathology scoring of these four survivors, we found that two of them had no detectable CFUs or histopathological lesions, while the other two had only mild lesions. This is compelling data for resistant animals, and under the assumption that these four individuals were truly survivors, our guinea pigs nicely show the three phases of TB infection that are typical of this animal model: 1) susceptible (die before 20 weeks), 2) expected (die between 20-40 weeks), and 3) resistant (never show signs of disease). More research needs to be done to determine whether the susceptible and resistant populations closely mimic humans infected with *M. tuberculosis* as well. Additionally, if we allowed these four survivors to continue on, we may have a true animal model of latency. The potential for translational research would be great, as this would provide another animal model of latent TB.

Chapter 5. Conclusions

Our hypothesis when starting this project was that there would be a parameter or set of parameters that were associated with the development of disease, and could be used to identify criteria for early endpoints in guinea pigs experimentally infected with *Mycobacterium tuberculosis.* After extensive data collection in two groups of guinea pigs exposed to *M. tuberculosis*—one group vaccinated with BCG, and one group treated with only saline—we found that identifying disease in guinea pigs was much more difficult than anticipated, although we did find subtle changes which could be used to assess disease. As expected, using three simple tests we did find that many commonly evaluated parameters in the blood and urine did change in a way that have potential to establish criteria for earlier intervention. Specifically, the blood urea nitrogen, urine specific gravity, serum proteins, alanine aminotransferase, and heterophils were significantly different between the groups, among the potential for use as early endpoints.

Additionally, our data corroborate earlier studies that indicate *M. tuberculosis* disease results in similar hematologic changes as seen in humans. Kim and colleagues found that human TB patients show a slowly progressive, mild, non-regenerative anemia without prominent leukocytosis, but often with neutrophilia. Saline-treated guinea pigs had a lower hematocrit and higher heterophils than BCG-vaccinated guinea pigs. Human TB patients show relatively normal liver function and an increase in serum immunoglobulins. Our guinea pig data parallels these findings with minimally changed liver values and an elevation in serum globulins with severe histopathologic liver lesions in saline-treated animals.

Although our study did not lead to concrete parameters that could be used to establish early endpoint criteria it did establish parameters, which could be monitored in the face of disease, as an additional tool for evaluators. Based on our study we recommend specifically monitoring serum BUN, ALT, globulins, hematocrit, and heterophils as biomarkers for disease.

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APPENDIX 1: SOPS

Growth of virulent *Mycobacterium tuberculosis*

Materials
Vial of *Mycobacterium tuberculosis*
Proskaur Beck Media
Lowenstein-Jensen Slants
Blood agar or Trypticase Soy Agar (TSA) plates
15 ml screw cap glass tubes
Either: Preferably a 160ml Corning Milk Dilution Bottle (narrow mouth) or a wide mouth 100ml Corning
    flask or a 75cm^2 tissue culture flask,.
Bacteria transfer loops
Incubator
Sonicator
Homogenizing tube with pestle and homogenizing apparatus
Glass vials with rubber stoppers and sealing rings
Labels

All procedures must be performed in a BSL-3 laboratory inside a Class II Biosafety hood.

Preparation of Seed Stocks
From one vial of *M. tuberculosis* (Mtb)
Transfer 1ml of the Mtb to a glass tube containing 9ml of Proskaur Beck (PB) medium, in a clear glass
tube.
Also streak a loop of the *M. tuberculosis* culture onto 1 or 2 slants of Lowenstein-Jensen media
Incubate slants at 37°C until confluent and then, once confluent, transfer to -80°C.

Incubate the Mtb in PB at 37°C without agitation
Check the culture to ensure the formation of a layer of organisms growing on the top of the liquid, this is
the pellicle
The remaining medium below the pellicle should be clear however there may be a ring of organisms at
the bottom of the tube
> **Passage 1:**
> When pellicle is confluent (cover the top surface of the liquid)
> Using a concentrically coiled loop, transfer one loop-full of the pellicle to a 160ml Corning Milk
> Dilution Bottle (narrow mouth) placed on its side or a wide mouth 100ml Corning flask
> containing PB medium (if no other containers are available a 75cm^2 flask can be used)
> (Note: glass is preferable to plastic when growing pellicle)
>
> Streak a loop of the pellicle/medium onto blood agar (or TSA) during the transfer to check for
> contamination
> Incubate 37°C, for 48-96hours
>
> In addition, transfer one loop-full into a new glass tube containing 9ml of PB medium (this is a
> "drop back". In case something goes wrong with the new culture you have something to back to
> without having to start over again)
> Incubate flask and drop back tube at 37°C without agitation
> Examine the cultures – as the pellicle grows the medium below should remain clear with some
> organisms visible.

Passage 2:
When pellicle is confluent
Using a concentrically coiled loop transfer one loop-full of the pellicle to a 160ml Corning Milk Dilution Bottle (narrow mouth) placed on its side or a wide mouth 100ml Corning flask containing PB medium
In addition, transfer one loop-full into a new glass tube containing 9ml of PB medium (the "drop back" culture)
Incubate at 37ºC without agitation

Streak a loop of the medium onto blood agar (or TSA)
Incubate 37ºC, for 48-96hours
Examine the cultures – as the pellicle grows the medium below should remain clear with some organisms visible.

Passage 3:
When pellicle is confluent
Using a concentrically coiled loop transfer one loop-full of the pellicle to a 160ml Corning Milk Dilution Bottle (narrow mouth) placed on its side or a wide mouth 100ml Corning flask containing PB medium
In addition, transfer one loop-full into a new glass tube containing 9ml of PB medium (the "drop back" culture)
Incubate at 37ºC without agitation
Examine the cultures – as the pellicle grows the medium below should remain clear with some organisms visible.

Streak a loop of the medium onto blood agar (or TSA)
Incubate 37ºC, for 48-96hours

Bottling Seed Stocks
When pellicle is confluent
Using a concentrically coiled loop transfer as much of the pellicle from the flask to a grinding tube containing approximately 5ml of PB medium
In addition, transfer one loop-full into a new glass tube containing 9ml of PB medium (the "drop back" culture)
Incubate at 37ºC without agitation

Streak a loop of the medium onto blood agar (or TSA)
Incubate 37ºC, for 48-96hours

Grind the solution for approximately 30-60 seconds
Place tube on ice for 10-15min to allow clumps to settle
Take off the top suspensions
Sonicate the suspension 3 x10 seconds each

Transfer organisms from the top layer (avoiding large clumps) to vials
Streak blood agar (or TSA) with seed stock culture several times throughout the bottling procedure
Seal vials
Label vials as seed stock with the strain name/number, initials of RA and the mm/yy

Store Seed Stocks at -80ºC

Note: The concentration of the Seed Stock is not required to prepare Working Stock, what is important is the quality of the Seed Stock, so that a virulent Mtb remains virulent.

Growth of Working Stocks for Virulent *Mycobacterium tuberculosis*

Materials
Vial of *Mycobacterium tuberculosis* Seed Stock
7H9 with OADC
Sterilized 20% Tween 80 solution
Proskaur Beck Media + 0.1% Tween
7H11 agar (quad plates)
Blood agar or Trypticase soy agar (TSA) plates
15 ml screw cap glass tubes
Wide mouth 100ml Corning flask
1 Liter Roller Bottles
2 Liter Erlenmeyer flask (autoclaved with magnetic stir bar inside)
Bacteria transfer loops
Incubator
Sonicator
Stir Plate
2ml Corning Transfer Syringes
Glass vials with rubber stoppers and sealing rings
70% Ethanol
Labels

All procedures must be performed in a BSL-3 laboratory inside a Class II Biosafety hood.

Passage 1 (from Seed Stock)
From one vial of Seed Stock aliquot 1 ml into 9ml of 7H9+OADC medium containing 0.1%
Tween 80
Incubate at 37ºC on a shaker
Every day: check culture by shaking tube – look for white swirl
After shaking a frothy layer is noticeable above the liquid, this is the Tween.
As the Mtb grows the amount of Tween is reduced and it may be necessary to add new Tween.
To add new Tween: add 1 ml of a 20% Tween-80 in PB solution to 1000ml of culture.
Over time the culture will turn a milky white color – this indicates growth

Passage 2 (from Seed Stock)
At approximately 10 to 14 days, when there is a milky white appearance to the culture transfer
5ml into 50ml of PB+0.1%Tween
Drop back (1ml into 9ml) and streak a loop of culture onto blood agar (or TSA)
Repeat culture conditions as above.

Passage 3 (from Seed Stock)
At approximately 10 to 14 days of incubation transfer 50 ml of culture to 500ml of
PB+0.1%Tween in a roller bottle
Drop back (5ml into 50ml) and streak a loop of culture onto blood agar (or TSA)
Incubate at 37ºC on a roller apparatus
Repeat culture conditions as above.

Approximately 16 hours prior to bottling work stocks, add 0.5ml of 20% Tween 80 to roller

bottle.

Incubate at 37ºC on a roller apparatus

Transfer to an erlenmeyer flask containing a large stir bar
(Pre-autoclave erlenmeyer flask with stir bar inside)
Sonicate 3 x 10 seconds

Bottling Working Stocks

To bottle working stocks, place the flask onto stirrer and using a 2ml Corning transfer syringe add 1 to 1.5ml of Mtb into vials.
Seal vials
Drop sealed vials into a beaker of 70% Ethanol
Let the vial sit in ethanol for 10 minutes
Remove from ethanol
Allow vials to dry
Label each vial with organism name, strain name (and TMC# if applicable), and the lot number (which should include the RA's initials and a date)

Store Working Stocks at -80ºC

Two to 3 days later take 3 to 4 vials and plate 10-fold dilutions (10^1 to 10^8) on 7H11 agar to determine the CFU/ml for the working stock.
The concentration should be between $7x10^7$ and $2x10^8$ CFU/ml.

Note: To maintain a consistent virulence among the different lots of Working Stocks made from the seed, the Working Stock should never be taken more than 6 passages from the Seed Stock.

Guinea pig aerosol challenge (Painter)

Purpose. To define a procedure for aerosol challenge with mycobacteria using the Madison (Generation III) infection chamber.

Scope. Colorado State University Mycobacteria Research Labs, a unit within the Department of Microbiology Immunology and Pathology.

Responsibility. Any scientific staff member(s) within the Mycobacteria Research Labs required to perform a guinea pig aerosol challenge.

Frequency. As needed.

History. This SOP was issued in order to establish a procedural continuity within the laboratory and among its scientific staff and documentable training in aerosol challenge with mycobacteria.

Note: Although this procedure can be accomplished by one experienced staff member, with two (preferably 3) staff members the procedure can be handled much more efficiently.

Procedure.

Materials:

Madison infection chamber	Infection basket(s)
Class II biosafety cabinet	2.5% Vesphene wash bottle
70% EtOH wash bottle	Absorbent bench-top paper
Paper towels	5 ml Falcon tube(s)
Small biohazard bags	Pipette boat
M. tuberculosis H37Rv	Ice chest with dry-ice
Sterile pyrogen free H20	50 ml conical tubes
20cc syringes	18G1½ needles
Tupperware container	Pen/log book
Tube rack(s)	24-well plate(s)
P1000 micropipette + tips	10 ml pipettes
1cc syringe	26G½ needle
P200 micropipette/P200 tips	Quad 7H11 agar plates
Timer	PAPR
Autoclaved glass nebulizer jars	Stainless-steel container(s)
Large/X-large biohazard bags	Disposable surgical gown

1. Sterilize the surfaces of the Class II Biosafety Cabinet with paper towels doused in 2.5% Vesphene (followed by re-wiping the surfaces with 70% EtOH).
2. Transport all materials from airlock inside the barrier, and take the *M. tuberculosis* H37Rv stock out of the dry-ice containing ice chest, and begin thawing the stock inside the biosafety cabinet (inside a 50 ml conical in a tube rack).
3. Load 15 ml of sterile pyrogen free H20 into a 20cc syringe affixed to an 18G1½ needle (this will be used for the test run for the Madison infection chamber).
4. Transport the test run syringe into the chamber room inside a Tupperware container (used for weighing guinea pigs).
5. Begin the test run in the Madison chamber (15 minute cycle):

a. Dispense the 15 ml of pyrogen free H_2O into the autoclaved glass nebulizer jar by removing the plug screw (Figure 1), unsheathing the needle, and placing the needle tip in through the orifice to rest against the side of the jar (so as not to create any aerosols when dispensing).
b. After dispensing the pyrogen free H_2O into the jar, re-fasten the plug screw!
c. *For the test run only*, unlatch the Control Box lid (Figure 2A), and check that the time delay relay, TD1, is set to 900 sec/ON DELAY, and that the second time delay, TD2, is set to 300 sec/INTERVAL DELAY (Figure 2B).
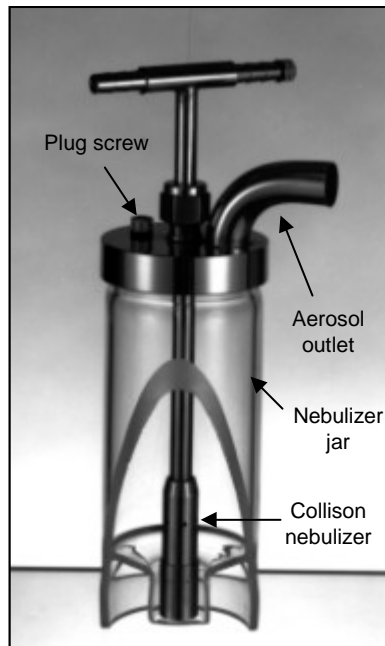

Figure 32. Diagram of the Collison nebulizer unit, complete with the surrounding glass nebulizer jar.

A   **B**
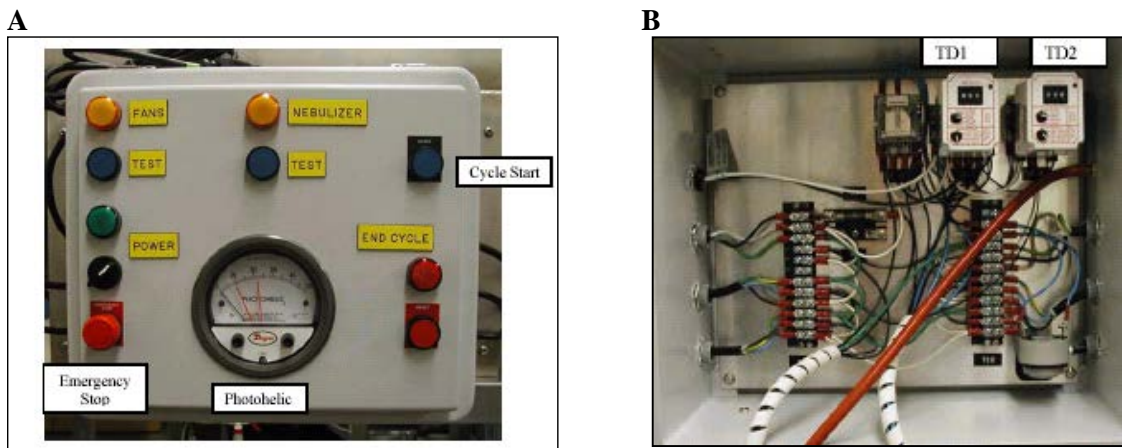

Figure 33. The exterior (Panel A) and interior (Panel B) of the Madison chamber Control Box.

d. Close/latch the Control Box lid, and supply power to the chamber system by depressing the outlet switch (white button on power strip), this powers the compressor/vacuum pump unit.
e. Check for the proper amount of airflow into the system (secondary air, ~45 L/min) indicated on the flow meter on the right side of the flow panel (Figure 3). Also, be sure

to check the Photohelic unit (Figure 2A) that the proper amount of vacuum is being maintained (should read 9-10 inches of H_2O).
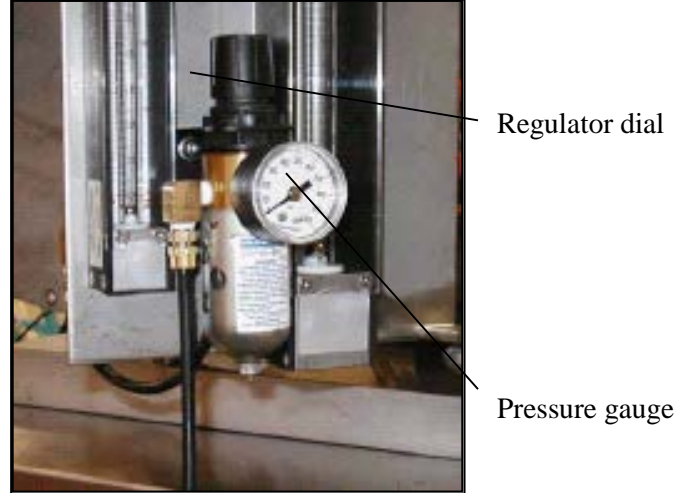

Regulator dial

Pressure gauge

Figure 34. Diagram of the flow panel. The flow meter on the right indicates the secondary airflow, or air being pumped through the system by the vacuum/compressor unit. The flow meter on the left indicates the primary airflow (the air being channeled through the nebulizer unit determined by the pressure regulator (at center).

     f. Turn on the Control Box "Power" switch by turning clockwise.

     g. Depress the Nebulizer "Test" button to check for the proper airflow into the nebulizer (~4 L/min) as indicated on the flow meter at left on flow panel (Figure 3).

     h. Unlatch the chamber door (8 latches around the perimeter) and open the door to visualize the mixing fans (Figure 4).

Figure 35. Diagram of the one of the mixing fans mounted on the inside of the chamber door.

     i. Test the mixing fan(s) operation by depressing the Fan "Test" button on the Control Box.

     j. If everything is operational, re-seal the airtight door with <u>all 8 latches</u>.

     k. Initiate the test run by depressing the "Cycle Start" button on the Control Box (Figure 2A).

     l. With a pen, log both the reading on the primary airflow meter into nebulizer left side of flow panel) & the reading on the pressure gauge Figure 3) beneath the regulator into the log book which is kept on the cart beneath the chamber.

     m. Also, before leaving the room, be sure that the nebulizer is fully functional by checking for spray coming out of each of the 3 jets of the nebulizer probe.

     n. Lastly, post the "Infection in progress Do Not Enter" sign over the door window.

6. While waiting for the *M. tuberculosis* H37Rv vial to thaw, label each of the corresponding guinea pig cage cards for the guinea pigs to be aerosolized by writing "aerosolized with ~100 CFU *M. tuberculosis* on <u>*date/initials*</u>" onto each cards.

7. Next, place the stainless steel container and fill with a 2.5% Vesphene solution next to the aerosol machine (all "used" nebulizer jars will be placed into Vesphene). After each run, remove jar

from Vesphene and wrap the jar in paper towels and place inside the nebulizer container and put generous amount of Vesphene on paper towels. Then place the lid on the container and put in a large clear bag for autoclaving.

8. After the *M. tuberculosis* H37Rv has thawed, place some absorbent bench-top paper inside the biosafety cabinet, don a disposable surgical gown, double glove, and fill one 50 ml conical tube with the 2.5% Vesphene solution (~25 ml).

Note: If two or more staff members are present, while the inoculum is being prepared, the other staff member(s) can load the guinea pigs to be challenged into the infection basket. This can be performed for the first two aerosol runs inside the guinea pig housing rooms (since both infection baskets have been autoclaved). However, once each basket has went through one infection cycle, for ALL other runs the guinea pigs need to be loaded into the infection baskets (as well as <u>un</u>loaded after the infection cycle) from the chamber room into their cages that are to go no further than the chamber ante-room. After all guinea pigs have been loaded/unloaded the cages on the cage cart/rack in the anteroom are to be removed and placed back into the housing room or temporarily stored in the hallway until the cycle is completed.

9. Then, pipette 11 ml of sterile pyrogen free H_2O (using a 10 ml pipette) into an additional 50 ml conical tube and close the cap on the "diluent tube" and stand in the tube rack.
10. Spray the top of the *M. tuberculosis* H37Rv vial with 70% EtOH.
11. Fit the 1cc syringe with 26G½ needle, insert needle into the vial, & invert vial.
12. Pull bacteria in and out of syringe at least 10×; do not froth.
13. Pull approximately 1 ml into the syringe; then, turn the bottle upright and pull approximately 100ul of air into the syringe (so that the vial does not "spit" when needle is removed); withdraw needle.
14. Slowly dispense the 1 ml into a 5 ml Falcon tube so as not to create an aerosol.
15. In a second 5 ml Falcon tube, perform the necessary dilution(s) to arrive at the working stock concentration needed to place into 11 ml of sterile pyrogen free H_2O such that the final concentration is 1×10^6 CFU/ml, *and that upon transferring this aliquot into the 11 ml of diluent this dilution does not exceed 1/10.*
16. Remove cap from the diluent 50 ml conical tube, and with the P1000 micropipette pull 900ul into a P1000 blue tip from the working stock and dispense into the diluent tube by placing the needle tip just below the meniscus of diluent, and slowly adding the bacteria to the 11 ml volume of pyrogen free H_2O.
17. Discard the P1000 tip CAREFULLY into the 2.5% Vesphene-containing pipette boat.
18. With the P1000, dispense 900ul into 7 consecutive wells on a 24-well plate
19. With a 10 ml pipette, mix the "inoculum" by repetitive pipetting.
20. With the P200, dilute 100ul of the "inoculum" into the first well on the 24-well plate, and serially dilute across the remaining 6 wells.
21. Using a 20cc syringe fitted with an 18G ½" needle, pull 10 ml of the "inoculum" from diluent tube into the syringe.
22. Plate 100ul from each well on the 24-well plate 10^{-1} thru 10^{-7} dilutions onto two 7H11 quad plates and place into the 37ºC incubator.
23. With the same 20cc syringe, transport the "Inoculum"-filled syringe (along with a timer) into the hallway inside the Tupperware container.
24. Put on the PAPR.
25. Transport the container with the syringe (and the autoclaved nebulizer jars if haven't done so already) into the chamber room.
26. When the "End cycle" indicator sounds for the test run, press "Reset" on the control box and take out 1 of the autoclaved nebulizers from their individual containers.

27. Unscrew the nebulizer jar used for the test run from the apparatus, and replace with a fresh autoclaved nebulizer jar (place the "used" nebulizer jar into the Vesphene-containing stainless-steel container).
28. After the nebulizer jar is screwed tight, remove the plug screw and repeat Step 5a/5b with the syringe containing the 10 ml of "inoculum".
29. Pull ~15 ml of 2.5% Vesphene (from the stainless steel container) inside the syringe and place back into the stainless-steel container.
    NOTE: Be sure to re-fasten the plug screw before proceeding!!
    NOTE: Be sure that the secondary doors, and the basket tabs are flush with the outer perimeter of the infection basket before performing Step 30.
30. Load the guinea pigs (inside the infection basket) into the chamber by aligning the cart to the opened chamber door, latching the lock-pin, and sliding the basket into the main cylinder of the chamber.
    NOTE: Be sure that the spacer is in place at the posterior (the spacer should be placed between the rear mixing fans and the infection basket) of the chamber before re-latching the chamber door.
31. Re-latch the door, and be sure that ALL the 8 latches are fastened on the door so as to create the necessary air-tight seal.
32. Re-check for the proper amount of airflow being channeled through the chamber (~45 L/min). Also, be sure to check that the vacuum gauge reads ~9-10 inches of H_2O (after opening/closing the chamber door this may take a couple of minutes).
33. Initiate the cycle by depressing the "Start" button, and set 15 min on the timer.
34. Again, be sure to check the 3 jets on the nebulizer probe for proper spray/dispersion before leaving the room.
35. Again, be sure that the "Infection in progress Do Not Enter" sign is visible over the door window.
36. Leave the room.
37. Once the "End cycle" indicator light/alarm sounds, depress the "Reset" button.
38. Open the chamber door and remove the infection basket.
39. Transfer each of the infected guinea pigs back into their cages by mobilizing the entire cage rack (or cart will do if only a few guinea pigs are being aerosolized) into the ante-room, and transferring each of the guinea pigs from the aerosol basket into place back into B125/B131.
40. For multiple runs, re-mix the inoculum inside the 5 ml Falcon tube, and repeat Steps 16 thru 39.
41. Place the "inoculum" syringe(s) into a sharps container.
42. After the final infection run, carefully unscrew the nebulizer jar from the apparatus, put in 2.5% Vesphene then wrap in paper towels, and place inside the autoclavable container put generous amount of 2.5% Vesphene solution on paper towel , and place the lid on the container. Put in a large clear bag for autoclaving.
43. Thoroughly, but gently wipe the entire stainless-steel nebulizer probe with paper towels doused in 2.5% Vesphene.
    NOTE: Do NOT press the paper towels too hard over the nebulizer probe. There are minute holes that paper towel filaments can plug and, thus, block the nebulizer jets.
44. Repeat Step 43 with a paper towel stack doused in 70% EtOH.
45. Pour approximately 20 ml of pyrogen free H_2O (from a sterile 50 ml conical tube) inside an additional autoclaved nebulizer jar and screw into the apparatus.
46. Check for the proper amount of airflow being channeled through the chamber as before (~45 L/min). Also, be sure to check that the vacuum gauge reads ~9-10 inches of H_2O.
47. Initialize the "Cleaning run" by depressing the "Start" button on the Control box.
48. Following the "Cleaning run", turn off all switches.
49. Replace the nebulizer jar with the final autoclaved nebulizer jar.
50. Place the "used" jar into the 2.5% Vesphene solution.
51. Close the lid to the container, place into a large biohazard bag, and mark "FRAGILE – glass nebulizer jars"!

52. Remove the infection basket(s) from the cart(s), bag with the X-large biohazard bags, and seal with autoclave tape.
53. Open the chamber door, and wipe inside of chamber apparatus with Vesphene (2.5%); repeat process with 70% EtOH.
54. Repeat Step 53 on the basket cart.
55. Sweep up, and dispose of any debris from animal cages, etc.
56. Remove the "Infection in progress Do Not Enter" sign from the door.
57. Leave the room, replace PAPR.
58. Leave packed nebulizer/aerosol baskets in B144 hallway for autoclaving.

Intramuscular injection of the guinea pig

Materials
Injectate
1- to 3-ml syringe with 26 1/2-G needle

1. Fill syringe with injectate and remove air bubbles.
2. For the guinea pig, restrain by securing the animal's head under the upper arm, supporting the body with the forearm and encircling the rump with the hand.
3. Two people may be necessary for intramuscular injections in guinea pigs, one to restrain the guinea pig and another to inject into the gluteal muscles.
3b. For the guinea pig, insert the needle into the gluteal muscles.
4. Aspirate briefly with the syringe before injecting to prevent intravenous or intra-arterial injection.
5. Inject with moderate pressure and speed to avoid tissue damage.

Intradermal injection of guinea pig

Materials
70% ethanol
Injectate
Clippers with #40 blade
Gauze sponge or swab
1.0 ml insulin syringe (28G x ½ in)

Method
1. Restrain the animal.
2. Wipe area with 70% ethanol on a gauze sponge or swab.
3. Fill syringe with injectate and remove air bubbles.
4. Pull the skin taut with one hand and insert the needle, bevel up and at a shallow angle, just under the superficial layer of epidermis.
5. Inject 100 microliters for each test antigen with a maximum of eight sites on the back or stomach of the guinea pig. Make sure the injection sites are spaced evenly.
   A bleb will appear at the injection site when needle placement is correct.

Subcutaneous implantation of a microchip in the guinea pig

Materials:
IPTT-300 Implantable microchip -temperature
At approximately 14 millimeters in length by 2 millimeters in diameter, the IPT-300 can be simply and humanely implanted within an animal. Designed to be minimally invasive, IPT-300 transponders are injected with a syringe-like action with a pre-sterilized disposable needle gently into an animal's tissue.

Procedure:
1. The syringe is pre-loaded with the microchip.
2. The microchip can either be implanted in the inguinal or interscapular area – the preferred method is inguinally.
2. For the guinea pig, place the animal on a clean towel and restrain by grasping the skin of the chosen implant area with the non-preferred hand.
3. Tent the skin with the thumb and forefinger and insert the needle under the skin.
4. Inject the microchip with moderate pressure and speed.

Preparaton of Proskauer and Beck liquid medium

Purpose: To define a procedure for preparation of Proskauer and Beck medium used in laboratory procedures.

Time: 30 minutes

Materials:

- 1-liter Erlenmeyer flask
- 0.22 µm sterile filter
- KH_2PO_4 (Mallinckordt #7100)
- Asparagine (Difco #214410)
- $MgSO_4 \cdot 7H_2O$ (Fisher #M63-500)
- Magnesium Citrate (ICN #212256)
- Glycerol (Fisher #G33-500)
- Tween 80 (optional for growth of dispersed cultures) (Mallinckrodt #2744)
- ddH_2O

Procedure:

1. Prepare the following:

KH_2PO_4	5.0 g
Asparagine	5.0 g
$MgSO_4 \cdot 7H_2O$	0.6 g
Magnesium Citrate	2.5 g
Glycerol	20.0 ml
Tween 80	0.5 ml
ddH_2O (Q.S.)	1,000 ml

2. Dissolve the above ingredients one at a time in 1-liter ddH2O.
3. Adjust the pH to 7.8 using 40% NaOH (about 3.5-5.0 ml per liter)
4. Autoclave at 121ºC for 15 minutes. Be sure to use the slow exhaust cycle.
5. The pH after autoclaving will be about 7.4.
6. Filter the medium through a 0.22µm filter to remove any precipitate.

Euthanasia of guinea pigs

Procedure:
1. Pull up 0.2 ml of ketamine into a syringe.
   *note ketamine is a controlled drug so you must be authorized to use
1. Add 0.15 ml of Xylazine to the syringe, first replacing the 26G1/2 needle with a sterile one so as not to contaminate the Ana Sed
2. Remove the guinea pig from the cage and inject the guinea pig IM in the hind leg area with the cocktail. Place the guinea pig back in the cage until it is completely anesthetized.
3. Wait until the guinea pig is completely anesthetized before continuing. You can tell that the guinea pig is sedated because it will be completely relaxed and will not try to right its self when placed on its back.
4. Once the guinea pig is anesthetized, place it on the necropsy board inside of the hood, then put together a 1 ml syringe with a 26G 1inch needle and draw up 0.9ml of the 1:1 diluted B-euthanasia to PBS solution.
5. Place your fingers on the guinea pig's chest. You should be able to feel where the heart is by its beating.
6. Insert the needle on the guinea pig's left side (your right side) of its chest and draw back. If you are in the heart you should see blood in the syringe. You want to inject on the left side of the guinea pig because you take the right lung lobes for viable count and histology and you don't want to damage them with the needle.
7. Slowly inject the solution into the heart. You do not want to move the needle during this time because you may pull it out of the heart or push it right through the heart. You should be able to see the heart stop beating and the front feet should go pale in color.
8. Once you are done injecting the solution, put the syringe into a 50ml conical tube containing Lysol. Draw up Lysol into syringe. Wait a few minutes before starting the necropsy. Before you begin, pinch all 4 feet either with your fingers or with you forceps. If there is no movement from the guinea pig you may begin the necropsy. If the guinea pig twitches or moves, wait a few more minutes and repeat the pinching. Do this until there is no movement from the guinea pig. Once there is no movement, you may begin the necropsy.